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Treponema denticola invasion into human gingival epithelial cells

Satoru Inagaki[a, b], Ryuta Kimizuka[a, b], Eitoyo Kokubu[a, b], Atsushi Saito[a, c], Kazuyuki Ishihara[a, b]*

[a]Oral Health Science Center, Tokyo Dental College, 2-9-18 Misaki-cho, Chiyoda-ku, Tokyo 101-0064, Japan
[b]Department of Microbiology ,Tokyo Dental College, 2-1-14 Misaki-cho, Chiyoda-ku, Tokyo 101-0061, Japan
[c]Department of Periodontology, Tokyo Dental College, 2-9-18 Misaki-cho, Chiyoda-ku, Tokyo 101-0061, Japan

*Corresponding author:
Kazuyuki Ishihara, Department of Microbiology, Tokyo Dental College
2-1-14 Misaki-cho, Chiyoda-ku, Tokyo 101-0061, Japan
Phone: +81-3-6380−9558, FAX: +81-3-6380−9578
e-mail: ishihara@tdc.ac.jp

Abstract

Host cell invasion is important for periodontal pathogens in evading host defenses and spreading into deeper areas of the periodontal tissue. *Treponema denticola* has been implicated in a number of potentially pathogenic processes, including periodontal tissue penetration. Here we tested the ability of *T. denticola* strains to invade human gingival epithelial cells (HGEC). After 2 h infection, intracellular location of *T. denticola* cells was confirmed by confocal laser scanning microscopy (CLSM). Results from an antibiotic protection assay following [^{3}H]uridine labeling indicated that invasion efficiency reached a maximum at 2 h after infection. Internalized *T. denticola* cells were still observed in HGEC at 24 h by CLSM. A dentilisin deficient mutant exhibited significantly decreased invasion ($p < 0.05$) compared with the wild-type strain. In inhibition assays, phenylmethylsulfonyl fluoride and metabolic inhibitors such as methyl-β-cyclodextrin and staurosporine significantly reduced *T. denticola* invasion. Under CLSM, *T. denticola* colocalized with GM-1 ganglioside-containing membrane microdomains in a cholesterol-dependent manner. These results indicated that *T. denticola* has the ability to invade into and survive within HGECs. Dentilisin activity of *T. denticola* and lipid rafts on HGEC appear to play important roles in this process.

Key words: Invasion, Gingival epithelial cell, Protease, Periodontitis, Spirochetes

1. Introduction

Periodontitis, one of the most common infectious diseases that afflict humans, is characterized by gingival inflammation, as well as loss of connective tissue and bone surrounding teeth, which eventually leads to tooth exfoliation [1]. A major etiological factor of this disease is infection by gram negative anaerobic rods such as *Porphyromonas gingivalis* and spirochetes represented by *Treponema denticola* [2]. *T. denticola* is frequently isolated from human periodontal lesions and its involvement in the pathogenesis and progression of periodontitis has been reported [3]. *P. gingivalis*, *Tannerella forsythia* and *T. denticola* were categorized as the "red complex" because these species have been frequently co-isolated together from lesions of chronic periodontitis [4].

So far, several virulence effects of *T. denticola* on host tissue or cells have been characterized [5]. The major outer sheath protein (Msp) induces innate immune responses through TLR2-MyD88 [6], affects the migration of neutrophils during chemotaxis [7] and also acts as a pore-forming cytotoxin [8]. Activation of the complement component C3 by the *T. denticola* surface protease dentilisin and inactivation of C3b by dentilisin and FhbB have been reported previously [9, 10]. Dentilisin was also reported to exhibit cytotoxity [8] and be involved in the migration of *T. denticola* into a basement membrane gel, Matrigel, obtained from an Engelbreth-Holm-Swarm transplantable mouse tumor [11].

Interactions between bacteria and their surrounding epithelia are critical factors in bacterial infections [12, 13]. Invasion allows the bacteria not only to evade host immune surveillance but also to spread into deeper tissues. The

invasion of periodontal tissues by oral bacteria is important in establishing a niche and exerting pathogenic effects on periodontal tissue [14, 15]. Cellular invasion by periodontal pathogens such as *P. gingivalis* has been implicated in the pathogenesis of periodontitis [16]. Invasion by *P. gingivalis* causes degradation of the integrin-related signaling molecules, paxillin and focal adhesion kinase, which disables cellular migration and proliferation [17].

Previous studies showed that spirochetes penetrate into periodontal tissue [18-22] *T. denticola* was shown to penetrate into epithelial cell monolayers [23], and dentilisin plays a critical role in this process by altering cellular tight junctions [24, 25]. In addition, detection of *T. denticola* in gingival crevicular epithelial cells from chronic periodontitis lesions was reported [22, 26]. *T. denticola* was detected in the gingival epithelial cells at 24 h after infection [27]. These reports suggested that *T. denticola* has the ability to penetrate to and invade into epithelial cells. However, little is known about the mechanism or factor involved in invasion of gingival epithelial cells by *T. denticola*. To better define the interaction between *T. denticola* and host cells, we investigated intracellular invasion of human gingival epithelial cells (HGEC) by *T. denticola*.

2. Materials and Methods

2.1 Bacterial strains and culture conditions

T. denticola ATCC 35405 [28], dentilisin deficient mutant K1 [29] and Msp deficient mutant DMSP3 [30] were grown in TYGVS medium under anaerobic conditions (10% CO_2, 10% H_2, 80% N_2) as described previously [31]. The

mutants constructed by allelic exchange mutagenesis were grown in the same medium containing 40 μg ml^{-1} erythromycin. *Escherichia coli* ATCC 11775 was grown in LB medium under anaerobic conditions and used as a control for antibiotic protection assays.

