Purification, crystallization and X-ray crystallographic studies of flagellin from *Pseudomonas aeruginosa*

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Flagellin constitutes the whip-like structure of the bacterial flagellum that is required for locomotion. Upon bacterial invasion into a host, flagellin functions as a pathogen-associated molecular pattern that is recognized by immune receptors, such as Toll-like receptor 5 (TLR5) and NAIP5/NLRC4, and activates host innate immunity against pathogens. Structural and biophysical studies of flagellins have been limited to those of *Salmonella* species. To better understand the functions of flagellin, it is necessary to study flagellins from other species. In this study, the overexpression, purification and crystallization of *Pseudomonas aeruginosa* flagellin that lacks the D0 domain (*paflagellin*- Δ D0) are reported. *paflagellin*- Δ D0 crystals diffracted to 2.15 Å resolution and belonged to space group *C*2, with one protein molecule in the asymmetric unit. Future structure-based functional studies of *paflagellin* would extend the knowledge of the TLR5 or NAIP5/NLRC4 activation mechanisms of flagellin and would make a significant contribution to the design of flagellin vaccines and antiradiation therapeutics.

1. Introduction

Bacterial flagella are whip-like appendages that provide bacteria with motility and chemotaxis. The flagellar filament is formed by helical polymerization of the flagellin protein. Flagellin plays a key role as a virulence factor in the adhesion and invasion of pathogenic flagellated bacteria into host epithelial cells (Ramos *et al.*, 2004). Flagellin also functions as a pathogen-associated molecular pattern in the host and is involved in the stimulation of host immune responses. Flagellin is detected by pattern-recognition receptors, such as Toll-like receptor 5 (TLR5) and NAIP5/NLRC4, and subsequently causes the host to activate innate immunity against pathogens (Takeda *et al.*, 2003; Franchi *et al.*, 2012). Moreover, flagellin has radioprotective activity in gastrointestinal tissues (Burdelya *et al.*, 2008). Thus, flagellin provides a valuable avenue for the development of antiviral vaccines and antiradiation therapeutics (Liu *et al.*, 2012).

Bacterial flagellins range in size from 28 to 65 kDa and comprise multiple domains (Yonekura *et al.*, 2003; Samatey *et al.*, 2001). All flagellins contain the D0 and D1 domains, which are highly conserved in diverse bacteria owing to their functional importance in flagellar filament formation. The D1 and D2 domains are targeted by animal TLR5 and NAIP5/NLRC4 and even by plant FLS2 (Kofoed & Vance, 2011; Gómez-Gómez & Boller, 2000; Hayashi *et al.*, 2001). Many types of flagellins have additional domains (D2 or D3 domains) that decorate the outside surface of the bacterial flagellar filament. In contrast to the conserved D0 and D1 domains, the D2 and D3 domains exhibit high variation in sequence and structure, as shown by the observation that the D2 domains from two serotypes of the same species, *Salmonella enterica* subspecies *enterica* serovar Typhimurium (*S. Typhimurium*) and *S. Dublin*, adopt completely different folds (Yoon *et al.*, 2012; Samatey *et al.*, 2001).

Extensive structural, biophysical and cellular studies have been carried out on flagellins of *Salmonella* species. The structures of *S. Typhimurium* flagellin revealed the molecular packing mechanism of the flagellar filament (Maki-Yonekura *et al.*, 2010; Samatey *et al.*, 2001; Yonekura *et al.*, 2003), and the structure of *S. Dublin* flagellin in complex with TLR5 provided deep insight into flagellin recognition by TLR5 (Yoon *et al.*, 2012). However, since structural information

is limited only to *Salmonella* flagellins, it is unclear whether the mechanism used by *Salmonella* flagellins is conserved in other bacteria. To extend our knowledge regarding the diversity of flagellin structures and the flagellin-mediated activation of immune receptors, we expressed and purified full-length and D0 domain-deleted flagellins (*paflagellin*-full and *paflagellin*- Δ D0, respectively) from the opportunistic pathogen *Pseudomonas aeruginosa*. The D0 and D1 domains of *paflagellin* share 51–53% sequence identity with those of *S. Typhimurium* and *S. Dublin* flagellins, whereas the variable domain does not exhibit any sequence similarity between *paflagellin* and *Salmonella* flagellins. *paflagellin*- Δ D0 was crystallized using PEG 6000 solutions and the *paflagellin*- Δ D0 crystals diffracted to 2.15 Å resolution.

