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The dimer formed by the periplasmic domain of EpsL from the Type 2 Secretion System of *Vibrio parahaemolyticus*

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Abstract

The Type 2 Secretion System (T2SS), occurring in many Gram-negative bacteria, is responsible for the transport of a diversity of proteins from the periplasm across the outer membrane into the extracellular space. In *Vibrio cholerae*, the T2SS secretes several unrelated proteins including the major virulence factor cholera toxin. The T2SS consists of three subassemblies, one of which is the Inner Membrane Complex which contains multiple copies of five proteins, including the bitopic membrane protein EpsL. Here we report the 2.3 Å resolution crystal structure of the periplasmic domain of EpsL (peri-EpsL) from *V. parahaemolyticus*, which is 56 % identical in sequence to its homolog in *V. cholerae*. The domain adopts a circular permutation of the “common” ferredoxin fold with two contiguous sub-domains. Remarkably, this permutation has so far only been observed once before: in the periplasmic domain of EpsM (peri-EpsM), another T2SS protein which interacts with EpsL. These two domains are 18 % identical in sequence which may indicate a common evolutionary origin. Both peri-EpsL and peri-EpsM form dimers, but the organization of the subunits in these dimers appears to be entirely different. We have previously shown that the cytoplasmic domain of EpsL is also dimeric and forms a heterotetramer with the first domain of the “secretion ATPase” EpsE. The latter enzyme is most likely hexameric. The possible consequences of the combination of the different symmetries of EpsE and EpsL for the architecture of the T2SS are discussed.

Keywords

Extracellular protein secretion; General secretion pathway; Cholera; ETEC; Ferredoxin Fold

1. Introduction

Gram-negative bacteria use sophisticated protein machineries for the extracellular transport of a wide variety of proteins including virulence factors (Cianciotto, 2005). Examples of secreted proteins are cholera toxin, the infective agent of the human pathogen *Vibrio cholerae*, and heat-labile enterotoxin, a major virulence factor of enterotoxigenic *E. coli* (ETEC). These hetero-hexameric AB₅ toxins are exported in folded form (Hirst and Holmgren, 1987) across the outer membrane by the “Type 2 Secretion System” (T2SS). The T2SS is called the “Extracellular protein secretion” (Eps) system in *V. cholerae* and related species, and the “General secretory

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pathway” (Gsp) system in ETEC and other species (Filloux, 2004; Johnson et al., 2006; Pugsley, 1993; Sandkvist, 2001a; Sandkvist, 2001b; Sandkvist et al., 1993; Sandkvist et al., 1997; Tauschek et al., 2002). The T2SS is a complex machinery spanning the inner and outer membranes and consists of 11 or more different proteins, with many of these present in multiple copies. The nomenclature of the T2SS proteins is also quite complex. In this paper, proteins from the Eps system from *Vibrio* species are referred to as “Eps” followed by a capital letter, while the non-*Vibrio* T2SS homologs will be called “Gsp” followed by the same capital letter. For instance, the T2SS protein EpsL in the various *Vibrio* species is called GspL in other species.

The T2SS can be thought to consist of three sub-assemblies: the Outer Membrane Complex, the Pseudopilus, and the Inner Membrane Platform (Filloux, 2004; Johnson et al., 2006; Py et al., 2001). The main component of the Outer Membrane Complex is EpsD, which is thought to open and close during protein secretion. The Pseudopilus consists of five different pseudopilins, EpsG, EpsH, EpsI, EpsJ and EpsK, with EpsG the most abundant and therefore called the “major pseudopilin” of the T2SS. The Inner Membrane Platform consists of five proteins: the two bitopic membrane proteins EpsL and EpsM; the membrane-anchored EpsC; the integral membrane protein EpsF; and the membrane-associated “secretion ATPase” EpsE in the cytosol. The focus of this paper is EpsL.

Several components of the T2SS are related to components of the type 4 pilin biogenesis (T4PB) system (Filloux, 2004). T4PB systems are responsible for a diversity of functions including pilus assembly and disassembly, protein export, DNA import and phage entry. Studies of T2SS proteins are therefore in principle also useful for increasing our understanding of the T4BP system, but there are considerable differences between the two systems as well, in particular regarding the Inner Membrane Platform. For instance, the T4BP system does not contain sequence homologs of GspM, GspC and neither of GspL (Filloux, 2004). However, a functional homolog of GspL appears to exist in the T4BP system (Crowther et al., 2005).

