

A streptococcal effector protein that inhibits *Porphyromonas gingivalis* biofilm development

Aaron B. Christopher,² Annette Arndt,¹ Carla Cugini² and Mary E. Davey²

Correspondence

Mary E. Davey
mdavey@forsyth.org

¹Institute of Microbiology and Biotechnology, University of Ulm, Ulm, Germany

²Department of Molecular Genetics, The Forsyth Institute, Boston, MA, USA

Dental plaque formation is a developmental process involving cooperation and competition within a diverse microbial community, approximately 70% of which is composed of an array of streptococci during the early stages of supragingival plaque formation. In this study, 79 cell-free culture supernatants from a variety of oral streptococci were screened to identify extracellular compounds that inhibit biofilm formation by the oral anaerobe *Porphyromonas gingivalis* strain 381. The majority of the streptococcal supernatants (61 isolates) resulted in lysis of *P. gingivalis* cells, and some (17 isolates) had no effect on cell viability, growth or biofilm formation. One strain, however, produced a supernatant that abolished biofilm formation without affecting growth rate. Analysis of this activity led to the discovery that a 48 kDa protein was responsible for the inhibition. Protein sequence identification and enzyme activity assays identified the effector protein as an arginine deiminase. To identify the mechanism(s) by which this protein inhibits biofilm formation, we began by examining the expression levels of genes encoding fimbrial subunits; surface structures known to be involved in biofilm development. Quantitative RT-PCR analysis revealed that exposure of *P. gingivalis* cells to this protein for 1 h resulted in the downregulation of genes encoding proteins that are the major subunits of two distinct types of thin, single-stranded fimbriae (*fimA* and *mfa1*). Furthermore, this downregulation occurred in the absence of arginine deiminase enzymic activity. Hence, our data indicate that *P. gingivalis* can sense this extracellular protein, produced by an oral streptococcus (*Streptococcus intermedius*), and respond by downregulating expression of cell-surface appendages required for attachment and biofilm development.

Received 16 June 2010
Revised 2 August 2010
Accepted 9 August 2010

INTRODUCTION

High cell density and microbial diversity are fundamental to the development and function of the oral biofilm (Kolenbrander *et al.*, 2002). The close proximity of cells within the biofilm facilitates cell–cell interactions, while the diversity offers a wide variety of metabolic functions, allowing the community to acquire nutrients and persist. During plaque formation, organisms affect the activities of one another via physical associations and through the release of extracellular molecules; the effects of these interactions range from mutualistic to antagonistic (Davey & Costerton, 2006; Socransky & Haffajee, 2000). Molecules that are excreted by bacteria into the environment or directly into host cells (through either non-classical or conventional secretion pathways), affecting gene expression of another organism, are often referred to as effectors. The primary objective of this study was to identify effectors produced by oral streptococci that modulate biofilm formation by the oral anaerobe *Porphyromonas gingivalis*.

P. gingivalis is a Gram-negative anaerobe that persists within the oral biofilm community. Outgrowth of this normally commensal organism is associated with severe periodontal disease, resulting in the destruction of tissues that support the gums and, ultimately, tooth loss (Choi *et al.*, 1990; Dzink *et al.*, 1988; Grossi *et al.*, 1994; Lamont & Yilmaz, 2002; Moore *et al.*, 1991). Several studies have shown that *P. gingivalis* can invade and multiply within gingival epithelial cells, as well as penetrate deeper epithelial cell layers, releasing the whole organism and/or virulence factors into the bloodstream (reviewed by Yilmaz, 2008). Given its largely benign lifestyle as a part of the normal microbiota, with its ability to shift and become highly destructive, *P. gingivalis* may be best classified as a ‘commensal pathogen’ (Falkow, 2006). Understanding the mechanisms that control the colonization and outgrowth of this anaerobe within the oral biofilm community is fundamental to understanding the development of periodontal disease.

Oral streptococci have the capacity to produce more interspecies modulators than any other bacterial group within the oral biofilm community (Kuramitsu *et al.*, 2007). Some, under certain growth conditions, produce

Abbreviations: ADI, arginine deiminase; DMSO, dimethyl sulfoxide; MWCO, molecular mass (weight) cut-off; μ LC, microcapillary liquid chromatography; qPCR, quantitative reverse-transcription PCR.

significant quantities of hydrogen peroxide, which has antagonistic effects on a wide variety of isolates (Barnard & Stinson, 1999; Jakubovics *et al.*, 2008; Kreth *et al.*, 2005). In addition, many streptococci produce bacteriocins that kill select organisms, often causing lysis. The most widely studied oral streptococcus species, *Streptococcus mutans*, produces five distinct peptide bacteriocins (mutacins I to V), while nine more putative bacteriocin-encoding genes have been identified in its genome (Chikindas *et al.*, 1995; Kuramitsu *et al.*, 2007; Qi *et al.*, 1999, 2001). In addition, another oral species, *Streptococcus salivarius*, produces the lantibiotic salivaricin A, which inhibits the growth of a variety of streptococcal species (Kuramitsu *et al.*, 2007; Upton *et al.*, 2001). Recently, a novel contact-dependent signalling system was identified between *P. gingivalis* and *Streptococcus cristatus*, involving a surface-associated arginine deiminase. Early studies discovered that a cell-wall protein of *S. cristatus* repressed expression of *fimA* (encoding the major subunit of long fimbriae) in *P. gingivalis* (Xie *et al.*, 2000). Later studies identified the protein as an arginine deiminase (Xie *et al.*, 2007).

In the present study, we describe the isolation and characterization of a secreted/excreted arginine deiminase isolated from culture supernatants of a clinical strain identified as *Streptococcus intermedius*. We show that this arginine deiminase represses expression of genes encoding proteins that are the major subunits of two different fimbrial structures present on the surface of *P. gingivalis*, the short (*mfa1*) and long fimbriae (*fimA*). We also show that this arginine deiminase prevents mono-species biofilm development by *P. gingivalis* strain 381.

