RESEARCH ARTICLE

Analysis of the Upper Respiratory Tract Microbiotas as the Source of the Lung and Gastric Microbiotas in Healthy Individuals

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ABSTRACT No studies have examined the relationships between bacterial communities along sites of the upper aerodigestive tract of an individual subject. Our objective was to perform an intrasubject and intersite analysis to determine the contributions of two upper mucosal sites (mouth and nose) as source communities for the bacterial microbiome of lower sites (lungs and stomach). Oral wash, bronchoalveolar lavage (BAL) fluid, nasal swab, and gastric aspirate samples were collected from 28 healthy subjects. Extensive analysis of controls and serial intrasubject BAL fluid samples demonstrated that sampling of the lungs by bronchoscopy was not confounded by oral microbiome contamination. By quantitative PCR, the oral cavity and stomach contained the highest bacterial signal levels and the nasal cavity and lungs contained much lower levels. Pyrosequencing of 16S rRNA gene amplicon libraries generated from these samples showed that the oral and gastric compartments had the greatest species richness, which was significantly greater in both than the richness measured in the lungs and nasal cavity. The bacterial communities of the lungs were significantly different from those of the mouth, nose, and stomach, while the greatest similarity was between the oral and gastric communities. However, the bacterial communities of healthy lungs shared significant membership with the mouth, but not the nose, and marked subject-subject variation was noted. In summary, microbial immigration from the oral cavity appears to be the significant source of the lung microbiome during health, but unlike the stomach, the lungs exhibit evidence of selective elimination of *Prevotella* bacteria derived from the upper airways.

IMPORTANCE We have demonstrated that the bacterial communities of the healthy lung overlapped those found in the mouth but were found at lower concentrations, with lower membership and a different community composition. The nasal microbiome, which was distinct from the oral microbiome, appeared to contribute little to the composition of the lung microbiome in healthy subjects. Our studies of the nasal, oral, lung, and stomach microbiomes within an individual illustrate the microbiological continuity of the aerodigestive tract in healthy adults and provide culture-independent microbiological support for the concept that microaspiration is common in healthy individuals.

Until recently, it has been generally held that the upper respiratory tract contains abundant bacteria while the lower respiratory tract is sterile when healthy (1–4). In light of this belief and the invasive nature of lower respiratory tract sampling by bronchoalveolar lavage (BAL), the Human Microbiome Project (HMP) did not include the lower respiratory tract in its microbiome surveys (5). However, the concept of lung sterility during health would be in sharp contrast to earlier evidence, some of which was described in the 1920s and was obtained by using radiotracers, that pharyngeal microaspiration is common in healthy individuals (6–8). Previous studies based on culture-independent molecular techniques have analyzed the lung bacterial microbiomes of healthy adult subjects obtained via BAL and readily identified bacterial sequences in BAL fluid, with the most common bacterial phyla being *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* (3, 9–14). The prominent genera in BAL fluid samples from healthy subjects include *Prevotella*, *Veillonella*, and *Streptococcus*. These genera are also detected in concurrently collected oral samples, but their relative abundance in BAL fluid generally differs from that in oral samples.

On the basis of accumulating data from many laboratories, we have recently proposed the concept that the entire respiratory tract should be considered a single ecosystem extending from the nasal and oral cavities to the alveoli, which includes gradients and niches that modulate microbiome dispersion, retention, survival, and proliferation (2, 15). Thus, the composition of the lung mi-
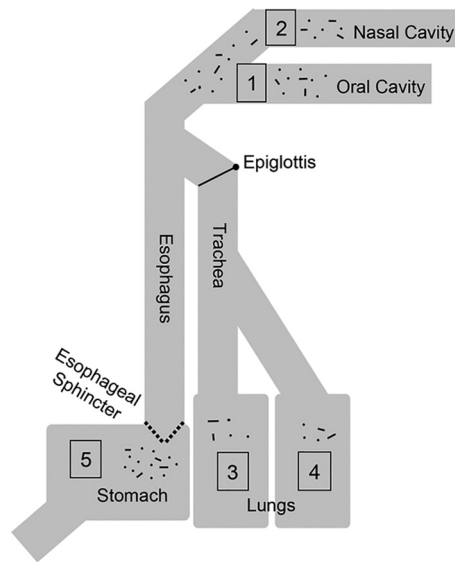
FIG 1 The aerodigestive tract. Schematic of the flow relationship between the oral and nasal cavities and the lungs and stomach. The numbers indicate the five sites sampled in this study.

crobiome will be determined by three factors: immigration, elimination, and the relative growth rates of its members. Anatomical and physiological considerations suggest that this model is also applicable to the microbiota of the stomach, with key differences. Just as the oral cavity is the site of the primary source community of the lung microbiota (acquired via microaspiration and inhalation), it is likewise the site of the primary source community of the gastric microbiota (acquired via swallowing). Direct mucosal dispersion of microbes from the nasal and oral cavities to both the lungs and the stomach, as parts of the contiguous aerodigestive tract, can occur (Fig. 1). The epiglottis acts as a major barrier to prevent saliva, food, and liquid from flowing into the trachea, diverting it to the esophagus. In contrast, microaerosols generated in the oral cavity will pass the epiglottis and be inhaled. The total flow of saliva out of the mouth via swallowing (roughly a liter per day) greatly exceeds that of microaspiration during health. Thus, the rate of immigration of the microbiome from the mouth to the stomach should far exceed that from the mouth to the airways. Similarly, the volume of saliva produced in the mouth greatly exceeds the secretions produced by the nasal mucosa. Thus, the "downstream" communities of the lungs and stomach would be expected to more closely resemble the source community of the mouth than that of the nose.

Despite these theoretical models, no studies have examined the relationships within an individual subject among the mouth, nasopharynx, lung, and stomach microbiotas in terms of species richness and the relative abundance of the bacterial microbiotas at those sites. Because it is well documented that there is significant subject-to-subject variation in their microbiome (3, 9–14), we collected (and compared) all of these samples from each study subject, thereby allowing each individual to serve as his/her own control. Our objective in performing this type of intrasubject and intersite analysis was to determine the contributions of the two upper mucosal sites (mouth and nose) as source communities to the bacterial microbiomes of two lower sites (lungs and stomach).

