RESEARCH ARTICLE

Biogeochemical Forces Shape the Composition and Physiology of Polymicrobial Communities in the Cystic Fibrosis Lung

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ABSTRACT **The cystic fibrosis (CF) lung contains thick mucus colonized by opportunistic pathogens which adapt to the CF lung environment over decades. The difficulty associated with sampling airways has impeded a thorough examination of the biochemical microhabitats these pathogens are exposed to. An indirect approach is to study the responses of microbial communities to these microhabitats, facilitated by high-throughput sequencing of microbial DNA and RNA from sputum samples. Microbial metagenomes and metatranscriptomes were sequenced from multiple CF patients, and the reads were assigned taxonomy and function through sequence homology to NCBI and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database hierarchies. For a comparison, saliva microbial metagenomes from the Human Microbiome Project (HMP) were also analyzed. These analyses identified that functions encoded and expressed by CF microbes were significantly enriched for amino acid catabolism, folate biosynthesis, and lipoic acid biosynthesis. The data indicate that the community uses oxidative phosphorylation as a major energy source but that terminal electron acceptors were diverse. Nitrate reduction was the most abundant anaerobic respiratory pathway, and genes for nitrate reductase were largely assigned to *Pseudomonas* and *Rothia*. Although many reductive pathways of the nitrogen cycle were present, the cycle was incomplete, because the oxidative pathways were absent. Due to the abundant amino acid catabolism and incomplete nitrogen cycle, the CF microbial community appears to accumulate ammonia. This finding was verified experimentally using a CF bronchiole culture model system. The data also revealed abundant sensing and transport of iron, ammonium, zinc, and other metals along with a low-oxygen environment. This study reveals the core biochemistry and physiology of the CF microbiome.**

IMPORTANCE **The cystic fibrosis (CF) microbial community is complex and adapts to the environmental conditions of the lung over the lifetime of a CF patient. This analysis illustrates the core functions of the CF microbial community in the context of CF lung biochemistry. There are many studies of the metabolism and physiology of individual microbes within the CF lung, but none that collectively analyze data from the whole microbiome. Understanding the core metabolism of microbes that inhabit the CF lung can provide new targets for novel therapies. The fundamental processes that CF pathogens rely on for survival may represent an Achilles heel for this pathogenic community. Novel therapies that are designed to disrupt understudied survival strategies of the CF microbial community may succeed against otherwise untreatable or antibiotic-resistant microbes.**

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The airways of people with cystic fibrosis (CF) are coated with an abnormally dense mucus layer. This mucus contains immune cells, principally neutrophils, and a diverse community of bacteria, microbial eukaryotes, and viruses (1–3). Bacteria that commonly infect CF lungs are difficult to treat because they have evolved resistance to most antibiotics, allowing them to persist throughout the life of the patient. Understanding the lung environment to which these microbes adapt is paramount for developing novel therapies that target the principal strategies of microbial persistence.

The physiological consequences of inherited mutations in the cystic fibrosis transmembrane receptor (CFTR) are complex; in the last 30 years, altered chloride transport leading to dehydrated airway surfaces has been the best-studied aspect of CF pathophysiology (4–6). More recently, decreased bicarbonate ion transport across mutant CFTR has been shown to disrupt electrostatic interactions necessary for mucus expansion, leading to dense immovable mucus in the CF airways (7). Other physiological aspects of the CF lung that have received attention are pH, iron and heavy metals, redox-active compounds such as phenazines, oxygen tension, nitrogen species, and carbon sources for microbial metabolism.

Several studies have reported that the pH of exhaled breath condensate is slightly acidic and further acidified during an acute disease flare known as an exacerbation (8, 9). A recent study using the porcine model of CF found that immediately upon birth, the airway surface fluid already has a lowered pH, which disrupts innate immune function (10). High concentrations of iron and the

iron storage protein ferritin have been detected in several studies (11–13). Redox-active phenazines derived from *Pseudomonas* have also been detected, increasing in concentration over time and as the disease worsens (14). These molecules impact CF lung microenvironments by producing destructive reactive oxygen species (ROS) in the presence of oxygen or enabling anaerobic survival under low oxygen (15, 16).

The oxygen concentration in obstructed airways decreases rapidly with depth (17) and does not penetrate static fluids effectively, resulting in anoxic microenvironments. Anoxia in obstructed CF airways is supported by direct measurements (17), the presence of anaerobes in sputum (18), and by the observation of anaerobic metabolism in laboratory microcosms inoculated with bacterial strains from CF patients (17, 20). Anoxia in the lung is counterintuitive, as the principal purpose of a healthy lung is to exchange oxygen.

Although it has not been assessed whether nitrogen compounds impact the functioning of CF microbial communities as a whole, studies of nitrogen species in the CF lung have found abundant ammonium and nitrate ions (21–23), as well as an abundance of nitrogen-rich amino acids (22, 24, 25). Artificial sputum cultures show that *P. aeruginosa* preferentially consumes a set of 5 amino acids and lactate as the carbon source (22, 26).

These direct measurements of CF lung physiology are fundamental to understanding how the opportunistic pathogens survive within the lung and further influence their biochemical environment. We now need detailed data that describe how the microbes respond to this local biochemistry in the structured lung environment, beyond what is already known for the principal pathogen, *Pseudomonas aeruginosa* (27–31). This is essential for a complete understanding of CF pathology, because recent studies of CF airways have shown that the lung contains a complex polymicrobial community that is not reflected in pure culture experiments (1, 32–34).

Microbes sense, respond, and adapt to the conditions that surround them, and their gene content and gene expression patterns provide evidence for these adaptations (35). Metagenomic sequencing of microbial communities can provide a collective view of adaptation and response at a community level, which is essential for a better understanding of microbial physiology in the CF lung. This is a powerful approach because it circumvents some difficulties associated with sampling poorly accessible areas to directly measure the CF lung biochemistry (36).

The goal of this study was to begin assembling a comprehensive view of the major physiological processes carried out by microbes in the CF lung in the framework of CF lung biochemical microenvironments (19, 33, 37). Sputum microbial DNA and RNA from multiple CF patients were sequenced to identify pathways that may be altered with the ultimate goal of controlling pathogen growth and improving the quality of life for patients. Because of the widespread occurrence of antibiotic resistance and the ubiquity of antibiotic resistance gene exchange, the exploration of alternative methods for controlling CF microbes is crucial for improving patient health and longevity.

RESULTS AND DISCUSSION

Microbial DNA and RNA were isolated from sputum samples taken from 6 CF patients at 2 to 4 time points (see Table S1 in the supplemental material), and sequenced with 454 GS-FLX technology as described in reference 1). Sputum samples were taken in patients at various disease states to provide a more collective view of the dynamic CF lung. These states included: during exacerbation, during and after antibiotic treatment, and during times of relative disease stability (samples described in Table S1).

The metagenome and metatranscriptome reads were analyzed as separate data sets using two strategies: analyzing each patient separately and combining the data from all patients. This allowed investigation of the between-patient variability while also increasing our power to detect more-complete functions and pathways. High-quality 454 sequence reads were matched against the KEGG (Kyoto Encyclopedia of Genes and Genomes) peptide database using tBLASTn, and the KEGG peptide BLAST output was then run through the HUMANnN pipeline (38) to produce normalized abundances of genes and pathways present in sputum samples. The KEGG database contains thousands of bacterial genomes, which are somewhat biased toward easily cultured organisms. However, the vast majority of organisms present in the CF lung microbiomes of patients in this study are available in the KEGG database, providing validity to the choice of this database (1). To probe how CF microbes respond to the biochemistry of the lung environment, we identified functions whose presence and expression were most abundant and enriched in CF sequencing data compared to a healthy human saliva control. Functions were predicted using several KEGG hierarchies that differ in their system of grouping similar functions: KEGG BRITE hierarchy, which is the most basic level hierarchy of the KEGG classification of higher-level biological systems; KEGG pathways (represented by identifiers starting with ko), which break down physiological functions into specific pathway networks or maps; KEGG modules (represented by identifiers starting with MO), which are similar to pathways but represent a set of manually defined functional units; and the KEGG Orthology hierarchy, which is at the level of individual genes. For all but the KEGG Orthology hierarchy, we were able to compare these data with oral samples from healthy individuals (from the Human Microbiome Project [HMP] database [http://www.hmpdacc.org/]).

