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Spirochete Periplasmic Flagella and Motility

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Abstract

Spirochetes have a unique structure, and as a result their motility is different from that of other bacteria. They also have a special attribute: spirochetes can swim in a highly viscous, gel-like medium, such as that found in connective tissue, that inhibits the motility of most other bacteria. In spirochetes, the organelles for motility, the periplasmic flagella, reside inside the cell within the periplasmic space. A given periplasmic flagellum is attached only at one end of the cell, and depending on the species, may or may not overlap in the center of the cell with those attached at the other end. The number of periplasmic flagella varies from species to species. These structures have been shown to be directly involved in spirochete motility, and they function by rotating within the periplasmic space. The mechanics of motility also vary among the spirochetes. In *Leptospira*, a motility model developed several years ago has been extensively tested, and the evidence supporting this model is convincing. *Borrelia burgdorferi* swims differently, and a model of its motility has been recently put forward. This model is based on analyzing the motion of swimming cells, high voltage electron microscopy of fixed cells, and mutant analysis. To better understand spirochete motility on a more molecular level, the proteins and genes involved in motility are being analyzed. Spirochete periplasmic flagellar filaments are among the most complex of bacterial flagella. They are composed of the FlaA sheath proteins, and in many species, multiple FlaB core proteins. Allelic exchange mutagenesis of the genes which encode these proteins is beginning to yield important information with respect to periplasmic flagellar structure and function. Although we are at an early stage with respect to analyzing the function, organization, and regulation of many of the genes involved in spirochete motility, unique aspects have already become evident. Future studies on spirochete motility should be exciting, as only recently have complete genome sequences and tools for allelic exchange mutagenesis become available.

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Introduction

Spirochetes are a medically significant but poorly understood group of bacteria. These organisms cause several diseases in both humans and animals. Those diseases that are most important to humans are syphilis (*Treponema pallidum*), Lyme disease (*Borrelia burgdorferi*), and leptospirosis (*Leptospira interrogans*). Spirochetes have also been implicated in periodontal disease (*Treponema* species including *Treponema denticola*). The human diseases are quite prevalent. Syphilis is a major health problem in several parts of the world. Leptospirosis is the most common worldwide waterborne zoonosis. Lyme disease is the most prevalent arthropod borne disease in the United States. Periodontal disease, which is the major cause of tooth loss, is among the most frequent human bacterial infections. Among livestock, *Brachyspira* (formerly called *Serpulina, Treponema*) *hyodysenteriae* causes swine dysentery and has major economic importance. A closely related spirochete, *Brachyspira pilosicoli*, is associated with human diarrheal disease in immuno-compromised individuals (Johnson, 1977; Canale-Parola, 1984; Barbour and Hayes, 1986; Harris *et al*., 1993; Trott *et al*., 1997). It should be emphasized that many spirochetes are free living and include, for example, *Spirochaeta*, *Spirosymplokos*, and *Leptospira biflexa*. In addition, others are commensals. To illustrate, the very large uncultivable *Cristispira* is found in the crystalline style of bivalve mollusks, and *Pillotina* and *Clevelandina* are found in the guts of termites and cockroaches (Harwood and Canale-Parola, 1984; Canale-Parola, 1984; Bermudes *et al*., 1988; Margulis *et al*., 1993). Only recently have some of the spirochete species from termites been able to be cultivated (Leadbetter *et al*., 1999).

Compared to what we know about other bacteria that cause disease, our information concerning the spirochetes is by far quite minimal for several reasons. First, the spirochetes in general are a fastidious group of bacteria. For example, *T. pallidum* and several of the oral spirochete species have yet to be continuously grown in the laboratory—the right growth medium and conditions have not been determined. Those spirochetes that can be grown have relatively long generation times (Johnson, 1977; Canale-Parola, 1977; Canale-Parola, 1984). Consequently, because these organisms are difficult to study, deciphering virulence factors is indeed quite challenging. Second, until recently, there has not been a means to selectively inactivate genes in the spirochetes, or even map a given mutation. Thus, it was not possible to rigorously determine the function of a given gene in the disease process. The accompanying articles in this issue of JMMB summarize the exciting new genetic tools and recent accomplishments for several spirochete species (see also ter Huurne *et al*., 1992; Rosey *et al*., 1995; Li *et al*., 1996; Bono *et al*., 2000).

The major emphasis of research in our laboratory and those of several of our colleagues has been trying to understand how spirochetes swim. As discussed below, motility is likely to play an important role in the disease process. It is important to emphasize that motility and

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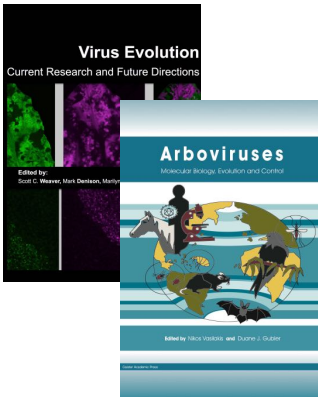

