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Purification, crystallization and preliminary X-ray crystallographic analysis of the C-terminal cytoplasmic domain of FlhB from *Salmonella typhimurium*

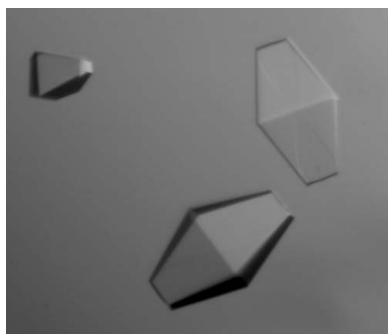
FlhB is a key protein in the regulation of protein export by the bacterial flagellar secretion system. It is composed of two domains: an N-terminal transmembrane domain and a C-terminal cytoplasmic domain (FlhBc). FlhBc from *Salmonella typhimurium* has been successfully crystallized using the vapour-diffusion method. The crystals diffracted to 2.45 Å resolution and belonged to space group $P4_22_12$, with unit-cell parameters $a = b = 49.06$, $c = 142.94$ Å. A selenomethionine-containing variant of FlhBc has also been crystallized in the same space group and was used for initial phase calculation by the multiwavelength anomalous dispersion (MAD) method.

1. Introduction

Many bacteria swim in a liquid environment by means of flagella. The bacterial flagellum is a long filament that protrudes out of the cell body. It is a huge complex structure made up of more than 30 different proteins (Macnab, 2003). The flagellum is divided into three major substructures: the basal body, the hook and the filament. Most of the flagellar proteins are localized outside the cell and are exported across the cytoplasmic membrane by the flagellum-specific secretion apparatus, which shares similarity to the bacterial type III secretion system that is used to secrete effectors into the host-cell cytoplasm (Aizawa, 2001; Blocker *et al.*, 2003). The flagellar type III secretion system is postulated to be located within the MS ring on the cytoplasmic side of the flagellar basal body. In the case of *Salmonella* it consists of six integral membrane proteins (FlhA, FlhB, FliO, FliP, FliQ and FliR) and three cytoplasmic proteins (FliH, FliI and FliJ) (Minamino & Macnab, 1999). The export apparatus is not only responsible for the translocation of flagellar proteins across the cell membrane but also regulates the order in which the proteins are exported. Upon completion of the hook, the secretion system switches substrate specificity from hook-type export to filament-type export. Three proteins, the membrane protein FlhB, the hook-length control protein FliK and Flk (also known as RflH or Fluke), play active roles in the switching (Hirano *et al.*, 1994; Kutsukake *et al.*, 1994; Williams *et al.*, 1996; Kutsukake, 1997; Aldridge *et al.*, 2006; Minamino *et al.*, 2009; Erhardt *et al.*, 2010).

Homologues of FlhB have been found in all bacterial type III secretion systems. The molecular weight of *Salmonella* FlhB is about 42 kDa and the protein consists of two domains: a hydrophobic N-terminal part consisting of 211 residues that is predicted to contain four transmembrane helices, and a C-terminal cytoplasmic domain, FlhBc, consisting of 172 residues (Minamino *et al.*, 1994). FlhBc has been shown to interact with the soluble components of the type III secretion system: FliH, FliI, FliJ and the cytoplasmic part of the membrane protein FlhA (Minamino & Macnab, 2000a; Zhu *et al.*, 2002).

The wild-type cytoplasmic domain of *Salmonella* FlhB undergoes autocatalytic cleavage between amino-acid residues Asn269 and Pro270 within a highly conserved NPTH sequence (Minamino & Macnab, 2000b; Zarivach *et al.*, 2008). This autocleavage is essential for the switching process (Fraser *et al.*, 2003; Ferris *et al.*, 2005).



Mutation of Asn269 to Ala prevents cleavage and locks the export apparatus in the hook-type specificity state.

To switch substrate specificity, FlhB receives a signal from FliK (Williams *et al.*, 1996; Minamino *et al.*, 2006). Cells with defective FliK have a very long hook (a so-called polyhook) without an attached filament (Hirano *et al.*, 1994). Suppression mutations of FlhB that can lead to switching even in the absence of FliK have been described (Kutsukake *et al.*, 1994; Williams *et al.*, 1996). All of these mutations are found exclusively in FlhBc, leading to the assumption of an important role of this part of the protein in export regulation. The interaction between FliK and FlhBc has been investigated by optical biosensing methods (Morris *et al.*, 2010). Recently, a model of the complex between FlhBc and the C-terminal domain of FliK has been proposed (Mizuno *et al.*, 2011).

