

Structural Characterization at the Atomic Level of a Molecular Nano-Machine: The State of the Art of *Helicobacter Pylori* Flagellum Organization

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Abstract: Motility is fundamental for success in colonization and survival of the human pathogen *H. pylori*, the bacterium that colonizes about half of the human population. Motility is achieved through flagella. In this review the present structural knowledge about the organization of *H. pylori* flagella is summarized, considering not only the structure of its proteins, but also what is known about flagella of other Gram-negative bacteria. Despite the limited amount of structural information about the *H. pylori* nano-machinery, sequence alignments and homology modeling allow to investigate the unique structural properties of several *H. pylori* flagellar proteins.

Keywords: *Helicobacter Pylori*, Flagellum, Motility, Secretion System

Introduction

The bacterium *Helicobacter pylori* colonizes the stomach of more than half of the world's population, being one of the most successful bacterial pathogens (Rothenbacher and Brenner, 2003). It was isolated and characterized at the beginning of the '80 s, (Marshall and Warren, 1984), thereby becoming the focus of intense research activity. It is a microaerophilic, non spore-forming and spiral-shaped Gram-negative bacterium, transmitted from human-to-human possibly by the fecal-oral or oral-oral route (Blaser, 1998). To date, several genomes of different strains have been completely sequenced, among them HP26695, J99 and G27 (Tomb *et al.*, 1997; Doig *et al.*, 1999; Oh *et al.*, 2006). A map of protein-protein interactions can be found through the PIMryder server (Rain *et al.*, 2001). The bacterial infection in humans is associated with a spectrum of disease outcomes, perhaps associated to potential selective advantages during long periods of the human history (Suerbaum and Michetti, 2002; Suerbaum and Josenhans, 2007). While many *H. pylori* infected individuals are clinically asymptomatic, most will exhibit some degree of gastritis. Approximately 10% of the infected subjects will develop more severe gastric pathologies, like peptic ulcer disease and atrophic

gastritis. Approximately 1% of infected individuals develop gastric adenocarcinoma and lymphoma of the Mucosa-Associated Lymphoid Tissue (MALT lymphoma) (Blaser, 1998; Suerbaum and Michetti, 2002). The severity of symptoms largely depends on the genetic diversity of the infecting strain and particularly on specific genotypes of virulence-associated genes, such as the *cag* Pathogen City Island (*cag*-PAI), encoding a T4SS which promotes delivery of the CagA effector into host cells, as well as a CagA-independent induction of interleukin-8 secretion via the host AP-1 and NF-κB signaling pathway (Backert and Selbach, 2008; Zanotti, 2011). *H. pylori* infections can be successfully cured with antibiotic treatment: Unfortunately, the available therapies are beginning to lose efficacy, because of the insurgence of antibiotic resistance.

H. Pylori's Flagellum

Success in colonization and survival of *H. pylori* is centered on the regulation of important virulence traits, such as motility, acid resistance, detoxification and metal ion homeostasis (Danielli and Scarlato, 2010). Motility is achieved through flagella. Flagella are of general relevance for many bacteria, but they are of paramount importance for *H. pylori*, as they are necessary for the survival of the bacterium in the stomach, particularly during the initial phases of infection (Josenhans and

Suerbaum, 2002; Ottemann and Lowenthal, 2002; Amieva and El-Omar, 2008). In fact, to survive and colonize the host the bacterium has to avoid the very acidic milieu of the stomach lumen and its periodic mechanical clearance. Flagella and outer membrane adhesins allow *H. pylori* to swim through the mucus layer and adhere to gastric epithelial cells. In contrast to many other Gram-negative bacteria, *Helicobacter* (and *Campylobacter*) species possess an unusual velocity in viscous media, possibly due to their helical shapes and to the presence of exclusively polar flagella (Lertsethtakarn *et al.*, 2011). Flagella are complex organelles composed of approximately 30 different proteins, but many others are necessary for flagella expression and assembly, for a total of at least 45 (listed in Table 1). Some of them have been identified as members of flagella, others are necessary for flagella assembly. The structural organization and control of flagella in Gram-negative bacteria have been thoroughly studied (Thomas *et al.*, 2006; Chevance and Hughes, 2008; Lertsethtakarn *et al.*, 2011; Paul *et al.*, 2011). A flagellum can be divided in two main portions, the hook-basal body and the extracellular filament (Fig. 1). The former, in turn, can be divided into three substructures: (i) The base, localized in the inner membrane and spanning to the cytoplasm; (ii) the rod and ring structures, located in the periplasm; and (iii) the hook, present on the surface.

The relevance of the flagellum is twofold: From one side, the knowledge of the detailed molecular architecture of *H. pylori* flagellum may allow to better fight the bacterium, since flagella are component essential for bacterium colonization of the host; on the other side, the flagellum represents an ideal example of a molecular machine and the mechanisms that bacteria have been able to devise in billion years of evolution to convert chemical into mechanical energy can eventually be copied by us for nano-technological purposes.

About the 3D structures of the proteins of *H. pylori* flagellum, only seven protein structures (including flagellar chaperons) have been determined and another nineteen molecular models can be constructed by homology modeling using an automated server (Phyre2, <http://www.sbg.bio.ic.ac.uk/phyre2/>), thanks to the structures of homologs from other species. In this review, what is known about the structure and organization of *H. pylori* flagellum, taking in consideration also what is known from flagella of other bacteria, will be summarized. All the protein mentioned in this review will refer to the bacteria strain HP26695.

The Bacterial Motor

The bacterial flagellar motor (BTM) is a rotatory nano-machine, able to self-assemble, that converts the energy, generally deriving from a flux of cations, into mechanical energy. The latter manifests itself in the

rotation of a long filament that allows the bacterium to move inside a viscous medium. Flagellar motor is generically composed by two elements, the stator and the rotor. The stator is non-covalently attached to the peptidoglycan layer. It interacts with the rotor, which is in contact with the MS-ring and is responsible of the torque generation (Macnab, 2003).

Several studies have been performed on the bacterial flagellar motor of *Escherichia coli* and *Salmonella sp.* and, despite the crystal structure of the separated components of the torque ring is known, more than one model of the BTM has been proposed (for a review, see for example (Stock *et al.*, 2012)).

Flagellar Basal Body Organization

In Gram-negative bacteria the basal body, which is embedded in the cell envelope, consists of a membrane ring, called MS-ring and a P-ring that passes through the periplasmic space and reach the outer part of the membrane until the L-ring (Fig. 1). The basal body has the role of transmitting the torque force from the motor to the filament (Chen *et al.*, 2011). Another feature common to most bacterial basal bodies is the presence of a Type 3 Secretion System (T3SS) export apparatus, which translocates unfolded flagellar proteins outside the membrane envelope through a central channel. It allows the assembly of the flagellum hook and of the filament beyond the membrane layer (Minamino and Macnab, 1999). It must be mentioned that flagellar systems and T3SS are evolutionary related each other (Abby and Rocha, 2012).

H. Pylori Stator Proteins

The stator is composed of two different transmembrane proteins, MotA and MotB, associated in a complex consisting in four copies of MotA and two of MotB (MotA₄MotB₂) (Terashima *et al.*, 2008). The stator main function is to create a proton gradient across the cytoplasmic membrane. The proton flow generates a potential difference that is used to generate a torque. The proton translocation through the MotA₄MotB₂ complex induces a conformational change in MotA cytoplasmic loop, which interests the rotor via electrostatic interaction with FliG (Chen *et al.*, 2011). There is very few information about MotA of *H. pylori*, but the structures of MotA and MotB are available for *Salmonella typhimurium*. MotA includes four transmembrane helices and a large cytoplasmic loop, with a charged residue on the last component of the structure that interacts with FliG (Zhou *et al.*, 1998). MotA transmembrane helices surround two MotB proteins. In particular, the transmembrane helix of MotB and two transmembrane helices of MotA constitute the proton channel (Blair and Berg, 1990; Stolz and Berg, 1991; Zhou *et al.*, 1998).

