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Virulence factors of *Treponema denticola*

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Spirochetes were first observed in the oral cavity in humans by van Leeuwenhoek (34, 101, 102, 182). Spirochetes are gram-negative microorganisms with long spiral-shaped cells which achieve motility by means of periplasmic flagella (Fig. 1). Members of the phylum "Spirochaetes" include the etiologic agents of syphilis, Lyme disease and other borrelial relapsing fevers. The phylum consists of Spirochaetaceae, Serpulinaceae and Leptospiraceae (3, 39). The first group consists of Spirochaeta, Borrelia, Clevelandina, Cristispira, Diplocalyx, Hollandina, Pillotina and Treponema. Among these, only the genus Treponema has been isolated from the oral cavity. Treponemes have been detected from a variety of environments such as the human oral cavity, the digestive tracts of cattle and termite guts (48, 53, 133, 177). Treponema share many characteristics with gram-positive bacteria, and recent phylogenetic studies have shown that spirochaetes form a single cluster distinct from both gram-positive and

gram-negative bacteria (63).

Eight species of treponema (*Treponema amylovorum, Treponema denticola, Treponema lecithinolyticum, Treponema maltophilum, Treponema medium, Treponema parvum, Treponema pectinovorum, Treponema socranskii* and "*Treponema vincentii*") have been identified from the oral cavity in humans (159, 160, 177, 188-190).   Recent studies employing molecular techniques have also detected as yet unclassified species (134).   Among these species, *T. denticola* has been extensively characterized in terms of its pathogenicity and involvement in the development of periodontitis.

Characteristics of *T. denticola*

The spiral shaped cells of this microorganism are covered with an outer sheath consisting of a fragile envelope-like structure (64).   Periplasmic flagella are located on the cytoplasmic membrane and are covered with the outer sheath.   *T. denticola* typically produces four flagella, which are intertwined around the cytoplasmic cylinder. Two originate at each end and they usually overlap in the middle of the bacterium. The major outer sheath protein (Msp) is the predominant protein in the outer sheath (114, 178).   The size of the Msp is usually approximately 53 kDa in *T. denticola* ATCC 33520, ATCC 33521, ATCC 35404 and ATCC 35405 (178); however, it is 64 kDa in some other strains (186).   Electron microscopy revealed a hexagonal array-type structure in the negative-stained *T. denticola* outer sheath due to the presence of the Msp (44, 114).   Other reports, however, suggested that this array was observed due to the structure of peptidoglycan (17).   Msp showed similarity with a family of 12 proteins, the *Treponema pallidum* repeat (Tpr) gene family (*tpr*A-L), based on a similarity search, whereas it showed no similarity with other spirochetes (187).   Tprs are candidate rare

outer membrane proteins and have been proposed to endow *T. pallidum* with the ability to alter its surface antigenicity (21, 91). As the database examined includes spirochetal sequences such as *Borrelia burgdorferi,* the Msp-Tpr gene family appears to be treponema-specific. The multiple paralogues in *T. pallidum* are believed to be involved in antigenic variation for evasion of host defenses.

A prolyl phenylalanine-specific peptidase dentilisin (chymotrypsin-like protease) is located on the surface of this microorganism (57, 173). This protease consists of a complex of three proteins. The protease domain is coded by *prtP*, which forms an operon with upstream genes (70). The gene upstream of *prtP* is designated as the protease complex associated gene *(prcA)* and the products of the *prcA-prtP* operon form the protease complex (69, 95). Recently, Bian et al. reported that an open reading frame upstream to *prcA* altered expression of *prcA* and *prtP* (11) The *prcA-prtP* operon was also identified in *T. lecithinolyticum* and *T. vincentii*, two canine species (29). Partial operons of 450- to 1,000-base fragments of the *prtP* genes were identified from *T. socranskii* subsp. 04 and four additional treponeme strains, respectively. The prevalence of paralogues suggests that dentilisin is a common ancestral protein among oral spirochaetes and that dentilisin evolved in each oral treponema. Phylogenetic analysis demonstrated that the sequences fall into two paralogous families. The treponema possessing the first family paralogues, including the sequence from *T. denticola*, can cleave N-succinyl-L-alanyl-Lalanyl-L-prolyl-L-phenylalanine *p*-nitroanilide, a synthethic substrate for dentilisin. Treponemes possessing the second paralog family do not cleave this substrate. However, Msp and dentilisin were reported to form a complex (40), and inactivation of dentilisin resulted in the absence of the high molecular weight

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oligomeric form of Msp (68).

The periplasmic filament is located just beneath the cytoplasmic membrane(115). A periplasmic filament-deficient mutant exhibited chains of treponemal cells and highly condensed chromosomal DNA compared to the homogeneous distribution of DNA throughout the cytoplasm in the wild-type. This suggests that these cytoplasmic filaments are involved in chromosome structure, segregation, or the cell division process.

The genome project (http://www.ncbi.nlm.nih.gov/nuccore/AE017226) yielded a great deal of relevant genetic information for *T. denticola.* The genome size of *T. denticola* is 2,843,201 bp and the GC content is 37.9% (153). It includes 2,786 predicted coding regions (CDS). The organization of the *T. denticola* chromosomal origin of replication differs from that typical for other bacteria, including other spirochetes (*Treponema pallidum*, *Borrelia burgdorferi* and *Leptospira interrogans*). The proteins predicted from 618 CDSs were common among pathogenic spirochetes. Approximately one-fourth of these CDSs have their best matches with genes of *T. pallidum* and, on average, these share only 53% amino acid identities. 1,268 CDSs have no matches in any of the genomes of other spirochetes. Their best matches are found among various sequences from gram-positive species such as Clostridium spp., Streptococcus spp., as well as *Fusobacterium nucleatum,* a primary colonizer during dental plaque formation, found in conjunction with *T. denticola* and *Porphyromonas gingivalis* and thought to be required for the colonization of the later two organisms (161). These include various membrane proteins, ATP-binding cassette (ABC) transporters, transcriptional regulators, and enzymes involved in amino acid metabolism as well as glycogen synthesis. Specifically, the number of ABC transporters is

extremely high in *T. denticola* compared with other microorganisms, suggesting that ABC transporters may play important roles in the colonization or virulence of this microorganism.