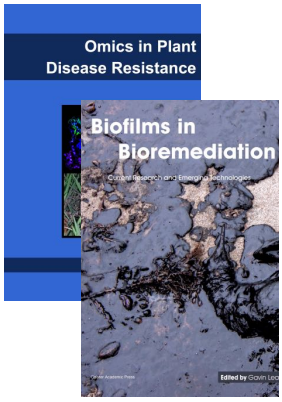

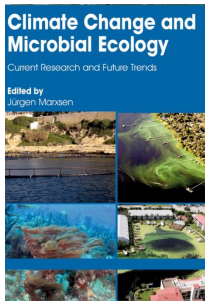

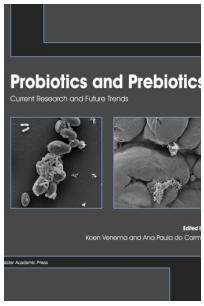

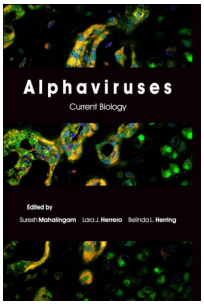

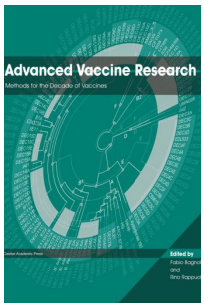

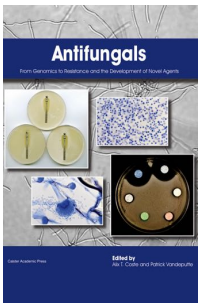

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directed movement such as chemotaxis are tied together. Bacteria have a sensory system, and they respond to the environment by changing direction and undergoing taxis (Manson, 1992; Armitage, 1992). The accompanying review by Lux *et al.* in this issue emphasizes the chemotaxis part of spirochete sensing. Here we address the mechanics, structures, and genes involved in spirochete motility. When we took this research direction several years ago, we realized that the results obtained would contribute to an understanding of these organisms, as so little was known about spirochete biology. We also know that basic research is important and fundamental in determining how bacteria cause disease. We anticipate that the knowledge gained will eventually be applied to the treatment and prevention of spirochetal diseases.

Review

The spirochetes are a very diverse group of bacteria. 16S ribosomal RNA gene sequence analysis indicates that the spirochetes evolved from a primordial spirochete and are an ancient phylum of the *Bacteria* (Canale-Parola, 1977; Woese, 1987; Paster *et al.*, 1991). These organisms have a helical or flat-wave morphology. The handedness of those spirochetes that are helical (right or left-handed) is species specific, and the organisms vary in shape and size. (A right-handed helix rotates clockwise [CW], and a left-handed helix rotates counter-clockwise [CCW], going away from an observer). Some spirochetes, such as the *Leptospira*, are so thin (0.12 μm in diameter) that they cannot be seen using bright-field microscopy. Others, such as the *Cristispira* and *Spirosymplokos* are so large (>100 μm long) that they approach the size of multicellular organisms (Holt, 1978; Carleton *et al.*, 1979; Stephan and Johnson, 1981; Canale-Parola, 1984; Charon *et al.*, 1991; Margulis *et al.*, 1993; Ruby *et al.*, 1997).

Spirochetes have common morphological attributes. Attached near each end of the protoplasmic cell cylinder are periplasmic flagella**,** which resemble flagella of other bacteria. One to several hundred periplasmic flagella are attached subterminally near each cell end and extend inward toward the center of the cell where they may or may not overlap. The number of periplasmic flagella, and whether they overlap at the center of the cell, depend on the species. For example, *Leptospira* has one periplasmic flagellum at each end, but *Cristispira* has over 100 (Holt, 1978; Canale-Parola, 1984). Surrounding both the protoplasmic cylinder and periplasmic flagella is an outer membrane sheath. In those species in which the periplasmic flagella have been purified and characterized, these organelles have been shown to have left-handed helices (Charon *et al.*, 1991; Charon *et al.*, 1992a). Thus, spirochetes differ from other bacteria in that the spirochete organelles for motility reside **inside** the cell and within the periplasmic space.

The common morphological structure of spirochetes allows these organisms to bore through highly viscous gel-like media, such as connective tissues, which inhibit the motility of most other bacteria (Armitage, 1992). Several investigators have found that *Leptospira* and other spirochetes increase in speed in media with a higher viscosity (Kaiser and Doetsch, 1975; Greenberg and Canale-Parola, 1977). Berg and Turner carefully analyzed the conditions for increased motility speed with viscosity (Berg and Turner, 1979). They found that compounds that increase the macroscopic but not the microscopic viscosity, i.e. gel-like media such as methylcellulose, enhance the speed of spirochetes. On the other hand, media conditions that increase both macroscopic and microscopic viscosity, such as Ficoll, have an opposite effect and decrease the speed of the organisms, and the organisms become quite sluggish. Thus, the concept developed that the spirochetes have a unique ability: they increase in speed in a highly viscous gel-like medium such as found with methylcellulose and connective tissue. Similar results demonstrating enhanced motility in viscous gel-like media have been described for *T. denticola* (Pietrantonio *et al.*, 1988; Ruby and Charon, 1998), *T. pallidum* (Van Weelden *et al.*, 1990), as well as for *B. burgdorferi* (Kimsey and Spielman, 1990; Goldstein *et al.*, 1994).

The hypothesis that motility is involved in the disease process has been examined. Sadziene found that a spontaneously occurring, non-motile mutant of *B. burgdorferi* was markedly hindered in its ability to penetrate human umbilical vein endothelial cell monolayers (Sadziene *et al.*, 1991). Although the site of the mutation (or mutations) in this mutant is presently unknown, the results give credence to the concept that motility is a virulence factor for these bacteria. Furthermore, periplasmic flagellar mutants of *B. hyodysenteriae,* which were still motile but had altered motility, were found to have a decreased ability to colonize and infect the intestines of mice and were avirulent in pigs (Kennedy *et al.*, 1995; Rosey *et al.*, 1996; Kennedy *et al.*, 1997). Such evidence reinforces the idea of a strong relationship between motility and disease.

Over the years, our laboratory has centered its efforts on answering four questions related to spirochete motility.

1. What are the mechanisms which allow these organisms to swim?
2. What are the structure and composition of the organelles—the periplasmic flagella—which augment motility?
3. What are the roles of the different periplasmic flagellar filament proteins in flagellar function, and how are they regulated?
4. What are the organization and function of the other genes involved in motility, and how is the expression of these genes controlled?

