molecular oral microbiology

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Community-based interference against integration of *Pseudomonas aeruginosa* into human salivary microbial biofilm

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SUMMARY

As part of the human gastrointestinal tract, the oral cavity represents a complex biological system and harbors diverse bacterial species. Unlike the gut microbiota, which is often considered a health asset, studies of the oral commensal microbiota have been largely limited to their implication in oral conditions such as dental caries and periodontal disease. Less emphasis has been given to their potential beneficial roles, especially the protective effects against oral colonization by foreign or pathogenic bacteria. In this study, we used salivary microbiota derived from healthy human subjects to investigate protective effects against colonization and integration of *Pseudomonas aeruginosa*, an opportunistic bacterial pathogen, into developing or pre-formed salivary biofilms. When co-cultivated in saliva medium, *P. aeruginosa* persisted in the planktonic phase, but failed to integrate into the salivary microbial community during biofilm formation. Furthermore, in saliva medium supplemented with sucrose, the oral microbiota inhibited the growth of *P. aeruginosa* by producing lactic acid. More interestingly, while pre-formed salivary biofilms were able to prevent *P. aeruginosa* colonization, the same biofilms recovered from mild chlorhexidine gluconate treatment displayed a shift in microbial composition and showed a drastic reduction in protection. Our study indicates that normal oral communities with balanced microbial compositions could be important in effectively preventing the integration of foreign or pathogenic bacterial species, such as *P. aeruginosa*.

INTRODUCTION

The human oral cavity harbors over 700 different bacterial species and is one of the most complex ecosystems ever described (Paster *et al.*, 2001, 2006; Aas *et al.*, 2005; Zaura *et al.*, 2009). Owing to its accessibility, the oral microbial community has become one of the best studied human microbial systems (Kolenbrander & London, 1993; Kolenbrander, 2000; Kolenbrander *et al.*, 2002, 2010; Kuramitsu *et al.*, 2007). Extensive work has investigated the development and formation of multispecies oral microbial biofilms (Kolenbrander *et al.*, 2010). This has revealed the antagonistic or synergistic inter-species interactions between resident bacteria within the community (Kuramitsu *et al.*, 2007). In turn, this has led to evaluating the implications of interspecies interactions in oral diseases such as dental caries and periodontitis (Marsh, 1994; Liljemark & Bloomquist, 1996).

Accumulating evidence indicates that the indigenous microbiotas play diverse roles in host physiology. The gastrointestinal-tract-associated microbiota, particularly in the gut, has important roles in maintaining human health (Guarner & Malagelada, 2003). Considered an 'exteriorized organ', the gut microbiota has been shown to contribute to human homeostasis with multiple functions, including harvesting energy (Sonnenburg *et al.*, 2005), training the immune system (Cebra, 1999), and protecting against epithelial cell injury (Rakoff-Nahoum *et al.*, 2004). More importantly, the gut-associated microbiota has also been implicated in preventing colonization of pathogenic microbes through bacterial interference (Guarner & Malagelada, 2003). Unlike the gut microbiota, which is normally considered a health asset (O'Hara & Shanahan, 2006), the oral microbiota is often implicated in oral diseases such as dental caries and periodontal disease. Although a protective role of the healthy oral microbiota against foreign or pathogenic bacteria has been suggested, evidence has largely been descriptive of dual species antagonisms between a pathogen and a specific commensal bacterium (Uehara *et al.*, 2001a,b), community-based interference and mechanistic studies are still lacking.

Studies of 16S ribosomal RNA have revealed that despite repeated exposure to a multitude of diverse bacterial species from different origins, the microbial compositions within oral cavities of healthy human subjects are relatively stable (Rasiah *et al.*, 2005; Zaura *et al.*, 2009). Although host factors have been implicated to play a significant role in shaping indigenous microbial communities (Rawls *et al.*, 2006), increasing evidence also suggests that the established oral microbial community might develop invasion resistance mechanisms to protect its domain and maintain its stability. Using a mouse oral microbial community *in vitro*, we have recently demonstrated that an existing microbial community exerts bacterial interference effects and imposes a selective pressure on incoming foreign bacterial species, independent of host-mediated selection (termed, the Community selection effect). The latter may indeed play a significant role in maintaining community stability and preventing foreign microbial colonization (He *et al.*, 2010a,b). In this study, we have established *in vitro* healthy human subject-derived salivary biofilms and tested their abilities to prevent the integration of *Pseudomonas aeruginosa*, an opportunistic pathogen that can be isolated from certain oral infections (Nord *et al.*, 1972). Furthermore, the effect of shifts in biofilm microbial composition on defense against invasive foreign bacteria was investigated.

METHODS

Bacterial strains and growth conditions

Pseudomonas aeruginosa PAO1 (laboratory strain), Pa060928 (a clinical isolate from an adult patient with cystic fibrosis) (Kus *et al.*, 2004), and salivary bacteria (S-mix) from healthy subjects were grown in salivary medium [75% filter-sterilized saliva, 25% brain–heart infusion (BHI) broth]. Cultures were incubated at 37°C under anaerobic conditions (nitrogen 85%, carbon dioxide 5%, and hydrogen 10%). Kanamycin (150 μg ml^{-1}) was supplied in the medium to select *P. aeruginosa* when needed.

Saliva collection

Saliva samples were collected from five healthy subjects, age 25–40 years under UCLA-IRB No. 09-08-068-02A. These individuals had no history of periodontal disease and had not used biocide-containing dentifrice for at least 6 months before saliva donation. Subjects had not been treated for any systemic disease nor were they taking any prescription or non-prescription medications.

Saliva collection for preparing saliva medium

Subjects were asked to refrain from any food or drink 2 h before donating saliva. Expectorated saliva (10 ml) was collected from each person in collection tubes. Saliva samples were pooled and centrifuged at 14,000 ***g*** for 5 min. The supernatant was filter-sterilized and used for preparing saliva medium and precoating 12-well plates.

Saliva collection for starting biofilms

On a separate day, subjects were asked to donate saliva; 2 ml was collected from each person as described in the previous section. Saliva samples were pooled and centrifuged at 2,600 ***g*** for 10 min to sediment large debris and eukaryotic cells. The supernatant containing salivary bacteria was referred to as S-mix and was used for setting up co-cultivation assays with *P. aeruginosa* and for starting salivary biofilms.

Isolation and identification of bacterial species from salivary samples

Pooled saliva was diluted in SHI medium, an optimal medium for culturing oral bacteria that has been shown to be able to sustain the growth of highly diverse *in vitro* microbial communities with similar microbial profiles to the original salivary microflora (Tian *et al.*, 2010). The diluted saliva was then seeded on SHI agar plates. The plates were incubated for 5 days at 37°C under anaerobic conditions. Potentially different bacterial colonies were picked from the plate based on differences in colony morphology, pigmentation and the incubation time needed for colonies to appear. Individual colonies were grown in SHI medium at 37°C under anaerobic conditions until turbid. Bacterial cells were collected, frozen stocks of each isolate were made and genomic DNA of each isolate was prepared using the MasterPure™ DNA purification kit (Epicentre, Madison, WI).

