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Effects of Nicotine on the Growth and Protein Expression of *Porphyromonas gingivalis*

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Tobacco smoking is considered one of the most significant environmental risk factors for destructive periodontal disease. The effect of smoking on periodontopathic microbiota has not yet been elucidated, as previous studies failed to identify a concrete relationship between periodontopathic microorganisms and smoking. However, it is likely that smoking, as an environmental stress factor, may affect the behavior of dental plaque microorganisms, ultimately leading to alteration of the host-parasite interaction. The goal of this study was to examine the effect of nicotine, a major component of tobacco, on the growth and protein expression of the crucial periodontal pathogen *Porphyromonas gingivalis*. The growth of *P. gingivalis* 381 was measured after bacterial cells were cultivated in liquid broth containing various nicotine concentrations. First, *P. gingivalis* cells were allowed to grow in the presence of a single dose of nicotine (the single exposure protocol) at 0, 1, 2, 4, and 8 mg/L, respectively. Second, *P. gingivalis* cells were exposed to five consecutive doses of nicotine (the multiple exposure protocol) at 0, 1, 2, and 4 mg/L, respectively. Bacterial growth was measured by optical density and protein expression was analyzed by SDS-PAGE and 2-D gel electrophoresis. In the single nicotine exposure protocol, it was observed that the growth of *P. gingivalis* 381 was inhibited by nicotine in a dose-dependent manner. In the multiple nicotine exposure protocol, the growth rate of *P. gingivalis* increased with each subsequent nicotine exposure, even though bacterial growth was also inhibited in a dose dependent fashion. SDS-PAGE and 2-D gel electrophoresis analyses revealed a minor change in the pattern of protein expression, showing differences in proteins with low molecular weights (around 20 kDa) on exposure to nicotine. The results of this study suggest that nicotine exerts an inhibitory effect on the growth of *P. gingivalis*, and has a potential to modulate protein expression in *P. gingivalis*.

Keywords: smoking, nicotine, protein expression, virulence, periodontal disease, oral anaerobic bacterium

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Introduction

Periodontal disease is arguably one of the most common infectious diseases affecting humans, and this chronic affliction can lead to destruction of the supporting structures of dentition and, ultimately, tooth loss (Williams, 1990). It has been established that dental plaque is an etiological agent of periodontal disease. Dental plaque is a biofilm consisting of more than 700 different bacterial species and their products (Kroes *et al.*, 1999; Paster *et al.*, 2001). Immunological and inflammatory responses by the host to dental plaque biofilm via host-parasite interaction are manifested by signs and symptoms of periodontal disease. The outcome of this interaction could also be modulated by other components known as risk factors or disease modifiers. These factors, either inherent (genetic) or acquired (environmental), may in turn significantly affect the initiation and progression of periodontal diseases of different types (Page *et al.*, 1997; Kinane and Hart, 2003; Loos *et al.*, 2005).

Tobacco smoking is considered to be one of the most important environmental risk factors for the initiation and the progression of periodontal disease (Palmer *et al.*, 2005). Over the past several decades, numerous epidemiological data have demonstrated that smoking represents an increased risk for periodontal disease (Albandar, 2002; Rivera-Hidalgo, 2003). The exact pathogenic mechanisms whereby smoking exerts its effect on periodontal disease are not completely understood, although it has been suggested that changes in the host immune and inflammatory responses may be partially responsible for periodontal disease in smokers (Obeid and Bercy, 2000).

The relationship between smoking and dental plaque microorganisms has not yet been clearly delineated. Although the effect of smoking on dental plaque development has not been clearly determined, it appeared that the rate of plaque formation was not affected by smoking (Bergstrom and Preber, 1986; Bostrom *et al.*, 2001). Some studies observed higher emergence of certain bacterial species in smokers, including *Porphyromonas gingivalis*, *Treponema denticola,* and *Tannerella forsythia* (Zambon *et al.*, 1996; Umeda *et al.*, 1998; Kamma *et al.*, 1999), while others failed to detect differences in the microbiota between smokers and nonmokers (Darby *et al.*, 2000; Bostrom *et al.*, 2001; Van der Velden *et al.*, 2003). *P. gingivalis* is a Gram-negative, non-

