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The Upper Respiratory Tract as a Microbial Source for Pulmonary Infections in Cystic Fibrosis

Parallels from Island Biogeography

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Abstract

A continuously mixed series of microbial communities inhabits various points of the respiratory tract, with community composition determined by distance from colonization sources, colonization rates, and extinction rates. Ecology and evolution theory developed in the context of biogeography is relevant to clinical microbiology and could reframe the interpretation of recent studies comparing communities from lung explant samples, sputum samples, and oropharyngeal swabs. We propose an island biogeography model of the microbial communities inhabiting different niches in human airways. Island biogeography as applied to communities separated by time and space is a useful parallel for exploring microbial colonization of healthy and diseased lungs, with the potential to inform our understanding of microbial community dynamics and the relevance of microbes detected in different sample types. In this perspective, we focus on the intermixed microbial communities inhabiting different regions of the airways of patients with cystic fibrosis.

> When we try to pick out anything by itself, we find it hitched to everything else in the Universe.
>
> —John Muir, *My First Summer in the Sierra*

Individual humans, and their organs and tissues, can be considered islands. Like the islands of the Galapagos, humans constitute spatially structured environments that offer microbes abundant ecological opportunity (Figure 1). The Human Microbiome Project (http://www.hmpdacc.org/) has shown that different organs and systems within the human body are inhabited by different species assemblages (1). Just as the islands of an archipelago are exposed to a reservoir of potential emigrants from the mainland, individual human organ systems are exposed to potential migrants from the surrounding milieu. Irrespective of specific habitat details, community assembly and stability are driven by dispersal rates and priority effects, with the order and timing of arrival influencing the fate of particular species (2, 3).

The diversity of species inhabiting the islands of an archipelago depends on how close each island is to a mainland (from which colonizing species are derived) and the rate that species go extinct (Figure 2). The theory of island biogeography, born in the 1960s (4), includes islands of many different sorts: islands as mountain tops, lakes, trees, and in fact any set of niches separated by time, space, or an environmental barrier. Although studies of island biogeography initially focused on ecology, there has long been awareness that patterns of species diversity cannot be fully understood outside the evolutionary processes that fuel diversification (5). Indeed, incorporation of evolutionary thinking into ecology has underpinned advances in understanding adaptive radiation, speciation, and opportunities for ecosystem restoration.

In extreme cases, for example after a forest fire, the process of community assembly is given special prominence (6). However, the same processes that lead

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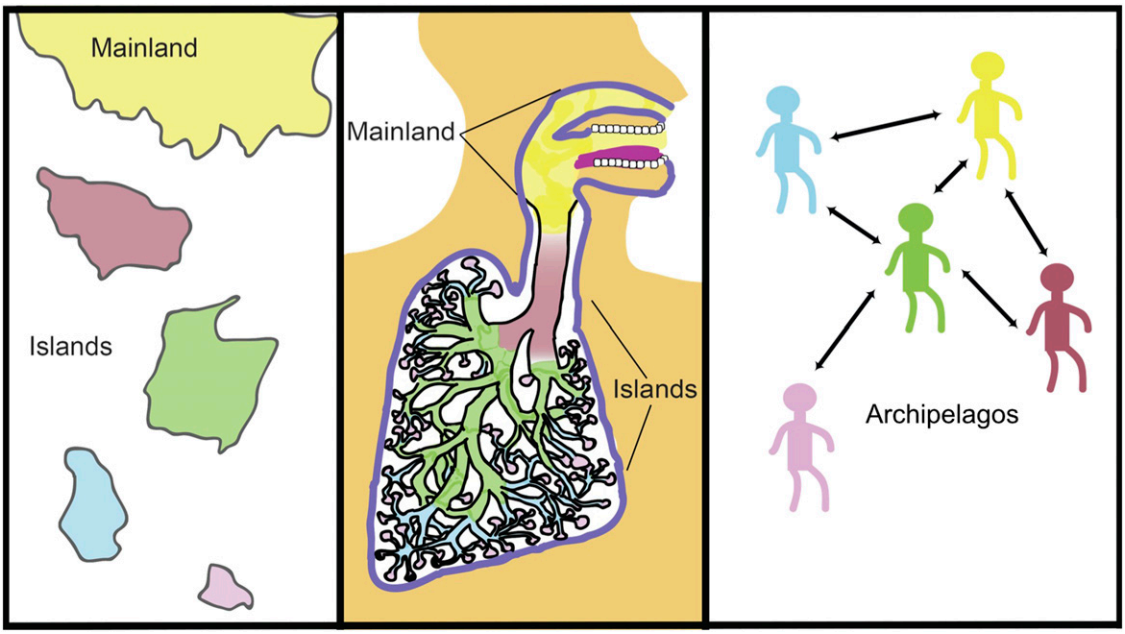

Figure 1. Parallels between island biogeography and polymicrobial lung colonization. (*Left*) In island biogeography theory, the mainland is the greatest source of species diversity, with individual island species composition depending on the distance from the mainland. (*Middle*) Human airway microbial colonization is likely to display a similar dependence on the distance from the mainland (largely the oral cavity, shown in *yellow*, which is the richest and most diverse source of microbes with proximity to the lung). (*Right*) Other people, along with the air, water, and other environments, are also important sources of microbes, which can immigrate to the islands in the human airways and influence the polymicrobial community.

to reestablishment of a forest on barren land may also govern the formation of host-associated microbial communities. Community assembly has important implications for health and disease (2, 3, 7). For example, the capacity for a pathogen to establish itself in a given niche is likely to depend on the presence (or absence) of competing microbes (8–12), the timing of arrival, and the history of colonization events. Establishment will also be affected by host factors, including the immune system, the availability and quality of nutrients (their spatial and temporal distribution), and the physical structure and properties of tissues and surfaces.

