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Crystallization and preliminary X-ray analysis of FlgA, a periplasmic protein essential for flagellar P-ring assembly

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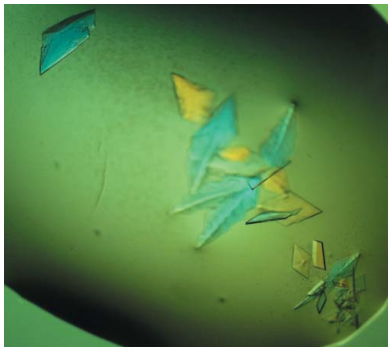

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Salmonella FlgA, a periplasmic protein essential for flagellar P-ring assembly, has been crystallized in two forms. The native protein crystallized in space group $C222$, with unit-cell parameters a = 107.5, b = 131.8, c = 49.4 Å, and diffracted to about 2.0 Å resolution (crystal form I). In this crystal, the asymmetric unit is likely to contain one molecule, with a solvent content of 66.8%. Selenomethionine-labelled FlgA protein crystallized in space group $C222_1$, with unit-cell parameters a = 53.2, b = 162.5, c = 103.5 Å, and diffracted to 2.7 Å resolution (crystal form II). In crystal form II, the asymmetric unit contained two molecules with a solvent content of 48.0%. The multiple-wavelength and single-wavelength anomalous dispersion methods allowed the visualization of the electron-density distributions of the form I and II crystals, respectively. The two maps suggested that FlgA is in two different conformations in the two crystals.

1. Introduction

The bacterial flagellum of *Salmonella typhimurium* is a large macromolecular protein complex that extends from the cytoplasm, penetrates both the inner and the outer membranes and extends into the extracellular space with a long helical filament that acts as a propeller (Berg, 2003; Macnab, 2003). It is composed of about 30 different proteins and is divided into three substructures: the basal body, the hook and the filament. The basal body is a sophisticated structure containing the MS ring, the LP ring and the rod. The LP ring, a molecular bushing supporting the rotation of the rod, firmly keeps the flagellum in place during its high-speed rotation. Three flagellar proteins involved in LP-ring formation, FlgH, FlgI and FlgA, have secretion signal sequences in their N-termini and are secreted into the periplasmic space by way of the general secretion pathway (Homma *et al.*, 1987; Jones *et al.*, 1989; Schoenhals & Macnab, 1996; Kutsukake *et al.*, 1994). FlgH and FlgI are structural components of the outer membrane L ring and the periplasmic P ring, respectively. No P-ring structure is observed by electron microscopy in negatively stained basal bodies isolated from *Salmonella flgA* or *flgI* knockout mutants (Kubori *et al.*, 1992). The overproduction of FlgI suppresses *flgA* mutation in *Salmonella* to some extent. It is thought that FlgA inhibits the premature protein oligomerization of FlgI and helps in the localization of FlgI around the rod prior to P-ring formation (Nambu & Kutsukake, 2000). However, the essential roles of FlgA during P-ring assembly are not fully understood. Peripheral flagellar proteins are also involved in the flagellar assembly process as well as in flagellar rotation. FliS and FliT are flagellar proteins that are known to be specific chaperones for FliC, the filament structural protein, and FliD, the filament-capping protein, respectively (Fraser *et al.*, 1999; Auvray *et al.*, 2001). These chaperone proteins, for which atomic structures have been solved, form binary complexes with their target proteins prior to protein export through the secretion channel (Evdokimov *et al.*, 2003; Imada *et al.*, 2010). In the needle complex of the virulence type 3 secretion system of *Shigella flexneri*, a secretin pilot protein, MxiM, binds to a secretin protein, MxiD, for proper localization to the outer membrane and stability in the periplasm (Schuch & Maurelli, 2001; Lario *et al.*, 2005).

Thus, FlgA seems to play important roles in flagellar P-ring assembly and revealing the structure of FlgA will therefore be of great interest in order to understand its functional mechanism during LP-ring formation. Here, we have successfully expressed and crystallized full-length *Salmonella* FlgA. X-ray diffraction analyses show promise for the structural determination of FlgA.

