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Analysis and Management of Plaques on Intraoral Artificial Dental Materials

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Analysis and Management of Plaques on Intraoral Artificial Dental Materials

A dissertation submitted in partial satisfaction

of the requirements for the degree Doctor of Philosophy

in Oral Biology

by

Tingxi Wu

2017

ABSTRACT OF THE DISSERTATION

Analysis and Management of Plaques on Intraoral Artificial Dental Materials

by

Tingxi Wu

Doctor of Philosophy in Oral Biology

University of California, Los Angeles, 2017

Professor Wenyuan Shi, Chair

Oral cavity contains diverse microorganisms including bacteria and fungi. These microbes are able to colonize on natural surfaces, including tooth and mucosa, as well as artificial surfaces, such as surfaces of denture, implants, and orthodontic braces. The microbial colonization and formation of biofilms on these biotic and abiotic surfaces may lead to local and systemic diseases such as caries, white spot lesion, gingivitis, halitosis, and denture stomatitis. However, microbial study related to intraoral artificial surfaces has drawn little attention in comparison to microbial study on natural oral surfaces. While

I learned my microbiology and molecular biology experimental skills through studying a dental caries related pathogen, *Streptococcus mutans*, my mian research projects have been focusing on studying plaque on the artificial surfaces and their associations with dental diseases with denture and orthodontic brackets as model systems. In order to study the mechanisms behind these artificial surface plaque associated oral infectious diseases, one would need to first characterize microbial compostion of these plaques as well as establish effective study models, then study the dynamic interactions between different pathogenic species within these plaques. Through understanding the molecular components involved in plaque formation and inter-species interactions, we may develop the tools to manage these diseases.

My thesis consists of three parts: Part I describes my initial work of learning microbiology and molecular biology technologies to discover and characterize a novel membrane associated protein (PrsA) in the major cariogenic pathogen, *Streptococcus mutans*, which is important for cellular aggregation and biofilm formation. In Part II, I applied the molecular biology tools to address clinic relevant issues with a focus on denture related oral microbial diseases, including characterization of the denture associated oral microbiome under healthy and diseased conditions and investigation of the molecular mechanism behind the potential pathogens associated with denture-associated microbial diseases. Part III covers my ongoing studies on orthodontic related oral microbial diseases after I joined orthodontic residency program. In this part, we

established a clinical-relevant high-thoughput screening assay to discover compounds that could block orthodontic bracket induced plaque formation, then formulate these biofilm-inhibitory compounds into a toothpaste for the prevention of bracket-induced plaque formation. The clinical efficacy of the toothpaste is being tested now. I also have another ongoing project to characterize the dynamic change of oral microbiomes in orthodontic patients before, during, after orthodontic treatments.

The dissertation of Tingxi Wu is approved.

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2017

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My deepest appreciation also goes to Drs.Kang Ting, Ben Wu, and Xuesong He for their important feedbacks on my research project and their strong support during my doctorate program. They are all brilliant scientists and wonderful mentors. While I was resident in orthodontic program, with Dr. Ting's guidance and support, I was able to successfully extend my research into orthodontic filed and got funded by American Association of Orthodontists Foundation (AAOF). I was also lucky to be mentored by Dr. Wu to work on the denture-related microbial study and one of the technologies we collaborated has already filed patent application.

I would like to thank Dr. Renate Lux who is caring, considerate and patient professors. She always patiently explained rationale behind my experiments in a simple and clear way to help me understand better. Dr. He is another caring and patient professor who was guiding me with every detail during my experiments and helped me master the important advanced technology in microbiologic field that I used in my research, also guiding me to revise my thesis.

I would also like to take the opportunity to express my deepest gratitude to Dr. Xuedong Zhou at West China School of Stomatology, who is my role model as the best clinician scientist and leader in dentistry. In particular, I wish to thank her constant guidance for my career development.

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- Conducting a Pilot Clinical Study to Evaluate the Safety and Efficacy of a Newly Developed Chemical Formula for Managing Bracket-Induced Plaque Formation (Principle Investigator)

B. Publications & Patents

- **T. Wu**. Status of Chinese Dentistry after earthquake disaster. Chinese Journal of Stomatological Information. 2008 17(5).
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PATENT APPLICATIONS

- 2016 A Chemical Formula for Managing Bracket-Induced Plaque Formation, U.S. Provisional Application Serial No. 62/329, 114

Part I

Chapter I

Exploring novel surface proteins of cariogenic pathogen *Streptococcus mutans*

The first part of my thesis focuses on the phenotypic and genetic study of caries pathogen *Streptococcus mutans*. The phenotypic and genetic study on disease-related bacteria has been used to characterize the important role of these microorganisms in disease pathogenesis, such as *Streptococcus mutans* in caries and *Fusobacterium nucleatum* in periodontitis (Cohen S 1973, Kolenbrander 2000). With the molecular genetic techniques such as DNA homologous recombination, we are able to characterize the correlated phenotype to reveal the function of this gene. After characterizing the function of the genes, we could further reveal the pathogenic aspect of this disease-associated bacteria.

S.mutans is the principal etiological agent of dental caries. Its surface proteins as well as the extracellular matrix are required for its colonization and pathogenesis in oral cavity. The surface proteins or secreted proteins are all having the signal peptide at the N-terminus to facilitate their transportation to the cell membrane. In order to find the uncharacterized surface proteins as well as the secreted proteins, signal peptide database (http://www.signalpeptide.de/index.php) has been searched. 15 proteins were found to have signal peptide, and 3 of them have not being studied, including membrane protein OxaA1, and OxaA2, and foldase protein PrsA. Based on previous studies, foldase protein PrsA has been studied, and its role in regulating folding and export of certain secreted proteins in *Bacillus* and *Lactococcus* has been demonstrated. In *B. subtilis,* the extracytoplasmically located PrsA has been shown to be critically important *in vivo* for the proper conformation of various exoproteins (Jacobs et al., 1993), and considered as an

essential rate-limiting component of the secretion machinery (Kontinen & Sarvas, 1993). It influences neither the expression nor the translocation of exoproteins but is required for their correct conformation and stability in the post-translocational phase of secretion (Hyyryläinen et al., 2001; Vitikainen et al., 2001). In *L. lactis*, the PrsA- like protein triggers the folding of the secreted lipase (Drouault et al., 2002). Over-expression of *B. subtilis* PrsA also resulted in increased heterologous protein expression in *L. lactis*, presumably by allowing more efficient protein folding (Lindholm et al., 2006).

However, no study has been done to reveal the function of PrsA in *S. mutans*. In chapter two, we focus on characterization of the function of PrsA in *S. mutants* by constructing a prsA-deficient strain and analyzing its phenotypes to explore the biological function of PrsA in *S. mutans.*

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Part I

Chapter II

Phenotypic characterization of the foldase homologue PrsA in *Streptococcus mutans*

This chapter has been published as the following:

L. Guo, T. Wu, W. Hu, et al. Phenotypic characterization of the foldase homologue *PrsA* in *Streptococcus mutans*. Mol Oral Microbiol. 2013 28 (2): 154-165

Abstract

Streptococcus mutans is generally considered to be the principal etiological agent for dental caries. Many of the proteins necessary for its colonization of the oral cavity and pathogenesis are exported to the cell surface or the extracellular matrix, a process that requires the assistance of the export machineries. Bioinformatic analysis revealed that the *S. mutans* genome contains a *prsA* gene, whose counterparts in other gram-positive bacteria, including *Bacillus* and *Lactococcus*, encode functions involved in protein post-export. In this study, we constructed a PrsA-deficient derivative of *S. mutans* and demonstrated that the *prsA* mutant displayed an altered cell wall/membrane protein profile as well as cell-surface-related phenotypes, including auto-aggregation, increased surface hydrophobicity and abnormal biofilm formation. Further analysis revealed that the disruption of the *prsA* gene resulted in reduced insoluble glucan production by cell surface localized glucosyltransferases, and mutacin as well as cell surface-display of a heterologous expressed GFP fusion to the cell surface protein SpaP. Our study suggested that PrsA in S. mutans encodes functions similar to those identified in *Bacillus*, and so is likely to be involved in protein post-export.

Introduction

As the primary etiological agent of dental caries in humans (Hamada & Slade, 1980), *Streptococcus mutans* relies on the activities of secreted or cell surface localized proteins to interact with other oral bacteria, colonize the oral cavity and exert its pathogenesis. Previous studies have shown that *S. mutans* employs various mechanisms to deliver proteins across the cell membrane. The general secretory (Sec) translocation channel is the major secretion apparatus in protein translocation across the cytoplasmic membrane (Fekkes & Driessen, 1999; Muller *et al*., 2001). In addition, *S. mutans* also contains certain specific secretion systems. For example, specific ATP-binding cassette transporters were found to be responsible for direct translocation of the bacteriocins across the cytoplasmic membrane (van Belkum *et al*., 1997). Most proteins translocated across the cell membrane via the above pathways are considered to be delivered in an unfolded conformation across the cytoplasmic membrane into the interface between cytoplasmic membrane and cell wall peptidoglycan (Sarvas *et al*., 2004; Matias & Beveridge, 2006, 2008). Due to the high cation concentration, as well as the low pH and high density of negative charge within the cytoplasmic membrane–cell wall peptidoglycan interface (Sarvas *et al*., 2004), protein chaperones as well as foldases are required to ensure the proper folding of these proteins after export. However, the components and their roles in the folding and stabilization of proteins to their active form are poorly characterized in *S. mutans*.

The foldase protein PrsA is found ubiquitously in the genomes of all gram-positive bacteria including *S. mutans*. In the Group A streptococci including *Streptococcus pyogenes*, PrsA was found to be required for the final maturation steps of SpeB, a pluripotent cysteine protease and an important virulence factor (Ma *et al*., 2006). The role of PrsA in assisting the folding and stability of exported proteins has been extensively studied in *Bacillus* and *Lactococcus*. In *Bacillus subtilis*, the extracytoplasmically located PrsA has been shown to be critically important *in vivo* for the proper conformation of various exoproteins (Jacobs *et al*., 1993), and considered as an essential rate-limiting component of the secretion machinery (Kontinen & Sarvas, 1993). It influences neither the expression nor the translocation of exoproteins but is required for their correct conformation and stability in the post-translocational phase of secretion (Hyyryläinen *et al*., 2001; Vitikainen *et al*., 2001). In *Lactococcus lactis*, the PrsA-like protein triggers the folding of the translocated lipase (Drouault *et al*., 2002). Over-expression of *B. subtilis* PrsA also resulted in increased heterologous protein expression in *L. lactis*, presumably by allowing more efficient protein folding (Lindholm *et al*., 2006).

In *S. mutans*, the predicted PrsA protein contains 333 amino acids with a molecular mass of ~36 kDa. It includes a predicted signal sequence at the N-terminus. However, no cellular function has been assigned to this protein. In this study, by constructing a *prsA*-deficient strain and performing an array of phenotypic analyses, we sought to investigate the biological functions of PrsA in *S. mutans*.

Methods

Bacterial strains and growth conditions

Escherichia coli strain DH5α was used for cloning as well as plasmid amplifications and grown in Luria–Bertani (LB) medium aerobically at 37°C. The *S. mutans* strain UA140 (wild-type), UA140 *prsA*-deficient strain and UA140 pHluorin-SpaP fusion strain and its corresponding *prsA*-deficient strain were cultured in Todd–Hewitt (TH) media (Difco, Franklin Lakes, NJ) at 37°C in the presence of 5% CO2. For selection of antibiotic-resistant colonies after genetic transformation, spectinomycin (100 μg ml−1 for *E. coli* or 800 μg ml−1 for *S. mutans*) or kanamycin (150 μg ml−1 for *E. coli* or 800 μg ml−1 for *S. mutans*) was added to the medium.

Strain construction

The open reading frame for the predicted *prsA* gene (GenBank accession no. AAN58382) was annotated in the *S. mutans* UA159 genome database (http://www.genome.ou.edu/smutans.html). BLASTn and BLASTp sequence homology analyses were performed using the BLAST network service of the National Center of Biotechnology Information (NCBI; Bethesda, MD).

The pFW5 vector (Podbielski *et al*., 1996) was employed for generating a mutant derivative of *S. mutans* wild-type strain UA140 (Qi *et al*., 2001) carrying a deletion in the *prsA* gene. The *S. mutans* UA140 genomic DNA served as a template to amplify the *prsA*

upstream region with the primer pair upF (5′-CCGCTCGAGCGCAAACCACATCCACAGGG), which contains a *Xho*I site incorporated at its 5′ end, and upR (5′-CCCAAGCTTCACAAGTCCTGTAGCAATCG), which has a *Hin*dIII site incorporated at its 5′ end, whereas the corresponding downstream region was obtained using downF (5′-ACATGCATGCCAGCAGCAAGCGGAAGTGGC), which carries a *Sph*I site incorporated at its 5′ end, and downR (5′-TCCCCCCGGGAGCATCATCACGGAAGTAAT) with a *Xma*I site incorporated at its 5′ end (the restriction enzyme recognition sites are underlined). The fragments were generated using *Pfu* polymerase (Stratagene, La Jolla, CA) and subsequently inserted into the two multiple cloning sites of the pFW5 vector, respectively. The resulting recombinant plasmid pFW5-*prsA* was confirmed by restriction analysis, polymerase chain reaction (PCR) amplification and DNA sequencing. Plasmid pFW5-*prsA* was then linearized using a unique *Nhe*I site in the vector backbone and transformed into *S. mutans* UA140 via competence-stimulating peptide (CSP) -induced natural transformation (Kreth *et al*., 2005). The CSP was a gift from C3 Jian Inc. (Los Angeles, CA). Transformants were selected on TH agar containing 800 μg ml−1 spectinomycin. The resulting *prsA* deletion mutant was confirmed by PCR and sequencing.

The GFP coding sequence was in-frame inserted via overlapping PCR between the second and third amino acids after the identified signal-peptide cleavage site of SpaP (Kelly *et al*., 1989), a surface protein antigen-encoding gene of *S. mutans*. The resulting

fragment was then ligated downstream of the lactate dehydrogenase gene (*ldh*) promoter (Merritt *et al*., 2005) and cloned into pFW5 vector to generate pFW5Φ (*ldh*p-leading sequence-*gfp-spaP*). The recombinant construct was transformed into *S. mutans* strain UA140 as well as the *prsA*-deficient mutant to generate the cell-surface-displayed GFP-SpaP fusion derivative (Guo *et al*., unpublished data). The integration of *gfp-spaP* into the chromosome of *S. mutans* via single crossover homologous recombination was confirmed by PCR, and cell surface localization of the GFP-SpaP fusion was revealed by Western blot analysis using an anti-GFP antibody (Guo *et al*., unpublished data).

General phenotypic characterization assays

Growth kinetics were measured for *S. mutans* UA140, the *prsA*-deficient strain and the strain containing the cell-surface-displayed GFP. Autolysis assay was also performed as previously described (Wen & Burne, 2002).

Hydrophobicity assay by the bacterial adherence to hydrocarbons

The bacterial hydrophobicity was measured using the bacterial adhesion to hydrocarbons (BATH) method as described previously (Dillon *et al*., 1986). Briefly, the bacterial cultures were dispersed by drawing up and expelling the bacterial suspension 10 times through a 26-gauge, 15.9-mm long needle (Dunning *et al*., 2008) then suspended in PUM buffer (K2HPO4, 16.87 gl−1; KH2PO4, 7.26 gl−1; MgSO4·7H2O, 0.2gl−1; urea, 1.8 gl−1; pH 7.1) to a final optical density at 600 nm (OD600) of 0.6. A 1.2-ml volume of the bacterial suspension was dispensed into each one of 12 round-bottom test tubes with

10-mm diameter. Four tubes, each containing a different volume (0.2, 0.15, 0.1 and 0.05 ml) of *N*-hexadecane, were kept at room temperature for 10 min, and vortex-mixed for 2 min followed by incubation at room temperature for 15 min to allow hydrocarbon separation. The absorbance at 400 nm of the aqueous phase was measured before and after treatment (Spectronic Genesys 5 UV-Visible Spectrophotometers) and results were recorded as the percentage absorbance of the aqueous phase after treatment relative to the initial absorbance of the bacterial suspension.

Atomic force microscopy force spectroscopy

Atomic force microscopy (AFM) force spectroscopy was performed to measure cell hydrophobicity. The AFM force-distance curves were obtained in deionized water using a combined inverted optical system (Bruker, Santa Barbara, CA). Oxide-sharpened microfabricated Si3N4 cantilevers with a spring constant of 0.01 N m−1 (MLCT; Bruker) were coated by electron beam thermal evaporation with a 5-nm-thick Cr layer followed by a 30-nm-thick Au layer (Sharma *et al*., 2009). Gold-coated cantilevers were immersed for 14 h in 1 mm solutions of HS (CH2)11CH3 in ethanol and then rinsed with ethanol. To probe *S. mutans* cell surface hydrophobicity, cells were grown on glass cover-slips for 3 h in TH medium supplemented with 0.5% sucrose. Surface immobilization of the bacterial cells was tested by gently imaging them at low forces (~200 pN) before the force curve measurements. Force–distance curves over a 400 × 400-nm area were obtained using hydrophobic tips with z-ramp size of 10 μm, 1024 × 1024 samples/line

and 0.5 Hz. The adhesion strength was calculated for each force curve using spip™ software (Image Metrology, Horsholm, Denmark).

Sonication-resistance analysis

The resistance of *S. mutans* UA140 and its *prsA*-deficient derivative to sonication was assayed by monitoring the bacterial viability after sonication treatment. Bacterial suspensions with a density of 109 colony-forming units (CFU)/ml were prepared from overnight cultures and subjected to sonication at a constant frequency of 22 kHz and output power of 10 watts for different periods of time ranging from 1 to 9 min. Cell viability after sonication treatment was determined by CFU counting on TH agar plates.

Early biofilm formation assay

To compare the sucrose-independent and sucrose-dependent adhesion abilities between *S. mutans* UA140 wild-type and the *prsA*-deficient mutant, overnight cultures of both strains were dispersed using needle and syringe, and resuspended in glucose or sucrose-supplemented (20 mm) minimal medium (Loo *et al*., 2000) to a final OD600 of 0.1. Four hundred microliters of bacterial suspension was added to the well of a 24-well flat-bottomed polystyrene microtiter plate (Corning, New York, NY). After 3 h of incubation in the presence of 5% CO2, the plates were rinsed with phosphate-buffered saline (PBS) three times to remove planktonic and loosely bound cells. The biofilms were detached using cell scrapers (Thermo, Rochester, NY) and clumps were broken up and dispersed

by needle and syringe. The biomass of early biofilm was then calculated as CFU ml−1 by viability counting on agar plates.

Scanning electron microscopy

Overnight cultures of *S. mutans* UA140 and its *prsA*-deficient derivative were harvested and resuspended in fresh TH medium to an OD600 of 0.6. A 100-fold dilution of the bacterial suspension into defined minimal medium supplemented with 0.5% (weight/volume) sucrose was then added to each well of six-well polystyrene microtiter plates in which sterile coverglasses had been placed. After 16 h of incubation, the medium containing the remaining planktonic cells was aspirated and the cover glasses were carefully rinsed twice with 1 ml PBS without disturbing the attached biofilms. The biofilms were fixed with 1% glutaraldehyde. After another wash with phosphate buffer, the samples were mounted on a stub with silver adhesive (Electron Microscopy Sciences, Hatfield, PA), sputter-coated with a 40-nm layer of platinum and examined with a scanning electron microscope (SEM) operating at 5 kV in the secondary electron mode (XL 30 S, FEG; FEI Company, Hillsboro, OR).

Sodium dodecyl sulfate—polyacrylamide gel electrophoresis

Cell wall/membrane was prepared from *S. mutans* as described by Yamashita *et al.,* (1998) with some modifications. Briefly, bacterial cells from the overnight cultures were collected by centrifugation. The cell pellets were resuspended in 50 mm Tris–HCl (pH 8.0) containing 1 mm phenylmethylsulfonyl fluoride (PMSF), transferred to a chilled 2-

ml microcentrifuge tube containing 425–600 μm diameter glass beads (Sigma, St Louis, MO) and disrupted with a Mini-Bead Beater homogenizer (Biospec Products, Bartlesville, OK) for 10 min. The glass beads were removed and undisrupted cells were separated by centrifugation at 2000 *g* for 10 min. The supernatants were further centrifuged at 150,000 *g* for 2 h to collect the crude cell wall fraction. The pellets were washed twice with warm distilled water and resuspended in 50 mm Tris–HCl (pH 8.0) containing 1 mm ethylenediaminetetraacetic acid, 1 mm PMSF, 10 mg l−1 RNase and 10 mg l−1 DNase and incubated at 37°C for 1 h. The samples were boiled in sodium dodecyl sulfate—polyacrylamide gel electrophoresis (SDS–PAGE) loading buffer for 10 min before being loaded on to 10% SDS–PAGE gels. The protein concentration was determined by the Bradford method with the Pierce BCA protein assay kit (Thermo, Rockford, IL).

