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Purification, crystallization and X-ray crystallographic analysis of the type VI secretion system accessory protein TagF from *Pseudomonas aeruginosa*

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TagF is the product of the type VI secretion system (T6SS) accessory gene F, which is capable of regulating T6SS assembly in *Pseudomonas aeruginosa*. Experimental results suggest that TagF may post-translationally regulate the T6SS through phosphatase activity, but the physiological functions of TagF are not yet understood. To provide structural insight into TagF function and the regulatory mechanisms controlling the T6SS, N-terminally His_6-tagged TagF was overexpressed, purified, and crystallized using hanging-drop vapor diffusion in a solution of 2.5 M NaCl and 0.1 M Bis-Tris propane (pH 7.0). X-ray diffraction data from TagF crystals was collected at a resolution of 2.7 Å. TagF crystals belonged to the space group $P2_12_12$, with unit cell parameters a = 93.7, b = 92.4, c = 151.1 Å, and $\alpha = \beta = \gamma = 90°$. TagF consists of four independent subunits in the asymmetric unit.

INTRODUCTION

Bacteria have evolved a variety of large molecular machines (Gerlach and Hensel, 2007). For example, gram-negative bacteria have developed complexes enabling the secretion of substrate into the extracellular space, through two cell membranes (Desvaux et al., 2009). These machines contribute to bacterial pathogenicity, survival, and horizontal gene transfer (Costa et al., 2015). The secretion systems in gram-negative bacteria have been classified into six types according to their structure and function (Rego et al., 2010), and studies on the molecular mechanisms underpinning their functions are underway.

The type VI secretion system (T6SS) was discovered in a study of the gene clusters of novel virulence factors in *Vibrio cholerae* (Pukatzki et al., 2006). In addition, *Pseudomonas aeruginosa* T6SS has been found to secrete Hcp, which was discovered in studies of the sputum of patients with cystic fibrosis (Hachani et al., 2011). T6SS is thought to play an important role in pathogenicity (Mougous et al., 2006). To date, T6SS has been found in approximately one-quarter of gram-negative bacteria, and it appears to have slightly different structural characteristics from species to species. The genes encoding T6SS components are designated Type Six Secretion A–M (TssA–M) (Shalom et al., 2007).

Recently, proteins encoded by the T6SS gene cluster were shown to regulate their assembly through post-translational mechanisms (Mougous et al., 2007). A number of accessory genes capable of regulating the expression of the tss gene cluster were identified and designated as type-six associated genes A–P (TagA–P). Although the function of most of the gene cluster is unknown, several lines of evidence suggest that T6SS assembly may be regulated by phosphorylation and dephosphorylation in *P. aeruginosa* (Mougous et al., 2007). For example, T6SS assembly can be promoted by TagH phosphorylation by the serine-threonine kinase PpkA, and the assembly of T6SS can be inhibited by TagH dephosphorylation via the serine-threonine phosphatase PppA (Hsu et al., 2009). Furthermore, assembly can be inhibited by TagF regardless of the presence of TagH (Silverman et al., 2011).

The TagF protein contains a domain of unknown function (DUF), and therefore belongs to the DUF2094 family. It is classified as TIGR03373 by the Institute for Genomic Research (Finn et al., 2006; Selengut et al., 2007). The TagF protein in *P. aeruginosa* is homologous to ImpM in *Rhizobium leguminosarum* and SciT in *Salmonella enterica*. Its function is unknown; however, in-frame deletion experiments suggest that TagF is probably involved in post-translational regulation through phosphatase activity (Silverman et al., 2011). Currently, no published structural report for TagF is available. In this study, to understand the specific structural features of TagF and to elucidate the molecular mechanism of T6SS assembly regulation by the *tagF* gene, we overexpressed, purified, and crystallized full-length TagF protein tagged with hexahistidine (His_6) at its N-terminus.

RESULTS AND DISCUSSION

N-terminal His_6-TagF was overexpressed in Escherichia coli and purified by affinity chromatography followed by size-exclusion

chromatography (SEC; Figure 1a). The SEC elution peak profile showed a single symmetric peak at an elution volume of 72.2 mL, which corresponded to a molecular weight of 51.0 kDa. As the calculated molecular weight of monomeric TagF is 23.9 kDa, this indicates that TagF exists as a dimer in solution (Figure 1b). The protein was subsequently concentrated to 33.1 mg/mL.

