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The crystal structure of a binary complex of two pseudopilins: EpsI and EpsJ from the Type 2 Secretion System of *Vibrio vulnificus*

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Abstract

Type II Secretion Systems (T2SS) translocate virulence factors from the periplasmic space of many pathogenic bacteria into the extracellular environment. The T2SS of *Vibrio cholerae* and related species is called the Extracellular protein secretion (Eps) system that consists of a core of multiple copies of eleven different proteins. The pseudopilins, EpsG, EpsH, EpsI, EpsJ and EpsK, are five T2SS proteins that are thought to assemble into a pseudopilus, which is assumed to interact with the outer membrane pore, and may actively participate in the export of proteins. We report here biochemical evidence that the minor pseudopilins EpsI and EpsJ from *Vibrio* species interact directly with one another. Moreover, the 2.3 Å resolution crystal structure of a complex of EpsI and EpsJ from *Vibrio vulnificus* represents the first atomic resolution structure of a complex of two different pseudopilin components from the T2SS. Both EpsI and EpsJ appear to be structural extremes within the family of Type 4a Pilin structures solved to date, with EpsI having the smallest, and EpsJ the largest, “variable pilin segment” seen thus far. A high degree of sequence conservation in the EpsI:EpsJ interface indicates that this heterodimer occurs in the T2SS of a large number of bacteria. The arrangement of EpsI and EpsJ in the heterodimer would correspond with a right-handed helical character of proteins assembled into a pseudopilus.

Keywords

General Secretion Pathway; Cholera; Pseudopilus; Type 4 Pilin Biogenesis

INTRODUCTION

Various toxins and virulence factors of Gram-negative bacteria are secreted from the periplasm into the extra-cellular environment by a sophisticated multi-protein machine, called the “Type 2 Secretion System” (T2SS) or the “General Secretory Pathway” (GSP), and in *Vibrio cholerae* the “Extracellular Protein Secretion” (Eps) system.^{1–6} Quite remarkably, the proteins are translocated by the T2SS in their folded state.^{7–11} The nomenclature of T2SS proteins from different species is rather complex.^{12,13} In the current paper, the eleven T2SS core proteins

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from the Eps system from *Vibrio* species are referred to as EpsC-EpsM. All non-*Vibrio* T2SS proteins will be referred to by a generic “Gsp” prefix followed by a capital letter, which indicates the specific T2SS protein. For example, EpsI and EpsJ are equivalent to GspI and GspJ from a species other than *Vibrio*.

The T2SS apparatus spans both the inner and outer membrane, and includes (i) a “protein conducting” pore in the outer membrane formed mainly by the “secretin” GspD; (ii) the “pseudopilins” in the periplasm (GspG, GspH, GspI, GspJ and GspK); (iii) several proteins in the inner membrane platform (GspC, GspF, GspL, GpsM); and (iv) an ATPase in the cytoplasm (GspE) which is associated with GpsL. The Eps system of *V. cholerae* consists of multiple copies of eleven different proteins, which secrete at least five virulence factors including a chitinase and the AB₅ heterohexamer of cholera toxin (CT).^{11,2} We have reported previously on crystal structures of several T2SS proteins (Refs14-19) to further our understanding of this machinery in three-dimensions at atomic resolution.

Many Gsp proteins share homology with components of the Type IV Pilus Biogenesis (T4PB) system.²⁰⁻²³ The T4PB system regulates the assembly, extension and retraction of Type 4 Pili (T4P) from the cell poles of many gram negative bacteria.²⁴ Type 4 pilins are the subunits of T4P fibers and are characterized by:²⁴⁻²⁷ (i) a pre-pilin leader sequence that is cleaved off by a pre-pilin peptidase yielding mature protein;^{28,29} (ii) an extended hydrophobic N-terminal α -helix, which is thought to anchor pilins into assembled pili and into a membrane when pili are not assembled;¹² and (iii) a conserved glutamate at position +5 of the mature pilin sequence thought to be involved in the assembly of pilins into a pilus. Type 4 pilins can be further divided into subclasses of Type 4a pilins (T4aP) and Type 4b pilins, with T4aP's displaying a shorter pre-pilin leader sequence and higher sequence conservation in the N-terminal α -helix than the Type 4b pilin subclass.^{26,27}

The T2SS components GspG, GspH, GspI, GspJ and GspK share several characteristic features with T4aP's and are therefore called “pseudopilins”. The pseudopilins are thought to assemble into a T4P-like “pseudopilus” that plays a key role in the T2SS.^{23,20} Sauvonnnet *et al.*³⁰ first reported the presence of a pseudopilus resulting from the overexpression of T2SS genes from *Klebsiella oxytoca*. Pseudopili are thought to function either as a retractable “plug” for the outer membrane pore, GspD, and/or as a “piston” that actively pushes secreted proteins through the GspD pore.^{31,32,30,33} Similarities between pseudopilins and T4aP are that they both contain a hydrophobic N-terminus²⁷ and a pre-pilin leader sequence that is cleaved off by a pre-pilin peptidase.^{34-37,29,38} The four pseudopilins GspG-J also contain the conserved Glu at position +5 of the mature pilin sequence^{39,12}, which is thought to play a role in pilin-pilin interactions in the assembled fiber.⁴⁰

In Type 4 Pili, one pilin component is predominant and therefore referred to as the “major pilin”. In addition, there are also several “minor pilin” components, which are thought to localize to the tip of pili, or to the inner and/or outer membranes, and likely mediate extension and retraction events associated with pilus function.^{41,21,42} Recently, the minor pilin PilX has been shown to be an integral component of the Pile pilus of *Neisseria meningitidis*.⁴³ Interactions between major and minor pilins, and between different minor pilins, are thought to be critical for the biogenesis and proper functioning of the T4PB system.

The organization of a T2SS pseudopilus is thought to be similar to that of a Type 4 Pilus, with GspG being referred to as the “major” pseudopilin, since several studies have shown that GspG is the predominant component of the pseudopilus, while GspH, GspI, GspJ and GspK are called “minor” pseudopilins.^{36,44,45,30,46,47,12,13} Nunn and Lory³⁶ reported a ratio of 16:1:1:4 for the GspG, GspH, GspI and GspJ pseudopilins of *P. aeruginosa*. The minor pseudopilins are thought to localize to the inner and/or outer membranes and regulate pseudopilus assembly

and function.⁴⁸⁻⁵⁰ All four minor pseudopilins have been shown to be essential for the functioning of the T2SS, with GspI required for pseudopilus assembly while the minor pseudopilins GspH, GspJ and GspK are dispensable for pseudopilus formation.^{30,46} Several studies have reported specific interactions between different pseudopilins. The major pseudopilin GspG of *P. aeruginosa* was reported to form homodimers and heterodimers with the minor pseudopilins GspH, GspI and GspJ.⁵¹ A yeast-two-hybrid study from *Erwinia chrysanthemni* reported a direct interaction between the minor pseudopilins GspI and GspJ.⁵² However, a Ni-affinity chromatography study from *X. campestris* reported that GspI and GspJ alone did not co-elute together, and instead required the presence of GspH to associate with one another.⁴⁸ Diverging results such as these, may result from variations between the T2SS's of different species, or may be due transient pseudopilin interactions that occur during different stages of T2SS assembly or functioning.^{12,13} Overall, specific interactions between different pseudopilins are likely to be critical for the biogenesis and assembly of T2SS pseudopili.

