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Proteomic analysis of *Porphyromonas gingivalis* exposed to nicotine and cotinine

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Background and Objective: Smokers are more predisposed than nonsmokers to infection with *Porphyromonas gingivalis*, one of the most important pathogens involved in the onset and development of periodontitis. It has also been observed that tobacco, and tobacco derivatives such as nicotine and cotinine, can induce modifications to *P. gingivalis* virulence. However, the effect of the major compounds derived from cigarettes on expression of protein by *P. gingivalis* is poorly understood. Therefore, this study aimed to evaluate and compare the effects of nicotine and cotinine on the *P. gingivalis* proteomic profile.

Material and Methods: Total proteins of *P. gingivalis* exposed to nicotine and cotinine were extracted and separated by two-dimensional electrophoresis. Proteins differentially expressed were successfully identified through liquid chromatography-mass spectrometry and primary sequence databases using MASCOT search engine, and gene ontology was carried out using DAVID tools.

Results: Of the approximately 410 protein spots that were reproducibly detected on each gel, 23 were differentially expressed in at least one of the treatments. A particular increase was seen in proteins involved in metabolism, virulence and acquisition of peptides, protein synthesis and folding, transcription and oxidative stress. Few proteins showed significant decreases in expression; those that did are involved in cell envelope biosynthesis and proteolysis and also in metabolism.

Conclusion: Our results characterized the changes in the proteome of *P. gingivalis* following exposure to nicotine and cotinine, suggesting that these substances may modulate, with minor changes, protein expression. The present study is, in part, a step toward understanding the potential smoke–pathogen interaction that may occur in smokers with periodontitis.

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Periodontal diseases are complex, multifactorial, polymicrobial infections characterized by inflammatory conditions that cause the destruction of tooth-supporting tissues. Furthermore, a possible connection is emerging between chronic periodontitis and serious systemic conditions, such as cardiovascular diseases (1–3), spontaneous preterm low birthweight (4,5), rheumatoid arthritis (6,7), diabetes (8) and respiratory infections (9). The development of periodontal diseases is a consequence of intricate interactions between the bacteria on periodontal sites and the immune and inflammatory reactions of the host. Among the periodontal bacteria associated with the etiology of periodontitis is the anaerobe *Porphyromonas gingivalis*, a pathogen that resides predominantly in

subgingival biofilms (10). This bacterium expresses several virulence factors, such as proteases, fimbriae, lipopolysaccharides and adhesins, which may cause tissue destruction and induce host inflammatory and immune responses (11).

Tobacco use is recognized as one of the most important risk factors for the development and progression of periodontal diseases and a further reduction in the response to periodontal therapy (12). Several studies comparing smokers with nonsmokers have shown that smokers have more alveolar bone loss, deeper periodontal pockets and higher levels of attachment and tooth loss (13–15). Tobacco smoke contains more than 4000 substances. Nicotine, one of the major Nicotine, one of the major components of tobacco (16), has a short blood half-life (± 2 h), whereas cotinine, the main metabolite of nicotine, has a longer blood half-life (± 19 h) (17,18). Because of the longer half-life of cotinine, this substance has been used as a biomarker for smoking status, and its presence in biological fluids indicates exposure to nicotine (19).

It is known that smoking has deleterious effects in the oral cavity, especially on periodontal tissues, and it has also been raised that nicotine negatively affects local cell populations (12). *In vitro* and *in vivo* studies have demonstrated that nicotine can affect various functions of human periodontal ligament fibroblasts (16,20–23), up-regulate the lipopolysaccharide-mediated secretion of prostaglandin E_2 by monocytes (24), stimulate osteoclast resorption (25), augment cytokine levels in nicotine-treated mice (26) and have other deleterious effects on the periodontal tissues.

Nicotine and cotinine concentrations are much higher in saliva and gingival crevicular fluid than in plasma (18,27). Therefore, it is assumed that the oral cavities of smokers, including the oral tissues and their microbiota, are exposed to high concentrations of nicotine and cotinine (12). However, few *in vitro* studies have evaluated the effects of tobacco, especially nicotine and cotinine, on oral bacteria. It was recently shown that *P. gingivalis* cells exposed to cigarette smoke induced a lower pro-inflammatory response from monocytes, presented alterations in the expression of genes related to virulence, oxidative stress and DNA repair, and promoted biofilm formation with *Streptococcus gordonii* (28,29). Although nicotine and cotinine neither reduce nor increase the *in vitro* viability of *P. gingivalis* (30), these substances may have other effects on these bacteria. Sayers *et al.* (31,32) reported that a synergic interaction between *P. gingivalis* toxins and nicotine or cotinine can occur. The colonization of epithelial cells by *P. gingivalis* may also be altered in the presence of nicotine or cotinine (33,34). Very recently, it was found that different concentrations of nicotine have the potential to modify the expression of low-mass proteins (35). Thus, it is possible that the increased severity of periodontitis in smokers may occur as a result of the influence of substances in tobacco on both host and microbial responses. Given these previous results, we hypothesized that nicotine and cotinine could affect protein production by *P. gingivalis* by reducing or increasing protein levels. Therefore, in view of the importance of *P. gingivalis,* nicotine and cotinine in periodontal disease, our study aimed to evaluate the effects of these tobacco substances on the *P. gingivalis* proteome.