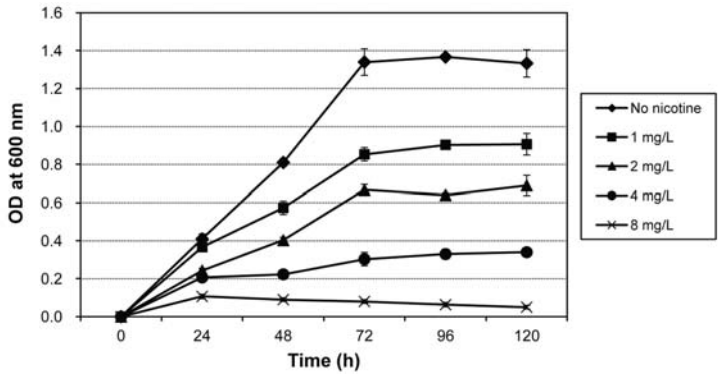

Fig. 1. The growth pattern of *P. gingivalis* 381 in the presence of nicotine (0. 1, 2, 4, and 8 mg/L). The error bars indicate the range of data, as the experiments were performed in triplicates.

pore-forming, anaerobic, black-pigmented bacterium, and has long been considered an important pathogen involved in the initiation and progression of periodontal disease, due to frequent recovery of this species from the lesion, and the presence of potent virulence factors (O'Brien-Simpson *et al.*, 2004; Holt and Ebersole, 2005), including fimbriae, hemagglutinins, a broad spectrum of proteases and other enzymes, lipopolyssacharides, and capsule.

Bacterial pathogens are equipped with sophisticated mechanisms for adapting to complex environmental changes and thereby ensure adequate growth and survival within the host (Finlay and Falkow, 1989, 1997). Recent studies showed that expression of virulence factors of *P. gingivalis* is regulated in response to environmental changes. It was found that fimbrial gene (*fimA*) activity was decreased by approximately 50% in response to hemin limitation and the presence of serum or saliva in the growth medium (Xie *et al.*, 1997). Iron and heme utilization by *P. gingivalis* adopts various schemes to ensure optimal nutrient uptake in response to environmental changes (Olczak *et al.*, 2005).

However, the effect of smoking, as an environmental stress factor on the virulence mechanisms, on physicochemical characteristics of dental plaque microorganisms, has not yet been adequately studied. Very recently, it was found that cigarette smoke extract (CSE) can modulate the expression of several virulence factors of *P. gingivalis* – including major and minor fimbrial antigens (FimA and Mfa1, respectively) and capsule – concomitant with a reduced pro-inflammatory potential of intact *P. gingivalis* (Bagaitkar *et al.*, 2009, 2010). These results suggest that smoking, as an environmental stress factor, may affect the behavior of *P. gingivalis*, leading to modification of the host-parasite interaction.

The purpose of this study was to assess the effects of nicotine, a major component of tobacco smoking, on modulation of the growth pattern and overall protein expression of *P. gingivalis*.

Materials and Methods

Bacterial strain and growth conditions

P. gingivalis 381 was purchased from ATCC (USA) and maintained on anaerobic blood agar plates (Anaerobe Systems, Hardy Diagnostics, USA) at 37°C under anaerobic conditions (80% N_2, 10% CO_2, 10% H_2), in a Forma 1025 Anaerobic Chamber (Thermo, USA). For liquid growth, bacterial cells were cultured in 3% (w/v) trypticase soy broth supplemented with 0.5% yeast extract, 5 mg/L hemin, and 1 mg/L menadione. *E. coli* DH5α was obtained from Promega (USA) and grown in LB medium, as described elsewhere. Nicotine (Acros Organics, Belgium) was added to the liquid broth at final concentrations of 0, 1, 2, 4, and 8 mg/L. The growth rates of *P. gingivalis* 381 were determined by measuring optical density at 600 nm, using a spectrophotometer.