Island Culture: Distinct Communities Intermingle in the Oropharynx–Airway–Lung Ecosystem

As islands that provide ecological opportunity to microbes, the unique environment of the lung in diseases such as cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD), and other inflammatory respiratory conditions are of special interest. Microbes immigrating to the lung come from various sources, including air, water, and food, but also from the oral cavity and other nearby islands, such as the sinuses and even the gastrointestinal tract. In the model proposed here, the oral cavity might be considered as the mainland, or the largest, most diverse proximal island, and various niches in the respiratory tract as islands (Figure 2). Like modern ecologists who find exceptions to the original island biogeography theory

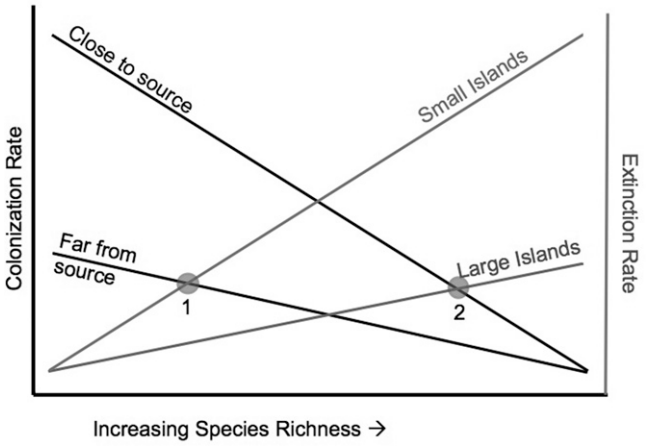

Figure 2. Classic island biogeography. The richness of species depends on the colonization rate (*left y-axis*) and the extinction rate (*right y-axis*). Migration through the trachea offers colonization opportunity to microbes from multiple sources, and impaired mucociliary clearance decreases the extinction rate. *Gray circle 1* represents a small distant island (i.e., the lung) with few species, whereas *gray circle 2* identifies the mainland or a large proximal island with high species diversity, such as the oral cavity. Diversity is composed of both the number of species and their distribution, or evenness, and can be indicated by different measures of species richness and frequency. The number of species, or species richness, is an indicator of diversity. The term diversity is used throughout this perspective as informed by the species richness, which can be predicted in the island biogeography model. Adapted by permission from Reference 4.

of the 1960s, it is necessary to acknowledge the influence of factors beyond the size and remoteness of island reservoirs on community diversity (as indicated by species richness) (13). In patients with CF, the immune system, interspecies interactions, and antibiotic pressure exert profound additional influences on microbial community structure.

Microbes traditionally inhabiting the oral cavity that are also capable of colonizing the lower airways are likely to be an important source of attack community microbes in lungs, as presented in the climax–attack model (14). The climax–attack model postulates that there are two major microbial or viral communities inhabiting the CF lung: a well-adapted, persistent climax community and an attack community composed of more virulent and transient microbes and viruses. Although the oral cavity hosts a large diversity of microbes, the most abundant species found deep in explanted lung samples from patients with end-stage disease are usually from a small group of known CF pathogens, including *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *Staphylococcus aureus*, and *Burkholderia cenocepacia* (15). Furthermore, well-studied clinical isolates of *P. aeruginosa* often harbor a subset of common mutations that reveal their adaptation to the CF lung environment (16–18). Recent evidence suggests a similar trend in *Rothia mucilaginosa*, a bacterium traditionally considered to be part of the oral microbial community (19).

Oral microbes that do not normally inhabit the lungs are likely to significantly impact lung pathophysiology through the interconnected oropharynx–airway–lung ecosystem. Even if those oral microbes remain in their endemic location (the oral cavity), they may still impact populations in the lung by producing metabolites that passively travel to the lungs (20–22) or by stimulating systemic host responses that could directly impact the deep airway microbial community. Additionally, given the potential for these oral microbes to emigrate to the lungs across the continuum of the respiratory system, these same microbes may be important competitors and thus regulators of lung-invading microbes. Bacteriophage and other agents of gene exchange may also transfer DNA-encoded traits (e.g., antibiotic resistance) (23, 24). Microbes within the oral cavity, and their migration from that source, are therefore likely to be important contributors to lung disease. It is critical that all such sources are identified and studied so that their connection with the CF lung can be understood.

Given the potential for mainland microbes to directly and indirectly affect lung communities, we argue that to understand the evolution of polymicrobial lung communities, and their related pathophysiologies, ecological dynamics must be acknowledged both within resident lung microbial communities and in neighboring niches.

Some have argued that oral commensals detected in sputum samples used to interrogate lung microbial communities represent contamination from the oral cavity (15, 25, 26). However, the degree to which oral microbes may emigrate and persist in lower airways is likely to vary from one patient to the next, depending on the presence of specific microbes as well as a variety of other environmental and host factors. Thus, tracking all airway microbes is important because oral microbes need not frequently form resident communities in the lower airways to profoundly influence polymicrobial community physiology.

