

S.G. Dashper, C.A. Seers, K.H. Tan,
and E.C. Reynolds*

Cooperative Research Centre for Oral Health, Melbourne Dental School and Bio21 Institute, The University of Melbourne, 720 Swanston Street, Victoria, 3010, Australia; *corresponding author, e.reynolds@unimelb.edu.au

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ABSTRACT

There is compelling evidence that treponemes are involved in the etiology of several chronic diseases, including chronic periodontitis as well as other forms of periodontal disease. There are interesting parallels with other chronic diseases caused by treponemes that may indicate similar virulence characteristics. Chronic periodontitis is a polymicrobial disease, and recent animal studies indicate that co-infection of *Treponema denticola* with other periodontal pathogens can enhance alveolar bone resorption. The bacterium has a suite of molecular determinants that could enable it to cause tissue damage and subvert the host immune response. In addition to this, it has several non-classic virulence determinants that enable it to interact with other pathogenic bacteria and the host in ways that are likely to promote disease progression. Recent advances, especially in molecular-based methodologies, have greatly improved our knowledge of this bacterium and its role in disease.

KEY WORDS: chronic periodontitis, polymicrobial biofilms, immunomodulation, outer sheath vesicles.

Virulence Factors of the Oral Spirochete *Treponema denticola*

THE TREPONEMES AND DISEASE

Oral treponemes, along with over 600 other bacterial species, exist as part of a polymicrobial biofilm accreted to the tooth surface in the gingival crevice (Kolenbrander *et al.*, 2002; Ellen and Galimanas, 2005; Dewhirst *et al.*, 2010). Treponemes play a role in the etiology of several chronic diseases of humans including syphilis and yaws (*Treponema pallidum*), periodontal diseases including chronic periodontitis and acute necrotizing ulcerative gingivitis (*Treponema denticola*, *Treponema lecithinolyticum*, *Treponema socranskii*, and others), and endodontic infections and some acute dental abscesses (Sela, 2001; Foschi *et al.*, 2006; Holt and Ebersole, 2006; Robertson and Smith, 2009). In addition, treponemes have been implicated in the development of chronic diseases of domestic animals, including periodontal diseases of dogs (Nordhoff *et al.*, 2008b), bovine digital dermatitis of dairy cattle (Choi *et al.*, 1997; Klitgaard *et al.*, 2008; Nordhoff *et al.*, 2008a), and contagious ovine digital dermatitis (Dhawi *et al.*, 2005; Sayers *et al.*, 2009).

Treponemes are members of the *Spirochaetes* phylum, a clade now believed to be distinct from both Gram-positive and Gram-negative bacteria, that is believed to have undergone extensive horizontal gene transfer with Archae and possibly with eukaryotic organisms (Ibba *et al.*, 1997; Brown *et al.*, 1998, 2001; Bond and Francklyn, 2000; Wolf *et al.*, 2001; Henz *et al.*, 2005; Paster and Dewhirst, 2006). It has been proposed that all treponemes descended from a common spirochetal ancestor and, as a taxon, have evolved a range of unique characteristics, including virulence determinants, metabolic pathways, solute transport systems, and surface-binding proteins due to their evolutionary trajectory (Paster and Dewhirst, 2000; Seshadri *et al.*, 2004). However, this hypothesis remains to be comprehensively tested. The sequencing of the complete genome of *T. pallidum* in 1998, *T. denticola* ATCC 35405 in 2004, and the recent release of the *T. lecithinolyticum* OMZ684T and *Treponema vincentii* ATCC 35580 sequences have already proven invaluable to treponeme research and will continue to illuminate the evolution and virulence characteristics of these species, especially as other genome sequences become available (Fraser *et al.*, 1998; Seshadri *et al.*, 2004; Dewhirst *et al.*, 2010; <http://www.jcvi.org/>, www.homd.org/). Currently, genomic comparisons with other treponemes and spirochetes are facilitating a more targeted selection of potential *T. denticola* virulence factors for detailed investigation.

The numbers of both cultivated and uncultivated *Treponema* phylotypes reported in recent years have rapidly increased, mainly due to the mass of data obtained from efforts to sequence genes encoding 16S rRNA (Choi *et al.*, 1994; Wyss *et al.*, 2004; Demirkan *et al.*, 2006; Molbak *et al.*, 2006; Nordhoff *et al.*, 2008a; Pringle *et al.*, 2008, 2009; Evans *et al.*, 2009; Sayers *et al.*, 2009; Yano *et al.*, 2009). There are currently 49 species of oral *Treponema* listed on the Human Oral Microbiome Database (Dewhirst *et al.*, 2010), the best-characterized being *T. denticola*, *Treponema amylovorum*, *T. lecithinolyticum*, *Treponema maltophilum*, *Treponema medium*, *Treponema parvum*,

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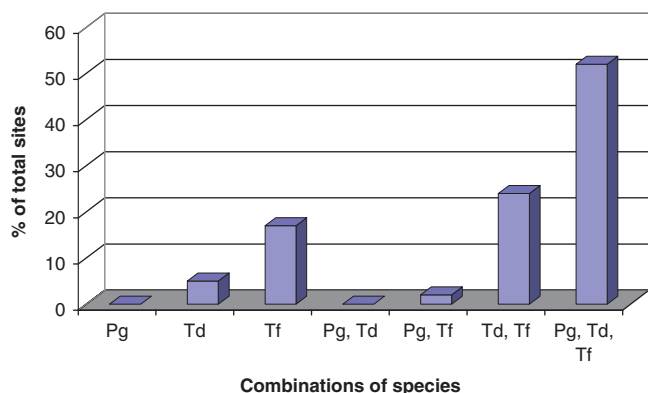


Figure 1. Combinations in which *P. gingivalis*, *T. denticola*, and *T. forsythia* were found in subgingival plaque that had quantifiable levels of at least one of these species, by real-time PCR, in 41 sites of 37 individuals previously diagnosed with chronic periodontitis. The majority of sites were colonized by all three species, and *P. gingivalis* was rarely detected without the presence of *T. denticola* and *T. forsythia*. The individuals had completed initial treatment and been on a maintenance program for a minimum of six months in the Specialist Periodontics Department of the Royal Melbourne Dental Hospital, Australia (Byrne *et al.*, 2009).

Treponema pectinovorum, *T. socranskii*, and *T. vincentii* (www.homd.org/).

