

Insertional Inactivation of the *prtP* Gene of *Treponema denticola* Confirms Dentilisin's Disruption of Epithelial Junctions

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Abstract

The purified chymotrypsin-like protease of *Treponema denticola*, designated dentilisin or PrtP (DDBJ accession no. D83264), can disrupt cell-cell junctions and impair the barrier function of epithelial monolayers *in vitro*. Serine protease inhibitors block these effects. Yet, the protease is apparently less significant in perturbing intracellular signaling pathways and cytoskeletal rearrangement in fibroblasts. The purpose of this study was to use a PrtP-deficient mutant of *T. denticola* to confirm that the cytopathic effects of whole bacteria and its outer membrane on epithelial cell junctions were primarily accounted for by the activity of this protease. The *prtP* gene of ATCC 35405 was inactivated by insertion of an erythromycin-resistance cassette, yielding mutant K1. In contrast to wildtype ATCC 35405, mutant K1 grew in tight cell aggregates; the cells had a disrupted outer sheath, as determined by electron microscopy. When compared by silver stained SDS-PAGE of sonicated extracts of whole cells, the extract of mutant K1 was missing a band at ~90 kDa that was present in the wildtype ATCC 35405 strain. Whole cells and Triton X-100 outer membrane (OM) extracts of K1 and the wildtype strains were compared 1) for SAAPNA degrading activity by a colorimetric assay, 2) for stress fiber disruption in human gingival fibroblasts (HGF) by fluorescence microscopy of TRITC-phalloidin stained cells, and 3) the OM extracts only for perturbation of HEP-2 epithelial monolayers by electrical cell-substrate impedance sensing (ECIS). Mutant K-1 cells and OM had no SAPPNA degrading activity that is characteristic of dentilisin. K1 cells had HGF stress fiber disrupting activity ($86 \pm 4.5\%$ of HGFs affected) equivalent to both 35405 wildtype strains ($84 \pm 3.9\%$ and $71 \pm 14.1\%$ of HGF, respectively). Yet, mutant K1 OM had diminished stress fiber disrupting activity ($12.9 \pm 4.6\%$ of HGF) compared with its parent 35405's OM ($94.6 \pm 2.9\%$). The major cytopathogenic difference

between the K1 mutant and wildtype strains was in their OM's effect on epithelial cell junctions. ATCC 35405 OM completely disrupted epithelial resistance in a concentration – dependent manner; mutant K1 OM had negligible effects. These data confirm that inactivation of the *prtP* gene completely reverses *T. denticola*'s disruption of epithelial junctions, but there are pleiotropic effects of the mutation that may account for its apparently diminished effects on the cytoskeleton of HGF when the cells were challenged with OM extracts.

Introduction

Treponema denticola is the most frequently cultivated oral spirochete. Clinical microbiology studies associate it among a few other oral anaerobes in the mixed gingival sulcus microbiota that contribute to the etiology of periodontitis in adults (Socransky *et al.*, 1998). Due to recent progress in the genetic transformation and mutation of *T. denticola*, it has become a very useful model for testing the function of treponemal proteins and virulence factors (Chi *et al.*, 1999). The outer sheath of *Treponema denticola* contains a few proteins that have been investigated for their cytopathogenicity in human cell culture systems (Fenno and McBride, 1998; Fenno *et al.*, 1998; Ellen 1999). Most prominent among these are the major surface protein, Msp, and a cell-associated serine protease, dentilisin or PrtP. The genes encoding both of these proteins have been sequenced (Ishihara *et al.*, 1996; Fenno *et al.*, 1996). The *prtP* gene of type strain ATCC 35405 has been inactivated by insertional mutagenesis, and the *prtP*-deficient mutant is relatively less virulent than its parent strain in a mouse abscess model (Ishihara *et al.*, 1998).

Whole cells of *T. denticola*, outer membrane (OM) extracts of *T. denticola*, purified OM proteins and protein complexes cause many cytopathic effects in cultured human cells (Fenno *et al.*, 1998; Ellen, 1999). Challenge of gingival fibroblasts with *T. denticola* cells and its OM perturb inositol phosphate and calcium signaling pathways that affect the actin cytoskeleton (Yang *et al.*, 1998; Ko *et al.*, 1998a). The fibroblasts experience changes in pools of F-actin and stress fibers; they round up, form surface blebs, and eventually detach from the substratum (Weinberg and Holt, 1990; Baehni *et al.*, 1992). Cultured epithelial cells challenged with *T. denticola* cells or OM extracts show evidence of perturbed volume regulation, shrink, and experience reduced expression of F-actin and junctional proteins like desmoplakin II (DeFilippo *et al.*, 1995; Uitto *et al.*, 1995). There is disruption of cell-cell junctions, desmosomes, and barrier functions of epithelium, both in monolayers and in multiple layers resembling stratified squamous epithelium (Uitto *et al.*, 1995; Ko *et al.*, 1998b). Such changes are characteristic of the sulcular epithelium of inflamed gingival tissues.

