

Comparative Analyses of the Bacterial Microbiota of the Human Nostril and Oropharynx

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ABSTRACT The nose and throat are important sites of pathogen colonization, yet the microbiota of both is relatively unexplored by culture-independent approaches. We examined the bacterial microbiota of the nostril and posterior wall of the oropharynx from seven healthy adults using two culture-independent methods, a 16S rRNA gene microarray (PhyloChip) and 16S rRNA gene clone libraries. While the bacterial microbiota of the oropharynx was richer than that of the nostril, the oropharyngeal microbiota varied less among participants than did nostril microbiota. A few phyla accounted for the majority of the bacteria detected at each site: *Firmicutes* and *Actinobacteria* in the nostril and *Firmicutes*, *Proteobacteria*, and *Bacteroidetes* in the oropharynx. Compared to culture-independent surveys of microbiota from other body sites, the microbiota of the nostril and oropharynx show distinct phylum-level distribution patterns, supporting niche-specific colonization at discrete anatomical sites. In the nostril, the distribution of *Actinobacteria* and *Firmicutes* was reminiscent of that of skin, though *Proteobacteria* were much less prevalent. The distribution of *Firmicutes*, *Proteobacteria*, and *Bacteroidetes* in the oropharynx was most similar to that in saliva, with more *Proteobacteria* than in the distal esophagus or mouth. While *Firmicutes* were prevalent at both sites, distinct families within this phylum dominated numerically in each. At both sites there was an inverse correlation between the prevalences of *Firmicutes* and another phylum: in the oropharynx, *Firmicutes* and *Proteobacteria*, and in the nostril, *Firmicutes* and *Actinobacteria*. In the nostril, this inverse correlation existed between the *Firmicutes* family *Staphylococcaceae* and *Actinobacteria* families, suggesting potential antagonism between these groups.

IMPORTANCE The human nose and throat, though connected, contain distinct niches that are important sites of colonization by pathogenic bacteria. For many of these pathogens, colonization increases the risk of infection. Most research on the microbiota of nose and throat habitats has focused on carriage of one or a few pathogens. We hypothesized that increased knowledge of the composition of the complex bacterial communities in which these pathogens reside would provide new insights into why some individuals become colonized with pathogens, while others do not. Indeed, in the nostril microbiota of participants, there was an inverse correlation between the prevalences of the *Staphylococcaceae* family (*Firmicutes*), whose members include important pathogens, and the *Corynebacteriaceae* and *Propionibacteriaceae* families (both *Actinobacteria*), whose members are more commonly benign commensals. An improved understanding of competitive bacterial colonization will increase our ability to define predispositions to pathogen carriage at these sites and the subsequent risk of infection.

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The outermost segment of the nose, the nostrils or anterior nares, is a transition zone from the skin to the nasal cavity. Like skin, the nostrils contain sebaceous glands, sweat glands, and hairs and are lined by a keratinized, stratified squamous epithelium more similar to that of skin than to the mucus-producing, ciliated, columnar epithelium of the nasal cavity (1). The nostrils help filter inhaled air, which contains low numbers of extremely diverse microbes (2, 3). In addition, the nostrils are exposed to microbes present in the drainage from the nasal cavity and sinuses. The throat, or pharynx, can be divided into three sections. Like the

nasal cavity, the nasopharynx (the upper region of the throat behind the nose) is lined by a ciliated, columnar epithelium. The oropharynx, located immediately behind the mouth, is lined by a nonkeratinized stratified squamous epithelium, as is the more distal laryngopharynx. The oropharynx is constantly exposed to both inhaled and ingested microbes, those cleared by mucociliary mechanisms from both the upper and lower respiratory tracts and those contained in saliva. The nostril and oropharynx are distinct habitats. While the pathogen *Staphylococcus aureus* colonizes both sites (1, 4–6), cultivation-based studies suggest that these sites

share few other common bacterial residents. This led us to explore the bacterial community compositions of the microbiota of these two habitats in conjunction with each other.

As mentioned above, most of the knowledge on nose and throat microbiota has been generated via cultivation and has focused on pathogen carriage. The nostrils are known to harbor bacteria from the genera *Corynebacterium*, *Propionibacterium*, and *Staphylococcus*, including the important pathogen *Staphylococcus aureus* (1). The adjacent nasal cavity appears dominated (at least by cultivation) by *Corynebacterium* spp. and *Staphylococcus* spp. (7). The oropharynx harbors species from the genera *Streptococcus*, *Haemophilus*, *Neisseria*, and to a lesser extent *Staphylococcus* and various anaerobic bacteria (1). It is the site of carriage of many important human pathogens, including *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Haemophilus influenzae*, *Neisseria meningitidis*, *Moraxella catarrhalis*, and *Staphylococcus aureus* (1, 4, 6). Three recent culture-independent surveys focused on skin or gastrointestinal microbiota included either the nostril or the throat (8–10), though none compared the microbiota of the nostril to that of the throat.

The recent application of culture-independent analyses to the healthy adult human mouth (11, 12), saliva (13), gastrointestinal tract (8, 14–17), vagina (18–20), outer ear (21), and skin (9, 10, 22–25) has revealed that hundreds of types of bacteria colonize various human body niches. These surveys indicate that a limited number of phyla account for the majority of bacteria present at each site, with phylum-level conservation among healthy humans (26). They also show a high degree of interpersonal variation in species-level bacterial community composition at each site.

