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, syphilis, 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 colonal mucus 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 (Δp) (Gouilbourse 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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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 activity.

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.

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.
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* (Kataoka et al., 1997; Greene and Stamm, 1997; Li et al., 1999) whose genome sequence is not yet completed. MCPs have also been found in the oral spirochete *T. denticola*, 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 chemotactants 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 (ΔΨ) 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 ΔΨ, but not ΔpH, inhibited chemotaxis responses without affecting motility. Application of a voltage clamp had the same effect as disruption of ΔΨ (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 an 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, *H₂O₂*, KCl, CaCl₂, 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 *H₂O₂*, 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
etiological 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 chemotactic stimulants, 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 leptospirae 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; Reijnjens 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, 1986).
Differential attachment to epithelial cell lines of different origin and confluence level was shown for the oral treponemes *T. denticola*, *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 mucus 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 (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 *Treponema* 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

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.

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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 µm). b) FISH using DDK4 Cyanine dye (Cy3) and TRE I Fluorescin-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 µm).

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, fliE (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 spheroplasts 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 therapeutical targets.

Acknowledgements

We wish to thank Dr. Igor Zhulin for providing exciting information on genomics of bacterial chemotaxis and his expert discussion of this topic, Jon P. Tsai for his unpublished results, and Michael Wagner for access to and help with the confocal laser scanning microscope. We would also like to thank Dr. Nyles Charon, Dr. Ulf B. Göbel, Dr. Howard Kuramitsu, Patrick Masson for illustrations, and all lab members for their great support. This work is supported by NIH grant DE12532 and GM54666 to Dr. Wenyuan Shi, and by grant O1K9318 from the Bundesministerium für Bildung und Forschung and the Körber European Research Award to Dr. Ulf B. Göbel, and the Humboldt Universität zu Berlin.

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