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The unique paradigm of spirochete motility and chemotaxis

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Abstract

Spirochete motility is enigmatic: It differs from the motility of most other bacteria in that the entire bacterium is involved in translocation in the absence of external appendages. Using the Lyme disease spirochete *Borrelia burgdorferi* (*Bb*) as a model system, we explore the current research on spirochete motility and chemotaxis. *Bb* has periplasmic flagella (PFs) subterminally attached to each end of the protoplasmic cell cylinder, and surrounding the cell is an outer membrane. These internal helically shaped PFs allow the spirochete to swim by generating backward-moving waves by rotation. Exciting advances using cryoelectron microscopy tomography are presented with respect to *in situ* analysis of cell, PF, and motor structure. In addition, advances in the dynamics of motility, chemotaxis, gene regulation, and the role of motility and chemotaxis in the life cycle of *Bb* are summarized. The results indicate that the motility paradigms of flagellated bacteria do not apply to these unique bacteria.

Keywords

Spirochete; *Borrelia*; Lyme disease; chemotaxis; motility; motor

INTRODUCTION

A century ago Clifford Dobell (1912) said “the movements of the Spirochaetes are still surrounded in mystery (23).” A half a century later Claes Weibull (1960) quoted Dobell and went on to say “it could be asked whether the situation has changed very much since those days (96).” After yet another half century, although progress has been made, many

intriguing questions about spirochete motility remain unanswered and some obscurity about their elegant motions still persists. Genomic analysis indicates that spirochetes are a monophyletic clade (69), so we expect that spirochete motility has some similarity across taxa. Several recent reviews have been published on spirochete motility and the reader is referred to these articles (15, 34, 48, 97). However, there has been some fresh and robust progress in several areas that focus on particular aspects of spirochete motility, especially on the spirochete *Borrelia burgdorferi* (*Bb*). This progress has been spurred by the increase in research interest in *Bb* because of the importance of the disease it causes and recent breakthroughs in genetic manipulation. Furthermore, owing to its small diameter, *Bb* is optimal for analysis employing the ground-breaking methodology of cryoelectron microscopy tomography (Cryo-ET).

***B. burgdorferi* life cycle, Lyme disease, and genomics**

Bb is the causative agent of the zoonosis referred to as Lyme disease (77, 82, 85). Small mammals such as mice and voles, and specific species of birds, serve as reservoirs of infection. Humans are accidental hosts. Transmission occurs via the bite of hard shell ticks of certain species of the genus *Ixodes*. In humans, Lyme disease has many manifestations, including a spreading rash (erythema migrans), acute and chronic arthritis, a skin disease (acrodermatitis), neurologic problems, and heart block. In the United States, Lyme disease is the most prevalent arthropod borne human infection. Ten different species have been identified in *Bb* and are referred to as the *sensu lato* species complex. The species that is the most studied is *Bb sensu stricto* (hereafter referred to as *Bb*); it is the most prevalent species associated with disease in North America. *Borrelia afzelii* and *Borrelia garinii* are the species most commonly associated with Lyme disease in Eurasia. The genomes of *Bb sensu lato* consist of one small, linear chromosome (approximately 950 kb) and a variable number (7–21) of circular and linear plasmids. The plasmids comprise approximately one third of the spirochete's total genetic material. Because of its small genome, its nutritional requirements are complex.

Structure of a spirochete

What is a spirochete? In general, spirochetes have a unique and distinct structure; in fact, spirochetes are one of the few phyla of bacteria that can be recognized based on morphology (Figure 1a)(69). Outermost is a lipid bilayer membrane (OM), and internal to the OM is the protoplasmic cell cylinder (PC). At each end of the PC are subterminally attached periplasmic flagella (PFs) that reside in the periplasmic space (PS). The PFs rotate in a manner similar to the flagella of externally flagellated bacteria (15, 16, 16, 33). The final shape of the cell, depending on the species, is either a helix or a flat-wave. The size of the spirochete, the number of PFs attached at each end, and whether the PFs overlap at the middle of the cell varies from species to species. The overall morphology of spirochetes, with their flagella located internally, raises the long standing questions: How do these bacteria swim and how do they carry out chemotaxis?

MORPHOLOGY

***B. burgdorferi* as a model spirochete**

Bb fits the morphological description of a spirochete (Figure 1b). Both light and high voltage transmission electron microscopy (TEM) indicate that *Bb* has a flat-wave morphology (32, 33) (Supplemental Figure 1, Supplemental Movie 1. See the Supplemental Material link in the online version of this article or at <http://www.annualreviews.org/>). This planar wave form is similar to that of the syphilis spirochete *Treponema pallidum* (22) but differs from the many helically shaped members of the phylum such as the Leptospiraceae (69). *Bb* is long and thin, with a length between 10–20 μm and a diameter of approximately

300 nm (32). Its shape is characterized as a planar, regular, periodic undulation of the cell body, with an amplitude of 0.78 μm and a wavelength of 2.83 μm .

Ultrastructure of *B. burgdorferi*

The fine structure and general composition of *Bb* has been analyzed using several approaches. The composition of the *Bb* OM is unusual. It lacks lipopolysaccharide, but contains lipids uncommon in bacteria such as cholesterol (8). Cryo-ET has been extensively used to analyze cell structure (17, 41, 51). The OM also has a large number and variety of surface exposed lipoproteins that are seen as a layer surrounding the cell by Cryo-ET (51). The OM has a width of 4 nm and tightly fits around the PC. At the PC surface, the peptidoglycan forms a wall approximately 6.8 nm wide (51). As discussed below, for the spirochete to form a dynamic flat-wave and carry out its remarkable motions as it swims, the wall must be astonishingly flexible. Little is known about the structure of the *Bb* peptidoglycan other than that it contains alanine, glycine, glutamic acid, and ornithine (7), and nothing is known about what imparts its highly flexible nature. Inside the wall layer is the 4 nm wide protoplasmic cell membrane which encloses the cytoplasm.

