Oral Microbiology Immunology 2009: 24: 304–309
Printed in Singapore. All rights reserved

ORAL MICROBIOLOGY AND IMMUNOLOGY

Prevotella intermedia ATCC 25611 targets host cell lamellipodia in epithelial cell adhesion and invasion

U. K. Gursoy[1,2,3], E. Könönen[2,4], V.-J. Uitto[1]

[1]Institute of Dentistry and Department of Oral and Maxillofacial Surgery, Helsinki University Central Hospital, University of Helsinki, Helsinki, Finland, [2]Department of Bacterial and Inflammatory Diseases, National Public Health Institute (KTL), Helsinki, Finland, [3]Department of Periodontology, Faculty of Dentistry, Cumhuriyet University, Sivas, Turkey, [4]Institute of Dentistry, University of Turku, Turku, Finland

Gursoy UK, Könönen E, Uitto V-J. Prevotella intermedia *ATCC 25611 targets host cell lamellipodia in epithelial cell adhesion and invasion.*
Oral Microbiol Immunol 2009: 24: 304–309. © 2009 John Wiley & Sons A/S.

Introduction: The *Prevotella intermedia* group bacteria, namely *P. intermedia*, *Prevotella nigrescens*, and *Prevotella pallens*, are phylogenetically closely related and potentially connected with oral and gastrointestinal tract disease pathogenesis. The aim of the present study was to examine whether these species differ in their capabilities of adhesion to and invasion of epithelial cells.
Methods: Adhesion and invasion were assayed by standard antibiotic/culture assays and fluorescent microscopy techniques. The effect of *Prevotella* strains on epithelial cell viability was measured using a commercial cell proliferation assay.
Results: The strains *P. intermedia* ATCC 25611 and *P. nigrescens* ATCC 33263 adhered to epithelial cells, the adhesion numbers of *P. intermedia* being twice as high as those of *P. nigrescens*. These strains invaded epithelial cells but invasion was weak. The adhesion of *P. intermedia* was specifically targeted to epithelial cell lamellipodia. The number of adhered *P. intermedia* cells increased or decreased when the formation of lamellipodia was stimulated or inhibited, respectively. None of the tested strains showed toxic effects on epithelial cells; a clinical *P. intermedia* strain even increased the number of viable cells by about 20%.
Conclusion: The results suggest that among the *P. intermedia* group bacteria, *P. intermedia* and *P. nigrescens* type strains can adhere to and invade epithelial cells, the capability of *P. intermedia* ATCC 25611^T being highest in this context. This strain proved to have a special affinity in binding to epithelial cell lamellipodia.

Key words: adhesion; invasion; lamellipodia; *Prevotella intermedia*

Ulvi Kahraman Gursoy, Institute of Dentistry, Biomedicum, University of Helsinki, PO Box 63, FI-00014 Helsinki, Finland
Tel.: +358 40 4194735;
fax: +358 9 19125371;
e-mail: ulvi.gursoy@helsinki.fi

Accepted for publication December 30, 2008

Most anaerobic infections are of endogenous origin, i.e. they are caused by members of the host's indigenous microbiota. Pigmented *Prevotella* species are clinically important anaerobic gram-negative bacteria involved in infections of the gastrointestinal tract and the oral cavity (10). The *Prevotella intermedia* group comprises *Prevotella intermedia*, *Prevotella nigrescens*, and *Prevotella pallens* (19). *P. intermedia* and *P. nigrescens* are biochemically similar so they should be classified as *P. intermedia sensu lato* if molecular methods have not been used for their separation (22). These species have been associated with various infections in humans, such as mediastinitis (4), atherosclerosis (31), noma (cancrum oris) lesions (8), and preterm birth (15). The *P. intermedia* group organisms are frequent members of the oral anaerobic microbiota from the early years of life (20, 22, 25). In the oral cavity, *P. intermedia* and *P. nigrescens* have been associated not only with periodontitis (24) but also with endodontic and odontogenic infections (1). However, it seems that *P. intermedia* has the strongest association with periodontitis, while *P. nigrescens* is found both in healthy and periodontally diseased mouths, and *P. pallens* has no clear disease association (19, 21, 25, 33).