RESULTS

Analysis of potential oral sample contamination of BAL fluid due to bronchoscopy. For 12 study subjects, we compared matched samples from the oral wash sample with the first BAL fluid sample obtained and the second BAL fluid sample obtained from a right middle lobe segment. The objective was to confirm that research bronchoscopy did not systematically carry over oral contamination into the lower airways. After insertion of the bronchoscope past the epiglottis into the first lobe for the first BAL, the bronchoscope was retracted slightly without pulling the tip of the bronchoscope more proximal than the midtrachea (well below the epiglottis) and then repositioned into the other lobe for the second BAL. Within each subject, we determined the similarity of the bacterial communities between the oral wash and the first return BAL fluid sample and between the oral wash and the second return BAL fluid sample by calculating the θ_{YC} distance ($1 - \theta_{YC}$) between the two comparisons. The oral-BAL fluid sample comparison showed that the first and second return BAL fluid samples did not differ significantly in terms of bacterial community composition (Fig. 2A). In addition, there was no difference in bacterial levels in the first and second BAL fluid sample as measured by quantitative PCR (qPCR) ($P > 0.05$, data not shown). The total bacterial load in the BAL fluid ($10^{3.5}$/5 ml) was greater than that found in the saline and preinsertion bronchoscope rinse controls ($<10^2$/5 ml) (Fig. 2B). These results were entirely consistent with the serial bronchoscopy study by Segal et al., which also concluded that bronchoscopy is a viable option for sampling of the lung microbiome (13). Furthermore, we have previously reported that, despite significant differences between the oral and nasal microbiomes, the route of insertion of the bronchoscope (oral insertion versus nasal insertion) also did not affect the microbiota of BAL fluid (16). Our data are also consistent with a previously published study by Morris et al. that demonstrated a significant difference between oral and BAL fluid microbiota communities in healthy subjects (17). Thus, sampling of the lungs by bronchoscopy was not confounded by oral microbiome contamination.

We also compared the compositions of the microbial communities in the first and second return BAL fluid samples of individuals in our healthy cohort. There were no statistically significant differences between the bacterial communities of the first and second return BAL fluid samples (analysis of molecular variance [AMOVA] = 0.963, permutational multivariate analysis of variance [PERMANOVA] = 0.967, Table 1). Thus, the analyses in this study combined the sequence data from the two BALs to generate a single data set for each subject prior to subsampling.

Bacterial levels in samples by qPCR. Our first analysis comparing the four contiguous sites was for total bacterial levels measured by qPCR targeting levels of 16S rRNA-encoding genes in the DNA. Similar to previously published studies, the total bacterial signal level in the BAL fluid was 100- to 1,000-fold lower than that found in the oral wash fluid (Fig. 2B). The BAL fluid samples also contained 10- to 100-fold lower bacterial signal levels than the gastric aspirate samples (Fig. 2B). We could readily detect bacteria on the nasal swabs ($\sim 10^4$ 16S rRNA gene copies/swab). Although these measurements roughly correspond to the expected levels of total bacterial colonization in each of these sites, it would not be accurate to draw conclusions about the differences in bacterial densities at the different sites due to disparity in sample types and dilution effects. However, the samples do provide valid snapshots
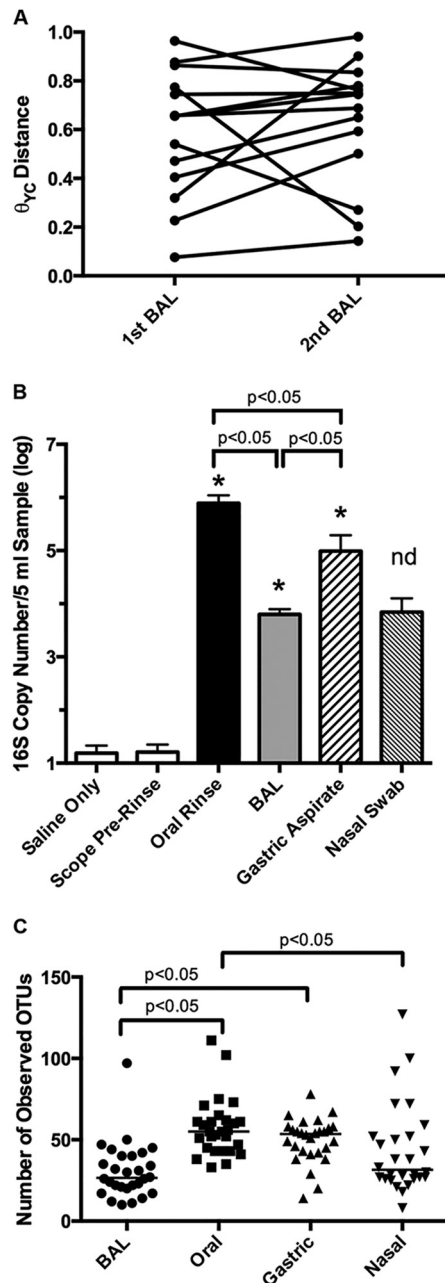
TABLE 1 Statistical significances of differences between sample types

Comparison	AMOVA[a]	PERMANOVA[b]	RDA[c]
Overall model	<0.001	0.001	0.005
BAL1 vs BAL2	0.963	0.967	
BAL1 vs oral	<0.001	0.001	
BAL2 vs oral	0.001	0.001	
BAL1 vs nasal	<0.001	0.001	
BAL2 vs nasal	<0.001	0.001	
BAL1 vs gastric	0.001	0.003	
BAL2 vs gastric	0.011	0.006	
BAL vs oral	0.001	0.001	
BAL vs nasal	<0.001	0.001	
BAL vs gastric	0.002	0.013	
Oral vs nasal	<0.001	0.001	
Oral vs gastric	0.016	0.002	
Nasal vs gastric	<0.001	0.001	
RDA1 (1st axis)			0.005
RDA2 (2nd axis)			0.005
RDA3 (3rd axis)			0.025

[a] Based on θ_{YC} distance values.
[b] Function adonis in R package vegan. Hellinger transformed distances were used (method, Euclidean).
[c] Function anova.cca in R package vegan. For axis testing, the setting by = "axis" was used.

