

# Chemotaxis in Pathogenic Spirochetes: Directed Movement Toward Targeting Tissues?

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## Abstract

**Chemotaxis is an important feature of motile organisms that allows navigation through various environments. It enables them to detect nutrients and to avoid unfavorable or dangerous conditions. Motility and chemotaxis are widely acknowledged as important virulence factors for pathogenic bacteria. In this review, we try to explore the role of chemotaxis in the pathogenesis of spirochetes. Chemotaxis might be involved in tissue identification and penetration, and represents a possible mechanism for evasion of the host's immune defense. The recent development of genetic tools for pathogenic spirochetes and "tracking" techniques, employing fluorescent *in situ* hybridization (FISH), could revolutionize our understanding of the importance of chemotaxis for infection and persistence of these bacteria in their host.**

## The Spirochetes

The bacterial order of spirochetes contains many members that are responsible for a variety of human and mammalian diseases, such as Lyme disease, relapsing fever, syphilis, leptospirosis, and periodontitis. Many of these organisms try to resist further study by being difficult or impossible to cultivate *in vitro*. Spirochetes are divided into eight different genera: *Borrelia*, *Brachyspira*, *Brevinema*, *Cristispira*, *Leptonema*, *Leptospira*, *Spirochaeta*, and *Treponema* (Olsen *et al.*, 2000). The genus *Borrelia*, which contains the causative agents of Lyme disease, relapsing fever, and borreliosis, seems to be comprised of "obligate" pathogens. All *Borrelia* species identified so far are pathogenic. *Brachyspira* require host-association as pathogens or parasites. Some members of *Brachyspira* that formerly belonged to the genus *Serpulina* (Ochiai *et al.*, 1997) are characterized by their attachment to the colonic mucous layer of mammalian or avian hosts. *Brachyspira* (*Serpulina*) *hyodysenteriae* is responsible for swine dysentery. *Brevinema* and *Cristispira* reside as parasites in small mammals or gastropods, respectively. Both *Leptonema* and *Leptospira* are found free-living in soil, freshwater and

marine environments, or associated with mammalian hosts. However, *Leptonema* is non-pathogenic, whereas *Leptospira* contains pathogenic species, such as *Leptospira interrogans*, the etiological agent of leptospirosis. All known members of *Treponema* live host-associated as parasites or pathogens. *Treponema pallidum* and *Treponema pertenue* cause syphilis and yaws, respectively. A variety of oral treponemes, such as *Treponema denticola*, are involved in gingivitis and periodontitis. *Spirochaeta* is found exclusively free-living in freshwater or marine habitats. This genus of spirochetes is the only one that does not contain members with a "host-requirement".

## Unique Motility of Spirochetes

Spirochetes have drawn a lot of attention not only because of their infectious potential, but also because they show interesting morphological and motility features. "Coiled hair", the literal meaning of spirochete, fits their appearance strikingly well (Figure 1). Very thin and long, helical-shaped in a corkscrew-like or flat-waved manner, these highly motile bacteria come in all sizes (0.1 – 3 x 5 –250 µm). They are the only known flagellated bacteria whose flagella do not protrude through the outer membrane but rather reside in the periplasm. Number and length of flagella varies between the different species, a characteristic that is commonly used as a tool for classification. Spirochetes exhibit a very unique kind of motility: the polar inserted periplasmic flagella rotate around the cytoplasmic cylinder and generate thrust (Berg, 1976; Charon *et al.*, 1992a). The resulting movement has been referred to as rotation about their longitudinal axis for spiral-shaped spirochetes (Canale-Parola, 1978) or snake-like as a planar helical wave for *Borrelia* (Goldstein *et al.*, 1994). This type of motility enables translocation in highly viscous environments, an adaptation to their lifestyle as pathogens/parasites in tissues or free-living as "mud-dwellers". In contrast, movement of most of the externally flagellated bacteria is greatly impaired under viscous conditions (Schneider and Doetsch, 1974).

Motility in spirochetes is powered by proton motive force ( $\Delta p$ ) (Goulbourne and Greenberg, 1980) as is common for other motile bacteria (Larsen *et al.*, 1974; Shioi *et al.*, 1978). Flagellar motors of both, spirochetes and externally flagellated bacteria, appear to rotate in either direction, clockwise (cw) or counterclockwise (ccw) (Charon *et al.*, 1992b; Berg and Anderson, 1973). Most of the genes and proteins that build the flagellar motor hook-basal body (HBB) complex in enteric bacteria (Macnab, 1996) are also present in spirochetes (Limberger *et al.*, 1994; Jwang *et al.*, 1995; Ge *et al.*, 1996; Li *et al.*, 1996; Limberger *et al.*, 1996; Heinzerling *et al.*, 1997; Fraser *et al.* 1997, 1998; Stamm and Bergen, 1999). Despite the unusual placement of the flagellar in the periplasm, the architecture of the spirochete HBB appears to be very similar to the motors of many flagellated bacteria (Brahamsha and Greenberg, 1988).