Despite their clinical importance, little is known about the basis of the virulence of the *P. intermedia* group bacteria. The host cell adhesion and invasion capability of bacteria is a prerequisite for the initiation of infections. Several bacteria, including *Brucella*, *Listeria*, *Salmonella*, and *Shigella*,

have the ability to adhere to and invade non-phagocytic cells (3, 11, 26). Some periodontal pathogens, such as *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans*, *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, and *P. intermedia* are known to invade epithelial cells (7, 13, 23, 27). Invasion of human cells by *P. intermedia* was reported to be weak and to occur in a strain-dependent manner (6, 7). The invasion of human cells by *P. nigrescens* has been shown in only one study (28), while no data exist on the adhesion to or invasion of human cells by *P. pallens*. Although the *P. intermedia* group bacteria are biochemically and phylogenetically closely related, their adherence to and invasion of epithelial cells may be a distinctive factor in their different virulence capabilities. In the present study, we aimed to demonstrate whether the *P. intermedia* group bacteria differ in their adherence to and invasion of epithelial cells.

Materials and methods

Bacterial and epithelial cell cultures

The *Prevotella* species and strains included the type strain and one clinical strain each of *P. intermedia* (ATCC 25611^T and AHN 8290), *P. nigrescens* (ATCC 33263^T and AHN 8293), and *P. pallens* (NCTC 13042^T and AHN 9283). The strains were revived from frozen (−70°C) stocks and subcultured for purity. Bacteria were grown on hemin-enriched (0.0005%) Brucella blood agar and incubated at 37°C in an anaerobic chamber for 3–5 days. Bacterial stocks for experiments were prepared by adjusting the optical density at 490 nm (OD_{490}) to 0.5 with phosphate-buffered saline (PBS).

HaCaT cells, a non-tumorigenic human skin keratinocyte line (generously provided by Hubert Fusenig, German Cancer Center, Heidelberg, Germany) were maintained as frozen stock and cultured in 5% CO_2 at 37°C in Dulbecco's modified Eagle's minimal essential medium (DMEM) containing 10% fetal calf serum, 1% L-glutamine, and 1% penicillin G. This cell line was selected, because it is well characterized and widely used as a model for human epithelial cell studies (2, 34). During incubation of HaCaT cells with bacteria, medium without antibiotics and fetal calf serum were used.

Analysis of *Prevotella* adhesion to and invasion of epithelial cells

The levels of adhesion and invasion were quantified by a standard antibiotic assay (7). In all experiments, HaCaT cells at 60–70% confluency were used. In the adhesion and invasion experiments, 2×10^5 HaCaT cells/well were plated on 24-well sterile culture plates and incubated at 37°C under 5% CO_2 for 24 h. Bacterial stocks for the experiments were prepared by adjusting the OD_{490} to 0.5 with PBS. Multiplicity of infection (MOI) was about 1 : 100 (10^5 epithelial cells with 10^7 bacteria). About 1 h before adding the *Prevotella* suspensions to the wells, 1 ml of fresh DMEM containing 1% L-glutamine was placed in each well. For the adhesion experiment, epithelial cell monolayers were incubated with each strain at 37°C in a CO_2 incubator for 4 h, then the wells were washed twice with sterile PBS, and HaCaT cells were osmotically lysed with water and sonication. An aliquot of 100 μl of each sonicate was inoculated on Brucella blood agar plates and incubated for 5 days. In a separate experiment, it was shown that the sonication used in this study did not affect bacterial viability. For the invasion assay, after 4 h incubation of HaCaT cells with *Prevotella* suspensions, the wells were washed twice with PBS, and further incubated with fresh medium containing gentamicin (300 μg/ml) and metronidazole (200 μg/ml) (both from Sigma-Aldrich, St Louis, MO) for an additional hour at 37°C to kill extracellular or epithelial cell surface-bound bacteria. This antibiotic concentration has been found to be effective on *Prevotella* species (7), and we also confirmed this by incubating the antibiotic-containing medium for 1 h with the *Prevotella*. No bacterial growth could be detected after this treatment. The wells were washed twice with PBS, and internal bacteria were released by lysis of the HaCaT cells. An aliquot of 100 μl from each sonicate was cultured on Brucella blood agar plates. All assays were performed as duplicates and the results were expressed as mean colony forming units (CFUs) per well recovered.

Adhesion and invasion of *Prevotella* were also analysed under fluorescence microscopy. HaCaT cells were plated in eight-well chamber slides (Lab-Tek II; Nunc A/S, Roskilde, Denmark). After reaching 60–70% confluency with an overnight incubation, the HaCaT cells were incubated with each test strain at 37°C under 5% CO_2 for 4 h. Subsequently, the cells were fixed with 4% paraformaldehyde and stained with a commercial DNA stain (Live/Dead Baclight bacterial viability kit; Molecular Probes Inc., Eugene, OR). The analyses were performed immediately after staining, using a fluorescence microscope (Olympus BX61; Olympus Optical Co. Ltd., Tokyo, Japan) at a magnification of 100× (with immersion oil). The fluorescent viability stain (Live/Dead Baclight; Molecular Probes Inc.) used in this study comprises two stains, propidium iodide and SYTO 9. The green fluorescent SYTO 9 stain labels both live and dead bacteria while the propidium iodide penetrates only bacteria with damaged membranes. Consequently, after killing the extracellular bacteria with a 1-h antibiotic treatment, bacteria with only green fluorescence were accepted as intracellular (29).

