



Meta-analytical Research Article

CONSOLIDATION OF THE MURBURN MODEL OF FLAGELLA-AIDED MOTILITY IN BACTERIA

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Howard Berg had proposed a 'rotary motor' explanatory paradigm for the flagella-assisted motility of bacteria (1), claiming that proton motive force (pmf) provided the powering in such propulsion in low Reynold's number regimes (2). Paul Boyer subsequently suggested a pmf-aided rotary functionalism for explaining Complex V's role in photo-/oxidative- phosphorylations in chloroplasts/mitochondria. Michael Behe had questioned the 'evolvability' of such self-assembled multi-molecular protein complexes with rotary functions, calling these proposals 'irreducibly complex'. Over the last 25 years, while championing a new stochastic cellular principle of life (complementing the deterministic principle of central dogma) called murburn concept, we discredited the idea of "pmf-assisted rotary functions" in cellular routines in the two systems above. Herein, we confirm some of our earlier predictions and provide further evidence/arguments supporting the murburn model of flagella-aided motility in bacteria: (i) the component proteins of C-ring and Mot proteins show large variations in monomeric constitution and amino acid sequences suggesting that they could not have evolved for rotary activity (3), (ii) the purported rotary fulcrum role of a single amino acid residue (MotB's D/E32) is untenable as it is not conserved in a wide variety of bacteria surveyed, (iii) the C-ring proteins and cytoplasm-side of MotA have a relatively higher concentration of charged residues that enable DRS recruitment/ utilization, and (iv) the basal module is a Type-3 secretory system ejecting fluid wherein MotAB complex enable efficient proton delivery for water formation and expulsion via the rod. **Biomed Rev 2024; 35: 39-70**

Keywords: murburn concept, bacterial flagella, proton motive force, biological rotary motor

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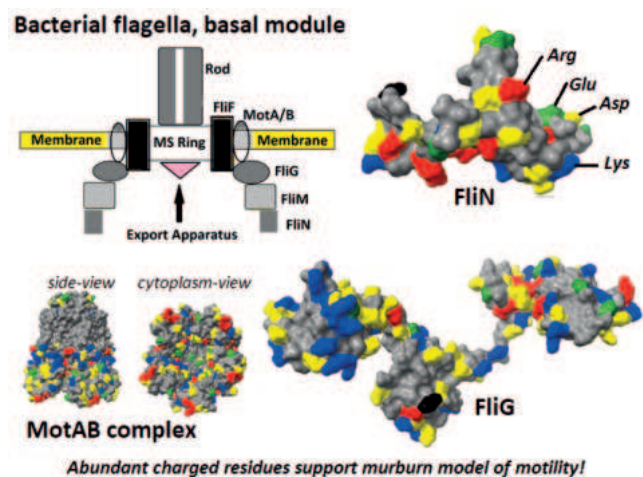
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TEXT HIGHLIGHTS

- * Proton motive force driven rotary model for bacterial flagellar movement is critiqued
- * The structure of basal module is a typical Type 3 secretory system
- * No conservation of key residues for rotation; but charged amino acids are plenty
- * Murburn model for flagellar motility depends on diffusible reactive species
- * Charged amino acid residues could catalyse conversion of diffusible reactive species
- * New perspectives for explaining experimental observations are provided

GRAPHIC HIGHLIGHTS



1. INTRODUCTION

Bacterial flagellar motility influences several aspects of host-pathogen interactions as well as bacterial virulence such as- survival, immune invasion, colonization and disease progression (4). It is a central process involved in pathology, antibiotic resistance and biofilm formation. Targeting bacterial flagellar motion could potentially combat bacterial pathogenesis and further complement existing treatment regimens against bacterial infections. Bacterial flagella are protein-based appendages that have been considered to be powered by a rotary motor. During chemotaxis, bacterial mobility allows for bacteria to move towards nutrients and enhance cell survival during hostile environments. Pathogenic bacteria such as *H. pylori* adhere to and penetrate gastric mucosa to establish ulcers. Motility is a key virulence factor for *V. cholerae*, which utilizes its flagella to navigate the intestinal mucosa and attach to intestinal epithelial cells (5).

Flagellin is recognized by toll-like receptor 5 (TLR5) located on immune cells to trigger an immune response and hence, some bacteria downregulate flagella and evade immune surveillance. *S. enterica* invades intestinal cells using flagella and later repress the expression of flagellar proteins to avoid evoking an immune response (6). *P. aeruginosa* utilizes flagella-based motility to initiate the development of biofilms during chronic lung infections and thus, protects bacteria from antibiotics as well as host defenses (7). Flagellar motility is also linked to antibiotic resistance as it aids in bacterial persistence, establishment of recurring infections and survival during antibiotic therapy. Mutant uropathogenic *E. coli* which are non-motile are less virulent when compared to the wild type motile strain (8). Targeting bacterial motility and flagellar assembly is an avenue for attenuating bacterial virulence without killing the bacteria. Molecules which target flagellar motility or chemotaxis decrease infection severity in preclinical studies (9).

The notion that ion-fluxes and proton motive force (or *pmf*) thereof could enable reproducible physiological rotary motion in biomolecular assemblies was perhaps first mooted in the bacterial flagellar system by Howard Berg (1, 2). This development could have been the stimulus for Paul Boyer's proposal of rotary functionalism in Complex V or F_0F_1 ATPase (10, 11).

With respect to the functioning of bacterial flagella, the powering mechanism that was pioneered and currently accepted in the community is deeply rooted in the "ion-pumping and trans-membrane potential (TMP) tapping" bioenergetics (12). It was proposed that electron transport proteins pump protons out, thereby creating a chemo-electrical potential difference (called the proton motive force or *pmf*), which is used to power a motor like function in the basal unit of bacteria. That is, quite akin to how an electric force is used to drive a motor or fluid flow is used to drive a turbine, the basal unit of bacterial flagellar system harnesses the *pmf* to rotate the internal components along with the long and slender bacterial filament outside, to provide torque and thrust, and enable a screwing type propulsion in a low Reynolds number milieu. This perception was seemingly justified by the microscopic observation of counter clockwise movements of the bacterial flagella, some "modified physiology" experimental observations and the arrangement of several proteins in radial symmetry in the flagellar basal module (2).

In his book (13), Michael Behe (1996) had questioned the classical textbook models of both bacterial flagellar and

Complex V mediated functions by calling them “irreducibly complex”. He had argued that it is highly unlikely that biological evolution could bring about the synthesis and functional aggregation of multiple proteins that have no other individual role attributed to them in routine physiology. He posited that such ordainment of functioning could only be indicative of creative intelligence at work. In simplified analogous terms, just like the making/functioning of a machine like electrical dynamo/motor or automobile cannot occur automatically/spontaneously in nature (but an intelligent person can make and peruse them!), it would require an intelligent agency to bring about the realization/assembly of components working with a complex mechanism as that of “bacterial flagellar motor” or “rotary ATP synthase”. Surely, accepting this proposal would challenge the concept of biological evolution! Unfortunately, the mainstream scientific community, rather than seeing the undeniable merit in Behe’s arguments, chose to disregard him and side with the “Apollonian Authorities” (<https://www.youtube.com/watch?v=NaVoGfSSSV8>). (We do not advocate “creative intelligence” theories, but merely point out that Behe had merit in the criticisms.) Unfortunately, anyone (e.g. Gilbert Ling, Ludwig Edelman, etc.) that questioned the ion-flux based popular narrative was ostracized and effectively silenced (14).

Over the past two and a half decades, under the umbrella of murzymes and murburn concept, our group has systematically attempted to incorporate diffusible reactive (oxygen) species or DR(O)S mediated catalyses, e-transfers and post-translational modifications into the array of order-ensuring routine cellular metabolic and physiological processes (15-17). In this purview, for the immediate maintenance of cellular functions, effective charge separation (or ECS) is the primary step in murzyme (any protein/biomolecule that generates or sustains or modulates or utilizes DRS) mediated biological processes, enabling the cell to work as a simple chemical engine (or SCE). Thus, murburn concept was proposed as a pivotal stochastic principle working at short time scales, augmenting the deterministic principle of central dogma that is applicable in the relatively longer time-terms (14). In doing so, we have provided novel thermodynamically, kinetically, mechanistically and evolutionarily (probabilistically) grounded explanations for fundamental PCHEMS (powering, coherence, homeostasis, electro-chemical and electro-mechanical sensing/responses) features of the cell operative in respiration, photosynthesis, thermogenesis, xenobiotic metabolism, cyanide toxicity, Na-K ion disparity, unusual dose responses, etc. [Manoj group’s work

till date, cited in (14)]. In the murburn purview, if cell theory were to be fundamental life principle, the “holistic intelligence of SCE” could only be attained at the cellular level and the scope for molecular machines functioning with intelligent design would be discounted. (As scientists, we merely make the framework for cause-consequence correlations in nature. Although we assume that biological evolution is justified by ample evidence, we remain open to considering arguments/findings that could counter such ideas.) Therefore, we had questioned any physiologically significant “rotary motor like functions” in Complex V (18) and bacterial flagella (19), and proposed tangible/viable murburn models instead. Via these works - particularly, the last paper (19):

(i) With over a dozen step-wise points, we demonstrated the untenable nature of ‘pmf/ion-pump based powering’ and the flawed rationale for interpretations in the ‘uncoupling experiments’ (the turning point in flagellar motility research) was brought out, thereby negating the founding premise of rotary functionalism. We also argued that if the filament rotated through the various trans-membrane rings, protons should easily leak through them!

(3) We highlighted the multitudes of models prevailing in the field (a clear indication of confusion and disagreements!) and exposed the chameleonic change of stators/motors attributions in the basal module [post the mutation experiments conducted by Zhou *et al.* (20)].

(iii) It was emphasized that the research community had not cross-checked the infeasibility of Left Hand Screw (LHS) structure of flagellar filament giving a pushing thrust upon counter-clockwise movement. We also pointed out that the vast number of flagellar filaments were rather flaccid and not rigid screw type structures. Also, we pointed out that the ion-flux based rotary movement is an unreal mirage like idea, quite unlike any known man-made systems or spontaneously evolved structures in nature!

(iv) Very importantly, we questioned the feasibility of purported rotations of $\sim 10^4$ per second in the basal module, stating the kinetic infeasibility. Also, we highlighted that the cross-sectional dimension of flagella was ~ 20 nm and the wavelength of light used for visualization/resolution of these filamentous structures was $>10 \times$ this size! We also brought to light a video published by Ali *et al.*, (2016), which clearly showed the “perceived rotation” of self-assembled (polymerized-flagellin) filaments tethered at one end (and lacking the basal module!) in shear stream flow (<https://doi.org/10.1103/APS.DFD.2016.GFM.V0097>). Since a tethered

filament cannot rotate (by rule!), the fact that “physiological rotation of bacterial filament was an optical illusion” was established.

Recently, the murburn model for bacterial motility was briefly consolidated post comparing/contrasting with the rotary model in an American Institute of Physics portal (21). In the current article, we present an elaborate logical and systematic follow through of the afore-mentioned works, to the test the validity of some of our predictions made regarding the structural and interactive dynamics of C-ring and MotA/B proteins. Our article also presents the interactive analysis of CheY protein (e.g. ‘conserved’ LSQAEIDALL sequence), discusses TIRF (total internal reflection fluorescence) experiments, various parameters’ effects on motility, etc., all of which provide hitherto unavailable insights and cutting-edge information in the field. In toto, our write-up argues for a paradigm-shift perception to better understand flagella-aided bacterial motility, and pays tributes to simple evolvable mechanism for electro-mechanical cellular workings.

2. METHODOLOGY

Protein sequences of the selected bacterial flagellar proteins - FliN, FliM, FliG (of the C-ring) as well as MotA and MotB (stator) were obtained from UniProt database in FASTA format. Since the question of evolutionary conservation of some key residues had to be addressed, very diverse bacterial species were chosen from different genera (and in some instances, 2-3 species from the same genera were randomly selected) to afford detailed comparison of a very diverse set of the selected flagellar proteins. Global alignment was carried out with EMBOSS-NEEDLE. The numbers of charged and hydrophilic amino acid residues – R/K/D/E/S/T were counted using ProtParam (ExPasy) tool. The total residue count of the full length proteins was also noted. This way, evolutionary conservation of the length, amino acid composition as well as distribution was compared by aligning the sequences of C-ring and stator proteins using ClustalOmega (ClustalW) on JalView. The aligned sequences were coloured based on their % residue conservation. Protein structural images were generated with UCSF ChimeraX.

