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# Purification, crystallization and preliminary X-ray crystallographic analysis of the C-terminal cytoplasmic domain of FlhB from *Aquifex aeolicus*

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FlhB is a key protein in the regulation of protein export by the bacterial flagellar secretion system. It is composed of two domains: an N-terminal transmembrane domain and a C-terminal cytoplasmic domain (FlhBc). Here, the crystallization and preliminary crystallographic analysis of FlhBc from *Aquifex aeolicus* are reported. Purified protein was crystallized using the vapour-diffusion technique. The crystals diffracted to 2.3 Å resolution and belonged to space group *C*2, with unit-cell parameters $a$ = 114.49, $b$ = 33.89, $c$ = 122.13 Å, $\beta$ = 107.53°.

## 1. Introduction

The flagellum is the most common motility organelle in the bacterial world. The bacterial flagellum is a long thin filament extending from the cytoplasm to the cell exterior. It can be divided into three major parts: the basal body, the hook and the filament (Macnab, 2003). Most flagellar proteins are localized outside the cell and are translocated across the cell membrane by the flagellum-specific secretion apparatus, which shares similarity to the bacterial type III secretion system (Aizawa, 2001; Blocker *et al.*, 2003). This protein export is strictly regulated. Upon completion of the hook, the secretion system switches substrate specificity from hook-type export to filament-type export. Two components, the hook-length control protein FliK and the membrane protein FlhB, have been found to be crucial for this switching (Hirano *et al.*, 1994; Kutsukake *et al.*, 1994; Williams *et al.*, 1996).

FlhB is an integral protein of the flagellar export apparatus and homologues of FlhB have been found in all bacterial type III secretion systems. The molecular weight of FlhB is about 40 kDa and the protein consists of two domains: a hydrophobic N-terminal part, consisting of ∼210 residues, which is predicted to contain four transmembrane helices, and a C-terminal cytoplasmic domain of ∼170 residues (Minamino *et al.*, 1994). The wild-type cytoplasmic domain of FlhB undergoes autocatalytic cleavage between amino-acid residues Asn and Pro within a highly conserved NPTH sequence (Minamino & Macnab, 2000; Zarivach *et al.*, 2008). This autocleavage is essential for the switching process (Fraser *et al.*, 2003; Ferris *et al.*, 2005). Mutation of the Asn residue to Ala prevents cleavage and locks the export apparatus in the hook-type specificity state.

Several structures of the cytoplasmic domain of FlhB paralogues from the bacterial needle type III secretion system have been published (Zarivach *et al.*, 2008; Deane *et al.*, 2008; Lountos *et al.*, 2009; Wiesand *et al.*, 2009).

In this paper, we describe the expression, purification and crystallization of the wild-type cleaved form of FlhBc from the flagellar export system of the hyperthermophilic bacterium *Aquifex aeolicus*.



## 2. Experimental procedures and results

### 2.1. Cloning, expression and purification

A DNA fragment encoding the cytoplasmic fragment of FlhB (Gene ID 1193710; amino-acid residues 213–350) was generated by PCR from genomic DNA of *A. aeolicus* as a template using the primers 5′-CAT ATG AAG ATA ATG ATG TCG AGA AGG GAA TTG-3′ and 5′-GCT CTT CCG CAG GCG TAA ACC TTT TTC

TTT TTG AAC-3′ containing *Nde*I and *Sap*I restriction sites. The amplified DNA fragment was purified on agarose gel and ligated into pGEM-T vector (Promega) using A–T base pairing. After confirming the sequence, the DNA fragment was digested from the vector with *Nde*I and *Sap*I, purified on agarose gel and cloned into the pTXB1 vector (New England BioLabs), generating an in-frame fusion with the intein and chitin-binding domain (CBD).

