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Review

Diving into the complexity of the spirochetal endoflagellum

Fabiana San Martin,^{1,2,8} Lenka Fule,^{2,3,8} Gregorio Iraola,^{4,5,6} Alejandro Buschiazzo,^{1,2,7,*} and Mathieu Picardeau ^{2,3,*}

Spirochaetes, a phylum that includes medically important pathogens such as the causative agents of Lyme disease, syphilis, and leptospirosis, are in many ways highly unique bacteria. Their cell morphology, subcellular organization, and metabolism reveal atypical features. Spirochetal motility is also singular, dependent on the presence of periplasmic flagella or endoflagella, inserted subterminally at cell poles and not penetrating the outer membrane and elongating outside the cell as in enterobacteria. In this review we present a comprehensive comparative genomics analysis of endoflagellar systems in spirochetes, highlighting recent findings on the flagellar basal body and filament. Continued progress in understanding the function and architecture of spirochetal flagella is uncovering paradigm-shifting mechanisms of bacterial motility.

Taxonomic diversity is uncovering a new picture of the structure and function of bacterial flagella

The flagellum is a complex nanomachine that allows bacteria to move at high speeds. Swimming is crucial for many pathogens to encounter their hosts, to penetrate, disseminate, and establish disease. Flagella are also involved in forms of adhesion and biofilm formation [1], and can be targeted by phages [2,3] and the host immune system [4]. The global architecture of the flagellum is well conserved among bacteria, and the systems from enteric bacteria, like *Escherichia coli* and *Salmonella enterica*, are among the best-studied models, constituting paradigms for bacterial motility. Most bacteria generate motility through flagellar gyration, powered by the rotation of the flagellar motor. Torque is subsequently transmitted through a hook to the flagellar filament that ultimately provokes thrust. The basal body of the bacterial flagellar motor is composed of the export apparatus, the L- (at the outer membrane) and P- (at the peptidoglycan interface) rings, the rod, MS-ring, and C-ring [5] (Figure 1A, Key figure). However, as a more diverse set of bacterial organisms are being studied, important differences to the archetype enteric flagellum have been steadily identified: Gram-positive bacteria lack P- and L-rings, flagellar filaments in *Vibrio cholerae* and *Helicobacter pylori* are enclosed by a membranous sheath [6], and flagella in spirochetes, called endoflagella, reside entirely inside the cell, within the periplasm.

Several important features distinguish spirochete endoflagella from the exoflagella of most other bacteria (Figure 1B). The spirochete basal body is associated with the inner membrane, and comprises a trans-inner membrane ring, and a rod that penetrates the peptidoglycan layer, but not penetrating the outer membrane. The basal body is also more complex in terms of protein composition as compared to bacteria with extracellular appendages [7], including unique structural features [8–10]. The periplasmic hook is heavily crosslinked via a self-catalyzed mechanism unique to spirochetes [11,12], instrumental in bearing with the higher torques that these bacteria typically produce [13]. Finally, the spirochetal flagellar filaments are not composed of a single flagellin protein, as in enterobacteria, but instead comprise core and sheath proteins, each

Highlights

Spirochetes are unique bacteria, exhibiting singular morphological and physiological features.

Spirochetal motility relies on endoflagella, which harbor a hook and filament confined within the periplasm, between the inner and outer cell membrane.

As opposed to the extensively studied models of exoflagellated organisms, including enteric bacteria, the periplasmic localization is seemingly linked to dragging the spiral- or wave-shaped cell body of spirochetes, thus propelling the organism.

Spirochetes have evolved high-torque motors, which include additional elements compared to exoflagellates. The hook and filament also reveal unique structures adapted to higher rotational forces.

A comprehensive genome analysis of flagellar components within spirochetes, and in comparison to exoflagellates, was a missing tool for the microbiology community.

¹Laboratory of Molecular and Structural Microbiology, Institut Pasteur de Montevideo, Montevideo, Uruguay

²Pasteur International Unit, Integrative Microbiology of Zoonotic Agents, Institut Pasteur de Montevideo, Montevideo, Uruguay/Institut Pasteur, Paris, France

³Institut Pasteur, Université Paris Cité, CNRS UMR 6047, Biology of Spirochetes Unit, Paris, France

⁴Microbial Genomics Laboratory, Institut Pasteur Montevideo, Montevideo, Montevideo, 11400 Uruguay

⁵Center for Integrative Biology, Universidad Mayor, Santiago de Chile, Chile

⁶Wellcome Sanger Institute, Hinxton, UK

⁷Institut Pasteur, Département de Microbiologie Paris, France



often present in several different isoforms [14,15], in some species eventually including more than ten proteins to assemble a very complex filament structure [16,17]. Added to the multiflagellin core, we have recently identified several novel sheath proteins in *Leptospira* filaments [18,19] organized asymmetrically onto the native supercoiled appendage. Previous reviews about spirochetal motility are mainly focused on the *Borrelia burgdorferi* model [7,20–24], generally overlooking the phylum Spirochaetes as a whole. Here, we analyze the genomes of representative genera spanning the phylum Spirochaetes, with special details of the genes that encode endoflagellar components. Providing a systematic insight into the architecture, function, and evolution of endoflagellar systems from these atypical bacteria will pave the way to visualizing and better understanding a new paradigm of bacterial motility.

⁸These authors contributed equally to this work

*Correspondence: alebus@pasteur.edu.uy (A. Buschiazzo) and mathieu.picardeau@pasteur.fr (M. Picardeau).

