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Structure of the minor pseudopilin XcpW from the *Pseudomonas aeruginosa* type II secretion system

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Pseudomonas aeruginosa utilizes the type II secretion machinery to transport virulence factors through the outer membrane into the extracellular space. Five proteins in the type II secretion system share sequence homology with pilin subunits of type IV pili and are called the pseudopilins. The major pseudopilin XcpT_G assembles into an intraperiplasmic pilus and is thought to act in a piston-like manner to push substrates through an outer membrane secretin. The other four minor pseudopilins, XcpU_H, XcpV_I, XcpW_J and XcpX_K, play less well defined roles in pseudopilus formation. It was recently discovered that these four minor pseudopilins form a quaternary complex that is presumed to initiate the formation of the pseudopilus and to localize to its tip. Here, the structure of XcpW_J was refined to 1.85 Å resolution. The structure revealed the type IVa pilin fold with an embellished variable antiparallel β -sheet as also found in the XcpW_J homologue enterotoxigenic *Escherichia coli* GspJ_W and the XcpU_H homologue *Vibrio cholerae* EpsU_H. It is proposed that the exposed surface of this sheet may cradle the long N-terminal α 1 helix of another pseudopilin. The final 31 amino acids of the XcpW_J structure are intrinsically disordered. Deletion of this unstructured region of XcpW_J did not prevent type II secretion *in vivo*.

1. Introduction

Many Gram-negative bacteria utilize the type II secretion system (T2SS) to secrete virulence factors. *Pseudomonas aeruginosa* uses its T2SS to secrete exotoxin A, phospholipase C, elastase, alkaline phosphatase and other substrates (Filloux, 2004). These exoproteins are translocated across the inner membrane *via* the Sec or twin-arginine translocation pathway followed by export across the outer membrane into the extracellular milieu *via* the T2SS (Pugsley, 1993; Voulhoux *et al.*, 2001).

P. aeruginosa uses 12 gene products, XcpA_O and XcpP_C–Z_M, to form the T2SS machinery commonly termed the secreton (Tomassen *et al.*, 1992). Five *xcp* gene products in *P. aeruginosa* contain short N-terminal leader peptides with sequence homology to subunits of type IV pili and are therefore referred to as pseudopilins (Peabody *et al.*, 2003). These are XcpT_G, XcpU_H, XcpV_I, XcpW_J and XcpX_K (where the subscripts reference the T2SS protein names in the non-*Pseudomonas* T2SS; for example, in XcpW_J the J refers to GspJ). This leader sequence on type IV pilins and T2S pseudopilins is removed by the prepilin peptidase XcpA_O, which cleaves between a conserved glycine at position –1 and a hydrophobic residue (often phenylalanine) at position +1.

XcpA_O is known as PilD in the context of type IV pilus assembly (Nunn & Lory, 1993). (Douzi *et al.*, 2009). Three of the four enterotoxigenic *Escherichia coli* (ETEC) T2SS homologues were also found to

The major pseudopilin XcpT_G is hypothesized to form an intraperiplasmic pilus, which acts as a piston to push substrates through the secretin XcpQ_D, the outer membrane pore (Filloux *et al.*, 1998). The energy for this process is generated by an inner membrane platform composed of XcpR_E, XcpS_F, XcpY_L and XcpZ_M (Filloux, 2004). The four low-abundance or minor pseudopilins XcpU_H, XcpV_I, XcpW_J and XcpX_K are believed to have accessory roles in T2S pseudopilus formation (Filloux *et al.*, 1998).