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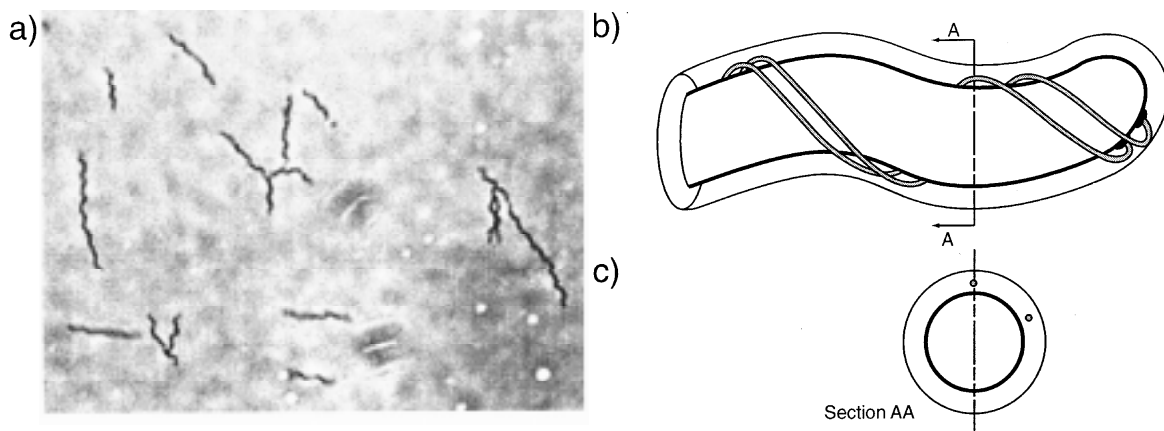


Figure 1. Cellular morphology of spirochetes. a) phase-contrast image (1000 x) of *T. denticola* ATCC35405 taken with a digital camera (SPOT 1.5.0, diagnostic instruments inc.) through a 100 x objective lens (Leica) b) schematic drawing of one spirochete cell end showing two periplasmic flagella wrapped around the cell body. c) schematic cross-section through the spirochete cell.

The motility pattern of spirochetes alternates between smooth swimming and flexing movements of variable duration (Greenberg *et al.*, 1985; Fosnaugh and Greenberg, 1988). The change in swimming direction is referred to as a reversal. In a reversal, the previously posterior end becomes the leading end of the cell. The frequent change of swimming in either direction and flexing is thought to be homologous to runs and tumbles in externally flagellated bacteria. In peritrichous flagellated bacteria, such as *Escherichia coli*, runs are generated by ccw rotation of the flagellar motor that allows formation of a thrust-generating flagellar bundle (Anderson, 1975). Tumbles are caused by disruption of the flagellar bundle upon cw rotation (Macnab and Ornston, 1977). Smooth swimming in spirochetes is believed to be the result of opposite rotation of the periplasmic flagella as viewed from the center of the cell. Flexing occurs when they spin in the same direction (Berg, 1976; Fosnaugh and Greenberg, 1988). A more detailed description of the unique motility of spirochetes is given by Li *et al.* in this issue of JMMB.

### Chemotactic Signal Transduction Pathways and the Related Genes

Motile bacteria are generally chemotactic which allows the organisms to perform directed movements in response to various environmental signals. Enteric bacteria, such as *E. coli* and *Salmonella typhimurium*, respond with movement towards nutrients, *e.g.* sugars and amino acids (positive chemotaxis), and with movement away from high/low pH or toxic compounds, *e.g.* benzoate and indole (negative chemotaxis). Over the past decades, an impressive amount of molecular information about chemotaxis in these enteric bacteria has been revealed (Stock and Surette, 1996). Chemotaxis is controlled by a set of chemotaxis proteins that modulate the tumble frequency of the bacterial cell.

In *E. coli*, environmental signals are transduced via a set of five membrane proteins, the methyl-accepting chemotaxis proteins (MCPs) (Figure 2a). The input signal is integrated into a motor response through the two-

component system CheA/CheY. CheA, the histidine kinase, is bound to the MCPs in a complex with CheW and communicates the signal to the response regulator CheY via phosphotransfer reactions. The phosphorylation level of CheY controls the direction of motor rotation. The flagellar motor spins naturally counterclockwise; binding of CheY~P to the flagellar motor switch reverses the rotation to clockwise and causes tumbling. Binding of chemoeffectors to the periplasmic portion of the MCPs induces a conformational change in the receptor that propagates across the membrane and regulates motor

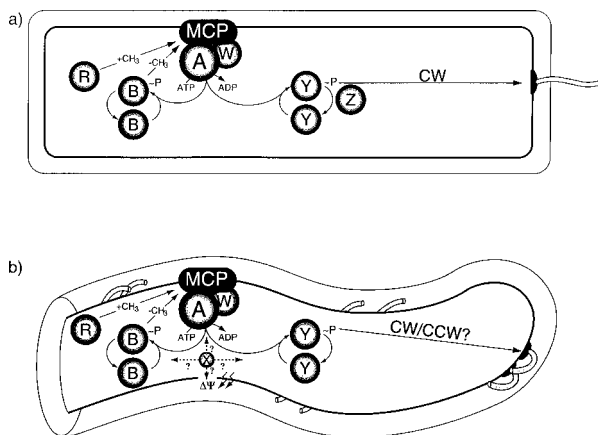


Figure 2. Bacterial signal transduction pathways. a) in *E. coli*: The proteins of the chemotactic signal transduction pathway (CheA=A, CheB=B, etc.), one of the chemoreceptors (MCP), and one of the 6-10 flagellar motors are indicated. See text for further explanation. b) hypothetical pathway in spirochetes: Signal transduction reactions very likely involve phosphotransfer reaction as shown for *E. coli*. However, it is not clear if "free" motors spin cw or ccw. Therefore the binding of CheY~P to the flagellar motor could result in cw rotation like in *E. coli* or in ccw rotation as described for *B. subtilis*. A membrane potential  $\Delta\Psi$  might be involved in signaling. CheX, a hypothetical chemotaxis protein, could be involved in transmission of this signal to the membrane, or interact with any of the other chemotaxis proteins. This pathway is lacking CheZ.

