

ROLE OF *TREPONEMA DENTICOLA* IN PERIODONTAL DISEASES

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ABSTRACT: Among periodontal anaerobic pathogens, the oral spirochetes, and especially *Treponema denticola*, have been associated with periodontal diseases such as early-onset periodontitis, necrotizing ulcerative gingivitis, and acute pericoronitis. Basic research as well as clinical evidence suggest that the prevalence of *T. denticola*, together with other proteolytic Gram-negative bacteria in high numbers in periodontal pockets, may play an important role in the progression of periodontal disease. The accumulation of these bacteria and their products in the pocket may render the surface lining periodontal cells highly susceptible to lysis and damage. *T. denticola* has been shown to adhere to fibroblasts and epithelial cells, as well as to extracellular matrix components present in periodontal tissues, and to produce several deleterious factors that may contribute to the virulence of the bacteria. These bacterial components include outer-sheath-associated peptidases, chymotrypsin-like and trypsin-like proteinases, hemolytic and hemagglutinating activities, adhesins that bind to matrix proteins and cells, and an outer-sheath protein with pore-forming properties. The effects of *T. denticola* whole cells and their products on a variety of host mucosal and immunological cells has been studied extensively (Fig. 1). The clinical data regarding the presence of *T. denticola* in periodontal health and disease, together with the basic research results involving the role of *T. denticola* factors and products in relation to periodontal diseases, are reviewed and discussed in this article.

Key words. *Treponema denticola*, periodontal diseases, adherence, motility, proteolytic enzymes, cytopathic effects.

(1) Introduction

Several species of spirochetes are known to be pathogenic in humans, including *Borrelia burgdorferi*, the etiologic agent of Lyme disease, *Treponema pallidum*, which causes venereal syphilis, and two other spirochetes related to *T. pallidum* which are the causative agents of yaws (*T. pertenue*) and pinta (*T. carateum*). *Leptospira*, a spirochete found in water and soil, may also be pathogenic to humans and other mammals (Canale-Parola, 1977; Holt, 1978). Treponemes are also part of the human normal oral flora. Being anaerobic, they reside mainly in the subgingival area. However, they may take hold in opportunistic infections such as periodontal diseases, which are destructive, inflammatory processes of the tooth attachment tissues, caused by Gram-negative proteolytic anaerobic bacteria (Gazi *et al.*, 1997). Various spirochetal morphotypes can be observed in periodontal pockets, but many of these morphotypes are as yet uncultivable (Moter *et al.*, 1998). One of the most studied oral spirochetes, *T. denticola* (Fig. 2), possesses the features needed for adherence, invasion, and damage of the periodontal tissues (Fenno and McBride, 1998). In part, these bacteria resemble the fastidious behavior of the known pathogenic spirochetes, and can be found in non-oral infections, *e.g.*, digital lesions in cattle (Choi *et al.*, 1997).

The recent complete *T. pallidum* genome sequence (Fraser *et al.*, 1998) revealed that the *T. pallidum* chromosome encodes 12 polypeptides with sequence relatedness to the major outer sheath of *T. denticola* (Centurion-Lara *et al.*, 1999). Comparison of the *T. pallidum* genome sequence with that of *B. burgdorferi* identified unique and common genes and substantiates the considerable diversity observed among pathogenic spirochetes. Defined genetic mutants of *T. denticola* were constructed by allelic replacement mutagenesis (Li and Kuramitsu, 1996; Li *et al.*, 1996), and a novel methyl-accepting chemotaxis

gene, *dmcB*, has been identified (Li *et al.*, 1999).

Furthermore, a new *Escherichia coli*-*T. denticola* shuttle vector, based on a naturally occurring spirochete plasmid, was recently constructed, and the heterologous *T. pallidum* *flaA* gene from the plasmid in *T. denticola* was expressed (Chi *et al.*, 1999). This new shuttle vector system may be useful in characterizing virulence factors from pathogenic spirochetes that cannot be cultured.

In the periodontal pocket, *T. denticola* are found close to the junctional epithelium, and were shown to adhere to host cells and tissues as well as to matrix proteins (Olsen, 1984; Sela *et al.*, 1987a; Weinberg and Holt, 1990; Holt and Bramanti, 1991; Uitto *et al.*, 1995; Leung *et al.*, 1996; Thomas, 1996). They are also able to adhere to other bacteria (Grenier, 1992a; Onagawa *et al.*, 1994; Kolenbrander *et al.*, 1995; Yao *et al.*, 1996), to collagen-binding proteins (Umemoto *et al.*, 1997), and to collagen used as a barrier for guided bone regeneration (Sela *et al.*, 1999). Of special interest are the bimodal and nutritional interactions between *T. denticola* and other periodontal bacteria, especially *P. gingivalis*, on the one hand (Grenier, 1992a,b; Yao *et al.*, 1996), and the antagonistic relationships of *T. denticola* with other bacteria, on the other (Grenier, 1996a). The presence of proteolytic enzymes and cytolytic factors in these micro-organisms (Uitto *et al.*, 1988, 1995; Holt and Bramanti, 1991; Syed *et al.*, 1993; Rosen *et al.*, 1994, 1995; Mäkinen and Mäkinen, 1997; Mikx, 1997), together with their high motility (Pietrantonio *et al.*, 1988), may contribute to the treponemes' possible invasive capability. Indeed, challenge of mice with *T. denticola* resulted in deep invasiveness and involvement of multiple tissues, including epithelial and connective tissues (Ebersole *et al.*, 1995). With the deterioration of the periodontal tissues during the development of periodontal diseases, a progressive increase in the percentage of gingival spirochetes can be seen (Listgarten and Levine, 1981; Loesche and Laughon, 1982).

A positive correlation has been established between the percentage of subgingival spirochetes and dental plaque index, gingival exudate, gingival index, bleeding tendency, pocket depth, connective tissue attachment loss, and periodontal disease index (Armitage *et al.*, 1982). Moreover, a significant reduction in the depths of individual pockets and in the degree of inflammation has been observed concomitantly with the decrease in the treponemal population following periodontal therapy (Loesche and Laughon, 1982). Studies also suggest that changes in antibody titers to specific oral treponemes may be associated with periodontal diseases (Jacobs *et al.*, 1980; Mangan *et al.*, 1982). Quantitative evidence for a positive relationship between *T. denticola* and severe periodontitis was established by Simonson *et al.* (1988). Shortly afterward, these researchers, using monoclonal antibodies specific for *T. denticola*, demonstrated a significant increase in the numbers of the organisms with disease severity (Simonson *et al.*, 1990). With the rapid development of the field of dental implantology, attention has been drawn to the microbial population of "peri-implantitis". Spirochetes were among the most frequently detected micro-organisms from patients with failing implants (Listgarten and Lai, 1999). The extensive search for possible relationships between periodontal bacteria and the pathogenic sequelae that accompany periodontal diseases has revealed the presence of several cell-modulating factors and effects in *T. denticola* (Fig. 1): suppression of fibroblast proliferation (Boehringer *et al.*, 1984); enhancement of collagen phagocytosis by gingival fibroblasts (Battikhi *et al.*, 1999); a monocyte-dependent suppressor for human lymphocyte response (Shenker *et al.*, 1984); an inhibitor of superoxide production by leukocytes (Sela *et al.*, 1987b, 1997); activation of IL-1 β (Beausejour *et al.*, 1997); bone resorption *in vitro* (Gopalsami *et al.*, 1993); and the activation of both the classic and the alternative pathways of human complement (Schenkein and Berry, 1991). *T. denticola* was further shown to perturb actin and actin-regulating pathways in host cells (Ellen, 1999).

These bacteria possess several lipoproteins and a specific lipopoligosaccharide molecule. An enriched lipoprotein fraction from *T. denticola* was shown to modulate oxygen-dependent and -independent mechanisms by human PMN (Sela *et al.*, 1997).

Interestingly, it was recently shown that although regarded as strict anaerobes, *T. denticola* have significant oxygen metabolism, even at the low levels of O₂ measured in periodontal pockets, and contain enzymes that offer at least moderate protection against damage by reactive oxygen species (Caldwell and Marquis, 1999).

The major surface protein and the surface-expressed chymotrypsin-like protease complex of *T. denticola* have been suggested to be mediators of the cytopathic effects typically seen in periodontal diseases (Fenno *et al.*, 1998a,b; Ishihara and Okuda, 1999). However, recent work (Caimano *et al.*, 1999) proposes that the major surface protein is predominantly periplasmic, with only limited surface exposure.

Several important issues concerning the potential virulence of *T. denticola* remain for further exploration: the possible existence of a biologically active lipopolysaccharide molecule, the exact location and function of the "major surface protein", and the genetic and pathogenic similarities between these bacteria and other disease-causing spirochetes. The ensuing discussion of clinical evidence and basic research studies will examine the role of *T. denticola* and their products in the cell and tissue destruction that accompanies periodontal diseases. Possible avenues of future research in the field of host-parasite inter-relationships in the periodontal environment will also be highlighted.