All five pseudopilins are essential for secretion, although the precise roles of the minor pseudopilins are still being investigated. The overproduction of XcpT_G leads to a hyperpseudopilus that extends past the outer membrane. In contrast to the requirement for all four minor pseudopilins for T2S, only XcpV_I is mandatory for the formation of the hyperpseudopilus (Durand *et al.*, 2005). The length of the hyperpseudopilus is controlled by the availability of XcpX_K (Durand *et al.*, 2005). It has recently been shown in a systematic protein–protein interaction study that the four *P. aeruginosa* minor pseudopilins XcpU_H, XcpW_J, XcpV_I and XcpX_K are able to form a quaternary complex that is proposed to be at the tip of the XcpT_G-containing pseudopilus

2. Materials and methods

2.1. Overexpression and purification of *P. aeruginosa* XcpW₁

The *xcpW_J* plasmid, pETG-20A-W_J, was constructed by cloning the coding region for the soluble periplasmic domain of XcpW_J (residues 22–231, lacking the N-terminal transmembrane helix; Fig. 1) into the Gateway (Invitrogen) pETG-20A vector as described previously (Douzi *et al.*, 2009). The expressed gene product includes an N-terminal thioredoxin followed by a six-residue histidine tag, nine amino acids encoded by the *attB1* site, a second histidine tag, a tobacco etch virus (TEV) protease cleavage site and the soluble domain of XcpW_J beginning with Arg22.

For each protein preparation, a fresh transformant of pETG-20A-W_J in *Escherichia coli* strain BL21 (DE3) pLysS (Promega) was inoculated into 100 ml Luria-Bertani (LB) medium containing 100 mg l⁻¹ ampicillin and 34 mg l⁻¹ chloramphenicol (LB^{amp,chl}). 25 ml aliquots of cultures grown overnight with shaking at 310 K were diluted into 1 l LB^{amp,chl} and shaken at 310 K until the OD₆₀₀ reached 0.4, at which point the temperature was lowered to 291 K. When the OD₆₀₀ reached 0.6, *xcpW* expression was induced with 1 mM IPTG and growth continued overnight at 291 K. Cells were harvested by centrifugation at 9000g for 20 min at 279 K. The cell pellet was plunged into liquid nitrogen and stored at 193 K. 7 g thawed cell pellet was homogenized in 35 ml 50 mM imida-

Figure 1
Sequence of *P. aeruginosa* XcpW_J. The primary sequence of XcpW_J includes leader-peptide (grey italics) and transmembrane-helix residues that were removed for soluble expression (grey bold), unobserved residues (regular text), amino acids creating the conserved and symmetric hydrophobic core (grey shading), α -helices and β -strands in the XcpW_J structure (cylinders and arrows, respectively) and the starting points for C-terminal deletions (black arrows and underlining). Following convention, numbering begins at the first residue of the mature protein (Phe +1); thus, the highly conserved glycine preceding the cleavage position is Gly -1. The sequence of GspJ_W is 36% identical to that of XcpW_J (asterisks and colons indicate identical and similar residues, respectively).

zole, 1× phosphate-buffered saline (PBS) and 250 U Benzonase Nuclease (Novagen). Cells were broken by two passes through a French press at 6.9 MPa and clarified by centrifugation at 58 500g for 30 min at 283 K. The supernatant was loaded onto a HisTrap FF 5 ml nickel-affinity resin column (Amersham Biosciences) equilibrated with 50 mM imidazole in PBS on an ÄKTApriime FPLC system. Following a wash with 30 column volumes, elution occurred during a gradient from 50 to 500 mM imidazole in PBS.

TEV protease was added to the purified fusion protein (32 µg ml⁻¹ final concentration). During overnight cleavage, the protein was dialyzed into 50 mM HEPES pH 7.5 plus 3 mM β-mercaptoethanol (BME) at 277 K. The cleaved XcpW_J was then loaded onto a nickel column equilibrated in 50 mM HEPES pH 7.5, which bound the (histidine-tagged) TEV protease and uncut protein. Flowthrough fractions that contained cleaved XcpW_J based on SDS-PAGE analysis were concentrated using a 3000 molecular-weight cutoff concentrator (Millipore) and loaded onto a Superdex 75 (Amersham Biosciences) sizing column for further purification. Purified XcpW_J was dialyzed overnight in 25 mM Tris pH 7.4. All protein samples were assessed for heterogeneity using dynamic light scattering. The polydispersity was generally around 25%.

