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ABSTRACT

The oral microbiome consists of a planktonic microbiome residing in saliva and an adhering microbiome (the biofilm adhering to oral hard and soft tissues). Here we hypothesized that possible differences in microbial composition of the planktonic and adhering oral microbiome on teeth can be related to the forces by which different bacterial species are attracted to the tooth surface. The relative presence of 7 oral bacterial species in saliva and biofilm collected from 10 healthy human volunteers was determined twice in each volunteer by denaturing-gradient-gel electrophoresis. Analysis of both microbiomes showed complete separation of the planktonic from the adhering oral microbiome. Next, adhesion forces of corresponding bacterial strains with saliva-coated enamel surfaces were measured by atomic force microscopy. Species that were found predominantly in the adhering microbiome had significantly higher adhesion forces to saliva-coated enamel (-0.60 to -1.05 nN) than did species mostly present in the planktonic microbiome (-0.40 to -0.55 nN). It is concluded that differences in composition of the planktonic and the adhering oral microbiome are due to small differences in the forces by which strains adhere to saliva-coated enamel, providing an important step in understanding site- and material-specific differences in the composition of biofilms in the oral cavity.

KEY WORDS: atomic force microscopy, denaturing gradient gel electrophoresis, biofilm, dental plaque, saliva, bacterial adhesion.

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Adhesion Forces and Composition of Planktonic and Adhering Oral Microbiomes

INTRODUCTION

The oral microbiome is a highly diverse and intricate system, consisting of more than 700 bacterial strains and species (Aas *et al.*, 2005), although remarkably higher estimates of the microbial diversity in the oral cavity have been reported as well (Keijser and Zaura, 2008). The oral microbiome is different for every individual and is specific to different sites and materials in the oral cavity. Minor disturbances in the delicate balance of the oral microbiome can lead to the onset of disease, such as caries and periodontitis (Marsh, 2006).

Oral bacteria reside either in a planktonic state, as in saliva, or in an adhering state, as in a biofilm on oral hard and soft surfaces. Although the oral microbiome is site-, material-, and subject-specific, there is the concept of a general core microbiome that plays an important role in the formation of oral biofilm (Aas *et al.*, 2005; Zaura *et al.*, 2009). The formation of an oral biofilm is initiated by the adhesion of initial colonizers (Hojo *et al.*, 2009; Kreth *et al.*, 2009; Kolenbrander *et al.*, 2010) to adsorbed salivary-conditioning films. As such, oral biofilm can be regarded as a transition of bacteria from the planktonic microbiome to the adhering microbiome on oral hard and soft surfaces. This transition is mediated by an interplay between ligand-receptor interactions and non-specific Lifshitz-Van der Waals, acid-base, and electrostatic forces.

Bacterial-probe atomic force microscopy (AFM) has enabled investigators to measure the forces by which bacteria are attracted to surfaces, and relatively small differences in adhesion forces have been demonstrated to have major implications with respect to the ability of bacteria to adhere to a surface (Liu *et al.*, 2006). Such observations led us to hypothesize that differences in the composition of the planktonic and adhering oral microbiome on oral hard surfaces can be related to the force by which different bacterial strains and species are attracted to the tooth surface.

The aim of this study was to verify the hypothesis that differences in the composition of the planktonic microbiome and adhering oral microbiome on oral hard surfaces can be related to the force by which different bacterial strains and species are attracted to the tooth surface. To this end, we first determined the strains and species predominantly present in the planktonic and adhering microbiomes of 10 healthy volunteers, using denaturing-gradient-gel electrophoresis (DGGE), and related their prevalence to the average adhesion forces of strain representatives of different species to saliva-coated enamel measured with bacterial-probe AFM.

MATERIALS & METHODS

Participants and Inclusion Criteria

Ten healthy volunteers (six females and four males, aged between 24 and 57 yrs) participated in this study. The study design was approved by the Medical Ethical Testing Committee of the University Medical Center Groningen (METc 2011/330), and all participants signed a declaration of informed consent. All volunteers considered themselves in good health and had a dentition with at least 16 natural elements. Volunteers who had used antibiotics up to 3 months prior to the study or had used a mouthrinse in the month prior to the study were excluded. Two wks before entering the study, volunteers were requested to brush their teeth according to their habitual oral hygiene, but the use of a standard, fluoridated toothpaste without antimicrobial claims (Prodent Softmint, Sara Lee Household & Bodycare, The Hague, The Netherlands) was imposed, as during the entire study.

