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Cloning, purification, crystallization and preliminary X-ray diffraction studies of *Escherichia coli* PapD-like protein (EcpD)

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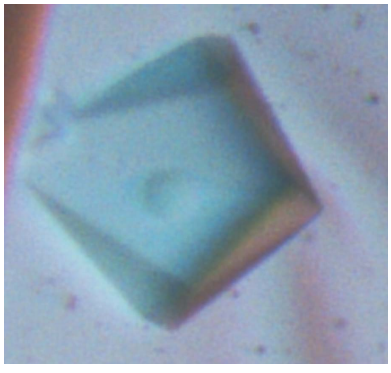
Many Gram-negative bacteria are characterized by hair-like proteinaceous appendages on their surface known as fimbriae. In uropathogenic strains of *Escherichia coli*, fimbriae mediate attachment by binding to receptors on the host cell, often contributing to virulence and disease. *E. coli* PapD-like protein (EcpD) is a periplasmic chaperone that plays an important role in the proper folding and guiding of Yad fimbrial proteins to the outer membrane usher protein in a process known as pilus biogenesis. EcpD is essential for pilus biogenesis in uropathogenic *E. coli* and plays an important role in virulence. In the present study, EcpD was cloned, overexpressed, purified and crystallized by the hanging-drop vapour-diffusion method. The crystals diffracted to 1.67 Å resolution and belonged to the orthorhombic space group $C222_1$, with unit-cell parameters a = 100.3, b = 127.6, c = 45.9 Å. There was a single molecule in the asymmetric unit and the corresponding Matthews coefficient was calculated to be 3.02 Å^3 Da^{-1}, with 59% solvent content. Initial phases were determined by molecular replacement.

1. Introduction

Many bacteria display proteinaceous appendages on their surface known as pili or fimbriae that are involved in a variety of functions such as attachment, invasion, colonization and biofilm formation (Telford *et al.*, 2006). Based on their biosynthetic pathways, these fimbriae have been categorized into five classes (Waksman & Hultgren, 2009). Of these five classes, the chaperone–usher (CU) pili often contribute to virulence and hence it is necessary to understand the role of these pili in host–pathogen interactions (Fronzes *et al.*, 2008). EcpD is a periplasmic chaperone of Yad CU pili which is known to play a significant role in pilus biogenesis and is prevalently found in uropathogenic *Escherichia coli* (UPEC), which predominantly infects bladder epithelial cells (Spurbeck *et al.*, 2011). EcpD shares ∼40% homology with the uropathogenic *E. coli* PapD protein (Raina *et al.*, 1993); it is supposed to be involved in the proper folding of Yad fimbrial proteins and in guiding them to the outer membrane usher in a systematic manner, thereby adding to the growing fimbriae (Korea *et al.*, 2010; Tønjum *et al.*, 1993). Previous studies have shown that the formation of Yad fimbriae is essential for the wild-type level of adhesion to the host epithelial cell, colonization and biofilm formation of UPEC (Spurbeck *et al.*, 2011).

Types I and P fimbriae are the most studied CU pili and a wealth of structural and functional information is available. Type I pili are found in uropathogenic as well as human commensal *E. coli* strains (Levine *et al.*, 1980). UPEC adheres even without the expression of either of these two pili, suggesting that an alternative mechanism is involved in adhesion (Miyazaki *et al.*, 2002). Recent studies have shown the importance of *yad* genes in adherence, as the deletion of *yad* genes increases cell motility. However, less is known about the fimbriae that are mainly found in UPEC and help in adhesion and infection (Spurbeck *et al.*, 2011). Considering the significant role of EcpD in such a highly orchestrated interplay of molecules, its structural and functional characterization is necessary in order to understand the mechanism of pilus biogenesis and its role in virulence. Furthermore, its study has potential for the structure-based rational design of inhibitors against EcpD that may help in counteracting



urinary-tract infections. Here, we report the cloning, overexpression, purification, crystallization conditions and preliminary X-ray studies of EcpD.