Epidemiology

The association of oral spirochetes with chronic periodontitis (4, 102), acute ulcerative necrotizing gingivitis (103, 105, 141) and necrotic root canals (30, 60, 122) has been reported. The association of *T. denticola* with periodontitis was suggested after the establishment of procedures for its isolation and molecular detection. *T. denticola* was detected from deciduous dentition and the detection rate increased according to age (87, 174). *T. denticola* was also reported to increase susceptibility to gingival inflammation (139). This microorganism is frequently isolated from human chronic periodontitis (140, 166, 174) and co-isolated with *P. gingivalis* and *Tannerella forsythia* (79, 85). The consortia of these species is known as the "red complex" (161). These epidemiological data agree with synergistic biofilm formation of *T. denticola* with *P. gingivalis* in vitro (56, 156, 193) . A recent report indicated that elevated salivary matrix metalloprotease (MMP)-8 and *T. denticola* biofilm levels displayed robust combinatorial characteristics in predicting periodontal disease severity (137). A detailed description of the role of *T. denticola* in the development of periodontitis was provided in a study by Ellen and Galimanas (38).

T. denticola was also detected from periapical endodontic lesions of asymptomatic teeth (165), teeth with carious lesions and necrotic pulp (157, 158, 181), periapical periodontitis lesions (45) and root-filled teeth with periapical lesions (51). Sakamoto et al. reported a prevalence of 66% for *T. denticola* in 90 cases of primary

endodontic infection and diverse Treponema species/phylotypes as part of a study of the microbiota associated with asymptomatic and symptomatic endodontic infections (148). Recent epidemiological data further suggested the potential involvement of periodontitis in cardiovascular disease (9, 10, 171).   In this regard, *T. denticola* was detected from atherosclerosis lesions (20, 71, 128) and Buerger disease(74).   Subgingival microorganisms have also been reported to cause bacteremia (6).   *T. denticola*-like spirochetes were also detected in lesions from digital dermatitis (23).   These epidemiological data suggest that *T. denticola* can be pathogenic and could be involved in the development of these diseases.

Virulence factors

The fastidious nature of *T. denticola* during cultivation has made it difficult to obtain insights into the mechanism of the virulence of this microorganism.   Molecular microbiological techniques are lowering this barrier and gradually revealing the role of this microorganism in the development of periodontitis.   The potential virulence factors of *T. denticola* are summarized in Table 1.   This microorganism colonizes the gingival crevice and multiplies using components of gingival crevicular fluid as an energy source.   The potential virulence factors of this microorganism include adherence factors, motility, evasion mechanisms from host defenses and cytotoxic factors for host tissues.   Previous analyses have characterized some, but not all, of these factors.   However, some of the molecules involved in its pathogenicity have not been fully characterized due to difficulties in isolating these molecules from cells of *T. denticola*.   The recent genome project has contributed to the identification of many of these factors, allowing us to reference its genome sequence in our investigations into the

mechanisms of treponeme virulence.

Adherence to host

The ability to adhere to host tissue is essential for human pathogens. Periodontopathic bacteria possess a variety of adherence factors (93, 170). Although *T. denticola* has no specific adherence structures such as fimbriae, this microorganism has been reported to have the ability to adhere to various surfaces of the oral cavity such as those of the tooth, host cells and other microorganisms in dental plaque biofilms (19, 28, 55, 130, 138, 185). This adherence ability of *T. denticola* is due to the variety of adherence factors on the surface of this microorganism. Collagen and cell adhesion molecules such as fibronectin and laminin are receptors for the bacterial colonization. The adherence of *T. denticola* to fibronectin has been most intensively characterized. Although *T. denticola* adhered to fibronectin and laminin in a tip-oriented manner, this activity was not inhibited by the RGDS peptide, which is a cell binding domain shared by fibronectin and laminin (31, 32). A 27 kDa type IV collagen binding protein of *T. denticolas* has also been reported (175). However the involved molecules remain to be clarified. For adhesion activity, however, several molecules for *T. denticola* attachment have been identified.

Msp

T. denticola binds to human gingival fibroblasts and the adherence of some strains was inhibited by addition of fibronectin (185). The ability of *T. denticola* to bind to human gingival fibroblasts is suggested to be caused by lectin-like proteins or mediated by host proteins. Antibody against the Msp protein of *T. denticola* strain GM1 inhibited the

adherence of this strain to human gingival fibroblasts by approximately 78% (186).

These results revealed that Msp is a fibronectin-binding protein involved in binding to

fibroblasts. Adherence of recombinant Msp to fibronectin and laminin was further

confirmed (42). Binding of Msp occurred preferentially to the N-terminal heparin

I/fibrin I domain of fibronectin (36). Analysis using recombinant proteins spanning

various regions of Msp, including the 203th-259th residues of Msp, showed similar

characteristics to that of whole recombinant Msp, with higher binding observed to

keratin, fibronectin and the 30-kDa fibronectin fragment. A recombinant N-terminal

Msp (rN-Msp) domain of 14-202 residues also binds to fibronectin but the C-terminal

Msp domain of 272-543 residues did not. These observations indicate that the binding

domain of Msp is located in the N-terminal half. In addition, exogenous recombinant

Msp enhanced adhesion of heterologous Treponema species, which also adhere to

fibronectin but show considerably lower binding levels than does *T. denticola* to

fibronectin, suggesting synergy in adherence among mixed populations of oral

spirochetes containing *T. denticola* (36).

As indicated above, Msp is usually 52 kDa in size. However, it was reported

to be 63 kDa in some strains (44, 185). The gene encoding Msp was identified and the

sequence of the 53 kDa Msp determined by Fenno et al. (42). The molecular mass of

Msp is 58,233 and its mature form is 56,151. The Msp coding sequence of *T.*

denticola ATCC35405 shows 94.6% identity with that of *T. denticola* ATCC33520,

which is a 53 kDa-type Msp, while it shows only 50.6% identity with the Msp of *T.*

denticola OKT, which is a 64 kDa-type Msp (44). Although there is high similarity

among the strains, antiserum against *T. denticola* ATCC33520 did not cross-react with *T.*

denticola ATCC 33521, ATCC 35404 or ATCC 35405 (178), whereas antiserum against

T. denticola ATCC 35405 reacted strongly with ATCC 35404 and only faintly with ATCC33520 (44). This suggests limited exposure of the Msp protein on the surface of some of these treponemes.

