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# Crystallization and preliminary X-ray crystallographic analysis of the N-terminal domain of XpsE protein from *Xanthomonas campestris*, an essential component of the type II protein-secretion machinery

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Secretion of pre-folded extracellular proteins across the outer membrane of Gram-negative bacteria is mainly assisted by the type II secretion machinery composed of 12–15 proteins. Here, the crystallization and preliminary analysis of one of the essential components of *Xanthomonas campestris* secretion machinery, the 21 kDa N-terminal domain of XpsE protein ($XpsE_N$), are reported. $XpsE_N$ has been crystallized at 277 K using PEG 400 as precipitant. These crystals belong to the tetragonal space group $P4_12_12$ (or $P4_32_12$), with unit-cell parameters $a = b = 56.1$, $c = 102.7$ Å. A 98.5% complete native data set from a frozen crystal has been collected to 2.0 Å resolution at 100 K with an overall $R_{merge}$ of 5.0%. The presence of one subunit of $XpsE_N$ per asymmetric unit gives a crystal volume per protein weight ($V_M$) of 1.92 Å$^3$ Da$^{-1}$ and a solvent content of 36.1%.

## 1. Introduction

The type II protein-secretion pathway (also known as the general secretion pathway) is widely used by Gram-negative bacteria, especially by animal and plant pathogens, for secreting hydrolytic enzymes or toxins across the bacterial outer membrane (Sandkvist, 2001). Secretion through this pathway differs from most other membrane-transport systems in that its substrates consist of folded proteins. The secretion machinery is composed of 12–15 conserved gene products. This gigantic supramolecular complex spans the entire periplasm and penetrates both the outer and the cytoplasmic membranes. Based on the current functional annotations and subcellular locations, the essential protein components of the *Xanthomonas campestris* pv. *campestris* type II protein-secretion machinery can be classified into four groups: (i) the outer membrane protein XpsD (Chen *et al.*, 1996), (ii) the pseudopilins XpsG, XpsH, XpsI, XpsJ and XpsK (Hu *et al.*, 2002), (iii) the integral cytoplasmic membrane proteins XpsF, XpsL, XpsM and XpsN (Lee *et al.*, 2001; Tsai *et al.*, 2002) and (iv) the non-membrane cytoplasmic protein XpsE.

Among these various protein components, XpsE is unique in two aspects: it is the only cytoplasmic protein and is the only protein component of this system with the potential to exhibit ATPase activity. XpsE belongs to the GspE-VirB11 superfamily of putative NTPases that are essential for type II (GspE) and IV (VirB11) secretion pathways (Planet *et al.*, 2001). Also included in the former are proteins involved in type IV pilus assembly (Christie, 2001; Mattick, 2002). Members of this superfamily are characterized by the presence of a Walker A motif for nucleotide binding in the C-terminal half of the polypeptide. The integrity of the nucleotide-binding motif is crucial for the functions of XpsE and its orthologues; protein secretion is greatly perturbed by mutations in this region (Turner *et al.*, 1993; Possot & Pugsley, 1994; Sandkvist *et al.*, 1995; Py *et al.*, 1999). Since the periplasmic space is in general short of energy sources such as ATP, it has been proposed that XpsE may function as an energy-generating component for protein secretion. Although isolated XpsE does not associate with the cytoplasmic membrane, it is recruited to the cytoplasmic face of the membrane-anchored secretion machinery by XpsL (Leu & Hu, unpublished results). It has been well established that protein–protein interactions between orthologues of XpsE and XpsL are required for secretion (Sandkvist *et al.*, 1995; Py *et al.*, 1999). Recent studies suggest that the XpsE–XpsL interaction is probably mediated by the N-terminal region of XpsE (Leu & Hu, unpublished results).

Results of limited proteolysis revealed a protease-sensitive site near the N-terminus of the XpsE protein (Hu, unpublished results). This protease-sensitive site coincides with a region in the EpsE protein (the XpsE orthologue in *Vibrio cholerae*) that divides it into an N-terminal and a C-terminal region. Species



