Heritability of Oral Microbial Species in Caries-Active and Caries-Free Twins

Patricia M. Corby,[1] Walter A. Bretz,[1] Thomas C. Hart,[3] Nicholas J. Schork,[2] J Wessel,[2] James Lyons-Weiler,[4] and Bruce J. Paster[5]

[1] *New York University College of Dentistry, New York, United States of America*
[2] *Scripps Genomic Medicine and The Scripps Research Institute and Center for Human Genetics and Genomics, University of California, San Diego, United States of America*
[3] *NIDCR/NIH, Division of Intramural Clinical Research, Bethesda, United States of America*
[4] *University of Pittsburgh, School of Medicine, Pittsburgh, United States of America*
[5] *The Forsyth Institute, Boston, United States of America*

Oral microbes that colonize in the mouths of humans contribute to disease susceptibility, but it is unclear if host genetic factors mediate colonization. We therefore tested the hypothesis that the levels at which oral microbes colonize in the mouth are heritable. Dental plaque biofilms were sampled from intact tooth surfaces of 118 caries-free twins. An additional 86 caries-active twins were sampled for plaque from carious lesions and intact tooth surfaces. Using a reverse capture checkerboard assay the relative abundance of 82 bacterial species was determined. An integrative computational predictive model determined microbial abundance patterns of microbial species in caries-free twins as compared to caries-active twins. Heritability estimates were calculated for the relative microbial abundance levels of the microbial species in both groups. The levels of 10 species were significantly different in healthy individuals than in caries-active individuals, including, *A. defectiva, S. parasanguinis, S. mitis/oralis, S. sanguinis, S. cristatus, S. salivarius, Streptococcus* sp. clone CH016, *G. morbillorum* and *G. haemolysans.* Moderate to high heritability estimates were found for these species (h^2 = 56%–80 %, $p < .0001$). Similarity of the overall oral microbial flora was also evident in caries-free twins from multivariate distance matrix regression analysis. It appears that genetic and/or familial factors significantly contribute to the colonization of oral beneficial species in twins.

Oral microbial species that populate and colonize the human mouth are known to contribute to disease (Loesche, 1986; Socransky & Haffajee 2005). The factors mediating levels of colonization are largely undocumented, but are likely to include factors that influence the host environment. Thus, it may be the case that inherited genetic factors that shape the human oral environment (e.g., amount and constituency of saliva, innate immune factors, etc.) influence the levels and patterns of colonization of oral microbial species. We therefore pursued an assessment of the heritability of the levels of colonization of a number of important oral microbial species using twin models (Neale et al., 1999).

We previously reported evidence from twin studies that genetic factors contribute to the salivary levels of mutans streptococci, a major caries pathogen (Corby et al., 2005a). A number of genetic factors including HLA genes have been reported to influence salivary mutans streptococci colonization and caries susceptibility in human and animal models (Abdus Salam et al., 2004; Acton et al., 1999; Ozawa et al., 2001; Suzuki et al., 1998). HLA genes are involved in a wide spectrum of immunologic responses, and are thus quite likely to play a substantial role in oral colonization and the establishment of the mutans streptococci in the oral environment (Wallengren et al., 2005).

It is also likely that indigenous oral microflora modulate risk for disease (Guarner & Malagelada, 2003). While disease-associated flora are recognized for a variety of conditions, we hypothesized that the indigenous microflora of caries-free children may be protective, thereby decreasing the risk for dental caries onset. In this light, we previously reported that caries susceptibility may be explained by the relative overabundance and/or underabundance in levels of disease-associated and health-associated microflora (Corby et al., 2005b). The purpose of this study was to assess the heritability of colonization of oral microbial species in biofilms of the primary dentition using twin models. Our findings suggest that many oral microbial species appear to be highly heritable. In addition, the overall oral microbial environment

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Address for correspondence: Patricia M. A. Corby,New York University, College of Dentistry, 345 East 24th Street, New York, NY 10010. E-mail: patricia.corby@nyu.edu

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appears to be radically different between caries-free and caries-active individuals.

