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PROTEINS OF TYPE II SECRETION SYSTEM IN VIBRIO CHOLERAE:
INTERACTION OF EPSC PROTEIN WITH
THE EPSD OUTER MEMBRANE PROTEIN

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PROTEINS OF TYPE II SECRETION SYSTEM IN *VIBRIO CHOLERAE*: INTERACTION OF EPSC PROTEIN WITH THE EPSD OUTER MEMBRANE PROTEIN

By

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ABSTRACT

PROTEINS OF TYPE II SECRETION SYSTEM IN *VIBRIO CHOLERAE*:
INTERACTION OF EPSC PROTEIN WITH
THE EPSD OUTER MEMBRANE PROTEIN

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Protein EpsD is the only protein of the type II secretion system located in the outer membrane of *Vibrio cholerae*. Strong evidence from other bacterial systems indicates that EpsD homologues constitute the pore through which exoproteins are transferred from the periplasm into extracellular medium. All other proteins essential for the secretion are located in the cytoplasmic membrane. In this thesis, I present experimental results indicating the interaction between EpsC protein and EpsD: (i) EpsD multimer is unstable in the absence of EpsC; (ii) only coexpression of *epsC* and *epsD in cis* can complement the *epsC* mutant to restore the secretion; (iii) in addition to *epsCD* genes, an open reading frame homologous to Hsp15 of *Escherichia coli* is required for this complementation.

Dedicated to my parents, Sungchoon Lee and Youngae Choi.

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KEY TO ABBREVIATIONS

AAA.....ATPases *a*ssociated with different cellular *a*ctivities
ABC.....ATP-Binding Cassette
ADP.....Adenosine 5'-Diphosphate
Ap^R.....Ampicillin Resistance
ATP.....Adenosine 5'-Triphosphate
ATPase.....ATP Hydrolase
BFP..... Bundle-forming Pili
bp.....base pair(s)
C-terminus.....Carboxyl terminus
Cm^R.....Chloramphenicol Resistance
CT.....Cholera Toxin
EDTA.....Ethylenediaminetetraacetic Acid
EHEC..... Enterohemorrhagic *Escherichia coli*
ELISA.....Enzyme-Linked Immunosorbent Assay
EM.....Electron Microscopy
EPEC.....Enteropathogenic *Escherichia coli*
eps.....*e*xtracellular *p*rotein *s*ecretion
ETEC..... Enterotoxigenic *Escherichia coli*
FRT..... FLP Recognition Target
GEP.....General Export Pathway
GFP.....Green Fluorescent Protein
GM1.....GM1 Ganglioside
GSP.....Genera Secretion Pathway
HAP.....Hemagglutinin/Protease
Hg^R.....Hygromycin B Resistance
$(His)_6$.....6X Histidine
IgA.....Immunoglobulin A
IgG.....Immunoglobulin G
IM.....Inner Membrane or Cytoplasmic Membrane
IPTG.....Isopropyl-β-D-ThioGalactopyranoside
kb.....kilobase(s)
kDa.....kilodalton(s)
Km^R.....Kanamycin resistance
LB.....Luria-Bertani medium
LT.....*Escherichia coli* Heat Labile Enterotoxin
MFP.....Membrane Fusion Protein
MTB.....Main Terminal Branch
MW.....Molecular Weight
N-terminus.....Amino terminus
NTA.....Nitriloacetic Acid
NTP.....Nucleoside-5' Triphosphate
NTPase.....NTP Hydrolase

OD_{650}.....Optical Density at 650nm
ORF.....Open Reading Frame
PAGE.....Polyacrylamide Gel Electrophoresis
PAI.....Pathogenicity Island
PBS.....10mM Phosphate Buffer oH7.4/136 mM NaCl
PBS-T.....PBS + 0.5% Tween 20
PCR.....Polymerase Chain Reaction
PDZ.....Post-synaptic density protein, Disc large and Zo-1 proteins
Pmx.....Polymyxin B Sulfate
PT.....Pertussis Toxin
SDS.....Sodium Dodecyl Sulfate
PAGE.....Poly Acrylamide Gel Electrophoresis
Sm^R.....Streptomycin Resistance
TCP..... Toxin-coregulated Pili
Tc^R.....Tetracyclin Resistance
Tfp.....Type IV Pili
TMD.....Trans-Membrane Domain
Tn5.....Transposon 5
TPS.....Two Partner Secretion
TTSS.....Type III Secretion System
T2SS.....Type II Secretion System
TFSS.....Type IV secretion System
VPI..... *Vibrio cholerae* Pathogenicity Island
WT.....Wild Type

CHAPTER 1

GENERAL INTRODUCTION

Mechanisms of Protein Secretion in Gram-negative Bacteria

Bacteria produce numerous kinds of proteins and some of them are secreted to the extracellular environment. Oftentimes these secreted proteins are critical for the virulence of bacterial pathogens or for survival of bacteria in different environments (40, 56, 57, 64, 98, 105, 106, 114, 121, 132, 197, 275, 277, 286, 301, 302, 307, 343). In Gram-negative bacteria, these extracellular proteins have to pass through the cytoplasmic and outer membranes (IM and OM, respectively) and the periplasmic space between the membranes that has been estimated to be 7-25nm wide (118). This distance is too large to allow the direct interaction of most IM and OM proteins During evolution, several distinct mechanisms have been developed to accommodate transfer processes of structurally and functionally diverse extracellular proteins (101, 142, 182, 208, 276, 318, 334, 335). In the past two decades five main pathways of extracellular protein secretion have been identified. The current knowledge of these secretion mechanisms in Gram-negative bacteria is summarized below.

Type I Secretion Pathway

Type I secretion system or ABC exporter is composed of three protein components, an ATP-binding cassette (ABC) protein, a membrane fusion protein (MFP) that spans the periplasm, and a porin-like OM protein (11, 22, 35, 82, 97, 177, 290). The secretion is independent of the Sec machinery and the proteins are transported across the two membranes in one step without generating any periplasmic intermediate (178). This pathway is exemplified by the secretion of

α-hemolysin (HlyA, 110kDa) in *Escherichia coli*, and the complex consists of HlyB (ATPase), HlyD (MFP) and OM protein TolC (145, 336).

The synthesis, activation and secretion of HlyA are determined by the *hlyCABD* operon, which is transcribed from a promoter located upstream of *hlyC* (145). TolC is not encoded in this gene cluster in *E. coli*, but several homologues of this OM component, widespread in Gram-negative bacteria, are found in the same gene cluster with two other components (1, 201, 336). HlyC, which is a fatty acid acyltransferase and required for hemolytic activity, is not involved in the secretion of HlyA. HlyB is inserted in the IM via hydrophobic α-helical transmembrane domains (TMD) (337). HlyD is anchored in the IM via a single TMD and possesses a large periplasmic domain within the carboxy-terminal 100 amino acids, which are highly conserved among MFPs. Thanabalu et al. (317) have shown interactions between the components by cross-linking experiments in the presence or absence of the substrate and protease accessibility experiments indicated conformational changes in each of the three exporter proteins HlyB, HlyD and TolC. HlyD and HlyB can be assembled into a stable IM complex in the absence of HlyA and TolC. Engagement of translocating substrate HlyA induces the IM complex to make a bridge, via a HlyD trimer, to the OM exit pore TolC, allowing direct passage of substrates across two membranes and the intervening periplasmic space through the TolC channel tunnel (11). This continuous conduit transiently connects the cell cytosol to the external environment through completely proteinaceous machinery. After the passage of hemolysin, the bridged components of the export channel revert to IM

and OM states (317). The crystal structure of TolC was determined to 2.1 Å resolution and the TolC homotrimer was seen as a tapered cylinder 140 Å long, extending from the extracellular side of the OM, through most of the periplasmic space, and ending in close proximity to the periplasmic side of the IM (177, 180). Recently, gating of the TolC tunnel has been shown to be achieved by an iris-like alignment of helices at the periplasmic tunnel entrance (12). Whereas the IM proteins HlyB and HlyD are specific components of the transport apparatus of *α*-hemolysin, TolC is a multifunctional protein, which also appears to be involved in the efflux of antibiotics, heavy metal ions, detergents, and solvent (242, 350). Very interestingly, TolC is also responsible for secretion of *E. coli* heat-stable enterotoxin I across the OM, which is translocated across the IM by the general export pathway (GEP) consisting of Sec proteins (107, 346, 347).

Other proteins secreted by the type I mechanism include proteins such as *Bordetella pertussis* adenylate cyclase, *Pasteurella haemolytica* leukotoxin, *Erwinia chrysanthemi* metalloprotease, *Pseudomonas fluorescens* lipase and proteases of *Serratia marcescens* and *Pseudomonas aeruginosa* (3, 11, 75, 88, 90, 117, 120, 242). Analysis of secretion via hybrid exporters in *S. marcescens*, which possesses multiple ABC exporter systems for secretion of several proteins, showed that specific interactions between the ABC protein and the MFP are required for the formation of active exporters (3). The secreted proteins have uncleavable C-terminal secretion signal specifically recognized by the ABC protein and it is believed that the secondary structure of this region is more important for function than primary sequence (22, 157, 179, 295). Fusions of this

signal to foreign proteins have been shown to promote secretion, although there seemed to be limitations related to the size and structure of the passenger (27, 89, 102, 145, 237).

Type II Secretion Pathway: Main Terminal Branch (MTB) of the General Secretion Pathway (GSP)

Type II secretion pathway (T2SS) is encoded by at least 12 genes to secrete different extracellular enzymes and toxins from a wide variety of Gram-negative bacteria (Table 1-1, next page) (104, 252, 258, 271, 278, 279). Since the sequence of *pul* gene cluster, which is responsible for the secretion of pullulanase from *Klebsiella oxytoca*, has been reported more than a decade ago, sets of homologous genes were subsequently discovered in several species of Gram-negative bacteria (78, 279). Some of well-studied T2SSs are from *K. oxytoca*: *pul* (76, 78), *Pseudomonas* species: *xcp* (4, 18, 72-74, 103, 104, 182, 192, 321), *Erwinia* species: *out* (127, 265), *Xanthomonas campestris*: *xps* (87, 152), *Aeromonas* species: *exe* (150, 161, 168) and *Vibrio cholerae*: *eps* (215, 280, 284). Functional T2SSs were also examined in enterohemorrhagic *E. coli* (EHEC) O157: *etp* (190, 289), enterotoxigenic *E. coli* (ETEC): *gsp* (147, 316), *Burkholderia* species: *gsp* (80, 99) and *Legionella pneumophila*: *lsp* (122, 203). A cluster of genes homologous to the type II secretion genes seems to be widespread among Gram-negative bacteria according to DNA sequence analysis from microbial genome data (279). Most of these organisms are animal and plant pathogens and the secretion is thought to play an important role in

BACTERIUM	PROTEIN	GENES																
Vibrio cholerae	CT Lipase Protease Chitinase Neuraminidase	*eps* *vcp*		B	A	C	D	E	F	G	H	I	J	K	L	M	N	D
Aeromonas hydrophila	Aerolysin	*exe* *tap*		B	A	C	D	E	F	G	H	I	J	K	L	M	N	D
Klebsiella oxytoca	Pullulanase	*pul*	S	B	A	C	D	E	F	G	H	I	J	K	L	M	N	O
Erwinia chrysanthemi	Cellulase Pectinase	*out*	S	B		C	D	E	F	G	H	I	J	K	L	M		O
Erwinia carotovora	Cellulase Pectinase	*out*	S	B		C	D	E	F	G	H	I	J	K	L	M	N	O
Pseudomonas aeruginosa	Exotoxin A Protease Elastase Lipase Type IV Pili	*xcp* *pil*	 P			P	Q Q	R B	S C	T A	U	V	W	X	Y	Z		A D
Xanthomonas campestris	Cellulase Pectinase	*xps*					D	E	F	G	H	I	J	K	L	M	N	O
Aeromonas salmonicida	Acetyl transferase	*exe*				C	D	E	F	G	H	I	J	K	L	M	N	
Escherichia coli	Chitinase	*gsp*			A	C	D	E	F	G	H	I	J	K	L	M		O
SUBCELLULAR LOCATION OF GENE PRODUCTS		C IM OM	 *	 * 	 * 	 * *	 *	* * 	 * 	 * *	 * *	 * *	 * *	 * *	 * *	 * 	 * 	 *

Table 1-1. Similarities between proteins essential for extracellular protein secretion and subcellular locations of each gene product. Homologous genes appear in the same column except for *pulA* of *K. oxytoca*, which is a structural gene for pullulanase and is not homologous to *epsA*, *exeA* and *gspA*, which are components of the GSP. Two different names, *pilD* and *xcpA*, are used to designate the same gene encoding a prepilin peptidase for the type IV pili (Tfp) and the type II secretion, respectively, in *P. aeruginosa*. *V. cholerae vcpD* is also called *pilD*. Many more unlinked genes are required in addition to the genes listed in the table for the Tfp biogenesis of *P. aeruginosa*. Abbreviations for subcellular locations are cytoplasm (C), inner membrane (IM), and outer membrane (OM). For orientation and spatial organization of each gene, refer to the reference (279).

pathogenesis since mutants, which are unable to secrete proteins, showed reduced pathogenicity or became totally avirulent (202, 240, 279). Although nomenclature of genes encoding the T2SSs are different depending on the organism, individual designations for homologues carry the same letter code except in *Pseudomonas* species (Table 1-1) (258, 279).

Unlike type I, type III, or type IV secretion pathways, which can transport proteins directly from the cytoplasm to the extracellular environment in a single step, complete secretion of type II substrates occurs in two distinct steps (260). The first step involves Sec-dependent translocation or GEP and it has been extensively studied in *E. coli* (68, 84, 93, 227, 252, 259). Six *sec* genes, *secA, secB, secD*, *secE*, *secF* and *secY*, have been shown to be required for processing of the signal peptide in precursor form of pullulanase and secretion in *pul* system of *Klebsiella* reconstituted in *E. coli* K12 (259). Sec homologues are expected to be present in all Gram-negative bacteria with GSP. Indeed, recently completed *V. cholerae* genome sequence does show homologues of Sec proteins (129). Therefore, substrate proteins of type II secretion pathway which contain N-terminal signal peptides are likely to be processed by signal peptidase when translocated across the IM via the Sec machinery (128, 248, 259). Following the removal of their signal peptides and release into the periplasm, they fold and assemble into translocation competent conformations to enter the T2SS and cross the OM (29, 33, 124, 128, 139, 255, 311, 329). When substrates of GSP are introduced into an *E. coli* strain, whose *gsp* genes are known not to be expressed under the normal laboratory growth conditions, or into

host organisms with mutation(s) in T2SS genes, they are found exclusively in the periplasm (108, 109, 210). Therefore, the type II secretion pathway actually refers to the second step or translocation of the periplasmic intermediates across the OM and is also referred to MTB of the GSP (252).

Recently, a novel type of T2SS involving twin-arginine translocation (Tat) pathway, rather than GEP, to translocate its substrates across the IM and to feed substrates into the secreton has been described for *P. aeruginosa* (328). Tat system operates in parallel to the Sec system to export periplasmic proteins and has remarkable ability to transport folded enzymes (21). Thus, *P. aeruginosa xcp* secreton recognizes elastase and exotoxin A, bearing Sec-dependent signal peptide, and phospholipase C, bearing a twin-arginine signal peptide, in the periplasm and release them across the OM via the same T2SS (193, 211, 212, 216, 328). Although T2SS genes are well conserved among members of proteobacteria, the transported proteins are diverse and most of them seem to be lacking any similarity in amino acid sequences and structures (279). Characterization of individual components and up to date information on T2SS will be further described in detail using *eps* system of *V. cholerae* as an example on page 21.

Type III Secretion Pathway

Type III secretion systems (TTSSs) are found in many plant and animal pathogens and they play a central role in the pathogenicity (9, 37, 43, 50, 60, 113-115, 156, 194, 325). Genes encoding the TTSS and genes encoding the

proteins released by the TTSS are generally located on virulence plasmids and pathogenicity islands (PAIs) (8, 59, 121, 136, 219, 344). More than 20 gene products are required for successful secretion of unfolded or partially folded proteins and at least nine are highly conserved between different bacterial species and probably constitute the core components of the secretion apparatus (126, 176, 198). Eight of these conserved proteins are homologous to components of the flagellar basal body or IM transport channel, which secretes structural components of the flagellum (2, 7, 20, 44, 125, 214, 247). However, the sequence and host cell activities of the secreted proteins differ in the individual pathogens (53, 115). Some of these proteins are effectors or virulence factors that are delivered by extracellular bacteria into the cytosol of the target cell while the others are translocators or translocons (discussed below), structural components that help the effectors to cross the membrane of the eukaryotic host cell (57, 315). The secretion is triggered by close physical contact between the bacterium and the host cell which initiates injection of effector molecules directly into the cytoplasm of eukaryotic host cells. Thus, it is designated contact-dependent secretion pathway (79, 194). In addition to cell contact, other factors such as environmental cues also play important roles in regulation of complex TTSS (110, 172, 218, 225). The effectors secreted by TTSS are very diverse and often resemble eukaryotic proteins, enabling them to interfere with signaling pathways and suppress host defenses (58, 61, 301, 304). Some of the effector proteins such as SopE from *Salmonella* are encoded by bacteriophage (31, 330). Secretion of some, if not all, effector proteins require

binding of distinct cytosolic chaperones, which stabilize their substrates and prevent premature interactions with other partners (20, 23, 158, 235, 236, 303, 340). Chaperones are also thought to present a secretion-competent conformation of the effector to the TTSS and loss of chaperone reduces secretion of their specific substrate proteins (20, 158, 303). One of a number of effectors from *Y. pseudotuberculosis*, YopE, has been suggested to possess several distinct domains required for secretion from the bacterial cell, release from the bacterial cell surface into the culture supernatant, formation of a stable complex with its chaperon, YerA, and traversing the eukaryotic membranes (288)

In the exemplary type III secretion of Yop proteins in *Yersinia* spp., the secretion signal is thought to be located either in the N-terminal 15-20 amino acid residues of Yops or in the mRNA rather than in the protein itself (6, 13, 14, 43, 52, 60, 198, 206, 207, 263, 288, 298). Supramolecular structures of TTSS in animal pathogens *Salmonella typhimurium*, *Shigella flexneri*, *Yersinia enterocolitica* and enteropathogenic *E. coli* (EPEC) have been shown to resemble a needle. The complex is composed of a cylindrical base that resembles flagellar basal bodies and anchors the whole structure to the IM and OM, and a needle-like structure which extends from the bacterial cell surfaces (28, 144, 173, 187, 188, 293, 314). One of the proteins comprising the needle complex belongs to the family of secretins, a group of OM proteins involved in the transport of various macromolecules and filamentous phages across the OM (116, 187). YscC of *Yersinia* has been shown to form ring-shaped multimeric structure in the OM as do the other secretins involved in Type II secretion, Type

IV pili (Tfp) biogenesis and filamentous phage extrusion (181). In EPEC, a sheath-like structure, EspA filament, is linked to the needle and may allow passage of effector proteins through the eukaryotic cell membrane, forming a physical bridge between the bacteria and the host cell (175, 245, 293).