Several structures of the cytoplasmic domains of FlhB paralogues from bacterial needle type III secretion systems have been published (Zarivach *et al.*, 2008; Deane *et al.*, 2008; Lountos *et al.*, 2009; Wiesand *et al.*, 2009). Recently, we reported the successful crystallization of FlhBc from the hyperthermophilic bacterium *Aquifex aeolicus* (Meshcheryakov *et al.*, 2011). *Salmonella* FlhBc and *Aquifex* FlhBc share 39% sequence identity (Fig. 1). However, the two proteins are evolutionarily very distant. Neither *fliK* nor *flk* were found in the *Aquifex* genome (Deckert *et al.*, 1998), suggesting that the regulation of secretion differs in *Salmonella* and *Aquifex*. Comparison of the two proteins may provide us with additional information about the mechanism of FlhB function. Here, we describe the crystallization and preliminary X-ray diffraction study of the wild-type cleaved form of FlhBc from *S. enterica* serovar Typhimurium.

2. Materials and methods

2.1. Cloning, expression and purification

A DNA fragment encoding the cytoplasmic fragment of *Salmonella* FlhB (GeneID 1253435; amino-acid residues 219–383) was generated by PCR from genomic DNA of *S. enterica* as a template using the primers 5'-CAT ATG AAA TTA CGC ATG TCG CGG CAG G-3' and 5'-GCT CTT CCG CAG GCA TCA GTA TTC TTC TCG TTC ATA AAA TC-3', which contain *NdeI* and *SapI* 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 *NdeI* and *SapI*, 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 BL21 (DE3) (Novagen). The transformed cells were cultured at 310 K to late exponential phase in 2 l Luria–Bertani medium containing 50 µg ml⁻¹ ampicillin. Expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside at an OD₆₀₀ of 0.7–0.8. After 4 h incubation at 310 K, the cells were harvested by centrifugation at 8000g for 15 min. Further purification of *Salmonella* FlhBc was performed as described in Meshcheryakov *et al.* (2011). A typical preparation gives about 20–30 mg pure protein per litre of *E. coli* culture. The final protein contained no non-native amino-acid residues, with the exception that the C-terminal Gly residue was replaced by Ala in order to facilitate self-cleavage of the intein.

Selenomethionine-substituted FlhBc was expressed in methionine-auxotrophic *E. coli* strain B834 (DE3) (Novagen) using SeMet core medium (Wako Pure Chemical Industries). The purification of SeMet FlhBc was the same as that for the native protein. The incorporation of selenomethionine was confirmed by MALDI–TOF mass spectrometry.

2.2. Crystallization

The purified protein was transferred into 10 mM sodium acetate pH 4.5, 50 mM NaCl by gel filtration and concentrated using an Amicon Ultra system (Millipore) with a molecular-weight cutoff of 10 kDa. The protein concentration was measured by UV spectroscopy at 280 nm using a calculated molar extinction coefficient of 22 460 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). Crystallization conditions were optimized by hanging-drop vapour diffusion using VDX plates (Hampton Research).

2.3. Data collection and processing

Diffraction data for both native FlhBc and SeMet-labelled FlhBc were collected on beamline BL44XU at SPring-8 using a MAR225 HE detector. Prior to data collection, the crystals were directly flash-cooled in their mother liquor using 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.

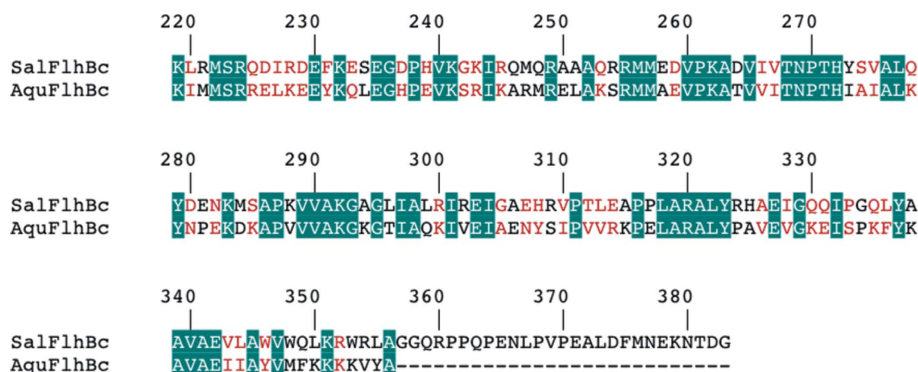


Figure 1

Sequence alignment of FlhBc from *S. typhimurium* (SalFlhBc) and *A. aeolicus* (AquaFlhBc). The numbering corresponds to *Salmonella* FlhB. Identical residues are shown with a teal background; similar residues are coloured red.