Spirochetes: spiral-shaped bacteria with a unique motility system

The phylum Spirochaetes includes bacteria that are ubiquitous and can adapt and colonize a wide range of environments (Box 1). The spirochetes are one of the few bacterial phyla whose cell morphology features reflect its phylogenetic relationships, thus forming a distinct line of evolution among bacteria [26]. Except for a few coccoid and nonmotile bacteria, such as *Sphaerochaeta* spp. [27], spirochetes are diderm, long, thin, and spiral-shaped motile cells. They have helical or flat-wave shapes such as in *Leptospira* and *Borrelia*, respectively. The cells are usually 0.1–3 μm in diameter and 3–20 μm in length, but giant spirochetes up to 150 μm in

Key Figure

Schematic representation of the endoflagella of *Borrelia* and *Leptospira* spp

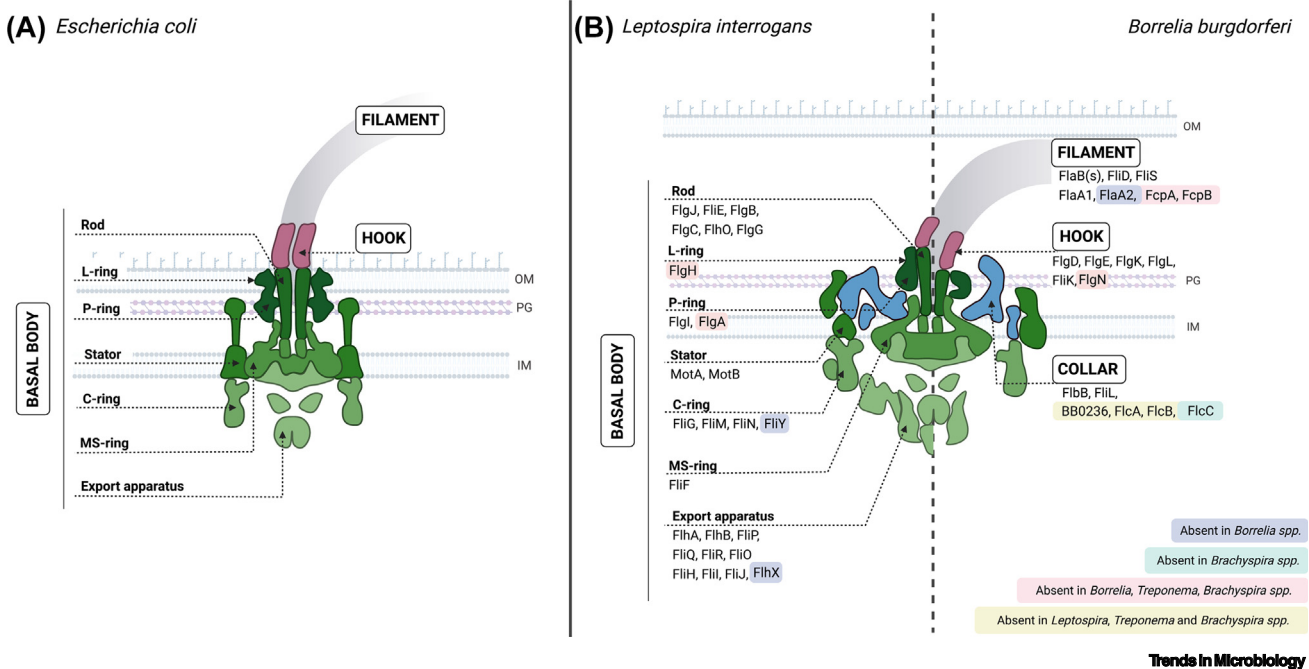


Figure 1. Schematic representation of the endoflagella of *Borrelia* and *Leptospira* spp. (A) and comparison with the *Escherichia coli* flagellum (B). Several flagellar proteins, such as MotA, MotB, FlgG, FlaB, or FlhA/FlhB, show apparent redundancy in some spirochetes but for convenience we kept only one paralog. A heatmap showing the distribution of flagellar proteins in the phylum Spirochaetes is shown in Figure 2.

Box 1. Ubiquity and diversity of spirochetes

The phylum of Spirochaetes is composed of a single class, the Spirochaetia, and four orders (Brachyspirales, Brevinematales, Leptospirales, and Spirochaetales) [25]. The Spirochaetales is the largest order, including several families and genera such as *Borrelia*, *Treponema*, *Cristispira*, and *Spirochaeta*. *Borrelia* spp. are arthropod-borne pathogens of humans, other mammals, and birds. *B. burgdorferi* and *Borrelia hermsii* are the agents of Lyme disease and relapsing fever, respectively. *Cristispira* spp. are found in the digestive tracts of marine and freshwater mollusks such as clams, mussels, and oysters. The genus *Treponema* includes the pathogens *Treponema pallidum* and *Treponema denticola*, the agents of syphilis and human periodontal disease, respectively. Most of the anaerobic spirochetes found in the hindguts of termites and wood-eating cockroaches belong to the genus *Treponema*. Among the order Brachyspirales, which is composed of a single family (Brachyspiraceae), the anaerobic spirochetes of the genus *Brachyspira*, including the agent of swine dysentery *Brachyspira hyodysenteriae*, colonize the intestines of various species of animals and birds. The order of Brevinematales and the family Brevinemataceae is composed of only one *Brevinema* species infecting shrews and mice. Among the order Leptospirales, the unique family Leptospiraceae is composed of the genera *Turneriella* and *Leptonema*, both composed of only one species, which are free-living bacteria, and the genus *Leptospira* which includes free-living saprophytes as well as pathogenic species that infect a wide range of mammals. Of note, most of the spirochetes described above are fastidious to grow *in vitro*, and several have not been amenable to grow in pure cultures.