Table 1. Chemotaxis Genes in Spirochetes

<i>T. pallidum</i>	<i>B. burgdorferi</i>	<i>E. coli</i>	Protein function in <i>E. coli</i>
<i>mcp-1</i>	<i>mcp-1</i>	<i>aer</i>	chemoreceptor
<i>mcp2-1</i>	<i>mcp-2</i>	<i>tar</i>	"
<i>mcp2-2</i>	<i>mcp-3</i>	<i>tap</i>	"
<i>mcp2-3</i>	<i>mcp-4</i>	<i>trg</i>	"
	<i>mcp-5</i>	<i>tsr</i>	"
<i>cheA</i>	<i>cheA-1</i> <i>cheA-2</i>	<i>cheA</i>	histidine kinase/ signal transduction
<i>cheY</i>	<i>cheY-1</i> <i>cheY-2</i> <i>cheY-3</i>	<i>cheY</i>	response regulator/ signal transduction
<i>cheW-1</i> <i>cheW-2</i>	<i>cheW-1</i> <i>cheW-2</i> <i>cheW-3</i>	<i>cheW</i>	purine-accepting chemotaxis protein/ connection MCP-CheA
<i>cheB</i>	<i>cheB-1</i> <i>cheB-2</i>	<i>cheB</i>	methylesterase/adaptation
<i>cheR</i>	<i>cheR-1</i> <i>cheR-2</i>	<i>cheR</i>	methyltransferase/adaptation
<i>cheX</i>	<i>cheX</i>	-	no homologue identified

responses by modulating CheA activity. Attractant binding decreases CheA autophosphorylation resulting in prolonged runs, whereas repellents stimulate CheA activity and thereby increase tumble frequency. Motor responses are enhanced by a phosphatase, CheZ, that specifically dephosphorylates CheY~P. Adaptation to the respective stimuli occurs through the action of CheR and CheB, which methylate and demethylate the cytoplasmic receptor domain, respectively. CheB also belongs to the response regulator family and can be activated upon phosphorylation by CheA.

The in-depth knowledge about chemotaxis in enteric bacteria may help us to better understand chemotaxis in spirochetes. Indeed, recent molecular studies on various pathogenic spirochetes, especially the whole genome sequence analysis of two pathogenic spirochetes, *B. burgdorferi* (Fraser *et al.*, 1997) and *T. pallidum* (Fraser *et al.*, 1998), revealed that many pathogenic spirochetes contain homologues of the genes necessary for chemotaxis responses in other bacterial systems (Table 1). This supports the idea that these pathogenic spirochetes perform chemotaxis and that their signal transduction may occur in a manner very similar to the known pathways of other bacteria.

Genomic information about the chemotaxis genes in *B. burgdorferi* and *T. pallidum* suggests an important role of chemotaxis in spirochetes. Both pathogens have a minimalistic chromosome that barely exceeds 1 Mb and lacks genes for many metabolic functions. However, the motility and chemotaxis genes take up more than 6% of the *T. pallidum* and about 5% of the slightly larger *B. burgdorferi* genome. *B. burgdorferi* even allows itself the luxury of two chemotaxis operons. One of the operons is closely related to the chemotaxis operons identified in other pathogenic spirochetes, such as *T. pallidum* or *T. denticola* (Greene and Stamm, 1999). The second chemotaxis operon shows the highest homologies to chemotaxis operons found in  $\alpha$ -proteobacteria and might have been

obtained through horizontal gene transfer (Igor Zhulin – unpublished). This opens up an interesting question: Does a pathogen like *B. burgdorferi*, whose lifecycle involves two completely different host environments (arthropod host tick and its various mammalian hosts) need two different signal transduction pathways for optimal adaptation to the respective hosts? Other pathogenic spirochetes, such as the above-mentioned treponemes, do not venture into changing their host environment dramatically and, therefore, might be perfectly fine with a single chemotaxis operon.

Multiple MCPs have been identified in *B. burgdorferi* and *T. pallidum* suggesting chemotaxis responses to various chemoattractants/repellents. This has already been confirmed for *B. burgdorferi* (Shi *et al.*, 1998). One MCP typically recognizes more than one chemoeffector. MCPs have also been found in the oral spirochete *T. denticola* (Kataoka *et al.*, 1997; Greene and Stamm, 1997; Li *et al.*, 1999) whose genome sequence is not yet completed.

No homologue for *cheZ* was found in *B. burgdorferi* or *T. pallidum*. *B. subtilis*, *Campylobacter jejuni*, *Helicobacter pylori*, and other motile bacteria, whose genome sequences are completed, also do not contain *cheZ*. This renders the involvement of CheZ in chemotactic signal transduction pathways that have been identified so far, the exception rather than the rule. The role and function of CheZ has only been demonstrated in the enteric bacteria, *E. coli* and *S. typhimurium*, and various *Pseudomonas ssp.* Instead a novel gene that might be involved in chemotaxis, *cheX*, was discovered in spirochetes. The *cheX* locus maps between *cheW* and *cheY*. It belongs in *T. denticola*, and very likely in *T. pallidum* and *B. burgdorferi* as well, to the same transcriptional unit as *cheA*, *cheW*, and *cheY* (Greene and Stamm, 1999). Interestingly, more detailed sequence analysis revealed that *cheX* might be related to *cheC* of *B. subtilis* (Ygor Zhulin – unpublished). A *cheX*-like gene was also found in *Thermatoga maritima*, a motile marine bacterium that lives in the extreme environment of

geothermally heated sea floors. It is interesting to note that these bacteria are relatively closely related in the phylogenetic tree. This similarity, in addition to the lack of *cheZ*, opens up the possibility that signal transduction in spirochetes exhibits similarities to the signal transduction pathway described for *B. subtilis* (Garrity and Ordal, 1995). A possible pathway is shown in Figure 2b.