For species identification, the universal bacterial 16S ribosomal DNA primer pair, 27F (5′-AGAGTT TGATCCTGGCTCAG-3′) and 1492R (5′-GGTTAC CTTGTTACGACTT-3′) (Martin-Laurent *et al.*, 2001), was used to generate an approximately 1500-base-pair amplicon. Each 50 μl polymerase chain reaction (PCR) mixture contained 10 ng genomic DNA, 200 μM of each dNTP, 4.0 mM $MgCl_2$, 100 nM of each primer, 5 μl 10× PCR buffer, and 2.5 U *Taq* polymerase (Invitrogen, Carlsbad, CA). The PCR conditions were as follows: 3 min at 94°C for initial denaturation and 27 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min and a final chain elongation at 72°C for 5 min. The PCR products were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA) and sequenced at the UCLA Core DNA Sequencing Facility using 27F and 1492R primers. The sequences obtained were subjected to nucleotide BLAST searches against the NCBI (http://blast.ncbi.nlm.nih.gov/) and Human Oral Microbiome Database (http://www.homd.org/index.php). Sequences with 98–100% identity to those deposited in the databases were considered to be positive for taxa identification.

Co-aggregation between *P. aeruginosa* and oral isolates

Co-aggregation assays were performed both in saliva medium and CAB buffer, which contains 150 mM NaCl, 1 mM Tris–HCl pH 8, 0.1 mM $CaCl_2$, 0.02% NaN_3 and 0.1 mM $MgCl_2$ as previously described (Cisar *et al.*, 1979). Bacterial cells were collected in the mid-exponential phase of growth, washed and re-suspended in CAB (or saliva medium) at an optical density at 600 nm of 1. Equal volumes of different bacterial species were added to the reaction tube, vortex-mixed for 10 s and graded on a 0–4 scale after 10 min based on the degree of co-aggregation (Kaplan *et al.*, 2009). A score of 0 was assigned for no visible co-aggregation and a score of 4 for complete sedimentation with a clear supernatant. No clumping of individual bacterial strains was observed in our experimental controls.

Co-cultivation of salivary bacteria and *P. aeruginosa* (PAO1 or Pa060928) in the presence or absence of sucrose or lactic acid

To enhance the attachment of oral microbes to the wells, 12-well plates were pre-coated with saliva as previously described (Tian *et al.*, 2010). Briefly, 100 μl filtered saliva with equal amounts of phosphate-buffered saline (PBS) was added to each well of the 12-well plate to pre-coat the wells. Plates were incubated at 37°C with their lids open for 1 h to dry the saliva coating, followed by sterilization under UV light for 1 h. Then, 150 μl fresh pooled saliva (S-mix) containing $\sim 10^6$ bacterial cells together with $\sim 10^4$ *P. aeruginosa* (PAO1 or Pa060928) cells, as determined by cell counting using a Petroff–Hausser chamber (Hausser Scientific, Horsham, PA), was inoculated into pre-coated wells containing 800 μl salivary medium supplemented with or without 0.02 and 0.05% sucrose, or 0.02% lactic acid. Plates were incubated at 37°C under anaerobic conditions for 24 h before samples (both planktonic and biofilm portion) were taken for viability counting, DNA isolation and PCR-Denaturing Gradient Gel Electrophoresis (DGGE) analysis.

Challenge of chlorhexidine gluconate-treated salivary biofilm with *P. aeruginosa* PAO1

Establishment of salivary biofilms

Pooled saliva (150 μl) was inoculated into pre-coated wells (12-well plates) containing 850 μl salivary medium. Plates were incubated at 37°C under anaerobic conditions overnight (16 h) to allow biofilm formation.

Chlorhexidine gluconate treatment of biofilm and challenge of biofilm with P. aeruginosa

Depending upon whether they would be subjected to chlorhexidine gluconate (CHX) treatment or challenged with *P. aeruginosa*, overnight salivary biofilms were divided into three groups. Group 1 (CHX-treated only): planktonic portion was carefully removed from each well without disturbing the biofilm. CHX (0.01%, 300 μl) was added to each well, and incubated for 15 or 30 s at room temperature before being carefully removed. Wells were immediately washed five times with 500 μl PBS followed by addition of 1 ml salivary medium. Group 2 (*P. aeruginosa*-challenged only): after removing the planktonic portion, 1 ml salivary medium was added to the wells. Group 3 (CHX-treated and *P. aeruginosa*-challenged): following CHX treatment and washing with PBS five times, 1 ml salivary medium was added to the wells. The 12-well plates containing the above three groups were incubated at 37°C under anaerobic conditions for 24 h to allow the CHX-treated group to recover. After the 24-h incubation, the planktonic portion of all three groups was removed, followed by the addition of 1 ml salivary medium to each well in Group 1, and 1 ml salivary medium containing $\sim 10^5$ *P. aeruginosa* cells to the wells in Group 2 and Group 3. The 12-well plates containing the above three groups were further incubated at 37°C under anaerobic conditions for 48 h, samples (both planktonic and biofilm portion) were taken at 24 and 48 h and subjected to viability counts, DNA isolation and PCR-denaturing gradient gel electrophoresis (DGGE) analysis.

Collection of planktonic and biofilm samples for viability counting and total DNA isolation

Samples were taken as follows: To collect the planktonic portion, the supernatant in the well was transferred to a 2-ml microfuge tube, 500 μl PBS was then used to gently wash the biofilm to collect loosely attached cells and combined with the supernatant. To harvest the biofilm portion, 500 μl PBS was added to the well, and a sterile spatula was used to meticulously scrape the biomass of the biofilm from the bottom of the wells. Collected cells were vortexed, 50 μl bacterial suspension was taken for each sample, subjected to serial dilution and seeded onto selective (kanamycin 150 μg ml^{-1}) or non-selective SHI agar plates. Plates were incubated at 37°C under anaerobic conditions for 5 days before colonies were counted to determine colony-forming units per millilitre. The remainder of the bacterial cell were collected from the planktonic and biofilm portion by centrifugation at 14,000 ***g*** for 5 min, and the cell pellets were further subjected to DNA isolation.

Ethidium monoazide bromide cross-linking

To prevent amplification of DNA from dead bacterial cells and to limit DNA-based PCR-DGGE community analysis to the viable fraction, the collected bacterial samples were treated with ethidium monoazide bromide (EMA) before DNA extraction. The EMA cross-linking was performed as described previously (Nocker & Camper, 2006). Briefly, EMA (Biotium, Hayward, CA) was dissolved in water at a stock concentration of 5 mg ml^{-1} and stored at −20°C in the dark. EMA was added to the culture samples to a final concentration of 100 μg ml^{-1} and samples were incubated in the dark for 5 min with occasional mixing. Samples were then incubated on ice and light-exposed for 1 min using a 650-W halogen light source placed about 20 cm from the samples. After photoinduced cross-linking, bacterial cells were collected by centrifugation at 5,000 ***g*** for 5 min, followed by total genomic DNA isolation.

