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Original Article

Metagenomics and metatranscriptomics: Windows on CF-associated viral and microbial communities

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Abstract

Background: Samples collected from CF patient airways often contain large amounts of host-derived nucleic acids that interfere with recovery and purification of microbial and viral nucleic acids. This study describes metagenomic and metatranscriptomic methods that address these issues.
Methods: Microbial and viral metagenomes, and microbial metatranscriptomes, were successfully prepared from sputum samples from five adult CF patients.
Results: Contaminating host DNA was dramatically reduced in the metagenomes. Each CF patient presented a unique microbiome; in some *Pseudomonas aeruginosa* was replaced by other opportunistic bacteria. Even though the taxonomic composition of the microbiomes is very different, the metabolic potentials encoded by the community are very similar. The viral communities were dominated by phages that infect major CF pathogens. The metatranscriptomes reveal differential expression of encoded metabolic potential with changing health status.
Conclusions: Microbial and viral metagenomics combined with microbial transcriptomics characterize the dynamic polymicrobial communities found in CF airways, revealing both the taxa present and their current metabolic activities. These approaches can facilitate the development of individualized treatment plans and novel therapeutic approaches.

Keywords: Cystic fibrosis; Viruses; Microbes; Metagenomics; Metatranscriptomics

1. Introduction

In the lungs of cystic fibrosis (CF) patients, the defective cystic fibrosis transmembrane regulator (CFTR) protein affects transepithelial ion transport, consequently hindering the normal airway clearance mechanisms [1,2]. The resultant static mucoid environment is colonized by a dynamic and complex community of microbes, viruses, and fungi (reviewed in LiPuma et al. [3]).

While standard microbial culture techniques had identified the key pathogens, more recent culture-independent approaches based on 16S rRNA gene sequencing revealed a much wider range of microbial species associated with CF lungs [4–9]. However, 16S rRNA-based methods are limited in taxonomic resolution and are subject to biases (summarized in Claesson et al. [10]); their

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predictions of metabolic activities are confined to those general functions known for the taxa, thus overlooking potentially important strain-specific variants. Metagenomics can overcome those limitations.

The metagenomic approach has been used to study viruses in human-associated environments such as blood [11], feces [12–14], and the lungs [15]. It has also been successfully used to characterize the viral communities in sputum samples from CF and non-CF individuals [15]. The presence of phages in CF airways is of particular relevance for clinical treatment, as environmental stress from the CF mucus and frequent antibiotic treatment is known to enhance phage mobility and promote the phage-mediated spread of antibiotic resistance genes in CF lungs [16,17].

On the other hand, it is challenging to generate microbial metagenomes from CF samples. One reason for this is that microbial DNA isolated from CF sputum or lung tissue samples usually contains a large amount of human DNA, often greater than 99% of the total DNA recovered [18–20]. Although some intact human cells may be present in the original sample, most of the contaminating DNA is extracellular and adsorbed to the surface of microbes, making isolation of pure microbial DNA particularly difficult.

Complementing metagenomics, metatranscriptomics characterizes the microbial genes expressed in an environment and can monitor shifts in their transcription or stability in response to perturbations, e.g., antibiotic treatments in CF patients. This approach has been used to investigate microbial community metabolism in marine [21–23] and soil [24,25] environments, but its application to host-associated microbes has been limited to a few instances [26,27] due to technical challenges (Supplementary Table 1). One such challenge is that messenger RNAs (mRNAs) account for only ~5% of total cellular RNA. Various rRNA depletion methods have been developed to enrich samples for mRNA (Supplementary Table 2). Concurrent application of multiple methods (e.g., mRNA-ONLY™, MICROB*E*xpress™ and MessageAmp™) can remove more of the rRNA in some instances, but efficacy remains limited, especially when working with partially degraded rRNA [28].

Metatranscriptomics of host-associated communities is particularly difficult. Amplification of the microbial RNA by methods that utilize synthetic polyadenylation is not applicable for samples that contain large amounts of eukaryotic mRNA. The appended poly-A tails reduce the amount of useful sequence data, especially when pyrosequencing technologies such as Roche/454 [29] are used. Due to the short half-life and small quantity of mRNA, sample filtration and manipulation with buffer should be avoided prior to RNA extraction. This inevitably causes an increase in host RNA contamination when dealing with host-associated microbial samples. In the recent microbial metatranscriptomic study of mule deer lymph nodes by Wittekindt et al. [26], 99.3% of the taxonomically assigned reads were host-derived and <0.01% were microbial.

Here we describe protocols to generate viral and microbial metagenomes, as well as microbial metatranscriptomes, from fresh CF sputum using 454 GS FLX Titanium pyrosequencing (Fig. 1). These methods target and enrich for viral and microbial DNA, as well as microbial mRNA, while minimizing contamination with host nucleic acids. This is the first study to simultaneously survey the microbiome, virome, and community metatranscriptome in any ecosystem.

2. Materials and methods

Note: A detailed standard protocol describing each step can be downloaded from www.coralandphage.org.

2.1. Sample collection

Eight sputum samples were collected from five CF volunteers (CF1 through CF5) at the Adult CF Clinic (San Diego, CA, USA) by expectoration into a sterile cup, with the exception of sample CF4-A that was a tracheal aspirate. All collection was in accordance with the University of California Institutional Review Board (HRPP 081500) and San Diego State University Institutional Review Board (SDSU IRB#2121). Clinical status at the time of collection was designated as *exacerbation* (prior to systemic antibiotic treatment), on *treatment* (during systemic antibiotic treatment), *post treatment* (upon completion of antibiotic treatment) or *stable* (when clinically stable and at their clinical and physiological baseline). Each sample was syringe-homogenized and divided into aliquots for metagenomic and metatranscriptomic analyses, culturing, and storage.

