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Overexpression, purification, crystallization and preliminary X-ray studies of *Vibrio cholerae* EpsG

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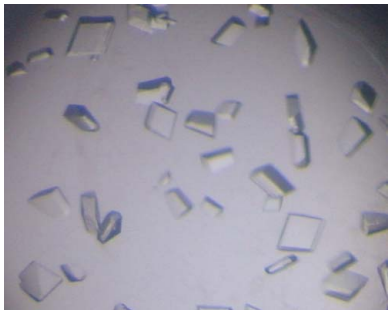

EpsG is the major pseudopilin protein of the *Vibrio cholerae* type II secretion system. An expression plasmid that encodes an N-terminally truncated form of EpsG with a C-terminal noncleavable His tag was constructed. Recombinant EpsG was expressed in *Escherichia coli*; the truncated protein was purified and crystallized by hanging-drop vapor diffusion against a reservoir containing 6 m*M* zinc sulfate, 60 m*M* MES pH 6.5, 15% PEG MME 550. The crystals diffracted X-rays to a resolution of 2.26 Å and belonged to space group $P2_1$, with unit-cell parameters a = 88.61, b = 70.02, c = 131.54 Å.

1. Introduction

Type II secretion is used by many pathogenic Proteobacteria to export hydrolytic enzymes and toxins to the extracellular milieu (Cianciotto, 2005). The proteins secreted by this system are synthesized as precursors and transported across the cytoplasmic membrane by the general secretion system. The N-terminal signal sequences are removed and the processed proteins are released into the periplasm, where they fold into their native conformation. The mature proteins are then targeted to the type II secretion system, where they are exported from the periplasm to the extracellular milieu. Mutants of *Vibrio cholerae* defective for type II secretion have reduced virulence and are unable to secrete the major virulence factor, cholera toxin (Sandkvist, 2001*a*,*b*).

The type II secreton is comprised of about 12 proteins, including one major and four minor pseudopilins. The proteins of the type II secreton are named Gsp in the general case and Eps in the *V. cholerae* case. The pseudopilins are homologous to type IV pilins and assemble in a similar fashion. However, the type II pseudopilus remains within the periplasm under normal conditions. The major pseudopilin of *V. cholerae* is EpsG, a 15 kDa protein whose multimerization forms 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 (EpsD) by repeated extension and retraction (Sandkvist, 2001*a*,*b*). The most conserved feature shared by both type IV pilins and type II pseudopilins is a long N-terminal α-helix. For example, an alignment of *V. cholerae* EpsG with the type IV pilus protein of *Pseudomonas putida* WC5358 showed 63% similarity and 17% identity between the 49 N-terminal amino acids of the mature proteins. The first 25 N-terminal amino acids after the cleaved signal sequence are highly hydrophobic and have the highest sequence conservation among these proteins. 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 and crystallization and the X-ray diffraction analysis of crystals of *V. cholerae* EpsG.

2. Materials and methods

2.1. Expression and purification of recombinant EpsG

An expression plasmid yielding high levels of recombinant EpsG was constructed by PCR amplification of chromosomal DNA from

V. cholerae strain TRH7000 (provided by Michael Bagdasarian; Hirst *et al.*, 1984). The nine N-terminal amino acids of the EpsG gene from *V. cholerae* comprise the signal sequence that is cleaved by prepilin peptidase upon transport to the periplasm. These residues as well as the first 25 highly hydrophobic N-terminal amino acids of the mature protein were replaced with a methionine by PCR amplification. The primers used were forward, 5′-GGAATTC**CATATG**GGTAACAA-AGAGAAAGCGGATCAACAG, and reverse, 5′-TCCG**CTCGAG**-TTGAAAATCTTGGATATTCCAGTTAC, with *Nde*I and *Xho*I sites marked in bold, respectively. The resulting fragment was digested using the restriction enzymes *Nde*I and *Xho*I and ligated into pET24a (Novagen). The final construct (ptEpsGcH6) expresses truncated EpsG with its C-terminus appended with LEHHHHHH, a short noncleavable His tag.