Table 1. *H. pylori* proteins involved in flagellum assembly

Localization	Protein name	Name for strain 26695	Typology	Role	Complex	Number of AA	PDB-ID structure
Export system	FliO	HP0583	Membrane protein	Protein associated with FlIP		293	
	FliP	HP0685		Protein associated with FliO and MSring		153	
	FliQ	HP1419		Export component	Associated with the previews three protein with FlhA and FlhB	88	
	FliR	HP0173		Associated with the MS ring		225	
	FlhA	HP1041		Target for soluble export proteins		733	3MYD
	FlhB	HP0770	Soluble Protein	Target for soluble proteins	The complex FliHIJ forms an ATPase complex Interaction with FliH	358	Model 35% (233-344 aa)
	FliH	HP0353		Negative regulator of FliI		258	Model 16% (76-257 aa)
	FliI	HP1420		ATPase; export protein outside of C-ring		434	Model 44% (17-434 aa)
	FliJ	HP0256		Putative protein		142	
	FlgJ	HP0245		Putative protein		105	
MS ring	FliF	HP0351	Transmembrane protein	Transmembrane component of the rotor	Associated with FlhA	389	
C ring	FliG	HP0352	Cytoplasmic protein	Rotor switch protein	Associated with MotA FliM	343	3USW; 3USY
	FliM	HP1031		Involved the rotor switch; binds the CheY-P protein	N-terminal interacts with FliF	354	4GC8
	FliN	HP0584		Flagellar export component; role in switching protein		106	Model 36% (37-119 aa)
Stator	FliY	HP1030	Transmembrane protein	Flagellar export component		283	Model 13% (1-172 aa)
	MotA	HP0815		Forms a proton channel through the membrane		210	
	MotB	HP0816			Associated with MotA	257	3SOY (cytoplasmic region)
P ring	FlgI	HP0246		Part of bushing		285	
L ring	FlgH	HP0325		Part of the bushing		237	
Proximal rod	FliE	HP1557		Has a role in rod stability during the growth; part of the export gate; MS ring rod junction protein		109	
Distal rod	FlgB	HP1559				140	
	FlgC	HP15558				161	
	FlgF	HP1092				269	
Hook-filament junction protein	FlgG	HP1585				262	
	FlgK	HP1119	Soluble	HAP1		606	Model 23% (71-564 aa)
Filament capping	FlgL	HP0295		HAP3		828	Model 30% (44-812 aa)
	FliD	HP0752		HAP2		685	
Hook cap	FlgD	HP0907	Soluble	Rod-modification protein; required for hook polymerization		301	Model 34% (125-266 aa)
Hook	FliK	HP0906		Hook length regulator		77	
	FlgE	HP0870		Hook protein	Helped by FlgD in Polymerization	718	Model 32% (93-678 aa)
Filament	FlaA	HP0601		Flagellin subunit	Polymerizes with FlaB	510	Model 26% (46-492 aa)
	FlaB	HP0115		Flagellin subunit		514	Model 29% (2-514 aa)
	FliS	HP0753	Cytosolic protein specific for FlaA, FlaB		Interacts with FliS	120	3K1H 3IQC; 3K1I
Regulatory protein	FlgM	HP1122		Encodes for the inhibitor of flagellum σ_{28} -factor		77	
	FliA	HP1032		Negative regulator of FlgM		255	
	FlgR	HP0730		Activates transcription with σ^{54} -factor		381	Model 40% (135-380 aa)
Chemotactic factor	CheY	HP1067		Chemotaxis	interact with FliM	124	3GWQ; 3HIF; 3HIG; 3HIE
Chaperon	FlgN	HP1457		Chaperon FlgKFlgL		210	Model 14% (72-208 aa)
	FliT	HP0754		Chaperon FliD		79	Model 29% (44-78 aa)
	FlgA	HP1477	Periplasmic protein	Chaperon involved in P-ring assembly		218	Model 18% (74-218 aa)
Flagellar Biosynthesis protein	FlhF	HP1035				459	Model 33% (190-449 aa)
Flagellar assembly factor	FliW1	HP1154				135	Model 21% (2-135 aa)
Paralysed flagellar protein	pFlA	HP1274				801	Model 30% (707-737 aa)
Flagellar assembly factor	FliW2	HP1377				129	Model 25% (1-121 aa)

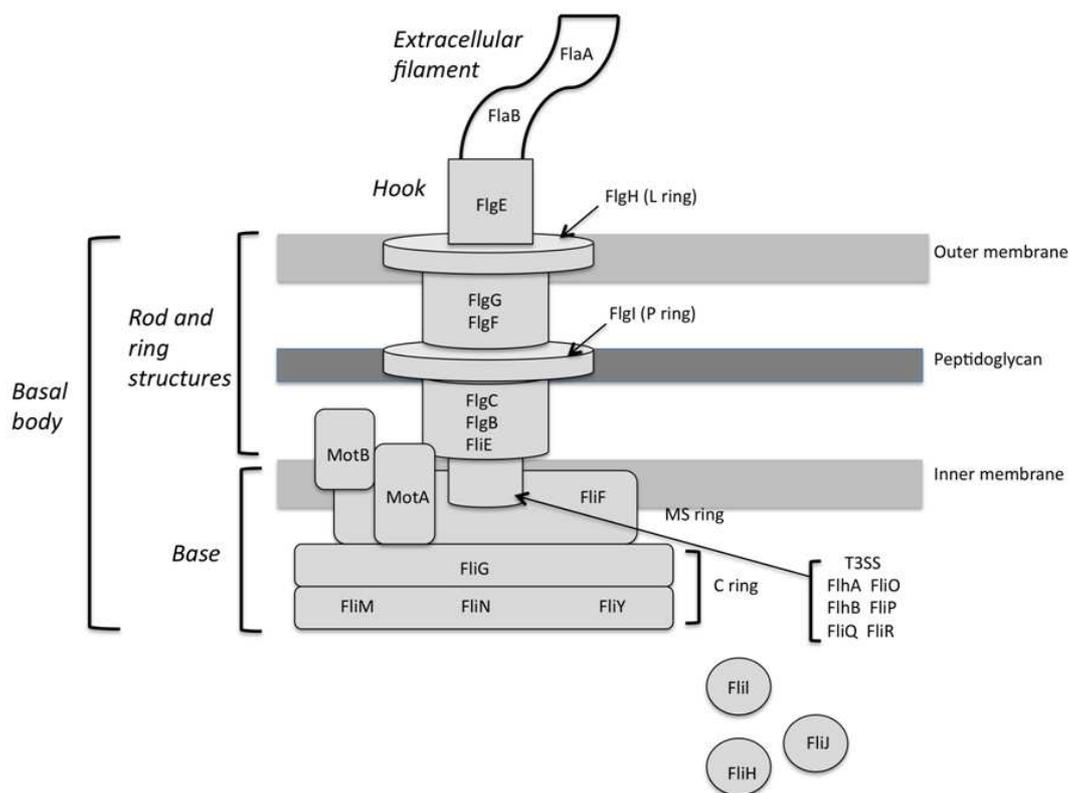


Fig. 1. General flagellar basal body (Scheme based on Lertsethtakarn *et al.*, 2011)

In *Salmonella*, MotB is divided in three different domains: a small cytoplasmic N-terminal domain (1-28); a single transmembrane helix (29-50) and a periplasmic C-terminal domain (51-209). MotA, due to its highly hydrophobic nature, has been modeled only for the middle domain, composed of around 60 amino acids (Fig. 2A).

Compared with MotA, MotB is much better characterized in *H. pylori* (Fig. 2B). O'Neill *et al.* (2011) have solved the structure of the middle and C-terminal part of the protein. The latter is composed of four pairs of alternating β -strands and α -helices, topologically arranged as $\beta\alpha\beta\alpha\beta\alpha$ and is thought to be involved in Peptidoglycan (PG) binding (Roujeinikova, 2008). In *H. pylori*, MotB is composed of a N-terminal cytoplasmic segment (residues 1-28), a single transmembrane helix (residues 29-50) and a large C-terminal periplasmic region (Muramoto and Macnab, 1998). The transmembrane helix contains an Aspartate residue (Asp33) that has been hypothesized to be involved in proton translocation across the cell membrane. Finally, residues 149-270 of MotB show sequence similarity to other OmpA-like proteins (O'Neill *et al.*, 2011). In solution, MotB behaves as a dimer; β -sheets of the two subunits associate through strand β_3 in an antiparallel edge-to-edge manner. The dimer is functional to peptidoglycan binding (Kojima *et al.*, 2009): It exposes the binding sites of the glycan chain on

opposite sites, in such a way that two different glycan chains can be bound simultaneously.

In *H. pylori*, the MotA₄MotB₂ complex is hypothesized to remain inactive inside the membrane until it is incorporated into the motor, extending the linkers connecting the transmembrane helices and the PG-binding domain. Notably, in the absence of the appropriate peptidoglycan maturation enzymes, MotB does not localize properly and this affects flagellar functionality (Roure *et al.*, 2012). The opening of the channel is triggered by the electrostatic interaction through MotA₄ complex and FliG rotor protein and induces the proton flow through the inner membrane of bacteria. The periplasmic P-ring has been proposed to be a binding site for the stator MotB₂ complex. Based on the crystal structure of MotB, the following conformational changes have been proposed: (i) Unfolding of the linkers connecting the transmembrane part of the MotB protein with the C-terminal one; ii) interaction with the PG layer; (iii) opening of the proton channel (O'Neill *et al.*, 2011) (Fig. 3). It has been shown that the anchoring of MotA/MotB complex is essential for the motor function (Blair and Berg 1991; Togashi *et al.*, 1997).

From the superposition of *H. pylori* MotB and the same protein from *Disulfobivros vulgaris* and *Salmonella* (Fig. 3C-D), it is noticeable how the N-terminal portion of the *H. pylori* protein assumes a very

different conformation with respect to the others. Another peculiar feature is the different orientation of the α -helix in *Salmonella* protein (amino acids from 265 to 274) with respect to the *H. pylori* one (amino acids from 231 to 250). These differences suggest a peculiar organization of *H. pylori* stator inside the membrane layer.

