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Cloning, purification, crystallization and preliminary X-ray studies of flagellar hook scaffolding protein FlgD from *Pseudomonas aeruginosa* PAO1

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FlgD regulates the assembly of the hook cap structure to prevent leakage of hook monomers into the medium and hook monomer polymerization and also plays a role in determination of the correct hook length, with the help of the FliK protein. In order to better elucidate the functions of FlgD in flagellar hook biosynthesis, the three-dimensional structure of FlgD is being determined by X-ray crystallography. Here, the expression, purification, crystallization and preliminary crystallographic analysis of FlgD from *P. aeruginosa* are reported. The crystal belonged to space group *I*222 and diffracted to a resolution of 2.5 Å, with unit-cell parameters a = 116.47, b = 118.71, c = 118.85 Å. The crystals are most likely to contain three molecules in the asymmetric unit, with a V_M value of 2.73 Å^3 Da^{-1}.

1. Introduction

Bacterial flagella, which are related to the virulence of opportunistic pathogens, have been examined in several animal models of infection (Feldman *et al.*, 1998). Different bacterial species have distinctive arrangements of flagella and unique flagellar antigens. These characteristics can be exploited in the identification of the infectious agents that may be involved in a given disease process. The bacterial flagellum, a complicated nanostructure protein machine which requires about 50 proteins for regulation and assembly, consists of three major substructures: the filament, the hook and the basal body (Soutourina & Bertin, 2003). FlgD functions as the hook scaffolding protein and is also considered to be the hook-capping protein and the basal body rod-modification protein. In addition, together with FliK, FlgD regulates the assembly of the hook cap structure to prevent the leakage of hook monomers into the medium and hook monomer polymerization as well playing a role in determination of the correct hook length (Ohnishi *et al.*, 1994). Studies of flagellar biosynthesis in *Escherichia coli* and *Salmonella enterica* suggest that the hook cap is a transient intermediate structure in hook biosynthesis and FlgD is not found in the mature flagellum (Pallen *et al.*, 2005). Because FlgD is required for hook regulation and assembly and therefore plays a critical role in bacterial pathogenesis, it has become the focus of much study.

To date, only the crystal structure of the C-terminal domain (residues 87–220) of FlgD from *Xanthomonas campestris* (XcFlgD; PDB code 3c12) has been determined. This structure comprises a novel hybrid fold consisting of a tudor-like domain interdigitated with a fibronectin type III domain (Kuo *et al.*, 2008). However, the FlgD C-terminal domain has not provided any clues about the function of the intact protein in the assembly and the regulation of this hook construction. In order to better understand the biological role of FlgD in flagellar hook biosynthesis, we have initiated the determination of its three-dimensional structure by X-ray crystallography. Here, we report the crystallization and preliminary X-ray study of FlgD from *Pseudomonas aeruginosa* PAO1. Structure determination will be pursued using experimental phasing methods.

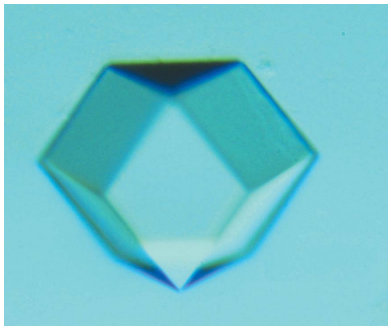

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Space group	*I*222
Unit-cell parameters (Å)	a = 116.47, b = 118.71, c = 118.85
Wavelength (Å)	1.5418
Resolution (Å)	60–2.5
No. of reflections (observed)	116248
No. of reflections (unique)	25001
Completeness (%)	96.1 (93.1)
$\langle I/\sigma(I)\rangle$	11.0 (2.9)
$R_{\rm merge}$† (%)	5.5 (26.6)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the ith measurement of an equivalent reflection with indices hkl.