Microbial metabolism in CF sputum. The presence and abundance of particular bacterial groups varied between patients, consistent with a previous report demonstrating that each patient harbors a unique microbial community (1) (Fig. 1A). However, at the level of the KEGG modules, a deep layer of microbial functional genetics and physiology, the top 25 module functions encoded by each patient's microbial metagenome showed a high degree of similarity (Fig. 1B). The variation in taxonomic abundances between patients was much larger than that for the functional abundances (taxon mean relative standard deviation [SD] of 198% [range, 57.1% to 316.2%]; function mean relative SD of 31.9% [range, 17.1% to 69.7%]). This demonstrates that while patient-to-patient microbiome taxonomic composition can vary greatly, there is conservation of lower-level functions within the CF lung microbial community, a phenomenon observed previously in the healthy human microbiome (39).

To examine the metabolism of CF microbes, the metagenome and metatranscriptome results were placed into the KEGG BRITE hierarchical classification system and compared with the results for saliva microbial metagenomes from five healthy humans from the HMP. Attention was paid to the most abundant pathways in CF, and these pathways were compared to those of saliva metagenomes as a reference. Comparison to healthy human saliva samples was included, because healthy lung sputum metagenomes

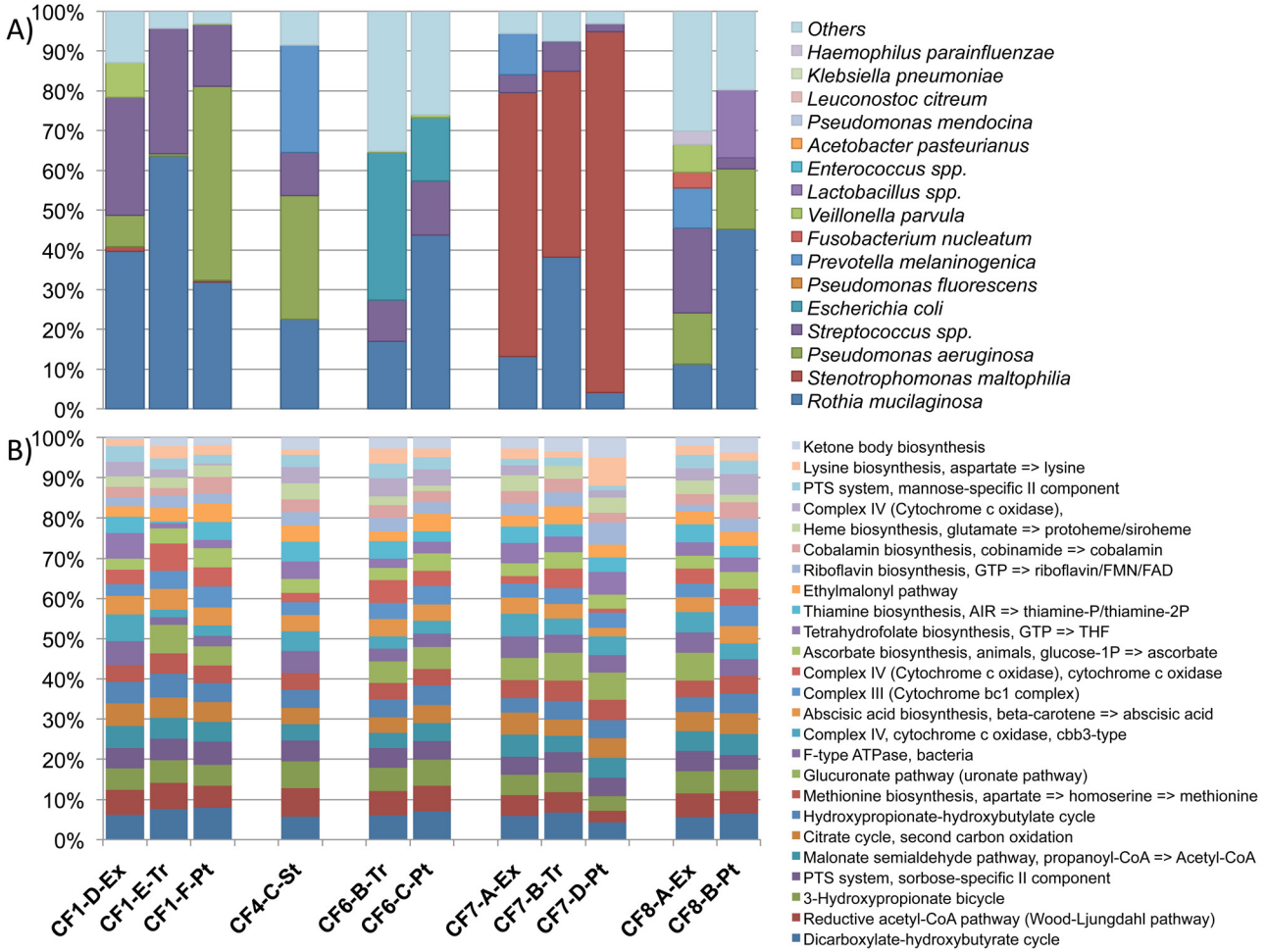

FIG 1 (A) Taxonomic distribution of metagenomic sequence reads per sputum sample and in the pooled data as determined by NCBI taxonomy mapping of reads. (B) Functional distribution of metagenomic sequence reads for individual sputum samples as determined by HUMAnN assignment to the top 25 KEGG (Kyoto Encyclopedia of Genes and Genomes) modules. PTS, phosphotransferase system; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; AIR, 5-aminoimidazole ribotide; thiamine-P, thiamine phosphate; THF, tetrahydrofolate; CoA, coenzyme A. Sample timepoint abbreviations: Ex, exacerbation; Tr, treatment; St, stable; Pt, post-treatment.

were not available and are not likely a comparable sample in a microbial community context. Healthy human lungs contain few bacteria, and there is an active debate about whether any bacteria present comprise a persistent resident microbiota distinct from upper airway microbiomes (38, 39). Furthermore, healthy human sputum samples are primarily saliva, as healthy lungs have little mucus and sputum, and saliva contains large amounts of some of the same microbes that are in CF sputum (40, 90, 91). Thus, any functional genetic differences inferred from healthy and CF sputum samples are not likely to be distinct from comparisons between CF sputum and healthy saliva samples. Pathways that were more abundant in the CF sputa compared with healthy saliva included nucleotide metabolism, metabolism of other amino acids, and biosynthesis of secondary metabolites (see Fig. S1 in the supplemental material). Nucleotide metabolism may reflect the abundance of free DNA in CF sputum (41), which is believed to come from neutrophil extracellular traps (42, 43) and bacterial biofilms (44). The elevated amino acid metabolism may reflect the abundant free amino acids in sputum (22, 24, 26), possibly due to the action of large amounts of human and microbial proteases in the CF lung (45, 46). The biosynthesis of secondary metabolites is likely a signature of competition and communication between microbes within the CF lung. For example, secondary metabolites such as phenazines provide *P. aeruginosa* with a competitive advantage in the CF microbial community; phenazines generate toxic reactive oxygen species (ROS) in the presence of oxygen and act as alternative electron acceptors under low-oxygen conditions (14, 47, 48).