Below we describe the progress made in trying to answer these questions. The reader is also referred to the published reviews on spirochete motility (Canale-Parola, 1978; Goldstein and Charon, 1988; Charon et al., 1992b), and the accompanying article by Lux *et al.*

1. By what Mechanism Do Spirochetes Swim?

The *Leptospiraceae* has for several years served as a model system for studying the dynamics of motility. It is among the easiest spirochete to grow and is structurally the least complex (Johnson and Harris, 1967; Holt, 1978). The cell cylinder forms a right-handed helix (Carleton *et al.*, 1979). Only one periplasmic flagellum is inserted subterminally at each end and is located along the center axis of the cell helix (Goldstein *et al.*, 1996). These periplasmic flagella are so short that they do not overlap–or even reach each other—at the center of the cell (Birch-Andersen *et al.*, 1973; Bromley and Charon, 1979). In a

translating cell, the posterior end is hook-shaped, and the anterior end is spiral shaped (Noguchi, 1918). When the cells reverse direction, the ends of the cell concomitantly change shape. In addition, non-translating cells are also seen: the ends of these cells are either both spiral- shaped or both hook-shaped (Berg *et al*., 1978). The early approach taken was to isolate non-motile mutants generated by chemical mutagenesis, and then compare the structure of the mutants with that of the parental strain. Although mutations in the non-motile mutants could not be mapped, comparisons of these mutants to the parental strain and motile revertants yielded critical information concerning their mechanism of motility. One class of non-motile mutants had ends that were straight, *i.e.* the ends were no longer hook- or spiral-shaped, but the cell body in this region was still helical. In addition, the mutants differed with respect to the shape of the periplasmic flagella. Periplasmic flagella from the parental strain and revertants coiled when observed by electron microscopy, but those of the mutants failed to coil (Bromley and Charon, 1979). Previously, the only evidence that the periplasmic flagella played a role in motility had been inferential, and this was based on their morphological similarity to the flagella of other bacteria (Nauman *et al*., 1969). These genetic results were the first which indicated that the periplasmic flagella were involved in spirochete motility. They also indicated that the periplasmic flagella influenced the shape of the ends of the cells. Subsequently, analyses of chemically induced, spontaneously occurring, and site directed mutants from several spirochete species have shown the importance of the periplasmic flagella in motility (Paster and Canale-Parola, 1980; Limberger and Charon, 1986a; Sadziene *et al*., 1991; Rosey *et al*., 1995; Rosey *et al*., 1996; Li *et al*., 1996; Ruby *et al*., 1997; C. Li, M. Motaleb, and N. Charon, unpublished).

The analysis of motility mutants of *Leptospiraceae* (strain *Leptonema illini*, formerly *Leptospira illini*), along with an analysis of swimming *Leptospira* in low viscosity and highly viscous gel-like media, led to a model for *Leptospira* motility (Berg, 1976; Goldstein and Charon, 1988). The periplasmic flagella rotate between the outer membrane sheath and the right-handed protoplasmic cylinder. The periplasmic flagellum is more rigid than the cell cylinder, causing the end of the cell to conform to the shape of its flagellum. As viewed from the **center** of the cell towards one of the cell ends, CCW rotation of a periplasmic flagellum results in the flagellum at that end forming a left-handed helix whose helical pitch and helical diameter are larger than those of the cell body. This form is referred to as the spiral-shaped end. On the other hand, rotation of that periplasmic flagellum in the CW direction results in the flagellum and cell body at that end being hook-shaped. When the periplasmic flagella are rotating in the same direction (both CCW or CW), the cell does not translate.

In the model, forward thrust results from two rotations. The model can be conveniently described from the viewpoint of an observer located **behind** a swimming cell. First, both periplasmic flagella rotate CCW. Secondly, the torque of the rotating flagella produces a CW counter-rotation of the right-handed helical cell body. Two modes of thrust are proposed. CCW rotation of the anterior periplasmic flagellum causes that end of the cell to gyrate (*i.e.* to bend in a circular manner without necessarily rotating); it generates a backward moving spiral-wave. This wave is predicted to be left-handed, and is sufficient for the cell to translate forward in a low viscosity medium. Concomitantly, CW rolling of the cell cylinder gives the organism the unique capacity to bore through gel-like media and connective tissue. The torque due to the roll of the cell cylinder is counter-balanced by the gyrations of the cell ends.

Three lines of evidence support this model. First, cells tethered to a glass surface by antibody coated latex beads were analyzed with the aid of a computer (Charon *et al.,* 1984). Viewed from above, cells with both ends spiral-shaped were found to rotate CW around the bead bound to the cover-glass, and CCW when bound to a slide. These results were interpreted as follows: a CCW gyration of the spiral- shaped ends interacts with the bottom surface (slide) causing a CCW rotation around the bead. A similar interaction with a top surface (cover glass) leads to a CW rotation around the bound bead. These results suggest that the spiral- end gyrates CCW as predicted by the model. Second, high speed cinematography was used to analyze the shapes and directions of rotation of swimming cells (Goldstein and Charon, 1990). Using this method, direct evidence confirmed that the spiral-shaped end was left-handed, that it gyrated CCW, and the cell rolled CW as predicted by the model. Finally, direct microscopic evidence indicated that the periplasmic flagella from several spirochete species rotate as predicted by the model (Charon *et al*., 1992a; Goldstein *et al*., 1994). In these experiments, high magnification dark-field microscopy and differential interference contrast light microscopy revealed that stationary phase cells of *Treponema*, *Borrelia*, and *Spirocheata* had protrusions which rotate. Certain motility mutants of *T. phagedenis* were found to form protrusions throughout their growth cycle with high efficiency (Charon et al. 1992a). We were unable to see such protrusions in *Leptospira*, perhaps because it is the only spirochete which has coiled rather than helical periplasmic flagella. Close examination of these protrusions with light and electron microscopy revealed that they were periplasmic flagella; they had the same helix pitch and handedness as purified periplasmic flagella, and electron microscopy confirmed that they were periplasmic flagella surrounded by a membrane. Taken together, the proposed model made certain predictions which were testable and were borne out by experimentation.

A similar approach was used in analyzing the more complex *Treponema phagedenis*. We found that the model for *Leptospira* motility applies to this spirochete as well (Charon *et al*., 1991). *T. phagedenis* has a right handed cell cylinder with 4-8 short periplasmic flagella attached subterminally at each end (Canale-Parola, 1984); they extend only a short distance towards the center of the cell and do not overlap. As with *Leptospira*, these structures cause the cell ends to be left-handed or irregularly shaped. However, CCW gyration of the bent-shaped ends does not yield sufficient thrust for the organism to swim in a low viscosity medium as the spiral-shaped end does for *Leptospira*: the gyration is either too slow or not large enough. Yet, because the cell is helical and is right-handed, *T. phagedenis* can translate in a highly viscous gel-like medium such as 1% methylcellulose with little slippage. It is the CW counter-rotation of the cell cylinder that we hypothesize is responsible for this translation.