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specificity was determined by the N-terminal region. The chimera constituting of the N-terminal region of EpsE and the C-terminal region of the ExeE protein (the XpsE orthologue in *Aeromonas hydrophila*) exhibited near-normal function in *V. cholerae*, whereas the reverse combination did not (Sandkvist *et al.*, 1995). Interestingly, despite its lower sequence similarity with other GspE proteins involved in type II secretion, the 21 kDa N-terminal region of the XpsE protein ($XpsE_N$, residues 1–152) shares significant similarity with those in the GspE subfamily involved in type IV pilus assembly. Collectively, the $XpsE_N$ polypeptide and its homologues are classified as a distinct protein family GSPII_E_N (PF05157) in the Pfam Protein Family Database (Bateman *et al.*, 2002). $XpsE_N$ is therefore a legitimate target for crystallographic studies. In sharp contrast to the progress of biochemical understanding of the type II protein-secretion pathway, no detailed three-dimensional structural information is currently available for this system. $XpsE_N$ has been cloned and overexpressed in *Escherichia coli*. As the first step toward structural studies on this complicated machinery, we have purified and crystallized $XpsE_N$. The crystallization conditions and preliminary X-ray crystallographic analysis are reported in this paper.

## 2. Experimental

### 2.1. Protein expression and purification

$XpsE_N$ was cloned into a T7 promoter-driven expression system (pET-15b vector, Invitrogen), allowing expression of six-histidine-tagged recombinant protein in *E. coli* BL21(DE3) cells. Protein expression was induced by the addition of 0.4 m*M* isopropyl-D-thiogalactopyranoside at an optical density ($OD_{595}$) of 0.6 and cell growth was continued for another 4 h at 310 K. The cells were harvested by centrifugation and the cell pellet was resuspended in lysis buffer (50 m*M* Tris–HCl pH 8.0, 300 m*M* NaCl, 0.5 m*M* PMSF, 10% glycerol and 5 m*M* $\beta$-mercaptoethanol). Following cell disruption (by Microfluidizer, Christison) and centrifugation, the clarified cell lysate was loaded onto an immobilized $Co^{2+}$ affinity column (Clontech). The bound $XpsE_N$ was eluted with buffer containing 150 m*M* imidazole. Fractions containing $XpsE_N$ were pooled and 3 *M* $(NH_4)_2SO_4$ was added to a final concentration of 1 *M*. The solution was loaded onto a HiPrep 16/10 Phenyl column (Amersham Biosciences) equilibrated in hydrophobic interaction buffer [20 m*M* Tris–HCl pH 8.0, 200 m*M* NaCl, 1.0 m*M* EDTA, 1 m*M* $\beta$-mercaptoethanol, 1 *M* $(NH_4)_2SO_4$] and $XpsE_N$ was eluted with a 1.0–0 *M* $(NH_4)_2SO_4$ gradient. Fractions containing $XpsE_N$ were pooled, concentrated by ultrafiltration using an Amicon Ultra device (Millipore; 10 kDa cutoff) and loaded onto a HiLoad 16/60 Superdex-200 size-exclusion column (Amersham Biosciences) equilibrated in gel-filtration buffer (20 m*M* Tris–HCl pH 8.0, 300 m*M* NaCl, 0.5 m*M* EDTA, 1 m*M* $\beta$-mercaptoethanol). The peak fractions containing $XpsE_N$ were pooled and concentrated by ultrafiltration to $\sim$20 mg ml$^{-1}$.

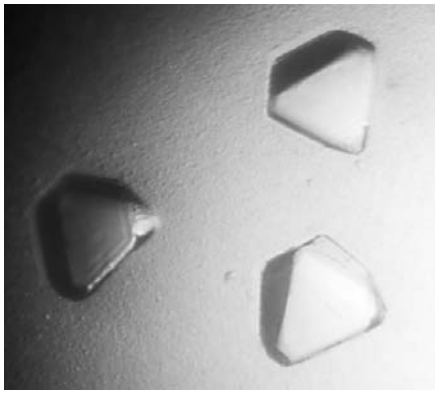

**Figure 1**
Crystals of the N-terminal domain of XpsE ($XpsE_N$) from *X. campestris*. Approximate dimensions of the crystals are 0.3 × 0.3 × 0.15 mm.

### 2.2. Crystallization and data collection

Initial crystallization trials were performed with commercially available kits (Hampton Research, USA) using the hanging-drop vapour-diffusion method. 2 µl protein solution (20 mg ml$^{-1}$ in gel-filtration buffer) and 2 µl reservoir solution were mixed and equilibrated against 500 µl reservoir solution at 277 K. Conditions that produced small crystals were then refined in a systematic manner by varying the pH and the concentration of precipitant. Diffraction-quality crystals were obtained using reservoir solution containing 0.08 *M* magnesium acetate, 0.05 *M* sodium cacodylate pH 6.5 and 15% PEG 400 (Fig. 1).

