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Expression, purification, crystallization and preliminary X-ray studies of *Vibrio cholerae* pseudopilin EpsH

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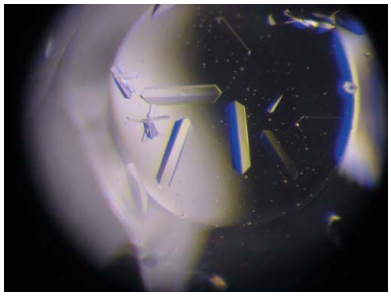

EpsH is a minor pseudopilin protein of the *Vibrio cholerae* type II secretion system. A truncated form of EpsH with a C-terminal noncleavable His tag was constructed and expressed in *Escherichia coli*, purified and crystallized by sitting-drop vapor diffusion. A complete data set was collected to 1.71 Å resolution. The crystals belonged to space group $P2_12_12_1$, with unit-cell parameters $a = 53.39$, $b = 71.11$, $c = 84.64$ Å. There were two protein molecules in the asymmetric unit, which gave a Matthews coefficient V_M of 2.1 Å^3 Da^{-1}, corresponding to 41.5% solvent content.

1. Introduction

The type II secreton is a nanomachine comprised of 12–15 proteins named Gsp or T2S in the general case and Eps in the *Vibrio cholerae* case (Johnson *et al.*, 2006). Proteins targeted to this system are first synthesized as precursors and transported across the cytoplasmic membrane by the Sec or Tat secretion systems (Hofstra & Witholt, 1984; Pugsley *et al.*, 1991; Voulhoux *et al.*, 2001). The N-terminal signal sequences are removed and the proteins are released into the periplasm (Marsh & Taylor, 1998). The folded proteins are then exported by the type II secreton to the extracellular milieu. Mutants of *V. cholerae* defective for type II secretion have reduced virulence and are unable to secrete cholera toxin (Sandkvist, 2001*a*,*b*).

Among the *V. cholerae* type II secreton proteins are the major pseudopilin EpsG and four minor pseudopilins EpsH, EpsI, EpsJ and EpsK (Sandkvist *et al.*, 1997). Pseudopilins are homologous to type IV pilins and are likely to assemble in a similar fashion (Filloux, 2004). However, the type II pseudopilus remains within the periplasm under normal conditions. EpsG is thought to multimerize, forming the shaft of the pseudopilus, a pilus-like structure that may act as a piston to push type II secreted proteins, such as mature cholera toxin, through the secreton pore by repeated extension and retraction (Sandkvist, 2001*a*,*b*). The recently reported structure of a complex composed of truncated forms of GspI, GspJ and GspK strongly suggests that these proteins form the tip of the pseudopilus (Korotkov & Hol, 2008). The location of EpsH within the pseudopilus has not been established, but modeling studies suggest that it could assemble at the tip, but not the base, of an EpsG shaft (Yanez *et al.*, 2008). The most conserved feature shared by both type IV pilins and type II pseudopilins is a long N-terminal α-helix. The first 25 N-terminal amino acids of the mature proteins are highly hydrophobic and have the highest sequence conservation among these proteins (Peabody *et al.*, 2003). The second half of this α-helix is part of a globular head domain that is unchanged when the N-terminal half of the helix is missing (Craig *et al.*, 2003; Hazes *et al.*, 2000; Kohler *et al.*, 2004). Here, we report the expression, purification, crystallization and X-ray diffraction analysis of crystals of the head domain of *V. cholerae* EpsH.

2. Materials and methods

2.1. Expression and purification of recombinant EpsH

A plasmid expressing truncated EpsH was constructed using the polymerase chain reaction with plasmid pMMB339 template

obtained from Michael Bagdasarian (Sandkvist *et al.*, 1994). Plasmid pMMB339 carries part of *epsD* as well as *epsE* through *epsN*. The primer sequences used were CGCCTC**CCATG*G***TCCGTCAAAGATAAA (forward) and GGTG**CTCGAG**CTCTTCATCACTTTCTC (reverse). Engineered restriction sites for *Nco*I in the forward primer and *Xho*I in the reverse primer are marked in bold and were used to insert the amplified fragment into the pET28a vector (Novagen). Site-directed mutagenesis was then employed to delete the italicized G residue. The final construct, ptEpsHcH6, encodes an N-terminally truncated EpsH protein with residues 27–188 of EpsH fused to the C-terminal His tag LEHHHHHH; after processing of the N-terminal methionine (Hirel *et al.*, 1989), the N-terminus of the 169-residue protein was Ser27. *Escherichia coli* BL21/λDE3 (Novagen) cultures were grown at 310 K with shaking at 250 rev min^{-1} in flasks of LB medium supplemented with 50 mg l^{-1} kanamycin and were induced at an optical density at 600 nm of 1.0 with IPTG at a final concentration of 1 m*M*. 6 h after induction, the cells were pelleted by centrifugation, resuspended in Ni–NTA buffer (50 m*M* NaH_2PO_4 pH 7.0, 300 m*M* NaCl, 5 m*M* imidazole) and lysed by sonication.