2.2. Crystallization conditions

Initial XcpW_J crystals were obtained using a sparse-matrix screen (JCSG, Qiagen). The crystals were grown at room temperature by vapour diffusion using the hanging-drop method (McPherson, 1982). The drops consisted of 1.5 µl protein solution at 17 mg ml⁻¹ and 1.5 µl reservoir solution. For optimized crystals, the reservoir solution was 0.1 M HEPES pH 7.5, 15 mM KCl, 7.5% PEG 8000 and 0.1 M ATP (from 1 M stock dissolved in 25 mM Tris pH 8.0). The crystals grown with the ATP additive were approximately 0.2 mm in size and tear-drop-shaped. The crystals were cryopreserved in mother liquor containing 30% ethylene glycol.

Crystals were harvested for mass-spectrometric analysis in several steps. Firstly, a drop containing needle-like XcpW_J crystals was transferred to a fresh glass cover slip. Mother liquor was slowly wicked from the crystals using absorbant paper. The crystals were resuspended in equilibrated mother liquor from the reservoir and this was also wicked away. The crystals were subsequently washed twice in 25 mM Tris-HCl pH 8.0 and then transferred into 25 µl filtered ddH₂O. This sample was analyzed for proteolysis of XcpW_J by matrix-assisted laser desorption/ionization mass spectrometry.

2.3. Data collection, processing and refinement

A 1.85 Å resolution native data set was collected on beamline 21-ID-G at the Argonne National Laboratory's Advanced Photon Source (APS) using a MAR 300 CCD detector. The crystals belonged to space group *P*2₁, with two monomers in the asymmetric unit. The diffraction data were integrated, scaled and merged using *HKL-2000* (Otwinowski & Minor, 1997). The structure was solved by molecular

Table 1

Crystallographic data collection and refinement of XcpW_J.

Values in parentheses are for the highest resolution shell.

Data collection	
Beamline	APS 21-ID-G
Wavelength (Å)	0.97856
Space group	<i>P</i> 2 ₁
Unit-cell parameters (Å, °)	<i>a</i> = 39.7, <i>b</i> = 82.9, <i>c</i> = 57.8, α = γ = 90.0, β = 105.4
Resolution (Å)	25.0–1.85 (1.88–1.85)
Unique reflections	30798
Multiplicity	5.2 (5.1)
<i>R</i> _{merge} [†]	0.054 (0.394)
Completeness (%)	99.8 (99.9)
Average <i>I</i> /σ(<i>I</i>)	35.6 (3.6)
Refinement	
Molecules per asymmetric unit	2
No. of protein atoms	2871
No. of solvent atoms	193
<i>R</i> _{work} / <i>R</i> _{free}	0.196/0.230 (0.224/0.254)
Wilson <i>B</i> (Å ²)	22.8
Average <i>B</i> overall (Å ²)	19.9
R.m.s.d.	
Bond lengths (Å)	0.010
Bond angles (°)	1.240
Solvent content (%)	45.7
E.s.u. [‡] (Å)	0.086
Ramachandran values	
Preferred regions (%)	94.2
Allowed regions (%)	5.8

[†] $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$. [‡] Standard uncertainty of positional parameters based on maximum likelihood.

replacement with *Phaser* (McCoy *et al.*, 2007) using GspJ_W (PDB entry 3ci0, chain *J*; Korotkov & Hol, 2008) as a model. The XcpW_J structure was built using *Auto-Rickshaw* (Panjikar *et al.*, 2005) with manual fitting in *Coot* (Emsley & Cowtan, 2004). The large loop region connecting β4 and β5 of XcpW_J could be traced in chain *B* (and was confirmed through the use of omit maps), but could not be fitted in chain *A* owing to poor electron density. The final structure was refined to 1.85 Å resolution using *REFMAC* v5.5.0072. Translation, libration and screw-rotation displacement (TLS) groups that were defined by the *TLSMD* server (Painter & Merritt, 2006) were also used in the refinement process. The final overall *R*_{work} and *R*_{free} were 19.6% and 23.0%, respectively, and the XcpW_J structure has 94.2% of residues in favoured regions of the Ramachandran plot, with no outliers (Table 1).