2.2 Gingival epithelial cell cultures

An established immortalized human gingival epithelial cell (HGEC) line, Ca9-22, was purchased from the Health Science Research Resources Bank (Osaka, Japan). Ca9-22 has been used in previous studies as a cultured model of HGECs [32, 33, 34]. Ca9-22 cells were maintained in Eagle's minimal essential medium (MEM) supplemented with glutamine (0.6 mg ml^{-1}), heat-inactivated 10 % fetal calf serum, and gentamicin (10 μg ml^{-1}) / amphotericin B (0.25 μg ml^{-1}) (Cascade Biologics, Portland, OR) at 37 °C in 5 % CO_2 humidified air. Cells were grown to near-confluence (95%) for the assays. For investigation of the invasion by *T. denticola,* these cells were cultured in the medium without gentamicin and amphotericin B.

2.3 Antibiotic protection assays

Invasion by *T. denticola* was quantitated by a standard antibiotic protection assay [35] with modifications for *T. denticola. T. denticola* ATCC35405, K1, DMSP3 and *E. coli* ATCC 11775 were labeled following incubation in medium containing 10 μCi ml^{-1} [^{3}H]uridine for 5 days. The multiplicity of infection (MOI) was calculated based on the number of cells per well at confluence. In our preliminary experiment, Ca9-22 cells were damaged at MOI 100 and the

invasion efficiency was very low at MOI 10. We used MOI of 100 in all subsequent experiments. Labeled bacteria (1×10^7) were added to the Ca9-22 monolayers (1×10^5) grown in the medium described above without antibiotics and incubated for 2 h (unless otherwise noted) under 5% CO_2 at 37°C. Following incubation, monolayers were washed twice with sterile PBS, and fresh medium containing gentamicin (300 μg ml^{-1}; Sigma-Aldrich, St. Louis, MO) and metronidazole (200 μg ml^{-1}; Sigma-Aldrich) was added for an additional 1 h to kill extracellular treponemes. The concentration the antibacterial agent was based on our previous experiment for *P. gingivalis* invasion [36] and before the quantitative assay, we confirmed by control experiments that the treatment was sufficient to kill external bacteria. Adherent cells were then washed with PBS before being lysed with sterile water. Internalized bacteria were counted using a liquid scintillation counter. Before the quantitative assay, we confirmed by control experiments that the treatment was sufficient to kill all external bacteria. As in our preliminary results, the doubling time for *T. denticola* in TYGVS medium was 24 h and multiplication of the bacteria in the tissue culture medium during the assay period was at most once. The level of invasion was expressed as the percentage of bacteria retrieved following cell lysis relative to the total number of bacteria initially added. The rate of survival of *T. denticola* in Ca9-22 was calculated as follows: Survival rate = (intracellular *T. denticola* at indicated time) / (*T. denticola* immediately after infection) x 100. All experiments were performed in duplicate or triplicate and repeated at least three times.

2.4 Confocal laser scanning microscopy (CLSM)

To observe both intracellular and extracellular *T. denticola* cells, a double-fluorescence technique was performed as described previously [37]. Briefly, mid-log phase cells of *T. denticola* were harvested and enumerated by optical density measurements at 660 nm. Ca9-22 cells were grown on coverslips in six-well tissue culture plates in the medium described above without antibiotics and infected with *T. denticola* cultivated under the conditions described above. The multiplicity of infection (m.o.i.) was calculated based on the number of cells per well at confluence. We used a m.o.i. of 100 in all subsequent experiments. Cells on overslips were fixed in 4% paraformaldehyde (Wako, Osaka, Japan) for 10 min, washed in phosphate buffered saline (PBS, pH 7.4), and then incubated with a rabbit anti-*T. denticola* serum [29] diluted 1:500 in PBS-0.5% bovine serum albumin (BSA) for 60 min. Following incubation, coverslips were washed three times with PBS and incubated with Alexa Fluor 488 (green fluorescent dye)-conjugated goat anti-rabbit immunoglobulin (Ig) G (Molecular Probes, Eugene, OR) diluted 1:500 for 30 min to visualize attached bacteria. Internalized bacteria were then stained by first permeabilizing Ca9-22 cells by dipping coverslips in 0.4% Triton X-100 solution for 5 min and then staining with the rabbit anti-*T. denticola* serum followed by Alexa Fluor 568 (red fluorescent dye)-conjugated with goat anti-rabbit IgG (Molecular Probes) diluted 1:500 as described above. Actin filaments were stained with Alexa Fluor 647 (blue fluorescent dye) conjugated to phalloidin (Molecular Probes) for 30 min according to the supplier's recommendations to visualize the cellular cytoskeleton. Coverslips mounted in a mounting medium were examined using

a laser scanning confocal microscope (model LSM510; Carl Zeiss MicroImaging, Göttingen, Germany). A series of 20-30 Z-stack images was scanned in increments using excitation wavelengths of 488, 561 and 633 nm. Images were analyzed using ZEN 2008 software (Carl Zeiss). Where appropriate, Z stacks of the X-Y sections of CLSM were processed to render a three-dimensional (3D) image using the 'Iso Surface' function of Imaris 7.0.0 software (Bitplane AG; Zurich, Switzerland).