Here, we report biochemical evidence that the globular domains of the minor pseudopilins EpsI and EpsJ from the T2SS of several *Vibrio* species, including *Vibrio cholerae*, interact directly with each other. This is confirmed by our 2.3 Å resolution crystal structure of the EpsI:EpsJ heterodimer from *V. vulnificus*. *V. vulnificus* is a salt-loving bacterium that can cause disease when eating contaminated seafood or when an open wound is exposed to seawater. In immunocompromised persons, *V. vulnificus* can infect the bloodstream, causing a severe and life-threatening illness.^{53,54} Secretion of by several proteins has been reported for *V. vulnificus*,^{55-58,53} and its prepilin peptidase vvpD shows 71 % sequence identity with the homologous peptidase vcpD from *V. cholerae*. Moreover, vvpD deletion mutants of *V. vulnificus* were defective in secretion of three enzymes: the cytolysin/hemolysin, a protease, and a chitinase.⁵⁹ Also the organization of the *eps* gene cluster is the same in all *Vibrio* species. Therefore it is likely that studies on components of the *V. vulnificus* T2SS are highly relevant for the T2SS from other *Vibrio* species, including *V. cholerae*.

So far, there were no reported three-dimensional structures of two different pseudopilin components from the T2SS in a complex, nor any structures of two different Type 4 Pilin components from the T4PB system forming a complex. Therefore, the *V. vulnificus* EpsI:EpsJ heterodimer structure described here provides key insight into protein-protein interactions that are likely critical to the biogenesis and function of pseudopili in the T2SS, and most likely also of Type 4a Pili in the T4PB system.

RESULTS

EpsI and EpsJ in solution

Truncating the first 24 N-terminal residues from *V. cholerae* EpsI and EpsJ yielded soluble proteins suitable for interaction studies. The interaction between *V. cholerae* EpsI and EpsJ was identified via Ni-affinity chromatography studies and by native gels (Figs. 1a,b). Gel-permeation chromatography measurements showed a peak for *V. cholerae* EpsI:EpsJ corresponding to a MW of approximately 30 kDa. The SDS-PAGE analysis of peak fractions suggests that EpsI and EpsJ co-elute at a ratio of approximately 1:1 (Figure 1(c)). Therefore we likely observe a heterodimer of EpsI:EpsJ consisting of one EpsI subunit and one EpsJ subunit, since the MW of truncated EpsI is 9.7 kDa and that of truncated EpsJ is 21.0 kDa. There is also a “shoulder” in front of the main peak on the size exclusion chromatography profile, indicating that a fraction of EpsI-EpsJ is in a higher order complex, possibly a heterotetramer (EpsI₂-EpsJ₂).

Crystal Structure Determination

Out of a total of 18 different EpsI:EpsJ constructs that were expressed, purified and tested for crystallization, only one variant (*V. vulnificus* EpsI with 24 N-terminal residues removed and *V. vulnificus* EpsJ, similarly truncated at the N-terminus plus a truncation at the C-terminus, with the double mutation E96T/K97T in the EpsI), yielded crystals suitable for structure determination. (Hereafter we will use “EpsI” and “EpsJ” for the truncated variants of these two proteins). These surface entropy reduction mutations were inspired by the work of the Derewenda group.^{60,61} *V. vulnificus* EpsI:EpsJ crystals of dimensions 200×100×50 μm belong to space group *P*1 and contain four VvEpsI:EpsJ heterodimers in the asymmetric unit. The VvEpsI:EpsJ structure was determined using Se-Met single-wavelength anomalous diffraction (SAD) phasing and refined to 2.3 Å resolution with an eventual $R_{\text{work}} = 18.7\%$, $R_{\text{free}} = 24.7\%$ and good geometry (Table 1). The final VvEpsI:EpsJ model contains a total of eight protein molecules with 954 residues, 595 water molecules and 2 chloride ions per asymmetric unit. The four VvEpsI:EpsJ heterodimers per asymmetric unit are very similar and their C $^{\alpha}$ carbons can be pairwise superimposed with an r.m.s. deviation between 0.3-0.5 Å.

The structure of EpsI

The fold of VvEpsI consists of one N-terminal α -helix (Val31-Leu53) followed by four anti-parallel β -strands (β 1- β 4) arranged within one β -sheet (Figures 2, 3). A loop connecting α 1 to β 1 of VvEpsI has several residues (Asn54-Leu58) that are either partially or fully disordered in several of the four VvEpsI monomers in the asymmetric unit. The EpsI fold appears to miss almost entirely the “variable segment” described in other pilin and pseudopilin structures. (Refs.^{62-66,19}).

A search for structurally homologous proteins using the EBI-SSM server^{67,68} failed to identify the major pseudopilin *K. oxytoca* GspG (PDB code 1T92)⁶⁶ as a related structure. Both VvEpsI and *K. oxytoca* GspG contain an N-terminal α -helix and one β -sheet with four antiparallel β -strands (Figure 4). However, the β -sheet topologies in VvEpsI and *K. oxytoca* GspG are such that only three β -strands (β 2, β 3 and β 4 in VvEpsI and β 1, β 2 and β 3 in *K. oxytoca* GspG) are equivalent in the two proteins (Figure 4). A superposition based on 22 residues within the three equivalent strands of VvEpsI and *K. oxytoca* GspG found a total of 42 equivalent C α residues with an r. m. s. deviation of 2.2 Å and 7% sequence identity. Residues that were structurally equivalent include 18 of the 24-residue long N-terminal α -helix of VvEpsI. This demonstrates that the angles between this helix and β -sheet I of VvEpsI and between the N-terminal helix and the β -sheet of *K. oxytoca* GspG are relatively similar.

In contrast, VvEpsI and the minor pseudopilin EpsH from *V. cholerae* (VcEpsH)¹⁹ are very different. A superposition based on 22 equivalent residues within the conserved C-terminal domains (i.e. β 2, β 3 and β 4 of VvEpsI and β 5, β 7 and β 8 of VcEpsH) yielded 26 structurally equivalent C α residues with an r. m. s. deviation of 1.4 Å and 19% sequence identity. In other words, only four additional residues followed the superposition operation of the C-terminal domain comparison. These four extra residues also reside within the C-terminal β -sheet of VvEpsI and VcEpsH. The lack of N-terminal residues that could be superimposed between the two structures is due to large differences in the angle between α 1 and the C-terminal β -sheets in VvEpsI and VcEpsH (Figure 4).