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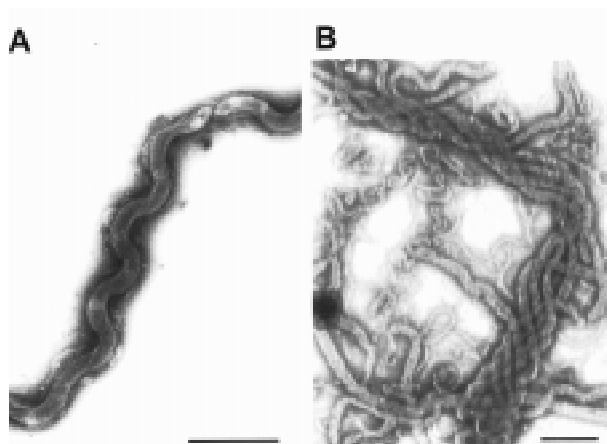


Figure 1. Negative-stained transmission electron photomicrographs of A) ATCC 35405 and B) *prtP*-inactivated mutant K1 (Bar = 1 μ m)

A protease with the characteristics of dentilisin appears to be responsible for some of the cytopathogenic effects of *T. denticola*. Experiments using specific protease inhibitors and/or heat at a temperature at which dentilisin is stable relative to other more labile proteins have indicated that the disruption of epithelial barriers and the degradation of endogenous fibronectin on the surface of fibroblasts by *T. denticola* are mostly due to its chymotrypsin-like, serine protease activity (Ellen *et al.*, 1994; Ko *et al.*, 1998b). In contrast, intracellular signaling events that promote actin rearrangement and the enhancement of actin-dependent collagen phagocytosis by fibroblasts seem to be perturbed by protein(s) phenotypically distinct from the protease (Ko *et al.*, 1998a; Yang *et al.*, 1998; Battikhi *et al.*, 1999). The purpose of this investigation was to use a PrtP-deficient isogenic mutant to test the hypothesis that disruption of epithelial junctions is mediated by dentilisin and that depolymerization of stress fibers in gingival fibroblasts is not.

Results and Discussion

Morphology and SAAPNA-Degrading Activity of the *prtP*-Inactivated Mutant K1

Insertional inactivation of the *prtP* gene caused differences in the cellular morphology of mutant K1 when compared with the wildtype strain ATCC 35405 (Figure 1). The mutant cells grew in tightly wound aggregates that were difficult to disperse. The outer sheath was diffuse and disrupted. The flagella extended through the remaining outer membrane material and thus did not have a periplasmic location that is characteristic for the flagella of wildtype spirochetes. These changes in the mutant K1 probably reflect gross cell structural outcomes due to the molecular disorganization of proteins comprising the outer sheath, as documented by Ishihara *et al.* (1998). They noted that in addition to loss of prolyl-phenylalanine-specific serine protease activity, mutant K1 differed from the parent strain in its growth characteristics, decreased hydrophobicity, and altered coaggregation with *Fusobacterium nucleatum*. Changes in physical properties of the outer sheath, as exemplified by the latter two observations and the protrusion of normally periplasmic flagella may account for