2. Materials and methods

2.1. Plasmid construction and protein purification

The DNA sequence encoding full-length FlgA was amplified by polymerase chain reaction (PCR) using chromosomal DNA from *S. enterica* serovar Typhimurium strain SJW1103 (Yamaguchi *et al.*, 1984). The PCR fragment was digested with *Nde*I and *Bam*HI and ligated into pET22b (Novagen) to create pHMK338, which encodes FlgA with a C-terminal hexahistidine tag connected by a vector-derived 15-amino-acid linker (denoted $FlgA_{HIS}$). The plasmid was transformed into *Escherichia coli* BL21 (DE3). The cells carrying the plasmid were grown in Luria–Bertani broth supplemented with 50 mg l^{-1} ampicillin at 310 K to a cell density of OD = 0.6–0.7 and protein expression was then induced with 1 m*M* isopropyl β-D-1-thiogalactopyranoside at 310 K for 2 h. The cells were harvested by centrifugation at 4000 rev min^{-1} in a JLA10.500 rotor (Beckman) for 10 min. The cell pellet was suspended in 20%(*w*/*v*) sucrose, 20 m*M* Tris–HCl pH 8.0, 0.3 *M* NaCl and kept at ambient temperature for 30 min. After harvesting the cells by centrifugation, the periplasmic fraction was released by suspending the pellet in 0.5 m*M* $MgCl_2$ and incubated on ice for 10 min. The periplasmic fraction was centrifuged at 40 000 rev min^{-1} in a 50.1 Ti rotor (Beckman) for 30 min and the supernatant was loaded onto a HisTrap column (GE Healthcare) equilibrated with 20 m*M* Tris–HCl pH 8.0. Elution was performed using a linear gradient of imidazole concentration from 0 to 0.5 *M* in the same buffer solution. The main peak fraction was dialyzed against 10 m*M* Tris–HCl pH 8.0 and applied onto a Superdex 75 gel-filtration column (GE Healthcare) pre-equilibrated with 10 m*M* HEPES pH 7.5 containing 100 m*M* NaCl. The protein eluted as a single peak and was concentrated to a final concentration of 20 mg ml^{-1} using a Vivaspin centrifugal filter device (Sartorius). The protein solution was kept as a stock solution at 277 K. The sample purity was checked by SDS–PAGE and the molecular mass of the purified protein was examined by matrix-assisted laser desorption ionization–time-of-flight (MALDI–TOF) mass spectrometry using a Voyager-DE/PRO spectrometer (Applied Biosystems). These experiments showed that the sample was pure and free from any protein degradation or contamination. Selenomethionine-labelled $FlgA_{HIS}$ (SeMet-$FlgA_{HIS}$) was produced using LeMaster medium (LeMaster & Richards, 1985) and was purified using the same method as used for the native protein. Finally, SeMet-$FlgA_{HIS}$ was concentrated to 5 mg ml^{-1} in 10 m*M* HEPES pH 7.0, 100 m*M* NaCl. Because its solubility is lower than the native protein, the addition of salt to the final buffer was essential.

2.2. Crystallization

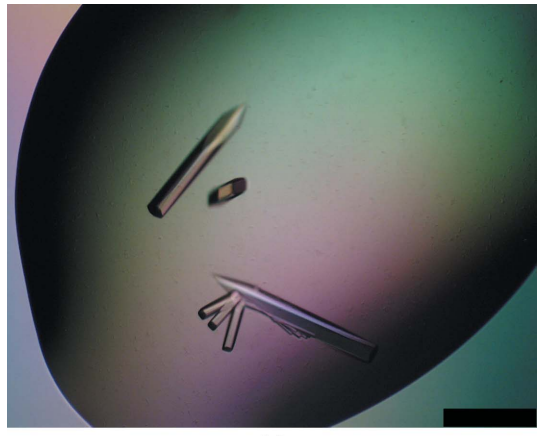
Initial crystallization trials were carried out using the Crystal Screen and Crystal Screen 2 (Hampton Research) or Wizard I, II and III (Emerald BioSystems) sparse-matrix kits. Drops were prepared by manually mixing 2 µl protein solution (5–10 mg ml^{-1}) and 2 µl reservoir solution and were equilibrated against 200 µl reservoir solution in CombiClover Jr Crystallization plates (Emerald BioSystems).

