Biochemical and Biophysical Research Communications xxx (2017) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Crystal structure of the flagellar chaperone FliS from *Bacillus cereus* and an invariant proline critical for FliS dimerization and flagellin recognition

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ARTICLE INFO

Article history:
Received 10 April 2017
Accepted 13 April 2017
Available online xxx

Keywords:
Bacillus cereus
BC1639
Flagellar chaperone
FliS
Crystal structure
Proline

ABSTRACT

FliS is a cytoplasmic flagellar chaperone for the flagellin, which polymerizes into filaments outside of the flagellated bacteria. Cytoplasmic interaction between FliS and flagellin is critical to retain the flagellin protein in a monomeric form, which is transported from the cytoplasm through the flagellar export apparatus to the extracellular space for filament assembly. Defects in the FliS protein directly diminish bacterial motility, pathogenicity, and viability. Although the overall structure of FliS is known, structural and mutational studies on FliS from other bacterial species are still required to reveal any unresolved biophysical features of FliS itself or functionally critical residues for flagellin recognition. Here, we present the crystal structure of FliS from *Bacillus cereus* (BcFliS) at 2.0 Å resolution. FliS possesses a highly dynamic N-terminal region, which is appended to the common four-helix bundle structure. An invariant proline residue (Pro17 in *B. cereus* FliS) was identified in all known FliS sequences between the N-terminal region and the four-helix bundle. The N-terminal proline residue functions as a helix breaker critical for FliS dimerization and flagellin recognition.

1. Introduction

More than 80% of known bacterial species contain a flagellum and respond to constantly changing environments by moving to a favorable place or by turning away from detrimental chemicals using the flagellum. In addition to cellular locomotion, the flagellum has been shown to mediate adhesion to substrates, biofilm formation, reduction of insoluble metal minerals, and pathogenicity [1,2].

The bacterial flagellum is a multimeric protein complex that is constructed from more than 30 different proteins via well-coordinated processes in designated space [3,4]. The flagellar structure can be subdivided into the basal body, the hook, and the filament from the cytoplasmic membrane through the cell wall to the extracellular space. The complexity of flagellar components and assembly locations indicates that flagellar construction is not a simple process but rather must be precisely coordinated in multiple sequential steps. In brief, first, flagellar component proteins are produced in the cytoplasm. Second, the components are delivered to designated cellular locations and assemble into the basal body and the hook. Next, the filament cap protein is exported on the top of the hook. Finally, ~20,000 copies of flagellin proteins are individually transported through the cell membrane and the cell wall to the extracellular space and are inserted under the cap protein where the flagellar filament forms [5,6].

During the flagellar system, it is critical to retain each protein in a monomeric form until flagellar assembly [5,7]. Cytoplasmic chaperones prevent component proteins from premature aggregation by binding to the protein in partially unfolded states. Furthermore, the chaperones facilitate the export of their cognate partners by directing them to the membrane-associated export protein, FliI. In *Salmonella enterica* serovar Typhimurium (the serovar designates subspecies of *S. enterica* that are defined by

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http://dx.doi.org/10.1016/j.bbrc.2017.04.070
0006-291X/

serological diagnosis against bacterial cell antigen) [8], a set of cytosolic chaperones, FlgN, FliS, and FliT, have been revealed to be specific for the hook-filament junction proteins (FlgK and FlgL), flagellin (FliC), and filament cap protein (FliD), respectively [9,10]. Defects in those flagellar chaperones impair flagellar structure and function, leading to the loss of bacterial mobility and pathogenicity and also to cell death.

Among flagellar chaperones, FliS is a small protein consisting of 122–135 amino acids. FliS functions in the cytoplasm and prevents flagellin from polymerization until it is delivered to the flagellar export apparatus [5,9]. In addition to the classic role of chaperone, FliS has also been recognized to be a transcriptional regulator of flagellar biosynthesis [11]. Structural studies of FliS from *Aquifex aeolicus* (AaFliS) and *Helicobacter pylori* (HpFliS) have demonstrated that FliS forms a four-helical bundle structure and recognizes the C-terminal residues of flagellin by inserting them into the inter-helical space [12,13]. However, other conserved, and thus potentially functionally significant, residues or regions of FliS have not been biophysically characterized for flagellar recognition. Furthermore, low amino acid sequence conservation (10–20% sequence identity) among FliS orthologs limits our understanding of FliS proteins from other bacterial species, and structure-based analyses of diverse FliS orthologs are required to determine whether the structure and function of FliS are conserved or divergent among bacteria.

Here, we present the 2.0 Å resolution crystal structure of the flagellin chaperone FliS from *Bacillus cereus* (BcFliS), which represents a FliS structure in gram-positive bacteria. Our structural and modeling studies, combined with structure-based mutational analysis, suggest that BcFliS is able to form a homodimer using the dynamic N-terminal helix. Moreover, comparative structural and sequence analyses allowed us to identify the invariant proline residue (Pro17 in BcFliS) as an N-terminal helix-breaker that facilitates flagellin binding.