length have also been described in the genera *Cristispira* and *Spirochaeta* [26]. The internal organelles of motility are called endoflagella, also referred to as axial fibrils/filaments, periplasmic flagella, or periplasmic fibrils/filaments [28]. Depending on the genus or species, spirochetes can have anything between one and hundreds of periplasmic flagella, inserted subterminally at each cell pole, and generally extending along most of the cell length, overlapping in the central region. *B. burgdorferi* has bundles of nine to 11 flagella arising from each pole and forming flat ribbons that wrap around the spirochete cell body [29]. *Treponema pallidum* and *T. denticola* have two to three periplasmic flagella that arise from each end of the cell, extending towards the center where they overlap [30,31]. The exceptions are the members of the family *Leptospiraceae*: they have a single periplasmic flagellum subterminally attached at each cell end, and their filaments are shorter, not overlapping towards the middle of the cells. In addition, and in contrast to *B. burgdorferi*, *Treponema phagedenis*, and *Brachyspira hyodysenteriae* – which display straight or slightly curved filaments [32–34] – the leptospiral periplasmic flagella adopt a highly curved supercoiled conformation once purified [35,36]. The rotation of these short, stiff, and supercoiled flagella in the periplasmic space, and their interaction with the cell body cylinder, generate very defined large-scale curved deformations of the cell body, resulting in the characteristic hook- and spiral-shaped ends, depending on the motors' rotational direction [18,37]. In *B. burgdorferi*, endoflagella not only induce undulations of the cell cylinder but also govern its shape [38]. The stiffness of the endoflagellar filament and its interaction with the outer membrane and the **protoplasmic cylinder** (see [Glossary](#)) drive a radically distinct way of swimming as opposed to the extensively studied models of exoflagellated organisms [39]. Spirochetes are capable of swimming in highly viscous, gel-like media that otherwise slow down or stop exoflagellated bacteria [40]. Furthermore, the swimming speeds of *Leptospira interrogans*, *Brachyspira pilosicoli*, and other spirochetes increase with **viscosity** [41,42]. Given that the motility of pathogenic spirochetes is crucial for host infection ([Box 2](#)), these swimming capabilities make spirochetes some of the most invasive mammalian pathogens among bacteria [23]. Recent studies have also shown that *Leptospira* spp. and *T. denticola* are capable of moving along surfaces by a mechanism called crawling, which may involve the flagella [43–45].

Uncovering the spirochetal flagellar proteins through genomic analysis

The main components of spirochetal endoflagella are highly conserved, yet some spirochetes have evolved genus-specific features ([Figure 1B](#)). Intriguingly, the evolutionary mechanisms that enabled the flagella from Spirochaetes to be confined within the periplasm are yet to be deciphered ([Box 3](#)). To better understand the evolutionary relationships of flagellar proteins within the phylum Spirochaetes we undertook a phylogenetic analysis by searching for homologous

Glossary

Collar: a large and complex spirochete-specific periplasmic structure that is anchored to the inner membrane and the MS ring. The collar may be crucial for the orientation of endoflagella in the periplasmic space.

Proton motive force: the electrochemical gradient of protons across a membrane due to a combination of the membrane potential and the concentration gradient of protons. It is used for various cellular processes, including flagellar rotation, ATP synthesis, and ion transport.

Protoplasmic cylinder: in bacteria, it comprises the cytoplasmic space, the inner membrane, and the peptidoglycan layer.

Stator units: the fixed component of the flagellar motor; it is an assembly of MotA–MotB complexes, also called force-generating units or torque-generating units, that power the rotation of the flagellar rotor, using energy derived from the transport of ions across the membrane.

Viscosity: the magnitude of the force required to shear a fluid. Water has a relatively low viscosity compared to gel-like media such as methylcellulose.

Box 2. Spirochetal motility and pathogenesis

Spirochetes are fastidious organisms to grow *in vitro* and to genetically manipulate. *T. pallidum*, for example, has been refractory to genetic manipulation until very recently [46]. In *B. burgdorferi*, several mutants have been generated, showing that motility and chemotaxis are required to invade and colonize both mammalian and tick hosts [22,47]. Similarly, analyses of *Leptospira* flagellar mutants, in animal models of the acute disease, have proved motility to be a key virulence determinant [16,48–50]. Flagellar mutants of *T. denticola*, the etiologic agent of periodontitis, were impaired in their ability to form dual-species biofilms with *Porphyromonas gingivalis* [51,52]. Analysis of motility of *B. burgdorferi* in the dermis of mice revealed new forms of motility, named lunging and wriggling [53]. Mathematical modeling predicts that *Borrelia* motility is the primary cause of the appearance and spread of the erythema migrans rash that characterizes Lyme disease [54]. *In vitro* studies have shown that *Leptospira* exhibits a back-and-forth movement when exposed to high viscosity environments, which could facilitate their ability to invade and spread during the infection [55]. It has been shown that *B. burgdorferi*, *T. pallidum*, and *L. interrogans* can cross the blood–brain barrier [56–58]. The syphilis agent is also able to cross the placental barrier, leading to fetal infection, and causing congenital syphilis [59]. *Leptospira* can also be found in placental tissue [60–62] and has long been known to affect pregnancy in cattle, but also in humans, leading to abortions and premature delivery [63,64] – which is consistent with the notion that *Leptospira* is capable of crossing the placenta barrier. In sum, pathogenic spirochetes can easily move through the dermis, break into and out of blood vessels, and can cross host barriers, but the exact mechanisms by which they do so remain elusive. Spirochetes are primarily extracellular bacteria, and translocation occurs predominantly via a trans-cellular route (crossing of pathogens through intercellular junctions) [47,65,66]. However, some reports suggest that *L. interrogans*, *T. denticola*, and *B. burgdorferi* can undergo intracellular invasion of different cell types [47,67,68].

sequences of 59 known flagellar proteins among 864 spirochetal genomes from 115 spirochetal species that belong to nine genera (Figure 2).

A heatmap depicting the presence/absence of flagellar proteins, and the degree of similarity among them, reveals an overall pattern that is largely in agreement with the phylogenetic tree (Figure 2). Excluding the coccoid and aflagellated *Sphaerochaeta coccoides* [27], which does not have flagellar proteins, 39/59 (66%) of the flagellar proteins are conserved among spirochetes (Figure 2). We can now clearly identify universal and genus-specific features for each of the major architectural elements of the spirochetal endoflagellum, namely the basal body, collar, hook and filament, as well as among flagellar regulators, as summarized below.