H. Pylori Switch Proteins

The switch complex is responsible of the torque reversal. It allows the bacterium to reorient its swimming, from Counterclockwise (CCW) to Clockwise (CW) (Lee *et al.*, 2010). The motion is controlled through a chemotactic signal, consisting in proteins CheW/CheA and CheY. In brief, the chemoreceptor CheW breaks its link to the histidine kinase CheA, which

auto-phosphorylates and becomes active, activating in turn the response regulator CheY. Once phosphorylated, the latter interacts with the N-terminal part of the rotor protein FliM that induces the switch from the CCW to CW (Sarkar *et al.*, 2010).

Proteins involved in the switching of the rotor have been identified for several Gram-negative bacteria, but the better characterized are the ones from *Salmonella*. The switch components of the flagellum are FliG, FliM and FliN, present in 26, 34 and 136 copies, respectively (Thomas *et al.*, 2001). They are arranged in a ring at the base of flagellum. FliG, the main protein involved in the torque generation, is directly in contact with the stator protein MotA, possibly through a single helix located in the C-terminal domain (Zhou *et al.*, 1998).

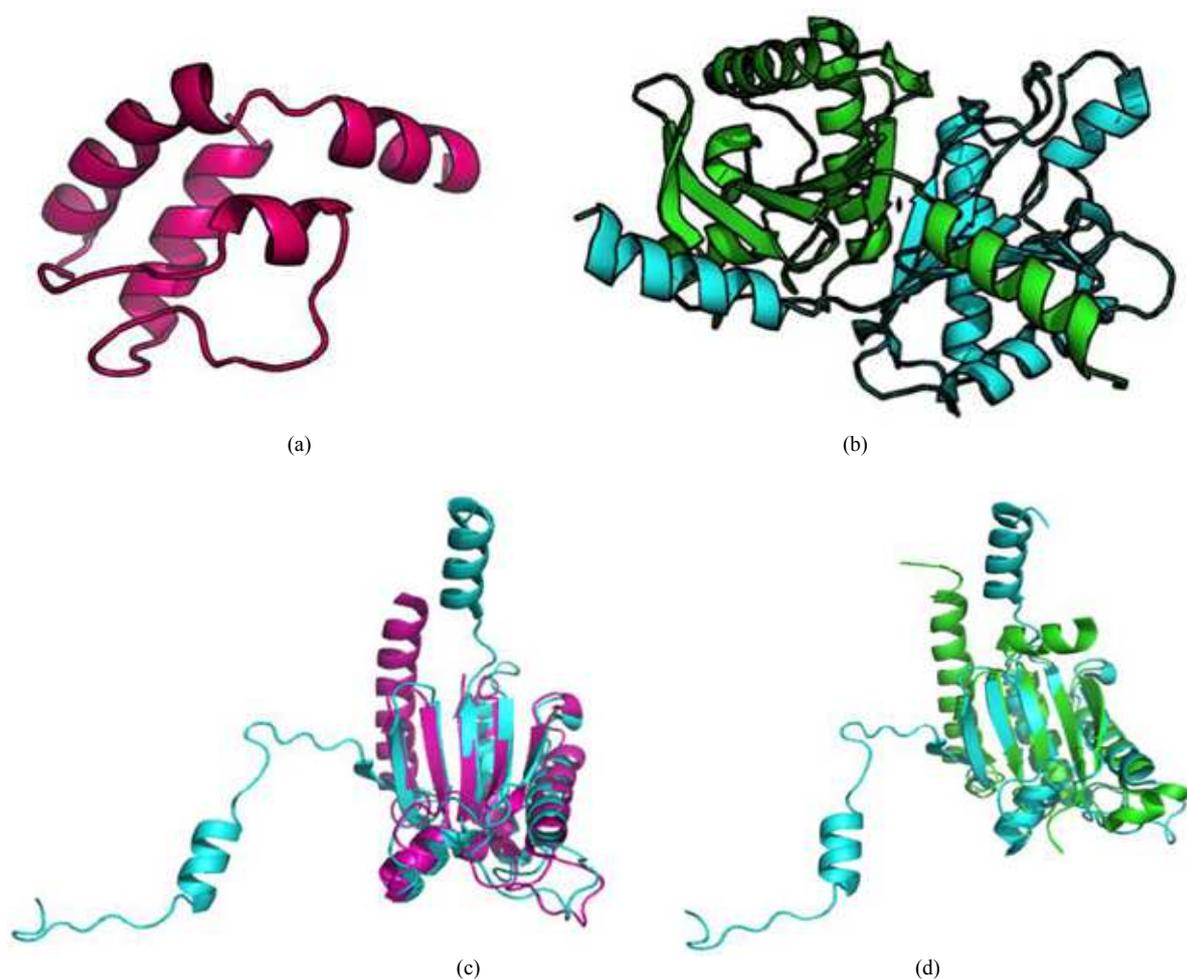


Fig 2. (A) MotA model (fragment from 82-148 amino acids) obtained by sequence homology by Phyre² server. The structure template used in Colicin IA (PDB ID: 4CII) from *E. coli*. (B) MotB cytoplasmatic domain from *H. pylori* (PDB ID: 3SOY). In solution is a dimer and shows the 2-fold axis is designed perpendicular to the plane. (C) Superimposition of 103 amino acids from *H. pylori* MotB C-terminal domain with *Desulfovibros vulgaris* MotB (RMS: 2.94 Å). (D) Superimposition of 108 amino acids from *H. pylori* MotB C-terminal domain with *Salmonella typhimurium* MotB C-terminal domain (RMS: 6.36 Å). Figures from 2 and 4-10 were produced using PyMol (PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC.)

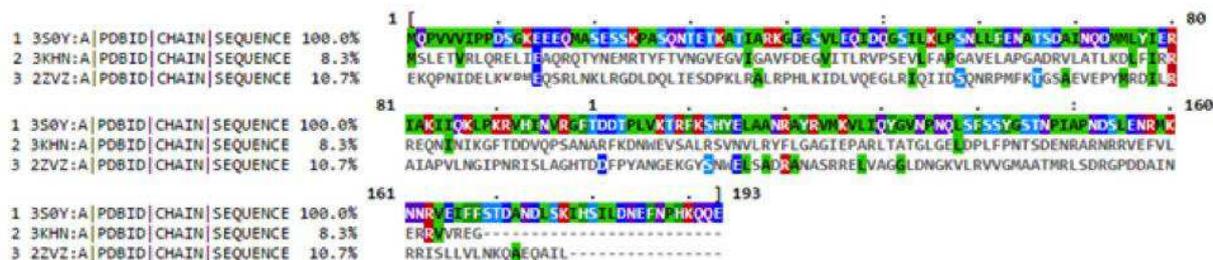
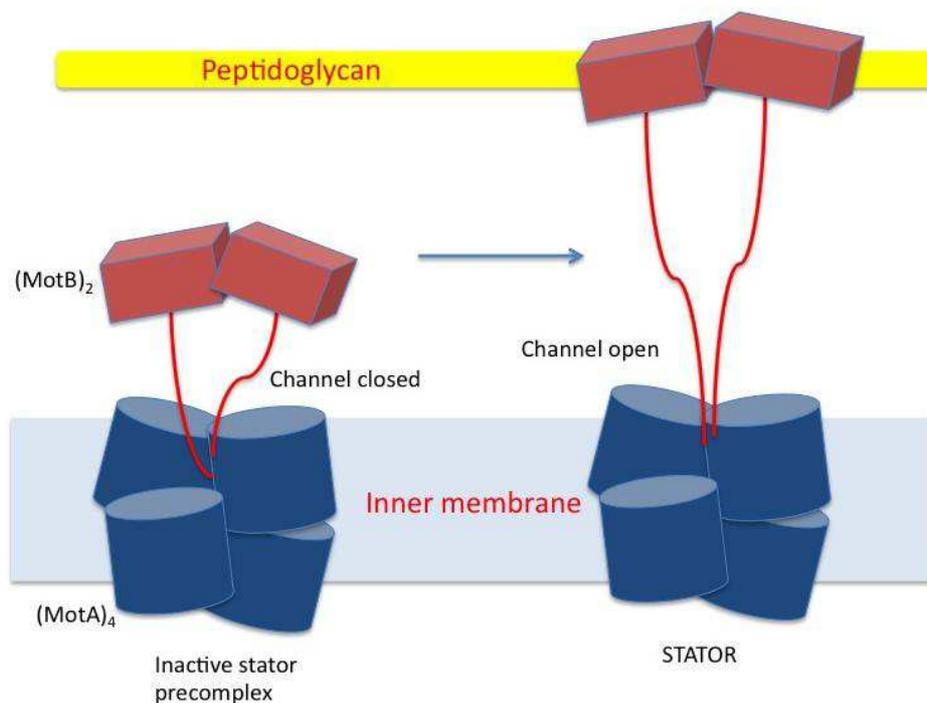


Fig. 3. Top: Hypothetical activation mechanism of MotA₄MotB₂ complex inside the membrane. The mechanism is specific for *H. pylori* (Scheme based on O'Neill *et al.*, 2011). Bottom: Comparison of the three MotB amino acidic sequence. In order: *H. pylori* MotB; *Desulfovibros vulgaris* MotB; *Salmonella typhimurium* MotB. Multiple alignment performed by MView (<http://www.ebi.ac.uk/Tools/msa/mview/>)

Several hypotheses about the exact position of FliG in the ring have been put forward; in particular, a huge number of interactions between FliG and FliM have been exploited. From 3D electron microscopy reconstruction of *Salmonella* C-ring, it seems reasonable to believe that FliG is located on the cytoplasmic face of the MS-ring. A relevant interaction with FliM and FliN has been hypothesized, owing to the tendency of FliG to be disordered in the absence of the C-ring.