The growth of *P. gingivalis* 381 after exposure to nicotine

In the single nicotine exposure scheme (Fig. 1), 5×10^7 *P. gingivalis* cells were added to the 20 ml liquid broth containing varying nicotine concentrations, i.e. 0, 1, 2, 4, and 8 mg/L, and cultivated for 5 days. The effect of nicotine on

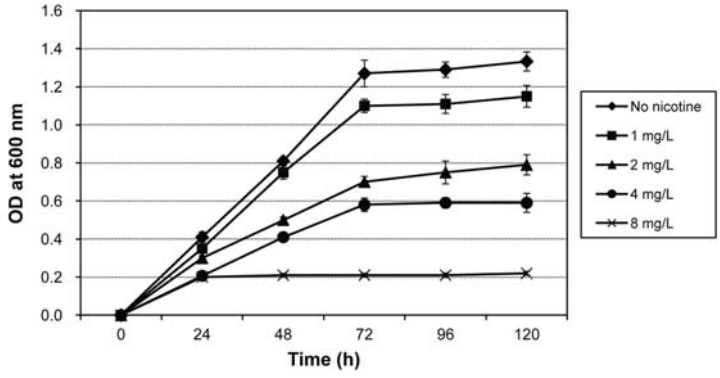

Fig. 2. The growth pattern of *P. gingivalis* 381 pre-exposed to nicotine. Bacterial cells were first exposed to 4 mg/L nicotine, and allowed to grow again in the fresh media containing different nicotine concentrations (0, 1, 2, 4, and 8 mg/L). The error bars indicate the range of data, as the experiments were performed in triplicates.

the growth pattern and protein expression of *P. gingivalis* was measured.

In the second experiment (Fig. 2), *P. gingivalis* cells were grown for 48 h by adding nicotine (4 mg/L), until they reached the mid-log phase, and the cells were washed three times with PBS to remove any residual nicotine. Then, washed bacterial cells were re-inoculated into the fresh medium containing different nicotine concentrations (0, 1, 2, and 4 mg/L) to determine whether pre-conditioning of *P. gingivalis* with a high concentration of nicotine affects the subsequent growth pattern.

In the multiple nicotine exposure scheme (Fig. 3), *P. gingivalis* cells were exposed to five consecutive doses of nicotine. Briefly, 5×10^7 bacterial cells were grown in the presence of nicotine (0, 1, 2, and 4 mg/L) for 48 h, and these nicotine-exposed bacterial cells (5×10^7) were then re-inoculated into fresh medium containing the same nicotine concentration. This procedure was repeated consecutively five times. This experiment was performed to compare the differences in the growth pattern of *P. gingivalis* cells when they were exposed to single or multiple doses of nicotine.

Protein concentration measurement

Total protein content was determined by using a Protein Assay kit (Bio-Rad), according to the manufacturer's directions.

SDS-PAGE analysis

Conventional SDS-PAGE (10%) analysis was performed according to standard protocols. The sample was solubilized in 0.0625 M Tris hydrochloride (pH 6.8) containing 3% sodium dodecyl sulfate (SDS), 20% glycerol, 0.005 M EDTA, and 1% 2-mercaptoethanol. Protease inhibitor (Sigma-Aldrich Co, UK) was added, as required, directly to the first dimension sample buffer. The samples were then heated at 100°C for 5 min and applied to the gel. Electrophoresis was carried out at room temperature and at a constant voltage (100 V).

2-D gel electrophoresis was performed, as described before (O'Farrell, 1975). Briefly, 10 µg of bacterial lysates were precipitated with a 2-D clean up kit (Bio-Rad) and solubilized in 185 µl rehydration buffer, containing 8 M urea, 2% CHAPS, 50 mM DTT, 0.2% Bio-Lyte 3/10 ampholytes, and 0.001% Bromophenol Blue. Then, the solubilized proteins were loaded onto 11 cm pH 3–10 immobilized pH gradient (IPG) strips (Bio-Rad). Active rehydration was performed at 50V, 20°C for 12 h. Isoelectric focusing (IEF, first dimension) was conducted on a Protean IEF cell (Bio-Rad), under the following conditions: 300 V for 30 min; 8,000 V (linear) for 2.5 h; 8,000 V (rapid) until 30,000 V-hr and 200 V for 1 h. The strips were equilibrated with 2% DTT and 2.5% iodoacetamide for 15 min each in equilibration buffer containing 6 M urea, 2% SDS, 0.375 M Tris-HCl (pH 8.8) and 20% glycerol. Equilibrated strips were placed on an 8–16% gradient gel (Bio-Rad) for vertical electrophoresis at 100 V constant voltage, from the front blue line to the bottom of the gel. Proteins on the gel were visualized by silver staining, as described previously (Wang *et al.*, 2002).