Material and methods

Bacterial culture conditions and treatments

For total protein analyses, *P. gingivalis* W83 was cultured in brain–heart infusion broth supplemented with hemin (5 μg/mL) (Sigma Chemical Co., Poole, UK) and menadione (1 μg/mL) (Sigma), under anaerobic conditions (10% CO_2, 10% H_2 and 80% N_2 – MiniMacs Anaerobic Workstation; Don Whitley Scientific, Shipley, UK) at 37°C. Three different bacterial growth conditions were used: (i) nicotine, 6 μg/mL; (ii) cotinine, 6 μg/mL; and (iii) control (bacterial growth without any substance). Both nicotine and cotinine were purchased from Sigma. After adding nicotine and cotinine to the culture medium, the pH was verified and all culture media were found to have similar pH values (approximately 7.40). Culture flasks containing 120 mL of brain–heart infusion medium received 6 mL of a standardized bacterial suspension adjusted with a spectrophotometer to a cell density of 40% transmittance (the final bacterial concentration in each flask was approximately 4×10^7 colony-forming units/mL). *P. gingivalis* was grown in the presence of nicotine or cotinine until an optical density of 1.4 at 660 nm was reached (late-logarithmic phase, approximately 18 h of growth). For the growth evaluations, *P. gingivalis* was cultured under the same conditions mentioned above. The growth evaluations were performed in triplicate. The optical densities in the growth assays were analyzed by analysis of variance (ANOVA). The statistical differences between the control and concentration groups were determined using Dunnett's test ($p < 0.05$).

Total protein extraction and two-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis

The method used for the extraction of total proteins was adapted from a previously described method (36). Briefly, *P. gingivalis* W83 was centrifuged at 8000 *g*, 4°C, for 16 min, and the supernatant was discarded. The proteins in the pellet were precipitated by adding 10% trichloroacetic acid (Sigma) and 0.07% 2-mercaptoethanol in ice-cold acetone (Merck & Co., Inc., Whitehouse Station, NJ, USA) and stored at −20°C for 1 h. After centrifugation at 13,000 *g* and 4°C for 15 min, the protein pellets were rinsed twice (1 h each at −20°C) with ice-cold acetone containing 0.07% 2-mercaptoethanol. The precipitated pellets were centrifuged at 16,000 *g* and 4°C for 20 min. The supernatant was removed, and the protein pellet was air-dried and solubilized in 1 mL of buffer {7 M urea, 2 M thiourea, 4% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS)

and 100 mM dithiothreitol}. Proteins were quantified using the Bradford method (37). Two-dimensional electrophoresis (2DE) of protein samples (750 μg of total protein) was conducted as previously described (38). A minimum of three biological replicates from each treatment were analyzed. Proteins were stained with Coomassie Brilliant Blue G-250 (Pittsburgh, PA, USA) (39).

Gel image analysis and spot detection

The gels were scanned, digitized at 300 dots per inch (dpi) and 16-bit depth resolution (UTA-1100 scanner, LABSCAN V5.0 software; GE Healthcare, Pittsburgh, PA, USA), and submitted to image analysis using IMAGEMASTER 2D software V4 (GE Amersham Biosciences). The protein sample replicates were normalized to quantify the spot intensity and to minimize analytical variation among the gels. Spots were compared based on their volume percentages in the total spot volume over the whole gel image (40). For each sample analyzed, the average spot volume of the three replicate gels was determined and normalized using the "total spot volume normalization" parameter (individual spot volume/ total spots volume × 100 = normalized spot volume). The spot volumes obtained for the control were compared with those observed for the other groups (i.e. those exposed to nicotine and cotinine). The data collected from the protein spot volumes were subjected to ANOVA using the general linear model of the SAS package (41). Statistical significance between the mean values was analyzed using Tukey's test ($p < 0.05$). Spots indicating up-regulation or down-regulation of proteins were excised from the gels and identified by liquid chromatography–tandem mass spectrometry (LC-MS/MS).