Glucan analysis

Overnight cultures of *S. mutans* strains were collected by centrifugation at 6000 *g* for 5 min. The pellets were washed twice with PBS and resuspended in minimal defined medium to a cell density of 108cells ml−1. Sucrose was added to the cell suspension to a final concentration of 100 mm. The cells were incubated at 37°C for 16 h in the presence of 5% CO2, and collected by centrifugation at 10,000 g for 10 min. Pellet and supernatant were used to assess the production of insoluble and soluble glucan, respectively. For soluble glucan analysis, the supernatant was precipitated by chilled ethanol. As for insoluble glucan analysis, the pellet was washed with distilled water three times to

remove the remaining soluble glucan; NaOH (1.0 m) was added and the alkali-soluble polysaccharides were precipitated with chilled ethanol. The amounts of glucan were measured by the phenol–sulfuric acid method (Dubois *et al*., 1956). A derivative of *S. mutans* UA140 lacking *gtfBC* (kindly provided by H. Kuramitsu, University of Buffalo, NY) was used as a negative control.

Mutacin IV production assay

Mutacin IV production was measured by deferred antagonism according to the protocol by Tsang *et al*. (2006). Five microlitres of *S. mutans* overnight culture was spotted onto TH agar plates and the plates were incubated at 37°C in the presence of 5% CO2. After 16 h incubation, 5 ml of a soft agar overlay containing *Streptococcus sanguinis* ATCC 10556 or *Streptococcus gordonii* DL1 at an OD600 of 0.1 as indicator strain was poured on top of the plates spotted with *S. mutans*. The growth inhibition zone of *S. sanguinis* or *S. gordonii* in the overlay agar was inspected after overnight incubation. The distance from the colony edge to the edge of the clearance zone was measured to calculate the inhibition area. Results are expressed as percentage of inhibition area induced by the *prsA* mutant relative to its parent strain.

Confocal laser scanning microscopy

The 3-h biofilms of *S. mutans* GFP-SpaP fusion derivatives of wild-type and the *prsA* mutant were formed according to the procedure described in the Early biofilm formation assay section, except a final OD600 of 0.3 was used for the mutant to normalize the

number of bacteria attached to the well. Biofilms were grown in medium supplemented with sucrose in each well of a sterile eight-well Lab-Tek™ Chambered Coverglass (Nalge Nunc International, Naperville, IL). The biomass of wild-type and *prsA* mutant strains was normalized according to their respective CFU counts. All biofilm images were collected with a Zeiss LSM 5 PASCAL confocal laser scanning microscope (CLSM) using LSM 5 PASCAL software (Zeiss, Jena, Germany). Excitation at 488 nm with an argon laser in combination with a 505–530-nm bandpass emission filter was used for GFP fluorescence imaging. The scanning module of the system was mounted on an inverted microscope (Axiovert 200M). A 40× oil-immersion objective (numerical aperture 1.3) was used for imaging. Image stacks (1024 × 1024-pixel tagged image file format) of eight randomly chosen spots were collected for each biofilm and quantified using the image analysis software COMSTAT. The fluorescence intensities in the biofilms of *S. mutans* GFP-SpaP strain and its *prsA*-deficient derivative were normalized to the number (CFU counts) of bacteria present in the well.

Results

Bioinformatic analysis revealed the presence of a *prsA* gene in the *S. mutans* genome. Sequence homology analysis showed that the *prsA* gene of *S. mutans* exhibited 69% homology to the one found in *Streptococcus agalactiae*, 67% homology to *Streptococcus pyogenes*, and 66% homology to *Streptococcus dysgalactiae* at the nucleotide level. The deduced PrsA amino acid sequences exhibited a homology of ~ 57–62% to other PrsA proteins among these streptococci, suggesting that PrsA from these streptococci are not well conserved. In addition, the PrsA from *S. mutans* displayed only 32% identity to the PrsA of *B. subtilis*. As there are two types of PrsA proteins differing in the presence or absence of peptidyl-prolyl *cis/trans*-isomerases (PPIase) signature motif, the PrsA protein sequence of *S. mutans* was further aligned with both the *B. subtilis* and *Listeria monocytogenes* PrsA sequence using the ClustalW tool (www. ebi.ac.uk/clustalw). However, the signature motif for PPIase is absent in *S. mutans* PrsA.

prsA deletion mutant auto-aggregates when grown as planktonic culture

The expression of the downstream gene of *prsA* is not affected by the *prsA* deletion mutant construction method (data not shown). The disruption of *prsA* did not affect cell viability either, and no significant difference in cell autolysis was observed between the *prsA* mutant and its parent strain (data not shown). However, the *prsA*-deficient strain displayed a striking auto-aggregation phenotype and the cells tended to clump and precipitate at the bottom of the glass tubes, whereas the wild-type strain showed a

uniformly turbid appearance in TH medium after overnight growth (Fig. 1A). Light microscopy observations showed that the mutant strain formed about 12 clumps per field of view, in contrast to the parent strain for which no clumps were observed (Fig. 1B,C).

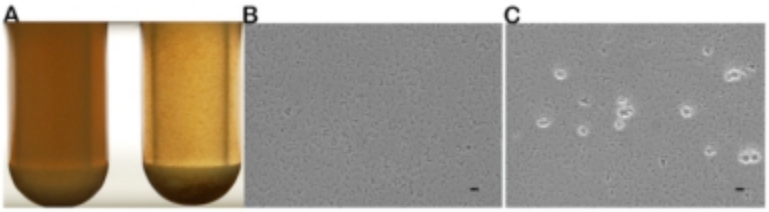

Figure 1. Cell morphological characteristics in Todd–Hewitt medium. Twenty-four-hour cultures of Streptococcus mutans UA140 (left) and the prsA-deficient strain (right; A). Light microscopic observation of a 24-h culture of S. mutans UA140 (magnification, ×400; B) and the prsA-deficient strain (C). The scale bar equals 10 μm.

prsA-deficient strain has altered cell surface characteristics

Enhanced auto-aggregation could result from altered cell surface properties, so we examined the cell surface hydrophobicity of the *prsA* mutant. Using the BATH assay, we demonstrated that the percentage absorbance of the aqueous phase in the *prsA* mutant after treatment with hydrocarbon relative to the initial absorbance was significantly less than that of the parent strain (37% vs 61%; Fig. 2A), suggesting an increased bacterial surface hydrophobicity in the *prsA*-deficient strain. Cell surface hydrophobicity was also quantitatively measured using chemically modified AFM. Fig. 2B shows the adhesion histograms and representative force curves obtained for the *prsA*-deficient and wild-type

strains. ‘Saw tooth-like’ force rupture events were observed in the retract regions of the force curves with hydrophobic tips (shown in inset images in Fig. 2B). Adhesion forces measurements showed higher adhesion forces over *prsA*-deficient cell surfaces compared with wild-type cell surfaces with mean values of 370 ± 17pN and 120 ± 12pN, respectively. These results confirm the qualitative findings obtained from the BATH assay at the single bacterial level and indicate that the *prsA*-deficient strain is about three-fold more hydrophobic compared with the wild-type ($P < 0.05$).

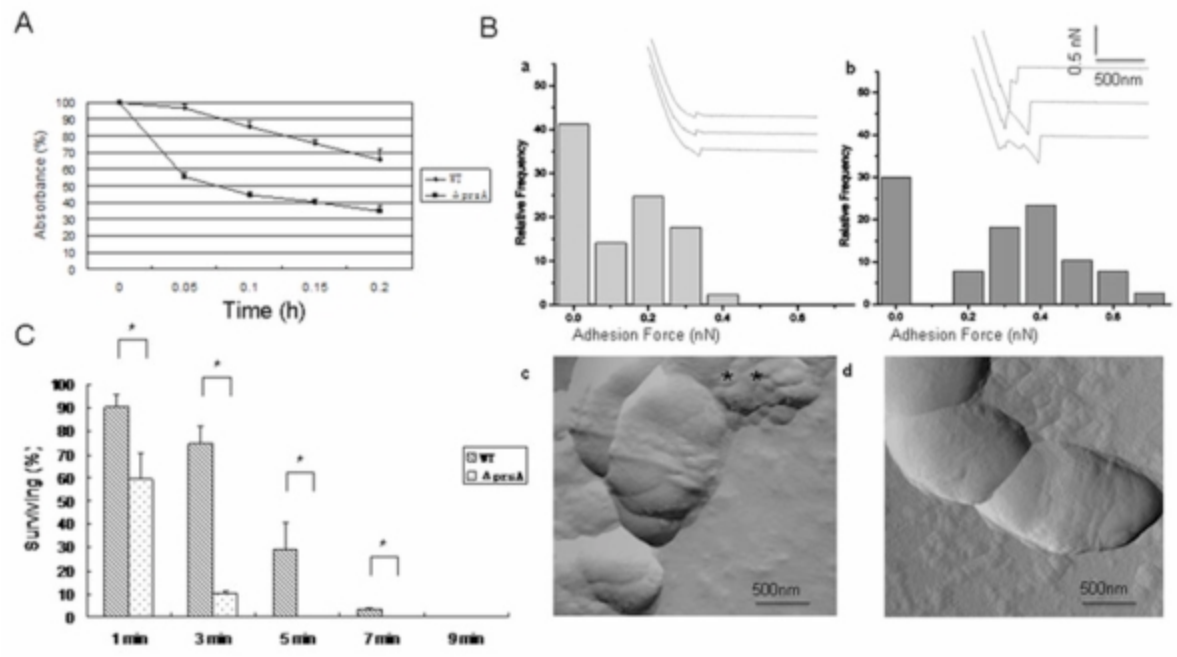

Figure 2. Cell surface characteristics. (A) Hydrophobicity: adherence of Streptococcus mutans UA140 wild-type and its prsA-deficient derivative to hexadecane in the bacterial adhesion to hydrocarbons assay. Results are expressed as percentage absorbance of the

aqueous phase after hexadecane treatment relative to the initial absorbance. Each point represents the mean of three independent experiments. (B) Atomic force microscopy (AFM) analysis: adhesion histograms and representative force curves (inset) recorded with hydrophobically modified tips on S. mutans UA140 (a) and the prsA-deficient strain (b) using a maximum applied force of 1 nN. The surface topographies of wild-type (c) and mutant strain (d) were also observed (amplitude images) using high-resolution AFM imaging. Two biological replicates were performed and representative images are shown. (C) Resistance of S. mutans UA140 and the prsA-deficient strain to sonication at a constant frequency of 22 kHz. Results are expressed as percentage of viable cells after sonication relative to untreated cells. Each point represents the mean ± SD of two independent measurements. The asterisk indicates that prsA-deficient strains were significantly less resistant to sonication than wild-type at the same treatment time-point (Student's t-test $P < 0.05$).

Sonication was used as a measure of the degree of physical cell membrane integrity. Results showed that, after 5 min of sonication treatment, the *prsA* mutant suffered a drastic reduction (about 300-fold) in viability compared with the wild type (about three-fold; Fig. 2C), indicating that deletion of the *prsA* gene renders cells more sensitive to sonication-induced lysis.

The prsA-deficient strain displays reduced early biofilm formation and forms overnight biofilms with aberrant architecture

Our data revealed that lack of *prsA* resulted in altered cell surface characteristics. As surface properties are important for cell adherence and biofilm formation, we further investigated the effect of PrsA on these phenotypes. Bacterial counts showed an almost 100-fold reduction in the number of attached *prsA*-deficient cells compared with the wild-type (Fig. 3A). Interestingly, when sucrose was replaced by glucose, both strains formed similar thin biofilms and the bottom of the well was not evenly covered with cells.

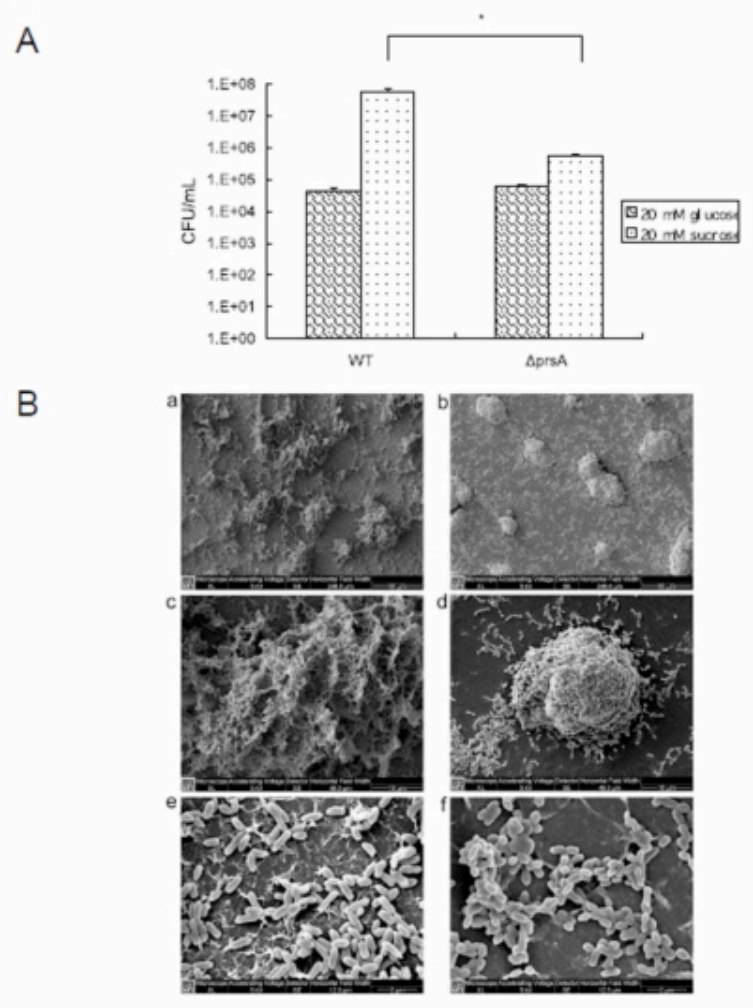

Figure 3. Biofilm formation characteristics. (A) Early biofilm formation of Streptococcus mutans UA140 wild-type and the prsA-deficient strains in minimal defined medium supplemented with glucose or sucrose. Each data point is the average of triplicate samples, and the error bars correspond to the standard deviations. The asterisk indicates that there were significantly fewer prsA mutant cells attached to the well of a 24-well flat-bottomed polystyrene microtiter plate than wild-type in the presence of sucrose (Student's t-test $P < 0.05$). (B) Scanning electron micrographs of S. mutans 16-h biofilms formed on glass surfaces. Streptococcus mutans UA140 wild-type biofilms (a,c,e); prsA-deficient strain biofilms (b,d,f). Magnifications, ×1000 (a,b), ×5000 (c,d) and ×20,000 (e,f).

The SEM analysis of biofilms grown on the surfaces of glass in defined medium with sucrose revealed that wild-type biofilms presented a uniform, sieve-like appearance with thick and compact layers of cells. In contrast, the mutant strain formed more compact microcolonies compared with its parent strain.

The prsA-deficient strain shows reduced insoluble glucan and mutacin IV production as well as the heterologous protein GFP-SpaP

PrsA has been shown to be involved in the post-export of a variety of exoproteins in *Bacillus* (Jacobs *et al*., 1993; Vitikainen *et al*., 2001), so we suspected that the deletion of *prsA* might affect the profile of cell wall/membrane proteins in *S. mutans* as well. As shown in Fig. 4A, the *prsA*-deficient strain displayed an altered cell wall/membrane

protein profile compared with the wild-type, with some protein bands showing increased intensity, whereas others exhibited an obvious reduction. As the effect of PrsA on cell wall/membrane proteins is relatively general, we chose several known exoproteins for further characterization.

The SEM imaging analysis revealed that the *prsA* mutant biofilm had less extracellular matrix than the wild-type (Fig. 3B). In *S. mutans*, cell-wall-associated glucosyltransferases (GTF) are responsible for synthesizing glucan, one of the main components of the extracellular matrix. As deletion of *prsA* could potentially affect translocation of GTF proteins to the cell surface and so alter glucan production, we determined the glucan production ability in both strains. Our results showed that the *prsA*-deficient strain produced less insoluble glucan compared with the wild-type (Fig. 4B), whereas no difference in soluble glucan production was observed.

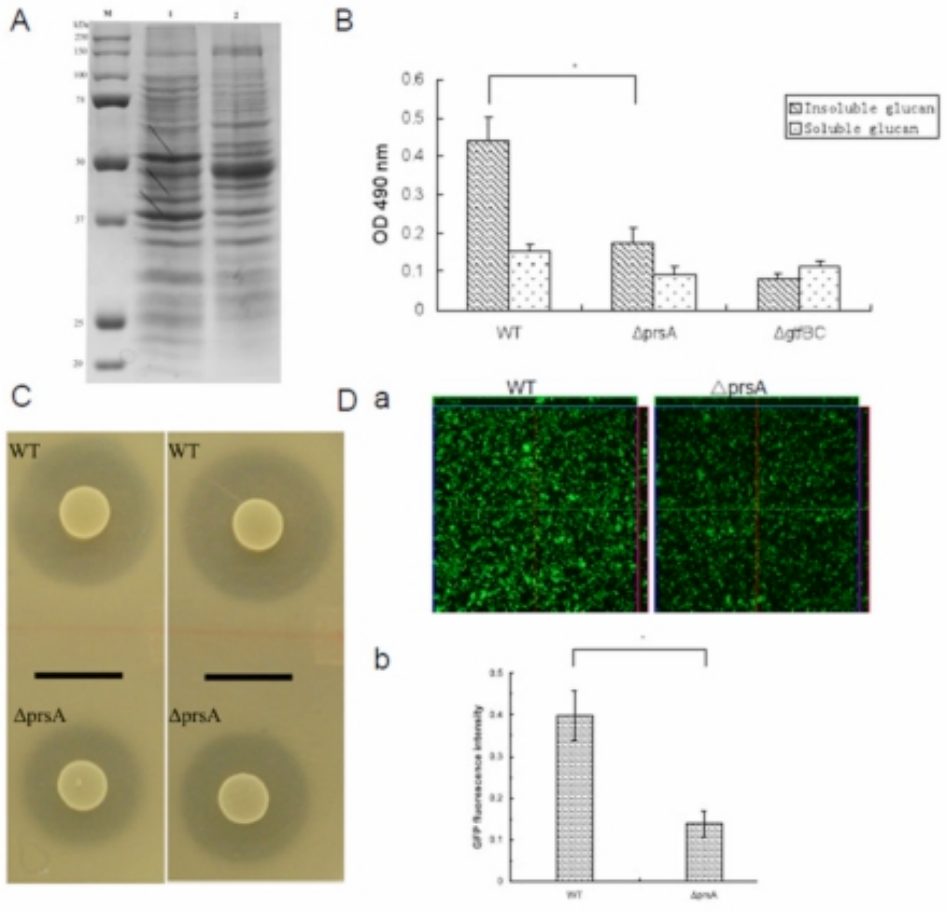

Figure 4. Analysis of PrsA-dependent phenotypes. (A) SDS–PAGE (10%) analysis of cell wall/membrane proteins from Streptococcus mutans UA140 and the prsA-deficient strain. Lane M, prestained protein markers (Bio-Rad, Hercules, CA); lane 1, S. mutans UA140; lane 2, UA140 prsA-deficient strain. Arrows indicate differentially expressed cell wall/membrane proteins. (B) Glucan production of S. mutans UA140 wild-type and the prsA-deficient strains in Todd–Hewitt medium supplemented with 100 mm sucrose. The S. mutans UA140 gtfBC-deficient strain was used as a negative control. Each data point

is the average of triplicate samples, and the error bars indicate standard deviations. The asterisk indicates that prsA-deficient strain produced significantly less insoluble glucan than wild-type (Student's t-test $P < 0.05$). (C) Drop inoculum deferred antagonism assay for S. mutans UA140 wild-type and the prsA-deficient strains. The clear zone indicates mutacin IV production. The indicator strains are Streptococcus sanguinis (left panel) and Streptococcus gordonii (right panel) respectively. (D) GFP fluorescent signals of 3-h biofilms of S. mutans wild-type and the prsA-deficient strain, both carrying surface-displayed GFP-SpaP fusion protein. (a) Confocal laser scanning microscopy analysis of surface-expressed GFP fluorescent signals within biofilms. (b) Quantification of GFP fluorescent signals within biofilms. The fluorescence intensities of S. mutans wild-type and the prsA-deficient biofilms were normalized to the number (colony-forming unit counts) of bacteria present in the well. The GFP expression percentage was calculated as the amount of fluorescence signal of prsA-deficient strain of compared with its parent strain.