Structure factors and coordinates have been deposited in the Protein Data Bank with code 3nje.

2.4. Construction of xcpW_J mutants

The *xcpW_J* alleles encoding wild-type XcpW_J (XcpW_Jwt), XcpW_JΔ195 and XcpW_JΔ201 (Fig. 1) were generated by PCR using the following oligonucleotides: XcpW_Jup (5'-ATAGG-ATCCGCGCCGCGGCGCGCCTCGTCGGTTTCCTCG-3') and XcpW_Jdown (5'-ATAAAGCTTCGACGCCGTTCTGC-CCGCGCCTCATTCCGG-3') for XcpW_Jwt, XcpW_Jup and XcpW_JΔ195down (5'-ATAAAGCTTTCAGAGCAGACGCCAGACGCGCACCAGCTTG-3') for XcpW_JΔ195 and XcpW_Jup and XcpW_JΔ201down (5'-ATAAAGCTTTC-ACTGCTTGAGCGGCGGATCGAGCAGACGC-3') for

Figure 2

Structural features of XcpW_J. (a) XcpW_J (ribbon representation with α -helices and β -strands indicated to highlight topology) displays the type IVa pilin fold consisting of a conserved N-terminal α 1 packed against the C-terminal antiparallel β -sheet (β 9– β 8– β 10– β 11). α 1 is flanked on the opposite side by a second antiparallel β -sheet (β 3– β 4– β 5– β 6– β 7). (b) Eight of the 17 residues making up the hydrophobic core are shown in stereoview. Phe74 and Phe86 are two of the three phenylalanine side chains that form the canopy of the hydrophobic core. Trp144 and Trp107 are two of the three residues that create the tryptophan ring in the protein core. Met177, Met48, Leu187 and Leu44 portray the duplication that is seen throughout the hydrophobic core of XcpW_J and GspJ_W.

XcpW_J Δ 201. The resulting DNA fragments were cloned into the pCR2.1 vector (Invitrogen) and sequenced. These fragments were further digested with *Xba*I–*Sac*I restriction enzymes and subcloned into the arabinose-inducible host-range vector pJN105 (Newman & Fuqua, 1999), leading to plasmids pXcpW_J, pXcpW_J Δ 195 and pXcpW_J Δ 201. Recombinant plasmids were introduced into the wild-type *P. aeruginosa* strain PA01 or its Δ xcpW_J derivative using the conjugative properties of pRK2013 (Figurski & Helinski, 1979). Transconjugants were selected on *Pseudomonas* isolation agar (Difco) supplemented with 50 μ g ml^{−1} gentamicin (Gm⁵⁰).

2.5. Analysis of xcpW_J mutants

Stable accumulation of the truncated forms of XcpW_J was tested in *P. aeruginosa*. Bacteria were grown at 303 K in TSB liquid medium (Difco) overnight with the addition of 2% L-arabinose (Ara). After overnight growth, the cells were collected and resuspended in SDS–PAGE sample buffer. Protein samples were analyzed as described in Voulhoux *et al.* (2001) on a 15% SDS–polyacrylamide gel (Bio-Rad III) followed by Western blotting using anti-XcpW_J primary antibody (1:5000; Douzi *et al.*, 2009).