Protein identification and analysis by LC quadrupole time-of-flight MS/MS

The proteins were digested according to a method previously described by Fiorani Celedon *et al*. (38). The peptides obtained from protein digestion were identified by capillary LC coupled to a quadrupole time-of-flight mass spectrometer (Q-TOF Ultima API mass spectrometer; Waters, Milford, MA, USA). Five microliters of the peptide solution was loaded onto a NanoEase trapping column (0.18 mm × 23.5 mm; Waters) for preconcentration, followed by peptide separation on a NanoEase Symmetry 300 C18 LC column (3.5 μm, 75 mm × 100 mm; Waters). The peptides were eluted in a 60-min linear gradient of solvent A [5% (v/v) acetonitrile, 0.1% (v/v) formic acid in water] and solvent B [95% (v/v) acetonitrile, 0.1% (v/v) formic acid in water] at a flow rate of 0.25 μL/min. A positive ion mode with a 3-kV needle voltage was used. The mass limit was set from 300 to 2000 mass-to-charge ratio (m/z), and the MS/MS spectra were obtained for the most intense peaks (≥ 15 counts). Multiply charged precursor ions were selected for fragmentation, and automated data-dependent acquisition was used for peptide sequencing with MASSLYNX software (Waters), switching from the MS mode to the MS/MS mode and then returning to the MS mode. The resulting fragmented spectra were processed using PROTEINLYNX software (V4.0; Waters). MASCOT MS/MS Ion Search (http://www.matrixscience.com) was used to compare the sequences with the MSDB, SwissProt and NCBInr databases. The combined MS-MS/MS searches were conducted with the following parameters: MS/MS mass tolerance at 0.5 Da; trypsin as the enzyme; peptide tolerance at 100 ppm; carbamidomethylation of cysteine (fixed modification); and methionine oxidation (variable modification). Only hits that were significant ($p < 0.05$) according to the MASCOT probability analysis were accepted. After protein identification, cellular role categories were verified using the TIGR (http://cmr.jcvi.org/tigr-scripts/CMR/CmrHomePage.cgi) database. Gene ontology analyses were then conducted using the DAVID (42) functional annotation clustering feature with the default databases (http://david.abcc.ncifcrf.gov/home.jsp).

Results

Culture growth

The growth patterns of *P. gingivalis* W83 were similar regardless of exposure to nicotine or cotinine. No statistically significant difference was observed between the control and the nicotine or cotinine groups (Fig. 1 ANOVA, Dunnett's test, $p > 0.05$).

Characterization of the *P. gingivalis* proteome with 2DE and protein identification by MS

To examine the effect of nicotine and cotinine on expression of *P. gingivalis* proteins, 2DE was performed. After 2D separation of the proteins of *P. gingivalis* using immobilized pH gradient (IPG) strips (linear strips of pH 4 to pH 7) and 12.5% polyacrylamide gels ($n = 9$), image analysis revealed 410–455 distinct spots. Most protein spots found in the gels were positioned between 20 and 100 kDa and had an isoelectric point (pI) of 4.5–6.5. IMAGEMASTER 2D software version 4 was used for comparative image analysis of the gels. The 2DE gels for each experimental group are shown in (Fig. 2A,B,C), and the locations of the spots showing a significant alteration in density in the treatment groups compared with the control are indicated. The comparison between the control and the nicotine/cotinine 2D gels showed that 23 spots were differentially expressed by twofold or more (ANOVA, Tukey's test, $p < 0.05$). For the nicotine group, 11 spots were up-regulated (2, 3, 4, 6, 7, 11, 12, 13, 14, 18 and 19) and four were down-regulated (1, 10, 16 and 23) ($p < 0.05$). When the cotinine group was analyzed, nine spots (2, 3, 4, 9, 13, 15, 17, 21 and 22) were more abundant and five spots (5, 8, 16, 20, 23) were less abundant in volume than the same spots in the control ($p < 0.05$). Only six spots were altered in volume in both treatments (spots 2, 3, 4, 13, 16 and 23). In Fig. 2D, enlarged partial 2D maps from the control, cotinine and nicotine groups show the expression of spots 1, 3, 4, 6 and 8.

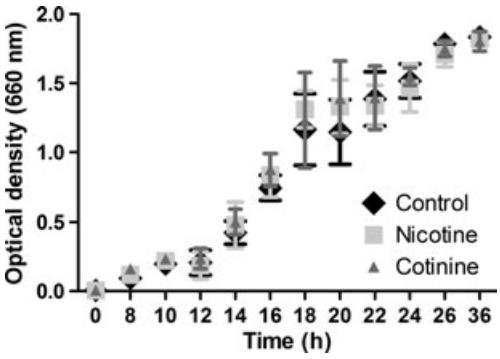

Fig. 1. Growth curves for *Porphyromonas gingivalis* exposed or not exposed for 36 h to 6 μg/mL of either nicotine or cotinine. No statistically significant differences were observed between control and nicotine or cotinine groups (ANOVA, Dunnett's test, $p > 0.05$).

The differentially expressed proteins ($n = 23$) were analyzed by LC-Q-TOF MS/MS. Protein sequencing showed a good correlation between the theoretical and experimental values for both the pI and the relative molecular mass. The data for the proteins with expression levels different from those observed for the control are shown in Tables 1 and 2. The means, standard deviations and intensity ratios for each protein spot after treatment are shown in Table 1. The proteins were grouped according to their molecular functions. The proteins with significantly altered production included molecules that participate in cell envelope biosynthesis (phosphomannomutase, spot 1), oxidative stress (OxyR, spot 2; and rubrerythrin, spot 3), transport (RagA, spot 4; and hypothetical tonB-linked outer membrane receptor PG50, spot 5), virulence (dipeptidyl aminopeptidase IV, spot 6; hypothetical protein, spot 7; and peptidase M20/M25/M40 family, spot 8), protein synthesis and folding [elongation factor Ts (EF-Ts), spot 9; ribosome recycling factor (RRF), spot 10; and translation elongation factor G (TEF-G), spot 11] and transcription (transcription termination factor Rho, spot 12). However, most of the proteins that exhibited altered expression levels were involved in metabolic processes such as central intermediary metabolism (acetyl-CoA hydrolase, spot 13), in the biosynthesis of coenzymes, prosthetic groups and carriers (oxidoreductase, spot 14; pyridoxal phosphate biosynthetic protein PdxJ, spot 15; and riboflavin synthase,