CLSM was also used to assess colocalization of *T. denticola* with a lipid raft marker, GM1 ganglioside. In this case, Ca9-22 cells grown in the medium described above without antibiotics were exposed to *T. denticola* at m.o.i. 100 as described above for 30 min at 37°C. Unattached bacteria were then removed by washing with PBS. After fixation, *T. denticola* attached on the epithelial cells was stained with rabbit anti-*T. denticola* serum followed by Alexa Fluor 488 (green fluorescent dye)-conjugated with goat anti-rabbit IgG (Molecular Probes) as described above, and the cell surface ganglioside GM1 was labeled with Alexa Fluor 568 (green fluorescent dye)-conjugated CTB (Molecular Probes; 1 $\mu g\ ml^{-1}$, 60 min). The cells were imaged as above and colocalization of bacteria with GM-1 was assessed.

2.5 Scanning electron microscopy (SEM)

Ca9-22 cells seeded on 12-mm-diameter glass coverslips for 24 - 48 h were infected with *T. denticola* in MEM without FCS and antibiotics for 30 to 60 min. After the preset time, cells were fixed in ice-cold 1.5% glutaraldehyde in 0.1M

sodium cacodylate buffer (pH 7.4) for 1 h and post-fixed with 1% osmium. The specimens were dehydrated with graded alcohols, critical-point-dried and subsequently sputter-coated with gold palladium. Ca9-22 cells infected with *T. denticola* were visualized by JOEL7400 SEM (Joel, Tokyo, Japan).

2.6 Inhibitors of bacterial and epithelial cell functions

To dissect the biochemical pathways involved in treponeme invasion, the effects of eucaryotic cell function inhibitors and protease inhibitors on *T. denticola* invasion were investigated. Inhibition experiments were performed as described previously [38, 39] with minor modifications. All chemicals were obtained from Sigma-Aldrich. To disorganize cytoskeletal architecture, cytochalasin D (1 μg ml^{-1}) and nocodazole (10 μM) were used. Methyl-β-cyclodextrin (MβCD;10 mM, cholesterol depletion drug) and wortmannin (100 nM, PI3K inhibitor) , cycloheximide (100μg ml^{-1}, inhibitor of protein biosynthesis in eukaryotes), staurosporine (1 μM, protein kinase inhibitor) and monodansylcadaverine (50 μM, inhibitor for receptor mediated endocytosis) were also used. To analyze the role of dentilisin in bacterial entry, the serine proteinase inhibitor phenylmethylsulfonyl fluoride (PMSF) was used. Cytochalasin D and staurosporine were preincubated with Ca9-22 cells for 30 min prior to addition of the bacteria and remained present throughout the invasion assay. Nocodazole was preincubated with the monolayers for 1 h on ice and then at 37°C for 30 min prior to bacterial infection. Cycloheximide was preincubated with the monolayers for 4 h before bacterial infection and the drug was present during the invasion assay. Protease inhibitors were preincubated with the bacteria for 30

min prior to the assay. All potential inhibitors were tested at the concentrations used for possible adverse effects on the epithelial cells by examining the morphology of the cells and the confluency of the monolayers.

2.7 In vitro wound healing assay

The effects of invasion on cell migration were also investigated according to the method of Inaba et al. [40]. Briefly, Ca9-22 cells were cultured until confluent. The cell layers were then scratched using a plastic tip and washed three times with MEM. The cells were then incubated with *T. denticola* for 2 h in MEM containing 10% serum in 5% CO_2 as described previously. External nonadherent cells were removed by washing the cells three times with MEM supplemented with glutamine (0.6 mg ml^{-1}), heat-inactivated 10 % fetal calf serum, and gentamicin (10 µg ml^{-1}) / amphotericin B (0.25 µg ml^{-1}) and incubated with the medium for 5 h. The closure rate for each scratched area was determined using Image J software (US National Institute of Health) (n = 9-10).

2.8 Statisitical analysis

Differences in invasion efficiency among strains of *T. denticola* and the effects of metabolic inhibitors were subjected to one way analysis of variance (ANOVA) and Tukey's multiple comparison test at a 5% level of significance using Prism 5.0 (GraphPad Software, La Jolla, CA).

3. Results

3.1 Demonstration of cellular invasion by T. denticola and the role of surface proteins

In the antibiotic protection assay, the invasion level of *T. denticola* ATCC35405 was 3% while that of *E. coli* was 0.01 (Table 1). CLSM following dual labeling of extracellular and intracellular bacteria was performed to verify the invasion of bacteria into HGEC. The invasion was confirmed by Z stack images in orthogonal views and 3D rendered images. When infected with the wild-type strain, invasion of Ca9-22 cells by *T. denticola* was observed (Fig. 1A) and cells containing multiple *T. denticola* cells were frequently observed in the composite images converted with IMARIS software (Fig. 1B). These data revealed that *T. denticola* ATCC35405 has the ability to invade into the cytoplasm of Ca9-22 cells.

Bacterial interaction with the cell surface of Ca9-22 was investigated by SEM. In Ca9-22 cells exposed for 1 h to *T. denticola*, we observed a number of treponeme-like structures on the cell surface and a portion of the spirochete cells appear to have invaded Ca9-22 cells in a polar manner (Fig. 1C).