2.2. Virome protocol

(Supplementary Note 1) Dithiothreitol was added to the diluted sputum to aid mucus dissolution. Viral particles were purified by cesium chloride (CsCl) density gradient ultracentrifugation as described in Thurber et al. [30]. For one sample (CF4-A), the density gradient purification step was omitted for comparison. Viral DNA was extracted using CTAB/phenol: chloroform, and amplified with Phi29 DNA polymerase.

2.3. Microbiome protocol

(Supplementary Note 1) Sputum samples were treated with β-mercaptoethanol to disrupt mucus. Pelleted bacterial cells were repeatedly washed and centrifuged, then treated with DNase to remove human DNA.

2.4. Microbial metatranscriptome protocol

(Supplementary Note 1) Microbial cells in the sputum aliquots were mechanically lysed by vortexing with zirconia beads in TRIzol® LS (Life Technologies, NY, USA). RNA was extracted using the Zymo Clean & Concentrator™ 25 kit (Zymo Research, Irvine, CA, USA) with the small RNA removal protocol variation and treated with RNase-free DNase I (Ambion, Life Technologies: Grant Island, NY, USA).

cDNA was generated using the WTA-2 kit (Sigma-Aldrich). The effect of nebulization on transcript length was assessed as described in Supplementary Note 2. Similarly, two rRNA depletion methods were tested: (i) the 'Ambion' method, i.e.,

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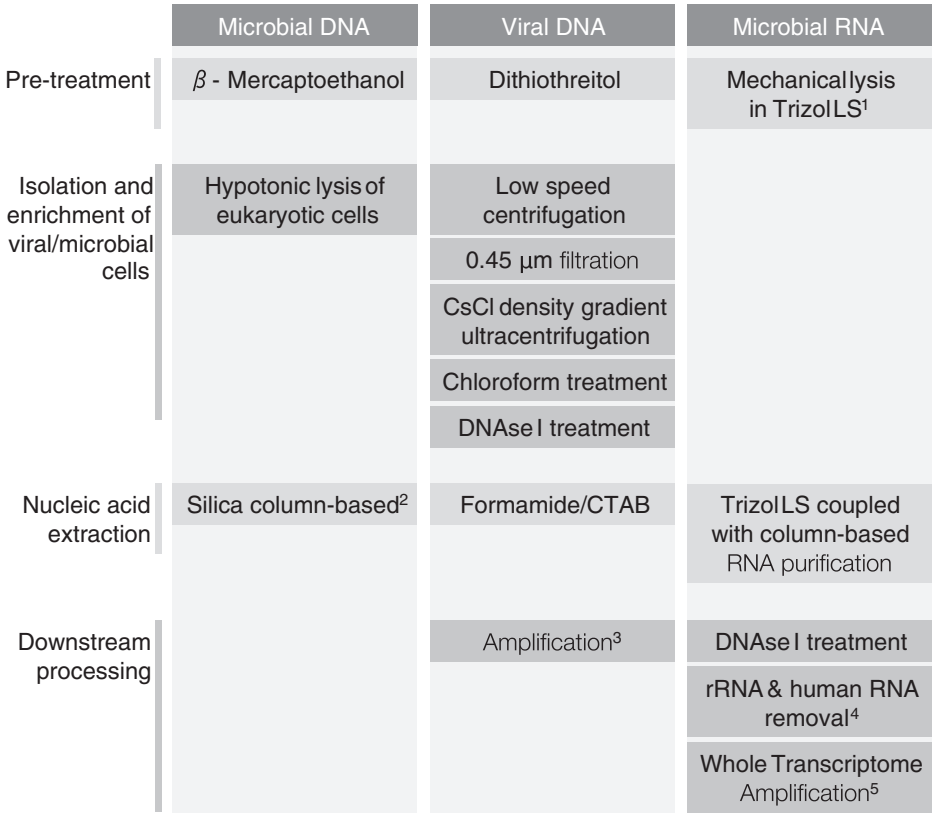

[1] Trizol LS, a more concentrated form of Trizol reagent, was used for liquid samples.
[2] The silica column-based NucleoSpin Tissue Kit (Machery-Nagel, Düren, Germany) with the Grampositive protocol.
[3] Amplification by phi29 DNA polymerase.
[4] Removal of microbial and human rRNA by the Epicentre Ribo-Zero™ Meta Kit.
[5] TransPlex™ Complete Whole-Transcriptome Amplification (WTA2) kit (Sigma-Aldrich: St. Louis, MO).

Fig. 1. Workflow for the preparation of CF sputum samples for microbiome, virome, and metatranscriptome sequencing.

MICROB*Enrich*™ and MICROB*Express*™, that removes bacterial rRNA as well as human rRNA and mRNA; and (ii) the Ribo-Zero™ method, i.e., Ribo-Zero™ rRNA Removal kit (Epidemiology version) (Epicentre, an Illumina company, Madison, WI) that removes bacterial and human rRNA.

2.5. Sequencing and data preprocessing/analysis

All samples were sequenced using the GS-FLX Titanium chemistry system. Primer tags in WTA amplified samples were removed using TagCleaner [31]. All datasets were preprocessed to remove duplicates and reads of low quality using PRINSEQ [32] (Supplementary Note 1). Metagenomic datasets were further screened and human-derived reads were removed using DeconSeq [33].