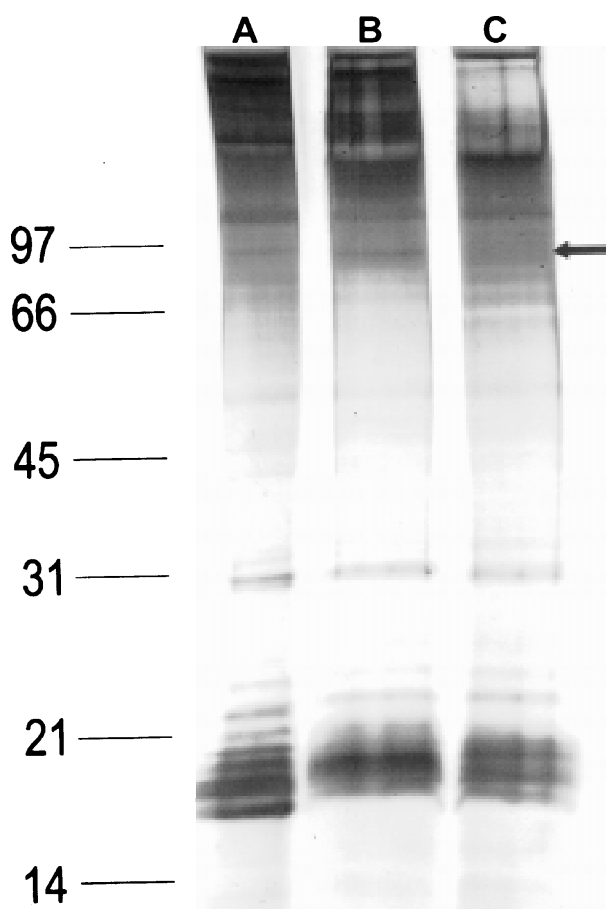


Figure 2. Silver stained PAGE gel of soluble whole cell sonicated extracts of lane A) ATCC 35405 (UToronto); lane B) ATCC 35405 (Tokyo); and lane C) PrtP-deficient mutant K1. The position of molecular size standards (broad-range SDS-PAGE standards, BioRad) are shown in kilodaltons at the left. The lane containing the extract of mutant K1, lane C, is missing a band at ~ 90 kDa that is found in the other two lanes (arrow).

Table 1. SAAPNA-Degrading Activity

<i>T. denticola</i> whole cells	Enzyme activity (μ g/ml) ¹
ATCC 35405 (UToronto) ²	1.00 \pm 0.02
Parent 35405 (Tokyo) ²	1.31 \pm 0.07
PrtP-deficient mutant K-1	0.01 \pm 0.03
<i>T. denticola</i> outer membrane extracts ³	
ATCC 35405 (whole cell internal standard)	1.23 \pm 0.04
ATCC 35405 (UToronto)	3.05 \pm 0.07
ATCC 35405 (UToronto) – sonic. ⁴	6.71 \pm 1.60
Parent 35405 (Tokyo Dent. Coll.)	2.37 \pm 0.01
Parent 35405 (Tokyo Dent. Coll.) – sonic.	2.71 \pm 0.07
PrtP-deficient mutant K-1	0.07 \pm 0.07
PrtP-deficient mutant K-1 – sonic.	0.07 \pm 0.07

¹SAAPNA-degrading activity equivalent to that of Sigma chymotrypsin standard in μ g/ml

²Strains designated 35405 were derived from the type strain but subcultured in different laboratories; mutant K-1 and its parent strain are from Tokyo Dental College, Chiba, Japan

³The concentration of OM extracts was adjusted to that extracted from whole cells of the internal standard (~2.5 \times 10⁹ cells/ml).

⁴Suspensions of OM extracts were dispersed by sonication immediately before use.

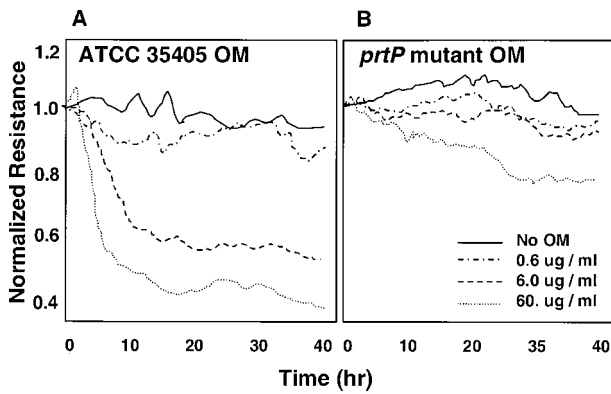


Figure 3. Normalized resistance measurements (retraced for illustration) of a confluent HEP-2 monolayer upon addition of different concentrations of OM extract of A) ATCC 35404, 20 hr data from Ko et al., 1998b; and B) *prtP*-inactivated mutant K1, 40 hr data.

the tightly bundled arrangement of K1 cells. Dentilisin is expressed in the outer sheath of *T. denticola* as part of a protein complex (Ishihara et al., 1996; Fenno et al., 1998) and interacts with Msp and possibly with other yet to be identified proteins. Thus, a mutation affecting the expression, post-translational modification, transport, or integration into the outer membrane of one of these proteins probably affects the surface organization or even the function of the other(s). The obvious pleiotropic effects arising from the insertional inactivation of the *prtP* gene, especially the tight clumping of whole cells, prompted us to use primarily the OM extracts for most of our cell biology experiments. Moreover, interpretation of cell challenge experiments and experimental infections in animals (Ishihara et al., 1998) must take the possibility of such pleiotropic effects into consideration.

