Motility and Chemotaxis in Tissue Penetration of Oral Epithelial Cell Layers by Treponema denticola

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The ability to penetrate tissue is an important virulence factor for pathogenic spirochetes. Previous studies have recognized the role of motility in allowing pathogenic spirochetes to invade tissues and migrate to sites favorable for bacterial proliferation. However, the nature of the movements, whether they are random or controlled by chemotaxis systems, has yet to be established. In this study, we addressed the role of motility and chemotaxis in tissue penetration by the periodontal disease-associated oral spirochete Treponema denticola using an oral epithelial cell line-based experimental approach. Wild-type T. denticola ATCC 35405 was found to penetrate the tissue layers effectively, whereas a nonmotile mutant was unable to overcome the tissue barrier. Interestingly, the chemotaxis mutants also showed impaired tissue penetration. A cheA mutant that is motile but lacks the central kinase of the chemotaxis pathway showed only about 2 to 3% of the wild-type penetration rate. The two known chemoreceptors of T. denticola, DmCA and DmCB, also appear to be involved in the invasion process. The dmc mutants were actively motile but exhibited reduced tissue penetration of about 30 and 10% of the wild-type behavior, respectively. These data suggest that not only motility but also chemotaxis is involved in the tissue penetration by T. denticola.

Motility is now widely recognized as a virulence factor for many pathogenic organisms (39). Movement of motile organisms is usually guided by a sophisticated chemotaxis system (29, 56). Motility and chemotaxis are known to allow bacteria efficient nutrient acquisition, avoidance of toxic substances, or translocation to optimal colonization sites. In a variety of pathogenic bacteria, including the human gastric and gastrointestinal pathogens Helicobacter pylori (5) and Campylobacter jejuni (57, 62), the cholera agent Vibrio cholerae (8–10), the fish pathogen Vibrio anguillarum (36–38) and the plant root pathogen Agrobacterium tumefaciens (13), chemotaxis appears to be an important factor for successful colonization of their respective hosts. Interestingly, a strong chemotactic response towards substances that are present at their site of infection has been demonstrated for many gastrointestinal pathogens, including H. pylori, V. cholerae, and the spirochete Brachyspira hyodysenteriae, each of which is highly attracted by mucin. It has been discussed that this feature enables H. pylori to direct itself against the gastric flow toward the epithelium (5). Fully motile but nonchemotactic mutant strains were shown to be avirulent even though they appeared to be able to persist in the stomach for an extended period of time.

Pathogenesis-associated spirochetes are motile bacteria that can be found in the most advanced regions of infected tissue (34). Previous studies have shown that the motility of spirochetes is a key virulence factor, since spirochete motility mutants fail to infect their host (18, 48). While it is evident that pathogenic spirochetes do move within the tissues of their respective hosts, it is still unclear whether these cellular movements are random or directed by chemotaxis systems. Genome sequence analyses of Borrelia burgdorferi, Treponema pallidum, and Treponema denticola revealed that these spirochetes not only have complete flagellum-based motility systems but also possess the genes necessary for chemotaxis that could direct the flagellar movement (6, 7, 11, 14, 22a, 54; see also data available at www.tigr.org). Earlier experiments in our laboratory as well as those of other groups have also indicated that pathogenic spirochetes do indeed perform chemotaxis towards substances present at their site of infection (15, 19, 23, 30, 33, 52, 63). Based on these findings, we have hypothesized that chemotaxis may play a role in guiding motility of pathogenic spirochetes during penetration and further invasion of the host tissues (28). In this study, we addressed this question by analyzing the tissue penetration ability of chemotaxis mutants of the oral spirochete T. denticola.

T. denticola is frequently isolated from inflamed sites of the periodontal pocket and is thought to be implicated in periodontal disease (27, 43, 49, 53, 61). Periodontal disease appears to be a very complex mixed infection involving virulence factors such as adhesion to the tissue, immune suppression, and tissue invasion and destruction (1). Several of these virulence factors have been characterized in T. denticola, including tissue-destroying enzymes that exhibit proteolytic, collagenolytic, or fibrinolytic activities (31, 46, 47, 60) and immune suppression (50, 51). Furthermore, the bacterium has been shown to be able to penetrate endothelial tissue layers (42).