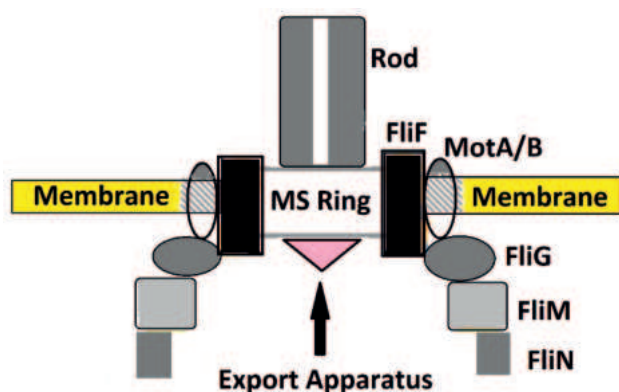
3. RESULTS AND DISCUSSION

3.1. Overview of bacterial flagellar proteins

The bacterial flagellar assembly has dozens of various types of monomers and hundreds of such individual components in a mammoth structure that weighs $\sim 2 \times 10^7$ Daltons (exclusive

of the long filament that protrude out). The polymerized filament of $\sim 3 \times 10^4$ flagellin monomers constitute a structure of molecular weight of $\sim 1.5 \times 10^9$ Daltons (22). The bacterial flagellum, on the whole, is made up of 30 different types of protein monomers, and excluding the chemotaxis-associated proteins, at least 11 different protein parts are known to be present in the purported motor unit of bacterial flagellar system (23). The key components of the bacterial (common to both Gram +ve and –ve) flagellar complex are (from inside to out): C-ring (cytoplasmic), MS ring and Mot proteins (membrane-embedded), rod (space from membrane to the exterior wall/membrane) and hook + filament (extracellular); along with the export apparatus lodged within the circular MS ring region (extending into the cytoplasm). The C-ring consists of FliM, FliN and FliG. MotA pentamer and MotB dimer together form a complex which is supposed to be responsible for proton conduit/channeling. FliF is present in the MS ring (or constitutes it), along with other proteins like FliA, FliB, FliO, FliP, FliQ and FliR. In Gram –ve bacteria, the P-ring contains FlgI, and L-ring has FlgH and distal rod has FlgG. The proximal rod is made up of FliE, FlgB, FlgC and FlgF. The hook consists of FlgI and FlgH, while the ATPase complex (the cytosolic components of the flagellar export system) comprises of FliH, FliJ and FliI. FliI is the subunit with ATPase activity and FliH is a negative regulator/modulator of ATPase activity of FliI (24). While the H^+ -utilizing bacterial flagellar system contains MotA and MotB, the Na^+ -system contains PomA and PomB proteins (25). Some bacteria such as marine *Vibrio* sp. rely on the much faster Na^+ -powered system. MotAB/PomAB systems are tethered to the peptidoglycan layer to form the stator. For a visual view of the structures and their simulation, the reader can peruse a highly popular youtube video given at the link: <https://www.youtube.com/watch?v=B7PMf7bBczQ>. A schematic of the overall juxtapositions of the basal part of the bacterial flagellar assembly (including the cytoplasmic C-ring and export apparatus piercing the membrane, membrane-embedded Mot proteins and MS ring, the rod filament, etc.) is shown in Figure 1. At the very outset, it becomes very counter-intuitive to imagine a cyclic movement of a few protons (in millisecond time ranges) could generate the adequate torque at the base of such a massive structure (with a purported Brownian ratcheting mechanism) to rotate the spiral/flaccid filamentous flagella, thereby leading the filaments into any type of synchronous/orchestrated undulations (which in turn push the bacteria onward), all without messing up the export assembly at the base! Regardless, we analyse the known

3.3. C-ring proteins (FliN, FliM and FliG) and their interactions



The C-ring of *E. coli* has three monomers: 102 FliN and 34 each of FliM and FliG (26, 29), with FliN being the most distal component from the membrane. It has been shown that FliG interacts with FliM, and FliM interacts with FliN. These three proteins of the C-ring are known to affect the rotation as well as switching of direction of the flagellar motor (30). Together with FliM and FliG, FliN is involved in the formation of a switch complex which binds to phosphorylated CheY, a chemotaxis protein, whose binding ultimately causes a conformational change in FliM, which is transduced to FliG (31). FliG supposedly serves as a switch protein (for clockwise and counter clockwise movements) and articulates with FliF (which is inserted into the membrane). FliG and FliF are supposed to interact with each other mostly through conserved hydrophobic interactions and the latter is known to act as a polymerization site for FliG (27). The purported flagellar motor switch in *Escherichia coli* and *Salmonella typhimurium* regulates the direction of flagellar rotation and hence controls swimming behaviour. The switch (FliG, FliM and FliN) is supposedly a complex apparatus that responds to signals transduced by the chemotaxis sensory signalling system during chemotactic behaviour (32). First, we analyse individual proteins, starting from FliN. Table 1 shows the analyses of FliN from over 20 different bacterial species which were aligned to check evolutionary conservation of the FliN protein and some salient structural aspects shown in Figure 2, with positively and negatively charged residues highlighted. The position of these residues is not conserved, as can be seen in the sequence analysis given in Item 1, Supplementary Information File, showing low identity/similarity scores. Several well-conserved hydrophobic residues form a nonpolar patch on the surface of the molecule that articulates with the FliM protein. A mutation in the hydrophobic patch affected flagellar assembly and switching, showing that this surface feature is important for FliN function (33). The chain length varied from 79 (*H. pylori*) to 179 (*A. fabrum*).

The second protein (from the distal side) is FliM, which serves to connect FliN and FliG. As expected, the sides connecting the monomers have hydrophobic patches (Figure 3), which stick the heteromers together. Table 2 shows moderate amounts of hydrophilic/charged residues and Item 2 of Supplementary Information shows the sequence analysis for conservation. It can be seen that the length of the chain varied from 315 to 374 residues, which is not expected if the protein served as crucial mechano-transducing components.

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Table 1. Analysis of amino acids (aa) in FliN.

No.	Organism	R	K	D	E	S	T	% (polar)	%(charged)
1	<i>Escherichia coli</i> (137)	8	5	13	8	10	9	38.7	24.8
2	<i>Pseudomonas aeruginosa</i> (157)	6	5	12	16	9	9	36.3	24.8
3	<i>Rhizobium meliloti</i> (198)	6	9	19	11	6	11	31.3	22.7
4	<i>Buchnera aphidicola</i> subsp. <i>Baizongia</i> (134)	2	17	12	7	8	6	38.8	28.4
5	<i>Buchnera aphidicola</i> subsp. <i>Acyrtosiphon</i> (133)	2	17	10	8	14	8	44.4	27.8
6	<i>Buchnera aphidicola</i> subsp. <i>Schizaphis</i> (133)	2	25	7	10	11	2	42.9	33.1
7	<i>Salmonella typhimurium</i> (137)	8	5	14	7	7	10	37.2	24.8
8	<i>Aquifex aeolicus</i> (112)	4	16	5	21	3	3	46.4	41.1
9	<i>Caulobacter vibrioides</i> (110)	5	3	10	9	5	4	32.7	24.5
10	<i>Agrobacterium fabrum</i> (179)	8	7	22	12	12	11	40.2	27.4
11	<i>Derxia gummosa</i> (138)	7	2	10	11	6	10	33.3	21.7
12	<i>Aeromonas hydrophila</i> (130)	7	6	11	14	6	6	38.5	29.2
13	<i>Pseudomonas</i> sp (155)	6	4	13	16	9	7	35.5	25.2
14	<i>Wigglesworthia glossinidia brevipalpis</i> (135)	9	16	14	16	6	5	48.9	40.7
15	<i>Sphingomonas</i> sp (118)	5	3	8	11	7	10	37.3	22.9
16	<i>Pseudomonas putida</i> (160)	6	4	10	19	12	6	35.6	24.4
17	<i>Candidatus Midichloria</i> (94)	2	10	8	8	4	4	38.3	29.8
18	<i>Cereibacter sphaeroides</i> (152)	20	2	7	11	7	12	38.8	26.3
19	<i>Escherichia fergusonii</i> (137)	8	4	13	8	9	10	38.0	24.1
20	<i>Proteus mirabilis</i> (136)	7	6	14	9	14	8	42.6	26.5
21	<i>Bacillus cereus</i> (119)	1	15	9	11	9	2	39.5	30.3
22	<i>Helicobacter pylori</i> (79)	5	11	5	17	7	4	62.0	48.1

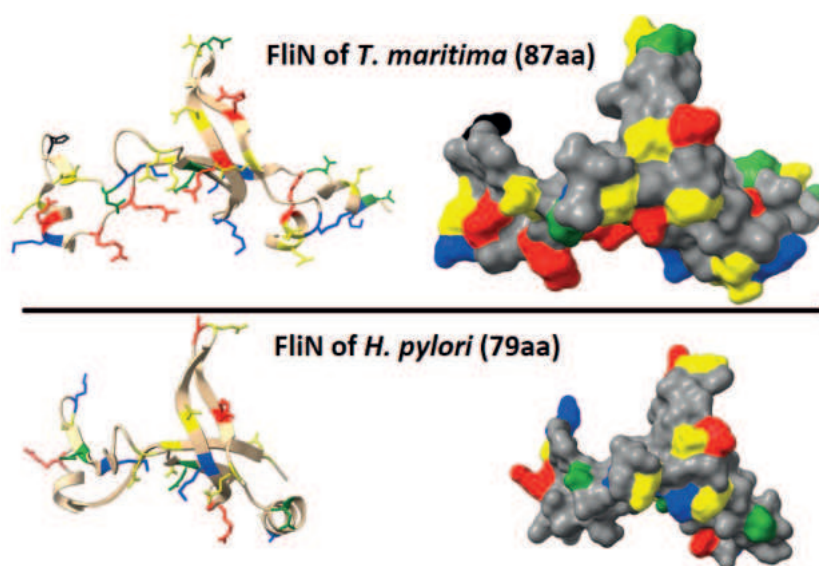
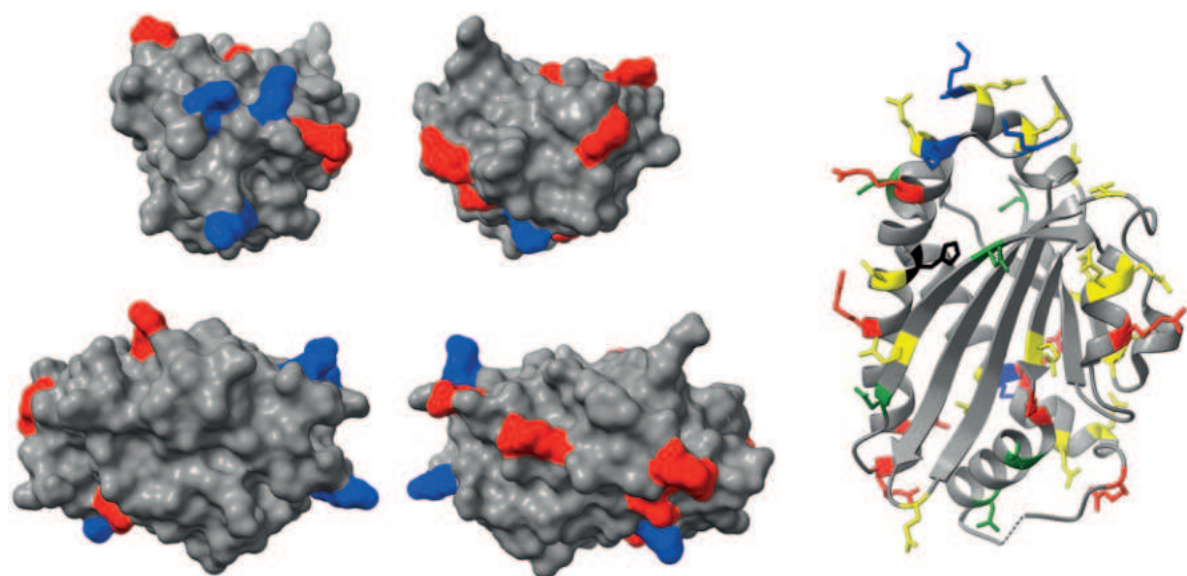


Figure 2. Fli N (PDB ID: 1YAB) *T. maritima* with R (coloured red), K (blue), H (black), D (green) and E (yellow) side chains. – has around 87 residues. R- 6, K- 6, D- 5, E- 10 and H-1. *H. pylori* FliN (5XRW)– has only 79 residues – There are around R- 4, K- 4, D- 3 and E- 7 AND no H residue. FliN is a tightly intertwined dimer composed mostly of beta sheet. More information on the structure of FliN can be obtained from the paper by Brown et al 2005 (33) who gave the crystal structure of the FliN dimer (PDB ID: 1YAB). In contrast, *E. coli* FliN contains about 137 residues (not shown).