Material and Methods

Subject Population and Sample

A total of 204 twins (80 monozygotic [MZ]; 124 dizygotic [DZ] twins) with an average age of 3.83 ± 2.55 years comprised the study group (Table 1). The MZ and DZ twin groups consisted of caries-free twins (n = 118, Surface-based Caries Prevalence Rate [SBCPR) = 0) and a group of highly caries-active twins with no existing restorative dental work (n = 86, mean SBCPR= 17.23% ± 10.7%; Table 2). Parents of the twins signed consent forms and the study protocol was approved by three different human subjects' institutional review boards contributing to the study.

Zygosity Assessment

DNA was extracted from peripheral venous blood samples, and zygosity was determined by genotyping all individuals for 8 highly-polymorphic DNA loci, as previously reported (Bretz et al., 2005). Briefly, polymorphic DNA marker loci were PCR amplified by standard methods, and marker allele sizes were determined using an ABI-377 automated DNA sequencer and analyzed by GENESCAN 2.1 (Applied Biosystems, Foster City, CA; Zhang et al., 2001). Zygosity determination confirmed 40 pairs of MZ twins and 62 pairs of DZ twins.

Dental Caries Examination and Plaque Sampling

Dental caries examination were performed according to NIDCR criteria (Kastle et al., 1996) modified to distinguish white spot lesions (incipient lesions) from cavitated carious lesions. Interproximal surface caries