(2) *T. denticola* in Periodontal Diseases

(2.1) HUMAN STUDIES

The most common anaerobic bacteria shown to dominate periodontal plaque associated with disease are *P. gingivalis*, *B. forsythus*, and *T. denticola*. Anaerobic micro-organisms, such as spirochetes and black-pigmented Bacteroides (classified recently as *Porphyromonas* and *Prevotella*), were identified more than 35 years ago as periodontal pathogens (MacDonald *et al.*, 1962). As the clinical periodontal parameters worsen, the number and percentage of spirochetes counted by microscopic techniques increase proportionately (Listgarten and Helldén, 1978; Lindhe *et al.*, 1980; Loesche and Laughon, 1982; Riviere *et al.*, 1995). The presence of *T. denticola* and unidentified spirochetes in healthy periodontal sites was also associated with an increased susceptibility to gingival inflammation (Riviere and DeRouen, 1998).

Spirochetes were also shown to be significantly elevated (in numbers and proportions) in dental plaques removed from untreated patients compared with those from patients receiving periodontal treatment. Spirochetes were the overwhelming microbial type in the plaques of adult periodontitis patients, averaging about 45% of the microscopic count (Loesche *et al.*, 1985), as well as in microbial samples from patients with acute pericoronitis (Weinberg *et al.*, 1986) and failing implants (54%) (Listgarten and Lai, 1999). Furthermore, the examination of intra-oral sources of species colonizing dental implants (Lee *et al.*, 1999) has revealed positive associations between *T. denticola* isolated from bacterial samples taken from implants and teeth at the same visit.

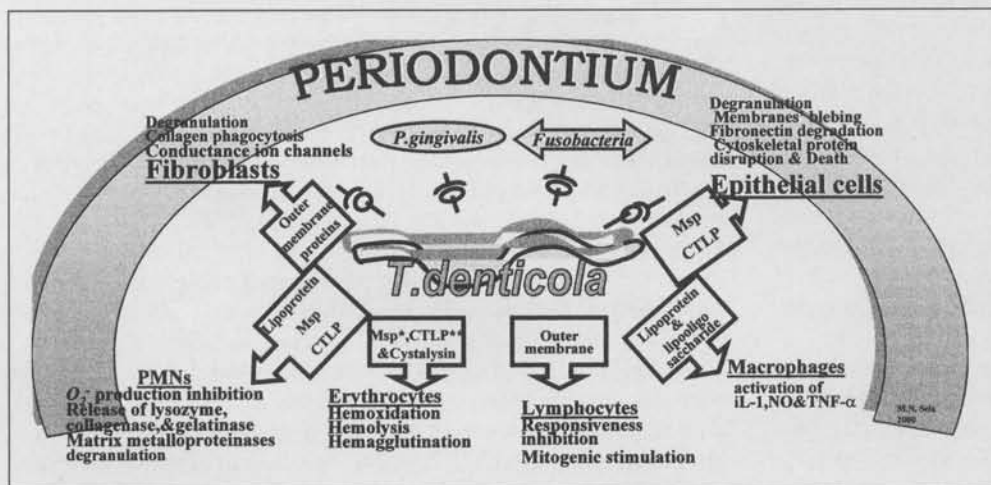


Figure 1. The effects of *T. denticola* and their metabolic products on mucosal and immunologic cells in the periodontal site. *T. denticola* adheres to (—○—) epithelial cells and fibroblasts as well as to *P. gingivalis* and *Fusobacteria*. Bacterial products (Msp* = major surface protein, CTLP** = chymotrypsin-like protease) act on (⇒) mucosal cells and on immune system cells, causing cell damage and release of cellular deleterious factors to the periodontal environment.

Species-specific nested PCR revealed that subjects with periodontitis harbored in their dental plaque strains of *T. denticola*, *Treponema amylovorum*, *Treponema maltophilum*, *Treponema medium*, *Treponema pectinovorum*, *Treponema socranskii*, and *Treponema vincentii* (Willis *et al.*, 1999).

Using DNA probes, polyclonal antibodies, and culture methods, investigators have also shown that *P. gingivalis*, *B. forsythus*, *T. denticola*, and other spirochetes were present in 80–100% of plaque samples removed prior to periodontal surgery (Loesche *et al.*, 1992). In a recent study aimed to determine the association between the levels of granulocyte elastase and prostaglandin E2 in the gingival crevicular fluid and the presence of periodontopathogens in untreated adult periodontitis (Jin *et al.*, 1999), the predominant combination of species detected was *P. gingivalis*, *P. intermedia*, *B. forsythus*, and *T. denticola*. This bacterial combination was significantly higher at periodontitis sites (68%) than at healthy (7%) or gingivitis sites (29%).

Especially noteworthy is an enzyme-linked immunosorbent assay procedure, developed with a monoclonal antibody specific for a serovariety of *T. denticola* (Simonson *et al.*, 1988). *T. denticola* was present at significantly elevated levels in plaque samples collected from deep-pocket sites of patients with severe periodontitis, relative to healthy controls and a group with moderate disease. These findings may be considered quantitative evidence of a positive relationship between *T. denticola* and severe periodontitis.

The *T. denticola* serovar C content from deep pockets of patients with severe periodontitis was found, by specific monoclonal antibodies, to be twice that of samples collected from pockets of patients with moderate periodontitis, or from healthy subjects (Simonson *et al.*, 1990). It has been recognized for some time that bacterial species exist in complexes in subgingival plaque. In an attempt to define such communities, investigators have used data from large numbers of plaque samples (13,000) and examined different clustering and ordination techniques (Socransky *et al.*, 1998). Five major complexes were consistently observed. One complex in particular, consisting of the tightly related group of *B. forsythus*, *P. gingivalis*, and *T. denticola*, related strikingly to clinical measures of periodontal disease, particularly pocket depth and bleeding on probing.

In a series of 993 subgingival microbial samples sent to a diagnostic microbiology laboratory, 196 samples could be identified as compatible with a clinical diagnosis of refractory, or recurrent, periodontitis (Listgarten *et al.*, 1993). In descending order of prevalence, the associated microbiota included: *B. forsythus* (84%), spirochetes (83%), motile rods (76%), *Fusobacterium* species (68%), and *P. gingivalis* (63%).

Localization of *P. gingivalis* and *T. denticola* in different areas of subgingival plaque from advanced adult periodontitis patients by means of sensitive immunogold/silver-staining and immuno-electron microscopy was also studied (Kigure *et al.*, 1995). Cells of both *P. gingivalis* and *T. denticola* were found predominantly in subgingival plaque located at depths of more than 4 mm in periodontal pockets. *T. denticola* cells were found in the surface layers of subgingival plaque, and *P. gingivalis* were predominant beneath them. However, in the deeper subgingival plaque, coexistence of *P. gingivalis* and *T. denticola* was observed. Therefore, the presence of *P. gingivalis* and *T. denticola* may provide useful information regarding the pattern of colonization of micro-organisms in the periodontal pocket and may be directly related to the deleterious changes seen in the pocket. On the other hand, several oral bacteria exhibiting antagonism

toward *T. denticola* could be demonstrated (Grenier, 1996a). These positive and negative interactions may have a significant role in the population shifts observed in subgingival sites.

In another development, checkerboard DNA-DNA hybridization technology was used to study the occurrence of 18 microbial species associated with various states of periodontal health and disease, in a sample of 148 Chinese subjects never before exposed to systematic dental therapeutic intervention (Papapanou *et al.*, 1997). An increase of *P. gingivalis*, *T. denticola*, and *B. forsythus* in deep pockets or progressing sites was found. It is important to note that colonization by *P. gingivalis*, *B. forsythus*, *Campylobacter rectus*, and *T. denticola*, at levels exceeding certain thresholds, entailed a significantly increased probability for an individual subject to harbor deep pockets or tooth sites with progressing disease. A contingency table of the frequency of *T. denticola* and *P. gingivalis* in subgingival plaque samples revealed a highly significant synergistic relationship between the bacteria. The finding that the occurrence of *T. denticola* apparently requires the presence of *P. gingivalis* (Simonson *et al.*, 1992) may also represent a synergistic relationship between these putative oral pathogens.

Taken together, the fact that cell-free culture filtrates of *P. gingivalis* stimulated the growth of strains of *T. denticola* (Nilius *et al.*, 1993), and since subgingival plaques from periodontal pockets colonized with *T. denticola* also contain *P. gingivalis*, suggested that certain extracellular products of *P. gingivalis* may act as growth factors for *T. denticola* in the periodontal pocket. Additional clinical studies on the coexistence of bacteria in periodontal sites by means of whole genomic DNA probes and checkerboard DNA-DNA hybridization (Haffajee *et al.*, 1998) revealed an etiologic role for *B. forsythus*, *P. gingivalis*, *T. denticola*, and *Selenomonas noxia* in adult periodontitis. Yet, when the presence of disease-associated bacteria in health-associated plaque was correlated with susceptibility to periodontitis over time (Riviere *et al.*, 1997), *T. denticola* was not detected in health-associated plaque from stable health sites and was detected in only three sites that progressed to periodontitis. Among several bacterial species which were found to be associated with early-onset periodontitis (EOP) in a population of young adults (Albandar *et al.*, 1997), of particular importance were *P. gingivalis* and *T. denticola*, which are suggested to play a significant role in severe and progressive forms of EOP. Similar findings, borne out in animal studies, will be discussed in greater detail later in this review.