Furthermore, the effect of the *prsA* deletion on mutacin IV, a well-known secreted peptide bacteriocin, was investigated. The deferred antagonism assay showed that the inhibition zone produced by the *prsA*-deficient strain was $49.67 \pm 4.20\%$ and $50.39 \pm 4.52\%$ of the one produced by the wild-type using *S. gordonii* DL1 and *S. sanguinis* ATCC 10556 as indicator strains (Fig. 4C).

To investigate whether PrsA facilitates the folding of exported heterologous proteins, we constructed derivatives of *S. mutans* wild-type and *prsA* mutant, both displaying a GFP protein on the cell surface via a fusion to SpaP. Our previous study showed that fusion of GFP to SpaP resulted in surface localization and efficient folding of GFP with proper function (unpublished). Therefore, the post-export folding of heterologous protein GFP can be analysed based on the fluorescence intensity of fused GFP. By quantifying image stacks of three randomly chosen biofilm spots, we found that the fluorescence intensity, and so the GFP surface display, was reduced in the *prsA* mutant biofilm (more than three-fold) compared with the wild-type (Fig. 4D).

Discussion

Streptococcus mutans secretes numerous proteins (enzymes)/peptides that play crucial roles in competing with other bacteria and establishing itself within the oral cavity via biofilm formation. The expression and activity of these exoproteins have been shown to be regulated at multiple levels, including post-translational and translocation regulation. In this study, we report the identification of PrsA, a predicted foldase that is involved in the post-export of a variety of proteins including membrane-associated proteins in *S. mutans*.

The auto-aggregation phenotype, as well as increased cell surface hydrophobicity and reduced resistance to mechanical breakage, indicated a substantial change in the cell surface structure and properties upon deletion of the *prsA* gene. This was further corroborated by our AFM data showing the 'saw tooth-like' force rupture patterns between hydrophobic AFM probes and *prsA*-deficient strains (Fig. 2B), which often reflects the unfolding of cell surface-bound proteins (Rief *et al*., 1998; Hu *et al*., 2011). As demonstrated in other gram-positive bacteria such as *Bacillus* and *Lactococcus*, PrsA acts as a chaperone to assist the folding and stability of exported proteins (Wahlström *et al*., 2003; Lindholm *et al*., 2006). Hence, the presence of unfolded proteins on the cell surface of the PrsA-deficient *S. mutans* derivative would be consistent with a role for PrsA as a cell surface chaperone in *S. mutans*, similar to findings in other species. The notion that the PrsA of *S. mutans* has roles similar to those extensively researched

especially in *B. subtilis* is further sustained by the observed differences in the cell wall/membrane protein profile of the *prsA* mutant compared with its parent strain (Fig. 4A). In a recent proteome analysis, PrsA-depleted *B. subtilis* cells were found to differentially secrete almost 200 proteins (Hyyryläinen *et al*., 2010).

Further phenotypic analysis of the PrsA-deficient *S. mutans* derivative indicated that one group of proteins affected by PrsA function was the glycosyl transferases of *S. mutans*. The *prsA*-deficient strain exhibited a significant decrease in insoluble glucan production, which resulted in reduced sucrose-dependent adhesion and biofilm formation (Fig. 3). It is well known that the majority of dental biofilm matrix is rich in polysaccharides (Paes Leme *et al*., 2006), of which glucan is one of the main components. This glucan-rich matrix could provide binding sites that promote accumulation of microorganisms on the tooth surface and further establishment of pathogenic biofilms (Koo *et al*., 2010). *Streptococcus mutans* encodes three GTFs. GtfB synthesizes mostly insoluble glucan, GtfC forms a mixture of insoluble and soluble glucan, while GtfD produces predominantly soluble glucan (Paes Leme *et al*., 2006). The insoluble glucan contributes to the glue-like characteristics of dental biofilms (Xiao *et al*., 2012). The *S. mutans* strains deficient in insoluble glucan production are essentially non-cariogenic in a rodent model (Yamashita *et al*., 1993). Although deletion of *prsA* resulted in a near 60% reduction in insoluble glucan production, it did not affect soluble glucan production, suggesting that PrsA might be involved in the post-export or function of GTF B and C, but not GTF D.

Mutacin is a known secreted bacteriocin and has been implicated as virulence factor of *S. mutans* (Kuramitsu, 1993). *Streptococcus mutans* strain UA140 could produce both mutacin I and IV (Qi *et al*., 2001). Our results indicated reduced mutacin IV in the *prsA* mutant. This correlates well with previous related studies in other gram-positive bacteria, where PrsA has been shown to be involved in the post-export of a variety of virulence factors. However, there was no difference in mutacin I between the wild-type and the *prsA* mutant (data not shown). In *Bacillus* species, α-amylase, β-glucannase and lipoprotein β-lactamase were exported in a PrsA-dependent manner (Jacobs *et al*., 1993; Vitikaninen *et al*., 2005), and overexpression of PrsA has been shown to increase the secretion of major bacillary exoenzymes (Kontinen & Sarvas, 1993). In Group A streptococcus, genomic disruption of *prsA* decreased the production of enzymatically active streptococcal pyrogenic exotoxin (SpeB) but not the level of the pro-SpeB zymogen (Ma *et al*., 2006).

The function of heterologous protein is mainly limited by the post-translational events including the inefficient translocation and folding of proteins and protease degradation (Tjalsma *et al*., 2004) and PrsA has been suggested to facilitate the folding of exported proteins into their final, mature conformation (Jacobs *et al*., 1993). In this study, we found that the fluorescence signal of a surface displayed GFP-SpaP fusion protein was reduced in the *S. mutans* strain lacking *prsA*. This result is consistent with observations that there was a positive correlation between the level of PrsA and the amount of a

surface-localized fusion protein consisting of *S. mutans* SpaP and the pertussis toxin S1 fragment following expression in the heterologous host *S. gordonii* (Davis *et al*., 2011). Furthermore, the level of PrsA was found to be linearly proportional to the protein secretion rate in *B. subtilis* (Vitikainen *et al*., 2001); whereas overproduction of PrsA could increase the stability of the amylase and the protective antigen from *B. subtilis* (Kontinen & Sarvas, 1993; Williams *et al*., 2003; Vitikaninen *et al*., 2005), and prevent degradation of proteins on the cell surface of *L. lactis* (Drouault *et al*., 2002).

GTFs contain N-terminal signal peptide which is thought to direct these proteins to the Sec secretory pathway (Navarre & Schneewind, 1999); while mutacin might be translocated by a specific ATP-binding transporter (van Belkum *et al*., 1997). The involvement of PrsA in GTF and mutacin IV production as demonstrated in this study suggested that, like foldase homologues identified in many other gram-positive bacteria, PrsA may work closely with cellular export machineries in assisting the folding of a variety of exoproteins in *S. mutans*.

Acknowledgements

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Part II

Chapter I

Managing denture biofilm related diseases

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Abstract

The oral cavity harbors more than 700 microbial species and is one of the most complex ecosystems ever described. While the majority of these microbes are considered commensal, some of them are responsible for oral infectious diseases such as dental caries, periodontitis, halitosis and stomatitis. The advancement of modern science has greatly furthered our understanding of oral microbes and their roles in host health and disease. It has also led to the development of new tools for early detection, effective treatment, and prevention of oral microbial infections. This perspective provides a general understanding of oral microbiology, and its clinical relationship to oral infectious diseases, with a specific focus on denture-related microbial infections. The perspective also discusses the potential for developing innovative interventions for managing denture-related disease based on recent advances in our understanding of oral microbiology and denture-associated biofilms.

Introduction

The association between microbes and oral diseases had long been suspected. Dr. W. D. Miller is generally recognized as the father of modern dental microbial pathogenesis. His 1890 seminal book titled *Microorganisms of the Human Mouth*[1] makes the first connection between bacteria in dental plaque and tooth decay, and remains a foundation of current under-standing of dental disease. For a long time, oral microbes had been indiscriminately regarded as pathogens. In fact, their removal from the oral cavity has become the main objective of dentists. Not until recently, did we realize that like microbes associated with other parts of the human body, most of the oral microorganisms are commensal and might have protective role in preventing the colonization of pathogens.[2,3] More importantly, increasing evidence suggests that oral infectious diseases such as dental caries and periodontitis are often the result of the disturbed host homeostasis, and an imbalanced oral microbial ecology often leads to overgrowth of otherwise low abundant opportunistic pathogens.[4,5]

Recent advances in molecular biological techniques are broadening our understanding of bacterial diversity and the societal community interactions which occur between species in the oral cavity.[6] This has led to tremendous advances in our understanding of oral microbiology and its involvement in health and disease, including tooth decay, gum diseases, as well as the diseases associated with artificial dental apparatus introduced through modern dentistry.[6,7]

Advancing understanding of oral microbiology through molecular biological approaches

Our bodies are home to a multitude of microbial organisms that form distinct microflora inhabiting the gut, skin, vagina and oral cavity. These microbial communities have been of great interest to scientists in recent years due to their impact on host health and disease. Increasing lines of evidence indicate that these commensal microbiotas have important metabolic, trophic, and protective functions and greatly affect the host's physiology and pathology.[8,9] For example, the importance of the gut flora in digesting unutilized substrates, training the immune system and protecting against epithelial cell injury is well appreciated,[10-12] and we are beginning to understand its potential role in systemic diseases, such as inflammatory bowel disease[13,14] and obesity.[15] Molecular biological tools have been critically important for identifying the diversity of these host-associated microbiotas, including the oral microbial community.[16] Prior to the availability of such tools, determining the diversity of complex microbiological communities, such as those of the oral microbiota had been essentially dependent on the ability to culture and identify individual organisms. However, we then realized that only a small fraction of the organisms comprising these microbial communities has been isolated.[6] In fact, accumulating lines of evidence suggested that there are extensive physical and metabolic interactions between different microbial species within the same community, which are essential for the growth and persistence of certain microbes[17] and make them recalcitrant to cultivation. The power of molecular biological approaches,

such as culture-independent 16S rRNA gene sequencing-based methods allows us to identify yet-uncultivable species and provides a more comprehensive and detailed inventory of human oral microbiota.[6]

The studies using culture-independent approaches have revealed the sheer magnitude of the diverse microbes, including yet-uncultivable species residing within the oral cavity.[18,19] The human mouth is estimated to harbor more than 700 different bacterial species, comprising one of the most complex microbial flora.[18] The diversity of microorganisms that inhabit the oral cavity includes bacteria, archaea, protozoa and fungi.[20,21] An interesting perspective regarding diversity of the oral flora is the presence of Archaea as a constituent.[22] Phylogenetically, Archaea is among the oldest known type of prokaryotes; it has previously been isolated from ocean bottom, and yet also appears to be a colonizer of the human oral cavity with yet-to-be-determined role in oral microbial ecology.[23] The diversity of the microbial flora reflects tremendous genetic information and immense bio-physiological potential that may have huge impact on host health and disease. If we consider that an average bacterial species has 2,000-6,000 genes, then an oral bacterial population of some 700 individual species represents a pool of over 1 million genes, 10 times more than human host genes. This provides the oral microbial environment with a huge quantity of information related to unique metabolic pathways, the generation and secretion of various factors that can control and modify their ecological niche, and factors that may impact function of the human host.

The structure of dental and denture plaque

Bacteria in the oral cavity often reside within biofilms, such as those that form dental and gingival plaque.[24] For edentulous and partially edentulous individuals who wear dentures, a denture-associated biofilm, or denture plaque, forms on the denture surface and could potentially serve as a reservoir of pathogenic microbes for infections.[7]

Dental and denture plaque are not simple matrices. They consist of a diverse collection of microbial species, and furthermore have a highly organized structure in which different species can occupy specific sites or niches within the biofilms.[25] During dental biofilm formation, bacteria that are early colonizers, such as Streptococci (i.e., *S. gordonii*, *S. oralis*, etc.) with specific adhesins can effectively bind to proteins deposited as a pellicle coat on the tooth surface. This is followed by the subsequent recruitment of intermediate and late colonizing species through cell-cell coadhesion *via* specific adhesin-receptor interactions.[25] These specific bacterial physical associations eventually generate a highly structured microbial community, which we recognize as dental plaque or biofilms.[5,6,26] Furthermore, bacterial species within dental biofilm are often engaged in extensive signaling and metabolic interactions to ensure their survival within the microbial community.[6]

Dental biofilms of healthy subjects harbor a commensal oral microbial community with properties that limit the invasive potential of opportunistic pathogens.[3,27] And like most ecological communities, once established, dental biofilm generally has a stable and

controlled population of different organisms and displays resilience to environmental disturbance.[28,29] However, as will be discussed in the following section, the microbial composition of denture biofilm flora and their pathogenic potentials could differ significantly from that of healthy dental biofilm, thus contributing to the pathogenesis of denture-biofilm related diseases, such as stomatitis.

Connections between microbes and oral diseases associated with denture wearing

Denture stomatitis is a common disorder in subjects wearing dentures, which are prostheses that provide important functional and esthetic improvements for edentulous and partially edentulous patients.[30,31] The disorder is characterized as inflammation and erythema of the oral mucosal areas covered by the denture. The current view regarding the etiology of denture stomatitis is that it is a multifactorial infectious disease. It involves a number of associative factors, including denture-induced trauma, continual denture wearing and denture plaque harboring pathogenic microbes, such as Candida.[30] Among those factors, the microbial biofilm formed on the denture surface plays a significant role in contributing to the disease pathogenic process. Whereas the normal commensal oral microbial community could prevent infection by interfering with the invasive potential of opportunistic pathogens, this is altered in the denture biofilm. Indeed, the microbial composition of the biofilm which forms on denture surfaces differrs significantly from that observed in the oral cavity of healthy individuals.[32,33] This could be due to the fact that denture wearing can alter normal oral physiology by affecting normal salivary flow that plays an important role in shaping the microbial

community.[34] Meanwhile, the older segment of the population often has a comparable higher proportion of denture wearer.[35] These individuals are more likely to have systemic health conditions and could have already had imbalanced oral microbial ecosystem due to disturbance in host homeostasis. Furthermore, a denture provides a unique abiotic surface for microbial colonization, which often leads to the development of a denture biofilm with microbial composition and structure different from normal oral biofilm.[7,32]

Compared to the normal oral and dental biofilms, denture biofilms are associated with a much higher occurrence of *Candida* yeasts, particularly *Candida albicans*.[36] *C. albicans* is a commensal fungal species commonly colonizing human mucosal surfaces. It co-exists with diverse oral microbial species and has long been adapted to the human host.[37] In healthy individuals, *C. albicans* is usually a minor component of their oral microfloras. However, under conditions of immune dysfunction or local predisposing factors such as poor oral hygiene or ill-fitted dentures, colonizing *C. albicans* can become an opportunistic pathogen. In these patients, *C. albicans* becomes more predominant and invasive; causing recurrent mucosal infections such as denture stomatitis.[38] The presence of *C. albicans* on denture and oral mucosal surfaces of denture wearers is positively associated with denture stomatitis.[39] The virulence factors of *C. albicans* have been well documented.[40] Among them, multiple host recognition biomolecules, such as Als1p and Hwp1p,[41,42] as well as the secreted enzymes, including Aspartyl proteinases (SAP)[43] and Phospholipase (PL)[44] have been shown to play important role in determining *C. albicans'* pathogenicity. Meanwhile, its polymorphic growth patterns[40] as well as

phenotypic switching[45] have also been implicated in contributing to its virulence. While *C.albicans* infection cannot be claimed as the single causal pathogen for inducing denture stomatitis, it has a strong associative presence when the disorder occurs, and its eradication from denture and mucosal surfaces is associated with reversal of the condition.[46,47] Hence, it is generally accepted that *C. albicans* is a main opportunistic pathogen which is involved in the development and pathogenesis of denture stomatitis. Meanwhile, certain bacterial species, such as *Prevotellasp*., *Veillonellasp*. and *Staphylococcus sp*. have been found to be enriched in denture biofilms,[48,49] although their potential role in denture stomatitis pathogenesis remains to be determined. More importantly, increasing lines of evidence indicate the extensive Candida-bacterial interactions, which could impact their pathogenicity.[37] For example, co-infection of *C. albicans* and *Staphylococcus aureus* has been shown to lead to increased mortality in animal model.[50] A better understanding of the physiology of Candida and bacteria co-existence and the inter-kingdom Candida-bacterial interactions would shed light on the impact of polymicrobial infection on the etiology of denture-related stomatitis.

Treatment of denture-related infectious disease

Plaque formed on the denture surface often serves as a reservoir of opportunistic pathogens, including *C. albicans* for infections. In addition to maintaining good general oral hygiene, the most recommended approach to managing and preventing microbial-related disease associated with denture use is for patients to maintain a high level of denture hygiene by appropriate cleaning.[51,52] Common approaches to denture cleaning

utilized by patients include brushing with abrasive cleansers, such as toothpastes, and washing or soaking dentures using commercial chemical cleansers with antimicrobial compounds designed for this purpose. The latter is preferred, as brushing with abrasive cleansers has been shown to be less effective for removal of the biofilm, and furthermore, can roughen the denture surfaces and result in more rapid bacterial adherence and biofilm growth.[7,53] However, as observed with other biofilms, a problematic issue associated with denture plaque is that it reduces the effectiveness of antimicrobial, including antifungal treatment.[54] The mechanisms by which biofilm environments enhance antimicrobial resistance are not fully understood. However, putative mechanisms likely include decreased ability of the antimicrobial agents to penetrate and diffuse within the biofilm matrix, protective functions conferred to the putatively susceptible bacteria due to slower growth rates and even changes in phenotype, and perhaps protective factors secreted by other microbes within the biofilm community which can degrade the applied antimicrobial agents.[55] In addition, the materials used in the manufacture of dentures can also affect adherence and colonization by microbes, including *C. albicans*, as well as impact the efficacy of antimicrobial treatment on the biofilm.[56]

Soaking dentures in an appropriate commercial cleanser has been shown to be effective in removing attached microbes without increasing surface roughness.[57] Overnight denture removal is also important for controlling denture plaque, as it isolates the denture from salivary secretion that provides nutrients for microbial growth of denture biofilm. In addition to maintaining denture hygiene, various antimicrobials, including imidazole

(clotrimazole, ketaconazole), triazole (fluconazole, itraconazole) and polyene (nystatin, amphotericin B) antifungals for treating Candida, and antibiotics for treating bacterial pathogens were also recommended for controlling denture-related mucosal infections.[58,59] More recently, a new antifungal therapeutic approach Photodynamic (PDT) therapy has been used to treat denture stomatitis.[60] PDT uses a photosensitizing agent and light of appropriate wavelength. The interaction between the photosensitizer and light in the presence of oxygen produces reactive species that induce cell damage and death.[61] In a recent clinical trial, PDT was shown to be as effective as topical nystatin in the treatment of denture stomatitis.[62] Since PDT can effectively kill Candida species, including strains resistant to conventional antifungal agents,[63] it has been regarded as a promising method for the treatment of dental stomatitis. Recurrence of stomatitis is frequently observed within short period of time after stop-ping antifungal treatment.[64] This is likely due to reinfection by residual pathogens that remain within plaque on dentures and are resistant to treatment. Meanwhile, many patients failed to respond to the usual treatment, largely due to the development of drug resistance of candida species. For patients with systemic diseases, such as type 2 diabetes mellitus or being immunocom-promised,[65] they often show less responsiveness to the treatment as well. When treating these patients, combined efforts including antifungal treatment and improving patients overall health status are critical in determining the outcome.[66]

The knowledge we are gaining from molecular biological studies of dental and denture biofilms is contributing to the development of novel therapeutic tools.[6,67] One approach

is to build on our ability to identify specific pathogenic organisms that inhabit the biofilm, and develop therapeutics that specifically target these organisms. An example of this approach undertaken by our research group is the development of STAMPs (Specifically Targeted Antimicrobial Peptides).[68,69] A typical STAMP consists of two functional moieties conjoined in a linear peptide sequence: a nonspecific antimicrobial peptide serves as the killing moiety, whereas a species-specific binding peptide provides specific binding to a selected pathogen and facilitates the targeted delivery of the attached antimicrobial peptide. The feasibility of this approach has been demonstrated by the development of C16G2, a STAMP specifically targeting *S.mutans*, the bacterium known to cause dental caries. C16G2 has been shown to remove *S. mutans* within *in vitro* multi-species biofilms with high efficacy and specificity,[68,70] and is under further animal and human evaluations.[71]The successful demonstration of this targeted approach could serve as proof-of-concept for applying this technology to the treatment of denture-related Candida infections.