Crystallization trials were performed at 4°C using the hanging-drop vapor diffusion method with over 500 different conditions from sparse-matrix screening solution kits. TagF crystals usually appeared in 1 day. The shape of these crystals was categorized as hexagonal-prism; the size of the crystals was 200–350 μm (Figure 2). Optimal crystals were obtained in a solution of 2.5 M NaCl and 0.1 M Bis-Tris propane (pH 7.0; Table 1). X-ray diffraction data of the optimized crystals were collected to 2.7 Å resolution, and indicated that TagF crystals belonged to space group $P2_12_12$, with unit cell parameters a = 93.7, b = 92.4, c = 151.1 Å, and α = β = γ = 90° (Figure 3). Based on the processed data, the crystal volume per protein mass (V_M) and Matthews coefficient values were 1308584.5 and 3.15 $Å^3$/Da, respectively. This indicated that there were four molecules in the asymmetric unit, with 60.9% solvent content (Matthews, 1968).

To obtain phase information, molecular replacement (MR) was performed using the uncharacterized protein PA0076 (PDB code: 2QNU) as an initial search model using Auto MR software in the PHENIX crystallographic software package (PHENIX; USA) (Adams et al., 2010). Currently, preliminary structure refinement and electron density map interpretation are in progress using PHENIX.refine and Wincoot softwares (Emsley and Cowtan, 2004). Detailed crystallographic information is shown in Table 2.

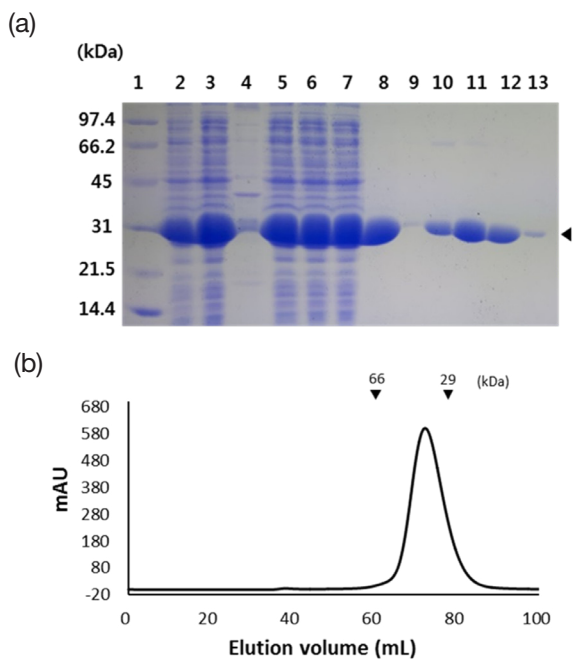

FIGURE 1 | Preparation of TagF protein (a) SDS-PAGE analysis of N-terminally His6-tagged TagF. The black arrowhead indicates the size of the target protein. Molecular weights of the standard size markers are shown on the left-hand side. Lanes are as follows: 1, marker; 2, cells induced with IPTG; 3, supernatant; 4, cell pellet; 5, flow-through; 6, wash with 20 mM imidazole; 7, wash with 25 mM imidazole; 8, elution with 250 mM imidazole; 9–13, fractions from the gel filtration peaks on the SEC profile. (b) SEC profile of His6-TagF. The standards were calibrated using albumin (66 kDa) and carbonic anhydrase (29 kDa) as indicated above the SEC profile.