In general, three different domains of FliG are known: A N-terminal domain, which binds FliF and acts as an anchor in the C-ring (Levenson *et al.*, 2012); a C-terminal domain involved in the electrostatic interaction

with MotA (Lloyd and Blair, 1997); a middle-domain associated with FliM through the sequence EHPQR, highly conserved in most of the flagellate organisms. Another binding site for FliM is present in FliG C-domain, but it is not clear yet how this interaction takes place. FliG and FliM are present in a 1:1 stoichiometry, according to a correspondence between FliG in the C-ring and FliF in the MS-ring. A 26-fold symmetry has been detected in both rings (Suzuki *et al.*, 2004).

Three different architectures of the bacterial flagellar motor have been proposed, based on the crystal structures of single proteins and/or on the EM images of *Salmonella* flagellum.

Model A

The first model (Paul *et al.*, 2011) locates FliG in the inner and the outer side of the C-ring, with the C-terminal part of the protein in the outer part. As seen in EM, the outer lobe of the electron density contains a 34-fold rotational symmetry, but only 26 FliG molecules are present. Model A proposes a solution to this contradiction. It assumes a different arrangement of FliG molecules aligned on the 34 FliM subunits. It seems reasonable that FliG molecules are separated by several gaps in the outer lobes. The main feature of this model is that FliG C-domain and FliG middle domain interact both with FliM. In particular, it is proposed that all 26 FliG C-domains are bound to FliM monomers. The remaining FliM monomers bind the FliG middle domains. Although the geometrical problem of rotor dimensions seems to be solved, a major problem of this model is that the free hydrophobic surface exposed to the solvent is quite high, around 21,000 Å².

Model B.

Stock *et al.* (2012) It imposes some intermolecular restraints between FliG proteins. In protein crystals, FliG-FliG interaction is mediated by an Armadillo Repeat Motifs (ARM), characterized by a three-helix fold. Each of these motifs is composed by FliG middle domain (ARMm) and FliG C-domain (ARMc). The interaction between ARMm-ARMc shields each monomer from the contact with the bulk solvent. The molecule is obliged to arrange in a righthanded super helix. So FliG can be divided in a globular N-terminal domain, ARM super helix and C-terminal domain (Stock *et al.*, 2012). The final restrain position of FliG confines the N-terminal domain in the inner lobe of C-ring, with helices involved in MS-ring binding. FliG C-domain is proximal to the membrane. Finally, FliG middle domain is arranged to form an ARM super helix (Lee *et al.*, 2010). Even though the model seems to give a better accord with EM data, no explanation is given about the symmetry mismatching.

Model C.

Stock *et al.* (2012) Model C was proposed after that the structure of FliG C-domain and middle domain were determined. Model C fits the X-ray structure in the EM electron density as does model B, but with some significant differences. For instance, the helix connecting the Cdomain and the middle domain is not in close contact with the middle domain. Indeed, it was observed that the close position is not physiological. Finally, the contact between ARMc and ARMm occurs in the same subunit.

These three different models imply three different switching mechanisms. Model A requires the rotation of

FliG C-domain, because of the direct binding with FliM. The turn reorients torquegenerating charges, which will cause rotational switching. The FliN-FliM C-domain interaction can be seen as a cooperative mechanism, which generate the torque after a chemotactic signal. On the other hand, in model B the motion is essentially due to the conformational changes in the helix connecting the FliG C-domain and FliG middle domain. The movement causes its adjacent FliG promoter rotate. Finally, in model C the movement is intrinsically similar to model B (Stock *et al.*, 2012).

Differently from other bacteria, *H. pylori* includes one more protein involved in flagellum switch regulation in its genome. The four motor switch proteins are identified as FliG (HP0352), FliM (HP1031), FliN (HP0584) and FliY (HP1030) (Fig. 4). Chen *et al.* (2011) demonstrated by some cryo-EM studies that the main core of C-ring in most of the bacteria is principally regulated by the presence of FliG, FliM and FliN, but not FliY. According to Lowenthal *et al.* (2009), FliY protein has an N-terminal domain that belongs to CheC/CheX/FliY/FliM family. Due to its feature, it is possible that FliY may substitute FliM in FliG C-domain interaction, but experimental confirmation is lacking. Following this hypothesis, the structural assembly of *H. pylori* flagellum should be barely different from the other organisms.

FliN (Fig. 5C) plays a structural role in building the flagellar architecture, too. It binds the flagellar export protein FliH and localizes itself with FliI and FliJ. In *H. pylori*, FliN C-terminal domain is fused with a phosphatase/CheC-like domain (McMurry *et al.*, 2006; Paul and Blair, 2006). Moreover, FliN is involved in the direct binding of the regulator protein CheY-P (Paul and Blair, 2006). Lowenthal *et al.* (2009) performed some alignment of FliN and FliY sequences taken from well characterized bacteria. They noticed highly conserved structural regions, even though some specific amino acids were not. Taking into account the differences, alignment data seem to suggest that both FliN and FliY are involved in export and protein-protein interaction, beyond the motility function. Moreover, it was evident that *fliY* and *fliN* mutants were only partially flagellated.

H. Pylori Flagellar Export Apparatus

The general assembly of the export apparatus resembles the Type III Secretion System (T3SS), a virulence factor common to most Gram-negative bacteria. The two systems usually share several homologous proteins (Cornelis, 2006). In fact, flagellar T3SS has been hypothesized of being also involved in secretion of virulence factors in *Campylobacter jejuni*, a species closely related to *H. pylori* (Roure *et al.*, 2012). Very few information are present in the literature about the flagellar export apparatus from *H. pylori*, but

common features with other bacteria can be detected. The export apparatus consists of six membrane proteins (FliO, FliP, FliQ, FliR; FlhA and FlhB) and three soluble ones (FliH, FliI and FliJ) (Minamino and Namba, 2004). All of them associate to the MS-ring and assemble together to build the export machinery. Among all the proteins listed, FlhA from *H. pylori* is the best characterized, especially the cytoplasmic domain, whose structure was solved in 2010 (Moore and Jia, 2010) (Fig. 4D). Biochemical studies have demonstrated a strong interaction between the membrane fragment of FlhA and FliF, which is the

unique component of MS-ring (McMurry *et al.*, 2004). Interactions between FlhA and FlgM, a protein involved in regulation of transcription of flagellum genes, have also been demonstrated (Colland *et al.*, 2001).

In analogy with its homolog in the T3SS, FlhA presents an N-terminal part inserted inside the cytoplasmic membrane, a C-terminal domain floating in the cytoplasm and a linker portion. The C-terminal domain contains a thioredoxin-like domain, a RNA recognition motif domain inserted into the thioredoxin-like domain, a helical domain and a C-terminal β/α -domain.