Finally, oral micro-organisms, including Gram-negative periodontal bacteria, are frequently associated with systemic disorders such as cardiovascular diseases (Beck and Offenbacher, 1998). Recently, *B. forsythus*, *P. gingivalis*, *Actinobacillus actinomycetemcomitans*, and *T. denticola* were detected at higher levels in pre-term low-birth-weight (PLBW) mothers, as compared with controls (Offenbacher *et al.*, 1998). Biochemical measures of maternal periodontal status and oral microbial burden may therefore be associated with PLBW.

In conclusion, research attempts from diverse groups using different clinical tools and experimental methods point to the presence of *T. denticola* in relatively high proportions in diseased human periodontal sites.

Portions of the data point to the existence of constant patterns of bacterial interactions, specifically between *T. denticola* and *P. gingivalis*. Such interactions may participate in the establishment of a potentially pathogenic subgingival plaque. Furthermore, several studies have suggested even more specific

interactions between *T. denticola* and *P. gingivalis* (Grenier, 1992a,b), including a strong co-aggregation reaction between these two periodontopathogens, while other black-pigmented oral bacterial species tested did not co-aggregate with *T. denticola* (Grenier, 1992a). Taken together, the presence of high numbers of *P. gingivalis* and *T. denticola* in affected sites, and the described interactions between these two pathogens, as well as their pathogenic properties and proteolytic capacity, may play an important role in the initiation and progression of periodontal diseases. It remains for future research to examine the issue of the mutual chemical and molecular effects of *P. gingivalis* and *T. denticola* in the development of these infectious diseases.

(2.2) ANIMAL STUDIES

Several studies on the microbiology of periodontal disease in animal models revealed the importance of *T. denticola* and their pathogenic capacity.

In dogs, the presence of a group of pathogen-related oral spirochetes as well as *T. denticola* and *T. socranskii* was determined by the use of specific monoclonal antibodies in an immunocytochemical microscopic assay (Riviere *et al.*, 1996). A significantly greater proportion of dogs with deep pockets had detectable treponemes, compared with dogs experiencing periodontal health.

The virulence characteristics of *T. denticola*, *T. socranskii*, *T. pectinovorum*, and *T. vincentii* following challenge infection of mice were also investigated (Kesavalu *et al.*, 1997). These micro-organisms induced dose-dependent subcutaneous abscesses that were similar in time of onset, lesion progression, and duration of healing. Only viable cells were capable of inducing these characteristic abscesses. Histological examination of the skin lesion revealed abscess formation in the subcutaneous tissues, and abundant spiral organisms were demonstrated to be present in the abscess. *T. denticola* were shown, in this animal model, to be able to invade the deep oral connective tissue layers (Ebersole *et al.*, 1995). Polymicrobial infections with *T. denticola* and *P. gingivalis* in the murine lesion model (Kesavalu *et al.*, 1998) revealed that, at high doses of *P. gingivalis*, the addition of *T. denticola* had no effect on the formation and size of the spreading lesion caused by this micro-organism. However, at low *P. gingivalis* challenge doses, *T. denticola* significantly enhanced the virulence of *P. gingivalis* compared with mono-infection of this micro-organism. *P. gingivalis* mutants, deficient or devoid of trypsin-like protease activity, were used for examination of the potential role of enzyme activity in the synergistic virulence. *T. denticola*-*P. gingivalis* complexes exhibit enhanced virulence in this model, and the trypsin-like protease activity was found to be important to *P. gingivalis* virulence expression in the polymicrobial challenge infection. The significance of these bacterial proteases in the etiology of periodontal diseases in humans is discussed later in this review. The characteristics of humoral immune responses to *T. denticola* following primary infection, re-infection, and active immunization were recently investigated, as was immune protection in mice (Kesavalu *et al.*, 1999a,b). Primary infection with *T. denticola* induced a significant serum IgG response compared with that of the control uninfected mice. The IgG response to re-infection was significantly higher than that for control mice and primary infection.

Mice actively immunized with formalin-killed treponemes developed serum antibody levels 7 to 8 times greater than those in animals after primary infection. A broad antigen reactivity of

the serum antibody was significantly increased with re-infection and active immunization. Furthermore, serum antibody was effective *in vitro* in immobilizing and clumping the bacteria, but did not inhibit growth or passively prevent the treponemal infection. It is suggested that humoral immune responses were not capable of resolving a *T. denticola* infection in the mice model.

The effects of environmental alteration on the virulence of *T. denticola* and *T. pectinovorum* were also assessed in a localized inflammatory abscess model in mice (Kesavalu *et al.*, 1999a). *In vitro*, growth of *T. denticola* and *T. pectinovorum*, as a function of modification of the cysteine concentration, significantly enhanced abscess formation and size. Furthermore, *T. denticola* induced significantly larger lesions in mice pre-treated with iron. Although not comparable with the pathologic events involved in bacterial-host reactions observed in the human periodontal environment and caused by members of the periodontal flora, the mice abscess may serve as an important model for the future investigation of the pathogenic capacity of *T. denticola* in relation to periodontal diseases.

(3) Adherence, Co-aggregation, and Locomotion

The development of mucosal infections by bacteria may depend on the ability of the causative agent to adhere to the mucosal cells and tissues. Furthermore, if bacterial invasion of the mucosa occurs, bacterial motility may appear as an important pathogenic tool. *T. denticola*, like other strains of treponemes, were shown to adhere to both host cells and matrix proteins through protein moieties *in vitro*.

The direct contact between *T. denticola* and the epithelium in the periodontal pockets triggered several research groups to study the adhesion of *T. denticola* to human gingival fibroblasts and epithelial cells. These studies are of interest in light of the proteolytic activity of these bacteria and the possible invasion of the connective tissue by *T. denticola*. It is important to note that the encounter of *T. denticola* and human gingival epithelial cells and fibroblasts *in vitro* is associated with major alterations in the cells' morphology, including cellular rounding and shrinkage (Baehni *et al.*, 1992; De Filippo *et al.*, 1995).

T. denticola was shown to adhere to fibroblasts and epithelial cells of different origins (Weinberg and Holt, 1990; Keulers *et al.*, 1993a,b; Ellen *et al.*, 1994a; Haapasalo *et al.*, 1996), as well as to extracellular matrix components present in periodontal tissues (Dawson and Ellen, 1990; Haapasalo *et al.*, 1991) and hyaluronan (hyaluronic acid) (Haapasalo *et al.*, 1996). Recently, it was shown that oral treponemes, including *T. denticola*, may adhere to endothelial cells and penetrate through endothelial cell monolayers *in vitro* (Peters *et al.*, 1999).

The adherence patterns of spirochetes, including *T. denticola*, are often in a polar orientation with their free end in motion (Dawson and Ellen, 1990, 1994; Ellen *et al.*, 1994a,b). This specific bacterial substrate contact pattern may play a role in the cytopathogenicity of *T. denticola* and their invasive ability. A polar clustering of adhesins in the bacterial fluid outer sheaths is the basis for tip adhesion, which was also shown in *T. pallidum* (Konishi *et al.*, 1986). The adherence of oral spirochetes, including *T. denticola*, to host cells and matrix proteins was thoroughly discussed by Thomas (1996). Since then, several studies have elaborated on these essential characteristics of the bacteria. Serotypes of *T. denticola*—*Treponema scoliodontum*, *T. socranskii*, and *T. vincentii*—were tested with monolayers of epithelial cells of human and canine origin (Carranza *et al.*, 1997). Attachment of oral treponemes was compared with attachment by *T. pal-*

lidum subsp. *pallidum*, and by the non-pathogen *Treponema phagedenis*.

The different serotypes of *T. denticola* had similar abilities to attach to epithelial cells, and the proportion of epithelial cells susceptible to attachment by oral spirochetes was strongly related to the confluence level of the monolayer.