2.3. Data collection

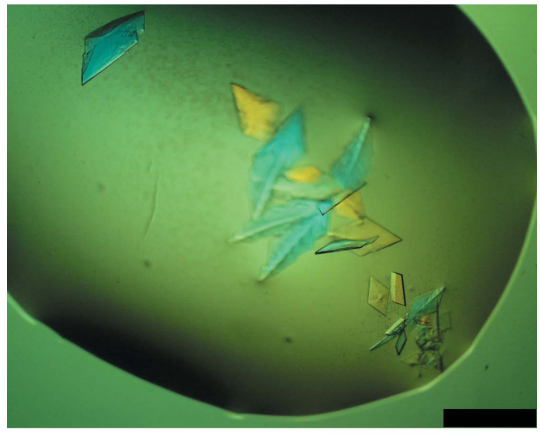
The native $FlgA_{HIS}$ crystals (form I) were picked up in nylon loops and plunged into liquid nitrogen without increasing the glycerol concentration in the drop. Heavy-atom derivative crystals of the protein were prepared by adding a one-tenth volume of $KPtCl_4$ stock solution (~100 m*M*) directly to the crystallization drops and the drop was equilibrated for 8 h. Prior to freezing, these crystals were kept in their reservoir solution for a couple of minutes. The SeMet-$FlgA_{HIS}$ crystals (form II) were soaked briefly in a 9:1 mixture of the reservoir and 2-methyl-2,4-pentanediol prior to cooling. X-ray diffraction data were collected on SPring-8 beamline BL41XU (Harima, Japan) at 100 K using a CCD detector. All diffraction data were processed with *MOSFLM* (Leslie, 2006) and scaled with *SCALA* (Winn *et al.*, 2011). For the form I crystal, the initial phase was calculated with *auto-SHARP* (Vonrhein *et al.*, 2007) using a multi-wavelength anomalous dispersion (MAD) data set from a Pt-derivative crystal. For the form II crystal, the initial phase was calculated with *PHENIX* (Adams *et al.*, 2010) using a single-wavelength anomalous dispersion (SAD) data set from an SeMet-labelled crystal. Electron-density maps were displayed using *Coot* (Emsley & Cowtan, 2004).

3. Results and discussion

The mature form of FlgA lacking the signal sequence and an N-terminally His-tagged protein were first overexpressed, but formed



(*a*)

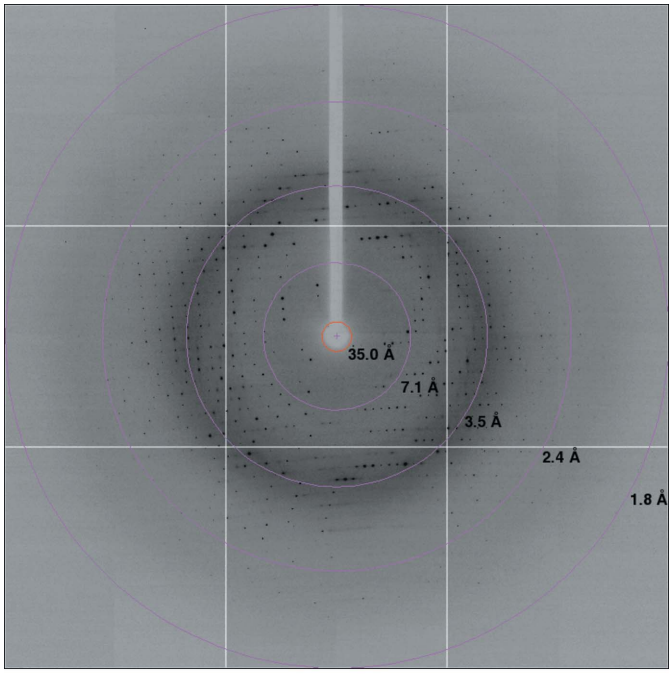


(*b*)