The abundances of metabolic pathways encoded by microbes inhabiting CF lungs, functionally annotated using the KEGG pathway hierarchy, were compared to the same pathways in healthy human saliva metagenomes from the HMP. The pathways enriched in CF lungs compared to healthy human saliva included D-glutamine and D-glutamate metabolism (ko00471), D-alanine metabolism (ko00473), valine, leucine, and isoleucine biosynthesis (ko00280), and folate biosynthesis (ko00790). Also enriched in CF lungs, but rare in saliva, were one carbon pool by folate (ko00670), aminoacyl-tRNA biosynthesis (ko00970), and peptidoglycan biosynthesis (ko00550) (Fig. 2). Analyzing the data at the level of individual samples allowed for an assessment of the

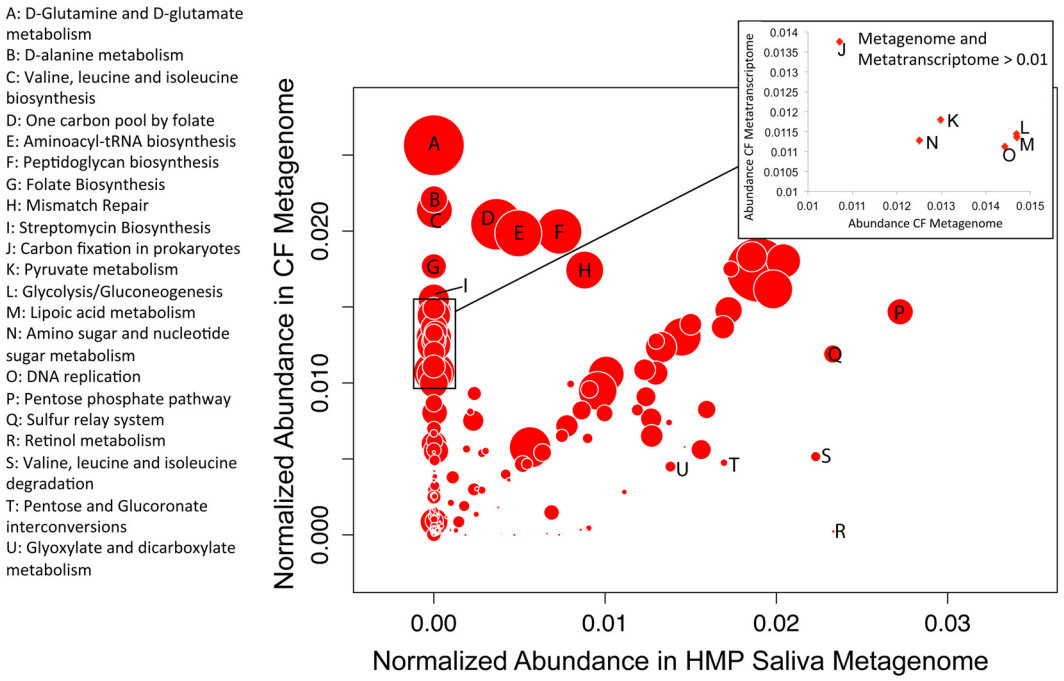

FIG 2 Comparison of normalized abundance data of KEGG pathways in metagenomes and metatranscriptomes from cystic fibrosis (CF) patients and HMP (Human Microbiome Project) healthy human saliva controls from the HUMAnN output. Each data point represents an individual pathway, and the radius of the circle is an indicator of its abundance in the CF metatranscriptome. (Inset) Plot that more easily visualizes CF elevated pathways in a region of the bubble plot with too much overlap. Here the plot depicts the normalized abundances of the pathways in the CF metagenomes and metatranscriptome only, and pathways with abundances greater than 0.01 in both are shown.

variability in pathway abundances based on the averaged relative standard deviations of each pathway across patients. Overall, there was little variation in the metagenome pathway abundances, while the transcriptome variation was often large (metagenome mean relative SD = 21.9%; transcriptome mean relative SD = 69.7%) (see Fig. S2a in the supplemental material). In particular, D-glutamine and D-glutamate metabolism was highly variable (relative SD = 115.4%), as one CF patient had a high abundance of this pathway, while other CF patients had zero. This may demonstrate the dynamic nature of transcriptional response, where the response to these amino acids may be large when the amino acids are available and absent when they are not. Other pathways with highly variable transcriptome abundance were D-alanine metabolism (relative SD = 98.2%), valine, leucine, and isoleucine biosynthesis (relative SD = 41.2%), one carbon pool by folate (relative SD = 109.0%), and streptomycin biosynthesis (relative SD = 106.8) (Fig. S2a). The functional similarity between patients' metagenomes demonstrates the conserved nature of the core functions identified as CF enriched; however, expression of these pathways is more variable between sputum samples.

The abundance of amino acid metabolism, including amino sugar and nucleotide metabolism (ko00520), is consistent with the results from the KEGG BRITE hierarchy (see Fig. S1 in the supplemental material). Also enriched in the CF metagenomes and metatranscriptomes were pathways for synthesis of particular amino acids (Fig. 2), which may indicate differential availability of amino acids. *P. aeruginosa* has been shown to evolve preferences for the transport and synthesis of certain amino acids, depending on their availability and energetic cost (26). The prevalence of mismatch repair enzymes may reflect exposure to ROS and high iron levels. In contrast, the pathways that were more prevalent in healthy human saliva samples than in the lungs of CF patients were those related to sulfur metabolism (ko04122), valine degradation, and the glyoxylate cycle (Fig. 2). This may reflect differences in the nutrients that oral microbes have access to (49, 50).

Amino acid metabolism was enriched in CF lungs versus healthy human saliva samples when the data were compared using both the higher-level KEGG BRITE hierarchy and the finer-scale KEGG pathway hierarchy, indicating that this may be an important driver of community assembly and function, in addition to its specific effects on lung chemistry. Microbes may be using these amino acids as preferential carbon sources primarily with deaminases producing ammonia. Several studies have demonstrated that CF-adapted *P. aeruginosa* metabolism and antimicrobial activity are dependent upon the presence of high concentrations of amino acids (24, 51, 52). *In vitro*, *P. aeruginosa* preferentially deaminates arginine in the absence of O_2 and NO_3, indicating that this amino acid may be important for anaerobic metabolism in the CF lung (53).

A major by-product of amino acid degradation is ammonia, and elevated ammonia levels have been detected in CF sputum (44; see below). Ammonia can act as a weak base, and its production may contribute to an increase in sputum pH. This is particularly important in the context of CF pathology, as the lowered pH of airway surface liquid (ASL) itself may be a cause of immune dysfunction (10, 54). Deamination of amino acids would contribute to increases of the ASL and sputum pH, which may actually ease the effect of pH on inflammation. Microbes that can raise the pH of the CF lung mucus may benefit themselves and contribute to a stabilization of their CF microenvironment, as this alkalinity

could neutralize lethal acidification from a combination of inflammation, lack of bicarbonate due to insufficient CFTR activity, and microbial fermentation products.

Other pathways clearly represented in the CF metagenomes were glycolysis/gluconeogenesis (ko00010) and pyruvate metabolism (ko00620) (Fig. 2). These pathways work together to function as the central means of energy generation during oxidative phosphorylation or fermentation, depending on the availability of electron acceptors. Two of the most highly expressed genes in the CF transcriptome (excluding those for translation or RNA processing) were NADH oxidoreductase (K03882) and the alpha-subunit of the F-type ATPase (K02126). This indicates that CF microbes may acquire ATP from well-known respiratory pathways with NADH as the electron donor.