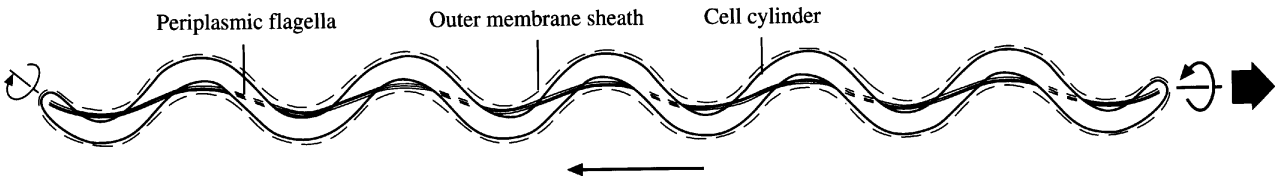

Figure 1. Proposed model of *B. burgdorferi* motility translating toward the right (wide arrow). The ridge, which is comprised of the periplasmic flagella, extends down the length of the cell, and is left-handed with respect to the cell axis. The periplasmic flagella rotate CCW as viewed from the back of the cell (thin arrow at anterior end). This rotation causes waves to move from the anterior to the posterior ends of the cell (center arrow). Concomitant with this rotation, the cell rolls CW about the body axis (thin arrow at posterior end).

T. denticola is considerably more complex, and at this time the dynamics of its motility are not understood in detail (Ruby *et al.*, 1997). *T. denticola* has approximately two periplasmic flagella attached at each end, and those at one end are long enough to overlap in the center of the cell with those attached to the other end (Canale-Parola, 1984). An analysis of a spontaneously occurring non-motile mutant, and a site directed mutant to the flagellar hook protein, have yielded important information with respect to mechanisms of motility. Both mutants were found to lack periplasmic flagella. Light microscopy revealed that repeatedly cloned wild-type cells consistently yielded two morphological types: one with a right-handed regular helical morphology, and the other with an irregular morphology. These irregularly shaped cells were helical with periodic perturbations superimposed on the cell helix. The mutants lacking the periplasmic flagella were only helically shaped with a shape identical to the wild-type regularly-helical cells. Thus, the irregular shaped morphology is related to the periplasmic flagella influencing the shape of the cell in the domain where they reside. In this case, that domain is the entire cell, not just the cell ends as with the other spirochete species. As with *T. phagedenis*, both the helically and irregularly shaped cells failed to translate in a low viscosity medium (Ruby *et al.*, 1997). However, in a gel-like medium with high macroscopic viscosity, the cells translate quite efficiently, especially if the temperature for observing the cells is raised from room temperature to 37°C (Klitorinos *et al.*, 1993; Ruby and Charon, 1998). It is known that purified periplasmic flagella of *T. phagedenis* and *T. denticola* are left-handed (Charon *et al.*, 1991; Charon *et al.*, 1992a). Thus, our working hypothesis is that in *T. denticola*, CCW rotation of the periplasmic flagella generates backward moving helical or irregularly shaped gyrating waves. These gyrating waves do not have sufficient thrust for noticeable translation in a low viscosity medium. However, the CCW rotation of the periplasmic flagella is expected to produce a CW counter-rotation of the cell cylinder. This rolling or rotation of the cell cylinder has been observed in *T. denticola* (Ruby and Charon, 1998). Because *T. denticola* translates quite efficiently in a gel-like medium, we propose that CW rolling of the cell cylinder is its main means of thrust.

B. burgdorferi is unique among the spirochetes. It has a flat-wave morphology (not helical; it is a planar wave) as revealed by analyzing the motion of swimming cells, and by high voltage electron microscopy (Goldstein *et al.*, 1994; Goldstein *et al.*, 1996). Cells swim with the flat-waves being propagated from the anterior end of a translating cell to the posterior. These flat-waves are reminiscent of eukaryotic cell motility and flagella. In *B. burgdorferi*, there are between seven and eleven periplasmic flagella attached near each end, and these filaments are so long that they overlap in the cell center (Barbour and Hayes, 1986). Surprisingly, mutants that lack the periplasmic flagella (Sadziene *et al.*, 1991) are rod- shaped (Goldstein *et al.*, 1994; Goldstein *et al.*, 1996). The initial rod-shaped mutant analyzed was a spontaneously occurring non-motile mutant; the site of the mutation in that mutant remains unknown (Sadziene *et al.*, 1991; Goldstein *et al.,* 1994; Goldstein *et al.,* 1996; Ge *et al.*, 1997a; Ge and Charon, 1997a). More recently, we used an efficient method of site directed mutagenesis (Bono *et al.,* 2000) directed to *flaB*, which encodes the major filament protein (M. Motaleb and N. Charon, unpublished). The mutants obtained lacked periplasmic flagella and were rod-shaped; the results indicate that the periplasmic flagella markedly dictate the shape of the entire cell.