PCR-DGGE analysis

Total genomic DNA of bacterial samples was isolated using the MasterPure™ DNA purification kit (Epicentre). DNA quality and quantity were determined by a Spectronic Genesys UV spectrophotometer at 260 nm and 280 nm (Spectronic Instruments, Inc. Rochester, NY). Amplification of bacterial 16S ribosomal RNA genes by PCR was carried out as described previously by Li *et al.* (2005). Briefly, the universal primer set, Bac1 (5′-CGCCCGCCGCGC CCCGCGCCCGTCCCGCCGCCCCCGCCCGACTA CGTGCCAGCAGCC-3′) and Bac2 (5′-GGACTAC CAGGGTATCTAATCC-3′) (Sheffield *et al.*, 1989), was used to amplify an approximately 300-base-pair internal fragment of the 16S ribosomal RNA gene. Each 50-μl PCR contained 100 ng purified genomic DNA, 40 pmol each primer, 200 μM of each dNTP, 4.0 mM $MgCl_2$, 5 μl 10× PCR buffer, and 2.5 U *Taq* DNA polymerase (Invitrogen). Cycling conditions were 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 30 s, with a

final extension period of 5 min at 72°C. The resulting PCR products were evaluated by electrophoresis through 1.0% agarose.

Polyacrylamide gels (8% acrylamide) were prepared with a denaturing urea/formamide gradient between 40% (containing 2.8 M urea and 16% formamide) and 70% (containing 4.9 M urea and 28% formamide). Approximately 300 ng PCR product was applied per lane. The gels were submerged in 1× TAE buffer (40 mM Tris base, 40 mM glacial acetic acid, 1 mM EDTA) and the PCR products were separated by electrophoresis for 17 h at 58°C using a fixed voltage of 60 V in a Bio-Rad DCode System (Bio-Rad Laboratories, Inc., Hercules, CA). After electrophoresis, the DNA bands were stained with 0.5 μg ml^{-1} ethidium bromide and DGGE profile images were digitally recorded using the Molecular Imager Gel Documentation system (Bio-Rad). DIVERSITY DATABASE Software (Bio-Rad) was used to assess the change in the relative intensity of bands corresponding to bacterial species of interest.

Identification of bacterial species from DGGE gel

The DNA bands of interest were excised from the DGGE gels and transferred to a 1.5-ml microfuge tube containing 20 μl sterile ddH_2O. Tubes were incubated at 4°C overnight before the recovered DNA samples were re-amplified with the universal primer set (Bac1 and Bac2). The PCR products were purified using the QIAquick PCR purification kit (Qiagen) and sequenced at the UCLA Core DNA Sequencing Facility. The sequences obtained were subjected to nucleotide BLAST searches against the NCBI (http://blast.ncbi.nlm.nih.gov/) and Human Oral Microbiome (http://www.homd.org/index.php) databases.

HPLC-ESI-MS analysis for detecting lactic acid in spent media

Overnight cultures of salivary bacteria in saliva medium supplied with and without 0.05% sucrose were harvested by centrifugation, spent media were collected, filtered through Millex®GP membranes (0.22 -μm pore size, Millipore, Billerica, MA), and subjected to high performance liquid chromatography-mass spectrometry (HPLC/MS) analysis. The HPLC/MS was performed on a Waters 2767 HPLC separation module equipped with an XBridge BEH130 C18 column (5 μm, 4.60 × 100 mm) in tandem with a Waters 3100 mass detector (Waters, Milford, MA). Separation of lactic acid was achieved using a gradient solvent system comprising ddH_2O-acetonitrile-trifluoric acid at 0.6 ml min^{-1} in the proportions 94.95 : 5 : 0.05 to 89.95 : 10 : 0.05 over 7 min, and these conditions were held for 5 min before returning to the initial conditions over 5 min, and equilibrating for 5 min. In-line MS was performed using a Waters 3100 single-quadrupole mass spectrometer with electrospray ionization (ESI) in the negative mode, specifically with a capillary voltage of −3 kV and cone voltage of − 30 V. Nitrogen was used for both the cone gas (160 l h^{-1}) and desolvation gas (650 l h^{-1}), with the source and desolvation temperatures being held at 150°C and 350°C, respectively. Total ion current chromatograms of samples ranging between 50 and 400 *m/z* were displayed, and selected ion monitoring (SIM) chromatograms were used to record the abundance of the deprotonated molecule of lactic acid ($[M\text{-}H]^-$) at *m/z* 89. Lactic acid in the spent medium was identified by comparing both the retention time and mass spectrum (*m/z* 50–110) with that of the standard lactic acid. The quantification was based on the abundance of identified peak area in SIM chromatogram of each sample against lactic acid standards (0.3–16.5 mM).

RESULTS

P. aeruginosa PAO1 was ineffective in integrating into oral microbial community during biofilm formation

Our previous *in vitro* study demonstrated that mouse oral cavity-derived microbial flora was able to prevent the integration of bacterial species of foreign origin (He *et al.*, 2010a). In an effort to further investigate human salivary microbiota protective ability, the opportunistic pathogen *P. aeruginosa* PAO1 was initially chosen as a representative non-oral resident and co-cultivated with salivary bacteria (S-mix). After 24-h incubation, the co-culture resulted in the formation of biofilm attached to the bottom of the well, as well as unattached bacterial cells in the planktonic portion. PCR-DGGE analysis revealed that the banding pattern of salivary bacteria within the biofilm sample was very close to that of the original saliva

sample, indicating that most of the salivary bacterial species were capable of integrating into surface-attached multispecies biofilm (Fig. 1, lanes 2 and 3). *Pseudomonas aeruginosa* PAO1 was capable of forming biofilm by itself under the condition used (see Supporting Information, Fig. S1). However, when co-cultivated with S-mix, its corresponding band was not easily detectable in the biofilm portion (Fig. 1, lane 3). Separately, there was a drastic increase in the relative intensity of the same band within the planktonic portion compared with the initial inoculum (Fig. 1, lane 4). Our results suggested that although *P. aeruginosa* PAO1 was able to persist when co-cultivated with salivary bacteria, it was ineffective in integrating and becoming an associated member of the surface-attached oral biofilm.