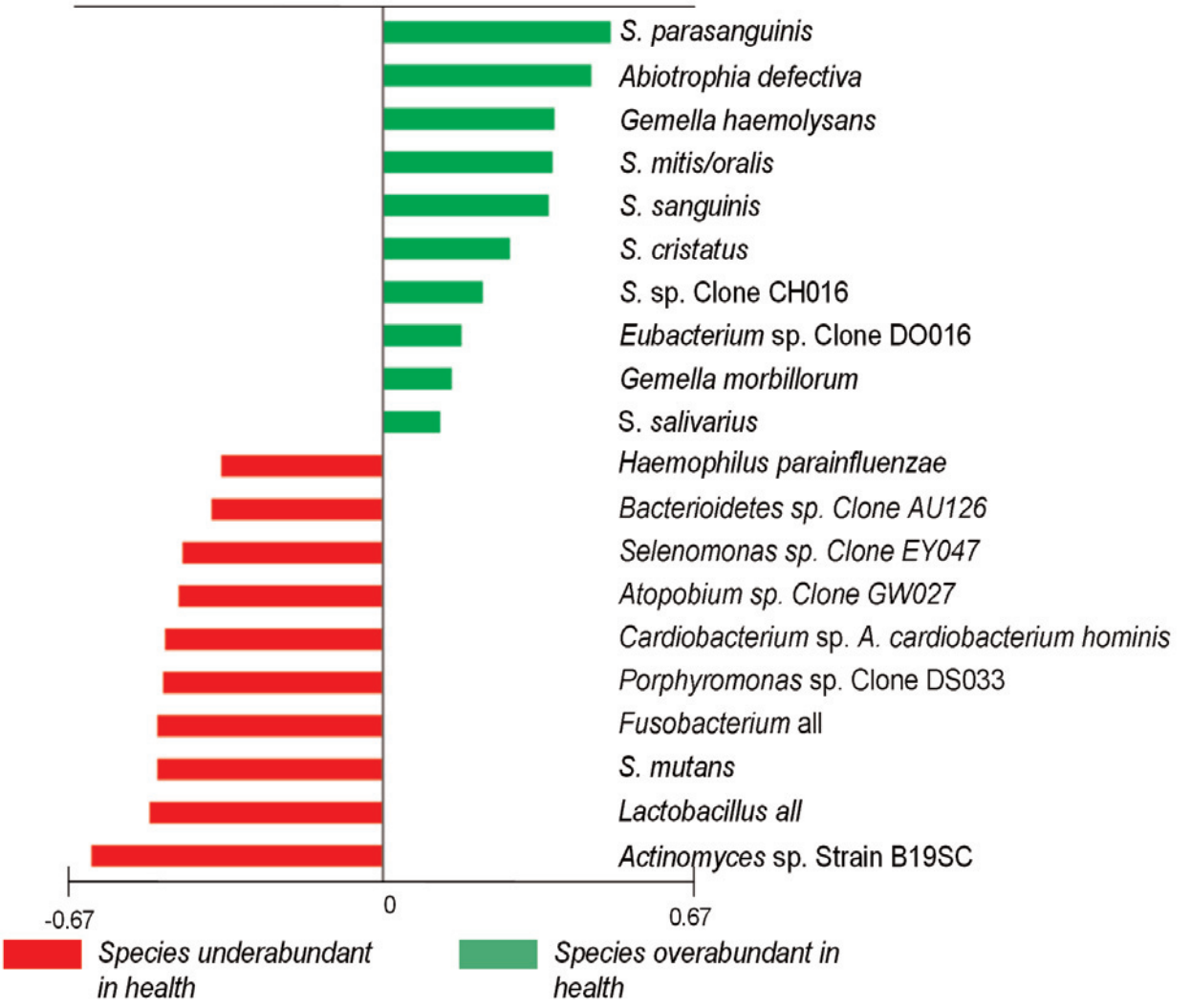

Figure 1

Significant species in caries-free twins compared to caries-active twins.

Note: Dental biofilm of caries-free subjects compared to combined sites of intact enamel, white-spot lesions, enamel lesions, and dentin lesions in caries-active subjects. Species that are overabundant in caries-free subjects relative to caries-active subjects are colored in green; species that are underabundant in caries-free subjects relative to caries-active subjects are colored in red.

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Table 1

Demographic Characteristics of Study Participants (n = 204)

	n	Mean age	Lower 95% CI	Upper 95% CI
Gender				
Female	100	3.80 (+ 2.48)	3.31	4.29
Male	104	3.77 (+ 2.46)	3.29	4.25
Zygosity				
MZ	80	3.77 (+ 2.28)	3.27	4.28
DZ	124	3.79 (+ 2.58)	3.33	4.25

Note: MZ = monozygotic twins, DZ = dizygotic twins

were assessed using digital imaging fiber-optic transillumination (DIFOTI, Irvington, NY). For caries-free twins, plaque samples were collected from three sites, including anterior and posterior teeth, which were pooled. Caries-active subjects had plaque samples collected separately (from different teeth) from three to four types of sites: surface of intact enamel, surface of white spot lesions, surface of initial enamel lesions, and excavated plaque from deep dentinal lesions. For intact enamel and white spot lesions, plaque was collected by swiping the tooth surface with a Stimudent (J&J, New York, NY). Plaque from cavitated lesions was collected by means of a sterile Gracey 1–2 curette (Hu-Friedy, Chicago, IL). A total of 448 plaque samples (118 from caries-free subjects and 330 from caries-active subjects) were used for analysis.

Isolation of Bacterial DNA

Plaque samples were placed inside a sterile microcentrifuge tube containing 50 µl of TE buffer (50 mM Tris-HCl, pH 7.6, 1 mM EDTA pH 8.0, 5% Tween 20, and 10mg/ml Proteinase K [Promega]). Samples were incubated at 55 °C for 2 h. The Proteinase K was then inactivated by heating at 95 °C for 10 min.

Amplification of Bacterial 16S rRNA Cistrons

The 16S rRNA genes were amplified under standard conditions using a universal forward primer and a universal reverse primer (Paster et al., 1998). One µl of the DNA template was added to 22.5µl of a PCR SuperMix (Invitrogen, Carlsbad, CA).

Checkerboard Hybridization

The abundance (levels) of 82 oral bacterial species or groups were detected using the reverse capture checkerboard hybridization assay (Corby et al., 2005b; Paster et al., 1998). Briefly, the amplified product for each sample was PCR amplified using a forward primer labeled with digoxigenin and a universally-conserved reverse primer. Standard chemifluorescence detection was performed using the Storm system (Amersham, Piscataway, NJ). For each hybridization spot on the membranes, bacterial signal levels were extracted from their background by applying spot edge detection methodology, and signal levels were calculated independently for each spot (ImageQuant software, Amersham, Piscataway, NJ). Two lanes in each run had the universal probes to serve as standards, and signal levels were converted to mean counts by comparison with standards on the membrane. Signal levels were then adjusted for abundance by comparing them to the universal probes. This approach allowed for computing the abundance of the target species individually by adjusting the DNA concentration in each sample.