The changes of protein expression in SDS-PAGE and 2D-gel separations were evaluated by visual inspection using unaided eyes.

Results

In the single nicotine exposure protocol, it was observed that the growth of *P. gingivalis* 381 was inhibited by nicotine in a dose-dependent manner, throughout the entire growth period (Fig. 1). Compared to the normal growth pattern observed with no nicotine exposure, *P. gingivalis* cells exposed to nicotine exhibited a significant decrease in their growth rate. Thus, it was observed that 8 mg/L of nicotine essentially blocked bacterial growth.

The bacterial cells pre-exposed to nicotine exhibited a trend of increased growth rate, compared to those not exposed to nicotine. *P. gingivalis* cells pre-exposed to 4 and 8 mg/L nicotine showed almost 2-fold increase in their growth rate at 48, 72, 96, and 120 h (Fig. 2), compared to unexposed *P. gingivalis*. There was also a slight increase in growth for the bacterial cells exposed to 1 and 2 mg/L nicotine.

In the next experiment adopting a multiple nicotine exposure protocol (Fig. 3), *P. gingivalis* growth increased at each subsequent nicotine exposure every 48 h, even though *P. gingivalis* growth was also inhibited in a dose dependent fashion.

On SDS-PAGE analysis, total protein profiles of *P. gingivalis* 381 revealed minor differences in proteins with molecular weight of around 20 kDa upon a single nicotine exposure at 1 mg/L and 2 mg/L (Fig. 4). Similarly, 2-D gel electrophoresis analysis of total cellular lysates of *P. gingivalis* 381 also showed minor differences in protein profiles when exposed to various concentrations of nicotine for 5 days, indicating an increased protein expression in low MW proteins (Fig. 5).

Discussion

The purpose of this study was to assess the effects of nicotine, a major component of tobacco smoke, on the growth pattern and protein expression of *P. gingivalis*. It is estimated that tobacco smoke contains more than 4,000 different compounds, including carbon monoxide, toxic substances, carcinogens and addictive psychoactive substances, such as nicotine (Tonetti, 1998). Nicotine is the major tobacco alkaloid exerting a variety of pharmacological activities. An average cigarette contains 10 to 14 mg of nicotine, and it is estimated that, on average, about 1 to 1.5 mg of nicotine is absorbed systemically during smoking (Benowitz and Jacob, 1984).

In the present study, nicotine was added to the culture medium at a final concentration of 1–8 mg/L. The amount of nicotine used in the experiments was determined based upon previous findings suggesting that the mean concentration of cotinine, a metabolite of nicotine, in saliva and gingival crevicular fluid was 0.4±0.2 mg/L and 2.5±2.4 mg/L, respectively (McGuire *et al.*, 1989).

As expected, the growth of *P. gingivalis* was inhibited by nicotine in a dose-dependent manner, and the growth was totally blocked by adding 8 mg/L nicotine (Fig. 1). This result

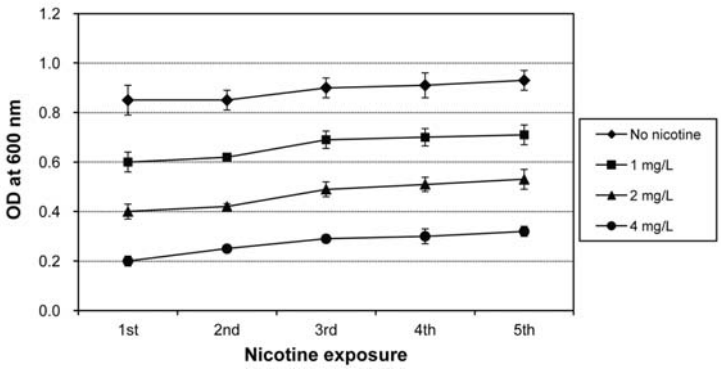

Fig. 3. The growth pattern of *P. gingivalis* 381 following multiple nicotine exposure. *P. gingivalis* cells were first exposed to different nicotine concentrations (0, 1, 2, and 4 mg/L), and allowed to grow in fresh medium containing the same nicotine concentrations. This procedure was repeated consecutively five times. The error bars indicate the range of data, as the experiments were performed in triplicates.