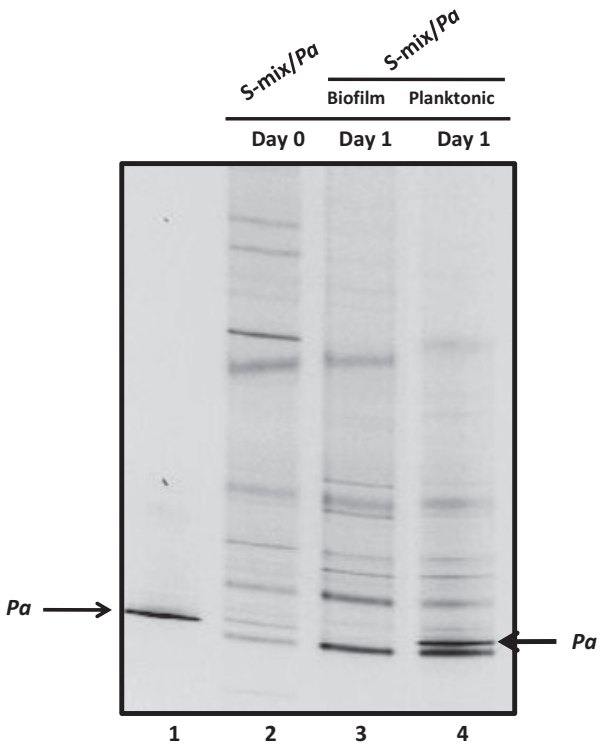

Figure 1 Polymerase chain reaction-denaturing gradient gel electrophoresis analysis of microbial profiles obtained from biofilm and planktonic portion of the co-culture of salivary bacteria (S-mix) and *Pseudomonas aeruginosa* (*Pa*) after 24 h of co-incubation. S-mix/*Pa* indicates co-culture of S-mix and *Pa*. The arrows indicate the DNA bands corresponding to *P. aeruginosa*. Two biological replicates were performed and a representative gel image is shown.

Effect of sucrose on the growth of *P. aeruginosa* PAO1 within the co-culture

Oral bacteria are frequently exposed to different fermentable carbohydrates. As sucrose is one of the sugars most often consumed by humans, we were interested in investigating its effect on the growth of *P. aeruginosa* when co-cultivated with salivary bacteria (S-mix). After a 24-h incubation, the cells from the biofilm and planktonic portions of the same well were combined and subjected to PCR-DGGE analysis. Results showed that the addition of 0.05% sucrose did not cause a significant change in banding patterns of salivary bacteria within the co-cultures. However, the intensity of the band representing *P. aeruginosa* PAO1 was greatly reduced in the co-culture containing 0.05% sucrose (Fig. 2A), suggesting that the viability of *P. aeruginosa* was severely affected. Viable count data showed that 24-h incubation with S-mix in the presence of sucrose resulted in more than 100-fold reduction in the total viable count of *P. aeruginosa*. In the absence of sucrose, the viable counts of *P. aeruginosa* increased about two orders of magnitude (Fig. 2B). We had already shown that *P. aeruginosa* PAO1 was ineffective in integrating into salivary biofilms (Fig. 1), so the *P. aeruginosa* detected most likely existed in the planktonic phase. It is worthwhile noting that the growth of *P. aeruginosa* was not affected when grown alone in the salivary medium supplemented with 0.05% sucrose, and the addition of sucrose resulted in a slight increase in the total viable counts of salivary bacteria compared with co-cultures without sucrose (data no shown).

Detection of lactic acid in the spent medium of salivary microbiota

Many oral bacterial species, including *Streptococcus* spp. and *Lactobacillus* spp., are able to produce lactic acid as the major metabolic end-product of carbohydrate fermentation. As a result of its antimicrobial property, particularly towards gram-negative bacteria, we reasoned that lactic acid might play a role in inhibiting the growth of *P. aeruginosa* cells when co-cultivated with salivary bacteria in the presence of sucrose. To test this, we performed HPLC/MS analysis on the spent medium of salivary bacteria grown in the salivary medium with and without sucrose. Lactic acid was detected in the spent medium supplemented with 0.05% sucrose by comparing its SIM chromatogram at *m*/*z* 89 (Fig. 3A) and the mass

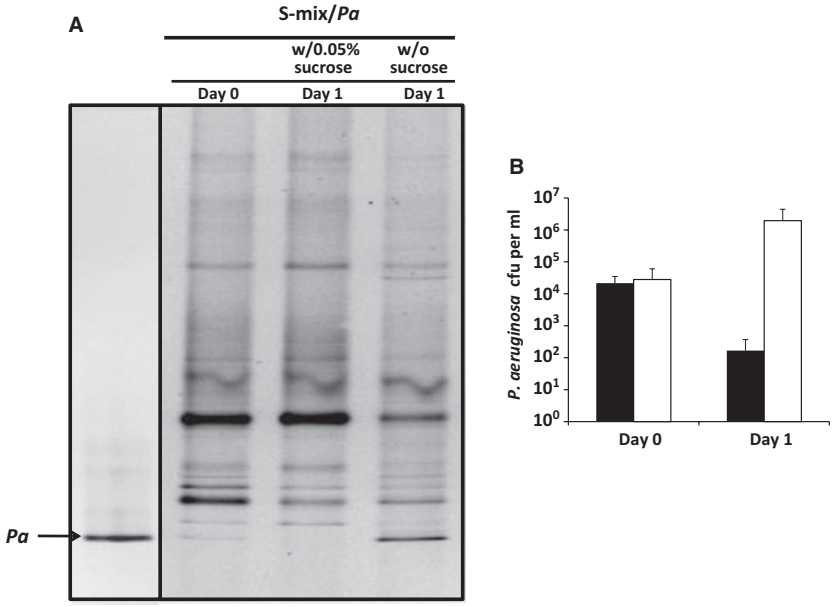

Figure 2 Effect of sucrose on the growth of *Pseudomonas aeruginosa* within the co-culture. (A) Salivary bacteria (S-mix) and *P. aeruginosa* (*Pa*) was co-cultivated in the presence and absence of 0.05% (weight/volume) sucrose for 24 h, DNA from total viable cells (including biofilm and planktonic portion) were isolated and subjected to polymerase chain reaction-denaturing gradient gel electrophoresis analysis. (B) Total co-culture samples (including planktonic and biofilm cells) were subjected to serial dilution and plated onto selective plates. Viable counts were monitored for *P. aeruginosa*. Black bars represent *P. aeruginosa* in the co-culture supplemented with sucrose; open bars represent *P. aeruginosa* in the co-culture without sucrose. Two biological replicates were performed and a representative gel image is shown. Three replicates were performed for each viability count assay. Average values mean + SD are plotted.

spectrum (Fig. 3B) with that of standard lactic acid (Fig. 3). After calculating the corresponding peak area against the standard, the concentration of lactic acid within spent medium (with sucrose) was determined to be 2.71 ± 0.95 mM. This was sufficient to inhibit the growth of *P. aeruginosa* (data not shown). Meanwhile, the absence of sucrose resulted in no significant amount of lactic acid being detected in the spent medium (Fig. 3A). It is worthwhile noting that the pH of fresh saliva medium was around 7.1, whereas the pH of the spent saliva medium containing 0.05% sucrose was ~6.6. We also showed that saliva medium with adjusted pH of 6.6 did not significantly affect the growth of *P. aeruginosa* compared with the original saliva medium (data not shown).

Inhibitory effect of lactic acid on the growth of *P. aeruginosa* PAO1 when co-cultivated with salivary bacteria

To further confirm the inhibitory effect of lactic acid towards *P. aeruginosa*, we added lactic acid to the *P. aeruginosa* PAO1/salivary bacteria co-culture to a final concentration 0.02% (2.2 mM). After a 24-h incubation, cells within the same well (including biofilm and unattached planktonic cells) were combined and subjected to PCR-DGGE analysis. The data revealed that, the sample from co-culture without addition of either sucrose or lactic acid contained the band representing *P. aeruginosa* PAO1 with high intensity (Fig. 4, lane 2), whereas samples prepared from co-cultures with the addition of sucrose or lactic acid displayed a much weaker *P. aeruginosa* band (Fig. 4, lanes 3–5).