Computation of Microbial Abundance Patterns

An integrative computational predictive model determined microbial abundance patterns of microbial species in caries-free twins as compared to caries-active twins. To identify microbial species associated with health, we compared biofilms from surfaces of intact enamel in the caries-free group with biofilms from all sites combined in the caries-active group: namely, biofilms of surfaces of intact enamel, white spot lesions, dentinal lesions, and deep dentinal lesions. It is important to point out that biofilm samples were not pooled for analysis, and that instead, a ratio was created to adjust bacterial levels within species by the total number of sites collected for each participant. Gene Expression Data Analysis software (Patel & Lyons-Weiler, 2004) was used for the classification of global patterns of microbial colonization and abundance levels in the two groups. Sensitivity and specificity of the model were measured using the area under the receiver/operator curve (ROC). Methods for computational analysis of microbial expression used in this study have been reported in detail elsewhere (Corby et al., 2005b). Significant oral health-associated species predicted by this model were used for computing heritability estimates.

Heritability Analyses

Estimates of heritability ($h^2 = V_G/V_P$, where V_G is additive genetic variance and V_P is total phenotypic variance) were obtained using the variance-component methodology implemented in the Sequential Oligogenic Linkage Analysis Routines (SOLAR) package, version 8.0, available at: http://www.sfbr.org.solar/. This method utilizes maximum likelihood estimation procedures to provide estimates of V_G and V_P assuming a bivariate normal distribution of phenotypes in twin pairs. The method also estimates the mean levels of relevant variables

Table 2

Disease Status by Zygozity (n = 204)

Zygosity	Caries-Active	Caries-Free	n (%)
MZ	34 (16.64%)	46 (22.55%)	80 (39.22%)
DZ	52 (25.49%)	72 (35.29%)	124 (60.78%)
n (%)	86 (42.16%)	118 (57.84%)	204 (100.00%)

Note: MZ = monozygotic twins, DZ = dizygotic twins

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accommodating a regression model that assumes a particular set of explanatory covariates (Almasy & Blangero, 1998). The null hypothesis (H_0: $V_G = 0$) of no heritability was tested by comparing the full model, which assumes genetic variation ($V_G \geq 0$), and a reduced model, which assumes no genetic variation (i.e., $V_G = 0$), via likelihood ratio tests. All heritability estimates were adjusted for age and gender. Additionally, we adjusted a final model for age, gender and disease status (caries-free vs. caries active twins).

Regression Analysis of Similarity Matrices

We also pursued Multivariate Distance Matrix Regression (MDMR) analyses of the oral health-associated microbial species abundance data (Zapala & Schork, 2006). Distance-based analyses are used routinely in the ecological literature to assess differences in species abundance between different ecological niches (MacArdle & Anderson, 2001; Legendre & Legendre, 1998). MDMR analysis can be used to relate variables to a large set of species abundance data, or more specifically, multivariate species abundance profiles, and hence was used by us to test hypotheses such as whether or not individuals that are caries-free have a more similar overall oral microbial species profile than individuals that are caries active, and whether or not younger children had a more similar oral microbial species profile than older children. Since MDMR is framed in standard linear models frameworks, we could also explore the relationship of multiple variables on species abundance profiles. To assess the distance/similarity of subjects with respect to oral microbial species profile we employed the Bray-Curtis distance measure (Legendre & Legendre, 1998).

Results

In order to determine if there were differences in the levels of individual microbial species present in the biofilm of caries-free subjects and in biofilms from caries–active subjects, analysis of global patterns in bacterial abundance levels were performed. The levels of about 20 species were significantly different in the caries-active and caries-free groups (Figure 1). The achieved classification error was statistically significant under ROC (Specificity = 0.84, Sensitivity = 0.85). Ten health-associated species were found to be significantly more abundant in the caries-free group

Table 3

Heritability Estimates of Microbial Species in Caries-Active and Caries-Free Children