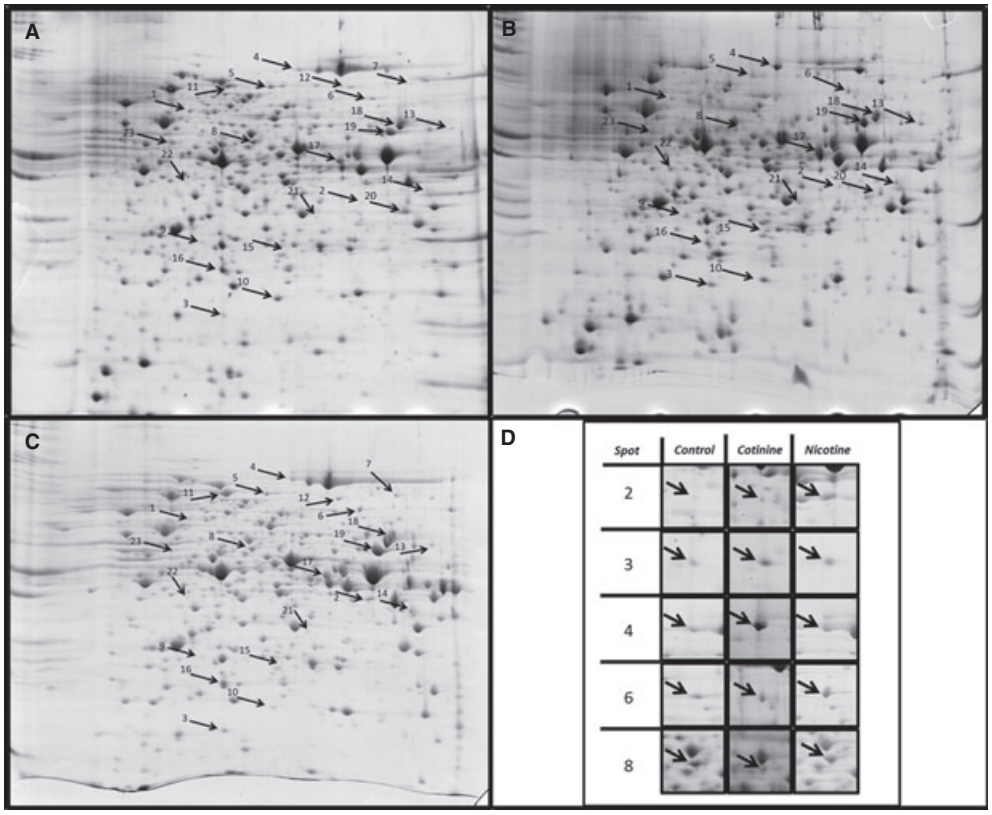

Fig. 2. Two-dimensional electrophoresis (2DE) gels of *Porphyromonas gingivalis* in control (A), cotinine (B), and nicotine (C) groups. The proteins identified in the current study (Table 2) are indicated. Enlarged partial 2DE gels (D) showing some of the differentially expressed protein spots (1, 3, 4, 6, and 8) when comparing the treatments with the control. Mean, SD, intensity ratios and statistical results for treatments and the control are shown in Table 1.

alpha subunit, spot 16), in energy production (2-amino-3-ketobutyrate CoA ligase, spot 17; delta-1-pyrroline-5-carboxylate dehydrogenase, spot 18; and 4-hydroxybutyryl-CoA dehydratase, spot 19), in fatty acid and phospholipid catabolism (acyl-CoA dehydrogenase, short-chain specific, spot 20) and in nucleotide biosynthesis, transport and catabolism (dihydroorotate dehydrogenase, spot 21). In addition, proteins with unknown functions (immunoreactive 42 kDa antigen PG33, spot 22; and immunoreactive 53 kDa antigen PG123, spot 23) were also identified.