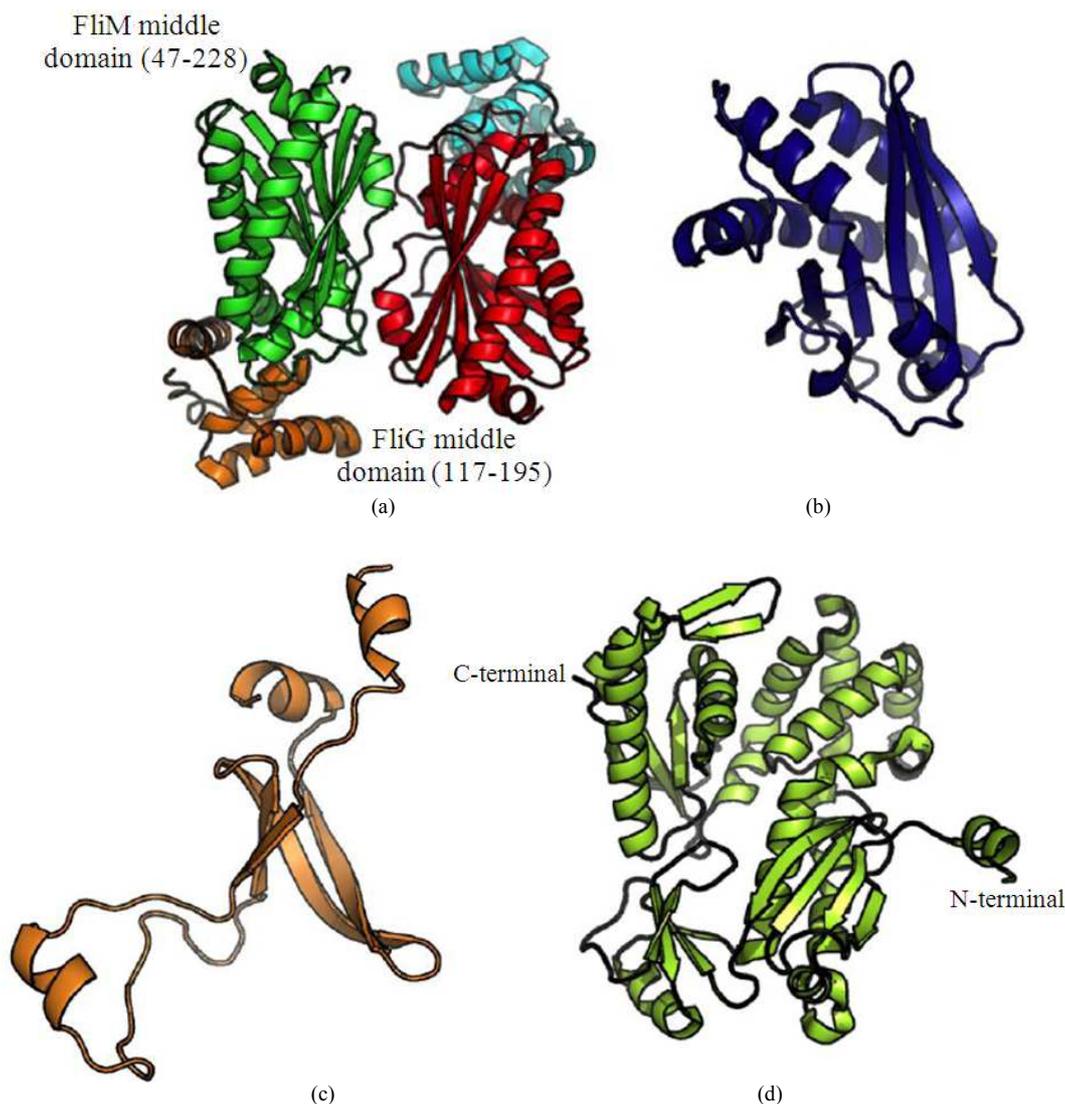


Fig. 4. (A) FliM (middle domain)-FliG (middle-domain) dimer from *H. pylori* (PDB ID: 4GC8). The binding is initially mediated through the binding to FliG middle and N-domains. On the other side, the affinity for the C-domain of FliG seems to be very low, suggesting that the primary domain for FliM is the middle domain of FliG (Lam 2013). (B) FliY structure model, built by Phyre2 server using FliM protein from *T. maritima* (PDB-ID: 2HP7, chain A as template). All the amino acids from 1 to 270 were modeled. (C) FliN model obtain by sequence homology by Phyre² server. FliN structure from *T. maritima* was used as template, with 43% of identity. (D) FlhA cytoplasmic domain (PDB ID: 3MYD)

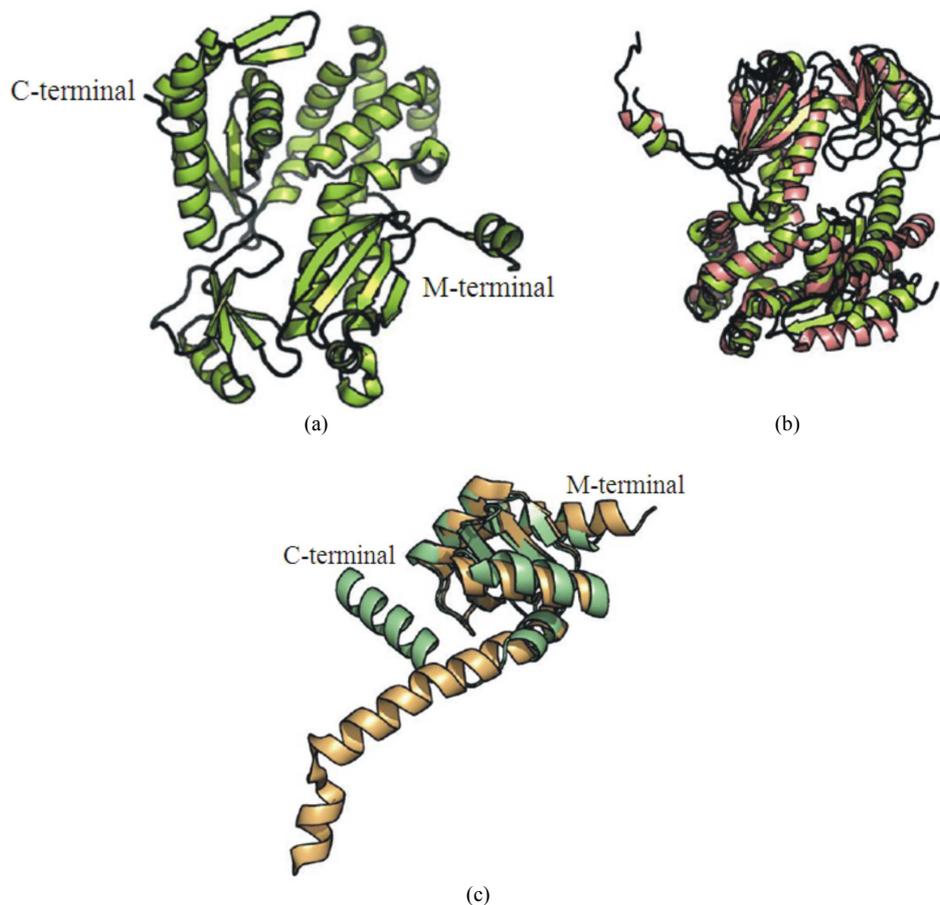


Fig. 5. (A) *H. pylori* FlhA cytoplasmic domain (amino acids from 385-732) (PDB ID: 3MYD). (B) Comparison of 309 residues of FlhA from *H. pylori* (lemon) and FlhA from *Salmonella* (amino acids from 347 to 692, PDB ID: 3A51) (salmon). RMS 3.79 Å from the superimposition. (C) Superimposition between *H. pylori* FlhB (pale green), modeled using cytoplasmic YscU protein from *Yesimia pestis* (PDB ID: 2JLHa) as template and *Salmonella* FlhB protein cytoplasmic domain (light orange). The alignment was carried out on 98 atoms with RMS 2.45 Å

The last is characterized by a high hydrophobic surface that likely suggests the presence of a ligand-binding site. The protein, crystallized in a closed conformation, presents the RNA recognition domain and the C-terminal domain in close contact. Most of the protein domains seem to be highly conserved in structures from different organisms. The thioredoxin-like and the helical domain seem to be the highly conserved in sequence. The most variable part is the linker segment, which nevertheless seems to be crucial in the protein correct conformation and position (Moore and Jia, 2010).

FlhB in *H. pylori* is protein HP0770. FlhB from *Salmonella typhimurium* has been demonstrated to play a structural role in the formation of the rod and the basal body of flagellum and to have a tendency length regulation of the hook (Minamino *et al.*, 1994; Minamino and Macnab, 1999). The hypothesis in *H. pylori* is confirmed by the study of mutated cells, which shows a flagellate, non-motile and non-pathogenic

behavior (Foynes *et al.*, 1999). From a structural point of view, comparing the cytoplasmic domain of *Salmonella* protein and *H. pylori* model protein, a significant divergence in the C-terminal portion is evident (Fig. 5C).

HP1419 shows high homology in sequence with FliQ from *Salmonella typhimurium*, which is demonstrated to be a membrane protein involved in the export flagellar apparatus (Ohnishi *et al.*, 1997). *H. pylori* *fliQ* mutants present a reduced ability of the bacterium to adhere to the gastric cells and the development of a non-motile species (Foynes *et al.*, 1999). The experimental evidences support the hypothesis that the protein could be involved in the structural building of the export flagellar apparatus and has an influential role in the bacterial adherence to the gastric membrane.

HP0685 is the membrane protein ortholog to *Salmonella typhimurium* FliP protein, better characterized. FliP is another flagellar export component, which in *Salmonella* assembles to form a component containing

five subunits (Fan *et al.*, 1997). FliP seems to be involved in the export stability of flagellin components, since *H. pylori* *fliP* mutants are not able to export flagellar components (Josenhans *et al.*, 2000).

FliO shows a large conservation in export apparatus from several different organisms. In *H. pylori*, its role is played by HP0583. *fliO* mutants show a no-flagellate nature (Tsang and Hoover, 2014). Since *fliO* knockout induces a decrease in FlhA and RpoN-regulon (protein involved in biogenesis of flagellar genes) expression in *H. pylori* cells, causing a reduced level of FlaA and off the hook protein FlgE, it has been postulated that FliO is essential for the transcription of RpoN-regulated flagellar genes. In *H. pylori*, FliO seems to present a large periplasmic N-terminal domain, that is absent in *Salmonella* protein.