Basal body

The flagellar export apparatus is part of the flagellar basal body, and is highly conserved among bacteria, including in spirochetes (Figure 1). It consists of a transmembrane **proton-motive-force**-driven export gate (composed by the trans-membrane proteins FlhA, FlhB, FlhO, FlhP, FlhQ,

Box 3. The evolutionary origin of periplasmic flagella

The flagellar export apparatus is highly conserved among bacterial species. Its trans-membrane portion shares architectural features and homologous protein components with the virulence-associated type 3 secretion system (T3SS, or injectisome) [20,69], while the cytoplasmic portion of the flagellar export apparatus is homologous to F- and V-type ATPases [70]. Although the T3SS had initially been proposed to be an ancestor of the bacterial flagellum [71], recent phylogenetic reconstructions of both appendages suggest that the T3SS derived from a flagellar ancestor that subsequently lost flagellar genes [69]. This reduction of the flagellar structure is consistent with the observations of disassembly of different components of the flagella such as the hook, filament, cytoplasmic switch complex and associated ATPase, in exoflagellated bacteria under certain conditions [72,73]. Cryo-electron tomography studies have revealed structural diversity among flagellar motors from different species, showcasing adaptations to distinct environmental conditions [74–76]. Spirochetes, for example, have a unique multiprotein complex within the flagellar motor, called the collar, which seems essential to withstand the higher torques that spirochetal flagella produce [77]. The flagellar machinery of exoflagellated bacteria appears to be more simple in comparison to the spirochetal, and could have therefore preceded the emergence of periplasmic flagella. Chevance *et al.* showed that conversion of extracellular flagella of *Salmonella* to periplasmic flagella can arise by single amino acid changes in the flagellar basal-body rod protein FlgG [78]. Similarly, the deletion of *flgT* and *flgO*, which encode proteins that form a part of the flagellar outer membrane complex in *Vibrio alginolyticus*, shifts external sheathed flagella into periplasmic endoflagella [79]. Bacteria with sheathed flagella, such as most *Helicobacter* and *Vibrio* spp., and to a fewer extent members of the *Plantomyces* phylum [6], could therefore be evolutionary intermediates in the transition between the external and the periplasmic flagellum. Taken together, these results suggest that few mutational events could hamper the flagella to penetrate the outer membrane, leading to periplasmic appendages.