suggests that nicotine is a potentially toxic substance inhibiting overall bacterial metabolism, resulting in modulation of bacterial growth in the formation of oral biofilms.

In the next experiment, it was found that the growth pattern of *P. gingivalis* cells pre-exposed to nicotine was slightly increased, up to 2-fold, compared to that of the bacterial cells not exposed to nicotine (Fig. 2). In another experiment, adopting a multiple nicotine exposure protocol, the growth of *P. gingivalis* 381 slightly increased after each subsequent nicotine exposure, repeated every 48 h (Fig. 3). These results suggest that *P. gingivalis* is able to adapt to nicotine exposure over time and develops tolerance to the inhibitory effect of nicotine. Since nicotine exposure is most likely chronic in smokers, we repeatedly exposed *P. gingivalis* to nicotine, in order to imitate the environment in smokers. It was expected that chronic exposure to nicotine would enhance bacterial cells' ability to cope with subsequent nicotine exposure. As expected, *P. gingivalis* cells previously exposed to nicotine showed a slightly increased growth rate, as they were repeatedly exposed to nicotine. This finding suggests that it is possible for *P. gingivalis* to exhibit increased growth in the periodontal pockets of smokers, where nicotine exposure is chronic. Ultimately, it can be assumed that, by this mechanism, smoking may lead to an increased pathogenicity mediated by *P. gingivalis* in the periodontal lesion.

The effect of cigarette components on the physiology and pathogenic potential of *P. gingivalis* was not adequately studied so far. Recently, it was observed that *P. gingivalis* exposed to 100 mg/L cotinine exhibited a significant increase in both association with, and invasion of, epithelial cells (Cogo *et al.*, 2009).

It is well established that bacteria are able to adapt to environmental changes for their survival. Bacterial cells preferentially express specific proteins in response to a variety of environmental stresses, including heat, oxidative stress, heavy metals and toxins (Mekalanos, 1992). Previous studies have shown that the expression of several virulence factors of *P. gingivalis* was environmentally regulated (Amano *et*

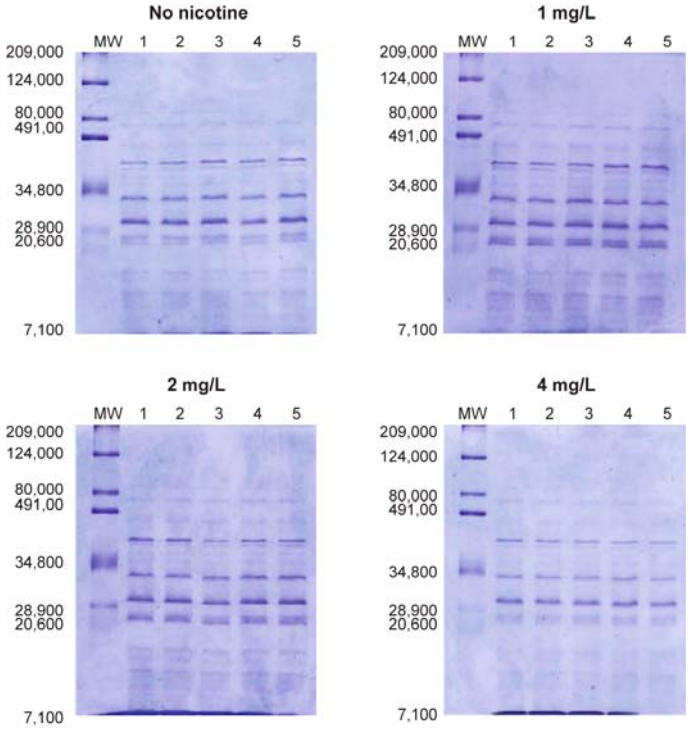

Fig. 4. SDS-PAGE analysis of total cellular lysates of *P. gingivalis* bacteria exposed to various nicotine concentrations (0, 1, 2, and 4 mg/L). Bacterial cells were grown in broth medium containing different nicotine concentration: no nicotine; 1 mg/L; 2 mg/L; and 4 mg/L. Molecular markers are shown in the left lane of each gel.