Sample Collection

Biofilm

Volunteers were asked to come to the laboratory for biofilm collection immediately after breakfast, without having brushed their teeth. Biofilm was collected, by means of a sterile hook and a cotton swab, from the left upper quadrant of the dentition (buccal, palatal, occlusal, and interproximal sides of the dentition). Biofilm was suspended in 1 mL sterile Reduced Transport Fluid (RTF) (Syed and Loesche, 1972), and all biofilm samples were immediately put on ice after collection and sonicated for 10 sec at 30 W (Vibra Cell model 375, Sonics and Materials Inc., Danbury, CT, USA) to suspend bacterial clumps. The samples were stored in RTF at -20°C for subsequent DGGE analysis.

Saliva

Volunteers were requested to collect 2 mL of unstimulated whole saliva. Saliva samples were put directly on ice after collection and sonicated 2 times for 10 sec at 30 W. Subsequently, samples were centrifuged at 18,000 g for 5 min (Eppendorf Centrifuge 5417R, Hamburg, Germany), supernatant was removed, and the pellet was re-suspended in 200 μ L TE buffer (10 mM Tris HCl, pH 7.5, 1 mM EDTA) and stored at -20°C for subsequent DGGE analysis.

Biofilm and saliva samples were taken twice from each volunteer, with a 6-week interval in between, and treated as separate samples.

DGGE Analyses of Biofilm and Saliva Samples

DNA extraction, PCR, and DGGE analysis are described in online Appendix I. To compare the gels, we added reference markers containing various species representing the oral microbiome in health and disease, namely: *Streptococcus mutans* ATCC10449, *Streptococcus sanguinis* ATCC10556, *Streptococcus sobrinus* ATCC33478, *Streptococcus salivarius* HB, *Streptococcus mitis* ATCC9811, *Streptococcus oralis* ATCC35307, and an isolate of *Lactobacillus* sp.

Gelcompar II (v6.5 Applied Maths, Sint-Martens-Latem, Belgium) was used for gel analysis. The presence of reference

species in all samples was analyzed by band-based matching, with 0.5% optimization and 0.5% band tolerance as accuracy settings. The presence of a band was taken as indicative of the presence of the reference species in the sample, regardless of the staining intensity. Dice's similarity coefficient was used to construct a similarity matrix. A dendrogram was calculated based on the non-weighted pair group method with arithmetic averages as a clustering algorithm (Signoretto *et al.*, 2010).

Atomic Force Microscopy Measurements

We used AFM to measure adhesion forces of selected bacterial strains to saliva-coated enamel surfaces. Nine oral bacterial strains, consisting of a combination of different laboratory strains and clinical isolates representative of the oral microbiome, were included in AFM force measurements: *S. mutans* ATCC700610, *S. mutans* NS, *S. sanguinis* ATCC10556, *S. sobrinus* HG1025, *S. salivarius* HB, *S. mitis* BMS, *S. mitis* ATCC9811, *S. oralis* J22, and *Lactobacillus acidophilus* JP and a *Lactobacillus* isolate. Bacteria were grown on blood-agar plates for 24 hrs at 37°C from frozen DMSO stock and inoculated in 10 mL Todd-Hewitt broth (Oxoid, Basingstoke, UK) for 24 hrs at 37°C in ambient air. The pre-culture was used to inoculate a main culture which was grown for 16 hrs. Bacterial harvesting was done by centrifugation (5,000 g, 5 min). Subsequently, the pellet was washed twice and re-suspended in demineralized water. Bacterial clumps were suspended by sonication for 3 x 10 sec at 30 W.

Human whole saliva, stimulated by the chewing of parafilm, was collected from both genders in ice-cooled beakers. All volunteers gave their informed consent, agreeing to the rules as stated by the Medical Ethical Testing Committee of the University Medical Center Groningen (letter 06-02-2009). The saliva was pooled, centrifuged to remove particulate debris, dialyzed against demineralized water, and subsequently lyophilized for storage. Lyophilized saliva was reconstituted at a concentration of 1.5 g/L in adhesion buffer (50 mM potassium chloride, 2 mM potassium phosphate, 1 mM calcium chloride, pH 6.8).

Enamel slabs were cut (0.6 x 0.6 x 0.2 cm) from the buccal surfaces of bovine incisors, ground, and polished, as described in online Appendix II. We created salivary-conditioning films by immersing the enamel slabs in reconstituted saliva for 16 hrs at 4°C. Before use in AFM experiments, the slabs were dipped 3 times in demineralized water to remove excess saliva.

Tipless AFM cantilevers (MP-010, Bruker, Billerica, MA, USA) with immobilized bacteria were prepared, and AFM was performed (BioScope Catalyst AFM, Bruker), as described in online Appendix III. For each combination of bacterial strains, at least 40 force-distance curves were recorded at randomly chosen spots with 2 to 4 bacterial probes and bacteria from at least 2 different cultures of each strain (for an example of a force-distance curve, see Appendix Fig. 1).