METHODS

Strains and culture conditions. Seventy-nine uncharacterized streptococci (isolated on blood agar plates from adolescent supra-gingival plaque samples in Dr Anne Tanner's laboratory at the Forsyth Institute) were grown overnight in Brain Heart Infusion (BHI) broth supplemented with 0.1% yeast extract. To obtain cell-free supernatants, the cultures (final pH 7.2–7.4) were grown to stationary phase, centrifuged to remove bacterial cells and then filter-sterilized. The isolates were maintained in a COY anaerobic chamber on trypticase soy agar plates supplemented with 5% defibrinated sheep blood (Northeast Laboratory Services, Waterville, ME, USA), 1 µg haemin ml⁻¹ and 1 µg menadione ml⁻¹ (BAPHK). *P. gingivalis* strain 381, obtained from Dr Howard Kuramitsu at State University of Buffalo (Buffalo, NY, USA), was stored in DMSO at -80 °C and revived on BAPHK plates, before transferring to Todd-Hewitt Broth containing 1 µg haemin ml⁻¹ and 1 µg menadione ml⁻¹ (THBHK).

Detection of biofilm inhibitory activity. The biofilm assay was performed as previously described (Davey, 2006; Davey & Duncan, 2006), with slight modifications. Briefly, THBHK broth cultures were inoculated from BAPHK plates of *P. gingivalis* strain 381 and grown anaerobically for 36 h. Broth cultures were then diluted in a chemically defined medium (CDM) developed for *P. gingivalis* (Milner *et al.*, 1996), supplemented with 1% tryptone, to OD₆₀₀ 0.2. Culture aliquots of 200 µl were then placed into 96-well polystyrene flat-bottom plates under aerobic conditions before anaerobic incubation (Pack-Anaero, Mitsubishi Gas Chemical

Company) at 37 °C for 24 h. The culture supernatant was then removed by inversion and shaking, and the plate was washed twice by immersion in distilled water. The biofilm-containing plates were air-dried, stained with 0.1% safranin for 15 min (100 µl per well) and washed twice more in distilled water. The amount of biofilm was quantified by solubilizing the safranin in 90% ethanol/1.0% SDS (200 µl per well) for 15 min before measuring absorbance (492 nm) with a plate reader.

To screen for biofilm inhibition by filter-sterilized spent supernatants, fractions from different stages of purification (see below) and aliquots of citrulline (final concentration 10 mM) were tested in triplicate by adding aliquots to the biofilm assay culture before anaerobic incubation. Through the dilution of a 2× concentrated CDM medium, samples of up to 100 µl (twofold dilution) were tested while maintaining the final nutrient concentration at 1× CDM.

Biofilm-inhibitor production analysis. To determine if the inhibitor was produced only during certain phases of growth, a production analysis was performed. BHI broth cultures of the *S. intermedius* isolate were grown (in triplicate) and incubated anaerobically at 37 °C for 24 h. Aliquots were withdrawn at regular intervals to measure cell density (OD₆₀₀) and to determine the titre of the effector in the culture supernatant. The activity was monitored using the biofilm assay, as described above. Cell-free spent supernatants that demonstrated activity at a dilution of 1:10 or greater were considered significant.

Protein purification. Our preliminary studies with protease (proteinase K) treatment indicated that the inhibitor (effector) was a protein; therefore, ammonium sulfate precipitation followed by ion-exchange chromatography was used to purify the effector. The *S. intermedius* isolate was grown anaerobically at 37 °C to stationary phase in one litre of BHI broth (final pH ~7.4). The cell-free supernatant was brought to 40% saturation with ammonium sulfate, stirred for 4 h at 4 °C and then centrifuged (23 200 g, 30 min). The centrifugate was brought to 65% saturation with ammonium sulfate and stirred overnight at 4 °C. The 40–65% ammonium sulfate precipitate was collected, dissolved in 25 ml 10 mM sodium phosphate buffer (pH 7.0) and desalted by overnight dialysis (10 kDa molecular mass (weight) cut-off, MWCO) in 10 mM sodium phosphate buffer (pH 7.0) at 4 °C. The material (8 ml) was then applied to a 30 ml DEAE Sepharose Fast Flow column (GE Healthcare) equilibrated with the same sodium phosphate buffer. The column was washed with 2 column volumes (CV) of equilibration buffer, one CV 100 mM NaCl and 3 CV 200 mM NaCl before activity was eluted in 3 CV 500 mM NaCl at a flow rate of 10 ml min⁻¹. The activity was recovered in a 30 ml fraction and desalted with a centrifugal filter device (Amicon Ultra-4, 10k, Millipore) along with buffer exchange to 20 mM, pH 6.0. A portion (0.5 ml) of the material was then applied to a Mono Q HR 5/5 FPLC column (Pharmacia) equilibrated with the Bistris/HCl buffer. The column was washed with 2 CV equilibration buffer followed by a linear NaCl gradient from 0 mM NaCl to 200 mM NaCl over 6 CV. The NaCl concentration was held at 200 mM for 4 CV, increased to 240 mM over 1 CV and kept at 240 mM for 2 CV. Activity was eluted with 240 mM NaCl at a flow rate of 1 ml min⁻¹ and recovered in a 1 ml fraction. All column purification steps were run at room temperature. The Bio-Rad Bradford reagent (Bradford, 1976) was used to quantify the protein content with bovine gamma globulin as a standard.

Protein size determination during purification. Amicon spin columns were used to approximate the mass of the effector protein. Cell-free spent supernatant was applied to a centrifugal filter device with a MWCO of 100 kDa (Amicon Ultra-4, 100k, Millipore). The retentate was kept for biofilm inhibition analysis while the flow-through was applied to another filter device with a MWCO of 50 kDa. This above process was repeated for filter devices with

MWCOs of 30, 10 and 5 kDa. All retentates and the 5 kDa flow-through were tested for activity on biofilm formation using the biofilm assay described above.

PAGE analysis. To assess the purity of the protein and isolate it for sequence analysis, we used SDS-PAGE and native PAGE, respectively. For SDS-PAGE analysis, the purified fractions were mixed in sample buffer, heated at 100 °C for 5 min and then aliquots were analysed using 12 % polyacrylamide gels. The samples were electrophoresed at a constant voltage (70 V) for approximately 4 h. Following electrophoresis, silver staining (Bio-Rad) was used for detection. For native PAGE analysis 10 % Tris/HCl (Bio-Rad) was used with Tris/glycine running buffer (25 mM Tris/HCl, 192 mM glycine, pH 8.3). The samples were electrophoresed at a constant voltage (200 V) for 35 min and colloidal Coomassie blue (Novex, Invitrogen) was used for staining.

Proteomic sequence analysis. To identify the effector, the protein was excised from a native polyacrylamide gel and extracted by electroelution. Identification of the protein was performed at the Harvard Microchemistry and Proteomics Analysis Facility by using microcapillary reverse-phase HPLC nano-electrospray tandem mass spectrometry (μ LC/MS/MS) on a Thermo LTQ-Orbitrap mass spectrometer.