Within the oral cavity, treponemes are most often associated with diseases of the periodontium. However, these treponemes are members of the normal oral microbiota of healthy individuals, albeit in very low numbers, and even those associated with disease cannot be considered frank pathogens. In this article, we will concentrate on the virulence characteristics of oral treponemes, and particularly *T. denticola*, in relation to chronic periodontitis. When considering the virulence characteristics of *T. denticola*, it is imperative to understand that it is part of a pathogenic bacterial consortium, and its interactions with other bacterial species are important for disease pathology. Therefore, the relationship between *T. denticola* and other bacteria involved in disease progression will also be addressed.

POLYMICROBIAL NATURE OF CHRONIC PERIODONTITIS

Chronic periodontitis is a polymicrobial disease that results from the overgrowth of a limited number of bacterial species that are normal members of the oral microbiota. It is widely accepted that *T. denticola*, *Porphyromonas gingivalis*, and *Tannerella forsythia* form a bacterial consortium, often referred to as the ‘Red Complex’, that is strongly associated with the clinical progression of chronic periodontitis (Lamont and Jenkinson, 1998; Socransky *et al.*, 1998; Holt and Ebersole, 2005). The levels of *P. gingivalis* and *T. denticola* in subgingival plaque have been shown to allow for prediction of periodontal breakdown in a longitudinal clinical trial (Byrne *et al.*, 2009). The unifying features of the Red Complex bacteria are their extracellular proteolytic activity, their complex anaerobic fermentations of amino acids, production of

toxic metabolites, and outer membrane (or sheath) vesicles. Of the three species, only the treponeme is motile and able to respond chemotactically to environmental stimuli. The *in vivo* interactions of these species are still poorly characterized, but some studies have indicated that *P. gingivalis* may be needed for *T. denticola* colonization and presence in subgingival plaque (Simonson *et al.*, 1992; Sela, 2001). A recent study of the bacterial composition of subgingival plaque in individuals with chronic periodontitis (Byrne *et al.*, 2009) showed that *P. gingivalis* and *T. denticola* and *T. forsythia* were routinely found together in subgingival plaque (Fig. 1). Interestingly, *P. gingivalis* or *T. denticola* were rarely found in subgingival plaque without *T. forsythia* (Fig. 1). Mineoka *et al.* (2008) have recently shown a similar relationship between *T. forsythia* and *P. gingivalis*. *T. forsythia* has also been found to be more prevalent than *P. gingivalis* in subgingival plaque (Haffajee *et al.*, 2006; Colombo *et al.*, 2009). This may suggest that *T. forsythia* colonizes plaque before *P. gingivalis* and *T. denticola*. Recently, Zijngje *et al.* (2010) used fluorescent *in situ* hybridization to show that *Tannerella sp.* were located in the intermediate layer of subgingival plaque, whereas *P. gingivalis* tended to be found in micro-colonies in the top layer, and *Treponemes* were found outside the top layer. These results are consistent with the proposal that *T. forsythia* may be a necessary precursor species for colonization by *T. denticola* and *P. gingivalis*. The interactions within the Red Complex species and with other newly identified species and the timing of colonization and proliferation clearly need further study.

DETERMINATION OF VIRULENCE - ANIMAL MODELS OF DISEASE

Virulence is defined as the capacity of a pathogen, usually a micro-organism, to cause disease. For the virulence factors of *T. denticola* to be determined, the role of this bacterium in disease must be clearly defined, and in recent times, animal models have been developed to examine this role. The animal model of disease allows key elements of the disease process to be investigated under the complex conditions found *in vivo*; however, the initiation of “disease” in animal models is, by necessity, induced and, of course, differs from natural pathogenesis in humans (Arnett and Viney, 2007). Animal models of chronic inflammatory diseases are problematic (Radbruch and Isaacs, 2009), especially for those diseases such as chronic periodontitis that are caused by an overgrowth of members of the normal microbiota. Despite these limitations, animal models of disease have proven invaluable for determining the pathogenicity of bacteria and providing insight into their potential virulence factors. Murine subcutaneous abscess models of disease have been used to investigate the virulence of *T. denticola* and other oral treponemes and the pathogenicity of polymicrobial infections (Kesavalu *et al.*, 1997; Gemmell *et al.*, 2002). As a subcutaneous mono-infection *T. denticola*, *T. pectinovorum*, or *T. vincentii* produced localized abscesses (Kesavalu *et al.*, 1997, 1998). In comparison, at similar inoculum sizes, *P. gingivalis* produced more severe, spreading ulcerative lesions remote from the site of infection (Kesavalu *et al.*, 1998; O’Brien-Simpson *et al.*, 2000). At high doses of *P. gingivalis* in the subcutaneous abscess

model, addition of *T. denticola* had no effect on the formation and size of the spreading lesion; however, at low doses of *P. gingivalis*, *T. denticola* co-infection significantly enhanced tissue damage (Kesavalu *et al.*, 1998). Analysis of these data indicates not only that the presence of *P. gingivalis* was needed for invasion and extensive tissue damage, but also that *T. denticola* greatly facilitated this process.

Although broadly predictive of disease, the relevance of these abscess models to chronic periodontitis has been questioned, and more appropriate models have been developed. In recent years, murine alveolar (periodontal) bone loss models have been adapted to test the virulence of *T. denticola* and polymicrobial infections. These studies reported that intra-oral inoculations with *T. denticola* resulted in colonization of the oral cavity of the animal, induction of a specific immune response, and significant alveolar bone loss (Kesavalu *et al.*, 2007; SF Lee *et al.*, 2009). Furthermore, a polymicrobial intra-oral inoculation of *P. gingivalis*, *T. denticola*, and *T. forsythia* at a 1:1:1 cell ratio with the same total number of bacterial cells caused significantly higher levels of bone resorption than those caused by mono-inoculations (Kesavalu *et al.*, 2007). In a recent study using a murine model of periodontal disease (O'Brien-Simpson *et al.*, 2005; Pathirana *et al.*, 2007), intra-oral inoculation with a 1:1 ratio of *T. denticola*:*P. gingivalis* cells caused the same level of alveolar bone loss as intra-oral inoculation with a 40-fold greater number of *P. gingivalis* cells inoculated alone (unpublished data). Analysis of these data together suggests a significant role of *T. denticola* in the tissue damage associated with chronic periodontitis, and demonstrates the importance of a polymicrobial infection for the initiation and progression of disease. The continuing development of these models will allow for the testing of *T. denticola* mutants lacking specific gene products, which will greatly improve our knowledge of *T. denticola* virulence factors.