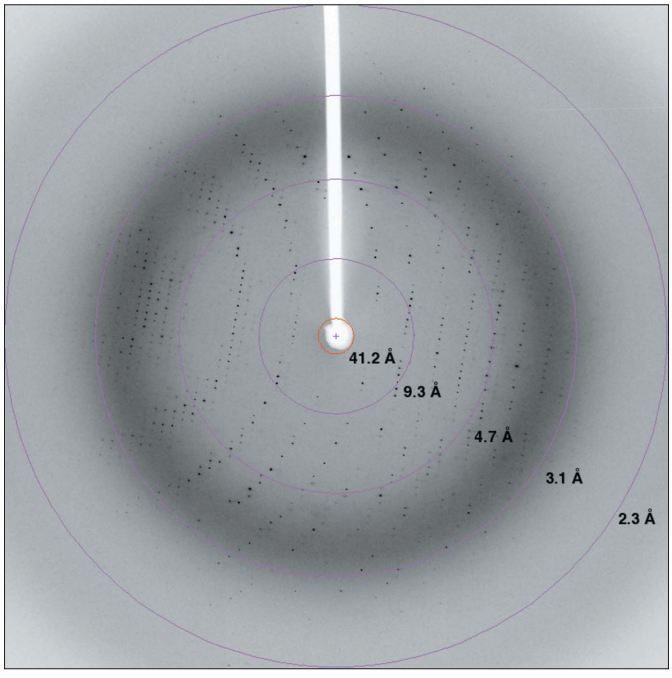
Figure 1
Two types of $FlgA_{HIS}$ crystals. (*a*) Rod-shaped crystals of $FlgA_{HIS}$ and (*b*) plate-shaped crystals of SeMet-$FlgA_{HIS}$ grown by hanging-drop vapour diffusion. The scale bars are 0.4 mm in length.

inclusion bodies in the cytoplasm of *E. coli* BL21 (DE3). To obtain soluble protein for crystallization, $FlgA_{HIS}$ was purified from the periplasmic fraction of the host cell. In order to localize the protein in the periplasm, where disulfide-bond formation occurs, the original secretion signal sequence of $FlgA_{HIS}$ was left unmodified in this construct. $FlgA_{HIS}$ was successfully expressed in the periplasm and the protein was purified to homogeneity by metal-chelating and gel-filtration chromatography.

All crystallization experiments were carried out at 289 K. Two types of crystals were obtained for the $FlgA_{HIS}$ protein and SeMet-labelled protein. Initially, small needle-like crystals of the native protein grew from two reservoir solutions. One solution was Crystal Screen condition No. 50 (Hampton Research) and consisted of 0.5 *M* lithium sulfate monohydrate, 15%(*w*/*v*) polyethylene glycol (PEG) 8000. The other solution, Wizard III condition No. 18 (Emerald BioSystems), consisted of 20%(*w*/*v*) PEG 6000, 0.1 *M* citric acid pH 4.0, 1.0 *M* lithium chloride. Crystals grew within 2–3 d by the sitting-