A more complete understanding of human microbiota begins with in-depth surveys of the bacterial community present in each niche. In identifying the bacteria present and determining their relative abundances, such surveys provide fundamental information on aspects of the microbiota that correlate with human health. For example, correlations are reported between health and microbiota compositions in obesity (27, 28) Crohn's disease (29, 30), periodontitis (31), or bacterial vaginosis (32, 33). Such surveys also serve as the foundation for identifying bacteria that might have significant influence on overall community composition and dynamics. The construction and sequencing of clone libraries of 16S rRNA genes from myriad sources have uncovered an immense diversity of bacteria. However, due to economic constraints, clone libraries cannot be feasibly applied for in-depth sampling of microbial communities. 16S rRNA gene microarrays offer an alternate approach. One such microarray, the PhyloChip (34, 35), possesses 500,000 probes and can detect approximately 8,500 bacterial taxa in a single experiment. On this array, a taxon is broadly defined as a cluster of 16S rRNA gene sequences with $\leq 3\%$ divergence (34). The PhyloChip has been used to examine bacterial community profiles from a number of different sample types, including mouse gastrointestinal tract (36) and human (37–39) samples. Comparison between the PhyloChip and 16S rRNA clone libraries indicates that the array is orders of magnitude more sensitive in its ability to identify diversity, detecting low-abundance taxa (0.01% of the community) even when the community is dominated by a small number of highly abundant microbes (2, 35).

Here we describe the application of the PhyloChip to profile the bacterial community composition of nostril and oropharyngeal samples from seven healthy adults. In addition, we con-

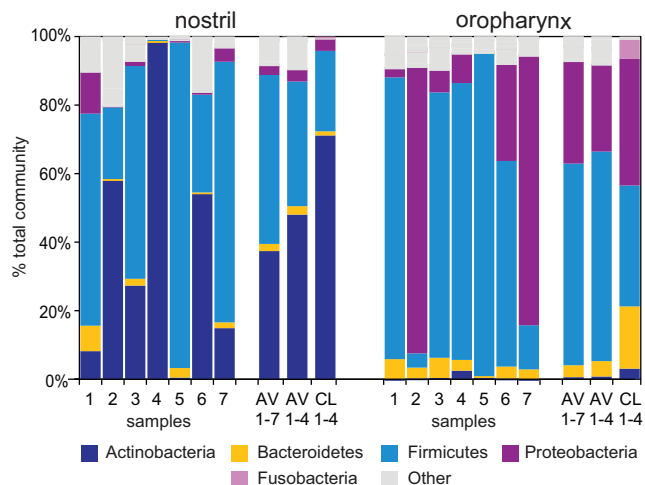


FIG 1 Bar graph showing the relative distributions of the major bacterial phyla in the nostril and oropharyngeal samples as detected with a PhyloChip. We used the microarray hybridization intensity to estimate the 16S rRNA gene copy number for each taxon detected on the array and then summed these to estimate the relative prevalence of each phylum in order to compare communities from all participants. Each bar labeled sample 1 to 7 represents 100% of the bacteria detected in a sample by the microarray analysis. Bars labeled AV 1-7 represent the average community composition detected from all 7 seven samples for a site by the microarray. Bars labeled AV 1-4 represent the average community composition detected by the microarray from samples 1 to 4. Bars labeled CL 1-4 represent the average of the relative abundances of phyla in the 16S rRNA gene clone libraries from samples 1 to 4.

structed and sequenced parallel 16S rRNA gene clone libraries from the samples of the first four participants to identify the most prevalent bacteria by 16S rRNA gene sequencing, as well as to provide a comparative method.

RESULTS

Phylum-level comparison of nostril and oropharyngeal bacterial communities. Paired mucosal surface swabs (one swab from each site) were collected from the nostril and the posterior wall of the oropharynx of seven healthy adults aged 26 through 45 years who had not taken antimicrobials in the preceding 2 months, were not pregnant, and were not acutely ill.

Taxonomy previously defined for the PhyloChip was used to classify bacteria detected using both methods (40, 41). Microarray analyses detected a total of 39 phyla from both sites, with 34 from the nostril and 38 from the oropharynx (see Fig. S1 in the supplemental material). 16S rRNA gene clone library analyses of samples from four of the seven participants identified eight phyla, six from the nostril and seven from the oropharynx (filled circles in Fig. S1 in the supplemental material).

An averaged phylum-level distribution pattern for each site demonstrated that both nostril and oropharyngeal microbiota have a phylum-level distribution distinct from those of other body sites (26). At both sites, a few phyla accounted for both the majority of the hybridization signal from the microarrays and the majority of cloned 16S rRNA gene sequences, with similar phylogenetic distribution patterns (Fig. 1; see Fig. S2A to C in the supplemental material), suggesting good concordance between these profiling approaches. From the nostril samples, these were *Firmicutes* and *Actinobacteria* (light blue and dark blue, respectively, in Fig. 1 and see Fig. S2C in the supplemental material). In

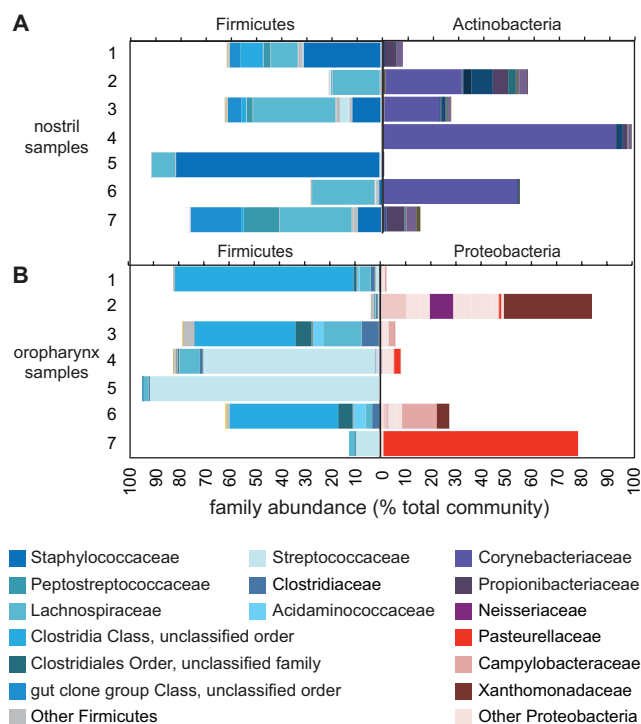


FIG 2 Relative abundances of the most common *Firmicutes* families compared to relative abundances of *Actinobacteria* families in nostril samples (A) and compared to relative abundances of *Proteobacteria* families in the oropharynx samples (B) as detected by PhyloChip analysis. For comparison, back-to-back graphs are shown for each site, each with the families from the specified phylum colored as indicated. We used the microarray hybridization intensity to estimate the 16S rRNA gene copy number for each taxon detected on the array and then summed these to estimate the relative abundance of each phylum.

the oropharynx, the most prevalent phyla were *Firmicutes*, *Proteobacteria*, and *Bacteroidetes* (light blue, maroon, and yellow, respectively, in Fig. 1 and see Fig. S2C). From the microarrays, the 16S rRNA copy number was estimated based on the fluorescence intensity of each taxon deemed present, to permit calculation of

the relative ratio of each phylum detected relative to the total bacteria detected (2). Interpersonal variation at the phylum level was evident, with the relative abundances of the core phyla at each site varying across samples.