Because microbes inhabiting CF lungs experience an oxygen gradient (17, 55), many CF microbes need diverse terminal electron acceptors to carry out oxidative phosphorylation. If an electron acceptor is unavailable, another alternative strategy is fermentation. Pyruvate metabolism (ko00620) is central to the switch between fermentative metabolism and oxidative phosphorylation. The fermentation pathways present in CF metagenomes and metatranscriptomes included alcoholic fermentation, lactic acid fermentation, and mixed-acid fermentations to acetate, butanediol, and butyrate (see Table S2 in the supplemental material). In our data, most reads corresponding to fermentation pathways mapped to *Streptococcus* spp., except for butanediol dehydrogenase, which also mapped to *Rothia* spp. (Fig. S3a). Thus, in the absence of oxygen and other terminal electron acceptors, fermentation may be favored by particular CF microbes or serve as an alternative for facultative microbes.

Lipoic acid metabolism was elevated in CF lungs and was present and expressed in all CF patients (Fig. 2; see Fig. S2a in the supplemental material). The abundance of this pathway may reflect the oxidative stress that microbial pathogens experience in the lung. Lipoic acid is a fat-soluble antioxidant and a potent quencher of ROS, such as superoxide, hydroxyl radical, and singlet oxygen (56). The CF lung environment is known to be high in ROS, due to immune cell activity in response to chronic microbial infection (57, 58) as well as phenazines (59), and antioxidant therapy has been proposed to alleviate pathology (60). Lipoic acid may serve a dual role as a ROS quencher for some bacteria and as a cofactor for metabolism by *Proteobacteria*, Gram-positive bacteria, and *P. aeruginosa* (56, 61). More specifically, dihydrolipoamide (the reduced form of lipoic acid) is a cofactor of pyruvate dehydrogenase, a central enzyme for the assimilation of pyruvate into anabolic pathways or the tricarboxylic acid (TCA) cycle. The abundance of lipoic acid metabolism may reflect the importance of this enzyme and its cofactor to CF microbial metabolism.

An unexpected finding was that all metagenomes had a high abundance of pathways for the synthesis of folate and tetrahydrofolate (Fig. 2), which were undetectable in the healthy human saliva metagenomes and not reported to be abundant in metagenomes from other HMP body locations (39). Because humans acquire folate from their diet, sulfonamide drugs were developed to target microbial enzymes required for folate biosynthesis. Sulfonamides such as sulfamethoxazole and trimethoprim are commonly used to treat CF infections (62), including several patients in this study. These and other sulfonamide drugs target the dihydropterate, dihydrofolate, and tetrahydrofolate steps of the biosynthesis pathway, which are carried out by products encoded by some of the most abundant genes in the CF metagenomes. Prolonged exposure to sulfonamide drugs may have selected for microbes with multiple copies or high expression of these genes in order to overcome the drug's effect on folate synthesis much like distribution of phosphate acquisition genes in *Prochlorococcus* is related to local phosphate concentrations (92).

Microbial respiration in the CF lung. Microbes responsible for earth's major biogeochemical cycles utilize aerobic and anaerobic respiration to harness the energy available in redox reactions (63). Energy generated from the oxidation of organic or inorganic compounds is coupled to reductive processes that accept the electrons generated from these oxidative reactions. Similarly, CF microbes oxidize organic or inorganic compounds during metabolism (55). If available, molecular oxygen is the electron acceptor of choice, and the aerobic and facultatively anaerobic bacteria in CF lungs can utilize this terminal electron acceptor. However, under the low-oxygen conditions thought to prevail in CF airways plugged with mucus (17, 19, 20), other oxidized compounds must serve this function. Our CF data are consistent with microbes sensing the varying availability of oxygen: the metagenomes contained anaerobic respiration response regulators (Gram-positive *resED* and Gram-negative *arcAB*), the *nreBC* low-oxygen sensor, and cytochrome *c* oxidase. All but *arcAB* were present in the metatranscriptome data set (see Table S5 in the supplemental material).

Genes present and expressed in the CF sputa provided insight into the primary electron acceptors and respiratory pathways utilized by CF microbes. Not surprisingly, the sequencing data indicate that CF microbes preferentially utilize the most electrochemically favorable terminal electron acceptors (Fig. 3). Nitrate reductase was the most abundant electron acceptor gene in the metagenome and transcriptome, consistent with *P. aeruginosa*'s proposed ability for denitrification in the lung (21, 55). This gene was abundant in all sputum metagenomes, but variable (0.0012 ± 0.00063 [mean $\pm$ SD]), due to the especially high abundance in sample CF4-C-St (see Fig. S2b in the supplemental material). Interestingly, the two different nitrate reductase genes had different taxonomic associations, the membrane-bound nitrate reductase *nar* reads were mostly assigned to *Rothia* spp., whereas the periplasmic nitrate reductase *nap* reads were assigned to *Pseudomonas* spp. and *Escherichia* spp. (Fig. 3 and Fig. S3b). The abundant potential for *Rothia* spp. to reduce nitrate in the CF lung may be relevant to the physiology of the overall pathogenic community. Genes encoding fumarate reductase and some reductases of sulfur species were also abundant in the sequence data (Fig. 3). Fumarate reductase was particularly abundant in the transcriptome, indicating that CF microbes use this organic terminal electron acceptor (Fig. 3), and the fumarate two-component sensor histidine kinase *dcuSR* was present and expressed (Table S5).

The origins of oxidized compounds that could serve as the substrates for these reductases are unknown. Nitrate may come from microbial nitrification or from the host via blood, lung tissue, or diet (64, 65). Oxidized sulfur species such as sulfate may be generated from mucin desulfuration (66), consistent with the presence and expression of the cysteine desulfuration gene *sufE* in the CF sputa (see Table S3 in the supplemental material). Fumarate is a central compound in many metabolic pathways, most notably the TCA cycle and amino acid breakdown, and is generated by succinate dehydrogenase, which was abundant in CF metagenomes and metatranscriptomes (Table S1).