Extracellular surface appendages named Hrp pili have also been observed in plant pathogenic bacteria such as *Erwinia amylovora*, *Pseudomonas syringae* and *Ralstonia solanacearum*, and they are thought to penetrate the plant cell wall (155, 176, 269, 326, 341). In *P. syringae* pv. *tomato*, *hrpA* gene, which encodes the major subunit of the Hrp pilus, is required for type III secretion *in vitro* and the pili were shown to serve as conduits to deliver proteins outside the cell (162, 163).

In several bacteria, transmembrane protein complexes termed translocons have been identified and they are predicted to be channels that are inserted into the host cell membrane since some of them have been shown to form pores (37, 38, 315). Effector proteins presumably pass through the eukaryotic cell membrane via such translocons. Therefore, bacteria defective in translocons are not able to inject effector proteins into the host cell cytoplasm and they are just secreted into the surrounding medium *in vitro* (37). Translocation via the type III pathway is Sec-independent and transit of effectors from the cytoplasm of the bacterium to that of the host cell is thought to occur in one step through the TTSS apparatus and the translocon.

Type IV Secretion Pathway

Type IV macromolecular transfer systems share a common ancestry with the conjugation machines of Gram-negative bacteria (36, 39, 46, 47, 64, 343). Type IV secretion systems (TFSSs) are able to transport proteins or nucleic acids and their substrates not only across the bacterial envelope, but also the plasma membrane or vacuolar vesicles of host cells. The family includes the *E. coli* conjugative pilus or transfer (Tra) systems of the conjugative plasmids pK101 (IncN), F (IncF) and RP4 (IncP); the *Agrobacterium tumefaciens* T-DNA transfer system required for exporting T-DNA to susceptible plant cells; the *B. pertussis ptl* system for secretion of pertussis toxin (PT); the *cag* system of *Helicobacter pylori* and *Rickettsia*, *Legionella* (*dot*/icm) and *Brucella* (*VirB*) systems for delivery of effector molecules to the eukaryotic cell cytoplasm or to the vacuolar membrane (19, 30, 36, 46, 64, 343).

TFSS of *A. tumefaciens*, which delivers not only T-DNA, but also effector proteins to plant cells, has been studied in most detail and serves as a model system. The genes of the 12 TFSS components from *A. tumefaciens*, *virB1* to *virB11* and *virD4*, are encoded in two operons on the Ti (tumor-inducing) plasmid and other type IV systems are composed of a complete or incomplete sets of genes homologous to *virB* and *virD4* (46, 64). The corresponding genes are often collinearly arranged in the respective operons. Genetic organization, function and localization of each member in several TFSSs are well described in several references (47, 63, 189). VirB proteins can be categorized into three functional groups: (i) proteins localized exocellulary forming the T-pilus or other

adhesive structures (VirB1, VirB2 and VirB5); (ii) putative channel components spanning the two membranes (VirB3, VirB6, VirB7, VirB8, VirB9 and VirB10); and (iii) cytoplasmic ATPases, including VirB4, VirB11 and VirD4 (47, 63). All of these proteins are probably assembled into a supramolecular structure and recent studies of protein-protein interaction involving yeast two-hybrid assay, peptide linkage mapping, protein fusions and detergent extraction will help to define the structure along with studies of protein localization (70, 81, 183, 338). VirB7, VirB8, VirB9, and VirB10, which form a structural and functional core, interact with each other and form a high molecular weight complex containing VirB7-10 that may be extracted with a detergent (183). Major T-pilus component VirB2 is associated with VirB5 and VirB7 (183). Several hypothetical models of *Agrobacterium* TFSS assembly were derived from the above results (64, 70, 183, 338). Further identification of protein-protein interactions will bring a more detailed structure of the type IV secretion machinery. The delivery routes and cellular consequences of various known effector proteins from *A. tumefaciens* and other Gram-negative bacteria have been reviewed recently (46, 47).

Despite the overall similarity in subunit composition between the T-DNA transfer and *ptl* secretion systems of *A. tumefaciens* and *B. pertussis*, respectively, the secretion route of PT from *B. pertussis* is thought to be quite different from the *A. tumefaciens* system. Substrates of *A. tumefaciens* TFSS, for example VirD2 and VirE2, lack signal sequences and it has been thought that the *virB* system and other conjugal DNA transfer systems transport their substrates across both bacterial membranes simultaneously in one step.

However, the secretion of PT from *B. pertussis* seems to be a two-step process as in the general secretion pathway (36). Each of the individual subunits of PT (S1-S5), which is a member of the AB_5 toxin family, is synthesized with their own signal sequence and they are likely to cross the inner membrane in a Sec-dependent manner. Holotoxin assembly in the periplasm is essential for effective secretion of PT across the outer membrane by the Ptl system (95, 306). Interestingly, unlike those of other AB_5 toxins, five B subunits of PT are not identical and PT B oligomer, which consists of one copy each of subunits S2, S3, and S5 and two copies of subunit S4, cannot be secreted effectively in the absence of S1, which corresponds to A component (95, 186). The PT, therefore, is different from the B subunit pentamers of cholera toxin (CT) or the *E. coli* heat labile enterotoxin (LT) , which can be secreted effectively by the type II secretion in the absence of A subunit (141).

There are several similarities between TTSSs and TFSSs of various pathogens: (i) TFSSs of some organisms are encoded in PAIs as in TTSSs (45, 63, 135); (ii) both PAI-encoded secretion systems are contact-dependent and directly deliver virulence factors or effectors to interfere with the functions of host cells with the exception of the *B. pertussis* Ptl system, which exports PT to the extracellular milieu (40); (iii) with the exception of the Ptl system, substrate translocation by TFSS probably does not require periplasmic intermediates as in TTSS (19). However, very recently Pantoja et al. (239)proposed a two-step model for the *Agrobacterium* TFSS as in the Ptl system and it needs to be clarified; (iv) surface appendages might be involved in translocating DNA and

effector proteins into eukaryotic host cells (16, 176). The Hrp pilus of TTSS in *P. syringae* can function as a conduit for protein delivery (162, 163). The TFSS-associated T-pilus has also been observed in *A. tumefaciens* and it has been hypothesized to serve as a channel for T-DNA transfer, but this has never been shown directly (111, 176, 189); (v) substrate-specific chaperones and coupling proteins are necessary for presentation of substrates to the mating channel (46); (vi) the TFSS secretes proteins which form a membrane channel or pore in the host cell membrane to aid transfer of DNA or effectors to eukaryotic cytoplasm, just like translocons of TTSS (37). *Agrobacterium* VirE2 forms a membrane channel that transfers the T-strand through the plant plasma membrane (86). VirE2 is one of the most abundant Vir proteins and it performs an unusually large number of functions as described by Ward and Zambryski (339).

Type V Secretion Pathway: Autotransporters

Descriptive designation for the type V secretion pathway is the autotransporter secretion system and it has been described as type IV in some past literatures, but recently the nomenclature for classification of secretion systems has been re-established by a group of researchers (133). As the name implies, the proteins secreted by this pathway mediate their own translocation across the OM and no other element is necessary for it (130, 132, 134). The best known member of this family is IgA1 proteases of *Neisseria gonorrhoeae* and the number of proteins that are secreted by an analogous mechanism is rapidly increasing (174). These proteins possess unusually long N-terminal

signal sequence and are thought to traverse the IM through Sec machinery just like the proteins secreted by the type II pathway. However, they differ in how they pass through the OM. In autotransporters, N-terminal signal sequence is followed by a passenger domain corresponding to the mature secreted protein, which is translocated to the cell surface through an OM pore formed by a C-terminal β-domain (174, 294, 327). After exiting the pore, the IgA1 proteases are cleaved by the catalytic activity of their serine protease active sites and release itself from the β-barrel structure (174). Therefore, it is assumed that all the requirements for secretion across the OM are contained within a single molecule and the secretion is probably energy-independent. Apparently, it is the simplest secretion mechanism for that reason and can be simply described as an all-in-one system.

In addition to the autoproteolytic processing of passenger domains for release into the external milieu, there can be two other outcomes depending on the autotransporter protein protruding through the OM. First, the protein may remain intact as a large polyprotein with a membrane-bound C-terminal domain and an N-terminal domain extending into the external milieu (62, 300, 322). Alternatively, the protein may be cleaved by an OM protease and the passenger domain may remain in contact with the surface via a non-covalent interaction with the β-domain (134, 305). Generally, β-domains of autotransporter proteins are highly conserved, consistent with their highly conserved function, although the passenger domains are widely divergent. There are several different types of autotransporter proteins secreted from diverse Gram-negative bacteria and they

can be grouped together according to functions of their passenger domains. Many of them are suspected to be involved in pathogenesis, but more studies have to be carried to determine their exact roles in virulence (132).

Recently, a new pathway called two-partner secretion (TPS) pathway has been described (160). As the name implies, TPS pathway involves a single accessory protein specifically devoted to the secretion of the exoprotein across the OM. The proteins being secreted, named TpsA family, are synthesized as precursors with an N-terminal cleavable signal peptide and has N-proximal module called the secretion domain, while the partner transporter proteins, named TpsB family, are channel-forming β-barrel proteins (160). Two partners are usually organized in an operon. The components of TPS are very similar to classic autotransporters, but the passenger protein and the pore-forming β-domain of the unlinked autotransporters are translated as two separate proteins rather than a single protein with two separate domains. For that reason, the term, 'unlinked autotransporter' has been proposed in place of TPS (130, 131, 159).

Mechanism of Type II Secretion in *Vibrio cholerae* And Its Significance in Cholera Pathogenesis

Cholera is a life-threatening epidemic disease that occurs worldwide (165). The main symptom of cholera is characterized by a severe watery diarrhea caused by ingestion of contaminated food or water containing *Vibrio cholerae*, which colonizes the small intestine after being ingested (96, 220). A

number of pathogenic factors produced by toxigenic *V. cholerae* are responsible for cholera pathogenesis and some of them are known to be secreted out of the cells. *V. cholerae* is a motile, Gram-negative curved rod-shaped bacterium belonging to the family Vibrionaceae and they are often found in estuarine and aquatic environments (266). *V. cholerae* genome contains two unique circular chromosomes that have been sequenced (129, 323). Genome of CTXΦ, 6.9-kb single-stranded covalently closed DNA filamentous bacteriophage, harboring *ctxAB*, which encodes cholera toxin (CT), is integrated into chromosome I or larger chromosome by phage lysogenic conversion (129, 332, 333). CT is responsible for profuse watery diarrhea and it contributes to the fecal-oral transmission of *V. cholerae* (292, 330). The gene encoding a member of the type IV pili (Tfp) family, toxin-coregulated pili (TCP), which are required for intestinal colonization and also serve as receptors for CTXΦ, is on *V. cholerae* pathogenicity island (VPI) (166, 332). VPI is a genome of another filamentous bacteriophage , VPIΦ and it is also on chromosome I (129, 167, 333). This is a good example demonstrating a critical role of bacteriophages in the emergence of pathogenic bacteria by horizontal gene transfer and also shows importance of bacteriophage-bacteriophage interactions in the evolution of pathogenic bacteria (31, 32, 221, 233, 330, 331). Along with two bacteriophage genomes encoding very important virulence factors, a cluster of T2SS genes designated *eps* (*e*xtracellular *p*rotein *s*ecretion), which is required for the secretion of CT and CTXΦ from *V. cholerae*, is also on the same chromosome (71, 129, 284, 333). In

addition to already mentioned genes, most genes known to be essential in cholera pathogenicity are encode by chromosome I (129).

Vibrio cholerae mutant strain that did not secrete CT has been reported more than 25 years ago (146), but actual genes that are responsible for the secretion have been identified within last 10 years (224, 284, 285). In addition to CT, which is a major virulence factor, T2SS of *V. cholerae*, identified by our laboratory, is also required for secretion of several different enzymes such as hemagglutinin/protease (HAP), endochitinase, lipase, and neuraminidase (55, 112, 234, 284, 285). Since they do not show any sequence similarity, it is still unknown how these heterologous proteins are recognized by the same machinery. Although the mode of action for CT is well studied, it is not well known how other potential virulence factors play roles in cholera pathogenesis (292).

CT and *E. coli* heat-labile enterotoxin (LT) share similar structural and functional properties due to ~80% protein sequence identity (292, 299). They are structurally AB_5 toxins composed of a single catalytically active A subunit and a pentamer of B subunits responsible for binding to receptors, and functionally belong to ADP-ribosylating exotoxins (94, 186, 238, 299). Biogenesis of CT and LT from *V. cholerae* is well characterized (138). The genes which encode the toxin subunits are organized into polycistronic operons. As depicted in Fig. 1-1 on page 39, individual A and B subunits of CT are synthesized in precursor polypeptides with N-terminal signal peptides in the cytoplasm before translocated across the IM via the Sec system (199). The signal sequences are proteolytically

removed by signal peptidase during the translocation of the polypeptides across the IM to yield mature protein in the periplasm (138). The mature A subunit (27.2 kDa) and five identical B subunits (11.6 kDa each) fold and assemble into the AB_5 holotoxin complex before being recognized by Eps secreton or type II secretion apparatus via specific recognition of B_5, which carries the putative secretion signal (139, 141, 243, 349). The B subunit pentamer can be secreted in the absence of A subunit, but A subunit alone will remain in the periplasm in the absence of B subunit (141). Therefore, A subunit is secreted only when it forms a multimer with the B subunit and the signal for OM translocation is probably contained in the tertiary conformation of the B_5 subunit (140, 141). However, the exact signal responsible for type II secretion is not known yet. The AB_5 complex is thought to be targeted somehow to the putative type II secretion pore, EpsD, in the OM to reach the extracellular environment. Other Eps proteins are expected to interact or communicate with each other and with EpsD to form an active secretion apparatus spanning the cell envelope or to bring the substrate proteins to the gate. Conformational changes caused by these interactions will probably regulate the secretion process. Since the multimeric exoproteins such as CT, with molecular weight of 84,000, are very large and the resident proteins of periplasm have to be distinguished from the substrate of the T2SS, the pore is expected to be gated, rather than being open at all times.

CT binds to the GM1 ganglioside on intestinal epithelial cells via B subunit pentamer and the A subunit ADP-ribosylates the stimulatory G protein of the

adenylate cyclase complex. The resulting elevation of cyclic AMP causes excessive secretion of salts and water leading to severe diarrhea followed by dehydration (165).

Components of Type II Secretion System

In *V. cholerae*, *eps* genes, *C* through *N*, which seem to be organized into an operon and a separate prepillin peptidase gene designated *vcpD/pilD* (*gspO* homologue) are required for type II secretion (112, 215, 284). Two more linked genes, *epsA* and *epsB*, which are separated from the *epsC-N* cluster, were identified by sequence similarity to *exeA* and *exeB*, which have been shown to play an important role in type II secretion of aerolysin in *A. hydrophila* (15, 161, 278). However, *epsA* and *epsB* genes do not seem to be essential for secretion (M. Sandkvist, personal communication). Although T2SS is required for translocation of proteins across the OM, most components of the apparatus are IM-associated proteins (Table 1-1). The only OM protein component in *V. cholerae* is EpsD. More than half of the T2SS components (EpsD, EpsE, EpsF, EpsG, EpsH, EpsI, EpsJ, EpsK and VcpD) are homologous to proteins required for the biogenesis of type IV pili (Tfp) or fimbriae (69, 191, 284). The proteins involved in Tfp assembly and T2SS also resemble proteins involved in filamentous phage assembly and natural competence or uptake of DNA (85, 273, 274).

Our laboratory has been characterizing individual components involved in type II secretion of *V. cholerae* and detecting interactions between its

components for several years. In this section, some of the well-characterized T2SS components will be discussed with the results of experiments done by us with *V. cholerae* system and also other researchers' reports using other Gram-negative bacteria as their T2SS model organisms. In the following section, assembly of the type II secretion apparatus will be discussed combining all the available data regarding interactions among type II secretion components from different organisms. Eps designation will be used where the information is derived directly from the study of *V. cholerae* while other designations or just one letter code representing homologues will be used if the information is from studies of other organisms.

Eps D protein

EpsD is an integral OM protein and association of this protein with the OM has been demonstrated by sucrose density gradient separation (77, 148, 153). This is the largest protein with 674 amino acid residues and predicted subunit molecular weight of ~ 73,000 Da among the Eps components (284). It is initially produced with a cleavable signal sequence before being processed to become a mature protein (77, 153). Protein D belongs to a family of OM proteins termed secretins, all of which form large complexes resistant to dissociation in sodium dodecyl sulfate (SDS) at 100° (25, 116). They are all involved in transport processes such as, type II and III secretions (YscC from *Y. pestis*, 48% similarity and 22% identity with OutD from *E. carotovora*), Tfp biogenesis (PilQ from *P. aeruginosa*, 26% identity with EpsD), filamentous phage extrusion (pIV from

M13, 54% similarity and 32% identity with OutD from *E. carotovora*), and DNA uptake (ComG1 from *Bacillus subtilis*, 61% similarity and 38% identity with OutD from *E. carotovora*) (83, 116, 170, 181, 265, 272). One of the secretins, XpsD in *X. campestris* is the only known lipoprotein among D homologues in the T2SS and at least part of it is exposed to the cell surface as suggested by results of trypsin digestion (153). Interestingly, its homologues PulD and XcpQ in *Klebsiella* and *Erwinia*, respectively, require a small peripherally associated OM lipoprotein PulS and OutS, respectively, for insertion into OM. They also stabilize protein D, although the majority of other type II systems, including that of *V. cholerae*, seem to lack this protein (66, 76, 123, 296). Similarly, a lipoprotein in *Neisseria gonorrhoeae*, PilP, is essential for Tfp biogenesis and is required for stability of PilQ homomultimer, which is a member of the secretin family (83). Similar OM lipoproteins are also required for the proper localization of secretins in TTSS (67).