Interruption of the *prtP* gene with the *ermF/ermAM* cassette interfered totally with the expression of dentilisin. Upon electrophoresis of sonic extracts, polyacrylamide gels of the K1 mutant lacked a polypeptide band with a mobility equivalent to ~90 kDa (Figure 2), which was close to the position seen for the active native protease but missing from K1 in zymograms published previously (Ishihara et al., 1998). The whole cells and OM extract of the K1 mutant used for cell challenge experiments in this project were clearly deficient in SAAPNA-degrading activity (Table 1).

Table 2. ECIS Data from Confluent Cell Layers of HEP-2 Cells Challenged with OM Extracts of ATCC 35405 and *prtP*-Inactivated Mutant K1

	Protein Conc. ($\mu\text{g/ml}$)	In-phase Voltage ¹ (mV)	% (of control)
Wildtype OM (20 hr) ²	0	7.82 \pm 0.52	100
	0.60	5.51 \pm 0.30	70
	6.00	4.54 \pm 0.53	58
	60.0	2.42 \pm 0.40	31
Mutant K1 OM (40 hr)	0	7.86 \pm 0.59	100
	0.60	7.58 \pm 0.44	96
	6.00	7.80 \pm 0.11	99
	60.0	5.47 \pm 0.98	69

¹Average in-phase voltage of cell layer \pm standard deviation (junctional resistance)

²Data for wildtype OM from Ko et al., 1998b

Identical peptidase activity has been ascribed in the past to a chymotrypsin-like protease complex of approximately 95 kDa. The protease encoded by *prtP* is expressed as a 72 kDa polypeptide, and its expression depends on a co-transcribed 43 kDa protein encoded by the gene immediately upstream (Ishihara et al., 1996; Ishihara unpublished data). The 72 kDa serine protease, PrtP, was designated 'dentilisin' to reflect its sequence similarity to proteases in the subtilisin family, especially among the well conserved catalytic residues (Ishihara et al., 1996). Originally, it was called a 'chymotrypsin-like' protease based mostly on its ability to degrade a broad range of native proteins and its pattern of sensitivity to enzyme inhibitors (Uitto et al., 1988; Grenier et al., 1990).

Diminished Disruption of Cell Junctions by the *prtP*-Deficient Mutant

In this paper, we have extended our previous work on *T. denticola* as the bacterial challenge in a new application of electrical cell-substrate impedance sensing (ECIS) (Ko et al., 1998b). The model relies on continuous, real-time collection of impedance data and analysis of variables contributing to the electrical resistance established by epithelial cells in monolayers grown directly on small electrode arrays in specially constructed dishes (Lo et al., 1995). Previous work in Uitto's and our laboratory identified the 'chymotrypsin-like' protease as a significant factor for breaching epithelial barriers (Uitto et al., 1995; Ko et al., 1998b). The conclusions were based on experiments in which cultured oral and laryngeal epithelial cell lines were challenged with the purified native protease or with *T. denticola* cells and OM extracts that had been pretreated with protease inhibitors. The results of our new experiments that compared the activity of OM extracts from the K1 mutant and wildtype strains in the ECIS model confirmed that dentilisin is essential for the disruption of epithelial junctions by *T. denticola* (Figures 3 and 4; Table 2). The OM extract of the wildtype strain caused a concentration-dependent reduction in electrical impedance within a 20 hour experimental period, whereas there was only a modest reduction in impedance by 40 hr exposure to the K1 mutant OM (Figure 3). The mean value for in-phase voltage (junctional resistance) taken at 40 hr cell exposure to mutant K1 OM extract showed a reduction only at the highest concentration, which was equivalent to 60 $\mu\text{g/ml}$ protein (Table 2). This was 100 times the concentration needed for the wildtype OM extract to achieve an equivalent reduction in junctional resistance and thus may reflect toxigenic effects of high concentration, long duration exposure to OM components other than dentilisin. In contrast, the mutation in *prtP* had no distinct effect on cell micromotion (data not shown). In experiments with a reverse design, in which the OM extracts were added to the electrode wells along with the cells, HEP-2 cells seeded in the presence of the K1 mutant OM were able to form a monolayer of high electrical resistance, whereas the cells never attached completely to form a confluent monolayer when challenged with the highest two concentrations of the wildtype OM (Figure 4).