3.2 Effects of incubation time on invasion and viability of T. denticola after invasion

To investigate survival of intracellular *T. denticola* ATCC35405 cells after infection, additional experiments were conducted to address the viability of *T. denticola* after invasion. Ca9-22 cells were incubated with *T. denticola* ATCC35405 for 120 min and the extracellular bacteria were killed with

antibiotics. After washing to remove the antibiotics and extracellular bacteria, the cells were maintained further for 24 h. Antibiotics were added again to kill any released extracellular bacteria. Following epithelial cell lysis, approximately half of the levels of [^{3}H]uridine were detected compared with 2 h infection (Fig. 2A). In addition, intact intracellular *T. denticola* cells were observed after 24 h by CLSM (Fig. 2B). In our preliminary results using a double-fluorescence technique with CLSM, *T. denticola* shaped cells in Ca9-22 (per 100 cells) were observed with similar levels within 2 days, however, it was reduced to less than 30% on day 3 (data not shown). The result suggests that internalized *T. denticola* were finally killed by endolysosomal degradation after couple of days. Collectively, these results suggested that *T. denticola* appears to be capable of remaining within HGEC for at least 24 hours.

3.3 Involvement of surface components for invasion by T. denticola

Major pathogenic factors on the surface of *T. denticola* such as Msp and dentilisin have the potential for involvement in invasion. In the Msp mutant, intracellular bacteria were detected (Fig. 1D); however, the numbers were similar level compared with the wild-type strain (Fig. 1A). In contrast, when infected with the dentilisin deficient mutant, adherent/internalized *T. denticola* were also observed (Fig. 1C), however the number of internalized treponema were very low (Fig. 1E). In the antibiotic protection assay, no difference was noted between the invasion efficiency of the msp-deficient mutant and the wild-type strain (Table 1). In contrast, the invasion level of the dentilisin deficient mutant as well as the wild type treated with PMSF was significantly lower than

that of the wild-type strain in the antibiotic protection assay ($P < 0.05$) (Table 1). For the dentilisin deficient mutant K1, only the adherent treponema were frequently observed on the surface of cell by CLSM (Fig. 1D).

These data revealed that dentilisin appears to be required for the invasion.

3.4 Metabolic requirements for invasion

To dissect the host biochemical pathways involved in *T. denticola* invasion, a group of host cell metabolic inhibitors were also utilized (Table 2). MβCD and staurosporine produced the most significant reductions in invasion, suggesting the involvement of plasma membrane cholesterol and activation of host tyrosine or threonine/serine kinases in the invasion process. Also, other mechanisms including actin rearrangement and receptor-mediated uptake are likely to be involved because wortmannin, cytochalasin D and monodansylcadaverine induced approximately 50% inhibition. Nocodazole and cycloheximide produced only slight inhibitions of invasion.

3.5 Cholesterol-dependent colocalization of T. denticola with lipid rafts

Since results from the inhibition assays suggested involvement of membrane cholesterol in host cell invasion by *T. denticola*, we sought to investigate the interaction between *T. denticola* and lipid rafts by staining with a raft marker, GM1 ganglioside. CSLM confirmed that *T. denticola* bacteria attached to or entered Ca9-22 cells, and revealed evident colocalization with GM1-containing membrane microdomains (Fig. 3A). In contrast, *T. denticola* did not colocalize

with GM1 in cholesterol-depleted cells (Fig. 3B).

3.6 In vitro wound healing assays

After Ca9-22 cells were scratched, cells migrated to cover the scratched area. Covered areas following migration of *T. denticola* ATCC35404 infected cells were significantly less compared with cells without infection, cells infected with, *T. denticola* K1 or *T. denticola* DMSP3 (Fig. 4). *T. denticola* DMSP3 infected cells also showed lower migration activity compared with cells without infection.

4. Discussion

In the present study, we demonstrated the invasion of HGEC by *T. denticola*. The results of protection assays and CSLM analyses clearly revealed internalization of *T. denticola* ATCC 35405 within the Ca9-22 cells. The internalization process was also observed by SEM. *T. denticola* has been reported to penetrate epithelial tissue monolayers via tight junctions [23, 24]. Our observations correlate with the detection of *T. denticola* in HGECs from patients with periodontitis [22, 41]. *T. denticola* was detected in the human periodontium by the PAS stain and its dentilisin activity could be detected in diseased human periodontium [42]. In our preliminary experiments, *T. denticola* could invade human umbilical vein endothelial cells and KB cells as well (unpublished observations). The invasion of gingiva by *T. denticola* in mice model also reported [43]. Recently, localization of *T. denticola* in human gingival epithelial cells at 24 h after infection were reported [27]. Therefore, it

appears that cellular invasion by *T. denticola* is not specific to Ca9-22 cells. *T. denticola* bacteria were observed in the cells at 24 h after infection. In the present study, adherent cell was not completely ruled our because we evaluated the invasion by measuring [^{3}H]uridine. However, in CSLM analysis the invasion level of wild type was frequent and the frequency of invasion was significantly reduced by staurosporin or MßCD. These results indicate that the results evaluated by [^{3}H]uridine reflect invasion of *T. denticola* although it has limitation because of measuring method.

In our preliminary kinetic assays, the entrance of *T. denticola* into gingival epithelial cells started soon after bacterial exposure and increased thereafter up to 2 h, suggesting that approximately 2 h is required to complete the invasion process. An additional 22 h of incubation (after killing of extracellular bacteria) resulted in about 56.1% retention of intracellular *T. denticola* estimated by [^{3}H]uridine levels (Fig. 3A). *T. denticola* cells were still found within the host cell 24 h after infection (Fig. 3B). We extended our CSLM observation further, but *T. denticola*-shaped cells did not show significant reduction for 2 days (data not shown). As for the intracellular fate, *T. denticola* was reported not to survive inside fibroblasts beyond 24h [44]. Previous report showed the resistance of *T. denticola* to endolysosomal degradation [27]. In *T. denticola* infected cells, the overall mean fluorescence intensity of the total *T. denticola* was not changed while that of *Fusobacterium nucleatum* was lost by 90% after 24h in *F. nucleatum* infetcted cells. *T. denticola* was suggested to limit fusion of lysosomes to phagosomes in human neutrophils [45]. It is possible that the *T. denticola* cells detected after day 1 were not viable but the limitation in the

fusion of lysozomes into phagosomes also results in the presence of *T. denticola* cells in the epithelial cells. Further analysis is required to confirm the viability and intracellular fate of *T. denticola*.