### Previous Studies on the Chemotactic Behavior in Spirochetes.

Not surprisingly, spirochetes – being motile and having all the necessary chemotaxis genes – are chemotactic. Most of our knowledge about spirochete chemotaxis is based on pioneering work on a free-living spirochete, *Spirochaeta aurantia*, by Greenberg, Canale-Parola and coworkers. Chemotaxis assays, such as swarm agar plates, capillary assay (Adler, 1973; Greenberg and Canale-Parola, 1977), and video analysis of cellular behavioral changes (Fosnaugh and Greenberg, 1988) have been applied to measure chemotactic responses in this spirochete. Through these assays, a variety of sugars (glucose, galactose, fucose, mannose, xylose, maltose, and glucosamine), but not amino acids have been identified as attractants (Greenberg and Canale-Parola, 1977). Repellents are alcohols such as ethanol or butanol, numerous acids, sulfide (Kaempf and Greenberg, 1990), and high oxygen concentrations (Greenberg and Canale-Parola, 1977). The addition of an attractant results in increased smooth swimming while addition of repellent causes increased flexing and reversal of swimming direction (Fosnaugh and Greenberg, 1988). The molecular mechanism of this chemotactic behavior is still largely unknown.

Methylation/demethylation of specific proteins upon addition/removal of chemoattractants has been demonstrated for *S. aurantia* (Kathariou and Greenberg, 1983). These proteins seem to be similar to the methyl-accepting chemotaxis proteins, the known chemoreceptors of other bacteria (Nowlin *et al.*, 1985). Other studies have indicated the additional involvement of a membrane potential ( $\Delta\Psi$ ) in chemotactic signal transduction, a feature that is not found in other known bacterial pathways (Goulbourne and Greenberg, 1981). The addition of attractants, such as D-glucose or D-xylose, resulted in a transient depolarization of the membrane, whereas non-attractant sugars did not show this effect. Disruption of  $\Delta\Psi$ , but not  $\Delta\text{pH}$ , inhibited chemotaxis responses without affecting motility. Application of a voltage clamp had the same effect as disruption of  $\Delta\Psi$  (Goulbourne and Greenberg, 1983). The authors speculated that the membrane potential might be needed to support fast transmission of the chemosensory information across the extraordinary long cell body of spirochetes.

The first chemotaxis mutants isolated and characterized in spirochetes were obtained in *S. aurantia* (Fosnaugh and Greenberg, 1989). These mutants fell into four different classes of phenotypes (*che-101*, *che-200*, *che-300*, *che-400*) with distinct motility patterns and altered chemotactic behavior. Their chemotactic properties were assessed using swarm plates and capillary assays, as well as video microscopy. Additionally, protein methylation and fluctuation in membrane potential was measured. The *che-101* mutant is non-chemotactic and exhibits altered

swimming behavior. This mutant seems to have a ratio of swimming to flexing that is similar to wild-type cells but rarely reverses the leading end after flexing. This result suggests that the ability to modulate the reversal frequency is crucial in spirochete chemotaxis. The *che-200* mutant flexes almost constantly and therefore resembles a tumbling mutant in *E. coli* (Parkinson and Houts, 1982). Similar to the *E. coli* phenotype, its chemotactic response is greatly reduced. The mutant phenotype of *che-300* is characterized by a high reversal frequency that results in impaired chemotaxis responses. The *che-400* mutant shows the swimming pattern of unstimulated wild-type cells but fails to respond to any kind of chemoeffector. A pseudorevertant, *che-401*, which reacts to certain stimuli, was isolated from *che-400*. However, this mutant showed prolonged adaptation times. All of these mutants showed surface protein methylation patterns indistinguishable from the wild type. In contrast, fluctuations in the membrane potential upon addition of attractant or non-attractant sugars differed radically from the wild-type behavior. These alterations could not be correlated with the various mutant phenotypes. Currently, these mutants remain the only mutants isolated in spirochetes that are affected in their swimming pattern and/or chemotactic response. It would be very interesting to further characterize these mutants and analyze them at a molecular level.

Although motility and chemotaxis are widely recognized as important virulence factors (Ottenmann and Miller, 1997), very little is known about chemotaxis in pathogenic spirochetes and its role in pathogenesis. The only report so far on chemotactic responses of *B. burgdorferi* identified serum as an attractant (Shi *et al.*, 1998). None of the known amino acids or a variety of carbohydrates tested elicited a response. However, high (> 8.5) or low (< 6.5) pH,  $\text{H}_2\text{O}_2$ , KCl,  $\text{CaCl}_2$ , ethanol, and butanol acted as repellents. Motility was optimal in buffer containing 0.15 M NaCl; higher and lower concentrations or different ions resulted in reduced motility. The authors discussed that this spectrum of attractants/repellents, in addition to the fact that NaCl was needed for motility, makes it likely that *B. burgdorferi* uses chemotaxis to direct its movement through tissues. The physiological conditions of the interstitial fluid meet the requirements for *B. burgdorferi* motility (high NaCl and pH 7.6). The attractant response to serum could be involved in the movement from tissues into the bloodstream and vice versa – a key element for infection. The repellent response to  $\text{H}_2\text{O}_2$ , a chemical released by neutrophils and macrophages, is also interesting. Chemotaxis might help *B. burgdorferi* to avoid the immune system of its host.

Qualitative experiments have been performed on the chemotactic behavior of *Leptospira interrogans*, a spirochete that causes leptospirosis (Yuri *et al.*, 1993). Virulent strains of this organism showed a positive chemotaxis response towards hemoglobin. This response was absent in avirulent derivatives of *L. interrogans* or saprophytic, non-virulent species, such as *L. biflexa*. Other chemoeffectors or conditions needed for optimal motility have not yet been identified. However, the observation that the virulent strains perform chemotaxis towards hemoglobin supports the idea that chemotaxis is involved in pathogenesis.