Family-level comparison of nostril and oropharyngeal bacterial communities. *Firmicutes* accounted for a large percentage of the bacteria present in both the nostril and oropharynx; however, the most abundant families of this phylum varied by site. In the nostrils, the *Staphylococcaceae* and *Lachnospiraceae* accounted for the majority of the *Firmicutes* detected by the array, while in the oropharynx, the majority of the signal was due to the *Streptococcaceae*, *Lachnospiraceae*, and an unclassified group of *Clostridia* (Fig. 2). Similarly, in the clone libraries, sequences from the family *Staphylococcaceae* were abundant in the nostril samples, and sequences from the families *Streptococcaceae* and the clostridial families *Acidaminococcaceae* and *Lachnospiraceae* were abundant in the oropharyngeal samples (see Fig. S3 in the supplemental material).

Inverse correlation between *Firmicutes* and another phylum in both sites. There was a strong inverse correlation in the relative prevalences of *Actinobacteria* and *Firmicutes* in nostril communities (Fig. 3A) (Pearson correlation coefficient = -0.95 , $P < 0.001$). At a finer level of phylogenetic resolution, this inverse correlation was evident between members of the *Firmicutes* family *Staphylococcaceae* and the *Actinobacteria* families *Corynebacteriaceae* and/or *Propionibacteriaceae* (Fig. 3B) (Spearman correlation coefficient = -0.93 , $P < 0.001$). Unlike with the phylum-level comparison, the latter relationship did not appear linear, as there was a sharp decrease in the prevalence of *Staphylococcaceae* once the combined prevalences of the *Actinobacteria* families rose above $\sim 22\%$ of the total community (Fig. 2). This apparent non-linearity was the reason for using the Spearman correlation coefficient rather than the Pearson correlation coefficient. Also, for the family-level comparison, the relative prevalence of each was \log_{10} transformed prior to regression analysis (Fig. 3B). A similar trend was observed in the data from the nostril clone libraries.

In the oropharyngeal communities, there was an inverse correlation in the relative prevalences of *Firmicutes* and *Proteobacte-*

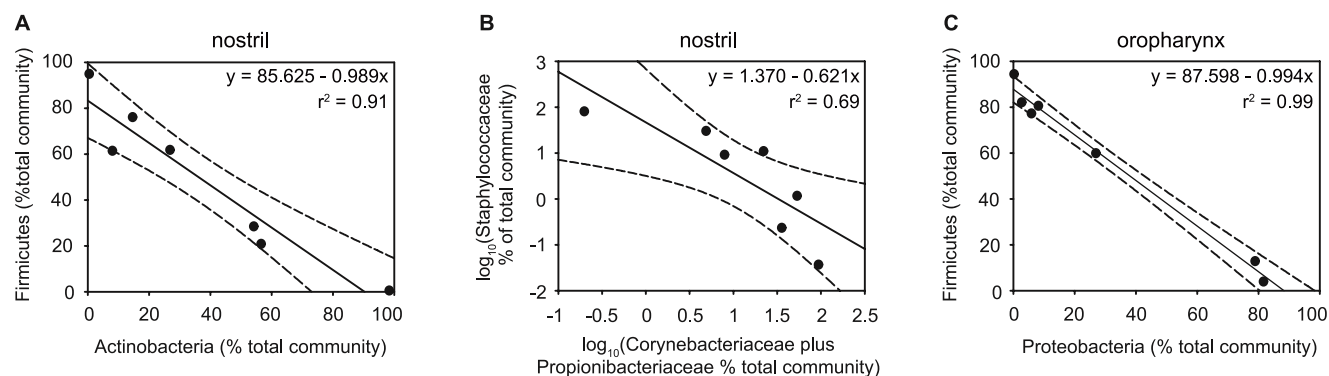


FIG 3 Inverse correlation between the relative prevalences of members of the phylum *Firmicutes* and another phylum at each site. Dashed lines indicate 95% confidence intervals. (A) Linear regression of the relative prevalences (percentages of the total community) of bacteria from the phylum *Firmicutes* and the phylum *Actinobacteria* in the nostril communities. Pearson correlation coefficient = -0.95 ; $P < 0.001$. $r^2 = 0.91$; $P < 0.001$. (B) Linear regression of the \log_{10} -transformed relative prevalences (percentages of total community) of the *Firmicutes* family *Staphylococcaceae* and the *Actinobacteria* families *Corynebacteriaceae* and *Propionibacteriaceae* in the nostril communities. Spearman correlation coefficient (of nontransformed data) = -0.93 ; $P < 0.001$. $r^2 = 0.69$; $P < 0.05$. (C) Linear regression of the relative prevalences (percentages of the total community) of bacteria from the phylum *Firmicutes* and the phylum *Proteobacteria* in the oropharyngeal communities. Pearson correlation coefficient = -0.994 ; $P < 0.001$. $r^2 = 0.99$; $P < 0.001$.

ria (Fig. 3C). We were unable to discern the potential underpinnings of this inverse correlation at a finer phylogenetic level.