Another type of oral treponeme which may be involved in periodontal diseases, *T. pectinovorum*, also binds firmly to epithelial cell monolayers in a concentration-dependent manner (Walker *et al.*, 1999a), and the binding depends on environmental factors. For example, increasing concentrations of fetal bovine serum inhibited binding, whereas *T. pectinovorum* membrane vesicles and co-incubation with *T. denticola* ATCC 35404 increased the number of cells bound to the monolayer. Co-infection of the epithelial cells with both *T. denticola* and *T. pectinovorum* did not involve any mutual interference in their attachment to the epithelial cells, suggesting that they do not compete for the same receptor on the host cell surface. Attachment of oral treponemes to epithelial cells may promote the colonization of the periodontal pocket, as well as the retention of colonies within plaque. The treponemes had a preference to attach to cells of low confluence fields, pointing to an increased ability to attach to cells which are actively dividing in areas of repair, such as inflamed periodontal pockets. Furthermore, attachment of oral treponemes to epithelial cell barriers may promote cytopathic effects and be the first step in bacterial invasion into the underlying tissues. *T. denticola* were also shown to have a high capacity to adhere to dental materials such as titanium and guided bone regeneration devices, especially bioabsorbable collagen barriers (Sela *et al.*, 1999). Bacterial plaque formation on such materials may have a crucial negative influence on the outcome of the treatment and related regeneration procedure.

The periodontal pocket ecosystem consists of numerous fastidious bacteria which co-exist in a delicate balance with the host cells and tissues. These close contacts in a very specific environment may lead to the development of specific inter-bacterial relationships.

Indeed, as shown above, substantial sources point to the existence of special inter-relationships between *P. gingivalis* and *T. denticola* in the periodontal pocket, including co-aggregation (Grenier, 1992b). Cells of *P. gingivalis* were further shown to be able to co-aggregate with strains of *T. denticola* and *T. socranskii* (Onagawa *et al.*, 1994). Strong interspecies adherence was demonstrated among *T. denticola*, *B. forsythus*, and *P. gingivalis*, and between these bacteria and the plaque organism *Streptococcus crista* (Yao *et al.*, 1996). The most important players in the bacterial interrelationships which occur during the development of the oral biofilm are the fusobacteria. Strains of *Treponema* spp., including members of all human oral species, were tested for co-aggregation with oral fusobacteria, non-oral fusobacteria, and additional strains of oral bacteria, including: actinobacilli, actinomycetes, capnocytophagae, eubacteria, porphyromonads, prevotellae, selenomonads, streptococci, and veillonellae. None of the treponemes co-aggregated with any of the oral strains or with the non-oral fusobacteria (Kolenbrander *et al.*, 1995). All treponemes—including *T. denticola*, *T. socranskii*, oral pectinolytic treponemes, *T. pectinovorum*, and *T. vincentii*—co-aggregated with at least one strain of the fusobacteria tested as partners, but no intragenetic co-aggregations were observed among the treponemes. In the more than 100 co-aggregations observed, the fusobacterial partner was heat-inactivated, while the treponemes were unaffected by heat treatment. Only the *T.*

denticola co-aggregations were inhibited by lactose and D-galactosamine, pointing to a possible existence of a specific adhesive site on the bacterial surface. Besides their interaction with *P. gingivalis*, *T. denticola* show specificity for oral fusobacteria as co-aggregation partners, a contact that may facilitate efficient metabolic communication, as shown for *P. gingivalis* and *T. denticola* (Simonson *et al.*, 1992), and enhance the proliferation of each cell in the progressively more severe stages of periodontal disease.

In bacterial species that are involved in cell adherence and tissue invasion, locomotion is considered a virulence factor. Strains of *T. denticola* were shown to move at different speeds in different conditions of viscosity (Pietrantonio *et al.*, 1988). The motility of *T. denticola* ATCC 33520 was shown to be temperature-dependent, and the speed of the bacteria was markedly increased at 35°C and related to the viscosity of the medium (Ruby *et al.*, 1998).

A motility locus containing 11 genes was identified in *T. denticola* (ATCC 35405), sequenced and analyzed (Stamm and Bergen, 1999). The order of these genes is identical to that of the corresponding region of the *T. pallidum* fla operon. Seven of the deduced fla proteins share significant homology with both *E. coli* and *Bacillus subtilis* proteins associated with flagellar structure and function. Reverse transcription-PCR analysis indicated that the *T. denticola* fla genes are transcribed as a single unit. A putative sigma-like promoter, identical to the *T. pallidum* fla promoter, was identified upstream of tap1. The *T. denticola* and *T. pallidum* fla operons, comprised of numerous motility-related genes, are therefore highly conserved. These results support a proposed phylogenetic relatedness of *T. denticola* and *T. pallidum*.

Stimulating advancement in locomotion analysis has been made by means of a recently developed system to generate specific mutants in *T. denticola*, built to determine if Tap1 was essential for motility (Limberger *et al.*, 1999). *T. denticola* tap1 and flanking DNA were identified, cloned, and sequenced, and a suicide plasmid that contained tap1, interrupted with an erythromycin resistance cassette, was constructed. Because of potential polar effects from this cassette, a second plasmid that contained tap1 interrupted with a modified erythromycin resistance cassette was constructed. Electroporation-mediated allelic exchange incorporated the interrupted tap1 genes into the *T. denticola* chromosome, creating Tap1-deficient mutants.

Reverse transcriptase-PCR revealed that the erythromycin resistance cassette within tap1 did not terminate fla operon transcription in either mutant. Moreover, the phenotypes of the two mutants were indistinguishable. These mutants lacked motion in liquid culture, were unable to spread on agar plates, and lacked flagellar filaments as determined by electron microscopy. Treponemal Tap1 is suggested to be analogous to FliK, which is involved in monitoring the flagellar hook length of *Salmonella typhimurium*. *T. denticola* also display chemotaxis *in vitro*, a property that may be of importance in their motility during tissue invasion. Chemotactic activity of motile bacteria is usually regulated by mcp and che genes. Methyl-accepting chemotaxis protein genes were isolated from *T. denticola* 35405, identified, and sequenced (Greene and Stamm, 1997; Kataoka *et al.*, 1997; Li *et al.*, 1999). Moreover, a chemotaxis gene cluster from *T. denticola* was cloned, sequenced, and analyzed (Greene and Stamm, 1999). The gene cluster contained three chemotaxis genes and an open reading frame that is homologous with that found in *T. pallidum* and *B. burgdorferi*. Active bacterial invasion into the periodontal tissues is a widely proposed hypothesis based on clinical observations, but has to be further proven by biochemi-

cal, immunological, and morphological means. Possible invasion of the oral mucosa by treponemes is most likely through intracellular spaces and not through the cells themselves, as shown *in vitro* for oral spirochetes sharing pathogen-restricted antigens with *T. pallidum* (Riviere *et al.*, 1991), and in *B. burgdorferi* (Ellen *et al.*, 1994a). In addition to their adherence capacity and high motility, *T. denticola*, like certain other periodontal pathogens, possess proteolytic enzymes that can degrade basement membrane proteins, thus aiding the bacteria in penetrating the mucosal barrier.

(4) Specific Bacterial Products

Several bacterial factors, manifesting specific cytotoxic effects, were demonstrated in *T. denticola*. One of these components, the 53-kDa major surface protein (Msp), was extensively studied in terms of its chemical and molecular structure and biological activities (Weinberg and Holt, 1991; Haapasalo *et al.*, 1992; Fenno *et al.*, 1996). The outer sheath protein was observed in an oligomeric form (Umemoto *et al.*, 1989; Fenno *et al.*, 1996). An additional well-characterized bacterial cytotoxic product is the chymotrypsin-like protease (CTLP) (Uitto *et al.*, 1995; Fenno *et al.*, 1998a). Interactions between Msp and CTLP in transport or assembly of the outer membrane complex were proposed (Fenno *et al.*, 1998b) following the construction of allelic replacement mutations at two sites in the Msp gene, and at one site in the CTLP locus. All mutant strains lacked the Msp hexagonal array outer membrane ultrastructure.

Both native and recombinant *T. denticola* major surface protein and the surface-expressed chymotrypsin-like protease complex were shown to adhere to, and gave indication of being cytotoxic toward, epithelial cells (Fenno *et al.*, 1997, 1998b). Electron microscope observations revealed that the Msp and CTLP were closely associated in the spirochete outer membranes. The multiple form of the CTLP-like activity (described below) (Rosen *et al.*, 1999a) also seems to be protein complexes formed by Msp and CTLP. Msp complex (partially purified outer membranes free of protease activity) was cytotoxic toward a variety of different cell types and caused lysis of erythrocytes. Pore-forming activities of recombinant Msp in black lipid model membrane assays and in HeLa cell membranes were similar to those reported for the native protein, supporting the hypothesis that Msp cytotoxicity was due to its pore-forming activity.