Periplasmic flagella *in situ*

Between 7–11 PFs are subterminally inserted at each end of the PC (Figures 1b, 2) (15, 37, 41, 100). These PFs are structurally similar to the flagella of other bacteria. Each PF is composed of a motor, hook, and filament. The flagellar motors are linearly inserted along the long axis of the cell at each end, and are 90 to 120 nm apart from each other (17, 41, 51, 100). The flagellar motor that is closest to the cell tip is approximately 150 nm from that end. Although previous TEM analyses indicate that the PFs form a bundle (32, 59), recent results with Cryo-ET indicate that the flagellar filaments instead form an elegant ribbon in the PS (17) (Figure 1b). The minimum thickness of the ribbon is equivalent to the diameter of a PF (16–18 nm) (37, 41), but the ribbon can be thicker and multilayered. The filaments are closely packed with approximately 3 nm of space between them. The ribbon also increases the thickness of the PS so that the OM bulges in its vicinity (17, 41, 51). Thus, in regions of the cell where there are no PFs, the PS thickness is approximately 16 nm. In contrast, the PS thickness is 42–49 nm in domains containing PFs (17, 51). High voltage TEM and Cryo-ET indicate that the ribbons wrap around the body axis (the longitudinal center of the cell, as if the cell is a sausage) in a right-handed sense (moving away from an observer, a right-handed helix spirals clockwise [CW], and a left-handed helix spirals counterclockwise [CCW]) (17, 32). However, along the cell axis (i.e., the abscissa, as the cell is in the form of a flat-wave, i.e., a sine-wave), the PFs form a left-handed helix with a helix pitch (a complete helical turn) of approximately 2.83 μm , which is equivalent to the periodic wave-length of the cell (32). Recent results suggest that in short cells (10 μm in length) there are more PFs in the cell center than in long cells (20 μm in length) (C. Li, unpublished). Evidently, the rate of growth of a given flagellar filament decreases as the cells become longer. Furthermore, the two opposing ribbons overlap in the central region. It is not clear whether the two ribbons form a continuous ribbon, or whether the ribbons are on opposite faces of the cell (32, 41, 89).

Periplasmic flagella structure and composition

Purified PFs have been analyzed in some detail. At neutral pH, PFs are left-handed and have a helix diameter of 0.28 μm and pitch of 1.48 μm ; however, a small fraction is seen with a helix diameter of 0.8 μm and a pitch of 2.0 μm (16, 24). The flagellar filaments consist of a major 41 kDa FlaB protein and a minor 38 kDa FlaA protein (15, 31). FlaB shows sequence identity to FlaB of other spirochetes and to the flagellar filament proteins of other bacteria such as FliC of *Escherichia coli*. Sequence analysis indicates that FlaB is likely to be excreted into the PS by the Type III flagellin-specific pathway (15). FlaA has sequence

identity to FlaA proteins that are present in PFs of other spirochetes. In contrast to FlaB, FlaA is likely to be excreted to the periplasm by the SecA-mediated pathway (15). In most spirochete species, FlaA forms a sheath that surrounds the FlaB core. The function of FlaA is presently unknown, but recent work on the spirochete *Brachyspira hyodysenteriae* is instructive (46). *flaA* mutants of *B. hyodysenteriae* have unsheathed PFs, are still motile, but swim more slowly than the wild-type. In addition, the PFs from these mutants have a different helix pitch and diameter and are less rigid than those from the wild-type. Thus, FlaA interacts with FlaB to augment filament shape and rigidity for optimal motility. However, in *Bb*, so little FlaA is made relative to FlaB that only a small region of the filament has a sheath (31). TEM analysis of the wild-type compared to the *flaA* mutant indicates that the FlaA sheath is localized adjacent to the flagellar hook (Shibata S., S. Aizawa, M. Motaleb, N. Charon, unpublished). Perhaps flagellar rigidity near this region is essential for optimal PF rotation and motility.

Cytoskeletal function of the periplasmic flagellar filaments

The analysis of specific mutants indicates that the PFs have a major role in the overall morphology of *Bb*. First, mutants in the following genes form a straight rod instead of a flat-wave (Supplemental Table 1): *flaB*, which encodes the major flagellin protein; *flgE*, which encodes the flagellar hook; *fliF*, the motor protein which encodes the motor MS ring; and *fliG2*, which encodes a motor protein of the switch complex (47, 59, 80). A mutant that overproduces the regulatory protein CsrA also forms a rod (see below) (89). Ultrastructure and western blot analyses indicate that the one common deficit of all these mutants is the lack of flagellar filaments and filament proteins. Thus, the ability to form flat waves positively correlates with the presence of flagellar filaments and FlaB. Second, a mutant was recently isolated that has a shorter cellular wave length and a smaller amplitude than that those of the wild-type (89). This mutant, which has a deletion in the *csrA* regulatory gene, produces PFs that are considerably longer than those of the wild-type. Cryo-ET analysis revealed that the flagellar ribbons in *csrA* mutants wrap around the PC more tightly than in wild-type cells with a smaller helix pitch and diameter. In addition, the two ribbons interdigitate with one another. These results indicate that increasing the length of PFs leads to cells with an altered flat-wave morphology (see below). Taken together, the results with the many filament-deficient mutants and the *csrA* deletion mutant strongly support the concept that the PFs influence cell shape by having a skeletal function.

Why is *B. burgdorferi* a flat-wave?

How do the PFs exert their skeletal function? Bacterial cells and flagella are elastic materials. When forces are applied, these structures deform, and when the force is removed, these structures revert back to the original shape. Since the PC of *Bb* is a straight rod when the PFs are not present, and purified PFs are helical, evidently the interaction between the two components leads to the flat-wave morphology. The helix pitch of the PFs *in situ* (2.83 μm) is markedly different from those that are purified away from the cell body (1.43 μm and 2.0 μm) (16, 24, 32). For the PFs to be contained within the PS, these structures must deform. The force that produces this bending of the PFs comes from the PC. Concomitantly, the PFs exert an equal but opposite force back onto the PC, which causes the cell to bend into its characteristic shape. Mathematical modeling, which takes into consideration the elastic properties of the PFs and PC, has shown that the balance between these two forces is a cell with a flat-wave shape—it is not intuitive! However, this force balance only conspires to produce the correct wavelength and amplitude if the PFs are in the helical conformation that is observed less frequently in purified PFs (helix diameter of 0.8 μm and a pitch of 2.0 μm) (24). How does the *csrA* deletion mutant that has longer PFs cause the cell to have a shorter wave-length and cell amplitude (89)? One possible explanation is that the increase in length of the PFs, and perhaps the interdigitation characteristic of the PF ribbons in the

mutant, cause the PFs to flip into the helical state that is observed more frequently in purified PFs. This change in preferred conformation of the PFs is predicted to produce a flat-wave shape with a smaller wavelength and amplitude.