Wavelengths for the MAD method were selected based on XAFS measurements. A complete SeMet-derivative data set was collected

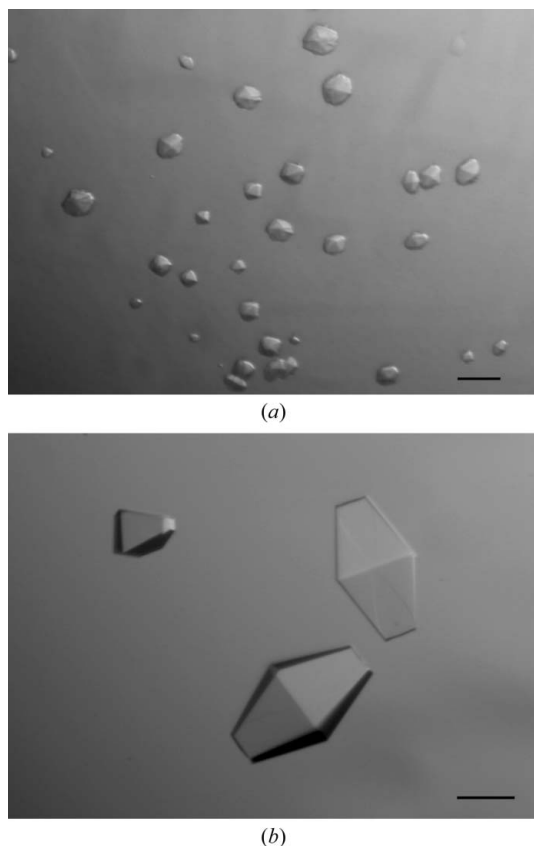


Figure 2 Crystals of *Salmonella* FlhBc grown in (a) 0.1 M MES pH 6.0, 0.2 M zinc acetate, 40% ethylene glycol and (b) 0.1 M sodium cacodylate pH 5.0, 0.2 M zinc acetate, 25% propylene glycol. Scale bar, 0.1 mm.

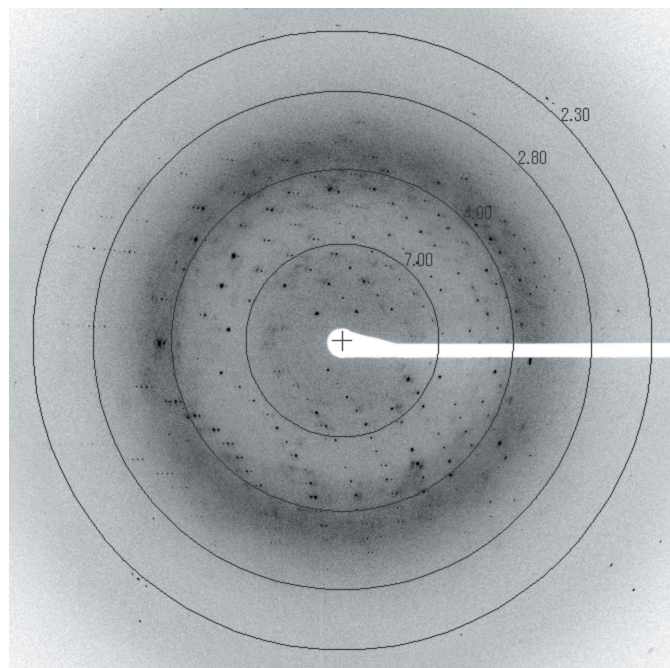


Figure 3 A typical X-ray diffraction pattern of a *Salmonella* FlhBc crystal. The oscillation range is 1°.

Table 1 Diffraction data statistics for FlhBc crystals. Values in parentheses are for the highest resolution shell.