FliM of *T. maritima* (334 aa)

Figure 3. FliM (PDB ID: 2HP7) from *T. maritima* is 334 AA long; The entire protein does not appear to have any cavities – it is almost like a barrel with alpha + beta secondary structures. FliM with only R (shown as red) and K (blue) residues depicted in the space-filling renditions. The ribbon depiction shows FliM with R (shown as red), K (blue), D (green), E (yellow) and H (black) residues. There are 8 R, 4 K, 6 D and 16 E residues.

Table 2. Analysis of amino acids (aa) in FliM.

No.	Organism	R	K	D	E	S	T	% (polar)	%(charged)
1	<i>Escherichia coli</i> (334)	25	9	17	26	19	15	33.2	23.1
2	<i>Rhizobium meliloti</i> (315)	25	7	21	22	23	19	37.1	23.8
3	<i>Buchnera aphidicola</i> subsp. <i>Baizongia</i> (334)	25	10	19	25	17	16	33.5	23.7
4	<i>Salmonella typhimurium</i> (334)	25	10	24	25	17	16	35.0	25.1
5	<i>Caulobacter vibrioides</i> (374)	31	8	28	26	22	17	35.3	24.9
6	<i>Agrobacterium fabrum</i> (321)	15	18	22	17	23	16	34.6	22.4
7	<i>Derxia gummosa</i> (342)	30	8	17	29	23	14	35.4	24.6
8	<i>Aeromonas hydrophila</i> (356)	27	15	27	38	15	17	39.0	30.1
9	<i>Sphingomonas</i> sp (340)	24	8	23	25	16	23	35.0	23.5
10	<i>Proteus mirabilis</i> (343)	23	13	24	25	17	17	34.7	24.8
11	<i>Helicobacter pylori</i> (354)	19	24	20	31	36	10	39.5	26.6

The third and last protein of C-ring is FliG, which is supposed to present two hooking facets to connect differently with the membrane-embedded protein components of the basal assembly (Figure 4). Analysis of the amino acids of interest is shown in Table 3. If this protein served as some kind of a switch, then we would expect high amount of conservation, as the complementary parts cannot vary. Instead, we found variable chain length (320-375), constituent amino acid residues and different electrostatic topography. Moreover, the conserved N-terminal LSQAEIDALL sequence which is known to act as the docking site for phosphorylated CheY, CheY-P (32), is not found in the FliM of a few bacterial species. A multiple sequence alignment of the purported CheY binding region is shown in Item 3, Supplementary Information.

Some species like *Buchnera aphidicola* subsp. Baizongia, *Buchnera aphidicola* subsp. schizaphis and *Rhizobium meliloti* do not possess this conserved sequence. Perhaps, analyses of all available FliM sequences could yield many more such outliers. Besides, CheY-P is known to bind with its phosphate bound side facing almost opposite to the region which interacts with FliM. This is not exactly a transfer of a phosphate group from CheY-P to FliM/the switch complex, but mild interaction of the CheY-P causes a binding-effected conformational switch which powerfully transduces to even FliG, and thus, changes the spin of the rotor, all when the rotor is spinning at several 100s of times per second! So, if the rotor changes from CCW to CW rotation owing to CheY-P binding, there are two pertinent questions: a) what prompts re-switching of rotation from CW back to CCW (Che-Y dissociation due to dephosphorylation by CheZ? – and if so, how does the interaction between Che-Y and FliM get affected?) and if so many CheY-P bind to the n-number of FliM present in the C-ring, is there simultaneous binding of several CheY-P, or sequential/step-wise binding of CheY-P to the FliM monomers? As questioned by Eisenbach and Caplan (35), the flagellar switch, we reckon, is still a mystery and so is the idea that the entire complex of proteins which are several thousand Daltons rotate at thousands of hertz only due to “non-existent” proton movements through MotAB.

Compared to small soluble protein controls (as their physiological roles are that of high affinity receptor-binding base hormones, and would not be anticipated to have DROS modulation activities) like human insulin (51 residues) or somatotropin (217 residues) that have ~21% charged amino acids, these basal module of flagella have significantly more K+R and D+E to recruit DRS/protons to bring about water formation. The general trend is that the charged (22-48%) and

polar (31-62%) residues are high in number. The species of *Magnetococcus marinus* is supposed to be highly motile (36), and it was found to have 38% charged residues in the FliN protein (Item 3, Supplementary Information). The numbers and positions of amino acids analyzed herein (Cys, His, Tyr, Gln, Asn are not shown) are not conserved, and there is no pattern of conservation either. If all these flagellar proteins (which form the C-ring) have to have evolved from one common ancestor, it seems that the evolution is extremely divergent, with all these proteins not having any common sequence – and yet, structurally, somehow have morphological similarities (as seen from crystal structures).

Figure 5 shows the affinity bound proteins of FliM-FliG and FliG-FliF respectively. In the latter (since two different domains are supposed to bind to FliF and Mot complex), there is little flexibility or scope for any clockwise or counter-clockwise rotary switching mechanism, as the topological complementarity is extensive. This will not allow FliG (which is bound to FliM/FliN in the cytoplasm and FliF on the membrane) complex to make any effective switch between its articulating facets/arms, and therefore, the FliG-FliF articulation must remain unchanging and inflexible. A schematic extension of this analysis is presented in Figure 6. As per Morimoto *et al.* (3), a crucial salt bridge pair between E98 of MotA and R281 of FliG, and R90 of MotA with D289 of FliG (and there is another D288 nearby) stabilizes the interaction between MotA and FliG. The aligned and conserved residues of FliG and MotA (wrt. *S. enterica* as the reference) were studied herein. Some anomalies with the numbers of the residues involved in electrostatic interaction between FliG and MotA are due to the variations in the positions of these residues in different bacterial species. The distance between the purported electrostatic pair of Arg284 and Glu292 (in *Aquifex aeolicus* flagellar proteins) is ~15.1 Å and the distance between their counterparts in MotA is 13.8 Å; somewhat agreeable. If we assume that the MotA binding arm of FliG is closest to the nearer periphery of MotA (as solicited for the best case scenario for CCW articulation) and that the FliF binding arm is taut and connected to the membrane-bound FliF (a condition absolutely necessary for the whole structure to rotate!), then the angle subtended by the FliG protein's arm (~47 Å in length) on the MotA plane would be ~90°. This would entail that for CW rotation, the FliG-MotA arm should somehow have ~78 Å length (a 66% increase from its inherent length to cover up for the distance of ~62 Å between the near and far sides of the purported electrostatic interaction sites on MotA)! Laxing or tightening any articulation would lose traction/tenor, causing

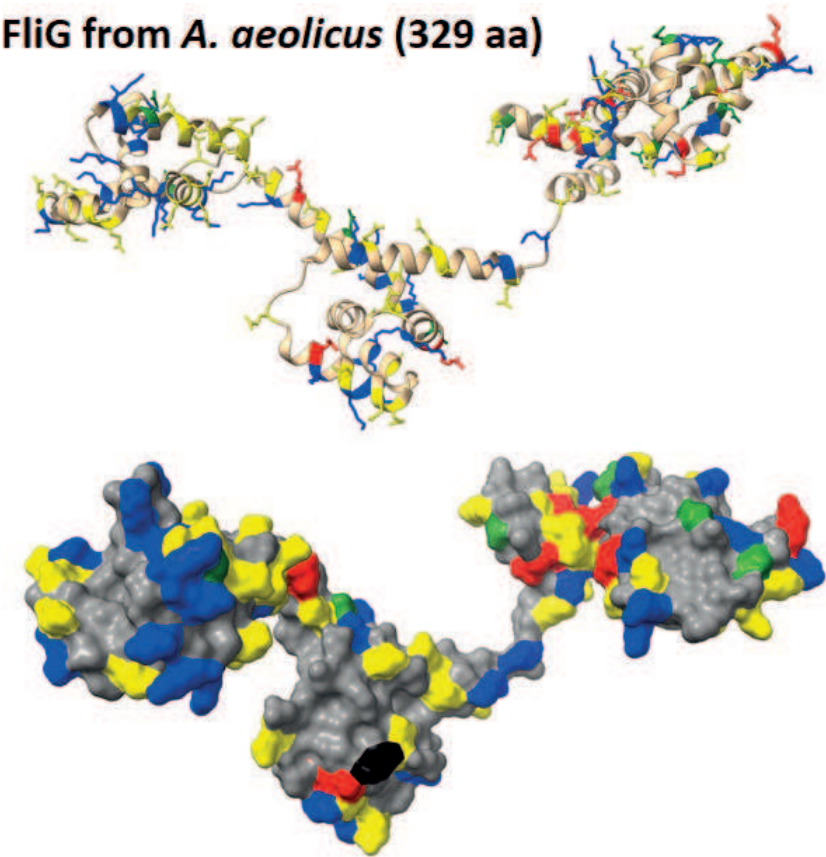


Figure 4. FliG from 3HJL (organism: *Aquifex aeolicus*). There are 11 R, 40 K, 14 D, 50 E and 1 H residue is found. Color code: R (coloured red), K (blue), D (green), E (yellow) and H (black) residues.

Table 3. Analysis of amino acids (aa) in FliG

No.	Organism	R	K	D	E	S	T	% (polar)	%(charged)
1	<i>Escherichia coli</i> (331)	23	12	22	33	16	17	37.2	27.2
2	<i>Rhizobium meliloti</i> (345)	21	16	19	32	25	13	36.5	25.5
3	<i>Buchnera aphidicola</i> subsp. <i>Baizongia</i> (320)	9	40	15	22	24	7	36.6	26.9
4	<i>Salmonella typhimurium</i> (331)	24	12	21	34	20	17	38.7	27.5
5	<i>Caulobacter vibrioides</i> (340)	22	19	24	32	20	13	38.2	28.5
6	<i>Agrobacterium fabrum</i> (375)	19	18	18	33	23	15	33.6	23.5
7	<i>Derris gummosa</i> (334)	17	25	26	34	16	15	39.8	30.5
8	<i>Aeromonas hydrophila</i> (356)	25	14	28	31	24	9	36.8	27.5
9	<i>Sphingomonas</i> sp (341)	30	7	20	30	24	9	35.2	25.5
10	<i>Proteus mirabilis</i> (332)	22	11	23	32	19	16	37.0	26.5
11	<i>Helicobacter pylori</i> (343)	11	32	18	34	20	22	39.9	27.7
12	<i>Thermotoga maritima</i> (335)	23	31	17	43	14	12	41.8	34.0

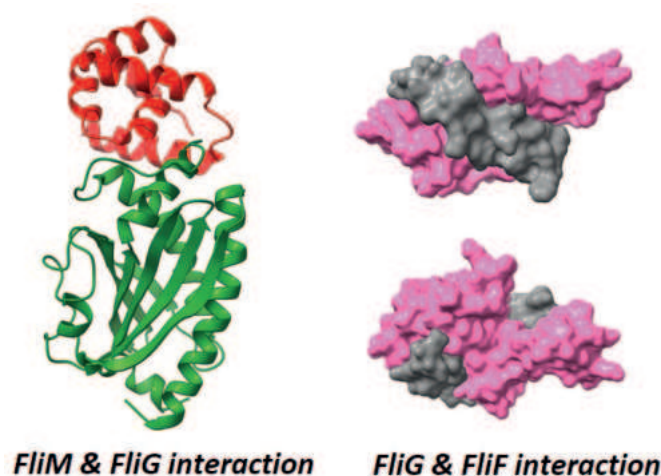


Figure 5. Left panel shows 4FQ0, the crystal structure for protein-protein association between FliM (green) and FliG (shown in red) from *H. pylori*. FliF-FliG complex (PDB ID: 5WUJ) from the same organism is shown in the right panel. The FliG protein in this crystal structure is 105-AA long (pink) and the part of FliF which interacts tightly with FliG is 37 AA long, coloured gray. Originally, FliG is ~330 amino acids long and FliF is ~530 amino acids long.