FIG 2 (A) Intrasubject similarity indices (θ_{YC} distance $[1 - \theta_{YC}]$) between the bacterial communities of the first return BAL fluid (BAL1) and oral wash samples, compared to the second return BAL fluid (BAL2) and oral wash samples from that same subject. Distances were based on a 3% OTU definition with subsampling of 700 sequences/sample. There was no statistically significant difference in the oral-BAL fluid sample comparison of the first and second return BAL fluid samples in terms of bacterial community composition. (B) 16S rRNA gene qPCR of DNA prepared from the samples in this study, as well as bronchoscope rinse saline and prebronchoscope saline. The number of copies of bacterial 16S rRNA genes per 5 ml of sample (saline, scope, BAL, oral, and gastric) or per (nasal) swab was measured by qPCR as described in Materials and Methods. Sample groups were compared by ANOVA and Tukey's multiple-comparison test. Data are the mean ± the standard error of the mean. *, $P < 0.05$ compared to saline only; other significant comparisons are shown in the graph; nd, not done (because the swab was of a different sample type). (C) Bacterial species richness of each site, as determined by calculating the number of OTUs (97% identity) per sample after subsampling of all samples to the same depth of 700 reads. Sample groups were compared by ANOVA and Tukey's multiple-comparison test.

of the relative abundances of bacteria within a site and can be compared at that level. Thus, the oral and gastric compartments contained the highest levels of bacteria and the nasal cavity and lungs contained much lower levels.

Numbers of observed OTUs at different sites. We next determined the richness of each site by calculating the number of operational taxonomic units (OTUs) per sample after subsampling all of the samples to the same depth, 700 reads. The oral and gastric samples had the highest OTU richness, which was significantly greater than that of the lung samples (Fig. 2C). The OTU richness of the nasal samples was very similar to that of the BAL fluid samples.

Analysis of lung, oral, gastric, and nasal microbial communities. The next objective was to compare the bacterial community composition of each of the four sites to the other sites. We used redundancy analysis (RDA) to visualize how the bacterial communities at each site relate to each other (Fig. 3) and to test whether significant amounts of variation could be explained by the differences between sampling sites (Table 1). The model proved to be significant ($P = 0.005$), with significant amounts of variation explained on all three axes (Table 1). Nasal communities separated from all other samples along the first RDA axis, and BAL fluid communities separated along the second RDA axis (Fig. 3A). The greatest similarity was between the oral and gastric communities (Table 1; Fig. 3B). The same relationships of the four sites were confirmed when tested by AMOVA or PERMANOVA (Table 1). Overall, these analyses demonstrated that the BAL fluid bacterial communities were significantly different from the oral wash, gastric aspirate, and nasal swab bacterial communities.

Intrasubject comparisons of lung, oral, gastric, and nasal bacterial communities. We also examined the similarity of bacterial community at each site in a given subject to either the oral or nasal community in that individual as the source community (Fig. 3C). For each subject, we calculated the θ_{YC} distances ($1 - \theta_{YC}$) among the bacterial communities in the BAL fluid, oral wash, gastric aspirate, and nasal swab samples from that subject
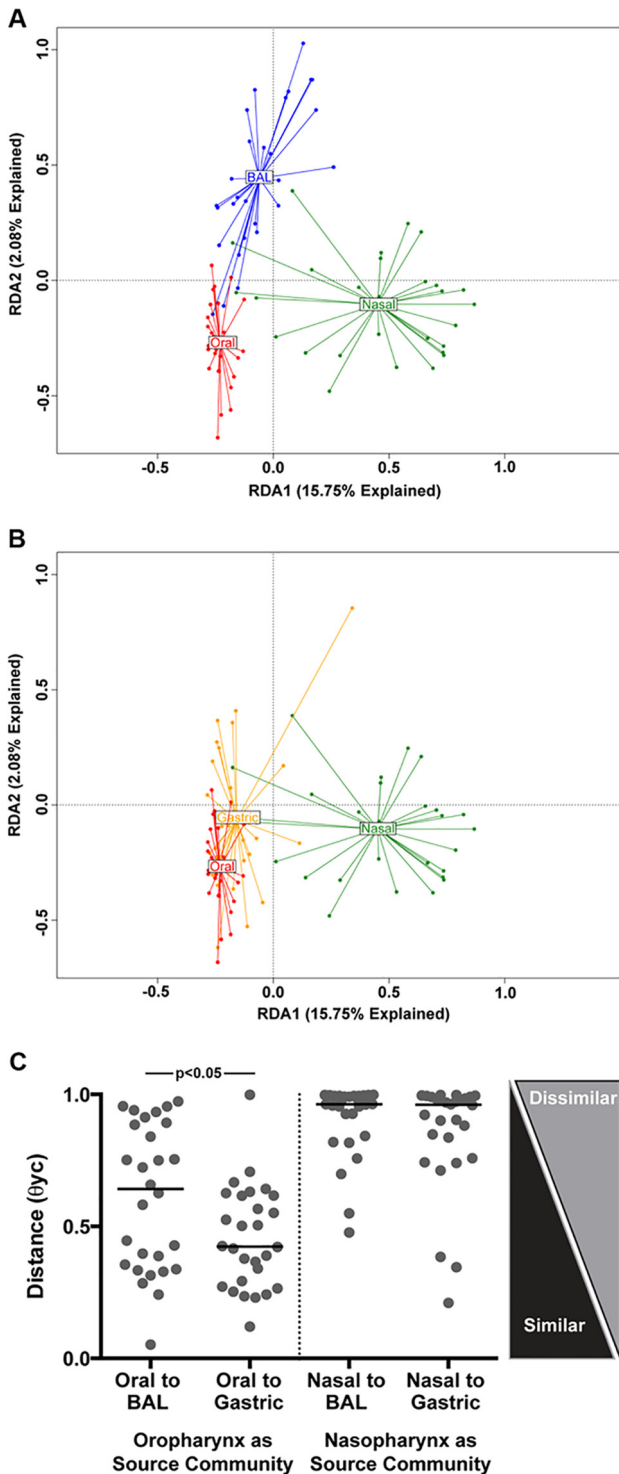
FIG 3 (A, B) Graphic representation of the results of an RDA of the bacterial samples isolated from each of the four sites to determine whether a significant amount of the variation can be explained by differences in sample location. (A) Comparison of nasal, oral, and lung samples in RDA1 versus RDA2. (B) Comparison of nasal, oral, and gastric samples on RDA1 versus RDA2. (C) Indices of intrasubject similarity (θ_{YC} distance [$1 - \theta_{YC}$]) between the bacterial communities of the two source sites (mouth and nose) and two target sites (lungs and stomach) of that subject. As shown, shorter θ_{YC} distances correspond to greater similarities between the bacterial communities of the samples indicated. Distances were based on a 3% OTU definition with subsampling of 700 sequences/sample.