On the other hand, in terms of function, Msp was reported not to be a surface-exposed protein based on the results of immunofluorescence analysis of treponemes embedded in agarose gel microdroplets (17). The data revealed that only minor portions of Msp are surface exposed. Immunoelectron microscopy of ultrathin-sectioned intact *T. denticola* using antibody against recombinant Msp also demonstrated that the preponderance of antigens was not associated with the outer membrane. These observations suggest that Msp locates within the *T. denticola* outer membrane and demonstrates, instead, that it is predominantly periplasmic, with only limited surface exposure. Jobin et al. demonstrated the surface exposure of Msp by antibogy against native Msp using the same method (76). As the surface of this microorganism is fragile, further investigations using multiple methods are required to confirm the surface exposure characteristics of the Msp molecule in intact *T. denticola*.

OppA (orthologue of oligopeptide transporter unit)

This 70 kDa protein has also been reported to bind to fibronectin (58, 176). The protein coding gene was isolated and designated *oppA* (43). OppA is an orthologue of the solute-binding proteins of Cluster 5 of the superfamily of bacterial ABC transporters, which primarily includes oligopeptide uptake systems. Downstream of *oppA, oppB, oppC, oppD* and *oppF* were detected, suggesting that these constitute a complete permerase system. An *oppA*-deficient mutant was not affected in binding to epithelial cells, and native OppA did not bind to immobilized substrates or epithelial cells

suggesting that this protein does not participate in direct adherence to cell bound receptors, although binding activity was shown at the protein level. It is possible that other proteins in addition to OppA is required by *T. denticola* for adherence to host cells. However, further analysis regarding its involvement in adherence to soluble proteins such as plasminogen and fibronectin in the colonization of epithelial cells is required.

Factor H-like protein 1 (FHL-1) binding proteins

Complement activation is caused by antigen-antibody complexes, pathogen surface material such as lipopolysaccharides, as well as binding of lectin to pathogen surfaces. Activation is regulated by host regulatory proteins, including C1 inhibitor, C4 binding protein, Factor H (FH), Factor I and CD59 (121). FH is a crucial negative regulator of the alternative pathway for complement activation (Fig. 2). FH acts as a competitor for factor B, binding to C3b to form convertases and is an essential cofactor for factor I-mediated degradation of C3b to iC3b and accelerates the decay of activated factor B (Bb) from C3/C5 convertases (146, 147). The FH protein family consists of FH, FH-like protein 1 (FHL-1), and five FH-related proteins designated FHR1 through FHR5 (78). FH comprises a series of short consensus repeats (SCRs) consisting of approximately 50-60 residues (199). FHL-1 is derived from factor H mRNA via alternative splicing and consists of the first seven SCRs of factor H plus four additional hydrophobic residues at its C terminus (47, 198). This protein is also involved in downregulation of C3b production, as well as FH. Adherence of microorganisms to these proteins has been demonstrated to facilitate evasion from alternative complement cascades and/or play roles in adherence to and invasion of host cells (198).

Accumulation of 50 kDa FHL-1 and an undefined 37 kDa protein was detected

on the surface of *T. denticola* incubated with FH/FHL-1 utilizing anti-FH/FHL-1 antibody; however, accumulation of 150 kDa FH was not detected (118). Accululation is induced by the 11.4 kDa FHL-1 binding protein of *T. denticola* and the protein was therefore designated as FhbB. FhbB is associated with the accumulation of FHL-1 in the wild-type; however, it binds to FH in the dentilisin deficient mutant (117). The protein is located on the surface of *T. denticola* and its levels were decreased with repeated washing. This protein binds only to FH recombinant fragment SCRs 1-7, which is located at the N-terminal of both FH and FLH-1 and not to SCR constructs spanning SCRs 8-15 or 16-20. FhbB was shown to bind to FHL-1 but not FH. The absence of significant binding to FH resulted from degradation of full size FH by dentilisin (117).

Truncation analyses of both the N- and C-terminal domains of the protein and random and site-directed mutation analyses suggested that the determinants which influence the FH/FHL-1 binding site of FhbB are distributed throughout the protein and that the binding site is not a simple contiguous linear sequence element which is consistent with earlier analyses in other microoorganisms (1, 65, 119). The lipoprotein acts as a cofactor of Factor 1 for degradation of C3b to iC3b. However, a protease of *T. denticola* also degrades C3b without FH or Factor I (117) and iC3b production was also reported (194). An increased binding of FH to apoptotic or necrotic cells has been described as well (172). It also has been demonstrated in pneumococci that binding of FHL-1 is important in the interaction between epithelial and endothelial cells (59, 136). These results suggest that FHL-1 binding by *T. denticola* facilitates adherence to FHL-1 present on anchorage-dependent cells and in the extracellular matrix.

Coaggregation

Coaggregation is a major strategy for colonization into dental plaque biofilms via adherence of cells to the surface of biofilms. *T. denticola* was co-isolated with *P. gingivalis* and *T. forsythia*, suggesting that coaggregation and synergy among these species are important in colonization by these microorganisms. In addition, enhancement of virulence by this consortium was reported (81, 184) . *T. denticola* has been reported to coaggregate with several members of dental plaque such as *P. gingivalis, T. forsythia* and *F. nucleatum,* (55, 89, 131, 196). A bimodal coaggregation reaction was suggested for coaggregation between *T. denticola* and *P. gingivalis* (55). Although binding of *P. gingivalis* to *T. denticola* was inhibited by arginine, such inhibition of binding was not observed after heat treatment of *P. gingivalis,* suggesting that a heat labile ligand which binds arginine residue is located on the surface of *P. gingivalis*. *T. denticola* coaggregated with purified hemmaglutinin, suggesting the involvement of hemmaglutinin on the surface of *P. gingivalis* in the coaggregation reaction(131). Recent molecular analysis further revealed several surface materials of *T. denticola* involved in coaggregation. For example, Msp was reported to be a candidate ligand for coaggregation reactions between *T. denticola* and *P. gingivalis* or *F. nucleatum.* Rosen et al. also reported that Msp is a glycoprotein and that Msp was involved in adherence of *T. denticola* to *P. gingivalis* and *F. nucleatum* via protein-protein and sugar-protein intereactions, respectively (142).