A Bridge to the Lung: Migration across the Trachea

The physical continuum of the trachea connects the oral cavity and the lungs. Indeed, lung samples from healthy humans are not sterile, and they occasionally contain microbes traditionally associated with the oral cavity (27–30). The density of bacteria in the oral cavity is orders of magnitude greater than the healthy lung (27). The composition and even the existence of microbial communities in healthy human lungs is an active area of research. Whether the upper and lower respiratory tracts of healthy people contain "tourists," which are quickly expelled, or whether there exist resident microbial communities is an important question (27, 29, 31–33). Either way, differences in commensal and pathogenic microbial load in the airways of healthy humans and those affected by various respiratory conditions are likely to be affected by the rate at which new types enter the system and the rate at which they fail to colonize or go extinct.

Healthy and diseased lungs are equally accessible to microbial migrants—they both contain a bridge across the trachea, and, significantly, migration may not always result in persistent colonization. Healthy lungs have coordinated mucociliary clearance (34) that forces microbes out, and this limits the time for adaptation and establishment. This means the extinction rates of microbes that reach the lower airways are much higher in healthy lungs; impaired mucociliary clearance will reduce extinction rates and lead to larger numbers of species colonizing the lower airways (Figure 2). Emigration of oral microbes to the lower airways due to aspiration is important in a number of respiratory conditions, in which a large fraction of infections are believed to have oral microbial etiology (35–39). Respiratory conditions associated with decreased extinction of microbes that reach the lower airways include smoking (31), vaccination and probiotics (40, 41), respiratory virus infection (42–44), pneumonia (36), HIV (45, 46), COPD (30, 47–49), and CF (50). In addition, improvements in oral hygiene practices in hospitals have been shown to prevent pneumonia (51). In newly transplanted lungs of patients with CF, strains of bacteria found in the sinuses are later found in the newly transplanted lungs, again suggesting that microbes in the sinuses have a route to the lower respiratory tract (52).

In CF, the epithelia lining the airways are covered by abnormally dense mucus, trapping the cilia and rendering them nonfunctional (53). In addition, lung-specific immune responses are impaired in CF, including dysfunctional alveolar macrophages and autophagy (54–56). Together, these defects allow the commonly observed opportunists, such as *P. aeruginosa* and *B. cenocepacia*, to persist in CF airways. Most patients harbor unique and persistent microbial communities, suggesting differences from patient to patient in exposure to microbial immigrants, or selective pressures, or both. Although the well-known opportunistic pathogens are clearly important, they do not exist in isolation; interactions with other community members have important implications (11, 57–59). Indeed, there are increasing examples of infections driven by an altered polymicrobial community, rather than a single pathogen, and these will likely influence how clinicians diagnose and treat infections (59, 60). Pneumonia, COPD, CF, chronic sinusitis, periodontitis, and

otitis media are examples of infections in which a commensal from one human site can emigrate to become a pathogen in another context (36, 51, 61–66). Like drifting seeds from a proximal island, exposure and survival rates of microbes from nearby habitats stand to profoundly influence microbial community composition and interactions.

Spoiled Sputum?

As sampling of the lungs of a living human generally requires passage through the oral cavity, the question of how frequently and how deeply oral microbes penetrate into the lower airways is unresolved. Invasive lower airway sampling methods such as bronchoalveolar lavage (BAL) enable careful sampling of a specific region of the lung with reduced potential for contamination, but these methods cannot be performed on a regular basis because of negative impacts on patients. Sputum and BAL samples are also limited in the extent to which they provide insight into spatial distribution of microbes within the airways. Currently, when typical CF pathogens are not found in CF sputum cultures, clinical microbiology labs report them as culture negative (67), essentially disregarding oral microbes as contaminants without clinical relevance. Given the interconnected topography of the oral cavity and the airways, it would not be surprising to learn that some microbial groups are found in both the oral and lung environments. Direct evidence that organisms considered oral contaminants are present in the large airway exists from early culture studies based on transtracheal aspirates, where mixing with oral microbiota is avoided (68). In addition, next-generation sequencing has often detected surprisingly high relative abundances of species such as *Streptococcus* spp., *R. mucilaginosa*, or *Gemella* spp. in large volumes of purulent sputum (milliliters to tens of milliliters) (11, 19, 69). Overall, although there are limitations associated with sputum samples, the fact that they can be obtained by noninvasive means, and that they show patterns of diversity similar to lower and upper airways (8, 15, 16, 34), suggest it is sensible to obtain as much information from sputum samples as possible.

To directly assess lung microbial communities, multiple studies have examined the microbes present in explant lung samples. These samples carry their own caveat—they are most often obtained from patients with end-stage disease and have been shown to contain significant regional differences in community composition and severely reduced diversity (30, 70, 71). In an attempt to reconcile the different caveats of sputum and explant lung samples in CF, a study by Goddard and colleagues in 2012 compared throat, sputum, and explant lung samples (average of all lobar bronchi) from the same patients with CF, close to the time of transplant surgery (15). This study concluded that next-generation sequencing of DNA in sputum samples inaccurately represents airway microbial communities and that oral microbes detected by this method should be regarded as contaminants.

We present a complementary analysis of Goddard and colleagues' 2012 data. Whereas Goddard and colleagues present the relative abundance of taxa as bar plots, we took the relative abundance data from their supplementary material and visualize an unsupervised random forest in a multidimensional scaling plot. The bar plots of Goddard and colleagues and our analysis both show that the overall microbial community composition of sputum samples closely resembles that of the explanted lung samples in half of the six samples tested (72) (Figure 3). In the other three patients, additional microbes traditionally considered oral commensals were found in sputum samples and were not found in the explanted lung samples. However, for all samples, the dominant microbe found in sputum was the same as the dominant microbe found in the explant lung samples. The lack of additional commensal microbes in three of the sputum samples may reflect antibiotic treatment and disease state rather than a lack of oral contamination. We therefore favor the interpretation that microbes considered as contaminants might be important components of CF lung ecology. We list some issues that warrant consideration.