Cultures of *Escherichia coli* BL21/λDE3 (Novagen) carrying ptEpsGcH6 were grown in flasks of LB medium supplemented with 50 mg l^{-1} kanamycin, induced at mid-log phase growth (optical density at 600 nm of 0.6–0.7) with IPTG at a final concentration of 1 m*M* and grown overnight (16–18 h for convenience, as yields were similar with a 6 h induction time) at 310 K with shaking at 250 rev min^{-1}. The cells were pelleted by centrifugation, washed once by resuspension in Vogel–Bonner medium E (Vogel & Bonner, 1956) and stored at 193 K.

The cells were thawed and resuspended in 1× BugBuster reagent with Lysonase (Novagen) and EDTA-free protease-inhibitor cocktail (Roche) and allowed to rock at 250 rev min^{-1} at 298 K for 45 min. Insoluble material was removed by centrifugation and the crude lysate was loaded onto a Talon resin column (BD Biosciences) equilibrated with 50 m*M* NaH_2PO_4 pH 7.0, 300 m*M* NaCl, 1 m*M* imidazole. The column was washed with 50 m*M* NaH_2PO_4 pH 7.0, 300 m*M* NaCl, 5 m*M* imidazole. Bound protein was then eluted with a 5–150 m*M* linear imidazole gradient. Fractions were analyzed by SDS–PAGE and those containing EpsG were pooled. The buffer was exchanged to EpsG storage buffer by dialysis with 3500 Da molecular-weight cutoff dialysis tubing (Spectra/Por) and EpsG protein was concentrated to ∼13 mg ml^{-1} in a 50 ml stirred-cell Amicon ultrafiltration unit under 448 kPa O_2-free N_2 using a 3500 Da molecular-weight cutoff membrane (Millipore). The EpsG storage buffer was 15 m*M* Tris pH 7.5, 15 m*M* NaCl; the protein was found to be stable in this buffer for several months when stored at 278 K. Prior to crystallization, the protein was further concentrated to 40 mg ml^{-1} using 3500 Da molecular-weight cutoff membrane MicroCon 0.5 ml centrifugal filter units (Amicon).

2.2. Crystallization screening and optimization

Initial screening was performed with the use of an IMPAX-5 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 Biosolutions) and used in 72-well microbatch plates (Hampton Research). In each well, 1 µl EpsG at a concentration of 40 mg ml^{-1} was mixed with 1 µl screening condition under a layer of Al's oil (a 1:1 mixture of paraffin oil and silicon oil). Plates were placed in a sealed plastic microbatch container at room temperature elevated above a 30 ml reservoir of 0.5 *M* or 2.5 *M* LiCl for high-salt and viscous screening conditions, respectively (following guidelines from the robot manufacturer, Douglas Instruments). The microbatch plate that yielded the initial EpsG crystals was equilibrated against 2.5 *M* LiCl. For optimization, EpsG crystal growth was reproduced manually using hanging-drop vapor diffusion with drops containing equal amounts of protein (1 µl) and reservoir (1 µl) solution on silanized glass cover slips suspended over the wells of VDX plates (Hampton Research). The wells contained 500 µl reservoir solution and were incubated at 278 K.

To produce untwinned crystals, the reservoirs of the VDX plate were partially covered with 200 µl paraffin oil. In addition, the starting conditions of the drop were altered by the addition of two volumes of water (4 µl). Finally, the reservoir solution was also diluted with water (300 µl reservoir solution plus 200 µl water). The diluted reservoir solution was 6 m*M* zinc sulfate, 60 m*M* MES pH 6.5, 15% PEG MME 550.

2.3. X-ray diffraction data collection and processing

Crystals were mounted in nylon loops (Hampton Research), flash-cooled by dipping them into liquid nitrogen and subsequently stored in liquid nitrogen. Data were collected at 100 K using a MAR CCD detector on beamline 32-ID at COM-CAT (Argonne National Laboratory, Argonne, Illinois, USA). Data were processed using *HKL*-2000 (Otwinowski & Minor, 1997).