Arginine deiminase assay. Protein sequence analysis indicated that the isolated protein was an arginine deiminase; therefore, enzymic assays were performed to verify this identification. The arginine deiminase activity was determined in 96-well plates, as previously described (Xie *et al.*, 2007).

Arginine deiminase treatment and RNA isolation. To examine the effect of the arginine deiminase protein on expression of genes (*fimA* and *mfa1*) encoding the major protein subunits of fimbriae, THBHK broth cultures of *P. gingivalis* strain 381 were grown anaerobically for 36 h, then diluted in a chemically defined medium (CDM) developed for *P. gingivalis* (Milner *et al.*, 1996), supplemented with 1 % tryptone, to OD₆₀₀ 0.2. The culture was then split in two, and one culture was exposed to a 1 : 50 dilution of the desalted and lyophilized final active Mono Q fraction (see Fig. 4, lane G). The control (treated with a 1 : 50 dilution of PBS) and the treated cultures were incubated anaerobically for 1 h at 37 °C. The cells were then pelleted and an aliquot of supernatant (100 μ l) was removed for testing for the presence of citrulline (monitoring of arginine deiminase enzymic activity). Total RNA was then extracted using the MasterPure RNA purification kit (Epicentre) according to the manufacturer's instructions. Contaminating DNA was removed by digestion with turbo DNase I (Ambion) followed by acid-phenol/chloroform extraction. The quality of the RNA was determined by agarose gel electrophoresis and the concentration was determined using a NanoDrop N-100 spectrophotometer (NanoDrop technologies). This treatment protocol and total RNA extraction were performed three times (three biological replicates).

Quantitative reverse transcription-PCR (qPCR). For cDNA generation, 1 μ g total RNA was reverse transcribed with random 9-mer primers using the MonstScript 1st-Strand cDNA Synthesis kit according to the manufacturer's protocol (Epicentre). Primers were designed using DNASTAR Lasergene software. The primer sequences used are as follows: for *fimA*, *fimA*-381F-qPCR 5'-CTGCCCG-ATGCTCCTC-3' and *fimA*-381R-qPCR 5'-CAAGTCGGCTCCGTT-TTTCTGA-3'; for *mfa1*, *mfa1*-381F-qPCR 5'-GGATATCAGATGG-GTTGTTGCTCA-3' and *mfa1*-381R-qPCR 5'-TGTATGGAAAGTG-CTGCTGGTAGG-3'. The product sizes for the *fimA* and the *mfa1* probes were 122 bp and 130 bp, respectively. The 16S rRNA gene was used as a reference, and the sequences of the forward and reverse primers used are as follows: 16S-Forw 5'-TGTTACAATGGGAGGG-

ACAAGGG-3' and 16S-Rev 5'-TTACTAGCGAATCCAGCTTCA-CGG-3'. qPCR was carried out with a Bio-Rad iCycler using SYBR Green Supermix (Bio-Rad). The qPCR consisted of an initial denaturation step at 95 °C for 3 min, followed by 50 cycles of 95 °C for 15 s, 60 °C for 15 s and 72 °C for 15 s. A melting curve profile for each amplified PCR product was determined using the following conditions: 95 °C for 1 min, 55 °C for 1 min, followed by 80 cycles of 10 s with a temperature increase of 0.5 °C for each cycle, starting at 55 °C.

Growth inhibition analysis. To determine if the biofilm inhibitor affected the growth rate of *P. gingivalis*, growth curves were determined in the presence and absence of the partially purified inhibitor (the active fraction obtained from the DEAE purification step described above; capable of biofilm inhibition up to a dilution of 1 : 40). To perform these experiments, a THBHK broth culture of *P. gingivalis* was grown anaerobically to early exponential phase (OD₆₀₀ ~0.3). Aliquots (4.75 ml) of the culture were mixed in triplicate with either 0.25 ml of pre-reduced 20 mM Bistris/HCl; pH 6 (buffer control), or 0.25 ml of the active DEAE fraction (dialysed against 20 mM Bistris/HCl; pH 6) to make a final dilution of 1 : 20. Cultures were then incubated anaerobically at 37 °C for 12 h, with aliquots being withdrawn at regular intervals to monitor growth, measured as OD₆₀₀.

Identification of the clinical isolate as *Streptococcus intermedius*. To identify the clinical isolate, sequencing of the 16S rRNA gene was performed and the sequence was compared to the Human Oral Microbiome Database (HOMD) by Dr Floyd Dewhirst's laboratory at The Forsyth Institute (<http://www.HOMD.org>) (Chen *et al.*, 2010; Dewhirst *et al.*, 2010). The clinical isolate was identified as a *Streptococcus intermedius* and the genome of this strain is presently being sequenced.

RESULTS

Screening of spent supernatants

Spent supernatants from 79 clinical streptococcal strains were tested (twofold and tenfold dilutions) for their ability to inhibit or enhance *P. gingivalis* biofilm formation. The majority of supernatants (61 isolates) resulted in the lysis of *P. gingivalis* cells and some (17 isolates) had no effect on cell viability, growth, or biofilm formation. However, one of the clinical strains (identified as *Streptococcus intermedius*) produced a supernatant that completely abolished biofilm formation at both a twofold and a tenfold dilution, with no effect on growth rate. Purification and characterization of the protein responsible for this extracellular activity is the focus of this report.