(Fig. 3C). In 16/28 subjects (57%), the bacterial communities in the lung and oral samples were relatively dissimilar (θ_{YC} distance, >0.5), and in 9/28 subjects (32%), the θ_{YC} distance was >0.75. Conversely, in 4/28 subjects (14%), the lung and oral communities were highly similar (θ_{YC} distance, <0.3). These nearly bimodal data are consistent with the reports that approximately half of healthy test subjects aspirate oral/nasal secretions in a given 24-h window (6–8). In contrast, the oral wash and gastric aspirate bacterial communities tended to be more similar within a subject, with only 1/28 subjects (3.6%) having a θ_{YC} distance of >0.7, as predicted, due to swallowing of saliva (and bacteria) from the mouth. For the nasal bacterial communities, >75% of the samples had a θ_{YC} distance of >0.7, whether the comparison was to BAL fluid or gastric communities (or oral communities; data not shown). Overall, the θ_{YC} distances between lung and oral bacterial communities in a subject ranged from similar to highly dissimilar, whereas the nasal bacterial communities were largely dissimilar from the BAL fluid communities.

Bacterial community membership in the oral cavity, nasal cavity, lungs, and stomach. To gain further insights into the community memberships of all four sites, the we classified OTUs taxonomically and then compared the average abundances of each site's bacterial communities (Fig. 4). The most dominant bacterial OTUs (in descending relative abundances) in the mouth, stomach, and lungs were classified as a *Prevotella* species, two different *Streptococcus* species, an unclassified member of the family *Pasteurellaceae*, a *Fusobacterium* species, and a *Neisseria* species. In contrast, the bacterial communities of the nasal cavity were dominated by three OTUs, classified as a *Staphylococcus* OTU, a *Corynebacterium* OTU, and a *Propionibacterium* OTU. Of these, the *Staphylococcus* and *Corynebacterium* OTUs were found almost exclusively in the nasal cavities of healthy individuals, while the *Propionibacterium* OTU was found in the lungs and stomach but not the mouth. Thus, the bacterial communities of the healthy lungs shared significant membership with the mouth but not the nose (Fig. 4); however, the relative abundance of the bacterial OTUs in the healthy lungs was different from that in the mouth, resulting in significantly different community structures, as well as different from that in the nose (Table 1; Fig. 3).

Analysis of *Prevotella* abundance as a surrogate measure of species elimination. In our final analysis, we compared microbial elimination of the stomach to that of the lungs. To accomplish this, we used a paired-specimen analysis of the *Prevotella* genus. We chose this genus because of its high relative abundance and broad distribution in the oral cavity, as well as the gastric and pulmonary compartments, and the presence of multiple *Prevotella* OTUs within a subject for comparison (Fig. 5). The relative abundances of the six dominant *Prevotella* OTUs in oral communities (the primary source community) were compared to the corresponding abundance of each OTU at the same subject's distal sites (stomach and lungs). This resulted in 168 data points in each analysis (28 subjects with six *Prevotella* OTUs per subject, Fig. 5). A 1:1 ratio of OTUs at the source and distal sites would be expected if elimination were balanced with immigration; in contrast, a lack of a 1:1 ratio would imply selectivity in elimination. Comparison of oral and gastric samples revealed no evidence of selective microbial elimination in the stomach. On average, the relative abundance of *Prevotella* OTUs in the stomach was comparable to that in the mouth, distributed symmetrically around the line of identity (Fig. 5A). While some OTUs within a subject were de-
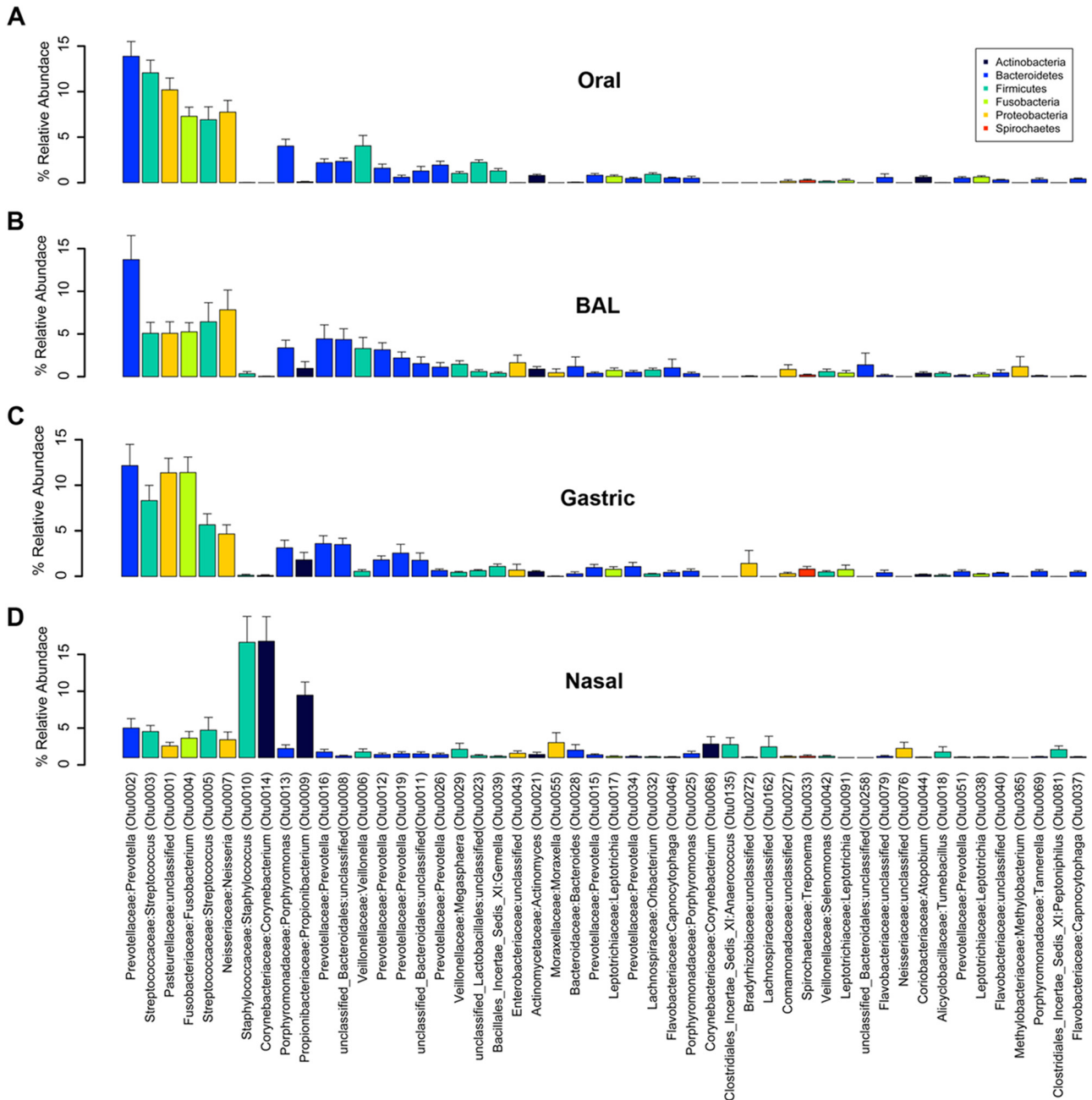
FIG 4 Rank abundance plots for each of the sampling locations based on the top 50 OTUs from the overall order (greatest to smallest) taken from all of the samples combined. The bars depict the mean ± the standard error of the mean. Bars are colored according to their phyla. The family, genus, and OTU identification of the bacterial community members are displayed along the *x* axis of panel D.