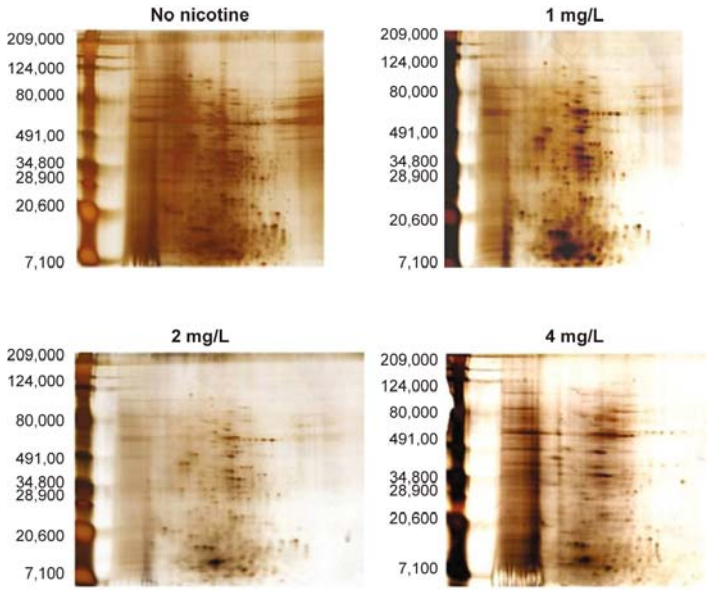

Fig. 5. 2-D gel electrophoresis analysis of total cellular lysates of *P. gingivalis* 381 after a single exposure to nicotine. It was found that increased protein expression occurs only in low MW proteins (around 20 kDa).

al., 1994; Percival *et al*., 1999; Pridmore *et al*., 1999; Forng *et al*., 2000). It is also well established that expression of virulence factors of *P. gingivalis* can be modulated in response to various growth conditions. Thus, in a mouse model, *P. gingivalis* exhibited increased proteolytic activity, and was therefore more virulent when growing under hemin-excess, than in hemin-limited conditions (McKee *et al*., 1986).

In this study, it was therefore expected that nicotine, as a toxic substance, could induce stress responses in *P. gingivalis*, leading to expression of stress-related proteins. The results suggested, however, that nicotine did not significantly affect total protein expression, as observed by SDS-PAGE and 2-D gel electrophoresis (Fig. 5), exhibiting changes only in the expression of proteins with low molecular weights, around 20 kDa. It was rather surprising that there was no major difference in protein expression of *P. gingivalis* after exposure to nicotine. Although the growth rate was inhibited in a dose-dependent manner, interestingly, the protein expression revealed only minor differences between the groups, as reflected in SDS-PAGE and 2-D gel electrophoresis analyses. These results suggest that nicotine has a potential to induce stress responses in *P. gingivalis*, affecting selected proteins. In order to find out whether this is a unique phenomenon for *P. gingivalis*, *E. coli* was grown overnight and exposed to different nicotine concentrations (0, 1, 2, and 4 mg/L), until bacterial growth reached a plateau (8 h). It was found that nicotine also affected protein expression in *E. coli* for selected proteins, as it inhibited the growth of bacterial cells, similar to *P. gingivalis* inhibition (data not shown). These results suggest that, in *P. gingivalis*, nicotine is able to inhibit the whole metabolism at a constant rate, but can modulate protein expression only in a limited fashion. However, it cannot be ruled out that a significant change in the protein profile actually occurred but was not detectable by visual inspection of gel electrophoresis.

Considering the limitations of the current study, it can only be concluded from our observations that nicotine has the potential to elicit a stress reaction, and therefore may serve as an environmental modulating factor for bacterial metabolism and survival.