Another pathogenic spirochete whose chemotactic abilities have been assessed is *B. hyodysenteriae*, the

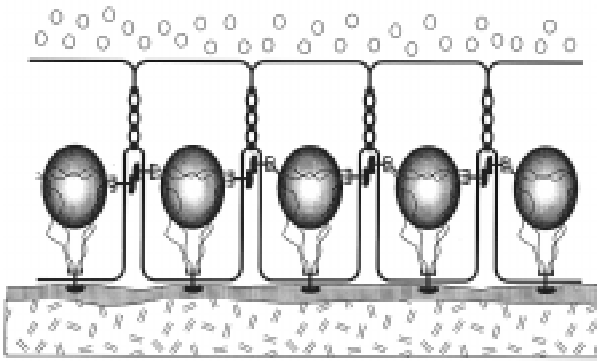


Figure 3. Epithelial cell layer. Schematic drawing showing epithelial cells that are interconnected by tight junctions (●) that make the tissue impermeable to even small molecules (○). Anchoring junctions (◄-E) join the cytoskeletal filaments (⌒) of the individual cells together and anchor them into the extracellular matrix (■). The underlying connecting tissue (//) is indicated as well.

etiologic agent of swine dysentery. This organism is closely associated with the intestinal mucosa of pigs. Large numbers of *B. hyodysenteriae* accumulated in capillaries filled with HGM (hog gastric mucin) (Kennedy *et al.*, 1988). The response toward hog mucin was specific: bovine mucin failed to elicit a response. Virulent strains of *B. hyodysenteriae* were significantly more chemotactic than avirulent ones (Milner and Sellwood, 1994). Later studies by Kennedy and Yancey (1996) confirmed the chemotactic response to mucin and identified a variety of other chemoattractants, such as the sugars fucose, galactose, and lactose, the amino acids serine and cysteine, and blood. Like *B. burgdorferi*, the motility of *B. hyodysenteriae* is also extremely sensitive to optimal NaCl concentration, exhibiting active motility only at physiological salt concentrations. Isolates of *Brachyspira (Serpulina) pilosicoli*, a relative of *B. hyodysenteriae* that causes colonic spirochetosis in humans and other animals, recognize serine or porcine gastric mucin as attractants depending on the organism from which they are isolated (Witters and Duhamel, 1999).

The oral spirochete *T. denticola* is involved in periodontal disease. Some preliminary studies suggest that it might exhibit chemotactic responses towards 11 different amino acids and sugars (Mayo *et al.*, 1990). These responses are greatly inhibited by addition of glucose to the growth medium, indicating that *T. denticola* might undergo catabolite repression that affects chemotactic behavior. Two open reading frames (ORFs), *dmcA* and *dmcB*, whose predicted protein sequences show homology to Tar and Tap, chemoreceptors of *E. coli*, and McpA of *B. subtilis*, have been identified in *T. denticola* (Kataoka *et al.*, 1997; Li *et al.* 1999). These proteins also cross-reacted with antiserum that was specific for the Trg-chemoreceptor of *E. coli*. *DmcA* and *DmcB* exhibit distinct methylation patterns that are absent in the null mutants. Interestingly, *DmcB* seems to affect methylation of *DmcA*, whereas the opposite is not the case. The *dmcA* and *dmcB* mutant strains of *T. denticola* are unable to migrate from nutrient poor conditions toward a nutrient rich (serum containing) environment in a qualitative chemotaxis assay.

### Perspective: Chemotaxis Toward Targeting Tissues?

Pathogenic spirochetes enter their host through mucous membranes (*Treponema*, occasionally *Leptospira*), direct injection into the host tissue via an arthropod vector, such as tick or lice (*Borrelia*), or simple ingestion (*Leptospira*, *Brachyspira (Serpulina)*). Invasive spirochetes, such as *Borrelia* or pathogenic leptospires and treponemes, multiply at their site of entry (primary lesion) and start spreading throughout the tissue in a motility dependent manner. Several investigators have addressed the importance of motility in spirochete pathogenesis. A non-motile mutant of *B. burgdorferi* turned out to be non-pathogenic and failed in tissue penetration (Sadziene *et al.*, 1991). Chemically inactivated *L. interrogans* is unable to adhere to or invade kidney fibroblasts or monocytes (Merien *et al.*, 1997). *Brachyspira (Serpulina)* is rarely invasive; it is pathogenic by simple attachment to the gastric mucosal cell layer. This attachment was also found to be motility dependent. A mutant of *B. hyodysenteriae* that lacks flagella was unable to successfully colonize the mucin layer of pig intestines (Rosey *et al.*, 1996).