Analysis of specific adhesion of bacteria to epithelial lamellipodia

HaCaT cells were grown to 60–70% confluency in eight-well chamber slides (Lab-Tek II; Nunc A/S). Cells were washed twice with PBS, and an aliquot of 300 μl fresh antibiotic-free and serum-free medium, containing either staurosporine (50 nM) or sodium orthovanadate (500 μM) (16), was placed in each well, and incubated at 37°C for 1 h. The anaerobic cultures of *P. intermedia* ATCC 25611^T, which was used as a test strain, were suspended in PBS and adjusted to an OD_{490} of 0.5. Epithelial cells were incubated for a further 1 h with bacteria in the presence of staurosporine and sodium orthovanadate at 37°C. Cells treated in the same way but without staurosporine or sodium orthovanadate were used as controls. At the end of the 1-h incubation with bacteria, epithelial and bacterial cells were analysed under a fluorescence microscope.

Epithelial cell viability assay

HaCaT cells were grown to 60–70% confluency in 96-well plates. Cells were washed twice with PBS, and fresh antibiotic-free and serum-free medium was placed in each well. Each *Prevotella* strain freshly collected from anaerobic cultures was suspended in PBS and adjusted to an OD_{490} of 0.5. The bacterial suspension was added to the culture medium at a 1 : 10 volume ratio. After incubation for 24 h, non-adherent bacteria were removed by several PBS washings, and the number of viable epithelial cells was assayed using the CellTiter 96 aqueous non-radioactive cell proliferation assay (Promega, Madison, WI). All tests were performed in triplicate, and the results were statistically analysed using Student's *t*-test.

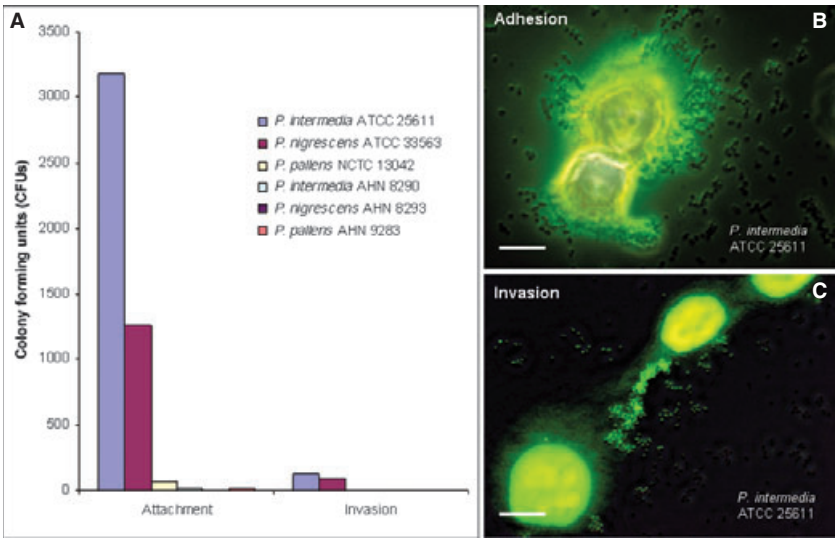

Fig. 1. Adhesion and invasion of *Prevotella* strains to epithelial cells. Bars indicate the mean number of adhered and invaded *Prevotella* [colony-forming units (CFU)/well] as calculated from triplicate tests. (A) A parallel test performed for fluorescent staining. Merged fluorescence and phase contrast pictures of the HaCaT cells show the adhesion (B) and invasion (C) of *Prevotella intermedia* ATCC 25611^T to HaCaT cells (bar represents 10 μm).