Minamino (Minamino, 2014) has reported a detailed description of the flagellar basal body of *Salmonella typhimorium*. According to his description, FlhA through its N-terminal domain, FliR and FliP associates with the MS-ring. Moreover FlhA binds also to FliO, FliP and FliQ, while FliO interact directly with FliP, which stabilizes the protein. Finally, a proximal position of FliR and FlhB proteins has been highlighted (Van Arnam *et al.*, 2004). A general interaction between the whole export apparatus and the MS-ring can be supposed in most of the flagellated organisms, which designs a multi-subunits complex ending with the channel of the export apparatus (Minamino, 2014).

Nothing is known about *H. pylori* ATPase proteins involved in the export process of hook and filament proteins outside the cell membrane, but some consideration can be proposed based on homology studies with *Salmonella* proteins. The proteins arranged in the export flagellar apparatus interact with the complex FliI-FliH_x, involved in the guide of substrates and ensuring a correct assembly of flagellum (Claret *et al.*, 2003). FliI is a peripheral membrane ATPase essential for the correct function of the export apparatus (Vogler *et al.*, 1991). It converts chemical energy, obtained from ATP hydrolysis, in the mechanical motion necessary for the protein export process. It is assembled as a homo-hexamer around the export gate, where it is able to bind to the cytoplasmic domain of FlhA and FlhB proteins (Claret *et al.*, 2003). FliI binds the FlgN chaperone as well, confirming its role in recognition of specific protein involved in the flagellum growth (Thomas *et al.*, 2004). FliH has an antagonist role in FliI regulation. FliH contrasts FliI oligomerization (Lane *et al.*, 2006) and ATPase activity, binding to the N-terminal region and forming the complex FliH₂-FliI (Minamino and Macnab, 2000). On the other hand, FliI is not able to dock correctly the export gate proteins if FliH is not linked, suggesting that FliH plays a crucial role in FliI

binding process (Minamino *et al.*, 2003). The models of *H. pylori* proteins are reported in Fig. 6A and B.

MS Ring Proteins

The MS-ring consists of FliF, which in *H. pylori* is supposed to be protein HP0351. The orthologous protein from *Salmonella* is well characterized: It assembles in a ring with dimension of 25 nm that is comparable with the M basal body. Moreover, an axial projection map of the ring reveal a central channel, involved in the export system of flagellins. The ring is kept together mostly by electrostatic forces (Suzuki *et al.*, 1998). Some attempts of homology modeling of FliF were performed to predict the hypothetical topological arrangement of the protein, but the model obtained was deprived of the C-terminal part.

The Periplasmic Rod and Rings

After a proper construction of all components of the base of the hook basal body, *Helicobacter pylori* Flagellar assembly continues with a formation of the periplasmic elements of the rod (FliE, FlgB, FlgC, FlgF and FlgG), the P-ring (FlgI) and the L-ring (FlgH) (Fig. 1).

Reconstruction of the basal body by the three-dimensional image implicates that the thin proximal portion of the rod is inserted into the cylindrical hollow (a distal portion of FliF) of the MS-ring. Genetic and biochemical data indicates a physical interactions between FliE and FlgB, suggesting that FliE has a role as a structural adapter between the MS-ring and the rod (Minamino *et al.*, 2000).

FlgG is placed in a distal part of the rod (Okino *et al.*, 1989). For FlgB, FlgC and FlgF it is not clear the order along the rod and the regulatory mechanisms that determines the rod length. *In vitro* trials (Saijo-Hamano *et al.*, 2004) did not yield in a successful self-assembly into the rod structure like in case of flagellin (Aizawa *et al.*, 1980) and other flagellar axial proteins (Kato *et al.*, 1982; Vonderviszt *et al.*, 1995; Furukawa *et al.*, 2002). The reason could lie in the lack of properly folded terminal regions responsible for the formation of the inner core of the axial structure.

FlgI and FlgH form around the rod the P- and the L-ring, respectively. Figure 1 shows a model where the P-ring is located into the peptidoglycan layer and the L-ring in the outer membrane of *H. pylori* flagellum (Lertsethtakarn *et al.*, 2011). The smooth and mechanically stable rotation of the rod and L- and P-rings requires two main properties: Rigid backbones and flexible surface-exposed side chains. It has been thought that the symmetry mismatch allows rod proteins to interact with circular symmetry elements of MS-ring (FliF protein) and L- and P-rings. In addition, this allows easy rotation of the rod inside the L- and P-rings.

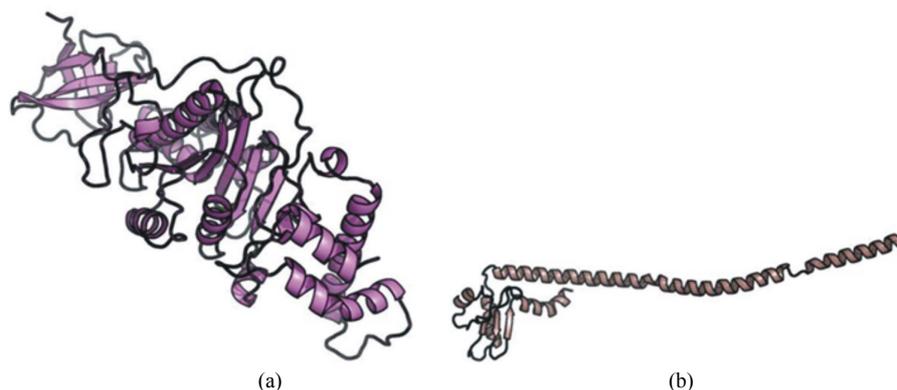


Fig. 6. (A) FliI model protein from *H. pylori* (amino acids from 17 to 434), obtained using the flagellar ATPase FliI from *Salmonella* (PDB ID: 2DPY). (B) FliH model protein (amino acids from 76 to 257), obtain using the yeast vacuolar ATPase heterodimeric ECG (head) as template (PDB ID: 4DL0)

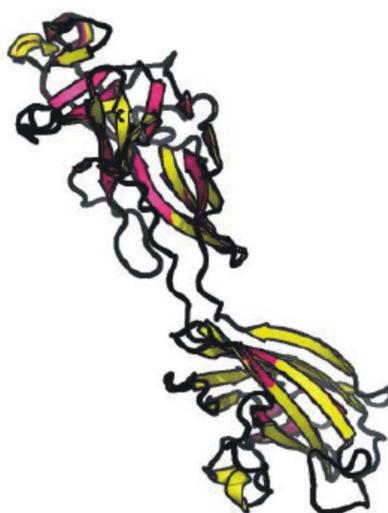


Fig. 7. Superimposition of a monomer of the crystal structure of FlgE from *S. typhimurium* (colored pink) and modeled 3D structure of FlgE from *H. pylori* (colored yellow). Superimposition is performed by PyMOL. (The model of the 3D structure of FlgE from *H. pylori* was built by the software Phyre² with a confidence and identity of 0.99 and 0.32, respectively. The r.m.s.d of the superimposition of 232 Ca atoms of these two structures is 1.344 Å. In both structures the main secondary structure elements are β -sheets which form two domains with an oval shape)

For the moment, the structural support about these proteins from *H. pylori* or other similar organisms (like *Salmonella sp.*, or *E. coli*) is still missing. Moreover, the reason why it is difficult to visualize the rod by electron microscopy is because the L-, P- and MS-ring usually cover the surface of the rod. Characterization of rod proteins in solution from *Salmonella typhimurium* showed that all rod proteins have tendency to aggregate (Saijo-Hamano *et al.*, 2004), a fact that by itself explain the lack of structural information on these proteins.

The Hook

The hook is a highly curved, tubular structure that bridges the basal body and the filament. It is composed

of about 120 copies of a single protein, FlgE. Until now, only the crystal structure of FlgE from *Salmonella enterica subsp. enterica* serovar Typhimurium (PDB ID 1WLG; (Samatey *et al.*, 2004)) has been determined at 1.8Å resolution (Fig. 7). The structure corresponds to residues 71-369 out of a total of 402. The crystallized fragment of FlgE lacks both the N- and C-terminal regions, since the full length protein formed filaments and thus failed to crystallize. The same behavior occurs during the crystallization of the full-length flagellin protein (see paragraph *Extracellular filament*). The group of authors that solved the crystal structure of FlgE built a model of the hook by using electron cryomicroscopy and image analysis, together with the docked crystal structure of FlgE. According to the density

map, the hook is composed of three domains: The outermost domain at the surface (7.5 nm), the middle domain (5-6 nm) and the inner core domain that forms a tube (1nm thick; 3 nm axial lumen). Samatey *et al.* (2004) assume that the terminal chains of FlgE are located in the inner core domain in a similar way to those of flagellin in the filament (Yonekura *et al.*, 2003).