Conclusions

The past decade has witnessed significant advances in our understanding of oral microbiota. We now better understand the structural and functional complexity of dental and denture plaque, and a strong connection between oral microbial ecology and host health and disease has been established. It is well known that the control of microbial pathogens, such *C. albicans* on dentures and in the oral cavity is critical for the oral health of denture wearers. Continued efforts using modern scientific methods will help us develop more diagnostic tools and therapeutic interventions for the identification, treatment and prevention of denture infections. New and improved approaches will be able to treat and control denture infections with less physical damage to denture surfaces by providing improved mechanisms for killing and removing microorganisms in the denture biofilm. We can envisage products that will have targeted killing of selective pathogens without affecting other commensal species within the same denture biofilm. Finally, we can also expect to see new products that will be able to enhance natural oral immunity, and provide cavity protection or control gingival disease in dentate individuals, and other inflammatory disorders in denture wearers.

Conflicts Of Interest

The authors declare that Wenyuan Shi is an employee of C3 Jian, Inc. which has licensed technologies from UC Regents that could be indirectly related to this research project.

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Part II

Chapter II

The denture-associated oral microbiome in health and stomatitis

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Abstract

While investigation of the microbiome on natural oral surfaces has generated a wealth of information, few studies have examined the microbial communities colonizing dentures and their relationship to oral health. To address this knowledge gap, we characterized the bacterial community associated with dentures and remaining teeth in healthy individuals and patients with denture stomatitis. The microbiome compositions of matched denture and tooth plaque samples of 10 healthy individuals and 9 stomatitis patients were determined by 16S rRNA gene pyrosequencing. The microbial communities colonizing dentures and remaining teeth in health and disease were very similar to each other. Matched denture and tooth samples from the same individuals shared a significantly higher percentage of identical phylotypes than random pairs of samples from different study participants. Despite these overall similarities, several bacterial phylotypes displayed discrete health- and stomatitis-associated denture colonization, while others were distinct in health and disease independently of the surface. Certain phylotypes exhibited differential colonization of dentures and teeth independently of denture health status. In conclusion, denture and natural tooth surfaces in health and stomatitis harbor similar bacterial communities. Individual-related rather than surface-specific factors play a significant role in the bacterial phylotype composition colonizing dentures and teeth. This individual-specific mutual influence on denture and tooth surface colonization could be an important factor in maintaining oral health in denture wearers. Discrete differences in colonization patterns for distinct genera and phylotypes warrant further studies

regarding their potential involvement or utility as specific indicators of health and disease development in denture-wearing individuals.

Introduction

Both oral health and the microbial inhabitants of the oral cavity, in particular, are increasingly being recognized for their role in overall human health and disease (1). Connections between oral microbial infections and numerous systemic disease conditions have been established (2), and oral biofilms have been suspected to serve as reservoirs for infectious disease agents (3). With its combination of soft tissue and hard surfaces, the oral cavity comprises a unique environment for microbial colonization. The combined efforts of a number of research groups have led to a comprehensive inventory of oral microorganisms (4,–6). In addition to the natural surfaces of the oral cavity, microorganisms can effectively form biofilms on the artificial hard surfaces that are introduced as part of dental restoration. Biofilm formation on restorative materials positively correlates with higher surface roughness and surface free energy (7). Among the biofilms colonizing artificial hard surfaces, those forming on dental implants have attracted a lot of attention in research. Healthy implants harbor very distinct microbiotas compared to teeth (8), and depending on the study, different microbial species have been suggested to be involved in peri-implant disease development (9). In contrast, the bacterial communities colonizing dentures, another important artificial surface present in the oral cavity of a significant part of the population, largely remain to be investigated. Even though some research groups have evaluated the denture-associated microbiota (10,–17), the majority of studies are focused on special aspects, including the specific groups of bacteria or the effect of cleaning agents.

Over 20% of the people over 65 years of age in the United States are missing all of their teeth. With the increasing proportion of elderly people in the population, this proportion is likely to rise (18, 19). The bacteria colonizing dentures comprise an important part of the human microbiome to be studied for their role in maintaining oral health in the elderly. Denture wearing has been associated with a number of microbial diseases, including denture-related mucosal tissue inflammation (denture stomatitis) (20) and malodor (21). Dentures are also suspected to serve as a reservoir for respiratory pathogens (3, 13) and thus lead to an increased risk of pulmonary infections (22). Despite the fact that microorganisms are the obvious suspects for most of the above denture-related diseases, little is known about their etiology. Most studies investigating denture-associated microorganisms focus on colonization with *Candida* sp., which is considered an important etiological agent for denture stomatitis (23), even though bacterial species have been implicated in this oral disease as well (11, 16, 24). Currently, only limited knowledge is available regarding the microbial composition of biofilms growing on dentures. Comparisons with the microbiota residing on natural oral surfaces to elucidate if and how the microorganisms colonizing the denture shape the microbiota of the oral cavity and vice versa remain to be performed. Very few studies have assessed bacterial denture colonization by using culture-independent clone library and checkerboard approaches (10, 14, 15, 17). To date, only one recent study has reported a next-generation sequencing analysis, which provided an initial predominantly class level analysis of the microbiota colonizing dentures, the respective mucosal surfaces, and selected remaining

teeth (12). While this is an important step toward understanding the bacterial component of health and disease in denture wearers, a recent comprehensive evaluation of different oral sites at the oligotype level has highlighted the importance of resolving communities at more detailed taxonomic levels to better understand the ecological and functional diversity of the microbiota relevant to health and disease (25). This level of analysis is still missing for the denture-associated microbiota and the corresponding microbial communities on the remaining teeth of denture wearers.

In this study, we performed a comprehensive genus and species level 16S rRNA gene sequencing-based analysis of the microbial biofilms colonizing dentures. Matched samples from dentures and remaining teeth from healthy individuals and those with denture stomatitis but no other oral diseases were used to allow comparison of patient- and surface-related factors. We investigated if the biofilms present on these different surfaces are distinct, if health- and disease-associated biofilm communities can be distinguished, and if microbial communities present on the different surfaces in the same patients influence each other. Identification of relevant disease-associated factors and microorganisms will enhance the ability of dentists to develop more targeted approaches for the treatment of denture-associated diseases.

Methods

Subject population and sample collection

Twenty adult denture-wearing volunteers with a minimum of four remaining teeth and one complete denture were recruited for this study under Institutional Review Board no. 2012-0004 to West China University (Chengdu, China). Ten individuals were healthy denture wearers, and 10 were patients with denture-associated stomatitis according to published guideline for diagnosis of denture stomatitis (55). The group of healthy denture wearers consisted of five women and five men with a mean age of 69.8 ± 4.7 years. Similarly, the group of stomatitis patients included five women and five men with a mean age of 61.1 ± 12.0 years. The study participants did not have any other active oral diseases such as caries or periodontitis. Eligible individuals were systemically healthy and not taking any prescription or nonprescription medication for at least 6 months. Additionally, the study participants had not used any biocide-containing toothpaste or denture cleanser for the past 6 months. Informed consent was obtained prior to sample collection and signed by study participants, as well as the clinicians and study personnel performing oral health evaluations, sample collection, and processing.

Study participants were asked to wear their dentures for at least 3 h and refrain from eating, drinking, and tooth or denture cleaning prior to plaque sampling. Plaque samples were collected by a trained dentist from the parts of the pink acrylic denture surface that was in contact with the oral mucosal surface by applying sterile toothpicks with a circular

motion. Using a similar circular motion, sterile toothpicks were also employed to obtain supragingival plaque from the remaining teeth. Care was taken to collect from the buccal surfaces of teeth that were not in direct contact with denture surfaces. Individual denture and tooth plaque samples, as well as control toothpicks, were placed into separate microcentrifuge tubes containing 0.5 ml of oxygen-reduced phosphate-buffered saline and immediately stored at −20°C until DNA extraction.

DNA extraction and sequencing

Genomic DNA was isolated from the collected plaque samples and the corresponding sterile toothpick controls with the DNeasy Blood and Tissue kit (Qiagen Inc., United States) as previously described (56) with the addition of bead beating for maximal cell lysis (29). DNA quality and quantity were determined with a NanoDrop 2000 spectrophotometer (Thermo, United States). After genomic DNA extraction and quantification, DNA concentrations of the samples were normalized to 2 to 6 ng/μl and PCR amplification was performed according to the protocol developed by the Human Microbiome Project for sequencing on the 454 FLX titanium platform (54). Briefly, hypervariable regions V1 to V3 of the 16S rRNA genes were amplified from purified genomic DNA with primers 27F (V1 primer, 5′AGAGTTTGATCCTGGCTCAG3′) and 534R (V3 primer, 5′ATTACCGCGGCTGCTGG3′) fitted with individual barcodes and the A adapter sequence (5′CCATCTCATCCCTGCGTGTCTCCGACTCAG3′) for the 534R primer and the B adapter (5′CCTATCCCCTGTGTGCCTTGGCAGTCTCAG3′) for the 27F primer and pooled for sequencing. The 16S rRNA gene amplicon libraries

were constructed and 454 pyrosequencing was performed at the J. Craig Venter Institute Joint Technology Center.

Microbial taxonomic composition analysis

The 454 pyrosequencing data were demultiplexed into the respective samples on the basis of the individual barcodes of each sample. After the bar codes were trimmed, a data cleaning process was applied to all of the samples with an in-house Perl program. Briefly, low-quality sequences containing bases with a Phred quality value of <20, were trimmed off the read ends. Sequences with a final read length of <300 bp or with ≥3% uncertain bases were removed. Suspected chimeras were identified and removed with ChimeraSlayer (57). The 16S rRNA sequences were clustered into operational taxonomic units (OTUs) at a 97% sequence similarity level with QIIME (58). OTUs were then annotated with taxonomic assignment by comparing the representative sequence of each OTU to the Human Oral Microbial Database references (16S rRNA gene RefSeq version 11.0) (59) with BLAST on the basis of the best match at >97% nucleotide sequence identity over at least 95% of the length of the query (56). The microbial community evenness and richness were measured by alpha diversity, and the similarity between individual microbial communities was measured by beta diversity. Alpha diversity (Shannon index), beta diversity (weighted and unweighted UniFrac), principal-coordinate analysis, and sequencing depth assessment by rarefaction analysis were calculated or performed with QIIME (58) at the OTU level. Genera present in at least two subjects at a relative abundance of >1% were included in further genus and species/phylotype level

analysis. The taxonomic composition of each sample was summarized at the genus and species levels.

Detection of Candida via PCR

The presence or absence of *Candida* in the samples collected from dentures was assessed with universal primers ITS1 (5′ CTTGTTATTTAGAGGAAGTAA 3′) and ITS2 (5′ GCTGCGTTCTTCATCATGC 3′) (60) under the following cycling conditions: 94°C for 11 min, followed by 35 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s with a final 30-min extension at 72°C.

Statistical analysis

Data are represented as mean ± the standard error of the mean (SEM) unless otherwise indicated. All data sets were examined for their distribution properties with the Shapiro-Wilk test prior to analysis. Statistical testing of differences in relative abundance of genus and species distribution between the different sample groups was tested with the Mann-Whitney U test for pairwise comparisons (dentures versus teeth; health versus stomatitis) and the Kruskal-Wallis test for comparison of multiple groups (dentures versus teeth in health and disease). Differences in the prevalence of genera and phylotypes between multiple groups were evaluated with Fisher's exact test. Bray-Curtis dissimilarity was employed to evaluate the copresence of bacterial phylotypes on dentures and teeth derived from the same individuals compared to dentures and teeth from different individuals and only dentures or only teeth from different individuals. The Bray-Curtis

dissimilarity results were further analyzed with a two-tailed unpaired *t* test and analysis of variance. All statistics were performed with the respective features in R Studio, Prism, and Excel, while the nonparametric multivariate analysis anosim in mothur (61) was used to test whether the microbiome similarities within groups are statistically significantly different from the similarities between groups. The *P* values were adjusted for multiple testing of microbial taxa with p.adjust in R by using the false-discovery rate (62).

Results

Patient characteristics, sample collection, and sequencing

Samples were collected individually from the dentures and remaining teeth of 10 healthy denture wearers and 10 patients with denture stomatitis who were otherwise free of oral microbial diseases (see Materials and Methods for further details). We identified the taxonomic composition of the oral microbiota by 454 pyrosequencing hypervariable regions V1 to V3 of the 16S rRNA genes for a total of forty samples (one denture- and one tooth-derived sample per patient collected as described in Materials and Methods). We obtained a total of 106,894 reads with a mean read length of 444 bp after removal of low-quality and short sequences, as well as chimeric sequences. One of the samples derived from the remaining teeth of a stomatitis patient that yielded <1,500 reads was excluded from further analysis together with the matching denture sample because of a lack of sufficient sequencing depth. An average of 2,739 reads (range, 1,692 to 4,975) was analyzed for each sample, which provides sufficient sequencing depth to capture the overall diversity of the samples (see Fig. S1 in the supplemental material).

The community structures of denture- and tooth-associated microbiomes in healthy and diseased denture wearers are very similar

The 16S rRNA gene sequences were processed as described in Materials and Methods. Twenty-six different genera representing a total of 136 different species/phylotypes were present in at least two subjects at a relative abundance of >1% (Fig. 1). Microbial

community evenness and richness (alpha diversity) at the genus level were very similar between dentures and remaining natural teeth in health, as well as disease (Fig. 2A). The microbial community structure (beta diversity) was not statistically significantly different among the four different groups (dentures-health; dentures-stomatitis; remaining teeth-health; remaining teeth-stomatitis), regardless of which aspect was examined (Fig. 2B; see Fig. S2 in the supplemental material). We then compared the individual taxa present in the samples derived from the dentures and natural teeth of the same individual with denture-tooth, denture-denture, and tooth-tooth combinations from different individuals by using Bray-Curtis dissimilarity, which, unlike UniFrac, does not include phylogenetic relatedness between community members into account. The average phylotype co-occurrence in the corresponding denture-tooth samples derived from the same study participants was significantly greater (lower Bray-Curtis index) than in the different sample combinations derived from different individuals (Fig. 2C). Thus, microbial colonization of different surfaces within an individual appears to be more similar than the same surface (dentures or remaining teeth) between individuals.

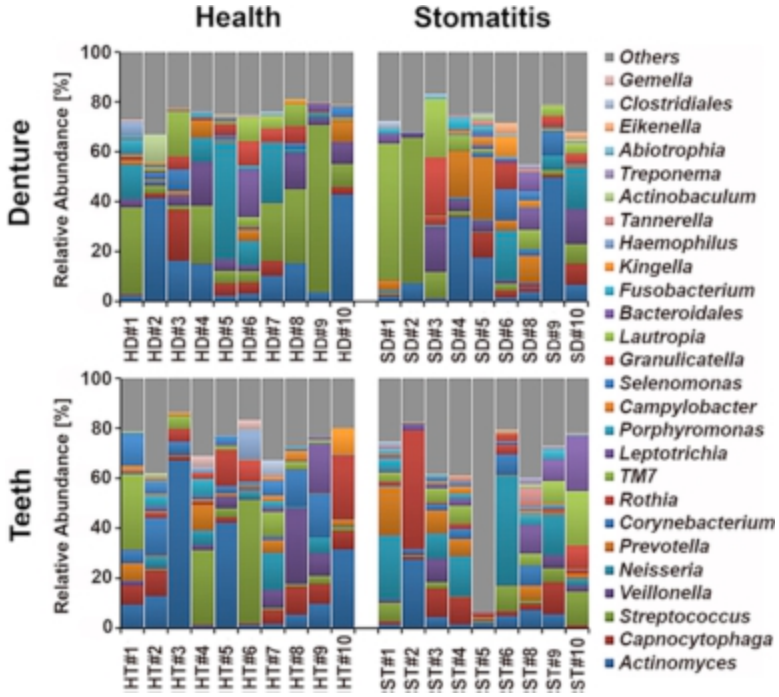

FIG 1 Genus level composition of denture- and tooth-associated microbiomes in health and stomatitis. The relative abundance at the genus level in denture (top) and tooth (bottom) samples collected from the same individuals are shown in the same order (H1 to H10, S1 to S6, and S8 to S10). The samples from healthy subjects are shown on the left, and those from stomatitis patients are shown on the right. HD, healthy denture; SD, stomatitis denture; HT, healthy remaining teeth; ST, stomatitis remaining teeth. Genera present with a >1% relative abundance in at least two patient samples were included in the analysis and are listed on the right with the respective color coding. Genera present at a <1% relative abundance or in only one patient sample were combined in the category "Others."

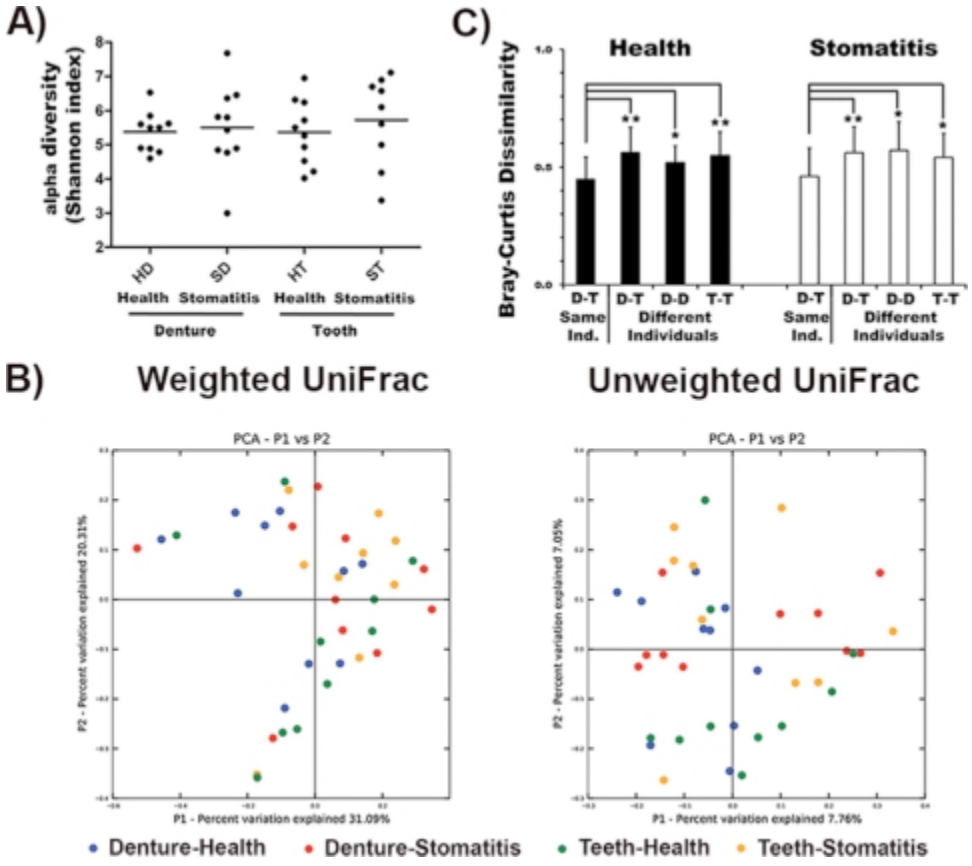

FIG 2 Microbial community structure analysis. (A) Alpha diversity (Shannon index) of the microbial community between dentures and teeth in health and disease, (B) Beta diversity (principal-coordinate analysis [PCoA]) of the microbial communities colonizing dentures (blue) or remaining teeth (green) of healthy individuals and stomatitis patients (red, dentures; yellow, remaining teeth), respectively. (C) Phylotype co-occurrence (calculated as Bray-Curtis dissimilarity) in denture and remaining-tooth samples from healthy individuals (black bars) and stomatitis patients (white bars). The

*phylotypes colonizing dentures (D) and teeth (T) were compared within the same individuals (D-T Same Ind.), between different individuals (D-T Different Individuals), and between denture (D-T Different Individuals) and tooth (T-T Different Individuals) samples from different individuals. *, $P < 0.05$; **, $P < 0.001$.*

Of the 26 genera identified (see Fig. S3A in the supplemental material), 25 were found in dentures, while 24 colonized teeth (see Fig. S3B) at the above cutoff criteria. *Eikenella* and *Abiotrophia* in significant numbers were absent from remaining teeth, while the genus *Gemella* was largely lacking on dentures. The genus *Actinomyces* was most pervasive on both surfaces, dentures and remaining teeth, followed by *Streptococcus*, *Veillonella*, *Capnocytophaga*, *Neisseria*, *Prevotella*, and *Corynebacterium*, all of which were identified in more than half of the samples tested, independently of the surface or health status. Other genera meeting the above cutoff criteria included *Rothia*, "*Candidatus* TM7" ("*Candidatus* G-1"), *Leptotrichia*, *Porphyromonas*, *Selenomonas*, *Campylobacter*, *Lautropia*, *Granulicatella*, *Haemophilus*, *Kingella*, *Fusobacterium*, *Bacteroidales* ("*Candidatus* G-5"), "*Candidatus* TM7" ("*Candidatus* G-5"), *Actinobaculum*, *Tannerella*, and *Clostridiales* ("*Candidatus* F-2," "*Candidatus* G-5"), as well as *Gemella*, *Eikenella*, and *Abiotrophia*. None of the apparent differences in prevalence or relative abundance were significant.