Dentilsin was also suggested to be involved in coaggregation. Long fimbriae (FimA) of *P. gingivalis* reacted with a 72 kDa-pI 5.3 or 5.4 *T. denticola* protein separated by 2-D electrophoresis and this protein was identified as dentilisin by N-terminal amino acid sequencing (61). A recombinant protein composed of residues

159-440 of the 772 amino acids of dentilisin was further shown to bind to *P. gingivalis* FimA, and more than 20 µg/ml FimA inhibited coaggregation between *P. gingivalis* and *T. denticola.* This result suggests that the dentilisin-long fimbriae interactions mediate the coaggregation reaction. However, dentilisin-deficient mutants coaggregated with *P. gingivalis* (68, 142), suggesting that other factors are also involved in the coaggregation reaction.

Leucine-rich repeats (LRRs) were reported to be involved in coaggregation of *T. denticola* with *T. forsythia.* LRRs are protein interaction motifs of 20-29 residues consisting of a high proportion of leucine residues. The major function of LRRs may be to provide a structural framework for the formation of protein-protein interactions (88). LrrA is a *T. denticola* ATCC 35405 protein containing LRRs (66). A LrrA-deficient mutant displayed reduced coaggregation with *T. forsythia* but was not altered in interactions with *P. gingivalis* or *F. nucleatum.* The leucine-rich-repeat protein (*B. forsythus* surface protein A, BspA) of *T. forsythia* also containss LRRs, and its corresponding deficient mutant also was attenuated in coaggregation with the spirochete. In addition, recombinant LrrA bound to BspA. These results suggested that LrrA-BspA interactions are involved in the coaggregation reaction between *T. denticola* and *T. forsythia*.

Immunomodulation

It was reported that levels of serum antibody to *T. denticola* were elevated in patients with chronic periodontitis (75). *T. denticola* was reported to induce a strong antibody response in young adults patients with localized juvenile periodontitis. On the other hand, levels of serum antibody against *T. denticola* in young adult patients with

generalized severe periodontitis were lower than those in healthy subjects (169).

Mangan et al. (113) reported that spirochetes comprised 20% of the total number of microorganisms detected in plaque from these patients, whereas no spirochetes were observed in plaque from the controls and the IgG and IgM titers of the patients were generally lower than the those of control subjects.   In the patients, only three of the IgG titers and two of the IgM titers of the patients were statistically lower than those of the controls.   In contrast, the patients had significantly higher IgA antibody titers to 7 *T. denticola* strains.   Lai et al reported no difference in antibody levels between chronic periodontitis patients and healthy adults (92).   However, several proteins, including Msp and dentilisin and the flagella of this microorganism were reported to be antigenic (18, 179).   Kesavalu et al. also reported a relationship between lesion size and immunization (82).   Primary infection with *T. denticola* induced a 400-fold serum immunoglobulin G (IgG) response compared to that in control uninfected mice.   The IgG response to reinfection was 20,000-fold higher than that in control mice.   Mice actively immunized with formalin-killed treponemes developed serum antibody levels seven- to eight-fold greater than those in animals after primary infection.   However, mice with primary infections or active immunization demonstrated no significant alterations in induction of lesions or decreases in abscess size   following challenge with *T. denticola*.   *T. denticola* was also reported to affect the migration of neutrophils (107).   The effects on neutrophils will be described in the section on the cytotoxic effects of Msp below.   These observations indicate that the immunomodulating activity of *T. denticola* is involved in the pathogenicity of this microorganism.

Immunosuppressive factors

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Immunosuppressive factors have been reported in several oral microorganisms, including *T. denticola* (154). Fractions with a molecular mass of approximately 100 kDa and 50 kDa from *T. denticola* suppressed human peripheral mononuclear cells (HPBMC) and fibroblasts, respectively (12, 155). The suppression of HPBMC depends on prostaglandin and hydrogen peroxide. We also reported that an approximately 100-kDa fraction of this microorganism suppressed mice lymphocyte proliferation induced by concanavalin A (73). This inhibitory activity was detected in *T. denticola* ATCC 33520 but not in *T. denticola* ATCC 35405. Lee *et al* further investigated the kinetics of this immunosuppressive activity (96). Fifty and 53 kDa spirochete immunoinhibitory proteins (Sip) isolated by ion exhange, chromatofocusing and gel filtration chromatography from sonicates of *T. denticola* inhibited activation of HPBMC in a dose-dependent.manner. Sip inhibited the progression of phyrohemagglutinin (PHA)-treated HPBMC beyond the G1 phase through CD69 and CD25, which are associated with transition through the G1 phase of the cell cycle. These results suggest that HPBMC are activated by PHA in the presence of Sip but inhibits the progress of HPBMC beyond the G1 phase. In addition, Sip-treated cells exhibited DNA fragmentation at 96 hr after exposure, whereas G1 arrest was observed at 72 hr. Therefore, it is possible that G1 arrest may precede the activation of the apoptotic cascade.

Dentilisin

T. denticola was reported to induce cytokine production (86, 124). The release of these cytokines was induced by cell surface components such as peptideglycan and lipopolysaccharide. Although the structure of the cell wall-cytoplasmic membrane of

T. denticola appears similar to that of a Gram-negative microorganism, the composition of its outer membrane lipid includes lipooligosaccharide (LOS) (150). Peptidoglycan isolated from *T. denticola* induced release of interleukin-1β (IL-1β), IL-6, tumor necrosis factor α (TNFα), regulated on activation normal T cell expressed and secreted (RANTES), IL-8, matrix metalloprotease-8 (MMP-8) and prostaglandin from macrophages (168). LOS also induced inflammatory cytokines (143, 167). The chemokine-inducing activity of this microorganism was further evaluated using human endothelial cells. *T. denticola* induced IL-8 and monocyte chemoattractant protein-1 (MCP-1) from human umbilical cord endothelial cells (129). In addition to inducing these cytokines, *T. denticola* also degrades them (120). *T. denticola* induced mRNA expression of IL-1β, TNF-α and IL-6 in peripheral blood mononuclear cells but levels of these cytokines in the medium were low. These cytokines were detected at high levels in the supernatant of a dentilisin-deficient mutant. MCP-1 and IL-8 were also degraded by dentilisin (129) indicating that dentilisin degraded the expressed cytokines. In addition, *T. denticola* was reported to be unable to induce IL-8 from human gingival epithelial cells (15). In that report, *T. denticola* was detected by Toll-like receptor 2 (TLR2) but the cells failed to respond appropriately. These activities result in disorder of normal immunoresponses and may be associated with the evasion of host defenses. Thus, activation and the resulting evasion from immunoresponses can lead to tissue destruction.