The C-terminal half, the β domain, is highly conserved among the secretins and is thought to be embedded in the OM (119). However, the N-terminus, which is variable and conserved only among related secretion pathways, is predicted to face the periplasm, where it might interact with other components of the secretion apparatus and/or with the secretable proteins (116). Although the β domain is important for multimer formation and/or stability, both the N and β domains are required for full multimerization (119). Some secretins were shown to form large, stable oligomers of ~12 subunits in the OM (42, 123, 170). PulD of *K. oxytoca* and XcpQ of *P. aeruginosa* for T2SS, YscC of *Y.*

enterocolitica and InvG of *S. typhimurium* for TTSS, PilQs of *P. aeruginosa* and *N. meningitidis* for Tfp biogenesis, and pIV required for the assembly and export of filamentous phage f1, have been shown to form ring-like structures by electron microscopic (EM) analyses (24, 34, 51, 65, 181, 205, 228). The cavity in these structures probably forms the channel that accommodates the transport of folded proteins, phages or pili (25). The sizes of these ring-shaped structures vary, ranging from 14 to 20 nm for outer diameters and 5 to 10 nm for inner diameters, but they are large enough to accommodate their own exoproteins that will go through the channels (25). Fusion of proteoliposomes containing the purified D complex with a planar lipid bilayer results in appearance of small, voltage-activated, ion-conducting channels (228). Thus the channel is expected to be gated for opening and closing.

EpsD has been shown to be required for secretion of both extracellular proteins and filamentous phage CTXΦ from *V. cholerae* (71). The genome of CTXΦ lacks a homologue of gene IV encoding the OM pore or secretin in the related filamentous phage of *E. coli*, f1. CTXΦ uses the host secretin, EpsD to exit from its host although other Eps components are not necessary for this process. Thus, EpsD plays a role both in pathogenicity and in horizontal transfer of a key virulence gene (71). Interestingly, certain strains of *A. hydrophila* have two different protein D homologues, ExeD, which is involved in the T2SS, and SspD, which is independent of Exe T2SS. SspD (S-protein secretion D) appears to be specific for secretion of S-protein or two-dimensional paracrystalline layer and does not appear to be involved in T2SS (320). In contrast, *P. aeruginosa*

also has two homologues of protein D, XcpQ involved in Xcp T2SS, and XqhA, which is not required for efficient secretion in the wild type, but is solely responsible for the residual export in Δ*xcpQ* strain (216). Unlike SspD from *A. hydrophila*, no other function has been discovered for XqhA except for its involvement in the type II secretion (216).

PilD/VcpD (O) Protein

O protein was initially identified as a prepilin leader peptidase involved in Tfp biogenesis (209). In *P. aeruginosa*, the gene (*pilD*/*xcpA*) encoding the peptidase is located adjacent to *pilA*, the pilin structural gene, along with other genes required for pilin assembly (229). The substrate of the enzyme is PilA, which is synthesized as a prepilin, containing leader sequence that is notably shorter than the typical signal sequences found in many substrate proteins of T2SS (229). The same gene turned out to be also required for secretion of extracellular proteins by the T2SS, which contains five consecutive prepilin-like componets (GspG-K, discussed below) (17, 240, 270, 311). Biochemistry of prepilin peptidase has been extensively studied by Lory's group (232, 308, 309, 312, 313). This integral IM protein is a single bifunctional enzyme, which catalyzes cleavage of a short basic leader sequence in both type IV prepilin and prepilin-like components of T2SS and N-methylates their exposed phenylalanine at the amino termini to generate mature proteins (17, 26, 191, 230, 231, 257, 264, 312, 313). Comparison of cleavages by signal peptidase for Sec-dependent signal peptide and by prepilin peptidase for type IV prepilin signal peptide shows

a few differences. Signal peptidase has its catalytic site in the periplasmic face of the IM and cleave after the hydrophobic region while prepilin peptidase has its catalytic site in the cytoplasmic face of the IM and cleave in front of the hydrophobic region (101). Interestingly, a homologue of O, ComC, is also present in DNA transformation system ComG of Gram-positive *Bacillus subtilis* (5, 49, 226). Though the region containing leader sequences are conserved among different organisms, the enzymatic activities seem to be quite specific to its native substrates, with some exceptions. For example, neither XcpA/PilD from *P. aeruginosa* nor ComC from *B. subtilis* is able to process prePulG of *K. oxytoca in vivo* (257). Without the processing, PulG cannot be transformed to mature protein, which is required for proper assembly into a subcellular structure in the T2SS (92). Thus, genes encoding prepilin peptidase for *P. aeruginosa* and *B. subtilis* cannot correct secretion defect of an *E. coli* strain carrying all *pul* genes except for *pulO*, while *pulO* gene complements this mutant *in trans* and its product processes PulG protein (257). In contrast to these data, *xcpA/pilD* mutant could be complemented by *pulO* (52% identity) and by *xpsO* (50% identity) from *X. campestris*, restoring the secretion of elastase in *P. aeruginosa* (18, 154, 257). Furthermore, PulO, XcpA and ComC can process type IV prepilin, PilE, from *Neisseria gonorrhoeae*, while EpsG and PulG from *V. cholerae* and *K. oxytoca*, respectively, can be processed by PilD from *N. gonorrhoeae* in *E. coli* (91, 92, 284). Further studies are required to determine the efficiency of processing in heterologous combinations of enzyme and substrate. Several bacteria with the T2SS also have the Tfp and the prepilin

peptidase, encoded by a gene near other genes required for Tfp biogenesis, is often shared between the two systems as in *Pseudomonas* species and *A. hydrophila* (244). However, if the O homologue gene is essential solely for T2SS, it is usually right next to the last gene of the T2SS operon, next to *M* or *N* gene as in *K. oxytoca* and *Erwinia* species, which seem to lack the Tfp (92, 204, 279). Lindeberg and Collmer suggested that *outO* of *E. chrysanthemi* is transcribed independently from the transcription units consisting of *outC* through *outM* (204).

In *V. cholerae*, there are two separate O homologues specific for Tfp or/and T2SS. First, TcpJ is required to yield the mature, export-competent form of the toxin-coregulated pilus (TCP), which is essential for colonization of the intestine by *V. cholerae*, but it cannot process the prepilin-like components involved in T2SS (169, 284). That led to the discovery of another prepilin peptidase VcpD/PilD that is absolutely necessary for both biogenesis of another Tfp and T2SS (112, 215). Processing of two prepilin-like components in T2SS, EpsG and EpsI, by PilD has been demonstrated (215, 284).

DNA composition analysis of the secretion genes of *V. cholerae* suggests that the *eps* genes are relatively old and that the *vcpD* gene has been acquired recently by horizontal gene transfer. This suggests possibility of loss of *epsO* gene, which was originally at the distal end of the *eps* cluster, due to the acquisition of *vcpD* resulting in redundancy.

EpsG, EpsH, EpsI, EpsJ and EpsK Proteins (Pseudopilins)

As mentioned above, *epsG-K* encode products having hydrophobic N-terminal α-helical regions that are highly homologous to leader sequences of the type IV pilin subunit of Gram-negative bacteria (10, 17, 18, 26, 143, 204, 217, 231, 256, 342). Similar prepilin-like proteins are also found in the DNA uptake system, ComG, of *B. subtilis* (217, 265). All these proteins are associated with IM, with the extreme N-terminus in the cytoplasm and the 16-18 conserved hydrophobic residues spanning the membrane (253, 264, 267, 310). However, majority of these proteins are targeted to the periplasm and some might even extend into the OM as shown with EpsG (148, 256, 264). In *P. aeruginosa*, relative amounts of XcpT (G), XcpU (H), XcpV (I), and XcpW (J) in membrane fractions have been estimated to be 16:1:1:4 (230, 264). Because of the similarity in sequence with type IV pilin subunit, these prepilin-like components or psuedopilins of T2SS have been thought to form a trans-periplasm pilus-like structure or pseudopilus, resembling a Tfp, to facilitate extracellular secretion (252). However, little data supported presence of this hypothetical structure. EpsG and some homologues of pseudopilins have been shown to be associated with both membranes by density gradient separation (18, 148, 230). Direct evidence for relocalization or assembly of multiprotein complex from processed pseudopilins has not been shown until PulG, which is the most abundant pseudopilin, has been demonstrated to assemble into pilus-like bundles by immunogold-labelling and EM when the 15 genes encoding the pullulanse secreton of *K. oxytoca* were expressed on a high copy number plasmid in *E. coli*

(253, 261, 287). Most components of T2SS, including all components with homologues in Tfp biogenesis, were necessary for PulG to form pili (287). G protein is probably the major component of pseudopilus (287). High level expression of the *pulG* gene interfered with pullulanse secretion, possibly due to the unnatural stoichiometry of the secretion machinery components preventing assembly of the proper secretion machinery (254). These results strongly suggest that pseudopilus or individual pseudopilins interact with other secreton components. Crosslinking analysis of *P. aeruginosa* revealed that major component of pseudopilus, XcpT (G) forms dimers with XcpU (H), XcpV (I), XcpW (J), and also with PilA, major subunit of Tfp (213). The authors suggested that these dimers could be the components of the secreton or intermediates involved in assembly of the secreton. One unexpected result in this report is that PilA is required for efficient secretion since *pilA* mutants, which are unable to express this protein, could not secrete extracellular proteins as efficiently as the wild type strain does. Similarly, a type IV fimbrial subunit gene, *fimA* of an anaerobic sheep pathogen, *Dichelobacter nodosus*, has been shown to be essential for pilus subunit production, protease secretion, and also natural competence (171). This shows close relationships among similar macromolecule transfer systems involving Tfp-like components and suggests possible roles of the pseduodpilus in the type II secretion process.

EpsE Protein

EpsE is another component of T2SS with its homologues in various macromolecular transport systems required for Tfp biogenesis (PilB from *P. aeruginosa*, 44% identity), TFSS (VirB11 from *A. tumefaciens*, 14% identity with PulE from *K. oxytoca*, 50% similarity and 24% identity with OutE from *E. carotovora* ssp.), and DNA transport in conjugation (TrbB from RP4 plasmid) and natural transformation (ComG1 from *B. subtilis*, 42% identity with PulE) (246, 249, 284). In addition, XpsE from *X. campestris* shows significant overall homology to the ABC exporter components, PrtD (47% similarity, 19% identity) and HlyB (45% similarity, 19% identity) from *E. chrysanthemi* and *E. coli*, respectively (87). These homologues are generally hydrophilic and lack obvious transmembrane domains and N-terminal export signals (184, 249). These proteins are characterized by presence of nucleotide-binding motifs called Walker A and B boxes, and for that reason they are putative NTPases, probably providing energy for the assembly of the secreton, gating of the OM channel, or for the secretion process itself (249). In addition to Walker boxes, they also possess two conserved regions designated as the Asp and His boxes (184, 246, 250, 268).

Although ATP binding and autophosphorylation or autokinase activity have been shown for purified EpsE protein, actual ATPase activity has not been reported for any T2SS (250, 281). Very recently, Sandkvist's lab was able to demonstrate ATPase activity by purified EpsE proteins (M. Sandkvist, personal communication). ATP hydrolysis by some homologues in other systems has also

been demonstrated. According to the phylogenetic analysis, T2SS E protein homologues are most closely related to putative NTPase proteins of TFSS although clear distinctions can be made between the two families, which are generally called PulE-VirB11 family (246). Two purified proteins of TFSS in *A. tumefaciens*, VirB4 and VirB11, have been reported to bind and hydrolyze ATP in addition to autophosphorylation activity for VirB11 (48, 297). According to Planet et al., VirB11 is evolutionarily closely related to EpsE homologues in T2SS, both possessing all four conserved domains mentioned above (246). Another purified protein, TrwD, encoded from conjugative transfer region of a IncW plasmid could hydrolyze ATP and intact Walker A box was essential for the function (268). Walker A box has also been shown to be essential for the function of EpsE and other T2SS homologues in secretion (250, 281). Furthermore, the mutated TrwD whose lysine residue in Walker A box has been replaced with glutamine showed dominant negative effect when introduced into a donor strain containing the wild type plasmid, decreasing conjugation frequency 1,000-fold (268). Similar phenomena of transdominance have been observed for EpsE and PulE (250, 281). These results indicate that those proteins with mutation in the Walker A boxes compete with the wild type proteins to interact with either itself or other components of secretion or conjugative machinery, suggesting that these proteins function as multimers (184, 281). Furthermore, it was shown that N-terminal region of *E* gene is required and is sufficient for transdominant effect (250, 281). Sandkvist et al. (281) showed that the N-terminal half of EpsE contains the domain that associates with EpsL. In the same line with that, they

also showed that the N-terminus of EpsE determines species specificity by demonstrating effective complementation of *epsE* mutant by a chimeric protein composed of the N-terminal half of EpsE and the C-terminal half of ExeE from *A. hydrophila*, but not by the whole ExeE or a chimera with N-terminal half of ExeE. Similar results have been shown for complementation of *P. aeruginosa* XcpR (E) mutants by the gene fusion composed of two different domains from two different XcpR's of *P. aeruginosa* and *P. putida* (74). These results suggested that species specificity or the reason ExeE cannot substitute for EpsE in *V. cholerae* is most likely due to its inability to interact with EpsL (281). EpsE is soluble and found in the cytoplasm when produced alone without other Eps components in *E. coli* but a fraction of EpsE is membrane associated in the presence of IM protein EpsL. Thus, EpsL is responsible for the association of EpsE with the IM in the native WT *V. cholerae* strain (249, 250, 281). A filamentous phage-encoded pI, which is not a part of the virus particle, might be equivalent to fused E and L components because it contains an essential nucleotide-binding motif in the cytoplasmic domain and it spans the IM. Upon binding of packaging signal in phage DNA, cytoplasmic domain of pI causes a conformational change in the periplasmic domains of pI and interacts with a secretin homologue pIV, which is not part of the virion, triggering pIV channel opening to allow phage particle to assemble and exit (273). Requirement of ATP hydrolysis for filamentous phage assembly has been demonstrated (100). Several reports suggest that E protein might be a homodimer although purified EpsE was shown to be a monomer (250, 280).

Interestingly, EM studies determined that above mentioned TrwD and two other related secretion NTPases (TrbB and HP0525) encoded by the conjugative transfer region of the broad host range plasmid RP4 and by the *cag* PAI (TFSS) of *H. pylori*, respectively, share similar hexameric ring structures (184). The hexameric ring with diameter of 12 nm seemed to be composed from a trimer of dimers and a central channel of about 3 nm in diameter was exhibited (184, 185). The authors suggested that these hexameric ring-structured proteins might aid or catalyze a repetitive step and successive rounds of NTP hydrolysis might support translocation of cognate substrat(s) across the IM (184). Crystal structure of binary complex of HP0525 bound to ADP suggested a model that this hexameric ATPase functions as an IM pore, the closure and opening of which is regulated by ATP binding and ADP release (348). However, utility of forming rings in the mechanisms of these AAA (ATPases associated with different cellular activities) ATPase is still poorly understood (241, 324).

Another indirect indication of EpsE's function as an ATPase providing energy comes from the study of its homologue PilT, from *N. gonorrhoeae* Tfp system (324). PilT protein, which is dispensable for Tfp biosynthesis, but is essential for both twitching motility and for the DNA uptake step of genetic transformation, has been demonstrated to be required for pilus retraction (222). Therefore, *pilT* mutations lead to defects in competence for natural transformation and in twitching motility despite the expression of structurally and morphologically normal Tfp (345). This observation supports a model in which

ATPase activity of PilT is responsible for cell movement and macromolecular transport (164).

Other Less Characterized Proteins (EpsC, EpsF, EpsL, EpsM, EpsN, and EpsA-EpsB)

None of these proteins are well-characterized structurally or functionally, but interactions between T2SS components seem to involve some of these proteins. According to the density gradient separation of *V. cholerae* membranes performed by Hough, the majority of EpsC fractionated with IM proteins, but significant amount of this protein also seemed to be associated with OM, suggesting direct or indirect association of this protein with the OM component(s) (148). Relatively little information is available about EpsC, which is the main subject of this thesis, and more discussion about EpsC and its homologues will be covered in the next chapter.

EpsF protein is another component of T2SS, which has similar components in Tfp biogenesis (PilC from *P. aeruginosa*, 29% identity) and other related systems (284). Similarly to homology between XpsE and ABC exporter components, it is interesting to see XpsF of *X. campestris* share a significant degree of homology with PrtE (46% similarity, 18% identity) and HlyD (42% similarity, 18% identity), MFP components of the type I secretion system from *E. chrysanthemi* and *E. coli*, respectively (87). This protein is also similar to ComG2 of *B. subtilis*, which is involved in DNA uptake (87). F protein has large hydrophobic domains and it may be a polytopic IM protein spanning the IM three times, with a large N-terminal tail and a large central loop being exposed in the

cytoplasm (87, 249, 265). Indeed, OutF from *E. carotovora* has been shown to have a small periplasmic loop and two larger cytoplasmic domains connected by three transmembrane regions by OutF-BlaM topological analysis and OutF may be involved in interactions on both sides of the IM (319). Thus, this might act as a platform for the assembly of a putative pseudopilus or secreton (265). EpsF with $(His)_6$-tags has been purified from *E. coli* by Hough (148) and 0.5% SDS was used to solubilize the EpsF protein in the insoluble fraction of the cell, while it was not extractable in 1% Triton X-100 with or without 10mM EDTA .