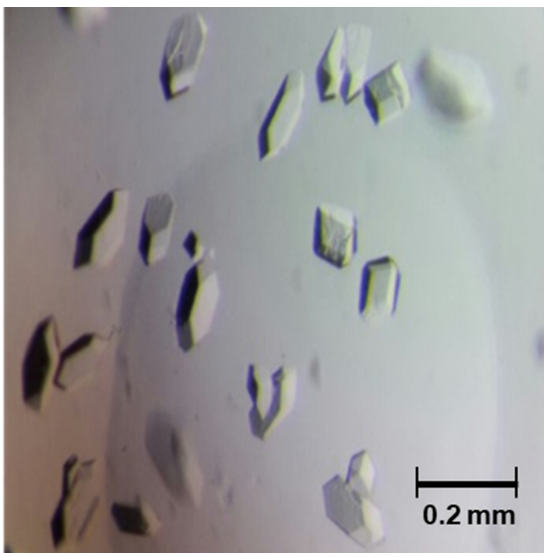

FIGURE 2 | Crystals of TagF from *P. aeruginosa*. Hexagonal-prism-shaped TagF crystals were obtained in a solution of 2.5 M NaCl and 0.1 M Bis-Tris propane (pH 7.0). The approximate dimensions of the crystals were 0.2 × 0.1 × 0.1 mm.

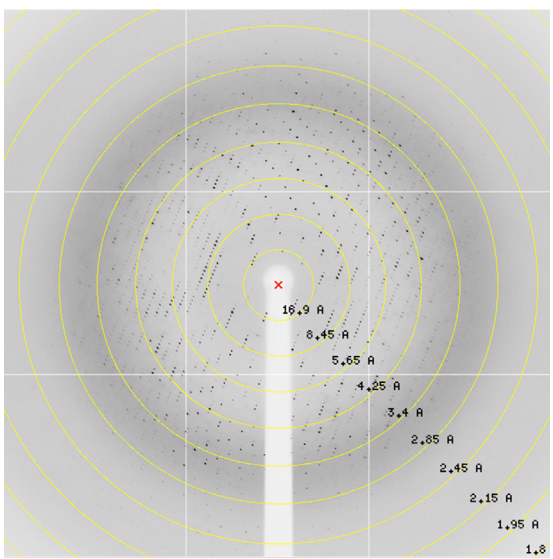

FIGURE 3 | A representative diffraction pattern of the TagF crystal. Diffraction image of a *P. aeruginosa* His_6-TagF crystal. The yellow circles and black text represent resolution ranges.

TABLE 1 | Crystallization

Method	Vapor diffusion
Plate type for screening	96-well sitting-drop crystallization plate, Art Robbins Instruments
Plate type for optimization	24-well plate, Hyundai Micro
Temperature (°C)	7
Protein concentration (mg/mL)	33.1
Composition of protein solution	20 mM Tris-HCl pH 7.6, 150 mM NaCl, 2 mM DTT
Composition of reservoir solution	2.5 M NaCl and 0.1 M Bis-Tris propane (pH 7.0)
Volume and ratio of drop	2 μL, 1:1
Volume of reservoir (μL)	500

TABLE 2 | Data collection and processing

	TagF
Diffraction source	Beamline 5C, PAL
Wavelength (Å)	0.9796
Temperature (°C)	-180
Detector	ADSC Quantum 270
Crystal-to-detector distance (mm)	300
Rotation range per image (°)	1
Total rotation range (°)	180
Exposure time per image (s)	1
Space group	$P2_12_12$
a, b, c (Å)	93.7, 92.4, 151.1
α, β, γ (°)	90, 90, 90
Resolution range (Å)	50.0–2.7
Total no. of reflections	872509
No. of unique reflections	37160
Completeness (%)	99.9 (100)
Multiplicity	6.2 (6.5)
$I / \sigma I$	37.0 (4.7)
R_{merge} (%)[b]	6.8 (53.9)
$R_{p.i.m}$ (%)	4.0 (27.5)
Overall B factor from Wilson plot (Å^2)	59.72

[a]The numbers in parentheses are statistics from the highest resolution shell. [b]Rmerge = Σ $|I_{obs} - I_{avg}| / I_{obs}$, where I_{obs} is the observed intensity of individual reflections and I_{avg} is averaged over symmetry equivalents.