EpsL is a 40 kDa bitopic inner-membrane T2SS protein. Its cytoplasmic domain binds to the cytoplasmic “secretion ATPase” EpsE (Sandkvist et al., 2000). EpsL also interacts with the inner membrane proteins EpsM and EpsF and with the pseudopilin EpsJ (Douet et al., 2004; Sandkvist et al., 1995; Sandkvist et al., 1999) thus bridging the Inner Membrane Platform with the Pseudopilus of the T2SS. So far, structural information at the atomic level has been published for the C-terminal domains of EpsE (Robien et al., 2003), the periplasmic domain of EpsM (peri-EpsM) (Abendroth et al., 2004b), the cytoplasmic domain of EpsL (Abendroth et al., 2004a), all the five pseudopilins (Kohler et al., 2004; Korotkov and Hol, 2008; Lam et al., 2009; Yanez et al., 2008a; Yanez et al., 2008b), the cytoplasmic domain of EpsL in complex with the N-terminal domain of EpsE (Abendroth et al., 2005), the two N-terminal domains from *Xanthomanas campestris* GspE (Chen et al., 2005), the PDZ domain of EpsC (Korotkov et al., 2006), the first cytoplasmic domain of EpsF (Abendroth et al., 2009), and the three N-terminal domains of GspD (Korotkov et al., 2009).

Here, we describe the 2.3 Å crystal structure of the periplasmic domain of EpsL (peri-EpsL) from *V. parahaemolyticus* (Vp), an organism occurring in brackish water which can cause gastrointestinal illness in humans when ingested. This species was chosen because of the difficulties encountered in our studies on the homolog of *V. cholerae*. The amino acid sequence identity of peri-EpsL for these two species is 56 %. The sequence identity of Vp peri-EpsL with the peri-GspL homolog from ETEC is 29 %. Therefore, structural studies on peri-EpsL from *V. parahaemolyticus* are of direct relevance for understanding the functioning of the T2SS in several important bacterial pathogens (Figure 1).

The structure reported here for Vp peri-EpsL reveals a surprising topological similarity between peri-EpsL and the periplasmic domain of EpsM (peri-EpsM), another protein from the Inner Membrane Platform. Both domains are cyclic permutations of the “ferredoxin fold” containing two similar contiguous domains, observed for the first time in peri-EpsM. Despite adopting similar folds, peri-EpsM and peri-EpsL form entirely different dimers. The cytoplasmic domain of EpsL is dimeric as well (Abendroth et al., 2004a; Abendroth et al., 2005). Since EpsL interacts with EpsE, which is most likely hexameric, possible modes of assembling EpsE and EpsL in the Inner Membrane Complex of the T2SS are discussed.

2. Materials & Methods

2.1 Cloning and Expression of *V. parahaemolyticus* peri-EpsL

The construct of *V. parahaemolyticus* peri-EpsL was created through PCR amplification from genomic DNA using the following primers:

sense: AAGGTCTCATATGTCTGGTGGCTCAACA

anti sense: GGTTCCTCGAGCTGACGCTTCAACAC

The PCR products were cloned in the pET21a(+) vector (Novagen) using *NdeI* and *XhoI* restriction sites.

Se-Met labeled *V. parahaemolyticus* peri-EpsL (Vp Per-EpsL), comprising residues 319-404 (see also Figure 2A), was prepared using methods as described (Van Duyne et al., 1993) by expression in BL21gold(DE3) *E. coli* cells. 20 ml of an overnight culture in Luria-Bertani broth (LB) containing 100 µl/ml carbenicillin were used after washing twice to inoculate a 1 l culture of M9 medium (50 mM Na / K-phosphate (pH 7.4), 0.5 g/l of sodium chloride, 1 g/l of ammonium chloride, 2 mM magnesium sulphate, 0.1 mM calcium chloride, 0.2% (w/v) glucose) containing 100 µg/ml of ampicillin. The cells were grown at 37°C. In order to inhibit the biosynthesis of methionine, thirty minutes before induction with 1 mM IPTG at $A_{600}=0.6$ an amino acid mixture of 125 mg each lysine-HCl, threonine, phenylalanine, and 65 mg each leucine, valine, and selenomethionine was added per liter of broth. The cells were harvested after over-night induction at 20°C and pelleted by centrifugation at 6000×g for 15 minutes.

2.2 Purification of *V. parahaemolyticus* peri-EpsL

The pelleted cells were suspended in 30 ml of lysis buffer: 50 mM Tris-HCl (pH 8.0), 150 mM sodium chloride, and 0.1 mM PMSF. The cells were lysed by sonication and the debris was pelleted by centrifugation at 20,000×g for 20 minutes. The lysate was purified by immobilized metal affinity chromatography: 3ml of Ni-NTA (Qiagen) resin were equilibrated with 20 mM Tris-HCl (pH 8.0), 150 mM sodium chloride, 0.1 mM PMSF, and 2 mM tris (2-carboxyethyl)-phosphate (TCEP) and then incubated with the clarified lysate at 4°C for 1 hour. Following incubation, the column was washed with 30 ml of the same buffer containing 15 mM imidazole and the target protein was eluted with 15 ml of 150 mM imidazole-containing buffer. The pooled fractions were concentrated to 5 ml by ultrafiltration (10 kDa cut-off, Millipore). Gel-permeation chromatography was carried out in 20 mM Tris-HCl (pH 8.0), 150 mM sodium chloride, 0.1 mM PMSF, and 2 mM TCEP buffer with Superdex S-200 resin in a XK 60/16 system (Amersham-Pharmacia). Peak fractions were pooled and concentrated to 7.5-30 mg/ml. Protein concentrations were determined through the Bradford assay (BioRad) at 595 nm using bovine serum albumin as a reference.