2. Experimental methods

2.1. Cloning

Genomic DNA was extracted from *E. coli* cells according to standard protocols and used as a template for PCR (Sambrook *et al.*, 1989). The following primers were designed with an *Nde*I restriction site in the forward primer and a *Bam*HI site in the reverse primer (restriction sites are shown in bold): forward primer, 5′-GGAATT-**CCATATGG**ACATTGTCATTTCGGGTACTCG-3′; reverse primer, 5′-CGC**GGATCC**TTACAGCCTGGCATTACCTTC-3′. The *ecpD* gene without its signal sequence (residues 1–25 from the N-terminus) was PCR-amplified and gel purified using a gel-extraction kit (Qiagen, Germany). The PCR product and the pET11a expression vector (Novagen) were both sequentially digested overnight with *Bam*HI and *Nde*I and gel purified. The purified PCR product was ligated with double-digested and purified pET11a in a 6:1 molar ratio and incubated overnight at 289 K. The ligation mixture was used to transform *E. coli* TOP10 cells and positive colonies were screened by colony PCR. The error-free sequence of the cloned *ecpD* gene was established by DNA sequencing (Macrogen, Republic of Korea). The pET11-EcpD construct with the following expression sequence (an N-terminal methionine followed by the EcpD-specific amino acids) was overexpressed: MDIVISGTRV IYKSDQKSVN VRLENKGNNP LLVQSWLDTG DDNAEPGSIT VPFTATPPVS RIDAKRGQTI KLMYTASTSL PKDRESVFWF NVLEVPPKPD AEKVANQSLL QLAFRTRIKL FYRPDGLKGN PSEAPLALKW FWSGSEGKAS LRVTNPTPYY VSFSSGDLEA SGKRYPIDVK MIAPFSDEVM KVNGLNGKAN SAKVHFYAIN DFGGAIEGNA RL.

2.2. Overexpression

The recombinant pET11a-EcpD construct was transformed into *E. coli* BL21 (DE3) CodonPlus for protein overexpression. A single colony was picked up from the freshly transformed LB agar plate, inoculated into 10 ml Luria–Bertani (LB) medium containing 100 g ml^{-1} ampicillin and incubated in a shaker incubator at 175 rev min^{-1} at 310 K. At a cell density (A_{600}) of ∼0.7, 2% of the primary inoculum was inoculated into 2 l LB medium containing 100 µg ml^{-1} ampicillin. The cells were induced with 1 m*M* isopropyl *β*-D-1-thiogalactopyranoside (IPTG) at an A_{600} of ∼0.6–0.8 and incubated at 310 K for 6 h. The cells were harvested by centrifugation at 5000*g* for 20 min.

2.3. Purification

The harvested cell pellet was resuspended in sonication buffer (20 m*M* phosphate buffer pH 8.5 containing 20 m*M* NaCl) containing a Complete protease-inhibitor cocktail tablet (Roche) and lysis was achieved by sonication. The lysate was centrifuged at 20 000*g* for 45 min to remove unlysed cells and debris. The recombinant EcpD carried no affinity tag. Since the theoretical pI of EcpD was calculated to be 9.3 (http://web.expasy.org/protparam), initial purification was achieved by the pH-dependent precipitation of contaminating proteins. The lysate supernatant was dialyzed against buffer consisting of 20 m*M* sodium phosphate pH 6.5, 20 m*M* NaCl. After 8 h of dialysis, the solution was centrifuged at 20 000*g* for 1 h to remove the precipitated contaminating proteins. The supernatant was then loaded onto a series of pre-equilibrated anion-exchange (QFF) and cation-exchange (SP-HP) columns (GE Healthcare, USA). The columns were washed with ten column volumes of dialysis buffer and elution was performed using a step gradient of increasing molar concentrations of NaCl. The fractions obtained were analyzed on 15% SDS–PAGE. Pure fractions were pooled and concentrated using 10 kDa molecular-weight cutoff centrifugal filters (Millipore). To assess the integrity and homogeneity, EcpD was further purified using a Sephadex 75 gel-filtration chromatography column (GE Healthcare). Fractions containing pure protein, based on SDS–PAGE, were pooled and concentrated to ∼1 m*M* (∼24.5 mg ml^{-1}) in a buffer consisting of 20 m*M* sodium phosphate pH 6.5, 20 m*M* NaCl.

2.4. Crystallization, data collection and processing

EcpD was crystallized using commercial screens from Molecular Dimensions. Initial hits were obtained using a condition from the PGA-LM Screen consisting of 0.1 *M* ammonium sulfate, 0.1 *M* sodium acetate, 0.3 *M* sodium formate, 30%(*w*/*v*) PEG 400, 3%(*w*/*v*) PGA-LM pH 5.0 at 291 K. Optimization was performed by varying the concentration of PGA-LM in the crystallization buffer [0, 3, 5 and 8%(*w*/*v*)]. Crystals were obtained in all concentrations of PGA-LM. Therefore, we selected the condition involving no PGA-LM for further optimization, which was performed by varying the pH and the