The preprocessed metagenomes were annotated using BLASTn against the NCBI nucleotide database. Sequences assigned to the phylum Chordata and to vector or synthetic sequences were identified and removed. Virome sequences were then compared against an in-house boutique viral database containing 4019 unique complete viral genomes using tBLASTx and normalized viral abundances were calculated. In the preprocessed metatranscriptomes, rRNA-like and non-rRNA reads were identified using BLASTn against the SILVA database [34]. Non-rRNA reads were annotated using BLASTx against the NCBI non-redundant protein database. For details of database generation and content, normalization, as well as BLAST parameters, see Supplementary Note 1.

2.6. Taxonomic assignments

The best hit was assigned to the alignment with the highest coverage, identity, and score values. Query sequences with no BLAST hits above the defined threshold were designated as *unassigned*. The diversity of microbiomes was calculated based on the number of bacterial species identified in the datasets (Supplementary Note 1).

2.7. Metabolic pathways

Sequences from the metagenomes and metatranscriptomes (excluding all Chordata, vector, and synthetic sequences) were compared against the KEGG protein database using BLASTx. (The CF1-A metatranscriptome was omitted due to insufficient data.) For each pathway, the best hits and their abundances were identified and normalized using HUMAnN [35] . Normalized pathway abundance values were used to calculate similarities between samples based on random forests [36] and

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to partition the samples by Partitioning Around Medoids (PAM) clustering [37].

2.8. Data accessibility

Sequence data was deposited in the NCBI Short Read Archive (SRA) with accession numbers SRP007749, SRP009392, and SRP009438.

3. Results and discussion

This is the first study to describe a comprehensive workflow (Fig. 1) for the generation of viromes, microbiomes, and microbial metatranscriptomes from any environment. The coupling of metagenomic and metatranscriptomic approaches provides an overview of both who is there and what they are doing, i.e., community taxonomy combined with the community's encoded and expressed functional diversity. For this work, viral metagenomes (viromes), microbial metagenomes (microbiomes), and microbial metatranscriptomes were generated from twelve fresh sputum samples that had been collected from five adult CF patients (Table 1).

3.1. Viruses in CF sputum

The metagenomic approach was successfully used previously to characterize viruses in CF lungs [15]. In this study, viromes were generated from eight sputum samples obtained from three CF patients (Table 1, Supplementary Table 3). Two methods for purifying virus-like particles (VLPs) from sputum were compared. Seven samples were purified by filtration and cesium chloride density gradient ultracentrifugation, followed by chloroform and DNase I treatment. This procedure yielded viromes that contained little (0.02%–3.7%) host-derived sequence (with one exception due possibly to its exceptionally high amount of mucins and free DNA, thus more viscous sputum; Supplementary Table 3). Omission of the density gradient ultracentrifugation step for the eighth sample (see Materials and methods) resulted in a virome with 97% host-derived sequence. Cesium chloride density gradient centrifugation, previously shown to recover the majority of

Table 1
Results summary for all viromes, microbiomes, and metatranscriptomes.

Patient ID	Time point	Date of collection	Health status	Metagenomes		Metatranscriptomes
				Microbial	Viral	Microbial
				% microbial sequences (number of microbial sequences)	% viral sequences [a] VLPs observed [b]	% non-rRNA sequences (number of non-rRNA sequences)
CF1	A	09/02/2010	Stable	N/A [c]	N/A [c]	10.7% [d] (738)
	B	10/18/2010	Stable	N/A [c]	N/A [c]	30.3% [d] (41,789)
	C	11/12/2010	On treatment	N/A [c]	N/A [c]	30.6% [d] (38,532)
	D	02/11/2011	Exacerbation	9% (14,691)	6.59% Yes	95.4% [e] (1900)
	E	02/24/2011	On treatment	58% (67,780)	31.99% Yes	N/A [c]
	F	03/14/2011	Post treatment	79% (40,825)	6.07% No	31.6% [e] (7971)
CF2	A	11/10/2010	On treatment	N/A [c]	N/A [c]	87.6% [d] (68,976)
CF3	A	11/10/2010	On treatment	N/A [c]	N/A [c]	89.6% [d] (93,375)
CF4	A	01/22/2011	Exacerbation	2% (3834)	0.90% [f] No	86.8% [d] (59,394)
	B	02/01/2011	Post treatment	0.2% (405)	6.77% No	99.1% [e] (32,446)
	C	03/20/2011	Stable	23% (41,636)	1.93% No	95.1% [e] (34,411)
CF5	A	10/07/2011	Exacerbation	2% (1034)	3.00% Yes	N/A [c]
	B	10/21/2011	Post treatment	1% (247)	3.78% Yes	N/A [c]

[a] Based on tBLASTx against viral genome database (threshold of 40% identity over at least 60% of the query sequence).
[b] Observation by epifluorescence microscopy of the viral fraction collected following cesium chloride density gradient centrifugation.
[c] Sample not available.
[d] Following rRNA depletion by the Ambion kits.
[e] Following rRNA depletion by the Ribo-Zero™ (Epidemiology) kit.
[f] Sample collected by filtration through 0.45 μm filter without cesium chloride density gradient ultracentrifugation.

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known phages [25] remains the method of choice for reducing host contamination when isolating viruses from complex samples such as CF sputum.

Analysis of the seven cesium chloride density-purified viromes using tBLASTx against the viral genome database identified more than 450 viral genotypes with each virome containing 319–456 genotypes (except CF4-C that contained only eight; Fig. 2a). Unknowns accounted for 49% to >99% of the total reads in most of the viromes (Supplementary Table 3), which is typical for viral metagenomes [15]. The exceptions were those samples highly contaminated by host sequences (CF4-A and CF4-C; Fig. 2a). The high number of "unknown" sequences implies the presence of novel viruses that cannot be identified by database similarity, as had been found in previous studies [11,15].