References

Albandar, J.M. 2002. Global risk factors and risk indicators for periodontal diseases. *Periodontol. 2000* **29**, 177–206.

Amano, A., Sharma, A., Sojar, H.T., Kuramitsu, H.K., and Genco, R.J. 1994. Effects of temperature stress on expression of fimbriae and superoxide dismutase by *Porphyromonas gingivalis*. *Infect. Immun.* **62**, 4682–4685.

Bagaitkar, J., Demuth, D.R., Daep, C.A., Renaud, D.E., Pierce, D.L., and Scott, D.A. 2010. Tobacco upregulates *P. gingivalis* fimbrial proteins which induce TLR2 hyposensitivity. *PLoS One* **5**, e9323.

Bagaitkar, J., Williams, L.R., Renaud, D.E., Bemakanakere, M.R., Martin, M., Scott, D.A., and Demuth, D.R. 2009. Tobacco-induced alterations to *Porphyromonas gingivalis*-host interactions. *Environ. Microbiol.* **11**, 1242–1253.

Benowitz, N.L. and Jacob, P.3rd. 1984. Daily intake of nicotine during cigarette smoking. *Clin. Pharmacol. Ther.* **35**, 499–504.

Bergstrom, J. and Preber, H. 1986. The influence of cigarette smoking on the development of experimental gingivitis. *J. Periodontal. Res.* **21**, 668–676.

Bostrom, L., Bergstrom, J., Dahlen, G., and Linder, L.E. 2001. Smoking and subgingival microflora in periodontal disease. *J. Clin. Periodontol.* **28**, 212–219.

Cogo, K., Calvi, B.M., Mariano, F.S., Franco, G.C., Goncalves, R.B., and Groppo, F.C. 2009. The effects of nicotine and cotinine on *Porphyromonas gingivalis* colonisation of epithelial cells. *Arch. Oral Biol.* **54**, 1061–1067.

Darby, I.B., Hodge, P.J., Riggio, M.P., and Kinane, D.F. 2000. Microbial comparison of smoker and non-smoker adult and early-onset periodontitis patients by polymerase chain reaction. *J. Clin. Periodontol.* **27**, 417–424.

Finlay, B.B. and Falkow, S. 1989. Common themes in microbial pathogenicity. *Microbiol. Rev.* **53**, 210–230.

Finlay, B.B. and Falkow, S. 1997. Common themes in microbial pathogenicity revisited. *Microbiol. Mol. Biol. Rev.* **61**, 136–169.

Forng, R.Y., Champagne, C., Simpson, W., and Genco, C.A. 2000. Environmental cues and gene expression in *Porphyromonas*

gingivalis and *Actinobacillus actinomycetemcomitans. Oral Dis.* **6**, 351–365.

Holt, S.C. and Ebersole, J.L. 2005. *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*: the "red complex", a prototype polybacterial pathogenic consortium in periodontitis. *Periodontol. 2000* **38**, 72–122.

Kamma, J.J., Nakou, M., and Baehni, P.C. 1999. Clinical and microbiological characteristics of smokers with early onset periodontitis. *J. Periodontal Res.* **34**, 25–33.

Kinane, D.F. and Hart, T.C. 2003. Genes and gene polymorphisms associated with periodontal disease. *Crit. Rev. Oral Biol. Med.* **14**, 430–449.

Kroes, I., Lepp, P.W., and Relman, D.A. 1999. Bacterial diversity within the human subgingival crevice. *Proc. Natl. Acad. Sci. USA* **96**, 14547–14552.

Loos, B.G., John, R.P., and Laine, M.L. 2005. Identification of genetic risk factors for periodontitis and possible mechanisms of action. *J. Clin. Periodontol.* **32 Suppl 6**, 159–179.

McGuire, J.R., McQuade, M.J., Rossmann, J.A., Garnick, J.J., Sutherland, D.E., Scheidt, M.J., and Van Dyke, T.E. 1989. Cotinine in saliva and gingival crevicular fluid of smokers with periodontal disease. *J. Periodontol.* **60**, 176–181.