INTERBACTERIAL BINDING AND LOCALIZATION OF *T. DENTICOLA* WITH OTHER SPECIES

T. denticola exists as part of a complex, structured multispecies biofilm (subgingival plaque) in the relatively protected region of the gingival crevice or periodontal pocket, which is largely protected from the shear forces associated with salivary flow and the effects of mastication. *T. denticola* tends to inhabit the deeper periodontal pockets and is not an early colonizer of subgingival plaque (Kolenbrander *et al.*, 2002). Treponemes, including *T. denticola*, have been reported to be located on the surface of the dense subgingival bacterial biofilms, at the interface of the biofilms and the gingival epithelium (Kigure *et al.*, 1995; Ellen and Galimanas, 2005; Zijng *et al.*, 2010).

Interspecies bacterial binding plays a vital role in biofilm development and in the co-location of bacterial species. *T. denticola* binds weakly to fusobacteria in an interaction mediated by the carbohydrate moiety of the *T. denticola* major sheath protein (Msp, see below) and the galactose-binding lectin receptor of the fusobacteria (Kolenbrander *et al.*, 1995; Rosen *et al.*, 2008; Kaplan *et al.*, 2009). *T. denticola* also binds to the commensal, early-colonizing *Streptococcus crista* (Yao *et al.*, 1996). These

interactions may be important for *T. denticola* to initially colonize sites and persist during health. *T. denticola* has been shown to bind specifically to both *P. gingivalis* and *T. forsythia* with similar avidity, which may explain the close association of the species (Fig. 2a), and these interactions are mediated to some extent by *T. denticola* dentilisin or Msp (see below). However, co-aggregation of the bacteria was not reduced in *T. denticola* dentilisin- or Msp-deficient mutants, and pre-incubation of *T. denticola* with *P. gingivalis* fimbriae did not prevent its ability to co-aggregate with *P. gingivalis* (Yao *et al.*, 1996; Ishihara *et al.*, 1998; Hashimoto *et al.*, 2003b; Rosen *et al.*, 2008). This indicates that this interaction is multimodal, involving different proteins and possibly carbohydrate moieties. This close association of species will enhance the repertoire of proteolytic and other enzymes available to each species.

LEUCINE-RICH REPEAT PROTEINS

A *T. denticola* leucine-rich repeat protein (LrrA) has recently been shown to play a role in binding to *T. forsythia*, but not to *P. gingivalis* or *F. nucleatum*, and to mediate binding to epithelial cells and to promote swarming (Ikegami *et al.*, 2004; Rosen *et al.*, 2008). Interestingly, Lrr proteins have been shown to be important for epithelial cell invasion and biofilm formation by *P. gingivalis*. They are also important for epithelial cell invasion and virulence in a mouse alveolar bone loss model by *T. forsythia* (Sharma *et al.*, 1998, 2005; Capestany *et al.*, 2006; Inagaki *et al.*, 2006; Dashper *et al.*, 2009). In both of these species, the leucine-rich repeat proteins are members of the CTD family of proteins that are secreted and attached to the surface by novel mechanisms (Seers *et al.*, 2006; Veith *et al.*, 2009). There are two characterized Lrr proteins in *P. gingivalis*. In the *T. forsythia* genome, one Lrr protein is characterized (BspA) and another 5 predicted. Six Lrr proteins are predicted in the *T. denticola* genome. Together, this suggests that this family of proteins plays an important role in the virulence of these species, and that these Lrr proteins warrant further attention.

METABOLIC COOPERATIVITY

Close association can minimize the dilution of metabolites and signaling molecules exchanged between interacting species, thereby facilitating efficient metabolic communication (Grenier, 1992). It is likely that some of the bacterial species to which oral treponemes bind have some form of metabolic interaction with them. Consistent with this concept, it has been shown that *T. denticola* and *P. gingivalis* display symbiosis in protein degradation, nutrient utilization, and growth promotion (Grenier, 1992; Nilius *et al.*, 1993; Kigure *et al.*, 1995; Hollmann and Van der Hoeven, 1999; Grenier and Mayrand, 2001; Yoneda *et al.*, 2005).

When grown together in continuous culture, *P. gingivalis* and *T. denticola* bound to each other (Fig. 2a) and developed a stable cell ratio of 6:1, respectively, as determined by real-time PCR (unpublished data). This indicates that they do not directly compete under these specific growth conditions, since if this were the case, one species would eventually displace the other.