Discussion

Since the mid-1990s, smoking has been recognized as one of the most important

Table 1. Differing expression of proteins from *Porphyromonas gingivalis* during treatment and their corresponding intensity ratio

Protein	Spot number	Control	Cotinine	Nicotine	Intensity ratio[1]	
					Cotinine	Nicotine
Cellular processes						
Cell envelope biosynthesis						
Phosphomannomutase, putative	1	0.127 (± 0.026)	0.108 (± 0.028)	0.056 (± 0.007)	0.85	0.44[a]
Oxidative stress						
Redox-sensitive transcriptional activator OxyR	2	0.013 (± 0.003)	0.032 (± 0.004)	0.034 (± 0.006)	2.50[a]	2.61[a]
Rubrerythrin	3	0.125 (± 0.013)	0.278 (± 0.043)	0.254 (± 0.051)	2.23[a]	2.03[a]
Transport and binding activity						
RagA protein	4	0.087 (± 0.001)	0.323 (± 0.170)	0.290 (± 0.086)	3.70[a]	3.33[a]
Hypothetical tonB-linked outer membrane receptor PG50	5	0.184 (± 0.033)	0.091 (± 0.019)	0.170 (± 0.006)	0.50[a,b]	0.92
Virulence and acquisition of peptides						
Dipeptidyl aminopeptidase IV, putative	6	0.119 (± 0.023)	0.130 (± 0.037)	0.242 (± 0.011)	1.10	2.04[a,b]
Hypothetical protein	7	0.032 (± 0.002)		0.154 (± 0.031)	–	4.82[a]
Peptidase, M20/M25/M40 family	8	0.341 (± 0.031)	0.160 (± 0.043)	0.281 (± 0.031)	0.47[a,b]	0.82
Information storage and processing						
Protein synthesis and folding						
Elongation factor Ts	9	0.059 (± 0.007)	0.128 (± 0.035)	0.061 (± 0.011)	2.16[a,b]	1.03
Ribosome recycling factor (ribosome-releasing factor)	10	0.356 (± 0.010)	0.330 (± 0.036)	0.145 (± 0.023)	0.93	0.41[a,b]
Translation elongation factor G, putative	11	0.473 (± 0.124)	–	0.988 (± 0.091)	–	2.09[a]
Transcription						
Transcription termination factor Rho	12	0.077 (± 0.014)	–	0.172 (± 0.028)	–	2.21[a]
Metabolism						
Central intermediary metabolism						
Acetyl-CoA hydrolase/transferase family protein	13	0.059 (± 0.020)	0.160 (± 0.026)	0.126 (± 0.009)	2.72[a]	2.14[a]
Coenzyme, prosthetic groups and carriers biosynthesis						
Oxidoreductase, putative	14	0.078 (± 0.021)	0.098 (± 0.025)	0.198 (± 0.057)	1.25	2.52[a,b]
Pyridoxal phosphate biosynthetic protein PdxJ	15	0.035 (± 0.009)	0.088 (± 0.022)	0.043 (± 0.010)	2.52[a,b]	1.22
Riboflavin synthase, alpha subunit	16	0.436 (± 0.017)	0.218 (± 0.020)	0.249 (± 0.073)	0.50[a]	0.57[a]
Energy production: amino acids and amines						
2-amino-3-ketobutyrate CoA ligase	17	0.378 (± 0.024)	0.944 (± 0.014)	0.463 (± 0.099)	2.50[a,b]	1.22
Delta-1-pyrroline-5-carboxylate dehydrogenase	18	0.855 (± 0.109)	0.613 (± 0.075)	1.796 (± 0.377)	0.72	2.1[a,b]
Energy production: fermentation						
4-hydroxybutyryl-CoA dehydratase	19	0.538 (± 0.116)	0.807 (± 0.139)	1.252 (± 0.204)	1.50	2.33[a,b]
Fatty acid and phospholid catabolism						
Acyl-CoA dehydrogenase, short-chain specific	20	0.419 (± 0.071)	0.164 (± 0.012)	–	0.40[a]	–
Nucleotide biosynthesis, transport and catabolism						
Dihydroorotate dehydrogenase	21	0.035 (± 0.004)	0.082 (± 0.018)	0.050 (± 0.014)	2.33[a]	1.42
Unknown function						
Immunoreactive 42-kDa antigen PG33	22	0.033 (± 0.011)	0.173 (± 0.011)	0.020 (± 0.009)	5.32[a,b]	0.62
Immunoreactive 53-kDa antigen PG123	23	0.285 (± 0.123)	0.142 (± 0.018)	0.091 (± 0.008)	0.50	0.32[a]

Values are given as mean (± SD).
[1]Normalized volume of spot in treatment/normalized volume of spot in control.
[a]Differences statistically significant between one treatment and control.
[b]Differences statically significant between the nicotine and cotinine.
Spots were concluded to be significantly up- or down-regulated when $p < 0.05$ (ANOVA, followed by Tukey's test).