Production of the effector protein in relation to growth phase

In order to assess whether cell density played a role in release of the activity into the culture supernatant, production of the inhibitor was determined during various phases of growth in BHI broth. A profile of growth in relation to inhibitory activity, and a dilution series of the spent supernatant (18 h culture) are shown in Fig. 1(a, b). High levels of inhibitory activity (from at least 1 : 10 dilution of spent supernatant) were detected as early as the

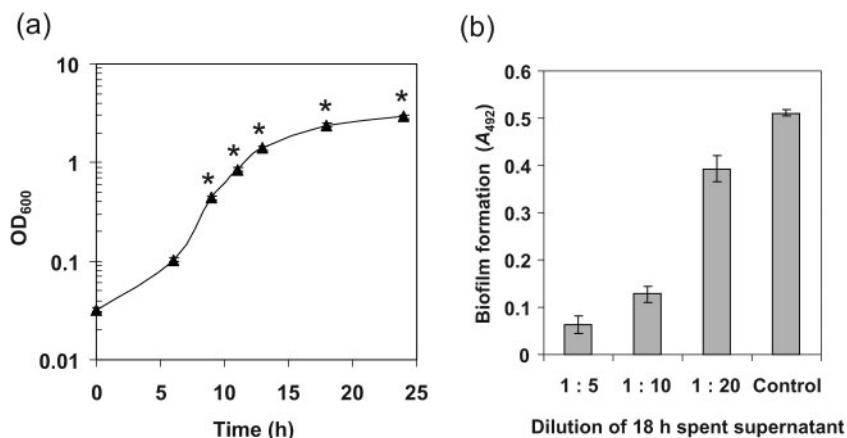


Fig. 1. Detection of biofilm inhibitory activity in the spent supernatant of the clinical strain of *S. intermedius* grown in BHI broth at 37 °C for 24 h. (a) Inhibitory activity (*) was detected in mid- and late-exponential growth and during stationary phase. (b) Effect of 18 h spent supernatant on biofilm formation. Supernatant obtained after 18 h anaerobic growth significantly inhibited *P. gingivalis* biofilm formation at all dilutions tested. Results are means \pm SD ($n=3$).

mid-exponential growth phase (OD_{600} 0.4) and throughout exponential growth (indicated by asterisks in Fig. 1a), paralleling the increase in biomass. The levels of activity reached a maximum as the culture entered stationary phase, again paralleling biomass levels.

Effect on growth rate

To determine whether biofilm inhibition was the result of growth restriction, the target organism, *P. gingivalis* strain 381, was grown in the presence (and absence) of the partially purified biofilm inhibitor (DEAE active fraction) at a 1:20 dilution, which is two times more concentrated than the dilution that inhibited biofilm formation. Growth was assessed at OD_{600} over time. The results, summarized in Fig. 2, show that although biofilm formation was inhibited with this fraction there was no effect on *P. gingivalis* growth.

Purification and characterization of the effector protein

During purification, size fractionation using centrifugal filter devices (Amicon) allowed for a rough estimate of the protein's mass. Activity was recovered in the 50 kDa filter device flow-through/30 kDa filter device retentate, while the other size fractions lacked activity (data not shown). The mass of the active compound was thereby estimated to be in the range between 30 and 50 kDa.

P. gingivalis biofilm assays were used to monitor purification of the inhibitory activity. Representative data from a biofilm assay are shown in Fig. 3(a). Dialysed fractions eluted from the DEAE column with 500 mM NaCl demonstrated strong inhibitory activity (Fig. 3a). This

fraction was then applied to a Mono Q column and of those fractions eluted at a linear NaCl gradient from 0 to 600 mM, activity was recovered in one fraction between 200 and 240 mM NaCl (Fig. 3a, Mono Q2 fraction). To further purify the protein, the active fraction from the first Mono Q column (Mono Q2) was applied to a second Mono Q column. This column was then run with a flatter gradient ranging from 200 to 240 mM NaCl. When the purity of the fractions obtained from the second Mono Q column was assessed by SDS-PAGE analysis, only one protein band was present in all lanes that were loaded with an active fraction (Fig. 3b). Conversely, this band

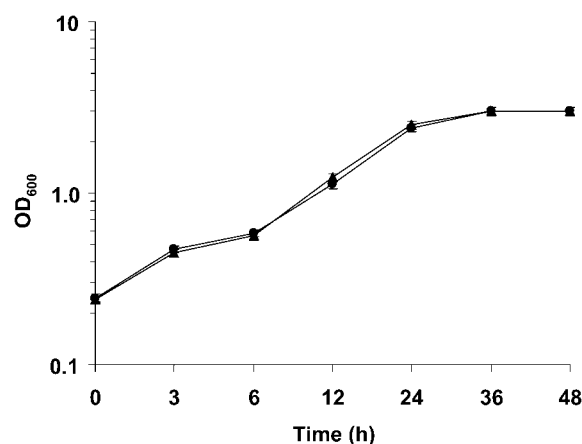


Fig. 2. Growth curves of *P. gingivalis* in the presence or absence of biofilm inhibitor. The dilution of partially purified biofilm inhibitor (DEAE fraction; Fig. 3b, lane B) was 20-fold diluted (●) compared to the control buffer (▲). The addition of the arginine deiminase had no effect on the growth rate of *P. gingivalis*.