In this study we addressed the role of motility and especially chemotaxis in tissue penetration of T. denticola. Several gene inactivation mutants in some of the motility- and chemotaxis-related genes have already been constructed by other investigators and in our laboratory (17, 24–26; R. Lux, J.-H. Sim, J. F. Tsai, and W. Shi, unpublished data). We also developed an oral epithelial cell line-based tissue penetration assay, since the oral epithelium constitutes the first barrier that T. denticola
must overcome to initiate its tissue invasion. By analyzing the tissue penetration ability of a set of defined motility and chemotaxis mutants, we obtained the first experimental evidence that not only cellular motility but also chemotaxis is important for *T. denticola* to penetrate tissue layers.

### MATERIALS AND METHODS

**Bacterial strains and growth conditions.** Strains used in this study and their relevant genotypes are listed in Table 1. *T. denticola* ATCC 35405 and its mutant derivatives HL51, HL50A1, and HL503 were kind gifts from Howard Kuramitsu (State University of New York, Buffalo), *T. phagedenis* biotype Reiter was a gift from Ulf B. Göbel (Humboldt University, Berlin, Germany), *Treponema pallidum* subsp. *pallidum* Nichols was maintained in one of our laboratories (J.N.M.) by rabbit testicular passage. The *cheA* mutant (RL101) was constructed through insertion of an *erm* cassette in the middle of the *cheA* gene according to the methods described by Li et al. (25). Detailed information about the *cheA* mutant will be published elsewhere (Lux et al., unpublished). All treponemes except *T. pallidum* were grown in TYGVS medium (35°C) at anaerobic conditions (85% N₂, 10% H₂, 5% CO₂). On the day of the experiment, *T. pallidum* was extracted from the orchi
des of an infected rabbit with heat-inactivated PBS (pH 7.2) as described above, because a considerable loss of motility of this spirochete was observed when no tissue was present. The bacteria were collected from the lower well and counted using a Petroff-Hausser bacterial counting chamber (Hausser Scientific Partnership, Horsham, Pa.). Penetration rates were calculated as the percentage of the total motile bacteria that penetrated through the system in the control experiment without tissue. In this control experiment, typically 50 to 80% of the cells that were initially added to the upper well migrated through the filter support of the system without a tissue barrier.

**Tissue embedding.** The filter of the Transwell system that the tissue layer was grown on was transferred from the culture medium into PBS containing 2% glutaraldehyde for cross-linking. The sample was further prefixed with 1% O₂-dehydrated with ethanol, and embedded in Epon. Sections 1 to 2 μm thick were mounted on slides and stained with toluidine blue.

**Microscopy.** Dark-field microscopy (Leitz, Wetzlar, Germany) at a ×1,000 magnification was used to examine the motility of the spirochetes and to count bacteria in the counting chamber at a ×200 magnification. Tissue sections were observed using phase-contrast microscopy at a ×400 magnification, and tissue layers were observed at a ×320 magnification with an inverted microscope (Leitz). Pictures were taken with a digital camera (SPOT; Diagnostic Instruments Inc., Sterling Heights, Mich.).

### RESULTS

Development of an oral epithelial cell line-based in vitro tissue penetration assay under anaerobic conditions. In vitro tissue penetration experiments have become a tool to assess invasiveness of pathogenic spirochetes. Strains that fail to penetrate tissue layers in vitro usually prove to be impaired in virulence as well (48). In vitro tissue penetration experiments for spirochetes are typically performed aerobically using endo
dermal tissue layers (4, 42, 58, 59) in the growth medium for the cell line used or multilayered intact tissue preparation of mouse abdominal cell walls (44, 45). Since the oral epithelium is the first barrier that *T. denticola* must overcome to initiate its tissue penetration, we decided to adapt this assay to an oral epithelial cell line to analyze the role of motility and chemotaxis for this oral spirochete.

Various human oral epithelial cell lines (such as SCC9, CAL27, and HOK-16B) were tested, and the immortalized human gingival keratinocyte cell line HOK-16B (40) was chosen for the in vitro tissue penetration assay. This cell line is able to form tight-junctioned tissue layers with a resistance of >10Ω. A thin section of a typical tissue layer at 14 to 17Ω is shown in Fig. 1. HOK-16B is normally grown aerobically in supplemented KBM medium. Unfortunately, under these growth conditions, the percentage of motile *T. denticola* cells dropped dramatically (Fig. 2). Good bacterial viability and motility, however, are crucial for the analysis of tissue penetration experiments. Therefore, we performed a series of experiments to search for conditions that ensured epithelial cell integrity as well as motility of *T. denticola*.