Hook and motor structure

The flagellar hook region in *Bb* and certain other spirochetes has some unique attributes. In other bacteria, the function of the hook is to serve as a universal joint connecting the flagellar filament to the motor (81). In *Bb*, the hook is a 61 nm long hollow tube consisting of approximately 120 FlgE units (80). As already mentioned, inactivation of *flgE* results in cells that lack flagellar filaments, are non-motile, and are rod shaped (80). In most bacteria, the FlgE subunits are held together by hydrophobic interactions that are readily dissociated by denaturing agents. Surprisingly, FlgE in *Bb* and other spirochetes forms a stable, high molecular weight complex (49, 80). Several lines of evidence indicate that FlgE subunits in *Bb* are covalently cross-linked (80) (K. Miller, M. Miller, N. Charon, unpublished). The flagellar hook and filament are likely to be under greater stress in spirochetes compared to other bacteria, as the PFs deforms the PC while they rotate (see below, dynamics of motility). Perhaps the hook proteins are cross-linked in spirochetes for added structural strength to perform this function.

The incredible methodology of Cryo-ET is being exploited in analyzing the intact *Bb* motor (42, 51), as well as other spirochetal motors (50, 66). The overall structures of spirochetal motors are similar to each other, consisting of the MS ring, the C ring, the rod, the export apparatus and the stator (Figure 3, Supplemental Movie 2). *Bb* exhibits a relatively large C ring ~57 nm in diameter, compared to the 45 nm C ring of *E. coli* and *S. enterica* serovar Typhimurium (90). This enlarged C ring shares similar features with the *S. enterica* structure: a cylindrical structure with a slightly bulky bottom and a Y-shaped extension at the top (90). Thus, the C ring is likely composed of multiple copies of proteins FliG, FliM and FliN arranged in a manner similar to that of *E. coli* and *S. enterica*.

The stator, encoded by the *motA* and *motB* genes, is the motor force generator embedded in the protoplasmic cell membrane. The visualization of the stators in many species of bacteria is difficult (19), and this difficulty may in part be the result of the stator being dynamic and that its functional units freely interchange between the motors ‘circulating’ in the protoplasmic cell membrane (43). In contrast, the stators of *Bb* and other spirochetes are clearly visualized (42, 50, 51, 66). Cryo-ET analysis of *motA* and *motB* mutants of *Bb* provide the first structural evidence that these genes encode the sixteen stators radially arranged around the rotor (Figure 3, Supplemental Movie 2) (X. Zhao, T. Boquoi, M. Motaleb, J. Liu, unpublished). Conceivably, the stators in *Bb* and other spirochetes are relatively stable, or unit interchange occurs without disrupting the overall arrangement of the stator. Remarkably, the cytoplasmic domain of the stator is adjacent to the C-terminal domain of the C-ring rotor protein FliG. This stator-rotor interaction evidently induces an unexpected conformational change of FliG, and this change is likely to be a fundamental mechanism for flagellar rotation (X. Zhao, T. Boquoi, M. Motaleb, J. Liu, unpublished).

The *Bb* flagellar motor is a remarkable and complicated nano-machine, consisting of at least 20 different proteins (Figure 3, Supplemental Movie 2). To understand the flagellar motor assembly and function, it requires the determination of the structural and functional roles of all flagellar proteins. Fortunately, many mutants in motor genes have been constructed either by transposon mutagenesis or in-frame deletion (Supplemental Figure 2, Supplemental Table 1). *fliE* mutants are defective in rod assembly, and *fliM* mutants are deficient in the middle and the bottom of the C ring (X. Zhao, T. Boquoi, A. Manne, M. Motaleb, J. Liu, unpublished). *fliI* and *fliH* mutants are defective in the assembly of the bottom part of the export apparatus (X. Zhao, T. Lin, S. Norris, unpublished). FliL proteins localize between

the stator and rotor, and these proteins are evidently involved in the proper orientation of the PFs within the PS (62). *flgI* mutants fail to form a hollow, torus-shaped structure around the rod. However, in contrast to other bacteria, these *flgI* mutants are still fully motile; evidently the P ring is not required for flagellar rotation (51). Instead, a spirochete specific “collar” may function as a bushing that facilitates the rotation of the flagellum without disruption of the surrounding peptidoglycan layer. The identities of the specific genes encoding the “collar” have yet to be identified. In sum, the structure of the *Bb* flagellar motor is being characterized in some detail, and specific motor proteins and function are being correlated with specific genes *in situ* for the first time.

DYNAMICS OF MOTILITY

Swimming *in vitro*

Given the structure of *Bb*, the obvious first question with respect to their motility is what do swimming cells look like? Also, given that the PFs are located in the PS, how does rotation of the PFs drive motility? The swimming behavior of *Bb* is more complicated than that of other bacteria as a consequence of their complex geometry. *Bb* has four motility modes based on the direction of flagellar rotation: two run modes (with either end leading), and two non-translational modes (Figure 4, Supplemental Movie 3). In an isotropic environment, a given cell runs at least 90% of the time (M. Motaleb and N. Charon, unpublished). During a run, waves are propagated from the front to the back at a frequency of 5–10 Hz at room temperature. In contrast to eukaryotic flagella, propagating waves are full size at the anterior end instead of starting as small bends and increasing in size. These waves propagate at a speed of 34 $\mu\text{m}/\text{sec}$ relative to the cell (33). The cells swim at a mean speed of 4.25 $\mu\text{m}/\text{sec}$ in a pure liquid (33). The ratio of the swim speed to wave propagation is 0.12, i.e., in the time it takes a wave to travel the length of the cell, the cell advances 12% of its length through the medium. Thus, as *Bb* swims, waves are clearly evident as they swim in a given direction.