Pa060928, a clinical *P. aeruginosa* isolate, was ineffective in integrating into salivary biofilm and suffered viability loss when co-cultivated with salivary bacteria in the presence of sucrose

To further demonstrate that the salivary bacterial community was effective in preventing the integration of pathogenic species, a clinical *P. aeruginosa* isolate (Pa060928) from an adult patient with cystic fibrosis, was co-cultivated with salivary microbiota. PCR-DGGE analysis revealed that the band representing

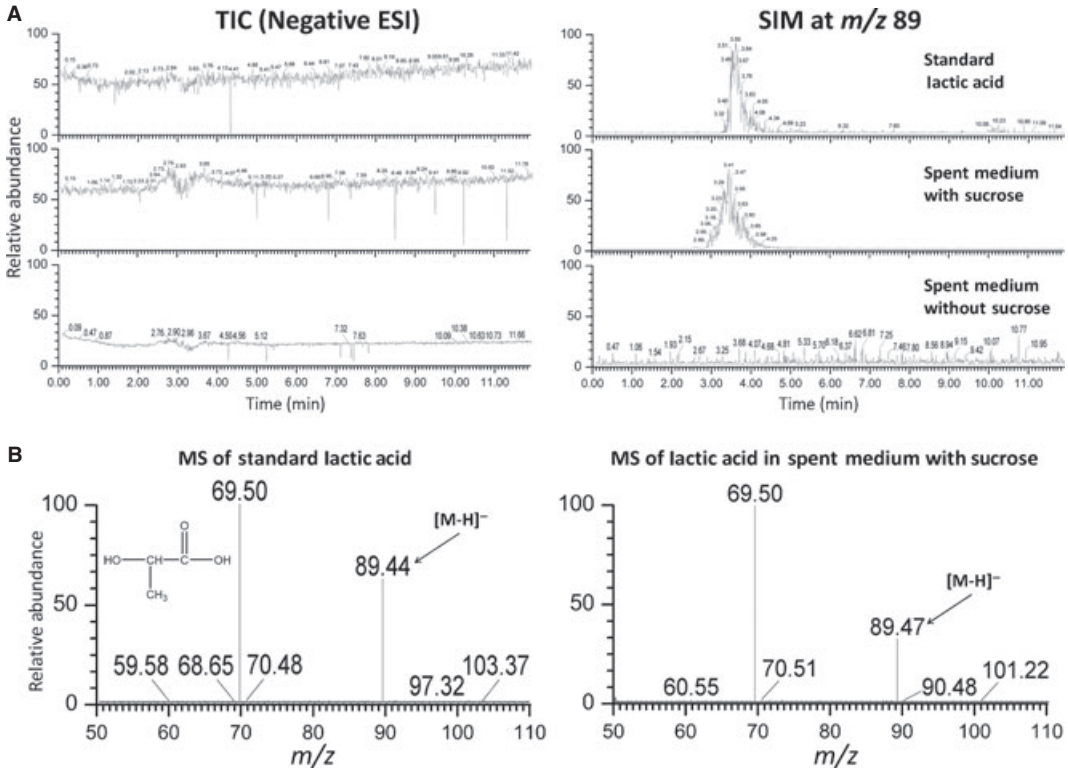

Figure 3 High-performance liquid chromatography/mass spectrometry analysis of the spent medium of oral flora grown in salivary medium supplemented with and without 0.05% sucrose. (A) Total ion current (left panel) and selected ion monitoring (right panel, with deprotonated molecular ion 89 *m/z*) chromatograms: top, a standard lactic acid sample at 3.25 mM prepared in ddH_2O; middle, spent medium with 0.05% sucrose; bottom, spent medium without sucrose. (B) Electrospray ionization-mass spectrometry of standard lactic acid (left) and corresponding peak in the spent medium with 0.05% sucrose (right).

Pa060928 became the most dominant one within the planktonic portion, whereas it was not easily detectable in the biofilm portion (Fig. 5A). This is corroborated by the viability data, which showed that after a 24-h co-cultivation, the majority of Pa060928 cells remained in the planktonic phase and those found within biofilm accounted for only <0.01% of the total *P. aeruginosa* cells (Fig. 5B). When sucrose was added to the co-culture medium, Pa060928 suffered drastic viability loss as revealed both by DGGE analysis and viable count (Fig. 5C,D), a phenomenon similar to that observed when the PAO1 strain was tested.

Co-aggregation between *P. aeruginosa* and oral isolates

In an effort to test the co-aggregation ability of *P. aeruginosa* with oral bacteria, we isolated major bacterial species from the saliva samples of healthy subjects, and their ability to adhere to *P. aeruginosa* (both the laboratory strain PAO1 and the clinical isolate Pa060928) was determined by a co-aggregation assay. Results showed that all the tested salivary bacterial isolates, including *Streptococcus* spp., *Lactobacillus* spp., *Actinomyces* spp. and *Prevotella* spp., demonstrated substantial levels (with co-aggregation scores of 2–4) of interspecies co-aggregation with *Fusobacterium nucleatum*, but no detectable co-aggregation was observed between *P. aeruginosa* (both PAO1 and Pa060928 strains) and oral isolates, including *F. nucleatum* (Table 1). Similar results were observed when co-aggregation assay was performed in saliva medium (see Supporting Information, Table S1).

CHX-induced shift in microbial profile within salivary biofilms resulted in reduced defense against *P. aeruginosa* integration

To test the protective capability of pre-formed salivary biofilm against integration by foreign bacteria, we

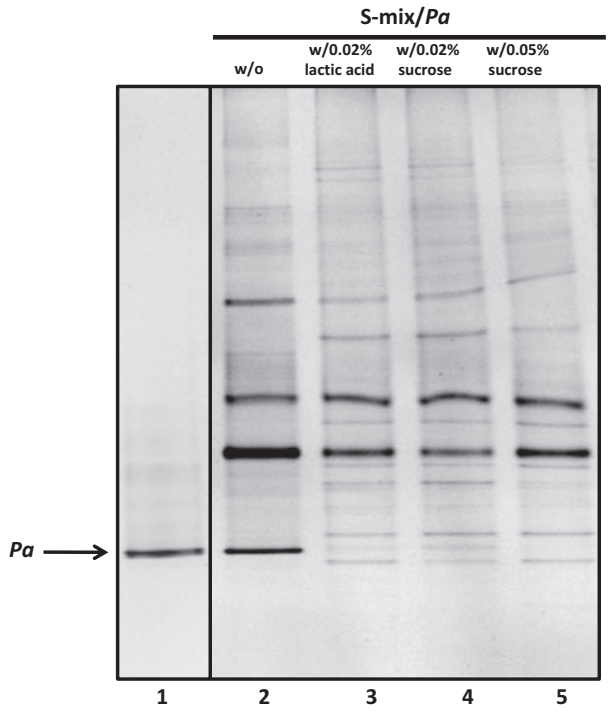

Figure 4 Effect of lactic acid on the growth of *Pseudomonas aeruginosa* within co-culture. Salivary bacteria (S-mix) and *P. aeruginosa* (*Pa*) was co-cultivated in the absence (lane 2), or the presence of 0.02% lactic acid (lane 3), 0.02% sucrose (lane 4) or 0.05% sucrose (lane 5) for 24 h, DNA from total viable cells (including planktonic and biofilm) was isolated and subjected to polymerase chain reaction-denaturing gradient gel electrophoresis analysis. Arrow indicates the band of *P. aeruginosa*. Two biological replicates were performed and a representative gel image is shown.