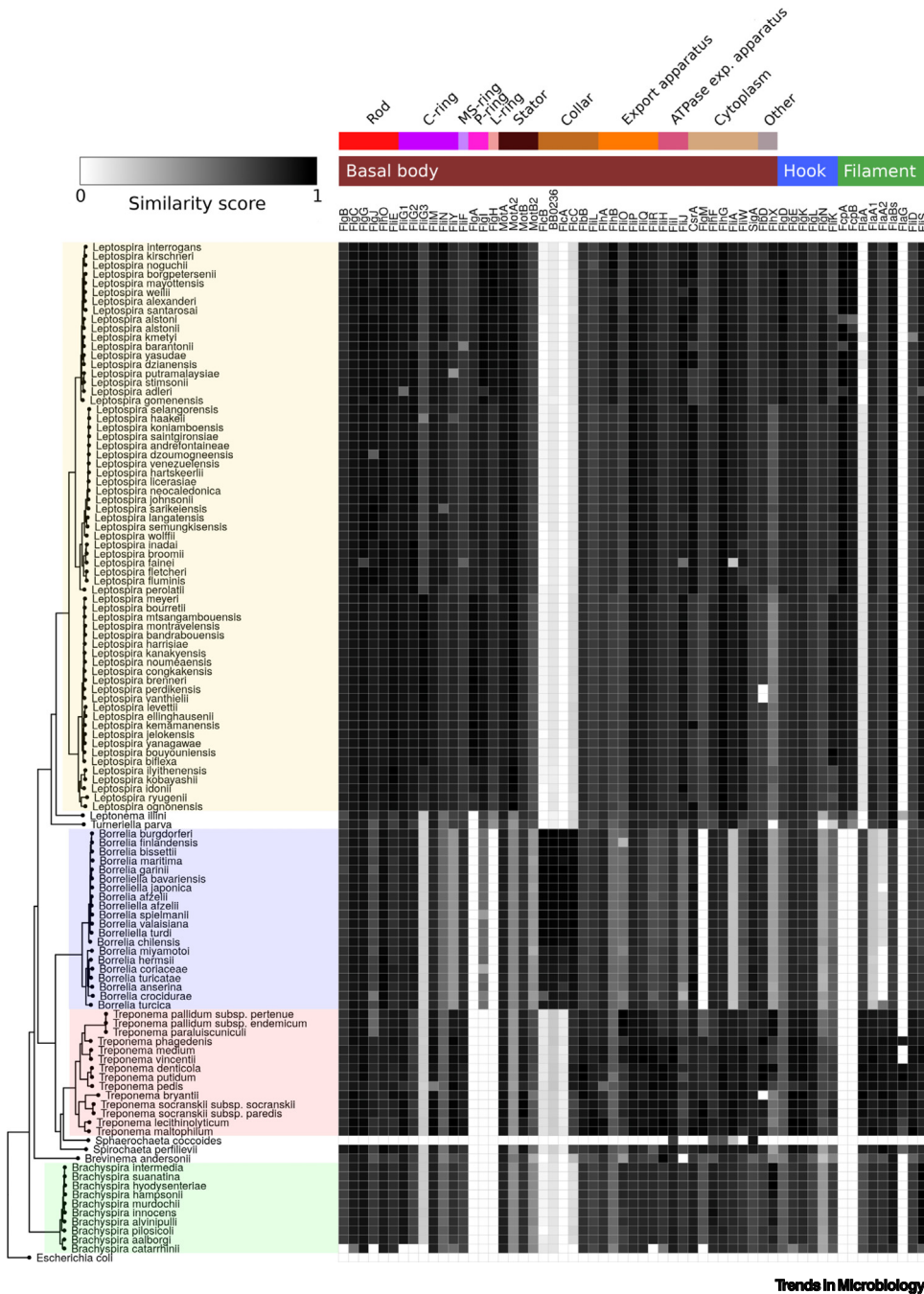


Figure 2. Distribution of flagellar protein-encoding genes in spirochetes. Distribution of flagellar protein-encoding genes in spirochetes. A heatmap is shown as a matrix, with columns corresponding to individual flagellar genes and lines corresponding to different species in the phylum of Spirochaetes. The heatmap color indicates low to high sequence similarity, with the extremes corresponding to absence (white) or presence (black) of the gene. The distribution was analyzed by first calculating manually-curated multiple amino acid sequence alignments of 59 known flagellar proteins; for convenience, we kept one FlaB (spirochetes encode between one and four FlaB isoforms). Then, Hidden Markov Models (HMMs) were calculated for each individual alignment using hmmbuild v3.3.2 [122]. All available genomes belonging to the phylum Spirochaetes ($n = 971$) were downloaded from the PATRIC database (www.patricbrc.org) and 864 genomes were kept for downstream analyses.

(Figure legend continued at the bottom of the next page.)

and FliR) and a cytoplasmic ATPase complex (formed by soluble components FliH, FliI, and FliJ in a 12:6:1 stoichiometry when assembled onto the flagellar appendage [80]). The assembled ATPase complex in spirochetes exhibits structural variations from the enterobacterial models [81]. Notably, each ATPase complex in *B. burgdorferi* appears to have a larger number of FliH subunits – which act as spokes that attach the hexameric FliI core to the rotating C-ring. The FliX protein, which can functionally substitute for the C-terminal domain of FliB in *H. pylori* [82], is also found in spirochetes but its function remains unknown. Previous studies in *B. burgdorferi* and *B. hermsii* show that the FliH–FliI complex is essential for motility and cell morphology but not for exporting rod, hook, and filament proteins [83,84]. In *Leptospira*, the transmembrane export gate is larger towards its cytoplasmic face, compared with those from other spirochetes [85], suggesting the presence of additional *Leptospira*-specific proteins. Similarly, cryo-electron tomography (cryoET) of *B. burgdorferi* flagella has recently revealed novel structures of the ATPase complex [81].

The *B. burgdorferi* motor has 16 **stator units**, in comparison to a maximum of 12 stator units in the motors of exoflagellated bacteria [10]. This is consistent with a larger motor (~80 nm of diameter in *Borrelia* vs. ~45 nm in *E. coli* and *Salmonella*) [81] and the higher torques that spirochetes ultimately achieve. The stator units in *Leptospira* comprise two copies of both MotA (MotA/MotA2) and MotB (MotB/MotB2). There seems to be no such redundancy in other spirochetes, which may be linked to the fact that *Leptospira biflexa* can translocate both H⁺ and Na⁺ ions as an energy source, while other spirochetes, including *Spirochaeta aurantia* and *B. burgdorferi*, use only protons [86]. In the C-ring, FliG is the protein most directly involved in interacting with the stator to generate torque. While there is only one isoform of FliG in most exoflagellated bacteria, two FliG paralogs are found in all spirochetes – except in *Leptospira*, which contains three paralogs. In *B. burgdorferi* only FliG2, and not FliG1, is present in the C-ring [10], and disruption of the *fliG1* gene did not affect flagellation, even though the swimming ability in highly viscous media is defective [50]. The function of the three FliG copies in *Leptospira* remains unknown. FliD (small flagellar operon protein D), which is present in all spirochetes, could be related to increasing the motor's torque as shown in the homologous SwrD (swarming motility protein D) from *Bacillus subtilis* [87].

The MS-ring constitutes the base of the rotor and is formed by multiple copies of FliF that polymerize forming a ring within the cytoplasmic membrane of bacteria, including in Spirochaetes. The P-ring protein FliG is present in *Leptospira*, *Leptonema*, *Turneriella*, and *Borrelia*, but is absent from *Treponema*, *Brevinema*, *Spirochaeta*, and *Brachyspira*. In *B. burgdorferi*, the P-ring, which appears to be masked by the collar (see below), is important for flagellar hook and/or filament assembly [88]. The periplasmic flagellar chaperone protein FliA, which is required for P-ring assembly in exoflagellated bacteria, is absent in all spirochetes except *Leptospira*. The L-ring protein FliH, which forms a circular complex traversing the outer membrane of exoflagellated Gram-negative bacteria, is found only in *Leptospira*, *Turneriella*, and *Leptonema* (Figures 1 and 2). In these spirochetes the function of FliH is still enigmatic since the structure it forms does not traverse the outer membrane, therefore lacking the bushing role ascribed to the L-ring in exoflagella [85]. A recent study suggests that the PL complex and its persistence is an ancient and conserved feature of the flagellar motor [89].

removing the ones not assigned to known species or from uncultivated samples. Each genome was reannotated with Prokka [123] and searched against the 59 flagellar HMMs using hmmsearch v3.3.2. We reported positive hits with e-value < 1 × 10⁻¹⁰. Resulting bitscores were scaled from 0 to 1 by normalizing by the highest score obtained for each flagellar protein. To put the results into an evolutionary context, a phylogenetic tree was built from the 864 spirochete and one *Escherichia coli* genome (outgroup) using gtdbtk v3.0 [124] based on a concatenated alignment of 120 universal bacterial marker genes. The resulting tree was collapsed at the species level, resulting in 115 species from genus *Leptospira* (n = 63), *Leptonema* (n = 1), *Turneriella* (n = 1), *Borrelia* (n = 21), *Treponema* (n = 15), *Sphaerochaeta* (n = 1), *Spirochaeta* (n = 1), *Brevinema* (n = 1), and *Brachyspira* (n = 10). For collapsed species, the mean normalized bitscore is shown for each protein. Visualizations were generated with the ggtree package [125]. Files, sequence alignments, and scripts to reproduce the analyses are available in the FigShare repository (<https://doi.org/10.6084/m9.figshare.19686282>).