2.3 Crystallization of *V. parahaemolyticus* peri-EpsL

Initial crystallization conditions were screened using commercial screens Wizard I and II (deCODE Biosystems), PEG-Ion, Index, and Salt RX (Hampton Research), using the sitting-drop method of vapor diffusion by mixing equal volumes (1–2 μ l) of precipitant and protein at protein concentrations of 7.5–30 mg/ml. Initial hits (Index 19, 67, and 68) were refined and yielded the optimized crystallization conditions: (a) 1.2–1.4 M K/Na phosphate; (b) 25–30 % PEG 3350, pH 7.5–8.0, and 200 mM $(\text{NH}_4)_2\text{SO}_4$, respectively. Crystals appeared from 5 days up to several weeks after setting up the drops. For vitrification of the crystals, two different protocols were established: (i) crystals were transferred directly into a buffer containing 30 % glycerol or sequentially transferred into buffers containing increasing amounts of glycerol (10 %, 20 %, and 30 %); and, (ii) crystals were transferred directly into a buffer containing 30 % ethylene glycol or sequentially transferred into buffers containing increasing amounts of ethylene glycol (10 %, 20 %, and 30 %). The crystals were then vitrified in liquid nitrogen.

2.4 Data collection and structure solution of *V. parahaemolyticus* peri-EpsL

Data of a single crystal were collected at a wavelength of 0.97924 Å on beam line 9.2 at SSRL (Stanford Synchrotron Radiation Laboratory). A fluorescence scan was carried out in order to optimize the wavelength for a strong anomalous signal. The data were indexed, integrated and scaled with the HKL2000 suite (Otwinowski and Minor, 1997). Sites were searched for at 2.7 Å resolution with ShelxD (Schneider and Sheldrick, 2002), the correct hand was determined with ShelxE. The program Sharp (Bricogne et al., 2003) was used for the refinement of sites at 2.3 Å resolution and for phasing. For density modification using Solomon (Abrahams et al., 1996) and DM (Cowtan and Zhang, 1999) were employed. The good quality of the resulting experimental map allowed ARP/wARP (Perrakis et al., 1999) or Resolve (Terwilliger, 2000) to build ~150 residues. The model was completed manually in Coot (Emsley and Cowtan, 2004) and refined with Refmac5 (Murshudov et al., 1997). Seven TLS groups per chain were determined using the TLSMD server (Painter and Merritt, 2006). Tight NCS restraints were maintained throughout the refinement. Therefore, care was taken to assign the free reflections in thin resolution shells. Two elongated pieces of density were tentatively modeled as ethylene glycol since this was the cryo-protectant, three spherical densities were modeled as phosphates, most likely originating from the crystallization buffer.

3. Results

3.1 The structure of *Vibrio parahaemolyticus* peri-EpsL

Based on the limited proteolysis results of the *V. cholerae* protein (See Supplementary Text), a construct of *V. parahaemolyticus* peri-EpsL spanning residues 319–404, which corresponds to residues 318–403 of *V. cholerae* peri-EpsL, was cloned and expressed as Se-Met protein (Figure 2A). Size exclusion chromatography of Vp peri-EpsL yields an molecular weight of approximately 17.1 kDa (Figure 2B) and dynamic light scattering data showed that peri-EpsL forms dimers of approximately 19–21 kDa in solution (Figure 2C). Since the molecular weight of a single chain of Vp peri-EpsL is 10.5 kDa this indicates that the protein forms dimers in solution. The crystals obtained allowed the structure to be solved by SeMet SAD methods. The structure of *V. parahaemolyticus* peri-EpsL has been refined at 2.3 Å resolution to an R_{work} of 21.4 % and an R_{free} of 25.6 % with good geometry (Table 1). There are two chains per asymmetric unit, with each chain consisting of residues Ser322 to Gln404 in the final structure, since the first three residues of the expressed protein was not represented by electron density in the maps obtained. Since density for Asp392 was weak in both chains, this residue is probably quite flexible and was not incorporated into the structure.