All *T. denticola* derivatives that were used in this study remained motile in unsupplemented KBM for more than 48 h (Fig. 2) if incubated anaerobically. Anaerobic conditions, how
ever, interrupt the electron transfer chain of eukaryotic cells due to lack of the end acceptor O₂, resulting in loss of energy and tissue integrity. Eukaryotic cells that lack a complete electron transfer chain can be rescued in the presence of pyruvate and uridine (21). We found that the pyruvate concentration (55 mg/liter) in the unsupplemented growth medium KBM was sufficient to keep HOK-16B cells healthy under anaerobic conditions. Therefore, tissues of HOK-16B cells were first grown aerobically in a 5% CO₂ atmosphere until they exhibited a tissue resistance of >10Ω, and then they were transferred to an anaerobic chamber containing 85% N₂, 10% H₂, and 5% CO₂.

### TABLE 1. Strains used in the study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype or description</th>
<th>Reference</th>
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<tbody>
<tr>
<td><em>T. denticola</em></td>
<td>ATCC 35405</td>
<td>Serovar A</td>
</tr>
<tr>
<td>HL51</td>
<td>ATCC 35405 <em>bsd::ermB ermAM</em></td>
<td>3</td>
</tr>
<tr>
<td>HL50A1</td>
<td>ATCC 35405 <em>adm::ermDermAM</em></td>
<td>25</td>
</tr>
<tr>
<td>HL503</td>
<td>ATCC 35405 <em>adm::ermDermAM</em></td>
<td>17</td>
</tr>
<tr>
<td>RL101</td>
<td>ATCC 35405 <em>cheA::ermDermAM</em></td>
<td>24</td>
</tr>
<tr>
<td><em>T. pallidum</em></td>
<td>Subsp. <em>pallidum</em>, Nichols strain</td>
<td></td>
</tr>
<tr>
<td><em>T. phagedenis</em></td>
<td>ATCC 51274</td>
<td>Biotype Reiter</td>
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The presence of pyruvate was sufficient to maintain tight junctions for more than 12 h. In the additional presence of uridine (110 mg/liter), the tissue cells were healthy for 2 to 3 days (data not shown). Based on these findings, we decided to use anaerobic coincubation of T. denticola ATCC 35405 and its various mutant derivatives with tight-junctioned tissue layers of HOK-16B in unsupplemented KBM as the experimental medium. Under these conditions, both the bacterial strains and the eukaryotic tissue appeared to remain viable for the duration of the experiment (8 to 10 h).

**Tissue penetration by wild-type T. denticola.** Using the assay developed above, we tested the tissue penetration ability of wild-type T. denticola. The pathogenic spirochete T. pallidum subsp. pallidum Nichols was used as a positive control, and T. phagedenis biotype Reiter, a spirochete that is not pathogenic to humans, was used as a negative control. Experiments with the microaerophilic T. pallidum were performed under both aerobic and anaerobic conditions. No significant difference in penetration rates was observed. As shown in Fig. 3, T. pallidum actively penetrated epithelial cell layers (about 14% of the cells pervaded the tissue in 10 h), whereas T. phagedenis was unable to overcome the tissue barrier. Wild-type T. denticola ATCC 35405 was also found to be able to penetrate oral epithelial tissue layers but only at about half the rate observed for T. pallidum (about 8% in 10 h) (Fig. 3). It is interesting that both T. denticola and T. pallidum exhibited an exponential increase of penetration efficiency over time, unlike the linear or hyperbolic increase that was described for their respective penetration of endothelial tissue layers (12, 42, 59).

We found that tissue penetration rates were also dependent on the tissue resistance (Fig. 4). The onset of detectable penetration was delayed with increased tissue resistance, but the penetration kinetics remained exponential (data not shown). It turned out, however, that the increased resistance was apparently due to the tissue growing into a multilayer rather than forming increased numbers of tight junctions. Tissues that consisted mainly of a monolayer had resistances of 10 to 13Ω. To rule out experimental ambiguities due to formation of cellular multilayers, tissue penetration rates of different strains were compared only for tissues that exhibited a resistance corresponding to predominantly monolayer or double-layer formation.