The C-ring, which includes the bidirectional rotor of the flagellar motor and the switch complex, typically comprises three proteins: FlgG, FlhM, and FlhN or FlhY (the latter two are mutually exclusive in most bacteria). However, as also seen in *Campylobacter* and *Helicobacter* [90,91], *Leptospira* species produce both FlhN and FlhY. Studies of *Campylobacter jejuni* indicate that these two proteins have distinct roles within the C-ring [90], while *L. interrogans* *flhY* and *flhM* mutants showed reduced motility and attenuation of virulence [92,93].

The rod is a short, hollow tube that connects the basal body to the hook, playing the role of a central shaft for the rotating appendage. It can be divided into an inner-membrane proximal portion, composed of four different proteins (six units of FlhE, five FlgB, six FlgC, and five FlgF), and a distal rod segment, which comprises 24 protomers of the protein FlgG [94]. In spirochetes, FlhO is the ortholog of FlgF [95]. The rod ends with a distal segment that comprises protomers of the protein FlgG. The distal rod is capped by the protein FlgJ on its tip, which, in enterobacteria, is a two-domain protein: an N-terminal portion involved in rod assembly, and a C-terminal muramidase domain engaged in peptidoglycan (PG) remodeling, a key event during flagellar biogenesis and PG wall-piercing [96]. Interestingly, in spirochetes, as well as in *Vibrio* and *C. jejuni*, FlgJ only comprises the N-terminal portion as a monodomain protein [97], implying that the muramidase domain is expressed as a separate protein. Work done with *B. burgdorferi* reveals singular features of both FlgJ and independent PG-remodeling enzymes. In contrast with the critical importance of FlgJ in Proteobacteria rod assembly, its influence on flagellation and motility is not substantial in *B. burgdorferi*, uncovering unique functional implications perhaps related to normal flagellar regulation [98]. A separate protein with PG-hydrolyzing activity, related to the C-terminal domain of FlgJ in two-domain variants of the protein, has recently been identified in *B. burgdorferi* [97]. This enzyme (encoded by *B. burgdorferi* *bb0259*) is indeed a lytic transglycosylase that interacts directly with FlgJ in the spirochete, enabling the flagella to penetrate through the PG layer and allow for proper filament assembly.

Collar

The flagellar basal body from spirochetes has a unique structure known as the **collar**, which is absent in exoflagellated bacteria. In *B. burgdorferi*, the collar is a large structure of ~71 nm in diameter and ~24 nm in height. Anchored in the cytoplasmic membrane and the MS-ring, the collar surrounds the central rod and the P-ring, interacting with the stator units and the FlhL protein. FlhL, in turn, interacts with the rotor, although its function is still not fully understood [7,99]. Several key components of the collar have been recently identified in *B. burgdorferi*: BB0326, also called periplasmic flagellar collar protein A (FlcA) [77], FlbB/BB0286 [100], BB0236 [101], BB0058 (FlcB), and BB0624 (FlcC) [8]. Combining *in situ* cryo-electron tomography (cryo-ET) studies of mutants, and protein–protein interaction data, it has been proposed that FlcA is closely associated with the membrane and forms a turbine-shaped element; FlcB forms a ring at the base of the collar; FlcC is located on top of the collar structure; and BB0236 assembles onto FlbB, which is located in the middle of the collar complex [8,100]. Although such structural complexity is not yet fully understood, several mutants have started to decipher the role that the collar exerts in the basal body. *flbB*, *bb0236*, *bb0362*, and *flhL* mutants produce fewer, shorter, and abnormal endoflagella, which can accommodate fewer stator complexes and are unusually tilted towards the cell pole [77]. Surprisingly, most of these proteins are not conserved in other spirochetes (Figures 1 and 2), strongly suggesting that other genus-specific collar proteins are yet to be identified. This is further confirmed by cryo-ET and subtomogram averaging data showing that the collar in *L. interrogans* is different from the structures of *B. burgdorferi* and *Treponema primitia* [7].

Hook

During hook assembly, the hook-capping protein FlgD and the hook protein FlgE are sequentially exported via the flagellar export apparatus. Recent studies demonstrate that, in spirochetes, subunits of FlgE self-catalyze a crosslinking reaction among different protomers, creating lysalanine covalent bonds that form a stable high-molecular-weight complex [11,12], likely conferring an unusually high robustness to the hook under stringent rotational stress, essential for spirochetal motility.

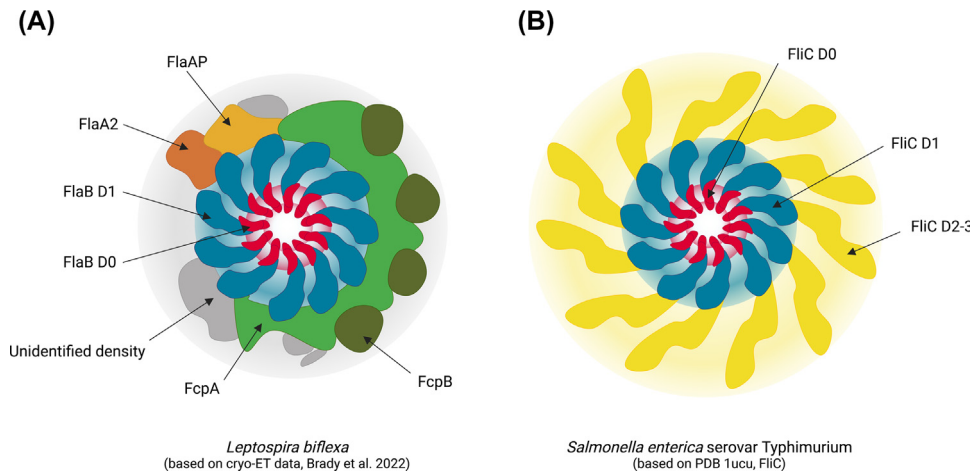
FlgE also plays a unique role in *B. burgdorferi*, regulating the expression of the filament proteins FlaA and FlaB [102]. Instead of affecting the transcription of filament-encoding genes, it is the translation and/or protein turnover of FlaA/B which is controlled. This mechanism appears to substitute the more classic cascades of motility gene transcriptional regulation exerted by dedicated sigma and anti-sigma factors such as σ^{28} /anti- σ^{28} (FlaA/FlgM), or σ^{54} (see the section 'Regulation of flagellar gene expression').