Nostril- and oropharynx-associated taxa detected using the microarray. At a finer phylogenetic level, PhyloChip analysis detected a total of 1,325 bacterial taxa from across all sites. Cumulatively, 911 bacterial taxa were detected from the nostril samples, and 1,066 were detected from the oropharyngeal samples (see Table S1 in the supplemental material). A comparison of the taxa from both sites identified 259 taxa unique to the nostril (highlighted blue in Table S1 in the supplemental material) and 414 taxa unique to the oropharynx (highlighted pink in Table S1). As shown in Fig. 4A, the majority of these taxa were detected at very low levels. Like all methods, the PhyloChip has limitations. As we did not attempt to validate the identification of these many rare taxa, the total number of taxa detected might be an overestimation. At each site there was a high degree of interpersonal variation in the taxon-level community composition (see Fig. S4 in the supplemental material).

Nostril and oropharyngeal bacterial microbiota based on 16S rRNA gene clone libraries. We also examined the microbiota of the nostril and oropharynx from four of the seven participants sampled using 16S rRNA gene clone libraries. The PhyloChip detects rare taxa that are unlikely to be detected in standard-sized clone libraries of 200 to 500 clones per sample, provided that there are probes for these taxa on the array. Clone libraries, however, permit direct identification of highly prevalent 16S rRNA gene sequences present in the sample. In total, from all four adults, we analyzed 719 nostril-derived clones and 666 oropharynx-derived clones. We detected 36 taxa (defined by clustering at 97% nucleotide identity) from the nostrils and 71 taxa from the oropharynx (see Fig. S2A and B in the supplemental material). At 97%, the Chao 1 value (an estimate of community richness) for nostrils was 50 taxa (standard deviation ± 7.2) and for the oropharynxes 120 taxa (SD ± 17). The 36 taxa present in the nostril samples clustered within five bacterial phyla: *Actinobacteria*, *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, and *Fusobacteria* (in addition to chloroplasts [see Fig. S2A in the supplemental material]). The 71 taxa detected in the oropharyngeal samples clustered within seven bacterial phyla: *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Fusobacteria*, *Actinobacteria*, TM7, and SR1 (see Fig. S2B). As stated above, the relative abundance of each phylum in each site was similar to that detected by the microarrays (Fig. 1 and see Fig. S2C in the supplemental material). Rarefaction analysis demonstrated that at 97% sequence clustering, the combined libraries for each site were beginning to saturate (see Fig. S2D). This is best explained by the large proportion of rare taxa detected using the microarray (each present at $<0.05\%$ of the estimated total 16S rRNA gene copy number based on hybridization signals, as shown in Fig. 4A), which are unlikely to appear within clone libraries of the size constructed for this study.

The microbiota of the oropharynx has greater richness than nostril microbiota. The richness (number of different taxa) and evenness (relative abundance of taxa) of a sampled community are often calculated in order to characterize and compare the sampled community with other communities. Using the microarray, the number of taxa per nostril sample (dark-gray bars on the left in Fig. 4A and B) varied from 125 to 778, with an average of 342.7 taxa per person (standard error of the mean [SEM], 86.8). The number of taxa per oropharyngeal sample (dark-gray bars on the right in Fig. 4A and B) varied from 444 to 788, with an average of

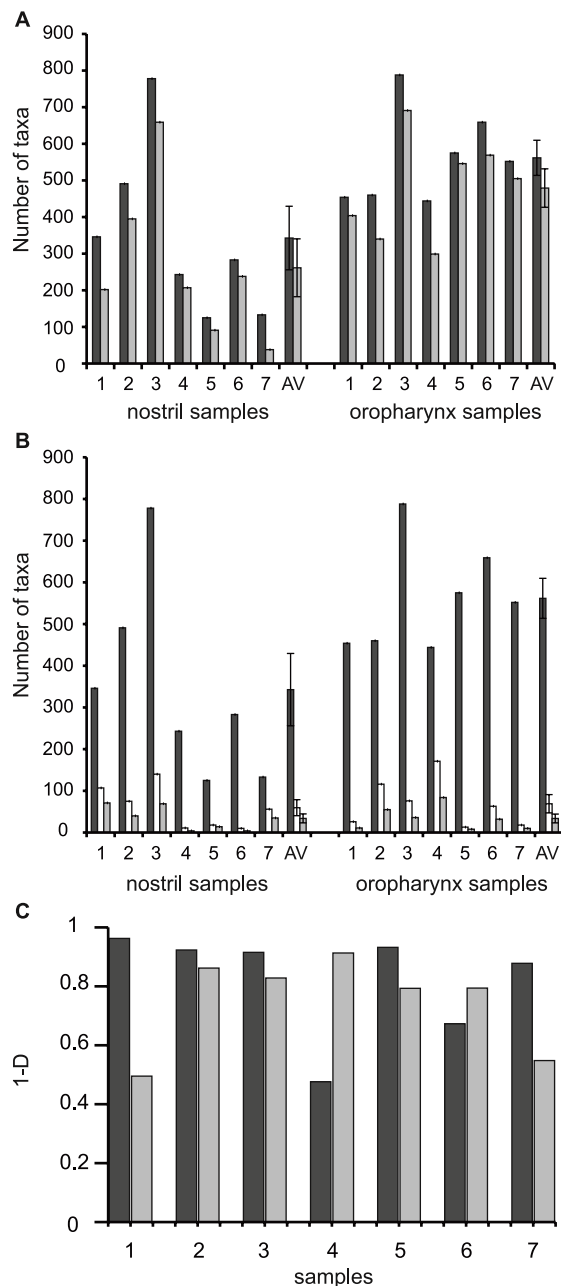


FIG 4 Taxonomic diversity detected by the microarray in samples from the nostril and oropharynx. (A) Total number of taxa detected in each sample and average number (AV) of taxa detected for each site (dark-gray bars). Numbers of taxa detected in each sample and on average per site (AV) that each made up $<0.05\%$ of the total community (light-gray bars) are shown. Error bars represent the standard errors of the means. (B) Numbers of taxa that constituted 100% (dark-gray bars), 95% (white bars), and 90% (light-gray bars) of the total bacteria detected by the microarray in each sample and on average per site (AV). Error bars represent standard errors of the means. (C) Simpson's index of diversity ($1 - D$) for each sample from the nostril (dark-gray bars) and the oropharynx (light-gray bars) calculated using the estimated 16S rRNA gene copy number derived from microarray hybridization intensity data. Data are graphed as $1 - D$, such that the higher the bar, the greater the diversity.