A comparison of the topology of VvEpsI with all known pseudopilin and T4aP structures (Figure 4) shows that all five previously solved structures, including a major and minor pseudopilin (*K. oxytoca* GspG⁶⁶ and VcEpsH¹⁹) from the T2SS, and three major Type 4a pilins from the T4PB system (*P. aeruginosa*^{PAK} PilA,⁶² *N. gonorrhoeae* Pile⁶² and *P. aeruginosa*^{K1224} PilA⁶⁵), share the same, yet very variable, Type 4a Pilin Fold, defined by a N-terminal α -helix (α 1), a variable segment, and a C-terminal β -sheet composed of four

structurally conserved anti-parallel β -strands^{26,27} (colored green in Figure 4). While the new sixth member of the T4aP family, VvEpsI, also contains an N-terminal α -helix and a C-terminal β -sheet, the topology of the VvEpsI β -sheet differs from the five remaining T4aP structures (Figure 4(b)). VvEpsI is missing the fourth of four structurally conserved β -strands within the T4aP fold and, instead, contains an extra β -strand (β 1), located prior to the first of three structurally conserved β -strands.

The program EBI-SSM⁶⁷ revealed that the closest structural homologue of VvEpsI is the C-terminal outer membrane translocator domain of the autotransporter Hia from *Haemophilus influenzae* (PDB code 2GR8).⁶⁹ The Hia translocator is a trimeric β -barrel, made up of three monomers, each with one α -helix followed by four anti-parallel β -strands, with the same topology as VvEpsI. One monomer of the C-terminal domain of HIA superimposes onto one monomer of VvEpsI with an r.m.s. deviation of 2.6 Å for 46 C α residues and 20% sequence identity. When a trimer of VvEpsI subunits is constructed by superimposing VvEpsI onto each monomer of the Hia trimer, significant clashes occur at the C-terminal end of the α -helix of VvEpsI. This suggests that VvEpsI most likely does not form a trimer similar to the one formed by the C-terminal outer membrane autotransporter domain of *H. influenzae* Hia.

The structure of EpsJ

The overall fold of VvEpsJ consists of one N-terminal α -helix (α 1) followed by 10 β -strands, of which 9 occur within two β -sheets, plus a short strand β 2' which is not part of a β -sheet (Figures 2, 3 and 4). Following the N-terminal helix α 1 of VvEpsJ, five strands (β 1- β 5) form anti-parallel β -sheet I, and four strands (β 6- β 9) form the second anti-parallel β -sheet II (Figures 3 and 4). A loop after strand β 2 forms a β -turn, which connects back to β 3 of β -sheet I. A second β -turn follows the first strand (β 6) of β -sheet II after which the chain proceeds as a short strand β 7, followed by a short loop that connects back to the second strand (β 8) of β -sheet II (Figure 3 and 4).

A search for structural homologues of VvEpsJ with EBI-SSM⁶⁷ failed to identify homologues that could be superimposed with an r.m.s. deviation of less than 3.5 Å. Like VvEpsI, VvEpsJ shares the N-terminal α -helix and three of the four structurally conserved β -strands with the T4aP fold (Figure 4). Also similar to VvEpsI, VvEpsJ is missing the fourth of the canonical anti-parallel β -strands that occurs in each of the five known T4aP-like structures previously mentioned. A superposition based on three strands of VvEpsJ (β 6, β 8 and β 9) onto the equivalent strands (β 1, β 2, and β 3) of the major pseudopilin *K. oxytoca* GspG⁶⁶ resulted in 29 equivalent residues, all located within the C-terminal β -sheet, with an r.m.s. deviation for the C α atoms of 2.9 Å and 7% sequence identity. The angle between α 1 and the conserved β -sheet of VvEpsJ and *K. oxytoca* GspG is relatively similar (Fig. 4a). However, the helix of VvEpsJ is slightly further away from the conserved β -sheet of VvEpsJ than is the case in *K. oxytoca* GspG. Therefore, residues from the N-terminal α -helix of VvEpsJ and *K. oxytoca* GspG were not added to the residues used for the superposition of these two pseudopilins.

A superposition based on three strands (β 6, β 8 and β 9) of VvEpsJ and the equivalent strands (β 5, β 7 and β 9 for VcEpsH) of the minor pseudopilin VcEpsH (Yanez *et al.*, submitted number), resulted in the superposition of 53 C α residues. However, the topologies of the β -sheet of VcEpsH and the variable β -sheet VvEpsJ differ since several strands (β 2, β 3 and β 4 of VvEpsJ and β 1, β 2 and β 4 of VcEpsH) run in opposite directions (Fig. 4(b)). Therefore, only 27 C α residues from the conserved β -sheet are structurally equivalent between these two minor pseudopilins, with a 2.2 Å r.m.s. deviation and 15% sequence identity. Overall, both VvEpsI and VvEpsJ appear to be extremes within the family of T4aP structures solved to date, with VvEpsI having the smallest, and VvEpsJ the largest “variable pilin segment” (Figure 4).

Sequence Conservation of EpsI and EpsJ

The EpsI/GspI and EpsJ/GspJ family sequence alignments with nine homologues of VvEpsI and VvEpsJ are shown in Figure 2. In the EpsI/GspI and EpsJ/GspJ families, 19% and 15% of the position are occupied by identical amino acid residues, respectively. The pairwise sequence identity varies between 71% to 34% for the EpsI/GspI family and between 72% to 37% within the EpsJ/GspJ family.

There are eleven invariant residues present within the globular domain of VvEpsI (Figure 2). The highest degree of sequence conservation in VvEpsI is in the N-terminal α -helix (α 1). For VvEpsJ, twenty-one residues are totally conserved within the VvEpsJ/GspJ family, excluding the 24 N-terminal residues that are not present in our structure. The N-terminal helix α 1 of VvEpsJ shows a lower degree of sequence conservation than α 1 of VvEpsI, and contains only two absolutely conserved residues (Gln45 and Asp54) and one residue that is always a positively charged residue in all EpsJ/GspJ variants (Arg40 in VvEpsJ) (Figure 2). Interestingly, sixteen of the twenty-one invariant residues in VvEpsJ²⁵⁻¹⁹⁸ are located within the variable pilin domain (Figure 2). Typically, the variable pilin domain of the T4aP's and the pseudopilins consists of the least conserved residues of the pilin sequence.⁶² In contrast, the variable pilin domain of VvEpsJ shows several regions of high sequence conservation (Figure 2).

Structure of the VvEpsI:EpsJ Heterodimer

The interface between VvEpsI and VvEpsJ (Figure 3(b)) buries 1450 Å² accessible solvent area, which is considerably above the mean of 983 Å² for heterodimer interfaces as analyzed by Jones and Thornton.⁷⁰ The gap volume index (GVI), a measure of the complementarity of surfaces,⁷⁰ of the VvEpsI:EpsJ heterodimer is 2.6 Å, which is essentially the same as the average for physiologically relevant heterodimers.⁷⁰ Fourteen residues in VvEpsI and eighteen residues in VvEpsJ (Figure 5(c)) are engaged in a large number of hydrophobic interactions and five H-bonds between VvEpsI and VvEpsJ. Importantly, several invariant residues are located in the EpsI:EpsJ interface, including I-Glu35, I-Ala68, I-Gly69, I-Asn46, I-Leu53, plus J-Gln45, J-Trp158 and J-Arg186 (Figures 2, 5 and 6).