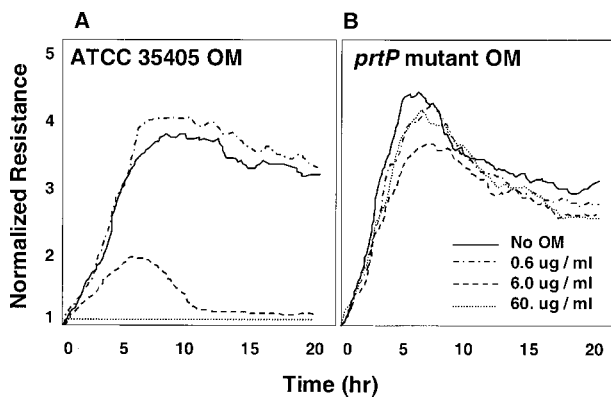


Figure 4. Normalized resistance measurements (retraced for illustration) of HEp-2 cell cultures that were seeded and grown in the presence of OM extracts of *T. denticola* strains A) ATCC 35405 and B) *prtP*-inactivated mutant K1.

Partially Diminished Effect of the *prtP*-Inactivated Mutant OM on Stress Fibers in HGF

Inactivation of the *prtP* gene had no effect on *T. denticola* whole cells' capacity to alter stress fiber patterns in cultured HGF. Cell suspensions of the mutant K1, even those dispersed repeatedly through a syringe needle, caused an increase in the percentage of HGF with altered stress fibers that was equivalent to that caused by suspensions of ATCC 35405 (UToronto) and 35405 (Tokyo), its parent strain (Figure 5). However, when the HGF were challenged with OM extracts, there was a reduced effect on stress fiber integrity by the OM of the K1 mutant compared with the OM extracts of the wildtype strains, suggesting that dentilisin can affect communication of stress fibers with the ECM in some way (Figure 6). This finding is inconsistent with our previous work in which we found that the serine protease inhibitor PMSF had no effect on the capacity of

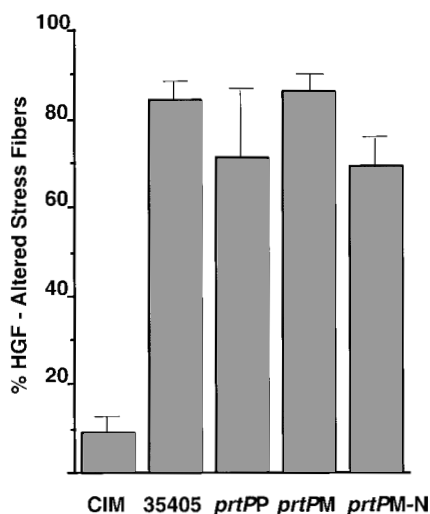


Figure 5. Histogram comparing the ability of *T. denticola* cell suspensions and treponeme-free CO₂-independent medium control (CIM) to disrupt TRITC-phalloidin stained stress fibers in human gingival fibroblasts (HGF). ATCC 35405, the UToronto derivative of the type strain; *prtPP*, ATCC 35405 (Tokyo), the parent strain; *prtPM*, mutant K1; and *prtPM-N*, syringe needle-dispersed mutant K1.

T. denticola to cause rapid cytoskeletal rearrangement under the same experimental conditions used herein (Yang *et al.*, 1998). Indeed, the OM of the wildtype strain interferes with neither the binding of collagen-coated beads to the dorsal surface of HGF nor Ca²⁺ flux through integrin-gated channels of their plasma membrane (Ko *et al.*, 1998a). Perhaps dentilisin released from the treponemal surface in shed OM fragments can more readily degrade noncollagenous ECM proteins at the edges of cell sheets or in the substratum. Its absence from the OM of the mutant K1 might, then, explain the reduction in stress fiber perturbation on this account. Indeed, PMSF is known to inhibit the subsequent detachment of HGF from the substratum that occurs in response to high densities of *T. denticola*, usually very late in the time period used in our stress fiber assay (Baehni *et al.*, 1992).

Although diminished, the OM of mutant K1 still retained much more stress fiber disrupting activity than the OM-free control, especially after the K1 OM suspension was dispersed by sonication. The relatively small size of the suspended OM particles, especially when sonicated, combined perhaps with the reduced hydrophobicity of the mutant's surface (Ishihara *et al.*, 1998), may foster the more rapid diffusion of its nonproteolytic active components between and under the HGF. Whole treponemes are rarely if ever detected under the HGF during the time period used in our assay (Baehni, 1992). Yet, shed outer membrane fragments have been detected by immunofluorescence between the cells in confluent epithelial cultures that had been challenged with suspensions of whole bacteria (Uitto *et al.*, 1995). Therefore, *T. denticola*'s rapid effect on the HGF cytoskeleton may be more an immediate cellular response to other OM proteins than an outcome secondary to direct proteolytic effects of dentilisin, which may be more significant at later stages. It is conceivable that one of these proteins might be Msp complex, which is known to diffuse into mammalian plasma membranes and to produce short-lived alterations in ion conductivity (Mathers *et al.*, 1996). Although potentially affected by pleiotropic outcomes of *prtP* inactivation, Msp or other bioactive proteins still retained in the OM may account for the residual stress fiber perturbing activity, albeit diminished, of the mutant K1 OM extract.