The cellular location and topology of the *T. denticola* polypeptide was recently investigated by ultrastructural methods, in light of the newly discovered fact that the *T. pallidum* genome encodes 12 orthologs of the *T. denticola* Msp (Caimano *et al.*, 1999), and the possibility that *T. pallidum* Msp orthologs [Tpr] perform analogous related functions. Freeze-fracture electron microscopy revealed that the *T. denticola* outer membrane contains heterogeneous transmembrane proteins but no array. In contrast, a lattice-like structure was observed in vesicles released from mildly sonicated treponemes (discussed later); combined EM and biochemical analyses demonstrated that this structure was the peptidoglycan sacculus. Immunoelectron microscopy identified substantial amounts of Msp in sonicated organisms. Intact treponemes exhibited that most of the antigen was unassociated with the outer membrane. In addition, immunofluorescence analysis of treponemes revealed that only minor portions of Msp are surface-exposed. These findings contradict the notion that Msp forms an array within the *T. denticola* outer membrane and suggest that Msp is predominantly periplasmic, with only limited sur-

face exposure. The possibility that CTLP might mediate adherence of *T. denticola* to epithelial cells is further supported by the fact that the protease inhibitor anti-CTLP IgG inhibited the attachment of *T. denticola* to these cells (Leung *et al.*, 1996). The enzymatic activity of the chymotrypsin-like protease of *T. denticola* was studied by several groups (Uitto *et al.*, 1988; Rosen *et al.*, 1994, 1995; Mäkinen KK *et al.*, 1995; Ishihara *et al.*, 1996; Fenno *et al.*, 1998a). It was found to hydrolyze transferrin, fibrinogen, alpha 1-antitrypsin, IgA, IgG, gelatin, bovine serum albumin collagen IV, fibronectin, and a synthetic peptide containing phenylalanine, as well as several additional host protease inhibitors (Grenier *et al.*, 1990; Rosen *et al.*, 1995; Grenier, 1996b; Uitto *et al.*, 1988), but did not degrade collagen I or synthetic substrates containing arginine or proline. The gene, *prtP*, coding for the prolyl-phenylalanine-specific protease of *T. denticola*, was recently cloned and sequenced (Ishihara *et al.*, 1996).

N-terminal amino acid sequences were determined for the 43- and 72-kDa proteins, and the genes coding for these proteins were isolated and sequenced. The protein appeared to be composed of a signal peptide region followed by a prosequence and the mature protein domain. The deduced amino acid sequence exhibited similarity with that of the *Bacillus subtilis* serine protease subtilisin, a chymotrypsin-like protease.

The outer cell envelope, or the periplasmic space of *T. denticola*, contains several additional novel proteinases and peptidases which may contribute to the tissue damage that accompanies periodontal diseases. These outer membrane-associated or secreted enzymes of *T. denticola* have been assumed to mediate cytopathic effects and are considered to be significant pathogenic factors in periodontal diseases. Several proteases or peptidases [a trypsin-like activity, a proline aminopeptidase, a proline iminopeptidase, and an endopeptidase (FLAG-PA)] of *T. denticola* have been described, and their pathogenic effects have been characterized (Ohta *et al.*, 1986; Mäkinen KK *et al.*, 1992, 1995, 1996; Mäkinen PL *et al.*, 1994, 1995; Rosen *et al.*, 1994, 1995, 1999a; Mäkinen KK and Mäkinen, 1996; Fenno *et al.*, 1997, 1998a,b; Mäkinen PL and Mäkinen, 1997; Mikx, 1997; Ishihara *et al.*, 1998). Examination of the enzyme profiles of 20 treponemal strains (Mikx, 1991)—including the oral species *T. denticola*, *T. vincentii*, and *T. pectinovorum*—revealed more exo- and endopeptidase activities in *T. denticola* than in the other species studied. The presence of peptidases in the outer cell structures of the treponemes points to the importance of these enzymes for the nutrition of the bacteria, and the possible peptidolytic processing of host tissue proteins and peptides to gradually smaller molecules by the bacteria.

The effects of pH, redox potential, O₂, and H₂ on the growth and proteolytic activity of *T. denticola* ATCC 33520 was studied in a chemostat at different growth rates (Mikx, 1997).

The phenylalanine peptidase activities seemed to be of greater importance for the growth of *T. denticola* ATCC 33520 than the rather low arginine and proline peptidase activities. The cell mass and proteolytic activity of *T. denticola* are modulated by the growth rate, the pH, and, to a lesser extent, the redox potential and presence of O₂. Stagnation of the exudate-flow influences these factors, and may lead to an increase in the spirochetal population and proteolysis of periodontal tissues. The dominance of the proline iminopeptidase among aminopeptidase activities present in *T. denticola* and the proposed location of the enzyme in the outer cell envelope suggest that it may have an important function in the propagation of damage to the cells within the inflamed human periodontal tis-

sues. The biological role of the proline iminopeptidase may be the termination of the overall peptidolytic cascade. Host tissue proteins and peptides may be first processed and inactivated by other peptidases possibly present within the same sites as the proline iminopeptidase, and then cleaved by the bacterial enzymes (Mäkinen *et al.*, 1996). In addition, a glutamyl-peptidase was found in these bacteria (Mäkinen *et al.*, 1994), as well as several fibrinolytic activities which were identified and characterized following phase partitioning with the non-ionic detergent Triton X-114 (Rosen *et al.*, 1994). The apparent molecular masses of these proteases ranged from 91 to 228 kDa. Zymograms of crude bacterial outer sheaths prepared by repeated freezing and thawing revealed that the proteases may be associated with this subcellular compartment. The proteases displayed substrate specificity toward fibrinogen, were susceptible to sulfhydryl group reagents, and had a pH optimum between 7 and 8. By immunoblotting using specific antibodies (Rosen *et al.*, 1999a), we have further identified the 160-, 190-, and 270-kDa outer sheath proteases of *T. denticola* ATCC 35404 to be multiple forms of the major 91-kDa phenylalanine protease (PAP). The purified PAP completely degraded keratin, but was unable to degrade native actin (Rosen *et al.*, 1999a).

The purified enzyme consisted of 3 polypeptides (38, 43, and 72 kDa), exhibited specificity for peptide bonds containing phenylalanine and proline, and was classified as a serine protease on the basis of inhibition studies. Naturally occurring protease inhibitors such as α 1-antitrypsin and α 1-antichymotrypsin had no effect on enzymatic activity. Multiple forms of the phenylalanine protease were also found in two other *T. denticola* strains studied: ATCC 33520 and the clinical isolate GM-1. Protein, proteolytic, and Western blot analyses with use of antibodies against the PAP and the bacterial major surface protein indicated that the 190- and 270-kDa proteases were protein complexes formed by the Msp and the PAP. The association of the Msp adhesin with a protease capable of degrading host native proteins may benefit the attainment of protein-based nutrients necessary to support the growth of the bacteria. These complexes may also play a role in the structural organization of the *T. denticola* outer sheath. The ability of whole cells of *T. denticola* ATCC 35405 to hydrolyze substance P, bradykinin, and angiotensin I was also examined for assessment of the role of the chymotrypsin-like membrane-associated proteinase in inactivation of bioactive peptides (Mäkinen PL *et al.*, 1995). Substance P was attacked primarily by the chymotrypsin-like proteinase. Bradykinin was cleaved by the endopeptidase (FAL-GPA-peptidase) and the prolyl oligopeptidase (POPase), while angiotensin I was rapidly converted to angiotensin II by the chymotrypsin-like protease, and both angiotensin I and angiotensin II were further hydrolyzed by the POPase. The outer membrane of *T. denticola* may therefore take part in the breakdown and inactivation of bioactive peptides and other peptidolytic catalyses in the periodontium. The enzyme's ability to hydrolyze humoral proteins and protease inhibitors may be involved in bacterial invasiveness and tissue destruction.

The role of the prolyl-phenylalanine-specific serine protease (dentalisin) in the physiology and virulence of *T. denticola* was examined by a recently constructed dentalisin-deficient mutant (Ishihara *et al.*, 1998).

Not only was no chymotrypsin-like protease activity detected in the dentalisin-deficient mutant, but also the high-molecular-mass oligomeric protein characteristic of the outer sheath of the organism was decreased in the mutant. Furthermore, the