In our experiments, the serine protease inhibitor PMSF inhibited invasion by *T. denticola* and a low level of invasion was observed in a dentilisin-deficient mutant. These results suggest that dentilisin is involved in invasion by *T. denticola.* In these experiments, we used [^{3}H]uridine to evaluate the numbers of *T. denticola* because it is virtually impossible to evaluate colony forming unit of relatively low numbers of *T. denticola*. The CLSM observation was supported by the results with the antibiotic protection assay. The invasion levels of the dentilisin deficient mutant and wild type cells were significantly different. With regard to the mechanism(s) by which dentilisin is involved in this cellular invasion process, dentilisin may contribute to the invasion process by facilitating adherence or stimulation of HGEC. Dentilisin has been reported to be involved in adherence to glutaraldehyde-fixed periodontal ligament epithelial cells [8]. It is possible that the reduction of adherence activity by deletion of dentilisin affected invasion, although further studies including the potential activation of lipid rafts by dentilisin are required.

Msp produces several effects on host cells, such as pore forming activity in host cell membranes, induction of acute Ca2+ transients and the disassembly of ventral actin in fibroblasts [46-48]. Thus, we speculated that Msp might also be involved in host cell invasion. However, our observation that the Msp deficient mutant exhibited similar invasive ability as the wild-type strain suggested that

Msp does not play key role in invasion.

Metabolic inhibition assays showed that invasion by *T. denticola* might require the participation of cholesterol and protein kinases (Table 2). Recently, accumulating evidence has suggested that lipid rafts serve as entry sites into host cells for some bacteria [49, 50]. To examine the possible involvement of lipid rafts in invasion by *T. denticola*, we perturbed raft formation by treating Ca9-22 cells with the lipid raft disrupting reagent, MßCD. *T. denticola* colocalization with lipid rafts was observed in untreated HGEC while colocalization was not observed in the MßCD treated cells (Fig. 3). However, adherence of *T. denticola* was not affected by MßCD treatment. Depletion of cellular cholesterol using MßCD also resulted in significant inhibition of *T. denticola* invasion as assessed by the protection assay (Table 2). These data implicate a significant role of lipid rafts in host cell invasion by *T. denticola*. Lipid rafts are reportedly involved in cholesterol homeostasis, endocytosis, and cell signalling [49, 51]. It was reported that uptake of *P. gingivalis* membrane vesicle coated beads by CHO cell requires lipid raft components for actin organization, with Rho GTPase Rac1[50, 52]. In our previous report, we indicated that lipid raft-mediated process was reported to be at least one of the potential mechanisms involved in fusobacterium-modulated host cell invasion by *P. gingivalis*[53]. *Treponema lecithinolyticum* induced IFN-ß expression and subsequent up-regulation of IP-10 and RANTES via TBK1/IRF-3/STAT-1 signaling secondary to lipid raft activation [54]. These reports suggest that component of lipid raft is involved in signal for internalization of *P. gingivalis* or cytokine production by oral pathogen. Inactivation of surface protease of *T. denticola* affected the rate of

invasion. In *T. denticola*, dentilisin and Msp were associated with surface structure [29]. It is possible that signal induction by activation of lipid rafts components by *T. denticola* surface component may be involved in invasion of HGEC. Possible activation of lipid rafts by dentilisin remains to be investigated.

Our results indicating that MßCD and staurosporine did not completely inhibit *T. denticola* invasion suggest that HGEC-*T. denticola* interactions may also involve non-raft membrane regions. Adherence of *T. denticola* to HGEC was also observed in MßCD treated cells. Our experiments using metabolic inhibitors further suggested the involvement of actin rearrangement in invasion but its contribution is relatively small. Further analysis of signal transduction in the infected cells is required in order to clarify the mechanism of invasion by this microorganism.

In the wound healing assay, the cells infected with *T. denticola* ATCC35405 attenuated the migration of the infected Ca9-22 cells compared to non-infected, msp deficient mutant and dentilisin deficient mutant infected cells. Proteolytic activity of *P. gingivalis* affects the migration of the epithelial cells after invasion of the cells [17]. Msp was reported to affect actin remodeling and reorganization in fibroblasts or neutrophils and is involved in the chemotaxis of neutrophils [7, 48]. These results indicated that dentilisin and Msp are involved in impairment of the migration of gingival epithelial cells. Msp and dentilisin were reported to organize into an oligomeric protein complex [8] and inactivation of dentilisin impaired oligomeric protein formation [29]. The migration inhibition was dramatically decreased in the presence of the Msp deficient mutant although the

spirochete invasion efficiency was similar. These results suggest that the migration inhibitory effect also requires Msp-dentilisin complex.

In summary, *T. denticola* invades HGEC and dentilisin is significantly involved in this process. Utilization of lipid rafts in HGEC and subsequent signal transduction are suggested to be involved in invasion. The ability of intracellular invasion by *T. denticola* to delay wound healing may also be an important virulence factor in the initiation and development of periodontal disease.