The Relevance of Dentilisin to Tissue Changes in Periodontal Disease

Maintenance of healthy periodontal tissues relies on homeostatic remodeling of gingival connective tissues and a selectively exclusive epithelial barrier that lines the gingival crevice. When pathogenic bacteria colonize the crevice in sufficient mass to overwhelm host defenses, their metabolic products and fragments shed from their surfaces cause a breach in the epithelium, and they initiate an inflammatory cascade that drives tissue responses toward net resorption. Cytoskeletal perturbation is a hallmark of *T. denticola*'s cytopathogenic effects on HGF (Ellen, 1999). This would be expected to alter the fibroblasts' physiologic capacity to phagocytose and remodel collagen (Battikhi *et al.*, 1999) and to migrate tractionally in contact with extracellular matrix (ECM) proteins. In this paper, some data, from the OM challenge experiments only, suggest that dentilisin may account for some of the bacterially induced actin rearrangement in HGF. Yet, such an interpretation raises serious caveats: 1) the pleiotropic

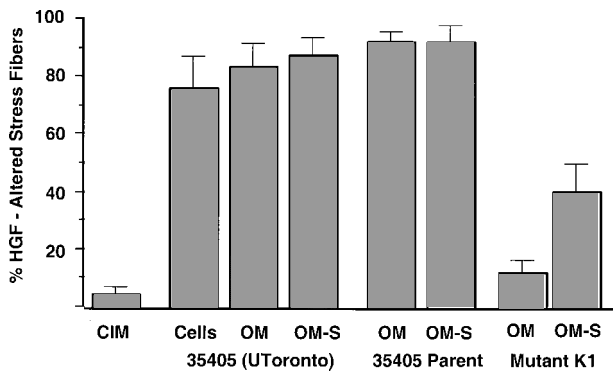


Figure 6. Histogram comparing the ability of *T. denticola* outer membrane (OM) extracts and treponeme-free CO₂-independent medium control (CIM) to perturb TRITC-phalloidin stained stress fibers in human gingival fibroblasts (HGF). Cells, an internal standard of ATCC 35405 (UToronto) cell suspension similar to that used in figure 5; OM-S, sonicated OM extract.

effects of *prtP* inactivation on the expression and organization of other OM proteins (Ishihara *et al.*, 1998); and 2) our previous findings that i) loss of stress fiber integrity, ii) changes in collagen phagocytosis, and iii) diminution of calcium and inositol phosphate responses to *T. denticola* OM extracts were all unaffected by the serine protease inhibitor PMSF, which blocked SAAPNA-degrading activity, and they were inhibited by heating at 60° C, a temperature at which SAAPNA-degrading activity is relatively stable (Yang *et al.*, 1998; Ko *et al.*, 1998a, Battikhi *et al.*, 1999). It is more likely that the intracellular effects of *T. denticola* dentilisin on the HGF stress fibers are secondary to its degradation of ECM proteins that communicate with the cell surface (Grenier, 1990; Ellen *et al.*, 1994), eventually leading to cell detachment from the substratum (Baehni *et al.*, 1992). Degradation of ECM components would be expected to interfere with the locomotion of HGF and their progenitors during wound healing and physiologic remodeling of the gingival connective tissues.

By virtue of their location at the interface of the subgingival bacterial biofilm and the gingiva, *T. denticola* and other treponemes are in a position to contribute significantly to direct damage to the epithelium. Evidence from several *in vitro* cell culture models that have tested whole bacteria, OM extracts and the purified native protease (Uitto *et al.*, 1995; DeFilippo *et al.*, 1995; Ko *et al.*, 1998b), including the comparison of OM extracts from a *prtP*-inactivated mutant and the wildtype strains in this study, demonstrate clearly that dentilisin has the capacity to disrupt cell-cell junctions in the epithelium. Moreover, the chronicity of gingivitis and periodontitis may be exacerbated by a damaged epithelium that is never allowed to heal into a functional barrier due to a lingering local infection. Our finding that proteolytic OM extracts from wildtype *T. denticola* ATCC 35405 interfered with the attainment of confluency and junctional resistance, whereas the OM of the *PrpP*-deficient mutant K1 was ineffective, is a clear demonstration that dentilisin has the capacity to impair the repair of a simple epithelium similar to that found in the gingiva.