Results

The tested *Prevotella* species and strains adhered to and invaded the epithelial cells to different extents. The adhered cell number per well was about 3000 for *P. intermedia* ATCC 25611^T and about 1200 for *P. nigrescens* ATCC 33563^T by the end of the fourth hour, while the numbers of other strains varied between 1 and 10 bacterial cells/well. Invasion was less limited, only the strains *P. intermedia* ATCC 25611^T and *P. nigrescens* ATCC 33563^T showing invasion (Fig. 1A). In the fluorescence microscopy analyses, the amounts of adhered and invasive cells of *P. intermedia* ATCC 25611^T were higher than those of other strains, being in correlation with the culture results. Overall, the culture technique gave much lower adhesion and invasion values, indicating the loss of bacterial viability as a result of contact with epithelial cells or during processing for the culture assay. *P. intermedia* ATCC 25611^T specifically adhered to lamellipodia-type protrusions of HaCaT cells, forming small chains. Some bacterial cells were found adherent to extracellular areas. A separate test, where the *Prevotella* strains were incubated in DMEM in 24-well plates for 4 h, then washed with PBS and sonicated after adding water to the wells, was performed to check whether adhesion numbers of *Prevotella* strains in culture tests included cells attached to the well surface. No bacteria grew in these cultures.

The viability of bacteria was around 90% for adherent and invasive bacteria based on the Live/Dead assay. No significant morphological changes were observed in HaCaT cells that *Prevotella* had bound to or invaded (Fig. 1B). The DNA stain also stained epithelial cell DNA and no difference in their viability was observed during *Prevotella* adherence or invasion. This finding was confirmed with a formazan cell proliferation assay (CellTiter 96; Promega). *Prevotella* had no toxic effect on HaCaT cells but, instead, tended to increase their numbers (Fig. 2).

The fluorescence and phase contrast microscopy analyses showed that the adhesion of *P. intermedia* ATCC 25611^T was not random but occurred specifically to HaCaT cell protrusions (Fig. 3A–C), unlike the adhesion of other *Prevotella* strains. *P. intermedia* ATCC 25611^T did not adhere to sites where epithelial cells were in contact with each other and had no lamellipodia (Fig. 3B,C). In contrast, it adhered to dying/dead cells, where the epithelial membrane was damaged and several actin filament-containing microspike-like structures were formed (Fig. 3D). To confirm this preferred adhesion of *P. intermedia* ATCC 25611^T, HaCaT cells were cultured with staurosporine or sodium orthovanadate to stimulate or inhibit lamellipodia formations, as previously described (16, 17). After incubation of HaCaT cells with these chemicals and with *P. intermedia* ATCC 25611^T for a further 1 h, prominent adhesion of *P. intermedia* ATCC 25611^T to the stimulated lamellipodia was seen, whereas inhibition of lamellipodia decreased its adhesion (Fig. 4).

Discussion

The role of *P. intermedia* in disease pathogenesis is usually related to its different virulence factors, for example, hemolytic, hemagglutinating, and proteolytic activities (5, 17). In the present study, where

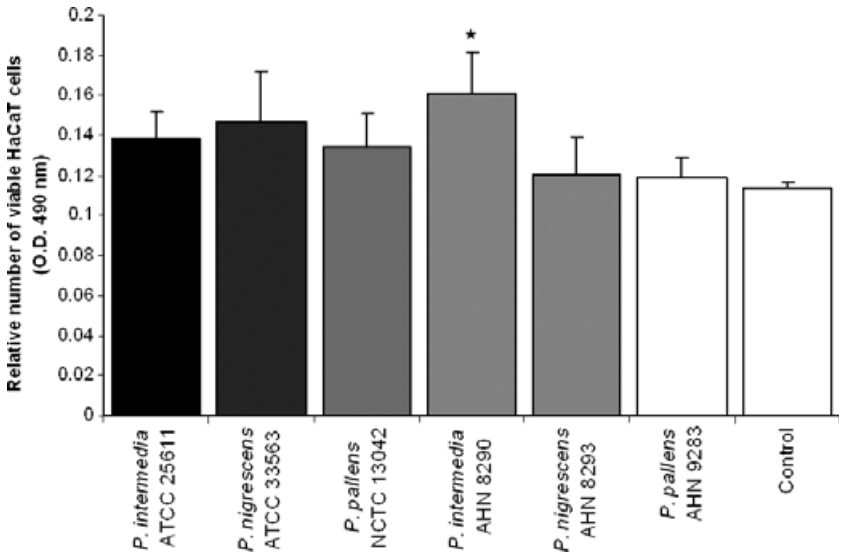

Fig. 2. Effect of *Prevotella* strains on epithelial cell viability. Following the culture of each strain with HaCaT cells for 24 h, the viable epithelial cell count was measured using the CellTiter 96 kit (Promega). Bars present the means and standard deviations for triplicate tests. The asterisk indicates that $P < 0.05$.