The majority of the viruses identified were phage, predominantly those that infect known CF pathogens. Their predicted bacterial hosts were tallied and the top 21 were used to construct predicted host range profiles (Fig. 2a). The profiles were highly similar between patients, and even more so for multiple samples from the same patient. They were dominated by phages that infect major CF pathogens such as *Streptococcus*, *Burkholderia*, *Mycobacterium*, *Enterobacteria*, and *Pseudomonas* genera. *Streptococcus* phage (particularly Dp-1) were found in high abundance in the samples with the greatest abundance (>30%) of *Streptococcus* spp. (i.e., CF1-D and CF1-E; Fig. 4a).

Streptococcus phage Dp-1 had been first isolated in 1975 from patients presenting with upper respiratory symptoms and was described as a virulent phage [38]. Here tBLASTx analysis

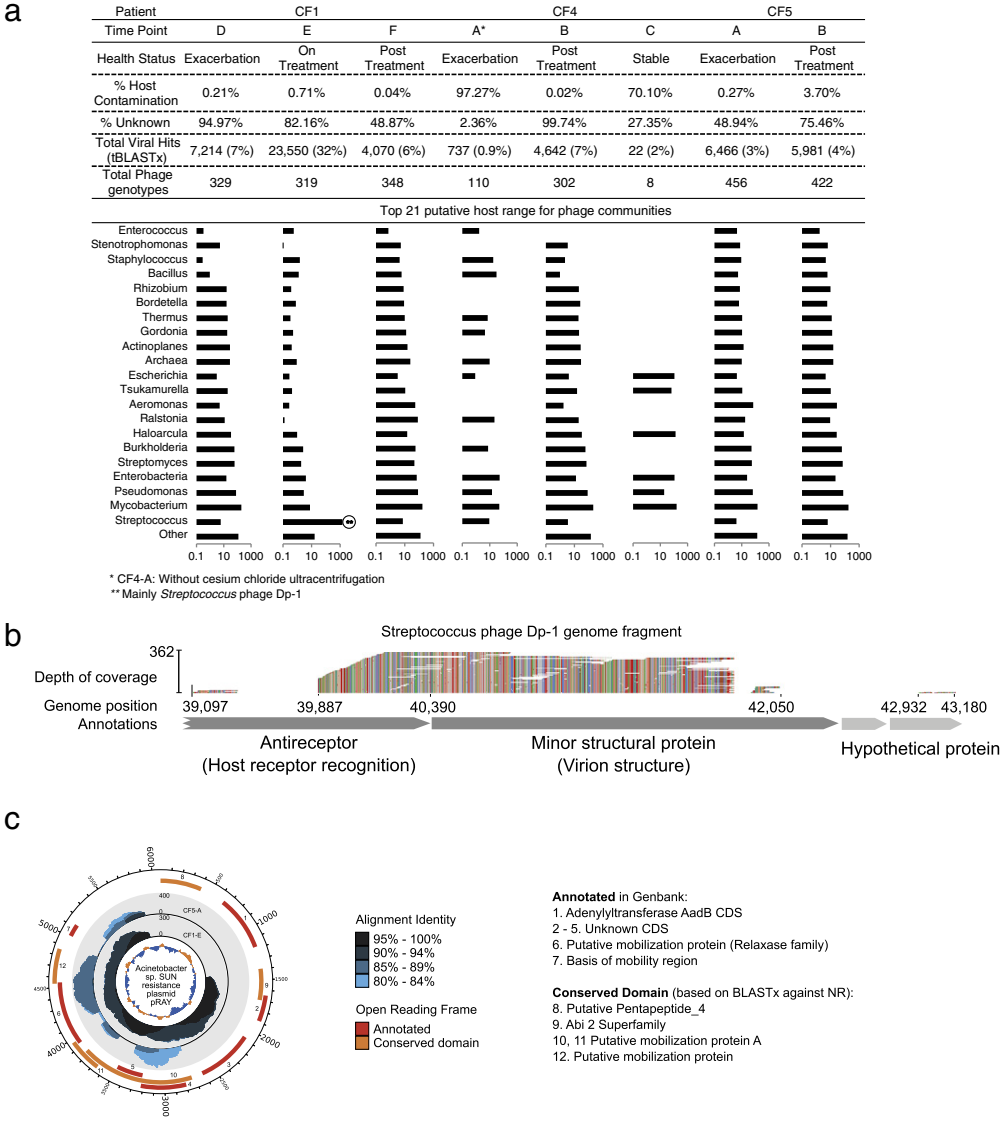

Fig. 2. Taxonomic analysis of CF viromes. (a) Putative host range profiles for phage communities. Each bar represents the sum of the normalized abundance values for all phage genotypes with the same putative bacterial host. Only the top 21 hosts are shown. (b) Nucleotide-level alignment of CF1-E virome sequences against a region of the *Streptococcus* phage Dp-1 genome. Depth of coverage was based on 90% nucleotide identity. The colors represent different nucleotides and demonstrate alignment quality. (c) Coverage plot of *Acinetobacter* sp. SUN resistance plasmid pRAY recovered from CF1-E and CF5-A.

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detected phage Dp-1 genes for DNA replication and packaging, host-receptor recognition, tail and capsid structural proteins, and host lysis (endolysin). The endolysin suggests possible top-down control of the *Streptococcus* spp. by lytic phage predation in these patients [39].

When reads from the CF1-E virome were mapped against the Dp-1 reference genome (GI:327198314), high depth of coverage was observed for a Dp-1 genome fragment in a 3 kbp region that codes for an antireceptor and a minor structural protein (Fig. 2b). Similarly, high coverage of regions of the *Acinetobacter* sp. SUN resistance plasmid pRAY was also observed in three samples (CF1-E, CF5-A, and CF5-B), including regions encoding a domain of the Abi-2 superfamily (proteins that confer resistance to phage infection) and mobilization proteins (Fig. 2c). Finding these short sequences from these two genomes highly enriched in the viromes implies that they must be present in many other genomes, as well, likely the result of active horizontal gene transfer (HGT) in CF lungs. HGT is an important mechanism by which microbes evolve and adapt to the CF lung environment [40], and phage can potentially facilitate this process.