Next, we performed a similar analysis comparing samples derived from healthy subjects with those obtained from patients with stomatitis regardless of the surface they originated

from (see Fig. S3C). The microbiomes of healthy subjects contained only 22 of the 26 genera included in the analysis. The missing genera were *Fusobacterium*, *Tannerella*, "*Candidatus* TM7" ("*Candidatus* G-5"), and *Eikenella*. The microbial communities isolated from stomatitis patients contained all of the genera, with the exception of *Gemella*. The only genus that exhibited significant disparities ($P = 0.026$) in relative abundance between health and disease independently of the surface was *Fusobacterium* (see Fig. S3C and S4A).

In additional comparisons, we further divided the samples into those obtained from the dentures and teeth of healthy individuals and patients with denture stomatitis (Fig. 3). The microbiome of dentures from stomatitis patients was most diverse and contained 24 different prevalent genera, while only 18 genera were found on the dentures of healthy individual. In each case, health and disease, the remaining teeth contained 21, albeit not identical, genera. Several of the genera that were present at a lower prevalence were predominantly detected in particular conditions. *Kingella* and *Gemella* mainly colonized the teeth of healthy denture wearers, while *Bacteroidales* ("*Candidatus* G-5") was absent. *Eikenella* was limited to diseased dentures. The genus *Porphyromonas* met the 1% relative abundance cutoff for all conditions except diseased dentures. In *Fusobacterium*, *Tannerella*, and "*Candidatus* TM7" ("*Candidatus* G-5"), which were found predominantly in patients with disease, no surface-dependent differences were detected.

Specific species/phylotypes exhibit distinct health- and disease-associated surface colonization of teeth and dentures

Further detailed species/phylotype level analysis was performed for genera that occurred in at least 10% of the samples to rule out the influence of phylotypes that occur in only one or two samples with very high relative abundance and thus are not representative of the condition. This analysis revealed that *Fusobacterium nucleatum* subsp. *animalis* was found almost exclusively on the dentures of stomatitis patients ($P = 0.016$). *F. nucleatum* subsp. *vincentii*, in contrast, was found mostly on the remaining teeth of stomatitis patients ($P = 0.0097$) (Fig. 4A), while *F. nucleatum* subsp. *polymorphum* and *F. periodonticum* were not significantly different on either surface in health or disease (data not shown). Even though *Streptococcus* did not stand out as a genus, several species, including *Streptococcus gordonii* ($P = 0.012$), *S. sanguinis* ($P = 0.049$), and *S. australis* ($P = 0.0215$), colonized the dentures of healthy denture wearers at significantly higher levels (Fig. 4B). Interestingly, *Porphyromonas* sp. strain HOT-279, a phylotype of a genus often associated with oral diseases (26), was significantly more abundant in health ($P = 0.048$) (see Fig. S4A). Most of the other representatives of this genus were strongly associated with teeth in stomatitis patients, albeit at a relatively low abundance. Surface-dependent differences irrespective of health/disease status were observed for *Campylobacter showae* ($P = 0.0173$), *Capnocytophaga* sp. strain HOT-329 ($P = 0.045$), and *P. melaninogenica* ($P = 0.026$), with the first two being present predominantly on teeth and the latter one being significantly more abundant on denture surfaces (see Fig. S4B).

In addition, each denture sample was evaluated for the presence of *Candida* via PCR. In the stomatitis patient group, *Candida* was detected in 90% of the samples compared to 50% of those from the healthy group (Table 1). Several bacterial species, including *Atopobium parvulum*, *Lachnospiraceae* sp. strain HOT-097, and *Veillonella atypica*, were present mainly in denture samples containing *Candida*, while *Leptotrichia* sp. strain HOT-212, with the exception of one patient, was present only in samples without *Candida*.

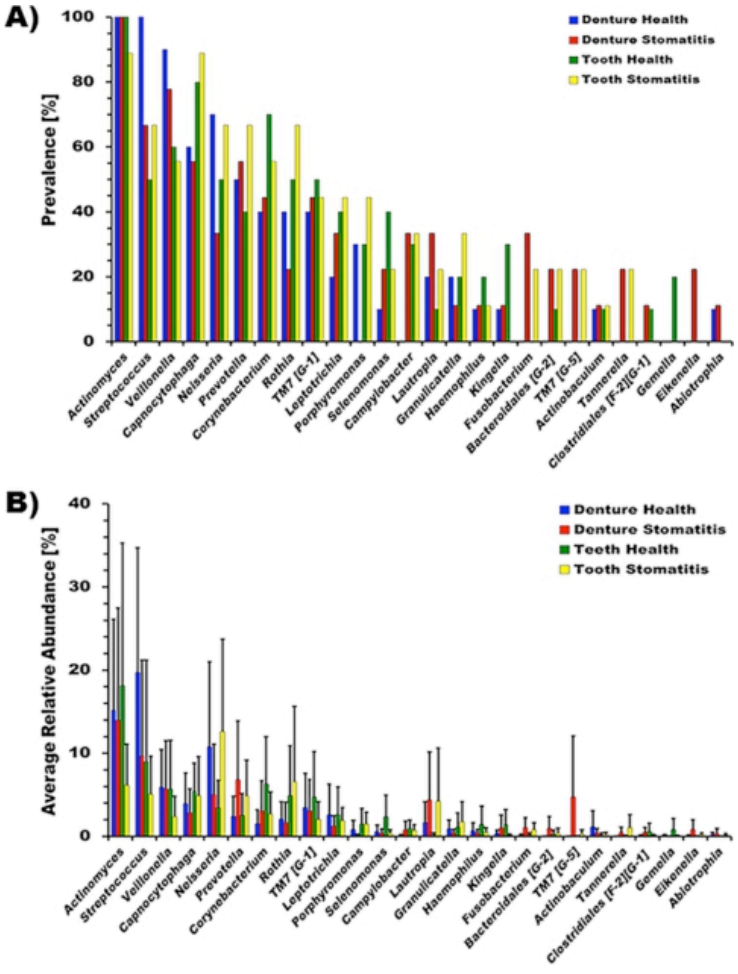

FIG 3 Genus prevalence and average relative abundance of denture- and tooth-associated microbiomes in health and stomatitis. Genera present at a >1% relative abundance in at least two patient samples were included in the analysis. Shown are the prevalence (A) and average relative abundance ± SEM (B) on dentures in health (blue bars), on dentures of stomatitis patients (red bars), on teeth in health (green bars), and on teeth in stomatitis patients (yellow bars). Numbers preceded by the letters G and F refer to as-yet-unnamed genera and families, respectively, within the phyla and orders indicated.

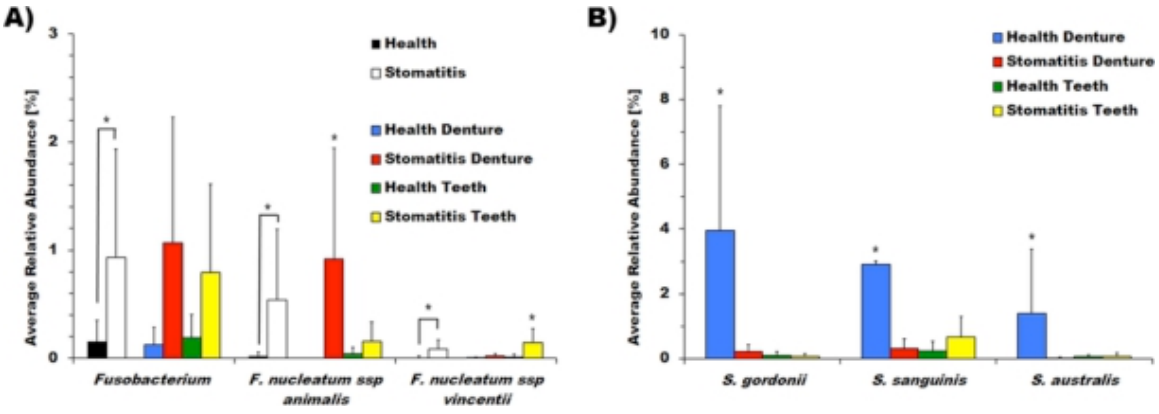

*FIG 4 Differential colonization of dentures or teeth in health and disease at the genus and phylotype levels. Shown is the mean relative abundance of the genus Fusobacterium, as well as F. nucleatum subsp. animalis and vincentii (A), S. gordonii, S. sanguinis, and S. australis (B). Samples are differentiated into health (black bars), disease (white bars), dentures in health (blue bars), dentures from stomatitis patients (red bars), teeth in health (green bars), and teeth from stomatitis patients (yellow bars). *, $P < 0.05$.*

TABLE 1

Evaluation of denture samples for the presence of *Candida* via PCR and co-occurrence with bacterial species/phylotypes

Microorganism	Healthy patients										Stomatitis patients								
	HD1	HD2	HD3	HD4	HD5	HD6	HD7	HD8	HD9	HD10	SD1	SD2	SD3	SD4	SD5	SD6	SD8	SD9	SD10
Candida	+[a]	−	−	+	−	−	+	+	−	+	+	+	+	+	+	+	+	−	+
Atopobium parvulum	+	−	−	+	−	−	−	−	−	+	+	+	−	+	+	+	+	−	−
Lachnospiraceae sp. strain HOT-097	+	−	−	−	−	−	−	−	−	+	+	+	−	+	−	−	+	−	+
Leptotrichia sp. strain HOT-212	+	+	−	−	+	+	−	−	+	−	−	−	−	−	−	−	−	+	−
Veillonella atypica	+	−	−	+	−	+	−	−	−	+	−	+	−	−	+	−	−	−	+

[a]Symbols: +, presence of *Candida* in sample; −, absence of *Candida* in sample (as determined by PCR). The presence or absence of bacterial species was derived from the 16S rRNA gene sequencing data.

Discussions

Denture stomatitis has largely been studied in the context of fungal infections, and even though there was a suggestion decades ago by Koopmans and coworkers "to pay more attention to the bacterial population in denture-induced stomatitis instead of focusing only on *Candida albicans*" (11), few studies have done so since. A detailed clone library analysis (10) provided a first glimpse into the diversity of bacterial phylotypes associated with dentures in health and disease, while a recent predominantly class and phylum level study (12) introduced modern sequencing approaches to the field. The data presented here comprise the first comprehensive genus and species level analysis of the bacteria residing in biofilms on the dentures and remaining teeth of healthy patients and those with stomatitis. Importantly, comparison of samples derived from the dentures and remaining teeth of the same individuals allowed us to analyze the possible mutual influence of the bacterial communities colonizing these different surfaces in health and disease.

Our initial genus level analysis revealed that, unlike the distinct health- and disease-associated microbiota reported previously for other oral diseases such as periodontitis (27,–30), the bacterial communities residing on dentures and remaining teeth in health and disease are rather similar to each other (Fig. 1). This lack of differences in bacterial community composition was also reflected by the respective alpha and beta diversities (Fig. 2A andB; see Fig. S2). Consistent with previous findings of large individual

dependent variations of the microbiome (29, 31, 32), we found that the phylotype composition of bacterial communities growing on dentures and those derived from remaining teeth were significantly more similar to each other (lower Bray-Curtis dissimilarity index) in samples derived from the same individual than in unrelated denture and tooth samples from different individuals (Fig. 2C). This is also reflected in our observation that only three species/phylotypes displayed significant differential surface colonization (see Fig. S4B). Considering this apparent strong mutual influence of bacteria colonizing dentures and teeth in the same individual, the health and integrity of remaining teeth could comprise an important factor in the mucosal health of denture wearers beyond their role in anchoring restorations and maintaining bone integrity. Similarly, the denture-associated oral mucosal health status could play a critical role in conserving remaining teeth.

Furthermore, the bacterial phylotype compositions present in biofilms collected from the same surface (dentures or remaining teeth) of different patients were significantly less similar to each other than to the communities identified on the matched denture-teeth samples in the same patients (Fig. 2C). This is interesting, since biofilm formation is thought to be rather surface dependent (7), and while overlap exists, different natural surfaces within the oral cavity are colonized by distinct communities (25). Our findings indicate that individual-specific factors can be more dominant determinants of the oral bacterial biofilm community composition than surfaces. One important host factor involved in this phenomenon could be saliva, which coats available natural, as well as

artificial, oral surfaces with a so-called pellicle (33). Saliva can display large variability between individuals (34, 35) and provides important adhesion proteins for bacterial attachment (36). In addition, our discovery that intrapatient factors could be stronger determinants of bacterial biofilm community composition than different surfaces emphasizes that grouping and pooling of samples from different people can influence analysis outcome.

Previous studies analyzing the bacteria colonizing dentures in health and disease are not conclusive. Similar to our results, a recent sequencing study (12) and older culture-based approaches (11, 16, 24) found no difference in the apparent microbial composition between healthy and stomatitis patients and noted only that the amount of plaque buildup is significantly greater in stomatitis patients. In contrast, a clone library-based culture-independent study (10) reported that the microbiota of biofilms colonizing dentures in health and disease are distinct. Furthermore, unlike our results detailed above, a recently published sequencing study (12) described a significant difference between the bacterial community compositions found on denture surfaces and those on remaining teeth. The apparent discrepancies between our findings and previous 16S rRNA gene-based sequencing studies can be due to many factors, ranging from geographical differences between patient populations to sample collection, sequencing parameters (choice of 16S rRNA target region, the sequencing platform used, available read length, and sequencing depth, among others), or DNA extraction and PCR protocols (37).

Not surprisingly, we found *Actinomyces*, *Capnocytophaga*, *Streptococcus*, *Veillonella*, and *Neisseria* to be most prevalent and abundant genera, independently of the surface or health/disease status, in all of our samples (Fig. 3; see Fig. S3). These genera are among the most predominant in the oral cavity and have been identified as major denture colonizers in previous culture-based and culture-independent studies (10,–12, 16). Especially the genera *Actinomyces* and *Streptococcus* are considered early colonizers of the oral cavity that readily attach to available surfaces, as well as each other (38, 39). They enable surface colonization of other microbial species, including *Capnocytophaga* and *Neisseria*, via physical binding, as well as metabolic interactions such as the metabolic interdependence between *Veillonella* and *Streptococcus* species (40, 41). Other prevalent genera present in the samples analyzed in our study include *Corynebacterium*, *Rothia*, several genera of "*Candidatus* TM7," and *Fusobacterium*. Most previous studies comparing denture plaque in health and disease did not identify these genera (10,–12), even though they were found to colonize denture teeth in a checkerboard study comparing natural tooth and denture colonization patterns (15). Consistent with earlier studies (10, 12, 23, 24, 42, 43), *Candida* was not limited to denture stomatitis samples, with fewer of the healthy samples being positive (Table 1). While previous class level analysis indicated that *Candida* colonization was positively correlated with lactobacilli and negatively correlated with *Fusobacteria*, this was not the case for the samples analyzed here. In our study, we observed a possible positive correlation for *A. parvulum*, *Lachnospiraceae* sp. strain HOT-097 ("*Candidatus* G-4"), *Veillonella atypica*, while *Leptotrichia* sp. strain HOT-212 was not present in samples containing *Candida*, with the

exception of one healthy patient. Since little is known about the interaction between these species and *Candida*, further study is needed to confirm the relevance of this observation. Despite the similarities on the biofilm community level, individual genera and species were significantly different in their occurrence on specific surfaces and/or the denture-related health/disease status of the patient. All members of the genus *Fusobacterium* had very low colonization rates on healthy dentures and health in general (Fig. 4A), even though the species *Streptococcus gordonii* and *S. sanguinis* that fusobacteria are known to attach to (44, 45) were present in significantly elevated numbers under this condition (Fig. 4B). These findings indicate that in the complex biofilm environment of the oral cavity, factors beyond the ability to bind to each other play important roles in microbial community dynamics and composition. Among the different *Fusobacterium* species and subspecies, *F. nucleatum* subsp. *polymorphum*, *F. nucleatum* subsp. *vincentii*, and *F. periodonticum* have previously been observed to increase more on natural teeth than on denture teeth (15). In our study, they exhibited a higher relative abundance on natural teeth in disease; however, this difference was only significant for *F. nucleatum* subsp. *vincentii* (Fig. 4A). Surprisingly, the colonization pattern of *F. nucleatum* subsp. *animalis* was completely different from that of all of the other fusobacterial species and subspecies identified, with a striking almost exclusive colonization of denture surfaces in stomatitis patients (Fig. 4A). This particular subspecies of *F. nucleatum* has been identified as an etiological agent in a case report connecting the oral microbiota with stillbirth (46), documented to be more prevalent in subgingival plaque and early periodontitis (47), as well as experimental gingivitis (48). Furthermore, *F. nucleatum* subsp. *animalis* is part of

the microbial signature in early detection of colorectal cancer (49) and the only fusobacterial species/subspecies found to overlap between the microorganisms isolated from the periodontal pocket and the atheromatous plaque in cardiac disease patients (50). Our finding of a strong association of *F. nucleatum* subsp. *animalis* with the inflammatory mucosal condition stomatitis in combination with the above findings by other research groups suggests that this *F. nucleatum* subspecies may be more pathogenic than others.

In addition to the prominent difference in *F. nucleatum* subsp. *animalis* distribution, which could be relevant for stomatitis etiology, other microorganisms displayed disparate surface- and/or health status-dependent colonization patterns. As already mentioned above, certain *Streptococcus* species displayed a significantly higher prevalence and abundance on dentures in healthy denture wearers than under all other conditions (Fig. 4B). Among these, *S. sanguinis* and *S. gordonii* have previously been associated with oral health (51,–53), and a recent study revealed a distinct, species-specific distribution of streptococci on the natural oral surfaces of healthy subjects (25). Additional evidence for the importance of analysis beyond the genus or phylum level provides the colonization pattern of the genera *Fusobacterium* and *Porphyromonas*. While their presence correlated strongly with health and disease independently of the surface when analyzed on the genus level (Fig. 4A; see Fig. S4A), detailed species/phylotype level analysis yielded a more differentiated picture. As already discussed above, the different representatives of the genus *Fusobacterium* present in our

samples displayed a distinct surface and denture health status-dependent distribution (Fig. 4A). Our finding that *Porphyromonas*, a genus typically associated with disease (26), exhibited a significant surface-independent health association (see Fig. S4A) provides an example of how the lack of taxonomic resolution could influence results and data interpretation. Phylotype level examination revealed that the genus *Porphyromonas* was predominantly represented in our samples by *Porphyromonas* sp. strain HOT-279, a phylotype that is abundant in healthy human subjects who participated in the Human Microbiome Project (25, 54). Additional, less abundant representatives of *Porphyromonas* (*P. gingivalis*, *P. endodontalis*, *P. catoniae*, and *Porphyromonas* sp. strain HOT-275) exhibited the "typical" disease association of this genus, as they were significantly correlated with the remaining dentition of stomatitis patients (see Fig. S4A). Since these distinct health- and disease-associated distribution patterns of individual representatives of certain genera are apparent only on the phylotype/species level, genus level microbiome analysis is not always sufficient to provide a comprehensive picture of the relevant players and higher-resolution analyses could be critical for in-depth understanding of the oral microbiome in health and disease.

In conclusion, our study suggests that the bacterial microbiota on dentures is highly similar in health and disease on the broader community level. This was also observed for dentures and remaining teeth independently of health status, especially in samples derived from the same individuals. The phylotype composition of the bacterial communities colonizing the dentures and remaining teeth of the same individuals are

largely reflective of each other, indicating a possible mutual influence of denture health status on the dentition and vice versa. The observed lack of distinct microbiota is consistent with most previous reports that in denture-associated oral diseases, the overall microbial load may have a greater impact on stomatitis development than the actual microbial composition of the mucosa-facing denture plaque. Despite these overall similarities, we were able to identify distinct species such as *F. nucleatum* subsp. *animalis* and several species of *Streptococcus* that were strongly associated with diseased and healthy denture samples, respectively. Our findings that significant differences in colonization were observed predominantly on the phylotype/species level highlight the importance of species/phylotype or even oligotype level analysis (25).