**Tissue penetration by T. denticola mutant strains defective in chemotaxis or motility.** Several defined motility and chemo-
taxis mutants have been previously constructed in other laboratories and in our laboratory, including strains that are nonmotile (flgE) or are defective in the central kinase of the general chemotaxis pathway (cheA) or one of the chemoreceptors (dmcA or dmcB). All these mutants were derived from wild-type T. denticola ATCC 35405. We examined these motility and chemotaxis mutants for their ability to penetrate oral epithelial cell layers. As expected, the nonmotile flgE mutant HL51 was unable to penetrate the epithelial tissue layer, confirming that motility is crucial for this feature (Fig. 5). Interestingly, the chemotaxis mutants (cheA, dmcA, and dmcB) showed significantly reduced penetration rates compared to the wild-type cells (Fig. 5A), even though they were fully motile throughout the experiment (Fig. 5B). The cheA mutant strain RL101 penetrated only at about 2 to 3% of the rates observed for the wild type. Both chemoreceptor mutants, the dmcA (HL0501) and dmcB (HL503) strains, also showed significantly decreased penetration rates, about 30 and 10% of the

FIG. 2. Motility of wild-type T. denticola ATCC 35405 in KBM for epithelial cell lines in a 5% CO2 atmosphere at 35°C (○) and under anaerobic conditions (85% N2, 10% H2, 5% CO2) at 35°C (□). Values for each condition were obtained in triplicate in two independent experiments. More than 600 cells were examined for cellular motility in each experiment. All mutant derivatives except the nonmotile strain HL53 showed similar results (data not shown).

FIG. 3. Time course of tissue penetration for different treponemata over 10 h. Diamonds, T. pallidum (n = 4); squares, T. denticola (n = 5); triangles, T. phagedenis (n = 4). The tissue resistance was about 12 to 13Ω.
wild-type behavior, respectively. These data show that not only cellular motility but also chemotaxis is important for T. denti-
cola to penetrate tissue layers.

**DISCUSSION**

The ability of spirochetes to invade tissue is an important virulence factor. It has been shown previously that B. burgdor-
feri, Leptospira interrogans, T. pallidum, and also T. denticola can cross barriers of endothelial cell layers whereas nonpatho-
genic species of the same genera are unable to do so (4, 42, 58, 59). T. pallidum and some oral spirochetes are apparently also able to invade the complex tissue of mouse abdominal wall preparations (44, 45) but interestingly only from the epithelial and not from the connective tissue side (44). Loss of motility was correlated with drastically reduced pathogenesis (48). As expected, the T. denticola derivative lacking flagella was com-
pletely unable to penetrate the tissue, supporting the idea that active bacterial movement and not passive translocation via endocytosis by the eukaryotic cells or other cellular processes is required for tissue invasion. This is in agreement with pre-
vious studies on other pathogenic spirochetes and a variety of other pathogenic bacteria that demonstrated the requirement of motility for virulence.

Although the role of chemotaxis in the tissue invasion pro-
cess of spirochetes has been considered (28), experiments ad-
ressing this issue have not yet been conducted. Chemotaxis has been described to be a virulence factor for some patho-
genic bacteria, such as H. pylori, C. jejuni, V. cholerae, and others (5, 8–10, 57, 62), whereas it appears to be only indirectly involved in the pathogenesis of Salmonella enterica serovar Typhimurium (16, 22). In this study, we provide evidence, for the first time, that chemotaxis is implicated in tissue penetra-
tion by T. denticola, since chemotaxis mutants were impaired in tissue penetration despite being motile. At present, however, we do not know the exact nature of the relationship between chemotaxis and tissue penetration. Chemotaxis could serve as a means to direct the bacterium into and through the tissue.

Chemotaxis mutants of V. cholerae were found to migrate into the mucus-filled intervillous spaces of rabbit intestines at a much lower rate than their wild-type parent strain (10). It is also conceivable that chemotaxis plays an indirect role by maintaining the motility pattern of reversals and flexing at a certain frequency. Alternatively, chemotaxis mutations may affect the expression of other virulence factors related to tissue penetration.