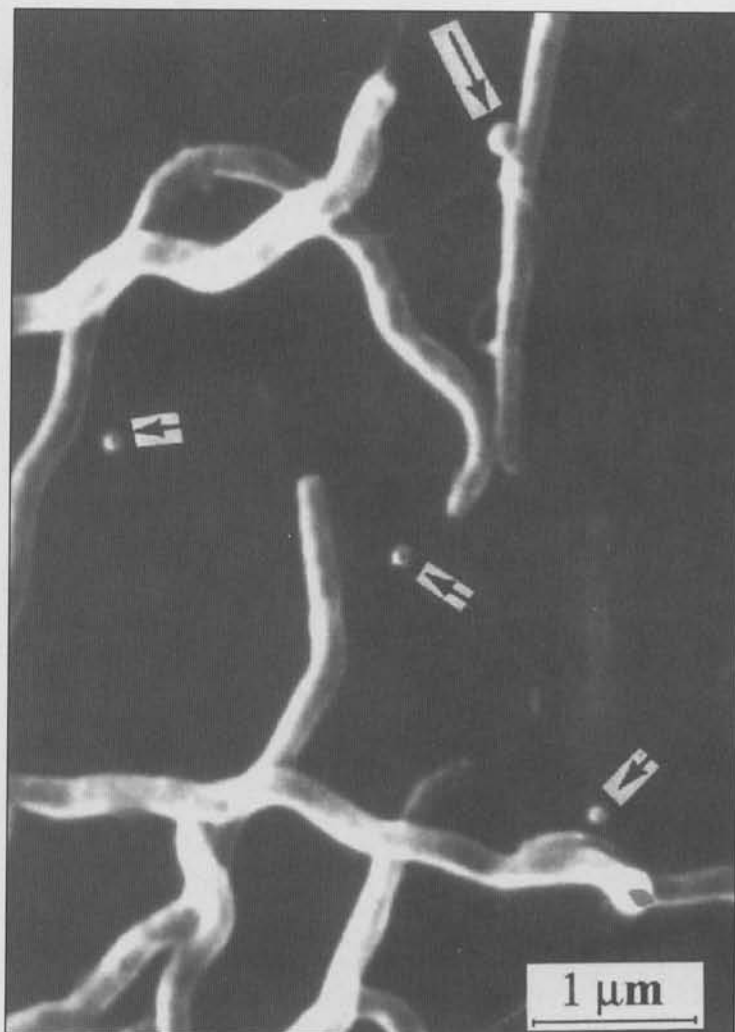


Figure 2. Scanning electron micrograph of *T. denticola* and their extracellular vesicles (\rightarrow).

hydrophobicity of the mutant was reduced and co-aggregation of the mutant with *Fusobacterium nucleatum* was enhanced compared with the wild-type organism. The virulence of the mutant in a mouse abscess model was attenuated in relation to that of the wild-type organism. These results indicate that dentalisin activity plays a major role in the structural organization of the outer sheath of *T. denticola*, while the loss of dentalisin activity and the structural change in the outer sheath affect bacterial pathogenic capacity (Ishihara *et al.*, 1988; Ishihara and Okuda, 1999).

A neutral phosphatase gene, *phoN*, from *T. denticola* ATCC 35405 was isolated from a clone bank constructed in the medium-copy-number plasmid vector (Ishihara and Kuramitsu, 1995).

Enzyme-containing extracellular vesicles (Fig. 2) can be seen bound to the bacterial surface or free in the surrounding medium of *T. denticola* (Rosen *et al.*, 1995). Protein and proteolytic patterns of the vesicles were found to be very similar to those of isolated *T. denticola* outer sheaths. They were enriched with the major outer sheath polypeptides (molecular sizes, 113 to 234 kDa) and with CTLP. A recent study of the enzymatic activity of periodontopathic bacteria claims that *T. denticola* was unable to degrade IgA, IgG, or albumin, while a strain of *P. gingivalis* completely hydrolyzed these proteins (Hollmann and

Van der Hoeven, 1999), suggesting that, in the periodontal pocket, *T. denticola* depends on proteinases from other bacteria for utilization of the available serum proteins. This is in accordance with the clinical data (see above) showing a close relationship between *T. denticola* and strongly proteolytic bacteria, such as *P. gingivalis* and *B. forsythus* (Pederson *et al.*, 1994). Of the anaerobic oral bacteria capable of degrading extracellular matrix proteins, *T. denticola* and *P. gingivalis* have been shown to possess the highest proteolytic activity (Uitto *et al.*, 1988). Both contain several surface membrane proteases that may be released in the bacterial vicinity through extracellular vesicles (Rosen *et al.*, 1995). While the *P. gingivalis* enzymes are cysteine proteinases with specificity toward arginine and lysine, the *T. denticola* outer sheath proteases appear to belong to the serine family. These differences in enzymatic specificity may be of great importance in the mutual co-existence of these two bacteria in the cytopathic events that accompany periodontal diseases. Furthermore, these enzymatic differences may be used in differentiating between the two periodontal pathogens in the periodontal pocket. In addition to the Msp and the enzymes discussed above, *T. denticola* possess several lipoproteins and a lipo-oligosaccharide (LOS) with a molecular mass of 14 kDa (Sela *et al.*, 1997). *T. denticola* ATCC 35404 and the clinical isolate GM-1 were extracted with Triton X-114. The extract was phase-separated and de-lipidated to remove non-covalently-bound lipid (de-lipidated lipoprotein = dLPP). Polyacrylamide electrophoretic separation followed by autoradiography of *T. denticola* ATCC 35404 showed [³H]-cis-9-octadecenoic acid incorporation in bands with apparent molecular masses of 14, 20, 26, 31, 38, 72, and 85 kDa and a broad band running from 113 kDa to the top of the gel. This last band resolved into a 53-kDa [³H]-cis-9-octadecenoic acid band when heated. In the GM-1 strain, [³H]-cis-9-octadecenoic acid incorporation was observed in the 116-kDa and 14-kDa bands. Chemical analysis showed that hexadecanoic acid was the predominant fatty acid bound to *T. denticola*. Comparison of the protein pattern of the detergent phase fraction with that of isolated outer sheaths from *T. denticola* ATCC 35404 (Rosen *et al.*, 1994) showed, in both fractions, high-molecular-weight oligomeric proteins that seemed to be enriched by different polypeptides. When either the Triton X 114 phase or the outer sheaths were heated, a major 53-kDa protein appeared in both cases. The same methodology was used to separate the major outer sheath protein from the 95-kDa protease (Fenno *et al.*, 1998a).

While Msp was found mainly in the aqueous phase, the protease partitioned in the detergent phase (Rosen *et al.*, 1994; Fenno *et al.*, 1998a). It is evident from these results that the high-molecular-weight [³H]-cis 9-octadecenoic acid-labeled proteins found in the Triton X-114 phase of strain ATCC 35404 are different from the Msp (which segregated to the aqueous phase). Furthermore, sequence analysis of the Msp had a typical prokaryotic signal sequence with a potential cleavage site for signal peptidase I (Fenno *et al.*, 1996). On the other hand, nucleotide sequence analysis of the DNA fragment encoding for the *T. denticola* Johnson 53-kDa protein revealed a signal peptide that was homologous to that of bacterial lipoproteins (Miyamoto *et al.*, 1991). A probe (tdpA) encoding for the 53-kDa polypeptide hybridizes intensively with DNA obtained from several *T. denticola* strains, including ATCC 35404, 35405, and 33520 (MacDougall and Saint Girons, 1995). Taken together, these findings suggest that more than one class of 53-kDa proteins exists in the bacteria. The effects of *T. denticola* lipoproteins

on host immune cells, *e.g.*, PMNs and macrophages, in relation to other pathogenic spirochetes will be discussed in the next sections. It was recently proposed that the LOS molecule extracted from several strains of *T. denticola* is a new type of outer membrane lipid (Schultz *et al.*, 1998). While the chemical analysis of LOS places this molecule close to lipoteichoic acids of Gram-positive bacteria, its biophysical membrane behavior is similar to that of LPS of Gram-negative bacteria. The presence of lipopolysaccharide seems to be diverse among spirochetal species. The stepladder banding observed in *E. coli* LPS following electrophoresis was found in *T. phagedensis* and *T. vincentii*. Several immunogenic low-molecular-weight LPS bands were reported in two anaerobic spirochetes: the swine enteropathogenic *Serpulina hyodysenteriae* and the non-pathogenic *Serpulina innocens*. While there is sustained evidence for the lack of LPS in *T. pallidum*, its presence in *B. burgdorferi* remains controversial.

Recently, lipopolysaccharide from the outer membrane of *T. pectinovorum* was isolated and chemically defined (Walker *et al.*, 1999b). LPS-like material was suggested to exist in *T. denticola* (Kurimoto *et al.*, 1990; Yotis *et al.*, 1995), based on the capacity of the material to activate the Limulus lysate clotting assay. An LPS-like material isolated from *T. denticola* was reported to react with sera from periodontitis patients (Yotis *et al.*, 1995). Nevertheless, *T. denticola* lacks the repetitive banding typical of electrophoresed Gram-negative bacterial LPS (Sela *et al.*, 1997). The fatty acid bands contained in a Triton X-114 phase following extraction of *T. denticola* (with the exception of the 14 kDa) were lipoproteins that are readily degraded by proteinase K, while phenol-water extraction of *T. denticola* ATCC 35404 rendered a water phase enriched with a [³H]-cis-9-octadecenoic acid-labeled 14-kDa compound that was resistant to proteolytic degradation. The purified band was readily stained by silver, suggesting that it is a lipo-oligosaccharide. Inasmuch as the defining of biological activities and chemical characteristics of *T. denticola* LPS-like material will certainly benefit from further investigation, the delicate balance between the specific chemical content of LPS, such as sugar moieties and lipid specificity, and the biological activities of this important molecule must be considered.

(5) Effect on Host Cells

As noted above, *T. denticola* produce several factors that may contribute to bacterial virulence. These include outer-sheath-associated peptidases, chymotrypsin-like and trypsin-like proteinases, hemolytic and hemagglutinating activities, adhesins that bind to matrix proteins and cells, and an outer sheath protein with pore-forming properties. The effects of *T. denticola* whole cells and their products on a variety of host mucosal and immunological cells are discussed below.