The archaeal virus BJ1 (GI: 119756985) was identified in every virome — the first finding of an archaeal virus in the lungs. The hypersaline surface liquid in CF airways may be ecologically similar to the hypersaline lake in Inner Mongolia where the virus was isolated [41]. Archaea were identified in low abundance in one microbiome (CF4-C; <0.1%) and all metatranscriptomes (<1%; data not shown), suggesting that they could play a role in the CF lung ecosystem. However, since more than 71% of the predicted ORFs for this archaeal virus show no similarity to any known genes, its genome sequence provides no clues as to what that role might be.

Eukaryotic DNA viruses in CF individuals have been shown to be dominated by a few viral genomes [15] that could potentially cause persistent infections, exacerbations, tumorigenesis, and poor clinical outcomes [42–44]. The eukaryotic viruses identified in a previous study of the lungs of CF patients included torque teno virus (TTV), retroviruses, and human herpesviruses [15]. In the current study, eukaryotic viruses, including human herpesviruses and retroviruses, were found in all samples (Supplementary Table 4), with torque teno viruses in high abundance in one sample (CF1-D).

Because RNA viruses are involved in the majority of respiratory infections, a filtration-based method was used to isolate RNA viruses from CF sputum (data not shown). However, this method was unable to recover identifiable RNA viruses, likely due their low abundances and technical challenges in their isolation.

3.2. Microbes in CF sputum

When characterizing a microbial community, metagenomics surpasses a 16S rRNA-based approach in that it [1] frequently permits high-confidence species-level taxonomic assignment; [2] allows prediction of specialized functional capabilities of the adapted community, rather than inferring function from taxonomy; and [3] avoids the bias inherent in the selection of any universal target for PCR amplification. However, preparations of 'microbial' DNA derived from CF sputum or lung tissue are typically dominated by human DNA that was extracellular or adsorbed to the microbes. Several standard methods, including separation of human and microbial cells by percoll gradients [45], treatment with DNase I, selective degradation of human DNA by ethidium bromide monoazide [46], and use of the MolYsis kit (Molzym Life Science), have failed to reduce human DNA in CF samples (personal communications). In this study, the most effective procedure was found to be a modification of the method described by Breitenstein et al. [18] that employs a combination of β-mercaptoethanol to reduce biofilm disulfide bonds, hypotonic lysis of eukaryotic cells, and DNase I treatment of soluble DNA (Supplementary Fig. 1; Supplementary Table 5). Sufficient microbial DNA was extracted by this procedure to make amplification prior to sequencing unnecessary, thus avoiding potential amplification bias.

The amount of human contamination (13%–97% of total preprocessed reads) was highly dependent on the sample properties. Samples collected from patients during exacerbations might be expected to contain higher levels of host DNA due to greater inflammation and neutrophil activity than those collected during and immediately following treatment. However, our metagenomic data showed no significant correlation between the fraction of host DNA and a patient's health status even though the amount of host DNA varies markedly among the metagenomes.

With high-throughput pyrosequencing, even a relatively small proportion of non-host sequence data can be sufficient to provide significant information. After the removal of eukaryotic reads, the microbiomes contained >75% bacterial reads (Table 1) and 2%–12% unknowns, with the remainder being artificial and cloning vectors or synthetic constructs (Supplementary Table 5). The number of bacterial species identified, including aerobes and anaerobes, ranged from 24 to 256 (Supplementary Table 6).

Each patient presented a unique microbial profile (Fig. 3a). The predominant groups persisted across the time points assayed but the relative abundance varied with exacerbations and antibiotic treatments. This suggests complex community dynamics in which the predominant groups adapt and persist, while others come and go in response to antibiotic treatment or other perturbations.

CF4 presented a classic CF lung microbiome where *P. aeruginosa* was one of the main players at all time points. In contrast, CF1 was colonized mainly by *Rothia mucilaginosa* and *Streptococcus* spp. during exacerbation. Effective treatments decreased *R. mucilaginosa*, thereby increasing the proportion of *P. aeruginosa*. The *Rothia dentocariosa* that colonized CF5 during exacerbations was eliminated by treatments, and the patient was subsequently colonized by *Pseudomonas fluorescence* instead of the common CF pathogen, *P. aeruginosa*. The microbiome profiles also showed that *P. aeruginosa* can be replaced as the main player by other opportunistic bacteria from the environment, as evidenced here by the colonization of (i) CF5, a landscape architect, by soil-dwelling *P. fluorescens*