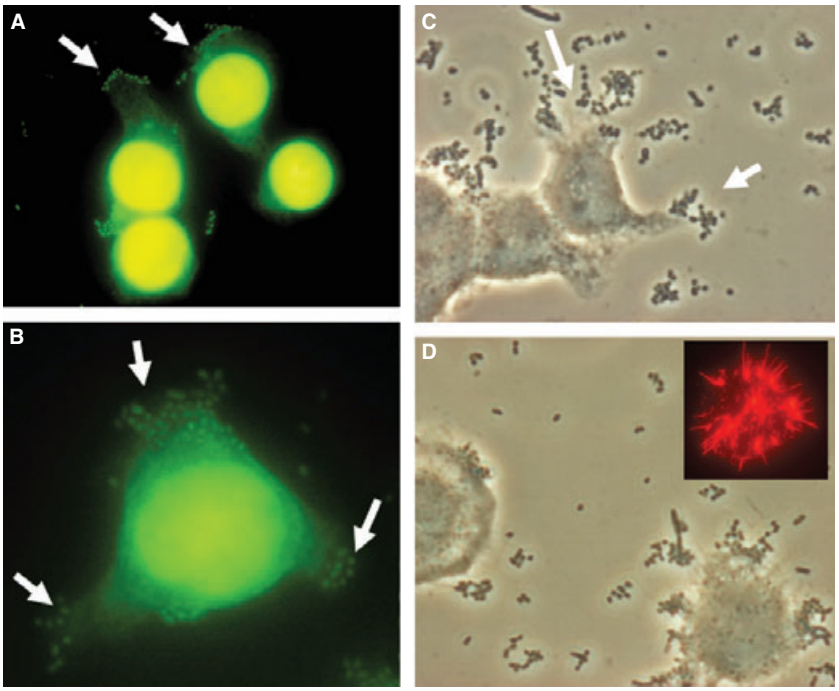

Fig. 3. Site-specific binding of *Prevotella intermedia* ATCC 25611^T to epithelial cells. Viable bacteria were added to HaCaT cell cultures and observed under a fluorescence microscope (A, B) and a phase-contrast microscope (C, D). The adhesion was specific to lamellipodia of the epithelial cells (A–C). Bacterial cells also bound to the peripheral extensions of dead cells (D). Arrows indicate bacteria adhered to lamellipodia.

the main target was to investigate whether the *P. intermedia* group bacteria differ in their adhesion and invasion capabilities, only type strains of *P. intermedia* and *P. nigrescens* adhered to and invaded cultured keratinocytes. *P. intermedia* ATCC 25611^T, but none of the other strains tested, formed chains at the adhesion sites of the keratinocytes.

Only a few studies in the literature analyse the *P. intermedia* group bacteria in this context (7, 28). Dorn et al. (7) found that invasion of epithelial cells by *P. intermedia* ATCC 25611^T was weak, the number of invasive bacteria being similar to our results (7). They suggested that *P. intermedia* ATCC 25611^T possesses type D fimbriae but not type C fimbriae. Indeed, lack of type C fimbriae could partly explain the differences in adhesion and invasion capabilities seen between the *Prevotella* species and strains tested in our study. In the study of Pantanella et al. (28), showing invasion and intracellular survival of *P. nigrescens*, carcinoma cells were used as the host cell type so their results are not comparable with ours, which were obtained using non-tumorigenic human keratinocytes. No studies on adhesion or