(5.1) FIBROBLASTS

Several strains of *T. denticola* bind to human gingival fibroblasts. Adherence of *T. denticola* is accomplished through their contact with specific plasma membrane molecules of these cells. It has been demonstrated that strains of *T. denticola* may adhere to extracellular proteins (synthesized by fibroblasts), like fibronectin, and also to their plasma membranes (Haapasalo *et al.*, 1991; Dawson and Ellen, 1994). Several cytopathic effects of *T. denticola* on fibroblasts have been shown: inhibition of proliferation (Boehringer *et al.*, 1984); cell detachment due to rapid cytoskeletal remodeling; and rounding of the cells and their shrinkage, as well as the induction of plasma membrane folds

or blebs (Baehni *et al.*, 1992). *T. denticola* 35405 were also shown to degrade endogenous plasma membrane fibronectin upon adhesion to human gingival fibroblasts (Ellen *et al.*, 1994b). More recent studies indicate that *T. denticola* cause re-arrangement of filamentous actin (F-actin) in human gingival fibroblasts (HGF). In addition to its major surface chymotrypsin-like properties, *T. denticola* express cytopathogenic activities that diminish the generation of inositol phosphates associated with cytoskeletal disruption in fibroblasts (Yang *et al.*, 1998). Moreover, non-lipopolysaccharide, non-chymotrypsin, heat-sensitive proteins in the *T. denticola* outer membrane can evidently inhibit both the release of calcium from internal stores and the uptake of calcium through the plasma membrane of gingival fibroblasts, possibly by interference with calcium release-activated channels (Ko *et al.*, 1998). Fibroblasts phagocytose extracellular collagen, as part of the pathway of collagen degradation in the physiological turnover of connective tissues.

Collagen phagocytosis by human gingival fibroblasts is regulated through protein kinase C and is also dependent upon cellular recognition and collagen structure, but not on the expression of collagenase (Knowles *et al.*, 1991).

Since *T. denticola* outer membrane (OM) extract perturbs actin filaments that are important structures in phagocytosis, its effect on collagen phagocytosis *in vitro* by human gingival fibroblasts (HGFs) was studied (Battikhi *et al.*, 1999) and the following findings reported: (1) Confluent HGFs pre-treated with *T. denticola* ATCC 35405 OM exhibited an increase in the percentage of collagen phagocytic cells (phagocytosis index [PI]), and in the number of beads *per* phagocytosing cell (phagocytic capacity [PC]); (2) this enhancement was swift and still evident after 1 day; (3) PI and PC of HGFs for bovine-serum-albumin (BSA)-coated beads were also increased, indicating a global increase in phagocytic processes; and (4) the *T. denticola* OM-induced increase in bead uptake was eliminated by heating of the OM and by depolymerization of actin filaments by cytochalasin D treatment of HGFs. The HGFs exhibited minimal detachment from the substratum. They did not take up propidium iodide, nor was there any change in their size, granularity, or content of sub-G1 DNA. These findings point to the presence of a heat-sensitive component in *T. denticola* OM extract, which subsequently stimulates the epithelial cells to phagocytize collagen and other endocytic processes, such as non-specific phagocytosis and pinocytosis.

(5.2) EPITHELIAL CELLS

Gingival epithelial cells act as the first line of defense of the periodontal mucosa. As indicated previously, cells of *T. denticola* are in close contact with the gingival epithelium, with some strains of the bacteria found readily adhering to different types of epithelial cells (Keulers *et al.*, 1993a,b; Thomas, 1996). Washed cells of *T. denticola* and their culture supernatants induced morphological damage and detachment of guinea pig ear epithelial cells to which the bacteria adhere (Reijntjens *et al.*, 1986).

Live *T. denticola* cells were shown to cause large intracellular vacuoles in spare cultures of migrating porcine periodontal ligament epithelial cells (Uitto *et al.*, 1995). Damaged treponemes were found inside membrane-bound vesicles; blebbing of membranes was also induced by the bacterial chymotrypsin-like proteinase. The whole bacteria and the protease caused the disorganization of cortical actin and actinin as well as degradation of pericellular fibronectin. Human KB epithelial cells (derived from epidermoid carcinoma) under-

went cell detachment, loss of cellular volume regulation associated with cytoskeletal protein disruption, and death upon contact with *T. denticola* (ATCC 35405) (De Filippo *et al.*, 1995). The peptidoglycan of the same strain of *T. denticola* was found to be highly toxic to porcine periodontal ligament epithelial cells (Grenier and Uitto, 1993). The 53-kDa major surface protein located in the outer membrane of *T. denticola* serovar a (ATCC 35405), free of the chymotrypsin-like proteinase, has both pore-forming activity and adhesin activity on HeLa cells (Mathers *et al.*, 1996). The fact that Msp binds both a putative epithelial cell-surface receptor and cytoplasmic proteins, and can form large conductance ion channels in the cytoplasmic membrane of epithelial cells, may contribute to the cytopathic effects of *T. denticola* on host epithelial cells. The ability of *T. denticola* and their products to adhere and cause damage to epithelial cells may initiate a sequence of events that may lead to the bacterial invasion of the deeper layers of the periodontal connective tissue, as observed in human and animal studies.

(5.3) LEUKOCYTES

Polymorphonuclear neutrophils (PMNs) are by far the dominant host-derived immune system cells in the gingival crevice and periodontal pocket. These phagocytes infiltrate through the gingival epithelium into the gingival crevicular fluid and protect the subgingival tissues from bacteria by various anti-bacterial mechanisms. The phagocytosis of *T. denticola* by human peripheral blood PMNs *in vitro* has been demonstrated (Lingaas *et al.*, 1983; Ding *et al.*, 1996, 1997).

The inhibition of superoxide production by human peripheral blood PMNs in the presence of oral treponemes, their cellular components, and their culture supernatants was investigated (Sela *et al.*, 1987b, 1997) and the following findings reported: (1) Superoxide production was inhibited 56% by a phenol extract of a human clinical isolate of *T. denticola*; (2) inhibition by culture supernatants of both the clinical isolate and a reference strain (ATCC 35404) was related to the bacterial phase of growth and viability, though inhibition also persisted in the decline phase; and (3) inhibition of superoxide production was not evident when either opsonized or non-opsonized whole spirochetes were reacted with PMNs. Therefore, the suppressive activity depended on the treponemes either being disrupted, or growing and releasing the inhibitory factor into the culture medium. It follows further that *T. denticola* possess factors which interfere with the activity of PMNs, and thereby may alter the inflammatory process in the diseased periodontal pocket. De-lipidated lipoprotein (dLPP) from strain ATCC 35404 caused an enhanced luminol-dependent chemiluminescence (LDCL) effect in human PMNs which could be related to protein concentration. The purified dLPP also induced lysozyme release by the PMNs by approximately 30% as compared with the release induced by FMLP. The release of beta-glucuronidase was not affected. The modulation of neutrophil activity was abolished by pre-incubation of dLPP with proteinase K. The fact that *T. denticola* possess several lipoproteins, including outer sheath oligomeric polypeptides and a lipo-oligosaccharide that may modulate immunologic cell functions, is of special importance in light of recent data on the role played by lipoproteins of *T. pallidum* and *B. burgdorferi* in the persistence of chronic inflammatory diseases. These bacteria, the etiological agents of syphilis and Lyme disease, possess lipoproteins as an inte-

gral part of their membranes. These bacterial factors were shown both to be potent immunomodulators *in vivo* and *in vitro*, and to be able to activate monocytes, macrophages, B-lymphocytes, and endothelial cells (Radolf *et al.*, 1988, 1991, 1994; Ma *et al.*, 1994). The fact that an enriched lipoprotein fraction from *T. denticola* modulates oxygen-dependent and -independent defense mechanisms against micro-organisms by human PMNs merits significant attention, in light of the presence of these bacteria and cells close to each other in the subgingival area. Further studies are needed to decipher the exact role of *T. denticola* lipoproteins and lipo-oligosaccharides in cell and tissue destruction.

Another aspect involved the study of the effects of *T. denticola* on degranulation of matrix metalloproteinases by human peripheral blood PMNs (Ding *et al.*, 1996, 1997). *T. denticola* 53-kDa outer membrane protein was found to act as an efficient inducer of matrix metalloproteinases. Both collagenase and gelatinase released from the PMNs were mostly in active forms. Release of cathepsin G and elastase was also observed with the 53-kDa-protein treatment. Lactate dehydrogenase was not released from PMNs by this treatment, indicating that the degranulation was specific and not caused by toxic effects of the bacterial protein. Electron microscopic examination of PMNs treated with the 53-kDa protein showed rapid vacuole formation and cell shape changes but no disintegration of the cells. Thus, *T. denticola* may participate in PMN-dependent extracellular matrix degradation in the periodontal pocket, by triggering the secretion and activation of matrix metalloproteinases.