Crystals of $XpsE_N$ were successfully frozen in liquid nitrogen using ethylene glycol as cryoprotectant. Specifically, these crystals were first transferred to a cryoprotectant solution consisting of reservoir buffer plus an additional 3% PEG 400 for 2 h. Crystals were transferred through a series of similar solutions containing 5, 10, 15 and 20% ethylene glycol over the course of $\sim$2 h. After soaking for 20 min in the last cryoprotectant solution, crystals were flash-frozen by plunging them into liquid nitrogen. X-ray diffraction data were collected from a flash-frozen crystal at 100 K using a Rigaku RU-H3R rotating-anode generator (Cu $K\alpha$, $\lambda$ = 1.5418 Å) fitted with Osmic Confocal Blue Optics and an R-AXIS IV++ image-plate detector. The crystal was rotated though a total of 150° with 1° oscillations. The data were processed and scaled using the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

**Table 1**
Data-collection statistics.

Values in parentheses refer to the highest resolution shell (2.07–2.00 Å).

| | |
|---|---|
| X-ray wavelength (Å) | 1.5418 |
| Space group | $P4_12_12$ (or $P4_32_12$) |
| Unit-cell parameters (Å) | $a = b = 56.1$, $c = 102.7$ |
| Resolution (Å) | 30.0–2.0 |
| No. measured reflections | 133240 |
| No. unique reflections | 11597 |
| Multiplicity | 11.5 |
| $R_{merge}$† | 5.0 (49.6) |
| Data completeness (%) | 98.5 (97.1) |
| $\langle I/\sigma(I)\rangle$ | 38.8 (4.5) |

† $R_{merge} = [\sum_h \sum_i |I(h,i) - \langle I(h)\rangle| / \sum_h \sum_i I(h,i)] \times 100$, where $I(h, i)$ is the intensity of the $i$th measurement of reflection $h$ and $\langle I(h)\rangle$ is the mean value of $I(h, i)$ for all $i$ measurements.

## 3. Results and discussion

The 21 kDa recombinant *X. campestris* $XpsE_N$ with an N-terminal six-histidine tag was overexpressed in *E. coli*, purified to apparent homogeneity and crystallized according to the methods described in §2. Crystals of $XpsE_N$ reached maximum dimensions of approximately 0.3 × 0.3 × 0.15 mm within 3–5 d (Fig. 1). As a sudden increase in ethylene glycol concentration led to crystal cracking, flash-freezing of these crystals was only possible when the slow step-transfer procedure described above was performed. Upon successful cryostabilization, they diffracted to better than 2.0 Å resolution using rotating-anode X-rays.

Preliminary processing of the diffraction pattern suggested that the crystals belong to the primitive tetragonal lattice system, with unit-cell parameters $a = b = 56.1$, $c = 102.7$ Å. Using a single crystal of $XpsE_N$, a total of 133 240 observations were collected at 100 K to give a data set which is 98.5% complete to 2.0 Å, with 11 597 unique reflections and an overall $R_{merge}$ of 5.0%. Although the $R_{merge}$ of 49.6% in the highest resolution shell (2.07–2.00 Å) seems alarmingly high, data at this resolution should still be useful given the corresponding $\langle I/\sigma(I)\rangle$ of 4.5 and the highly redundant nature of this data set.

Additional analysis and systematic absences indicate the space group to be $P4_12_12$ (or $P4_32_12$). Consistent with this assignment, a crystallographic fourfold axis and five crystallographic twofold axes were revealed from the self-rotation function using the *GLRF* program (Tong & Rossmann, 1990). Because no internal symmetry was expected for $XpsE_N$, there should be one $XpsE_N$ molecule per asymmetric unit, corresponding to a crystal volume per protein mass ($V_M$) of 1.92 $Å^3$ $Da^{-1}$ and a solvent content of about 36.1%; both values are consistent with those of protein crystals. The data-collection statistics are summarized in Table 1. The phase problem will be solved by applying the selenium-based multi-wavelength anomalous diffraction technique, as no homologous structure is currently available. To this end, selenomethionine-derivatized $XpsE_N$ crystals have been produced. In addition, a site-directed $XpsE_N$ mutant in which two leucine residues were replaced by methionines has been constructed, purified and crystallized to augment the number of ordered selenium sites ($XpsE_N$ has three methionine residues) and hence the anomalous scattering signal in order to facilitate structure determination.

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