EpsL homologues share a single hydrophobic region located in the C-terminal half of each protein. The distribution of positively charged residues in the proximity of this hydrophobic region suggests that the larger N-terminal domain is cytoplasmic and the rest of the polypeptide is periplasmic (149, 262, 265). Cell fractionation studies showed that these L homologue proteins are mainly associated with IM (148, 262, 319). Contradictory to this prediction, while most of EpsL was fractionated with the IM, some of them were also fractionated with the OM fractions (148). EpsL forms dimers and stabilizes EpsE protein by attracting it to the IM for interaction (281, 282).

EpsM homologues display a single hydrophobic segment, preceded by two or more positively charge residues, which is located in the N-terminal regions of these proteins. This region might function as a signal sequence or a membrane anchor (262, 265). An interactive relationship between the L and the M proteins were suggested by observation of mutual stabilization or proteolytic degradation of one protein in the absence of the other protein (195, 223, 282).

Physical interaction between two proteins has been strengthened further by co-immunoprecipitation experiments (251, 282). We also observed co-elution of EpsL with $(His)_6$-tagged EpsM from the metal affinity chromatography column (unpublished results). Sandkvist et al. (283) demonstrated that the first half of the C-terminal half of EpsL, which is predicted to be transmembrane sequence, is likely to be involved in its complex formation with EpsM and suggested that these two proteins probably interact through their transmembrane regions. EpsM has also been shown to form dimers as EpsL does (282).

EpsN homologues are not found in every organism with T2SS (see Table 1-1). These proteins contain an N-terminal hydrophobic region and are probably anchored in the IM, while majority of the proteins are exposed to the periplasm (196, 262, 264, 265). Significant studies about this protein have been carried out only in *X. campestris*. The gene encoding the XpsN protein has been identified by the location of the gene, which is right downstream of XpsM, and the protein has been shown to have a molecular weight similar to other N proteins of the T2SS (196). In accordance with the topology prediction, major part of the XpsN seemed to face the periplasm, and the possibility of interaction between XpsN and OM protein XpsD has been suggested by Lee et al. (196). They showed co-immunoprecipitation of XpsN with XpsD and also co-elution of XpsN with $(His)_6$-tagged XpsD from the metal affinity chromatography column, suggesting XpsN protein indeed forms a stable complex with XpsD protein. The authors also found that C-terminal region of XpsD protein is involved in complex formation with XpsN. In addition to the association with XpsD, IM protein XpsN seems to

be associated with XpsL and XpsM, and XpsL-XpsM complex formation is enhanced and stabilized by XpsN (195). Inhibition of secretion was observed by overproduction of wild type XpsL and XpsM together, but not separately, in the wild type strain of *X. campestris* (195). These data suggest the presence of trimeric complex composed of XpsL, XpsM and XpsN. Therefore, XpsN protein is a candidate for a bridge component connecting the IM XpsL-XpsM complex of the secreton to the OM exit pore and its role may be similar to TonB that serves as the energy transducer for the uptake of iron-siderophore complexes and vitamin B12 in *E. coli* as the authors suggested (195). TonB opens gated pores for the inward translocation of ligands across the OM. Similarly to XpsN, TonB is an IM protein with its N-terminus in the cytoplasm, signal sequence anchored in the IM, and most of the protein extending into the periplasm. TonB is thought to shuttle between the IM and the OM to contact both IM and OM components triggering conformational changes (41, 137, 200). If it is true that N protein has a similar role as TonB, it must be a very important essential component of T2SS, but PulN of *K. oxytoca* is dispensable for secretion and organisms such as *E. chrysanthemi* and *P. aeruginosa* do not even have N homologue (Table 1) (251). Thus, it was suggested that C homologues, whose molecular size and membrane topology are similar to those of the N protein, might play a similar role (195). Interestingly, C homologue has not been identified in *X. campestris* and it makes the hypothesis stronger.

Although some T2SSs possess protein B, only *V. cholerae* and *A. hydrophila* possess both A and B proteins among well-characterized organisms

(161). ExeA from *A. hydrophila* is a hydrophilic protein with a consensus ATP binding site (161). ExeB bears sequence as well as topological similarity to TonB mentioned earlier (151). ExeA and ExeB forms a stable complex and ExeAB complex is required to assemble and/or stabilize the ExeD secretin in the OM (15). In *E. chrysanthemi*, fractionation experiments indicated that OutB could be associated with the OM through its C-terminal part and an interaction between OutB and OutD has been suggested by several experiments (54). By analogy to TonB, ExeAB may act to transduce metabolic energy to the opening of a secretion pore in the OM (151). In contrast to ExeAB and OutB, PulB in *K. oxytoca* is not required for secretion (251, 252). EpsA and EpsB of *V. cholerae* have not been studied yet.

Assembly and Polar Localization of Eps Complex in *V. cholerae* cells

Since multiple proteins are required for T2SS, it is reasonable to anticipate that these proteins would form a multimer complex acting as a secretion apparatus. Since T2SS is responsible for the transport of proteins from the periplasm across the OM to the extracellular milieu, it has been thought that the secretion machinery will span the periplasm, thus envisioning a channel-like structure connecting the IM and the OM (Fig. 1-1). Researchers are now trying to figure out interactions between proteins involved in the T2SS to dissect the structure of this yet hypothetical apparatus and quite a number of protein-protein interactions have been detected.

However, the location of the secretion apparatus assembly in the cell has never been questioned before. Recently, a very elegant study, demonstrating the location of secretion apparatus on single poles of *V. cholerae* cells was carried out by Scott et al. (291). This study employed real-time monitoring of green fluorescent protein (GFP) fused to EpsM to locate the Eps complex at the old pole after cell division. They also visualized secretion of the protease from the site of the polar Eps apparatus, confirming the coincidence of the locations for Eps complex assembly and the secretion. Interestingly, detection of GFP reporter fused to the EpsL, which is known to interact with EpsM, required EpsM

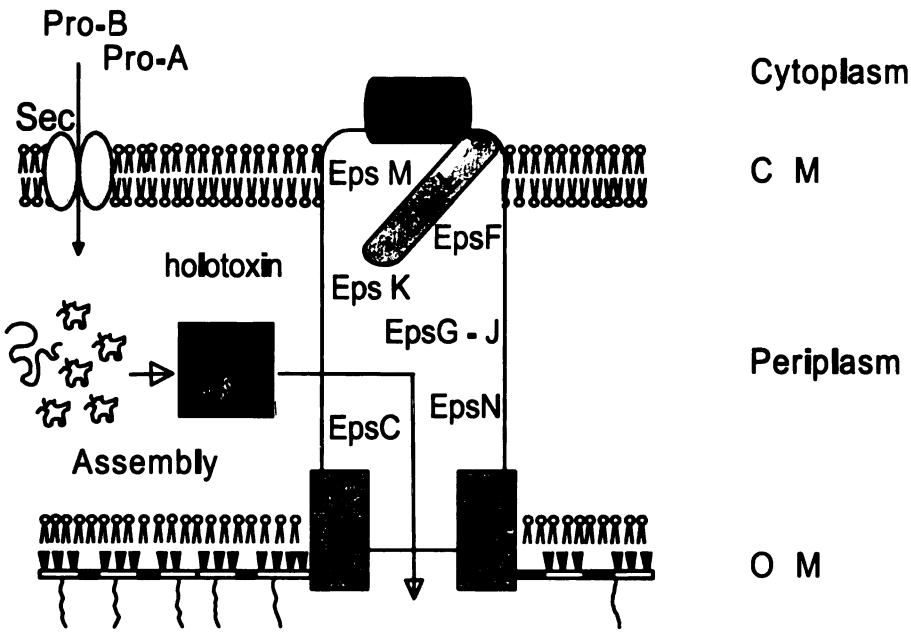

FIG. 1-1 Assembly and secretion of cholera toxin shown with a schematic hypothetical model of Eps type II secretion multiprotein complex in *V. cholerae*, suggesting protein-protein interactions between the components.

expression for polar localization, while EpsM could localize to the pole in the absence of other Eps components in *E. coli* (291). These results suggest that EpsM carries information for polar localization. However, they pose other interesting questions about the subcellular structure of bacterial cell. For example, what directs EpsM protein to its polar location? Polar secretion would be more efficient in delivering virulence factors such as CT and enzymes directly to the specific target rather than wasting proteins and energy by secreting all over the surface of the cells. TCP is known to be required for colonization and CT expression, thus a possible scenario would be that *V. cholerae* cells first attach to its target in the small intestine, then produce and secrete CT and other virulence factors in a directed manner. Still there are many questions to be answered to figure out how the cells deliver virulence factors to the specific target and this finding will provide important insights for pathogenesis of *V. cholerae* for future research. Localization of EpsC protein in the cell is also under investigation by Sandkvist's group and the result would provide more information valuable for understanding the role of the EpsC in the Eps complex.

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CHAPTER 2

COMPLEMENTATION OF THE *epsC* MUTANT INDICATES INTERACTIONS BETWEEN EPSC AND EPSD

Abstract

The human intestinal pathogen *Vibrio cholerae*, which is responsible for the severe diarrheal disease cholera, secretes cholera toxin and a number of degradative enzymes via type II or general secretion pathway, which is common in Gram-negative bacteria. Secretion of these extracellular proteins plays an important role in pathogenesis. The secretion process requires products from a cluster of *eps* genes, *C-N*, in addition to prepilin peptidase, which is required for the processing of some Eps proteins. Requirement of these Eps proteins for the secretion has been proven by inability of several *eps* mutants to secrete and restoration of secretion when complemented by copies of these genes *in trans*. One of the mutants, with Tn5 insertion in *epsC*, could not be complemented by copies of *epsC in trans*. Here we show that this mutant can be complemented very efficiently by providing *epsCD in cis*, but not by *epsC* and *epsD* genes provided *in trans* to each other. We also found that the amount of multimeric EpsD proteins is severely reduced in the *epsC* mutant and the restoration of the secretion through the complementation by *epsCD* is accompanied by reappearance of EpsD multimers. Overexpression of *epsD* in the mutant can also restore the EpsD multimers, but not the secretion. These results indicate that EpsC is required for the stability of EpsD, not for the formation of multimeric EpsD complex. The failure of complementation by *epsC* and *epsD in trans* suggests that coexpression of these two genes is important for stoichiometry, likely required for the assembly of the secretion apparatus.

Introduction

Vibrio cholerae secretes a number of proteins including cholera toxin (CT) and degradative enzymes via the type II secretion system (T2SS) involving *eps* products. Our group has shown the requirement of these genes for secretion by demonstrating inability of secretion in *V. cholerae* strains mutated in *epsC*, *epsD*, *epsE*, *epsF*, *epsG*, *epsL* and *epsM* (36). Secretion was restored when a clone of the corresponding gene was provided *in trans* to each mutant with the exception of the *epsC* mutant, which could not be complemented with a plasmid containing *epsC* gene. However, when a cosmid containing all *eps* genes was introduced to the *epsC* mutant, the secretion defect could be relieved.

EpsC is approximately 33.5 kDa protein with 305 amino acid residues (36). According to the sequence analysis of EpsC and its homologues from other species, N-terminal 29 residues are cytoplasmic, residues 30~50 are potentially transmembrane domain, and the rest are probably in the periplasm. (4, 12, 14, 29, 38). These domains have been dissected into smaller domains in some homologues of EpsC and significance of each segment has been studied (discussed below). Cytoplasmic N-terminus of EpsC homologues seems to be not essential for function and specificity as it can be deleted or exchanged without a loss of function (4, 6, 29). The transmembrane domain is necessary for function of the C proteins and inner membrane (IM)-anchoring has been considered important to promote interactions with other secretion components (4, 14). For XcpP (C), an EpsC homologue in *Pseudomonas aeruginosa*, the membrane emerging periplasmic region, 35 residues at the C-end of the

transmembrane domain, was not exchangeable with the corresponding domain of OutC from *Erwinia chrysanthemi* and this region has been suggested to be involved in the specificity of the XcpP (C) protein (14).

Analysis of all sequences from EpsC homologues identified presence of PDZ domains (named after the three eukaryotic proteins, Post-synaptic density protein, Disc large and Zo-1 proteins) in these proteins, except for *P. aeruginosa* and *P. alcaligenes* , which contain coiled-coil domains in place of PDZ domains for XcpP (C) (14, 25, 26). They are present in the C-terminal half region of the periplasmic domain. PDZ domains were first discovered in eukaryotic proteins and many proteins containing PDZ domains are associated with the cytoplasmic membrane, in which the PDZ domains mediate the assembly of multiprotein complexes that may initiate signal transduction (26, 28). Both PDZ domains and coiled-coils are thought to be involved in protein-protein interactions and PDZ domains within GspC proteins were suggested to be involved in recognition of specific signals in the secreted proteins and/or interaction between the components of secreton (28).

Topology studies of OutC, PulD, and XcpP (C) from *Erwinia carotovora*, *Klebsiella oxytoca*, and *P. aeruginosa*, respectively, by fusion with topology probes, *blaM* and alkaline phosphatase (PhoA), revealed that C proteins are really IM proteins with majority of the protein targeted to the periplasm, in agreement with the predictions resulting from the sequence analysis (5, 12, 38). When PulC-PhoA fusion protein was subjected to isopycnic sucrose density centrifugation of membrane vesicles, small amounts were also detected in the

OM fractions while majority was detected in the IM fractions (12). EpsC associated with OM has also been observed when IM and OM proteins of *V. cholerae* were separated by sucrose density gradient, raising the possibility of interactions between EpsC and OM protein(s), possibly EpsD (17). Furthermore, indications of interactions between EpsC and OM protein EpsD have been demonstrated by co-immunoprecipitation with or without crosslinking and also by instability of EpsD protein when expressed alone in *E. coli*, in the absence of EpsC (17). *Cis* expression of *epsC* and *epsD* in the same plasmid increased the amount of EpsD detected dramatically when the whole cell samples of *E. coli* expressing *epsCD* were subjected to SDS-PAGE and subsequently detected with polyclonal anti-EpsD antibodies (17). A number of researchers studying T2SS with other Gram-negative bacteria also suggested possibility of the protein-protein interaction between EpsC and EpsD homologues based on several observations: (i) in *P. aeruginosa*, only *xcpP* (C) and *xcpQ* (D) genes are organized into a single operon being transcribed in the opposite direction compared to the orientation of other Type II secretion genes. This raises the possibility of coordinated action of the corresponding proteins (1, 4); (ii) XcpP (C) was unstable in an *xcpQ* (D) mutant (4); (iii) in *E. chrysanthemi*, OutC and OutD have been proposed as gatekeepers of species-specific secretion (see Discussion) (18).

In *P. aeruginosa*, decrease in the stability of XcpP (C) has been observed in the absence of XcpY (L) or XcpZ (M) and this suggested interactions between XcpP (C) and the already described and well-established XcpYZ (LM) complex

(14, 20). Similar results were also observed for the *V. cholerae* Eps system homologues (35) (M. Sandkvist, personal communication). XcpZ (M)-dependent stability of XcpY (L) has also been shown to be modulated by XcpP (C) and its N-terminal transmembrane domain seemed to play an important role in the stability of the XcpY-XcpZ complex (14, 32). These results suggest three partner subcomplex composed of XcpP (C), XcpY (L), and XcpZ (M) along with the results by Possot et al. (30), who demonstrated co-immunoprecipitation of PulC with PulE, PulL, and PulM. Presence of another multimeric complex composed of E, L, M and F proteins has also been suggested (31, 33). Thus, it is very likely that EpsC homologues interact directly or indirectly with all these IM proteins to form a multiprotein complex.

In this report, we characterize the *epsC* gene and its product. By constructing a new cosmid harboring all *eps* genes and with the *epsC* gene replaced by Cm^R cassette, we wanted to study the effect of the *epsC* mutation on stability of other Eps proteins in *E. coli* in parallel with the study of the *V. cholerae* chromosomal *epsC* mutant mentioned earlier. We also introduced several different plasmids containing different lengths or combinations of *eps* genes to the *epsC* mutant to determine the minimal requirement for complementation to restore the secretion. The results indicate that EpsC is very important for stability and/or function of EpsD and the requirement of *epsCD in cis* for complementation of the *epsC* mutant suggest protein-protein interaction between EpsC and EpsD.

Materials and Methods

Bacterial strains, plasmids and culture conditions. Bacterial strains and plasmids used are listed in Table 2-1. *E. coli* strains were grown in Luria-Bertani (LB) broth and *V. cholerae* strains were grown in LB, M9, or syncase medium supplemented with 100 μg/ml thymine at 30°C, 37°C or 43°C. Antibiotics were used at the following concentrations: ampicillin (Ap), 100 μg/ml; kanamycin (Km), 100 μg/ml (50 μg/ml for *V. cholerae* Km^R insertion mutants); chloramphenicol (Cm), 25 μg/ml; tetracycline (Tc), 10μg/ml (1μg/ml for *V. cholerae*); polymyxin (Pm), 100μg/ml; streptomycin (Sm), 100μg/ml. Plasmids were introduced to the cells by conjugation or electrotransformation.

Construction of *epsC*-6X histidine fusion. To construct a clone of *epsC* fused to 6X histidine tags at the C-terminus, *epsC* gene was polymerase chain reaction (PCR)-amplified with *Pfu* DNA polymerase (Stratagene, La Jolla, CA) using primers EpsC1 (5'-ATGGAATTTAA-ACAACTTCC-3') and EpsC2 (5'-CGC**GGATCC**AAATTGAATA- TATACATCAT-3', *BamH*I site in bold) with genomic DNA of *V. cholerae* TRH7000 as a template. The following touchdown temperature cycle was used for amplification: 94°C, 2 min; 8 cycles of 94°C, 15 sec, 58°C (-1°C/cycle), 15 sec and 72°C, 45 sec; 17 cycles of 94°C, 15 sec, 50°C, 15 sec, and 72°C, 45 sec; 72°C, 7 min; and 4°C. The amplified DNA fragment was digested with *BamH*I (New England Biolabs, Beverly, MA) and inserted between the blunt-ended *Sph*I and the *Bgl*II site of the vector plasmid pQE70 (Qiagen, Chatsworth, CA)).