How are the waves generated, and what is the basis for the non-translational forms? Everything points to the PFs. We know that they are essential for motility, they rotate, they are helical shaped, and they have a skeletal function. Although it has not been directly proven, the following model is proposed (Movie S4) (32) (15): During a run, the PFs of the anterior ribbon are predicted to rotate CCW, and those of the posterior ribbon rotate in the opposite CW direction (as a frame of reference, the PFs are viewed from their distal end to where they insert in the PC). Thus, the PFs of the two ribbons rotate asymmetrically relative to one another during a run. This rotation causes backwardmoving waves to be propagated down the length of the cell. Reversals occur when the PFs of both ribbons change the direction of rotation. In addition, recent mathematical modeling suggests that during translation, the rotating PFs are not in direct contact with the PC, but rather there is a thin layer of fluid that separates these filaments from the PC (102). Forces between the PFs and the PC are therefore mediated by viscous forces in the fluid present in the PS. This hypothesis is based on the known elasticity of the PFs, PC, and the dynamics of swimming cells. The modeling predicts that the thin layer of fluid in the PS is essential for the smooth backward propagating waves noted on translating cells. Otherwise, if the PFs directly interacted with the PC, the PFs would be predicted to easily tangle up with one another and the propagating wave would not be as regular as is observed. Several questions arise with the overall model of translating *Bb*. If the PFs are rotating CCW along the axis of the cell as viewed from behind the cell, is the cell rolling about the body axis CW to balance that torque as predicted (15, 32)? What is happening to the OM? Cryo-ET indicates that it is held very close to the PC and even bulges in the vicinity of the PF ribbon. How is the tight-fitting achieved, especially in light of evidence that the OM is a lipid bilayer? Are there bonds that form between the OM and PC, and are those bonds broken as the PFs rotate?

In the non-translational modes, the prediction is that the PFs from both ribbons rotate in the same direction: both CW or both CCW. This stopped interval is referred to as a flex and is thought to be analogous to the tumble seen in externally flagellated bacteria. The cells often become quite distorted during this interval and may bend in the middle. It is difficult to distinguish between cells with PFs at both ends rotating CCW and those with PFs both rotating CW. It is not clear exactly what is occurring in the PS that causes the cell to form a distorted morphology. Do the PFs from the opposite end wind around one another to cause the distorted shape? In other spirochete species, the distortion associated with flexing only occurs if the PFs overlap in the center of the cell (33), so this is a possibility.

The description of swimming given above largely comes from motility assays in liquid environments. Many of the natural environments that *Bb* encounters do not behave like a liquid. For example, spirochetes are deposited into the dermis of the mammal via the saliva of the tick. The dermis of the mammal is largely composed of a cross-linked collagen network. This gel-like environment responds to external forces from the bacterium with a combination of elastic and viscous forces; i.e., it is a visco-elastic material. Liquid media, on the other hand, only responds with viscous forces. One of the first investigations into the effect of viscoelasticity on the motility of *Bb* examined swimming in solutions of methylcellulose or hyaluronic acid (39). Swimming speed increases substantially as the concentration of methylcellulose or hyaluronic acid is increased. The increase in swimming speed with the viscoelasticity of the environment suggested that *Bb* motility may be optimized for migration through host tissue. In addition, *Bb*, like several other spirochete species, can translate in highly viscoelastic medium that markedly slow or stop the motility of other flagellated bacteria. Liquid media and methylcellulose solutions, though, are poor facsimiles for many of the natural environments that *Bb* encounters. For one, collagen in the dermis forms a gel-like network (i.e., it is more elastic than viscous), whereas low concentration methylcellulose solutions (less than a few percent) are viscoelastic fluids (i.e., more viscous than elastic) (12). The natural environments are differentiated further from liquid media and methylcellulose solutions because they contain cells and various extra-cellular matrix components, such as collagen, fibronectin, and decorin, to which *Bb* binds. *Bb* in gelatin exhibited four motility states, which are determined by transient adhesion between the bacterium and the matrix (M. Harman, S. M. Dunham-Ems, M. J. Caimano, A. A. Belperron, L. K. Bockenstedt, H. Fu, J. D. Radolf, and C. W. Wolgemuth, submitted). In addition to adhering to these substrates, spirochetes also migrate through the matrix, even though the pores in the gelatin matrices are significantly smaller than the diameter of the bacteria. As in previous reports, the undulation and migration speed of the bacteria depend strongly on the physical properties of the environment; however, the bacteria always swim slower in gelatin than in liquid media. Therefore, the unique motility mechanism of *Bb* enables it to penetrate dense tissues in its hosts, but the speed of the bacteria may not be enhanced in these natural environments.

Swimming *in vivo*

We are at an early stage in sorting out the role of *Bb* motility *in vivo*. One of the major breakthroughs is the ability to track green fluorescent protein (GFP)-tagged virulent *Bb* *in vivo* using intravital microscopy (IVM) and fluorescence spinning disc confocal microscopy. Soon after injection of *Bb* in mice, spirochetes are seen interacting with capillaries and veins (58). The movement of the organisms is stop and go, with the stops characterized by adherence to the endothelium. Approximately 90% of interactions with the endothelium are less than 1 sec, and about 10% are 1–20 sec. These interactions are mediated in part by the spirochete surface protein BBK32 and host fibronectin and glycosaminoglycans (67). In contrast, approximately one per cent of the cells are stationary with one end attached and partially embedded in the endothelium, often at the endothelial

cell junctures, with the other end gyrating. These stationary cells resemble the often seen tethered spirochetes whereby the cells adhere to stationary ligands via an OM surface associated component yet remain motile (18). The attached spirochetes eventually escape the endothelial cell lining and penetrate into connective tissue. In the liver, the spirochetes first interact with Kupffer cells, which then trap, immobilize, engulf the spirochetes, and then present antigens to the iNKT cells (44). The role of motility and chemotaxis in both the adherence and penetration through blood vessels, and in the interaction of immune cells in the liver is likely to be important. Future experiments with specific motility and chemotaxis mutants should be definitive.

Some remarkable imaging studies have examined GFP tagged *Bb* in the tick host both before and after feeding on mice (26). In unfed nymphs, *Bb* cells are non-motile and distributed throughout the midgut. By 72 hours after feeding on mice, the density of *Bb* is high; however, the spirochetes remain non-motile but are viable. Remarkably, the presence of a diffusible factor(s) in the tick midgut is likely to be responsible for their non-motility. At 72 hours, only a small number of the spirochetes traverse the midgut basement membrane, enter the hemocoel, and colonize the salivary glands for transmission to mammals. These invasive spirochetes are motile. Why is *Bb* motility specifically inhibited in the tick midgut? Perhaps the tick developed a means of defense by inhibiting *Bb* motility to keep the infection localized. Future experiments to characterize the inhibitory factors should be enlightening.