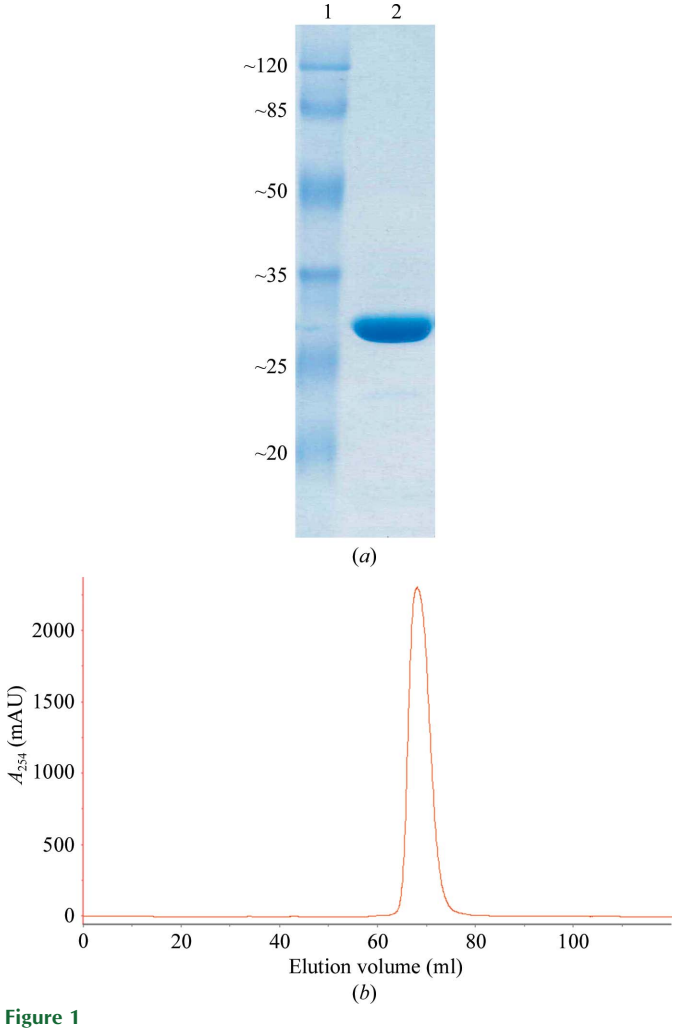


Figure 1
(*a*) 15% SDS–PAGE showing the electrophoretic profile of EcpD. Lane 1, protein molecular-weight marker (labelled in kDa); lane 2, purified EcpD fraction. (*b*) Chromatogram showing the elution profile of EcpD in gel-filtration chromatography

Table 1
Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

Space group	$C222_1$
Unit-cell parameters (Å, °)	a = 100.3, b = 127.6, c = 45.9, $\alpha = \beta = \gamma = 90$
Matthews coefficient (Å^3 Da^{-1})	3.01
Solvent content (%)	59.13
Data-collection temperature (K)	100
Detector	R-AXIS IV^{++}
Wavelength (Å)	1.5418
Resolution (Å)	50.0–1.67 (1.73–1.67)
Unique reflections	34473 (3383)
Multiplicity	12.8 (11.7)
$\langle I/\sigma(I)\rangle$	66.4 (4.9)
Completeness (%)	99.8 (99.7)
$R_{\rm merge}$† (%)	3.8 (45.4)
Total No. of reflections	440508
Overall B factor from Wilson plot (Å^2)	25.03
Mosaicity (°)	0.8

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

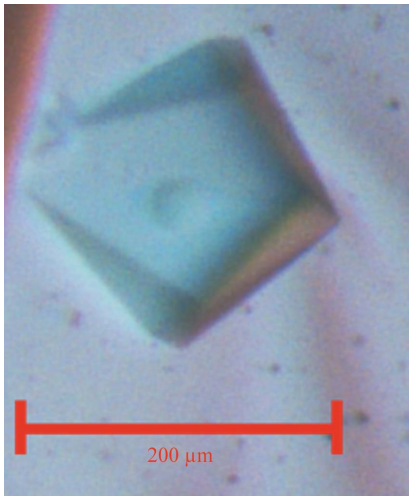


Figure 2
Morphology of the EcpD crystal. The approximate dimensions of the orthorhombic crystals were 200 × 200 × 125 µm.