	Caries-Active (n = 86)			Caries-Free (n = 118)			All (n = 204)		
Strain	h^2	SE	*p* value	h^2	SE	*p* value	h^2	SE	*p* value
Actinomyces sp strain B19SC	72.88	10	< . 0001	47.30	.12	.0011	72.96	.06	< .0001
S. mutans	46.85	.16	.0100	29.05	.16	.0459	35.26	.11	.0029
Lactobacillus all	13.36	.17	.2256	NC			22.59	.15	.0743
Fusobacterium all	52.83	.29	.0562	NC			NC		
Cardiobact sp. A. Cardiobact hominis	51.23	.14	.0021	NC			81.73	.04	< .0001
Selenomonas sp. Clone AA024	14.56	.25	.2817	NC			25.79	.11	.0126
Porphyromonas sp. Clone DS033	57.33	.14	.0014	NC			30.89	.12	.0069
Atopobium sp clone GW027	NC			NC			74.18	.05	< .0001
Haemophilus parainfluenzae	16.48	.20	.2102	22.02	.14	.0704	58.58	.08	< .0001
Bacterioidetes sp clone AU126	20.75	.20	.1604	NC			53.79	.09	< .0001
S. parasanguinis	53.23	.13	.0011	63.73	.09	< .0001	58.56	.07	< .0001
Abiotrophia defectiva	45.45	.12	.0017	43.32	.13	.0023	57.28	.08	< .0001
Gemella haemolysans	29.73	.17	.0561	33.17	.13	.0127	59.84	.08	< .0001
S. mitis/oralis	62.60	.10	< .0001	63.00	.08	< .0001	60.54	.07	< .0001
S. sanguinis	64.46	.11	< .0001	71.22	.08	< .0001	59.94	.07	< .0001
S. sp. group H6	90.99	.03	< .0001	63.48	.10	< .0001	61.37	.08	< .0001
S. cristatus	14.82	.18	.2082	56.38	.12	.0002	56.14	.08	< .0001
S. sp. clone CH016	89.64	.04	< .0001	62.07	.12	.0001	59.47	.08	< .0001
Eubacterium sp. Clone DO016	5.19	.19	.3932	42.04	.14	.0041	30.02	.11	.0085
Kingella sp caries clone DE012/ Neisseria polysaccharea	74.07	.09	< .0001	45.83	.12	.0012	31.12	.11	.0045
Gemella morbillorum	72.73	.11	.0003	58.95	.11	< .0001	79.97	.04	< .0001
S. salivarius	45.45	.18	.0132	41.14	.13	.0004	58.47	.08	< .0001

Note: h^2, heritability, NC, not calculable due to undetectable levels.

[a] Heritability estimates adjusted for age and gender

[b] Heritability estimates adjusted for age, gender and disease status

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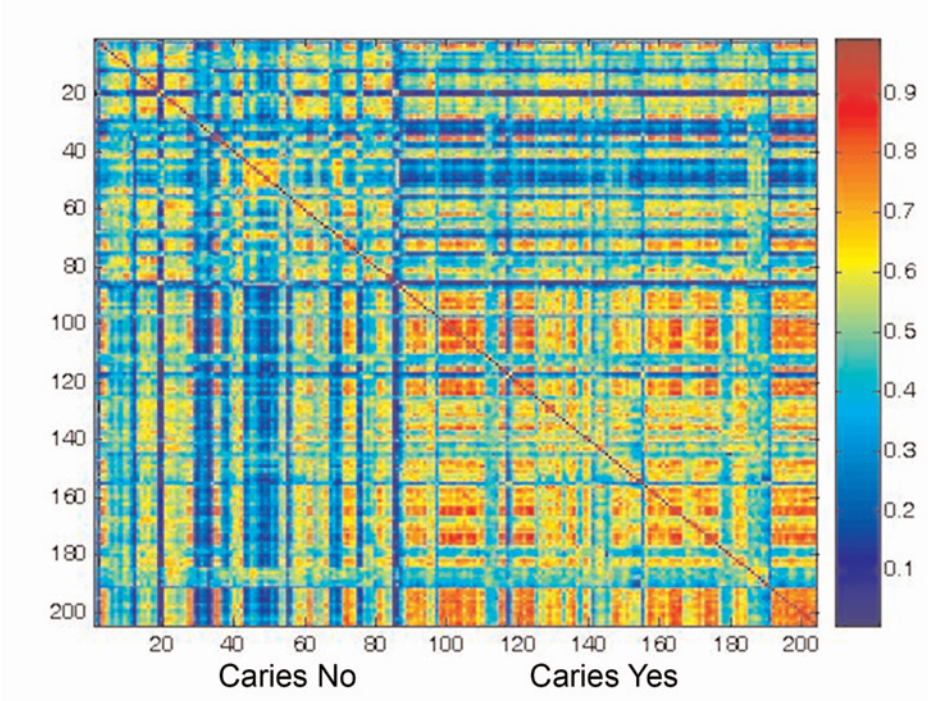