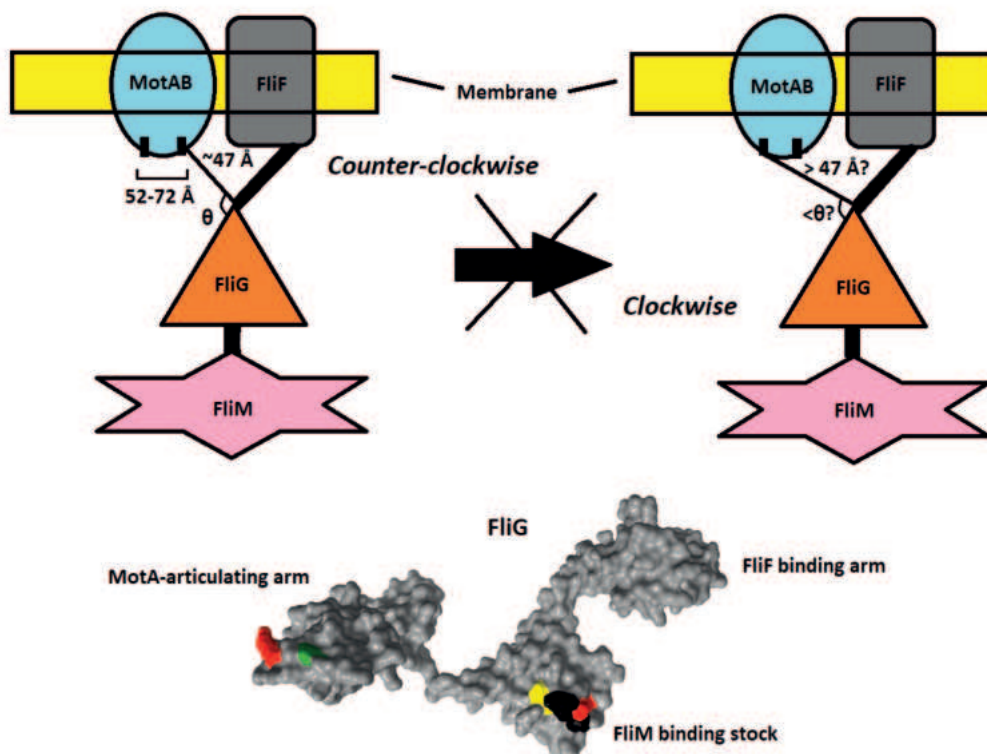


Figure 6. In the top panel, a schematic is presented for the purported interaction of the various proteins involved. The figure is not drawn to scale and the proteins are given distinct shapes and colors for demarcation purposes. In the lower panel, the red and green residues are R284 and D292 purportedly interacting with MotA via electrostatic interactions (which lie at the C-terminal domain) of the FliG protein of *Aquifex aeolicus*. The middle knot/domain has a conserved EHPQ-R motif which is responsible for interaction with FliM. E125, H126, P127 and Q128 are coloured black and R162 is coloured red.

either a destabilizing pull at other structures or lead to loss of leverage at the other hand. Further, the electrostatic interactions of E98 of MotA with R281 of FliG and R90 of MotA with D289 of FliG is not by any measure a smooth or round surface, but is very rugged (and not like a ball and socket complementary arrangement- and would the electrostatic interaction between a pair of residues effect rotations?). This cannot serve like a gear (as perceived by some) nor can it serve as an electrostatic pull-push mechanism based on the complementarity in shape or even charge. Furthermore, there is no rhyme or reason why a couple of electrostatic bridges between the MotA and FliG proteins would make the C-ring rotate! Therefore, the proposals in recent literature appear to be unrealistic extrapolations that were speculated to justify the Apollonian mandate of rotary movements in the basal module (37, 38). A mere look at the FliG protein with its highly flexible hinges (for the two arms that connect to FliF and MotA; and a base for articulating with FliM) suggests that this protein merely holds the other three proteins relatively intact with respect to one another! Such an articulation can only aid catalytic or networking function, not mechanical switching function. It is also unforeseeable as to how CheY binding or modification (phosphorylation) of the C-ring components could enable conformation and articulation switches in this setup.

When analyzing the conservation of FliG's key residues of R281 and D289 by alignment using ClustalOmega, it was found that R281 is mostly conserved in all species, except for *Buchnera sp.*, and when analyzing the -3 to +3 residues of the aligned sequences, it was evident that neither R nor K was seen. The -3, -2, -1, 0 (where R281 is supposed to be), +1, +2 and +3 residues are M, S, L, E, N, T and N, respectively. Similarly, *Buchnera sp.* bucked the trend of having a conserved D289, as it possessed I, K, N, Y, C, S and K as the -3, -2, -1, 0 (where D289 is supposed to be), +1, +2 and +3 residues, respectively. Since these residues are not conserved in all the species analyzed, and there exist anomalies such as *Buchnera sp.*, we may not deem that these two key residues are absolutely mandatory for articulation with MotA (even though these residues are conserved in several bacterial species). As full length FliG structure is available for only one species, we could not go onto analysis of different FliG-MotA pairs from varied bacterial species.

3.4. MotA/B proteins

Mutation studies of the acidic residues of various proteins in the basal module had revealed that most were not critically needed for flagellar function (20). The residue of D32 in

MotB was yet supposed to be the sole fulcrum relaying proton that enabled the MotB monomers to rotate within the MotA enclosure (37, 38) (Santiveri *et al* 2020; Wadhwa & Berg 2022). Figures 7 and 8 show the various views of MotA/B complex structure, the latter highlighting various charged amino acids on the proteins. Analysis of the Mot proteins structure does not show any evidence for rotability based on a proton conduit. There is no snug-fitting of the two MotB monomers and their pentameric MotA counterparts that would be expected. The MotB protein varied from 230 amino acids long (*Agrobacterium fabrum*) to 657 (*Pseudoalteromonas sp. Xi13*), indicating little conservation based on size (which would be absolutely essential for mechanical components!). Table 4 presents some results of alignment of various bacterial MotB proteins with *E. coli* D32 as the reference residue (Item 4, SI gives the actual sequence details). A comparison of overall sequence alignment of diverse bacterial MotB proteins is given in Item 5, SI. From any type of sequence analysis, it was noted that any D or E residue was not conserved across all the flagella-aided motile bacteria. Specifically, *Agrobacterium* does not have any amino acid residues that can be compared with reference to *E. coli* D32 (from -4 to +4 range). If we check ± 1 and ± 2 residues in organisms which do not possess a conserved D32 residue (with respect to *E. coli* D32), we can see that *Rhizobium* does not have any D or E residue in that vicinity. *Rhizobium*, *Sulfospirillum*, *Methylobacter*, *Mucilaginibacter*, *Dyella*, *Methylophilus*, *Pedobacter*, *Harlarcobacter*, *Rhodanobacter* and *Pseudoalteromonas* do not have any D or E residues from -4 to +4 range with reference to D32 of *E. coli*. Also, multiple De/E residues can be noted in some organisms' MotB. How could this enable rotation? Hence, a clear conclusion can be made that D or E is not present in all organisms at the exact location in MotB and therefore, this residue cannot be aiding any purported rotary function. Besides, one can see that analysis of the surface of MotA shows the presence of diverse charged residues (R, K, D and E) along its surface, and that there is significant variation in both the numbers and position of these charged residues (Figure 8). The analysis holds good even for *Vibrio* species, which purportedly uses Na⁺ ions (Figure 8, bottom panel). Figure 9 presents a detailed view of the location of the two amino acids on Mot A of *A. aeolicus* and a comparison with some other bacteria. Clearly, there is little logic for any kind of complementarity or pairing. Therefore, we do not think that specific electrostatic interaction of MotA-FliG pair and its relevance for rotary movement thereof is of major consequence in the bacterial flagellar function.

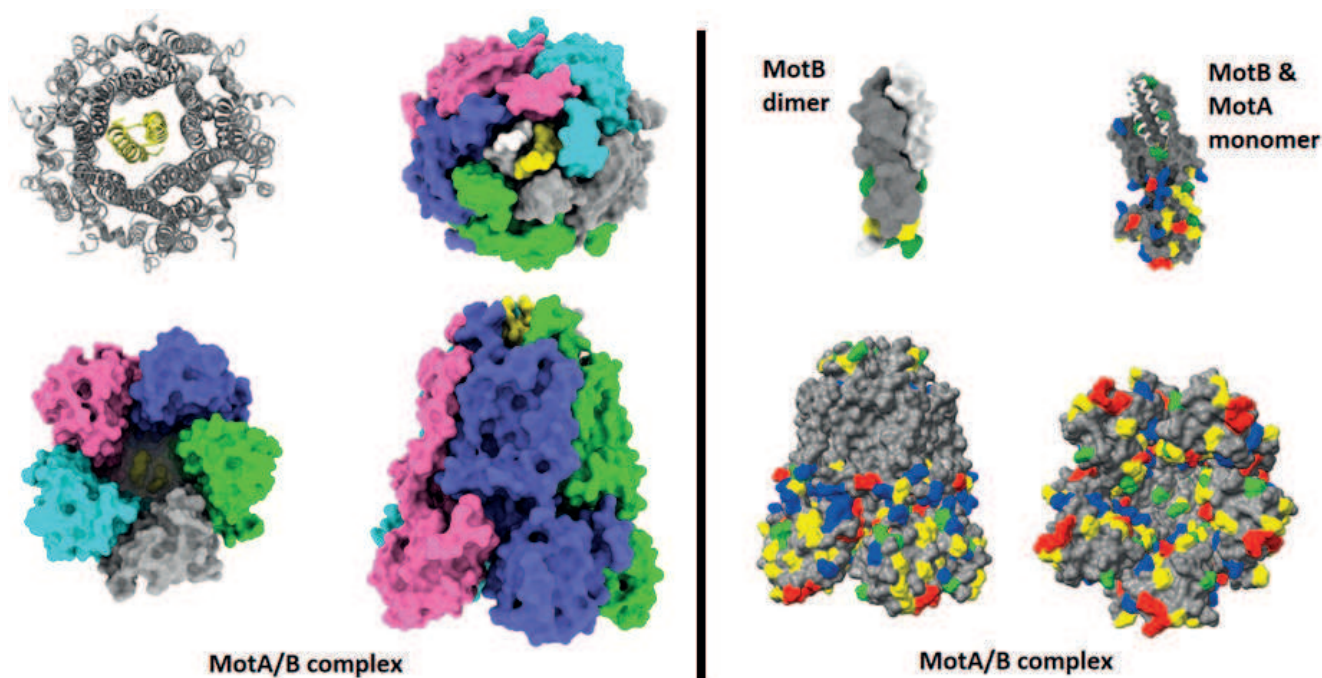


Figure 7. Mot protein complex of *C. jejuni* (6YKM) obtained using single particle EM. **Left panel:** Top left: Ribbon view of the chains of MotA (gray) as a pentamer with MotB dimer at the centre (two yellow chains interacting with each other). In the second image (top), the space filling extra-membrane view of the MotAB complex is shown with MotB dimers depicted in white and yellow to differentiate them, and MotA chains are given different colours to show the 5 distinct MotA chains. Bottom left: The intra-membrane view of MotAB complex (showing the cavity inside) is shown, with the tip of the MotB dimer having the D22 residue at the end located inside the MotA pentamer. Bottom right: The side view (length) of the protein complex. **Right panel:** Top left: MotB complex, with D coloured green and E coloured yellow. Two D and one E residue are present along the entire protein of 291 AA length. Top right: Only MotA (258 AA length) with chains of MotB (white) and the 'key' so-called conserved D residues coloured green, alongside MotA monomer with its internal surface having highly concentrated R and K residue distribution. Bottom left: The outer side of MotA trans-membrane region is completely devoid of polar residues, and all charged residues are concentrated towards the broad side, with positively charged (predominantly lysine) forming a ring at the end of membrane and the IMS tapering end presenting some D/E residues. Bottom right: The cytoplasm view of MotAB complex shows positively and negatively charged residues arranged in a radial fashion along the five monomers of MotA.