McKee, A.S., McDermid, A.S., Baskerville, A., Dowsett, A.B., Ellwood, D.C., and Marsh, P.D. 1986. Effect of hemin on the physiology and virulence of *Bacteroides gingivalis* W50. *Infect. Immun.* **52**, 349–355.

Mekalanos, J.J. 1992. Environmental signals controlling expression of virulence determinants in bacteria. *J. Bacteriol.* **174**, 1–7.

O'Brien-Simpson, N.M., Veith, P.D., Dashper, S.G., and Reynolds, E.C. 2004. Antigens of bacteria associated with periodontitis. *Periodontol. 2000* **35**, 101–134.

O'Farrell, P.H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **250**, 4007–4021.

Obeid, P. and Bercy, P. 2000. Effects of smoking on periodontal health: a review. *Adv. Ther.* **17**, 230–237.

Olczak, T., Simpson, W., Liu, X., and Genco, C.A. 2005. Iron and heme utilization in *Porphyromonas gingivalis*. *FEMS Microbiol. Rev.* **29**, 119–144.

Page, R.C., Offenbacher, S., Schroeder, H.E., Seymour, G.J., and Kornman, K.S. 1997. Advances in the pathogenesis of periodontitis: summary of developments, clinical implications and future directions. *Periodontol. 2000* **14**, 216–248.

Palmer, R.M., Wilson, R.F., Hasan, A.S., and Scott, D.A. 2005. Mechanisms of action of environmental factors–tobacco smoking 1. *J. Clin. Periodontol.* **32 Suppl 6**, 180–195.

Paster, B.J., Boches, S.K., Galvin, J.L., Ericson, R.E., Lau, C.N., Levanos, V.A., Sahasrabudhe, A., and Dewhirst, F.E. 2001. Bacterial diversity in human subgingival plaque. *J. Bacteriol.* **183**, 3770–3783.

Percival, R.S., Marsh, P.D., Devine, D.A., Rangarajan, M., duse-Opoku, J., Shepherd, P., and Curtis, M.A. 1999. Effect of temperature on growth, hemagglutination, and protease activity of *Porphyromonas gingivalis*. *Infect. Immun.* **67**, 1917–1921.

Pridmore, A.M., Devine, D.A., Bonass, W.A., and Silley, P. 1999. Influence of sample preparation technique on two-dimensional gel electrophoresis of proteins from *Porphyromonas gingivalis*. *Lett. Appl. Microbiol.* **28**, 245–249.

Rivera-Hidalgo, F. 2003. Smoking and periodontal disease. *Periodontol. 2000.* **32**, 50–58.

Tonetti, M.S. 1998. Cigarette smoking and periodontal diseases: etiology and management of disease. *Ann. Periodontol.* **3**, 88–101.

Umeda, M., Chen, C., Bakker, I., Contreras, A., Morrison, J.L., and Slots, J. 1998. Risk indicators for harboring periodontal pathogens. *J. Periodontol.* **69**, 1111–1118.

Van der Velden, U., Varoufaki, A., Hutter, J.W., Xu, L., Timmerman, M.F., Van Winkelhoff, A.J., and Loos, B.G. 2003. Effect of smoking and periodontal treatment on the subgingival microflora. *J. Clin. Periodontol.* **30**, 603–610.

Wang, H., Kachman, M.T., Schwartz, D.R., Cho, K.R., and Lubman, D.M. 2002. A protein molecular weight map of ES2 clear cell ovarian carcinoma cells using a two-dimensional liquid separations/mass mapping technique. *Electrophoresis* **23**, 3168-3181.

Williams, R.C. 1990. Periodontal disease. *N. Engl. J. Med.* **322**, 373–382.

Xie, H., Cai, S., and Lamont, R.J. 1997. Environmental regulation of fimbrial gene expression in *Porphyromonas gingivalis*. *Infect. Immun.* **65**, 2265–2271.

Zambon, J.J., Grossi, S.G., Machtei, E.E., Ho, A.W., Dunford, R., and Genco, R.J. 1996. Cigarette smoking increases the risk for subgingival infection with periodontal pathogens. *J. Periodontol.* **67**, 1050–1054.