During invasion of the host, the bloodstream is often used as a means for transportation. In order to get there from the initial site of infection, many barriers, the epithelial and endothelial cell layers, must be overcome. Tight junctions interconnect cells in these tissue types and make the cell layer impermeable even to very small molecules (Figure 3). Pathogenic spirochetes penetrate these tissues by actively invading these intercellular junctions and/or the cells in a motility dependent manner (Thomas *et al.*, 1988; Comstock and Thomas, 1989; Szczepanski *et al.*, 1990; Thomas and Higbie, 1990; Riviere *et al.*, 1991; Sadziene *et al.*, 1991; Haake and Lovett, 1994). Mechanisms to facilitate tissue penetration involve disruption or rearrangement of the cytoskeleton (Baehni *et al.*, 1992; De Filippo *et al.*, 1995), proteolytic, fibrinolytic, and collagenolytic activities (Nitzan *et al.*, 1978; Mäkinen *et al.*, 1986; Ohta *et al.*, 1986; Uitto *et al.*, 1988; Rosen *et al.*, 1994, 1995; Coleman *et al.*, 1995), and inhibition of wound healing factors, such as fibroblast or endothelial cell proliferation (Taichman *et al.*, 1984; Boehringer *et al.*, 1984). Avirulent forms seem to be unable to penetrate tissue or cause damage to individual cells. In addition to migration through the tissue layers within the extracellular matrix *Borrelia ssp.* (Ma *et al.*, 1991; Hechemy *et al.*, 1992; Weiss *et al.*, 1997) and *L. interrogans* (Thomas and Higbie, 1990; Merien *et al.*, 1997) actively invade cells and can be detected alive inside the cytoplasm of the host cell surrounded by a shielding membrane. *Treponema* in contrast is rarely found alive inside of cells. They seem to get there accidentally and prefer to stay in the surrounding matrix during tissue penetration (Thomas *et al.*, 1988; De Filippo *et al.*, 1995).

The first step of invasion most likely involves adherence to the tissue. Specific adherence to different types of tissue has been demonstrated for some of the oral treponemes (Olsen, I., 1984; Reijntjens *et al.*, 1986; Camargo *et al.*, 1996), *T. pallidum* (Fitzgerald *et al.*, 1975; Fitzgerald *et al.*, 1977; Thomas *et al.*, 1986; Konishi *et al.*, 1986; Thomas *et al.*, 1988), *Borrelia* (Thomas and Comstock, 1989; Hechemy *et al.*, 1989; Kurtti *et al.*, 1993; Isaacs, 1994), and *Leptospira* (Tsuchimoto *et al.*, 1984; Vinh *et al.*, 1984; Ballard *et al.*, 1986; Ito and Yanagawa,

1987). Differential attachment to epithelial cell lines of different origin and confluence level was shown for the oral treponemes *T. denticola*, *T. socolodontum*, *T. socranskii*, and *T. vincentii*, and *T. pallidum* (Carranza *et al.*, 1997). This indicates that spirochetes might be able to recognize and distinguish certain tissues. Chemotaxis could be involved in directing the spirochetes towards the target tissue.

In later stages of infection, most of the pathogenic spirochetes manifest themselves preferentially in certain types of tissues, even though they are sporadically found in any type of organ. *B. burgdorferi* is thought to persist primarily in the joints, but also appears frequently in the central nervous system (CNS) and the brain. *T. pallidum* shows a preference for mucous tissues, as found in the mouth or the anogenital region, especially in the secondary stage of infection. This human pathogen is also known to commonly cause birth defects by transplacental transmission, and to invade the CNS, the brain, the heart and the musculoskeletal system. *L. interrogans* manifests itself in kidneys and liver, whereas the oral treponemes typically establish infection in the periodontal pocket. *Brachyspira* (*Serpulina*), the only pathogenic genus of spirochetes that causes inflammation by attachment to enterocytes, resides in the mucin layer of the colon.

How do these pathogens navigate through tissues, enter and leave the bloodstream, identify cell types and their "target" tissue? Even though motility has become more and more acknowledged as an important virulence factor, very little is known about the involvement of chemotaxis in spirochete pathogenesis. However, the idea that these invasive bacteria use directed movement towards target sites, rather than random "trial and error" appears to be a logical possibility.

The interesting lifecycle of *Borrelia* strongly suggests the involvement of a sensory system that allows directed movement as a response to physiological changes of their arthropod host. While the tick enjoys its "bloodmeal", *B. burgdorferi* starts migrating, via the hemolymph, from the gut into the salivary gland (Zung *et al.*, 1989). A plausible explanation for this behavior is the involvement of chemotaxis in this process. *B. burgdorferi* resides in the tick gut where the chemical/physiological signal, *i.e.* uptake of blood, appears first. In response to this environmental signal, the pathogen starts migrating toward the salivary gland. The saliva will deliver it right into its next host, the mammal the tick is feeding on. It is hard to imagine how this fine-tuned event could be performed successfully through erratic movement without the guidance of a chemosensory system. The presence of multiple chemoreceptors and signal transduction pathways in this spirochete strongly support the hypothesis that chemotaxis is an important element of its pathogenic lifecycle.

Another example of chemotaxis involvement in spirochete pathogenesis has been demonstrated in *B. hyodysenteriae*. The non-motile *flaA1 flaB1* double mutant of this pathogen is severely impaired in colonization of mucosal surfaces of the gastrointestinal tract (Rosey *et al.*, 1996). The surprisingly dense and exclusive association of *B. hyodysenteriae* with the mucin layer of pigs, along with the observation of a specific chemotaxis response to mucin, led some investigators to the conclusion that motility and chemotaxis might be key elements in the pathogenesis of this organism (Kennedy *et al.*, 1988).

Recently, fluorescence *in situ* hybridization (FISH) (Moter and Göbel, 2000) in combination with confocal laser scanning microscopy (CLSM) was adapted to monitor the distribution of pathogenic spirochetes in their host environment (Moter *et al.*, 1998; Boye *et al.*, 1998; Jensen *et al.*, 1998; Jensen *et al.*, 2000). Extensive FISH studies have been performed on cows with digital dermatitis (DD), a chronic ulcerative disease of cattle (Moter *et al.*, 1998). Spirochetes of the genus *Treponema* are possibly involved in the etiology of this disease (Read *et al.*, 1992). These treponemes are closely related to the oral treponemes that are associated with human periodontitis (Choi *et al.*, 1997). FISH is an impressive technique that allows demonstration of the aggressive invasiveness of spirochetes (Figure 4a). Typically, spirochetes are found in deeper layers of the tissue than any other bacterial species. Their distribution appears non-random, but rather oriented toward the dermis, thus supporting the idea that chemotaxis may be involved in tissue penetration. Through the destructive nature of their advancement into these parts of the tissue they also enable other bacteria to further extend their range of colonization.