CHEMOTAXIS

Chemotaxis (movement toward or away from a chemical stimulus) in spirochetes is unique. Bacteria undergo a biased random walk during chemotaxis, and this walk is achieved by modulating the direction of rotation or speed of flagellar rotation (73, 84, 94). A two-component regulatory system mediates the direction or speed of flagellar rotation, and this variation leads to the biased walk up a gradient of attractant or away from a repellent. In the paradigmatic chemotaxis model of *E. coli* and *S. enterica*, variation in motor direction is determined as follows: The response regulator CheY is phosphorylated by the histidine kinase CheA to form CheY-P. CheA is part of the polarly located methyl-accepting chemotaxis protein (MCP) receptor signal complexes. The probability that CheA phosphorylates CheY is a function of the occupancy level of an attractant or repellent that binds directly or indirectly to the MCPs in the PS. CheY-P diffuses through the cytoplasm and interacts with the flagellar switch protein FliM, causing the motor rotational biases to shift from the default rotation of CCW to CW. If all the motors rotate CCW, the cell swims. If one of the motors rotates CW, the cell tumbles (92). The motors are within 1 μm of the MCP complexes. Thus, CheY-P can diffuse and bind to the motors within 0.1 sec (93). The CheY-P formed has phosphatase activity associated with it. However, dephosphorylation of CheY-P, which restores the default CCW behavior such that the cell can immediately respond to changes in the environment, is enhanced by the action of the CheY-P-specific phosphatase CheZ.

Chemotaxis in *Bb* is different from this and other well-studied bacterial chemotaxis models. Two conundrums are evident. First, these spirochetes have two sets of the flagellar motors: one at each cell end. As noted above, these sets of motors are located at a considerable distance from one another (between 10–20 μm). In addition, recent results using fluorescent antibodies and Cryo-ET indicate that the MCP complexes are subpolarly located at each cell end (100) (Figure 2). If CheY-P is formed at one end of the cell at these complexes, it could readily diffuse to the adjacent motors. However, given a cell length of 10 μm , it would take at least 10 seconds for CheY-P to diffuse to the motors at the other end of the cell (64). Because the speed of these bacteria is at least 4 μm per second (33), simple diffusion of

CheY-P to the opposite end of the cell to coordinate the rotation of the PFs is unlikely. Furthermore, in *Bb*, instead of runs and tumbling, there are four swim configurations based on the direction of flagellar rotation (Figure 4), all of which contribute to a higher level of complexity in swim behavior than most other bacteria. Thus, the first conundrum: how does *Bb* coordinate the rotation of the PFs within the ribbons at both cell ends to effect chemotaxis?

Second, for the bacteria to swim in a given direction, the motors at each end must rotate in the opposite direction from those at the other end, i.e., if the PFs in one ribbon rotate CCW, the others rotate CW. This asymmetrical rotation is markedly different from the swimming behavior of *E. coli* and *S. enterica* whereby all the motors rotate in the same direction during translation. Furthermore, as discussed below, in the absence of a functional CheY response regulator, *Bb* constantly runs. Thus, the second conundrum, what is the basis for asymmetrical rotation of the PFs during translational swimming in the absence CheY?

Although significant progress has been made in understanding *Bb* chemotaxis, a palpable molecular model putting it all together is still in the developmental stage. To address the above questions, a capillary tube chemotaxis assay was developed using flow cytometry to enumerate cells, and several chemically defined chemoattractants were identified (4, 60). These attractants include *N*-acetylglucosamine, glucosamine, glucosamine dimers, and glutamate. The assay allowed for testing whether specific genes are involved in chemotaxis. *Bb* has multiple copies of chemotaxis genes including the following: 6 *mcp* (2 lack membrane spanning regions), 2 *cheA*, 3 *cheY*, 2 *cheB*, 2 *cheR*, and 3 *cheW* (29). It has only one CheY-P phosphatase gene commonly found in bacteria, *cheX*, which has a similar activity as *cheZ* found in *E. coli* (61, 71). The results of extensive gene targeting analysis indicated that the chemotaxis pathway involves MCPs, CheW3, CheA2, CheY3, and CheX (Supplemental Figure 2, Supplemental Table 1). Biochemical analysis supports this conclusion: CheA2 readily phosphorylates CheY3 (64), and CheX dephosphorylates CheY3-P (61, 71). Interestingly, the half-life of the CheY3-P is 10 minutes (64), which is considerably longer than most CheY-P of other bacteria such as *E. coli*, which is a few seconds (36). Most important, because CheY plays such a pivotal role in the chemotaxis of all known species of bacteria, CheY3 is concluded to be the key chemotaxis response regulator in *Bb*.

An analysis of the swim behaviors of the chemotaxis mutants may yield a clue to the basis of asymmetrical rotation of the flagellar ribbons during translational motility. Both *cheA2* and *cheY3* mutants constantly run in one direction (45, 64) (Supplemental Table 1, Supplemental Movie 3). These results are similar to those of other bacteria such as *E. coli* and *S. enterica*; *cheA* and *cheY* mutants in these bacteria also constantly run. However, in *Bb* the motors rotate asymmetrically at one end relative to the other during a run, whereas in *E. coli* all the motors are rotating CCW. Evidently, in the default state, i.e., no CheY3-P being present, the motors at either cell end are different from the other, as they rotate in opposite directions. Perhaps there is a protein that interacts with motors at one cell end such that in the default state, the PFs rotate in the opposite direction relative to those at the other end. There is precedence for protein localization of this type, as in some bacteria specific proteins localize at the old cell end and not at the new cell end (6). A candidate protein is FliG1 (47). *Bb* and many other spirochetes have two *fliG* genes encoded in their genomes, *fliG1* and *fliG2* (29, 47). FliG is involved in motor rotation in other bacteria, is essential for flagellar assembly and motility, and it plays a major role in determining the direction of motor rotation (52). In *Bb*, *fliG2* likely plays this role, as mutants in this gene are non-motile and lack PFs; FliG2 has all the essential sequence and structural domains common to other bacterial FliGs (47). However, FliG1 lacks some of the essential residues common to FliG of other bacteria. Mutants in *fliG1* are still motile but are unable to swim in highly viscous

media containing methylcellulose; only one of the cell ends is seen to be able to gyrate. Interestingly, GFP tagged FliG1 localizes at only one cell end (47). It will be interesting to determine if FliG1 is in part responsible for the asymmetrical rotation of the PFs.