2. Materials and methods

2.1. Cloning of expression vectors for *paflagellin*-full and *paflagellin*- Δ D0

The *fliC* gene encoding *paflagellin*-full (residues 1–386) was cloned into a modified pET49b vector (pET49bm) that contains an N-terminal His₆ tag and a thrombin cleavage site (Hong *et al.*, 2012; Yoon *et al.*, 2012). The *paflagellin* gene was amplified by PCR from the genomic DNA of *P. aeruginosa* (ATCC 15522). PCR products were cleaved by the restriction enzymes *Bam*HI and *Xho*I and ligated into pET49bm vector backbone DNAs that had been treated with *Bam*HI and *Sal*I. The resultant ligation products were transformed into *Escherichia coli* strain DH5 α and plated on LB medium containing 50 μ g ml⁻¹ kanamycin. The sequence of the insert in the clones was verified by DNA sequencing. The *paflagellin*- Δ D0 expression vector was constructed by amplification of the central region of *paflagellin* (residues 55–341) by PCR from the *paflagellin*-full-pET49bm vector and subsequently by *Bam*HI/*Xho*I digestion and ligation into pET49bm.

2.2. Expression and purification of *paflagellin*

paflagellin proteins were expressed in *E. coli* strain BL21 (DE3). The cells were grown at 37°C in LB medium containing 50 μ g ml⁻¹ kanamycin. Protein expression was induced using 1 mM IPTG when the OD₆₀₀ (optical density at 600 nm) reached ~0.8. The cells were allowed to grow for 3 h at 37°C and were then collected by centrifugation.

Cells from a 600 ml culture were resuspended in 30 ml 50 mM Tris pH 8.0, 200 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF)

and lysed by sonication. The lysate was cleared by centrifugation and the supernatant was incubated with 5 ml Ni-NTA agarose resin (Qiagen) in the presence of 10 mM imidazole. The mixture was applied onto a glass Econo-Column (Bio-Rad) and unbound protein was washed with 200 ml 50 mM Tris pH 8.0, 200 mM NaCl, 10 mM imidazole. Bound *paflagellin* protein was eluted with 10 ml 50 mM Tris pH 8.0, 200 mM NaCl, 250 mM imidazole. The eluate was dialyzed against 20 mM HEPES pH 7.4. The resulting *paflagellin* protein (~70 mg in 10 ml) was digested with 100 U thrombin (Merck) at room temperature for 3 h to cleave the N-terminal His₆ tag and was further purified by anion-exchange chromatography using a Mono Q 10/100 GL column (GE Healthcare). The column was washed with 20 mM HEPES pH 7.4 and *paflagellin* protein was eluted at a conductivity of ~15 mS cm⁻¹ with eight column volumes of a linear NaCl gradient (0.0–0.5 M). The homogeneity of the purified *paflagellin*- Δ D0 protein was examined by gel-filtration chromatography. *paflagellin*- Δ D0 protein or gel-filtration standards (Bio-Rad) in 300 μ l were injected into a Superdex 200 10/300 gel-filtration column. Gel-filtration chromatography (GE Healthcare) was carried out in a running buffer consisting of 20 mM HEPES pH 7.4, 150 mM NaCl at a flow rate of 0.5 ml min⁻¹ at room temperature.

2.3. Crystallization of *paflagellin*- Δ D0

Crystallization conditions for *paflagellin*- Δ D0 were screened using The JCSG Core Suites kit (Qiagen) by the sitting-drop vapour-diffusion method. Initial crystals were obtained in diverse PEG conditions. After optimization of crystallization conditions, the best crystals were obtained in a drop consisting of 0.5 μ l 15 mg ml⁻¹ *paflagellin*- Δ D0 protein in 20 mM HEPES pH 7.4, 150 mM NaCl and 0.5 μ l well solution consisting of 26% (w/v) PEG 6000, 0.1 M bicine pH 9.0, 4% 1,1,1,3,3,3-hexafluoro-2-propanol at 18°C. Crystals started to be visible in ~6 h and grew fully in 2 d.