How do the periplasmic flagella dictate the flat-wave morphology of the *B. burgdoferi* cell, and how does their rotation enable the cell to swim? High voltage electron microscopy was used to analyze the cell shape, and to attempt to understand the relationship of the periplasmic flagella to the flat-wave morphology and motility. This microscope allows high resolution of thick cell sections, and consequently three dimensional reconstruction of the cell is possible (Goldstein *et al.*, 1996). The results indicated that in *B. burgdorferi*, the periplasmic flagella form a bundle and wrap around the body-helix in a right-handed sense, but are left- handed in space (see below). The rod-shaped cell body was found to be distorted into a flat sine or meander-like wave, and the helix pitch and helix diameter of the helical periplasmic flagellar bundle was stretched to a larger size than described for the isolated organelles. The helix pitch of the bundle equaled the wavelength of the flat wave. Based on these results and the motion analysis described above, we proposed a motility model of *B. burgdorferi* (Figure 1) (Goldstein *et al.*, 1994; Goldstein *et al.*, 1996). This model states that in translating cells, the left-handed periplasmic flagella form a bundle that rotates CCW around the cell axis (*i.e.* the x axis of the cell wave) as viewed from behind translating cells. As a result, backward moving flat waves are generated: the rotating helical periplasmic flagella essentially function as a worm gear and interact with the cell cylinder; this gear pushes the waves from the anterior end to the posterior end allows the organisms to translate forward. Concomitant with the generation of these waves and to balance this motion, the cell rolls CW around the body axis (*i.e.* the center of the cell body–if the cell body is a wavy sausage, the body axis

runs along the center of the sausage). The evidence for this model is not as complete as that for *Leptospira*. We know that the periplasmic flagella do rotate in *B. burgdorferi*, but direct evidence that these organelles generate the backward waves as described above has not been obtained. In addition, proof that the cell rolls CW about the body axis during translation is technically difficult to determine and has also not been forthcoming.

The examination of spirochete motility would not be complete without discussion of the model proposed by Berg several years ago (Berg, 1976). He observed that irregularly shaped *Spirochaeta aurantia* translated in low viscosity media with the cell rotating completely about the cell axis. Based on these and other results, he proposed that the periplasmic flagella rotated between the outer membrane sheath and cell cylinder. He envisioned that the outer sheath rotated in one direction, and the cell cylinder rotated in the opposite direction. Forward thrust is said to be obtained by two mechanisms. First, rolling of the cell cylinder in one direction allows cell to swim forward, in a highly viscous gel-like environment with little slippage. Second, rotation of the sheath in the opposite direction produces a forward component of thrust. This model may apply to *S. aurantia* (Fosnaugh and Greenberg, 1988; Fosnaugh and Greenberg, 1989), but firm evidence that the sheath rotates in the opposite direction relative to the cell cylinder has not been obtained for any spirochete. In addition, at least for some spirochetes, evidence using latex beads attached to antigens of the outer membrane sheath on live cells indicates that this structure is fluid in a manner similar to most lipid bilayer membranes and not rigid (Charon *et al.,* 1981; Charon *et al*., 1984). Attempts to attach latex beads to *S. aurantia* outer membranes to test this hypothesis have not been successful (E. P. Greenberg and N. W. Charon, unpublished).

2. What is the Composition and Structure of the Periplasmic Flagellar Filament?

The protein composition and the structure of spirochete periplasmic flagella are atypical compared to the flagella of other bacteria. In fact, it is among the most complex of bacterial flagellar filaments. In many bacterial species, flagellar filaments often consist of only one protein species. Results from several laboratories have shown that purified periplasmic flagella consist of several different proteins (Bharier and Allis, 1974; Sand Petersen *et al*., 1981; Limberger and Charon, 1986a; Radolf *et al*., 1986; Norris *et al*., 1988; Brahamsha and Greenberg, 1988; Trueba *et al*., 1992; Koopman *et al*., 1992; Ruby *et al*., 1997). Two classes of periplasmic flagellum proteins have been defined: Class A and Class B (also referred to as FlaA and FlaB) (Norris *et al.,* 1988; Brahamsha and Greenberg, 1989). FlaA proteins are 37-39 kDa, and are similar between species based on amino acid sequence determination and antigenicity. Because FlaA have their N-terminal amino sequence cleaved, they are likely to be exported to the periplasmic space via the SecA dependent pathway (Norris *et al*., 1988; Brahamsha and Greenberg, 1989; Isaacs and Radolf, 1990; Charon *et al*., 1992b; Norris and *Treponema pallidum* polypeptide research group, 1993). Generally, one or two different FlaA proteins are found in a given species. FlaB proteins are usually 33-39 kDa, although in *B. burgdorferi* FlaB is 41 kDa. When periplasmic flagella are purified, there are generally two to four different FlaB proteins from a given species. The exception again is *B. burgdorferi,* which has only one FlaB protein. Each FlaB protein in a given species is encoded by a separate gene (Pallesen and Hindersson, 1989; Champion *et al*., 1990; Fraser *et al*., 1998; C. Li and N. Charon, unpublished). FlaB proteins antigenically cross react between one another in a given species, and also between species (Limberger and Charon, 1986b; Brahamsha and Greenberg, 1988; Norris *et al*., 1988; Norris and *Treponema pallidum* polypeptide research group, 1993). In addition, N-terminal and gene sequence analyses indicate that FlaB proteins are well conserved among spirochete species (Norris and *Treponema pallidum* polypeptide research group, 1993). Because the N-terminal region of FlaB proteins is not cleaved, these proteins are likely exported to the periplasmic space through the basal bodies via a Type III secretory pathway (Norris *et al*., 1988). FlaB proteins have sequence similarities to flagellin of other bacteria, especially at their N and C terminal regions; thus, they are assumed to function by forming the essential part of the flagellar filament (Norris *et al.,* 1988; Norris and *Treponema pallidum* polypeptide research group, 1993; Wilson and Beveridge, 1993). There are no amino acid sequence similarities between FlaA and FlaB proteins.

3. What is the Role of the Different Filament Proteins in Flagellar Function, and How are They Regulated?

Studies are now ongoing to determine the roles of FlaA and the multiple FlaB proteins in periplasmic flagella structure and motility. Research in a number of laboratories have shown that FlaA forms a sheath around the FlaB flagellar core (Cockayne *et al*., 1987; Brahamsha and Greenberg, 1988; Blanco *et al*., 1988; C. Li and N. Charon, unpublished), but it is not clear what role these proteins play in periplasmic flagellar shape and motility. In addition, it is not known how the FlaB proteins are arranged in a given periplasmic flagellum. For this approach, *B. hyodysenteriae* is being used as a model system, as allelic exchange mutagenesis using two selectable markers has been developed in this species of spirochete (Rosey *et al*., 1995; Rosey *et al*., 1996; Kennedy *et al*., 1997). *B. hyodysenteriae* strain B204 has one FlaA and three FlaB proteins. Single mutants with a mutation in each of the flagellar filament genes were constructed using kanamycin (*kan*) and chloramphenicol (*cat*) resistance cassettes. Each of the single mutants was found to be motile and produce periplasmic flagella (Rosey *et al*., 1995; C. Li and N. Charon, unpublished). Qualitative differences in motility among the mutants relative to the wild-type were found, but quantitative data with respect to such parameters as cell speed and reversal frequency have not been obtained (Rosey *et al*., 1995). Some of the mutants were less virulent than the wild-type in a murine model of dysentery and in pigs; and it was concluded that full motility is required for optimal virulence (Rosey *et al*., 1996; Kennedy *et al*., 1995; Kennedy *et al*., 1997).