Figure 2

Heatmap representation of the similarity of the oral microbial species profiles.

Note: Each cell in the matrix reflects the similarity of two individuals, i, and j, where i, j=1 to 204 (the number of subjects) with respect to oral microbial species profile. The more 'hot' or red the color of the cell, the more similar the subjects are, whereas the more 'cold' or blue the less similar the subjects are. Note that the matrix has been ordered such that individuals with caries are listed as individuals 1-86 and individuals without caries are listed as individuals 87-204. Also note that twins are ordered next to each other. From the figure it is clear that individuals without caries have similar oral microbial species profiles and these profiles are different from individuals with caries.

(Figure 1-species depicted in red) and conversely less abundant in the caries-active group. Those in descending order of overabundance were *S. parasanguinis*, *A. defectiva, G. haemolysans*, *S. mitis/oralis*, *S. sanguinis, S. cristatus, Streptococcus* sp. clone CH016, *Eubacterium* sp. clone DO016, *Gemella morbillorum*, and *S. salivarius*. Interestingly, species that were abundant in caries-active subjects relative to caries-free are known cariogenic species, i.e. *Streptococcus mutans* and lactobacilli (Figure 1; species depicted in green).

Heritability estimates (Table 3) were moderate to high for oral species, especially known or hypothesized beneficial species, when they were abundant in caries-free twins, and, notably, when they were underabundant in caries-active twins, as can be seen from our analyses involving individual models for MZ and DZ twins that were adjusted for age and gender (Tables 1 & 2).

Moreover, Table 3 also provides overall heritability estimates for the oral species when models were adjusted for age, gender, and disease status (i.e., we analyzed caries-free and caries-active individuals together). From these analyses, estimates of total heritability indicated low, yet significant, values for *Eubacterium* sp. clone DO016 (h^2 = 30%), moderate to high values for *S. parasanguinis*, *A. defectiva, G. haemolysans*, *Streptococcus* sp. clone CH016, *S. mitis/oralis*, *S. sanguinis, S. cristatus*, and *S. salivarius* (range of h^2 = 56%–60%, p < .0001), and high values for *Gemella morbillorum* (h^2 = 80%, p < .0001). Consistent with our findings was the fact that *S. mutans*, a well known cariogenic species had a moderate heritable (h^2 = 47%, p = .01) component in caries-active children (Table 3) which could not be observed in caries-free children.

MDMR analysis was pursued in order to identify factors that could explain the greater or lesser similarity of overall oral microbial profiles among study subjects. It was found that whether an individual was caries-free or not could explain 13.8% (p = .002) of the variation in oral microbial profile similarity exhibited by study participants. Age could explain an additional 4.4% of the variation (p = .044). Figure 2 provides a heatmap representation of the similarity of the oral beneficial microbial profiles of the subjects ordered by caries status. It is evident that individuals that are caries-free have similar oral microbial profiles that are unlike the profiles of individuals with caries.

Discussion

Results of this study indicate that the relative levels of a subset of 10 microbial species within tooth-related biofilms correlates with the caries-free state. These 10 microbial species are more abundant in caries–free individuals than they are in caries-active individuals.