The VvEpsI:EpsJ interface can be considered as having three components:

- 1) "Helix-helix" interactions (Figure 5(d)) between six residues along α 1 of VvEpsJ (Arg37, Thr38, Leu41, Leu44, Gln45 and Leu48) and six residues along α 1 of VvEpsI (Val31, Leu34, Glu35, Met38, Phe39, Met42). Nine of the twelve "helix-helix" contact residues are either 100% conserved or consistently a hydrophobic residue within ten EpsI/GspI and EpsJ/GspJ homologues.
- 2) "Strand-helix" interactions between six residues (Tyr181, Ile184, Ala185, Arg186, Thr187, Leu189) along the third strand (β 9) of the conserved β -sheet II of VvEpsJ and six residues (Glu35, Phe39, Met42, Asn46, Ala48, Met50) along α 1 of VvEpsI. Here, there are both hydrophobic and polar contacts, as the 12 contact residues in VvEpsI and VvEpsJ form four H-bonds and five Van der Waals contacts between VvEpsI and VvEpsJ. Especially interesting is the invariant I-Asn46, whose sidechain forms multiple H-bonds with atoms of VvEpsJ. The O^{δ1} of I-Asn46 is engaged in hydrogen bonds with the guanidinium group of the invariant J-Arg186 and with the main chain N of J-Thr187. Moreover, the N^{δ2} of I-Asn46 forms an H-bond with the main chain oxygen of J-Ala185. In addition, four of the five "strand-helix" Van-der-Waals interactions are between residues that have a hydrophobic nature within all ten EpsI/GspI and EpsJ/GspJ homologues. Therefore, all hydrogen bonds, and four of the five hydrophobic interactions between β 10 of VvEpsJ and α 1 of VvEpsI are consistently present within the EpsI/GspI and EpsJ/GspJ homologues shown in Figure 2.

3) “Strand-strand” interactions between four residues (Tyr181, Lys183, Ala185, Ile184) from strand $\beta 9$ of VvEpsJ and three residues (Leu67, Ala68, Gly69) from strand $\beta 1$ of VvEpsI. Here, one H-bond is formed between a main chain N of an invariant I-Ala68 and a main chain oxygen of J-Lys183. In addition, Van-der-Waals interactions occur between I-Leu67 and J-Ala185, I-Ala68 and J-Ile184, and I-Gly69 and J-Tyr181. All six residues involved in these Van-der-Waals contacts are hydrophobic in all ten EpsI/GspI and EpsJ/GspJ homologues.

Overall, the interface between VvEpsI and VvEpsJ is highly conserved with a large number of hydrophobic residues involved in heterodimer formation (Figure 5(c)). An electrostatic surface representation of the interaction surface of VvEpsI and VvEpsJ (Figure 5(b)) shows that a small negatively charged surface region on VvEpsI contacts a small positively charged surface patch on VvEpsJ, with the invariant VvEpsI-Asp45, which is either an aspartate or a glutamate in all ten EpsI/GspI homologues (Figure 2), playing a central role. Therefore, in addition to hydrophobic interactions, electrostatic complementarity between VvEpsI and VvEpsJ appears to be a key element of the EpsI:EpsJ interface.

The “inside” and “top” views of the VvEpsI:EpsJ heterodimer show that VvEpsI and VvEpsJ form a tight complex with the two conserved β -sheets of these pseudopilins arranged like “wings” with a significant cleft between the two pseudopilins (Fig. 6). While the sequence conservation of the “outside” surface of the VvEpsI:EpsJ heterodimer is relatively low the “inside” surface of VvEpsI:EpsJ shows a higher degree of sequence conservation (not shown). The “inside” surface of the VvEpsI:EpsJ heterodimer appears to contain several conserved residues provided by both EpsI and EpsJ (Figure 6(a)) which may indicate an important function for this convoluted surface area of the heterodimer.

The crystallographic EpsI:EpsJ heterotetramer

One asymmetric unit of the *P1* crystals contains eight molecules of VvEpsI and VvEpsJ arranged into two I_2J_2 heterotetramers. The two I_2J_2 tetramers are nearly identical and can be superimposed with an r. m. s. deviation of 0.4 Å for all C α atoms.

The largest protein-protein interface within the heterotetramer occurs between VvEpsJ and VvEpsJ' and buries 1860 Å² with a gap volume index ⁷¹ of 3.0 Å. Twenty-six residues in VvEpsJ and the same residues in VvEpsJ' form a large number of hydrophobic contacts and three H-bonds (residues colored in red - Figure 2). There are two ions present in the VvEpsI:EpsJ crystal structure. One ion is coordinated between the N^ζ1 of J-Arg93 and the N^ζ1 of J'-Arg93, with N to chloride distances between 3.1-3.3 Å. The second ion is coordinated in the equivalent position, between J and J', of the second I_2J_2 tetramer in the asymmetric unit. Because of the presence of 100 mM MnCl₂ in the crystallization buffer this ion was modeled as a chloride ion. Interestingly, EpsJ-Arg93 is consistently an arginine within the family of ten EpsJ/GspJ homologues shown Figure 2.

The interface between VvEpsI and VvEpsJ' (which is obviously the same as the interface between VvEpsI' and VvEpsJ) buries 530 Å² with, however, a very large gap volume index ⁷⁰ of 7.0 Å involving nine VvEpsI and six VvEpsJ residues (Fig. 2), and is therefore not likely contributing significantly to the stability of the heterotetramer.

DISCUSSION

Crystal Structure

The crystal structure determination of the VvEpsI:EpsJ heterodimer was far from straightforward and only succeeded by applying a combination of: (i) species variation; (ii) surface mutagenesis; and (iii) truncation of C-terminal residues (See Materials and Methods).

The Surface Entropy Reduction mutations in EpsI (E96T, K97T) were crucial in obtaining the X-ray diffraction quality crystals since the two threonines are situated in the loop between $\beta 3$ and $\beta 4$ of EpsI and crystal contacts in all 4 copies in asymmetric unit. These two surface mutations are approximately 16 Å removed from the VvEpsI:EpsJ interface and are not expected to affect the EpsI:EpsJ heterodimer interactions in a significant way. Eventually a 2.3 Å structure was obtained with eight polypeptide chains, and nearly 1000 residues, in the asymmetric unit showing four essentially identical EpsI:EpsJ heterodimers with tight intersubunit interactions.

The EpsI:EpsJ Heterodimer

We determined through both *in vitro* experiments and by solving a crystal structure that a complex forms between the minor pseudopilins EpsI and EpsJ from *V. cholerae* and *V. vulnificus*, which does not require the presence of the first twenty-four N-terminal residues of these proteins. Our results agree with a yeast-two hybrid study by Douet *et al.*⁵² who reported interactions between EpsI and EpsJ from *E. chrysanthemi* using very similar truncated variants of GspI and GspJ as in our current study.

An analysis of the sequence conservation of the VvEpsI:EpsJ interface shows that twelve of the fifteen hydrophobic contacts and all five H-bonds between VvEpsI and VvEpsJ are invariant within a family of ten EpsI/GspI and EpsJ/GspJ homologues (Figs. 2 and 5). There is therefore a high probability that the observed *V. vulnificus* EpsI:EpsJ heterodimer (Fig. 3) is of physiological importance in the T2SS apparatus of a large number of species.