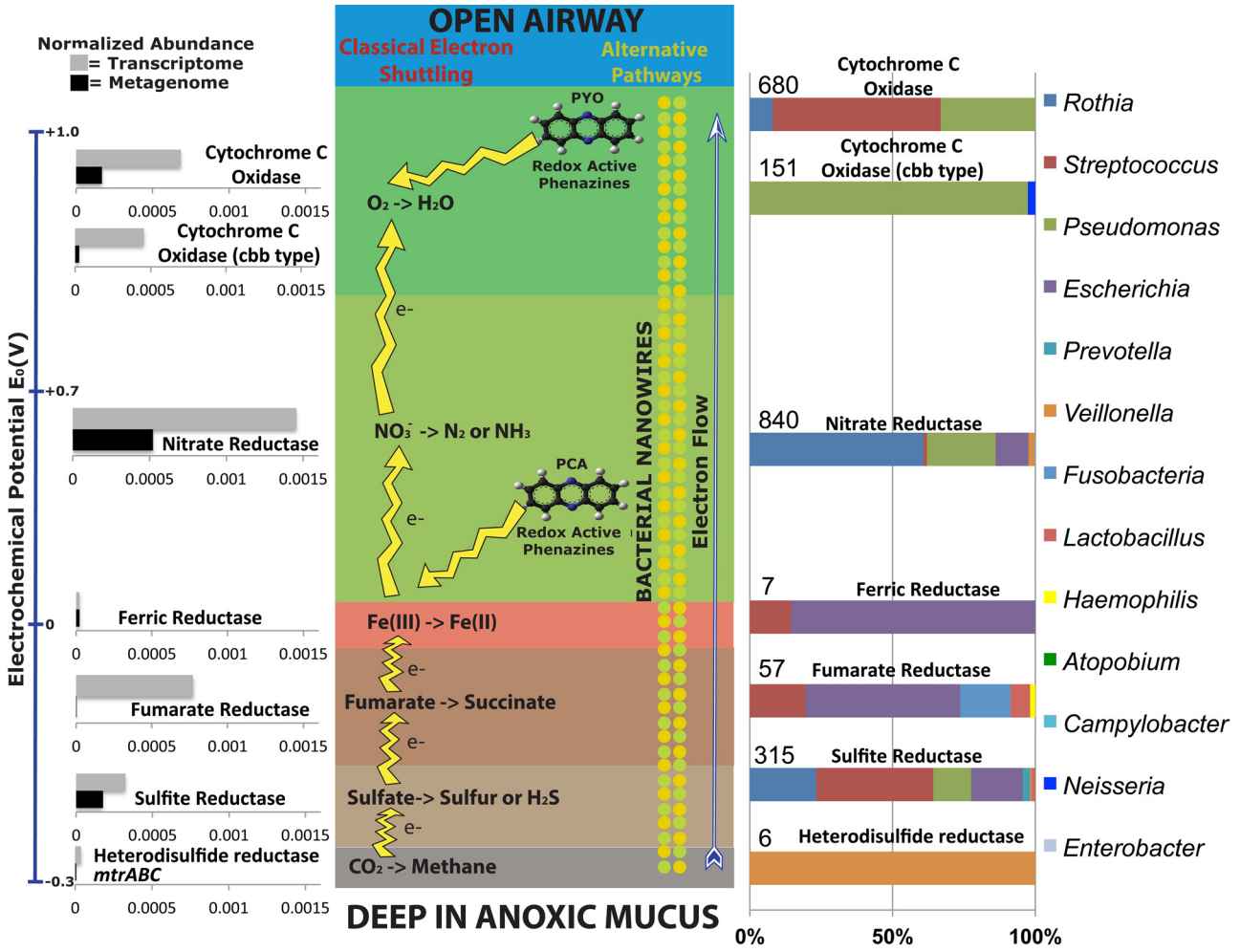

FIG 3 Theoretical electron transfer tower showing normalized abundances of microbial terminal respiratory reductase genes in the metatranscriptomes (gray bars) and metagenomes (black bars) from CF sputum samples. The total number of KEGG orthology hits for each gene from the HUMAnN output is normalized to the total number of hits to KEGG orthologs. The reductases are ordered on the basis of their approximate electrochemical potential and in the context of depth within CF airway mucus from the open airway. The size of the area containing each process is an approximation of its occurrence within the CF lung to aid visualization. Phenazines and nanowires are included as alternative electron shuttles (14, 71). Taxonomic information for the mapping of the sequence reads is also included with the number of reads that were mapped to a KEGG genome. PYO, pyocyanin; PCA, phenazine-1-carboxylic acid.

The terminal reductase cytochrome *c* oxidase was also present and expressed in the CF sputa, indicating that the CF microbes are utilizing O_2 as a terminal electron acceptor (Fig. 3). Interestingly, there was a high abundance of the *cbb*-type cytochrome *c* oxidase; this enzyme is known to have a higher affinity for O_2 and is preferentially used by *P. aeruginosa* in low oxygen (67, 68), which would be useful for aerobically respiring microbes living in the microaerobic conditions of mucus. Accordingly, *cbb*-type cytochrome *c* oxidase reads were assigned almost entirely to *Pseudomonas* spp., indicating that this may be a vitally important enzyme to the physiology of *P. aeruginosa* within the CF lung. The high transcription of cytochrome *c* oxidase may indicate that oxygen remains a major electron sink in CF lungs even in the microaerobic conditions of the CF lung. The source of oxygen in CF mucus is inhaled air and possible leaching from epithelial cells in lung capillaries. Overall, this analysis indicates that microbes in the CF lung are capable of respiring both aerobically and anaerobically, with the most genetic potential for anaerobic respiration with nitrate. This indicates the importance of this enzyme and its substrate to the physiology of microbes inhabiting CF lungs. Future studies that focus on identifying the major source of nitrate in the CF lung may inform therapeutic options, as manipulation of nitrate is likely to significantly alter microbial metabolism.

The availability of alternative terminal electron acceptors may be a major determinant of the growth rate for the CF microbial community. Although oxygen is surely present in microenvironments of CF sputum, there is mounting evidence that much of the lung mucus has a low oxygen concentration (17, 19, 20). A problem arises if oxidized compounds capable of accepting electrons are not readily available. In other environments, the completion of biogeochemical cycles requires oxidation of reduced compounds by specialized microbes (63). For example, nitrification is carried out by nitrifying archaea and soil bacteria such as *Nitrosomonas* spp. and *Nitrosobacter* spp. These types of chemotrophs are not found in the CF lung (1). Thus, a likely factor governing the maximum growth rate of CF microbes is that they are predominantly stuck in a reduced environment, which inhibits respiration and the generation of a proton motive force. Limited access to oxygen

or alternative terminal electron acceptors and the absence of bacteria that complete the complementary oxidative steps of these biogeochemical cycles invariably trap CF microbes in a reduced state. These microbes cannot escape this condition without the external input of available electron donors or molecules to balance the cellular redox state. Specialized metabolites like phenazines (14, 59, 61) may provide the required electron acceptors to enable the recycling of redox states and maintenance of redox balance, thereby completing cellular respiration. Phenazines may shuttle electrons to oxygen in the absence of other available electron acceptors (Fig. 3) (61). Alternative means of transferring electrons from redox reactions are also possible, e.g., bacterial appendages that can serve as nanowires (69) (Fig. 3). Existing in a highly reduced environment is likely a constant battle for CF microbes as they fight to maintain a redox balance while waiting for influxes of electron acceptors. This may explain the success of *P. aeruginosa* in CF disease, due to its ability to alleviate redox stress by producing phenazines that act as electron acceptors (61, 70).

Nitrogen cycling and sulfur reduction in the CF lung. Reduction of nitrogen oxide species (NOx) is one of the most energetically favorable pathways of anaerobic respiration in the microbial world (63). Though denitrification in *P. aeruginosa* has received some attention (21, 27, 29), and concentrations of nitrogen species have been measured in sputum, the nitrogen cycle and its relation to CF disease remain unexplored. Nitrate and ammonia have been shown to be elevated in CF lung fluids compared to non-CF controls (21, 23, 54), whereas nitric oxide and expression of human nitric oxide synthase are decreased compared to other inflammatory airway diseases (23, 71, 72). Outside of *P. aeruginosa* (21, 27), there are few data on how CF pathogens utilize NOx species *in vivo* to provide energy for their metabolism.

The normalized abundances of individual nitrogen cycle genes were calculated to determine which pathways are most likely to function in the CF lung. Genes devoted to amino acid breakdown (deamination) and assimilation of ammonia were the most abundant nitrogen cycle genes in the CF metagenomes and were also transcribed (Fig. 4). The abundance of ammonia assimilation transcripts was highly variable between sputum samples (see Fig. S2c in the supplemental material), indicating that it is likely a temporal response possibly due to influx of this compound. The abundance of amino acid metabolism genes is consistent with the KEGG analyses discussed above (Fig. 2), supporting the idea that amino acids are major carbon and nitrogen sources for CF microbes, and their deamination may contribute substantially to the high levels of ammonia in CF sputum (21, 22). High levels of ammonia were produced by *P. aeruginosa in vitro* and were reduced by antipseudomonal therapy *in vivo* (21), indicating that *P. aeruginosa* may be a major contributor to this species of the nitrogen cycle.