Table 2. Identification of proteins differently expressed in the treatments

Protein	Spot number	TIGR locus	Accession number	Score[a]	M_r[b]	pI[c]	Sequence coverage (%)[d]	Matched peptides[e]
Cellular processes								
Cell envelope biosynthesis and degradation/evasion								
Phosphomannomutase, putative	1	PG 2010	Q7MTF4	217	61,587	5.23	14	7
Oxidative stress								
Redox-sensitive transcriptional activator OxyR (OxyR)	2	PG 0270	Q7MXD3	55	35,408	5.88	14	4
Rubrerythrin	3	PG 0195	RUBY	293	22,670	5.83	28	15
Transport and binding activity								
RagA protein	4	PG 0185	Q7MXJ7	851	112,362	6.07	28	40
Hypothetical tonB-linked outer membrane receptor PG50	5	PG 0707	Q9KIB4	93	94,510	5.58	4	4
Virulence and acquisition of peptides								
Dipeptidyl aminopeptidase IV, putative	6	PG 1361	Q7MUW6	992	82,670	6.16	45	63
Hypothetical protein	7	PG 2029	Q7MTD8	137	98,046	8.36	10	8
Peptidase, M20/M25/M40 family	8	PG 0561	Q7MWN9	259	50,677	5.42	20	10
Protein synthesis and folding								
Elongation factor Ts	9	PG 0378	gi34540207	637	30,282	5.31	59	22
Ribosome recycling factor (ribosome-releasing factor, RRF)	10	PG 1901	RRF	263	20,773	5.61	20	11
Translation elongation factor G, putative	11	PG 0933	Q7MVV0	636	80,533	5.33	31	20
Transcription								
Transcription termination factor Rho	12	PG 0332	Q7MX79	665	72,309	5.89	35	54
Metabolism								
Central intermediary metabolism								
Acetyl-CoA hydrolase/transferase family protein	13	PG 1013	Q7MVN7	62	54,983	6.18	11	8
Coenzyme, prosthetic groups and carriers biosynthesis								
Oxidoreductase, putative	14	PG 0430	Q7MWZ8	52	37,409	6.27	16	4
Pyridoxal phosphate biosynthetic protein PdxJ	15	PG 0630	gi34540436	77	27,047	5.58	26	4
Riboflavin synthase, alpha subunit	16	PG 0733	Q7MW99	624	22,636	5.30	75	34
Energy production: amino acids and amines								
2-amino-3-ketobutyrate CoA ligase	17	PG 0481	Q7MWV5	354	44,490	5.70	19	7
Delta-1-pyrroline-5-carboxylate dehydrogenase	18	PG 1269	Q7MV36	807	60,211	6.20	40	53
Energy production: fermentation								
4-hydroxybutyryl-CoA dehydratase	19	PG 0692	Q7MWD1	640	54,513	6.00	54	44
Fatty acid and phospholid catabolism								
Acyl-CoA dehydrogenase, short-chain specific	20	PG 1076	Q7MVI5	243	42,283	6.21	26	9
Nucleotide biosynthesis, transport and catabolism								
Dihydroorotate dehydrogenase	21	PG 1065	Q7MVJ6	86	33,327	5.69	11	3
Unknown function								
Immunoreactive 42 kDa antigen PG33	22	PG 0694	gi34540489	229	42,596	7.68	22	8
Immunoreactive 53 kDa antigen PG123	23	PG 2167	Q9X6S8	69	53,605	9.00	11	5

[a]Score given to the results obtained from Mascot Search.
[b]M_r, relative molecular mass (in Da).
[c]pI, isoeletric point.
[d]Coverage of peptides sequenced.
[e]Number of peptides matches for tandem mass spectrometry (MS/MS).

risk factors for the development of periodontitis (12,17,43–45), and nicotine, one of the major compounds of tobacco, has been investigated as being a key substance associated with the negative effects of smoking on periodontal cells (16,20–23). *P. gingivalis* is also strongly associated with the etiology of periodontitis (46,47). Nevertheless, the interactions between *P. gingivalis* and cigarette-derived components are not fully understood. Because *P. gingivalis* is exposed to nicotine and cotinine in the oral cavity through the smoking habit of the host, it was hypothesized that this microorganism might develop mechanisms to respond to the changing environment. In the present study, the ability of nicotine and cotinine to modify the *P. gingivalis* proteome was evaluated and 23 protein spots from *P. gingivalis* were found to be significantly altered in the presence of nicotine and/or cotinine *in vitro*.

The concentrations of nicotine and cotinine used in this study were similar

to the levels found in saliva and in gingival crevicular fluid and were therefore considered adequate to evaluate their effects. The mean nicotine yield in cigarette smoke can vary from 1.10 to 3.40 mg per cigarette, based on the Massachusetts smoking regimen for all cigarette brand styles and major market categories in 1997–2005 (48). The concentration of nicotine in the plasma of smokers generally ranges from 10 to 50 ng/mL (49), while that of cotinine is about 250–300 ng/mL (18). Ryder *et al*. (50) observed nicotine concentrations in saliva and gingival crevicular fluid of 1.821 (± 0.609) μg/ml and 5.961 (± 0.771) μg/mL, respectively, in samples collected from smokers immediately after smoking one cigarette. The mean levels of cotinine reported in the saliva and the gingival crevicular fluid of cigarette smokers ranged from 7.978 to 15.027 μg/mL and from 2.259 to 3.186 μg/mL, respectively (44).