2. Materials and methods

2.1. Cloning and expression

The gene encoding FlgD was amplified by the polymerase chain reaction using *P. aeruginosa* PAO1 genomic DNA as the template and primers that contained *Nde*I and *Xho*I restriction sites. Amplified DNA was digested using the *Nde*I and *Xho*I endonucleases and was then inserted into *Nde*I/*Xho*I-digested pET28a to generate pET28*FlgD*-His6. The complete nucleotide sequence of the insert was confirmed by DNA-sequence analysis on an ABI 3100 DNA Sequencer (Applied Biosystems). The expression construct contained two additional amino residues, Met-Glu, as a cloning artifact together with the N-terminal 6×His tag encoded by the vector. The recombinant expression vector was then introduced into *E. coli* BL21 (DE3) cells. 2 l of cells was grown at 310 K in LB medium containing 50 mg l^{-1} kanamycin until the $OD_{600\,nm}$ reached 0.4; protein expression was then induced with 0.5 m*M* isopropyl *β*-D-1-thiogalactopyranoside (IPTG) for 4 h and the cells were harvested by centrifugation for 10 min at 4000*g* and 277 K.

2.2. Purification

The cell pellet was resuspended in 50 ml lysis buffer containing 20 m*M* Tris–HCl pH 8.0 and 300 m*M* sodium chloride and lysed by sonication. The lysate was centrifuged at 15 000*g* for 30 min at 277 K and the cell debris was discarded. The supernatant was applied onto a 4 ml Ni^{2+}–NTA (Qiagen) column pre-equilibrated with lysis buffer. The column was washed with 40 ml lysis buffer plus 10 m*M* imidazole, followed by 40 ml each of lysis buffer plus 20 m*M* imidazole and lysis buffer plus 40 m*M* imidazole. The FlgD protein was then eluted from the column using elution buffer containing 20 m*M* Tris–HCl pH 8.0 and 200 m*M* imidazole. The Ni^{2+}–NTA eluate was then loaded onto a DEAE Sepharose Fast Flow (Amersham Bioscience) column equilibrated with 20 m*M* Tris–HCl pH 8.0 for further purification. Recombinant proteins were eluted with a linear gradient of 0–500 m*M* sodium chloride in 20 m*M* Tris–HCl pH 8.0. Fractions containing FlgD were identified using SDS–PAGE and Coomassie staining. Pooled fractions were concentrated and buffer-exchanged to 5 m*M* Tris–HCl pH 8.0, 50 m*M* NaCl using a Millipore Amicon concentrator with 10 kDa cutoff membrane for subsequent crystallization. The final purified protein concentration was 20 mg ml^{-1}.

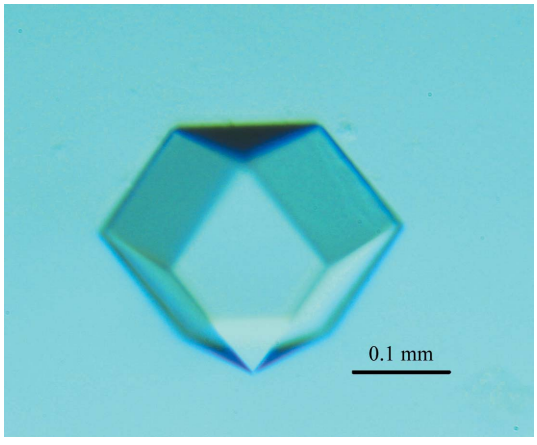

Figure 1
Crystal of FlgD from *P. aeruginosa* as grown by the hanging-drop method. The average dimensions of the crystals were 0.5 × 0.3 × 0.3 mm.

2.3. Crystallization

Preliminary screening of crystallization conditions was performed using the hanging-drop vapour-diffusion method with Crystal Screens I and II (Hampton Research) and PEG Screen kits. The crystallization experiments consisted of 1.0 µl protein solution (containing the His tag) and 1.0 µl reservoir solution. The drops were equilibrated against 400 µl reservoir solution at 293 K. After 7 d, small crystals of FlgD were observed using reservoir conditions consisting of 12%(*w*/*v*) polyethylene glycol (PEG) 20 000, 0.1 *M* MES pH 6.5. Further optimization of the conditions with PEGs of different molecular weights at various concentrations gave good diffraction-quality crystals using 15–20% PEG 6000 in the presence of 0.1 *M* MES pH 6.60–6.80. Crystals appeared after 3 d equilibration against the crystallization solution and grew to full size (0.5 × 0.3 × 0.3 mm) in 10 d (Fig. 1).