The effect of a given mutation on periplasmic flagella shape and composition is being examined for *B. hyodysenteriae* (C. Li and N. Charon, unpublished). Recent results indicate that periplasmic flagella purified from each of the *flaA* and *flaB* mutants contain all the proteins except the one harboring the targeted mutation. Thus, loss of one

σ^{28} like promoters found in the regulation of spirochete filament protein synthesis

	-35		-10
B. subtilis consensus	**TAAA** ----------	N-16 ----------	G**CCGATA**T
T. phagedenis flaB2	**TTAA** ----------	N-15 ----------	T**CCGATA**C
T. pallidum flaB2 *	**TCAA** ----------	N-15 ----------	T**CCGATA**C
T. pallidum flaB1, B3 *	**TCAA** ----------	N-15 ----------	T**CCGAT**TT
B. hyodysenteriae flaB1	**TTAA** ----------	N-16 ----------	A**CCGATA**A

σ^{70} like promoters found in the regulation of spirochete filament protein synthesis

	- 35		-10
E. coli consensus	**TT**GACA ----------	N-16 ----------	**TATAAT**
S. aurantia flaA	**TT**GACA ----------	N-16 ----------	**TATAAT**
B. hyodysenteriae flaA	**TT**GACA ----------	N-17 ----------	**TATA**AA
B. hyodysenteriae flaB3	**TT**ACTT ----------	N-15 ----------	**TATAAT**
T. pallidum flaA	**TT**GACA----------	N-16 ----------	**TATACT**
B. burgdorferi flaA	**TT**AAAG ----------	N-17 ----------	**TAAATT**
B. burgdorferi flaB	**TT**CTTT ----------	N-17 ----------	**TAT**TC**T**

Figure 2. Sequence of motility gene promoters. Only those promoters involved with periplasmic flagella filament synthesis are listed. In each case, the promoter sequence has been identified by primer extension (Limberger *et al.*, 1992; Ge *et al.*, 1997a; Ge and Charon, 1997b; C. Li and N. Charon, unpublished; Isaacs and Radolf, 1990; Parales and Greenberg, 1993) except those marked with an asterisk. Bold residues indicate conserved bases as compared to the consensus sigma 28 or sigma sequence.

of the proteins did not noticeably influence periplasmic flagella assembly. Analysis of periplasmic flagella from each of the mutants using dark-field microscopy revealed that the *flaA* mutant had periplasmic flagella with a helix pitch and helix diameter different from those of the wild-type. In addition, the periplasmic flagella were thinner than those of the wild-type, again indicating that FlaA forms a sheath around the FlaB core. The *flaB* mutants had periplasmic flagella with the shape almost identical to those of the wild-type. The results indicate that FlaA influences the helical shape of the periplasmic flagella. Because bacterial flagella are often quasi-rigid and undergo helical transformations (Hasegawa *et al.*, 1998), perhaps the sheath augments stabilization of the FlaB core into one of these configurations, and this configuration is optimal for thrust as it rotates between the outer membrane sheath and cell cylinder.

The analysis of double mutants of *B. hyodysenteriae* has yielded insight into the function of the FlaA and FlaB proteins. The mutants constructed include the following: *flaA-flaB1*, *flaA-flaB2*, *flaA- flaB3*, *flaB1-flaB3* and *flaB1-flaB2* (Rosey *et al.*, 1996; C. Li and N. Charon, unpublished). Periplasmic flagella isolated from the first two mutants were found to have a helix pitch and helix diameter markedly different from all of the single mutants and the wild-type. The simplest explanation is that FlaA interacts with FlaB1 and FlaB2 and thus all three proteins reside on a given periplasmic flagellum. Preliminary evidence indicates that only the *flaB1-flaB2* double mutant is non-motile, and of the double mutants isolated, it is the only one that lacked intact periplasmic flagella. These results imply that there is an overlap in function with respect to FlaB1 and FlaB2 in flagellar assembly and in motility.

As previously mentioned, *B. burgdorferi* is unusual in that its periplasmic flagella consist of only one FlaA and one FlaB (Fraser *et al.*, 1997; Ge *et al.*, 1998). Using a gentle lysis technique to purify the periplasmic flagella, FlaA was found to be associated with FlaB; previously, FlaB was thought to be the only flagellar filament protein in this species. Because the periplasmic flagella are comprised of considerably less FlaA than FlaB, it is unlikely that FlaA forms a sheath along the entire flagellar length. Recently, inactivation of *flaB* using a *kan* resistant cassette resulted in *B. burgdoferi* being non-motile, aflagellate, and as discussed previously, rod shaped (M. Motaleb and N. Charon, unpublished). Thus, FlaB plays essential roles in both cell shape and in motility in this species.