METHODS

Preparation of the TagF protein

Genomic DNA from the *P. aeruginosa* strain DSM 50071 was obtained from the Korean Collection for Type Cultures. Full-length TagF was amplified by polymerase chain reaction (PCR) using Pfu-X DNA polymerase (Solgent, Republic of Korea). Oligonucleotide primers used in the study were purchased from Cosmogenetech Inc. The amplified fragment was digested with the restriction enzymes NdeI and XhoI (R006S and R007S, respectively; Enzynomics, Republic of Korea) for 3 h at 37°C in a water bath, and ligated into the pET28a vector using T4 ligase (M0202S; Roche, Germany) overnight at 20°C. This vector inserted a hexahistidine (His_6)-tag at the N-terminus of the target protein. The ligated plasmid was then transformed into the *E. coli* strain DH5α, and transformants were confirmed using colony PCR. The TagF plasmid was transformed into E. coli BL21 (DE3) Star cells, which were grown at 37°C to an optical density at 600 nm (OD_{600}) of approximately 0.7 in LB medium (Ambrothia, Republic of Korea) containing 50 mg/L kanamycin (Applichem, USA). Following induction with 0.3 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; Calbiochem, Germany), the cells were further grown for 16 h at 20°C, then harvested by centrifugation at 5000 × g for 20 min at 4°C. The cell pellet was resuspended in buffer containing 250 mM NaCl (Applichem, USA), 5% glycerol (Affymetrix, USA), 0.2% Triton X-100 (Sigma-Aldrich, USA), 10 mM β-mercaptoethanol (BioBasic, Canada), 0.2 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich, USA), and 20 mM Tris (pH 8.0; Sigma-Aldrich, USA). Cells were disrupted by ultrasonication with 3 s on/off pulses for 20 min. Cell debris was removed by centrifugation at 15000 × g for 50 min, and the supernatant was bound to Ni-NTA agarose (Qiagen, Germany) for 90 min at 4°C. After washing with buffer A (200 mM NaCl, 50 mM Tris, pH 8.0) containing 20 mM imidazole (Sigma-Aldrich, USA), the bound proteins were eluted with 250 mM imidazole in buffer A. SEC was performed on the purified proteins using a HiPrep 16/60 Sephacryl S-300 HR column (GE Healthcare, Canada). The SEC buffer contained 150 mM NaCl, 2 mM dithiothreitol (DTT; Calbiochem, Germany), and 20 mM Tris (pH 7.5). Following SEC, proteins were stored at −80°C pending crystallization trials. The purified proteins were assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis using a 15% acrylamide gel, which showed a single band corresponding to the calculated molecular weight of the target protein.

Crystallization

All crystallization trials were performed at 4°C using the sitting-drop vapor diffusion method in 96-well sitting-drop plates (Art Robbins Instruments, USA). Over 500 different conditions from sparse-matrix screening solution kits were tested to identify optimal crystallization conditions. The kits used included PEG/Ion (HR2-126 and -098), Index (HR2-144), Salt Rx 1/2 (HR2-107 and -109), and Crystal Screen 1/2 (HR2-110 and -112), all from Hampton Research (USA), in addition to Wizard (CS-311, Jena Bioscience, Germany). N-terminal His_6-TagF crystals grew within 1 day in drops containing equal volumes (1 μL) of protein sample (50 mg/mL in 150 mM NaCl, 2 mM DTT, and 20 mM Tris [pH 7.5]) and reservoir solution (2.5 M NaCl, 0.1 M imidazole [pH 8.0], 3.2 M NaCl, and 0.1 M Bis-Tris propane [pH 7.0]). To improve the crystals, additional screening was performed using the Additive (HR2-428, Hampton Research, USA) and Detergent (HR2-406, from Hampton Research, USA) screening kits.

Data collection and processing

Prior to data collection, 30% glycerol was added to the reservoir solutions as a cryoprotectant, and crystals were flash-cooled in liquid nitrogen. All diffraction datasets were collected at 100 K on beamline 5C of the Pohang Accelerator Laboratory (PAL; Republic of Korea) using a Quantum 270 CCD detector (USA) (Park et al., 2017). Data were processed using the HKL2000 software suite. Experimental electron density maps were obtained by MR methods using PHENIX software, version 1.9 and were

interpreted using the Coot program (PHENIX; USA) (Adams et al., 2010; Emsley and Cowtan, 2004).

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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