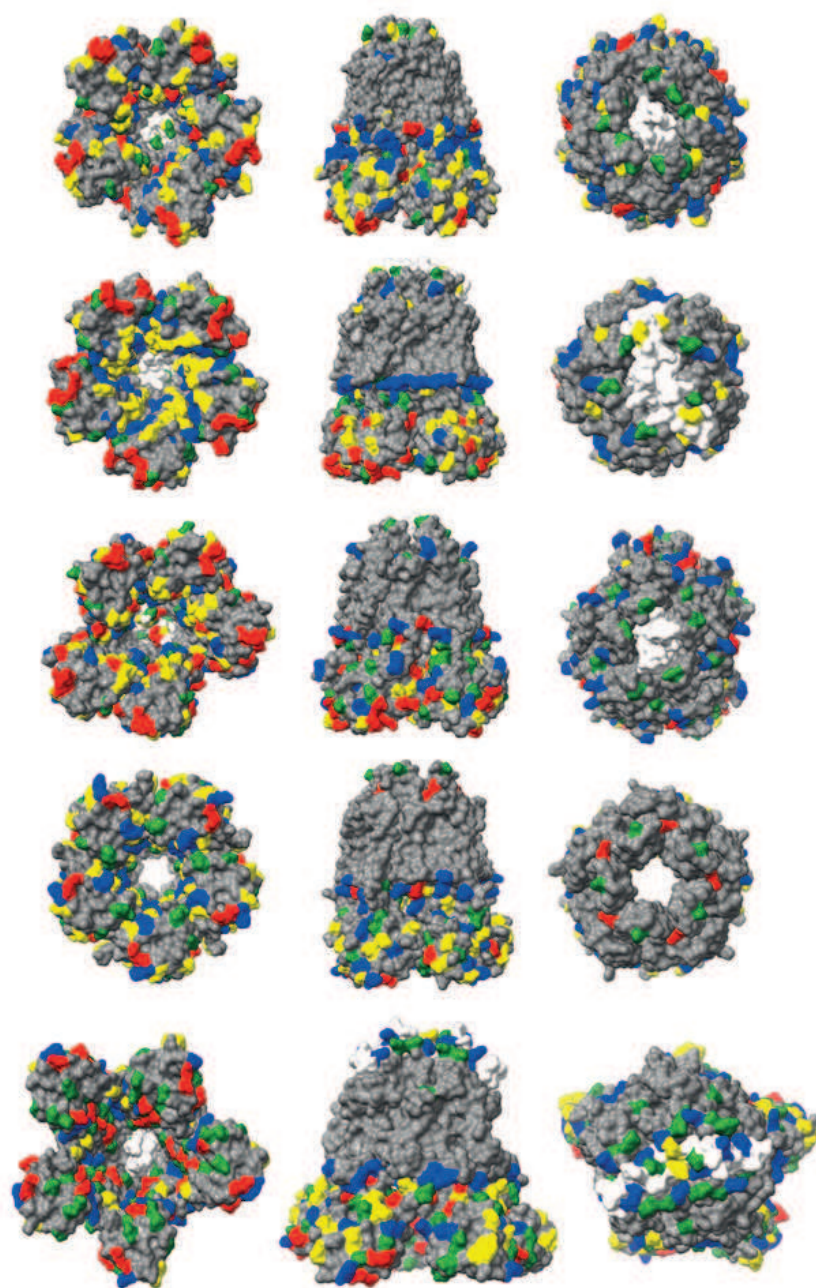


Figure 8. *Top row (left to right) – MotAB from Bacillus Subtilis (6YSL); Second row – MotAB from Campylobacter jejuni (6YKM); Third row – MotAB from Clostridium sporogenes (6YSF) and Fourth row – MotA alone from Aquifex aeolicus (8GQY). Fifth row – In the Na⁺-dependent system of MotAB (flagellar Na⁺-driven stator unit) from Vibrio alginolyticus (8BRD), such common features can be seen – a high distribution of R/K/D/E residues at the cytoplasmic side, complete lack of those residues in the membrane-associated region of the protein complexes, and lower distribution of these residues towards the other side of the complex. Colour codes: R- red; K- blue, D - green and E- yellow. All left panels show the bottom side of the complex (oriented towards the bacterial cytosol), middle panels show the side view of the entire complex and all right panels show the view of the complex from the top side. In the top three rows, MotB is coloured white. In all these complexes, the membrane domains lack any R/K/D/E residues, while the cytoplasmic side (all left panels) contains large numbers of these residues. These are very few in number in the top side (towards periplasmic side), as can be seen in all right panels. In some crystal structures, MotB appears to come out of the MotA pentameric complex and is exposed to the outside (as seen in the right side panel of row 2).*

Table 4. Analysis of MotB alignment with respect to *E. coli* D32

No.	Organism	-4	-3	-2	-1	#32	+1	+2	+3	+4
1	<i>Escherichia coli</i>	×	×	×	×	D	×	×	×	×
2	<i>Agrobacterium fabrum</i>	-	-	-	-	-	-	-	-	×
3	<i>Rhizobium meliloti</i>	×	×	×	×	×	×	×	×	×
4	<i>Veillonella denticariosi</i>	×	D	×	×	×	×	×	×	E
5	<i>Sulfurospirillum</i> sp.	×	×	×	×	×	×	×	×	×
6	<i>Aurantiacibacter gangjinensis</i>	×	E	D	×	E	×	D	×	×
7	<i>Methylomonas denitrificans</i>	×	×	×	×	×	×	×	×	×
8	<i>Mucilaginibacter</i> sp. 44-25	×	×	×	×	×	×	×	×	×
9	<i>Pacificimonas flava</i>	×	×	D	×	E	×	E	×	E
10	<i>Flavobacterium</i> sp.	D	×	×	×	×	×	×	×	×
11	<i>Dyella jiangningensis</i>	×	×	×	×	×	×	×	×	×
12	<i>Methylophilus</i> sp.	×	×	×	×	×	×	×	×	×
13	<i>Kangiella sediminilitoris</i>	×	E	×	×	×	×	D	×	×
14	<i>Blastomonas fulva</i>	×	E	D	×	×	×	D	×	×
15	<i>Qipengyuania flava</i>	×	E	D	×	×	×	D	×	×
16	<i>Pedobacter paludis</i>	×	×	×	×	×	×	×	×	×
17	<i>Alteromonadaceae bacterium</i>	D	D	×	×	×	×	×	×	×
18	<i>Halarcobacter ebronensis</i>	×	×	×	×	×	×	×	×	×
19	<i>Rhodanobacter thiooxydans</i>	×	×	×	×	×	×	×	×	×
20	<i>Pseudoalteromonas</i> sp.	×	×	×	×	×	×	×	×	×

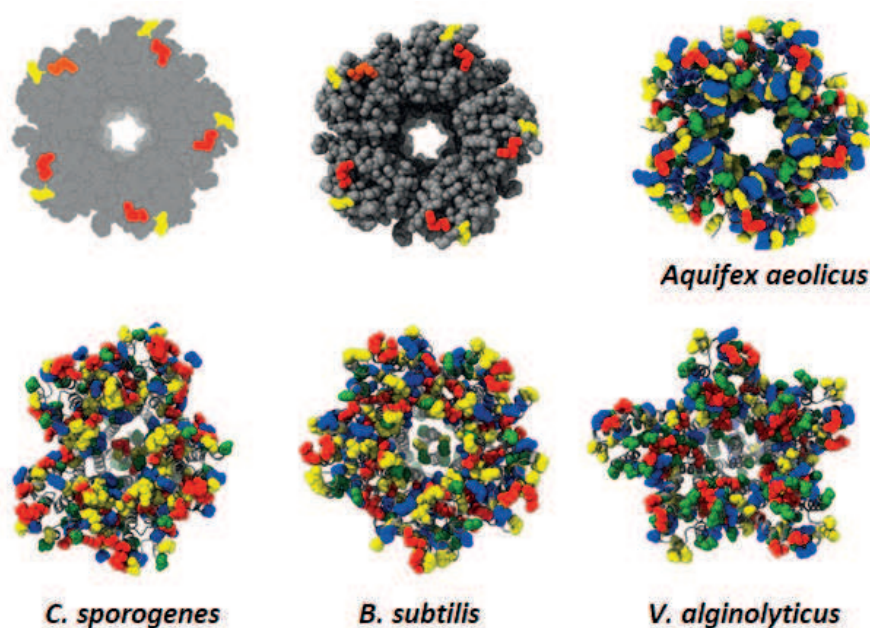


Figure 9. In the top left panel, the crucial (and conserved) R88 and E90 residues of *Aquifex aeolicus* MotA (PDB ID: 8QGY) which are involved in salt bridge formation (and thus, electrostatic attraction) between MotA and FliG are shown. R is coloured red and E is coloured yellow. Bottom left – MotA of *Clostridium sporogenes* in the MotAB complex (PDB ID: 6YSF), bottom middle – MotA of *Bacillus subtilis* (PDB ID: 6YSL) and bottom right – MotA of *Na⁺-driven Vibrio alginolyticus* (PDB ID: 8BRD). In all these panels, there is a wide distribution of charged residues R-red, K (blue), D (green) and E (yellow) residues (whose atoms are coloured as spheres).

3.5. Overviews of various proteins involved

Table 5 shows the primary chain lengths of the various flagellar proteins of bacteria. There is little consistency or trend/ratio among the various proteins. This indicates that evolutionarily, the proteins are not required to have good correlation with each other. This is not expected of mechanoproteins that are supposed to articulate and directly depend on each other.

While hydronium-water-amino acid hydrogen bond energy is in the range of 8-29 kJ/mol, the electrostatic interactions between positively and negative charged amino acids falls in the range of 42-84 kJ/mol. It is unforeseeable as to how hydrogen bonds making and breaking within the cell could lead to the higher electrostatic bonds being formed and broken while the module undergoes rotation. It must be remembered that since the classical rotary motor explanation is based in proton cycling and not based in chemical reactions per se, these kind of thought lines (besides the arguments we presented against the pmf logic earlier!) render the ion-flux based logic redundant. Most important aspect that the current rotary model does not address is: how could the hydrophobic interactions between the FliF (MS ring) and the lipid membrane (and the other several ring proteins bound to the rod) be broken by a few protons coming in through a highly “open” like structure of MotAB complex? This question can be asked in another perspective: If the rod is free to rotate due to the busing actions of the various rings through which the rod pierces the membrane and wall, why can't the protons gain access and exit freely from the cytoplasm? This question/criterion is of fundamental importance, which was overlooked by the researchers in this field, and coupled with the illusion of rotation (and some other coincidental experimental observations), the acceptance on the rotary model proposal was an unfortunate outcome.

3.6. Fresh perspectives

Hitherto, trans-membrane potential (TMP) dynamics resulting from membrane-protein based ion-pumping/channeling is considered as the central logic for electrochemical and electromechanical works done by cellular systems. This statement stands justified by the strings of Nobel Prizes awarded to: Alan Hodgkin – Andrew Huxley (neuron impulse relay, 1963), Peter Mitchell (powering of bioenergetic phosphorylations, 1978), Erwin Neher – Bert Sakmann (current flowing via isolated ion-channel, 1991), Paul Boyer (proton-flux mediated protein conformation change, 1997), Jens Skou (Na-K ion pumping, 1997), Roderick Mackinnon (rationale for high throughput trans-membrane

Table 5. Comparison of amino acid chain length among articulating components

No.	Organism	FliN	FliM	FliG	FliF	MotB
1	<i>Escherichia coli</i>	137	334	331	552	308
2	<i>Rhizobium meliloti</i>	198	315	345	557	292
3	<i>Buchnera aphidicola</i> subsp. <i>Baizongia</i>	134	334	320	555	x
4	<i>Salmonella typhimurium</i>	137	334	331	560	309
5	<i>Caulobacter vibrioides</i>	110	374	340	536	x
6	<i>Agrobacterium fabrum</i>	179	321	375	566	230
7	<i>Derxia gummosa</i>	130	342	335	x	x
8	<i>Aeromonas hydrophila</i>	138	356	346	x	299
9	<i>Sphingomonas sp</i>	118	340	341	507	358
10	<i>Proteus mirabilis</i>	136	343	332	x	x
11	<i>Helicobacter pylori</i>	79	354	343	567	257

x- not available

cation selectivity, 2003), etc. Therefore, classical cell biology perceptions solicit the operational relevance of “intelligent gambits” and “sophisticated molecular motors” at the onset of cellular life, which we successfully question in our recent pursuits (14).

In the first part of our work on this topic (19), we had provided reasons to doubt the “bacterial flagella motor” model and mooted an alternate explanation in murburn concept. Herein, we demonstrated that practically, most structural details elaborated in support of the rotary motor type functionalism of bacterial flagella are not conserved and thus, do not hold water. There is little scope for the simultaneous rotation of the three components of [MotB (2 units), then MotA (5 units)] x 11 to 18 of MotAB complex and consequently 32 to 36 FliG in the C-ring (with >100 FliN!). Also, there is no conceivable way (MotB-FliG) electrostatic interactions can enable rotation/switching directions because: (i) the energetics don't add up, ii) the MotA complex is a pentamer (symmetry mandate for gaining opposite directionality would dictate a hexamer), (iii) spatial analysis shows dimensional constraint for FliG. That is, as FliG is structurally articulated with FliM of the C-ring and FliF on the membrane, it CANNOT: (i) effectively interact with both the near and far peripheries of MotA (a proposal recently mooted to explain clockwise and counter-clockwise rotations!) and ii) rotate while bound to FliF (which aggregates to form the circular MS ring) embedded in the lipid with high-energy lipid-protein hydrophobic interactions! Therefore, the rotation of C-ring + FliF can be discounted. Lowe *et al*'s statement that “the flagellar bundles rotate more rapidly than formerly

supposed, at rates that increase linearly with temperature” (39) suits well in a murburn reinterpreted explanation. While pumping out protons into the periplasm would be envisaged to enable a quicker equilibration with external ambience (leading to loss of pmf!), the production of DRS and their interactions thereof would be higher at elevated temperatures.