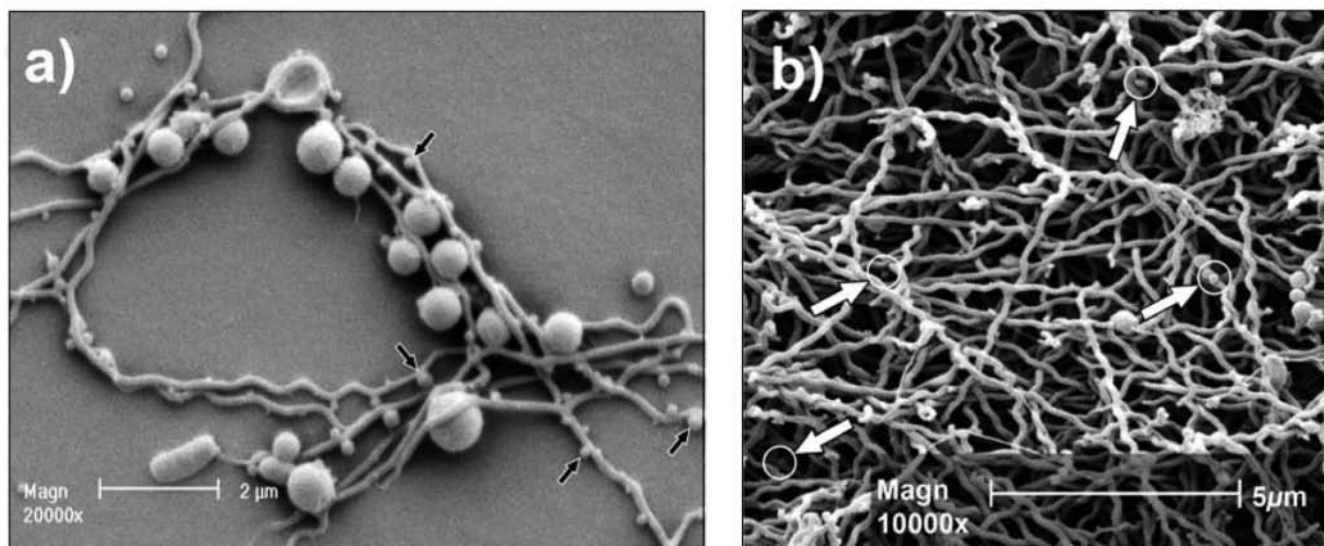


Figure 2. Electron micrographic images of *T. denticola* co-cultured with *P. gingivalis* and as a monospecies biofilm. **(a)** Electron micrograph of *P. gingivalis* and *T. denticola* growing in continuous co-culture, showing the intimate association of the bacteria and the putative vesicles on the surface of the treponeme (arrows). The bacteria were co-cultured under conditions similar to those described previously for the culture of *T. denticola* in Oral Bacterial Growth Medium (Orth *et al.*, 2010) and *P. gingivalis* (Ang *et al.*, 2008). *T. denticola* was initially inoculated into the chemostat, and once cell density had stabilized, *P. gingivalis* was added to the continuous culture. **(b)** Electron micrograph of *T. denticola* biofilm formed on a glass rod submerged in continuous culture after 14 days' incubation as described previously (Mitchell *et al.*, 2010). Outer sheath vesicles are indicated by arrows. Morphological variations of *T. denticola* cells in the form of "spherical bodies" can also be seen in this micrograph.

METABOLIC END-PRODUCTS

Relatively little is known regarding the metabolism of *T. denticola*, and many of its metabolic pathways are likely to be novel (Rother *et al.*, 2001; Seshadri *et al.*, 2004; Chu *et al.*, 2008). It primarily utilizes serine, alanine, cysteine, and glycine when grown *in vitro* and generates fermentation products including acetate, lactate, succinate, formate, pyruvate, ethanol, carbon dioxide, hydrogen sulfide (H_2S), and ammonia (Hespell and Canale-Parola, 1971). These metabolites can accumulate in high concentrations and could influence the composition of bacterial species within the polymicrobial biofilm as well as affecting host tissue (Carlsson, 1997; Kuramitsu *et al.*, 2007). These compounds can penetrate periodontal tissue and disrupt host cell activity and the host immune response (Tonetti *et al.*, 1987; Bartold *et al.*, 1991; Eftimiadi *et al.*, 1993; Kurita-Ochiai *et al.*, 1995; Carlsson, 1997; Niederman *et al.*, 1997; Kuramitsu *et al.*, 2007). The short-chain fatty-acid fermentation products have been suggested to play a role in disease progression (Niederman *et al.*, 1997). Volatile sulfur compounds such as methyl mercaptan and H_2S , highly toxic metabolites produced by *T. denticola* and other oral bacteria, are thought to play an important role in periodontal disease (Yoshimura *et al.*, 2000). Methyl mercaptan is derived from methionine by the action of methionine- α -deamino- γ -mercaptomethane lyase (METase), which has been shown to be present in both *T. denticola* and *P. gingivalis*, but is absent in mammals (Yoshimura *et al.*, 2000; Fukamachi *et al.*, 2005). Methyl mercaptan reduces protein synthesis by human gingival fibroblasts and inhibits cell migration in periodontal ligament cells (Johnson *et al.*, 1992; Lancero *et al.*, 1996). Mice infected with a *P. gingivalis* mutant lacking METase had a

significantly higher survival rate relative to the wild-type strain W83 (Yoshimura *et al.*, 2000). *T. denticola* METase has a much higher affinity for methionine than *P. gingivalis* METase, indicating that, at low concentrations, *T. denticola* may be the major producer of methyl mercaptan (Fukamachi *et al.*, 2005).

Hydrogen sulfide is a major metabolic end-product of the fermentation of cysteine by some anaerobic bacteria. H_2S is cytotoxic for a variety of host cells, including gingival fibroblasts and epithelial cells (Beauchamp *et al.*, 1984; Reiffenstein *et al.*, 1992; Chu *et al.*, 1999; Yoshimura *et al.*, 2000). *T. denticola* is the only known oral bacterium to contain the three-step pathway required to produce H_2S , from the abundant host tripeptide, glutathione (Chu *et al.*, 2008). This pathway contains cysteinylglycine, γ -glutamyltransferase, and the novel enzyme, cystalysin (Makinen and Makinen, 1997; Chu *et al.*, 2002). H_2S production *via* cystalysin has been shown to be the mechanism by which *T. denticola* disrupts erythrocyte membranes, thereby explaining its original description as a hemolysin (Chu *et al.*, 1995). H_2S also has both pro- and anti-inflammatory effects (Kimura, 2009), and a recent study by Chen *et al.* showed that H_2S production by *P. gingivalis* stimulated production of the pro-inflammatory cytokine interleukin-8 (IL-8) by gingival epithelial cells (Chen *et al.*, 2010). The stimulation of both pro- and anti-inflammatory mediators may serve to dysregulate the host's defense.

BIOFILMS

Survival and virulence of oral treponemes are dependent on their ability to form biofilms, grow in this milieu, interact with the other species in the biofilm, and, presumably, escape from

the biofilm, depending on environmental conditions. Direct microscopy of freshly prepared samples of periodontal pocket plaque most often reveals planktonic spirochetes of diverse size swimming among a rich variety of other highly motile bacterial species. The surface of the adjacent biofilm usually has a concentrated sessile population of the same morphotypes (Ellen and Galimanas, 2005; Zijngje *et al.*, 2010).

When co-cultured, *P. gingivalis* and *T. denticola* form significantly more biofilm compared with monoculture, with the resultant polymicrobial biofilms adhering more tightly to the substratum than the monospecific biofilms (Yamada *et al.*, 2005). Molecules involved in polymicrobial biofilm formation have been examined in a panel of *T. denticola* mutants. A *T. denticola* CfpA-deficient mutant, which lacks cytoplasmic filament, was unable to form a mixed biofilm with *P. gingivalis*, while an immotile FlgE mutant and an Msp-deficient mutant displayed reduced biofilm-forming ability with *P. gingivalis* (Yamada *et al.*, 2005).