Each Vp peri-EpsL chain folds as a compact globular unit and consists of two α -helices and four β -strands forming an anti-parallel β -sheet (Figure 1, 2D). The secondary structures

elements of the C-terminal periplasmic domain are labeled $C_{\alpha 1}$, $C_{\alpha 2}$ etc for the helices and $C_{\beta A}$, $C_{\beta B}$, etc for the strands in order to distinguish these secondary structure elements in the C-terminal periplasmic domain from helices $\alpha 1$, etc. and strands βA etc. in the cytoplasmic domain of EpsL (Abendroth et al., 2004a; Abendroth et al., 2005). The order of secondary structure elements is $C_{\alpha 1}C_{\beta A}C_{\beta B}-C_{\alpha 2}C_{\beta C}C_{\beta D}$. Each of the $\alpha\beta$ -units forms a compact sub-domain, with the two $\alpha\beta$ -halves related by a pseudo twofold axis that runs vertical to the β -strands such that the two helices are on the same side of the sheet (Figure 2D). The fold of Vp peri-EpsL is an untypical variant of the ferredoxin fold. While the generic ferredoxin-fold also consists of four α -helices and two β -strands and also forms two $\alpha\beta$ -units, the first of these $\alpha\beta$ -units is discontinuous and made up from an N-terminal α -helix and two β -strands from the C-terminus of the polypeptide chain. In contrast, each $\alpha\beta$ -unit in peri-EpsL is formed by a contiguous chain.

Although the two $\alpha\beta$ -units in peri-EpsL adopt a similar fold they are very different in sequence. Using the INDONESIA program (Madsen et al., 2002) the two domains superimpose with an r.m.s. deviation of ~ 1.8 Å for C^{α} atoms, with only one out 37 aligned residues being identical.

3.2 The dimer of peri-EpsL

Vp peri-EpsL forms dimers in the crystals, in agreement with the results of size exclusion chromatography and dynamic light scattering experiments (Figures 2B,C). The contacts between the two protomers in the asymmetric unit bury 872 Å² solvent accessible surface. This is the result, however, of rather limited interactions between the $\alpha 1$ -helices across the non-crystallographic twofold (not shown). An “A-A’ dimer” is formed by a crystallographic dyad and buries 1440 Å² solvent accessible surface, a considerably larger amount than between the two subunits in the asymmetric unit. Two anti-parallel β -strands from the two protomers in the A-A’ dimer form an anti-parallel arrangement, resulting in an eight-stranded β -sheet in the dimer (Figure 2D). In addition to the polar interactions of residues in strand $C_{\beta 1}$ (Thr347-Gly353) with the equivalent strand $C_{\beta 1}'$ of the other subunit in the dimer, a large portion of the dimer interface is hydrophobic and involves residues Leu328 and Leu331 from helix $C_{\alpha 1}$, and Phe349 and Tyr351 from strand $C_{\beta 1}$. Also, Thr323 from helix $C_{\alpha 1}$ and residues Ile346, Thr347, Gly353, are involved in polar interactions between A and A’. The A-A’ dimer interface ΔG , as calculated by PISA (Krissinel and Henrick, 2007), is -10.8 kcal/mol which is much more favorable than the $+0.5$ kcal/mol calculated for the interaction between the A and B subunits in the asymmetric unit. The A-A’ dimer is therefore most likely the dimer corresponding to that observed in solution, and will hereafter be referred to as the “peri-EpsL dimer”, shown in Figure 2D.

3.3 Dimers of peri-EpsL T2SS homologs

Several surface features of the Vp peri-EpsL dimer are quite striking. For instance, when viewed perpendicular along the twofold axis towards the β -sheet, a cluster of four positively charged residues is seen near the twofold, which is formed by two Lys 350 and two Arg 359 residues provided by two subunits (Figure 2E). The positive charge of these residues is conserved in the family of *Vibrio* EpsL sequences (Figure 1B) but not in the broader GspL family (Figure 1A). It could therefore be that these residues have a specific role in the T2SS of *Vibrio* species. A striking hydrophobic feature occurs on the other side of the Vp peri-EpsL dimer, where helices $\alpha 1$ are approaching each other (Figure 2E). The residues mainly responsible for this hydrophobic patch are Ala 326, Ala 332, Ala 333, Pro335 and Ala 336, from both subunits. Inspection of sequence alignments (Figure 1) shows that these residues are only poorly conserved, even among *Vibrio* species.

The interface residues in the Vp peri-EpsL dimer are, on the other hand, well conserved in the GspL family. For instance, Leu 328 is a Leu or Ile, once a Thr and once a Gly (Figure 1A). Leu 331 shows very little variability and is either a Leu or Met; Phe 349 is always a Phe, Ile or Leu; Tyr 351 either a Tyr, Phe or Trp; Ile 346 near the start of strand β -1 is a Ile, Leu, Val, or Met, and only once a Pro. Hence it seems likely that the Vp peri-EpsL dimer is conserved across the GspL family.

3.4 The closest structural homolog of peri-EpsL is peri-EpsM

Searches in the PDB for structural homologs, using the DALI (Holm and Sander, 1993), SSM (Krissinel and Henrick, 2004) and INDONESIA (Madsen et al., 2002) search algorithms, revealed, most unexpectedly, that the periplasmic domain of EpsM (Abendroth et al., 2004b) is the closest structural homolog of peri-EpsL with a DALI Z-score of 7.0 and an r.m.s. deviation of 2.1 Å for 71 structurally equivalent residues (Figure 3). Peri-EpsM is the only protein in the search with the same permutation of the ferredoxin fold as observed in peri-EpsL. While the fold and permutation of secondary structure elements of peri-EpsL and peri-EpsM are conserved (Figure 3), the structure-based sequence alignment of these two periplasmic domains yields 13 identical amino acids out of 71 equivalent residues, or ~ 18 % identity.