tected exclusively in the mouth, a comparable number of OTUs were detected exclusively in the stomach (Fig. 5A). Thus, we found no evidence of selective elimination of *Prevotella* from the stomach. In marked contrast, the relative abundance of *Prevotella* OTUs was not symmetrically distributed around the line of identity (Fig. 5B). This observation is consistent with the idea that selective elimination of *Prevotella* is occurring in the lungs (and inconsistent with the conclusion that BAL fluid communities are attributable to simple bronchoscopic carry-over). Numerous *Prevotella* OTUs of moderate to high abun-

dance in oral communities within a subject were below the limit of detection in BAL fluid communities (<0.5%), whereas the converse was not observed (Fig. 5B). Furthermore, when a *Prevotella* OTU could be detected in the oral wash sample, that same OTU was below detectable levels in 44% of BAL fluid samples but only 15% of gastric samples. If a *Prevotella* OTU was below the detectable level in an oral sample, it could be found 44% of the time in the gastric aspirate but only 4% of the time in the BAL fluid. Thus, the lung, unlike the stomach, exhibits evidence of selective elimination of *Prevotella* species,
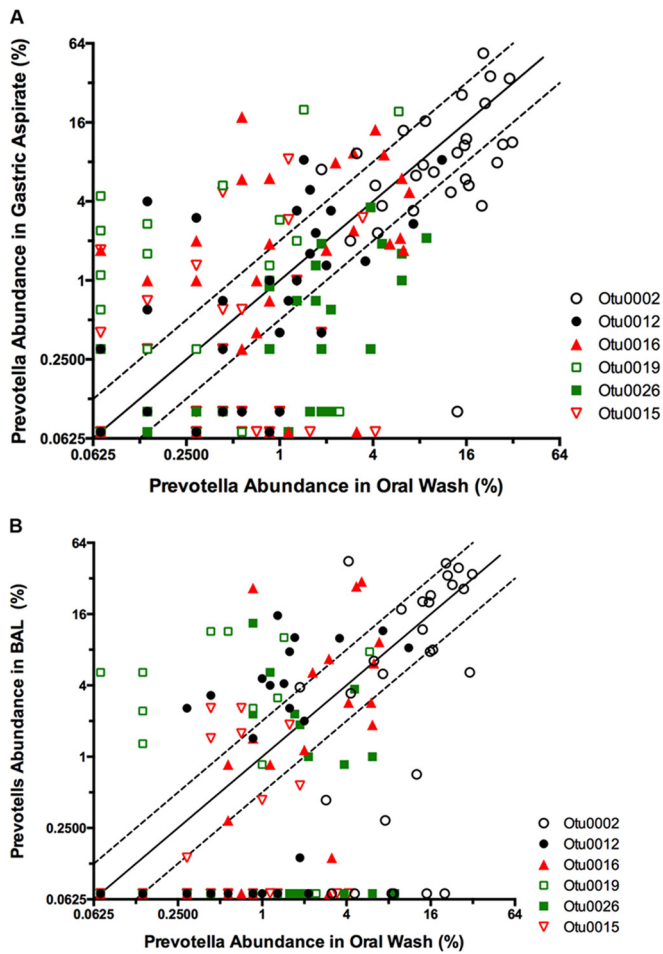
FIG 5 Paired analysis of *Prevotella* abundance distribution in the oral wash of an individual and the abundance of that same OTU in the gastric aspirate (A) and BAL fluid (B) of the same individual. *Prevotella* OTUs were determined as described in Materials and Methods, and all of the samples from all of the sites were subsampled to 700 reads. For this analysis, any OTU below the limit of detection was assigned an abundance of 0.07%. The solid line indicates a 1:1 ratio of abundance in the oral wash compared to the gastric aspirate (A) or BAL fluid (B), and the dotted lines are 2:1 and 1:2 ratios. Six *Prevotella* OTUs were detected in the analysis, and the abundance of each OTU in each of the 28 subjects is displayed on the graphs (168 data points/graph). The oral-BAL fluid sample and oral-gastric sample data sets were significantly different from each other ($P = 0.003$). The mean ratio within the oral-gastric sample data set was 1.18:1, while the mean ratio within the oral-BAL fluid sample data set was 2.04:1. Other abundance comparisons: 11.4% of the observations in the oral-BAL fluid sample data set were at a ratio of 64:1 or greater, and 31.0% were at 8:1 or higher, while only 2.4% of the observations in the oral-gastric sample data set were 64:1 or greater and only 14.3% were 8:1 or greater.

one of the most abundant members of the bacterial community in the aerodigestive tracts of healthy individuals.