At the concentrations tested, our results showed that neither nicotine nor cotinine interfered with *P. gingivalis* growth. This finding is in agreement with previous data (30). Although no changes in the growth of *P. gingivalis* in culture (with or without nicotine and cotinine) were found, its protein expression profile was considerably altered. Changes in protein expression without alterations in *P. gingivalis* growth were previously observed when this bacterium was exposed to epithelial cells (51). Recently, slight up-regulation of the expression of low-molecular-weight proteins by *P. gingivalis* cultures exposed to 0, 1, 2 and 4 mg/L (0, 1, 2 and 4 μg/mL) of nicotine for 5 d was reported (35). In the present study, we also found that nicotine caused minor alterations in the production of protein from *P. gingivalis*, including proteins of around 20 kDa (spots 3, 4, 10 and 16). However, this previous study did not show which spots were altered in 2DE gels and did not identify the proteins represented by these spots. Furthermore, the protein expression in 2DE gels was evaluated by means of visual inspection using naked eyes instead of by image-analysis software (35). Therefore, further comparisons between our results and those reported in this previous study are difficult because of differences in methodology.

Gene expression in *P. gingivalis* exposed to cigarette smoke extract (CSE) containing 500–4000 ng/mL (0.5–4 μg/mL) of nicotine equivalents was also characterized through microarray analysis and quantitative real-time RT-PCR (28). Cigarette smoking promoted changes in the expression of *P. gingivalis* genes: 58 genes were up-regulated and 46 were down-regulated, representing approximately 4.7% and 2% of the total genome, respectively. Multiple genes from several predicted operons were stimulated, such as major fimbrial operon and an operon encoding outer membrane antigenic proteins. In addition, genes related to virulence (specifically some proteases and an efflux transporter), genes encoding cell-surface polypeptides, putative lipoprotein genes involved in *fimA* co-expression and genes encoding DNA replication and repair proteins, were also stimulated. Genes participating in capsular biosynthesis (*capK* and PG0117), a gene that regulates the expression of minor and major fimbrial operons (*fimS*) and a tonB-dependent hemoglobin receptor gene (*hmuR*) were some of the genes that were down-regulated upon exposure to CSE. Compared with the present study, the expression of proteins encoded by these genes was unaltered in the presence of nicotine or cotinine. Although this previous study provides somewhat different data in comparison with the present study, its findings also suggest that cigarette substances may affect genes involved in different cellular processes, including genes associated with virulence aspects. In addition to the transcriptome analysis, these authors also evaluated the production of cell-surface and outer membrane proteins using sodium dodecyl sulfate–polyacrylamide gel electrophoresis and MS. RagA, RagB and PG0179 proteins were found at higher levels; however, interestingly, the genes encoding these proteins were not stimulated as observed in the microarray analysis. RagA protein was also overproduced in the nicotine and cotinine groups in the present study. As concluded by this previous study, cigarette substances may sometimes affect the production of protein without promoting changes in gene expression, by interfering in some post-transcriptional processes (28).

Of the 15 spots altered in the nicotine-treatment group, one *P. gingivalis* protein – phosphomannomutase (PMM), which is involved in cell envelope biosynthesis – was down-regulated. PMM plays a crucial role in the synthesis of cell envelope components, such as surface polysaccharides and lipopolysaccharide (LPS), in several bacterial species. This enzyme has been reported to participate in the LPS biosynthetic pathways of *Vibrio furnissii* (52) and *Pseudomonas aeruginosa* (53). Thus, PMM may contribute to the production of LPS by *P. gingivalis* and participate in fructose, mannose, amino sugar and nucleotide sugar metabolism. Despite its importance, the PMM enzyme was down-regulated (spot 1) in the presence of nicotine. This reduction in the expression of PMM may reduce energy waste. Nevertheless, low levels of PMM could cause a decrease in bacterial viability (although such a decrease was not evident in the present study) and in host cell evasion.

Both spots identified as oxidative stress class proteins were up-regulated in the presence of nicotine and cotinine. The redox-sensitive transcriptional activator, OxyR (spot 2), and rubrerythrin (spot 3) were more abundant in the experimental groups than in the control group. These proteins play an essential role in protecting *P. gingivalis* from oxidative stress (54–57), enabling this bacterium to survive within periodontal pockets despite occasional exposure to aerobic conditions (58). In fact, OxyR regulates the transcription of oxidative-stress-related genes under anaerobic and aerobic conditions (55,57). Although the bacterial cultures were not exposed to an aerobic environment in the present study, nicotine and cotinine enhanced the production of two proteins involved in the protection against reactive oxygen species during the oxidative stress response. Nicotine has

been reported to be an oxidative agent (59–62); however, no information on the effects of cotinine on oxidative stress can be found in the scientific literature. The up-regulation of oxidative stress proteins in *P. gingivalis* could be a strategy for neutralizing damaging oxidants that may be formed after exposure to nicotine and cotinine.