challenged 24-h saliva biofilms with *P. aeruginosa* PAO1 and monitored the microbial profiles using PCR-DGGE analysis. Results showed that even after 48 h of co-incubation, there was no detectable *P. aeruginosa* band within the salivary biofilm [Fig. 6: samples with CHX treatment (–) and *P. aeruginosa* challenge (+)], suggesting that the pre-formed biofilms were effective in preventing *P. aeruginosa* integration.

We further investigated the effect of shifts in microbial composition within the same biofilms on their defense capability. By mildly treating pre-formed salivary biofilms with diluted (0.01%) CHX followed by 24-h re-growth, we generated a community with a drastic shift in the microbial profile and reduction (<20%) in the biodiversity [Fig. 6: sample with CHX treatment (+)], while still maintaining similar numbers of viable cells compared with non-treated biofilms (data not shown). The treatment resulted in reduced population of certain oral microbes, e.g. *Streptococcus* spp. (Fig. 6, O1), and clonal expansion of bacterial species that were less susceptible to CHX, such as *Neisseria* spp. (Fig. 6, O3) as indicated by increased band intensities at 24 and 48 h. Meanwhile, the abundance of certain oral residents, including *Porphyromonas* spp. (Fig. 6, O2) and *Peptostreptococcus* spp. (Fig. 6, O4) remained relatively stable. When challenged with *P. aeruginosa* cells, CHX-treated biofilms suffered drastically reduced protective ability in preventing *P. aeruginosa* from colonizing the biofilm, as indicated by the increased intensity of the band representing *P. aeruginosa* within the biofilm samples taken 24 and 48 h after CHX treatment/*P. aeruginosa* challenge [Fig. 6: samples with CHX treatment (+) and *P. aeruginosa* challenge(+)]. For CHX-treated samples, longer treatment time (30 s) and longer incubation time (48 h) resulted in a more intense signal of the *P. aeruginosa* band recovered from biofilm samples (Fig. 6).

DISCUSSION

The interaction between the human indigenous microbiota and exogenously acquired pathogens has been the subject of continuous investigation for the past few decades (Sanders, 1969; Brook, 1999; Reid *et al.*, 2001). It has been speculated that indigenous microbiota may enhance the host ability to resist infection, and the notion that it may be manipulated to the host's advantage makes the topic an interesting one (Reid *et al.*, 2001; Falagas *et al.*, 2008). Unlike the indigenous microbiota associated with other parts of the human body, such as the intestine, the upper respiratory tract and the female genital tract, whose beneficial and protective roles have been well studied (Larsen, 1993; Brook, 2005; O'Hara & Shanahan, 2006), the demonstration and investigation of oral microbial community-based bacterial interference in preventing pathogenic or foreign microbial colonization is still lacking.

Despite repeated exposure to various bacteria from the nose, the respiratory and intestinal tract, as well as contaminated water and food sources, the microbial composition within oral cavities of healthy subjects is relatively stable (Rasiah *et al.*, 2005; Zaura *et al.*, 2009); However, patients with certain oral disease conditions have been shown to carry altered

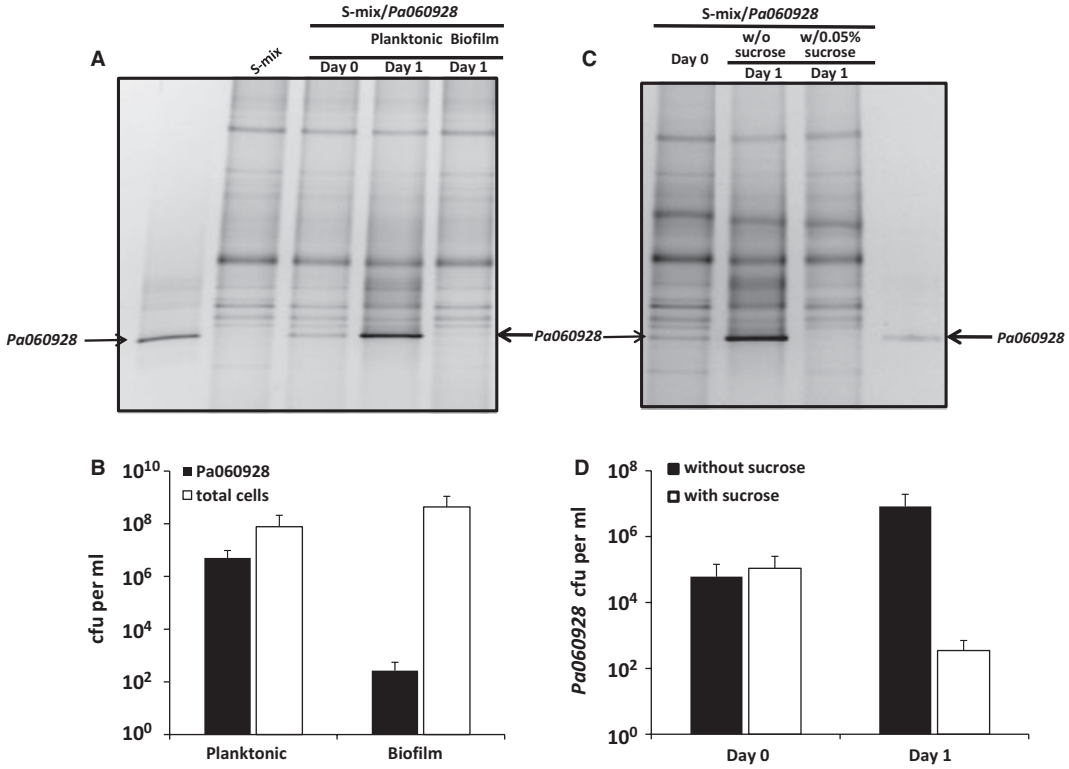

Figure 5 Co-cultivation of Pa060928 with salivary bacteria (S-mix) in the absence and presence of sucrose. (A) Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analysis of microbial profiles obtained from biofilm and planktonic portion of the co-culture after 24-h co-incubation. S-mix/Pa060928 indicates co-culture of S-mix and Pa060928. The arrows indicate the DNA bands corresponding to Pa060928. Three biological replicates were performed and a representative gel image is shown. (B) Viability count of Pa060928 and total bacteria within planktonic and biofilm portions after a 24-h incubation in saliva medium. Three replicates were performed for each viability count assay. Average values + SD are plotted. (C) PCR-DGGE analysis of total bacterial profile (including planktonic and biofilm portion) after 24-h co-cultivation in the absence and presence of 0.05% sucrose. Three biological replicates were performed and a representative gel image is shown. (D) Viability count of Pa060928 within the co-culture (combining planktonic and biofilm portion) after a 24-h incubation in the absence and presence of sucrose. Black bars represent Pa060928 in the co-culture without sucrose; open bars represent Pa060928 in the co-culture supplemented with 0.05% sucrose. Three replicates were performed for each viability count assay. Average values + SD are plotted.

oral microbial communities and are prone to colonization by bacteria of foreign origin (Leung *et al.*, 1998; Almståhl *et al.*, 2008). These intriguing phenomena suggest that the normal commensal oral microbial communities might play beneficial roles and exert protective functions against pathogenic/foreign colonization.