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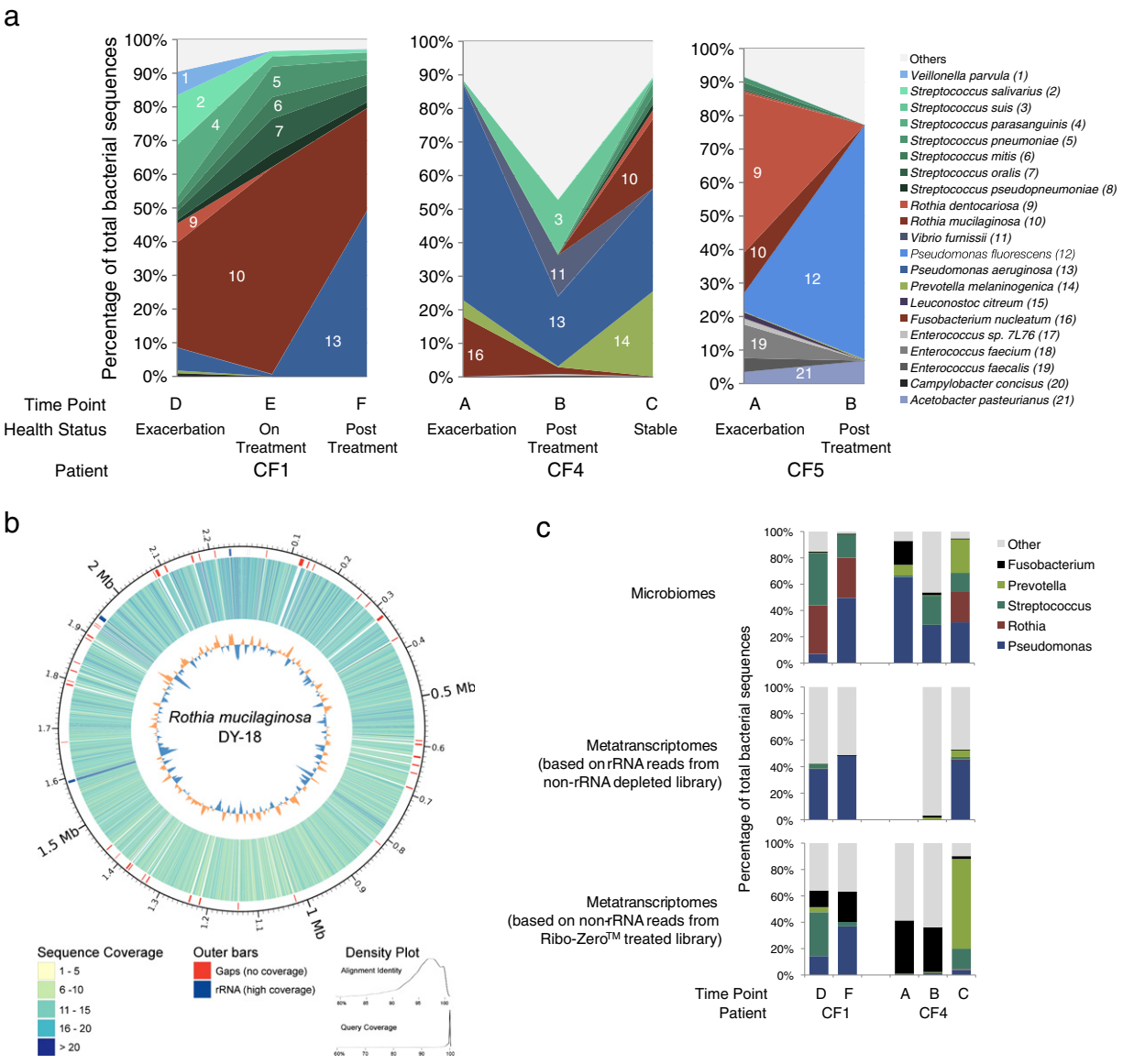

Fig. 3. Taxonomic analysis of the microbial communities in three CF patients across multiple time points. (a) Species-level comparisons between microbiomes. Identification was based on unique best hits using BLASTn against the NCBI nucleotide database. All species shown from the same genus are assigned similar colors. (b) Sequence coverage of the *Rothia mucilaginosa* DY-18 genome by reads from the CF1-E microbiome. (c) Genus-level comparisons between microbiomes and metatranscriptomes. (The CF4-A taxonomy is not shown here because an rRNA removal kit was used during metatranscriptome preparation.)

instead of the more common CF pathogen, *P. aeruginosa*, and (ii) CF1 by the oral flora *R. mucilaginosa* (Fig. 3a). By going beyond the traditional tracking of particular recognized CF pathogens, metagenomics offers the possibility of personalized clinical treatment plans.

In some situations, metagenomics can yield in-depth genomic analysis [47,48]. In this study, the CF1-E microbiome provided 7.8× average coverage over 93.56% of the genome of the most abundant species, *R. mucilaginosa*. Mapping of short reads against the reference genome DY-18 (GI: 283133067) (Fig. 3b) revealed only 41 gaps that were >1000 bp. Of those gaps, almost 20% were located in non-coding regions, 20% in regions annotated as hypothetical proteins, and the rest in genes of known function (Supplementary Table 7). In-depth analysis and interpretation of the genomic changes will be presented elsewhere (manuscript in preparation).

3.3. Evaluation of microbial metatranscriptome preparation

A high quality metatranscriptome contains relatively few rRNA reads and an unbiased sampling of RNAs of various lengths. Nebulization, the first step during preparation of a sequencing library, is a potential source of transcript size-induced bias since the size of our cDNA ranged from 50 to 4000 bp (Supplementary Fig. 2). While nebulization of high molecular weight DNA creates random fragments, application

to lower molecular weight cDNA may result in non-uniform coverage or the loss of short transcripts [49].

Here, the effect of nebulization on transcript length was tested on four samples (Supplementary Note 1). There was no difference in the relative translated protein length profiles with and without nebulization (Supplementary Fig. 3). The median translated polypeptide length (345–412 amino acids; Supplementary Table 8) is in the high range of previously described microbial protein lengths [50], possibly due to the use of the small RNA removal protocol in the RNA Clean & Concentrator ™ kit (Zymo).

Nebulization also did not affect the relative proportion of rRNA-like and non-rRNA reads, although the proportion of rRNA varied from patient to patient (9.4% to 70.8%; Fig. 4a). A reduced rRNA fraction was often associated with an increased proportion of eukaryote and unidentified reads.