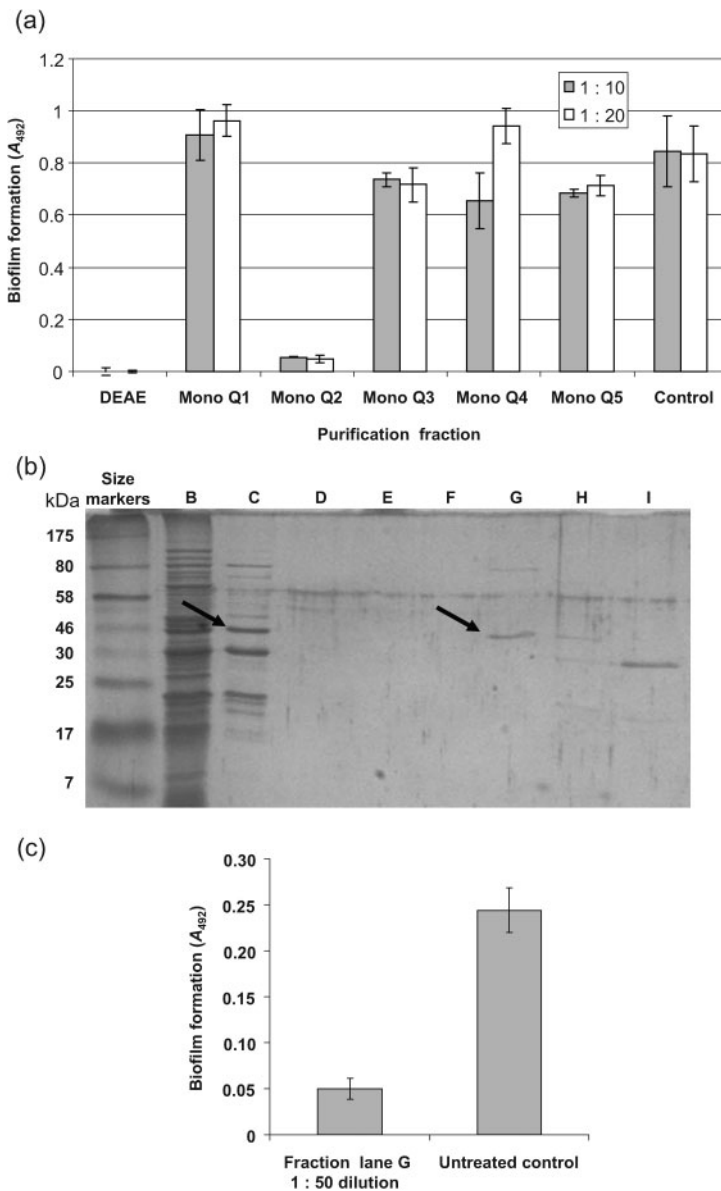


Fig. 3. (a) Biofilm assays were used to monitor the biofilm inhibitory activity during purification from the spent supernatant. Samples of the fraction eluted from the DEAE column using 500 mM NaCl (DEAE) and fractions eluted from a Mono Q column using between 0 and 600 mM NaCl (Mono Q 1–5) were tested in the biofilm assay, in triplicate, at dilutions of 1:10 and 1:20. Note: the active fraction applied to Mono Q2 eluted from the Mono Q column within the 200 to 240 mM NaCl range. Results are means \pm SD ($n=3$). (b) Samples of the active fraction from the DEAE column (lane B) and of the subsequent fractions from the Mono Q columns (lanes C–H) were submitted to SDS-PAGE analysis. Proteins were detected with silver staining. The material applied to lane B corresponds to the fraction tested in the biofilm assay in (a) (DEAE). Lane C contains an active fraction of all material eluted from 200 to 240 mM NaCl [corresponding to material applied in (a) Mono Q 2], while lanes D–I contain material eluted serially within the 200 to 240 mM NaCl range, at a flatter gradient. Of these, the greatest activity was observed in the material applied to lane G, which contained a prominent \sim 48 kDa band (marked with arrow). (c) Effect of a 1:50 dilution (final concentration $\leq 3.8 \mu\text{g}$ arginine deiminase ml^{-1}) of the purified fraction (lane G) on *P. gingivalis* biofilm formation. The results show a significant inhibition ($P < 0.0001$) of biofilm formation when compared to the untreated control. Results are means \pm SD ($n=3$).

(\sim 48 kDa) was absent in all lanes that were loaded with an inactive fraction. The most pure fraction (Fig. 3b, lane G) contained one other protein at \sim 80 kDa. However, our studies had already determined that the active protein was between 30 and 50 kDa; therefore, the 48 kDa protein was isolated and analysed. This fraction was highly active, inhibiting *P. gingivalis* biofilm formation out to a 1:80 dilution. Inhibition at a 1:50 dilution (final arginine deiminase concentration, $\leq 3.8 \mu\text{g ml}^{-1}$) is shown in Fig. 3(c).

Native PAGE was used to isolate the active protein for identification, and protein sequence analysis (see below) identified the 48 kDa band as an arginine deiminase. The identification was then confirmed with an enzymic assay for arginine deiminase (see Fig. 4).

Proteomic sequence analysis

The 48 kDa protein band was excised from a native polyacrylamide gel and the protein extracted by electro-elution. Identification of the protein was determined at the Harvard Microchemistry and Proteomics Analysis Facility by $\mu\text{LC/MS/MS}$ on a Thermo LTQ-Orbitrap mass spectrometer. The highest similarity was to arginine deiminases (ArcA) of oral streptococci, i.e. *Streptococcus gordonii* (strain Challis/ATCC 35105) and *Streptococcus sanguinis* (strain SK36). Forty-eight cleavage products (50.1% coverage) matched ArcA from *S. gordonii*. The sequences of all of these trypsin cleavage products aligned to the *S. gordonii* ArcA protein coding region (data not shown), as well as four additional trypsin cleavage products that matched *S. sanguinis*. We concluded that the purified

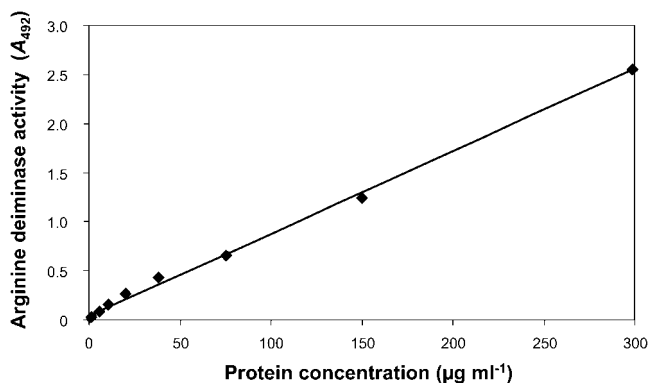


Fig. 4. Arginine deiminase activity of purified protein. Serial twofold dilutions (highest concentration 300 µg ml⁻¹) of the *S. intermedius* purified protein were tested in a colorimetric assay for arginine deiminase activity. The enzymic activity of the purified protein demonstrated a linear dependence on the protein concentration.

effector protein was an arginine deiminase; this was confirmed with an enzyme assay for arginine deiminase activity, corroborating the sequence data.

Expression analysis of *fimA* and *mfa1* in response to arginine deiminase exposure

Previous studies reported that a cell-wall-associated arginine deiminase produced by the oral streptococcus *S. cristatus* caused a downregulation of fimbriae expression in a cell-to-cell contact dependent manner. Since both short and long fimbriae have been shown to play a key role in *P. gingivalis* biofilm development, we hypothesized that the arginine deiminase isolated in our studies may also be acting, in part, by downregulating the expression of fimbriae. Therefore, qPCR was used to examine the expression of the *fimA* and the *mfa1* genes in response to arginine deiminase exposure for 1 h. A 1:50 dilution of the final Mono Q purified fraction, corresponding to a final arginine deiminase concentration of ≤ 3.8 µg ml⁻¹, was used in this experiment. As shown in Fig. 5, expression of both *fimA* and *mfa1* were significantly downregulated when *P. gingivalis* strain 381 cells were exposed to the arginine deiminase produced by *S. intermedius*.