Msp and Lipooligosaccharide (LOS)

Several reports indicated that *T. denticola* activated host cells via TLR (5, 126, 144). In these reports, *T. denticola* was indicated to activate innate immunity via TLR2. This

activity was attenuated by anti-TLR2 antibody and the response was absent in TLR2-deficient cells (126, 144).   Activation was also observed with *T. vincentii* and *T. medium* (5) and the glycolipid of *T. multophilum*-activated TLR2 (132).   *T. denticola* was reported to activate HPBMC via mitogen-activated protein (MAP) kinases   such as extracellular signal-regulated kinase (ERK)1/2 and p38 (144).   In that study, *T. denticola* induced innate immunity via TLR2 but not TLR4 (144).   Recent reports also indicated that *T .denticola* cells and Msp induced innate immune responses of macrophages through TLR2- myeloid differentiation factor 88 (MyD88), whereas LOS induced a macrophage response through TLR4-MyD88 (126).   This study also indicated that the presence of γ interferon (γ-IFN) or of high numbers of *T. denticola* circumvented the requirement for TLR2 for the macrophage response to *T. denticola* , although the response was still dependent on MyD88.   In contrast, synergy with γ-IFN did not alter the TLR-dependence of the response to the *T. denticola* surface components LOS and MSP, despite enhanced sensitivity.   Therefore, these reports suggest that *T. denticola* activates the immune system via two different pathways and that certain cytokines modulate this activation.

Resistance to defensins

Antibacterial peptides play an important role in the initial host defense against microbial attack.   Epithelial cells produce antibacterial peptides such as β-defensin which protect against colonization by microorganisms.   These small cationic peptides interact with negatively-charged cell wall components of bacteria and fungi, disrupting membrane integrity (49).   β-defensin are also active against oral microbes, including periodontitis-related bacteria (33).   However, *T. denticola* is resistant to human

β-defensin-1 and-2 (14). The protease inhibitor chymostatin did not enhance killing of

T. denticola by human β-defensin-2, suggesting that resistance to human β-defensin-2

does not depend on the protease activity of *T. denticola*. Most *T. denticola* strains are

resistant to human β-defensin-1-3 and *T. lecithinolyticum, T. multophilum* as well as *T.

socranskii* are resistant to human β-defensin-1 and -2 (16). Dentilisin activity and

reduced binding of human β-defensin to the bacterial surface by serum protein were not

associated with this resistance. However, binding ofhuman β-defensin-2 and -3 to *T.

denticola* was significantly lower than that of susceptible organisms, suggesting that the

unusual outer membrane composition of *T. denticola* may reduce cationic peptide

binding. In addition, a proton motive force inhibitor, carbonyl cyanide

3-chlorophenylhydrazone, increased the susceptibility of *T. denticola* to killing by

human β-defensin-3, suggesting a potential role for efflux pumps in resistance to this

peptide, although three inhibitors of bacterial ATP-binding cassette efflux pumps had no

effect on susceptibility of *T. denticola* to human β-defensin-2 or -3. These

observations indicate that *T. denticola* evades attachment by antibacterial peptides by its

unique surface structure and efflux pumps.

Factors involved in cytotoxic effects

Lipooligosaccharides (LOS)

Some components in the outer membrane have the ability to induce bone resorption (52).

Tartrate-resistant acid phosphatase (TRAP, a marker of osteoclasts)-positive cell levels

were increased by addition of 10 μg/ml sonicates or 0.1-10 μg/ml LOS to cocultures of

mouse calvaria and bone marrow cells in a dose-dependent manner (24). The LOS of

T. denticola up-regulated the receptor activator of nuclear factor κB (RANKL) and

down-regulated osteoprotegerin (OPG).   These effects were eliminated in the presence of polymixin B.   LOS also induced prostaglandin E2 and this effect was also inhibited by polymixin B.   Moreover, the addition of indomethacin reduced the number of TRAP-positive cells in this system.   These results suggest that the LOS of *T. denticola* activates osteoclast differentiation via alterations in the equilibrium between RANKL and OPG.   As this microorganism is also reported to induce IL-1β, IL-6 and TNFα, these cytokines may contribute to bone resorption.

Msp

In addition to the adherence activity of Msp, its cytotoxic activity on pig ear epithelial cells was also reported (138).   The Msp protein of *T. denticola* ATCC 35405 was suggested to have a membrane topology similar to that of outer membrane porins and pore-forming cytotoxins of Gram-negative bacteria (42).   The channel-forming activity of *T. denticola* Msp has also been reported (37, 116) and this activity was   confirmed using recombinant Msp (40).

        This outer membrane protein of *T. denticola* was also reported to diminish the generation of inositol phosphates during the time course associated with significant cytoskeletal disruption in fibroblasts (195).   In addition, Msp treatment inhibited the migration of fibroblasts and neutrophils (2).   In Msp-treated cells, immunofluorescent staining revealed that disassembly of actin filaments toward the center of the cell occurred simultaneously with de novo subcortical actin filament assembly.   The mechanism of this microorganism's effects on fibroblasts was further analyzed using Msp.   After incubation of Msp with fibroblasts, Msp associated with the fibroblast surface and intense Msp staining was clustered in one area of the cell after 30-min

incubation. Adherence of Msp disturbs calcium signaling in human fibroblasts by uncoupling store-operated channels (183). This may cause two potential pathological responses: (i) Msp uncouples store-operated Ca^{2+} channels of fibroblasts, apparently conformationally, by promoting actin assembly subadjacent to the plasma membrane; and (ii) Msp-induced subcortical actin filament assembly which inhibits β_1 integrin affinity modulation that normally accompanies the engagement of extracellular collagen. The latter was confirmed using Rat-2 fibroblasts (8). This effect may disrupt cell signaling via Ca^{2+} and could be involved in the pathogenicity of *T. denticola*. Msp was reported to regulate actin assembly of fibroblasts by inducing phosphorylation of multiple kinases in pathways that respond to extracellular agonists and regulate actin assembly. Thirty-four kinases were significantly activated, including p38 and ERK 1/2, suggesting that *T. denticola* Msp may exert transient stress on fibroblasts through activation of MAP kinase pathways. (77). The regulation of epithelial cells via kinase pathways was also reported (97). ERK1/2 activation appears to lead to increased epithelial cell proliferation and survival in the presence of moderate concentrations of *T. denticola*, while cell death is induced by both p38-mediated apoptosis and high concentrations of bacterial toxic substances causing cell membrane damage.