The molecular ruler FliK regulates the mature hook's length before FlgK and FlgL can be exported to form the hook-filament junction. This assembly process is essentially conserved in spirochetes [95] even though the cytosolic export chaperone protein FlgN (which binds specifically to the hook junction proteins FlgK and FlgL) is found only in *Leptospira*. In fact, the only chaperone which is conserved in spirochetes is FliS (described as a FliC chaperone in *E. coli*), which may here serve as a general chaperone with broad substrate specificity, at least partly substituting for FlgN and FliT (the latter is a FliD chaperone in *E. coli*) [103]. Of note, and for unclear reasons, the hook on top of the motor is wider (a diameter of ~21 nm) in *Leptospira* spp. than in *B. burgdorferi* (~16 nm) [85].

Filament

Flagellar filament assembly starts with the incorporation of a capping protein onto the tip of the nascent filament, a feature that spans bacteria, including spirochetes [104,105]. The filament cap protein, FliD, then serves as a flagellin-chaperoning component during filament assembly, also securing the filament's stability. The periplasmic flagellar filament in spirochetes was originally thought to be composed of a core of polymerized flagellin protomers (FlaBs), surrounded by a symmetric proteinaceous sheath of FlaA subunits [40]. Both FlaA and FlaB are essential to achieve full motility and virulence in many spirochetes [34,38,48,106,107]. Automatic genome annotations have resulted in some inconsistencies regarding gene nomenclature of flagellar multigene families. Such is the case for the several *flaA* and *flaB* variants found in different spirochete species, with numbering schemes that occasionally assign identical names to otherwise non-orthologous genes. The use of common gene names based on phylogenetic grounds is essential to facilitate comparison of data from different laboratories.

Spirochetal filaments express one (*Borrelia* spp.) or up to four different FlaBs (*Leptospira* spp.) which are orthologous to FliC, the widely known flagellin from *Salmonella* (Figure 3). The 3D structure of flagellins typically comprises four distinct domains. The N- and C-terminal regions are extremely conserved and fold into domains D0 and D1 that allow flagellins to polymerize into protofilaments; while domains D2 and D3, in the center, are less conserved among different bacterial taxons [108]. In spirochetal filaments, the FlaB core follows the same 11-protofilament helical organization shared by all bacteria, but each FlaB protomer lacks the external D2 and D3 domains (Figure 3A), resulting in a thinner tubular structure (~15 nm outer diameter vs. ~21 nm in *Salmonella*). FlaBs are O-glycosylated, as shown in *T. pallidum* and *T. denticola* [109,110], and strongly suggested to be present in *Leptospira* as well, where high-resolution cryo-EM maps of purified filaments show features compatible with saccharide moieties bound



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Figure 3. Cross-sectional view of the flagellar filament from *Salmonella* and *Leptospira*. Schematic comparison highlighting the protein composition of (A) the *L. biflexa* endoflagellar filament adapted from Brady *et al.* [19]; vs (B) the *Salmonella* exoflagellar filament. Abbreviations: cryo-ET, cryo-electron tomography; PDB, Protein Data Bank accession number

to conserved Ser/Thr glycosylation sites in the sequence [19]. The FlaB proteins are likely exported via the flagellar protein export apparatus, as flagellins typically do.

The endoflagellar filaments exhibit a proteinaceous sheath that surrounds the FlaB flagellin core. Early on, one or two isoforms of FlaA proteins were identified as the constituents of such a sheath [40]. When present, the *FlaA1* and *FlaA2* isoforms are homologous, albeit displaying low sequence identity (<30%). The two genes form an operon [48], probably as a result of gene duplication and diversification. As shown in the heatmap, *Brevinema*, *Brachyspira*, *Treponema*, and *Spirochaeta* also have another FlaA isoform. Despite the relative low sequence identities, another *Leptospira* conserved protein-encoding gene exhibits similarities to FlaA1 and FlaA2, suggesting the presence of a third paralog in *Leptospira* spp. Further work is needed to check if the three isoforms are expressed and associated with the flagellar filament in these spirochetes.

Additional sheath proteins, FcpA and FcpB, have been identified more recently in *Leptospira* [16,17]. These two proteins are Leptospiraceae-specific (also found in *Turneriella* and *Leptonema*) (Figure 2), with no homology between the two, and with FcpA showing a novel protein fold previously absent in the entire Protein Data Bank. Both FcpA and FcpB are localized asymmetrically on the convex, and not on the concave, side of the curved filament [16–19]. The singularly asymmetric recruitment of all these sheath proteins, FlaAs and Fcps, plays a key role in enforcing the strong supercoiling that *Leptospira* filaments typically exhibit. It is worth stressing that such supercoiling is essential to normal flagellar function and cell motility [35]. The concave side of *Leptospira* filaments is composed of FlaA2 [18,19] and most likely FlaA1. *In vitro* pull-down assays have shown that FcpA interacts with FlaA2 and FlaB1 [111], which is consistent with our latest high-resolution cryo-EM data of assembled filaments [18,19]. Additional, yet poorly characterized, filament proteins of the sheath have been recently identified in *Leptospira*, such as FlaAP (FlaA2-associated protein) [19], and likely several other components still annotated as hypothetical proteins (Figure 3A). A FlaG-homolog is also found in some *Treponema* species [112], FlaG being present in numerous exoflagellated bacteria. All spirochetal sheath proteins are likely exported to the periplasmic space via the SecA secretion pathway, as their N-termini include signal peptide sequences.