Even though the folds of the peri-EpsL and peri-EpsM domains are similar, their dimer arrangements are unrelated (Figure 3). In peri-EpsM, the dimer-interface of 916 Å² as calculated by PISA is formed by extensive contacts between residues from helices α 2 and α 2'. Despite the close proximity in space, residues from strands β 3 and β 3' in the interface, do not interact with one another leaving a cleft with a hydrophobic bottom in which an extra density from an as yet unknown molecule is clearly visible (Abendroth et al., 2004b). The peri-EpsL dimer interface is entirely different than that of the peri-EpsM dimer, with the former made up by residues from the N-terminal helices $C\alpha$ 1 and $C\alpha$ 1' and the N-terminal strands $C\beta$ A and $C\beta$ A', while the latter is made up by the C-terminal helices α 2 and α 2' without involving β -strand interactions. (Figure 3).

4. Discussion

4.1. "Fundamental ferredoxin" folds in EpsL and EpsM

As mentioned above, peri-EpsL folds into an unusual variant of the ferredoxin-fold. The ferredoxin-like fold is shared by an enormous variety of proteins (there are 44 superfamilies in the current SCOP database, see also (Abendroth et al., 2004b)). It is most intriguing that the $\alpha\beta\beta$ - $\alpha\beta\beta$ permutation of the ferredoxin fold found in peri-EpsL has so far only been observed for the periplasmic domain of EpsM (Abendroth et al., 2004b), the protein that binds to EpsL in the type 2 secretion system (Johnson et al., 2006; Sandkvist et al., 1999). In these variants of the ferredoxin fold, a pseudo twofold axis relates the two halves. In the vast majority of the structures with a ferredoxin fold, the chain goes forth and back between the two halves, resulting in a first domain made up of two discontinuous segments of the polypeptide chain. In the ferredoxin fold of peri-EpsL and peri-EpsM, the two $\alpha\beta\beta$ units in the first and second domains are both continuous. In that sense, this variant is simpler than the common ferredoxin fold, and we propose therefore to call it the "fundamental ferredoxin fold". The overall degree of sequence identity between peri-EpsL and peri-EpsM is ~ 18 % (Section 3.4) which might be a reflection of a common ancestor. In contrast, the cytoplasmic domains of these two bitopic inner membrane T2SS proteins have nothing in common whatsoever: the N-terminal domain of EpsM is only approximately 20 residues long, while the cytoplasmic domain of EpsL comprises about 240 residues and adopts a fold which is an unusual variant of the actin topology (Abendroth et al., 2004a; Abendroth et al., 2005).

4.2 Interactions of EpsM and EpsL in the periplasm

Several investigations by the Sandkvist group have shed light on the interactions between EpsL and EpsM: (i) residues 84-99 of *V. cholerae* EpsM, forming the N-terminal helix $\alpha 1$, are required for stable interactions with *V. cholerae* EpsL (Johnson et al., 2007), and (ii) the region formed by residues 216-296 of EpsL interacts with EpsM (Sandkvist et al., 2000). The latter residues are not covered by our peri-EpsL structure (Figures 1, 2A), hence this structure cannot provide detailed information about the mode of interaction of these two periplasmic domains of the T2SS. Since the EpsL-contacting N-terminal helix of EpsM resides in the periplasm, it is likely that the EpsM-contacting residues of EpsL also occur in the periplasm. This most likely then comprises all or parts of the region from residue ~ 276 , the end of the TM helix, to residue ~ 296 , the end of region implied by biochemical studies (Sandkvist et al., 2000), which is located approximately 10 residues before the beginning of the peri-EpsL domain. Since both peri-EpsM and peri-EpsL form dimers (Figure 3), a global suggestion as to how the two periplasmic parts of EpsM and EpsL may be assembled is that the twofold axes of the two dimers coincide. This possibility is sketched very schematically in Figure 4 (to be discussed in more detail in the next Section) with the EpsL dimer in green colors and EpsM in shades of yellow. The full details of the interactions of EpsL and EpsM in the periplasm obviously still require further investigations.

4.3 Dimers, Hexamers and Architecture of the T2SS Inner Membrane Complex

The Vp peri-EpsL dimer is likely to be representative for the T2SS of other species given the high degree of conservation of interface residues observed in the *V. parahaemolyticus* dimer (Section 3.2) and the yeast two-hybrid studies on the periplasmic domain of *Erwinia chrysanthemi* GspL which indicated that this domain interacts with itself (Py et al., 1999). The observations that the periplasmic domain of EpsL forms a dimer in solution (Figures 2B,C) and in crystals (Figure 2D), and that the cytoplasmic domain of EpsL (cyto-EpsL) forms a dimer in crystals (Abendroth et al., 2004a; Abendroth et al., 2005) and the cytoplasmic domain of the homolog from *E. chrysanthemi* forms homo-multimers according to yeast-two hybrid studies (Py et al., 1999), suggest that also full-length EpsL forms dimers in the T2SS.