Of utmost importance to the nitrogen cycle are the energy-generating reactions (Fig. 4). These reactions include the denitrification of NOx to N_2 (or to various intermediates), the dissimilatory nitrate reduction to ammonia (DNRA) pathway that reduces NOx to ammonium, and the oxidative pathway nitrification. Denitrification has been associated with *P. aeruginosa* (55), and DNRA is favored when C/N ratios are high (73, 74), which is likely the case in the CF lung environment. In our sequencing data, genes encoding components of these two pathways were highly abundant and approximately equally expressed. Active transcription of all intermediate genes in denitrification were also

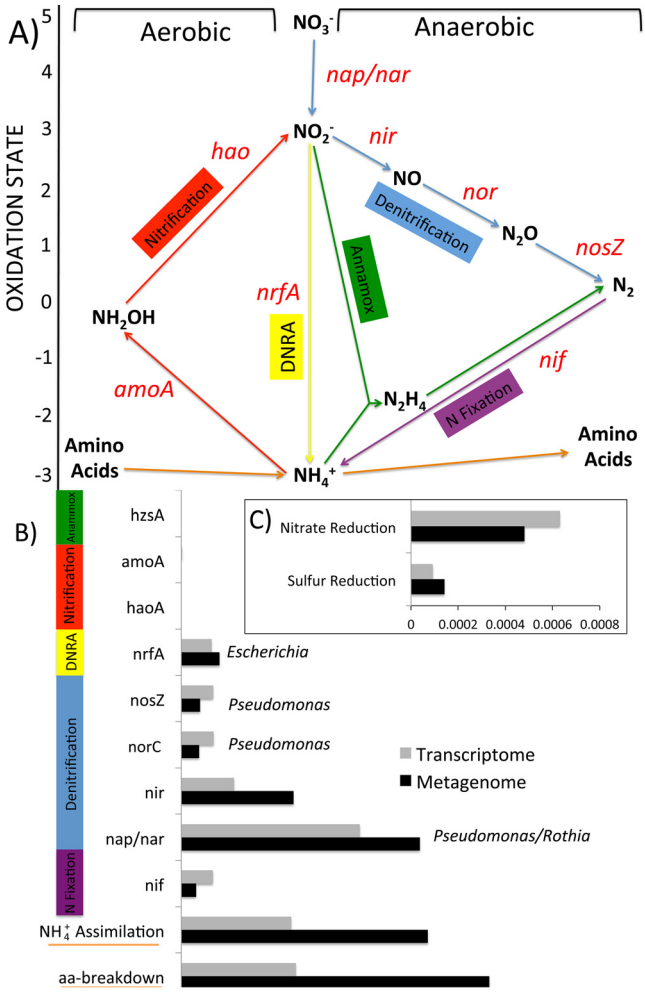

FIG 4 (A to C) Nitrogen cycle (A), normalized abundance data from HUMAnN output of the KEGG orthology for each gene in the cycle (B), and comparison of nitrate and sulfur reduction abundances (C). Gray and black bars represent gene abundances in the metatranscriptome and metagenome, respectively. The oxidation state of each nitrogen species is indicated. Amino acid breakdown and ammonia assimilation are also shown, but these pathways are a sum of a number of key genes (see Table S1 in the supplemental material) and occur both aerobically and anaerobically. Genus name taxonomic information is included for genes that were mapped to particular organisms. The normalized abundance of genes for nitrate reduction and sulfur reduction is also included for comparison. Their normalized abundance has been averaged based on their representative genes in the KEGG Orthology database. DNRA, dissimilatory nitrate reduction to ammonia; aa-breakdown, amino acid breakdown.

detected (Fig. 4), indicating that N_2 gas could be produced in the CF lung. Reads to the denitrification intermediate genes *nor* and *noz* were exclusively mapped to *Pseudomonas* spp., indicating that this species may be responsible for complete denitrification, while reads to the DNRA gene *nrfA* were mostly mapped to *Escherichia* (Fig. 4; see Fig. S3c in the supplemental material). Interestingly, the periplasmic nitrate reductase *nap* was expressed at a higher level than *nar*, though less abundant in the metagenomes. *Escherichia coli* preferentially uses NapA under low-nitrate conditions (75), consistent with this enzyme's adeptness at scavenging nitrate and the phylogenetic distribution of hits to this enzyme (Fig. S3c) (76). These results indicate that both of these reductive pathways

may be used by the CF microbial community. Complete denitrification may contribute to the decreased levels of NO detected in CF sputum compared to other inflammatory airway diseases, as this nitrogen species is reduced during denitrification (21, 23, 71, 72). Absent in the sequence data were genes devoted to nitrification, indicating that this oxidative process which completes the N cycle does not likely occur in the CF lung.

Surprisingly, nitrogenase (*nif*) was abundant and expressed in CF sputa (Fig. 4). This enzyme, most commonly associated with bacteria living in soil, is responsible for fixing (or assimilating) nitrogen, i.e., making atmospheric N_2 available for incorporation into biological molecules by converting it into ammonia. Manual analysis of the phylogenetic association of the *nifH* reads in our data showed that the hits were to *Rothia mucilaginosa* DY-18 nitrogenase subunit NifH (49 reads; average hit length = 360 bp; average identity = 96%). *Actinobacteria* spp. have been found to have the minimum components of nitrogenase (77); therefore, *Rothia* spp. may be capable of fixing nitrogen in the CF lung, but determining whether this *nifH* is properly annotated and functional is required.

Genes responsible for sulfur reduction were also present in the CF metagenomes, and sulfite reductase was particularly enriched in the transcriptome (see Table S3 in the supplemental material). Taxonomic composition of sulfur reduction genes is equally distributed in these samples (Fig. S3d). Collectively, however, sulfur reduction genes were less abundant than the comparable denitrification genes (Fig. 4). This indicates that sulfur reduction can be a source of energy generation for CF microbes but that more genetic potential is devoted to using N species to generate energy apart from aerobic respiration and fermentation.

Measurement of nitrogen oxides (NOx) in sputum and an *in vitro* culture model. To test the hypothesis that ammonia accumulates in the CF lung mucus due to microbial metabolism from an incomplete N cycle and amino acid breakdown, several nitrogen species were measured in sputum samples from 7 CF patients (two of them are included in the sequencing study). In sputum samples, nitrate, nitrite, and ammonia were detectable at relatively equal concentrations, indicating that there was a source of all three NOx and the system was equilibrated (Fig. 5). However, when the same sputum samples were inoculated into a sterile culture model of a CF bronchiole, almost all NOx species were present as ammonia after 48 h of incubation (Fig. 5). This suggests that microbes from the CF lung rapidly reduce nitrate and nitrite and produce ammonia. This finding was supported by our sequence analysis, which did not detect the oxidative pathways of the N cycle that replenish nitrate in environmental ecosystems. Thus, the nitrate detected in the sputum samples (18, 43; this study) is not likely from microbial nitrification which occurs in environmental ecosystems. Although the *in vitro* microcosm does not completely recapitulate the *in vivo* lung environment, these data provide evidence that N cycling in the CF lung may rely on input of oxidized NOx species from the host or other external sources.

The accumulation of high levels of ammonia may be a problem for CF microbes because it could slow their rate of growth. Thus, the assimilation of ammonia to more oxidized forms of nitrogen through amino acid synthesis and other anabolic pathways is a crucial step for microbes to avoid ammonia toxicity. Indeed, our transcriptomic data indicated that these enzymes are transcribed (Fig. 4).

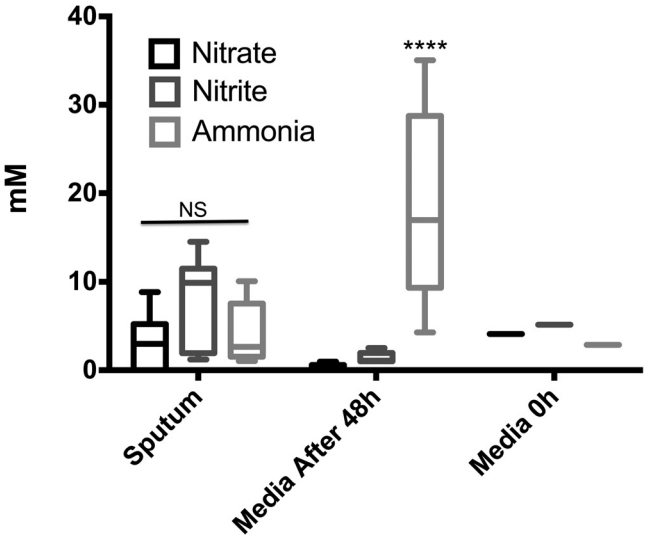

FIG 5 Levels of ammonia, nitrate, and nitrite in CF sputum samples and artificial sputum media (ASM) before and after inoculation and incubation with the same sputum samples. A one-way analysis of variance (ANOVA) test for statistical significance was performed on data from the sputum samples and media (after 48 h of incubation). Values that were not significantly different are indicated by the bar labeled NS. Values that were significantly different (P value of <0.0001) are indicated by four asterisks (P value of 0.164 or >0.05). NS, not significant.