RagA (spot 4) was highly expressed in the nicotine- and cotinine-treated groups, while cotinine reduced the expression of another protein linked to outer membrane transport activity, the hypothetical tonB-linked outer membrane receptor PG50 (spot 5). Both proteins participate in inorganic ion transport and metabolism, and their respective genes were classified, by the DAVID gene clustering analyses, as being very highly related. In agreement with the present findings, RagA production has been shown to increase in *P. gingivalis* exposed to CSE (28). Previously known as an immunodominant surface antigen, RagA has been identified in the sera of patients with periodontal disease (63). This protein has homology to TonB-linked outer-membrane receptors, which are involved in the recognition and active uptake of a specific carbohydrate or glycoprotein and iron acquisition in *P. gingivalis* (64). The *ragA* locus arose by horizontal gene transfer and may be a significant virulence factor in *P. gingivalis* (65).

In the present study, both nicotine and cotinine also affected the expression levels of three proteases. Cotinine reduced the levels of the peptidase M20/M25/M40 (spot 8) family, in contrast to nicotine, which promoted higher levels of dipeptidyl aminopeptidase IV putative protein (spot 6) and a hypothetical protein (spot 7) with proteolytic function. In summary, nicotine appeared to stimulate the production of proteins related to bacterial virulence. However, cotinine up-regulated only one protein (RagA) and down-regulated two proteins (peptidase M20/M25/M4 and hypothetical tonB-linked PG50) involved in virulence processes. The down-regulation of some virulence factors of *P. gingivalis* under different culture conditions has been reported previously. When internalized in gingival epithelial cells *in vitro*, *P. gingivalis* reduced the expression of some proteases to avoid host cell damage and apoptotic cell death (66). Under aerobic conditions, *P. gingivalis* overexpresses the superoxide dismutase (*sod*) gene (related to the oxidative stress response) but represses the expression of FimA, a virulence factor responsible for cell adhesion and invasion (67). As mentioned in these previous studies, the decreased expression of some genes or proteins may preserve bacterial energy or prepare it for new growth conditions. In the present study, the presence of cotinine may be a stress factor, and *P. gingivalis* may have down-regulated some apparently nonessential proteins to preserve energy. By contrast, other proteins associated with virulence were overexpressed. These proteins were probably up-regulated to perform proteolysis and to recycle amino acids for protein synthesis as well as to control the bacterial response to a stressful condition. In fact, the effect of either nicotine or cotinine on protease expression may not cause an extreme change in the proteolytic activity of *P. gingivalis* because 42 proteases have been identified in its genome sequence (68).

Most of the proteins involved in transcription (transcription termination factor Rho, spot 12) and protein synthesis and folding (EF-Ts, spot 9; and TEF-G, spot 11) were up-regulated in the experimental groups, except for the (RRF, spot 10). This up-regulation profile was expected because the expression of many bacterial proteins was increased by the treatments. An increase in RRF was also expected because this protein dissociates the ribosome complex after translation and releases it to initiate another translation cycle (69). EF-Ts, which was up-regulated by the cotinine treatment, regulates translation elongation, acting as a steric chaperone for folding and protection against growth inhibition, as previously described in *Escherichia coli* (70). TEF-G, another protein up-regulated by nicotine, has GTPase activity and can bind nucleic acids, purines, guanyl nucleotides, ribonucleotides and guanyl ribonucleotide. It is also reported to be a chaperone in *E. coli* and may be involved in protein folding and protection against stress, in addition to its role in translation (71). The possible role of EF-Ts and TEF-G as chaperones may explain their higher levels under the stressful condition of bacterial exposure to nicotine or cotinine.

The addition of nicotine or cotinine to *P. gingivalis* cultures promoted a significant alteration in some enzymes involved in metabolism, up-regulating most of them. These enzymes are associated with the metabolism of amino acids, coenzymes, fatty acids, prosthetic groups and carriers, the synthesis of phospholipids and nucleotides and the production of energy. Special attention must be given to the proteins involved in energy production. They play an important role in bacterial metabolic pathways, such as the citrate cycle (oxidoreductase, putative, spot 14), riboflavin metabolism (riboflavin synthase, alpha subunit, spot 16), glycine, serine and threonine metabolism (2-amino-3-ketobutyrate CoA ligase, spot 17), alanine, aspartate and glutamate metabolism, arginine and proline metabolism (delta-1-pyrroline-5-carboxylate dehydrogenase, spot 18), benzoate degradation via hydroxylation (4-hydroxybutyryl-CoA dehydratase, spot 19) and pyrimidine metabolism (dihydroorotate dehydrogenase, spot 21). Generally, this up-regulation may allow the bacterium to produce more energy to preserve all bacterial processes, including virulence. However, considering their complexity and the involvement of several proteins in each metabolic process, these processes have probably not changed drastically.

In summary, the present study showed that the expression levels of some proteins were altered in the presence of nicotine and cotinine. Indeed, the *P. gingivalis* proteome responds to nicotine and cotinine in a specific manner, and virulence, oxidative stress and metabolism factors were differentially expressed. A greater number of changes may be found in the protein production when *P. gingivalis* is exposed to nicotine and cotinine in the host environment than under the conditions described

here. However, further studies are required to elucidate the exact roles of these substances and their physiological relevance in oral bacteria.

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