Using an *in vitro* model of mouse gastrointestinal-tract-associated microflora, we demonstrated that the oral microbiota was able to prevent the integration of bacterial species originating from the gut (He *et al.*, 2010a). In the present study, we further investigated the protective role of the human oral microbiota by establishing saliva-derived biofilms and testing their ability to prevent the integration by two *P. aeruginosa* strains: the PAO1 laboratory strain and Pa060928, a clinical isolate from a patient with cystic fibrosis. Saliva medium was used for cultivation to mimic nutritional conditions within the oral cavity. We demonstrated that although both *P. aeruginosa* strains were able to persist during co-cultivation with oral flora in saliva medium, they mainly existed in the planktonic phase and were ineffective in integrating into surface-attached oral microbial communities during biofilm formation (Figs 1 and 5).

Development into structured oral biofilms requires co-aggregation between different bacterial species and attachment to the extracellular matrix (Lamont & Jenkinson, 2000; Kolenbrander *et al.*, 2010). Co-aggregation among indigenous oral bacterial species

Table 1 Co-aggregation between *Pseudomonas aeruginosa* and oral isolates[1] in co-aggregation buffer

Strains	*Fusobacterium nucleatum* (oral isolate)	*Pseudomonas aeruginosa* PAO1	*Pseudomonas aeruginosa* Pa060928
Gram-positive oral isolate			
Streptococcus mitis	4	0	0
Streptococcus salivarius	4	0	0
Streptococcus australis	3	0	0
Streptococcus parasanguinis	4	0	0
Lactobacillus casei	2	0	0
Actinomyces naeslundii	3	0	0
Gram-negative oral isolate			
Prevotella denticola	2	0	0
Fusobacteirum nucleatum	0	0	0
Non-oral isolate			
Escherichia coli	0	0	0

[1]The method for assigning co-aggregation scores is described in the Methods section.

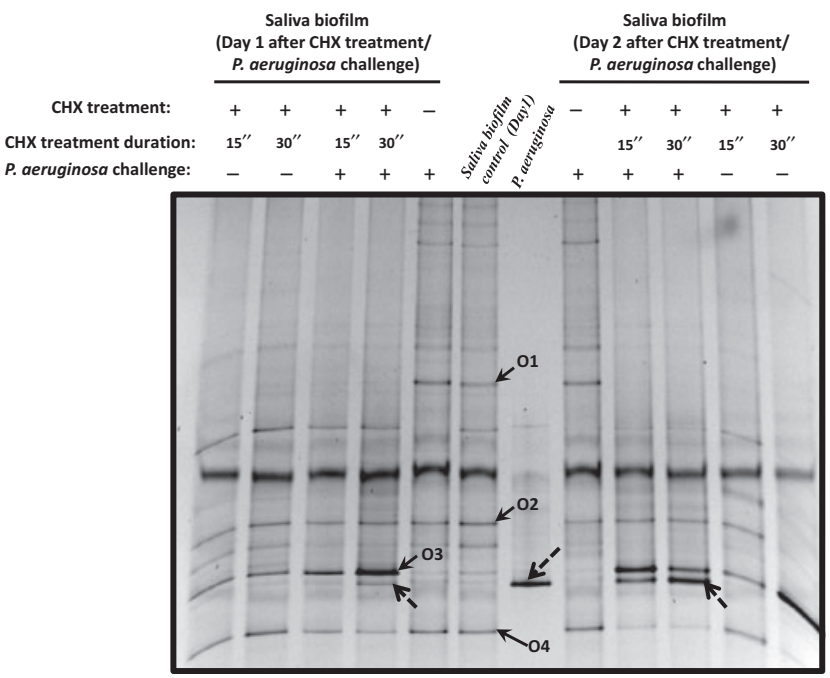

Figure 6 Polymerase chain reaction-denaturing gradient gel electrophoresis analysis of cycloheximide gluconate (CHX) treatment effect on biofilm's capability to prevent *Pseudomonas aeruginosa* PAO1 colonization. Pre-formed (24-h-old) saliva biofilm was subjected to single-dose CHX treatment for 15 or 30 s, followed by 24 h of re-growth. The recovered biofilm was challenged with *P. aeruginosa* and followed by extended incubation for 48 h. The microbial profiles of the biofilms were monitored by PCR-DGGE analysis. CHX treatment or *P. aeruginosa* challenge is indicated by (+) and (–) represents samples without CHX treatment or *P. aeruginosa* challenge. Bacterial species corresponding to the bands of interest were identified as: O1, *Streptococcus* spp.; O2, *Porphyromonas* spp.; O3, *Neisseria* spp.; O4, *Peptostreptococcus stomatis*. Dotted arrow indicates *P. aeruginosa* PAO1. Two biological replicates were performed and a representative gel image is shown.

is a fairly common phenomenon. It has been demonstrated that while oral bacterial species do not co-aggregate well with non-oral bacteria, such as intestinal species (Ledder *et al.*, 2008), all of the approximately 1,000 oral bacterial strains examined so far have at least one co-aggregation partner. Indeed, co-aggregation plays a pivotal role in the formation of structured multispecies oral biofilms

(Kolenbrander *et al.*, 2010). As non-oral commensal bacteria, neither the *P. aeruginosa* laboratory strain PAO1 nor the clinical isolate Pa060928 was able to co-aggregate with any of the tested oral bacterial species isolated from healthy subjects in this study (Table 1), including the bridging organism *F. nucleatum*, which has been suggested to play critical roles in facilitating the development of oral community because of its ability to co-aggregate with a variety of oral bacteria (Kolenbrander *et al.*, 2010). Our results suggested that the inability of *P. aeruginosa* to adhere to oral bacterial species might contribute to its ineffectiveness in integrating into the developing salivary biofilm.