The similarity between the crystal structure of FlgE from *Salmonella typhimurium* and the modeled 3D structure of FlgE from *H. pylori* is shown in Fig. 7. In the domain 2 of the modeled HpFlgE, the portion from Ala 528 to Val 532 includes a small α -helix that is absent in the StFlgE. In addition, a α -helix is present from Gly 652 to Ser 654 in domain 1 of HpFlgE, whereas the corresponding residues of StFlgE are flexible and form a loop. The protein sequence length of FlgE from *H. pylori* is 718 amino acids, almost double in size than the orthologue protein from other bacteria. It is interesting to highlight that *E. coli* and *Salmonella* share a sequence identity of 88% for FlgE, while in the case of *H. pylori* the identity with *E. coli* and *Salmonella* protein sequence is only 36 and 35%, respectively. The main part of the *H. pylori* sequence that is missing in other organisms is from the residues 200 to 443. Even though it is not clear why this protein requires around 250 additional amino acids, this property could be mandatory for the proper hook assembly in the extreme environment to which *H. pylori* is exposed and the crystal structure of FlgE from *H. pylori* would possibly give some clues about it.

The proper hook assembly of *H. pylori* needs other proteins, like FliK, FlgD and FlhB. FliK is supposed to be involved in measurement of a correct hook length (Ryan *et al.*, 2005; Kamal *et al.*, 2007). The predicted role of FlgD is that of controlling the number of FlgE monomers that are used for the hook growing (Kubori *et al.*, 1992). Ohnishi *et al.* (1997) demonstrated that FlgD is displaced by FlgK prior to filament formation, suggesting it is present only as an intermediate during the hook polymerization. FlhB (part of T3SS) is located in the inner membrane and helps the hook formation by interacting with the C-domain of FliK. When this interaction occurs, it induces a signal for the termination of the export of proteins involved in hook assembly and a signal for the export of proteins necessary for filament formation (Minamino *et al.*, 2009).

H. pylori FlgD is composed of 301 amino acid residues. By now, two crystal structures of FlgD have been solved, from *P. aeruginosa* (PDB ID 3OSV; Zhou *et al.*, 2011) and *X. campestris* (PDB ID 3C12; Kuo *et al.*, 2008). Both structures do not include the N-terminal domain, largely flexible. A similar behavior occurs in *H. pylori* FlgD, whose crystals have been

grown by our group. A study of FlgD from *E. coli* (Weber-Sparenberg *et al.*, 2006) indicated that the first 71 N-terminal residues represent a signal for the export into the flagellar channel. The superimposition of FlgD structures is given within Fig. 8. The structure of HpFlgD was modeled by the software Phyre² (identity with the template 34%) limited to part of the protein from residues 125 to 266. HpFlgD superimposes onto the PaFlgD structure with a r.m.s.d. of 3.4Å² for 89 aligned Ca atoms (Fig. 8A) and onto the XcFlgD structure with a calculated r.m.s.d. of 6.7Å² for 82 aligned Ca atoms (Fig. 8B). As in other two homologs, modeled HpFlgD is composed of two domains, mainly consisting of β -sheets and flexible loops. The difference is that HpFlgD comprises two α -helices in domain 1 that are not found in PaFlgD and XcFlgD. In addition, HpFlgD forms a small α -helix at the N terminal part like in XcFlgD, which on the contrary is not found in the Pa homolog.

There are also two proteins in between the hook and the filament, called *hook associated proteins*-FlgK and FlgL. These proteins form a very short hook-filament junction zone, important for adapting these two mechanically different structures. The hook is relatively flexible, while the filament works as a propeller and for this reason has much more rigid structure. According to this fact, those two protein should share structural characteristics similar to the hook and filament proteins. In fact, FlgK crystallized from *Salmonella typhimurium* is rich in both α - and β -structural elements (PDB ID 2D4Y), while FlgL is mainly composed of α -helices (PDB ID 2D4X). Modeled HpFlgK and HpFlgL have the same composition in terms of the secondary structure elements (Fig. 9A and 9C). Moreover, the superimposition of HpFlgK onto the StFlgK gives a calculated r.m.s.d. of 4.9Å² for 342 aligned Ca atoms (Fig. 9B), while the superimposition of HpFlgL onto the StFlgL shows a higher r.m.s.d. of 11.4Å² for 149 aligned Ca atoms (Fig. 9D). The latter is explained by the presence in HpFlgL of two extra β -strands with respect to the *Salmonella* orthologue.

The Extracellular Filament

The complete biosynthesis of the flagellum ends with the assembly of a filament. Its function is that of a helical propeller, whose movement is generated by the flagellar motor. *H. pylori* filament is composed of the major flagellin FlaA, the minor flagellin FlaB, co-expressed in a different amount, and a filament-associated cap protein, FliD.

In some other organisms (like *P. aeruginosa* and *Salmonella*) the filament is built through the assembly of a single FliC flagellin (Maki-Yonekura *et al.*, 2010; Song and Yoon, 2014). Electron cryomicroscopy data

have shown (Yonekura *et al.*, 2003) that there are 11 protofilaments involved in filament assembly and

each filament is formed by the polymerization of a single FliC monomer.

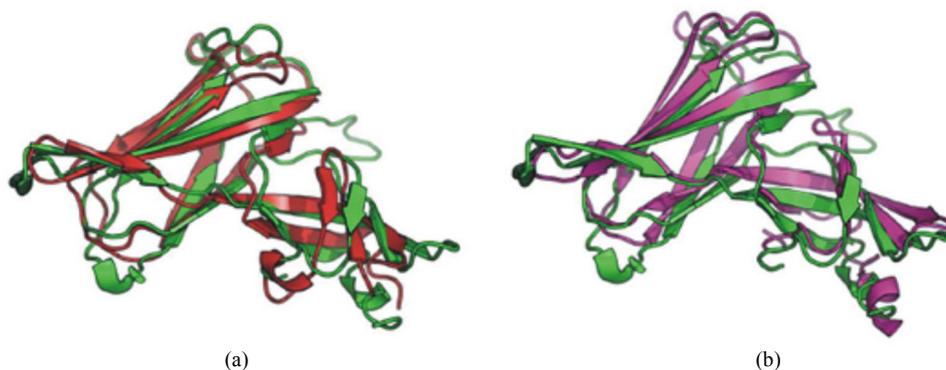


Fig. 8. Superimposition of a modeled 3D structure of FlgD from *H. pylori* (colored green) and (A) a monomer of the crystal structure of FlgD from *P. aeruginosa* (colored red) and (B) a monomer of the crystal structure of FlgD from *X. campestris* (colored purple)

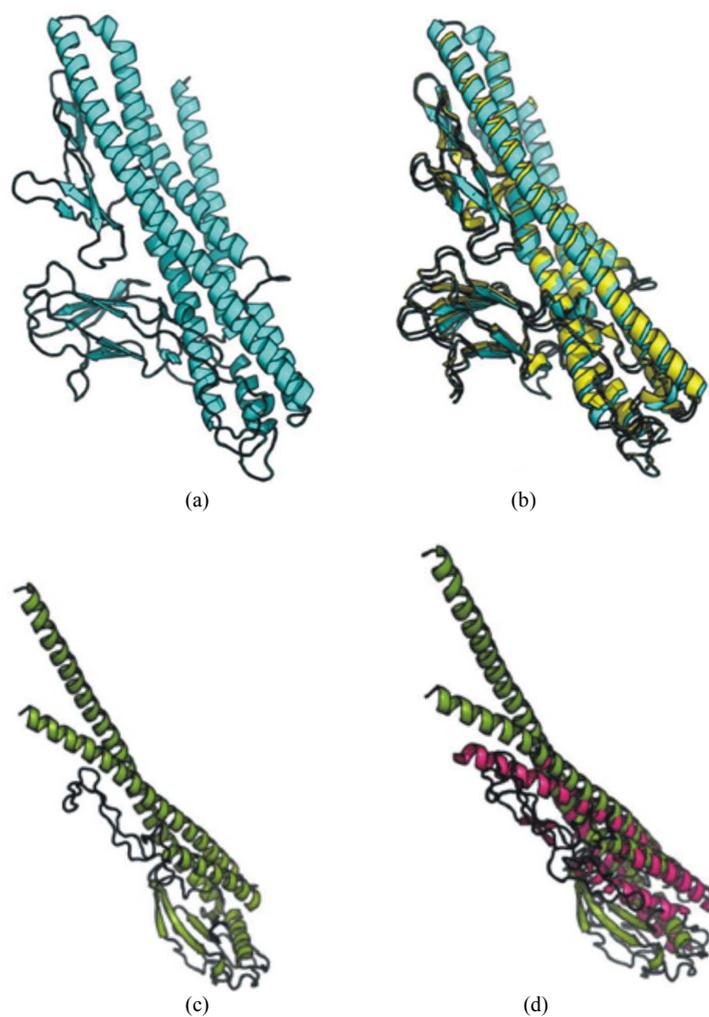


Fig. 9. (A) Model of the 3D structure of HpFlgK. (B) Superimposition of a modeled structure of FlgK from *H. pylori* (blue) and a crystal structure of *S. typhimurium* (yellow). (C) Model of the 3D structure of HpFlgL (D) Superimposition of a modeled structure of FlgL from *H. pylori* (green) and a crystal structure of *S. typhimurium* (magenta)

H. pylori flagellins are composed of 510 and 514 amino acids of FlaA and FlaB, respectively. The protein sequence identity between these two flagellins in *H. pylori* is 61%. Both flagellins are essential for *H. pylori* motility (Josenhans *et al.*, 1995). It is thought that the amount of expression of the *H. pylori* FlaA/FlaB ratio is keen on environmental factors (as viscosity or pH), providing a mechanism that allows bacteria to create the best filament properties for motility in a certain condition. The proposed models of HpFlaA and HpFlaB are given in the Fig. 10. Sequence identities of the models with the template are 26 and 29%, respectively. From Figure 11 it is evident that both Hp-flagellins share similar characteristics. Superposition of HpFlaA onto HpFlaB show a r.m.s.d. of 2.1Å for 235 aligned C α atoms. The comparison of the known crystal structure of FliC protein from *P. aeruginosa* and *H. pylori* flagellins (Fig. 11B and 11C) show slight differences in their structural arrangement.