An analysis of promoters of the *flaA* and *flaB* genes reveal both similarities and differences in the regulation of flagellin gene expression compared to that of other bacteria. In other bacteria, transcription of the flagellin gene *fliC* relies upon the motility and chemotaxis specific transcription initiation factor, sigma 28 (Kutsukake and Iino, 1994), and in some cases sigma 54 (Anderson *et al.*, 1995). In addition, the intracellular level of the anti-sigma 28 factor FlgM controls the activity of sigma 28 (Macnab, 1996; Hughes *et al.*, 1993). The start sites of transcription for many *flaA* and *flaB* promoters have been recently mapped by primer

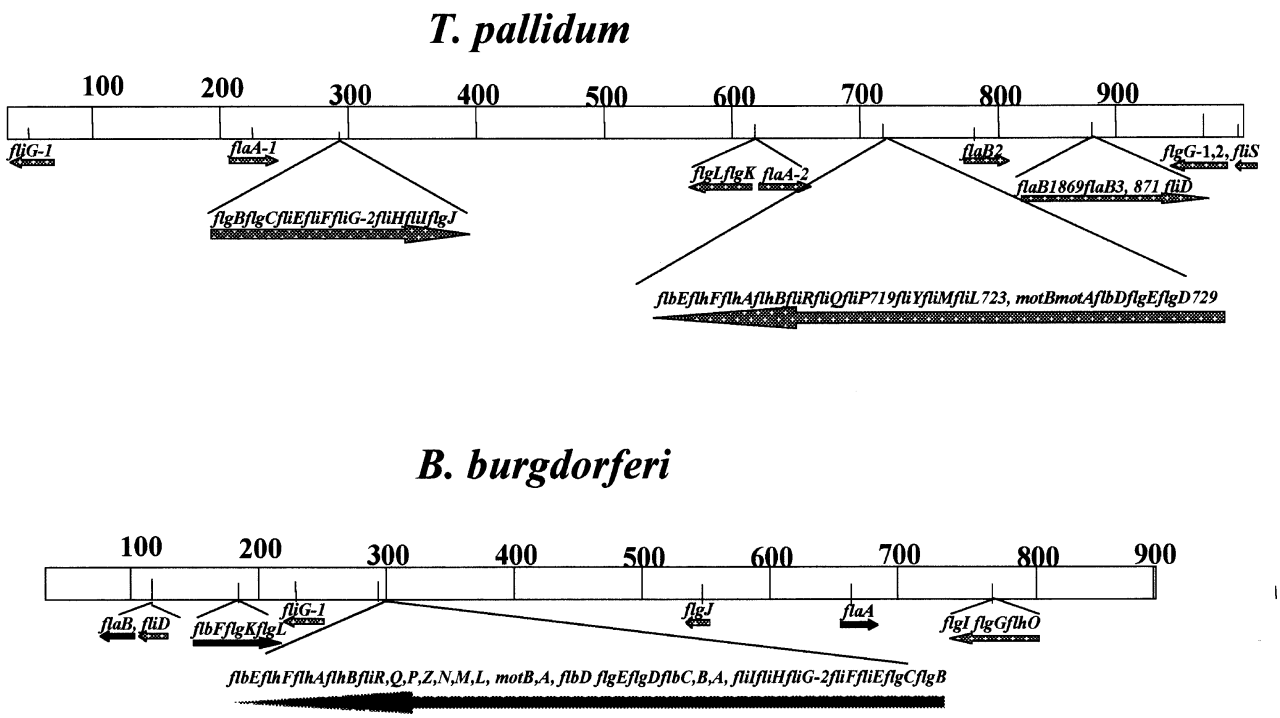

Figure 3. Organization of the motility genes of *T. pallidum* and *B. burgdorferi* (Fraser *et al.*, 1997; Ge *et al.*, 1997a; Ge *et a*l., 1997b; Ge and Charon, 1997a; Ge and Charon, 1997b; Fraser et al., 1998). Black arrows indicate direction of transcription of genes, and promoters have been identified by primer extension and operons by RT-PCR (Ge and Charon, 1997a; Ge and Charon, 1997b; Ge *et al.*, 1997a; Ge *et al.*, 1997b). Grey stippled arrows indicate presumed promoters, operons, and direction of transcription based solely from sequence analysis.

extension. As shown in Figure 2, most of the *flaB* genes in a number of spirochete species are initiated by the sigma 28 consensus sequences. These results indicate that the promoter sequences for FlaB proteins in spirochetes, and FliC in other bacteria, are similar. However, there are two exceptions: the single *flaB* gene in *B. burgdorferi* (Ge *et al.*, 1997a), and the *flaB3* gene in *B. hyodysenteriae* (C. Li and N. Charon, unpublished). Both are initiated by the housekeeping transcription factor sigma 70. Interestingly, the homolog of *flgM* has not been identified in either of the two spirochete genomes that have been sequenced, *i.e. B. burgdorferi* and *T. pallidum* (Fraser *et al.*, 1997; Fraser *et al.*, 1998). This result would be expected for *B. burgdorferi,* as no sigma 28 promoter recognition sequences have been detected for *flaB* and any of its motility genes (see below). In addition, a gene encoding the sigma 28 factor has yet to be detected in its genome. However, sequence analysis suggests that *T. pallidum* uses sigma 28 to initiate transcription of FlaB proteins, and a sigma 28 homolog has been identified in its genome (Fraser *et al.*, 1998). Because FlgM is a small protein in other bacteria and shows weak homology between species, it could still be present in *T. pallidum* but not yet identified. Alternatively, it is conceivable that there is no FlgM in *T. pallidum,* and motility genes are regulated in a unique manner. In contrast to *flaB*, *flaA* genes from all spirochetes studied are initiated by sigma 70 promoters (Brahamsha and Greenberg, 1989; Koopman *et al.*, 1992; Parales and Greenberg, 1993; Ge and Charon ,1997b).

4. What Are the Organization and Function of the Other Genes Involved in Motility, and How is the Expression of These Genes Controlled?

Several laboratories have been analyzing the organization and structure of the myriad (over 30) motility and flagellar genes found in spirochetes (Limberger *et al.*, 1992; Hardham *et al.*, 1995; Ge *et al.*, 1996; Heinzerling *et al.*, 1997; Ge *et al.*, 1997a; Ge *et al.*, 1997b; Ge and Charon, 1997a; Izard *et al.*, 1999; Limberger *et al.*, 1999). Here we attempt to summarize some of this work, and the recent results obtained from the genome sequences of *T. pallidum* and *B. burgdorferi* (Fraser *et al.*, 1997; Fraser *et al.*, 1998) (Figure 3).