Statistics

DGGE profiles were assessed based on the predominant presence of bacterial species in the planktonic or adhering microbiome. Therefore, a three-way mixed-effects analysis of variance (ANOVA) model was fitted to the bacterial presence among

Figure 1. Clustering tree from the DGGE profiles of the adhering and planktonic oral microbiomes, indicated as “biofilm” and “saliva”, respectively, as taken from ten healthy volunteers, on 2 different occasions from each volunteer (individual volunteers are indicated by numbers). Clustering tree was based on a band-based percentage similarity matrix.

volunteers to investigate the effect of the microbiome for each species separately at a significance level of .05. The relative presence of species (adhering microbiome minus planktonic microbiome) in the model was taken at random to be able to overcome the sparse data for the relative large number of species, and to address possible correlations, the microbiome and period were taken as fixed effects. The relative presence of species was estimated and accompanied with a 95% confidence interval. The residuals were investigated to evaluate the model fit.

Adhesion forces of individual bacterial strains displayed a skewed distribution (Shapiro-Wilk test, $p < .01$) and are presented as medians. Species-averaged adhesion forces were calculated as weighted averages over the different strains used in AFM to represent a given species and are presented as means with standard errors. SAS v9.3 (SAS Institute Inc., Cary, NC, USA) and SPSS v20.0 (IBM Corp., Armonk, NY, USA) were used to conduct statistical analysis.

RESULTS

The planktonic and adhering oral microbiomes of nine out of the ten volunteers separated at the main branch of the clustering tree (Fig. 1) of their DGGE profiles (Appendix Fig. 2) at 36% similarity to each other. Samples taken on different occasions from each volunteer showed remarkably high similarities, on average 75%, indicating no periodic carry-over effects.

We further analyzed this difference in microbial composition between the microbiomes by determining the species predominantly present in the adhering and planktonic microbiomes (Fig. 2). *S. mutans*, *S. sanguinis*, and *Lactobacillus* strains were predominantly present in the adhering microbiome of the volunteers. *S. mutans* was not detected in saliva samples of any of the volunteers, but was present in biofilm samples of 50% of all volunteers. *S. sanguinis* was present in 30% of all saliva samples, but was found in 85% of all biofilm samples. No

Figure 2. The percentages of volunteers carrying specific bacterial species in their adhering and planktonic oral microbiomes, indicated as “biofilm” and “saliva”, respectively. Error bars denote SD over ten volunteers, each measured on 2 occasions. Statistically significant differences in species occurrence in saliva and biofilm ($p < .05$) according to a three-way mixed-effects ANOVA model, corrected for periodic effects, are indicated by an asterisk (*). The residuals of the model do not demonstrate outliers, and the distribution is close to normal, implying an appropriate description of the observed bacterial presence.

Lactobacillus strains were found in any of the saliva samples and only in 30% of all biofilm samples.

Bacteria significantly more present in saliva samples were identified as *S. sobrinus* (60% in saliva samples vs. 35% in biofilm samples) and *S. salivarius* (90% in saliva samples vs. 35% in biofilm samples). In nine out of ten volunteers, *S. mitis* and *S. oralis* were found in both microbiomes.

The relative presence (percentage of volunteers with the species present in the adhering microbiome minus the percentage occurrence in the planktonic microbiome) of different species toward the adhering microbiome increased with their adhesion forces to saliva-coated enamel (Fig. 3). For species predominantly present in the adhering microbiome, species-averaged adhesion forces varied from -0.60 nN to -1.05 nN. *S. sanguinis* and *Lactobacillus* sp. showed the highest adhesion forces, followed by *S. mutans*. Bacteria predominantly present in the planktonic microbiome, *S. salivarius* and *S. sobrinus*, had lower species-averaged adhesion forces between -0.40 and -0.55 nN. Bacterial species without a predominant presence in either microbiome, *S. mitis* and *S. oralis*, had adhesion forces in the range of -0.60 nN to -0.80 nN. Note that in online Appendix Fig. 3, we present the adhesion forces of the individual strains used in AFM to represent a given species and obtain species-averaged adhesion forces.