The phenotypes of other specific mutants make the picture quite complicated. The *cheX* mutant constantly flexes (61), which is analogous to the constantly tumbling *cheZ* mutants of *E. coli* (10). A *cheX* mutant is predicted to have a higher CheY3-P than the wild-type. One expectation of the protein localization model is that cells with a high CheY3-P should also run, not flex. For example, those motors that run CW in the default state should reverse and rotate CCW under high CheY3-P concentration, and those motors that run CCW in the default state should run CW. Perhaps there is another CheY-P phosphatase that has not been identified in *Bb*, and as result, the *cheX* mutant could have an intermediate level of CheY3-P that results in flexing. In *Bacillus subtilis*, FliY and CheC have CheY-P phosphatase activity, but homologs of these genes are lacking in *Bb* (65). *Bb* also has a gene encoding CheD. CheD in other bacteria augments the phosphatase activity of CheC, binds to and deamidates glutamine residues on MCPs, and regulates the activity of CheA kinase. Mutants in *cheD* in other bacteria have a decreased activity of CheA kinase leading to lower levels of CheY-P (74). CheD may have a similar function in *Bb*, as a *cheD* mutant has a non-chemotactic, constantly running phenotype, which is similar to the *cheY3* and *cheA2* mutants that are also expected to decrease CheY3-P concentration (M. Motaleb and N. Charon, unpublished). However, because CheD in other bacteria has additional activities besides activation of the CheA-kinase, its role in *Bb* chemotaxis is presently unclear. The second messenger, 3',5'-cyclic-diguanosine monophosphate (c-di-GMP) is emerging as a major factor influencing motility and possibly chemotaxis (see section on regulation, below). Certain mutants involved in c-di-GMP synthesis, degradation, and effector binding have aberrant swim behavior, such that some mutants constantly run (*pdeA*), while others have a high flex rate (*pdeB*) (86, 87). c-di-GMP and its effector protein are known to bind to the flagellar motor protein in other bacteria and influence the direction and speed of flagellar rotation (9, 21, 27, 70). At this time it is difficult to sort out how c-di-GMP influences *Bb* swimming behavior on a molecular level.

The results accumulated point toward the following conclusions: First, CheY3 is the major response regulator, and the pathway leading to its phosphorylation and dephosphorylation involves MCPs, CheW3, CheA2, CheX, and possibly CheD. Second, the asymmetrical rotation of the PFs in the default state can best be explained by differences in the motors at each end of the cell, but support for this hypothesis awaits experimental evidence. Third, it is too early to understand the basis of flagellar coordination mediated by CheY3 that results in chemotaxis, although many hypotheses are conceivable. As previously mentioned, diffusion of CheY3-P from one cell end to the other is too slow to coordinate the rotation of the PFs at the distal end. As an alternative, perhaps there is a cytoskeletal structure whereby CheY-P is able to move rapidly from one end to the other. There is precedence for this possibility: In *Myxococcus xanthus*, the protein AglZ mediates gliding motility by moving from one cell end to the other by way of the MreB cytoskeleton (55). Because completion of this internal cell migration of AglZ is on the order of several minutes, a CheY3 transport system in *Bb* would have to be considerably faster. Another possibility for coordinating the PFs at each end relates to a possible mechanosensing mechanism. Perhaps there is an interaction of the PFs at one end of the cell with those at the other such that rotation of the PFs at that end influences the rotation of the PFs at the other end. This possibility is conceivable, as the PFs in *Bb* overlap in the center of the cell. However, this hypothesis does not apply to all spirochetes, as *L. interrogans* has PFs that are short and do not overlap (97), yet these spirochetes are chemotactic (105) (N. Takahashi and N. Charon, unpublished). CheY3-P could act at another, unknown site such that the membrane potential is altered when the cell is undergoing chemotaxis, and this change in potential might allow

coordination of the PFs (35). Alternatively, perhaps CheY3-P together with c-di-GMP coordinate PF rotation. Finally, perhaps there is no internal signal that coordinates the motors at both cell ends: Flagellar coordination and chemotaxis are achieved by the attractant binding to either one or both of the MCP clusters at the cell ends. The change in CheY3-P concentration generated by this binding specifically affects the direction of rotation of the motors that are adjacent to those MCPs. For example, if the attractant binds the MCPs at one cell end, it causes the motors only at that end of the cell to change their direction of rotation, and the cell flexes. In contrast, if attractant molecules simultaneously bind to the MCPs at both cell ends, the motors at both ends change directions and the cell runs. In closing, now that specific compounds that serve as attractants are known, and that CheY3 is the functional response regulator, the basis for coordination of rotation of the PFs for chemotaxis can finally be determined.

TRANSCRIPTIONAL AND TRANSLATIONAL REGULATION OF MOTILITY AND CHEMOTAXIS GENES

The genetic map indicating the genes that are involved in motility and chemotaxis is presented in Supplemental Figure 2. Most motility genes involved in motility are present in single copies (the only exception is *flhG*, the motor control protein). As previously noted, there are multiple copies of chemotaxis genes. The analysis of mutants described above indicates that the *Bb* cluster *cheA2-cheW3-cheX-cheY3* is involved in chemotaxis under standard laboratory conditions. This cluster is closely related to chemotaxis gene systems in the other spirochetes and is probably inherited from a common ancestor (15, 45, 98). The other cluster consisting of *cheW2-bb0566-cheA1-cheB2-bb0569-cheY2* may be a recent gene transfer from the Proteobacteria (45, 98). The function of this second cluster is not known, but it may be involved in chemotaxis under different environmental conditions.