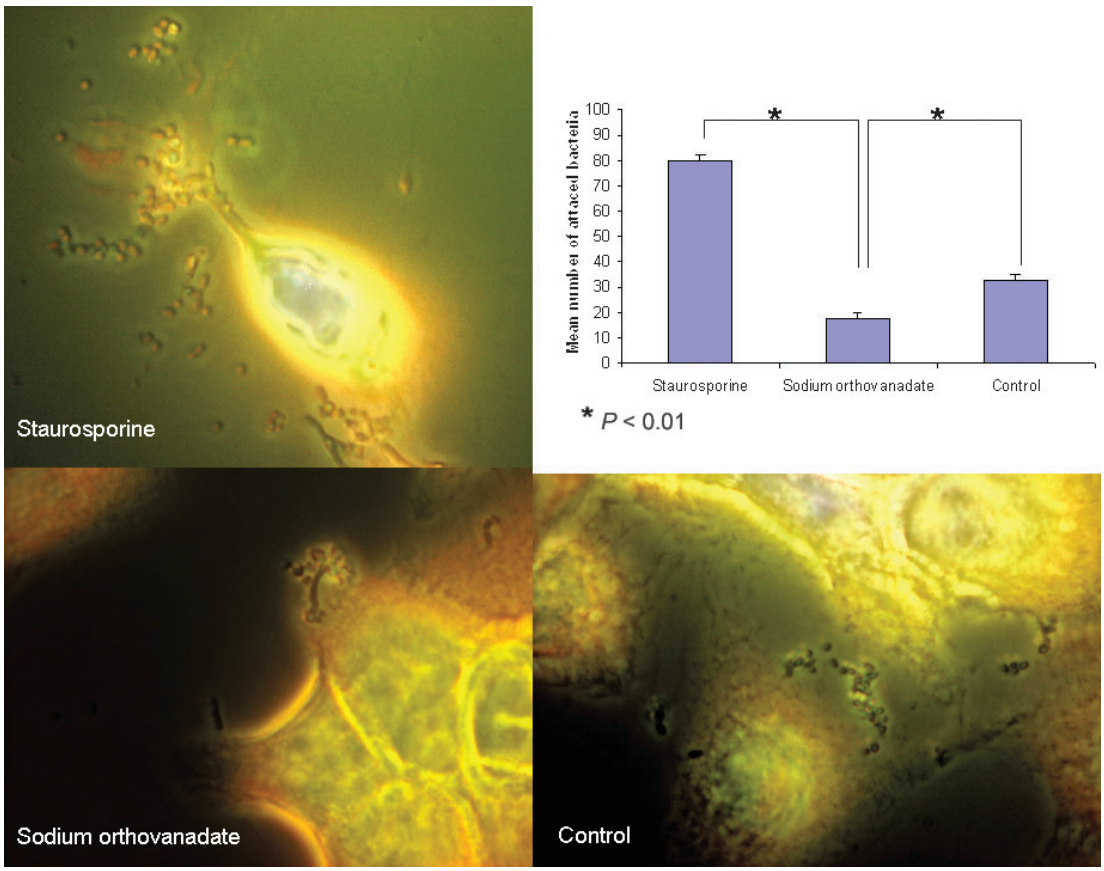

Fig. 4. Adhesion of *Prevotella intermedia* ATCC 25611^T to HaCaT cells after stimulation and inhibition of epithelial cell lamellipodia formation with staurosporine and sodium orthovanadate, respectively. The asterisk indicates that $P < 0.01$ (A).

invasion of *P. pallens* are available for comparisons.

Microscopic examinations demonstrated that *P. intermedia* ATCC 25611^T had a preference for specific regions of the host cell membrane and the rest of the cell membrane remained relatively free of bacteria. These preferred regions were lamellipodia, which are thin sheet-like processes that periodically extend especially during migration. For example, during the epithelial wound healing, keratinocyte migration starts with the formation of long lamellipodia (17). A similar cell behavior is thought to play an important role in the morphogenesis of tissues during periodontal disease and regeneration processes (18). In the present study, stimulation of lamellipodia production increased the number of bacteria attached to the host cells. Bacterial binding to the tips of the epithelial lamellipodia may disrupt the functions of localizing and harnessing actin polymerization for cell motility. In the pathogenesis of periodontal disease, during the formation of periodontal pockets, the lamellipodia of migrating epithelial cells may become targets for *P. intermedia* binding. Bacterial binding to the lamellipodia of gingival epithelial cells may also disrupt tissue repair by hindering the cell–cell and cell–matrix contacts. Bacteria usually target cellular adhesion molecules, such as integrin, fibronectin, and laminin, to adhere to and invade host cells (14). Provisional extracellular matrix secreted by migrating keratinocytes contains fibronectin and laminin-5 (35). Within the limits of the present study, it is not possible, however, to demonstrate which molecule(s) on the lamellipodium surface is the target for *Prevotella* binding.

In the present study, *Prevotella* adhered to dead epithelial cells. This has been reported previously, and suggested as being an adhesion characteristic for another periodontal pathogen, *P. gingivalis* (29). Microscopic examination revealed that damaged or dead epithelial cells lost their contact with the neighboring cells and shrank, leaving adhesion filaments in their periphery. According to our results, actin filaments stayed intact even after epithelial cell death. Dead cells may release a number of cellular components, increasing bacterial adhesion (29). Part of the epithelial cell lining of inflamed periodontal pockets may express the cell extensions upon dying which may provide *P. intermedia* another opportunity to adhere and invade the pocket epithelium (30).

In the microscopic analysis of the infected cultures, we observed some bacteria that were not in contact with epithelial cells. During the migration, epithelial cells leave laminin-5 and fibronectin footprints behind (12, 35). These extracellular matrix depositions on migration areas could explain the *Prevotella* binding on empty surfaces. In the continuation of the present study and as part of our new project, we tested the laminin-5 and fibronectin binding of *Prevotella* by coating glass microscope slide surfaces with these matrix proteins. We found that *Prevotella* (especially *P. intermedia*) strains have a high affinity to bind laminin-5 and fibronectin, which may explain in part both the binding of bacteria to extracellular areas and the binding to lamellipodia.