First, the careful study of explanted lungs presented by Goddard and colleagues provides valuable information about the end-stage disease–associated microbial community DNA found in CF explanted lungs. However, end-stage disease communities are not representative of microbial community composition and activity during earlier stages. One of the most consistent signatures of CF airway microbial community evolution from longitudinal sputum sampling is the increase in antibiotic-resistant pathogen load as disease state becomes more severe (15, 50, 73–75). Furthermore, by the time a lung is surgically removed, many of the airways have undergone bronchiectasis and become clogged, no longer exchanging air with upper airways. Substantially reduced microbial diversity is expected

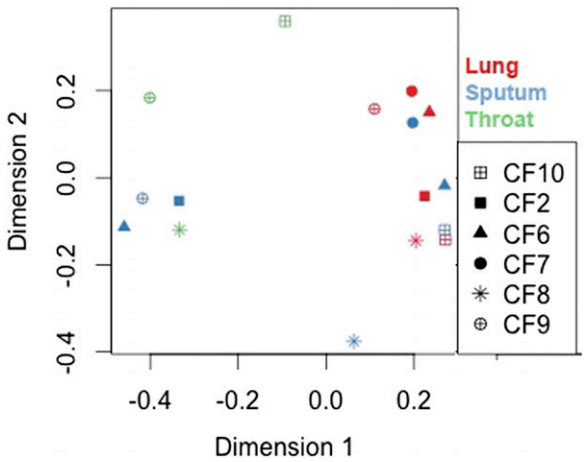

Figure 3. Multidimensional scaling of an unsupervised Random Forest comparing the relative abundance of taxa derived from 16S sequencing of lung explant samples (*red*) with sputum samples (*blue*) and oropharyngeal swabs (*green*) from six patients with cystic fibrosis (CF) (15). Shared community composition leads to clustering of sputum and lung samples in most cases, whereas some sputum and throat samples cluster together. Data from Reference 15; analysis conducted in R with the package Random Forest (72).

in explant samples and is very different from the microbial community composition in the airways of younger patients with CF with less severe disease (76). Community interactions involving the more diverse repertoire of microbes found in younger, earlier-stage patients are integral to the evolution of the community toward a less diverse, more antibiotic-resistant state found at the end stage of respiratory diseases and should be studied in greater detail.

Second, sputum may come into contact with oral microbes independent of the collection of the sputum sample. Sputum may accumulate in areas between the throat and the lobar bronchi in some patients, and oral microbes may inhabit these sites. Half of the six sputum samples reported by Goddard and colleagues had a low abundance of oral microbes. This dearth of microbes may have been due to long-term antibiotic use and acute antibiotic treatment after an exacerbation (70, 73, 75, 77). The other three sputum samples did contain a greater abundance of oral microbiota compared with the explant samples, matching microbes identified in throat swabs. The presence of oral microbes could be significant to disease progression.

Third, the authors note that they made no attempt to separate intact microbes from the surrounding material, neither in the sputum samples nor in the explanted lung samples. This is relevant because *P. aeruginosa* is known to rely on large amounts of extracellular DNA from coordinated cell death for biofilm formation (78). This DNA could accumulate over time and exaggerate the apparent abundance of *Pseudomonas* relative to other microbes, especially in a context like the lower airways of patients with severe disease, where little disruption of the microbial communities would take place (79). In many explanted lungs, the authors did in fact detect some microbes typically considered oral commensals (such as *Streptococcus* spp.) but at much lower abundances than in sputum. If the relative abundance of *Pseudomonas* was exaggerated by the sampling method, then the low but nonzero relative abundances of oral microbes could be much higher than originally considered by the authors. However, it is important to note that high abundance does not equal ecosystem importance, as some oral microbes may be able to influence lung microbial communities even at very low abundances (64, 65).

Moving Forward: Understanding Colonization and Migration Events in Respiratory Infections

Further study is required to understand the dynamics of airway microbial communities that are relevant to disease. Human airways are exposed to microbes from many sources. Although the persistent microbial community of the adjacent oral cavity is likely to be a dominant source, additional sources include nearby humans (other archipelagos, in Figure 1), animals, the home, water, food, and other environmental reservoirs capable of creating airborne particulate matter. By analogy to the island biogeography example, the oral cavity can be thought of as a mainland species reservoir; the communities that become established in these locations can have a profound effect on the prospective colonizers of more remote islands further down the chain. Understanding where reservoirs of relevant microbes exist could help avoid or delay colonization, inform therapeutic strategies, and potentially improve clinical outcomes.

Fortunately, modern omics profiling—next generation sequencing, proteomic, and metabolomics measurements—now allows the identities and activities of both chronic microbial communities and drifting microbial seeds to be identified with unprecedented depth and breadth. New technologies and decreasing costs allow for measurement of taxonomic identities, genome content, and transcriptomic, proteomic, and metabolomic activity of microbes in sputum samples. Insights into how movement and changes in activity of lung and oral microbes may precipitate exacerbations and other pathophysiological changes should be possible. The next major challenge is designing careful studies so that the large amounts of new data can give a clear picture of the spatial and temporal dynamics of these communities and lead to understanding of how these dynamics impact clinical outcomes. The biological question should dictate the most appropriate sampling approach. Standardization of sample collection and processing protocols is essential to producing data that can be compared, regardless of sample type.