Environmental sensing and transport in CF microbes. To assess the chemistry of the environment sensed by CF microbes, the abundance of transporters and environmental sensing pathways (e.g., two-component systems) was quantified using the KEGG module hierarchy (see Fig. S3 in the supplemental material). The abundance of the peptide/nickel transporters (M00239) is consistent with the utilization of amino acids as carbon sources (discussed above). The abundance of spermidine/putrescine transporters indicate that there may be amino acid catabolism occurring as these compounds are principal by-products of this process. Sugar transporter modules and phosphate transporters (M00222) were also abundant (Fig. S3)

Specific genes for ABC transporters (ko02010) and sensor histidine kinases (ko02020) were searched for in the sequence data. The bicarbonate transporter was present and transcribed, indicating that CF microbes are capable of scavenging bicarbonate and possibly reducing the already low bicarbonate concentrations in the CF ASL (78). The zinc transporter *znu* was also present, as was its sensor histidine kinase (see Table S4 and Table S6 in the supplemental material), enabling transport of zinc under conditions of elevated zinc concentrations in CF sputum (79). The manganese starvation two-component system was also present and expressed (Table S5). Elevated concentrations of zinc have been shown to disrupt manganese transport, starving cells of this element (80). The CF lung has also been found to be elevated in copper (79), and accordingly, the sensor histidine kinase that responds to copper, *cusSR*, was also present and expressed (no ABC transporter for copper is available in the KEGG database) (Table S6). Given the fact that nitrate could be used by CF microbes (discussed below), it was surprising that the KEGG nitrate transport module was not detected (Table S4). However, the two-component nitrate sensor system *narQPXL* was present and ex-

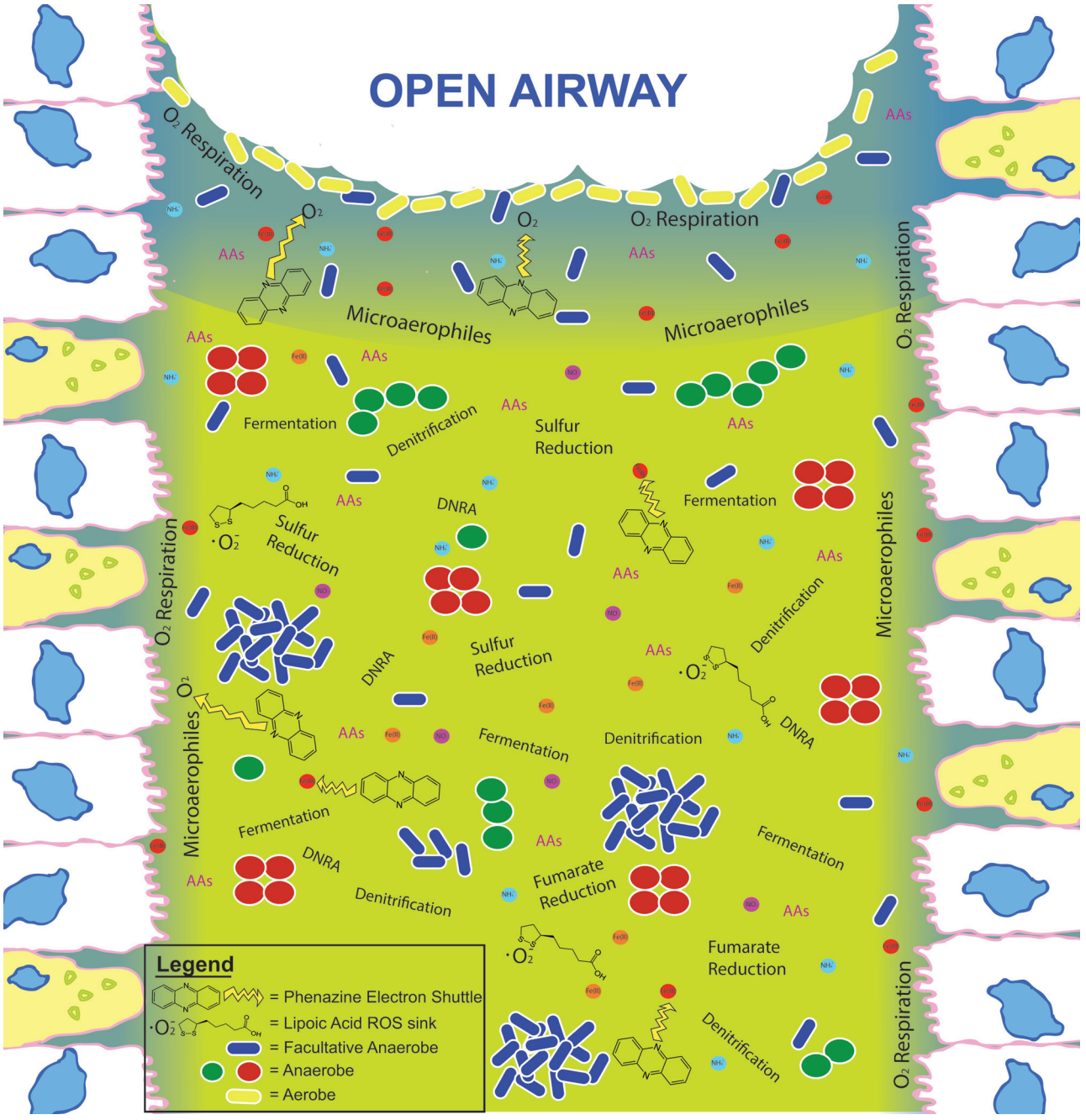

FIG 6 Illustration of a theoretical mucus-filled CF bronchiole inferred from the major signatures of the sequencing data described. The green section represents anaerobic mucus, while the blue faded area represents theoretical oxygen penetration. Ions are depicted as circles where Fe(III) is red, Fe(II) is orange, NH_4^+ is blue, and NO_3^- is purple. The epithelial and goblet cell layers of the bronchiole are also depicted. AAs, amino acids.

pressed, indicating that the microbes are sensing nitrate (Table S5).

Other abundant ion transporters in both metagenomes and the metatranscriptomes included those for iron, ammonium, and chloride (see Table S4 in the supplemental material). The chemical state of iron is particularly relevant to microbes in CF. In the CF lung environment, with low oxygen, low pH, and abundant reductants such as phenazines, much of the free iron pool is present as Fe(II) instead of Fe(III) (81). Assessing individual iron transporters for both chemical states of iron showed that both are present and expressed, with Fe(II) transporters expressed 5-fold higher than Fe(III) transporters when also considering abundances in the metagenome (Table S4). Transporters for bound iron, including those for heme and siderophores, were also detected (Table S4). Siderophore transporters were the most abundant iron transporter in our data set, indicating that CF bacteria may rely more on chelated iron.

Some genes are known to be transcribed during acid or alkaline

stress in model microbes such as *E. coli* and *Bacillus subtilis* (82–85). In the CF sequence data, response to both acidic and basic pH was observed in the metatranscriptome. The detection of responses to both alkalinity and acidity may result from varied responses to the known heterogeneity of microenvironments in CF sputum (28), where some microbes experience acidity and others experience alkalinity. The main by-products of the two central catabolic pathways, amino acid breakdown and fermentation, have opposite effects on environmental pH. Breaking down amino acids tends to raise pH due to ammonia production, whereas fermentation lowers pH by producing reduced organic acids such as lactic acid. If both pathways occur simultaneously in the microbial community, their synergistic effect would be the production of a more neutral environment than if they occurred separately. Tracing the pH response through different states of disease in a CF patient is an important next step in understanding microbial physiology during disease flares.