Table 2-1 Strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
V. cholerae		
TRH7000	ElTor *thy* Hg^r Δ(*ctxA-ctxB*)	(16)
VB12 (PU6)	TRH7000 *epsC*::Km^R	(24)
E. coli		
MC1061	F⁻ *araD*139 Δ(*ara-leu*)7697 Δ(*lac*)X74 *rpsL hsdR2 mcrA mcrB1*	(7)
TG1	*supE thi-1* Δ(*lac-proAB*) Δ(*mcrB-hsdSM*)*5* (r_k-m_k-) [F' *traD36 proAB lacI*^{q}ZΔ*M15*]	Amersham
XL1-Blue MRF'	Δ(*mcrA*)183Δ(*mcrCB-hsdSMR-mrr*)173 *endA1 supE44 thi*-1 *recA*1 *gyrA*96 *relA*1 *lac* [F' *proAB lacI*^{q}ZΔM15 Tn10 (Tc^R)	Stratagene
Plasmids		
E26	Cosmid pLARF5::*epsC* to *epsN*	(36)
pAR3	Arabinose-inducible expression vector	(27)
pKD3	Template plasmid for Cm^R	(10)
pKD46	Red recombinase expression vector	(10)
pMMB207/208	Broad-host-range cloning vectors; Cm^R	(22)
pMMB503	Broad-host-range cloning vector; Sm^R	(21)
pMMB611	$epsC_{(His)6}$ in pQE70	This study
pMMB741	pMMB503::*epsC*	(17)
pMMB771	pMMB207::*epsD*	This study
pMMB781	pMMB208::*epsC*	This study
pMMB790	pAR3 Cm^R replaced by Km^R	This study
pMMB794	pMMB790::*epsD*	This study
pMMB799	pMMB207::*epsCD* with ~1.2 kb upstream sequence	This study
pMMB831	E26 *epsC*::Cm^R	This study
pMMB835	pMMB208::*epsCD'*	This study
pQE70	Cloning vector inserting C-terminal 6X His, Ap^R	Qiagen
pWD615	*etxB* (*E. coli* heat-labile enterotoxin B subunit gene), Tc^R	(9)

Purification of EpsC protein and generation of antibodies. *E. coli* TG1 carrying plasmid pMMB611 [$epsC_{(His)6}$] was grown with shaking in 2 liters of LB medium containing 100 μg/ml ampicillin at 37°C. At OD_{650nm} of 0.3, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.05 mM and growth was continued overnight. Cells were harvested by centrifugation, suspended in Buffer I (50 mM $NaPO_4$ buffer pH 8.0, 300 mM NaCl), and lysed by sonication in the presence of 1.0 mg/ml lysozyme. DNase I was added to a final concentration of 10 U/ml in the presence of 10 mM $MgCl_2$ and incubated at room temperature for 10 min. Cell membranes were separated by centrifugation for 30 min at 70,000 x g and extracted with Buffer II (50 mM $NaPO_4$ buffer pH 8.0, 300 mM NaCl, 5% Glycerol, 0.5% Triton X-100, 20 mM imidazole). Insoluble material was removed by centrifugation for 30 min at 70,000 x g and the supernatant was applied to a 25.0-ml metal chelate column of POROS 20MC (PerSeptive Biosystems, University Park, MA) charged with Ni^{2+} and equilibrated with Buffer II. After washing the column with Buffer II until the OD_{280nm} = 0.05, adsorbed proteins were eluted with a linear gradient of 20 – 300 mM imidazole in Buffer I. Protein EpsC eluted at approximately 130mM imidazole and was visualized by separating the proteins on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie Blue staining. Using the purified $EpsC_{(His)6}$ protein, EpsC-specific antibodies were raised in rabbits according to Harlow and Lane (15).

Quantitative determination of LT B-subunit pentamer secretion. The gene *etxB*, encoding the B subunit of LT, was introduced into *V. cholerae* on plasmid pWD615 (8). LT B-subunit pentamers present in the growth medium and sonicated cells were determined by GM1 enzyme-linked immunosorbent assay (ELISA) (37) as described previously (23).

Construction of in-frame insertion mutants of E26 cosmid. To inactivate *epsC* gene in cosmid E26, containing *epsC* through *epsN*, procedures described by Datsenko and Wanner were followed (10). PCR products were generated by using a pair of primers, pKD3-C1 (GCGTAAAAAGTACAGAA-AGGAATAACGTGGAAATTTggtgtaggctggagctgcttc) and pKD3-C2 (TCGTTAC-TCGCCTTAGCGTTAAAATTGAATATATACcatatgaatatcctccttag), which include homology extension sequences in the *epsC* gene (capital letters) and 20-nt priming sequences (small letters) for pKD3 as template. The linear PCR products containing Cm^R gene with homology extension were gel-purified, digested with *Dpn*I before electrotransformation into *E. coli* MC1060 harboring recombinase expression plasmid pKD46 and E26.

Extraction of proteins from *V. cholerae* and *E. coli*. *E. coli* or *V. cholerae* cells grown in liquid medium were harvested by centrifuging in a Beckman JA 20 rotor at 6,000 rpm for 10 min at 4°C. The cell pellet was resuspended in B-PER bacterial protein extraction reagent (Pierce, Rockville, IL) and then the suspension was centrifuged in a Beckman JA 20 rotor at 14,000

rpm for 15 min at 4°C to collect the soluble proteins from the supernatant. Both IM and OM proteins were soluble in the B-PER bacterial protein extraction reagent.

Gel electrophoresis and immunoblotting. B-PER extracts or whole cells in sample buffer containing 2% sodium dodecyl sulfate (SDS) and 1% β-mercaptoethanol were heated at 100°C for 5 min and proteins were separated by 10% or 12% SDS-PAGE and transferred onto BA-S 83 nitrocellulose (Schleicher and Schuell, Keene, NH) by discontinuous semi-dry electroblotting using the TransBlot SD apparatus (Bio-Rad, Hercules, CA). Gels were transferred for 1 hr at ~2 mA/cm^2. Membranes were blocked with blocking buffer, 5% skim milk in 1X PBS-T (PBS + 0.5% Tween 20) for 30 min at room temperature or overnight at 4°C. Blots were incubated with rabbit-raised polyclonal primary antibodies specific for the Eps protein at appropriate dilutions for 1 hr at room temperature. PBS-washed blots were incubated with secondary antibodies, peroxidase-labeled goat anti-rabbit immunoglobuoin G (IgG) (Pierce), at 1:20,000 dilution in blocking buffer for 1 hr. After washing, peroxidase activity on the blot was visualized with SuperSingal WestPico chemiluminescent substrate (Pierce) followed by exposure to Kodak Biomax MR film.

Results

Purification of histidine-tagged EpsC protein and detection of EpsC by anti-EpsC antibodies. Upon induction with IPTG the cells of *E. coli* TG1 carrying plasmid pMMB611 produced a protein of the apparent molecular weight (MW) of 33.5 kDa. When Triton X-100 extracts from the membranes of these cells were chromatographed on the Ni^{2+}-NTA Sepharose column, this protein was eluted at approximately 130mM imidazole. The purified $EpsC_{(His)6}$ migrated on the SDS-PAGE as a single, 33.5 kDa band that could be visualized with Coomassie Blue staining (Fig. 2-1 A, lane 2). This MW agreed well with that predicted for EpsC based on the nucleotide sequence of its gene (36). The $epsC_{(His)6}$ gene could not complement an *epsC* mutant of *V. cholerae* for the secretion of toxin, similarly as the previously reported for the wild type *epsC* gene (36). Interestingly, when *epsC* gene was overexpressed in *E. coli*, several protein bands were detected by the immunoblot with anti-EpsC antibodies (Fig. 2-1 B, lane 3). This was in contrast to the results obtained in wild type *V. cholerae* where only a single EpsC band was observed upon reaction with the same antiserum (Fig. 2-1B, lane 1). This indicates that in the absence of other Eps proteins EpsC is unstable and presumably undergoes proteolytic cleavage.

EpsC stabilizes EpsD when expressed *in trans* in *E. coli*. In the previous work (17), when *epsD* gene was expressed in *E. coli* under bacteriophage T7 Φ10 promoter control, the protein was hardly detectable by

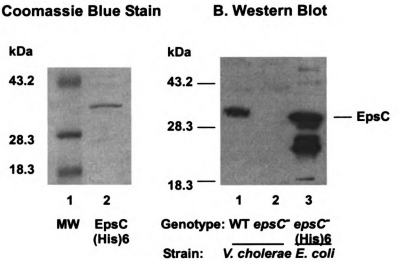


FIG. 2-1 Purified EpsC and detection of EpsC by anti-EpsC antibodies. (A) SDS-PAGE of purified $EpsC_{(His)6}$ after Coomassie Blue staining. Lanes: 1, MW=molecular weight standard: 2, purified $EpsC_{(His)6}$. (B) Western blot analysis of EpsC and $EpsC_{(His)6}$ with anti-$EpsC_{(His)6}$ antibodies. Lanes: 1, a single band for EpsC in the whole cell sample of wild type *V. cholerae*; 2, absence of EpsC in the *V. cholerae epsC* mutant; 3, multiple $EpsC_{(His)6}$ bands, presumably degraded products of $EpsC_{(His)6}$ from *E. coli* expressing $epsC_{(His)6}$ from pMMB611. (Lanes in B, 1=VB1, 2=VB12, 3=CB2077)

Western blot with anti-EpsD antibodies. However, coexpression of *epsCD in cis* from the same promoter increased the amount of EpsD protein considerably (17). Hough (17) interpreted these results as protection of EpsD protein by EpsC from proteolysis. However, it is also possible that *epsD* expression requires *epsC* expression. To distinguish these possibilities, I placed *epsD* under the *ara* promoter (pMMB794) in the presence (pMMB741) or absence (pMMB503) of the *epsC*, which was expressed from the *tac* promoter in *E. coli*. Whole cell samples in the SDS sample buffer were subjected to separation by SDS-PAGE followed by Western blot with anti-EpsD antibodies. As shown in lanes 1 and 2 of Fig. 2-2, the *ara* promoter was very tightly regulated, and in the absence of

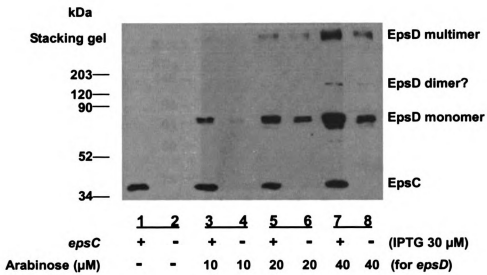


FIG. 2-2 Stabilization of EpsD by EpsC *in trans*. *E. coli* strains harboring two plasmids, pMMB741 for *epsC* expression (lanes 1, 3, 5, and 7) or empty vector pMMB503 (lanes 2, 4, 6, and 8), and pMMB794 for *epsD* expression. EpsC was produced in a constant level by induction with 30μM IPTG, and EpsD was produced by induction with increasing concentrations of arabinose. Pairwise comparison of EpsD level with or without EpsC shows the effect of EpsC protein on the stability of EpsD protein (compare lanes 3 with 4, 5 with 6, and 7 with 8). As more arabinose was added to induce the *epsD* gene, more EpsD monomer, probable dimer, and multimer forms are observed. (Lanes 1, 3, 5, and 7=CB2413, lanes 2, 4, 6, and 8=CB2450)

arabinose, EpsD was not produced. As the arabinose concentration increased the amount of EpsD detected also increased (compare lanes 1, 3, 5 and 7, with lanes 2, 4, 6 and 8, Fig. 2-2). Not only monomer, but also multimer forms of EpsD were observed. These observations suggest that increased amount of EpsD detected in the presence of EpsC is due to interaction at the protein level, but not to the improved transcription of the *epsD* gene in the presence of the *epsC* gene.

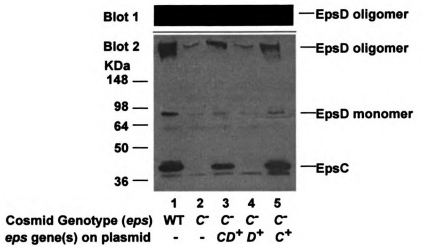


FIG. 2-3 Stability of EpsD is affected by the *epsC* mutation in the cosmid (*epsC-epsN*) when expressed in *E. coli*. Although expression of both *epsC* and *epsCD* from pMMB781 and pMMB799, respectively, could raise the EpsD protein amount, it could not be determined which plasmid was more effective from this experiment since two different blots with the same samples showed different results (lanes 3 and 5 for blots 1 and 2). (Lanes 1=CB2464, 2=CB2478, 3=CB2534, 4=CB2533, 5=CB2532)

Effect of the *epsC* mutation in a cosmid harboring *epsC-N* on the steady-state level of cosmid-expressed EpsD protein in *E. coli*. Since EpsD has been shown to be unstable in the absence of EpsC supplied *in trans*, we wanted to observe the effect of *epsC* mutation on EpsD in a cosmid system containing all *eps* genes, *C-N*, to determine if the presence of other Eps proteins make difference for EpsD in the absence of EpsC. Compared to the wild type cosmid E26 (*epsC-N*) (lane 1), pMMB831 (E26 *epsC*::Cm) (lane 2) showed much less EpsD and addition of the *epsC*-expressing plasmid pMMB781 to the mutant (lane 5) increased the EpsD level without IPTG induction (Fig. 2-3). pMMB799, a plasmid containing *epsCD*, was also introduced to the mutant to see if it would make any difference compared to the introduction of *epsC* alone (lane 3). It also

increased the amount of detected EpsD compared to the EpsD level in the *epsC* mutant (lane 3). However, it was hard to determine whether the plasmid expressing the *epsCD* was more efficient than the plasmid expressing only *epsC* because the results from two immunoblot experiments with the same samples were different even though both plasmids were regulated without IPTG induction. According to blot 1, *epsCD* was more effective than *epsC* alone to restore the EpsD level, but result on the blot 2 was opposite (Fig. 2-3). This discrepancy may be due to the inconsistent transfer of large multimeric proteins to the blot since multimeric EpsD proteins could not be separated well by SDS-PAGE, but stayed in the stacking gel, although the difference between the wild type and the *epsC* mutant was always reproducible. Additional copies of *epsD* gene expressed from pMMB771 in the *epsC* mutant cosmid-harboring *E. coli* did not affect the amount of EpsD (lane 4, Fig. 2-3).

Effect of the *epsC* mutation on the steady-state level of EpsD protein in *V. cholerae*. Since the stability of EpsD was affected by the *epsC* mutation of E26 cosmid in *E. coli* (Figs. 2-2 and 2-3), we expected that the *V. cholerae epsC* mutant (VB12) would also contain less EpsD compared to the wild type cells. To verify this, proteins extracted from the previously constructed *V. cholerae epsC* mutant and the wild type cells were subjected to Western blot analysis with anti-EpsD antibodies (23, 36). In *V. cholerae*, EpsD protein was detected exclusively as a multimeric form, which is probably closer to the native form of this protein (Fig. 2-4). As expected, compared to the wild type (lane 1), the *epsC* mutant

(lane 2) contained an extremely low amount of EpsD (Fig. 2-4), often not detectable when less amount of samples was loaded and/or with shorter film exposure. Supply *in trans* of the *epsC* gene (pMMB781) under the control of the *tac* promoter without IPTG induction to the mutant could increase the amount of EpsD, but not to the wild type level (Fig. 2-4, lane 3). IPTG induction of the *tac* promoter-controlled *epsC* gene did not further increase the level of EpsD, although it produced more EpsC when analyzed by immunoblot with anti-EpsC antibodies (data not shown). This is probably because the amount of EpsC encoded by the chromosomal gene of the wild type *V. cholerae* and the EpsC encoded by the plasmid without IPTG induction were similar (lanes 1 and 3 in the panel for EpsC). These results confirmed that the lack of EpsC affected stability of EpsD protein in *V. cholerae*.

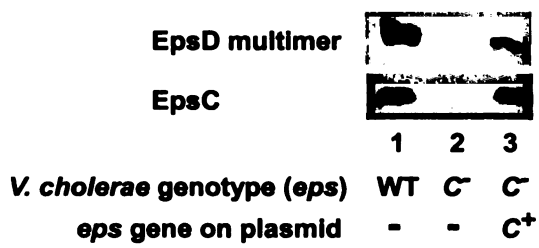


FIG 2-4 Effect of the *epsC* mutation on steady-state level of EpsD protein in *V. cholerae*. B-PER extracts of *V. cholerae* strains were separated by SDS-PAGE and subsequently immunoblotted with the anti-EpsD antibodies after transfer to nitrocellulose membrane. The same blot was re-probed with anti-EpsC antibodies to confirm the presence of EpsC. EpsD protein is almost undetectable in the *epsC* mutant (lane 2), but it can be restored when complemented with *epsC* gene (lane 3) although not quite up to the wild type level (lane 1). (Lanes 1=VB1, 2=VB12, 3=VB339)

Steady-state level of EpsD multimer is not affected by absence of *epsE*, *epsF*, *epsG*, *epsL*, and *epsM* in *V. cholerae* genome. Since the *epsC* mutant affected the formation of EpsD multimer and there was an indication of interaction between EpsD and EpsL (17), the ability of multimeric EpsD formation was examined in various available mutants to see whether absence of any other Eps proteins can also affect the formation of EpsD multimer. All mutants tested, except for two different *epsC* mutants, could form the EpsD multimer and no difference was observed in levels of EpsD protein compared to the wild type strain of *V. cholerae* (Fig. 2-5). These results do not exclude the possibility of interactions between the tested Eps proteins and EpsD protein, but suggest the absolute requirement of EpsC for the stability of EpsD, indicating a very close relationship between the two proteins.