After centrifugation, the crude lysate was loaded onto a 15 ml Ni–NTA column (BD Biosciences). The column was washed first with 6 *M* urea, 50 m*M* NaH_2PO_4 pH 7.0, 300 m*M* NaCl, 20 m*M* imidazole (while we were able to obtain crystals without the use of this denaturing step, its inclusion resulted in higher purity), then with 50 m*M* NaH_2PO_4 pH 7.0, 600 m*M* NaCl, 20 m*M* imidazole and then with Ni–NTA buffer and was finally eluted with Ni–NTA buffer and a linear imidazole gradient from 5 to 250 m*M* imidazole. Fractions were analyzed by SDS–PAGE (Fig. 1) and those containing EpsH were pooled and dialyzed in 3500 molecular-weight cutoff dialysis tubing (Spectra/Por) against 15 m*M* Tris pH 7.5, 100 m*M* NaCl. The EpsH protein was stable for several months when stored at 278 K in this buffer. Prior to crystallization, the protein was concentrated to 15 mg ml^{-1} using 0.5 ml Microcon centrifugal filter units with YM-3 membranes (Millipore).

2.2. Crystallization screening and optimization

Initial and optimization additive screening was performed by sitting-drop vapor diffusion with the use of an ORYX-4 crystallization robot (Douglas Instruments, UK). Reservoir solutions were prepared corresponding to Crystal Screens I and II (Hampton Research) as well as Wizard Screens I and II (Emerald BioSystems) and 96-well Crystal EX vapor-diffusion plates (Corning) were used. In each sitting drop, 1 µl EpsH at a concentration of 15 mg ml^{-1} was mixed with 1 µl screening condition drawn from the 70 µl reservoir that it was to be equilibrated against. The plate was then sealed with clear tape (Henkel Consumer Adhesives) to allow equilibration. Optimizations were performed using the Opti-Salts suite (Qiagen) as a 10% additive screen; 7 µl of each additive was mixed with 63 µl of the initial screening condition and 1 µl of this reservoir solution was then added to 1 µl protein solution in the sitting drop.

2.3. X-ray diffraction data collection and processing

Crystals were mounted in nylon loops (Hampton Research), flash-cooled by dipping in liquid nitrogen and subsequently stored in liquid nitrogen. Data were collected at 100 K using a MAR CCD detector at the LS-CAT 21-ID-D beamline at the Advanced Photon Source and were processed using *HKL*-2000 (Otwinowski & Minor, 1997).

3. Results and discussion

As the EpsH protein is insoluble in its full-length mature form, we worked with a recombinant truncation. The plasmid ptEpsHcH6 was constructed and expresses recombinant EpsH with a truncation of 26 amino acids from the N-terminus of the mature protein and with its C-terminus appended with an eight-residue His tag, LEHHHHHH. Metal-affinity chromatography was used to produce electrophoretically pure recombinant EpsH protein. In some preparations an additional band was observed, but this did not affect crystal growth. Crystallization screening of the protein produced small crystals that formed in 2 d in Crystal Screen I (Hampton) condition 37 (8% PEG 4000, 0.1 *M* sodium acetate pH 4.6) and consistently stuck to the plastic surfaces of the plate's wells. This made harvesting the crystals challenging, as they had to be prised off the well surfaces without incurring damage. Crystal trials with variation of the PEG 4000 and sodium acetate concentrations suggested that the initial concentrations were optimal.

The Opti-Salts suite used as a 10% additive screen produced visibly larger crystals with Opti-Salt 63 (0.1 *M* Tris–HCl pH 8.5, 1.75 *M* sodium formate) as an additive, thus arriving at a final crystallization reservoir solution of 7% PEG 4000, 90 m*M* sodium acetate pH 4.6, 10 m*M* Tris–HCl and 175 m*M* sodium formate.