EpsL is known to interact in the cytosol with EpsE, a “secretion ATPase” associated with the Inner Membrane Platform of the T2SS (Abendroth et al., 2005; Sandkvist et al., 1995; Shiue et al., 2006). The major domains of EpsE are N1, N2, C1 and C2, where we ignore for simplicity the small but essential metal-binding C_M domain (Possot and Pugsley, 1997; Robien et al., 2003). The precise multimeric nature of EpsE and its T2SS family members has been hard to establish. The crystal structure of *V. cholerae* EpsE missing the first domain, hereafter called also “ $\Delta N1$ -EpsE”, revealed a 6_1 helical arrangement of subunits (Robien et al., 2003). Biochemical studies (Camberg and Sandkvist, 2005) provided evidence for hexamer formation in solution but hexamers represented only a small fraction of the total EpsE population. However, the monomer-hexamer equilibrium could obviously be greatly affected by interactions of GspE with lipids and partners in the T2SS. Interestingly, in *X. campestris*, GspE hexamer formation is influenced by the binding of ATP analogs (Shiue et al., 2006).

It is also useful to look at other secretion ATPases, related to EpsE but not from T2SSs, which have been studied crystallographically (Savvides, 2007). Closest in sequence to the N2-C1-C2 domains of EpsE are the secretion ATPases GspE from *Archaeoglobus fulgidus* (Yamagata and Tainer, 2007) and PilT from *Aquifex aeolicus* (Satyshur et al., 2007). GspE and PilT share 22 and 34 % sequence identity with the N2-C1-C2 domains of *V. cholerae* EpsE, respectively. These studies indicate that these non-T2SS homologs of EpsE can form hexameric arrangements with cyclic C_6 symmetry, and also that these proteins can adopt multiple different conformations while remaining assembled as hexamers but with either C_2 or C_3 symmetry (Satyshur et al., 2007; Yamagata and Tainer, 2007). No structural information is available of

partners of the secretion ATPases GspE and PilT, but for the T2SS three crystal structures involving EpsE's partner EpsL are presently available: (i) the cytoplasmic domain of EpsL by itself (Abendroth et al., 2004a); (ii) the same domain in complex with "N1-EpsE", the first domain of EpsE (Abendroth et al., 2005); and, (iii) the periplasmic domain of EpsL reported in the current paper (Figure 2D). It is therefore of interest to look into the way in which a likely hexamer of the N2-C1-C2 domains of GspE may interact via their N1 domains with dimers of GspL in the Inner Membrane Platform of the T2SS. Additional key observations to be taken into account are:

- i. The linker between the N1 and N2 domains of GspE is sensitive to trypsin digestion and therefore probably highly flexible (Abendroth et al., 2005);
- ii. This flexible N1-N2 linker is approximately 18 residues long (Abendroth et al., 2005; Robien et al., 2003) which means that it can span a distance of 40 or more Ångstroms when in extended conformations;
- iii. The heterotetramer of the cytoplasmic domain of EpsL in complex with the most N-terminal N1 domain of the secretion ATPases EpsE (N1-EpsE), contains at its center a very similar dimer as seen in the structure of cyto-EpsL alone, with the N1-EpsE domains at the periphery of the heterotetramer (Abendroth et al., 2005). For convenience, this assembly of two cyto-EpsL domains plus two N1-EpsE domains will hereafter also be called the "heterotetramer".

Different options for combining a GspE hexamer with GspL dimers, while fulfilling the conditions that N1-GspE and cyto-GspL assemble as heterotetramers, that the TM helices of GspL cross the inner membrane, and that the periplasmic domains of GspL form dimers, include:

- i. the dimer axis of the "first" heterotetramer coincides with the sixfold axis of the hexamer. The heterotetramer would then "straddle" the hexamer, and is linked to two N2-C1-C2-GspE domains which are on opposite sides of the GspE-hexamer. This would be a possible arrangement for the addition of the first heterotetramer to the hexamer, but adding the second and third heterotetramer sharing the same twofold axis of the entire assembly is much less self-evident. The transmembrane helices of GspL are likely to clash in such an arrangement with the many N1-GspE and cyto-GspL domains crowded near the approximate twofold axis of the assembly (Supplementary Figure S1);
- ii. only one heterotetramer is associated with the hexamer, with their twofold and sixfold axes approximately aligned as outlined above, leaving the four remaining N1 domains of the GspE hexamer unoccupied – which would be a rather unusual arrangement (Supplementary Figure S1 top);
- iii. each heterotetramer is linked to two adjacent N2-C1-C2 domains in the hexamer, as sketched in Figure 4. The long linkers between the N1 and N2 domains in the cytoplasm make it possible to orient each of the heterotetramers such that the two TM helices of GspL in each heterotetramer can cross the inner membrane and allow dimerization of the periplasmic domains of GspL. In contrast to option (i) above, there is no difficulty in such an arrangement for the two TM helices of GspL to cross the inner membrane, since the three heterotetramers per assembly are far apart from each other.