Experimental Procedure

Bacterial Strains, Culture Conditions, Electron Microscopy, SDS-PAGE, and Preparation of OM Extracts

Cultures of the *T. denticola* type strain ATCC 35405 were derived from the culture collection of both the University of Toronto (UToronto) and Tokyo Dental College (Tokyo). ATCC 35405 (Tokyo) served as the parent strain for insertional mutagenesis of the *prtP* gene, yielding a dentilisin-deficient mutant K1 (*prtP::Em^r*). Mutant K1 was constructed as a result of a double-crossover event following electroporation with a *prtP*-inactivated DNA fragment containing an *ermF-ermAM* cassette inserted between internal *KpnI* and *PstI* restriction sites (Ishihara *et al.*, 1998), according to the procedure of Li *et al.* (1996).

The strains were grown anaerobically in a complex spirochete broth medium containing brain heart infusion, tryptic peptone, yeast extract, and volatile fatty acids and supplemented with rabbit serum as previously described (Dawson and Ellen, 1990). For electron microscopy, a 10- μ l sample of a washed bacterial suspension was mixed with 5 μ l H₂O containing 0.05% bacitracin. A 300-mesh nickel grid that had been coated with Formvar and carbon was floated on the suspension for 2 min. Excess liquid was removed with filter paper, and the sample was negatively stained with either 1% methylamine tungstate (figure 1A) or 2% phosphotungstic acid (figure 1B). The samples were examined with a Philips 400T transmission electron microscope at 80 kV (Dawson and Ellen, 1994).

For SDS-PAGE, 20 ml bacterial cultures of ATCC 35405 (UToronto), ATCC 35405 (Tokyo), and mutant K1 were washed, then transferred in a small volume to 1.5 ml microtubes. After an additional centrifugation step, the pellets were resuspended in 1.0 ml cold 0.01 M phosphate buffered saline, pH 7.2 (PBS), containing 1 mM benzamide, 1 mM EDTA, 2 mM PMSF, and 0.4 mM TLCK to inhibit protease activity. The cells were sonicated for 6 x 10 sec with a Kontes cell disruptor at power setting 6. The supernatants were adjusted for protein concentration (Bio-Rad assay), mixed 1:1 with sample buffer containing 5% β -mercaptoethanol, and applied without boiling to 12% polyacrylamide gels.

OM extracts were prepared as described previously (Yang *et al.*, 1998). Briefly, the bacteria were harvested at late stationary phase, washed, resuspended in PBS containing 10 mM MgCl₂, and extracted in this buffer containing 0.2% Triton X-100. After repeated centrifugation at 12,000 x g, the supernatant was dialyzed for several days until the OM precipitated, and it was centrifuged at 25,000 x g for 45 min. The pellet was resuspended in the original volume of distilled water and stored at -20° C until used. The dry weight of the extract was determined after freeze-drying, and the protein content was determined (BioRad assay) using bovine albumin (Sigma Chemical Co., St. Louis, Mo.) as a standard.

T. denticola ATCC 35405 cell suspensions (2.5 x 10⁹ per ml) and OM extracts were suspended in CO₂-independent medium (CIM, GIBCO) and tested for peptidase activity typical for the serine protease dentilisin, using the chromogenic peptide *N*-succinyl-L-alanyl-L-prolyl-L-phenylalanine-*p*-nitroanilide (SAAPNA) (Ellen *et al.*, 1994). Bacteria- and OM-free CIM served as the negative control, and the value obtained for its absorbance at 405 nm was subtracted from all other values when calculating SAAPNA degrading activity. For comparisons, the concentration of OM extract was adjusted to contain a dry weight equivalent to that which was extracted from the same quantity of bacterial cells used in the assay, and the data were standardized against the SAAPNA-degrading activity of chymotrypsin (Sigma) at known concentrations.

Cell Culture

HEp-2 epithelial cells were grown as monolayers in T-75 flasks (Costar, Mississauga, ON) containing alpha minimal essential medium (α MEM) supplemented with 15% heat-inactivated fetal bovine serum and a 1:10 (v/v) dilution of an antibiotic solution (0.17 % penicillin V (w/v), 0.1% gentamicin sulphate (w/v) and 0.01 mg/ml amphotericin: Sigma) (Ko *et al.*, 1998b). Human gingival fibroblast (HGF) cultures were established from primary human tissue explants as described previously (Arora *et al.*, 1994). The cells were cultured in α MEM containing 400 U/ml of penicillin G and 10% (v/v) heat-inactivated fetal bovine serum. HEp-2 and HGF cells were maintained at 37° C in a 5% CO₂ incubator and passaged with 0.05% trypsin.