Effective mRNA enrichment by rRNA removal using subtractive hybridization (e.g., the MICROB*Enrich*™ from Ambion) had been previously demonstrated on synthetic microbial communities [28]. However, efficacy depended on the integrity of the RNA and community composition. Here, four samples (CF1-D, CF1-F, CF4-B, and CF4-C) were used to compare two hybridization-based commercial rRNA removal kits. Each sample was divided into three aliquots for alternative treatments: (A) MICROB*Enrich*™+MICROB*E*xpress™ (**A**mbion); (E) Early access version of Ribo-Zero™ rRNA removal kit - Epidemiology (**E**picentre); and (N) **N**o treatment. In total, these twelve metatranscriptomes yielded 425,523 reads (105 Mbp), 48 – 77% of which were retained after data preprocessing (average read length=252 bp).

With either treatment, the proportion of rRNA was reduced significantly for CF1-D but minimally for CF1-F (Fig. 4b, Supplementary Table 9). Of the two treatment methods, the Ribo-Zero™ is more effective in eukaryotic rRNA removal as evidenced by CF4-B and CF4-C. In these samples that contained a larger proportion of eukaryotic reads, the Ribo-Zero™ treatment removed 96% and 90% of the rRNA, while the Ambion treatments increased the relative rRNA content.

Notably these rRNA removal methods were markedly less effective for sample CF1-F. Even with Ribo-Zero™, the most effective for all other samples, the treatment yielded only a 5% reduction in total rRNA. This inter-sample variation in rRNA removal could reflect differences in the microbial community present, the quality of extracted RNA, the accessibility of rRNAs for probe hybridization, and/or the degree of homology between the designed rRNA probes and the unknown community members.

Use of either rRNA depletion treatment precludes subsequent rRNA-based analysis of the sample because both methods distort the apparent relative abundances of microbial taxa (Supplementary Fig. 4). Bias was apparent for all samples

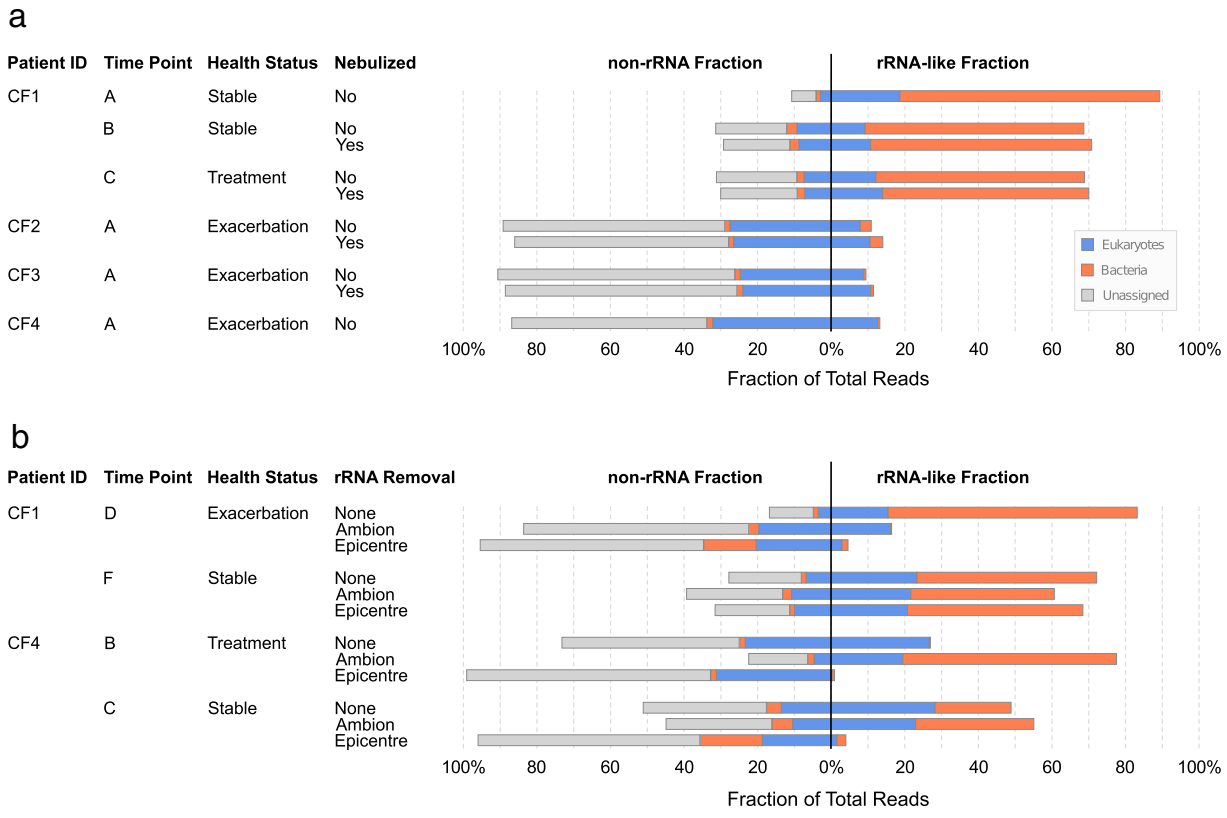

Fig. 4. Evaluation of the effects of nebulization and rRNA depletion on the relative amounts of rRNA and non-rRNA in metatranscriptomes. (a) Effects of nebulization. All samples were treated by the Ambion rRNA depletion kits. (b) Comparison of rRNA depletion methods. "Ambion" method uses a combination of MICROB*Enrich*™+MICROB*Express*™; "Epicentre" method uses Ribo-Zero™ rRNA Removal kit (Epidemiology version).