Msp inhibited formyl-methionyl-leucyl-phenylalanine (fMLP)-induced chemotaxis, phagocytosis of immunoglobulin G-coated microspheres, fMLP-stimulated calcium transients and actin assembly (135). Neutrophil activation by chemoattractant peptides depends on highly regulated signaling pathways responsible for the recruitment of active small guanosine triphosphatase (GTPases) such as Rac and the coordinated production of phospholipid products by phosphatidylinositol 3 (PI3) kinase at the plasma membrane (123, 162). Msp pretreatment of neutrophils inhibited both

polarization and chemotactic migration in response to fMLP.   Native Msp selectively inhibited fMLP-stimulated Ras-related C3 botulinum toxin substrate 1 (Rac1) activation in a concentration-dependent manner but did not affect Rac2, cell division cycle 42 (cdc42) or ras homolog gene family member A (RhoA) activation (107).   Msp inhibited the polarized accumulation of activated Rac and PI3-kinase products upon exposure to fMLP.   These findings indicate that *T. denticola* Msp inhibition of neutrophil polarity may be due to the selective suppression of the Rac1 pathway.   The lack of detectable effects of Msp pretreatment on the activation of Rac2, Cdc42 and RhoA shows that these cells could still detect the fMLP chemoattractant, although the secondary signaling mechanism linking this stimulus to normal Rac1 activation and polarity was evidently impaired.   The inhibition of migration by Msp was also demonstrated *in vivo* using murine neutrophils expressing enhanced green fluorescent protein (E-GFP) in a murine experimental peritonitis model (162).

Dentilisin

Dentilisin degrades synthetic substrates prolylphenylalanine and prolyl-leucine (70). However, it also degrades natural substrates as well (111).   The natural substrates of this protease are transferrin, fibronectin, fibrinogen, laminin, gelatin, IgG, IgA, α1-antitrypsin, type IV collagen and human complement 3 (173, 194).   *T. denticola* was reported to have the ability to also penetrate human tissue (106).   Its activity against fibronectin, laminin and type IV collagen may be involved in its ability to penetrate tissue.   However, immunization against this microorganism did not alter its abscess-forming activity (82).   Hydrolysis of IgG and IgA may be associated with such mechanisms for evasion of host defenses.

The adherence activity of *T. denticola* to fibrinogen was reduced in a dentilisin-deficient mutant (7). In addtion, a fibrinogen-binding protein was isolated using a fibrinogen-linked sepharose column. The isolated protein was approximately 100 kDa without boiling and 80, 48, and 45 kDa after boiling in SDS polyacrylamide gel electrophoresis (SDS-PAGE). Eight peptides were detected from the 100 kDa protein. Five of them were identified as components of PrtP, PrcA1 and PrcA2 while the other three were not identified. The results of an overlay assay revealed that fibrinogen only bound to a 100-kDa protein but did not bind to the dissociated molecules, suggesting that a complex of PrcA and PrtP is required for binding. *T. denticola* binds to Aα and Bβ fragments but not to the γ fragment of fibrinogen and a dentilisin-deficient mutant did not adhere to these fragments. In addition, dentilisin activity extended thrombin clotting time. These results suggested that binding and hydrolysis of fibrinogen caused longer clotting times and aided in the avoidance of host-mediated killing.

As described above, dentilisin degrades inflammatory cytokines (120). It has been reported that *T. denticola* has the ability to also activate complement (149). Dentilisin hydrolyzed a fragment of human C3 (Fig. 2) and induced activation of human polymorphonuclear cells (194). This activation induced the release of MMP 9 from polymorphonuclear cells and MMP-9 was reported to be involved in the progression of periodontitis (90, 108). Its activation may be involved in tissue destruction by this microorganism. In addition, iC3b, which was degraded from C3b when complement was incubated with *T. denticola* ATCC 35405, was elevated compared to incubation with a dentilisin-deficient mutant. This result suggested that dentilisin attenuated activation of complement via degradation of C3b. In addition, *T. denticola* bound

FH or FHL-1 utilizing Fhb (117).   These effects play an important role in its evasion of the bacterocidal activity of complement.

The *in vivo* pathogenicity of dentilisin was further evaluated in an abscess-forming animal model.   A model system for evaluating the pathogenicity of *T. denticola* was developed by Kesavalu et al. (35, 84) and is now frequently used.   In this model, the infection of mice with *T. denticola* resulted in the involvement of multiple tissues, including epithelial and connective tissues.   As well as appearing to invade muscle layers and deeper tissues, lesion sizes with *T. denticola* were smaller than that with *P. gingivalis* or *Aggregatibacter actinomycetemcomitnas*.   However, the time required for resolving lesions with *T. denticola* was longer than that with *P. gingivalis* or *A. actinomycetemcomitans* (35).   In this model, the lesion size induced by the *T. denticola* wild type strain was significantly larger than that of the dentilisin-deficient mutant (68).   It is possible that dentilisin is involved in the invasion of *T. denticola* into muscle layers and deeper tissues and that Msp is involved in the longer time required for resolving lesions due to its effects on neutrophils and fibrobrasts as described above.

Cystalysin

Cystalysin is a hemolytic protein capable of hemooxidizing haemoglobin and causing lysis of human erythrocytes (26).   Cysteine desulphydrase activity, which is also expressed by cystalysin, is required for the removal of the sulphyhydryl and amino groups from sulphur compounds to produce pyrvate, an energy source as well as ammonia and hydrogen sulphide, which are highly toxic to eukaryotic cells (25).   This activity is also involved in the metabolism of glutathione.   The addition of cystalysin

and L-cysteine to human periodontal ligament cells induces apoptosis and among the products of the metabolism of cysteine, only hydrogen sulfide induced apoptosis in human cells (197)

Other enzymes

Trypsin-like activity was identified by Ohta (127) and this oligopeptidase of *T. denticola* is coded by *opdB* (41).   This activity has been used to detect the red complex (72, 104) of periodontopathogens.   An analysis using *opdB* indicated that this protein was required for growth of *T. denticola*.   However, its role in the virulence of *T. denticola* remains to be clarified.   Peptidases such as dipeptidyl peptidase IV (50), endo-acting proline-specific oligopeptidase (112), proline iminopeptidase (109) and other enzymes such as gamma-glyttamyl transferase and phosphatase have also been described (67, 110, 125).   However, their possible roles in pathogenicity still remain to be demonstrated.