The regulation of the motility and chemotaxis genes of *Bb* is unique. In other bacteria, there is cascade control of gene regulation of motility gene expression (20). For example, at least 50 genes are involved in the motility and chemotaxis of *E. coli* and *S. enterica*, and these genes fall into three classes (class I, II and III), which are under the tight regulation of a transcriptional hierarchy. Within this regulatory cascade, the class I master regulator (FlhDC), in conjunction with the house-keeping sigma factor (σ^{70}), direct RNA polymerase to initiate the transcription of the class II genes. Class II genes encode the structural proteins involved in the motor-hook complexes, and two regulatory elements: the flagellum-specific sigma factor FliA (σ^{28}), and the antagonist of FliA, (anti- σ^{28}), FlgM. Prior to the completion of the hook assembly, FlgM binds to FliA to prevent premature synthesis of the class III genes encoding the flagellin and the chemotaxis proteins. Upon completion of the hook structure, FlgM is excreted by the flagellum-export apparatus, thereby allowing FliA to initiate the transcription of the class III genes. The last step allows for completion of flagellar assembly and chemotaxis gene expression. In contrast, *in silico* analysis indicates that no FliA, FlgM, or σ^{28} promoter consensus sequences are present in the genome of *Bb* (15, 29). All of the motility and chemotaxis genes identified thus far fall under the regulation of the house-keeping σ^{70} (Supplemental Figure 2). These and other results indicate that *Bb* does not employ a transcription cascade to regulate its motility genes. Instead, these genes are primarily regulated by a post-transcriptional mechanism. Two key studies stand out that lead to this conclusion. First, in a *flaB* filament mutant, the amount of FlaA synthesized is only 13% of the wild-type level despite equivalent levels of *flaA* transcript (63). Second, in a *flgE* mutant, FlaA and FlaB accumulation are decreased by more than 80%, whereas the levels of their respective encoding mRNA are equivalent to those of the wild-type (80). Furthermore, although FlaA is slowly degraded in the *flgE* mutant, there is no turnover of FlaB. The conclusion reached is that translational control and not protein turnover is

responsible for the lack of accumulation of FlaB and possibly that of FlaA in the *flgE* mutant.

Recent experiments on the *Bb* carbon storage regulator A (CsrA) indicate that it is a major regulator of translational control of FlaB (89). The Csr system is present in many bacterial species (3, 76), and its importance in carbon metabolism, virulence, biofilm formation, and motility is well established (2, 53, 76). In *E. coli*, the Csr system is comprised of an RNA binding protein, CsrA, two non-coding RNAs (CsrB and CsrC), and a regulatory protein, CsrD. CsrA functions through binding to the consensus sequence RUACARGGAUGU, which is present within the leader region of its targeted transcripts and subsequently regulates gene expression post-transcriptionally (25, 57). In *E. coli*, CsrA positively regulates the flagellar synthesis by serving as an activator of *flhDC* expression (95). However, in *B. subtilis*, CsrA has an opposite effect, as it negatively regulates flagellin synthesis at a post-transcriptional level by binding to mRNA and inhibiting translation (101). In a similar manner, *Bb* CsrA is a negative regulator of FlaB. CsrA binds to two sites present in the leader region of the *flaB* transcript with one of them overlapping the Shine-Dalgarno (SD) sequence. Binding of CsrA to these regions leads to translation inhibition of FlaB, presumably via blocking access of ribosomes to the SD sequence of *flaB* mRNA. Thus, the amount of FlaB in a cell may be controlled on the translational level by CsrA (89).

The two component regulatory system called HK1/Rrp2 is involved in motility gene expression in *Bb* (40, 68, 99). Acetyl phosphate, an intermediate product from acetate metabolism, autophosphorylates Rrp2, the response regulator of the Hk2/Rrp2 and in doing so activates RpoN, a ⁵⁴ transcription factor. RpoN in turn upregulates RpoS (the Rrp2-RpoN-RpoS pathway) (99). CsrA serves as a repressor of phosphoacetyltransferase (Pta), one of the key proteins in acetate metabolism, and indirectly modulates the level of acetyl-phosphate in *Bb* as well as the subsequent activation of the Rrp2-RpoN-RpoS pathway (88). Microarray analysis of mutants in *rrp2*, *rpoN*, and *rpoS* has revealed a potential role for the Rrp2-RpoN-RpoS network in the regulation of chemotaxis gene expression (14, 28, 68). For instance, the transcription of eight chemotaxis genes is regulated by the Rrp2-RpoN-RpoS network (68). Interesting, Rrp2-RpoN-RpoS activity is maximally induced under conditions that mimic the mammalian host environment (103). Regulation of chemotaxis proteins via the Rrp2 pathway may allow the spirochete to modulate its chemotaxis genes expression while being transmitted between different hosts to aid in colonization as well as dissemination.

Recent studies in *Bb* indicate a link between the signaling molecule second messenger c-di-GMP and cell motility (40, 72, 86, 87). In *Bb*, the c-di-GMP metabolism pathway consists of Rrp1, a sole-diguanylate cyclase (40, 79), two phosphodiesterases PdeA and PdeB (86, 87), and PlzA (30, 72), a c-di-GMP binding protein. A mutation in any of the genes of the c-di-GMP pathway alters both cell motility and chemotaxis, but many different phenotypes are generated. For example, disruption of Rrp1 causes cells to constantly run and have an attenuated chemotactic response (40). Mutations of in the phosphodiesterase genes result in two different phenotypes: a *pdeA* mutant runs and pauses but fails to reverse, and a *pdeB* mutant has increased flexing frequency (86, 87). A *plzA* mutant has a defect in motility in agar but has the wild-type swimming behavior (72). Remarkably, the *plzApdeB* double mutant constantly flexes (86). Taken together, c-di-GMP clearly affects motor function in *Bb*, but as previously stated, how it is all integrated is unclear.

In summary, our understating of the regulation of chemotaxis and motility gene expression in *Bb* is still at its infancy. Translational control of motility gene expression appears to be crucial, with CsrA and c-di-GMP emerging as major regulators of chemotaxis and motility. Many questions remain unanswered. What is the interplay between the identified regulatory

elements in modulating gene expression? How do environmental cues play a role in differential gene expression within the two hosts, and what are those cues?

VIRULENCE AS RELATED TO CHEMOTAXIS AND MOTILITY

The role of motility and chemotaxis during the infection and the disease processes have been examined in several species of pathogenic bacteria. In many species, mutant analysis indicates that motility and chemotaxis are essential for infection and invasion (13, 38, 56, 104). Among the spirochetes, motility deficient mutants of *B. hyodysenteriae* are attenuated in a mouse model of swine dysentery (78). In *Treponema denticola*, motility and chemotaxis deficient mutants are less invasive than their parent in an oral epithelial cell line based model (54). Recent results suggest that motility is essential for *L. interrogans* to cause disease in the hamster model for leptospirosis (E. Wunder and A. Ko, unpublished).