There are at least 36 motility genes (not including chemotaxis genes) in *B. burgdorferi* and in *T. pallidum*: they comprise at least 3-4% of all the deduced open reading frames. The presence of such a high percentage of motility genes emphasizes the importance of motility and taxis for the survival of the spirochetes in nature. In analyzing the motility genes, both the gene order and the deduced amino acid sequences are most similar to those of other spirochete species, followed by *Bacillus subtilis*. These results again suggest the common evolutionary ancestry of spirochetes. Because spirochete motility genes have such a high homology to the motility genes of other bacteria, this basic apparatus and mechanism required for flagellar rotation are also well conserved and present in spirochetes. In fact, some spirochetal motility genes are so similar to those of *E. coli* and *S. typhimurium* that their expression in

these bacteria results in negative complementation effects on flagella synthesis and motility (Ge *et al*., 1997b; Heinzerling *et al*., 1997). A likely explanation for this negative complementation is that the spirochetal motility proteins compete and interfere with their counterparts with respect to assembly of flagellar components (Ge *et al*., 1997b; Heinzerling *et al*., 1997).

The motility genes are clustered very tightly with little intervening sequences between genes. In *E. coli* there are 13 motility operons (Macnab, 1996), but preliminary results indicate that there are only eight motility operons in *B. burgdorferi,* and nine to ten in *T. pallidum*. The control of expression of these genes is presently poorly understood, but has been most studied in *B. burgdorferi*. In *B. burgdorferi*, there is a remarkably large motility gene cluster of 21 kb comprising 26 open reading frames (Ge *et al*., 1997b). Its gene order is similar to that of a gene cluster found in *B. subtilis*. Primer extension and RT- PCR analysis indicate that this cluster is initiated by a single sigma 70 like promoter which maps upstream of the rod protein *flgB* within the *hslU* gene. Hopefully, gene inactivation studies will reveal whether there are other transcription start sites in this gene cluster, as it is quite unusual for such a large operon to be controlled by one promoter.

Some of the proposed motility genes are unique. For example, there are a number of genes (in *B. burgdorferi* designated as *flb* genes), which cluster with other motility genes but have no obvious homologs in other bacteria (Fraser *et al*., 1997;Ge *et al*., 1997b). The functions of these genes are presently unknown, but further analysis of these genes should be revealing. For example, Limberger *et al*. inactivated the *tap1* gene of unknown function in *T. denticola* by allelic exchange mutagenesis (Limberger *et al.,* 1999). *tap1* mapped within a large motility gene cluster. The phenotype of this mutant revealed that it formed polyhooks similar to the *fliK* mutants of *S. typhimurium*. Further analysis of the Tap1 deduced amino acid sequence revealed a weak homology to FliK of *S. typhimurium*. Thus, in some cases, homologs of other motility genes may be present in spirochetes, but are not yet recognized. On the other hand, spirochetes must control the rotation of the periplasmic flagella at both ends of the cell so that these organelles can rotate in opposite directions as viewed from the center of the translating cell. Along these lines, spirochete chemotaxis differs from that of other bacteria, as a membrane potential is involved in its chemotactic response; this response presumably involves coordination of the direction of rotation of the periplasmic flagella at both cell ends (Goulbourne, Jr. and Greenberg, 1981; Fosnaugh and Greenberg, 1988; Fosnaugh and Greenberg, 1989). Perhaps the genes of unknown function that map within the motility gene clusters are involved in this coordination. There are also some duplications of genes which are unexpected. For example, there are two *fliG* basal body switch gene homologs in both *B. burgdorferi* and *T. pallidum*; only one *fliG* is generally present in other bacteria (Macnab, 1996). Each of the *fliG* homologs are in different operons. The function of each of these *fliG* genes is presently unknown, but they could be related to the opposite polarity of flagellar rotation as discussed above.

The control of motility gene expression is clearly different in *B. burgdorferi* compared to that of other bacteria. In other bacteria there is a cascade control of motility gene expression: These genes are regulated in an ordered sequence of three classes (Macnab, 1996). Late genes, which encode the flagellar genes, chemotaxis genes, and some of the basal body genes are transcribed under the control of sigma 28. A detailed transcriptional analysis of motility genes of *B. burgdorferi* using primer extension analysis and RT-PCR has shown that four of the operons so far studied are initiated by sigma 70, but not by sigma 28 (Ge *et al*., 1997a; Ge *et al*., 1997b; Ge and Charon, 1997c). These results are consistent with the analysis of its complete genome: only sigma 70 and sigma 54 factors were found in *B. burgdorferi*; no sigma 28 factors were identified (Fraser *et al*., 1997).

The basis for sigma 70 like control of periplasmic flagella synthesis and motility in *B. burgdorferi* is poorly understood, but it could be related to the ecological niches of this organism (Ge *et al.,* 1997b). *B. burgdorferi* synthesizes periplasmic flagella throughout its life-cycle: serological evidence indicates that periplasmic flagella proteins are synthesized in the tick and during animal and human infection. In other bacteria, such as *E. coli* and *Bordetella pertussis*, environmental conditions markedly influence flagellar synthesis; these and other bacteria shut off flagellar synthesis under certain environmental conditions (Armitage, 1992; Akerley *et al*., 1995; Macnab, 1996). Perhaps motility is so vital to *B. burgdorferi* in its environmental habitats that it has evolved a unique flagellar synthesis control mechanism. Conceivably, the direct involvement of the housekeeping protein sigma 70 in flagellar gene expression could be an indicator of the importance of motility in all phases of growth and survival in nature.

Conclusion and Future Directions

A few years back, the comment was made that "spirochetes come in from the cold" as the genome of *B. burgdorferi* was published (Barbour , 1998). Now that genetic tools have been recently developed to inactivate specific genes in several spirochete species (see accompanying articles in this volume), we are now in a position to sort out what makes spirochetes so unique. Progress has been made over the last several years in understanding spirochete structure and their mechanisms of motility. As discussed in this review, there is much to be done in sorting out the regulation and function of individual genes involved in motility, and how spirochetes coordinate the rotation of their periplasmic flagella to effect directed movement.

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