However, we do not negate direct observations such as: (i) beads attached to filament tracing circular or elliptical movements, and ii) the cell body appears to rotate or revolve with low frequencies (< 10 Hz) upon tethering the flagella on to the slide. In the first case, we argue that though the bacterial body or flagellar filament might show some revolution or spiraling movements, this observation cannot be taken as evidence for any physiological rotary function within the basal module. This observation can also be accounted for by water rushing out at the hook and such a forceful flow introducing the outcomes observed thereof. In the second case, we propose/infer that the low frequency random bi-directional revolutions/rotations are non-physiological. [See Howard Berg demonstrating the counter-logic here: <https://www.youtube.com/watch?v=ioA1yuIA-t8>. It can be seen that holding the toy mouse by the arm or by the body did not change the revolutions!] This is because in such cases, the tethering and forced expulsion of hot water could damage the assembly whereby the flagellar rod could be freed from the leak-proofing ring membrane system, setting the rod/filament loose at the various rings that secure the rod's exit through membranes/wall. In some of the mutant strains that show only unidirectional rotation upon such tethering, the internal structure would have a constraint that would permit an asymmetric operation. (It must be borne in mind that mutations in a protein need not affect only the perceived function, but it can alter several other aspects of the system wherein the protein might be involved! Therefore, mutation studies do not affirm roles with certainty but can only rule out obligatory roles for the protein!) Such findings and arguments cannot be taken as a support for the claim that the flagellar components in the basal module rotated at frequencies approaching ten thousand hertz in physiological conditions. (If the C-ring rotated at >1500 Hz, it dictates that MotAB complex must rotate much faster, based on gearing principles!) If the flagellum indeed rotated at the base, then the body proper cannot rotate in the opposite direction, as it would imply a loose and useless articulation between the basal module and membrane, one that cannot provide torque or thrust. But it has been noted physiologically that bacterial body proper could exhibit spiral movement,

which could be better explained by the hydrodynamics resulting out of a backward of water expulsion along a spiral filament. The murburn model is better capable of explaining the movements in diverse filament morphologies whereas the rotary model would be envisaged to afford thrust (of very low amounts) only with a rigid-spiral flagellum. Further, herein, we ratified some of the requisites our earlier proposals by showing that both C-ring and MotAB complex are studded with high amounts of charged residues that enable efficient water formation at the interface, which can be forcefully expelled via the ATP-dependent Type 3 secretory system components.

We fail to understand how the much smaller protons (which are ~ 0.00002 Å) or the larger Na ions (which are ~ 2 Å) dynamically produce traction within the purported rotary logic of snug-fit MotB dimer within the MotA pentamer (Figure 7 & 8), in very similar structured MotA/B complexes of *E. coli* versus *Vibrio* species. Also, how could the cylindrical filament shaft rotate piercing through multiple membrane/wall surfaces retaining cellular integrity, when all that is needed is a 0.00002 Å space for protons to seep through? We denounce the flagellar motor perception, which did not consider such fundamental criteria and was built on far-fetched extrapolations. At this juncture, we are also not in a position to comment on the methodology or interpretation rationales of sophisticated techniques like that of TIRF (Total Internal Reflection Fluorescence) wherein MotB is tagged to GFP (a 28 KDa protein), and photobleaching loss and recovery are analysed (40). We cannot fathom how/why the incorporation of GFP (a protein of an average dimension of ~ 3.3 nm) onto a MotB monomer would not affect its articulation with MotA and rotary function (twirling the C-ring) thereafter. It appears that the zeal to demonstrate some surreal ideas seem to lead Apollonian pursuits into self-contradicting premises!

We believe that the efforts to understand bacterial motility were waylaid by the choice of bacteria with unipolar helical flagella, as these systems are not the general/common case scenario across the diverse bacteria. Though Purcell showed and argued that the high viscous forces prevail over the low inertial forces in the low Reynolds number realms, a spiralling flagellar movement (misinterpreted as rotation, owing to inadequate resolution!) need not result only owing to Magnus effect. A viscous fluid shear flow (as seen in the video of Ali *et al.*, 2016 - <https://gfm.aps.org/meetings/dfd-2016/57da1549b8ac3117910009ed>) can cause a cylindrical shaft to apparently exhibit a pseudo-rotary movement, resulting because of the interplay of the helical structure

and fluid flow along the pipe, and this could be perceived as rotational. In such a scenario, when the water expulsion at the base stops (i.e. owing to change in the internal metabolic activities, the DRS-mediated production of water goes down), an aberrant perception of clockwise movement can also result, which could revert to counter-clockwise movement once the water flows again; and this tangible explanation agrees with the murburn model. In CheY-P mutants (41), the rotational direction reversal has been noted with changes in hydrostatic pressure. Our inference is also supported by the fact that cutting down the length of the filaments to a micrometer (as in tethering assays in the field) significantly lowers cellular motility. Recently, it was also demonstrated that the filament lengths affected rotation, a dominant filament was important for the CCW movement (42), and this agrees with the murburn model. In a flagellum-tethered cell setup, only a few cells rotate and this may be due to the fact that the tethering may be due to the reason that tethering is not efficient (as opposed to the rotary model's assumption that multiple filaments could be tethered!). Murburn model could potentially also reason that the cell body rotates in a direction countering the flagellar movement because of back-pressure. We would explore in greater details if the mathematical fluid dynamics advocated by Spagnolie & Lauga (43) could potentially support the murburn model, regarding the curved path taken by a swimming cell (rather than a straight one) and also explain the diversity of flagellar structures and distribution known among bacteria.

CheY, the chemotaxis signalling protein which is known to bind to the conserved N-terminal motif of FliN, is considered to bind with 20 folds higher affinity when phosphorylated, when compared to its non-phosphorylated counterpart (34). (There are claims that phosphorylation of Asp-57 causes a local adjustment of the carboxyl side chain and a repositioning of the Lys-109 side chain, which is supposed to drastically change CheY's ability to interact with the C-ring and enable switching.) Since the full structure of FliM is not available (bound to CheY-P and CheY), it is difficult to tell whether there is such a drastic change in the structure of the switch complex, which would even translate to alteration of FliG structure. The main chains of activated and unactivated CheY are very similar in structure and there is no drastic change in the traces, as can be seen in Figure 10. Similarly, there is not much significant change in the domain of FliM which is deemed to be crucial for the binding switch in rotation owing to CheY binding. A bigger question is, why does un-activated CheY also bind to FliM, in the first place? Another thing

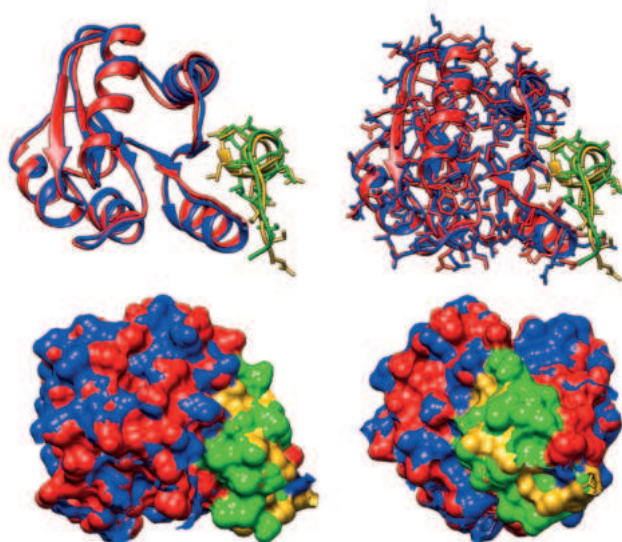


Figure 10.: The superimposed structures of unphosphorylated CheY from *E. coli* (PDB ID: 2b1J – Dyer & Dahlquist, 2000) and activated CheY (crystallized with BeF₃, an analog of phosphorylated CheY from *E. coli*; PDB ID: 1F4V – Lee et al 2001), as well as the respective FliM N-terminal regions (which contain the ‘conserved’ LSQAEIDALL motif) are compared using Chimera 1.14 MatchMaker tool. The top left image shows the main chain views of activated CheY (blue), unphosphorylated CheY (red colour), N-term of FliM from 2BIJ (green) and the N-term of FliM from 1F4V (gold). The top right image shows the side chains also, apart from main chains of the proteins. The bottom two images show the surface views from different angles.

to keep in mind is that we have found that some bacterial strains do not have this ‘conserved motif’ of LSQAEIDALL. An inference cannot be from these superimposed structures that phosphorylated CheY has higher binding affinity when compared to the non-phosphorylated protein. The interpretation that phosphorylation of FliN/M/G by CheY-P could change rotations (44) could be a coincidental one, as the C-ring locale could be a hub of murzyme phosphorylations and CheY/CheY-P could affect water expulsions.

The stochastic murburn proposal (which does not need any specific arrangement or any exact residue located at a particular locus of the protein to be conserved like the deterministic rotational model but only requires a preponderance of charged/polar residues at the proteins that line the interface) explains why the flagella system wasn't ‘selected out’ by natural selection over the course of evolution. It is highly

improbable to have residues of several proteins conserved together (which the current model deems) through millions of years of evolution (especially by making several variations of the flagella system in different bacterial species). Thus, the murburn model can explain the evolution and the observed diversity of the flagellar system components.

4. SUMMARY AND CONCLUSIONS

Figures 5 and 6 and Tables 4 and 5 depicting the proteins' structural features and non-conservation of essential residues (or overall features) across the bacterial species clearly counter the claim that these components are involved in and evolved for rotary function. The schematics of the arrangement of proteins shown in Figure 1 agree with the components known of bacterial secretory systems embedded in proteins, in this case, Type 3 (45). This crucial aspect addresses the discussion in the last paragraph of Section IV E. FliM merely serves as a connecting module between FliN and FliG and therefore, lacks other significant attributes. Further, Figures 2, 4, and 7-9 present qualitative and visually tangible data for FliN, FliG and MotA supporting the murburn model (which posits that charged residues enhance DRS recruitment and conversion at the interface) and Tables 1 and 3 provide quantitatively verifiable data supporting the preponderance of the charged residues. The meta-analysis of amino acid composition of *Helicobacter pylori* (a gut pathogen that causes ulcers) and *Escherichia coli* (largely, a gut commensal that may lead to some infections) as given in Tables 1 through 3 is very insightful. It is well known that *H. pylori* is more motile than *E. coli* (46). Comparison of all the cytoplasmic protein components of the bacterial flagellar system (FliN, FliM and FliG) show a significantly higher percentage of polar/charged residues in *H. pylori*. This finding is a yet strong support for the murburn model, which predicts motility to be directly correlated with DROS to water conversion, which is directly dependent upon the charged/polar residues.

Although we are confident that the bacterial flagellar assembly is not a physiological rotary system and murburn model is not "irreducible complex", we are far away from explaining the actual details of how it functions (e.g., in the spirochaete system or even some of the known observations with certain mutants). Details as to the site and modality of water expulsion, the simulation of movements with hydrodynamics, etc. needs to be done to get better grasp on the mechanism. But solving of a complex problem is based on addressing the core aspects

and pointing out the pivotal points of the system and with our last manuscript in the field (19) and the current one, we have achieved the minimal requisite towards the same.