(5.4) LYMPHOCYTES

It has been proposed that impaired host defense may play a role in the pathogenesis of periodontal diseases. The possible effect of periodontopathic bacteria on immunosuppression may alter the interactions between the host's local defense mechanisms and the bacteria. Lymphocyte blastogenic responses of patients with moderate to advanced periodontitis and of orally healthy individuals to different spirochetes, including isolates of *T. denticola*, were assayed (Mangan *et al.*, 1982).

The blastogenic responses to the spirochetes were not statistically different between patients and controls. On the other hand, sonicates of several strains of *T. denticola* caused a dose-dependent inhibition of human lymphocyte responsiveness to Con A, PHA, PWM, and a recall antigen (Shenker *et al.*, 1984). Suppression involved alterations in DNA, RNA, and protein synthesis, with no effect on cell viability, and was dependent on the presence of adherent monocytes; removal of these cells prevented spirochete-induced suppression of lymphocyte proliferation. Furthermore, the combination of indomethacin and catalase was able to reverse the inhibitory effects of the spirochete extracts, demonstrating a requirement for both prostaglandins and hydrogen peroxide. The outer membrane (OM) from *T. denticola* ATCC 35405, ATCC 33521, and ATCC 35404, representing serovars a, b, and c, respectively, as well as from two fresh isolates of *T. denticola*, was active in the mitogenic stimulation of mouse spleen cultures, but to a somewhat lesser extent than purified lipopolysaccharide (LPS) from *E. coli* (Schade *et al.*, 1998). Polymyxin B inhibited the response only partially. Unheated OM abrogated mitogenic activity of *E. coli* LPS, but heated preparations enhanced the mitogenic activity of *E. coli* LPS, suggesting the presence of a heat-labile cytolytic factor associated with *T. denticola* OM in addition to a

putative lipopolysaccharide and/or heat-stable lipoprotein.

T. denticola may have a pro-inflammatory role in periodontal diseases, since it can cleave pro-IL-1 β to yield two fragments with molecular masses of 18 and 19 kDa (Beausejour *et al.*, 1997). Cleavage products showed a dose-dependent biological activity in a thymocyte proliferation bioassay, an activity that was inhibited by anti-IL-1 β neutralizing antibodies. The presence of *T. denticola* and their proteases in periodontal sites may therefore play an additional important role in the inflammatory nature of periodontal diseases by activating pro-IL-1 β .

(5.5) ERYTHROCYTES

Hemolysin is considered a potent virulence factor in many Gram-positive and Gram-negative pathogenic bacteria. Production of hemolysin by periodontal pathogens such as *T. denticola* and *P. gingivalis* provides these organisms with heme-containing molecules necessary for their growth. *T. denticola* were shown to agglutinate and lyse human red blood cells. Both activities were cell-associated, heat-labile, and produced during the exponential growth phase of the bacteria. Hemagglutination was reduced in the presence of D-glucosamine, EDTA, or sodium salicylate, whereas hemolytic activity was affected by calcium chloride, magnesium chloride, EDTA, or sodium salicylate. These biological activities may favor a higher concentration of hemin-containing compounds in periodontal sites, and therefore represent potential bacterial virulence mechanisms (Grenier, 1991). The *T. denticola* cell-bound and growth-phase-related hemagglutinating activity was further found to act on erythrocytes of human, equine, bovine, and rabbit origin. The agglutinin was suggested to be a glycoprotein, like lectin, that recognizes sialic acid as a receptor (Mikx and Keulers, 1992).

Investigators isolated two hemolysin genes from *T. denticola* strains ATCC 35404 and GM-1 (Karunakaran and Holt, 1994) by screening genomic DNA libraries of *T. denticola* on sheep blood agar plates, pointing to the fact that the two examined strains possess identical hemolysin genes. A 46-kDa protein (Cystalysin), isolated from the cytosol of *T. denticola*, was capable of both cysteine-dependent hemoxidation and of hemolysis of human and sheep red blood cells (Chu and Holt, 1994; Chu *et al.*, 1994a,b, 1995, 1997, 1999a,b). The activities were found to be associated with the red blood cell membrane and were characteristic of a cysteine desulfhydrase. Substrates for the enzyme enhanced its interaction with whole red blood cells as well as with isolated and purified red blood cell ghosts.

These findings suggest that the interaction of cystalysin with the red blood cell membrane results in the chemical oxidation of the hemoglobin molecule, as well as in an alteration in the red blood cell membrane itself. This 46-kDa hemolytic protein from *T. denticola* ATCC 35404 was overexpressed in *E. coli* (Chu *et al.*, 1995). Both the native and recombinant 46-kDa proteins were purified to homogeneity, and showed identical biological and functional characteristics.

In addition to its biological function of lysing erythrocytes and hemoxidizing hemoglobin to methemoglobin, cystalysin was also capable of removing the sulfhydryl and amino groups from selected S-containing compounds (*e.g.*, cysteine), producing H₂S, NH₃, and pyruvate (Chu *et al.*, 1999b). Moreover, the same group of researchers demonstrated recently that cystalysin acts as an unregulated L-cysteine catabolizing enzyme, with the resulting H₂S production being essential for this atypical hemolytic activity (Kurzban *et al.*, 1999). It is hypothesized

that since large amounts of H₂S have been found in deep periodontal pockets, cystalysin may also function *in vivo* as an important virulence molecule (Chu *et al.*, 1999b). The inflamed periodontal tissues have a tendency to bleed almost spontaneously, leading to the presence of vast amounts of erythrocytes, which may undergo hemolysis by periodontal bacteria, thereby providing the latter with essential nutrients.

(5.6) MACROPHAGES

The enriched lipoprotein fraction (dLPP) from *T. denticola* ATCC 35404 was found to stimulate the production of nitric oxide (NO), tumor necrosis factor alpha (TNF- α), and interleukin-1 (IL-1) by mouse macrophages in a dose-dependent manner (Rosen *et al.*, 1999b). dLPP-mediated macrophage activation was unaffected by amounts of polymyxin B that neutralized the induction produced by bacterial LPS. dLPP also induced NO and TNF- α secretion from macrophages isolated from endotoxin-unresponsive mice, to an extent similar to the stimulation produced in endotoxin-responsive mice. Purified *T. denticola* lipo-oligosaccharide (LOS) also produced a concentration-dependent activation of NO and TNF- α in LPS-responsive and non-responsive mouse macrophages. However, macrophage activation by LOS was inhibited by polymyxin B. One should bear in mind that the host response, which is essentially aimed at protection from bacterial damage, may also take part in periodontal tissue destruction. TNF- α and IL-1 β , which induce bone resorption, were found in high levels in gingival tissues and crevicular fluid of patients with periodontal disease, and their presence was correlated with disease activity (Stashenko *et al.*, 1991).

Summary and Future Directions

The bulk of information concerning the adherence, motility, invasion, and cytopathic effects of *T. denticola* (summarized in Fig. 1) is impressive. These bacteria, together with other Gram-negative anaerobes, especially *P. gingivalis*, clearly possess the potential armaments needed for at least part of the cellular and tissue damage seen during the development of periodontal disease. Part of the avenues of studies currently under way with regard to *T. denticola* pathogenicity may soon lead to a better understanding of the molecular basis of the interrelationships between these bacteria and the periodontal apparatus. Increased appreciation is already emerging through mutant construction and genetic transformation, as well as *via* investigation of the interactions between bacterial proteins and periodontal cell surfaces on a molecular level. The studies showing pore-forming activity by *T. denticola* Msp, and induction of large conductance ion channels in mucosal cells by the Msp complex, together with the binding of the Msp complex to a cell-surface receptor, are of strategic significance, since bacterial interaction with the cells may lead to cell membrane permeabilization and cell death.

Nonetheless, the place of Msp/Tpr orthologs in treponemal physiology and disease pathogenesis was recently challenged in light of the finding that Msp is predominantly periplasmic, with only limited surface exposure. *T. denticola* membrane proteins and proteolytic enzymes can also be found in the bacterial vicinity and in bacterial extracellular vesicles. The fact that *T. denticola* and their proteases, present in periodontal sites, can cleave pro-IL-1 to yield two fragments is very intriguing, and may suggest that *T. denticola* have a pro-inflammatory role in periodontal diseases. Although the question of active bacterial invasion into the

periodontal connective tissue still remains open for exploration and confirmation, the high motility of *T. denticola* together with their ability to release highly proteolytic vesicles may provide these bacteria with an effective mechanism of penetration through tissues and tissue damage. The presence of bacterial extensions in the form of outer sheath vesicles that contain at least part of the bacterial noxious products may have an additional harmful effect on the periodontal tissues and therefore requires attention in future studies. Since many of the spirochetal morphotypes observed in periodontitis patients are as yet uncultivable, effort has to be put into the assessment of these unknown bacteria in relation to the propagation of periodontal diseases.

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