In addition, we hypothesized that this change in *P. gingivalis* gene expression could be triggered by arginine deiminase enzymic activity (i.e. production of citrulline or removal of arginine). To investigate this hypothesis, we tested for citrulline production (using the final step of the arginine deiminase assay) after 1 h exposure to the enzyme and no citrulline was detected (limit of detection in the linear range is 1.4 mM), indicating that enzymic activity did not occur during exposure, yet signalling was detected. Furthermore, the addition of citrulline (10 mM) did not inhibit *P. gingivalis* biofilm formation (data not shown). Overall, our data are consistent with previous reported

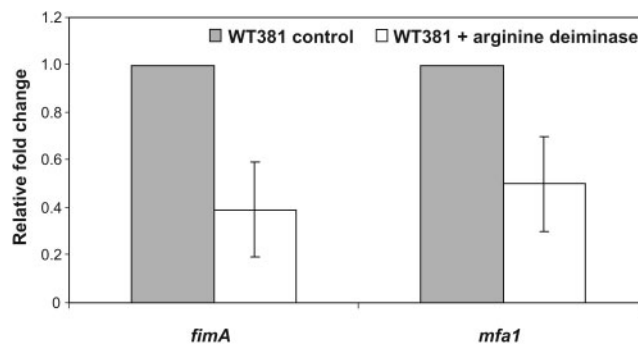


Fig. 5. qPCR analysis of the *fimA* (0.41 ± 0.2-fold change, *P*-value 0.006) and *mfa1* (0.50 ± 0.2-fold change, *P*-value 0.011) genes in *P. gingivalis* cells exposed to the purified arginine deiminase. A 1:50 dilution of the protein corresponds to ≤ 3.8 µg arginine deiminase ml⁻¹. The data presented are means of three separate datasets. Error bars represent SEM.

data showing that enzymic activity is not required for signalling.

DISCUSSION

A key determinant that regulates colonization and virulence in *P. gingivalis* is the expression of fimbriae. Two distinct fimbrial structures are present on the surface of *P. gingivalis* cells: the long fimbriae, primarily consisting of FimA protein subunits, and the short fimbriae, composed of the Mfa1 subunit. Studies have demonstrated a role for long fimbriae in adherence to and invasion of gingival epithelial cells, bone resorption, co-aggregation with other oral isolates, and biofilm formation (Amano *et al.*, 2004; Hamada *et al.*, 1998; Lamont & Jenkinson, 1998). Interestingly, it was recently shown that the short fimbriae, unlike the long fimbriae, are required for auto-aggregation (Lin *et al.*, 2006), indicating that these two surface structures have distinctive functions. The *in vivo* signals that trigger the expression of genes involved in the biogenesis of fimbriae are not known; however, it has been shown that expression is affected by environmental and nutritional parameters. Expression of *fimA* is repressed under elevated temperatures or when iron is limiting (Amano *et al.*, 1994; McKee *et al.*, 1986; Xie *et al.*, 1997). In addition, the molecular mechanism involved in the regulation of *fimA* expression is beginning to be understood. A two-component system (FimS-FimR) that regulates expression has been identified (Hayashi *et al.*, 2000; Watanabe-Kato *et al.*, 1998; Watanabe *et al.*, 1996), and recently it was shown that the response regulator of this two-component system (FimR) controls the expression of these genes at the transcription level (Nishikawa *et al.*, 2004). In regard to *mfa1*, expression of this gene has been shown to be affected by cell-to-cell contact with a variety of oral streptococci; either upregulation or downregulation was observed, depending on the species (Park *et al.*, 2006).

Likewise, *fimA* expression has been shown to be affected by cell-to-cell contact, specifically with *S. cristatus*, and an arginine deiminase on the surface of the cell was shown to be responsible for this change in expression (Xie *et al.*, 2007). Furthermore, those data indicate that downregulation occurred in the absence of arginine deiminase enzymic activity.

Here, we show that the transcription of both *fimA* and *mfa1* was downregulated when *P. gingivalis* strain 381 was exposed for 1 h to an arginine deiminase purified from the supernatant of a *S. intermedius* clinical isolate and, as for the arginine deiminase isolated from *S. cristatus*, enzymic activity is not involved in signalling. Unfortunately, the addition of arginine deiminase inhibitors (lysine, formamidine and aminoguanidine) at recommended concentrations led to growth defects of *P. gingivalis* so we were not able to employ these reagents in our analyses. Our future intentions are to generate an inactive form of the arginine deiminase and compare the response of *P. gingivalis* to the enzymically active and inactive forms. Our current working hypothesis is that this arginine deiminase protein is secreted and acts as an effector, possibly signalling via an interaction with a receptor on the cell surface of *P. gingivalis*.

Arginine deiminase is the first enzyme in an anaerobic energy-generating pathway (the ADI pathway) that catalyses the conversion of L-arginine to ornithine, ammonia and carbon dioxide with the concomitant synthesis of ATP (Cunin *et al.*, 1986). Arginine deiminase catalyses the hydrolysis of L-arginine to L-citrulline and ammonia. The release of ammonia from this reaction can neutralize acids produced through glycolysis, thereby raising the pH of the environment. The increase in pH resulting from arginine catabolism by oral streptococci (as well as other oral isolates) is believed to play an important role in inhibiting tooth decay and in modulating the microbial composition of the oral biofilm (Burne & Marquis, 2000; Nascimento *et al.*, 2009). Interestingly, all of the enzymes in the ADI pathway, along with a variety of other proteins typically thought of as being cytoplasmically localized, have been identified as being cell-surface associated in a diverse number of streptococci (Cole *et al.*, 2005), including the arginine deiminase of *S. cristatus* (Xie *et al.*, 2000; Xie *et al.*, 2007). Furthermore, ADI enzymes have been detected in culture supernatants of streptococci (Lei *et al.*, 2000). Interestingly, these proteins lack a secretion signal sequence or an LPXTG membrane-anchoring sequence; hence, the mechanism by which these enzymes are transported or localized to the cell surface is unknown. It has been proposed that an internal sequence, a post-translational acylation, or an association with another secreted protein may be involved (Cole *et al.*, 2005). Furthermore, if these proteins are localized to the cell surface and then released, the mechanism of release from the cell surface is unknown. The protein sequence data we obtained from our analysis indicate that the extracellular arginine deiminase we identified is intact, i.e. cleavage products from both the C terminus and the N terminus of the protein-coding region

were identified, indicating that the protein is not likely to have been cleaved from the cell surface. Furthermore, the protein was detected during mid-exponential growth. This would indicate that the protein is either secreted or released by an unknown mechanism. Further studies are required to determine how this protein makes it to the supernatant.