561.7 taxa per community sampled (SEM, 48). The oropharyngeal bacterial microbiota had a greater average number of taxa per sample than did the nostril microbiota (t test, $P < 0.05$). The clone

libraries also demonstrated greater richness in the oropharyngeal microbiota, with an average of 33 taxa per oropharynx versus 15 taxa per nostril (*t* test, $P < 0.05$) (see Fig. S5A in the supplemental material).

The microarray detected a large number of taxa in each sample that were present at low levels, each accounting for $<0.05\%$ of the total estimated 16S rRNA gene copy number as determined from the hybridization signal (light-gray bars in Fig. 4A). In the nostril samples, an average of 71% of the taxa in each sample were present at $<0.05\%$ each (SEM, 7.9%), and in the oropharynx, an average of 84% of the taxa detected were present at $<0.05\%$ each (SEM, 3.8%).

The evenness of the communities varied from person to person, with as few as 11 taxa and as many as 171 taxa accounting for 95% of the total estimated 16S rRNA gene copy number, as determined from the hybridization signals for sampled communities (white bars in Fig. 4B). The average number of taxa representing 90% of the community detected with the microarray (light-gray bars in Fig. 4B) was similar to the average number of taxa per community detected by the clone libraries for each site (see Fig. S5A in the supplemental material).

To assess the diversity (both the richness and evenness) of each community, we used Simpson's index of diversity (D) (Fig. 4C; see Fig. S5B in the supplemental material). Simpson's diversity index indicates the probability that any two bacteria detected in a sample are of the same species (taxon) (42). Person-to-person variation in whether the nostril or oropharynx harbored greater diversity (greater value of $1 - D$), as measured by Simpson's index, was indicated by both the microarray (Fig. 4C) and the clone library (see Fig. S5B in the supplemental material) data.

Interpersonal comparison of nostril and oropharyngeal microbiota. To explore potential relationships between sampled communities without *a priori* expectations, we used correspondence analysis (CoA). This analysis revealed that the bacterial communities analyzed by microarray grouped by site and not by individual, with nostril and oropharyngeal communities clustering separately (Fig. 5A and B). Correspondence axis 1 is graphed against both axes 2 and 3 to illustrate the grouping between sites and also the relationship of communities from the same site. Neither nostril nor oropharyngeal communities appeared to be more closely related among themselves by this analysis.

To better discern the relationship between bacterial communities from the same site but different individuals, we performed cluster analysis using weighted UniFrac, which takes into account both phylogenetic relationships and relative abundances of taxa within each community (43). Each terminal branch of the dendrogram represents the community from one sample as detected by the microarray (i.e., one individual, one site) (Fig. 5C). Again, the nostril and oropharyngeal communities clustered separately, supporting the conclusion that one individual's nostril microbiota is more similar to another individual's nostril microbiota than to his/her own oropharyngeal microbiota. Similar results were obtained using unweighted UniFrac (data not shown). No discernable pattern emerged from within-site gender or age comparisons in the CoA and UniFrac analyses (data not shown). A comparison of the weighted UniFrac distances within all the nostril samples and within all the oropharyngeal samples indicated that the oropharyngeal microbiota was less varied among the seven individuals than was the nostril microbiota (Fig. 5D). Again, similar results were obtained using unweighted UniFrac (data not

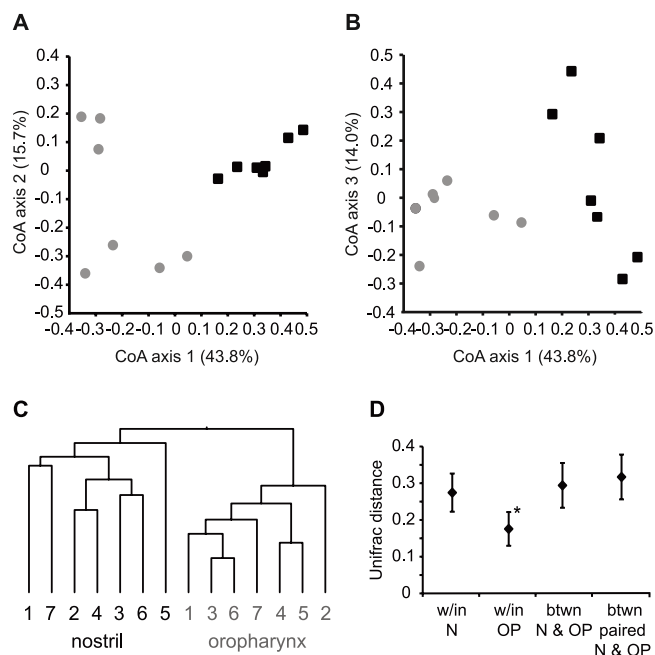


FIG 5 Bacterial communities grouped by site and not by individual. (A and B) Correspondence analysis of the total microarray hybridization profile from each sample performed in MeV v4.4. Black squares, nostrils; gray circles, oropharynges. For clarity the data are shown in two dimensions, with axis 1 graphed against both axis 2 (A) and axis 3 (B). The percentage in parentheses for each axis indicates the percent variation that is explained by that axis. (C and D) Weighted UniFrac analysis of the total microarray hybridization profile from each sample. (C) Each terminal branch represents the total bacterial community detected from one person's sample from the specified site. All nodes were recovered at 100% using the Jackknife method. (D) UniFrac distances measured within the nostril microbiota samples (w/in N), within the oropharyngeal microbiota samples (w/in OP), between all the nostril and oropharyngeal samples (btwn N and OP), and between the paired nostril and oropharyngeal microbiota for each individual person (btwn paired N and OP). *, statistically different from values for the others as determined by one-way ANOVA with Tukey's test (set to 0.05) on pair-wise UniFrac values. Error bars represent standard deviations.

shown). There was no statistical difference found in the weighted UniFrac distances when communities were compared between all 14 samples, nostril and oropharynx, versus between the paired nostril and oropharyngeal microbiota from each individual (Fig. 5D).