Obtaining a model system for biofilm growth of pure cultures of *T. denticola* has been reported as problematic (Vesey and Kuramitsu, 2004), and, as a result, little work has been conducted on *T. denticola* biofilms. To determine the effects of biofilm growth on *T. denticola*, investigators have recently developed a continuous-culture system that allowed for the cultivation of biofilm and planktonic cells simultaneously in the one vessel. This system was then used specifically to determine the effects of biofilm growth relative to the planktonic state. In the biofilm, 126 *T. denticola* genes were differentially expressed, with a fold change of 1.5 or greater (Mitchell *et al.*, 2010). In biofilm cells, there was an up-regulation of genes encoding several putative virulence factors, including cystalysin and the outer membrane dentilisin protease lipoprotein complex, toxin-antitoxin systems, and a family of putative transposases (Mitchell *et al.*, 2010).

TOXIN-ANTITOXIN SYSTEMS

Toxin-antitoxin (TA) systems consist of both a toxin, which inhibits essential cell components, and an antitoxin, which counteracts the toxin. The antitoxin component is generally labile, requiring constant synthesis to remain active in the cell, whereas the toxin component is stable. There is a growing body of evidence suggesting that TA systems are involved in programmed cell death, bacterial stringent response to amino acid starvation, and reversible bacteriostasis (persistence or dormancy), and that these and other roles influence biofilm formation (Kim *et al.*, 2009; Makarova *et al.*, 2009). *T. denticola* 35405 contains 33 predicted TA systems, and a high proportion of these (25/33) showed an increase in expression when the bacterium was grown as a monospecific biofilm (Mitchell *et al.*, 2010). The large number of these systems in *T. denticola* and their increased levels of expression in mature biofilm suggest that not only may they play a role in biofilm persistence, but they may also represent a mechanism of resistance to various environmental assaults, including diverse antibiotics and other drugs (Lewis, 2000; Jayaraman, 2008).

TRANSPOSASES

T. denticola possesses a large, unusual family of genes with significant similarity to transposases. Transposases are enzymes that "cut and paste" mobile genetic elements from one position to another within the genome. Twenty-five of the 35 putative transposase genes were significantly up-regulated in the biofilm relative to planktonic cells. This could lead to extensive chromosomal re-arrangement, resulting in the development of population-level diversity, or may represent a novel gene-regulatory mechanism (Mitchell *et al.*, 2010). A functional lysogenic bacteriophage, ϕ td1, was discovered during *T. denticola* biofilm growth that may be capable of transferring virulence-related genes through horizontal gene transfer. The bacteriophage is 37,920 bp in length and has a GC content of ~37%, similar to that of *T. denticola* (Mitchell *et al.*, 2010). Analysis of these data together indicates that there is a higher potential for genetic mobility in *T. denticola* when growing as a biofilm, and that these systems are important for biofilm persistence and therefore virulence of this bacterium.

OUTER SHEATH PROTEINS

Cell-surface components, especially proteins, act as the sensors and effectors of interactions with the host and other bacteria and are largely the targets of the host adaptive immune response. One of the unifying features of the Red Complex bacteria that are responsible for chronic periodontitis progression is their high levels of extracellular proteolytic activity that is mediated by cell-surface-located proteases. Since the outer membrane of *T. denticola* is significantly different from that of Gram-negative bacteria, it is usually referred to as the outer sheath.

DENTILISIN

Dentilisin has been proposed to be a major *T. denticola* virulence factor, since it is an active cell-surface-located protease that cleaves at phenylalanyl/alanyl and prolyl/alanyl bonds (Uitto *et al.*, 1988a,b; Grenier *et al.*, 1990; Makinen *et al.*, 1995; Ishihara *et al.*, 1996; Beausejour *et al.*, 1997). It contributes to disease progression by disrupting or modulating intercellular host signaling pathways and degrading host cell matrix proteins. Dentilisin potentially allows for penetration of epithelial cell layers by *T. denticola* by degradation of intercellular adhesion proteins (Chi *et al.*, 2003) and modulates host cell immune responses by degradation of interleukin-1 β (IL-1 β), IL-6, tumor necrosis factor alpha (TNF- α), and monocyte chemoattractant protein 1 (Miyamoto *et al.*, 2006; Okuda *et al.*, 2007). The protease occurs as part of a complex of three proteins, the 72-kDa subtilisin-like protease (dentilisin, PrtP, chymotrypsin-like protease) and two auxiliary proteins, PrcA1 (~40 kDa) and PrcA2 (~30 kDa) (Lee *et al.*, 2002; Ishihara *et al.*, 2004). Recent work has shown that the dentilisin protease complex is encoded by an operon containing three genes, *prcB*, *prcA*, and *prtP* (Bian *et al.*, 2005). The protein encoded by *prcA* is hydrolyzed to produce two proteins (PrcA1 and PrcA2) of various sizes, depending on

the strain. *prcB* is expressed at a lower level than *prtP* and *prcA*, and although recombinant PrcB associates with PrtP, its role in complex formation and activity is currently unknown (Bian *et al.*, 2005). Dentilisin may also play a role in processing of other cell-surface components, such as Msp, and is proposed to associate with this protein. As noted above, dentilisin is also proposed to interact with the fimbriae of *P. gingivalis* and participate in the co-aggregation of the bacteria. The dentilisin-PrcA complex has been compared with the major virulence factors of *P. gingivalis*, the RgpA-Kgp proteinase-adhesin complexes, since each complex adheres to and degrades fibrinogen (Bamford *et al.*, 2007). It has been suggested that *T. denticola* and *P. gingivalis* synergistically hydrolyze fibrinogen, thereby promoting vascular disruption, bleeding, and inflammation and retarding tissue repair (Bamford *et al.*, 2007).

TRYPSIN-LIKE PROTEASE ACTIVITY

Trypsin-like protease activity has been reported from *T. denticola* (Ohta *et al.*, 1986). The responsible enzyme, oligopeptidase B, is encoded by *opdB* (TDE2140), has similarity to prolyl oligopeptidases, and has been shown to cleave only C-terminal to Arg-residues (Fenno *et al.*, 2001; Lee and Fenno, 2004). In addition to the 78-kDa OpdB described by Fenno *et al.* (2001), a second putative OpdB (TDE1195) of the same mass and sharing 45% amino acid sequence similarity (28% identity) was found by Veith *et al.* (2009). Although the activity of this putative protease is unknown, it is possible that the second OpdB may be a Lys-specific protease, which together would explain the trypsin-like proteolytic activity described for *T. denticola*. Per cell, *T. denticola* 35405 has approximately ten-fold less Arg-specific activity than *P. gingivalis* W50 when tested with the chromogenic substrate BApNA (unpublished).