DISCUSSION

This study, which analyzed the upper aerodigestive tract of 28 healthy human subjects, is the first to compare the mouth, nasal, gastric, and lung bacterial microbiota within the same individual. We demonstrated that the bacterial communities of the healthy lung overlapped those found in the mouth but were found at lower concentrations, with lower membership and a different community composition. The nasal microbiome, which was dis-

tinct from the oral microbiome, appeared to contribute little to the composition of the lung microbiome in healthy subjects. The stomach microbiome was far more similar in all study subjects to that of the mouth in bacterial concentrations, membership/diversity, and composition, as expected, due to swallowing of saliva (and bacteria) from the mouth. Intrasubject analysis of *Prevotella* OTU abundances in the mouth compared to the lungs and stomach supports the concept that the mouth is a major source community for the lungs and stomach microbiomes (Fig. 1); however, the lungs exhibit selective elimination of *Prevotella* species, one of the most abundant bacterial community members in the aerodigestive tracts of healthy individuals. Within an individual, the similarity between the oral and lung microbiomes varied widely, with some individuals having a lung microbiome that was almost identical to their oral microbiome, similar to a previous report (9), while in others the two sites were markedly different. Our nearly bimodal data are consistent with the reports that approximately half of healthy test subjects aspirate oral/nasal secretions in a given 24-h window (6–8). Overall, our data support the concept that microaspiration is common in healthy individuals and illustrate the microbiological continuity of the aerodigestive tract in healthy adults.

Neither the lung nor the stomach was included in the microbiome surveys of healthy individuals for the HMP (5), but other studies have begun to fill in those gaps, focusing on either the lungs or the stomach (1–4, 17–20). The detection of high levels of mouth-resident bacteria in the stomach is consistent with previous reports (18–20). This is also the expected result of the swallowing of saliva (and bacteria) from the mouth. One limitation of culture-independent analysis is that it cannot distinguish live from recently killed bacteria. However, even with this caveat, our study lays the foundation for future investigations of anatomy and unique environmental niches of the upper aerodigestive tract as factors that shape the lung microbiota during health and disease.

Our comparison of nasal, oral, lung, and gastric microbiotas within the same individuals provides culture-independent microbiological support for the concept that microaspiration is common in healthy individuals. Huxley and colleagues used radioactive indium to study pharyngeal aspiration during sleep in 20 healthy subjects and 10 patients with depressed consciousness. Almost half of the healthy subjects and 70% of those with depressed consciousness aspirated during deep sleep. Those normal subjects who did not aspirate were noted to sleep poorly (7). Similarly, Gleeson and colleagues performed a study in which a radioactive Tc tracer was deposited into the nasopharynxes of 10 healthy subjects through a small catheter during electroencephalogram-documented sleep and standard lung scans were conducted immediately following final awakening. They documented that microaspiration occurs commonly in healthy young men during sleep, is unrelated to sleep quality, and is variable within subjects who were studied on more than one occasion. They concluded that the "quantity aspirated is of an order of magnitude likely to contain bacterial organisms in physiologically significant quantities" (6). Furthermore, as discussed by Quinn and Meyer, healthy human subjects, as well as other mammals, aspirate small amounts of liquids from the upper airways into the lower airways (8).

We did not detect a significant contribution of the nasal microbiome to that of the lungs during health (of the three major OTUs in the nasal cavity, only low levels of the *Propionibacterium*

OTU were readily detected). *Staphylococcus*, *Corynebacterium*, and *Propionibacterium* species are also dominant members of the skin microbiota and have been previously reported as members of the nasal cavity microbiota (5, 21–23). Our study raises the possibilities that (i) the earlier tracer studies were far more sensitive for tracking nasal-oral-airway dispersion, (ii) nose-associated bacteria are more firmly attached than the intranasal liquid radiotracers, and (iii) the design of these studies, both of which involved the insertion of a catheter via the nose into the posterior nasopharynx, actually more closely simulates aspiration of saliva in the supine position. During health, there is a constant flow of liquid from the mouth (saliva) while there is much less liquid flow from the nose into the throat. During states of ill health, such as viral upper respiratory tract infections, allergies, or sinus infections, the liquid flow from the nasal cavity increases, and this clearly has the potential to affect the lung microbiome, consistent with studies of rhinovirus infection and asthmatics that highlighted changes in the lung microbiome under these conditions (3, 11, 24, 25).

The stomach and lung present very different ecological niches for bacterial survival, yet both are highly restrictive. *Helicobacter* and *Lactobacillus* are two bacterial genera that are well adapted for growth at low pH and have routinely been reported by culture from healthy human stomachs (18). Of note, we only observed *Lactobacillus* at a >0.5% abundance in the gastric samples of one of our subjects. This exact same protocol and primers can detect *Lactobacillus* in our mock-treated controls, as well as the mouse intestinal tract (26). Thus, the lack of *Lactobacillus* likely reflects differences in sampling. The pH should exert some selective pressure, favoring the retention of acid-tolerant bacteria such as *Lactobacillus*; however, the fact that we did not observe such a selective pressure likely reflects the fact that immigration is constant and at a high level (swallowing of saliva), compared to the levels of indigenous colonization of the stomach and the gastric aspirate is largely sampling the upper compartment of the stomach. In contrast to the stomach, no such bacteria have been reported by culture for healthy human lungs. Culture-independent surveys of the stomach and lungs have revealed a microbiome that is much more diverse than that reported by culture alone (1–4, 17–20). Identifying the reasons for this discrepancy between culture-based and culture-independent studies is an active area of investigation (27) and very likely involves the adaptation of immigrant bacteria to the metabolically restricted and harsh environments of the stomach and lower airways.

The high shared membership of bacterial species between the lung microbiome and that of the mouth in our study and others, contrasting with that reported for air, suggests that microaspiration and direct mucosal translocation contribute more to microbial immigration than does inhalation of bacteria (6–8, 28–31). Microbes are cleared from the respiratory tract via mucociliary clearance, coughing (frequent even among healthy subjects [32]), and innate and adaptive immunity. The distal alveoli are bathed in pulmonary surfactant, which also has bacteriostatic activity against some bacterial strains, further creating selective pressure on reproducing communities (33). Thus, the "steady state" of the lung microbiome during health is likely one of constant influx, constant elimination, and unfavorable growth conditions.

Our data support the concept that immigration is the significant driver of "maintenance" of the lung microbiota during health. Healthy individuals with very similar oral and BAL fluid microbiome community structures may simply have experienced

recent aspiration events or may have some anatomical abnormalities. In addition to being strongly influenced by immigration and weakly influenced by the relative reproduction rates of community members, the community membership of the lung microbiome is strongly influenced by selective elimination, which is absent from the gastric microbiome. Our empirical evidence of these ecological determinants is consistent with the known anatomical, physiological, and immunological features of the respiratory tract.