Although by no means certain, the EpsI:EpsJ dimer observed (Fig. 6) may reflect features of interactions between pseudopilins assembled into a pseudopilus, where the N-terminal helices are generally believed to come together near the fiber axis.^{63,66,72,27} The N-terminal parts of the N-terminal α -helix of the pseudopilins are probably crucially important for the interactions between adjacent pseudopilin molecules in the pseudopilus center, but the high degree of sequence identity of this region (mostly consisting of aliphatic residues, see e.g. Hansen and Forest,²⁷ may not provide enough information for specific assembly of the pseudopilins. Thus, the specificity of assembly is probably provided mainly by the globular part of the pseudopilin molecules, and our EpsI:EpsJ heterodimer may be a first high-resolution structure revealing such specific interactions in the case of two minor pseudopilins.

The shape of the EpsI:EpsJ heterodimer is remarkable (Fig. 6). The C-terminal β -sheets of the two pseudopilins point in a similar direction, roughly perpendicular to the N-terminal helices, creating a deep crevice between them of irregular shape. Several residues on the surface of this crevice are highly conserved (Fig. 6a). Since the Y2H studies of Douet *et al.*⁵² reported that, in *E. chrysanthemi*, the homolog of EpsJ not only interacts with the homolog of EpsI, but also with the homologs of EpsD and EpsL, it is well possible that one or more of these proteins bind to the EpsI:EpsJ dimer in this crevice. Since not all T2SS periplasmic domains were included in this Y2H study, other T2SS proteins may (also) interact with this deep crevice during the assembly or functioning of this dynamic secretion apparatus.

The crystallographic EpsI₂:EpsJ₂ heterotetramer

The crystal structure contains in the asymmetric unit an EpsI₂:EpsJ₂ tetramer of approximately 65 kDa. The question remains whether this heterotetramer is of relevance for the functioning or formation of the T2SS. The EpsJ:EpsJ' interface within a tetramer buries a solvent accessible surface of 1860 Å². The GVI of 2.99 Å for the EpsJ:EpsJ' interface is somewhat at the high end of the expected GVI range for physiological dimers.^{71,70} An analysis of the sequence conservation of the EpsJ:EpsJ' interface shows that of the twenty-six VvEpsJ residues involved in the inter-subunit contacts, sixteen show low to moderate sequence conservation within the

EpsJ/GspJ family (Fig. 2). Overall, the sequence conservation of the EpsI:EpsJ' interface is significantly lower than that of the EpsI:EpsJ heterodimer interface. In addition, the helices in one IJ dimer make an angle of $\sim 60^\circ$ with that of the other IJ dimer in the tetramer (Fig. 3(a)). The cryo-electron microscopy structure of the PilE pilus from *N. gonorrhoeae*⁶³ indicates that the N-terminal helices of all pilin subunits within one pilus are approximately parallel to one another and also to the direction of the fiber axis. The organization of the four helices in the I₂J₂ tetramer therefore does not resemble the arrangement of the helices of four subsequent subunits in the PilE fiber model. Taking all these factors together, it is unlikely that the crystallographic EpsI₂:EpsJ₂ tetramer is physiologically relevant.

The EpsI:EpsJ dimer and the Pseudopilus

There are two Type 4-like Pilin models described in the literature, both obtained from cryo-electron microscopy studies. One model is for GspG from *K. oxytoca*, with a resolution of 25 Å,⁶⁶ and the other is for PilE from *N. gonorrhoeae*, with a resolution of 12.5 Å.⁶³ While the *K. oxytoca* GspG model suggests a left-handed one-start helix assembly for the pseudopilus, the model of the type 4 pilus formed by PilE is right-handed.

The number of EpsI and EpsJ molecules in an assembled pseudopilus and their precise location are still largely unknown. However, Nunn and Lory³⁶ reported that in the pseudopilus of *P. aeruginosa* the EpsI and EpsJ homologs are present in the pseudopilus, although in much smaller numbers than the EpsG homolog. The minor pseudopilins have been located in the inner and outer membrane and regulate assembly and function of the pseudopilus.^{49,50,48} Interestingly, in *X. campestris* Ni-affinity chromatography experiments have indicated that GspG interacts with GspI.⁴⁸ Therefore the EpsI:EpsJ dimer observed in the crystals is perhaps transiently, and possibly permanently, a component of the T2SS pseudopilus. If this is correct, then the observed EpsI:EpsJ heterodimer allows us to evaluate whether the pseudopilus is a right- or a left-handed arrangement of subunits. If the following two assumptions are made: (i) the N-terminal helices of the EpsI:EpsJ heterodimer roughly correspond with the direction of the pseudopilus fiber axis, as is the case in the pilus model of Craig et al.⁶³ and, although to a lesser degree, also in the model of Kohler et al.⁶⁶ and, (ii) the EpsI:EpsJ heterodimer resembles the arrangement of two neighboring EpsG major pseudopilins in the pseudopilus, then, the VvEpsI:EpsJ heterodimer corresponds with VvEpsJ in the n-th position and VvEpsI in the (n + 1)-the position of a right handed arrangement of pseudopilus-forming subunits (Figure 7). Therefore, our structure agrees in general terms with the cryo-electron microscopy model of a pilin formed by PilE pilins from the T4PB system in *Neisseria*.⁶³ Obviously, further experiments are required to definitely settle this point.

There are similarities and differences between the helix-helix interactions in our VvEpsI:EpsJ heterodimer and the PilE helix-helix interactions in the pilus model of Craig et al.⁶³ The similarities, in addition to the right-handedness of the assemblies, are the relative positions of the helices parallel to the fiber axis. The helices of VvEpsI and VvEpsJ are shifted parallel to the helix axis with respect to one another by approximately 12 Å (Fig. 7). Adjacent subunits within the PilE pilus electron microscopy model are similarly shifted with respect to one another. In the PilE pilus model, the N-termini of adjacent subunits are positioned such that the conserved Glu5 of the subunit in the nth position forms a salt bridge of the Phe1 of the subunit in n+1 position. Extending the truncated α -helices in the VvEpsI:EpsJ heterodimer (Fig. 6D) towards the N-terminus can be accomplished without any clashes (data not shown). This extension also suggests that in the full length proteins, the amino group of Phe1 in VvEpsI might be positioned near the conserved Glu5 of VvEpsJ as is also the case in the PilE pilus model⁵⁴.