2.4. Data collection and processing

Crystals were transferred to a cryoprotectant solution consisting of 0.08 *M* MES pH 6.60–6.80, 16% PEG 6000 and 20% glycerol. X-ray diffraction data were collected from a single crystal using a Rigaku RU-200 rotating-anode X-ray generator equipped with Osmic

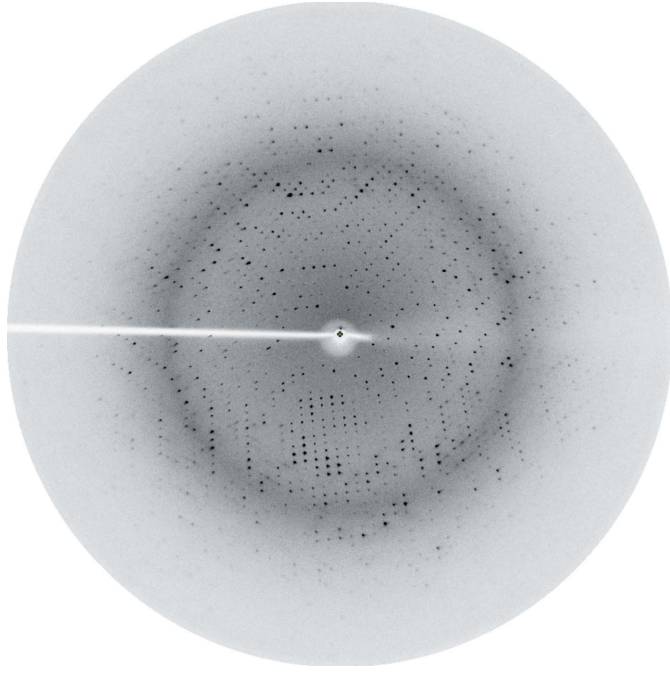

Figure 2
X-ray diffraction image from a native *P. aeruginosa* FlgD crystal recorded on a MAR 345 image plate. The edge of the detector corresponds to a resolution of 2.2 Å.

focusing mirrors and a MAR Research image-plate system (diameter 345 mm). The crystal-to-detector distance was 200 mm. All images were collected at 100 K using a 1° oscillation angle and an exposure time of 600 s per frame (see Fig. 2). The data set was integrated and reduced using *MOSFLM* (Leslie, 1992) and *SCALA* from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994). Table 1 lists the final data-collection and processing statistics.

3. Analysis and discussion of preliminary X-ray diffraction results

A complete diffraction data set was collected to 2.4 Å resolution from a single crystal and the data-collection statistics are reported in Table 1. A total of 361 418 measured reflections in the resolution range 50–2.4 Å were merged into 38 942 unique reflections with an $R_{\rm merge}$ of 6.7%. Analysis of the diffraction intensities indicated that the most plausible space group was body-centered orthorhombic *I*222, with unit-cell parameters a = 116.46, b = 118.72, c = 118.86 Å. Based on the molecular weight of FlgD (25 kDa) and space group *I*222, it was assumed that the crystal contained three molecules per asymmetric unit. The assumption gives a $V_{\rm M}$ value of 2.73 Å^3 Da^{-1} and a solvent content of 55% (Matthews, 1968). Molecular replacement using *AMoRe* (Navaza, 2001), *Phaser* (McCoy *et al.*, 2005) and *MOLREP* (Vagin & Teplyakov, 2000) was carried out using the XcFlgD structure as a search model (PDB code 3c12; Kuo *et al.*, 2008). The homologous XcFlgD has 30% sequence identity to the C-terminal domain of FlgD from *P. aeruginosa* PAO1. However, efforts to determine the structure using molecular replacement with XcFlgD have proved to be unsuccessful. We are now in the process of producing selenomethionylated FlgD protein in order to use multi-wavelength anomalous dispersion methods for structure determination (the FlgD protein contains six methionine residues).

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