As a result of the present study, as well as previous studies by Xie *et al.* (2000, 2007), it is becoming evident that *P. gingivalis* is able to sense arginine deiminase proteins produced by oral streptococci and respond by altering the expression of key surface structures that are required for colonization. Although this is not the first report of an arginine deiminase produced by an oral streptococcus that signals *P. gingivalis* to downregulate fimbriae expression, this is believed to be the first report of an extracellular form of arginine deiminase being able to downregulate fimbriae expression, thus providing an added dimension to this inter-species communication. In addition, although the previously reported arginine deiminase did not affect expression of *mfa1* in strain 33277, our data indicate that the protein described here affects expression of both *fimA* and *mfa1* in strain 381; thus this effector protein may trigger a different response in different strains of *P. gingivalis*. It was recently discovered that another bacterium that is an indigenous member of the human oral microbiota (*Granulicatella elegans*) also produces an extracellular arginine deiminase that inhibits the proliferation of mitogen-stimulated human peripheral blood mononuclear cells (Kanamoto *et al.*, 2007). Growth inhibition of various human cell lines has also been reported for arginine deiminases from *Streptococcus pyogenes* and *Mycoplasma arginini*, and although some of this growth-inhibitory activity has been attributed to the depletion of arginine, in studies of the *M. arginini* arginine deiminase the effect was observed even in arginine-free medium, again supporting the model that signalling is not merely due to the lack of arginine in the medium or the influence of arginine deiminase-yielded end products (Degnan *et al.*, 1998; Gong *et al.*, 1999, 2000). Thus, these arginine deiminases appear to be moonlighting as effectors not only between different genera of prokaryotes, but also between prokaryotic and eukaryotic cells. Our future studies are designed to further our understanding of this signalling mechanism.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Colgate Palmolive Company.

REFERENCES

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

- Amano, A., Nakagawa, I., Okahashi, N. & Hamada, N. (2004). Variations of *Porphyromonas gingivalis* fimbriae in relation to microbial pathogenesis. *J Periodontol Res* **39**, 136–142.
- Barnard, J. P. & Stinson, M. W. (1999). Influence of environmental conditions on hydrogen peroxide formation by *Streptococcus gordonii*. *Infect Immun* **67**, 6558–6564.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.
- Burne, R. A. & Marquis, R. E. (2000). Alkali production by oral bacteria and protection against dental caries. *FEMS Microbiol Lett* **193**, 1–6.
- Chen, T., Yu, W.-H., Izard, J., Baranova, O. V., Lakshmanan, A. & Dewhirst, F. E. (2010). The Human Oral Microbiome Database: a web accessible resource for investigating oral microbe taxonomic and genomic information. *Database* (in press), doi:10.1093/database/baq013
- Chikindas, M. L., Novak, J., Driessen, A. J., Konings, W. N., Schilling, K. M. & Caufield, P. W. (1995). Mutacin II, a bactericidal antibiotic from *Streptococcus mutans*. *Antimicrob Agents Chemother* **39**, 2656–2660.
- Choi, J. I., Nakagawa, T., Yamada, S., Takazoe, I. & Okuda, K. (1990). Clinical, microbiological and immunological studies on recurrent periodontal disease. *J Clin Periodontol* **17**, 426–434.
- Cole, J. N., Ramirez, R. D., Currie, B. J., Cordwell, S. J., Djordjevic, S. P. & Walker, M. J. (2005). Surface analyses and immune reactivities of major cell wall-associated proteins of group A streptococcus. *Infect Immun* **73**, 3137–3146.
- Cunin, R., Glansdorff, N., Pierard, A. & Stalon, V. (1986). Biosynthesis and metabolism of arginine in bacteria. *Microbiol Rev* **50**, 314–352.
- Davey, M. E. (2006). Techniques for the growth of *Porphyromonas gingivalis* biofilms. *Periodontol* **42**, 27–35.
- Davey, M. E. & Costerton, J. W. (2006). Molecular genetics analyses of biofilm formation in oral isolates. *Periodontol* **42**, 13–26.
- Davey, M. E. & Duncan, M. J. (2006). Enhanced biofilm formation and loss of capsule synthesis: deletion of a putative glycosyltransferase in *Porphyromonas gingivalis*. *J Bacteriol* **188**, 5510–5523.
- Degnan, B. A., Palmer, J. M., Robson, T., Jones, C. E., Fischer, M., Glanville, M., Mellor, G. D., Diamond, A. G., Kehoe, M. A. & Goodacre, J. A. (1998). Inhibition of human peripheral blood mononuclear cell proliferation by *Streptococcus pyogenes* cell extract is associated with arginine deiminase activity. *Infect Immun* **66**, 3050–3058.
- Dewhirst, F. E., Chen, T., Izard, J., Paster, B. J., Tanner, A. C. R., Yu, W.-H., Lakshmanan, A. & Wade, W. G. (2010). The Human Oral Microbiome. *J Bacteriol* (in press), doi: 10.1128/JB.00542-10.
- Dzink, J. L., Socransky, S. S. & Haffajee, A. D. (1988). The predominant cultivable microbiota of active and inactive lesions of destructive periodontal diseases. *J Clin Periodontol* **15**, 316–323.
- Falkow, S. (2006). Is persistent bacterial infection good for your health? *Cell* **124**, 699–702.
- Gong, H., Zolzer, F., von Recklinghausen, G., Rossler, J., Breit, S., Havers, W., Fotsis, T. & Schweigerer, L. (1999). Arginine deiminase inhibits cell proliferation by arresting cell cycle and inducing apoptosis. *Biochem Biophys Res Commun* **261**, 10–14.
- Gong, H., Zolzer, F., von Recklinghausen, G., Havers, W. & Schweigerer, L. (2000). Arginine deiminase inhibits proliferation of human leukemia cells more potently than asparaginase by inducing cell cycle arrest and apoptosis. *Leukemia* **14**, 826–829.
- Grossi, S. G., Zambon, J. J., Ho, A. W., Koch, G., Dunford, R. G., Machtei, E. E., Norderyd, O. M. & Genco, R. J. (1994). Assessment of risk for periodontal disease. I. Risk indicators for attachment loss. *J Periodontol* **65**, 260–267.
- Hamada, S., Amano, A., Kimura, S., Nakagawa, I., Kawabata, S. & Morisaki, I. (1998). The importance of fimbriae in the virulence and ecology of some oral bacteria. *Oral Microbiol Immunol* **13**, 129–138.
- Hayashi, J., Nishikawa, K., Hirano, R., Noguchi, T. & Yoshimura, F. (2000). Identification of a two-component signal transduction system involved in fimbriation of *Porphyromonas gingivalis*. *Microbiol Immunol* **44**, 279–282.
- Jakubovics, N. S., Gill, S. R., Vickerman, M. M. & Kolenbrander, P. E. (2008). Role of hydrogen peroxide in competition and cooperation between *Streptococcus gordonii* and *Actinomyces naeslundii*. *FEMS Microbiol Ecol* **66**, 637–644.
- Kanamoto, T., Sato, S., Nakashima, H. & Inoue, M. (2007). Proliferation of mitogen-stimulated human peripheral blood mononuclear cells is inhibited by extracellular arginine deiminase of *Granulicatella elegans* isolated from the human mouth. *J Infect Chemother* **13**, 353–355.
- Kolenbrander, P. E., Andersen, R. N., Bleher, D. S., Eglund, P. G., Foster, J. S. & Palmer, R. J., Jr (2002). Communication among oral bacteria. *Microbiol Mol Biol Rev* **66**, 486–505.
- Kreth, J., Merritt, J., Shi, W. & Qi, F. (2005). Competition and coexistence between *Streptococcus mutans* and *Streptococcus sanguinis* in the dental biofilm. *J Bacteriol* **187**, 7193–7203.
- Kuramitsu, H. K., He, X., Lux, R., Anderson, M. H. & Shi, W. (2007). Interspecies interactions within oral microbial communities. *Microbiol Mol Biol Rev* **71**, 653–670.
- Lamont, R. J. & Jenkinson, H. F. (1998). Life below the gum line: pathogenic mechanisms of *Porphyromonas gingivalis*. *Microbiol Mol Biol Rev* **62**, 1244–1263.
- Lamont, R. J. & Yilmaz, O. (2002). In or out: the invasiveness of oral bacteria. *Periodontol* **30**, 61–69.
- Lei, B., Mackie, S., Lukomski, S. & Musser, J. M. (2000). Identification and immunogenicity of group A *Streptococcus* culture supernatant proteins. *Infect Immun* **68**, 6807–6818.
- Lin, X., Wu, J. & Xie, H. (2006). *Porphyromonas gingivalis* minor fimbriae are required for cell–cell interactions. *Infect Immun* **74**, 6011–6015.
- McKee, A. S., McDermid, A. S., Baskerville, A., Dowsett, A. B., Ellwood, D. C. & Marsh, P. D. (1986). Effect of hemin on the physiology and virulence of *Bacteroides gingivalis* W50. *Infect Immun* **52**, 349–355.
- Milner, P., Batten, J. E. & Curtis, M. A. (1996). Development of a simple chemically defined medium for *Porphyromonas gingivalis*: requirement for alpha-ketoglutarate. *FEMS Microbiol Lett* **140**, 125–130.
- Moore, W. E., Moore, L. H., Ranney, R. R., Smibert, R. M., Burmeister, J. A. & Schenkein, H. A. (1991). The microflora of periodontal sites showing active destructive progression. *J Clin Periodontol* **18**, 729–739.
- Nascimento, M. M., Gordan, V. V., Garvan, C. W., Browngardt, C. M. & Burne, R. A. (2009). Correlations of oral bacterial arginine and urea catabolism with caries experience. *Oral Microbiol Immunol* **24**, 89–95.
- Nishikawa, K., Yoshimura, F. & Duncan, M. J. (2004). A regulation cascade controls expression of *Porphyromonas gingivalis* fimbriae via the FimR response regulator. *Mol Microbiol* **54**, 546–560.
- Park, Y., James, C. E., Yoshimura, F. & Lamont, R. J. (2006). Expression of the short fimbriae of *Porphyromonas gingivalis* is regulated in oral bacterial consortia. *FEMS Microbiol Lett* **262**, 65–71.