2.4. X-ray data collection and analysis

paflagellin- Δ D0 crystals (50 $\times$ 500 $\times$ 5 μ m) were soaked in a solution consisting of 28% (w/v) PEG 6000, 0.1 M bicine pH 9.0 for ~10 min and then in 28% (w/v) PEG 6000, 0.1 M bicine pH 9.0, 25% ethylene glycol for ~10 min for cryoprotection. Crystals were harvested in nylon loops and flash-cooled at -173°C in a cryo-stream. A total of 360 frames of X-ray diffraction images were collected with 0.5° oscillation, an exposure time of 3 s per frame and a crystal-to-detector distance of 230 mm using an ADSC Quantum 270 detector on beamline 7A of the Pohang Accelerator Laboratory (Republic of Korea). Diffraction data were processed using the *HKL*-2000 package (Otwinowski & Minor, 1997).

Figure 1
SDS-PAGE analysis of purified *paflagellin*-full (lane 2; calculated molecular weight ~40 kDa) and *paflagellin*- Δ D0 (lane 3; calculated molecular weight ~29 kDa) proteins with molecular-weight markers (lane 1; labelled in kDa).

Figure 2
Gel-filtration analysis of the purified *paflagellin*- Δ D0 protein using a Superdex 200 10/300 column. Peaks corresponding to gel-filtration standards (bovine thyroglobulin, 670 kDa; bovine γ -globulin, 158 kDa; chicken ovalbumin, 44 kDa; horse myoglobin, 17 kDa) are represented by vertical lines.

Figure 3
paflagellin-ΔD0 crystals in a drop consisting of 0.5 μ l 15 mg ml⁻¹ *paflagellin-ΔD0* protein in 20 mM HEPES pH 7.4, 150 mM NaCl and 0.5 μ l well solution consisting of 26% (w/v) PEG 6000, 0.1 M bicine pH 9.0, 4% 1,1,1,3,3,3-hexafluoro-2-propanol.

3. Results and discussion

To determine the crystal structure of *paflagellin*, *paflagellin*-full protein was expressed in an *E. coli* cell expression system and purified by Ni-NTA affinity chromatography and anion-exchange chromatography (Fig. 1). *paflagellin*-full did not yield diffraction-quality crystals, potentially owing to the high flexibility of the D0 domain (Vonderviszt *et al.*, 1989).

To improve the crystallizability and X-ray diffraction quality, the D0 domain was deleted from *paflagellin*. *paflagellin-ΔD0* was expressed and purified in an identical manner to *paflagellin*-full, with a final yield of ~40 mg from 1 l of culture (Fig. 1). Purified *paflagellin-ΔD0* protein eluted as a single peak in gel-filtration chromatography, demonstrating the homogeneity and purity of the sample (Fig. 2). Although the calculated molecular weight of *paflagellin-ΔD0* is ~29 kDa, its elution volume was similar to that of the 44 kDa protein standard. The irregular mobility of *paflagellin-ΔD0* can be explained by the substantially elongated structure of the flagellin D1 domain.

paflagellin-ΔD0 was crystallized in PEG 6000/hexafluoro-2-propanol solution at pH 9.0 (Fig. 3). The *paflagellin-ΔD0* crystals diffracted to 2.15 Å resolution and belonged to space group *C2*, with unit-cell parameters $a = 98.88$, $b = 61.01$, $c = 51.53$ Å, $\beta = 90.54^\circ$

(Table 1). Matthews coefficient probability analysis indicates that the asymmetric unit contains one copy of *paflagellin-ΔD0* (Matthews coefficient V_M of 2.64 Å³ Da⁻¹; 53.4% solvent content; Matthews, 1968; Kantardjieff & Rupp, 2003). The structure determination of *paflagellin-ΔD0* by *de novo* phasing and structure analysis will be described elsewhere.

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