The main difference between our VvEpsI:EpsJ heterodimer and the PilE pilus model is the distance between helix axes of adjacent subunits. The helices of VvEpsI and VvEpsJ are about

4 Å closer together than the helices of adjacent PilE subunits in positions *n* and (*n*+1) of the PilE pilus model (data not shown). This difference can have several causes, including: (i) the VvEpsI:EpsJ hetero-dimer represents an arrangement of minor pseudopilins reflecting a “tightening” or “tapering” off at the base and/or tip of a pseudopilus during or after assembly, and therefore is not a reflection of all aspects of the arrangement of the major pseudopilins in the pseudopilus, nor of the major pilins in the Type 4A pilus; (ii) the organization of subunits in a T2SS pseudopilus and a Type IVa pilus are similar in orientation, yet adjacent helices are not at similar distances from each other; (iii) the PilE model needs to be adapted and has in actual fact a tighter arrangement of helices than proposed; and, (iv) the EpsI:EpsJ dimer observed in our crystal structure might not be a permanent or transient component of the T2SS pseudopilus but is located somewhere else in the T2SS protein translocation machinery. Further studies will be required to determine which of these options, or other ones, are correct.

MATERIALS AND METHODS

Cloning and Expression

In order to obtain soluble domains of VcEpsI and VcEpsJ suitable for interaction studies, the first 24 N-terminal residues of the mature (post-peptidase cleavage) sequence were deleted from the sequence of each protein. These truncated versions of VcEpsI and VcEpsJ were amplified from codon +25 relative to the pre-pilin peptidase cleavage site by the polymerase chain reaction (PCR) using *V.cholerae* genomic DNA as a template.

The following primers were used: VcEpsI forward 5'-CCTCATGAGTCAACACATCAATACGGTC-3', VcEpsI reverse 5'-CGCTCGAGGTTCCGCCACATAGCTACGG-3', VcEpsJ forward 5'-GAGATATACATATGAATCAAGTCCAACGCAGC-3', VcEpsJ reverse 5'-GGCTCGAGTTAGCCCGCATTTTCAAC-3'. The restriction sites are underlined. VcEpsI was cloned into pET-21d(+) vector (Novagen) using *NcoI* and *XhoI* restriction sites and contained a C-terminal non-cleavable hexahistidine (His₆) tag sequence. VcEpsJ was cloned into pET-22b(+) vector (Novagen) using *NdeI* and *XhoI* restriction sites and had no tag.

To obtain protein variants suitable for crystallization, genes of truncated VvEpsI and VvEpsJ were amplified by PCR using *V. vulnificus* genomic DNA as a template. The following primers were used: VvEpsI forward 5'-CCCGTCATGAGCCAGCACATCAACACCG-3', VvEpsI reverse 5'-CACTGCTAGCCACATAGCTTCTCACCG-3', VvEpsJ forward 5'-GGAGATATACATATGAATCAGGTGCAACGCAGCAATG-3', VvEpsJ reverse 5'-GGTTCTCGAGTTAGCCATTGTTGCCTGCTC-3'. PCR products were ligated into two different multiple cloning sites (MCS) into a single pCDF-CT vector using *NcoI* and *NheI* restriction sites for the first MCS, and *NdeI* and *XhoI* restriction sites for the second MCS. pCDF-CT vector is a modified pCDFDuet-1 vector (Novagen) with a C-terminal His₆ tag and a TEV cleavage site in MCS1. The bi-cistronic plasmid was then transformed into *E. coli* BL21 (DE3) (Novagen) expression cells. Selenomethionine-labeled VvEpsI:EpsJ complex was obtained by inoculating 2ml of an overnight culture into 1 L of Luria-Bertani broth (LB) containing 50 mg/ml of streptomycin. Cells were grown at 30 C for 3 hours to A₆₀₀=0.6 and pelleted by centrifugation at 5000g for 15 minutes. Pellets were re-suspended into M9 minimal medium supplemented with selenomethionine containing 50 mg/ml of streptomycin. Cells were induced with 0.5 mM IPTG, grown at 25 C and harvested after 4 hours. Cells were pelleted by centrifugation at 5000g for 20 minutes, frozen and stored at -80 C.

Purification

Cell pellets were thawed and re-suspended in 20 mM Tris-HCl (pH 7.8), 250mM NaCl, complete EDTA-free Protease Inhibitor Cocktail (Roche). Cells were lysed with a French Press

and centrifuged at 20,000 g for 40 min. 5 ml of Ni-NTA resin (Qiagen) were equilibrated with wash buffer containing 20 mM imidazole, 20 mM Tris-HCl (pH 7.8), and 250 mM sodium chloride. The clear lysate was loaded onto the Ni-NTA column, and was washed with the same buffer. To remove the His tag, TEV protease was added and the mixture incubated at 4° C overnight, followed by a second pass over the Ni-NTA column and eluted with wash buffer containing 250 mM imidazole. Samples were further purified using a Superdex 75 HR10/30 size-exclusion column (Amersham Biosciences) equilibrated in 20 mM Tris-HCl (pH 7.8), 250 mM NaCl, 1 mM EDTA. The pure protein was concentrated to 10-12 mg/ml for crystallization.

Surface Entropy Mutations, Truncations and Crystallization

Crystals of VvEpsI:EpsJ appeared overnight in 20% PEG 3350, 200 mM NaBr and 0.1 M Bis-Tris Propane pH 6.5. Crystals were improved and grew to dimensions of 200x50x30 μ m after optimization with temperature, additives and by crystallizing over oil. However, diffraction was poor ($\sim$ 6 Å) and anisotropic when tested at the SSRL beamline 9.2.

Next, using the secondary structure prediction by *Disopred*⁷³ and the VvEpsI/GspI and VvEpsJ/GspJ family sequence alignments (Figure 2), six different single surface entropy mutations, and 4 different double surface entropy mutations on either VvEpsI or VvEpsJ were selected. The required site directed mutagenesis was carried out with the QuickChange procedure (Stratagene) The resultant variant genes were used for protein expression, followed by protein purification, using methods essentially as described above, and by setting up various crystallization screens. A new crystal form was observed from the double mutation (E96T, K97T) in VvEpsI. Crystals of this variant were improved by adding 10 mM of CdCl₂ and grown to final dimensions of 150x150x30 μ m. These crystals diffracted to a slightly higher resolution of 3.0 Å, however, suffered severe radiation damage and a structure determination was not possible.

Prior limited proteolysis studies by Yanez *et al.* (unpublished results) indicated that the final 10 C-terminal residues of *V. cholerae* VvEpsJ are easily susceptible to proteolysis and therefore were probably solvent accessible and flexible. Secondary structure prediction of *V. vulnificus* VvEpsJ (not shown) and the VvEpsJ/GspJ family sequence alignment (Figure 2) confirmed that the C-terminus of *V. vulnificus* VvEpsJ is likely to be disordered. Therefore, 4 variants of VvEpsI(E96T/K97T):EpsJ were cloned with 14, 21, 25 and 28 residues truncated from the C-terminal sequence of *V. vulnificus* VvEpsJ. The variant with 28 C-terminal residues truncated from VvEpsJ failed to express. Of the three remaining VvEpsI(E96T/K97T):EpsJ variants, only one variant, missing 21 C-terminal residues, yielded crystals suitable for structure determination. Crystals appeared overnight in 10-15% PEG4000 and 0.1 M MnCl₂. Crystals were soaked for 10 seconds in stepwise increments containing 5%, 10% and 15% of cryoprotectant containing crystallization solution plus PEG 400. Finally, crystals were flash-frozen in liquid nitrogen and sent for data collection.