Electrical Cell-Substrate Impedance Sensing (ECIS)

ECIS is an application of biophysical principles that allow for continuous, real-time impedance measurements of cell monolayers, in which changes in cell shape, junctional resistance, cell-substrate separation, and cellular micromotion can be determined by applying mathematical models of data analysis (Lo *et al.*, 1995). The OM extracts were compared for their effects on epithelial cell junctional integrity by ECIS, using conditions previously described in detail (Ko *et al.*, 1998b). Briefly, HEp-2 cells were harvested 48 h before each experiment, and 5 x 10⁴ cells were plated in each well of a multiple electrode array construction designed specifically for ECIS. Cell density was kept at ~10⁵ cells per cm². The electrode arrays, lock-in amplifier,

and software were obtained from Applied Biophysics (Troy, NY) (Giaever and Keese, 1993; Pei *et al.*, 1994). After 48 h in culture, the confluence and viability of the cell monolayer was confirmed by light microscopy and electrically as impedance data obtained from the cell-covered and cell-free reference electrodes. Stable impedance measurements were confirmed at the beginning of each experiment. After 30 min recording of baseline impedance, dilutions of the OM extracts were added to the wells containing the electrode arrays bearing the HEP-2 cells. The data presented as in- and out-of-phase voltage with the applied signal were converted as resistance and capacitance connected in series. The electrical impedance of each ECIS well was measured every minute for cell attachment/junctional contact studies, every 20 minutes for frequency scan measurements, and every second for micromotion. For this study, the junctional resistance between cells was the major variable calculated. In one experimental series, the *T. denticola* wildtype and K1 mutant OM extracts were added along with seeded cells, and their effect on cell attachment and the subsequent attainment of a stable electrical impedance pattern typical of established junctional resistance in cell confluence was compared. Cellular micromotion was also determined. The voltage data of each well was measured every second until 1024 points had been acquired. The time series data were analyzed by calculating the normalized variance (Lo *et al.*, 1993). To minimize experimental variability, micromotion for each treatment group was expressed as a variance ratio obtained by dividing its normalized variance by that of the control group, which did not contain OM extract (Ko *et al.*, 1998b).

HGF Stress Fiber Assay

The ability of *T. denticola* whole cell and OM suspensions to cause rearrangement of filamentous (F-) actin in HGF was determined by a quantitative assay that we have described previously (Yang *et al.*, 1998), with slight modification. HGF were grown in 24-well dishes, each well containing one circular glass coverslip (no. 1, 12-mm diameter, Fisher Scientific). Prior to the addition of bacteria or OM extract, each well was washed once with CIM. The treponemes were suspended to a cell density of 2×10^9 bacteria/ml; 0.5 ml was added to each well in triplicate. Due to the growth of mutant K1 in tight aggregates, suspensions of this strain were also prepared by passing clumped bacterial suspensions forcefully and repeatedly through a narrow gauge syringe needle. The OM extracts were added to the wells at a final concentration equivalent to 0.27 mg/ml dry weight and 60 µg/ml protein (Ko *et al.*, 1998a). Control wells received CIM without bacteria or OM extract. The dishes were incubated at 37° C for 80 min. The CIM was removed, and the cells on the coverslips were fixed in 3.75% formaldehyde in PBS for 60 min at ambient temperature, and then washed twice in PBS. The HGF were permeabilized and labeled for F-actin with a solution containing 0.6 U/ml rhodamine-phalloidin (Molecular Probes, Eugene, Or) in 0.1% Nonidet P-40 (Sigma) in PBS. Two hundred fifty microliters was added per well, and the dishes were incubated at room temperature for 60 min. The coverslips were washed twice in PBS, rinsed in deionized H₂O, dried and mounted immediately on glass slides with an anti-fade mounting medium (Yang *et al.*, 1998). Each of two hundred HGF cells per sample was examined by fluorescence microscopy at a magnification of 400x (Leitz Dialux equipped for epifluorescence) and scored dichotomously according to preset criteria for presenting either a normal or altered stress fiber pattern. The outcome was expressed as the percentage of cells with altered stress fibers. The samples were coded to obscure their identity. The method of counting the frequency of cells with altered stress fibers was found to have a correlation of 0.89 ($p < 0.01$) with a confocal microscopy method in which F-actin depolymerization was quantified by measuring mean TRITC-phalloidin fluorescence per cell by scanning individual, serial optical sections of HGF after challenge with *T. denticola* (Yang *et al.*, 1998).

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