The *Treponemes* that cause DD also appear to penetrate the tissue layers by migrating in the intercellular space rather than invading the cells, as documented previously for other treponemal species by electron microscopy (Thomas *et al.*, 1988; De Filippo *et al.*, 1995). Interestingly, individual cells that are located in deeper not yet completely invaded parts of the tissue appear to become a target for part of the spirochete population (Figure 4b). It is not yet clear what distinguishes this cell from the surrounding tissue; nevertheless, it seems to specifically attract spirochetes. The cell could have been damaged or gotten killed by "pioneering" spirochetes and is releasing factors that are recognized as a chemoattractant. Previous studies using light and transmission electron microscopy have indicated a close association of these spirochetes with necrotic cells (Choi *et al.*, 1997). Selective probes, in combination with FISH, also allow specific identification of one species within a multitude of organisms. This has been demonstrated by the specific *in vivo* identification of *B. hyodysenteriae* or *B. pilosicoli* within a biopsy containing a variety of different *Brachyspira* *ssp* (Boye *et al.*, 1998; Jensen *et al.*, 2000).

Tracking of fluorescent labeled spirochetes *in vivo* using CLSM would be another interesting option to address the invasiveness of pathogenic spirochetes. However, the application of the green fluorescent protein (GFP) that has become a powerful tool for *in vivo* fluorescent labeling (Prasher, 1995) is limited to aerobically growing organisms because of its strict requirement of oxygen for fluorescence. Another technique that was originally developed for *in vivo* fluorescent labeling of mammalian cells has recently been adapted in our lab for the staining of the anaerobic oral spirochetes *T. denticola* (Jon P. Tsai – unpublished, Figure 5). The modified protocol allows long-term fluorescent labeling of this spirochete by incorporation of the hydrophobic fluorescent dye PKH67 (Sigma) into the outer sheath. This method appears not to affect viability or motility of *T. denticola*.

The recent development of genetic inactivation tools for some species of pathogenic spirochetes, such as *B. burgdorferi*, *B. hyodysenteriae*, and *T. denticola* (Samuels *et al.*, 1994; ter Huurne *et al.*, 1992; Li *et al.*, 1996), now