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A computational biology method was used to accommodate simultaneous analysis, by allowing cross-bacterial comparisons, of the abundance of bacterial levels between the healthy and the diseased groups. Several of the overabundant species in caries-free subjects relative to caries-active subjects are well known oral health-associated species which are involved in beneficial functions, that is, *S. sanguinis, S. salivarius,* and *S. mitis/oralis* (Loesche et al., 1984; Tanzer et al., 2001), and other species such as *S. parasanguinis* and *S. cristatus* are strongly associated with health (Figure 1). *Gemella* sp. are reported to be associated with extra-oral infections but do not seem to pose a threat to the dental tissues (Gimigliano et al., 2005). *A. defectiva* is also associated with extra-oral infections and its role in dental caries or health remains unknown (Paju et al., 2004). *Eubacterium* sp. clone DO016 and *Streptococcus* sp. clone CH016 are novel species that have recently been characterized, but again no definitive role in dental disease or health has been attributed to these species (Paster et al., 2001). A recent study evaluated the microbial flora of individuals that were free of caries (Aas et al., 2005), and employed culture-independent molecular techniques to determine the species present on biofilms of intact tooth surfaces. Those included *S. mitis*, *S. parasanguinis*, *S. sanguinis*, *G. haemolysans*, and *A. defectiva*, among others, thus corroborating our findings (Figure 1).

While previous studies have reported the association of certain microbial species with caries and with health (Becker et al., 2002; Munson et al., 2004; Tanzer et al., 2001), we found that changes in the relative abundance of these microflora are associated with health and disease (Corby et al., 2005b; Figure 1). Taking into account this paradigm, we evaluated these oral beneficial species in caries-active and caries-free twins, which permitted us to estimate the heritability not only in the presence, but also in the relative levels (abundance) of these microbes. To our knowledge, this is the first study to determine heritability estimates for the abundance of oral microbial species in caries-free twins relative to caries-active twins.

Individual analysis of heritability estimates for the 10 microbial species most correlated with a caries-free dentition (health), proved to be moderately-highly heritable (Table 3). We emphasize, however, that heritability estimates provide only estimates of the overall contribution of heritable versus environmental factors, where environment refers to any non-heritable factors. Thus, it may be the case that the estimate of heritability really subsumes shared environmental factors as well as shared parental-environmental and biological factors (Boomsma, et al., 2002; Martin et al., 1997; Townsend, et al., 2003). Notably, heritability estimates of the same beneficial species, which were found to be underabundant in caries-active individuals, also exhibited moderate to high heritability values (Table 3). After adjustment of the final model for disease status for all twins (Table 3), the results indicated moderate to high heritability for the relative abundance of the majority of species (h^2 = 56% to 80%) in these models (Table 3). Thus, heritability estimates determined by this study indicate that the relative abundance of beneficial oral microbial species is in part determined by the host genome. We further postulate that in caries-active children, host genetics and/or environmental factors (diet, antimicrobials) act to suppress the oral beneficial flora, thereby allowing for the overgrowth of cariogenic species. Collectively, these findings suggest that the relative abundance of oral beneficial species in tooth biofilms is partially under genetic modulation in health and disease.

A number of beneficial oral species were found to be significantly associated with health in this population. Very little is known about the way in which these beneficial species interact in biofilms and their role in preventing establishment and outgrowth of disease-associated pathogenic flora. Based on our findings, we hypothesize that a specific consortium of beneficial species may confer enhanced caries-resistance.

MDMR analysis was used in this study to test the hypothesis of whether or not individuals that are caries-free have a more similar oral microbial species profile than individuals that are caries active, and whether or not age influenced these microbial profiles. Heatmap analysis (Figure 2) clearly showed similarity of oral beneficial species in caries-free twins. Essentially, it appears that there are unique microbial 'ecologies' that develop in oral environments with and without caries that are shaped, in part, by host genetic factors, as well as the age of an individual. It is conceivable that environmental selective and progressive pressures may alter this beneficial microbial signature in late childhood, thereby increasing the risk for developing dental caries. Therefore, the 'beneficial' oral-associated microorganisms might profoundly influence oral health in early childhood by suppressing disease-associated microbial communities.

Understanding the genetic mechanisms that confer colonization of oral beneficial microbial species and protection for the host may help elucidate the relationship between salivary protein profile, dietary practices, microbial colonization, and dental caries. Moreover, identifying the microbial profiles associated with health and understanding the underlying mechanisms governing these profiles may allow for the development of strategies to manipulate microbial communities to prevent disease, as well as to maintain or restore health.

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