After 24 h of incubation, no toxic effects by *Prevotella* strains on epithelial cells were seen. Instead, the incubation with bacteria increased the epithelial cell numbers in a strain-dependent manner. Loss of cell cycle control by increased proliferation may affect periodontal tissue repair and initiate bacterial penetration into periodontal tissues. In addition, increased proliferation of epithelial cells will diminish their role in the signaling network during the immune response against colonizing bacteria. To obtain access into non-phagocytic cells and to replicate intracellularly, microbes need to modify some host cell functions (11, 20). For example, *Escherichia coli* forces the host cell to differentiate and rearrange the cytoskeleton for its invasion and intracellular replication, without exerting toxic effects on the host cell (9). A similar behavior has been observed for two oral bacteria, *A. actinomycetemcomitans* and *F. nucleatum* (13, 27). *P. nigrescens* has been described as causing the loss of epithelial cell adherence in human cell cultures (28). In the present study, no morphological changes or loss of cellular adherence were seen in the epithelial cells infected with the *P. intermedia* group bacteria.

The influence of laboratory handling and isolation conditions on the *Prevotella* strains should be considered when the differences in adhesion and invasion numbers between the clinical and type strains are studied. A recent study by Silva et al. (32) showed that *P. intermedia* ATCC 25611 has the highest tolerance to oxygen exposure when compared with *P. nigrescens* ATCC 33563 and some clinical *P. intermedia* strains. The test conditions we applied were aerobic, and the type strain has been handled in laboratory conditions for longer than the clinical isolates. Consequently, increased tolerance to oxygen exposure of the type strain may partly explain its increased adhesion.

In conclusion, the *P. intermedia* and *P. nigrescens* seem to adhere to and invade epithelial cells in a strain-dependent manner. The adhesion of *P. intermedia* ATCC 25611^T to epithelial cells may occur via the site-specific binding ability of the bacteria to the host cell lamellipodia.

References

1. Baumgartner JC, Watkins BJ, Bae KS, Xia T. Association of black-pigmented bacteria with endodontic infections. J Endod 1999: **25**: 413–415.
2. Boukamp P, Petrussevska RT, Breitkreutz D, Hornung J, Markham A, Fusenig NE. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. J Cell Biol 1988: **106**: 761–771.
3. Boyle AC, Finlay BB. Bacterial pathogenesis: exploiting cellular adherence. Curr Opin Cell Biol 2003: **15**: 633–639.
4. Brook I. The role of anaerobic bacteria in mediastinitis. Drugs 2006: **66**: 315–320.
5. Cookson AL, Wray A, Handley PS, Jacob AE. An investigation into the use of SDS–PAGE of cell surface extracts and proteolytic activity to differentiate *Prevotella nigrescens* and *Prevotella intermedia*. FEMS Microbiol Lett 1996: **136**: 109–115.
6. Dorn BR, Dunn WA Jr, Progulske-Fox A. Invasion of human coronary artery cells by periodontal pathogens. Infect Immun 1999: **67**: 5792–5798.
7. Dorn BR, Leung KP, Progulske-Fox A. Invasion of human oral epithelial cells by *Prevotella intermedia*. Infect Immun 1998: **66**: 6054–6057.
8. Enwonwu CO, Falkler WA, Idigbe EO. Oro-facial gangrene (noma/cancrum oris): pathogenetic mechanisms. Crit Rev Oral Biol Med 2000: **11**: 159–171.
9. Eto DS, Sundsbak JL, Mulvey MA. Actin-gated intracellular growth and resurgence of uropathogenic *Escherichia coli*. Cell Microbiol 2006: **8**: 704–707.
10. Finegold SM, Strong CA, McTeague M, Marina M. The importance of black-pigmented gram-negative anaerobes in human infections. FEMS Immunol Med Microbiol 1993: **6**: 77–82.
11. Goto T, Yoshinari M, Kobayashi S, Tanaka T. The initial attachment and subsequent behavior of osteoblastic cells and oral epithelial cells on titanium. Biomed Mater Eng 2004: **14**: 537–544.
12. Gruenheid S, Finlay BB. Microbial pathogenesis and cytoskeletal function. Nature 2003: **422**: 775–781.
13. Gursoy UK, Könönen E, Uitto VJ. Intracellular replication of fusobacteria requires new actin filament formation of epithelial cells. APMIS 2008: **116**: 1063–1070.
14. Hauck CR, Agerer F, Muenzner P, Schmitter T. Cellular adhesion molecules as targets for bacterial infection. Eur J Cell Biol 2006: **85**: 235–242.
15. Hill GB. Preterm birth: associations with genital and possibly oral microflora. Ann Periodontol 1998: **3**: 222–223.