The island biogeography analogy can be used to develop criteria for better understanding the relationships among traditionally "oral" and traditionally "CF pathogen" microbes. There may be cases where sputum samples are contaminated, and there may also be cases where "oral" microbes become established deeper in the CF lung. For example, *Rothia mucilaginosa*, a microbe traditionally found in the oral cavity, has been consistently found in high numbers in CF sputum, and in explant samples in some cases (19). Perhaps these cases could be distinguished by deep longitudinal sampling. Evidence of oral microbes becoming established in the lung could include (*1*) persistence over time, (*2*) stable abundance, and (*3*) genetic adaptation. These three lines of evidence are all predicted by island biogeography and evolutionary theory. Additional methods may also decipher the contributions of all airway microbes to the community dynamics. So far, little change in measurable density or composition of microbiota has been observed preceding CF disease flares known as pulmonary exacerbations (73, 77, 80). However, breath gas analysis and metabolite analysis of sputum samples may capture a valuable snapshot of metabolic activity that reflects the physiology of both the human host and microbes (20, 22, 81). Transcriptomic and proteomic analyses may also give deeper insight into not only the taxonomy of microbes present but also their activities. Finally, new imaging techniques may eventually allow a noninvasive window into the spatial distribution of sputum accumulation within the airways of patients (82).

In conclusion, understanding polymicrobial community dynamics in health and disease is a compelling challenge in 21st century medicine, with consequences for infection diagnosis and treatment. Applying island biogeography theory to microbial communities inhabiting niches in the human airways informs our interpretation of the role of microbes found in different human sample types. When microbes that are typical of the oral cavity are found in sputum or BAL samples from patients with CF, they should be considered potential immigrants, rather than contaminants. Regardless of whether microbial seeds originate from the mainland oral cavity or an adjacent island in the airways, they have the capacity to alter

the dynamics of the polymicrobial community that impacts the patient's quality of life. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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References