Chemotaxis and gene expression

The motility of *T. denticola* is dependent upon genes coding for periplasmic flagella and chemotaxis.   The flagellar filament of *T. denticola* consists of three core proteins (FlaB1, FlaB2, and FlaB3) and a major sheath protein, FlaA (145). In this regard, *flgE* is a flagellar hook protein gene and   *fliG* codes for a major protein implicated in flagellum assembly. *flgB, flgC, fliE* and *fliF* encode the basal body genes for the flagella while *filH* and *filI* enode flagellar export proteins.   The flagella operon includes tap1, *flgD, flgE,* orf4, *motA, motB, fliL, fliM, fliY*, orf10 and *flip,* which are thought to be related to motility and the environmental sensing functions of *T. denticola*

(100, 164). Stimuli from outside of the cell are sensed by membrane-spanning methyl-accepting chemotaxis proteins (Mcps) and the signals are transformed into appropriate motor responses by the two component system, CheA/Y. The stimulus is communicated to the motor via phosphotransfer reactions from CheA to the response regulator CheY, which controls the direction of flagellar motor rotation according to its phosphorylation level. Repellents increase the autophosphorylation of CheA, whereas attractants decrease this activity.

The *cheX* gene is unique to the phylum of spirochetes. Li *et al* inactivated *flaE* (99) and the resulting mutant did not express flagella and lost its locomotive capability. Chemotaxis has also been demonstrated for *T. denticola* (22) and *cheA*, *cheY* and *cheX* are a gene cluster responsible for this property in *T. denticola* (54). *T. denticola* also expresses *dmcA* and *dmcB* genes which are orthologues of methyl-accepting chemotaxis protein genes (80). The *dmcA* mutant was shown to be defective in chemotaxis toward nutrient-containing media. This defect was also observed with the *dmcB* mutant (98). These chemotaxis properties may play a role in tissue invasion since *flaA* and *cheA* mutants were attenuated in tissue penetration (106). The *dmcA* and *dmcB* mutants were also motile but exhibited reduced tissue penetration relative to the wild-type strain. This result indicates that motility, as well as chemotaxis, are involved in tissue penetration. The *flgE* mutant was also defective in mixed biofilm forming activity with *P. gingivalis* (180).

Acquisition of Fe^{+}

Acquisition of the Fe ion is essential for growth of microorganisms. However, up to now, few reports have characterized such systems in treponemes. Fifty and 35 kDa

lactoferrin binding proteins and 47 kDa and 44 kDa hemin binding proteins were reported in *T. denticola* (27, 151, 152, 163). These proteins play an important role in acquisition of Fe ions because these ions are sequestered by lactoferrin or transferrin (62). A 47 kDa hemin binding protein was produced constitutively and a 44 kDa protein (HbpA) was up-regulated under the iron-restricted growth conditions used for strains GM-1, MS-25, ATCC 33520 and ATCC 33404. The identification of *hbpA* revealed two genes, *hbpA* and *hbpB,* located in tandem and possessing 49% identity (191). A Hbp-deficient mutant reached lower cell densities compared with the wild-type strain in ron-limited media, indicating that this protein was important for iron acquisition (192).

T. denticola* has a genetic locus with significant homology with an operon in *T. pallidum* (13). *troABCD* and its transcriptional regulator *troR* of *T. pallidum* encodes components for a cation transport system and is involved in uptake of Fe^+. TroR of *T. denticola* was shown to be a manganese- and iron-dependent transcriptional repressor using an *Escherichia coli* reporter construct and *T. denticola.* The iron acquisition system is sometimes associated with virulence expression in some bacteria such as *Salmonella typhymurium.* Further analysis is required to evaluate the association of this system with the pathogenicity of *T. denticola.*

Animal models

In vivo investigations into the mechanism of the pathogenicity of *T. denticola* in periodontitis have been limited previously but now animal models for this microorganism have been established recently. Two oral infection models have been developed using mice and rats. In the mouse model (94), *T. denticola* was suspended

in phosphate-buffered saline supplemented with 6 mM L-cysteine HCl and inoculated into the oral cavity once a week, seven times. At 71 days after the first infection, the mice were euthanized and alveolar bone loss evaluated. *T. denticola* was detected at 37, 50 and 71 days and the infected mice showed greater bone resorption than mock-infected mice, indicating that significant bone loss had occurred. In infected mice, sera reacted to 11 *T. denticola* protein bands, including dentilisin and Msp, and the serum IL-10 levels in the infected mice showed a dramatic decrease. In the rat model, the effect of polymicrobial infection using *P. gingivalis*, *T. denticola*, *T. forsythia* and *F. nucleatum* was investigated (83). In this model, mixed infection with *T. denticola, P. gingivalis* and *T. forsythia* showed a significantly greater alveolar bone resorption compared to the results observed with monoinfection with each species. Interestingly, antibody against *T. forsythia* was elicited after infection with *T. denticola* alone.

As periodontitis has been reported to influence several systemic diseases, the involvement of *T. denticola* in systemic diseases was also investigated. *T. denticola* may spread to internal organs via the blood stream. Foschi et al investigated the dissemination of *T. denticola* from local foci (46). After infection by *T. denticola* in the root canal, its DNA was detected in the spleens of wild-type mice and the spleens, hearts, and brains in severe combined immunodeficiency mice. *T. denticola* also enhanced the pheumonia caused by *P. gingivalis* in a mouse model (86).

Conclusions

Recent analysis of *T. denticola* using molecular genetic techniques has begun to reveal the molecular basis for the physiology and pathogenicity of this microorganism.