The recombinant vector containing the gene for the FlhBc-intein-CBD fusion protein was transformed into *Escherichia coli* strain Rosetta (DE3) (Novagen). The transformed cells were cultured at 310 K to late exponential phase in 5 l Luria–Bertani medium containing 50 µg ml$^{-1}$ ampicillin and 34 µg ml$^{-1}$ chloramphenicol. Expression was induced with 0.5 m*M* isopropyl *β*-D-1-thiogalactopyranoside at an $OD_{600}$ of 0.7–0.8. After 4 h incubation at 310 K, the cells were harvested by centrifugation at 8000*g* for 15 min and suspended in 200 ml buffer *A* (40 m*M* Tris–HCl pH 8.0, 1 *M* NaCl, 2 m*M* EDTA, 20% glycerol). The suspended cells were sonicated on ice. After ultracentrifugation (100 000*g*, 1 h at 277 K), the supernatant was applied onto a 30 ml Chitin Beads column (New England BioLabs) equilibrated with buffer *A*. The column was washed with 200 ml buffer *A* and then with 100 ml buffer *A* supplemented with 0.1 *M* DTT to induce on-column cleavage. The *A. aeolicus* FlhBc protein was eluted with buffer *A* after 16 h incubation at 278 K. The eluted protein was dialyzed against 50 m*M* sodium acetate pH 4.5, 100 m*M* NaCl and applied onto an SP HP column (GE Healthcare) equilibrated with the same buffer. The protein was eluted with a linear gradient of NaCl from 0.1 to 1.2 *M* in 50 m*M* sodium acetate pH 4.5. Fractions containing pure FlhBc were pooled together and the protein was transferred to 10 m*M* sodium acetate pH 4.5, 100 m*M* NaCl by size-exclusion chromatography using Superdex75 (GE Healthcare). The final protein does not contain any non-native amino-acid residues. A typical preparation gives about 2–3 mg pure protein per litre of *E. coli* culture.

### 2.2. Crystallization

After gel filtration, the purified protein was concentrated to 10 mg ml$^{-1}$ using an Amicon Ultra system with a molecular-weight cutoff of 10 kDa (Millipore). The concentration of the protein was measured by UV spectroscopy at 280 nm using a calculated molar extinction coefficient of 10 430 $M^{-1}$ cm$^{-1}$.

Preliminary screening of crystallization conditions was carried out by the sitting-drop vapour-diffusion method (150 nl protein solution was mixed with 150 nl reservoir solution and equilibrated against 120 µl reservoir solution) at 293 K in 96-well plates using an automated nanolitre liquid-handling system (Mosquito, TTP Labtech) and the following screening kits: Crystal Screen and Crystal Screen 2 (Hampton Research), Wizard I, II and III and Cryo I and II (Emerald BioSystems). Crystals of FlhBc were obtained using condition No. 10 of Wizard III (0.2 *M* sodium thiocyanate, 20% PEG 3350). Crystals appeared in 3–4 weeks after setup of the drop and grew to maximum dimensions of 0.5 × 0.2 × 0.05 mm within a week (Fig. 1).

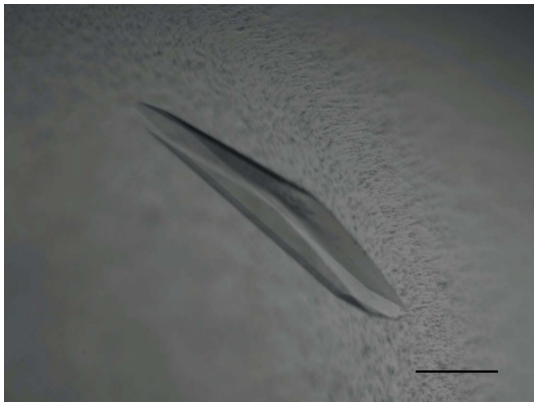

**Figure 1**
Crystals of FlhBc from *A. aeolicus*. The scale bar is 0.1 mm in length.

**Table 1**
Diffraction data statistics for FlhBc crystals.

Values in parentheses are for the highest resolution shell.