16. Koivisto L, Alavian K, Hakkinen L, Pelech S, McCulloch CA, Larjava H. Glycogen synthase kinase-3 regulates formation of long lamellipodia in human keratinocytes. J Cell Sci 2003: **116**: 3749–3760.
17. Koivisto L, Häkkinen L, Matsumoto K, McCulloch CA, Yamada KM, Larjava H. Glycogen synthase kinase-3 regulates cytoskeleton and translocation of Rac1 in long cellular extensions of human keratinocytes. Exp Cell Res 2004: **293**: 68–80.
18. Kolega J. Effects of mechanical tension on protrusive activity and microfilament and intermediate filament organization in an epidermal epithelium moving in culture. J Cell Biol 1986: **102**: 1400–1411.
19. Könönen E, Eerola E, Frandsen EVG et al. Phylogenetic characterization and proposal of a new pigmented species to the genus *Prevotella*: *Prevotella pallens* sp. nov. Int J Syst Bacteriol 1998: **48**: 47–51.
20. Könönen E, Kanervo A, Takala A, Asikainen S, Jousimies-Somer H. Establishment of oral anaerobes during the first year of life. J Dent Res 1999: **78**: 1634–1639.
21. Könönen E, Paju S, Pussinen PJ et al. Population based study of salivary carriage of periodontal pathogens in adults. J Clin Microbiol 2007: **45**: 2446–2451.
22. Könönen E, Wolf J, Frandsen EVG, Poulsen K, Jousimies-Somer H, Asikainen S. The *Prevotella intermedia* group organisms in young children and their mothers as related to maternal periodontal status. J Periodontal Res 2000: **35**: 329–334.
23. Lamont RJ, Chan A, Belton CM, Izutsu K, Vasel D, Weinberg A. *Porphyromonas gingivalis* invasion of gingival epithelial cells. Infect Immun 1995: **63**: 3878–3885.
24. Lopez NJ. Occurrence of *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Prevotella intermedia* in progressive adult periodontitis. J Periodontol 2000: **71**: 948–954.
25. Mättö J, Saarela M, von Troil-Linden B et al. Distribution and genetic analysis of oral *Prevotella intermedia* and *Prevotella nigrescens*. Oral Microbiol Immunol 1996: **11**: 96–102.
26. Méresse S, Unsworth KE, Habermann A et al. Remodeling of the actin cytoskeleton is essential for replication of intravacuolar *Salmonella*. Cell Microbiol 2001: **3**: 567–577.
27. Meyer DH, Lippmann JE, Fives-Taylor PM. Invasion of epithelial cells by *Actinobacillus actinomycetemcomitans*: a dynamic, multistep process. Infect Immun 1996: **64**: 2988–2997.
28. Pantanella F, Petrone G, Passariello C, Selan L, Thaller MC, Valenti P. Invasion of human cultured cells by *Prevotella nigrescens*. Int J Immunopathol Pharmacol 1999: **12**: 143–148.
29. Papaioannou W, van Steenberghe D, Cassiman JJ, Van Eldere J, Quirynen M. Comparison of fluorescence microscopy and culture assays to quantitate adhesion of *Porphyromonas gingivalis* to mono- and multi-layered pocket epithelium cultures. J Periodontol 1999: **70**: 618–625.
30. Pöllänen MT, Salonen JI, Uitto VJ. Structure and function of tooth–epithelial interface in gingival crevicular fluid. Periodontol 2000 2003: **31**: 12–31.
31. Pucar A, Milasin J, Lekovic V et al. Correlation between atherosclerosis and periodontal putative pathogenic bacterial infections in coronary and internal mammary arteries. J Periodontol 2007: **78**: 677–682.
32. Silva VL, Carvalho MAR, Nicoli JR, Farias LM. Aerotolerance of human clinical isolates of *Prevotella* spp. J Appl Microbiol 2003: **94**: 701–707.
33. Socransky SS, Haffajee AD. Periodontal microbial ecology. Periodontol 2000 2005: **38**: 135–187.
34. Uitto VJ, Baillie D, Wu Q et al. *Fusobacterium nucleatum* increases collagenase 3 production and migration of epithelial cells. Infect Immun 2005: **73**: 1171–1179.
35. Uitto VJ, Larjava H, Peltonen J, Brunette DM. Expression of fibronectin and integrins in cultured periodontal ligament epithelial cells. J Dent Res 1992: **71**: 1203–1211.