3. Results and discussion

As the EpsG protein was insoluble in its full-length mature form, we worked with a recombinant truncation. The plasmid ptEpsGcH6 was constructed and expresses recombinant EpsG with a truncation of 25 amino acids from the N-terminus of the mature protein and with its C-terminus appended with LEHHHHHH (Fig. 1). After metal-affinity chromatography, the protein was electrophoretically pure (Fig. 1). Only one of the tested conditions, Crystal Screen II condition No. 27 (10 m*M* zinc sulfate heptahydrate, 0.1 *M* MES pH 6.5, 25% PEG MME 550) yielded crystals after incubation at 278 K for about

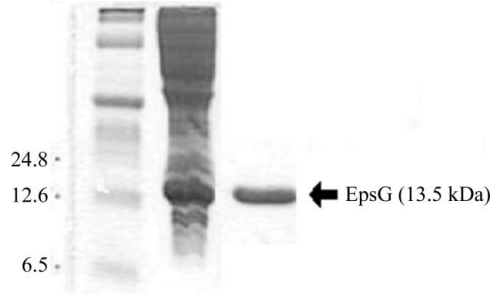

Figure 1
SDS–PAGE analysis of purified recombinant EpsG. Lane 1, molecular-weight markers (kDa); lane 2, crude lysate; lane 3, purified recombinant EpsG.

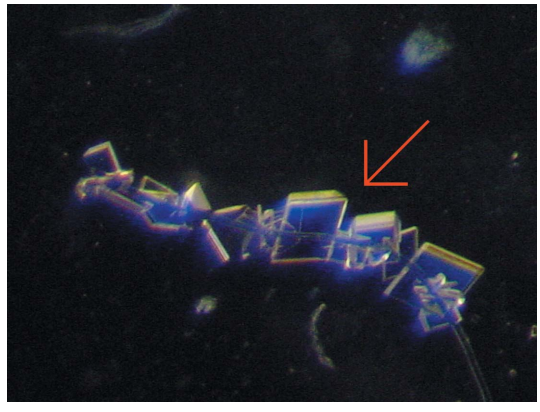

Figure 2
Twinned crystal of EpsG. The red arrow points towards a crystal that appears to have two plates sharing one face.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell (2.34–2.26 Å).

Wavelength (Å)	1.278
Resolution (Å)	30–2.26
No. of reflections	597503 (49930)
No. of unique reflections	143508 (13872)
Multiplicity	4.2 (3.6)
$R_{\rm merge}$ (%)	5.8 (32.5)
Completeness (%)	99.5 (96.6)
$\langle I\rangle/\langle\sigma(I)\rangle$	22.8 (3.6)
Mosaicity	0.30

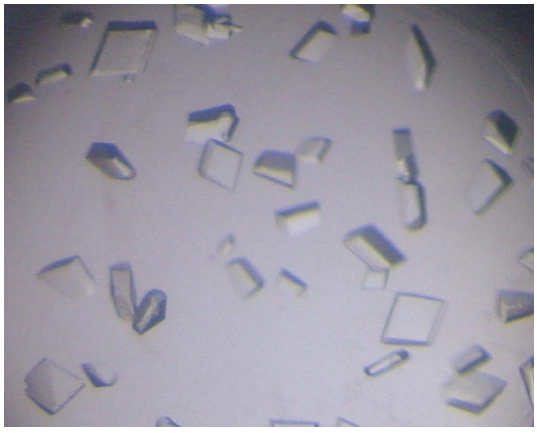

Figure 3
Optimized EpsG crystals. Typical crystal dimensions were 0.8 × 0.8 × 0.1 mm.

one week. Under the initial conditions used for optimization, EpsG crystals grew more rapidly (∼2 d) and often appeared to be visibly twinned, having the appearance of two plates with a shared face (Fig. 2). Twinning was confirmed by analyzing data sets using the *phenix.xtriage* program from the *PHENIX* suite (Zwart *et al.*, 2005). Most of the crystals appeared to be visibly twinned, especially the largest and thickest crystals. We were able to separate the conjoined plates using a nylon loop, but not without damaging the crystals.

Three approaches were combined to successfully produce larger untwinned crystals (Fig. 3). To reduce the rate of equilibration, the reservoirs of the VDX plate were partially covered with paraffin oil. In addition, the starting conditions of the drop were altered by the addition of two volumes of water. Finally, the reservoir solution was also diluted with water. Cryoprotection was achieved by adding 400 µl glycerol to the reservoirs and allowing the hanging drops to re-equilibrate, dehydrating and thus concentrating the PEG MME 550 in the drop to the level needed for cryoprotection. The resulting diffraction-quality crystals belonged to space group $P2_1$, with unit-cell parameters a = 88.61, b = 70.02, c = 131.54 Å (Table 1).

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