1. Grice EA, Segre JA. The human microbiome: our second genome. *Annu Rev Genomics Hum Genet* 2012;13:151–170.
2. Robinson CJ, Bohannan BJM, Young VB. From structure to function: the ecology of host-associated microbial communities. *Microbiol Mol Biol Rev* 2010;74:453–476.
3. Gonzalez A, Clemente JC, Shade A, Metcalf JL, Song S, Prithiviraj B, Palmer BE, Knight R. Our microbial selves: what ecology can teach us. *EMBO Rep* 2011;12:775–784.
4. MacArthur RH, Wilson EO. The theory of island biogeography. Princeton, NJ: Princeton University Press; 1967.
5. Darwin C. The origin of species (Modern Library Series). London: John Murray; 1859.
6. Ferrenberg S, O'Neill SP, Knelman JE, Todd B, Duggan S, Bradley D, Robinson T, Schmidt SK, Townsend AR, Williams MW, *et al*. Changes in assembly processes in soil bacterial communities following a wildfire disturbance. *ISME J* 2013;7:1102–1111.
7. Costello EK, Stagaman K, Dethlefsen L, Bohannan BJM, Relman DA. The application of ecological theory toward an understanding of the human microbiome. *Science* 2012;336:1255–1262.
8. Khoruts A, Dicksved J, Jansson JK, Sadowsky MJ. Changes in the composition of the human fecal microbiome after bacteriotherapy for recurrent Clostridium difficile-associated diarrhea. *J Clin Gastroenterol* 2010;44:354–360.
9. Murray JL, Connell JL, Stacy A, Turner KH, Whiteley M. Mechanisms of synergy in polymicrobial infections. *J Microbiol* 2014;52:188–199.
10. Kuramitsu HK, He X, Lux R, Anderson MH, Shi W. Interspecies interactions within oral microbial communities. *Microbiol Mol Biol Rev* 2007;71:653–670.
11. Sibley CD, Parkins MD, Rabin HR, Duan K, Norgaard JC, Surette MG. A polymicrobial perspective of pulmonary infections exposes an enigmatic pathogen in cystic fibrosis patients. *Proc Natl Acad Sci USA* 2008;105:15070–15075.
12. Korgaonkar A, Trivedi U, Rumbaugh KP, Whiteley M. Community surveillance enhances Pseudomonas aeruginosa virulence during polymicrobial infection. *Proc Natl Acad Sci USA* 2013;110:1059–1064.
13. Lomolino MV. A call for a new paradigm of island biogeography. *Glob Ecol Biogeogr* 2000;9:1–6.
14. Conrad D, Haynes M, Salamon P, Rainey PB, Youle M, Rohwer F. Cystic fibrosis therapy: a community ecology perspective. *Am J Respir Cell Mol Biol* 2013;48:150–156.
15. Goddard AF, Staudinger BJ, Dowd SE, Joshi-Datar A, Wolcott RD, Aitken ML, Fligner CL, Singh PK. Direct sampling of cystic fibrosis lungs indicates that DNA-based analyses of upper-airway specimens can misrepresent lung microbiota. *Proc Natl Acad Sci USA* 2012;109:13769–13774.
16. Hogardt M, Heesemann J. Microevolution of Pseudomonas aeruginosa to a chronic pathogen of the cystic fibrosis lung. *Curr Top Microbiol Immunol* 2013;358:91–118.
17. Lieberman TD, Michel JB, Aingaran M, Potter-Bynoe G, Roux D, Davis MR Jr, Skurnik D, Leiby N, LiPuma JJ, Goldberg JB, *et al*. Parallel bacterial evolution within multiple patients identifies candidate pathogenicity genes. *Nat Genet* 2011;43:1275–1280.
18. Marvig RL, Johansen HK, Molin S, Jelsbak L. Genome analysis of a transmissible lineage of pseudomonas aeruginosa reveals pathoadaptive mutations and distinct evolutionary paths of hypermutators. *PLoS Genet* 2013;9:e1003741.
19. Lim YW, Schmieder R, Haynes M, Furlan M, Matthews TD, Whiteson K, Poole SJ, Hayes CS, Low DA, Maughan H, *et al*. Mechanistic model of Rothia mucilaginosa adaptation toward persistence in the CF lung, based on a genome reconstructed from metagenomic data. *PLoS ONE* 2013;8:e64285.
20. Whiteson KL, Meinardi S, Lim YW, Schmieder R, Maughan H, Quinn R, Blake DR, Conrad D, Rohwer F. Breath gas metabolites and bacterial metagenomes from cystic fibrosis airways indicate active pH neutral 2,3-butanedione fermentation. *ISME J* (In press)
21. Venkataraman A, Rosenbaum MA, Werner JJ, Winans SC, Angenent LT. Metabolite transfer with the fermentation product 2,3-butanediol enhances virulence by Pseudomonas aeruginosa. *ISME J* (In press)
22. Twomey KB, Alston M, An SQ, O'Connell OJ, McCarthy Y, Swarbreck D, Febrer M, Dow JM, Plant BJ, Ryan RP. Microbiota and metabolite profiling reveal specific alterations in bacterial community structure and environment in the cystic fibrosis airway during exacerbation. *PLoS ONE* 2013;8:e82432.
23. Willner D, Furlan M, Haynes M, Schmieder R, Angly FE, Silva J, Tammadoni S, Nosrat B, Conrad D, Rohwer F. Metagenomic analysis of respiratory tract DNA viral communities in cystic fibrosis and non-cystic fibrosis individuals. *PLoS ONE* 2009;4:e7370.
24. Willner D, Haynes MR, Furlan M, Hanson N, Kirby B, Lim YW, Rainey PB, Schmieder R, Youle M, Conrad D, *et al*. Case studies of the spatial heterogeneity of DNA viruses in the cystic fibrosis lung. *Am J Respir Cell Mol Biol* 2012;46:127–131.
25. Sethi S. Bacteria in exacerbations of chronic obstructive pulmonary disease: phenomenon or epiphenomenon? *Proc Am Thorac Soc* 2004;1:109–114.
26. Spada EL, Tinivella A, Carli S, Zaccaria S, Lusuardi M, Sbaffi A, Donner CF. Proposal of an easy method to improve routine sputum bacteriology. *Respiration* 1989;56:137–146.
27. Charlson ES, Bittinger K, Haas AR, Fitzgerald AS, Frank I, Yadav A, Bushman FD, Collman RG. Topographical continuity of bacterial populations in the healthy human respiratory tract. *Am J Respir Crit Care Med* 2011;184:957–963.
28. Charlson ES, Bittinger K, Chen J, Diamond JM, Li H, Collman RG, Bushman FD. Assessing bacterial populations in the lung by replicate analysis of samples from the upper and lower respiratory tracts. *PLoS ONE* 2012;7:e42786.
29. Beck JM, Young VB, Huffnagle GB. The microbiome of the lung. *Transl Res* 2012;160:258–266.
30. Erb-Downward JR, Thompson DL, Han MK, Freeman CM, McCloskey L, Schmidt LA, Young VB, Toews GB, Curtis JL, Sundaram B, *et al*. Analysis of the lung microbiome in the "healthy" smoker and in COPD. *PLoS ONE* 2011;6:e16384.
31. Morris A, Beck JM, Schloss PD, Campbell TB, Crothers K, Curtis JL, Flores SC, Fontenot AP, Ghedin E, Huang L, *et al*.; Lung HIV Microbiome Project. Comparison of the respiratory microbiome in healthy nonsmokers and smokers. *Am J Respir Crit Care Med* 2013; 187:1067–1075.
32. Borewicz K, Pragman AA, Kim HB, Hertz M, Wendt C, Isaacson RE. Longitudinal analysis of the lung microbiome in lung transplantation. *FEMS Microbiol Lett* 2013;339:57–65.
33. Pezzulo AA, Kelly PH, Nassar BS, Rutland CJ, Gansemer ND, Dohrn CL, Costello AJ, Stoltz DA, Zabner J. Abundant DNase I sensitive bacterial DNA in healthy porcine lungs: implications for the lung microbiome. *Appl Environ Microbiol* 2013;79:5936–5941.
34. Chilvers MA, Rutman A, O'Callaghan C. Functional analysis of cilia and ciliated epithelial ultrastructure in healthy children and young adults. *Thorax* 2003;58:333–338.
35. Ben-David I, Price SE, Bortz DM, Greineder CF, Cohen SE, Bauer AL, Jackson TL, Younger JG. Dynamics of intrapulmonary bacterial growth in a murine model of repeated microaspiration. *Am J Respir Cell Mol Biol* 2005;33:476–482.
36. Bousbia S, Papazian L, Saux P, Forel JM, Auffray JP, MartinC, Raoult D, La Scola B. Repertoire of intensive care unit pneumonia microbiota. *PLoS ONE* 2012;7:e32486.
37. Gleeson K, Eggli DF, Maxwell SL. Quantitative aspiration during sleep in normal subjects. *Chest* 1997;111:1266–1272.