Structure Determination

An EXAFS scan was performed to optimize the wavelength for the anomalous signal. Data sets were collected at the 8.2.1 beamline of the Advanced Light Source, Berkeley, on a tiled 3 $\times$ 3 ADSC Quantum 315 detector. Data were reduced and scaled with HKL2000⁷⁴ for finding the Se sites and for initial refinement. For the final refinement, reflections were indexed and integrated with MOSFLM⁷⁵ and scaled with SCALA²⁹ using the automated Wedger script (J. Holton, unpublished results). The same set of the reflections was set aside for the calculation of the free *R*-factor.

The structure of VvEpsI:EpsJ was solved by Se-SAD using SOLVE⁷⁶, which located 30 out of the 40 expected Se sites per asymmetric unit. RESOLVE⁷⁶ was used for initial density

modification and automatic model building. The map obtained from RESOLVE⁷⁶ was used for automatic model building in Arp/Warp.⁷⁷ The protein model was improved by visual inspection and manual model building using the graphics program Coot.⁷⁸ The program REFMAC5⁷⁹ was used for TLS refinement with 4 TLS groups per chain, which were determined by the TLS motion determination (TLSMD) server.⁸⁰ The stereochemical quality of the model was verified using PROCHECK.⁸¹ Final structure determination and refinement statistics are listed in Table 1.

A total of 954 out of 1092 residues were built into the electron density for the final VvEpsI:EpsJ model. The degree of disorder differs somewhat in the four EpsI:EpsJ heterodimers per asymmetric units. In the dimer used for the description of the structure the following residues are not included: 1) the N-terminal residues 25-29 of VvEpsI, 2) N-terminal residues 25-31 of VvEpsJ, 3) C-terminal residues 114-117 of VvEpsI, 4) Loop residues 67-71 and 132-133 in VvEpsJ.

Characterization of VvEpsI:EpsJ in solution

The purity and molecular weight of the eluted protein was checked by SDS-PAGE analysis on a 4-20% gradient gel. The assembly state of soluble VvEpsI:EpsJ was investigated by analytical gel filtration analysis. Analytical size exclusion chromatography was performed using a Superdex 75 HR 10/30 (GE Healthcare) column. The column was calibrated with as molecular weight standards: Bovine Serum Albumin (67 kDa), Chicken Ovalbumin (43 kDa), Myoglobin (17.6 kDa) and Ribonuclease A (13.7 kDa).

Sequence and Structural analysis of VvEpsI:EpsJ

A multiple sequence alignment was made using the Multalin server⁸² and the *ESPript* server was used to render the alignment.⁸³ The program DSSP⁸⁴ was used to assign the secondary-structure. The *Protein-Protein Interaction Server*⁷⁰ was utilized to calculate buried solvent accessible surface areas (ASA) and to identify hydrophobic contacts and hydrogen bonds between adjacent molecules in the crystal lattice. Polar atoms within a distance range of 2.5-3.5 Å with proper hydrogen-bond geometry were considered to form hydrogen bonds. The gap volume index (GVI) was defined by: $GA (\text{\AA}) = \text{gap volume between molecules} (\text{\AA}^3) / \text{interface ASA} (\text{\AA}^2)$ (per complex), and was calculated by the Protein-Protein Interaction Server.⁷⁰ Superpositions were carried out using the Secondary Structure (SSM) Superposition Method⁶⁷ from the CCP4 suite. For plotting the degree of sequence conservation onto the surface of the VvEpsI:EpsJ structure the CONSURF⁸⁵ server was used. All figures were generated using PYMOL.⁸⁶

Protein Data Bank accession codes

The atomic coordinates and structure factors have been deposited in the RCSB Protein Data Bank and are available under accession code 2RET.

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Figure 1. The interaction between soluble domains of EpsI and EpsJ from *V. cholerae*

(a) An SDS-PAGE gel of a non-tagged soluble domain of VcEpsJ with the first 24 residues truncated co-eluting with a His-tagged soluble domain of similarly truncated VcEpsI. Lysate from three liters of cell culture expressing truncated VcEpsJ were mixed with lysate from one liter of cell culture expressing VcEpsI-His₆. The mixed cell lysate (Lane 1) was then applied to a column containing 1 ml of Ni-NTA resin. The flowthrough (Lane 2) and a subsequent 10 ml wash (Lane 3) with 20 mM imidazole were collected. Bound protein was eluted (Lane 4) with 200 mM imidazole. Molecular weight markers are indicated on the left.

(b) A native PAGE gel of VcEpsI (Lane 1), VcEpsJ (Lane 2) and EpsI-EpsJ complex (Lane 3). Approximately 2 mgs of purified protein was loaded per lane.

(c) A Size Exclusion Chromatography profile of the VcEpsI-EpsJ complex on a Superdex75 column. The positions of molecular weight standards are indicated by arrows: 1 - bovine serum albumin (67 kDa), 2 - chicken ovalbumin (43 kDa), 3 - myoglobin (17.6 kDa) and 4 - ribonuclease A (13.7 kDa). The insert represents an SDS-PAGE gel of the peak fraction

showing that the soluble domains of VcEpsI and VcEpsJ maintain an approximately stoichiometric complex during purification. The same molecular weight markers are used as in (a).

Figure 2. EpsI/GspI and EpsJ/GspJ family sequence alignment

A Multalin⁸² alignment of VvEpsI and VvEpsJ with nine EpsI/GspI and EpsJ/GspJ homologues. A black triangle denotes the start of the constructs for VvEpsI and VvEpsJ used for crystallization. The secondary structure elements as identified by DSSP⁸⁴ are shown on top, with the conserved N-terminal α -helix in blue, the variable segment in purple and the conserved β -sheet depicted in green. Invariant residues are highlighted with a red background, while residues that are conserved in all but 1 or 2 homologues are highlighted in pink. Residues that are a hydrophobic residue in all 10 homologues are highlighted orange. Yellow background denotes medium sequence conservation and white background denotes poor sequence conservation. In the lower two lines, contact residues that form Van-der-Waals contacts in the

EpsI-EpsJ interface are represented by triangles and residues that form intermolecular H-bonds are denoted by stars. Contact residues in the EpsI:EpsJ interface are labeled with triangles and stars in blue, those in the EpsI:EpsJ' interface in green, and those in the EpsJ:EpsJ' interface in red. The solvent accessibility, as assigned by ESPRIT⁸³ on the basis of the VvEpsI:EpsJ structure, is denoted by the bar *acc*, with dark blue meaning residues that are highly accessible, cyan meaning residues that are partially accessible and white denoting residues that are buried.

Figure 3. The crystal structure of the *V. vulnificus* EpsI:EpsJ complex

(a) Ribbon diagram of one VvEpsI₂:EpsJ₂ heterotetramer. VvEpsI colored in blue, VvEpsI' in cyan, VvEpsJ in brown and VvEpsJ' in red. One chloride ion, located between VvEpsJ and VvEpsJ', is shown in green.

(b) A "top" view of one VvEpsI₂:EpsJ₂ heterotetramer with buried accessible surfaces (in Å²) of the intersubunit contacts indicated.