- Qi, F., Chen, P. & Caufield, P. W. (1999). Purification of mutacin III from group III *Streptococcus mutans* UA787 and genetic analyses of mutacin III biosynthesis genes. *Appl Environ Microbiol* **65**, 3880–3887.
- Qi, F., Chen, P. & Caufield, P. W. (2001). The group I strain of *Streptococcus mutans*, UA140, produces both the lantibiotic mutacin I and a nonlantibiotic bacteriocin, mutacin IV. *Appl Environ Microbiol* **67**, 15–21.
- Socransky, S. S. & Haffajee, A. D. (2000). Dental biofilms: difficult therapeutic targets. *Periodontol 2000* **28**, 12–55.
- Upton, M., Tagg, J. R., Wescombe, P. & Jenkinson, H. F. (2001). Intra- and interspecies signaling between *Streptococcus salivarius* and *Streptococcus pyogenes* mediated by SalA and SalA1 lantibiotic peptides. *J Bacteriol* **183**, 3931–3938.
- Watanabe, K., Onoe, T., Ozeki, M., Shimizu, Y., Sakayori, T., Nakamura, H. & Yoshimura, F. (1996). Sequence and product analyses of the four genes downstream from the fimbriin gene (*fimA*) of the oral anaerobe *Porphyromonas gingivalis*. *Microbiol Immunol* **40**, 725–734.
- Watanabe-Kato, T., Hayashi, J. I., Terazawa, Y., Hoover, C. I., Nakayama, K., Hibi, E., Kawakami, N., Ikeda, T., Nakamura, H. & other authors (1998). Isolation and characterization of transposon-induced mutants of *Porphyromonas gingivalis* deficient in fimbriation. *Microb Pathog* **24**, 25–35.
- Xie, H., Cai, S. & Lamont, R. J. (1997). Environmental regulation of fimbrial gene expression in *Porphyromonas gingivalis*. *Infect Immun* **65**, 2265–2271.
- Xie, H., Cook, G. S., Costerton, J. W., Bruce, G., Rose, T. M. & Lamont, R. J. (2000). Intergeneric communication in dental plaque biofilms. *J Bacteriol* **182**, 7067–7069.
- Xie, H., Lin, X., Wang, B. Y., Wu, J. & Lamont, R. J. (2007). Identification of a signalling molecule involved in bacterial intergeneric communication. *Microbiology* **153**, 3228–3234.
- Yilmaz, O. (2008). The chronicles of *Porphyromonas gingivalis*: the microbium, the human oral epithelium and their interplay. *Microbiology* **154**, 2897–2903.

Edited by: R. Palmer