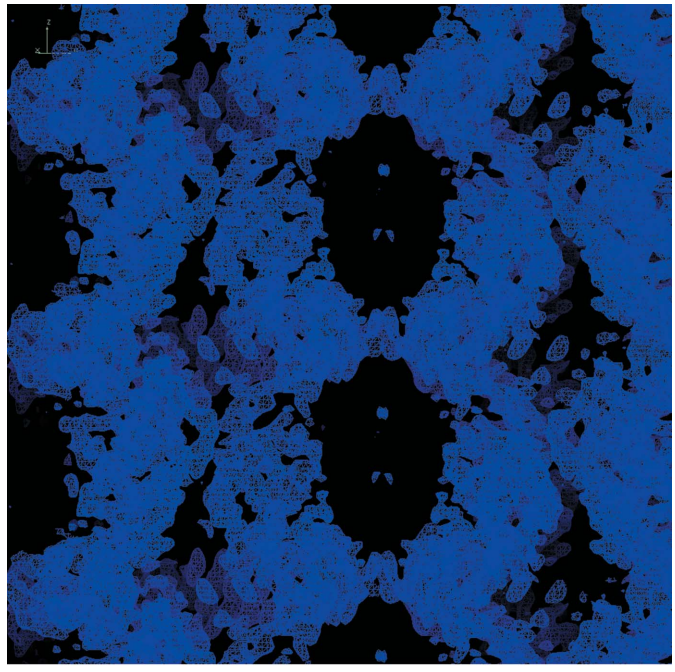


(*a*)

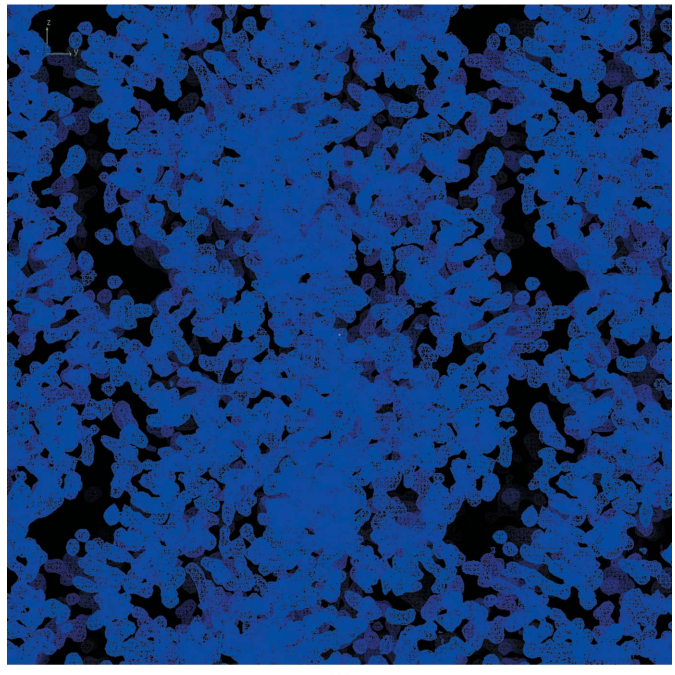


(*b*)

Figure 2
X-ray diffraction patterns (*a*) from a crystal of $FlgA_{HIS}$ recorded with an ADSC Q-315 CCD detector and (*b*) from a crystal of SeMet-$FlgA_{HIS}$ recorded with a MAR225HE detector.



(*a*)



(*b*)

Figure 3
$2F_o - F_c$ electron-density maps calculated using (*a*) experimentally determined phases from the Pt-derivative data at 3.0 Å resolution and (*b*) the SeMet-derivative data at 2.7 Å resolution. Both are contoured at 1.0σ.

Table 1
Summary of the data-collection statistics for $FlgA_{HIS}$ crystals.

Values in parentheses are for the highest resolution shell.