38. Marik PE. Aspiration pneumonitis and aspiration pneumonia. *N Engl J Med* 2001;344:665–671.
39. Scannapieco FA. Role of oral bacteria in respiratory infection. *J Periodontol* 1999;70:793–802.
40. Mina MJ, McCullers JA, Klugman KP. Live attenuated influenza vaccine enhances colonization of Streptococcus pneumoniae and Staphylococcus aureus in mice. *MBio* 2014;5:e01040–13.
41. Licciardi PV, Toh ZQ, Dunne E, Wong SS, Mulholland EK, Tang M, Robins-Browne RM, Satzke C. Protecting against pneumococcal disease: critical interactions between probiotics and the airway microbiome. *PLoS Pathog* 2012;8:e1002652.
42. Bosch AATM, Biesbroek G, Trzcinski K, Sanders EAM, Bogaert D. Viral and bacterial interactions in the upper respiratory tract. *PLoS Pathog* 2013;9:e1003057.
43. van den Bergh MR, Biesbroek G, Rossen JWA, de Steenhuijsen Piters WAA, Bosch AATM, van Gils EJM, Wang X, Boonacker CWB, Veenhoven RH, Bruin JP, *et al*. Associations between pathogens in the upper respiratory tract of young children: interplay between viruses and bacteria. *PLoS ONE* 2012;7:e47711.
44. Chertow DS, Memoli MJ. Bacterial coinfection in influenza: a grand rounds review. *JAMA* 2013;309:275–282.
45. Iwai S, Fei M, Huang D, Fong S, Subramanian A, Grieco K, Lynch SV, Huang L. Oral and airway microbiota in HIV-infected pneumonia patients. *J Clin Microbiol* 2012;50:2995–3002.
46. Lozupone C, Cota-Gomez A, Palmer BE, Linderman DJ, Charlson ES, Sodergren E, Mitreva M, Abubucker S, Martin J, Yao G, *et al*.; Lung HIV Microbiome Project. Widespread colonization of the lung by Tropheryma whipplei in HIV infection. *Am J Respir Crit Care Med* 2013;187:1110–1117.
47. Pragman AA, Kim HB, Reilly CS, Wendt C, Isaacson RE. The lung microbiome in moderate and severe chronic obstructive pulmonary disease. *PLoS ONE* 2012;7:e47305.
48. Sze MA, Dimitriu PA, Hayashi S, Elliott WM, McDonough JE, Gosselink JV, Cooper J, Sin DD, Mohn WW, Hogg JC. The lung tissue microbiome in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2012;185:1073–1080.
49. Sze MA, Hogg JC, Sin DD. Bacterial microbiome of lungs in COPD. *Int J Chron Obstruct Pulmon Dis* 2014;9:229–238.
50. Lynch SV, Bruce KD. The cystic fibrosis airway microbiome. *Cold Spring Harb Perspect Med* 2013;3:a009738.
51. Quinn B, Baker DL, Cohen S, Stewart JL, Lima CA, Parise C. Basic nursing care to prevent nonventilator hospital-acquired pneumonia. *J Nurs Scholarsh* 2014;46:11–19.
52. Ciofu O, Johansen HK, Aanaes K, Wassermann T, Alhede M, von Buchwald C, Høiby N. P. aeruginosa in the paranasal sinuses and transplanted lungs have similar adaptive mutations as isolates from chronically infected CF lungs. *J Cyst Fibros* 2013;12:729–736.
53. Horváth G, Sorscher EJ. Luminal fluid tonicity regulates airway ciliary beating by altering membrane stretch and intracellular calcium. *Cell Motil Cytoskeleton* 2008;65:469–475.
54. Simonin-Le Jeune K, Le Jeune A, Jouneau S, Belleguic C, Roux PF, Jaguin M, Dimanche-Boitre MT, Lecureur V, Leclercq C, Desrues B, *et al*. Impaired functions of macrophage from cystic fibrosis patients: CD11b, TLR-5 decrease and sCD14, inflammatory cytokines increase. *PLoS ONE* 2013;8:e75667.
55. Junkins RD, McCormick C, Lin TJ. The emerging potential of autophagy-based therapies in the treatment of cystic fibrosis lung infections. *Autophagy* 2014;10:538–547.
56. Mayer ML, Blohmke CJ, Falsafi R, Fjell CD, Madera L, Turvey SE, Hancock REW. Rescue of dysfunctional autophagy attenuates hyperinflammatory responses from cystic fibrosis cells. *J Immunol* 2013;190:1227–1238.
57. Duan K, Dammel C, Stein J, Rabin H, Surette MG. Modulation of Pseudomonas aeruginosa gene expression by host microflora through interspecies communication. *Mol Microbiol* 2003;50:1477–1491.
58. Rabin HR, Surette MG. The cystic fibrosis airway microbiome. *Curr Opin Pulm Med* 2012;18:622–627.
59. Rogers GB, Hoffman LR, Carroll MP, Bruce KD. Interpreting infective microbiota: the importance of an ecological perspective. *Trends Microbiol* 2013;21:271–276.
60. Friedrich MJ. Microbiome project seeks to understand human body's microscopic residents. *JAMA* 2008;300:777–778.
61. Gomes-Filho IS, Passos JS, Seixas da Cruz S. Respiratory disease and the role of oral bacteria. *J Oral Microbiol* 2010;2.