incubation temperature. Diffraction-quality crystals were obtained using the hanging-drop vapour-diffusion method at 277 K by mixing 1 µl protein solution and 1 µl well solution [0.1 *M* ammonium sulfate, 0.1 *M* sodium acetate, 0.3 *M* sodium formate, 30%(*w*/*v*) PEG 400 pH 4.6] and equilibrating against 400 µl well solution. A single crystal was flash-cooled in a stream of nitrogen gas at 100 K. Data sets were collected at 100 K using Cu $K\alpha$ radiation (λ = 1.54 Å) on a Rigaku FR-E+ SuperBright microfocus rotating-anode (dual-wavelength; Cu and Cr) X-ray generator operated at 45 kV and 55 mA and equipped with an R-AXIS IV^{++} detector. A total of 360 frames were collected in 1° oscillation steps with 40 s exposure per frame. The crystal-to-detector distance was set to 100 mm. The data set was processed and scaled using the *HKL*-2000 package (Otwinowski & Minor, 1997). Initial phases were determined by the molecular-replacement (MR) method with the program *Phaser* in the *CCP*4 suite (Winn *et al.*, 2011) using the CupB2 chaperone from *Pseudomonas aeruginosa* (PDB entry 3q48; Cai *et al.*, 2011) as the search model. The relevant data statistics are summarized in Table 1.

3. Results and discussion

EcpD was successfully cloned and the protein was overexpressed in a soluble form. The yield was about 50 mg per litre of culture. Since the recombinant protein carried no affinity tag, purification was achieved using a combination of pI-based precipitation and anion-exchange and cation-exchange chromatography. The oligomeric status and homogeneity of the protein were analyzed by gel-filtration chromatography and the chromatogram revealed the monomeric nature of EcpD (Fig. 1). EcpD crystallized in the orthorhombic space group $C222_1$, with unit-cell parameters a = 100.3, b = 127.6, c = 45.9 Å (Fig. 2). The crystals diffracted to 1.67 Å resolution (Fig. 3). The Matthews coefficient was calculated to be 3.02 Å^3 Da^{-1}, which corresponds to a single molecule in the asymmetric unit with an estimated solvent content of 59.1% (Matthews, 1968). The MR search model CupB2 chaperone from *P. aeruginosa* (PDB entry 3q48; Cai *et al.*, 2011) has 40% sequence identity to the target sequence and a possible MR solution was obtained using the *Phaser* MR routine in *CCP*4. The MR solution revealed good crystal packing without any

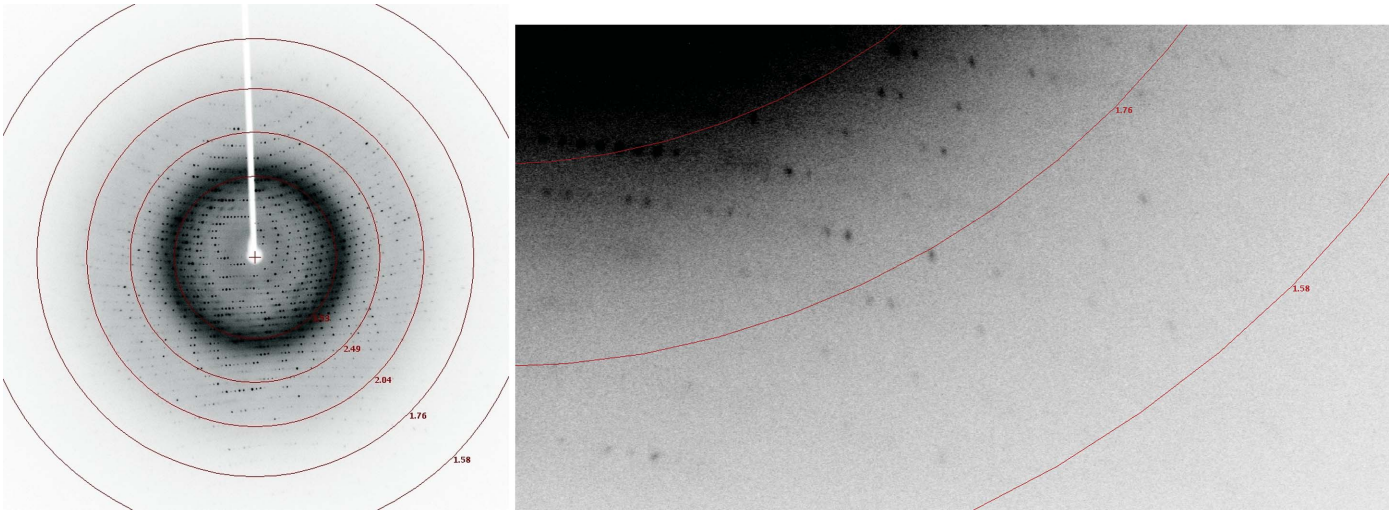


Figure 3
Representative X-ray diffraction image of the orthorhombic crystal of EcpD. A small region of the image is enlarged for clarity. The red concentric circles show the resolution rings (labelled in Å).

clashes between symmetry-related molecules. Efforts are under way to build and refine the model.

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