Conclusions. The data reported here provide an indirect view of CF biogeochemistry based on the strategies encoded and expressed by the resident bacterial community, augmented by a direct assessment of nitrogen species in CF sputa. The biochemistry of CF lungs is characterized by aerobic and anaerobic respiration, fermentation, catabolism and synthesis of amino acids, production of ROS quenching molecules, and a system that appears to accumulate ammonia in a reduced environment. The taxonomic distribution of the major energy-generating pathways indicates that in the presence of oxygen, most major CF pathogens in these samples will respire. As oxygen is depleted deeper in the mucus, microaerobic respiration by *P. aeruginosa* may occur, and then anaerobic metabolism via denitrification in *Rothia* spp. and *Pseudomonas* spp. or sulfur reduction in a diversity of bacteria may occur deeper in the mucus plugs. Furthermore, the less energetically productive fermentation pathways carried out by *Streptococcus* spp. and other microbes such as *Prevotella* spp. may occur simultaneously or be favored when other pathways are not possible. An overall schematic of the biochemistry occurring in a mucus-filled CF bronchiole is shown in Fig. 6.

These biochemical signatures are relevant to the development of novel treatment methods. CF pathogens evolve in their host over decades and become highly adapted to the CF lung environment. The data shown here may represent a mix of well-adapted CF climax community microbes and the invading attack community with more aggressive survival strategies as proposed in the climax-attack model by Conrad et al. (19). A better understanding of this environment, and the selective pressures it places on the microbes, will improve our ability to manipulate the environment in favor of the human host. Antibiotic resistance traits can be shared between all members of a microbial community, and the high levels of antibiotic resistance in many CF pathogens compromise the efficacy of antibiotic therapies.

The development of novel treatments in combination with existing antibiotic therapy may improve treatment overall. For example, antiprotease therapies could be developed to target CF microbes that feed on amino acids as principal carbon sources. Likewise, identifying the source of nitrate in the CF lung may provide a way of limiting microbial access to it, and hyperbaric oxygen therapy may disrupt anaerobic respiration and CF obligate anaerobes. Ultimately, this analysis has shed light on the ways in which the CF microbial community responds to local lung biochemistry, providing useful background information to help guide the development of novel therapies.

MATERIALS AND METHODS

Generation and analysis of sequencing data. Microbial DNA and RNA were extracted from CF sputum samples (see Table S1 in the supplemental material), and microbial metagenomes and metatranscriptomes were sequenced using 454 Roche technology as described in a previous study (1). Briefly, DNA was extracted after hypotonic lysis to aid in removal of human and extracellular DNA. Sputum samples were split into aliquots for transcriptomes, and for one aliquot from each sample, the RNA was subjected to mRNA enrichment using the Zymo Clean & Concentrator 25 kit (Zymo Research, Irvine, CA) and treated with RNase-free DNase (1). All samples were collected in compliance with the University of California Institutional Review Board (HRPP 081500) and San Diego State University Institutional Review Board (SDSU IRB no. 2121) requirements. This analysis includes new data from CF patients along with samples from CF patients previously published (1, 93). Raw reads were dereplicated, and sequences were removed if they were <60 bp long or had a mean quality score of less than 15 using Prinseq. Sequences of human origin were filtered out using Deconseq (86, 87). Ten individual samples with >20,000 reads, pooled microbial metagenome (approximately 654,000 reads of ~450 bp from 454 Roche sequencing) and metatranscriptome data (approximately 200,000 reads of ~450 bp from 454 Roche sequencing) were processed using the HUMAnN pipeline (38) as follows. (i) A BLASTx search was conducted with default parameters, including an E value of 10 against the KEGG v54 peptide database including >1,500 prokaryotes. (ii) HUMAnN uses the top 20 best BLASTx hits for each read to calculate a weighted sum of hits to each KEGG orthologous gene group based on hit quality (inverse BLASTx *P* value) and normalized by hit gene length. (iii) Pathway information is constructed using a combination of MinPath, elimination of pathways from taxa that are not present in the sample, and gap filling of enzymes that are likely missing based on abundances of hits inside each pathway (http://huttenhower.sph.harvard.edu/humann). The metagenomes and metatranscriptomes were pooled due to the tendency for HUMAnN normalization processes to overrepresent the abundance of certain pathways in low-sequence-coverage samples. The output was analyzed using multiple KEGG database hierarchies, including the KEGG BRITE, pathway, module, and individual gene orthologies (derived from the 04b, 03a, and 03b output files from the HUMAnN pipeline and the KEGG BRITE hierarchy). When the KEGG Orthology hierarchy was used for individual genes of interest, all individual gene abundances were normalized to the total number of hits the pooled data set had to the KEGG Orthology database. The HMP saliva metagenomes were obtained from the NIH HMP database website (http://www.hmpdacc.org/HMMRC/). The data obtained contained normalized abundance of KEGG pathways and modules from 5 healthy human saliva metagenomes; thus, comparisons could be made to our CF sequence data only at the level of KEGG pathways and modules (KEGG Orthology abundances were not publically available).

Phylogenetic read mapping. The taxonomy of each sequence read was assigned to the best BLASTn hit against the NCBI nr database (using a minimum cutoff of 40% identity and 60% coverage of the hit gene) by the method of Lim et al. (1). To assess which microbes encoded the metabolic genes identified in the metagenomes and metatranscriptomes, we selected 180 genomes from the KEGG Organism database that corresponded to taxa previously observed in our CF sputum samples. Stringent BLASTn (E value of 1e−40 and an alignment of at least 80 bp with 90% identity) was used to query each CF microbial metagenome sequence read against BLAST databases containing KEGG nucleotide sequences of functions of interest, using the KEGG Orthology gene identifiers in the KEGG fasta headers to select the sequences of functions of interest. The KEGG organism identifier of the top hit for each sequence read was retained, and a table with the distributions of hits across bacteria for each KEGG orthology of interest was created.

***In vitro* culture model and NOx measurement.** Artificial sputum medium (ASMDM formulation), originally described in reference 88, was modified to include 3 μg/ml ferritin as an alternate iron source to more appropriately reflect the conditions of the CF lung (ASMRQ medium). CF sputum samples were cultured in glass capillary tubes (75 mm in length and with an internal diameter of 1.4 mm) (Thermo Fischer Scientific Inc.) containing ASMRQ medium. The ASMRQ medium was prepared in a total volume of 500 μl and inoculated with 50 μl of a homogenized sputum sample. The capillary tubes were then filled with this mixture using capillary action, sealed at the bottom end, and incubated at 37°C and 100% humidity for 48 h. After incubation, the medium was removed from the tube, and debris was pelleted using a microcentrifuge. Nitrate and nitrite levels were measured using the Greiss reagent (89), and ammonia was measured using Nessler's reagent with appropriate ammonium chloride standards.

Nucleotide sequence accession numbers. Sequence data were deposited in the Sequence Read Archive (SRA) under accession no. SRP009392 (see http://trace.ddbj.nig.ac.jp/DRASearch/study?acc=SRP009392).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00956-13/-/DCSupplemental.

Figure S1, PDF file, 0.1 MB.
Figure S2, PDF file, 0.1 MB.
Figure S3, PDF file, 0.1 MB.
Figure S4, PDF file, 0.1 MB.
Table S1, DOCX file, 0.1 MB.
Table S2, DOCX file, 0.1 MB.
Table S3, DOCX file, 0.1 MB.
Table S4, DOCX file, 0.1 MB.
Table S5, DOCX file, 0.1 MB.
Table S6, DOCX file, 0.1 MB.

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