Noting that the crystallization condition contains PEG 4000 (Sato *et al.*, 2006), cryoprotection was achieved by the addition of 2–3 µl

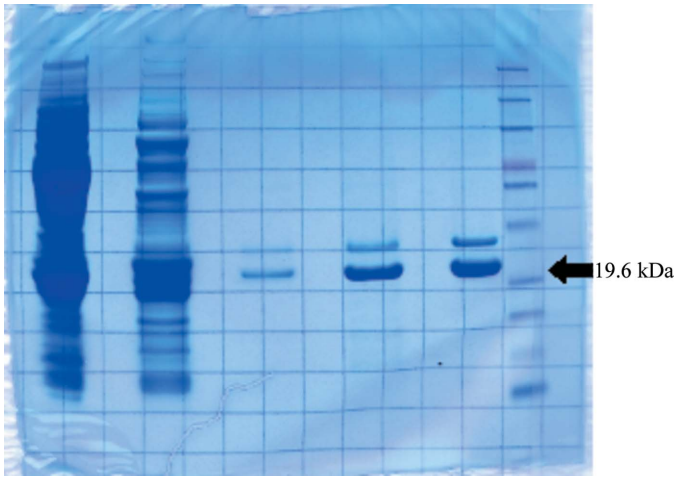

Figure 1
EpsH SDS–PAGE gel showing from left to right: crude lysate, cleared lysate, Ni–NTA column eluate, molecular-weight standards (BioRad).

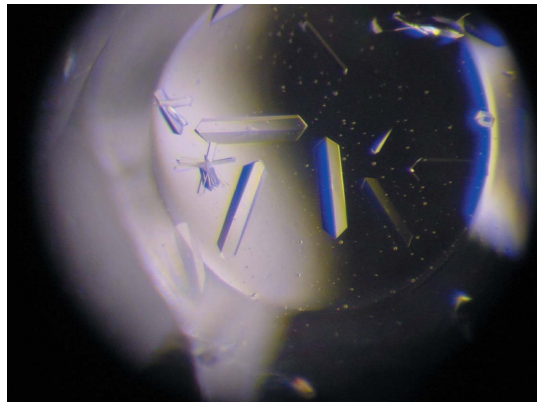

Figure 2
Crystals of EpsH. The longest crystals pictured here are approximately 0.9 × 0.2 × 0.2 mm in size, which was typical for the crystals harvested.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell (1.78–1.71 Å).

Wavelength (Å)	0.978
Resolution (Å)	1.71
No. of reflections	171098 (15229)
No. of unique reflections	71903 (6922)
Multiplicity	2.4 (2.2)
$R_{\rm merge}$ (%)†	4.7 (39.9)
Completeness (%)	95.5 (92.1)
Mean $I/\sigma(I)$	19.1 (3.3)
Mosaicity (°)	0.485

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the ith replicate of reflection hkl and $\langle I(hkl)\rangle$ is the mean value.

PEG 4000 to the sitting drop and 10–20 µl glycerol to the well followed by sealing with tape for 10–30 min prior to harvesting. This forced the dehydration of the crystal drop, resulting in concentration of the cryoprotectant PEG 4000. The resulting diffraction-quality crystals (Fig. 2) belonged to space group $P2_12_12_1$, with unit-cell parameters $a = 53.39$, $b = 71.11$, $c = 84.64$ Å (Table 1). There were two protein molecules in the asymmetric unit, which gave a Matthews coefficient $V_{\rm M}$ of 2.1 Å^3 Da^{-1}, corresponding to 41.5% solvent content (Matthews, 1968).

A truncated *V. cholerae* EpsH protein has previously been crystallized (Yanez *et al.*, 2008). These authors used a similar construct with the same C-terminus but with an additional three amino acids missing from the N-terminus. These crystals also belonged to space group $P2_12_12_1$ and had similar unit-cell parameters $a = 53.3$, $b = 70.4$, $c = 85.1$ Å. These crystals diffracted to 2.0 Å resolution, while the crystals reported here diffracted to 1.71 Å resolution.

The molecular-replacement program *MOLREP* (Vagin & Teplyakov, 1997) was employed for phasing using chain *A* of the reported 2 Å resolution structure (PDB code 2qv8; Yanez *et al.*, 2008) as the search model. *MOLREP* produced an initial solution with two monomers in the asymmetric unit, an *R* factor of 41.0%, a correlation coefficient of 60.3% and a contrast of 11.28 when limited to reflections of 3.0 Å and higher. A similar initial solution with an *R* factor of 47.8%, a correlation coefficient of 66.8% and a contrast of 29.16 was obtained when using the full range of reflections (1.7 Å and higher).

It is hoped that the smaller truncation and the use of different crystallization conditions will not only yield a higher resolution structure, but also one containing regions that are not visible in 2qv8 upon further refinement and modeling, with further insight into the crystal contacts and flexibility of EpsH.

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