Similar to the studies of Segal et al., we could identify individuals with high versus low *Prevotella* levels in the lungs (13), and when our results are combined with those of the previous study, a model begins to emerge that suggests that subclinical lung inflammation may create an environment that promotes retention of *Prevotella* in the lungs. Alternatively, the presence of *Prevotella* may stimulate subclinical lung inflammation, as suggested by Larsen et al. (34, 35). The latter investigators demonstrated that *Haemophilus*, *Moraxella*, and *Prevotella* species can all induce activation of human monocyte-derived dendritic cells (DCs), but asthma- and chronic obstructive lung disease (COPD)-associated pathogenic *Haemophilus* and *Moraxella* bacteria induce 3- to 5-fold higher production of the interleukin-23 (IL-23), IL-12p70, and IL-10 cytokines than the "commensal" *Prevotella* bacteria and also induce more pulmonary inflammation when introduced into mice. Interestingly, coculture experiments found that *Prevotella* species were able to reduce *Haemophilus influenzae*-induced IL-12p70 but not IL-10 in DCs (35). All together, these studies raise the possibility that subclinical lung inflammation associated with the presence of *Prevotella* may protect the lungs from overexuberant inflammatory responses.

MATERIALS AND METHODS

Subject demographics and sample collection. The University of Michigan and Veterans Affairs Ann Arbor health care system institutional review boards approved this study. All subjects provided written consent. The subjects were clinically well and could not have received antibacterials or corticosteroids in the 3 months prior to sampling. Detailed exclusion criteria were described previously (17). The demographics of the 28 subjects included in this study are presented in Table 2 (FEV$_1$% = FEV$_1$/FVC ratio, where FEV$_1$ is the volume that has been exhaled at the end of the first second of forced expiration and FVC is the forced vital capacity [the vital capacity from a maximally forced expiratory effort]).

The same bottle of freshly opened sterile 0.9% saline (Baxter Healthcare Corporation) was used for all sample collection from a given participant. Two saline control samples were collected for each bronchoscopy in specimen cups: an aliquot of saline directly from the bottle (neat saline) and a 10-ml sample of saline that was collected after injection through the bronchoscope with a sterile syringe (scope saline).

Oral washes were performed at the start of the bronchoscopy study visit, before any topical anesthesia or sedation, by having the participant gargle for 30 s with 20 ml sterile saline and then immediately expectorate into a sterile specimen cup. The scope saline, neat saline, and oral wash

TABLE 2 Study subject demographics

Parameter	Value
Total no.	28.0
No. of females/males	19/9
No. (%) who never smoked	20.0 (71.4)
No. (%) of current smokers	4.0 (14.3)
No. (%) of former smokers	4.0 (14.3)
FEV$_1$% range (mean ± SD	59.0–120.0 (102.0 ± 17.7)

samples were placed on ice. After the oral wash was completed, subjects were asked to gargle with 10 to 20 ml of Listerine antiseptic mouthwash for 20 s.

Bronchoscopy was performed under moderate conscious intravenous sedation conducted in accordance with standard safety and monitoring procedures for clinically indicated bronchoscopy, with some modifications to reduce possible contamination of the lower respiratory tract during sample collection. Participants were premedicated with diphenhydramine, midazolam, and fentanyl, followed by topical lidocaine. The bronchoscope was advanced via the mouth to a position just above the vocal cords. Lidocaine (4%) was slowly injected around the glottis as 4 aliquots of 1 ml each, attempting when possible to have the participant oppose the cords by phonating the letter E to maximize exposure to the vocal cords and minimize penetration of the medication below the cords. The bronchoscope was then advanced rapidly, without suction and as much as possible without additional lidocaine in the lower airways, into the wedged position in a subsegment of the right middle lobe and lingula.

BAL was performed by installation of saline in 30-ml aliquots that were immediately aspirated under gentle manual suction to a total of 300 ml (150 ml for each lung segment). After insertion of the bronchoscope past the epiglottis into the first lobe (usually the left one) for the first BAL, the bronchoscope was then retracted slightly without pulling the tip of the bronchoscope more proximal than the midtrachea and then repositioned into the other lobe for the second BAL. During BAL, the participant underwent gentle mechanical percussion with a pneumatic chest percussor to maximize the yield of alveolar cells for ancillary analysis of the host immune response. BAL fluid specimens from the two lung segments were collected in separate specimen cups and stored on ice.

After both BALs were completed and while the subject was still sedated, the bronchoscope was withdrawn to a position immediately superior to the glottis, where it was stabilized at the mouth with the vocal cords in constant view. An 18-gauge sterile gastric tube was introduced via the mouth, and the participant's head was flexed gently. Passage of the tube posterior to the larynx into the esophagus was observed directly to exclude entry into the trachea. The gastric tube was advanced to 40 to 45 cm, and its correct placement was confirmed by auscultation of air injected with a syringe. The bronchoscope was then withdrawn. Next, 50 ml saline was instilled through the tube and immediately aspirated with the syringe. The return was collected and placed on ice, and the gastric tube was removed.

Finally, while the participant was still sedated, the posterior nasopharynx was swabbed gently with a single sterile swab introduced via the nares without lubrication or topical anesthetic. The tip of the swab was cut with sterilized scissors and placed into a 2-ml UltraClean Fecal DNA Isolation kit (Mo Bio Laboratories, Inc.).

Sample processing and DNA isolation. Samples were transferred to the laboratory on ice. For saline controls, oral wash samples, BAL fluid samples, and gastric aspirate samples, 1 ml was transferred to a dry bead tube (Mo Bio Laboratories, Inc.), the tubes were centrifuged for 2 min at ~16,000 × g, and the supernatant was removed. This was repeated until 5 ml saline control, 5 ml BAL fluid, 5 ml oral wash, or 10 ml gastric aspirate had been transferred to the dry bead tube. Samples were then stored at −80°C until DNA isolation.

