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Crystal structure of truncated FlgD from the human pathogen *Helicobacter pylori*

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ABSTRACT

Flagellin component D (FlgD) participates in the assembly of flagella, helical tubular structures that provide motility in non-filamentous bacteria. FlgD guides and controls the polymerization of FlgE that builds the hook, a short curved and hollow cylinder that connects the flagellar basal body spanning the cell envelope to the protruding filament. Crystal structures of truncated forms of *Helicobacter pylori* FlgD from two different strains in two space groups, *I*422 and *P*2, are reported here, at 2.2 Å and 2.8 Å resolution, respectively. Analogously to *Pseudomonas aeruginosa* and *Xanthomonas campestris* FlgD proteins, crystallization experiments set up for the full length protein resulted in crystals of a truncated form, lacking both N- and C-terminus ends. The crystal structures of the central domain show that the monomer is composed of a tudor and a fibronectin type III domain. The full length *Hp*FlgD contains a long N-terminal signal region, probably partially flexible, a central globular region and a C-terminal segment with a peculiar repetitive pattern of amino acids. The spatial orientation of the two domains in *Hp*FlgD differs from that of the homologous FlgD family members, *P. aeruginosa* and *X. campestris*. This difference together with the observation that *Hp*FlgD assembles into tetramers, both in the solution and in the two crystal forms, strongly suggests that significant differences exist in the molecular organization of the flagella in different bacterial species.

1. Introduction

Helicobacter pylori is a Gram-negative pathogen able to colonize the human stomach and is responsible for several gastric pathologies, including gastritis, peptic ulcer, gastric adenocarcinoma and MALT lymphoma (Kusters et al., 2006; Rothenbacher and Brenner, 2003; Suerbaum and Michetti, 2002). In order to survive in the hard stomach environment and to permanently colonize it, *H. pylori* has to move through the mucous layer and adhere to gastric epithelial cells, in particular during the initial phases of the infection (Amieva and El-Omar, 2008; Josenhans and Suerbaum, 2002), and in doing so it has to rely on flagella (Lertsethtakarn et al., 2011; Ottemann and Lowenthal, 2002).

The flagellum is a rotatory nano-machine that can be described as composed by two portions, the extracellular filament and the hook-basal body. The latter represents the flagellar motor that converts the chemical into mechanical energy (Thomas et al., 2006). The assembly of the flagella and the stator requires proper interactions with the peptidoglycan layer through which the organelle has to pass for externalization (Roure et al., 2012). These authors demonstrated that even though the flagella were correctly assembled, lack of the appropriate lytic transglycosylase (MltD) resulted in the incorrect localization of the flagellar motor protein *Hp*MotB to the bacterial pole and with a loss of motility. The hook, which is a tubular structure that connects the basal body to the extracellular filament, is made of about 120 copies of a single protein, called FlgE. FlgE is a protein that tends to form filaments and, for this reason, it needs the presence of other proteins, FliK, FlhB and FlgD, in order to properly assemble the hook outside the external membrane of the bacterium (Macnab, 2003). FlgD in *Salmonella typhimurium* is predicted to control the correct number of FlgE monomers that form the hook (Kubori et al., 1992). In this

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bacterium FlgD is absolutely needed for the assembly of the flagellar hook, but it has not been detected in the mature flagellum (Ohnishi et al., 1994). FlgD, like the majority of the flagellar extracellular components, is exported via a specific type III secretion system (TTSS), located at the base of the flagellum. After assembly of the basal body in the cytoplasmic membrane and the C-ring in the cytoplasmic space, the flagellar export apparatus is built within the C-ring and gets ready to export the axial components, among which is FlgD, to construct the rod, hook, hook-filament and the long filament, according to this order (Erhardt et al., 2010). FlgD is associated to the apparatus when the rod is completed, but it is discarded as soon as the hook is completed, i.e. when FlgK is added to the hook. During this process the interaction between FlgE and FlgD may take place (Moriya et al., 2011).

Two crystal structures of FlgD from other bacteria have been determined: *Pseudomonas aeruginosa* (*Pa*FlgD, PDB ID 3OSV (Zhou et al., 2011) and *Xanthomonas campestris* (*Xc*FlgD, PDB ID 3C12 (Kuo et al., 2008). Both structures lack the N-terminal domain, which is predicted to be largely flexible, at least for the isolated proteins. A study of FlgD from *Escherichia coli* (Weber-Sparenberg et al., 2006) indicated that the first 71 N-terminal residues represent a signal for the export of the protein through a type III secretion system (TTSS) into the flagellar channel. TTSS is an essential part of the flagellum apparatus that allows the proper export of the proteins necessary for the assembly of the flagellum itself.

In this paper we describe the crystal structure of truncated FlgD from *H. pylori*, which presents specific differences with respect to the ortholog protein from other Gram-negative bacteria.

2. Materials and methods

2.1. Molecular cloning

The HP0907 gene was cloned from two different *H. pylori* strains, 26695 and G27. Cloning from different strains was performed since the *Hp*FlgD sequence analysis by the *PSIPRED* bionformatic tool (http://bioinf.cs.ucl.ac.uk/psipred/) predicted 15 extra disordered aminoacids (Pro273 to Lys287) of the protein from strain G27 with respect to strain 26,695 (Fig. 1). To gain a more successful crystallization outcome, two different constructs have been PCR-amplified starting from the purified genomic DNA of the *H. pylori* strain G27 or 26,695. Both *Hp*FlgD constructs have been inserted into the pETite (Lucigen) pre-processed linearized free-enzyme plasmid vector in frame with a C-terminal His tag, using 5′ primer – GGA GAT ATA CAT ATG GCT ATT GAT TTA GCA GAA G – (forward) and 3′ – GTG ATG GTG GTG ATG ATG TGC TGT CTC TTT AGG GG – (reverse).