STATEMENTS AND DECLARATIONS

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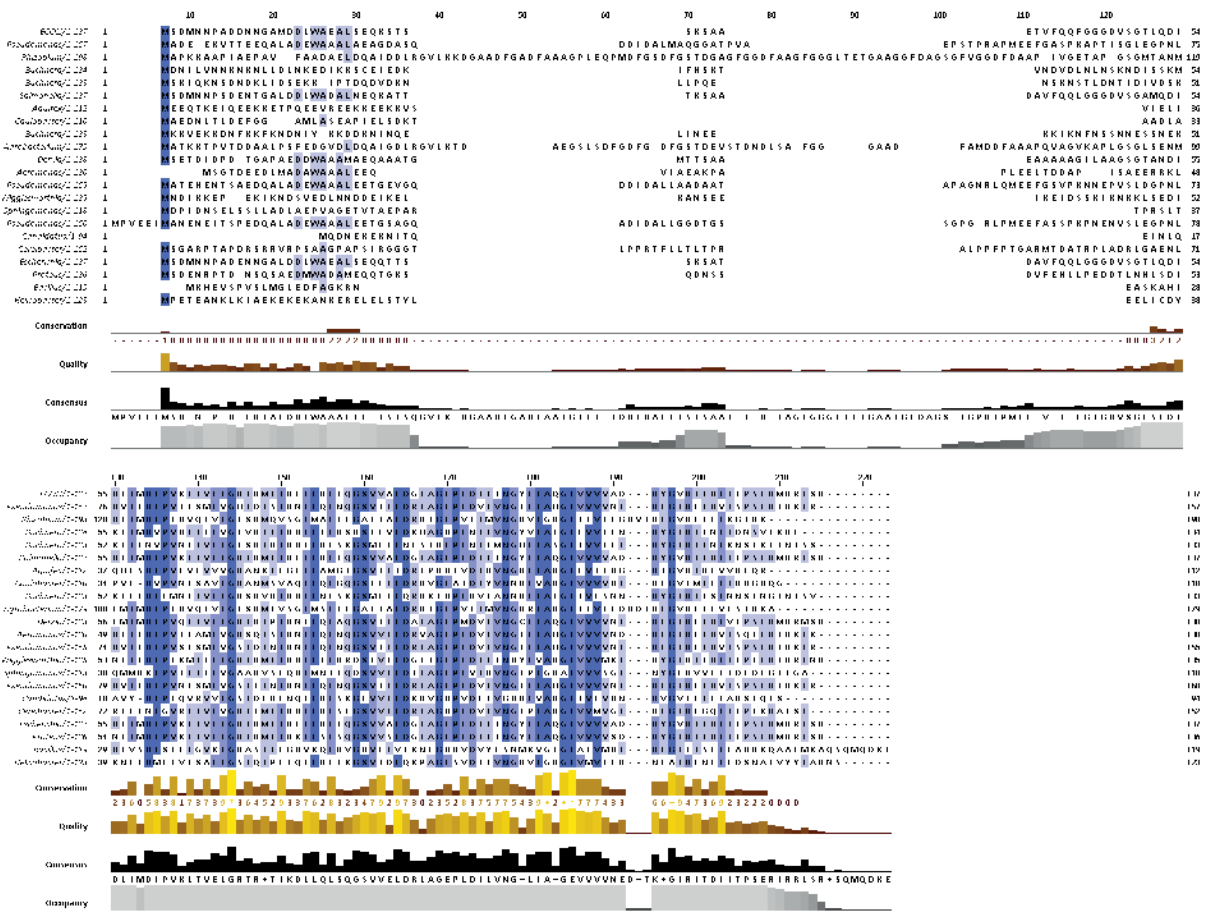
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Supplementary Information: Item 1. Alignment of FliN proteins



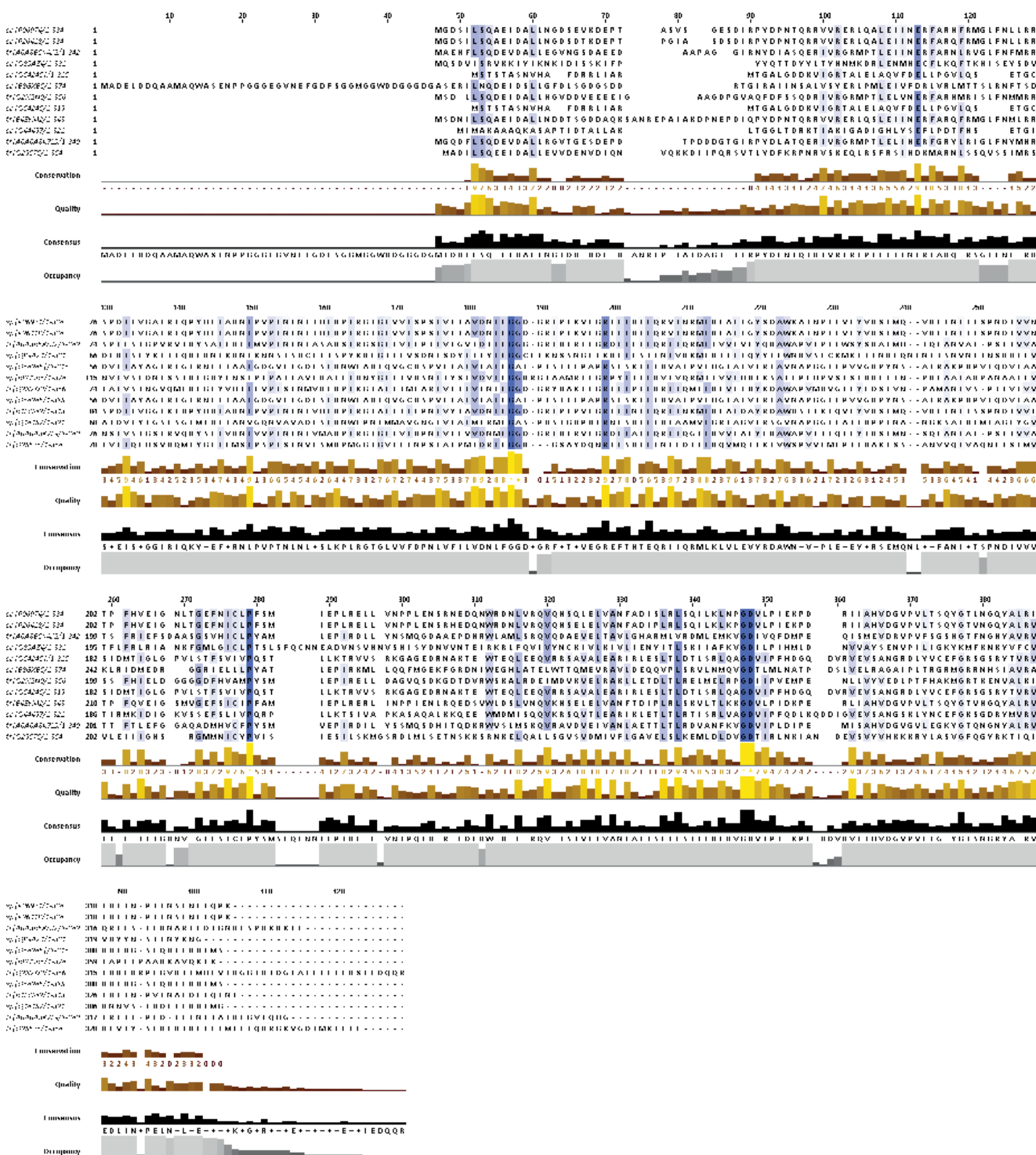
Eg., *E. coli* D55 (130 position on top of the second tier of analyzed sequences) is mostly conserved in other species. So is the next D residue (130-140 range). Some more E and D residues appear to be conserved. Other than that, no positively charged residues seem to be conserved.

Global alignment of diverse FliN

Organism	Global alignment (%)		
	identity	similarity	gaps
<i>Escherichia coli</i> (137)	Control		
<i>Pseudomonas aeruginosa</i> (157)	39.6	59.7	15.1
<i>Rhizobium meliloti</i> (198)	21.5	35.4	39.7
<i>Buchnera aphidicola</i> subsp. <i>Baizongia</i> (134)	34.2	52.3	18.1
<i>Buchnera aphidicola</i> subsp. <i>Acyrthosiphon</i> (133)	33.1	57.7	9.9
<i>Buchnera aphidicola</i> subsp. <i>Schizaphis</i> (133)	25.3	42.6	33.3
<i>Salmonella typhimurium</i> (137)	89.8	97.1	0.0
<i>Aquifex aeolicus</i> (112)	27.1	45.7	22.1
<i>Caulobacter vibrioides</i> (110)	35.0	54.0	19.7
<i>Agrobacterium fabrum</i> (179)	21.3	39.6	43.6

Organism	Global alignment (%)		
	identity	similarity	gaps
<i>Derrxia gummosa</i> (138)	25.3	42.6	33.3
<i>Aeromonas hydrophila</i> (130)	43.5	63.0	6.5
<i>Pseudomonas</i> sp (155)	41.8	60.1	15.2
<i>Wigglesworthia glossinidia brevipalpis</i> (135)	47.1	70.3	2.9
<i>Sphingomonas</i> sp (118)	32.2	49.7	21.7
<i>Pseudomonas putida</i> (160)	39.8	58.4	15.5
<i>Candidatus Midichloria</i> (94)	20.8	38.9	45.0
<i>Cereibacter sphaeroides</i> (152)	27.2	38.7	48.7
<i>Escherichia fergusonii</i> (137)	94.2	97.8	0.0
<i>Proteus mirabilis</i> (136)	67.2	80.3	0.7
<i>Bacillus cereus</i> (119)	19.3	41.4	23.4
<i>Helicobacter pylori</i> (117)	23.1	44.8	18.2

Supplementary Information: Item 2. Alignment of FlIM proteins

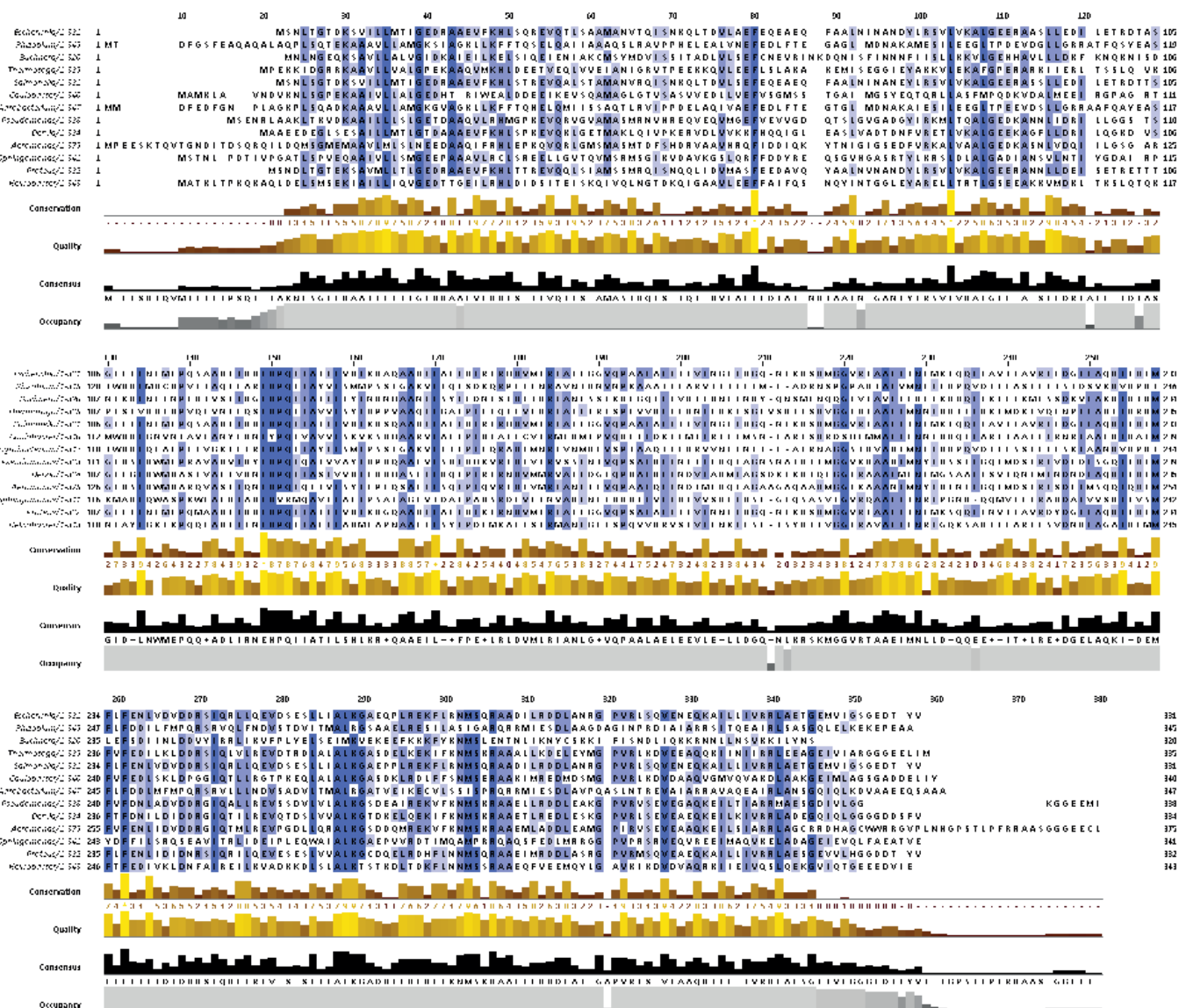


There is little residue conservation – there does not seem to be any evolutionary conservation. A bigger mystery is – how did these proteins ‘evolve’, with so many gaps and sequence dissimilarities and yet are able to perform the same function?