MAJOR SHEATH PROTEIN

Msp, a member of the Tpr (*T. pallidum* repeat) protein family, is the most abundant protein in the *T. denticola* outer membrane (or sheath) and is one of the most-studied *T. denticola* proteins. It is a β -barrel, integral outer sheath protein that acts as a porin and has surface-exposed loops that are able to bind to a variety of host proteins. Msp has been proposed to mediate colonization of host tissues and has cytopathic pore-forming activity against cultured epithelial cells (Fenno and McBride, 1998; Ellen, 2006).

Msp appears to be a ubiquitous outer sheath protein in oral treponemes, although there is considerable variation in the sequence among species and strains (Fenno *et al.*, 1997; Lee *et al.*, 2005). The *T. denticola* Msps can be divided into 3 domains, the highly conserved 203-residue N-terminal and 271-residue C-terminal domain, plus a central variable region of ~70 residues (Edwards *et al.*, 2005). Differences in Msp may be a defining element of strain serotype, because the strain-specific Msp correlates with different serotypes (Capone *et al.*, 2008). Edwards *et al.* showed that the variable region contains the dominant B-cell epitopes for animals immunized with *T. denticola* cells. A recombinant Msp protein and fragments expressed

in *E. coli* demonstrated that, in addition to its known binding to laminin and immobilized fibronectin, Msp also binds keratin, collagen Type 1, fibrinogen, hyaluronic acid, and heparin. The major binding epitopes were located in the N-terminal domain of the protein, and this region of the protein was surface-exposed (Edwards *et al.*, 2005).

Msp is one of the immunodominant *T. denticola* antigens recognized by human serum antibodies (Capone *et al.*, 2008) and murine anti-*T. denticola* polyclonal antibodies (SF Lee *et al.*, 2009; Veith *et al.*, 2009). Numerous studies have shed light on the mechanisms by which Msp interaction leads to actin remodeling and reorganization in host cells, and how this is likely to impair neutrophil chemotaxis and phagocytic activity (Batista da Silva *et al.*, 2004; Puthengady *et al.*, 2006; Amin *et al.*, 2007; Jobin *et al.*, 2007; Magalhães *et al.*, 2008). In contrast to the studies that showed induction of IL-8 expression from various cells following exposure to Msp, Brissette *et al.* (2008) showed that exposure to *T. denticola* did not induce IL-8 production by primary gingival epithelial cells (PGEC). IL-8 induces migration of neutrophils to sites of infection. Dysregulation of efficient neutrophil activation and migration is one means by which *T. denticola* could evade the host immune response and persist at a site. The *T. lecithinolyticum* Msp stimulates the up-regulation of signaling pathways in monocytes, inducing IFN- β expression and IFN-stimulated genes (SH Lee *et al.*, 2009).

Tp92 is a 92-kDa *T. pallidum* subspecies *pallidum* surface-exposed antigen that induces a protective immune response (Cameron *et al.*, 2000). Homologs of Tp92 have been identified in *T. maltophilum*, *T. lecithinolyticum*, *T. socranskii*, and *T. denticola*. Antisera raised to recombinant *T. denticola* Td92 have been used to show surface exposure of Td92 and inhibition of *T. denticola* binding to KB epithelial cells (Jun *et al.*, 2008). All oral treponeme Tp92 homologs induced production of various cytokines, cyclooxygenase-2 (COX-2), and prostaglandin E₂ from THP-1 cells and periodontal ligament cells, indicating a pro-inflammatory response.

LIPOPROTEINS

Lipoproteins are the most abundant membrane-associated proteins found in spirochetes, and *T. denticola* 35405 is predicted to have 166 of them, the highest number for any of the sequenced spirochetes (Setubal *et al.*, 2006). Lipoproteins are acylated at the N-terminus of the mature protein, and this anchors the protein to a membrane. Veith *et al.* (2009) detected 20 lipoproteins under a single set of growth conditions, including 11 uncharacterized putative lipoproteins, 2 hemin-binding proteins (HbpA, HbpB), 2 putative extracellular solute-binding lipoproteins, 4 putative oligopeptide/dipeptide ABC transporter peptide-binding proteins, and the dentilisin complex-associated polypeptide (PrcA). Fourteen lipoprotein genes had significant changes in expression when *T. denticola* was grown as a biofilm, including the outer membrane hemin-binding protein A that, along with 3 other putative lipoprotein encoding genes, was down-regulated in the biofilm (Mitchell *et al.*, 2010). Analysis of these data indicates the importance of lipoproteins to the treponeme lifestyle.

OppA is a 70-kDa cell-surface, membrane-associated lipoprotein that has significant similarity to the solute binding protein of a highly conserved ATP-binding cassette-type transporter involved in peptide uptake and environmental signaling in a wide range of bacteria (Fenno *et al.*, 2000). OppA can bind soluble host proteins such as plasminogen and fibrinogen, but not immobilized insoluble host proteins or epithelial cells. It has been proposed to act as an adhesin and to help decorate the surface of *T. denticola* with host proteins as a means of avoiding or delaying immune recognition. Group A streptococci and *Streptococcus equi subsp.* have been shown to be cleared more slowly from a host when coated with fibrinogen (Whitnack and Beachey, 1985; Poirier *et al.*, 1989; Boschwitz and Timoney, 1994; Ellen, 2006; Bamford *et al.*, 2007).

FhbB is a small (11.4 kDa) surface-exposed *T. denticola* lipoprotein that binds complement regulatory proteins of the factor H (FH) family. By using *T. denticola* dentilisin-deficient mutants, it was determined that after binding FH, it is cleaved by dentilisin to yield a FH subfragment of ~50 kDa. FH bound to dentilisin-deficient mutants was not cleaved and retained its ability to serve as a co-factor for factor I in the cleavage of C3b. The role of this unique binding and cleavage in *T. denticola* virulence is as yet unresolved, but it is likely to be linked to epithelial cell binding and invasion, subversion of the complement cascade, or tissue invasion (McDowell *et al.*, 2007, 2009).