Secreted protein profiles were analyzed from *P. aeruginosa* strains grown as described above. Cells and extracellular medium were separated by centrifugation; proteins contained in the supernatants were precipitated by adding trichloroacetic acid [15% (w/v) final concentration] and incubating for 2 h at 277 K. Samples were subsequently centrifuged (30 min at 15 000g), the pellets were washed with 90% (v/v) acetone, resuspended in SDS–PAGE sample buffer and analyzed as described in Voulhoux *et al.* (2001) on a 12% SDS–polyacrylamide gel stained with Coomassie Blue.

For functional secretion assays, *P. aeruginosa* strains were grown overnight in liquid medium at 310 K. Culture samples were plated on (Gm⁵⁰, 2% Ara) plates. Protease secretion was tested on TSA (Difco) plates containing 1.5% dried milk, with the zone of clearing indicating the secretion of active protease. For the detection of lipase secretion, lipid agar plates were used. Lipid agar is a minimal medium containing olive oil as the sole carbon source (Kagami *et al.*, 1998).

3. Results and discussion

3.1. The structure of XcpW_J

We have solved and refined the crystal structure of a soluble construct of XcpW_J (Fig. 1), one of the minor pseudopilins in the *P. aeruginosa* T2SS. The electron density for the XcpW_J structure was clearly defined from Arg35 through Trp91 in chain A and from Gln37 through Leu102 in chain B. Residues Gln103–Gln200 had well defined electron density for both monomers in the asymmetric unit. The remaining 31 residues at the C-terminus of XcpW_J could not be modelled and therefore are not included in the final coordinates. Mass spectrometry of washed crystals indicated that 30 of these C-terminal amino acids (along with eight N-terminal amino acids) had been cleaved during crystallization (data not shown). This sequence contains 50% proline or glycine residues (Fig. 1), which are likely to be the cause of intrinsic disorder in this region and may have contributed to the proteolytic susceptibility (Radivojac *et al.*, 2004).

The XcpW_J structure revealed the typical type IVa pilin fold distinguished by a long N-terminal α -helix that packs against an antiparallel β -sheet (β 9– β 8– β 10– β 11; Fig. 2*a*). β 9 makes only four nonstandard main-chain hydrogen bonds with β 8 main-chain atoms, while β 8– β 10– β 11 form the more canonical conserved sheet. XcpW_J contains a complex domain inserted between these two conserved structural elements consisting of a five-stranded antiparallel β -sheet (β 3– β 4– β 5– β 6– β 7). Several long excursions between the strands give XcpW_J its distinctive surface shape. Such an insertion in the structurally variable position between α 1 and the conserved β -sheet (given the moniker ' $\alpha\beta$ loop' in type IVa pilins; Craig *et al.*, 2003) is also seen in the ETEC GspJ_W, *V. vulnificus* EpsJ and *V. cholerae* EpsH_U minor pseudopilin structures (Korotkov & Hol, 2008; Yanez *et al.*, 2008*a,b*), although the sheet topology differs between J_W and H_U pseudopilins. This region has been called the 'variable sheet' to distinguish it from the 'conserved sheet' seen in every type IV pilin and pseudopilin structure solved to date.

An intriguing feature of the XcpW_J structure is an internally symmetric hydrophobic core (Fig. 2*b*). The residues that make up this symmetry are Leu44 on the N-terminal α -helix and Leu187 on β 11, Leu55 on the N-terminal α -helix and Leu179 on β 10, Leu114 on β 6 and Leu142 on β 8, Met48 on the N-terminal α -helix and Met177 on β 10, and Val84 on β 4 and Val175 on β 10. There are also three phenylalanine residues that form a canopy over the hydrophobic core. These are Phe74 on β 3, Phe86 on β 4 and Phe146 on β 8. Along with these hydrophobic residues there are three tryptophan residues in a ring within the core: Trp107 on β 5, Trp144 on β 8 and Trp191 on β 11. These internally symmetric hydrophobic residues are conserved in ETEC GspJ_W (Fig. 1). It has been shown recently that many, if not all, major T2SS pseudopilins rely on calcium for stability, unlike type IVa pilin subunits, which contain disulfide bridges (Korotkov *et al.*, 2009). The well packed interior and lack of metal ions in the XcpW_J and GspJ_W structures lead us to believe these pseudopilins rely completely on their hydrophobic cores for stability.