Global alignment of FliM

Organism	Global alignment (%)		
	identity	similarity	gaps
<i>Escherichia coli</i> (334)	Control		
<i>Derxia gummosa</i> (342)	42.9	62.3	4.1
<i>Rhizobium meliloti</i> (315)	19.8	33.9	31.0
<i>Buchnera aphidicola</i> subsp. <i>Baizongia</i> (334)	21.1	39.4	15.3
<i>Salmonella typhimurium</i> (334)	95.5	97.9	0.0
<i>Caulobacter vibrioides</i> (374)	20.8	40.3	18.5
<i>Agrobacterium fabrum</i> (321)	17.7	30.7	39.1
<i>Aeromonas hydrophila</i> (356)	33.7	56.8	12.5
<i>Sphingomonas</i> sp (340)	48.1	65.7	2.3
<i>Proteus mirabilis</i> (343)	73.8	85.1	2.6
<i>Helicobacter pylori</i> (354)	25.3	46.0	15.1

Supplementary Information: Item 3. Alignment of FliG proteins

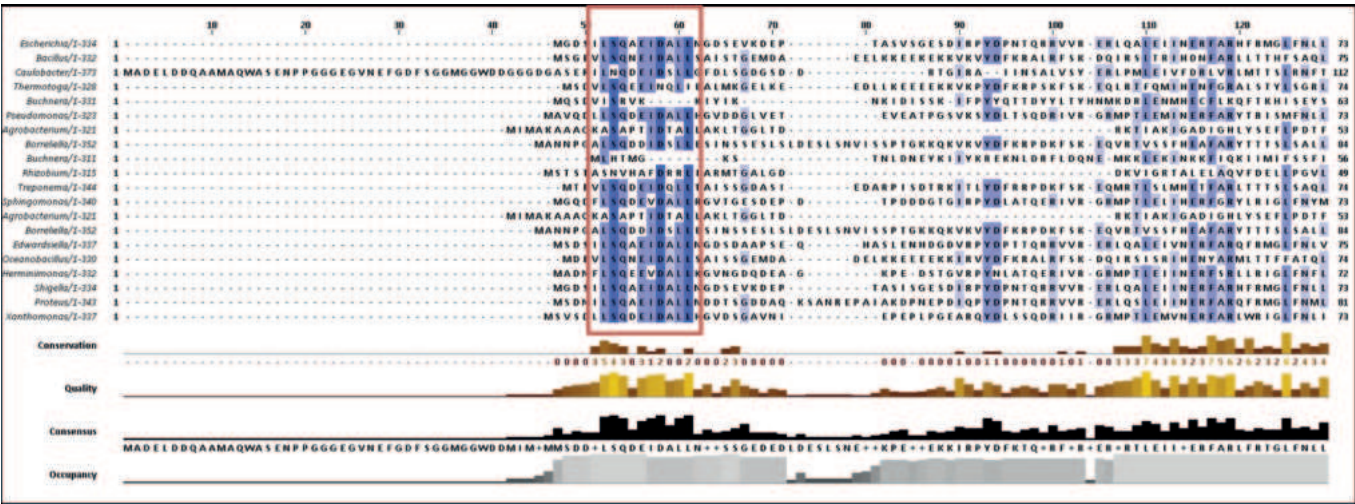


EHPQ (close to 150) and ALKG (280-290) stretches seem to be conserved and there are some places where R and K, as well as other amino acids have conservation scores of upto 60-80%.

Global alignment of FliG

Organism	Global alignment (%)		
	identity	similarity	gaps
<i>Escherichia coli</i> (331)	Control		
<i>Rhizobium meliloti</i> (345)	22.5	46.6	10.1
<i>Buchnera aphidicola</i> subsp. <i>Baizongia</i> (320)	14.7	27.0	50.5
<i>Salmonella typhimurium</i> (331)	47.3	48.1	51.0
<i>Caulobacter vibrioides</i> (340)	15.3	25.5	53.8
<i>Agrobacterium fabrum</i> (375)	26.2	46.5	11.1
<i>Pseudomonas aeruginosa</i> ()	21.5	34.2	51.8
<i>Derxia gummosa</i> (334)	49.3	72.8	1.5
<i>Aeromonas hydrophila</i> (356)	33.7	56.8	12.5
<i>Sphingomonas</i> sp (341)	13.6	24.4	53.0
<i>Proteus mirabilis</i> (332)	79.8	93.1	0.3
<i>Helicobacter pylori</i> (343)	14.7	26.8	53.0

A multiple sequence alignment of the CheY binding region

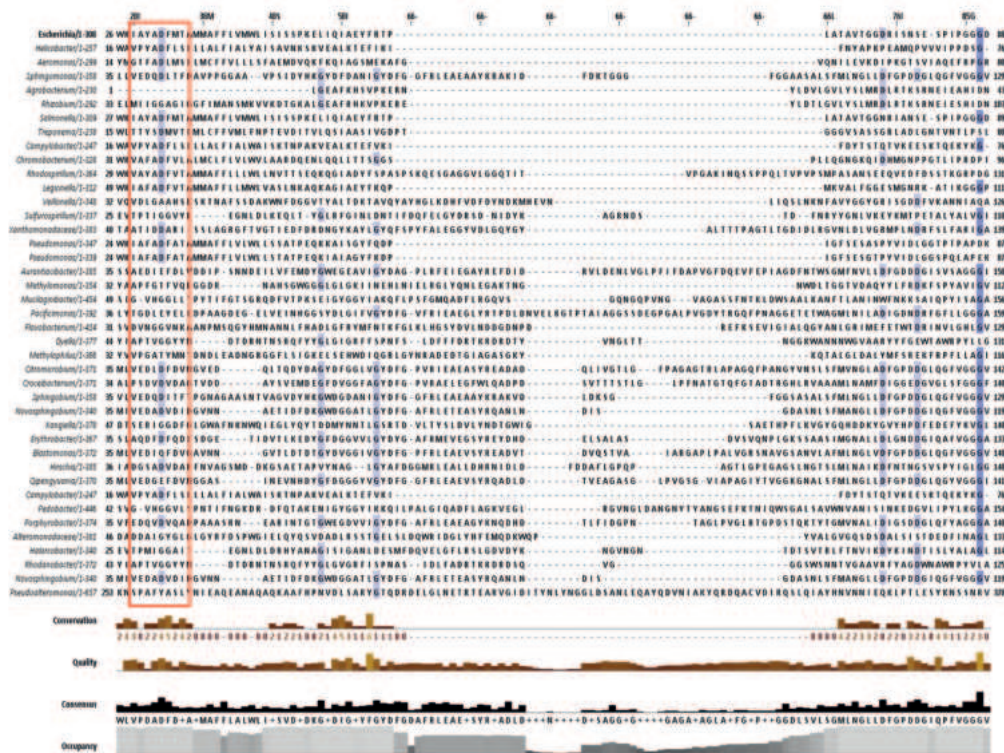


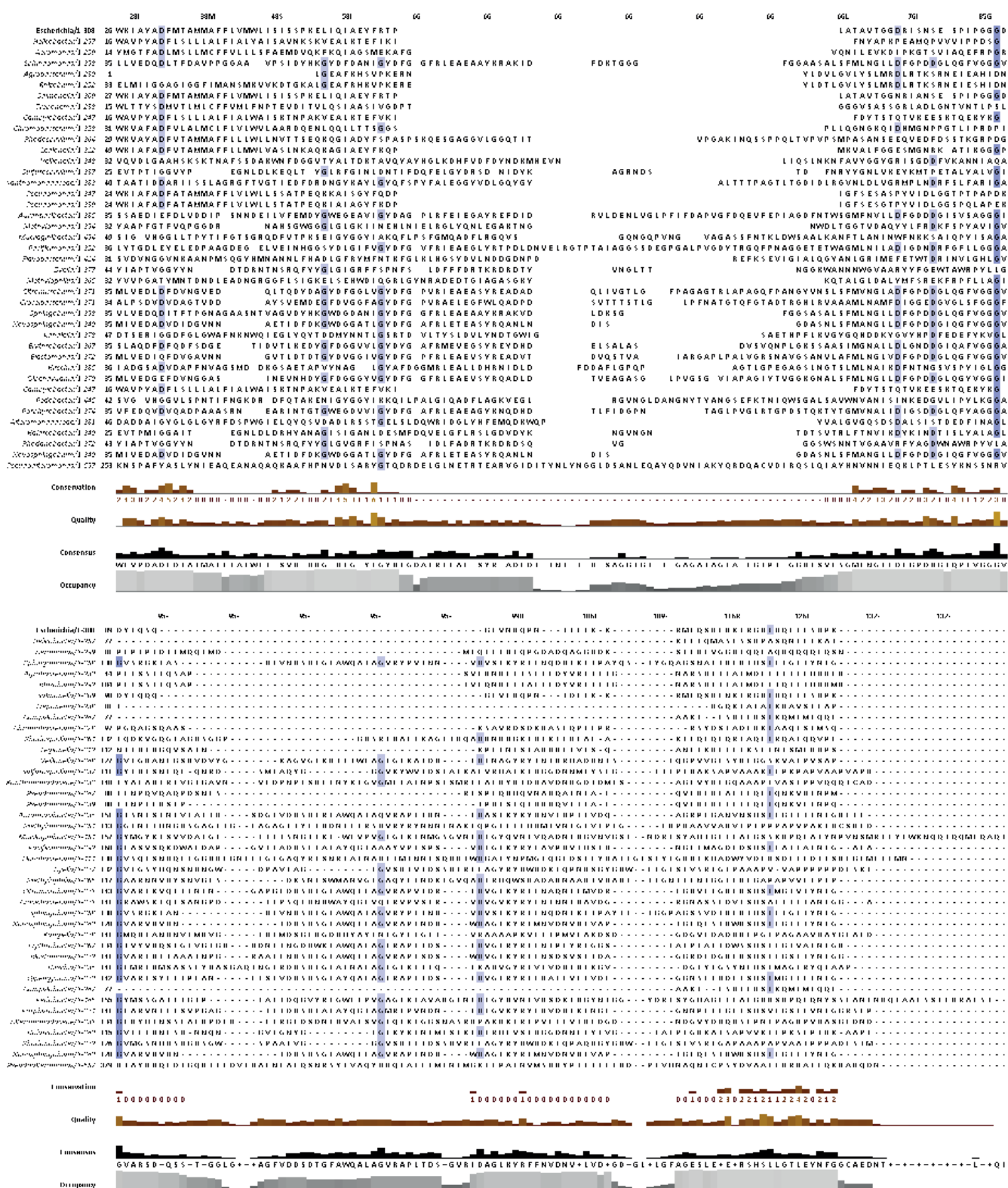
FliN, FliM, FliG and MotB of *Magnetococcus marinus*. a highly motile bacterium

Magnetococcus marinus

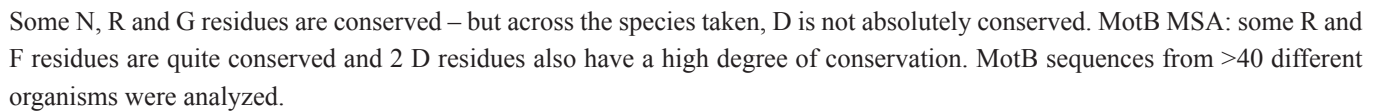
Protein	R	K	D	E	S	T	%(polar)	%(Charged)
FliN (137)	8	7	20	17	9	2	46.0	38.0
FliM (332)	14	18	22	29	18	20	36.4	25.0
FliG (336)	15	25	24	40	17	12	39.6	31.0
MotB (332)	13	25	33	13	22	15	37.6	26.1

Supplementary Information: Item 4. Alignment of Mot B proteins with respect to D32









Supplementary Information: Item 6. FlIF analysis



FlIF conservation analysis- JalView

Amino acid analysis of FliF

No.	Organism	R	K	D	E	S	T	% (polar)	%(charged)
1	<i>Escherichia coli</i> (552)	40	19	24	37	45	22	25.7	21.7
2	<i>Rhizobium meliloti</i> (557)	34	18	31	37	41	26	26.2	21.5
3	<i>Buchnera aphidicola</i> subsp. <i>Baizongia</i> (555)	18	54	21	34	66	13	25.2	22.9
4	<i>Salmonella typhimurium</i> (560)	39	19	25	36	41	33	27.1	21.3
5	<i>Caulobacter vibrioides</i> (536)	25	29	31	39	37	29	28.5	23.1
6	<i>Agrobacterium fabrum</i> (375)	34	21	21	31	46	33	37.3	28.5
7	<i>Derxia gummosa</i> (334)	-	-	-	-	-	-		
8	<i>Aeromonas hydrophila</i> (356)	-	-	-	-	-	-		
9	<i>Sphingomonas</i> sp (341)	32	16	38	35	41	28	43.7	35.5
10	<i>Proteus mirabilis</i> (332)	-	-	-	-	-	-		
11	<i>Helicobacter pylori</i> (343)	11	32	18	34	20	22	34.1	27.7
12	<i>Thermotoga maritima</i> (335)	-	-	-	-	-	-		

- sequence not available