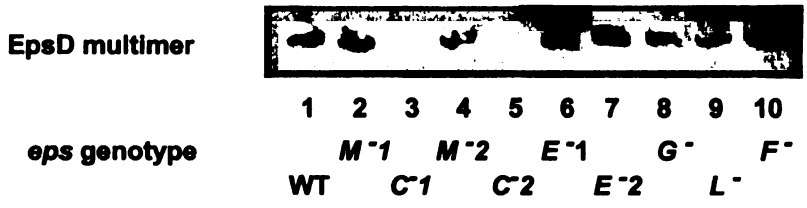
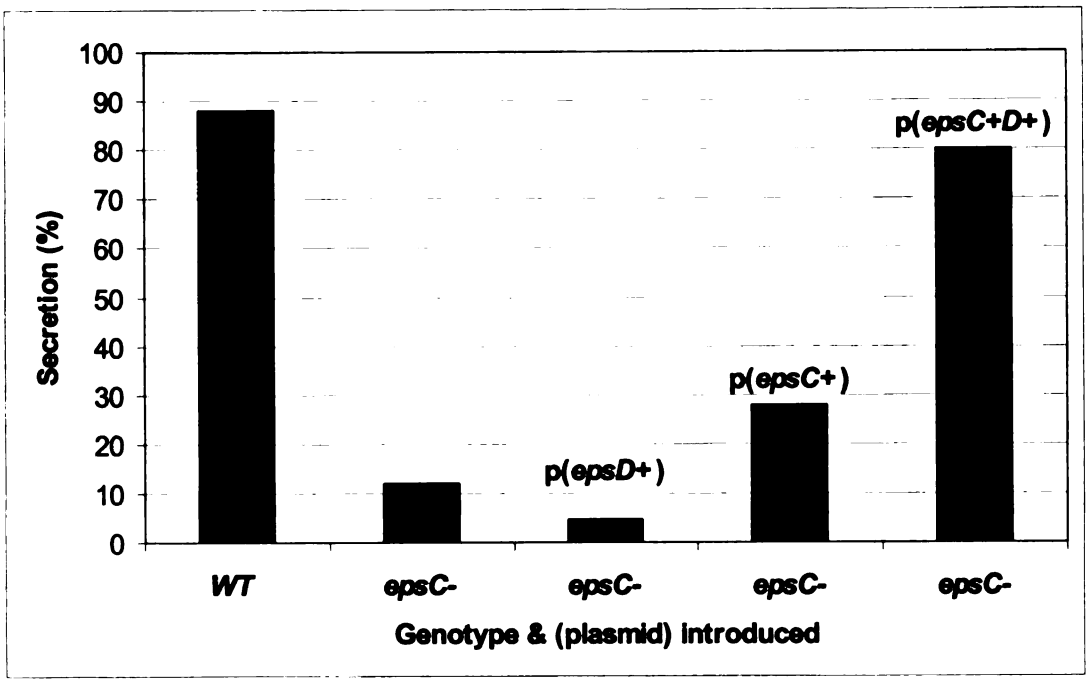


FIG. 2-5 Effect of various *eps* mutants on the stability of EpsD. Two different *epsC* mutants, generated by Tn5 insertion, are virtually missing EpsD protein (lanes 3 and 5) while the wild type (lane 1) and all other mutants shown seem to produce similar amounts of EpsD. (Lanes 1=VB1, 2=VB9, 3=VB10, 4=VB11, 5=VB12, 6=VB35, 7=VB36, 8=VB37, 9=VB106, 10=VB109)

Effective complementation of the *epsC* mutant by the *epsCD* expressed *in cis* and restoration of EpsD multimer in and LT secretion from *V. cholerae* cells. Previously, this *epsC* mutant created by insertion of Tn5, was the only mutant that could not be complemented by its own gene while *epsD*, *epsE*, *epsF*, *epsG*, *epsL* and *epsM* mutants were successfully complemented by their own genes to restore the secretion of the plasmid-encoded *E. coli* LT B subunit (2, 19, 36). The plasmid initially used for the complementation of the *epsC* mutant possesses about 200 bp upstream and 700 bp downstream sequences in addition to the *epsC* gene, thus including the sequence encoding about one third of EpsD (36). The above results of EpsC stabilizing the EpsD multimeric protein and the requirement of the *outCD* to restore secretion from the *outD* mutant in *E. chrysanthemi* were quite encouraging to try complementing the *epsC* mutant with *epsCD* (18). As shown in Fig. 2-6A (next page), while pMMB781 expressing *epsC* alone could not restore the secretion from the *epsC* mutant, pMMB799 expressing *epsCD in cis* could restore the secretion to the wild type level (4^{th} vs. 5^{th} bars on the graph). However, supply of the plasmid expressing the *epsC* only to the mutant consistently resulted in unignorable increases of LT secretion, compared to the mutant itself in several trials (2^{nd} vs. 4^{th} bars in Fig. 2-6A, not all data shown). This probably correlates with results demonstrating partial restoration of EpsD by copies of plasmid-encoded *epsC* in both the chromosomal and the cosmid *epsC* mutants (Figs. 2-3 and 2-4). Since the *epsC* mutant retained so little EpsD, a Western blot experiment with anti-EpsD antibodies as probes was performed to see if the restoration of the

A. Restoration of secretion by *epsCD*



B. Restoration of EpsD by *epsCD*

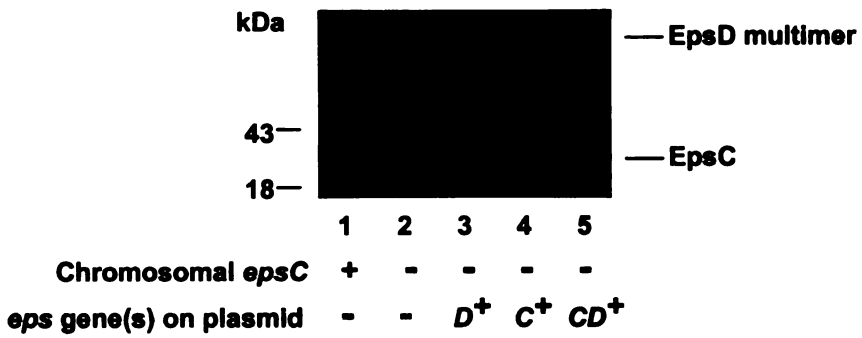


FIG. 2-6 Complementation of the *epsC* mutant by a plasmid expressing *epsCD*. (A) Secretion level is restored to the wild type level only when *epsCD* (pMMB799) is provided to the mutant. LT B subunit pentamers present in the growth medium and sonicated cells were determined by the GM1 enzyme-linked immunosorbent assay (ELISA). (B) Effective secretion is correlated with the restoration of EpsD protein in the mutant when *epsCD* is provided. Western blot was initially detected with anti-EpsD antibodies and then re-probed with anti-EpsC antibodies. (Lanes 1=VB101, 2=VB345, 3=VB347, 4=VB348, 5=VB349)

secretion accompanies the restoration of EpsD multimer. As shown in Fig. 2-6B, the amount of EpsD in the *epsC* mutant was restored to the level comparable to the wild type level in the presence of the plasmid that could restore the secretion (lane 5). Therefore, there is a correlation between the presences of EpsD multimer and the ability of the cells to secrete. It has been shown that overexpression of *epsD* gene can produce the multimeric form of EpsD as well as the monomer in *E. coli* (data not shown). When pMMB771 expressing *epsD* only was introduced into the *epsC* mutant, although the *epsD* gene was expressed from the same vector plasmid used to express *epsC* and also *epsCD* without IPTG induction, EpsD was not detected in the *epsC* mutant, thus failing to restore the secretion (Fig. 2-6A and B, 3rd samples). That is not really surprising considering that EpsD is unstable in the absence of EpsC. Therefore, it can be concluded that the *epsC* mutant requires *epsCD* to restore the formation of EpsD multimer and secretion, indicating that both EpsC and EpsD contribute equally to the formation and/or the function of the secretion apparatus, probably interacting each other.

Failure of the complementation for the *epsC* mutant by *epsC* and *epsD* expressed *in trans* in *V. cholerae*. As mentioned earlier, the formation of the multimeric EpsD is probably very important for assembly and/or integrity of the secretion apparatus. It was found that pMMB835 containing the *epsC* and about a third of *epsD* in tandem could restore the EpsD multimer almost to the wild type level in the *epsC* mutant, but this plasmid could not rescue the

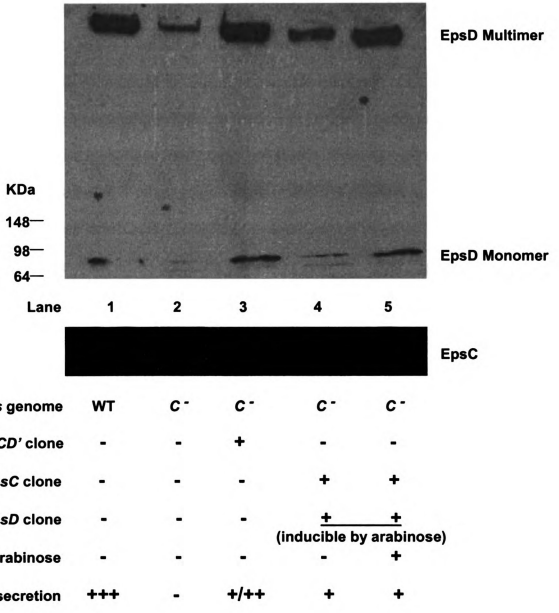


FIG. 2-7 ***V. cholerae epsC*** **mutant cannot be complemented by the clones of** ***epsC*** **and** ***epsD*** **supplied** ***in trans.*** Supplying additional copies of *epsD in trans* or even partial sequences of *epsD* at the 5' region *in cis*, in addition to the *epsC* gene, can help formation of EpsD multimers in the *epsC* mutant, but they are not functional, thus failing to restore the secretion to the full extent compared to the wild type and the *epsC* mutant supplied with *epsCD in cis*. Secretion levels of these mutants, which can restore the EpsD almost to the wild type level, are similar to the *epsC* mutant strain supplied with *epsC* alone (see Fig. 2-6A). (Lanes 1=VB1, 2=VB12, 3=VB366, 4 and 5=VB367)

secretion defect of the mutant, although the strain secreted some amount of LT B subunit similar to the level accomplished by complementation of the *epsC* mutant by the *epsC* clone alone (Fig. 2-7, lane 3). This means in spite of the correlation between the multimeric EpsD formation and restoration of the secretion, presence of the multimeric EpsD does not always indicate the functionality of the EpsD associated with other components of the T2SS apparatus. According to the results mentioned earlier, *epsC* alone could not complement the *epsC* mutant completely, but could restore the secretion slightly. Also *epsD* alone could restore the EpsD multimer if the expression was induced although it cannot restore the secretion, indicating absolute requirement of *epsC* to complement the *epsC* mutant. When *epsCD* was expressed from the same plasmid (pMMB799), it could efficiently complement the *epsC* mutant. All these results lead to an important question with a highly probable expectation. What if the *epsC* and the *epsD* genes were expressed *in trans* from the separate plasmids in the *epsC* mutant? Providing a functional clone of *epsC* and the *epsD* clone, which is able to restore formation of EpsD, in combination seems very tempting for complementation of the *epsC* mutant. So *tac* promoter-controlled *epsC* (pMMB781) and *araB* promoter-regulated *epsD* (pMMB794) were provided separately to the *epsC* mutant. The results were similar to that of the *epsC* mutant, harboring the *epsC* clone (pMMB781) only, which could consistently restore the secretion level slightly, about 10 to 20% more than the level of the *epsC* mutant (4^{th} sample, Fig. 2-6A). Induction of the *epsD* expression with arabinose did not make any difference for secretion in the presence of *epsC*,

which was constitutively expressed from the *tac* promoter (lanes 4 and 5, the panel for EpsC in Fig. 2-7) although it did increase the amount of EpsD multimer to the level comparable to the wild type level (Fig. 2-7, lanes 4 and 5). These results show that the addition of the *epsD* to the *epsC* mutant harboring a clone of the *epsC* does not help functionally and the multimeric forms of EpsD detected on the blot are not functionally viable forms. It would be interesting to investigate the structure of nonfunctional EpsD multimers generated by these strains. Alternatively, the EpsD might produce a native complex, but cannot be regulated properly to open the channel due to the ineffective coordination of conformation change caused directly or indirectly by the EpsC. The main reason for the failure of complementation by *in trans* expression of *epsC* and *epsD* separately, while the *epsC* mutant can be complemented effectively by coexpression of the *epsCD* *in cis*, is probably due to the lack of the fine tuning between EpsC and EpsD that can be achieved when coexpressed from the same promoter *in cis*.

Analysis of the *epsD* mutant and complementation of the *epsD* mutant. Our efforts to construct an *epsD* mutant in El Tor strain of *V. cholerae* TRH7000 were never successful, but there have been reports by other researchers, involving the *epsD* mutants in other strains (2, 11). The reason for the failure of the *epsD* mutant generation is probably due to impaired growth caused by EpsD deficiency as indicated by other researchers (11). This explanation is also supported by the nature of those *epsD* mutants acquired. They are mutants generated by the Km^R cassette insertion only 30 amino acids

away from the C-terminus of EpsD. Although these mutants are viable, they are all severely impaired for the secretion as measured by CT secretion (2, 11). We received one of the mutant strains, Bah-2 *epsD:: Km* along with the wild type Bah-2 strain, from Sandkvist and used them to characterize the mutant (11). First, we checked to see if they are still able to form the multimer in the absence of the N-terminus. According to the Western blot analysis with anti-EpsD antibodies, the bands appearing as multimeric form in the stacking gel were apparently same for both the wild type and the *epsD* mutant (data not shown). Although the actual EpsD protein cannot be examined thoroughly, it is very probable that the EpsD multimer formed by the *epsD* mutant is defective in an unknown manner since the secretion is known to be severely affected. Alternatively, the non-encoded or Km^R-inserted region might inhibit interaction of EpsD with EpsC or other Eps proteins, preventing the opening of the channel. Secondly, we checked to see if the mutation of the *epsD* affects other Eps proteins. Again, Western blots with available anti-Eps protein antibodies were performed to measure the levels of EpsC, EpsF, EpsG, EpsL, and EpsM proteins in the mutant. No difference was detected between the wild type Bah-2 and the Bah-2 *epsD:: Km* (data not shown). Thirdly, since Bah-2 strain is non-toxigenic, the authors who used this strain could not measure the secretion in terms of percentage for the toxin secreted into the medium, but could show reduced amount of hemagglutin/protease secreted from the *epsD* mutant compared to the wild type (11). Therefore, pWD615 encoding the LT B subunit was transformed into Bah-2 strains to measure the amount of LT B pentamer secreted into the

Strain	Genotype	Plasmid	IPTG	Secretion (%)
VB372	WT	-	-	93%
VB373	*epsD*$^-$	-	-	30%
VB375	*epsD*$^-$	*epsD*$^+$	-	30%
VB375	*epsD*$^-$	*epsD*$^+$	+	88%
VB376	*epsD*$^-$	*epsC*$^+$*D*$^+$	-	54%

Table 2-2 Complementation of the Bah-2 *epsD::Km* mutant strain. The *epsD* mutant's secretion is restored when the supplied copies of *epsD* gene from pMMB771 is induced with IPTG, but when it is not induced no change is observed for the secretion compared to the mutant. Introduction of *epsCD* by pMMB799 to the mutant can help restore the secretion to some extent, but not to the wild type level. In separate experiments, it has been shown that the induction of *epsCD* with IPTG can also complement this mutant as efficiently as the *epsD* expressed by IPTG induction.

growth medium by GM1-ELISA and also introduced plasmids expressing *epsD* (pMMB771) and *epsCD* (pMMB799) into the mutant strain to check for complementation. The results are shown in Table 2-2. Compared to the wild type (93%), the *epsD* mutant's secretion was very much reduced (30%). Introduction of the plasmid expressing *epsD*, the same plasmid used in the complementation of the *epsC* mutant, into the *epsD* mutant did not restore the secretion without IPTG induction, but IPTG induction of the same plasmid led to the very effective complementation. These results indicate that the amount of EpsD produced from the plasmid is probably not enough to complement or even if the *epsD* is expressed, as shown before, it may be unstable and disappears although there is a chromosomal copy of *epsC* apparently producing the same amount of EpsC as the wild type. Unlike the *epsC* mutant, the *epsD* mutant does not seem to require the coexpression of the

epsCD and the *epsD* alone is sufficient to complement the mutant as long as it produces enough EpsD as also shown by other researchers (2, 11). Since the uninduced *epsD* clone was not able to complement the *epsD* mutant, it was interesting to see the results of complementation by a plasmid expressing *epsCD*. It could complement the mutant, but not as effectively as induced *epsD* alone (table 2-2). Two possible reasons for this phenomenon are: (i) expression of *epsCD* is somewhat inhibitory to the *epsD* mutant because of additional copies of *epsC* expressed, causing stoichiometry imbalance; or (ii) *epsCD* does not produce enough EpsD necessary for the complementation. To verify this, *epsCD* under the contol of *tac* promoter was induced with IPTG in the *epsD* mutant. The data collected from several independent experiments suggest that induction of the *epsCD* can complement the *epsD* mutant as effectively as the IPTG-induced *epsD* under *tac* promoter (near 100% secretion). Therefore, in contrast to the results of the *epsC* mutant complementation, the *epsD* mutant does not require *epsCD* to restore the secretion and additional copies of *epsC* does not interfere with the assembly of the secretion apparatus or with the secretion.