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References

[1] Pihlstrom BL, Michalowicz BS, Johnson NW. Periodontal diseases. Lancet. 2005;366:1809-20.

[2] Teles RP, Haffajee AD, Socransky SS. Microbiological goals of periodontal therapy. Periodontol 2000. 2006;42:180-218.

[3] Ellen RP, Galimanas VB. Spirochetes at the forefront of periodontal infections. Periodontol 2000. 2005;38:13-32.

[4] Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RLJ. Microbial complexes in subgingival plaque. J Clin Periodontol. 1998;25:134-44.

[5] Ishihara K. Virulence factors of *Treponema denticola*. Periodontol 2000. 2010;54:117-35.

[6] Nussbaum G, Ben-Adi S, Genzler T, Sela M, Rosen G. Involvement of Toll-like receptors 2 and 4 in the innate immune response to *Treponema denticola* and its outer sheath components. Infect Immun. 2009;77:3939-47.

[7] Puthengady Thomas B, Sun CX, Bajenova E, Ellen RP, Glogauer M. Modulation of human neutrophil functions in vitro by *Treponema denticola* major outer sheath protein. Infect Immun. 2006;74:1954-7.

[8] Fenno JC, Hannam PM, Leung WK, Tamura M, Uitto VJ, McBride BC. Cytopathic effects of the major surface protein and the chymotrypsinlike protease of *Treponema denticola*. Infect Immun. 1998;66:1869-77.

[9] Yamazaki T, Miyamoto M, Yamada S, Okuda K, Ishihara K. Surface protease of *Treponema denticola* hydrolyzes C3 and influences function of polymorphonuclear leukocytes. Microbes Infect. 2006;8:1758-63.

[10] McDowell JV, Huang B, Fenno JC, Marconi RT. Analysis of a unique interaction between the complement regulatory protein factor H and the periodontal pathogen *Treponema denticola*. Infect Immun. 2009;77:1417-25.

[11] Grenier D, Uitto VJ, McBride BC. Cellular location of a *Treponema denticola* chymotrypsinlike protease and importance of the protease in

migration through the basement membrane. Infect Immun. 1990;58:347-51.

[12] Finlay BB, Cossart P. Exploitation of mammalian host cell functions by bacterial pathogens. Science. 1997;276:718-25.

[13] Meyer DH, Mintz KP, Fives-Taylor PM. Models of invasion of enteric and periodontal pathogens into epithelial cells: a comparative analysis. Crit Rev Oral Biol Med. 1997;8:389-409.

[14] Tribble GD, Lamont RJ. Bacterial invasion of epithelial cells and spreading in periodontal tissue. Periodontol 2000. 2010;52:68-83.

[15] Amano A. Disruption of epithelial barrier and impairment of cellular function by *Porphyromonas gingivalis*. Front Biosci. 2007;12:3965-74.

[16] Amano A, Furuta N, Tsuda K. Host membrane trafficking for conveyance of intracellular oral pathogens. Periodontol 2000. 2010;52:84-93.

[17] Nakagawa I, Inaba H, Yamamura T, Kato T, Kawai S, Ooshima T, et al. Invasion of epithelial cells and proteolysis of cellular focal adhesion components by distinct types of *Porphyromonas gingivalis* fimbriae. Infect Immun. 2006;74:3773-82.

[18] Carranza FA, Jr., Saglie R, Newman MG, Valentin PL. Scanning and transmission electron microscopic study of tissue-invading microorganisms in localized juvenile periodontitis. J Periodontol. 1983;54:598-617.

[19] Listgarten MA, Lewis DW. The distribution of spirochetes in the lesion of acute necrotizing ulcerative gingivitis: an electron microscopic and statistical survey. J Periodontol. 1967;38:379-86.

[20] Manor A, Lebendiger M, Shiffer A, Tovel H. Bacterial invasion of periodontal tissues in advanced periodontitis in humans. J Periodontol. 1984;55:567-73.

[21] Riviere GR, Weisz KS, Adams DF, Thomas DD. Pathogen-related oral spirochetes from dental plaque are invasive. Infect Immun. 1991;59:3377-80.

[22] Colombo AV, da Silva CM, Haffajee A, Colombo AP. Identification of intracellular oral species within human crevicular epithelial cells from

subjects with chronic periodontitis by fluorescence in situ hybridization. J Periodontal Res. 2007;42:236-43.

[23] Lux R, Miller JN, Park NH, Shi W. Motility and chemotaxis in tissue penetration of oral epithelial cell layers by *Treponema denticola*. Infect Immun. 2001;69:6276-83.

[24] Chi B, Qi M, Kuramitsu HK. Role of dentilisin in*Treponema denticola* epithelial cell layer penetration. Res Microbiol. 2003;154:637-43.

[25] Ellen RP, Ko KS, Lo CM, Grove DA, Ishihara K. Insertional inactivation of the *prtP* gene of *Treponema denticola* confirms dentilisin's disruption of epithelial junctions. J Mol Microbiol Biotechnol. 2000;2:581-6.

[26] Ji S, Shin JE, Kim YC, Choi Y. Intracellular degradation of *Fusobacterium nucleatum* in human gingival epithelial cells. Mol Cells. 2010;30:519-26.

[27] Shin J, Choi Y. The fate of *Treponema denticola* within human gingival epithelial cells. Mol Oral Microbiol. 2012;27:471-82.

[28] Chan EC, Siboo R, Keng T, Psarra N, Hurley R, Cheng SL, et al. *Treponema denticola* (ex Brumpt 1925) sp. nov., nom. rev., and identification of new spirochete isolates from periodontal pockets. Int J Syst Bacteriol. 1993;43:196-203.