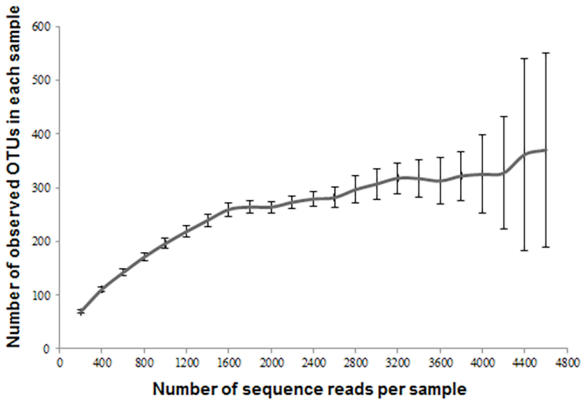

FIGURE S1 *Rarefaction curve of V1 to V3 16S rRNA gene sequences after application of the quality control and cleanup procedures described in Materials and Methods. Data are presented as mean values ± the SEM.*

Supplemental Figure 2

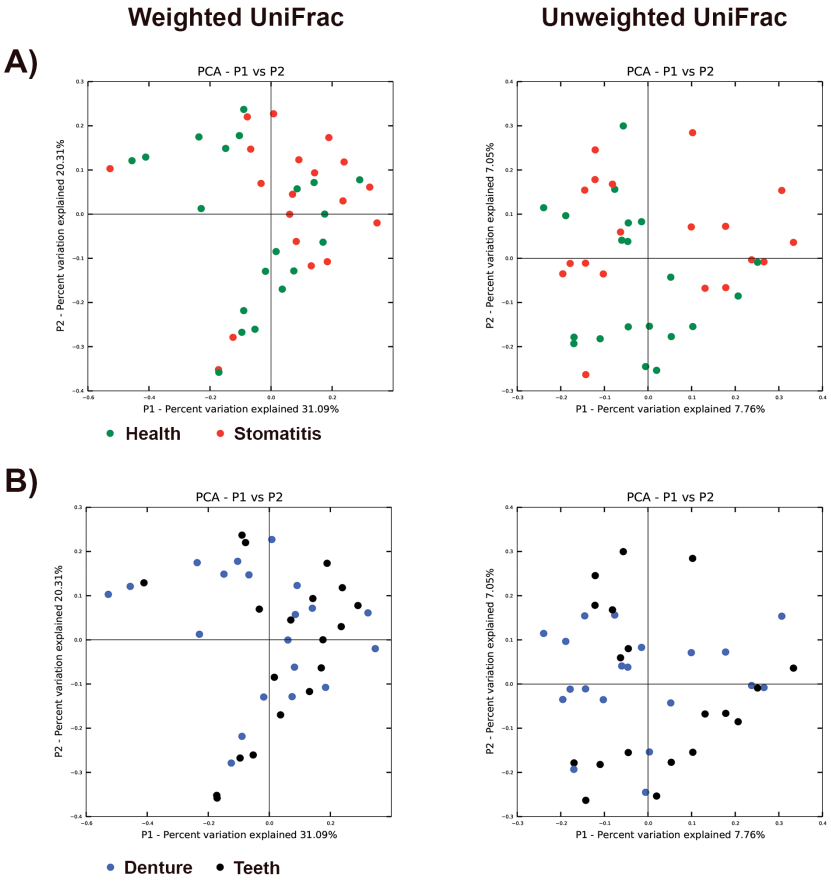

Figure S2 *Beta diversity (principal-coordinate analysis) of the microbial communities colonizing healthy individuals (green) and stomatitis patients (red) irrespective of the surface (A) and colonizing dentures (blue) and remaining teeth (black) irrespective of denture-related oral health status (B).*

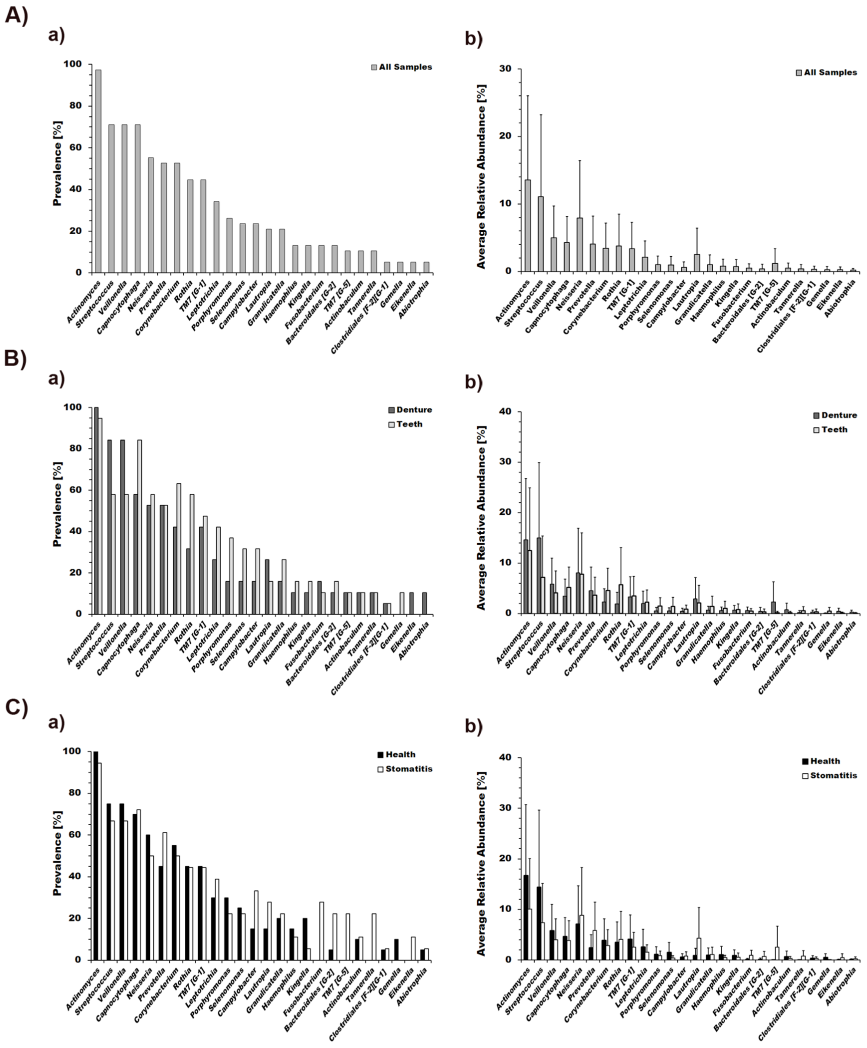

Figure S3 *Genera present at a >1% relative abundance in at least two patient samples were included in the analysis. Shown are the prevalence (a) and average relative abundance ± SEM (b) of all samples (medium gray bars) (A), differentiated into dentures*

*(dark gray bars) and teeth (light gray bars) (B), and in health (black bars) and stomatitis (white bars) (C). *, P < 0.05.*

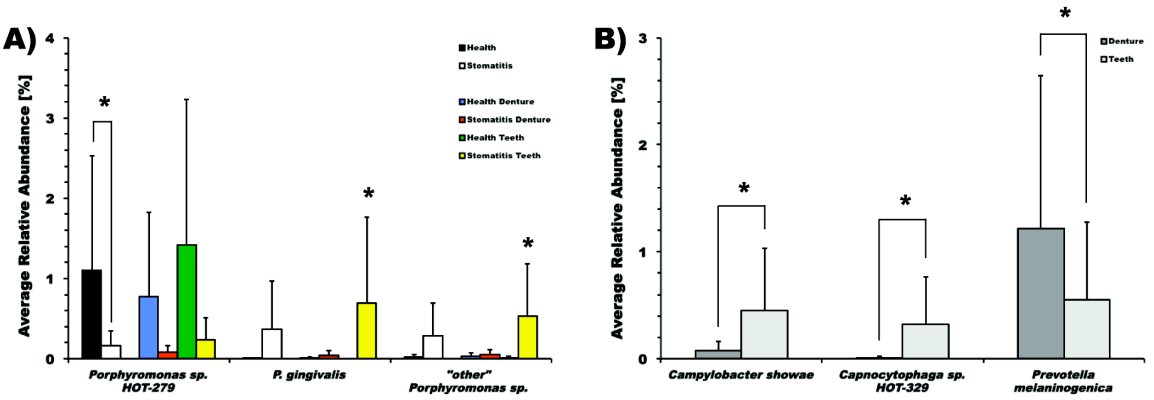

Figure S4 *Health and disease status and surface-dependent differential colonization at the genus and phylotype levels. Shown are the mean relative abundances of Porphyromonas sp. strain HOT-279, P. gingivalis and other disease-related Porphyromonas bacteria (P. cantoniae, P. endodontalis, and Porphyromonas sp. strain HOT-275 combined) (A) and Campylobacter showae, Capnocytophaga sp. strain HOT-329, and P. melaninogenica (B). Samples are differentiated into health (black bars), disease (white bars), dentures (dark gray bars), teeth (light gray bars), dentures in health (blue bars), dentures from stomatitis patients (red bars), teeth in health (green bars), and teeth from stomatitis patients (yellow bars). *, P < 0.05.*

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Part II

Chapter III

Cellular components mediating coadherence of *Candida albicans* and *Fusobacterium nucleatum*

This chapter has been published as the following:

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Abstract

Candida albicans is an opportunistic fungal pathogen found as part of the normal oral flora. It can be coisolated with *Fusobacterium nucleatum*, an opportunistic bacterial pathogen, from oral disease sites, such as those involved in refractory periodontitis and pulp necrosis. The physical coadherence between these 2 clinically important microbes has been well documented and suggested to play a role in facilitating their oral colonization and colocalization and contributing to polymicrobial pathogenesis. Previous studies indicated that the physical interaction between *C. albicans* and *F. nucleatum* was mediated by the carbohydrate components on the surface of *C. albicans* and the protein components on the *Fusobaterium* cell surface. However, the identities of the components involved still remain elusive. This study was aimed at identifying the genetic determinants involved in coaggregation between the 2 species. By screening a *C. albicans* SN152 mutant library and a panel of *F. nucleatum* 23726 outer membrane protein mutants, we identified *FLO9*, which encodes a putative adhesin-like cell wall mannoprotein of *C. albicans* and *radD*, an arginine-inhibitable adhesin-encoding gene in *F. nucleatum* that is involved in interspecies coadherence. Consistent with these findings, we demonstrated that the strong coaggregation between wild-type *F. nucleatum* 23726 and *C. albicans* SN152 in an *in vitro* assay could be greatly inhibited by arginine and mannose. Our study also suggested a complex multifaceted mechanism underlying physical interaction between *C. albicans* and *F. nucleatum* and for the first time revealed the identity of major genetic components involved in mediating the coaggregation. These

observations provide useful knowledge for developing new targeted treatments for disrupting interactions between these 2 clinically relevant pathogens.

Introduction

Candida albicans is a commensal fungal species commonly colonizing human mucosal surfaces, which can be isolated from the oral cavity of as many as 60% of healthy adult subjects (Odds et al. 1988; Glick and Siegel 1999). It coexists with diverse oral microbial species and has long been adapted to the human host (Morales and Hogan 2010). In healthy individuals, *C. albicans* is usually a minor component of their oral microbial flora. However, under conditions of immune dysfunction or local predisposing factors such as poor oral hygiene or ill-fitted dentures, colonizing *C. albicans* strains can become opportunistic pathogens. In these patients, *C. albicans* becomes more predominant and invasive, causing recurrent mucosal infection, such as denture stomatitis or life-threatening disseminated infections (Boerlin et al. 1995; Coleman et al. 1997; Perlroth et al. 2007; Azie et al. 2012).

The colonization of *C. albicans* in the human oral cavity can be achieved through its adherence to a variety of salivary pellicle components, including proline-rich proteins and statherin (Calderone and Braun 1991). Moreover, it is able to directly bind to the oral epithelial cells through adhesion/ligand interactions (Salgado et al. 2011). In addition to oral tissue surfaces, increasing evidence suggests that *C. albicans* could establish themselves by incorporation into existing biofilms through adherence to a variety of oral bacterial species including streptococci, actinomyces, and fusobacteria (Shirtliff et al. 2009).

Fusobacterium nucleatum is another opportunistic oral pathogen that has been implicated in periodontal disease (Signat et al. 2011). Best known for its ability to adhere to a diverse range of oral bacteria, it is considered a "bridging" organism that links early commensal colonizers and late colonizing species, many of which are periodontal pathogens; therefore, it plays an important role in the succession of genera in oral polymicrobial communities (Kolenbrander 2000; Rickard et al. 2003). Other than interacting with bacterial species, fusobacteria can establish direct physical association with eukaryotic microbes such as fungi. The coadherence between *F. nucleatum* and *C. albicans* has been well documented and has been implicated in facilitating the colonization of *C. albicans* within the oral cavity (Grimaudo and Nesbitt 1997; Jabra-Rizk et al. 1999). Likewise, the coisolation of *C. albicans* with *F. nucleatum* from a variety of oral disease sites—including refractory periodontitis, such as therapy-resistant apical periodontitis (Waltimo et al. 1997) and pulp necrosis (Lana et al. 2001)—further suggested their interaction as well as involvement in polymicrobial pathogenesis.

Previous studies suggested that the coadherence between *C. albicans* and *F. nucleatum* was mediated by the carbohydrate components on the surface of *C. albicans* and the protein components on the *Fusobacterium* cell surface (Grimaudo and Nesbitt 1997; Jabra-Rizk et al. 1999). However, the identities of the components involved remain elusive. In the current study, by screening a *C. albicans* mutant library and a panel of *F.*

nucleatum outer membrane protein mutants, we aimed to identify the genetic components involved in coadherence between these 2 clinically relevant opportunistic pathogens.

Methods

Strains and Growth Conditions

Wild-type strain *F. nucleatum* 23726 and its mutant derivatives lacking one of the large fusobacterial outer membrane proteins—including Fn0254, Fn0387, Fn1449 (Fap2), Fn1526 (RadD), Fn1554, Fn1893, Fn2047, or Fn2058 (Aim1; Kaplan et al. 2009; Kaplan et al. 2010; Gur et al. 2015)—were cultivated in Columbia broth (Difco, Detroit, MI, USA) at 37 °C anaerobically (nitrogen 90%, carbon dioxide 5%, hydrogen 5%).

A *C. albicans* SN152 mutant library was purchased from the University of California, San Francisco (Noble et al. 2010). The library contains 674 homozygous deletion mutants covering roughly 11% of the *C. albicans* genome. *C. albicans* wild-type and mutants were precultured in yeast peptone dextrose (YPD) medium (yeast extract, 10 g/L; peptone, 20 g/L; glucose, 20 g/L) at 30 °C in an atmosphere of 5% CO_2 in air. Hyphal growth of *C. albicans* was induced by inoculating overnight cultures in fresh YPD medium supplemented with 20% fetal bovine serum and aerobically incubated at 37 °C until the OD600 reached 0.2.

Coaggregation Assay

Coaggregation assays were preformed in modified coaggregation buffer (CAB) as previously described (He et al. 2012). Since hyphae-displaying *C. albicans* SN152 cells tend to precipitate even in the absence of coaggregating bacterial partner species, direct

visual microscopic observation of coaggregation between *C. albicans* and *F. nucleatum* cells was performed. Briefly, exponential bacterial cells and hypha-displaying candida cells were collected and resuspended in CAB to an OD_{600} of 1 and 2, respectively. Equal volumes (250 μL) of bacterial and candida cells were mixed in reaction tubes. The tubes were vigorously vortexed for 30 s and allowed to stand at room temperature for 30 min. Coaggregating samples were taken and observed using light microscopy; images were taken from 10 random fields of view. For each coaggregation sample, 10 random candida hypha-displaying cells were chosen, and the attached fusobacterial cells were counted for each cell. Based on the average number (n) of attached bacterial cells per 20 μm of hyphal body length, coaggregation was graded on a 0 to 4 scale: score 4, $n \geq 20$; score 3, $10 \leq n < 20$; score 2, $5 \leq n < 10$; score 1, $1 \leq n < 5$; score 0, when no attached bacterial cells could be observed.

Fluorescence-based Coaggregation Assay

To achieve a more quantitative and sensitive readout, a fluorescence-based coaggregation assay was developed (see Appendix for detailed experimental procedure). Briefly, Syto 9 green fluorescent nucleic acid dye–stained fusobacterial cells were used to set up coaggregation assays with *Candida*. Coaggregation mixtures were then washed to remove unattached fusobacterial cells. The coaggregation between *Candida* and *Fusobacterium* was quantified by measuring the intensity of the florescence signal retained on the *Candida* cell surface.

Screening a C. albicans SN152 Library for Mutants Defective in Coaggregating with F. nucleatum 23726

Two microliters of each mutant from a *C. albicans* SN152 mutant library stored in 96-well plates was transferred to plates containing 200 μL of fresh YPD medium in each well. Plates were incubated at 30 °C in an atmosphere of 5% CO_2 in air. After overnight growth, 60 mutant strains showing growth defects (with cell density <0.1 at OD_{600}) were excluded while the rest of the mutants were spun down and resuspended in fresh YPD medium supplemented with 20% fetal bovine serum. The plates were next further incubated aerobically at 37 °C for 4 h to induce hyphal growth. *C. albicans* cells were resuspended in CAB to a final OD_{600} of 0.5 to 1. Meanwhile, exponential-phase *F. nucleatum* cells were resuspended in CAB to a final OD_{600} of 1. One hundred microliters of bacterial cells were then added into each well of 96-well plates containing *C. albicans* mutants and mixed by pipetting. Plates were incubated at room temperature for 30 min before samples were taken and aggregation was observed under a light microscope. Mutants defective in coaggregating with wild-type *F. nucleatum* were confirmed by fluorescence-based coaggregation assays.

Inhibition of Coaggregation between F. nucleatum and C. albicans

By using the coaggregation assay described above, the following sugars or amino acids at a final concentration of 100 mM (Grimaudo and Nesbitt 1997) were tested for their ability to inhibit coaggregation between *C. albicans* and *F. nucleatum*: D-galactose, D-lactose, D-mannose, L-leucine, N-acetyl-glucosamine, and L-arginine.

Recruitment Assay

Recruitment assays were designed and performed to test the ability of coaggregation-defective *C. albicans* and *F. nucleatum* mutants identified in this study to bind to monospecies biofilms of their wild-type interacting partner. Detailed procedures are provided in the Appendix. Briefly, planktonic cells of *C. albicans* (wild type and mutants) or *F. nucleatum* (wild type and mutants) were added to the monospecies biofilm of their wild-type interacting partner, and their ability to bind to the biofilm cells was determined by cell counting.

Results

C. albicans SN152 Mutant FLO9 Exhibits Reduced Ability in Coaggregating with F. nucleatum

The coaggregating ability between *C. albicans* SN152 and *F. nucleatum* 23726 was tested using cell suspensions in CAB. Coaggregation was monitored and recorded by light microscopy and fluorescence-based assays. Wild-type *C. albicans* SN152 hyphal cells and *F. nucleatum* 23726 displayed a strong coaggregation phenotype (Fig. 1, panel 2), while no aggregation was detected between the yeast form of *C. albicans* and *F. nucleatum* under the same coaggregating conditions tested (data not shown). To identify the genetic components of *C. albicans* involved in its coaggregation with *F. nucleatum*, a homozygous *C. albicans* SN152 gene deletion library (Noble et al. 2010) was screened for mutants that had normal growth but were defective in coadherence with *F. nucleatum*. The library contains 674 homozygous deletion mutants covering roughly 11% of the *C. albicans* genome. The screen led to the discovery of 1 mutant, *C. albicans* SN152 FLO9, which displayed the most drastically reduced ability in coaggregating with *F. nucleatum*. As revealed by microscopic observation, the surface of the FLO9 mutant was only sparsely decorated with *F. nucleatum* (Fig. 1, panel 7), as compared with wild-type *C. albicans*, whose hyphal surface was extensively covered by the coaggregating bacterial partner. Fluorescence-based coaggregation assay showed that, when compared with the wild type, the fluorescence signal retained decreased >60%, suggesting a severe reduction in the number of attached *F. nucleatum* cells (Fig. 2). *FLO9* encodes a putative

adhesin-like cell wall mannoprotein (Noble et al. 2010), and our data suggested its involvement in mediating physical interaction with *F. nucleatum*.