DISCUSSION

This paper demonstrates the validity of our hypothesis that differences in the composition of planktonic and adhering oral microbiome on oral hard surfaces are related to the forces by which different bacterial species adhere to the tooth surface, i.e., saliva-coated enamel. Bacterial adhesion forces were higher for species predominantly residing in the adhering microbiome than for species mostly present in the planktonic microbiome. To the

best of our knowledge, this is the first time that such an influence of bacterial adhesion forces on the composition of oral biofilm vs. bacterial composition in saliva has been demonstrated. As an important additional observation, residence in the planktonic or adhering microbiome appears to be dictated by species-averaged adhesion forces that range between -0.40 and -1.05 nN. Species with adhesion forces stronger than -0.55 nN end up in the adhering microbiome, while species with smaller adhesion forces become members of the planktonic microbiome. A very similar adhesion-force value of -0.5 nN has been suggested to dictate whether *E. coli* would become a predominant member of the adhering urogenital microbiome (Liu *et al.*, 2006). It is amazing how such small differences in bacterial adhesion forces can select strains from saliva to become members of the adhering microbiome. Earlier, it had already been found that initial colonizers of dental hard surfaces possess adhesion forces to saliva-coated enamel that are only 0.1 nN stronger than those of later-colonizing, more cariogenic strains (Mei *et al.*, 2009). With respect to *Staphylococcus aureus* strains, a difference in adhesion force of 0.28 nN appears to dictate whether or not a strain can invade mammalian cells (Yongsunthorn *et al.*, 2007). Such observations endorse the concept that bacteria adhere to a surface according to their own specific characteristics, such as their specific hydrogen-bonding capability and ability to form ligand-receptor bonds (Gibbons *et al.*, 1985; Nobbs *et al.*, 2011). In the first instance, it appears as a limitation of our study that other interactions between bacteria, such as co-adhesion between different strains and species, are not included in the analysis. However, selection of appropriate co-adhesion partners also seems governed by the magnitude of the adhesion forces between the co-adhesion partners (Postollec *et al.*, 2006), similar to how the adhering microbiome arises from adhesion-force-governed selection from the planktonic microbiome, as we show in this article.

The complete separation of the adhering and planktonic oral microbiomes as found in our study (Fig. 1) is in line with literature results, showing that biofilm and saliva samples display statistically significant clustering profiles, with no significant differences between PCR-DGGE profiles of males and females (Ling *et al.*, 2010). *S. mitis* and *S. oralis* were found in 90% of our volunteers (Fig. 2) and are indeed known to account for a major proportion of all *Streptococcus* spp. (Diaz *et al.*, 2012) in the oral cavity. Note that DNA analysis in PCR-DGGE can yield multiple and overlapping bands (Janse *et al.*, 2004; Kušar and Avguštin, 2012), as in the present case for *S. mitis* and *S. oralis*, making it impossible to discriminate between these 2 species (Appendix Fig. 2). Levels of *S. mutans* and *S. sobrinus* in the adhering microbiome relative to each other are in line with reports in the literature, where the latter is rarely found in higher numbers than *S. mutans* (Beighton, 2005; Mäkinen *et al.*, 2005). Finally, the complete absence of subgingival species like *Porphyromonas gingivalis* in all samples (online Appendix Fig. 2) is supported by literature as well (Aas *et al.*, 2005). This agreement with literature data demonstrates that the group of volunteers used can be considered as a representative sample and justifies its use to support evidence for our hypothesis that the composition of the adhering microbiome is determined by the adhesion-force values of different strains and species.

Figure 3. The relative presence of different species in human volunteers as a function of the species-averaged adhesion force to saliva-coated enamel surfaces, separated with respect to their predominant occurrence in the planktonic (saliva) or adhering (biofilm) microbiome. Adhesion forces are displayed as mean values over all representatives of a given species used in the AFM part of this study with standard errors. Linear regression was significant at $p < .05$, and 95% confidence intervals of the mean are indicated by 2 outer lines.

Interestingly, some species (*S. mutans*, *Lactobacillus* sp.) were found only in the adhering microbiome on oral hard surfaces, whereas the planktonic microbiome naturally constitutes the species' origin. This supports our conclusion that the ability of a strain to adhere determines its presence in either the adhering or planktonic microbiome. Evidently, in cases of strains present below the detection limit of DGGE (Ashimoto *et al.*, 1996), stronger adhesion forces lead to selection, followed by a detectable presence of these strains in the adhering microbiome on oral hard surfaces. These observations support ongoing discussion in the literature that the microbial composition of saliva is of minimal value for the prediction of dental caries and should be used only as an associative instead of a causal relationship (Van Houte, 1993; Van Palenstein Helderman *et al.*, 2001; Gamboa *et al.*, 2004; Beighton, 2005).

The correlation between adhesion forces and the predominant presence of bacterial strains and species in either the salivary or adhering microbiome, demonstrated in this research, is new and provides an important step in our understanding of the site- and material-specific differences in composition of biofilms in the oral cavity.

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