Discussion

Type II secretion system in *V. cholerae* requires a concerted action of at least 12 genes. The products of each of these genes are located in the cytoplasmic membrane except for the EpsD protein which is in the outer membrane. It is reasonable to expect that at least some of the Eps proteins interact with one another and probably form some kind of a secretion apparatus. Indeed, the interactions of EpsE, EpsL and EpsM have been demonstrated in the previous work (33-35). The question of how the proteins located in the cytoplasmic membrane may cooperate with the protein EpsD, which is believed to constitute the exit pore in the outer membrane, has been asked in this work. It has been shown that EpsD is protected by the presence of EpsC both in *E. coli*, in the absence of other Eps proteins and in *V. cholerae*. In the *epsC* mutant, the concentration of EpsD protein is so low that it cannot be detected by immunoblot anlaysis. However, in the presence of EpsC protein, a considerable increase of EpsD protein is observed. The expression of *epsC* gene alone in the *epsC* mutant cannot restore the secretion of toxin, but when both *epsC* and *epsD* genes are expressed *in cis*, the secretion is restored. These experiments cannot rule out the possibility that the *epsC* mutation tested is polar. However, no evidence of polarity could be observed on the other *eps* genes located downstream of *epsD* and these genes were not essential for the complementation of the *epsC* mutation. We believe, thus, that the results showing the protection of EpsD by EpsC and the results from complementation of the *epsC* mutant suggest the interaction of the EpsC and EpsD proteins.

Previously, Lindeberg et al. (18) suggested possible interactions between OutC and OutD, homologues of EpsC and EpsD, in *Erwinia chrysenthemi* based on the indirect observation that the OutC homologues are among the least similar of the Out proteins (50% amino acid identity) (14). These authors could complement individual *E. chrysanthemi out* genes (homologues of the *eps* genes) by homologous genes from *Erwinia carotovora*. However, the *outC* and *outD* mutations could not be complemented by individual *E. carotovora* homologues. In order to complement these mutations, appropriate wild type *outC* and *outD* homologues had to be expressed together. Complementations requiring expression of more than one genes *in cis* have also been reported for the type IV secretion system of *Agrobacterium tumefaciens* (3, 13). These results suggest that sometimes precise stoichiometry can be achieved only by coexpressing genes involved in the assembly of multi-protein complexes such as the type II and IV secretion systems. Therefore, organization of these genes as an operon is thought to be efficient for fine tuning of the components involved in the assembly of the secretion apparatus.

It is at present unclear why *epsC* and *epsD* genes have to be present *in cis* configuration in order to complement the *epsC* mutant. It is possible that these proteins start interaction during the translation of the *epsD*. Alternatively, another gene may be present on the DNA fragment carrying *epsC-epsD* cluster and separation of these two genes onto different vector plasmids disrupts this gene. Further work on this problem is clearly required to decide between these possibilities.

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CHAPTER 3

A NEW GENE REQUIRED FOR TYPE II SECRETION IN *VIBRIO CHOLERAE*

Abstract

Out and Pul type II secretion systems from *Erwinia chrysanthemi* and *Klebsiella oxytoca* respectively, are the only systems that have been successfully reconstituted in *Escherichia coli*, which does not secrete proteins normally. Introduction of their corresponding substrate proteins make the *E. coli* strain with reconstituted genes to secrete them to the extracellular milieu. No other type II secretion system of many other Gram-negative bacteria has been reported to be reconstituted in *E. coli*. Eps system of *Vibrio cholerae* could not enable *E. coli* to secrete introduced proteins when reconstituted with prepilin peptidase gene, *vcpD*. Therefore, depending on the organism, some extra genes may be required for the efficient secretion in addition to the known type II secretion genes. During the course of studying the complementation of the *epsC* mutant, an unknown open reading frame (ORF) located upstream of the *epsC* gene was found to be required, in addition to *epsCD*, in the supplied plasmid to complement this mutant. The ORF is homologous to heat shock protein, *hsp15* of *E. coli* according to the sequence and homologues are highly conserved in prokaryotes, including other Gram-negative bacteria with the type II secretion system. Recently found Hsp15 is a ribosome-associated heat shock protein with a novel RNA-binding motif. It has been proposed that Hsp15 takes part in the translational cycle although its function has not been studied in detail yet. Here, we show that this Hsp15 homologue is necessary for the type II secretion function of *V. cholerae* by employing various plasmids with insertion or deletion mutations of this ORF in complementation experiments with the *epsC* mutant.

Introduction

With the increasing number of genome sequences available for many bacteria, type II secretion genes have been found in many bacteria. Generally, these genes are well conserved in sequence and organization, but there are exceptions (18). For example, C and N homologues are missing in *Xanthomonas campestris* and *Erwinia chrysanthemi*, respectively, and the gene organizations or the orientations of some genes are different in *Pseudomonas aeruginosa* and *X. campestris* compared to the type II secretion genes in other organisms. Out and Pul systems of *E. chrysanthemi* and *Klebsiella oxytoca*, respectively, are the only two type II secretion systems that have been successfully reconstituted in *E. coli* to secrete their own proteins produced from the supplied plasmids (6, 9). Therefore, in these organisms, the known type II secretion genes are minimal requirement for secretion although auxiliary association of other genes with the type II secretion genes cannot be ruled out completely. However, the type II secretion system of *Vibrio cholerae* could not enable *E. coli* to secrete introduced proteins when all *eps* genes and *vcpD* gene were reconstituted (unpublished data). This raises a possibility that yet unidentified gene might also be required for the type II secretion in *V. cholerae*. .

We showed efficient complementation of the *epsC* mutant by providing *epsCD* in a plasmid (described in chapter 2). This plasmid, pMMB799, also contains additional ~1.2 kb sequences upstream of *epsC* (Fig. 3-1). We constructed a new plasmid to remove the upstream sequence and tested the plasmid to complement the *epsC* mutant. Unexpectedly, this plasmid,

pMMB800, containing *epsCD* with only ~200 bp upstream sequence, but ~1 kb sequence deleted, could not complement the *epsC* mutant. Analysis of the upstream sequence revealed a full open reading frame (ORF) and another partial open reading frame (ORF2), further upstream of *epsC*, which are transcribed in opposite orientation to the *eps* transcription (Fig. 3-1).

Sequence analysis of this ORF identified a homologue of this protein in *E. coli*. The homologue, recently found Hsp15, is very abundant heat shock protein which binds nucleic acids, is ribosome-associated, and contains a novel RNA-binding motif (11, 12, 20). Its synthesis is induced massively at the RNA level upon temperature shift and its specific target for binding has been identified to be free 50S ribosomal subunit only when it is not a part of the 70S ribosome. It has been proposed that Hsp15 plays a role in recognition and repair of 50S particles, dissociated from the ribosome by error, which is likely to be prevalent under heat shock conditions (11). Thus, Hsp15 is functionally different from many other heat shock proteins known to act as molecular chaperones on protein substrates (11, 12).

In this report, we show that putative Hsp15 ORF is required to complement the *Vibrio epsC* mutant in addition to *epsCD*. Therefore, we suggest that the gene encoding the putative Hsp15 is a new gene required for type II secretion in *V. cholerae*.

Materials and methods

Bacterial strains and plasmids, and culture conditions. Bacterial strains and plasmids used are listed in Table 3-1.

Table 3-1 Strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
V. cholerae		
TRH7000	ElTor *thy* Hg^R Δ(*ctxA-ctxB*)	(10)
VB12 (PU6)	TRH7000 *epsC*::Km^R	(16)
E. coli		
MC1061	F^- *araD*139 Δ(*ara-leu*)7697 Δ(*lac*)X74 *rpsL hsdR2 mcrA mcrB1*	(2)
BL21(DE3)	F^- ompT T7 gene 1 under lacp control on λ prophage	(21)
Plasmids		
pCP20	FLP recombinase expression vector, Ap^R, Cm^R Temperature sensitive	(5)
pKD4	Template plasmid for Km^R	(5)
pKD46	Red recombinase expression vector, Ap^R Temperature sensitive	(5)
pMMB207/208	Broad-host-range cloning vector; Cm^R	(14)
pMMB781	pMMB208::*epsC*	Chapter 2
pMMB799	pMMB207::~1.2 kb upstream sequence and *epsCD*	Chapter 2
pMMB800	pMMB207::~200 bp upstream sequence and *epsCD*	This study
pMMB815	pT7-6::Hsp15 ORF in pMMB799	This study
pMMB843	pMMB207::~400 bp upstream sequence and epsCD	This study
pMMB844	pMMB799 Hsp15 ORF::Km^R	This study
pMMB845	pMMB799 ΔHsp15 ORF	This study
pT7-6	T7 Φ10 promoter, Ap^R	(23)
pWD615	*etxB* (*E. coli* heat-labile enterotoxin B subunit gene), Tc^R	(4)

Construction of in-frame insertion/deletion mutants of pMMB799 plasmid. To insert a Km^R cassette into and inactivate the ORF in plasmid pMMB799, procedures by Datsenko and Wanner have been followed as described in chapter 2 (5). Km^R – containing PCR products were generated by using a pair of primers, S-Prim1 (5'-CCAGCGCAGAAGAGGTGAGACTGGAT-AAATGGCTGTGGgtgtaggctggagctgcttc-3') and S-Prim2 (5'-TGAATTTGAGTAG-ATCTCGGCGCTGTTTCTTGTCatatgaatatcctccttag-3'), which include homology extension sequences in the ORF (capital letters) and 20-nt priming sequences (small letters) for pKD4 as template (Fig. 3-1). After getting transformants containing a plasmid (pMMB844) whose putative Hsp15 ORF is replaced by Km^R cassette, they were transformed with pCP20, which acts on directly repeated FRT (FLP recognition target) sites flanking the Km^R gene, to eliminate the resistance gene. Ampicillin-resistant transformants were selected at 30°C and then colony-purified at 43°C to remove Km^R gene and pCP20. The final transformants with a new plasmid pMMB845, which is derived from pMMB799 by deletion of Hsp15 ORF, were checked for the loss of ampicillin and kanamycin resistance.

Construction of pMMB843. To construct a plasmid containing *epsCD* with the C-terminal half of the Hsp15 ORF removed, corresponding region was PCR amplified using primers EPSC upst-2 (5'-GC**TCTAGA**GCATGCTTG-ACGCAATGTGATTTGGG-3', *Xba*I site in bold) and EPSD2 (5'-CGC**GGATCC**T-TGCTTGGGTTCCATCTG-3', *BamH*I site in bold) with E26 cosmid DNA as

template (Fig. 3-1). Both pMMB208 vector and the insert PCR product were digested with *BamH*I and *Xba*I, and ligated with ligase (Invitrogen, Carlsbad, CA).

Expression of Hsp15 ORF under bacteriophage T7 Φ10 promoter control. pMMB815, pT7-6 containing the Hsp15 ORF (Fig. 3-1), was introduced into BL21(DE3) and the strain was grown in LB supplemented with ampicillin at 37°C. When the OD_{650} of the culture became ~0.5, 50μM IPTG was added to induce the expression and the cells were harvested after 1.5 hours. The cells resuspended in sample buffer, containing β-mercaptoethanol, were subjected to 12% SDS-PAGE to separate proteins. The proteins on the gel were visualized by Coomassie Blue staining.

Quantitative determination of LT B subunit pentamer secretion, extraction of proteins, gel electrophoresis and immunoblotting were as described in chapter 2.

Results

Putative Hsp15 ORF (~380bp) located upstream of *epsC* gene, in addition to the *epsCD*, is required for complementation of the *V. cholerae* *epsC* mutant. In chapter 2, we showed effective complementation of the *epsC* mutant by pMMB799 expressing *epsCD*. The plasmid also contains additional ~1.2 kb sequence, which is located upstream of the *epsC* gene (Fig. 3-1, next page). When pMMB800, which contains only ~200 bp upstream sequence of the *epsC* in addition to *epsCD*, was introduced into the *epsC* it could not restore the secretion of LT. We found that the expression levels of both *epsC* and *epsD* from pMMB800 is much higher than the expression levels from pMMB799 without IPTG induction (Fig. 3-2, page 113). There have been reports demonstrating inhibition of secretion from the wild type strain by overexpressing one of the components. For example, overproduction of PulG blocks secretion and drastically reduce the cellular levels of PulC, PulE, PulL, and PulM as well as PulD in *K. oxytoca* (17). Therefore we suspected overproduction of EpsC and EpsD might be the reason for the complementation failure. To verify our hypothesis, pMMB799 and pMMB800 were transformed into the wild type *V. cholerae* and the secretion of LT was measured. Neither of the plasmids affected the secretion from the wild type *V. cholerae* (data not shown). Therefore, it is not likely that overexpression of *epsCD* is responsible for the failure of the complementation by pMMB800.

According to the analysis of the sequence upstream of *epsC*, there is a ~380 bp ORF that could encode a 128 amino acid residue protein with homology

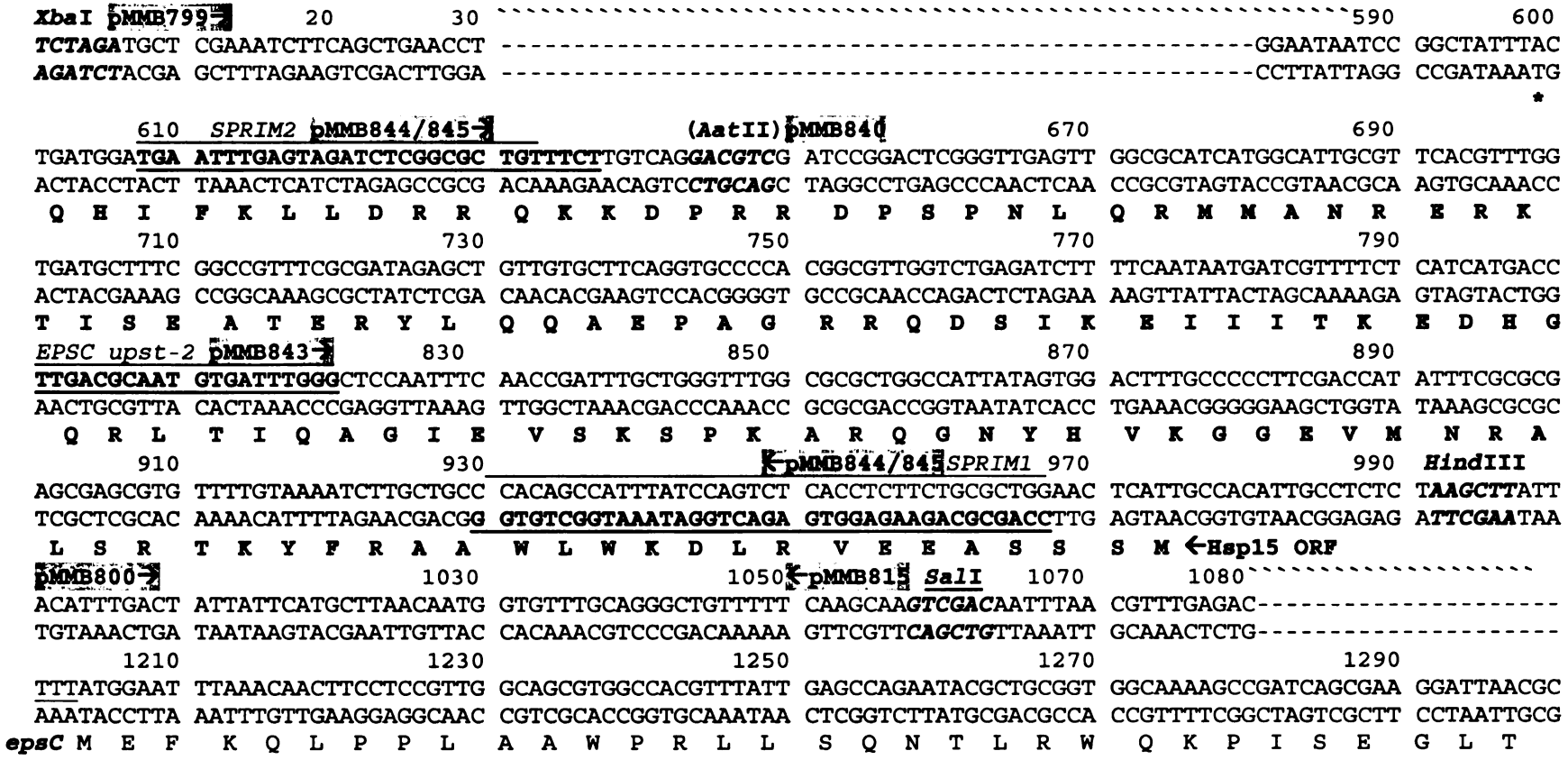


FIG. 3-1 Upstream sequence of *epsC*, including an ORF with amino acid sequence (in bold). Primer sequences, used for PCR when constructing new plasmids, are **underlined**. Restriction sites used for plasmid constructions appear in ***bold*** letters. pMMB840 was created by *Aat*II blunt ending and religation. pMMB843 contains a partial ORF in addition to *epsCD*. In pMMB844, ORF is replaced with Km^R gene and removal of the Km^R gene gave a birth to pMMB845, in which the ORF is deleted. pMMB815 used for expression of the ORF under bacteriophage T7 Φ10 promoter control has been cloned by inserting *Xba*I (position 1) and *Sal*I (position 1063) fragment into pT7-6. See the text for detail.