OUTER SHEATH VESICLES

Gram-negative bacteria have long been known to produce outer membrane vesicles (OMVs) (Devoe and Gilchrist, 1973; Nowotny *et al.*, 1982; Grenier and Mayrand, 1987). Initially thought to be a result of random blebbing of the outer membrane, or sheath, producing small spherical vesicles of 50-100 nm in diameter, more recent studies have revealed that OMV formation is a highly regulated process which may increase the fitness of the bacterium in response to environmental cues (Wensink and Witholt, 1981; Kato *et al.*, 2002; Wai *et al.*, 2003). OMVs are considered potent virulence factors, since they possess adhesins, toxins, and proteolytic enzymes, can mediate bacterial aggregation and invasion, are cytotoxic, and can modulate the host immune response (Kuehn and Kesty, 2005). Besides affecting the host, OMVs may be important for securing a niche in the competitive environment of subgingival plaque by eliminating competitors *via* the delivery of proteases and toxins (Kadurugamuwa and Beveridge, 1996; Allan and Beveridge, 2003). OMVs also facilitate the remote delivery of labile signaling molecules and prevent their degradation by other microorganisms (Z Li *et al.*, 1996; Mashburn and Whiteley, 2005).

T. denticola outer sheath vesicles (OSVs) have been proposed as a long-range virulence factor that can penetrate tissues more readily than the bacterium itself (Cimasoni and McBride, 1987; Cockayne *et al.*, 1989; Weinberg and Holt, 1991; Kuehn and Kesty, 2005). Application of *T. denticola* OSVs to Hep-2 epithelial cell monolayers disrupted the tight junctions, which might facilitate penetration into underlying tissues (Chi *et al.*, 2003). However, the involvement of treponemal OSVs in disease remains to be properly explored. When these bacteria are grown in continuous co-culture with *P. gingivalis*, scanning

electron microscopic analysis revealed not only their close association, but also the presence of OSV/OMVs on the surface of *T. denticola* (Fig. 2a). OSVs were also detected when *T. denticola* was grown as a monospecific biofilm (Fig. 2b). Recently, it was demonstrated that *T. denticola* lipooligosaccharide (LOS) and Msp induce macrophage tolerance to further stimulation (Nussbaum *et al.*, 2009). Considering these components are found on *T. denticola* OSVs, initial exposure of macrophages to these OSVs might limit the clearance of the bacterium during subsequent encounters.

MOTILITY

Motility and chemotaxis, like biofilm formation and interspecies co-operation, are not considered to be classic virulence factors of bacteria. However, in the context of *T. denticola* and its role in disease progression, it is likely that they are essential for the virulence of the bacterium. Of the sequenced treponemes, 5-6% of the genome is dedicated to motility and chemotaxis, implying its importance for survival and disease progression in the host.

PERIPLASMIC FLAGELLA

Unlike the exposed flagella of most motile bacterial species, the spirochetal flagella are located within the periplasmic space between the outer sheath and cytoplasmic membrane (Canale-Parola, 1978; Holt, 1978). In *Treponema*, up to 15 periplasmic flagella (PF) originate from each pole of the cell, entwine the cytoplasmic cylinder, and overlap at the cell center (Watson *et al.*, 1951; Bergey and Holt, 1984). The organization of PF in the peptidoglycan-containing periplasm as well as other native cellular structures has recently been resolved by cryo-electron tomography (Izard *et al.*, 2008, 2009). PF facilitate translocation in highly viscous environments which would usually slow or immobilize most externally flagellated bacteria (Fenno and McBride, 1998). PF are protected from the immobilization effects of flagella-specific antibodies produced by the host response to infection (Charon and Goldstein, 2002). Cytoplasmic filaments are cytoplasmic structures located directly beneath the PF. A CfpA-deficient mutant displayed reduced spreading ability compared with wild-type strains, implicating involvement of cytoplasmic filaments in motility (Izard *et al.*, 2001; Vesey and Kuramitsu, 2004).

CHEMOTAXIS

Chemotaxis allows motile bacteria to respond to environmental stimuli in a positive or negative manner. Genome sequencing and bioinformatic analyses predicted that *T. denticola* and *T. pallidum* possess a complete set of chemotaxis proteins required for signal perception, transduction, and adaptation. Environmental stimuli are detected through methyl-accepting chemotaxis proteins (MCPs) that traverse the inner membrane. These signals are transduced *via* the cytosolic chemotaxis proteins CheA, CheW, and CheY that modulate the direction of flagellar motor rotation. Adaptation in *T. denticola* is achieved *via* methylation/demethylation of the MCPs by the regulatory proteins, CheR and CheB, in a manner similar to that of well-studied organisms such as *E. coli* (Sim *et al.*, 2005). Surprisingly high numbers of

MCPs were identified in the *T. denticola* genome, suggesting chemotaxis responses toward a variety of compounds that might reflect the complexity of its ecological niche (Seshadri *et al.*, 2004). Chemoattractants for *T. denticola* include glucose, serum, and albumin, which are possible indicators of damaged host tissues (Umamoto *et al.*, 2001; Ruby *et al.*, 2008). Allelic replacement mutagenesis of two MCP encoding genes, *dmcA* and *dmcB*, resulted in a decreased chemotactic response to serum and albumin (Kataoka *et al.*, 1997; Li *et al.*, 1999). As expected, a *cheA* knock-out mutant, which lacks the central kinase of the chemotaxis pathway, failed to respond chemotactically to serum (Lux *et al.*, 2002).

EPITHELIAL MODEL SYSTEMS

The ability of *T. denticola* to penetrate and migrate through cultured tissue has been used as a model system to assess virulence (Lux *et al.*, 2001). The immotile *flgE* flagella mutant (H Li *et al.*, 1996) lost the ability to penetrate cultured layers formed by human gingival keratinocytes. The importance of a functional chemotaxis pathway for *T. denticola* virulence was underscored by the finding that a *cheA* mutant (Lux *et al.*, 2002), as well as *dmcA* and *dmcB* mutants lacking specific MCPs, displayed significantly impaired tissue penetration despite retaining motility (Kataoka *et al.*, 1997; Li *et al.*, 1999). These studies implied that chemotaxis and motility may be significant for *T. denticola*, and presumably other oral treponemes, in the invasion of gingival tissues. Transcriptome analysis of *T. pallidum* showed significant up-regulation of genes involved in chemotaxis during infection (Smajs *et al.*, 2005).

TISSUE PENETRATION AND EPITHELIAL CELL INVASION

The role of bacterial tissue invasion and internalization by epithelial cells in the progression of periodontitis is still unclear. There appears to be little evidence of extensive tissue penetration by *T. denticola* in human chronic periodontitis; however, *T. denticola* has been detected between junctional epithelium cells, which are normally tightly associated (Saglie *et al.*, 1982). Biopsy of tissue from canine periodontal lesions showed extensive tissue penetration by treponemes (Nordhoff *et al.*, 2008b).