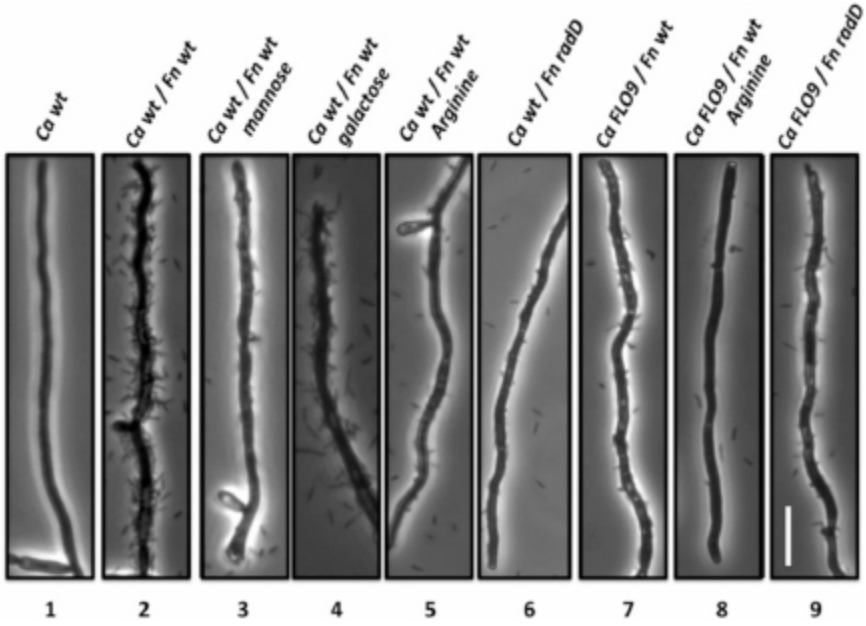

Figure 1. *Microscopic observation of coaggregation between Candida albicans (Ca) and Fusobacterium nucleatum (Fn) under different treatments. For each interacting pair, the pictures of 10 random Ca cells were taken and 1 representative image shown. The scale bar is 10 µm. wt, wild type.*

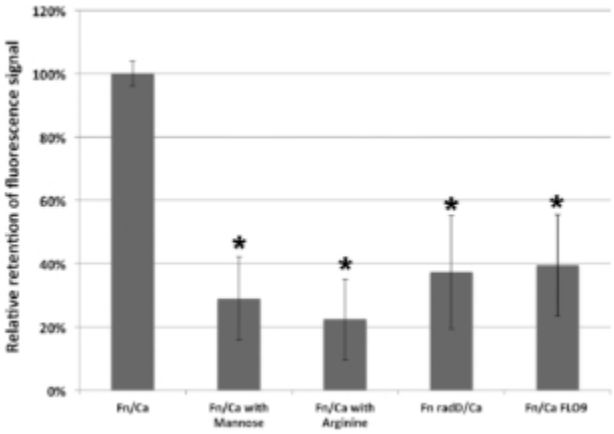

Figure 2. *Coaggregation between Candida albicans (Ca) and Fusobacterium nucleatum (Fn). Fluorescence-based coaggregation assay (as described in Materials and Methods) was performed for Fn and Ca, both wild type and mutants. The result was represented as the percentage of fluorescence signal retained compared with the control group containing wild-type Ca and Fn as a coaggregating pair. Average values ± SD are shown. Asterisk indicates the significant difference between the testing group and the control group (wild-type Fn/Ca; $P < 0.05$, t test). Note: The absolute number of relative fluorescence units represented by 100% was 2,500, corresponding to about 2×10^6 colony-forming units per milliliter of Fn based on the experimental procedure described in the Materials and Methods.*

F. nucleatum RadD Mutant Is Defective in Coaggregating with C. albicans SN152

A previous study indicated the involvement of the protein components on the *Fusobaterium* cell surface in its coaggregation with *C. albicans* (Grimaudo and Nesbitt 1997). In this study, a panel of *F. nucleatum* outer membrane protein mutants generated in our laboratory (Kaplan et al. 2009; Kaplan et al. 2010) was tested for their coaggregating ability with *C. albicans* SN152. The results showed that among the 8 mutants tested, RadD was the only mutant defective in binding to the hypha-displaying *C. albicans* cells (Fig. 1, panel 6), with a >60% reduction in fluorescence signal recovered from the *C. albicans*–bound fusobacterial cells as compared with the wild type (Fig. 2). *RadD* encodes an arginine-inhibitable adhesin that has been shown to be required for interspecies coadherence between fusobacteria and oral streptococci

(Edwards et al. 2007; Kaplan et al. 2009). Our data suggested that it also plays a role in mediating interaction with *C. albicans.*

We further tested the coaggregation phenotype between *F. nucleatum* RadD and *C. albicans* FLO9. Microscopic observation and fluorescence-based assay did not reveal any significant difference in coaggregation between the interacting pairs with either one or both mutant partners. In addition, there was visible residual coaggregation between fusobacterial and *Candida* cells when RadD and FLO9 mutants were used as interacting pair (Fig. 1, panel 9).

Mannose- and Arginine-inhibitable Coaggregation between *C. albicansand F. nucleatum*

Mutant screening revealed that the mannoprotein FLO9 from *C. albicans* and arginine-inhibitable RadD from *F. nucleatum* are involved in the interspecies coaggregation. These findings suggested that the observed strong coadherence phenotype between *C. albicans* and *F. nucleatum* might be mannose/arginine inhibitable. To confirm this, mannose and arginine, among other carbohydrates and amino acids, were tested for their ability in inhibiting the coadherence. The results showed that the presence of 0.1M mannose or arginine resulted in a drastic reduction in the coaggregation between the *Candida* and *Fusobacterium* pair, while other carbohydrates or amino acids tested did not have a significant effect on the coaggregation (Table). This was revealed by microscopic observation as well as the more quantitative fluorescence-based coaggregation assay showing about 70% reduction in the signal retention vs. the control (Fig. 1, panels 3, 5;

Fig. 2), indicating the reduced number of *F. nucleatum* cells attached to *Candida* hyphal cells. Moreover, the addition of other monosaccharides (e.g., D-galactose) and amino acids (including L-leucine) had no apparent inhibitory effects on the coaggregation (Fig. 1, panel 4; Table).

Table. Effect of Treatments on Coaggregation between *Candida albicans* and *Fusobacterium nucleatum*.

Coaggregating Pair	No Treatment	D-lactose	D-galactose	D-mannose	L-leucine	L-arginine	N-acetyl-glucosamine
Ca wt / *Fn* wt	4	4	4	2	4	2	4
Ca FLO9 / *Fn* wt	2	2	2	2	2	2	2
Ca wt / *Fn* RadD	2	2	2	2	2	2	2
Ca FLO9 / *Fn* RadD	2	2	2	2	2	2	2

For each coaggregation sample, 10 random hyphae-displaying *C. albicans* (*Ca*) cells were chosen; the attached *F. nucleatum* (*Fn*) cells were counted. Based on the average number (*n*) of attached bacterial cells per 20 μm of hyphal body length, coaggregation was graded on a 0 to 4 scale: score 4, $n \geq 20$; score 3, $10 \leq n < 20$; score 2, $5 \leq n < 10$; score 1, $1 \leq n < 5$; score 0, no attached bacterial cells could be found.

C. albicans SN152 FLO9 and F. nucleatum RadD Displayed Reduced Ability to Adhere to the Surface Attached Biofilm Cells of Their Respective Interacting Partners

Our data showed that *C. albicans* and *F. nucleatum* were able to coaggregate under planktonic conditions, while mutation of *FLO9* in *C. albicans* and *radD* in *F. nucleatum* rendered the mutants defective in interspecies binding. To further investigate the observed FLO9- and RadD-dependent coadherence property between *C. albicans* and *F. nucleatum* under biofilm conditions, *C. albicans* SN152 FLO9 and *F. nucleatum* RadD mutants were tested for their ability to adhere to monobiofilms of wild-type *F. nucleatum* and *C. albicans*, respectively. The results showed that, compared with its wild-type counterparts, *F. nucleatum* RadD and *C. albicans* SN152 FLO9 displayed significantly reduced capabilities (with about 50% and 70% reductions, respectively) to adhere to monospecies biofilms of their respective wild-type interacting partners (Fig. 3).

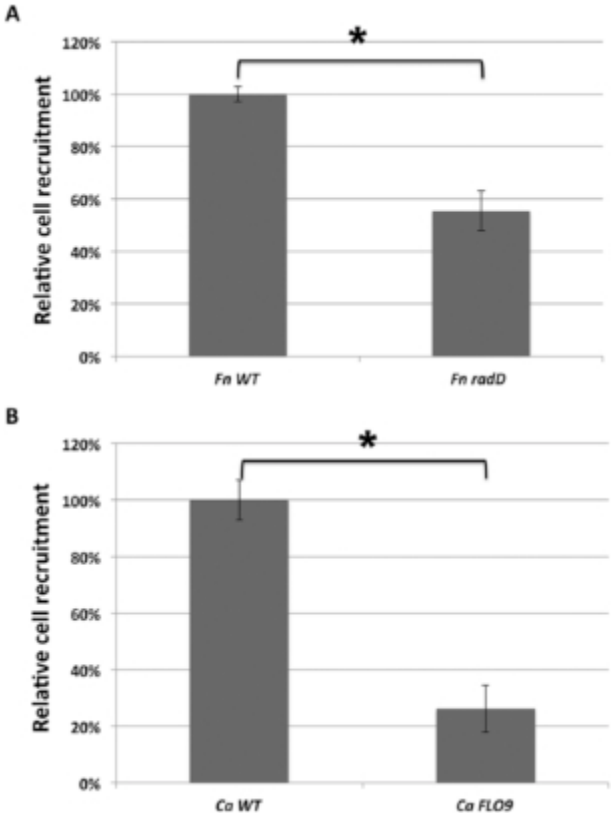

Figure 3. *Binding of Candida albicans (Ca) and Fusobacterium nucleatum (Fn) to monospecies biofilm of their interacting partner species. Recruitment assay (as described in Materials and Methods) was performed to test the ability of Fn RadD and Ca FLO9 mutant to adhere to monospecies biofilm of wild type (WT) Ca (**A**) and Fn (**B**), respectively. The result was represented as the percentage of mutant cells adhered to biofilm of its coaggregating partner compared with the WT control. Average values ± SD are shown. Asterisk indicates significant difference between the 2 sample sets ($P < 0.05$, t test). Note: The absolute number of cells represented by 100% was about 4×10^7 colony-forming units per milliliter for Fn (A) and 2×10^6 colony-forming units per milliliter for Ca (B) based on the experimental procedure described in Materials and Methods.*

Discussion

Coadhesion between different microbial species is a common feature of the oral microbiome. It plays a crucial role in colonization of the oral cavity, contributes to the formation of highly structured multispecies communities, and ultimately affects polymicrobial pathogenesis (Kolenbrander 1988, 1989; Kolenbrander et al. 1999; Kolenbrander et al. 2006; Peters et al. 2012). Such physical interspecies association not only exists among bacterial species but has also been observed for 2 clinically relevant opportunistic pathogens of different kingdoms, *C. albicans* and *F. nucleatum* (Grimaudo and Nesbitt 1997; Jabra-Rizk et al. 1999). Knowledge of the major genetic determinants involved in coadherence between these 2 microbes may help shed light on *Candida* succession and possible polymicrobial disease processes involving these 2 species.

Microscopic observations and fluorescence-based quantitative coaggregation assays clearly demonstrated the strong coadherence phenotype between the bacterial species *F. nucleatum* 23726 and the eukaryotic microbe *C. albicans* SN152 in the hyphal form (Fig. 1, panel 2; Fig. 2). Screening of a *C. albicans* SN152 mutant library resulted in the identification of the *FLO9* gene, which is critical in establishing physical interaction with *F. nucleatum. FLO9* encodes a putative adhesin-like cell wall mannoprotein in *C. albicans* (Boisrame et al. 2011). Mutation in this gene does not affect its colony morphology or vegetative growth, nor does it affect competitive fitness in a mouse intravenous infection model (Noble et al. 2010). However, this mutant displayed a

drastically reduced ability in coadherence with *F. nucleatum* as revealed by coaggregation assays, suggesting its role in mediating the observed duo-species interaction (Fig. 1, panel 7; Fig. 2). The identification of the mannoprotein FLO9 as the surface component mediating the coadherence of *C. albicans* with *F. nucleatum* was further confirmed by our observation that the coaggregation phenotype was mannose inhibitable (Fig. 1, panel 3). This is consistent with previous reports showing that the binding of *F. nucleatum* strains to *Candida* species, including *C. albicans* and *C. dubliniensis*, could be inhibited by addition of mannose but not other carbohydrates tested (Grimaudo and Nesbitt 1997; Jabra-Rizk et al. 1999).

C. albicans has a multilayered cell wall composed of an outer layer of proteins glycosylated with N- or O-linked mannosyl residues and an inner skeletal layer of beta-glucans and chitin (Gow and Hube 2012). The mannoproteins have been shown to play important roles in diverse cellular functions, such as acting as specific immunodeterminants (Gow and Hube 2012), binding to host-secreted macromolecules (Tronchin et al. 1988), as well as functioning as adhesins to mediate the direct *Candida*-bacteria and *Candida*–host cell recognitions (Salgado et al. 2011; Dutton et al. 2014). In *C. albicans, mnt1* and *mnt2* encode partially redundant α-1, 2-mannosyltransferases that catalyze the addition of the second and third mannose residues in an O-linked mannose pentamer, while *och1* encodes a α-1, 6-mannosyltransferase that is involved in N-glycan outer chain branch addition. In a recent study, Dutton et al. (2014) demonstrated that O-mannosylation via Mnt, specifically Mnt1, is critical for hyphal adhesion functions

required for coadherence between *C. albicans* and *Streptococcus gordonii*. In our study, a *mnt1* mutant displayed a normal coaggregation phenotype with *F. nucleatum*, while the *och1* mutant showed drastically reduced coaggregation with *F. nucleatum* (Appendix Fig. 1) even though this mutant was not initially identified via library screening due to its growth defects. Our data suggested that N-linked glycosylation could be more important for FLO9 in determining its binding ability with its counterpart in *F. nucleatum*. Furthermore, FLO9 was predicted to be hyphally regulated and expressed during hyphal growth (http://www.candidagenome.org). This is in agreement with our data showing that the coadherence can be observed only between *F. nucleatum* and *C. albicans* hyphal, but not yeast cells.

Our study further revealed that RadD, an outer member protein in *F. nucleatum* 23726, is also involved in mediating the coadherence between fusobacterial and candidal cells. RadD is an arginine-binding protein (Edwards et al. 2007) and has been shown to be an adhesin required for the coadherence of *F. nucleatum* with gram-positive oral bacterial species, such as streptococci (Kaplan et al. 2009). More important, our data showed that no additive effects in the reduction of coaggregation can be observed when *F. nucleatum* RadD and *C. albicans* FLO9 were tested as a pair compared with interacting pairs containing either mutant (Fig. 1, panels 6 to 9). These data strongly suggested that RadD is acting as the counterpart component for FLO9 in *C. albicans* and that these 2 components could directly interact with each other and mediate coadherence. Consistent with this finding, exogenous arginine was able to achieve a similar inhibitory effect on

the coaggregation between *F. nucleatum* and *C. albicans* compared with mannose (Fig. 1, panels 3 and 5; Table). Previous studies also suggested that other than a mannan-containing receptor on the *C. albicans*, the coadherence between *F. nucleatum* and *C. albicans* involves a heat-liable component on *F. nucleatum* (Grimaudo and Nesbitt 1997). Meanwhile, sequence analysis of RadD revealed a domain close to the C-terminus (1513-1707aa) with homology to a sequence encoding a bacteriophage tail fiber. Previous studies showed that the adsorption of bacteriophage can be achieved through the binding of tail fibers to the polymannose O-antigens on the *Escherichia coli* cell surface (Heller and Braun 1982). These observations implied that RadD potentially has binding sites for both mannose and arginine. It could act as a receptor and bind to the specific mannan residue on the FLO9 mannoprotein on the surface of *C. albicans* to mediate physical interaction. Meanwhile, the binding of arginine might induce a conformational change in the binding pocket for mannose and result in the disruption of the coadherence between the 2 species. Further studies are needed to determine the respective binding sites in RadD for mannose and arginine.

It is worthwhile to point out that, in a study by Jabra-Rizk et al. (1999), the coaggregation between *C. albicans* and *F. nucleatum* was mannose inhibitable, while arginine had no such effects. In that study, *F. nucleatum* ATCC 49256 was tested—a *F. nucleatum* subsp. *vincentii* strain instead of a *F. nucleatum* subsp. *nucleatum* strain. Furthermore, the *C. albicans* strains used by Jabra-Rizk et al. were all clinical isolates, and they displayed different phenotypes when coaggregating with *F. nucleatum* ATCC 49256. Although

FLO9 is conserved among many *Candida* species and *C. albicans* subspecies, RadD homologues have been found in different *Fusobacterium* species and subspecies, including *F. nucleatum* subsp. *vincentii.* It remains to be determined if these homologues could display differential adhesion/ligand specificities. Our recent study showed that different *F. nucleatum* subspecies display significantly differential coaggregation abilities with the same *Streptococcus mutans* strain (unpublished data), suggesting the involvement of different adhesins or the differential binding specificity of homologous adhesins in different subspecies when interacting with their partner species. Thus, it is likely that the observed difference in arginine inhibitability of the coaggregation could be due to the different strains used. The observed strain specificity in interspecies coadhesion could have an impact on host susceptibility to disease and/or the effectiveness of coadherence preventive measures.

In addition, residual coaggregation was observed even when *F. nucleatum* RadD and *C. albicans* FLO9 were used as interacting pairs (Fig. 1, panel 9), which suggested the presence of other receptor/ligand pairs playing minor roles in mediating the observed interspecies coadherence. This is consistent with the multimodal nature of adhesion of *C. albicans* in its coadherence with other oral species, such as *S. gordonii* (Holmes et al. 1996; Silverman et al. 2010) as well as host cells (Calderone and Braun 1991).

We also demonstrated that compared with their wild-type counterparts, both *F. nucleatum* RadD and *C. albicans* FLO9 were defective in coadhering to the monospecies

biofilms of wild-type *C. albicans* and *F. nucleatum*, respectively (Fig. 3; Appendix Fig. 2). Oral colonization is a crucial step in bacterial/fungal infections, which can be achieved via directly binding to host tissue surfaces or coadhering to the microbes that have already been established within surface-attached biofilm. Increasing evidence suggests that the coadherence between *Candida* and bacterial species could promote the formation of mix-species biofilms and affect polymicrobial pathogenicity (Adam et al. 2002; Fox et al. 2014; Xu et al. 2014). Thus, intergenic interaction between *C. albicans* and *F. nucleatum*, 2 clinically relevant oral species, not only contributes to their succession in the oral cavity but could also ultimately affect their pathogenicity during polymicrobial infections and so warrants further investigation. In addition, further characterization of the interacting cellular components could help develop new targeted treatments to interfere with the coadherence, a process that could play an important role in polymicrobial pathogenesis involving these 2 microbes.

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Part III

Chapter I

Development of a chemical formula with anti-plaque activity for orthodontic patients

Abstract

Orthodontic brackets alter tooth surface topology and hamper effective oral hygiene, which often cause heavy accumulation of dental plaques in the area, leading to the negative consequences such as decalcification of enamel surface (white spot lesions), caries, as well as gingival inflammation. So far, brushing is the main tool to mechanically remove dental plaque, but unfortunately, it is not an effective tool, especially for the bracket-induced plaque, making it a major problem in the orthodontic care. In this chapter, we focused our research effort to explore the new ways to inhibit bracket-induced plaque formation. In particular, we developed an innovative work hypothesis on finding the key amino acids and sugar monomers and analogs that could interfere or block polypeptides or polysaccharides based microbial adherence and biofilm formation during bracket-induced plaque formation. As shown in this chapter, we first successfully developed an effective screening assay, which led to the discovery of several chemical compounds with strong inhibitory efforts against bracket-induced plaque formation. These compounds were found to be safe under laboratory safety tests and have now been formulated into toothpaste with good *in vitro* efficacy. This would lead to a possible new therapeutic tool that could prevent and treat brackets associated, plaque-causing oral health problems.