For option (iii) above, there are two variants possible for dimerization of full length GspL. In a first variant, the *same* two GspL chains form the periplasmic dimer *and* the cytoplasmic dimer, as depicted in Figure 4. Alternatively, the periplasmic GspL dimer might be formed by GspL chains from two *different* cytoplasmic GspL dimers. In both variants, six GspL chains are combined with six GspE chains giving a GspE₆:GspL₆ stoichiometry.

Although no dimer formation has been observed by cyto-EpsL domains in solution (Abendroth et al., 2004a; Abendroth et al., 2005), the fact that cyto-GspL interacts with itself in yeast two-hybrid studies and forms dimers in crystals (Abendroth et al., 2004a; Abendroth et al., 2005; Py et al., 1999), combined with the observations that peri-GspL forms dimers in yeast two-hybrid studies (Py et al., 1999), in solution (Figures 2B,C) and in crystals (Figure 2D), makes it likely that cyto-EpsL forms dimers in full-length EpsL. Of course, whether one of the above-sketched options, or still other arrangements of GspE and GspL subunits, actually occurs in the inner membrane of the assembled T2SS, still needs experimental confirmation. In addition, GspL interacts with other T2SS proteins, including GspM, GspJ and GspF (Douet et al., 2004; Michel et al., 1998; Py et al., 1999; Py et al., 2001; Sandkvist et al., 1995; Sandkvist et al., 1999; Sandkvist et al., 2000; Shiue et al., 2006), in ways which still have to be unraveled.

Supplementary Material

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Acknowledgments

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Figure 1. Family Sequence alignments of peri-GspL domains

A. Representative examples from a broad family of GspL sequences, starting from about ten residues prior to the predicted TM helix (green bar). Shown are the *Vibrio parahaemolyticus*, *Vibrio cholerae*, *Vibrio vulnificus*, ETEC, EPEC, EHEC, *Aeromonas hydrophila*, *Klebsiella oxytoca*, *Erwinia chrysanthemi* and *Pseudomonas aeruginosa* GspL homologs. Note that the nomenclature for *P. aeruginosa* GspL homolog is XcpY (Filloux, 2004; Johnson et al., 2006). Horizontal arrows indicate β -strands and spirals α -helices in the peri-EpsL domain running from residue 319 to 403 in *V. parahaemolyticus* EpsL. The bar underneath the alignment indicates the degree by which residues are buried in the monomer. White is not solvent accessible; light blue intermediately accessible; dark-blue accessible.

Black asterisks underneath the alignment indicate key residues in the peri-EpsL dimer interface. Residues in white on red background are identical in all sequences shown; residues in black on a yellow background are homologous throughout the family of sequences depicted.

B. Representative examples of peri-EpsL sequences from a diversity of *Vibrio* species.

The secondary structure elements, interface residues and conservation of amino acids are indicated in a similar manner as in Figure 1A.

Figure 2. Structure of *V. parahaemolyticus* peri-EpsL

A. Domain diagram of *C* and *Vibrio cholerae* EpsL.

Crystal structures of the cytoplasmic domain have been determined previously for *V. cholerae* EpsL (Abendroth et al., 2004a; Abendroth et al., 2004b) with PDB codes 1w97 and 2bh1. The construct 319-404 of *V. parahaemolyticus* used in the current study which revealed the conformation of residues 322-404 of peri-EpsL are also indicated. In green the predicted transmembrane helix.

B. Size exclusion chromatography of Vp peri-EpsL. Size exclusion chromatogram, using Superdex 75 HR10/30 of a solution of 3.8 mg/ml *V. parahaemolyticus* peri-EpsL in 20 mM Tris-HCl buffer, pH 8.0, 150 mM NaCl. The peak eluted at 12.74 mL (blue line). In black letter

horizontally the elution volume in mL; in red lines and letters fraction numbers. Vertically the absorption at 280 nm in milli absorption units (mAU). Using a calibration curve based on five standard proteins, the estimated molecular weight of Vp peri-EpsL is 17.1 kDa.

C. Dynamic light scattering by Vp peri-EpsL. Dynamic light scattering profile of a solution of 3.8 mg/ml *V. parahaemolyticus* EpsL in 20 mM Tris-HCl buffer, pH 8.0, 150 mM NaCl, 150 mM imidazole, using a DynaPro instrument from Protein Solutions. Vertically the scattering intensity; horizontally the dynamic radius R_h in nm. The polydispersity of the solution is 12.5 %. The estimated molecular weight of Vp peri-EpsL is 18.9 kDa based on the dynamic radius, and 21.4 kDa by the volume shape hydration method, using software provided by the instrument manufacturer.