	$FlgA_{HIS}$	Pt-derivative $FlgA_{HIS}$			SeMet-$FlgA_{HIS}$
	Native	Peak	Inflection	Remote	Peak
Space group	*C*222	*C*222			$C222_1$
Unit-cell parameters (Å)	a = 107.5, b = 131.8, c = 49.4	a = 107.9, b = 130.5, c = 49.4			a = 53.2, b = 162.5, c = 103.5
Wavelength (Å)	0.98300	1.07153	1.07188	1.09074	0.97895
Resolution (Å)	34.5–1.95 (2.06–1.95)	36.4–3.00 (3.16–3.00)	36.5–3.00 (3.16–3.00)	36.6–3.00 (3.16–3.00)	40.6–2.70 (2.85–2.70)
Observations	102216 (15052)	44909 (6734)	45126 (6757)	44537 (6745)	82254 (12094)
Unique reflections	25669 (3686)	7298 (1052)	7319 (1053)	7341 (1064)	12420 (1828)
Completeness (%)	98.7 (98.3)	99.7 (100)	99.8 (100)	99.7 (99.9)	98.7 (99.7)
Multiplicity	4.0 (4.1)	6.2 (6.4)	6.2 (6.4)	6.1 (6.3)	6.6 (6.6)
$\langle I/\sigma(I)\rangle$	12.3 (3.4)	14.3 (3.9)	14.9 (4.4)	15.8 (3.3)	14.3 (3.8)
R_{merge}† (%)	6.9 (37.2)	7.0 (47.3)	7.0 (39.3)	6.4 (47.6)	8.9 (44.3)
R_{anom}‡ (%)	—	6.7 (17.6)	5.2 (16.0)	3.7 (19.3)	4.7 (17.6)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle|/\sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith observation of reflection hkl and $\langle I(hkl)\rangle$ is the weighted average intensity for all observations i of reflection hkl. ‡ $R_{anom} = \sum_{hkl}|I(hkl+) - I(hkl-)|/\sum_{hkl}[I(hkl+) + I(hkl-)]$, where $I(hkl+)$ and $I(hkl-)$ correspond to the averaged intensities of each Friedel pair for reflection hkl.

drop vapour-diffusion method. After optimization of the conditions by the hanging-drop vapour-diffusion method, rod-shaped $FlgA_{HIS}$ crystals grew in 18%(*w*/*v*) PEG 2000, 0.1 *M* citric acid pH 5.4, 0.8 *M* lithium chloride, 18%(*w*/*v*) glycerol (crystal form I; Fig. 1*a*). The form I crystal belonged to space group *C*222, with unit-cell parameters a = 107.5, b = 131.8, c = 49.4 Å, and a native data set was collected at 1.95 Å resolution (Fig. 2*a*). Assuming that one molecule of $FlgA_{HIS}$ was present in the asymmetric unit, the Matthews coefficient V_M was 3.71 $Å^3\,Da^{-1}$, corresponding to a solvent content of 66.8% (Matthews *et al.*, 1968).

In the early stages of the crystallization experiment the refinement of crystallization conditions for SeMet-$FlgA_{HIS}$ was hampered by the high nucleation rate for crystallization and this prompted us to prepare heavy-atom derivative crystals in order to solve the phase problem. A MAD data set was collected from a Pt-derivative $FlgA_{HIS}$ crystal at 3.0 Å resolution at three different wavelengths. However, plate-shaped crystals of SeMet-$FlgA_{HIS}$ grew in 10%(*w*/*v*) PEG 6000, 0.05 *M* citric acid pH 4.2, 1.0 *M* lithium chloride using the hanging-drop vapour-diffusion method with the microseeding technique (crystal form II; Fig. 1*b*). The form II crystal belonged to space group $C222_1$, with unit-cell parameters a = 53.2, b = 162.5, c = 103.5 Å, and a SAD data set was collected at 2.7 Å resolution (Fig. 2*b*). Assuming that two molecules of SeMet-$FlgA_{HIS}$ were present in the asymmetric unit, the Matthews coefficient V_M was 2.37 $Å^3\,Da^{-1}$, corresponding to a solvent content of 48.0% (Matthews *et al.*, 1968). The data statistics of these crystals are summarized in Table 1.

An initial electron-density map of the form I crystal, which was calculated using the MAD data set from the Pt-derivative crystal, showed interpretable features of protein secondary structure for model building (Fig. 3*a*). The electron-density map calculated from the SAD data set of the SeMet-$FlgA_{HIS}$ form II crystal also showed clear density; however, the overall features of the density were quite distinct from those of the form I crystal, indicating that structural changes are caused by the different crystal packing (Fig. 3*b*). Two cysteine residues in FlgA seem to be involved in the formation of a disulfide bond that stabilizes its native structure. The details of these structures, which are in the final stages of refinement, will be discussed elsewhere.

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