Procedures for gene inactivation provide a basis for characterizing the virulence factors of *T. denticola*.   Some mechanisms of virulence such as the induction and degradation of cytokines are similar to other periodontopathic bacteria while other mechanisms such as inhibition of migration of fibroblasts and neutrophiles are unique to *T. denticola*. The unique virulence factors of *T. denticola* appear to play roles in their association with chronic periodontitis.   The effects of *T. denticola* on neutrophiles may lead to incomplete eradication of periodontopathic bacteria including *T. denticola* and similar effects on fibroblasts may result in the retardation of wound healing.   The analyses of cell signaling induced by this microorganism may help explain their effects on host cell dynamics.   Further detailed analysis regarding the receptors and cascades of cell signaling induced by *T. denticola* are clearly necessary.

The recent development of animal models also greatly improved our understanding of the pathogenicity of *T. denticola.*   These models will allow for a more detailed analysis of the virulence of *T. denticola* as well as their interaction with other periodontopathic bacteria in the development of periodontitis.   Such models should also provide a better understanding of the precise nature of periodontopathic oral biofilms as well as how their formation could be regulated.

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Table 1

Potential virulence factor of *T. denticola*

Virulence factor	Gene(s)	Activity	Reference
Adherence			
Major outer sheath protein (Msp)	*msp*	Adherence to fibronectin and laminin	40, 42
OppA (orthologue)	*oppA*	Adherence to fibronectin and plasminogen	41
FH-like binding protein	*fhbB*	Adherence to FH/FHL-1	118, 119
Dentilisin	*prtP*	Ligand for coaggregation with *P. gingivalis*	61
Leucine-rich repeat A	*lrrA*	Ligand for coaggregation with *T. forsythia*	66
Immunomodulateion			

Immunosuppresive factor (Sip)	-	Induce G1 arrest in human peripheral blood mononuculear cell	96
Dentilisin	*prtP*	C3 activation	194
		Hydrolysis of human IL-1ß, IL-6, IL-8, MCP-1 and TNF α	120
		Hydrolysis of human IgG and IgA	70
Msp	*msp*	Activate innate immunity via TLR2	126
Lipooligosaccaride	-	Activate innate immunity via TLR4	126
		Induction of TNF α and nitric oxide from mice macrophage	143
		Induction of IL-1β, IL-6, IL-8, MCP-1, nitric oxide, MMP-8 and PGE2 from human gingival fibroblast	167
Peptideglycan	-	Induce release of IL-1ß, IL-6, IL-8, TNF α, RANTES, MMP-8	168

		and prostaglandinE2	
Efflux pump?	-	Resistance to hbD-1 and -2	14, 16
Cytotoxicity			
Msp	*msp*	Chanel-forming activity	137, 116
		Cytoskeletal disruption in fibroblast	2, 77, 97, 183, 195
		fMLP-induced chemotaxis , phagocytosis of neutrophil	107, 123, 135, 162
Dentilisin	*prtP*	Degradation of host protein (fibronectin, laminin, type VI collagen, α1-anti-trypsin etc.)	70
		Binding and degradation of fibrinogen	126

Dentilisin	*prtP*	Degradation of host protein (fibronectin, laminin, type VI collagen, α1-anti-trypsin etc.)	70
		Binding and degradation of fibrinogen	126
		Induction of MMP-8 production via activation of C3	194
Cystalisin		Involves hemolysis and hemooxidizing haemoglobin	26
		Cysteine desulphydrase activity	
Tissue penetration			
Periplasmic flagella	*flaB1-3, flaA, fliE-G, fliL, fliM, fliY*	Locomotive movement	99, 100, 145, 164

|			
Chemotaxis	*cheA, cheX,*		54, 80, 98,
(sensor/signal transduction)	*cheY*		99, 100,
	dmcA, dmcB		106, 145,
			164, 180
Acquisition of metal			
	troA-D, troR	Fe+ acquisition	62

Figure legend

Fig. 1. Structure of *T. denticola*

Periplasmic flagella is embedded inside periplasmic space of *T. denticola*. Major outer membrane protein is the major component of outer sheath and consists complex with its surface protein dentilisin.

Fig. 2　Involvement of surface component of T. denticola to complement activation.

In alternative pathway, Factor B binds to C3 and the complex is cleaved by factor D. C3b accumulates bacterial surface and involved in opsonization or activation of terminal complement components. Factor H and I regulate complement pathway. Factor H binds to C3b and compete with factor B to displace Bb from the convertase in addition to acting as cofactor for factor I.

Factor I　regulates complement by cleaving C3b. Dentilisin involves in activation of complement pathway by cleaving C3. FHL-1 binding protein (FHbB) binds to Factor H.
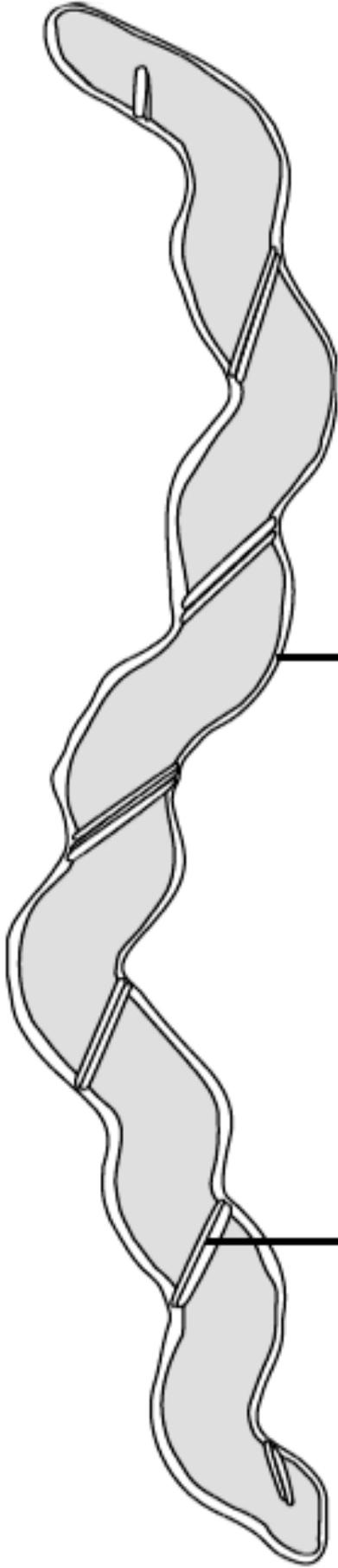
Periplasmic flagella

Outer sheath
Major outer sheath protein
Protease (dentilisin)