3.2. Features of the variable sheet

The variable sheet of XcpW_J is framed by small structural elements that create a polar gully on the surface of the monomer (Fig. 3). 'Overhang 1' is formed by α 2, 'overhang 2' is the β 1– β 2 hairpin, the 'back door' is the α 3 helix between β 9 and β 10 and the 'floor' is the loop between β 3 and β 4. Three of these are structurally distinct between XcpW_J and GspJ_W, which otherwise have very similar folds as evidenced by their r.m.s.d. of 1.2 Å over the 701 most structurally similar atoms (Fig. 3; DeLano, 2002). α 2 of XcpW_J is absent in GspJ_W, α 3 replaces a loop in GspJ_W and the minimal 'floor' in XcpW_J is a short helix in GspJ_W. Nine of the 11 negatively charged residues found in XcpW_J but not GspJ_W (Glu66, Asp69, Glu79, Asp81, Asp137, Asp160, Glu161, Glu165 and Glu169; Fig. 1) are located within the loops that surround the variable sheet. Altering these acidic residues to uncharged residues could define their importance in maintaining the Xcp quaternary complex stability or substrate recognition. In addition to these

Figure 3

Elements of the variable sheet of XcpW_J and ETEC GspJ_W. The strong structural similarity between XcpW_J (pink) and GspJ_W (blue) includes the pilin fold and variable β -sheet. Notable differences between XcpW_J and GspJ_W include α 2 (overhang 1), the α 3 helix between β 9 and β 10 in XcpW_J (back door), the small α 2 helix in GspJ_W that is lacking between β 3 and β 4 of the XcpW_J (floor) and the different orientation of the loop between XcpW_J β 4 and β 5 (flap). The exposed surface of the variable sheet is polar, as seen in the electrostatic potential of XcpW_J estimated within PyMOL (DeLano, 2002; insert on lower left in identical orientation to the cartoon, with red negative and blue positive regions ranging from -76 to $+76$ kT/e).

structural distinctions around the variable sheet, the large flap between $\beta 4$ and $\beta 5$ of XcpW_J is in a different conformation to that in the GspJ_W structure (Fig. 3).

Why does XcpW_J have a variable β -sheet? In every major (pseudo)pilin the conserved β -sheet cradles the long N-terminal $\alpha 1$ helix and the repetition of these two structural elements over tens to hundreds of subunits allows filament

formation. We speculate that the variable second sheet in XcpW_J may create a Janus-faced exterior surface for the similar packing of the exposed side of a second pseudopilin $\alpha 1$ helix. This helix could belong to another minor pseudopilin such as XcpU_H or the major pseudopilin XcpT_G itself. In this regard, it is interesting to note that the XcpU_H and XcpT_G major helices are amphipathic, with a charged face that could

potentially complement the polar surface of XcpW_J (Fig. 3, inset). By extension, we suggest the same role for the variable sheet predicted for XcpU_H based on its homology to EpsH_U (Yanez *et al.*, 2008b). Such packing would interrupt the continuity of the pseudopilus helix and provide a mechanism both for XcpU_H–XcpW_J interaction (Douzi *et al.*, 2009) and for the transition from the complex of pseudopilins to the repetitive downward assembly of XcpT_G.