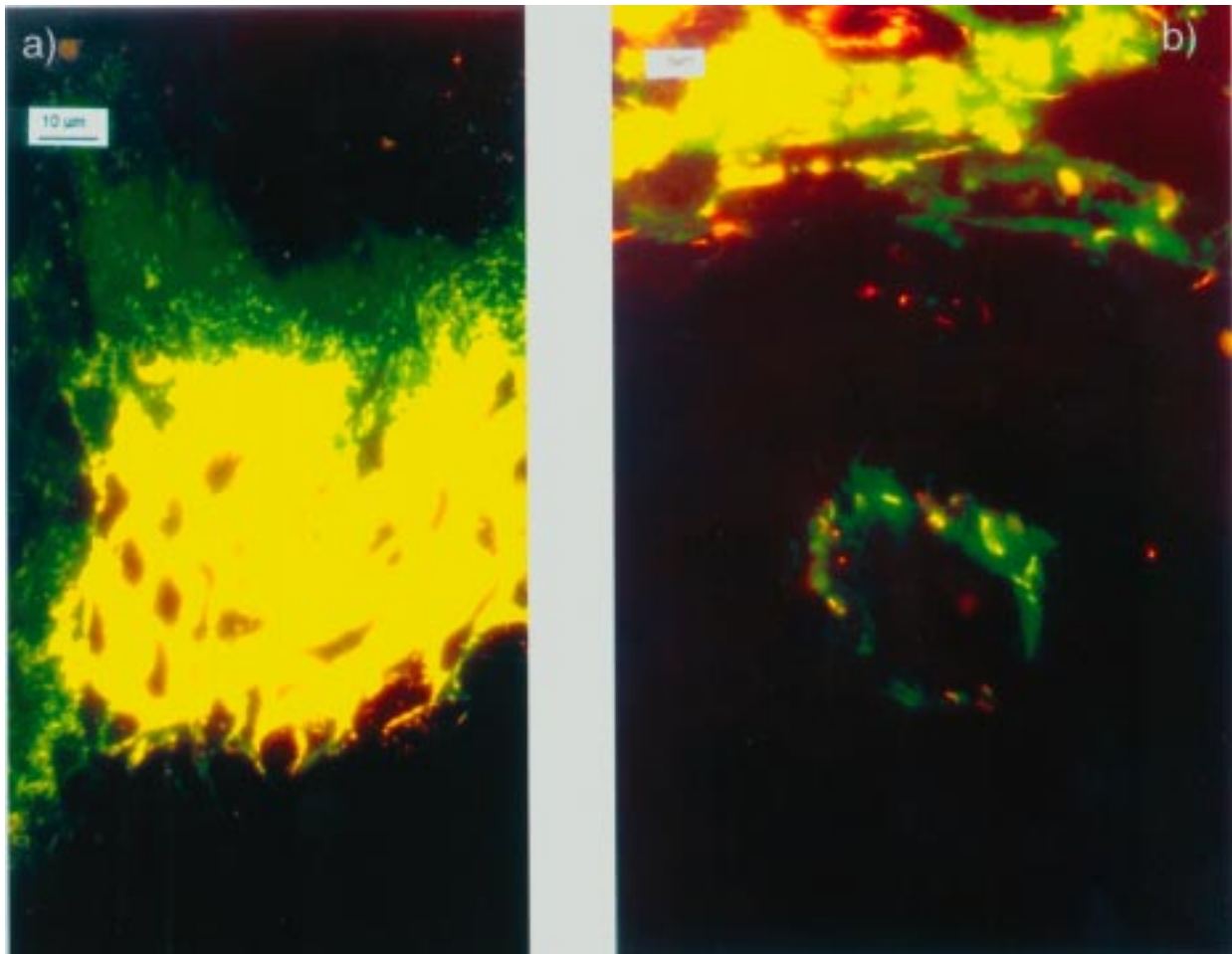


Figure 4. Spatial distribution of spirochetes within a tissue as shown by CLSM. a) Simultaneous hybridization with bacterial probe EUB338 FITC and DDK4Cy3, a probe specific for treponemes associated with digital dermatitis. Large numbers of treponemes are visible in the intercellular spaces of the epidermis. Note the stratification with EUB338-positive bacteria predominantly at the outside (upper part of the microphotograph) and DDK4-positive treponemes within stratum corneum and the stratum spinosum. (Bar 10  $\mu\text{m}$ ). b) FISH using DDK4 Cyanine dye (Cy3) and TRE I Fluorescein-Isothiocyanate (FITC), a probe specific for oral treponemes of phylogenetic group I, most of which are as yet uncultured. At higher magnification, single spirochetes are visible that seem to invade the tissue through the intercellular spaces. TRE I-positive and DDK4-positive treponemes surrounding an epithelial cell (with permission, A. Moter and U.B. Göbel, J. Microbiol. Meth., 2000. Bars, 5  $\mu\text{m}$ ).

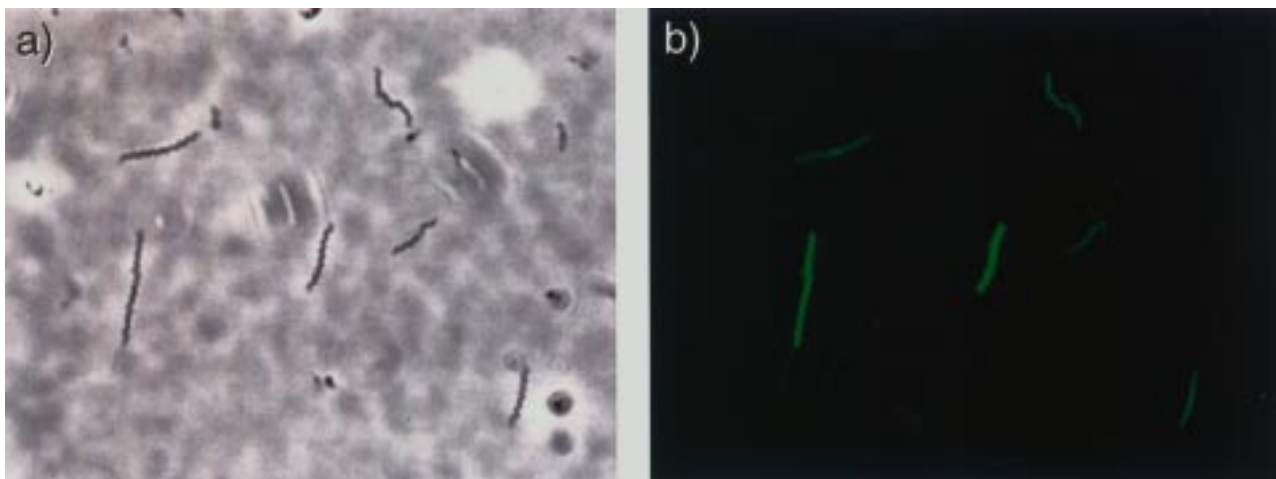


Figure 5. Fluorescent labeled spirochetes using PHK67. a) Phase contrast image of PHK67 fluorescent labeled *T. denticola* ATCC35405 taken as figure 1 a. b) Fluorescent image of the same cells.

enables the construction of specific chemotaxis mutants. These mutants will allow further exploration of the interesting possibility that chemotaxis is used for guidance of these virulent bacteria through the complex environment of their various hosts.

Several investigators have already addressed the role of some motility-related genes. As discussed previously, the genes *flaA1* and *flaB1* have been inactivated in *B. hyodysenteriae* (Rosey et al., 1995, Kennedy et al., 1997). The respective double mutant was constructed and shown to be deficient in pathogenesis (Rosey et al., 1996). Similarly, *flgE* (flagellar hook) and *tap1* (hook length control) mutants have been created in *T. denticola* (Li et al., 1996; Limberger et al., 1999). The only mutants in spirochetes that directly effect the chemotactic signal transduction pathway, rather than the motility apparatus, were also constructed in *T. denticola* (Kataoka et al., 1997; Li et al., 1999). Knockout mutants of *dmcA* and *dmcB*, the genes that encode MCPs, are defective in chemotaxis as mentioned previously.

Genome analysis will facilitate the application of these gene inactivation methods to create specific mutants that are defective in the general signal transduction (*cheA*, *cheW*, or *cheY*) or various chemoreceptors. In addition, the nature of the mysterious *cheX* might be finally revealed. These defined mutants can then be analyzed for pathogenic potential through various *in vitro* and *in vivo* assays that have been successfully applied to study other virulence factors. FISH and other fluorescent labeling techniques will allow specific tracking of these mutants during the experiment using fluorescence confocal microscopy. Such studies are expected to yield valuable information regarding the complex nature of spirochete infection and possibly lead to the development of novel therapeutic targets.

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