Finally, it is interesting to stress that flagellins that form the tubular filament have a structure similar to that of FlgE proteins in the hook, despite the fact that the hook and flagellin proteins have clearly different amino acid sequences.

The crystal structures of some chaperones of proteins of *H. pylori* filament are known: FliS (PDB ID 3IQC), HP1076 (PDB ID 3K1H) and their complex (Fig. 12; PDB ID 3K1I). The role of HP1076 is not clear, but it is known that this protein appears only in *Campylobacter*-related species. Structural studies of FliS-HP1076 complex suggest a role of HP1076 in flagellar biosynthesis. From Fig. 12 it is possible to see that the contacts between these two proteins are formed by

helical stacking, with a measured association constant of $1.5 \times 10^7 \text{ M}^{-1}$ (Lam *et al.*, 2010).

The role of FliS would be in controlling the premature polymerization of filament proteins. The 100-residues of the C-terminal part are essential for FliS interaction with FlaB. The large (61%) sequence identity between FlaA and FlaB in *H. pylori* suggests that the same interaction could occur between the FliS-FlaA and FliS-FlaB complex. It is interesting that the activity of FliS protein in *H. pylori* is not limited to the flagellin proteins. Moreover, it has been hypothesized that FliS can interact with other flagellar proteins, like hook-filament junction protein FlgK and filament cap protein FliD (Lam *et al.*, 2010). The importance of FliD goes outside its chaperon role, since it is a possible marker for serologic diagnosis of *H. pylori* infection (Khalifeh Gholi *et al.*, 2013).

Flagellar proteins glycosylation

A significant number of *H. pylori*'s proteins are glycosylated (Champasa *et al.*, 2014). Among them, flagellins FlaA and FlaB are heavily glycosylated with the nine-carbon sugar pseudaminic acid (Schirm *et al.*, 2003), a post-translational modification absolutely essential for the formation of functional flagella. In fact, deletion of any of the enzymes involved in pseudaminic biosynthetic pathways results in non-motile bacteria (Schirm *et al.*, 2003). In this respect, inhibition of enzymes of the latter metabolic pathway could be a key to indirectly target *H. pylori* motility and consequently stop colonization.

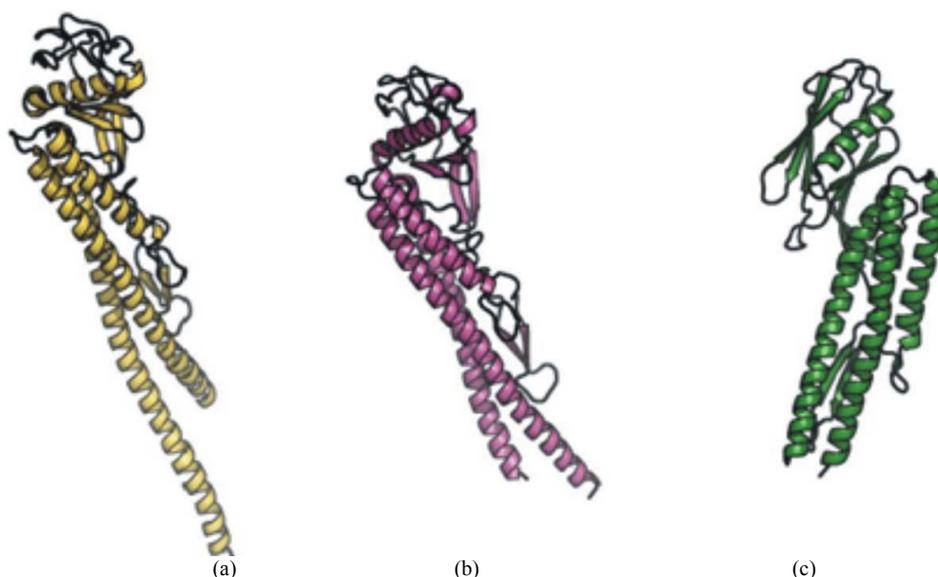


Fig. 10. (A) Modeled 3D structure of FlaA (yellow orange color) from *H. pylori*. (B) Modeled 3D structure of FlaB (purple color) from *H. pylori* (C) Crystal structure of FliC (green color) from *P. aeruginosa* (PDB ID; 4NX9). The models of the 3D structures were built by Phyre² software

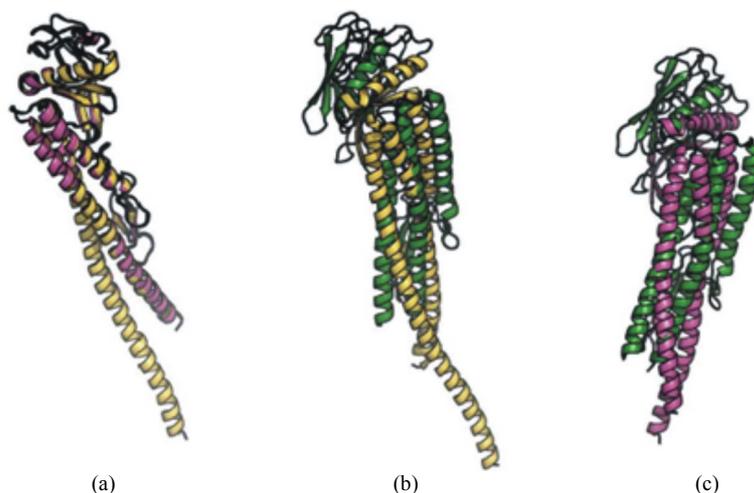


Fig. 11. Superimposition of A) modeled *H. pylori* flagellins- FlaA (colored yellow-orange) and FlaB (colored purple). B) A monomer of the crystal structure of FliC from *P. aeruginosa* (colored green) and modeled 3D structure of FlaA from *H. pylori* (colored yellow orange) (r.m.s.d = 8.364 Å for 217 Ca atoms). C) A monomer of the crystal structure of FliC from *P. aeruginosa* (colored green) and modeled 3D structure of FlaB from *H. pylori* (colored purple) (r.m.s.d. = 7.852 Å for 240 Ca atoms)

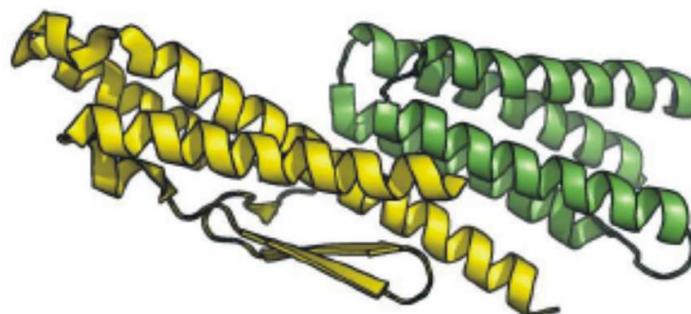


Fig. 12. Structure of the FliS-HP1076 complex. FliS and HP1076 are colored green and yellow, respectively

Conclusion

Although the structural information about the bacteria flagella are known from species like *Salmonella typhimurium* and *Escherichia coli*, *H. pylori* flagellum is still in the relatively early stages of structural investigation. For the moment, the crystal structures of the C-ring proteins *HpFliG*, *HpFliM* and their complex, crystal structure of the cytosolic domain of T3SS protein *HpFlhA*, crystal structure of a stator component of the flagellar motor *HpMotB*, crystal structure of the *HpFliS* flagellin chaperon and its complex with HP1076 have been solved. We still lack the structure of the *H. pylori* proteins that build the periplasmic rod and rings, the hook and the extracellular filament. Nevertheless, only nineteen structural models could be constructed according to the existing crystal structures of orthologs from other species. The interest in structural investigations of the *H. pylori* flagellar proteins could, together with the understanding of flagellar gene transcription and chemotactic response, explain how this bacteria achieve a functional rotary nano-machine

required for swimming to the more favorable environment during the infection processes.

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Author’s Contributions

All three authors contributed to write the paper.

Ethics

There are no ethical issues.

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