DISCUSSION

We utilized two different 16S rRNA gene-based culture-independent techniques to survey the bacterial composition of the microbiota sampled from healthy adult nostril and oropharynx. The number of participants in this study ($n = 7$) was comparable to those in the majority of initial culture-independent surveys of microbiota from different body sites (11, 15, 16, 19, 22, 24), although small in comparison to the hundreds to thousands of individuals included in epidemiological surveys. Nostril and oropharyngeal microbiota each had a distinct phylum-level distribution pattern, which was robust across methods (Fig. 1 and see Fig. S2C in the supplemental material). Similar to what occurred at other body sites, there was a large degree of interpersonal variation in taxon-level community compositions, along with some variation at the phylum level. This suggests that multi-

ple species might be capable of performing discrete and essential functions and that functional redundancy within communities might serve as a hallmark of community stability and potentially as a biomarker for human health (28, 44).

An inverse correlation between *Firmicutes* and *Actinobacteria* in nostril microbiota. In the nostril, the numerical dominance of *Actinobacteria* and *Firmicutes* was reminiscent of skin; however, *Proteobacteria* were much less common (10, 22, 24, 25). The prevalences of *Firmicutes* and *Actinobacteria* were inversely correlated, and this was statistically significant across the seven nostril samples. At the family level, this inverse correlation existed between the *Firmicutes* family *Staphylococcaceae* and the *Actinobacteria* families *Corynebacteriaceae* and *Propionibacteriaceae*, suggesting possible antagonism between these groups. The *Staphylococcaceae* include important community-acquired and nosocomial pathogens, e.g., *S. aureus* and coagulase-negative *Staphylococcus* spp. The observed inverse correlation raises the intriguing possibility that nostril bacterial communities in which *Actinobacteria* are most prevalent might protect against carriage of *Staphylococcus* spp., including the pathogen *S. aureus*. Consistent with this, based on cultivation of nasal bacteria from 156 healthy adults, Uehara and colleagues observed lower rates of *S. aureus* carriage in persons colonized with *Corynebacterium* (8.5%) than in those without (44.6%), though *Staphylococcus* spp. other than *S. aureus* were detected by cultivation in all individuals (45). Another cultivation-based study of *S. aureus* nostril carriage in 216 adults reports similar findings (46). Multiple possible explanations exist for such an inverse correlation, from variations in host factors, such as attachment sites and substrates, to various forms of bacterial competition, such as competition for common attachment sites and small-molecule-mediated inhibition. In the future, it will be exciting to elucidate the molecular mechanisms that drive this observed inverse correlation.

Comparison of nostril microbiota compositions from culture-independent surveys. A recent survey of skin microbiota using 16S rRNA gene clone libraries included naris (nostril) samples from 10 healthy adults and found that *Actinobacteria* were the most abundant sequences from this site (10). We speculate that differences in methodology might account for the increased prevalence of *Firmicutes* in our samples, for example, slightly different exclusion criteria, dry versus wet swabs, and differences in protocols for DNA preparation and 16S rRNA gene amplification. It seems less likely that differences in participant characteristics were contributory, as both surveys included adults of similar ages, 20 to 41 years (10) versus 26 to 45 years, and both surveys were performed in similar geographic locations (cities on the east coast of the United States). Another recent survey of the microbiota from multiple adult body sites using bar-coded tag pyrosequencing of 16S rRNA genes also included the nares (9). All but one of the participants was 30 to 35 years of age, and all lived in Boulder, CO, with a mix of males and females. In agreement with results presented here, this survey detected both *Actinobacteria* and *Firmicutes* as the most prevalent phyla in nostril samples. As with our clone library results, the nostril bacterial communities were all numerically dominated by members of the *Actinobacteria*. The one notable difference is that the *Actinobacteria* were overwhelmingly (>60%) from the family *Propionibacteriaceae* (9), whereas we observed a large number of both *Corynebacteriaceae* and *Propionibacteriaceae* among the *Actinobacteria* detected using both

methods (Fig. 2, and see Fig. S3 in the supplemental material). Again, methodological differences might account for this.

The phylum-level bacterial composition in the oropharynx differs from that in the esophagus and mouth but is similar to that in saliva. The increased presence of Gram-negative bacteria, particularly from the phylum *Proteobacteria*, in the oropharynx compared to their presence in the nostril is consistent with cultivation data. Compared to other human sites analyzed with culture-independent methods, the *Proteobacteria* signal from the oropharynx is rivaled only by those from skin (10, 22, 24–26) and saliva (13). The distal esophagus microbiota is numerically dominated by *Firmicutes* and *Bacteroidetes* of genera similar to those found in the oropharynx but with many fewer *Proteobacteria* (16). The healthy mouth likewise is host to an abundance of *Firmicutes*; in one study, *Firmicutes* were out of proportion to any other phylum present (11, 26), and in another, the phylum-level distribution pattern was similar to that of saliva (12). Of the human body niches analyzed with culture-independent methods, our clone library results from the oropharyngeal microbiota showed a phylum-level composition pattern most similar to that of saliva. The composition of phyla in the salivary microbiota detected by 16S rRNA gene clone libraries from 120 individuals (with ~120 16S rRNA gene sequences per person) is as follows: *Firmicutes*, ~37.8%; *Proteobacteria*, ~28%; *Bacteroidetes*, ~20%; *Actinobacteria*, ~7%; and others, 7.2% (13). A recent survey of gut microbiota using bar-coded tag pyrosequencing of 16S rRNA gene amplicon pools included the throat (8). Only ~5% of the sequences from their throat samples ($4.7\% \pm 3.4$) cluster in the phylum *Proteobacteria*, whereas ~15% (14.5 ± 3.9) cluster within the *Actinobacteria* (8). Differences in methods are likely to account for these different observations. Another possible source of variation is differences in the sampled populations. The six participants in the study by Andersson and colleagues both were older and had underlying medical conditions (three aged 42 to 73 years with duodenal ulcer and three controls aged 70 to 75 years with dyspepsia) (8). These differences suggest a need for surveys of healthy respiratory tract microbiota from a greater number of individuals with a broad age range.