[29] Ishihara K, Kuramitsu HK, Miura T, Okuda K. Dentilisin activity affects the organization of the outer sheath of *Treponema denticola*. J Bacteriol. 1998;180:3837-44.

[30] Sano Y, Okamoto-Shibayama K, Tanaka K, Ito R, Shintani S, Yakushiji M, et al. Dentilisin involvement in coaggregation between *Treponema denticola* and *Tannerella forsythia*. Anaerobe. 2014;30:45-50.

[31] Ishihara K, Kuramitsu HK. Cloning and expression of a neutral phosphatase gene from *Treponema denticola*. Infect Immun. 1995;63:1147-52.

[32] Horikoshi M, Kimura Y, Nagura H, Ono T, Ito H. A new human cell line derived from human carcinoma of the gingiva. I. Its establishment and morphological studies. Jpn J Oral Surg. 1974;20:100-6 in Japanese.

[33] Hirose K, Isogai E, Mizugai H, Ueda I. Adhesion of *Porphyromonas gingivalis* fimbriae to human gingival cell line Ca9-22. Oral Microbiol Immunol. 1996;11:402-6.

[34] Takeuchi H, Setoguchi T, Machigashira M, Kanbara K, Izumi Y. Hydrogen sulfide inhibits cell proliferation and induces cell cycle arrest via an elevated $p21^{Cip1}$ level in Ca9-22 cells. J Periodontal Res. 2008;43:90-5.

[35] Furuta N, Fujita N, Noda T, Yoshimori T, Amano A. Combinational soluble N-ethylmaleimide-sensitive factor attachment protein receptor proteins VAMP8 and Vti1b mediate fusion of antimicrobial and canonical autophagosomes with lysosomes. Mol Biol Cell. 2010;21:1001-10.

[36] Saito A, Inagaki S, Ishihara K. Differential ability of periodontopathic bacteria to modulate invasion of human gingival epithelial cells by *Porphyromonas gingivalis*. Microb Pathog. 2009;47:329-33.

[37] Inagaki S, Onishi S, Kuramitsu HK, Sharma A. Porphyromonas gingivalisvesicles enhance attachment, and the leucine-rich repeat BspA protein is required for invasion of epithelial cells by "*Tannerella forsythia*". Infect Immun. 2006;74:5023-8.

[38] Smart EJ, Anderson RG. Alterations in membrane cholesterol that affect structure and function of caveolae. Methods Enzymol. 2002;353:131-9.

[39] Rosenshine I, Ruschkowski S, Finlay BB. Inhibitors of cytoskeletal function and signal transduction to study bacterial invasion. Methods Enzymol. 1994;236:467-76.

[40] Inaba H, Kawai S, Nakayama K, Okahashi N, Amano A. Effect of enamel matrix derivative on periodontal ligament cells in vitro is diminished by *Porphyromonas gingivalis*. J Periodontol. 2004;75:858-65.

[41] Colombo AV, Silva CM, Haffajee A, Colombo AP. Identification of oral bacteria associated with crevicular epithelial cells from chronic periodontitis lesions. J Med Microbiol. 2006;55:609-15.

[42] Marttila E, Jarvensivu A, Sorsa T, Grenier D, Richardson M, Kari K, et al. Intracellular localization of *Treponema denticola* chymotrypsin-like proteinase in chronic periodontitis. J Oral Microbiol. 2014;6.

[43] Velsko IM, Chukkapalli SS, Rivera-Kweh MF, Zheng D, Aukhil I, Lucas AR, et al. Periodontal pathogens invade gingiva and aortic adventitia and elicit inflammasome activation in alphavbeta6 integrin-deficient mice. Infect Immun. 2015.

[44] Miao D, Godovikova V, Qian X, Seshadrinathan S, Kapila YL, Fenno, JC. *Treponema denticola* upregulates MMP-2 activation in periodontal ligament cells: Interplay between epigenetics and periodontal infection. Arch Oral Biol. 2014;59: 1056-1064.

[45] Boehringer H, Berthold PH, Taichman NS. Studies on the interaction of human neutrophils with plaque spirochetes. J Periodontal Res. 1986;21:195-209.

[46] Egli C, Leung WK, Muller KH, Hancock RE, McBride BC. Pore-forming properties of the major 53-kilodalton surface antigen from the outer sheath of *Treponema denticola*. Infect Immun. 1993;61:1694-9.

[47] Wang Q, Ko KS, Kapus A, McCulloch CA, Ellen RP. A spirochete surface protein uncouples store-operated calcium channels in fibroblasts: a novel cytotoxic mechanism. J Biol Chem. 2001;276:23056-64.

[48] Amin M, Ho AC, Lin JY, Batista da Silva AP, Glogauer M, Ellen RP. Induction of de novo subcortical actin filament assembly by *Treponema denticola* major outer sheath protein. Infect Immun. 2004;72:3650-4.

[49] Lafont F, Abrami L, van der Goot FG. Bacterial subversion of lipid rafts. Curr Opin Microbiol. 2004;7:4-10.

[50] Tsuda K, Furuta N, Inaba H, Kawai S, Hanada K, Yoshimori T, et al. Functional analysis of alpha5beta1 integrin and lipid rafts in invasion of epithelial cells by *Porphyromonas gingivalis* using fluorescent beads coated with bacterial membrane vesicles. Cell Struct Funct. 2008;33:123-32.

[51] Riethmüller J, Riehle A, Grassme H, Gulbins E. Membrane rafts in host-pathogen interactions. Biochim Biophys Acta. 2006;1758:2139-47.