(c) A close-up view of one VvEpsI:EpsJ heterodimer, in the same orientation as the heterotetramer shown in (A) above, with VvEpsI colored in dark blue and VvEpsJ colored in brown.

(d) The VvEpsI:EpsJ heterodimer shown in (C) rotated by 180° with each of the secondary structure elements in VvEpsI and VvEpsJ labeled. Two small stretches of residues in VvEpsJ (67-71 and 132 - 133) that could not be included in the model due to disorder, are denoted by a dotted black line.

Figure 4. Comparison of *V. vulnificus* EpsI and EpsJ soluble domains with known Type 4a-like Pilin structures

(a) The minor pseudopilins VvEpsI and VvEpsJ are shown schematically along side the equivalent structures of a minor pseudopilin EpsH from *V. cholerae* (Yanez et. al., submitted) and of the major pseudopilin GspG from *K. oxytoca* (PDB code 1T92)⁶⁶. To facilitate comparison, all structures are shown with the same orientation of the conserved β -sheet colored in green.

(b) Topology diagrams of the globular domains of four T2SS pseudopilins with known structure (*V. vulnificus* EpsI, *V. vulnificus* EpsJ, *V. cholerae* EpsM, *K. oxytoca* GspG) and of the three T4BP pilins PilA^{PAK} from *P. aeruginosa* (PDB code 1DZO)⁶⁵, PilE from *N. gonorrhoeae* (PDB code 2HI2)⁶⁴, and PilA^{K1224} from *P. aeruginosa* (PDB code 1RGO)⁸⁷. The red box contains the new structures of VvEpsI and VvEpsJ. Both these minor pseudopilins exhibit an “atypical fold” compared to known Type 4a Pilin structures as described in the text.

Figure 5. The *V. vulnificus* EpsI:EpsJ Interface

VvEpsI and VvEpsJ are shown in the same “butterfly” orientation in all figures (a)-(e).

(a) The contact surface of the VvEpsI:EpsJ interface. N-terminally truncated VvEpsJ in yellow and N-terminally truncated VvEpsI in blue. All residues that form hydrophobic contacts between VvEpsJ and VvEpsI are shown in green, while residues that form polar contacts between VvEpsJ and VvEpsI are colored blue.

(b) An electrostatic representation of the VvEpsI:EpsJ interface. The I:J interface is largely hydrophobic, with a small negatively charged region in VvEpsI that contacts a small positively charged surface in VvEpsJ.

(c) A cartoon representation of the VvEpsI:EpsJ interface showing the sequence conservation of the contact residues in VvEpsI and VvEpsJ. The cartoon representation of VvEpsJ is colored according to the Type 4a Pilin structural elements: the N-terminal helix in blue, the variable segment green and the C-terminal β -sheet purple. The cartoon representation of VvEpsI is colored in grey. The contact residues are denoted by spheres. Contact residues in VvEpsJ are labeled with spheres that are colored according to the Type 4a Pilin structural elements. Contact residues in VvEpsI colored according to the VvEpsJ residues with which they interact. Contact residues are labeled according to their sequence conservation within the EpsI/GspI and EpsJ/GspJ families as follows: invariant residues by a red box; hydrophobic residues labeled with a red residue name and number; residues with moderate sequence conservation labeled in yellow, and residues with low sequence conservation labeled in black. One residue, VvEpsI-Asp45, a conserved negatively charged interface residue, is labeled in pink.

(d) The “helix-helix” interactions between VvEpsI and VvEpsJ. The helix of VvEpsJ colored in yellow, the helix of VvEpsI in purple. Six residues from the helix of VvEpsJ form hydrophobic interactions with six residues along the helix of VvEpsI. The residues, shown in stick representation, are labeled according to their sequence conservation as in **(c)** above.

Figure 6. The shape and sequence conservation of the EpsI:EpsJ heterodimer

(a) Stereo view of the VvEpsI:EpsJ heterodimer shown in the same orientation as in figure 3. VvEpsI and VvEpsJ are colored by element and per chain: oxygens and nitrogens in red and blue, respectively; carbons in VvEpsI in green; and carbons in VvEpsJ in yellow. Conserved residues facing the viewer in the deep crevice are Asp45, Arg106, Tyr108 of EpsI, and Gln45 and Gln57 of EpsJ. In addition, Leu 48 and Leu 189 of EpsJ are consistently hydrophobic in the EpsJ/GspJ family (Fig. 2).

(b) The heterodimer in **(a)** above is rotated about the x-axis by 90° to show the “top” view of one VvEpsI:EpsJ heterodimer. This representation shows the large crevice formed between the two pseudopilins VvEpsI and VvEpsJ.

Figure 7. The VvEpsI:EpsJ heterodimer corresponds with a right handed one-start helical pseudopilin assembly

(a) A view of the VvEpsI:EpsJ heterodimer approximately perpendicular to the two N-terminal helices of VvEpsI and VvEpsJ with the N-termini of the helices at the lower part of the figure. In the crystal structure the N-terminal helix of VvEpsI is shifted “upwards” by about 12 Å with respect to VvEpsJ. These helices are thought to run approximately parallel to the pseudopilus fiber axis assuming that the T2SS pseudopilus shares this feature with the pilus model of Craig et al. 2006⁶³. VvEpsJ is depicted in brown, VvEpsI colored in blue.

(b) View of the VvEpsI:EpsJ heterodimer approximately parallel to the axes of the N-terminal helices, approximately 90 degrees rotated with respect to **(A)** above, with the N-termini of the helices in front. Since the helix axes are probably aligned roughly parallel to the pseudopilus helix axis, the symmetry operation relating EpsJ and EpsI corresponds with a right-handed rotation as indicated by the arrow.

Table 1

Data collection and refinement statistics

Data collection	
Wavelength (Å)	0.97903
Space group	P1
<i>a</i> (Å)	63.28
<i>b</i> (Å)	79.14
<i>c</i> (Å)	82.07
α	65.00°
β	69.19°
γ	69.50°
Resolution (Å)	20-2.20 (2.28-2.21) ²
Completeness (%)	97.5 (91.8)
Redundancy	3.8 (2.7)
R _{sym} (%)	8.5 (50.1)
R _{meas} (%)	9.9 (61.7)
I/ σ	8.9 (2.3)
No. reflections measured	239741
No. unique reflections	63933
Refinement¹	
Resolution (Å)	20-2.21
No. of reflections	60065
Molecules per asu	8
Residues per asu	954
Atoms	8286
Waters per asu	595
Chloride ions per asu	2
R _{work} (%)	18.7
R _{free} (%)	24.7
R.m.s.d bond lengths (Å ²)	0.015
R.m.s.d. bond angles (Å ²)	1.461
B-average (Å ²)	40.5
B-water (Å ²)	43.6
Wilson B factor (Å ²)	39.5
Ramachandran - preferred ³	96.7% (874 residues)
Ramachandran - allowed ³	2.9% (26 residues)
Ramachandran - outliers ³	0.4% (4 residues)

¹ Refinement statistics are based on Refmac579

² Values in parenthesis refer to outer shell

³ According to the program PROCHECK⁸¹