At the phylum level, from both the nostril and oropharynx, *Firmicutes* were detected as a greater proportion of the total community by using the microarrays than by using the clone libraries. For both, we followed the same protocols, used the same bacterial DNA mixture, and, in most cases, used the same amplicon pool. The differences observed suggest either that the microarray overrepresented *Firmicutes* or that the clone libraries underrepresented them for these sites. The relative proportions of probes on the microarray for *Firmicutes* versus *Bacteroidetes* and *Actinobacteria* might have contributed to the difference in prevalence of these phyla as detected by each method. Alternatively, some have speculated that cloning through *Escherichia coli* might lead to a slight decrease in detection of AT-rich organisms, i.e., *Firmicutes*, though to our knowledge, this has never been directly demonstrated. In fact, a recent assessment of the underrepresentation of marine SAR11 biodiversity based on techniques that rely on cloning through *E. coli* (fosmid and bacterial artificial chromosome [BAC] libraries) suggests that the underrepresentation of this low-GC-content group using these methods is unlikely to be due to its AT richness (47).

Combined 16S rRNA gene-based approaches to study microbiota composition. All of the 16S rRNA gene-based techniques

likely have specific biases and strengths, though generally similar patterns are expected from each; thus, a combined approach offers advantages. Most molecular analyses of human microbiota to date have utilized 16S rRNA gene clone libraries, and we used both this method and the PhyloChip to analyze samples from the first four participants. The number of clones per person per site was similar to those of other human body site clone libraries reported (10, 13, 14, 16, 22, 24). The number of taxa identified by the PhyloChip was much greater than the number identified from 16S rRNA gene clone libraries and was at least comparable to what might be expected using 454 pyrosequencing of the 16S rRNA gene. However, by using the microarray, we were able to analyze a larger number of individuals than would have been possible at the time for a cost comparable with pyrosequencing. While the microarray does not identify previously unreported taxa, the combined approach with the microarrays and 16S rRNA gene clone libraries enabled identification of predominant members of sampled communities via their 16S rRNA gene sequence. Furthermore, the total hybridization pattern of each chip can be used as a community “signature” for analyses comparing the communities (e.g., beta diversity).

As mentioned in the results, the exact number of taxa detected by the microarray is best viewed as an estimate. That said, both the nostril and oropharynx are continually exposed to a large number of environmental bacteria via inhaled air. Additionally, the oropharynx is exposed to microbes present in food and liquids. Thus, it is not surprising that a wide variety of bacteria associated with outside sources were detected at very low levels in samples from each site. Both a larger data set and validation of the presence of rare taxa via other methods will be required to discern if any of these rare taxa are long-term residents of these sites, or if these are simply transiently present.

Bacterial microbiota of the nostril compared to that of the oropharynx. Analyses of the microbiota sampled from the nostril and oropharynx revealed that the bacterial communities grouped by site and not by individual, similar to what has been observed for other body sites (9). The differences we observed largely reflected disparities in each taxon’s signal abundance at each site. As both the oropharynx and nostril receive drainage from common sources (nasopharynx, sinuses, and nasal cavity), it is not surprising that there was a large overlap in the taxa detected. Variation might arise then from different sources; bacteria in the oropharynx could be introduced via the mouth, saliva, and ingestions, whereas the nostrils filter air before it reaches the oropharynx. We postulate that the differences in bacterial microbiota compositions from the two sites are largely due to differences in niche environments, such as substrate and surface differences (e.g., keratin and sebum in the nostril), the slightly lower temperature of the nostrils, and the expected variations in pH between the sites (not measured in this study) (1).

Among the seven adults sampled, there was more conservation among the oropharyngeal microbiota compositions than among the nostril microbiota compositions based on a comparison of UniFrac distances within all the nostril and oropharynx samples (Fig. 5D). Previously, throat microbiota was shown to have less interpersonal variation than the stomach or fecal microbiota (8). Studies analyzing bacterial communities from a number of different body sites from a larger number of individuals will be required to determine if, in general, oropharyngeal microbiota demonstrate more interpersonal compositional conservation. Unlike with the gender differences in palmar skin microbiota composi-

tions (23), we did not observe any differences in the nostril or oropharyngeal microbiota compositions that correlated with gender among the individuals sampled, although our sample size might have impacted this assessment. This survey of the microbiota of the nostril and oropharynx from seven healthy adults contributes to the growing understanding of the composition of healthy human microbiota and its interpersonal variation. Such surveys are a necessary foundation for future research aimed at identifying the impact of various perturbations, e.g., antibiotics, vaccines, infections, diseases, and medical interventions, on the ecology of human-associated microbial communities and correlations between human health and microbiota composition.

MATERIALS AND METHODS

Participant enrollment. We enrolled seven healthy adult volunteers, four male and three female, whose ages were 26 through 45 years. After receiving an explanation of the study and details about sample collection, all provided verbal consent prior to participation. Exclusion criteria for this pilot study were as follows: (i) use of any antimicrobials within the past 2 months, (ii) pregnancy, (iii) any intercurrent illness, and (iv) an age less than 21 years or greater than 65 years. The Institutional Review Board (IRB) at Children’s Hospital Boston ceded review to the IRB at Harvard Medical School, which approved this study.