Introduction

Oral cavity harbors over 700 microbial species (see most updated list at Human Oral Microbiome Database, http://www.homd.org). These bacteria adhere to surfaces of various oral tissues (such as tooth, oral mucosa, tongue) and form complex multiple species biofilms (also commonly known as dental plaque). While majority of these bacteria are commensal, some oral microbial species are pathogens responsible for various oral diseases, including dental caries and periodontitis (Hardie JM 1992, Patil S 2013). So far, brushing based mechanical cleaning is the main tool to remove these dental plaques on native oral tissues (both teeth and gums), which is already shown to be ineffective (Boyd RL 1983, Costa MR 2007, Klukowska M 2011, Laher A 2003), making dental caries and periodontal diseases are still the most epidemic diseases in orthodontic patients (Alexander SA 1991, Boke F 2014, Consolaro A 2013, Lucchese A 2013, Tufekci E 2011, van Gastel J 2007, Zachrisson S 1972). In addition, several other methods have been tried such as fluoride, chlorhexidine delivered in the form of toothpaste, mouth rinse, varnish and gel (Baygin O 2013, Santamaria M 2014, van der Kaaij NC 2015). Nevertheless, plaque induced disease is still an unsolved problem, and novel methods are still urgently needed for orthodontic patients (Ren Y 2014). This study is focusing on managing orthodontic bracket induced plaques and their associated oral diseases. Orthodontic treatment is highly popular among juveniles and adults (AAO 2012, Christopherson EA 2009). As part of the orthodontic treatment, brackets need to be adhered to tooth enamel surfaces to provide the holding bases for wires. One of the key

unintended side-effects for fixed brackets on teeth surfaces is the alteration of tooth surface topology from smooth surfaces to new uneven, non-smooth junctions between teeth and brackets, making them the perfect places for much enhanced microbial biofilm formations, as documented by various previous studies (Freitas AO 2014, Sukontapatipark W 2001) and illustrated in Fig. 1a. As mentioned above, removing dental plaque on smooth teeth surfaces by brushing is already not an easy job, adding brackets and wires on these surfaces will further hamper effective oral hygiene (Boyd RL 1983, Costa MR 2007, Klukowska M 2011, Laher A 2003). Consequently, it is well known clinical fact that a large portion of patients undergoing orthodontic treatments suffer from the oral health problems associated with bracket-induced plaque formation, including white spot lesions and gingivitis, as illustrated in Figs. 1b and 1c.

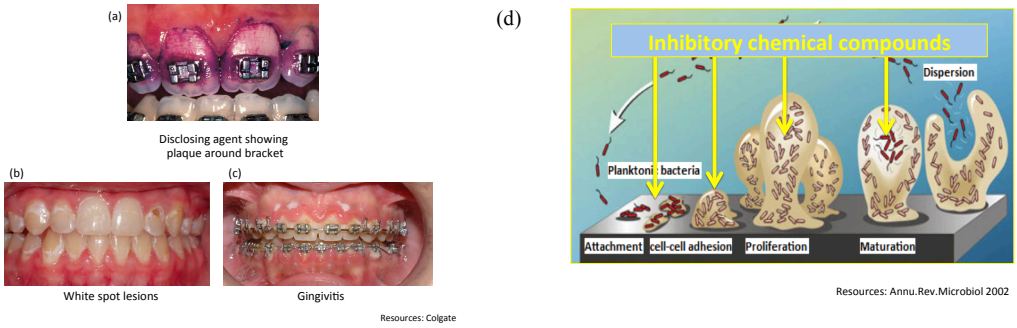

Fig. 1. Left panel: bracket-induced plaque formation (a) and its associated white spot lesions (b) and gingivitis (c); right panel: d) biofilm formation during bracket-induced plaque formation

As illustrated in Fig. 1d, bracket-induced plaque formation involves complex microbial adherence and biofilm formation that are heavily depended on polypeptides or polysaccharides on bacterial surfaces (Nobbs 2009, Peyyala R. 2013). Based on this fact, we developed an innovative working hypothesis for screening the key amino acids and sugar monomers and analogs that can interfere or block polypeptides or polysaccharides based microbial adherence and biofilm formation during bracket-induced plaque formation, as illustrated in Fig. 2.

a)

b)

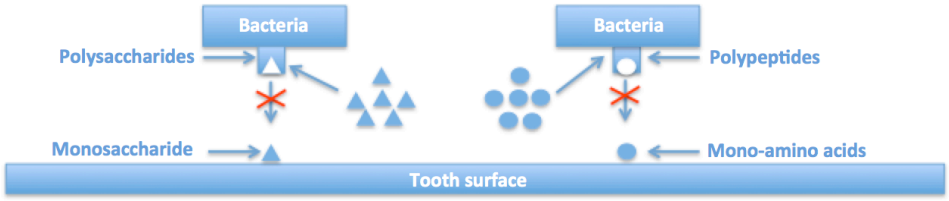

Fig. 2. The novel theory behind our working hypothesis on discovering compounds with inhibitory efforts against bracket-induced plaque formation

Methods

Culture multiwell plates and Hydroxyapatite discs

Sterile culture plate (Thermo Fisher Scientific) was used for establishing initial screening assay. Hydroxyapatite (HA) discs with bracket was used for secondary screening. HA disc used in this study were manufactured from Himed (Old Bethpage, NY). Orthodontic stainless steel bracket (3M-Unitek) was bonded to HA discs with composite (3M-Unitek).

Strains and biofilm growth conditions

Saliva stocks were used from Shi lab, which was collected by Tian and colleagues (Tian Y 2010). Saliva stock was inoculated into Shi medium at the ratio of 1:100. HA disc with bracket was sterilized under UV light for 1hr before inoculations. Amino acids, sugar monomers and analogs as potential anti-plaque compound were added into culture medium as experiment group. Control group was without adding these compounds. Plates with/without HA disc were then incubated at 37°C under anaerobic conditions for 16 hrs to allow biofilm formation. The biofilms were carefully washed twice with PBS. After the washing steps, biofilms grown on HA disc or plates were harvested for DNA extraction and PCR-DGGE analysis to obtain the bacterial profile of the original saliva.

DNA extraction and DGGE analysis

DNA was isolated using the DNeasy Blood and Tissue Kit (Qiagen Inc., Maryland, USA). Amplification of bacterial 16S genes and DGGE analysis was performed as

described in a previously published protocol (Tian Y 2010). The universal primer set Bac1 and Bac2 was used to amplify an approximately 300-base-pair (bp) internal fragment of the 16s ribosomal gene.

Crystal violet staining

Biofilms formed on the HA discs/Plastic wells were stained with crystal violet as described previously (Sharma A 2005). Briefly, HA discs/Plastic wells were washed twice with phosphate-buffered saline (PBS), air dried for 30 min, and stained with 0.4% (w/v) crystal violet in distilled water for 20 min. The stained surfaces were gently washed three times with sterilized distilled water and air dried for 30 min before being photographed. Images were taken with a D50 digital camera (Nikon, Japan). To elute the bound CV in biofilms, 200 uL of 95% ethanol was added to each well and the plate was left to stand on the bench for 30 minutes. Lastly, the plate is read with a microplate reader at 540 nm.

Formula development

After finding the inhibitory compounds that has strong inhibitory effect, different combinations of these compounds at different ratios have been tested following the same protocol as screening the inhibitory compounds. The mixture of these compounds with maximum bioactivity against bracket-induced plaque formation will be selected as new formula.

Cytotoxicity assay

Human Oral Keratinocytes-16B cells were used in this study. Media only group(with out cells), Positive Control Group (cells treated by lysis buffer), Test Group (cells treated by new formula), and Negative Control Group (cells without any treatment) were set up. The same amount of cells was attached to 96 well plates except the media only group, and the plates incubated under 37°C. Following the protocol from Promega CytoTox96-nonradioactive-cytotoxicity-assay, the cytotoxic activity from the new formula was compared to the positive control group.

Results And Discussion

Development of an in vitro bracket induced biofilm model system

As shown in Fig. 3a, brackets were adhered to small discs (13 mm in diameter) made of hydroxyapatite (HA) as the base supporting material. The culture multiwell plates or these discs fitted into 12 wells tissue culture plates were used for the growth of saliva-derived plaques. One unique feature of this study is to use a new culture medium developed in our laboratory (Shi-medium) (Tian Y 2010) which allows the *in vitro* grown biofilm very similar to original human plaque as Figure 4 shows that the microbial composition of these *in vitro* grown saliva derived plaque on discs were almost identical to original human saliva samples, indicating that this is a good screening assay which can mimic *in vivo* conditions.

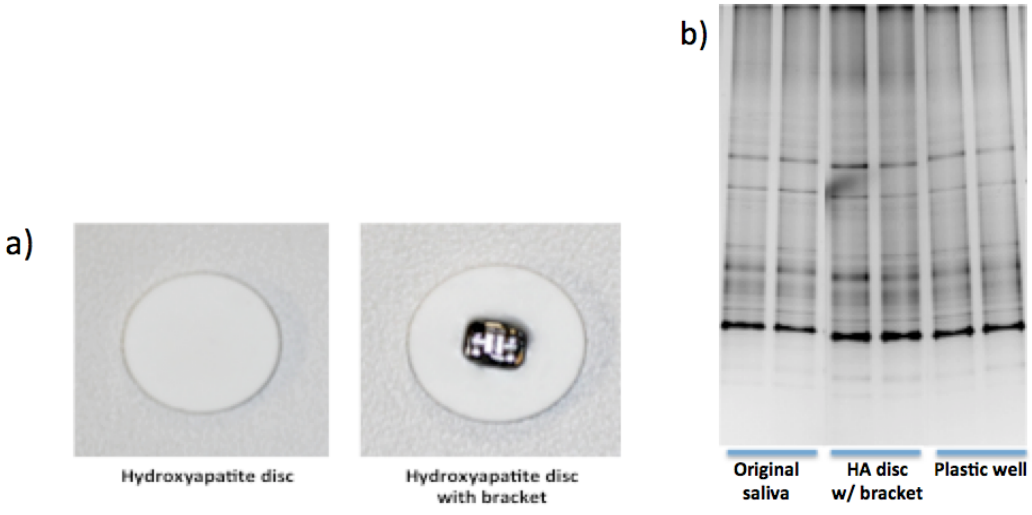

Fig. 3. a) HA discs with adhered brackets; b) Plaque microbial compositions in original saliva samples vs in vitro grown saliva derived plaque on discs analyzed by Denature Gradient Gel (DGGE)

Discovery of compounds with inhibitory efforts against bracket induced plaque formation

In vitro bracket induced biofilm model system was developed that can structurally and functionally mimic the *in vivo* clinical reality. Amino acids, sugar monomers and analogs were screened using this model system, which led to the discovery of several chemical compounds with strong inhibitory efforts against bracket-induced plaque formation. These compounds were found to be safe under laboratory safety tests and have now been formulated into toothpaste with good *in vitro* efficacy.

We used a mixed pool of human saliva samples collected from 10 volunteers to develop *in vitro* grown plaques on the model system developed above. Amino acids and sugar monomers and analogs selected chemicals mostly from FDA Generally Recognized As Safe (GRAS) list (http://www.fda.gov/Food/IngredientsPackagingLabeling/-GRAS/SCOGS/default.htm) were used for the screening. The anti-biofilm inhibitory function of these chemicals was assayed with a crystal violet assay (Sharma A 2005), as illustrated in Fig. 4a. The chemicals with more than 10X inhibitory effects (which is highly statistically significant) were selected for further studies. Several amino acids and sugars, including L-Arg, DL-Lys, DL-Ala, D-Thr and N-acyl-Glucosamine, showed the strongest inhibitory efforts (Fig. 4b).

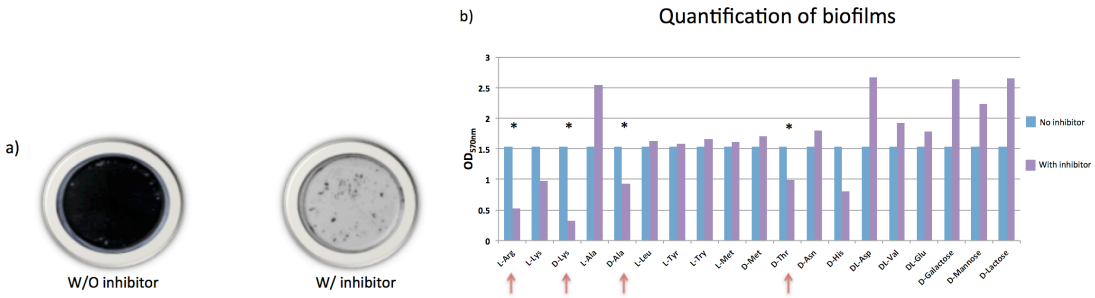

Fig. 4. a) An example showing a chemical with inhibitory effort against plaque formation in vitro; b) In vitro efficacy of the chemical mixture against plaque formation in vitro.

Formulating a toothpaste with safety and efficacy against bracket-induced plaque formation

We spent extensive efforts in combining these individual chemical compounds in different ratios to create a safe and effective mixture (L-Arg, L-Lys, L-Ala) with maximum bioactivity against bracket-induced plaque formation. This chemical mixture was then formulated into a toothpaste format with retained bioactivity as shown in Fig. 5a,5b. Additionally, we conducted *in vitro* cytotoxicity study and found no detectable toxicity issues.

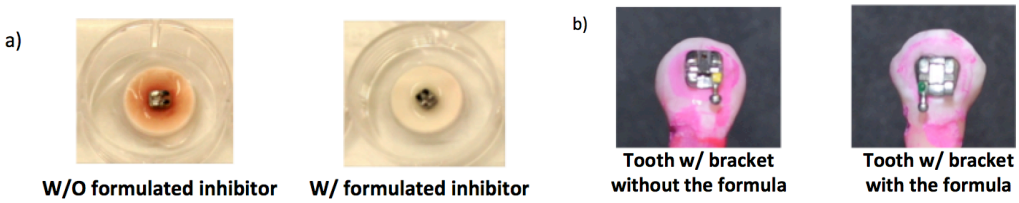

Fig. 5. a) An example showing chemical with inhibitory effort against bracket induced plaque formation in vitro; b) In vitro efficacy of the chemical mixture against bracket induced plaque formation in vitro.

In summary, using our developed screening assay, we discovered several chemical compounds with strong inhibitory efforts against bracket-induced plaque formation. These compounds were found to be safe under laboratory safety tests and have now been formulated into toothpaste with good *in vitro* efficacy. Its possible *in vivo* clinical efficacy is being explored in the following chapter.

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Part III

Chapter II

Ongoing study and future study

Ongoing study 1: Testing the clinical efficacy of the newly developed toothpaste

Following up with previous study in chapter I, the newly developed formula with anti-plaque activity has been formulated into a toothpaste. The newly developed toothpaste will be tested in the coming randomized double-blinded placebo-control pilot clinic study. The goal of this pilot clinic study is to investigate how the use of a commonly used toothpaste (Crest Regular) and this new toothpaste for their effects on plaque accumulation/gingival index and oral bacteria in orthodontic patients. Performing a study specifically in orthodontic patients is important to better understand plaque accumulation and oral health changes induced by brackets. The study will enroll 60 adolescent subjects (10-15 years of age) into the study. Prior to commencing enrollment of subjects, a safety only cohort of 4 adolescent subjects will be conducted first. For this study, clinic visits for subjects include Initial Visit 1 (day 0), and Follow-up Visits (Days 7 and 14). Clinic visits for Study subjects include Initial Visit 1 (day 0), and Follow-up Visits (Days 7 and 14). Subjects enrolled in the Adolescent Safety Only Cohort will be assessed for safety parameters only. Safety monitoring at day 0, 7 and 14 will include vital signs, intraoral assessments of hard and soft tissues, targeted physical examination, and collection of adverse events during study visits and unscheduled telephone contacts. If no safety concerns are identified, enrollment of adolescent subjects receiving 14 days of study materials administration will be initiated. The study will compare the study material administration vs placebo. The study subjects will be randomly assigned into two groups (treatment or placebo) using an interactive web response system (IWRS). Before dosing of study material, eligible subjects will undergo professional dental examination,

consultation and plaque examination using Trace Plaque Disclosing Liquid (DentaKit Item #: DK-R719). Quantitative plaque scores will be recorded according to Quigley-Hein plaque index. Subjects will then be subjected to plaque removal at the dental chair and then to be provided study materials (take-home toothpaste and Oral-B toothbrush) to be used at home twice a day over the next 14 days. To evaluate the anti-plaque effort, study subjects will be recalled to the clinic and examined for bracket-induced plaque formation using the same Plaque Disclosing Liquid (DentaKit Item #: DK-R719) based protocol at day 7, 14 and 30 respectively. This pilot clinical study outlined in this proposal will validate the clinical safety and efficacy of this newly formulated toothpaste against bracket-induced plaque accumulation, providing a new tool against bracket-induced dental diseases and greatly improving orthodontic care. The positive clinical data would further validate the working hypothesis, which could be used to develop additional therapeutic tools against other plaque related dental diseases.

After extensive interactions with FDA office and UCLA Office of the Human Research Protection Program, we now successfully achieved IRB approval (IRB# 17-000474) and will start the pilot clinical study in the coming weeks.

Ongoing study 2: Understanding the microbiome changes during orthodontic treatments and their possible connections with dental diseases

Orthodontic treatment with appliances such as metal or ceramic braces, or the newer clear aligners, can result in an imbalance in this ecosystem and lead to an increased risk of

white spot lesions, caries, or periodontal disease. While it is well known that orthodontic treatment can lead to these complications, the underlying microbial causes have not been well studied, including metal/ceramic braces and clear aligners. In this ongoing study, we investigate the microbial community in plaque and saliva of orthodontic patients, and analyze how the microbial community changes over time, before and after orthodontic treatment. We hypothesize that there will be significant changes in the balance of microbial species during orthodontic treatment. Increased understanding of the microbial species present during orthodontic treatment, and how orthodontic treatment changes the oral microbial community, will provide the base for further studying molecular mechanism of these braces-associated oral microbial diseases, enhance the ability of dentists to develop more targeted approaches for the treatment of braces-associated diseases, and lead to better prevention for orthodontic-related complications.

In this ongoing study, 300 orthodontic patients presenting to the UCLA orthodontic clinic will be recruited. Patients currently have not yet commenced treatment will be identified and recruited.

The inclusion criteria is:

1) Currently a patient at the orthodontic clinic at UCLA.

2) Actively using or wearing, or in preparation to wear or use, one of the following orthodontic appliances: metal brackets, ceramic brackets, or clear aligners.

3) Males and females, older than 9 years old, inclusive, at the time the Assent and

Informed Consent Form is signed.

4) Participants will be in good general health according to their medical and dental history, and clinical judgment of the clinical investigator.

5) Participants will be able to understand the study and study procedures; the parents of subjects who are under 18 will be able to understand the Information Sheet and provide Oral Consent.

6) Participants will be considered for participation regardless of ethnicity or gender.

7) Participants will be able to communicate with the investigator, understand and comply with the instructions.

The exclusion criteria is:

1) Participants who have active caries lesion(s) and generalized rampant dental caries.

2) Participants with chronic systemic diseases or medical conditions that will influence their ability or compromise normal immune function to participate in the proposed study (i.e. cancer treatment, HIV, rheumatic conditions, history of oral candidiasis)

3) Participants who have open sores or ulcerations in the month.

4) Participants who have significantly reduced saliva production.

5) Participants who have had radiation therapy to the head and neck region of the body.

6) Participants who smoke.

7) Participants who underwent anti-inflammatory, scaling and root planning therapy within the last six months.

8) Participants who are pregnant.

9) Participants who currently use antibiotics or have used in the last 30 days.

10) Participants who, in the opinion of the investigators, would not comply with the study procedures.

11) Participants who have advanced periodontal disease.

At each regularly scheduled orthodontic visit thereafter, including follow-up visits after orthodontic treatment is completed, plaque and saliva will be collected, plaque index and gingiva index will be recorded. Plaque and saliva samples will be analyzed by 16S rRNA gene 454 sequencing-based analysis at comprehensive genus and species level.

This study has been approved by Institutional Review Board (IRB#16-001258). So far, I have already recruited and followed up with 8 Invisalign patients and 27 metal brackets patients. In future, the new orthodontics residents and PhD students in Dr. Shi's lab will continue recruiting more patients into this study and further investigate if the biofilms present on different time point are distinct, if biofilm communities during orthodontic treatment are having disease-associated factors and microorganisms. This study will provide the base for further study of molecular mechanism of these braces-associated oral microbial diseases and also enhance the ability of dentists to develop more targeted approaches for the treatment of braces-associated diseases.

CONCLUSION REMARKS

The main accomplishment of this dissertation is to apply molecular biology tools to address basic scientific issues as well as clinic issues related to dental plaques. First part of my thesis successfully investigated the function of membrane protein PrsA which is involving in protein transportation of *S. mutans*. In the second part of my thesis, we were the first group to have characterized the denture-associated microbiota and found the microbial cellular components involving into the possible association of denture-related halitosis and stomatitis. In the third part of my thesis, I have contributed to develop a chemical formula and toothpaste with anti-plaque effects that is being tested for its *in vivo* clinical efficacy in near future. We can envision several future research directions based on the current study. 1) In denture study, the discovered cellular components involved in the inter-species interaction could be the potential therapeutic targets for further study. 2) In orthodontic study, after characterizing the profile of oral microbiome in orthodontic patients, identification of relevant disease-associated pathogens would allow us to better manage dental plaque associated diseases during orthodontic treatments.