For DNA isolation, 750 μl PowerSoil DNA kit bead solution (Mo Bio) and 60 μl PowerSoil DNA kit solution C1were added to each Dry Bead Tube (containing a nasal swab or the pellet from 5 ml saline control, 5 ml BAL fluid, 5 ml oral wash, or 10 ml gastric aspirate). Samples were bead beaten for 2 min at the Homogenize setting of a Mini-BeadBeater-8 (Bio-Spec Products) and centrifuged for 30 s at 10,000 × g. We then continued with the PowerSoil DNA Isolation kit protocol (Mo Bio) starting with step 7 (transfer supernatant to a clean 2-ml collection tube) or transferred samples into a 1-ml collection plate to continue with the PowerSoil-htp 96 Well Soil DNA Isolation kit protocol (Mo Bio) starting with step 10 (add 250 μl solution C2 to each well).

qPCR. Quantification of bacterial 16S rRNA-encoding genes was performed by real-time PCR with TaqMan hydrolysis probes on a Roche 480

LightCycler. The primers used, targeting the V1 and V2 regions of the 16S rRNA-encoding gene, were 5′ AGAGTTTGATCCTGGCTCAG 3′ (forward) and 5′ CTGCTGCCTYCCGTA 3′ (reverse), and the probe used was 5′-FAM-TA+ACA+CATG+CA+AGTC+GA-BHQ1-3′ (where FAM is 6-carboxyfluorescein, locked nucleic acid nucleotides are indicated by preceding plus signs, and BHQ1 is Black Hole Quencher 1) (9, 36). After an initial denaturation for 5 min at 95°C, 40 cycles of amplification for 30 s at 94°C, 30 s at 50°C, and 30 s at 72°C were performed. A final elongation step of 72°C for 2 min was performed. Final cooling was performed at 4°C.

Microbiota analysis by pyrosequencing. Libraries of V3V5 16S rRNA gene amplicons were constructed on the basis of the HMP protocol (http://www.hmpdacc.org/doc/16S_Sequencing_SOP_4.2.2.pdf) as described previously (37, 38), except under the following PCR conditions. The PCR started with 2 min at 95°C, which was followed by (i) 20 cycles of a touchdown PCR for 20 s at 95°C, 30 s at the annealing temperature (which was 60°C in the first cycle and dropped 0.5°C with each cycle), and 5 min at 72°C and then (ii) 20 cycles of a standard PCR with 20 s at 95°C, 30 s at 50°C, and 5 min at 72°C. Large-volume Lib-L emPCRs (Roche 454) were performed, and 454 sequencing was performed at the University of Michigan with the GS FLX titanium platform (Roche) in accordance with the manufacturer's instructions.

Sequence processing and low-biomass controls. Sequences were processed with mothur v.1.30.0 (39, 40) as described previously (37, 38, 41) through the step used for removal of sequences classified as chloroplast, mitochondria, *Archaea*, *Eukaryota*, or unknown kingdom. However, the fasta, names, and groups files (six of each, two from each sequencing run) were concatenated into a single fasta, names, and groups file, respectively, after the trim.seqs step. To control for equipment- and reagent-derived DNA that could contaminate the low-bacterial-biomass BAL fluid samples, we collected and sequenced controls from (i) scope saline, (ii) neat saline, and (iii) reagents used for DNA extraction. A customized R script based on the neutral model (42) was then used to identify and remove individual BAL fluid sequences that that likely arose by contamination from neat saline, scope saline, DNA isolation reagents, or PCR reagents (17). This model uses the relative abundance of a sequence read in the controls to calculate the probability of its detection in BAL fluid samples because of carryover. This analysis was performed prior to clustering of the sequences into OTUs at 97% similarity. Sequence reads falling within the confidence intervals of the model (neutrally distributed from controls) and outside the lower bounds of the confidence intervals (enriched in controls) were considered to be contaminating sequences and were removed from the analysis of all BAL fluid samples. Only sequences outside the upper bounds of the confidence interval with the controls as the source (overrepresented in BAL fluid samples) and unique to BAL fluid (not detected in controls) were retained for subsequent analyses.

Sequence analysis. Sequences were clustered by mothur into OTUs defined as ≥3% difference between OTUs using the average-neighbor algorithm. Consensus taxonomic classifications were generated for each OTU based on modified Ribosomal Database Project reference files. The make.Shared command was used to produce a table (shared file) of the number of sequence reads assigned to each OTU in each sample. Seven hundred sequences from each sample were subsampled, and samples with fewer than 700 sequences were discarded. A complete sample set (BAL1, BAL2, oral wash, gastric aspirate, and nasal swab), with a minimum of 700 sequences/sample, was required for each subject included in the analysis. The shared file was used to calculate the θ_{YC} distance $(1 - \theta_{YC})$ between bacterial communities.

For analyses in which BAL1 and BAL2 were combined (identified as BAL), the sequences of each OTU in the BAL1 and BAL2 samples were added together for each subject prior to subsampling at 700. Seven hundred sequences per sample were then subsampled from the shared file, and samples with fewer than 700 sequences were discarded. A complete sample set (BAL fluid, oral wash, gastric aspirate, and nasal swab), with a

minimum of 700 sequences/sample, was required for each subject included in the analysis. This shared file was used to calculate the number of OTUs observed in each sample, AMOVA based on θ_{YC}, PERMANOVA, redundancy analysis (RDA), relative abundances of OTUs and the θ_{YC} distances $(1 - \theta_{YC})$ between bacterial communities in all of the samples and within each subject.

Statistical analysis. Statistical analyses were performed with Prism 5 (GraphPad Software) for analysis of variance (ANOVA), *t* test, and regression analysis; mothur for AMOVA (43); and vegan and R for all diversity, rank abundance, and ordination analyses. The θ_{YC} distance $(1 - \theta_{YC})$ between bacterial communities measures differences in community structure (44) and was selected because it weighs rare and abundant OTUs more evenly than other metrics such as Bray-Curtis or Morisita-Horn (39). Using the shared file, we calculated θ_{YC} distances $(1 - \theta_{YC})$ between communities with mothur (http://www.mothur.org/wiki/Thetayc), where S_T is the total number of OTUs in samples P and Q, p_i is the relative abundance of OTU i in sample P, and q_i is the relative abundance of OTU i in sample Q. The θ_{YC} distance $(1 - \theta_{YC})$ incorporates the relative abundances of shared and nonshared OTUs of two communities. For data display by ordination, RDA (the constrained form of principal-component analysis) was used to focused on the variation that can be explained by the question of interest (are the microbial communities different at different sampling locations). ANOVA-like permutation testing of constrained ordinations, including RDA, was performed with the anova.cca function in the R package vegan. Significant differences in community membership identified via constrained ordination were confirmed by PERMANOVA via the Adonis function in vegan.

BioProject accession numbers. The sequences determined in this study are available at the NIH Sequence Read Archive (http://www.ncbi.nlm.nih.gov/bioproject) under BioProject accession numbers PRJNA263948 and PRJNA269493.

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