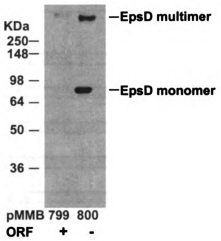
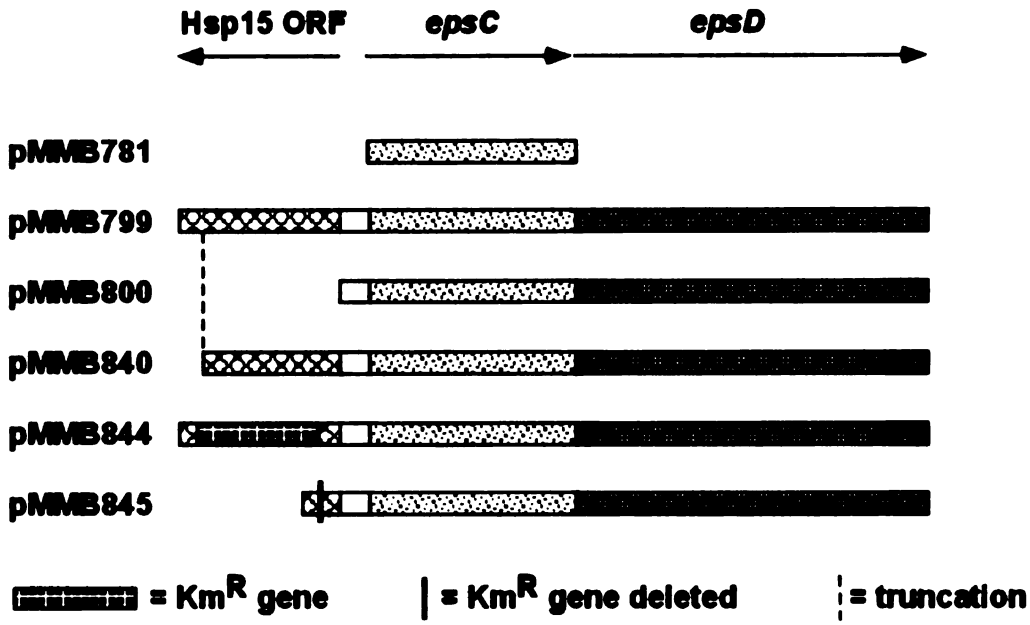


FIG. 3-2 Expression of *epsC* and *epsD* from pMMB800 lacking the ORF is much stronger than that of pMMB799 with the ORF. B-PER bacterial protein extraction reagent-extracted samples of *E. coli* harboring the plasmids were separated on SDS-PAGE and subsequently immunoblotted with anti-EpsD antibodies after proteins were transferred to the membrane. Both plasmids are under the control of *tac* promoter and they were not induced with IPTG. Both monomers and multimers of EpsD (in the stacking gel) are shown. EpsC is not shown here. (Strains pMMB799=CB2416, pMMB800=CB2417)

to a heat shock protein Hsp15 of *E. coli*, raising an alternative possibility. Is the Hsp15 ORF necessary for the secretion? To test this, different plasmids derived from pMMB799 and with alterations in this ORF were constructed and introduced into the *epsC* mutant (Figs. 3-1 and 3-3A). The results of complementations in terms of LT B subunit secretion percentage are shown in Fig. 3-3B (next page). The percentage of the secreted LT B for the *epsC* mutant was only 18% while the wild type *V. cholerae* secreted almost 100% of LT B to the extracellular milieu (strains 1 and 2). As shown in chapter 2, expression of *epsC* alone by pMMB781 in this mutant could increase the secretion level only slightly (36%), whereas the complementation by pMMB799, containing *epsCD* and ~1.2 kb upstream

A.



B.

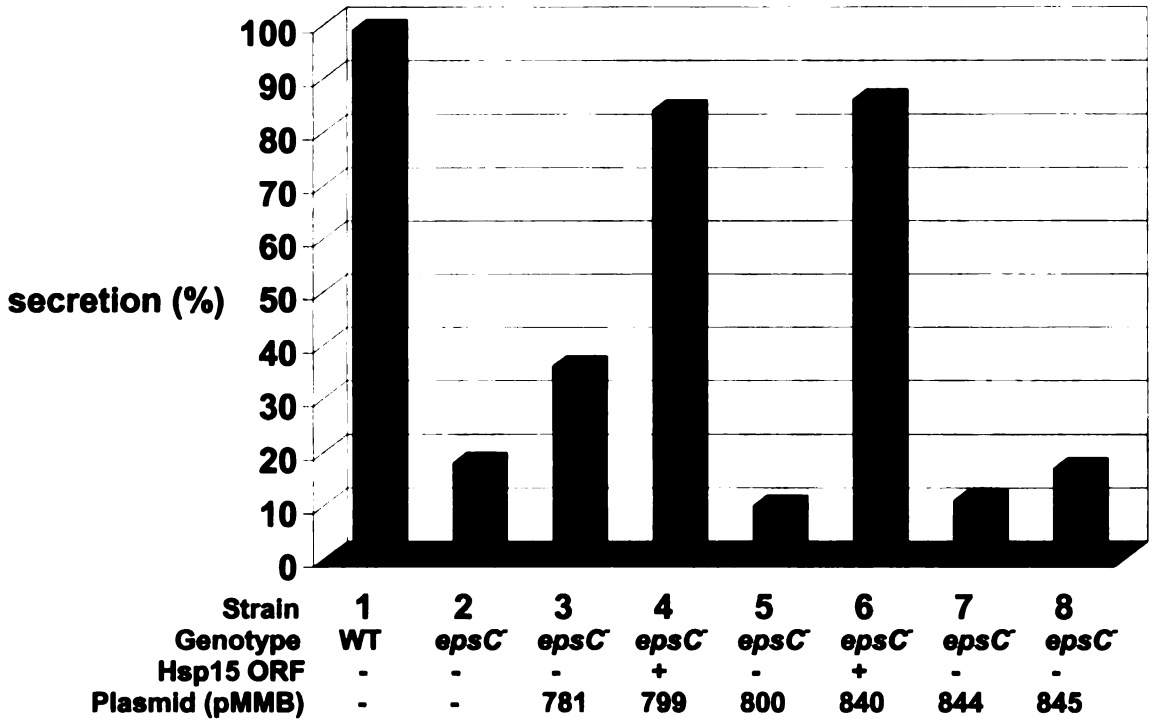


FIG. 3-3 A putative Hsp15 ORF is required in a plasmid expressing *epsCD* to restore secretion of the *epsC* mutant. (A) Schematic representation of various plasmids used to complement the *V. cholerae epsC* mutant. (B) Secretion of LT B subunit from various strains of *V. cholerae* as measured by GM1 ELISA (16, 22) and effect of the ORF in the plasmid on secretion in the *epsC* mutant. (Strains 1=VB101, 2=VB345, 3=VB348, 4=VB349, 5=VB385, 6=VB396, 7=VB403, 8=VB404)

sequence, could restore the secretion to the level comparable to that found in the wild type strain (strains 3 and 4). Plasmid pMMB800, containing only ~200 bp upstream sequence lost the ability to complement. The plasmid pMMB840 contains a frameshift in the last 17 residues of the Hsp15 ORF and it was constructed by digesting pMMB799 with *Aat*II, blunt ending and religating the plasmid. The resulting plasmid results in an early termination codon only after two amino acid residues from the *AatII* cleavage site, thus losing about one tenth of amino acid residues at the C-terminus of the protein encoded by the ORF. Although we do not know whether this alteration would change the biochemical function of this protein, it is possible that such protein would still be active. According to the complementation data, pMMB840 could restore the secretion from the *epsC* mutant as effectively as pMMB799 (Fig. 3-3B). This suggests that the frameshift mutation near the C-terminus of the ORF does not affect the protein function. The plasmids, pMMB844 (ORF is replaced by the Km^R gene) and pMMB845 (derived from pMMB844 and the ORF is completely deleted), could not complement the *epsC* mutant or could not rescue its secretion defect (Fig. 3-3B). In an independently performed experiment to complement the *epsC* mutant by pMMB843 whose ORF is truncated in the middle of the protein (Fig. 3-1), the secretion percentage was only 28%, thus failing to complement the mutant. All these data strongly indicate that the putative Hsp15 ORF is required in addition to *epsCD* to complement the *epsC* mutant. These results also suggest importance of this ORF in the type II secretion and indirectly show its requirement for the type II secretion in *V. cholerae*. Direct evidence of this ORF's

requirement in the type II secretion awaits the mutagenesis of this ORF in the genome of *V. cholerae*.

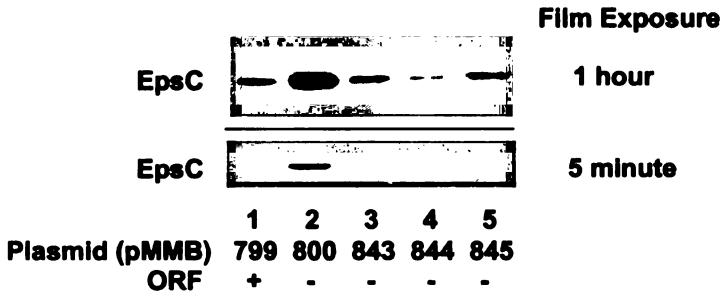


FIG. 3-4 Expression levels of *epsC* in various plasmids show that only pMMB800 overproduce EpsC, suggesting the inability of plasmids pMMB800, pMMB843, pMMB844 and pMMB846 to complement the *epsC* mutant is not due to the overproduction of EpsC and EpsD.

Effect of the Hsp15 ORF on expression of *epsCD*. To understand the reasons why pMMB800 produces much higher levels of EpsC and EpsD these levels were checked in several pMMB799 derivatives in *E. coli*. As shown in Fig. 3-4, all plasmids except for pMMB800 produced about the same level of EpsC according to the intensity of the band on the blot. These included plasmids pMMB843, pMMB844 and pMMB855 which do not contain the complete Hsp15 ORF (Fig. 3-1 and lanes 3, 4 and 5 in Fig. 3-4). These results suggest that the overproduction of EpsC and EpsD from pMMB800 is not directly due to the absence of Hsp15 ORF or of its product. If the ORF is the real gene, the promoter of the ORF must be present upstream of the ORF. Thus, one common point for pMMB799, pMMB843, pMMB844 and pMMB855 is that they all contain

the intact putative promoter for the Hsp15 ORF while pMMB800 is likely not to contain the complete promoter (Fig. 3-1). Therefore, it seems the reason for the difference in expression levels between pMMB800 and other plasmids is due to the presence of a putative promoter for the Hsp15 ORF acting in opposite direction of *tac* promoter in the vector which expresses *epsCD* genes (see Fig. 3-1). It has been known that counteracting promoters can affect the expression of a gene from the plasmid. This means the expression level of *epsCD* from pMMB800 is not actually overexpression, but normal while all other plasmids, including pMMB799, pMMB843, pMMB844, and pMMB845, express less than the normal level because of the presence of the promoter for the putative Hsp15 ORF.

Putative Hsp15 ORF expression under the control of the bacteriophage T7 Φ10 promoter. To test if the putative Hsp15 ORF of *V. cholerae* is expressible, it was cloned into the pT7-6 expression vector and induced with IPTG. *E. coli* cells harboring this plasmid were resuspended in sample buffer containing β-mercaptoethanol and the proteins were separated by SDS-PAGE. As shown in Fig. 3-5 (next page), the Coomassie Blue-stained gel shows distinct bands for the *E. coli* with the ORF-containing plasmid (lanes 1, 2, 5, 6, 7 and 8), while negative control, which contains the empty pT-6 vector plasmid, does not show this band. According to Korber et al., the molecular mass values of the Hsp15 homologues in Gram-negative organisms are in the range of 15.1-15.5 kDa (12). The predicted MW of the putative Hsp15 ORF is 14.85 kDa according to the

sequence analysis. The band on the gel, according to the molecular weight standard used, shows somewhat bigger molecular mass, but it is still pretty close to the expected size. Oftentimes, protein migration rate is affected by several factors such as folding or disulfide bonds and the actual molecular mass might not be reflected correctly on the SDS-PAGE.

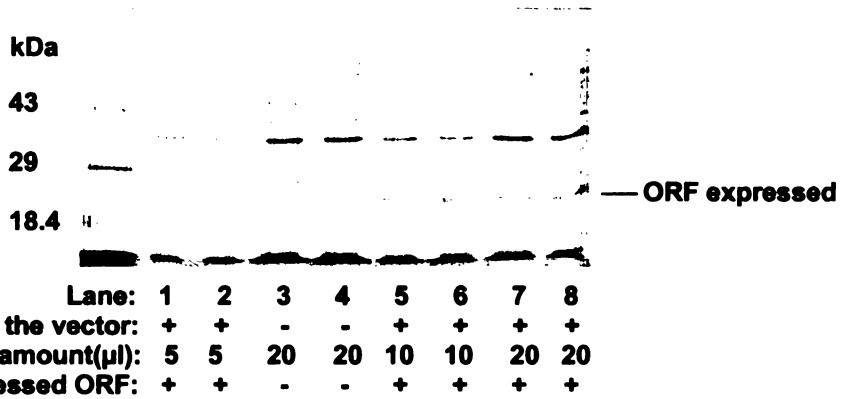


Fig. 3-5 Expression of the putative Hsp15 homologue ORF of *V. cholerae* under the control of the bacteriophage T7 Φ10 promoter. Compared to the proteins expressed from pT7-6 in *E. coli* BL21(DE3) (CB2457), pT7-6::ORF (CB2456) expresses an additional distinct protein and the band appears between 18.4 and 29 kDa standards.

Discussion

In this report, we identified a new ORF that may be important for type II secretion of *V. cholerae*. As shown in the genetic complementation analysis for the *epsC* mutant, only the plasmids containing this Hsp15 ORF in addition to *epsCD* could complement for secretion restoration when introduced into the mutant. Since site-directed mutagenesis in *V. cholerae* is still technically not possible, we still do not have a mutation affecting putative *hsp15* gene in this host. Thus, the crucial experiment showing whether the function of *hsp15* is essential for toxin secretion or whether the gene might have an auxiliary role in this process awaits further research.

At present, the role of the putative Hsp15 ORF is unclear. The *epsC* mutant should have intact wild type Hsp15 ORF in the genome. Expression of *epsC* and *epsD* from plasmids may result in some quantitative imbalance in the stoichiometry of the secretion apparatus and the amount of Hsp15 produced from the chromosomal gene may not be enough to complement. Since EpsC and EpsD interact and the absence of the ORF affects their function, it is likely that this ORF is involved in the regulation or fine tuning of these two components so they can play their role in the secretion apparatus properly in combination with other proteins. The main reason for the *epsC* mutant failing to secrete is caused not only by the absence of EpsC but also by insufficient level of EpsD. Therefore, to complement this mutant, very precise and well-balanced expression of *epsCD* is important and the ORF might be necessary for that. It is

possible that this Hsp15 might actually interact with EpsC-EpsD complex and its expression *in cis* may be essential for its action.

Although Hsp15 of *E. coli* has been studied to some extent, its homologues in other Gram-negative bacteria were identified by sequence similarity and none of them has been characterized yet. Thus, they are still listed as hypothetical proteins. One interesting point is that the location of the *hsp*15 gene is well conserved among a few bacteria with the type II secretion system. As seen in the literature, type II secretion system is quite well conserved, but there are some variations of gene organization (18). Among them, type II secretion systems of *V. cholerae*, *Aeromonas* and *Shewanella* species are most closely conserved in terms of gene organization and they all have the gene homologous to *hsp15* gene right upstream of type II secretion gene *C.* Therefore, it is quite probable that the homologues of Hsp15 in these organisms might play a role in the secretion. Although, Hsp15 is a heat shock protein, the role of heat shock proteins might be essential not only after heat shock but also under normal conditions (11).

The indication that Hsp15 homologue of *V. cholerae* is somehow associated with EpsC-D complex and might play a role in the type II secretion is further supported by presence of several D protein-associated proteins in other organisms. In *Erwinia* and *K. oxytoca*, S protein is required for outer membrane insertion, assembly and stability of the D protein (7, 8, 15, 19). In *A. hydrophila*, ExeAB complex is required for the localization and assembly of the ExeD multimer (1). Similarly, in *E. chrysanthemi*, OutB interacts with OutD (3). In *X.*

campestris which lacks EpsC homologue, XpsN protein has been shown to be associated with OM protein XpsD (13). This shows that D protein, which forms OM pore, interact with several proteins, suggesting that in addition to the interaction with the C protein, interactions with other proteins are also necessary to control the gated channel. Since the multimeric D protein channel, through which substrate proteins of the type II secretion system pass, is such an important part of the secretion apparatus, it seems to involve several interactions to communicate with other mostly IM proteins.

In conclusion, we have identified a new gene, whose product might be important for secretion. It may be involved in the functions of the EpsC, EpsD or of the EpsC-EpsD complex. Further study is required to understand the role of this protein in the function of Eps apparatus.

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CHAPTER 4

CONCLUSION

For the past decade our lab has been trying to understand the mechanism of type II protein secretion in *Vibrio cholerae*. Since the identification of a cluster of *eps* genes, which are required for type II protein secretion, we have been characterizing individual proteins and also identifying interactions between the proteins making up the secretion system. The only outer membrane component of *V. cholerae*, EpsD homologues have been characterized well structurally by electron microscopy studies and a role for EpsD as the exit pore through which the proteins are secreted, has been proposed (1, 4). However, the link between the outer membrane pore and the cytoplasmic membrane has been missing. In this thesis, I present three experimental results, two results directly supporting the interaction between EpsD and the periplasm-spanning cytoplasmic membrane protein EpsC:

(i) EpsD multimer is unstable in the absence of EpsC and vulnerable to proteolytic degradation (Figs. 2-2, 2-3 and 2-4);

(ii) EpsD can be stabilized by providing EpsC *in trans* (Figs 2-2, 2-3 and 2-4) but to form functionally active EpsD, EpsC has to be supplied *in cis* with EpsD (Figs. 2-6 and 2-7). This was demonstrated by effective complementation of the *V. cholerae epsC* mutant achieved only by supplying a plasmid expressing *epsCD* and ~1.2 kb sequence located upstream of the *epsC* gene (Fig. 2-6).

(iii) Not only the *epsCD*, but also an open reading frame (ORF) in the upstream sequence seems to play an important role in type II secretion of *V. cholerae* (Fig. 3-3). The ORF is a homologue of Hsp15 in

Escherichia coli, which is a novel ribosome-associated heat shock protein and the homologues are also found in many other Gram-negative bacteria (2, 3, 5).

The actual function of Hsp15 is unclear and its homologues have never been studied yet in organisms other than *E. coli*. Therefore, it is hard to predict the function of this putative Hsp15 ORF in *V. cholerae* from the known information of Hsp15 in *E. coli*. Although a direct evidence for its requirement in the secretion was not shown, its requirement for the complementation of the *epsC* mutant along with *epsCD* suggest that this Hsp15 homologue might be involved in interactions with EpsC and/or EpsD. The fact that at least two more species, *Aeromonas* and *Shewanella*, have the same conserved genome organization of this ORF with the type II secretion genes makes the involvement of this ORF in the secretion process more likely. Further characterization of this ORF will be worthwhile to help understand the type II secretion of *V. cholerae*.

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