2. Materials and methods

2.1. Preparation of recombinant BcFliS protein

The *bc1639* gene that encodes BcFliS protein residues 1–122 was amplified by PCR from the genomic DNA of *B. cereus* ATCC14579 using primers containing *Nde*I or *Sal*I restriction enzyme sites (Table S1) and ligated into a modified pET28a vector (pET28am) [14] that contains the N-terminal His_6-affinity tag and thrombin cleavage site. Identity of the cloned BcFliS plasmid was confirmed by DNA sequencing.

Recombinant BcFliS protein was overexpressed in the *E. coli* strain BL21 (DE3) by addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 18 °C for ~16 h. Cells were harvested by centrifugation and sonicated in phosphate-buffered saline (PBS, pH 7.4) containing 10 mM imidazole. The cell lysate was cleared by centrifugation (~25,000 × g). The soluble BcFliS protein was incubated with Ni-NTA agarose resin (Qiagen) and competitively eluted using 250 mM imidazole in PBS and dialyzed against 50 mM Tris, pH 7.4/50 mM NaCl/5 mM β-mercaptoethanol (βME).

The N-terminal His_6 tag used for affinity purification was removed by thrombin. The tag-free BcFliS protein was further purified by anion-exchange chromatography using a Mono Q 10/100 GL column (GE Healthcare) in 50 mM Tris, pH 7.4. Protein elution was performed by a linear NaCl gradient and BcFliS was eluted at a conductivity of ~30 mS cm^{-1}. Fractions were analyzed by SDS-PAGE. BcFliS fractions were concentrated to ~12.5 mg/ml for crystallization screening.

Selenomethionine-incorporated BcFliS protein (SeMet-BcFliS) was prepared for single-wavelength anomalous experiments as described [15], and purified in a manner similar to native BcFliS protein.

BcFliS mutant constructs ($BcFliS^{\Delta HN}$, a deletion mutant in which the N-terminal helix residues 1–12 were removed; $BcFliS^{DM}$, a double mutant with Asn16 and Pro17 mutated to alanine residues; $BcFliS^{P17A}$, a single mutant in which Pro17 was replaced by alanine) (Table S1) were generated using the QuikChange site-directed mutagenesis method (Agilent) and the mutations were confirmed by DNA sequencing. BcFliS mutant proteins were produced in an identical manner to the wild type BcFliS.

2.2. BcFliS crystallization and X-ray diffraction data collection

The crystallization conditions of SeMet-BcFliS were screened by the sitting-drop vapor-diffusion method at 18 °C. SeMet-BcFliS crystals were obtained in 1.5 M ammonium sulfate and 4% isopropanol. For X-ray diffraction data collection, SeMet-BcFliS crystals were cryoprotected in a crystallization solution supplemented with 35% glycerol. A single crystal was flash-frozen under the cryostream at −173 °C. X-ray diffraction was performed at beamline 7A of the Pohang Accelerator Laboratory (PAL). Diffraction data were indexed, integrated, and scaled using the HKL2000 package [16]. X-ray diffraction statistics are shown in Table S2.

2.3. Structure determination of BcFliS

The phases for the BcFliS structure were experimentally obtained by the SAD method using the AutoSol program in the Phenix suite [17]. The initial model of SeMet-BcFliS was built on the SAD map by the Coot program [18]. The final BcFliS structure was built by iterative cycles of rebuilding and refinement using the Coot and Refmac5 programs, respectively [19,20]. The final model that includes BcFliS residues 1–121 and 69 water molecules exhibited excellent stereochemistry with no Ramachandran outliers [21]. Structure refinement statistics are shown in Table S2.

2.4. Native PAGE analysis of interaction between BcFliS and flagellin

The interactions between BcFliS and flagellin were monitored by native PAGE. The *bc1658* and *bc1659* genes amplified from the genomic DNA of *B. cereus* ATCC14579 and inserted into modified pET28am plasmid (Table S1). The flagellin proteins were overexpressed and purified similarly to BcFliS.

Purified BcFliS and flagellin proteins were mixed at various molar ratios, incubated at 18 °C for 30 min, and then loaded onto an 8% polyacrylamide gel. Native PAGE was carried out at 18 °C for 2 h at 90 V and the gel was stained with Coomassie brilliant blue for visualization.

3. Results and discussion

3.1. Overall structure of BcFliS

For the structural study of BcFliS, a recombinant protein encoded by the *bc1639* gene was produced and crystallized. The crystal structure of BcFliS was solved by SAD phasing using SeMet-BcFliS crystal and refined to 2.0 Å resolution (Table S2). The asymmetric unit contains one chain of BcFliS (Fig. 1A). The BcFliS structure covers residues 1–121 from the entire molecule (residues 1–122) and adopts a four α-helix bundle (H1–H4) with dimensions of ~54 Å × 24 Å × 22 Å with an extended α-helix (HN) at the N-terminus. The BcFliS structure presents a topology of HN (residues 1–12) - H1 (residues 17–40) - H2 (residues 44–64) - H3 (residues 71–94) - H4 (residues 100–121). Single-turn 3_{10} helices are also observed in residues 38–40, 71–73, 91–93, and 118–120.

Please cite this article in press as: C. Lee, et al., Crystal structure of the flagellar chaperone FliS from *Bacillus cereus* and an invariant proline critical for FliS dimerization and flagellin recognition, Biochemical and Biophysical Research Communications (2017), http://dx.doi.org/10.1016/j.bbrc.2017.04.070