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except CF1-F; neither rRNA depletion method had significant effect on that sample.

3.4. Microbial taxonomy three ways

Three methods were used to determine the relative abundances of the microbial genera present within a patient sample: [1] annotation of microbiomes; [2] 16S rRNA-based annotation of metatranscriptomes; and [3] annotation of metatranscriptomes based on encoded protein sequences. The marked differences observed among the three (Fig. 3c) indicate that some community members are more transcriptionally active, thus contribute more to community metabolism than their relative numbers would predict. This is further evidenced by functional characterization (see below).

3.5. Community metabolic profiles

Whereas metagenomics surveys the functional capabilities encoded by members of the microbial commmunity, adding metatranscriptomic data provides insights into the current metabolic activities, insights that can assist in tailoring an effective treatment. To compare these approaches, the viromes, microbiomes, and metatranscriptomes were functionally annotated using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. A total of 216 metabolic pathways and 212 modules (collection of functional units) were identified. Multidimensional scaling (MDS) and clustering of all datasets based on the normalized abundance value (see Materials and methods)

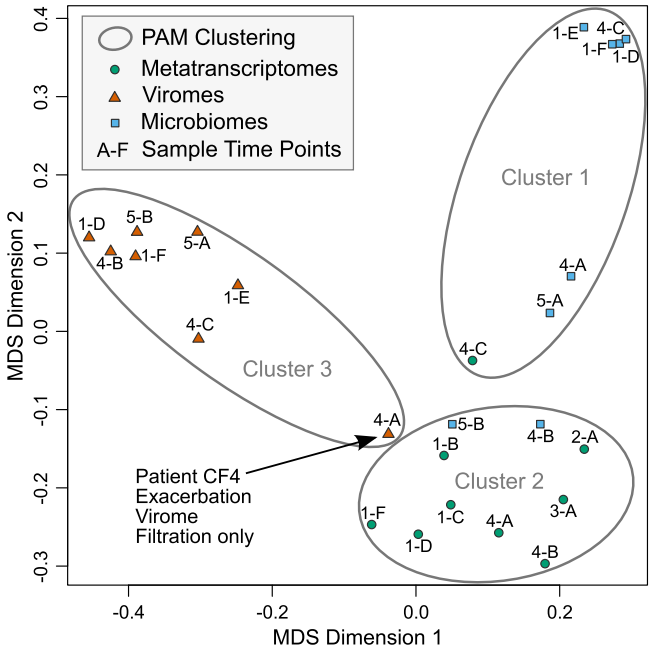

Fig. 5. Comparison of KEGG metabolic pathways identified in viromes, microbiomes, and metatranscriptomes as shown by multidimensional scaling (MDS). Grouping by Partitioning Around Medoids (PAM) clustering placed all samples in the appropriate cluster with the exception of the CF4-C metatranscriptome. CF1-A was omitted from both analyses due to insufficient data.

yielded three distinct groups, thus showing that a different view of community metabolism can be obtained from each method (Fig. 5). The results demonstrate the internal consistency of each method. The few exceptions were (i) the clustering of the CF4-C metatranscriptome with the microbiomes; (ii) the clustering of the CF4-B and CF5-B microbiomes with the metatranscriptomes attributable to the low number of reads; (iii) the CF4-A virome, purified without the gradient utracentrifugation, appearing as an outlier in the virome cluster.

Overall, the metabolic profiles derived from the microbiomes were the most similar between patients as well as between time points for each patient (Supplementary Fig. 5), indicating a shared pool of metabolic genes required for survival in the CF environment. The greatest variation, likely reflecting specialized adaptations within the viral and microbial communities, is seen in the principal component analysis (PCA) plot (Supplementary Fig. 6).

3.6. Clinical implications

The picture of dynamic and diverse polymicrobial communities presented here deviates from classic CF clinical profiles derived from culturing, thereby challenging one-size-fits-all treatment regimes. For example, current treatments targeting the classic CF pathogen, *P. aeruginosa*, would not be effective against *P. fluorescence*, *R. mucilaginosa*, or *R. dentocariosa* — all of which were abundant in these microbiomes. The ability to identify the resident viruses and microbes that could potentially trigger exacerbation events makes effective individual treatment plans a possibility, including intervention based on predicted disease progression. Ongoing surveillance can monitor inter-patient transmission and inform infection control measures. In addition, shifting the focus from pathogen taxonomy to the community metabolisms associated with periods of stability and exacerbation opens the door to novel therapeutic approaches that change the airway environment to favor less pathogenic communities.

3.7. Summary

The combination of metagenomic and metatranscriptomic approaches demonstrated here can provide insight into the complex and dynamic interations between the host and both the microbial and viral communities present in CF lungs.

- The methods described successfully recover viral DNA, microbial DNA, and microbial mRNA from CF sputum, while minimizing contamination with host nucleic acids.
- Of the viruses identified in the virome reads, most are phage that infect major CF pathogens. These likely include vectors for clinically-significant microbe-microbe horizontal gene transfer. However, the majority of virome reads are “unknown,” thus potentially novel viruses.
- To identify the microbes present, the microbiomes were annotated using BLASTn against the NCBI nucleotide database. Each CF patient possessed a unique microbial profile that shifted over time and sometimes reflected the

acquisition of persistent opportunistic bacteria from the environment. High genome coverage for the most abundant species allowed in-depth genomic analysis.

- The third concurrent approach, microbial metatranscriptomics, monitors the active community metabolism, as opposed to the metabolic potential encoded in the genomes. Of the three measures, the metatranscriptomes showed the greatest variation between patients and over time, thus is best able to capture the dynamic nature of these complex communities.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jcf.2012.07.009.

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