2.2. Expression and purification of HpFlgD_G27 and HpFlgD_26695

E. coli BL21(DE3) cells were transformed with pETite-*Hp*FlgD_G27 or pETite-*Hp*FlgD_26695 and grown at 310 K in 2 L Luria Bertani (LB) medium supplemented with 30 μg mL^{-1} kanamycin. When the OD_{600} of 0.6 was reached the protein expression was induced by adding 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) at 301 K. After 4 h of induction, cells were harvested by centrifugation and resuspended in buffer (20 mM Tris-base pH 7.5, 150 mM NaCl, 2 mM phenylmethylsulfonyl fluoride (PMSF)) and disrupted with One Shot Cell breakage system (Constant System Ltd., UK; 1.36 kbar). Afterwards, the lysate was clarified by centrifugation (30 min at 40,000 g) and the soluble fraction was loaded onto a 1 mL HisTrap column (GE Healthcare) equilibrated with buffer (20 mM Tris-base pH 7.5, 150 mM NaCl). After washing with buffer supplemented with 20 mM imidazole, the protein was eluted using an imidazole gradient (20–500 mM). The protein was concentrated by ultrafiltration (Vivaspin 15R 10,000 MWCO, Sartorius) and further purified by using the size-exclusion column Superdex 200 16/600 GL (GE Healthcare). The purity of the sample was verified by electrophoresis under denaturating conditions (SDS-PAGE).

Expression of recombinant seleno-methionine derivatized *Hp*FlgD_26695 was performed by using the non-methionine auxotrophic *E. coli* BL21 strain (DE3). The bacteria were grown in minimal medium M9 supplemented with 2% glucose, vitamin B_1, amino acids and L-seleno-methionine (as described in (Li et al., 2011)). The purification procedure for the methionine-labeled protein was the same as described for native *Hp*FlgD.

2.3. Characterization in solution

The monodispersity of the protein was evaluated at a concentration of 36 mg mL^{-1} and at 298 K by dynamic light scattering (DLS, Zetasizer Nano ZS, Malvern Instruments Ltd). In order to examine the oligomeric state of the protein, the analytical size-exclusion chromatography of the diluted protein (1 mg mL^{-1}) in combination with multi angle light scattering (MALS, Astra 6.1, miniDAWN TREOS) was carried out by using a Superdex 200 10/300 GL (GE Healthcare) column equilibrated with buffer containing 20 mM Tris-base pH 7.5, 150 mM NaCl. The same size-exclusion protocol was applied for the protein molecular weight standards: vitamin B12 (1.35 kDa), myoglobin (17 kDa), ovalbumin (44 kDa) and γ globulin (158 kDa). The void volume was determined by using Blue Dextran (GE Healthcare).

Secondary structure analysis of diluted *Hp*FlgD was performed by circular dichroism (CD) (see in Pulić et al. (2016) "submitted").

The presence of the His tag at the C-terminus of the full length *Hp*FlgD_26695 and crystallized *Hp*FlgD_26695 was evaluated with anti-His antibodies (Western blotting technique), see in Pulić et al. (2016) "submitted".

2.4. Crystallization and data collection

The purified native *Hp*FlgD_26695 and *Hp*FlgD_G27 were concentrated to 17.5 mg mL^{-1} and 30 mg mL^{-1}, respectively. Crystallization trials were set out by the sitting drop vapor diffusion technique with the Oryx 8 crystallization robot (Douglas Instruments). 1 μl drops were prepared by mixing the protein and precipitant solutions in a ratio 1:1, and equilibrated against 75 μl of the mother liquor in the reservoir. Two different crystal forms were obtained after approximately 2 months (see Fig. 2 in Pulić et al. (2016) "submitted"). Monoclinic crystals of *Hp*FlgD_G27 (*Hp*FlgD_m) were grown from the screening solution 4 of the PACT suite (Qiagen) (0.1 M SPG pH 7.0, 25% PEG 1500) at 293 K, while tetragonal crystals (*Hp*FlgD_t) of the construct *Hp*FlgD_26695 were obtained from the screening solution 79 of the same screening kit (0.2 M Na acetate, 0.1 M Bis-Tris propane pH 7.5, 20% PEG 3350) at 277 K. In order to solve the phase problem, seleno-methionine derivatized *Hp*FlgD_26695 was crystallized (concentration of 15 mg mL^{-1}) at 277 K from solution 4 of the PACT suite. Monoclinic crystals were directly cooled in liquid nitrogen, whilst the tetragonal crystals were cryoprotected by using the precipitant solution supplemented with 20% of ethylene glycol. Diffraction data of native *Hp*FlgD_m were measured at the ID14-4 beamline of the European Synchrotron Radiation Facility (ESRF, Grenoble, France) and they belong to the *P*2 space group (Table 1). Crystals of both native and seleno-methionine *Hp*FlgD_t belong to the tetragonal space group *I*422 (Table 1). Diffraction data of this second crystal form were measured at the PXIII beamline of the Swiss Synchrotron Light Source (SLS, Villigen, Switzerland). A fluorescence emission scan at the selenium edge was performed to optimize the energy for anomalous measurements. Diffraction data of

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