For the oral bacterium T. denticola, the epithelium of the gingival tissue constitutes a natural site of entry into its host. In this study, we established an in vitro tissue penetration assay for an immortalized human keratinocyte cell line. Tissue layers of this cell line maintained integrity even under anaerobic conditions for more than 48 h. Wild-type T. denticola was able to penetrate this tissue layer at rates that are comparable to those described for penetration of endothelial cell layers (42) (Fig. 3). T. pallidum, which we used as a positive penetration control, showed only about half of the penetration that was observed by other investigators for endothelial monolayers (12, 59). As expected, the nonpathogenic T. phagedenis was unable to overcome the epithelial barrier. Interestingly, the time course for penetration seems to be exponential rather than linear or following a saturation curve as found for pene-
tration of endothelial tissues (12, 42, 59). This finding implies that during penetration certain events increase the likeliness that other spirochetes penetrate the tissue. These events could involve tissue destruction that facilitates entry into the tissue. This possible tissue destruction, however, has to be very lim-
ited, because a significant loss of tissue resistance that would correlate with massive tissue damage was never observed dur-
ing the experiments. This exponential increase in tissue pene-
tration could be explained if chemotaxis-guided targeting of damaged tissue is involved in the penetration process. Mole-
cles that are released by injured or diseased tissue cells could attract the bacteria towards these weakened spots, thus facili-
tating penetration.

The hypothesis that chemoattraction could play a role in

*FIG. 4. Correlation of penetration rate and tissue resistance. Penetration rates were determined after 8 h of coincubation of the tissues with wild-type T. denticola. Tissue resistance was measured before and after the experiment.*
tissue penetration is supported by the result that a mutant strain lacking CheA, the central kinase in chemotactic signal transduction, is severely impaired in tissue penetration. It exhibits only 2 to 3% of the wild-type penetration rates. This mutant is fully motile but unable to respond to a mix of nutrients (Lux et al., unpublished). The lack of a chemotaxis response would disable the mutant to detect "weak spots" within the tissue, and each bacterium would randomly try to migrate into the cell layer rather than taking advantage of existing "passages." The motility pattern of this mutant, however, also appears to have a greatly decreased reversal frequency compared to that of the wild type (data not shown). This leaves open the possibility that a certain frequency of reversal might be necessary for efficient migration through a tissue. A more detailed analysis of the influence of motility patterns on the tissue penetration ability of T. denticola is in progress.

Two other mutant strains, HL0501 and HL503, that were tested are lacking the DmcA and DmcB chemoreceptors, respectively. Both mutants have reduced penetration rates but are not as severely impaired as the cheA mutant. DmcB appears to have a greater influence on tissue penetration than DmcA. It was shown previously, however, that loss of DmcB has a dominant effect on DmcA methylation (24). This additive effect could explain why the DmcB mutant strain has a signif-

FIG. 5. Tissue penetration rates (A) and motility (B) of wild-type T. denticola (Td), T. pallidum (Tp), and T. phagedenis (Tph) and various T. denticola motility and chemotaxis mutant strains. Values were determined after 8 h of coincubation of the bacteria with the tissue. Tissues used for these experiments exhibited resistances between 11 and 14Ω. More than 600 cells of each strain were examined for cellular motility.
ificant lower penetration rate than the DmcA mutant strain. These chemoreceptors were previously shown to be involved in migration towards nutrients (17, 24). A BLAST search of the unfinished genome sequence of T. denticola ATCC 35405 (www.tigr.org) revealed the existence of nine more open reading frames that contain the highly conserved domain described for methyl-accepting chemotaxis proteins (55) and therefore possibly encode chemoreceptors. Therefore, it appears unlikely that inactivation of one or two chemoreceptors would completely abolish the response to attractants. The possible presence of more than two chemoreceptors in T. denticola could explain why tissue penetration of HL0501 (dmcA) and HL503 (dmcB) is reduced but to a lesser extent than in the generally nonchemotactic cheA mutant RL101. The dmcA mutant HL501 exhibits a motility pattern that is similar to that of wild type. In contrast, HL503, which lacks dmcB, appears to have an elevated reversal frequency. As both RL101 and the wild-type HL0501 exhibits a motility pattern that is similar to that of the DmcA mutant strain. RL101 and HL0501, which exhibit the greatest decrease in tissue penetration rates, also differ in their motility pattern from the wild type, we cannot rule out the possibility that this altered motility pattern also affects tissue penetration.

We are currently in the process of constructing other chemotaxis mutants that allow potential determination of the impact of reversal frequency on tissue penetration. A cheRB double mutant of Escherichia coli that exhibits a wild type-like swimming behavior but is greatly impaired in chemotactic signaling has been described (20, 41). Single inactivation of either gene locks the bacterium in an extremely tumbling (cheB) or smooth (cheR) swimming pattern (41). Mutations in these genes might result in similar phenotypes in T. denticola and be useful in elucidating the importance of swimming pattern versus chemoa trophy in tissue penetration.

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