As an opportunistic pathogen, *P. aeruginosa* can be frequently isolated from certain oral infection sites (Nord *et al.*, 1972) and is one of the most common pathogens identified within the oral cavity and sputum of patients with cystic fibrosis (Komiyama *et al.*, 1985) and ventilator-associated pneumonia (Bonten *et al.*, 1999). Interestingly, in the case of cystic fibrosis, *P. aeruginosa* isolates have been shown to effectively co-aggregate with certain oral isolates obtained from the same patients (Komiyama *et al.*, 1987), further indicating that co-aggregation might play an important role in determining if *P. aeruginosa* can integrate into developing oral microbial communities. However, the cystic fibrosis clinical isolate Pa060928 used in this study was unable to co-aggregate with any of the oral isolates from healthy subjects tested (Table 1). One possible explanation could be that in patients with cystic fibrosis, the changes in oral ecological conditions could result in the colonization of specific oral bacterial strains with distinct outer membrane characteristics, which could allow them to interact and co-aggregate with certain *P. aeruginosa* strains.

Another interesting finding of this study was that in the presence of sucrose, the saliva-derived microbiota was not only able to prevent *P. aeruginosa* from integrating into biofilms, but it could also inhibit the growth of *P. aeruginosa* within the planktonic portion of the co-culture by producing lactic acid (Figs 2, 3 and 5). As an antimicrobial agent, lactic acid is able to inhibit the growth of many bacteria, particularly gram-negative species of the families *Enterobacteriaceae* and *Pseudomonadaceae* (Ray & Sandine, 1992; Alakomi *et al.*, 2000). One aspect of its antibacterial action is to lower the pH, which by itself might have certain inhibitory effects (Pasricha *et al.*, 1979). However, this is unlikely to be the key factor in our study because the high buffering capacity of salivary medium used in this study did not result in a significant drop in pH, even when lactic acid could be detected at millimolar levels in the spent medium (data not shown). Another important antimicrobial property of lactic acid is its ability, in the undissociated form, to permeabilize gram-negative bacteria by disrupting the outer membrane. It is also capable of penetrating the cytoplasmic membrane, resulting in reduced intracellular pH and disruption of the transmembrane proton motive force (Ray & Sandine, 1992). Lactic acid produced by probiotic *Lactobacillus* spp. has been shown to play an important role in fending off pathogenic *Salmonella* strains in the intestinal tract (Fayol-Messaoudi *et al.*, 2005). In the oral cavity, gram-positive microbes are the major species detected in saliva and supra-gingival plaque where the first encounter between exogenous gram-negative pathogens, such as *P. aeruginosa*, and oral microbes takes place. The fact that many oral bacterial species, including *Streptococcus* spp. and *Lactobacillus* spp., are able to produce lactic acid as the major metabolic end-product of carbohydrate fermentation (Dashper & Reynolds, 1996; Ljungh & Wadström, 2006) strongly suggests that it could be one of the defense mechanisms used by the oral microbiota to inhibit the growth and prevent the colonization of exogenous gram-negative pathogens, such as *P. aeruginosa*, when fermentable sugars are available.

From the community perspective, the production of lactic acid could be a double-edged sword: although it inhibits pathogens, it might also have negative effects on certain residents within the community. How the residential bacteria cope with this situation is not clear and is currently under investigation. However, a recent report showed that the co-aggregation with *Actinomyces naeslundii* protected another oral resident, *Streptococcus gordonii*, from oxidative damage by the catalase production by *A. naeslundii* (Jakubovics *et al.*, 2008). This suggests that co-aggregation between two oral bacterial species might help some species better tolerate the environmental stress experienced within multispecies communities.

Our results demonstrated that pre-formed salivary biofilms were effective in preventing *P. aeruginosa* colonization (Fig. 6). For a bacterium to successfully colonize the pre-existing biofilm microbial community,

it needs to overcome the invasion resistance developed by the existing flora. The invasion resistance often includes: (i) depletion of attachment sites, (ii) production of antibiotic substance, or (iii) the establishment of a restrictive physiological microenvironment, such as altered pH (Bernet *et al.*, 1994; Liévin *et al.*, 2000; Kreth *et al.*, 2005). Although the underlying mechanism is unclear and currently under investigation, recent studies have demonstrated that, depending on the sequence of inoculation, preformed *Streptococcus sanguinis* biofilms can prevent the integration of later *Streptococcus mutans* strains by producing the antimicrobial agent hydrogen peroxide (Kreth *et al.*, 2005). In the context of this study, it is possible that the presence of certain species in the established oral biofilm could exert inhibitory effects against *P. aeruginosa.* Our results are consistent with the report showing that the presence of existing oral biofilms can greatly reduce *S. mutans* colonization (Li *et al.*, 2010).

The most intriguing finding of our study was that a shift in salivary microbial composition, resulting from mild CHX treatment, led to a drastic reduction in the ability of the biofilm to inhibit *P. aeruginosa* colonization (Fig. 6). CHX is a cationic bis-biguanide biocide with broad-spectrum antibacterial activity that can induce concentration-dependent growth inhibition (Hugo & Longworth, 1966) and has been shown to be able to induce a bacterial profile change in existing microbial floras (McBain *et al.*, 2003). In our study, a low concentration of CHX was used to generate a microbial community with a shifted population profile.

The observed reduction in the biofilm's protective capability was not a result of the reduction in the total microbe population within the biofilm because 24-h re-growth allowed the bacterial counts of the microbial community to recover to pre-treatment levels (data not shown). After treatment, previously minor species, such as *Neisseria* spp., became dominant whereas *Streptococcus* spp. suffered severe reduction in population within the biofilm. The change in microbial composition could potentially affect community dynamics, including its invasion resistance and its response to the presence of bacteria of foreign origin (Ley *et al.*, 2006).

Furthermore, the reduced defense ability could also result from the decrease in biodiversity within the biofilm. In other ecosystems, it has been observed that species-rich communities are more resistant to invasion by exotic species than species-poor communities (Elton, 1958). This is also in agreement with our previous studies showing the maximum inhibitory effect exerted by an *in vitro* mouse oral microbial community toward a gut bacterium was achieved when the whole community was involved, suggesting a community-based antagonistic action (He *et al.*, 2010a). Our data corroborated documented reports, which show that microbial composition within oral cavities of healthy subjects are relatively stable (Rasiah *et al.*, 2005; Zaura *et al.*, 2009) and suggest that patients with altered oral microbial communities may be more susceptible to colonization by bacteria of foreign origins (Leung *et al.*, 1998; Almståhl *et al.*, 2008).

The human oral cavity has evolved complex and sophisticated mechanisms to fend off bacterial pathogens, including physical barriers, protective immunity conferred by the mucosal lining (Walker, 2004), and defense components within saliva (Tabak, 2006). Our study supports the notion that bacterial interference exerted by the oral commensal microbiota could also play a significant role in protecting against the colonization by foreign or pathogenic bacteria. In this regard, a normal and balanced oral commensal microbiota can greatly contribute to ecological stability.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Biofilm formation of salivary bacteria (S-mix) and *Pseudomonas aeruginosa* strain (PAO1 and Pa060928) in the presence and absence of 0.05% sucrose in saliva medium.

Table S1. Co-aggregation between *Pseudomonas aeruginosa* and oral isolates[1] in saliva medium.

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