	SeMet derivative		Native
	Peak	Edge	
Space group	$P4_22_12$	$P4_22_12$	$P4_22_12$
Unit-cell parameters (Å)			
$a = b$	48.75	48.82	49.06
c	143.08	143.2	142.94
Wavelength (Å)	0.978941	0.979187	0.9
No. of images	180	180	100
Resolution (Å)	48.75–2.80 (2.95–2.80)	48.82–2.90 (3.06–2.90)	40.45–2.45 (2.58–2.45)
Completeness (%)	99.8 (100)	99.8 (100)	98.8 (100)
Total reflections	63640 (9113)	57393 (8460)	52654 (7798)
Unique reflections	4714 (647)	4279 (601)	6875 (984)
Multiplicity	13.5 (14.1)	13.4 (14.1)	7.7 (7.9)
$R_{\text{merge}}^{\dagger}$ (%)	6.8 (34.0)	6.4 (32.3)	7.5 (38.0)
Mean $I/\sigma(I)$	21.4 (7.5)	23.3 (7.9)	16.2 (5.7)

$\dagger R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$, 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.

from a single crystal at wavelengths corresponding to the peak (0.97894 Å), the inflection point (0.97919 Å), a low remote (0.99490 Å) and a high remote (0.96396 Å). Diffraction data were indexed, integrated and scaled with *MOSFLM* (Leslie, 2006) and *SCALA* (Evans, 2006) from the *CCP4* package (Winn *et al.*, 2011).

3. Results

Microcrystals of *Salmonella* FlhBc were obtained using condition No. 26 of Cryo II (0.1 M MES pH 6.0, 0.2 M zinc acetate, 40% ethylene glycol; Fig. 2a). However, the crystals showed no diffraction even using a synchrotron X-ray source. Diffraction-quality crystals were obtained after optimization of each component of the initial crystallization condition. The final crystallization conditions were 0.1 M sodium cacodylate pH 5.0, 0.2 M zinc acetate, 25% propylene glycol. Crystals appeared in 2–3 d after setup of the drop and grew to maximum dimensions of 0.3 × 0.1 × 0.1 mm within a week (Fig. 2b).

The crystals diffracted to 2.45 Å resolution (Fig. 3) and belonged to space group $P4_22_12$, with unit-cell parameters $a = b = 49.06$, $c = 142.94$ Å, $\alpha = \beta = \gamma = 90.0^\circ$. Assuming one FlhBc molecule per

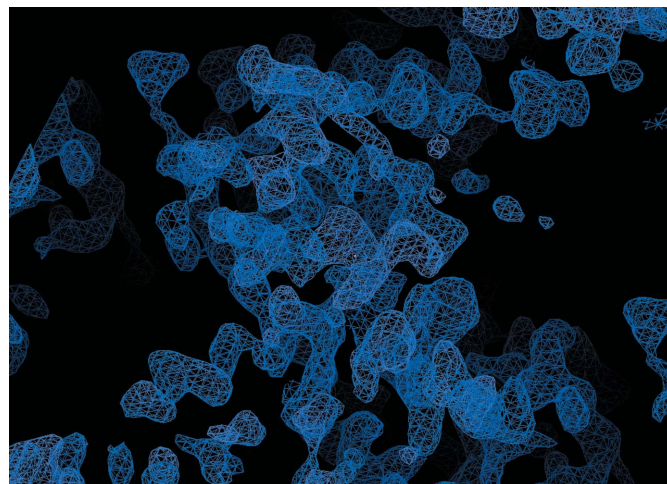


Figure 4 Experimental electron-density map at a contour level of 1.5σ calculated at a resolution of 2.8 Å.

asymmetric unit, the Matthews coefficient (Matthews, 1968) was calculated to be $2.31 \text{ \AA}^3 \text{ Da}^{-1}$, which corresponds to a solvent content of 46.8%.

Selenomethionine-labelled FlhBc was prepared in order to perform a MAD experiment. The SeMet FlhBc crystals grew under slightly different conditions (0.1 M sodium cacodylate pH 5.0, 0.3 M zinc acetate, 20% propylene glycol) with a similar morphology to the native FlhBc crystals. These crystals belonged to space group $P4_22_12$, with similar unit-cell parameters to the native protein (Table 1). A multiple-wavelength anomalous diffraction data set for the SeMet crystal was collected at four different wavelengths, but only two of them (peak and inflection point) were useful for phase calculation. Phase calculation and density modification were performed using *SHELXD* (Sheldrick, 2008), which resulted in an interpretable map (Fig. 4). Automatic model building with *Buccaneer* (Cowtan, 2008; Winn *et al.*, 2011) provided an approximately 70% complete model. Manual model building and refinement are currently under way.

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