| | |
|---|---|
| Space group | *C*2 |
| Unit-cell parameters (Å, °) | $a$ = 114.49, $b$ = 33.89, $c$ = 122.13, $\alpha = \gamma$ = 90.0, $\beta$ = 107.53 |
| Wavelength (Å) | 0.9 |
| No. of images | 200 |
| Resolution (Å) | 40–2.3 (2.42–2.30) |
| Completeness (%) | 97.7 (98.7) |
| Total reflections | 83364 (12279) |
| Unique reflections | 19822 (2873) |
| Multiplicity | 4.2 (4.3) |
| $R_{\rm merge}$† (%) | 5.2 (63.7) |
| Mean $I/\sigma(I)$ | 11.2 (1.6) |

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

### 2.3. Data collection and processing

X-ray diffraction data were collected on beamline BL44XU at SPring-8 using a MAR225HE detector (Fig. 2). Prior to data collection, the crystals were briefly soaked in a cryoprotectant solution consisting of 0.2 *M* sodium thiocyanate, 20% PEG 3350 and 15% glycerol. Subsequently, crystals were mounted on a nylon loop and flash-cooled in liquid nitrogen. X-ray diffraction data were recorded with an oscillation angle of 1°, an exposure time of 1 s per frame and a crystal-to-detector distance of 270 mm. Data sets were processed and

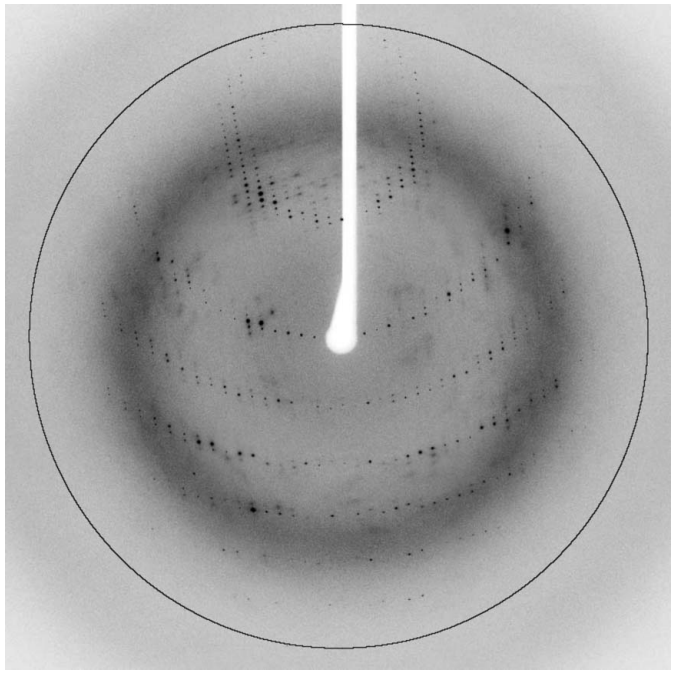

**Figure 2**
A typical X-ray diffraction pattern from an FlhBc crystal. The oscillation range is 1°. The ring indicates a resolution of 2.4 Å.

scaled using the *HKL*-2000 package (Otwinowski & Minor, 1997) and data statistics are given in Table 1. The crystals belonged to space group *C*2, with unit-cell parameters $a = 114.49$, $b = 33.89$, $c = 122.13$ Å, $\alpha = \gamma = 90.0$, $\beta = 107.53^{\circ}$. Calculation of the Matthews coefficient (Matthews, 1968) indicated that there could be between two and four molecules in the asymmetric unit, with Matthews coefficients of 3.5 and 1.8 $\text{Å}^3\,\text{Da}^{-1}$, respectively. This would correspond to a solvent content varying between 65.8 and 31.7%, respectively. In order to further investigate the number of molecules in the asymmetric unit, we calculated several self-rotation functions using *POLARRFN* (Kabsch, 1976) from the *CCP*4 package (Collaborative Computational Project, Number 4, 1994). However, these calculations were inconclusive. The number of molecules in the asymmetric unit will be determined clearly when the structure is solved.

Efforts are currently under way to solve the structure by molecular replacement using one of the structures of the FlhBc paralogues from the bacterial needle system as a search model.

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