3.3. Functional interrogation of the C-terminal tail

The C-terminal end of XcpW_J features a uniquely long extension among the GspJ_W pseudopilins (Fig. 1). When XcpW_J is

Figure 4

Analysis of the position and function of the C-terminal disordered region. (a) Structural alignment of XcpW_J (pink) with the ETEC GspI_V (yellow)–GspJ_W (blue)–GspK_X (cyan) complex suggests the disordered C-terminus of XcpW_J could lie near or in the cleft between GspJ_W and GspK_X. (b) XcpW_JΔ195 and XcpW_JΔ201 are produced in ΔxcpW_J when carried on a plasmid and expressed from the arabinose-inducible promoter, as seen in this Western blot using anti-XcpW_J serum against whole cell lysates. (c) Complementation of the ΔxcpW_J mutant by either truncated xcpW_J gene restored protein secretion to the extracellular medium. The major Xcp T2SS-dependent substrate elastase (LasB) is indicated in this Coomassie-stained SDS–polyacrylamide gel of culture supernatants. (d) Elastase and (e) lipase secretion on skim milk or lipid agar plates, respectively, is restored when the ΔxcpW_J mutant is complemented by one of the two truncated forms of XcpW_J. The halo around the colony on the skim milk plate corresponds to milk degradation owing to elastase activity (c). The lipid agar plate contains a minimal medium on which only T2S-proficient strains grow (d).

structurally aligned with GspJ_w in the GspI_v–GspJ_w–GspK_x ternary complex (Korotkov & Hol, 2008) it appears that the C-terminal extension could lie within the groove between GspK_x and GspJ_w (Fig. 4a), suggesting that it might play a role in holding the minor pseudopilin complex together. Since it is difficult to predict the structural organization of this 31-amino-acid intrinsically disordered tail or any interaction that it may make with other minor pseudopilins or T2SS components, we created XcpW_j variants missing 37 or 31 amino acids and tested their functionality *in vivo* (Figs. 4b–4e). Both truncated forms of XcpW_j, XcpW_jΔ195 and XcpW_jΔ201, were produced with their expected molecular weight (Fig. 4b). Both restored wild-type secretion profiles when used to complement an *xcpW_j* deletion strain (Fig. 4c). In addition, the extracellular activity of two T2SS-dependent substrates, elastase (Fig. 4d) and lipase (Fig. 4e), is restored with either truncated form of XcpW_j. Although we cannot rule out a subtle effect in recognition or chaperoning of a subset of the Xcp secretion substrates, these data uncover no obvious differences between full-length and C-terminally truncated XcpW_j. We conclude that the Pro/Gly-rich tail of XcpW_j is not required for functional interaction with the rest of the Xcp secretin.

We can now also further interpret results from experiments on XpsJ_w, the XcpW_j homolog from the *Xanthamonis campestris* T2SS. Kuo *et al.* (2005) found that the truncation of up to 14 amino acids from the C-terminus of XpsJ_w had a less than twofold effect on the secretion of amylase from *X. campestris*, but removal of 17 or more amino acids impaired amylase secretion and proper cellular localization of XpsJ_w (Kuo *et al.*, 2005). In light of our findings, it is notable that XpsJ_w has an 11-amino-acid Pro/Gly-rich tail which seems to be dispensable in the amylase-secretion system, whereas the inactive truncations remove part of the last predicted β-strand in the conserved sheet.

Our physiological result that the Pro/Gly tail is not needed for T2S despite the juxtaposition of the tail of XcpW_j with XcpX_k in the structural model (Fig. 4a) is well correlated with the observation that the soluble construct of XcpW_j interacts with soluble domains of XcpU_H and XcpV_I but not XcpX_k (Douzi *et al.*, 2009). It may be that the *P. aeruginosa* XcpW_j–XcpX_k interaction differs somewhat from that of the crystal-line interaction of ETEC GspJ_w–GspK_x (Fig. 3a; Korotkov *et al.*, 2009). Future mutational work should elucidate which regions of XcpW_j are required for Xcp complex formation. Definitive identification of the disposition of each minor pseudopilin within the T2SS will await the structure determination of the XcpU_H–XcpV_I–XcpW_j–XcpX_k quaternary complex.

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