D. The Vp peri-EpsL dimer in the crystal. The direction of view is approximately parallel to the crystallographic twofold (indicated by a yellow elongated ellipse) generating the dimer (see also text) with the α -helices in front and the β -sheet in the back. The subunits A' and A are positioned left and right, respectively. β -strands are shown in red, α -helices in blue. The order of secondary structure elements in each subunit is $\alpha\beta\beta-\alpha\beta\beta$. The electron density for the loop between helix $C_{\alpha 2}$ and strand $C_{\beta D}$ was too weak and hence was not built. The anti-parallel four-stranded β -sheet per subunit is extended to form an eight-stranded anti-parallel β -sheet in the dimer.

E. Surface features of the dimer. *Right*, same direction of view as in C above along the (yellow) twofold axis, revealing a hydrophobic patch near the twofold axis at the dimer interface formed by residues from helices $C_{\alpha 1}$ and $C_{\alpha 1'}$. *Left*, view rotated 180 degrees compared to right, with a positively charged region close to the twofold axis near the dimer interface.

Figure 3. Comparison of EpsL and EpsM dimers

The *V. parahaemolyticus* EpsL and *V. cholerae* EpsM (Abendroth et al., 2004b) dimers side by side. Subunits A in the two molecules are aligned such that similarity in topology is evident and at the same time the differences between the dimers are clear. The EpsL dimers interact via helices $C\alpha1$ and $C\alpha1'$, and via the anti-parallel strands $C\beta1$ and $C\beta1'$. EpsM dimers, in contrast, interact via contacts between helices $\alpha2$ and $\alpha2'$.

Figure 4. Schematic drawing of possible architectures of the GspE:GspL subcomplex in the Inner Membrane Platform of the T2SS

The view is from the cytoplasm outwards. The hexamer of GspE (purple) and the three dimers of cyto-GspL (shades of green per dimer) shown in the center are in the cytoplasm. Predicted TM-helices of GspL are shown as hatched rods, located in the inner membrane. On the outside are depicted three peri-GspL dimers in the periplasm (in darker shades of green), which, in three dimensions, could be much closer together than this schematic suggests. A tentative position of the peri-GspM dimer, with its twofold axis aligned with that of the peri-EpsL dimer, is depicted in light and darker yellow. N1 = the N1 domain of GspE; N2-C1-C2 = the N2-C1-C2 domains of GspE; c-L = N-terminal cytoplasmic domain of GspL; p-L = C-terminal periplasmic domain of GspL; p-M = periplasmic domain of GspM. In the left lower part of the drawing, the buried solvent accessible surfaces in the N1-GspE:c-GspL, c-GspL:c-GspL, p-GspL:p-GspL and p-GspM:p-GspM interfaces are indicated. In the left upper part of the figure the approximate number of residues in different linkers is indicated. Comma's are added to the central ring of GspE N2-C1-C2 domains, and to the left upper N1-domains of GspE and both domains of the GspL and GspM dimers, to show the different symmetry relationships: approximate cyclic C6 symmetry for the GspE hexamer, dimeric symmetry for the cytoplasmic domains of GspL and the associated N1-GspE domains as well as for the periplasmic domains of GspL and GspM. This implies that the linkers between N1 and N2 domains of GspE have to adopt two quite different conformations in order to connect two adjacent N2-C1-C2 domains in the hexamer with the N1-domains in the N1-GspE:c-GspL heterotetramer. These linkers are shown in slightly different shades of color (Of course, in actual fact, the six N1-N2 linkers might have all six quite different conformations). The schematics are based, amongst others,

on the crystal structure of the dimer of *V. parahemolyticus* peri-EpsL (Figures 2D) and on several previous crystal structures (Abendroth et al., 2004a; Abendroth et al., 2004b; Abendroth et al., 2005; Robien et al., 2003). See also Section 4.3 in the main text.

Table1

Data collection	SSRL 9-2	
Wave length	0.97924Å	
Space group	P6 ₂	
Unit cell (Å)	a=b=87.71Å, c=45.59Å	
Mosaicity	0.5°	
	Overall	Highest shell
Resolution (Å)	50-2.30	2.35-2.30
hkl (measured)	101,999	6,477
Hkl (unique)	9,031	571
Completeness (%)	100.0	100.0
Redundancy	11.3	11.3
$\langle I \rangle / \langle \sigma I \rangle$	18.1	5.0
R-merge (%)	7.9	64.9
R-meas (%)	8.2	67.7
Phasing		
Number of Se sites	4	
FOM (SHARP)	0.37	0.17
FOM (DM)	0.85	0.73
Refinement (50 – 2.3Å)		
R-work (%)	21.75	
R-free (%)	25.61	
RMSD bonds (Å ²)	0.017	
RMSD angles (deg.)	1.615	
RMSD chiral (Å ³)	0.095	
Model validation		
Ramachandran outliers	0 (0%)	
Ramachandran allowed	0 (0%)	
Ramachandran preferred	152 (100%)	