Invasion of, or internalization by, epithelial cells provides oral bacteria with a nutrient-rich environment that is partially protected from the host immune system. While not immediately contributing to disease, *T. denticola* may use the intracellular locale to persist safely and possibly replicate (Colombo *et al.*, 2007; Johnson *et al.*, 2008). During treatment of disease, intracellular bacteria are less likely to be physically removed by scaling and root planing and are more resistant to antibiotics. Furthermore, this intracellular population could constitute a reservoir of bacteria for the repopulation of treated subgingival sites in refractory periodontitis (Tribble and Lamont, 2010).

LIPOLIGOSACCHARIDES (LOS)

LPS, also called endotoxin, forms the outer leaflet of the Gram-negative bacterial outer membrane. The treponemal outer sheath

does not have a typical LPS, and the treponemes lack genes encoding the necessary enzymes for LPS synthesis (Fraser *et al.*, 1998; Schultz *et al.*, 1998; Norris and Weinstock, 2000; Schröder *et al.*, 2000; Hashimoto *et al.*, 2003a; Seshadri *et al.*, 2004). The treponeme outer sheath (membrane) contains lipooligosaccharides (LOS), however, that have functional similarities to LPS. The *T. denticola* LOS have a diacylglycerol lipid anchor plus a core region consisting of hexose-hexosamine-hexose, but lack heptose, 3-deoxy-D-manno-2-octulosonic acid, and β -hydroxy fatty acids, which are core components of LPS. Strikingly, the presence of a glycerol-hexose unit and two glycerol-hexadecanoic acid fragments in the lipid anchor indicates a glycolipid membrane anchor typically found in the lipoteichoic acid of Gram-positive bacteria (Schultz *et al.*, 1998).

IMMUNOMODULATION AND IMMUNO-EVASION

The host innate immune system is the first line of defense against bacterial infection. Inhibition of components of the innate immune system is obviously of great advantage to a bacterium that has been shown to associate closely with epithelial cells *in vivo*. *T. denticola* can evade aspects of the innate immune defense by preventing efficient binding of antimicrobial peptides, such as β -defensins, that are produced by epithelial cells, and by inducing rapid efflux of some host defense peptides which enter the cytoplasm (Brissette and Lukehart, 2007). Recently, *T. denticola* has also been shown to suppress the production of β -defensin 3 by human gingival epithelial cells (Shin *et al.*, 2010).

TLR-MEDIATED RESPONSES

Individual Toll-like receptors (TLRs) on the surfaces of host cells recognize structurally conserved, pathogen-associated molecular patterns, such as LPS, flagellin, and peptidoglycan. TLR activation leads to induction of inflammation-related genes important for limiting infection. However, dysregulation of the inflammatory response can lead to over-expression and continued expression of inflammatory mediators that can result in the tissue destruction characteristic of periodontitis.

Bacterial LPS is sensed by host epithelial cells and macrophages *via* binding to TLR4 that triggers a series of intracellular signaling systems, leading ultimately to production of inflammatory mediators and migration of macrophages and neutrophils to a site of infection (Dauphinee and Karsan, 2006). *T. denticola* LOS stimulates macrophages *via* TLR4 and MyD88 (myeloid differentiation primary response gene 88) and induces macrophage tolerance to LPS. *T. denticola* LOS also stimulates fibroblasts, inducing them to produce a variety of inflammatory mediators, interleukin-6 (IL-6), IL-8, monocyte chemoattractant 1, nitric oxide, and prostaglandin E₂, as well as matrix metalloproteinase 3 (MMP-3) (Tanabe *et al.*, 2008). Several signaling proteins have been shown to be phosphorylated following LOS stimulation of fibroblasts, including Fos, NF- κ B p50, and NF- κ B p65, indicating an inflammatory response.

In addition to LOS, other *T. denticola* cellular components can stimulate an immune response. Whole *T. denticola* cells and

Msp can activate macrophages in a TLR2-MyD88-dependent process, resulting in TNF- α production in a dose-dependent manner. These macrophages also became tolerant to stimulation by enterobacterial LPS (Nussbaum *et al.*, 2009). The LPS tolerance induced by *T. denticola* components during polymicrobial infection or proliferation may be a general mechanism to evade bacterial clearance. In addition to induction of innate immune responses by TLR2 and TLR4 receptors, *T. denticola* cells also induce innate immune responses via a TLR2/TLR6 heterodimer with mitogen-activated protein kinases (MAPKs), extracellular regulated protein-serine kinases 1 and 2 (ERK1/2), and p38, playing major roles in the resulting pro- and anti-inflammatory cytokine production (Ruby *et al.*, 2007).

T. denticola peptidoglycan can also stimulate factors of innate immunity in human macrophage-like cells, inducing inflammatory mediators including TNF- α , IL-1 β , IL-6, IL-8, and the chemokine RANTES (regulated upon activation, normal T-cell expressed, and secreted) and matrix metalloproteinase 9 (MMP-9) (Tanabe *et al.*, 2009).

Immunohistochemistry of gingival tissues taken from patients undergoing periodontal therapy revealed high levels of TLR2 and TLR5 receptors in comparison with healthy tissues and that gingival epithelial cells can be stimulated to produce IL-1 β and TNF- α in response to stimulation by the TLR2 and TLR5 ligands HKLM and flagellin (from *Salmonella typhimurium*), respectively (Beklen *et al.*, 2009). The authors suggested that these antigens would also be present in other periodontal pathogens, such as the flagellin of *T. denticola*, and these could also stimulate host epithelial cells via TLR5 and thus may promote inflammation.

CONCLUSION

Treponemes, including *T. denticola*, are able to suppress host responses to LPS, which may allow for the persistence of the bacterial consortia found associated with periodontal disease. Furthermore, *T. denticola* LOS has been shown to stimulate osteoclastogenesis and matrix metalloproteinase expression, which could exacerbate periodontal pathology (Choi *et al.*, 2003). The characteristics of *T. denticola* that represent its major virulence factors in chronic periodontitis are: its motility and chemotaxis, which enable the bacterium to rapidly colonize new sites, penetrate deep periodontal pockets, and penetrate epithelial layers; its ability to interact synergistically with other periodontal pathogens on several levels; its ability to produce cytotoxic metabolites; and its ability to form biofilms and a range of cell-surface proteins to dysregulate the host defense to help protect the subgingival biofilm and cause host tissue destruction.

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