Sample collection and DNA extraction. Separate mucosal swabs were collected from one nostril and from the posterior wall of the oropharynx of each participant and rapidly frozen at -80°C (BBL CultureSwab; Becton, Dickinson and Co.). The posterior wall of the oropharynx was swabbed without touching the tonsils, uvula, tongue, or other oral structures. For nucleic acid extraction, the top of the swab was aseptically snipped off into a sterile 2-ml lysing matrix B tube (MP Biomedicals) containing 600 μl buffer RLT Plus (Qiagen) with 2-mercaptoethanol (Sigma-Aldrich, Inc.). After bead beating (30 s at 5.5 m/s), genomic DNA was purified from sample supernatants using the AllPrep DNA/RNA kit (Qiagen, 2005).

PCR amplification and purification of 16S rRNA genes. To minimize potential PCR amplification bias, we amplified 16S rRNA genes from DNA extracts using a temperature gradient (48°C to 56°C) in eight replicate reactions with the bacteria-specific 16S primer set 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTGTACGACTT-3′) (48) as previously described (39). For each sample, amplified products were pooled, purified by isopropanol precipitation, and quantified by gel electrophoresis using a 2% E-gel with a low-mass-DNA quantification ladder (Invitrogen Corp.).

16S rRNA gene clone library construction and analysis. To construct the clone libraries for nostril and oropharyngeal samples from the first four participants, amplicon pools were ligated and cloned using the standard protocol from the TOPO TA cloning kit for sequencing (Invitrogen). Individual cloned 16S rRNA gene sequences were first amplified using the M13F and M13R primers (TOPO TA cloning kit for sequencing manual) and then sequenced from the 5′ end with the 27F primer using an ABI3700 (Applied Biosystems, Inc.). After primer and vector sequences were removed, the 16S rRNA gene sequences were trimmed by removing any leading and trailing bases that contained ambiguities and for which confidence was less than 25%, and the chromatogram of each sequence was manually inspected for any remaining base caller errors by using Sequencher (Gene Codes Corp.). Sequences with a minimal length of 500 bp were then grouped based on $\geq 97\%$ sequence identity. The 97% clustering was done to facilitate comparison with taxa detected by the PhyloChip, for which a taxon is broadly defined as a cluster of 16S rRNA gene sequences with $\leq 3\%$ divergence (34). Sequences were aligned using NAST on the Greengenes website (40, 49). Putative chimeras were identified using ChimeraCheck in RDP and Bellerophon and discarded from the data set (50). Grouped 16S rRNA gene cloned sequences were compared to sequences in two databases, NCBI and RDP, using sequence alignment (BLAST) to

identify the best-named matches. The NAST-aligned sequences, along with the best BLAST matches retrieved from the two databases for individual cloned sequences, were imported into the Greengenes database using the ARB software suite (40, 51). Sequences were added to the universal ARB dendrogram using the ARB parsimony algorithm with a Lane mask filter (48). Cloned sequences and their closest named reference sequence(s) were then retrieved and assembled using parsimony into the trees shown for the clone libraries. Ultimately, there were 141, 261, 176, and 141 clones from individual nostril samples and 199, 217, 171, and 79 clones from individual oropharyngeal samples, for a total of 719 nostril-derived sequences and 666 oropharynx-derived sequences.

Hybridization of pooled PCR amplicons to the PhyloChip. We spiked 250 ng of pooled 16S rRNA gene amplicon from each sample with a mix containing known concentrations of control amplicons to permit normalization of interarray variation (2). The combined mixture for each sample was then fragmented, biotin labeled, and hybridized to the PhyloChip (version G2; Affymetrix) as previously described (2, 34, 35). PhyloChips were washed, stained, and scanned using a GeneArray scanner (Affymetrix) as previously described (34). Each scan was captured using standard Affymetrix software (GeneChip Microarray Analysis Suite, version 5.1), and array data were processed as previously described (2, 34, 35). On the PhyloChip, each taxon was represented with a minimum of 11 probe pairs, and some were represented with up to 55. As previously described, a taxon was considered to be “present” in a sample when the number of positive probe pairs divided by the total number of probe pairs in a probe set was equal to or greater than 0.9 (34). Hybridization values (fluorescence intensity) for each taxon were calculated as a trimmed average (with maximum and minimum values removed before averaging) (34). Hybridization values were converted to estimated gene copy numbers using a formula derived from a Latin square assay as described previously (2).

Analysis of PhyloChip data. All of the taxa detected by the PhyloChip from all 14 samples were added using parsimony to the existing phylogenetic tree based on the ARB parsimony tree delivered with the Greengenes ARB database (October 2006 release) (40). To compare the numerically dominant phyla from each body site, correlation coefficients and linear regression were performed using Sigma Plot 11. We performed weighted and unweighted UniFrac analyses using the neighbor-joining tree of all taxa represented on the PhyloChip that have >1,200-bp 16S rRNA sequences (40, 43, 52, 53; file bacteria.6190.tree at http://greengenes.lbl.gov/Download/Taxonomic_Outlines/). A *t* test on the UniFrac distance matrix was used to determine if the UniFrac distances were on average significantly different for the bacterial communities detected in the two body sites. To determine if the community compositions within and between oropharynx and nostril groups were different, we used one-way analysis of variance (ANOVA) with Tukey's test (set to 0.05) on pair-wise UniFrac values.

To determine how bacterial community compositions varied across samples, we also compared total hybridization profiles for each sample using correspondence analysis (CoA) in MeV v4.4 (54). Analysis was done on the log₂-transformed hybridization intensity data for each sample, with a percentage cutoff filter set to 0.02%.

16S rRNA gene sequence accession numbers. Sequences from clone libraries grouped at 99% identity (53 from nostril and 109 from oropharynx) were deposited in the NCBI database with GenBank accession numbers HM172637 to HM172798.

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SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00129-10/-/DCSupplemental>.

Table S1, PDF file, 0.43 MB.
Figure S1, EPS file, 0.32 MB.
Figure S2, EPS file, 1.26 MB.
Figure S3, EPS file, 0.45 MB.
Figure S4, EPS file, 2.80 MB.
Figure S5, EPS file, 0.65 MB.

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