Regulation of flagellar gene expression

Multiple checkpoints occur to efficiently coordinate flagellar gene expression with protein assembly during flagellar biogenesis. In *B. burgdorferi* only one sigma-factor (σ^{70} , also named SigA or RpoD) is present to transcribe flagellar genes [21]. Many of the flagellar genes appear to be constitutively expressed, and *B. burgdorferi* regulates the synthesis of some motility proteins at the post-transcriptional level [21,84] as described above. In this species, the earliest flagellar structures to be synthesized include the MS-ring, the C-ring, the stators, and the export apparatus. Their synthesis and successful assembly are, in turn, required for the transcription of other genes that encode the rod, the P-ring, and the hook proteins [95]. Notably absent from *Borrelia* spp. [21], the alternative σ^{28} factor FliA (also named RpoF), and its anti-sigma factor FlgM, are indeed expressed in other spirochetes, and likely involved in defining the transcriptional regulatory hierarchy of flagellar genes as in other bacteria [15] (Figure 2).

FliH and FliG, which are involved in the control of spatial and numerical regulation of bacterial flagella, also appear to play a role in the regulation of flagellar gene expression at the transcriptional and post-translational levels in *L. biflexa* and *B. burgdorferi*, respectively [113,114]. The Carbon Storage Regulator A (CsrA), together with the flagellar assembly protein FliW, are both present in Spirochaetes. These proteins usually regulate gene expression at the post-transcriptional level by binding to mRNA targets, and affecting mRNA stability and translation. It has been shown that CsrA acts as a regulator of the expression of *flaBs* in *Leptospira* and *B. burgdorferi* [115,116].

Finally, the second messengers, like c-di-GMP and other cyclic nucleotides, also play an important role in controlling motility in many spirochetes [117–120], in several cases regulating key protein–protein interactions. Much work is needed to uncover the details of the regulatory pathways in which cyclic nucleotides are involved in spirochetes, as well as the molecular mechanisms implicated that still remain unknown.

Concluding remarks and future perspectives

Since the *Trends in Microbiology* review from Pallen *et al.* in 2005 [103], which used the genome sequences of only three representative spirochetes (i.e., *B. burgdorferi* str. B31, *T. pallidum* subsp. pallidum str. Nichols, and *L. interrogans* serovar Lai str. 56601), we now have access to thousands of genome sequences and have made huge advances in understanding the structure and function of many components of spirochetal endoflagella, using protein crystallography, cryo-EM and genetic approaches.

The global architecture of the spirochetal endoflagellum is well conserved, and most of the individual flagellar protein components are indeed highly conserved among spirochetes. However, in this review we have found numerous and significant differences among the different spirochetal genera, which are likely linked to genus-specific morphological features such as the stiffness, length, and number of flagellar filaments, as well as their interaction with the cell body cylinder and the outer membrane. Swimming motility thus involves different variations of the endoflagellar-driven mechanism, as exhibited by *Treponema*, *Leptospira/Leptonema*, and *Borrelia* [39]. Spirochetes are a highly diverse group of bacteria (Box1) and their ability to adapt to different niches/habitats may have influenced the evolution of their genomes. For example, *T. pallidum* and *B. burgdorferi* have a minimal genome in comparison to *Leptospira* spp. (~1–1.5 Mb vs. ~4 Mb) and this might constitute an additional constraint with particular impact on the number of flagellar genes.

Continued efforts over the next few years will fill in the remaining gaps (see [Outstanding questions](#)). What are the molecular determinants that lead to filament asymmetry in *Leptospira*?

Outstanding questions

What are the evolutionary mechanisms that enabled flagella to be confined within the periplasm in spirochetes? Why do they not penetrate the outer membrane and elongate outside the cell, as in enterobacteria?

What molecular connections, if any, explain how the motor-generated torque, transmitted to the flagellar filament, induces undulation and/or deformation of the whole cell body, eventually propelling spirochetes with a forward thrust?

How do pathogenic spirochetes cross tissue barriers?

What are the mechanisms involved in regulating flagellar rotation in spirochetes, especially according to media exhibiting highly disparate viscosities?

What is the reason behind *Leptospira*'s greater complexity along most of its flagellar substructures compared to other bacteria, including other spirochetes?

Several spirochetal flagellar proteins, such as MotA, MotB, FliG, FlaA, FlaB, or FliN/FliY, show apparent redundancy in the form of several paralogous isoforms – is this actually the case?

What is the structural basis, and potential physiologic role, for filament asymmetry in *Leptospira* flagella? And what is the fundamental role for such a rich complexity in the composition of the sheath? Is this filament asymmetry shared in other spirochetes?

Is such asymmetry relevant to other spirochetes? Also, many flagellar proteins remain to be identified, including collar proteins in spirochetes other than *Borrelia*. Half (three out of six) of the recently identified collar or collar-associated proteins in *B. burgdorferi* [8,77,100,101] are not conserved in other spirochetes. Conversely, *Leptospira* exhibits a more complex filament architecture, with at least ten different proteins, instead of two in *B. burgdorferi* and five in *T. pallidum* and *B. hyodysenteriae*. Are there additional filament proteins to be identified in the latter organisms, or actual differences relating to disparate motility mechanisms waiting to be uncovered? We also observe some redundancy among flagellar proteins, such as in the cases of MotA, MotB, FliG, FlaB, etc; and an important focus for future studies will be to decipher whether the paralog proteins have distinct functions.

The success of spirochetes as pathogens is explained by their spiral shape and endoflagellar motility, which enable them to cross connective tissues and barriers very efficiently and swiftly, contributing to their escape from the host's immune system. A better knowledge of the moving function of the flagellar apparatus is essential to understanding the motility and the pathogenesis of these atypical bacteria, and also to enable novel strategies to treat or prevent these infectious diseases. It has been shown that the nonmotile attenuated *fcpA* mutant in *L. interrogans* is an attractive candidate for efficacious and universal vaccines protecting against leptospirosis in humans and animals [121].

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Declaration of interests

No interests are declared.

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