[52] Furuta N, Tsuda K, Omori H, Yoshimori T, Yoshimura F, Amano A. *Porphyromonas gingivalis* outer membrane vesicles enter human epithelial cells via an endocytic pathway and are sorted to lysosomal compartments. Infect Immun. 2009;77:4187-96.

[53] Saito A, Kokubu E, Inagaki S, Imamura K, Kita D, Lamont RJ, et al. Porphyromonas gingivalisentry into gingival epithelial cells modulated by

Fusobacterium nucleatumis dependent on lipid rafts. Microb Pathog. 2012;53:234-42.

[54] Lee SH, Kim JS, Jun HK, Lee HR, Lee D, Choi BK. The major outer membrane protein of a periodontopathogen induces IFN-beta and IFN-stimulated genes in monocytes via lipid raft and TANK-binding kinase 1/IFN regulatory factor-3. J Immunol. 2009;182:5823-35.

Figure legends

Figure 1.

Invasion of *T. denticola* into HGEC visualized by CLSM following dual labeling and SEM images of *T. denticola* entering HGEC. Z stack of *T. denticola* invasion images in an orthogonal view by CLSM following dual labeling *T. denticola* ATCC35405 (A), *T. denticola* K1 (D) and *T. denticola* DMSP3 (E) invading Ca9-22 cells were stained red, while external bacteria were detected as green-yellow. The Ca9-22 cytoskeleton stained with phalloidin appeared blue. *T. denticola* ATCC35405 invasion composite images converted with "Iso surface" functions of the "Surpass" option on IMARIS software (B). SEM image of *T. denticola* penetrating into Ca9-22 cells from its ends (arrows) after 30-minutes exposure (C). Bar = 1μm.

Figure 2.

A: Kinetic parameters of *T. denticola* ATCC35405 invasion of HGEC. Percent invasion was calculated from the levels of bacteria recovered intracellularly after different incubation times. Error bars represent standard deviations (n=3). Bacteria were incubated with the epithelial cells for 2 h and the extracellular bacteria were killed with antibiotics. After washing to remove the antibiotics and extracellular bacteria, the cells were maintained for 0 h, 2 h, 10 h and 22 h. Survival rate was calculated as follows: Survavalrate = (live intracellular Td at indicated time) / (live Td at immediately after infection) x 100

B: Merged Z-plane image of *T. denticola* invading into HGEC 24 h after

infection. Ca9-22 cells were infected with *T. denticola* for 2 h, treated with antibiotics and incubated for 22 h.

Figure 3.

T. denticola colocalizes with lipid rafts in a cholesterol-dependent manner.

HGEC cultured on glass coverslips, were pretreated for 30 min at 37°C with medium only (A) or with 10 mM MßCD (B) to deplete cholesterol. Both groups were subsequently exposed to *T. denticola* (MOI= 100:1). Infection was allowed to proceed for 30 min, unattached bacteria removed by washing, followed by cell fixation and staining for GM1 (lipid raft marker) with Alexa Fluor 594-labelled CTB. The cells were then examined by CLSM.

Figure 4. Influence of infection by *T. denticola* on migration and proliferation of epithelial cells. Confluent layers of Ca9-22 cells were scratched with plastic tips and infected with *T. denticola* at m.o.i.100 for 2 h in MEM containing 10% serum. External nonadherent *T. denticola* were removed by washing the cells three times with MEM supplemented with antibiotics and epithelial cells were allowed to proliferate in the medium for 3 h. Values (wound closure rate) are shown as the means ± standard deviations (n = 9).

* $P < 0.05$ compared with cells without infection, † $P < 0.05$ compared with cells infected with *T. denticola* K1, § $P < 0.05$ compared with cells infected with *T. denticola* DMSP3

Table 1. *T. denticola* invasion of HGEC and the role of *T. denticola* surface proteins

Strain	Invasion level (%)
T. denticola ATCC 35405	3.00 ± 0.84
T. denticola ATCC 35405 with PMSF	1.14 ± 0.29*
T. denticola K1	1.31 ± 0.39*
T. denticola DMSP3	2.36 ± 0.25
E. coli ATCC 11775	0.01 ± 0.00*

The invasion level was expressed as the percentage of bacteria retrieved following antibiotic killing of external bacteria and HGEC lysis relative to the total number of input bacteria. Results reported as invasion levels (means ± standard errors) are representative of three independent experiments. *, $P < 0.05$ for comparison with *T. denticola* ATCC35405 invasion. *E. coli* ATCC 11775 was used as negative control.

Table 2. Effects of metabolic inhibitors on invasion by *T. denticola* 35405.

Inhibitor	Concentration	Target	Invasion (%)
Cytochalasin D	2 μg/ml	Actin polymerization	64.1 ± 2.9*
Cycloheximide	200 μg/ml	*de novo* protein synthesis	74.5 ± 3.7*
Staurosporine	5 μM	Protein kinase	40.6 ± 0.4*
Nocodazole	10 μM	Microtuble formation	80.1 ± 5.5*
Monodansylcadaverine	50 μM	Receptor binding	60.4 ± 1.4*
Wortmannin	10 μM	PI3Kinase	54.8 ± 2.3*
MβCD	10 mM	Cholesterol	33.8 ± 5.7*
w/o Inhibitor			100.0

Expressed as the percentage of the control level without any inhibitor (mean ± standard deviation). * $P < 0.05$ for comparison with *T. denticola* ATCC35405 invasion without inhibitor