Motility and chemotaxis of *Bb* are likely to be important factors for *Bb* to survive in nature. Genomic analysis suggests that over 50 genes (5–6% of the genome) are potentially involved in motility and chemotaxis (15, 29). In addition, approximately 10% of the total cellular protein is FlaB (63). These results imply *Bb* dedicates a significant proportion of its energy to motility and chemotaxis, and they further support the concept that these processes are necessary for the survival of the spirochete. In addition, during mammalian infection, and when growing *Bb* under conditions that mimic in vivo conditions, motility and chemotaxis proteins are up-regulated and some are among the most potent immunogens (5) (75, 91). Evidently the synthesis of these proteins is not turned off soon after infection as in some species of bacteria (1).

Motility and chemotaxis are likely to be important for *Bb* to participate in several steps in completing the host-vector cycle. First, after transmission from the tick to a vertebrate host, *Bb* disseminates through skin and migrates to an appropriate target tissue such as the joint. These sites allow for persistence and evasion of the expanding adaptive immune response. Second, after residing in the vertebrate host for weeks to years, *Bb* possibly detects the presence of feeding ticks, and then migrates to those sites to enter the blood meal of those ticks. Finally, during the blood meal of an infected tick, the spirochetes need to migrate from the tick gut to the salivary glands to restart the cycle.

Although using a genetic approach to determine the contribution of motility and chemotaxis to the life cycle of *Bb* is difficult (15, 62), significant progress is being made. One key study focused on the presumed motor protein *fliG1* gene from a virulent strain of *Bb* (11, 47). Mutations in this gene result in cells deficient in motility and are unable to establish an infection in mice. These studies are the first to show that motility is involved in the virulence of *Bb*. In another ongoing study, targeted mutants in *flaB* from a virulent strain of *Bb* are non-motile as expected, but are also non-infectious in mice (S. Sultan, M. Motaleb, P. Stewart, P. Rosa, N. Charon, unpublished). These preliminary results, coupled with those with the *fliG1* mutant suggest that motility plays a critical role in the disease process.

Recent results suggest that chemotaxis may also be involved in *Bb* virulence. *Bb* has been shown to be attracted to tick salivary extract (83). A non-chemotactic *cheY3* mutant is unable to establish infection in mice (M. Motaleb, unpublished). Similarly, a c-di-GMP *pdeA* mutant that constantly runs is also unable to infect mice (87). However, these preliminary studies suggest that chemotaxis is required for *Bb* infections. We expect that the analysis of other motility and chemotaxis mutants will lead to a better understanding of these processes with respect to tick transmission and mammalian infection and disease.

Conclusion

In this review, we summarized the developments made in understanding spirochete motility and chemotaxis with *Bb* chosen as the model. Significant progress has been made since the times of Dobell and Weibell, but many questions still remain. Spirochetes cause dreaded diseases that are prevalent throughout the world including syphilis, leptospirosis, relapsing fever, and Lyme disease. The tragedy is that so little is known about these terrifying bacteria: We cannot even grow the syphilis spirochete in the laboratory, and only a handful of laboratories in the world are doing basic studies on *T. pallidum*. We can only hope that in another half century (or sooner!) sufficient progress in understanding the biology of these enigmatic pathogens leads to new means of disease prevention and treatment such that these diseases eliminated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Terms

Bb	<i>Borrelia burgdorferi</i>
Cryo-ET	Cryoelectron microscopy tomography
CW	Clockwise
CCW	Counter-clockwise
c-di-GMP	3',-5'-cyclic-diguanosine monophosphate
OM	Outer membrane
MCP	Methyl-accepting chemotaxis protein
PF	Periplasmic flagellum
PC	Protoplasmic cell cylinder
PS	Protoplasmic space
TEM	Transmission electron microscopy
GFP	Green fluorescent protein

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Related Resources

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SUMMARY POINTS AND FUTURE ISSUES

1. Cryo-ET allows for exquisite detail analysis of the *Bb* flagellar motor. What are the functions of each of the motor proteins in generating flagellar rotation?
2. When *Bb* swims in one direction in the absence of the response regulator CheY3-P, it rotates the PFs of the polar ribbons asymmetrically. What is the molecular basis for asymmetrical flagellar rotation in the absence of CheY3?
3. *Bb* is so long that chemotaxis models in other bacteria do not directly apply. What is the molecular basis of chemotaxis in *Bb*? What controls the direction of flagellar rotation?
4. *Bb* lacks the cascade control of motility and chemotaxis gene expression and relies on translational control. What are the details? How do CsrA and c-di-GMP exert their activities?
5. Initial gene targeting experiments indicate that motility and chemotaxis play an important role in the life cycle of *Bb* in both tick and mammalian hosts. What are the precise steps for their involvement?

Figure 1.

(A) Longitudinal diagram of a typical spirochete. Note that the periplasmic flagella (PFs) overlap in the cell center. (B) Cross-section diagram of *Borrelia burgdorferi* (Bb) illustrating the component parts. Note that seven PFs form a tightly packed ribbon that causes the outer membrane to bulge.

Figure 2.

Cellular architecture of one end of *Bb* revealed by Cryo-ET. A 3-dimensional model was generated by manually segmenting the outer membrane (light green), protoplasmic cell membrane (green), flagellar motors and filaments (blue), MCP array (red), and outer surface proteins (yellow). Reprinted with permission with modification from Reference (100).

Figure 3.

A 3-D reconstruction of the *Bb* flagellar motor. The major components (the rod, the stator, the P ring, and the MS ring) are labeled, with CM representing the protoplasmic cell membrane. The C ring is composed of FliG, FliM and FliN. "Collar" is a spirochete specific feature. The export apparatus is divided into three separated densities, although the boundary between the MS ring and the export apparatus is not obvious from the figure.

Figure 4.

Swimming cells of *Bb* as a function of direction of rotation of the PFs. Blue arrows at cell ends indicate direction of swimming. Curved arrows indicate direction of rotation of the PFs. For simplification, only one PF is shown attached at each end of protoplasmic cell cylinder. The top two panels show translational forms, and the bottom two-panels show nontranslational forms.