62. Murphy TF, Parameswaran GI, Parameswaran GI. Moraxella catarrhalis, a human respiratory tract pathogen. *Clin Infect Dis* 2009;49:124–131.
63. Pettigrew MM, Laufer AS, Gent JF, Kong Y, Fennie KP, Metlay JP. Upper respiratory tract microbial communities, acute otitis media pathogens, and antibiotic use in healthy and sick children. *Appl Environ Microbiol* 2012;78:6262–6270.
64. Duran-Pinedo AE, Chen T, Teles R, Starr JR, Wang X, Krishnan K, Frias-Lopez J. Community-wide transcriptome of the oral microbiome in subjects with and without periodontitis. *ISME J* (In press)
65. Hajishengallis G, Liang S, Payne MA, Hashim A, Jotwani R, Eskan MA, McIntosh ML, Alsam A, Kirkwood KL, Lambris JD, *et al*. Low-abundance biofilm species orchestrates inflammatory periodontal disease through the commensal microbiota and complement. *Cell Host Microbe* 2011;10:497–506.
66. Paju S, Scannapieco FA. Oral biofilms, periodontitis, and pulmonary infections. *Oral Dis* 2007;13:508–512.
67. Burns JL, Rolain JM. Culture-based diagnostic microbiology in cystic fibrosis: can we simplify the complexity? *J Cyst Fibros* 2014;13:1–9.
68. Brook I, Fink R. Transtracheal aspiration in pulmonary infection in children with cystic fibrosis. *Eur J Respir Dis* 1983;64:51–57.
69. Carmody LA, Zhao J, Schloss PD, Petrosino JF, Murray S, Young VB, Li JZ, LiPuma JJ. Changes in cystic fibrosis airway microbiota at pulmonary exacerbation. *Ann Am Thorac Soc* 2013;10:179–187.
70. Blainey PC, Milla CE, Cornfield DN, Quake SR. Quantitative analysis of the human airway microbial ecology reveals a pervasive signature for cystic fibrosis. *Sci Transl Med* 2012;4:153ra130.
71. Willner D, Haynes MR, Furlan M, Schmieder R, Lim YW, Rainey PB, Rohwer F, Conrad D. Spatial distribution of microbial communities in the cystic fibrosis lung. *ISME J* 2012;6:471–474.
72. Liaw A, Wiener M. Classification and regression by randomForest. *R News* 2002;2:18–22.
73. Fodor AA, Klem ER, Gilpin DF, Elborn JS, Boucher RC, Tunney MM, Wolfgang MC. The adult cystic fibrosis airway microbiota is stable over time and infection type, and highly resilient to antibiotic treatment of exacerbations. *PLoS ONE* 2012;7:e45001.
74. Price KE, Hampton TH, Gifford AH, Dolben EL, Hogan DA, Morrison HG, Sogin ML, O'Toole GA. Unique microbial communities persist in individual cystic fibrosis patients throughout a clinical exacerbation. *Microbiome* 2013; 1: 27.
75. Zhao J, Schloss PD, Kalikin LM, Carmody LA, Foster BK, Petrosino JF, Cavalcoli JD, VanDevanter DR, Murray S, Li JZ, *et al*. Decade-long bacterial community dynamics in cystic fibrosis airways. *Proc Natl Acad Sci USA* 2012;109:5809–5814.
76. Cox MJ, Allgaier M, Taylor B, Baek MS, Huang YJ, Daly RA, Karaoz U, Andersen GL, Brown R, Fujimura KE, *et al*. Airway microbiota and pathogen abundance in age-stratified cystic fibrosis patients. *PLoS ONE* 2010;5:e11044.
77. Stressmann FA, Rogers GB, van der Gast CJ, Marsh P, Vermeer LS, Carroll MP, Hoffman L, Daniels TWV, Patel N, Forbes B, *et al*. Long-term cultivation-independent microbial diversity analysis demonstrates that bacterial communities infecting the adult cystic fibrosis lung show stability and resilience. *Thorax* 2012;67:867–873.
78. Ma L, Conover M, Lu H, Parsek MR, Bayles K, Wozniak DJ. Assembly and development of the Pseudomonas aeruginosa biofilm matrix. *PLoS Pathog* 2009;5:e1000354.
79. Villarreal JV, Jungfer C, Obst U, Schwartz T. DNase I and Proteinase K eliminate DNA from injured or dead bacteria but not from living bacteria in microbial reference systems and natural drinking water biofilms for subsequent molecular biology analyses. *J Microbiol Methods* 2013;94:161–169.
80. Stressmann FA, Rogers GB, Marsh P, Lilley AK, Daniels TWV, Carroll MP, Hoffman LR, Jones G, Allen CE, Patel N, *et al*. Does bacterial density in cystic fibrosis sputum increase prior to pulmonary exacerbation? *J Cyst Fibros* 2011;10:357–365.
81. Montuschi P, Paris D, Melck D, Lucidi V, Ciabattoni G, Raia V, Calabrese C, Bush A, Barnes PJ, Motta A. NMR spectroscopy metabolomic profiling of exhaled breath condensate in patients with stable and unstable cystic fibrosis. *Thorax* 2012;67:222–228.
82. Zarei S, Mirtar A, Rohwer F, Conrad DJ, Theilmann RJ, Salamon P. Mucus distribution model in a lung with cystic fibrosis. *Comput Math Methods Med* 2012;2012:970809.