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Evidence and role for bacterial mucin degradation in cystic fibrosis airway disease

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1 **Chronic respiratory infections are composed of complex microbial communities that incite**
2 **persistent inflammation and airway damage. Despite the high density of bacteria that**
3 **colonize the airways, nutrient sources that sustain bacterial growth *in vivo* are unknown.**
4 **Here we examine the role of respiratory mucins in the ecological dynamics of the cystic**
5 **fibrosis lung microbiota. While *P. aeruginosa* was unable to efficiently utilize mucins,**
6 **saliva-derived anaerobes stimulated the growth of opportunistic pathogens when provided**
7 **mucins as the sole carbon source. The fermentative metabolisms of these oral anaerobes**
8 **generated amino acids and short chain fatty acids (propionate and acetate) during mucin**
9 **enrichment *in vitro*, which were also found within expectorated sputum from CF patients.**
10 **The significance of these findings was supported by *in vivo* *P. aeruginosa* gene expression,**
11 **which revealed a heightened response to propionate. Given that propionate is exclusively**
12 **derived from bacterial fermentation, these data support a central role for mucin**
13 **fermentation in the carbon flux of the lower airways. More specifically, commensal oral**
14 **bacteria may contribute to airway disease by degrading mucins, in turn providing**
15 **nutrients for pathogens otherwise unable to obtain carbon in the lung.**

16 Culture-independent surveys of the lung microbiome have revealed a far more complex
17 bacterial community than previously appreciated^{1,2,3}. Both in health and disease, the airways
18 harbor a diverse microbiota comprised of multiple taxa infrequently detected by clinical lab
19 culture. While the temporal dynamics of these communities and their association with disease
20 states have been studied in detail, the *in vivo* host environment, and microbial metabolisms
21 therein, is relatively understudied. For example, the means by which lung pathogens obtain
22 sufficient growth energy is not known. The accumulation of mucus secretions in the airways of
23 cystic fibrosis patients represent a potentially abundant bioavailable nutrient source; the major

1 macromolecular constituents, mucins, are a large reservoir of both carbon and nitrogen, and have
2 been measured at concentrations of up to 10 g/L in sputum⁴. These high molecular weight
3 glycoproteins are highly recalcitrant to bacterial degradation⁵, yet, they can serve as a major
4 nutrient source for niche-specific microbiota of the gut and oral cavity. For example, oral
5 streptococci produce a variety of glycolytic and proteolytic enzymes that liberate bioavailable
6 carbohydrates from salivary mucins⁶. In turn, these primary degraders modify the metabolic
7 landscape of the oral cavity and stimulate the growth of secondary colonizers⁷. Similar
8 interactions between commensal gut microbiota are also known⁸. By comparison, the role of
9 airway mucins as a nutrient source for opportunistic pathogens is poorly understood. Here, we
10 investigated mucins as a primary airway carbon source and characterized their potential to
11 sustain pathogen growth. Our results demonstrate that the primary pathogen of CF, *Pseudomonas*
12 *aeruginosa*, is unable to use mucins directly. This lack of growth raised the question: can other
13 members of the CF microbiota degrade mucins and alter the nutritional reservoir available for
14 pathogen growth? We subsequently found that co-culture of pathogens with oral-derived
15 anaerobes can stimulate pathogen growth using mucins as a sole carbon source. Finally, we
16 revealed that *P. aeruginosa* expresses pathways for the catabolism of mucin-derived
17 fermentation byproducts *in vivo*, strongly supporting a pathogenic role for commensal anaerobes
18 in the progression of CF lung disease.

19 ***Pseudomonas aeruginosa utilizes mucin inefficiently as a sole carbon source.***

20 We first tested whether mucins alone could sustain the growth of *P. aeruginosa*. To do
21 so, we assayed strain PA14 for growth in a defined medium containing intact mucins from
22 porcine gastric mucin (PGM) as the sole carbon source (Figure 1a). Interestingly, 10g/L mucin
23 (sugar equivalent of ~45mM glucose assuming 80% sugar by weight) only resulted in a moderate

1 OD₆₀₀ gain of 0.2 after 20 h. By contrast, PA14 grew to 0.8 OD on glucose (15mM), which
2 underscores the inability of PA14 to efficiently break down and utilize mucin glycoproteins.

3 Given that the PA14 laboratory strain was isolated from a burn wound⁹, we reasoned that
4 lung-derived clinical isolates would show enhanced growth on mucins. Growth yield of clinical
5 isolates was assayed long past the onset of stationary phase (60 h) to ensure there was no long
6 lag-phase or slow growth phenotypes. Under these conditions, CF-derived *P. aeruginosa* strains
7 CI46 and CI47 grew to an even lower density on mucin relative to PA14 (Figure 1b). Growth on
8 mucin was also compared to growth with glucose alone, with and without the addition of an
9 amino acid supplement to account for auxotrophy, a common trait among CF isolates¹⁰. CI47
10 grew well with and without amino acids while CI46 grew poorly on glucose alone, suggesting an
11 auxotrophy that was corrected by the supplement. The ability of the clinical isolates to grow on
12 glucose supplemented with amino acids to a greater extent than the more nutrient-dense mucin
13 medium suggested that the lack of robust growth on mucin was not due to a slow-growth
14 phenotype. Rather, these data suggest that *P. aeruginosa*, including isolates derived from the
15 mucin-rich CF lung environment, are unable to efficiently utilize these complex glycoproteins in
16 monoculture.

17 **Saliva-derived mucin fermenting bacteria support the growth of opportunistic pathogens *in***
18 ***vitro*.**

19 The inefficient use of mucins by *P. aeruginosa* motivated us to consider recent culture-
20 independent studies of the lung microbiota for insights into the carbon flux of the airways.
21 Notably, obligately anaerobic taxa associated with the oral cavity (*e.g. Prevotella, Veillonella*)
22 have gained attention for their abundance in both healthy and diseased lungs¹¹⁻¹³. A role for these
23 organisms in CF disease is controversial^{14,15}; however, they have been characterized for their

1 ability to degrade and ferment both salivary and intestinal mucins^{16,17}, in turn supporting the
2 growth of secondary colonizers. Based on these observations, we hypothesized that oral-
3 associated taxa alter the metabolic landscape of the airways by degrading respiratory mucins.
4 More specifically, we predicted that the fermentative degradation of mucin glycoproteins
5 liberates sugars, amino acids and short chain fatty acids (SCFAs) that are readily utilizable by *P.*
6 *aeruginosa* and other lung pathogens¹⁸.

7 To test whether fermenting taxa could stimulate CF pathogen growth, a saliva-derived
8 bacterial community was first enriched on mucin (Supplementary Figure 1). The enrichment
9 culture was then used to inoculate an anaerobic minimal mucin medium supplemented with 1.0%
10 agar to mimic a high viscosity sputum gel¹⁹ (Figure 2a). Once solidified, PA14 was resuspended
11 in phosphate-buffered saline agar without mucin, and was overlaid on top of the mucin-enriched
12 bacterial community. Under these conditions, PA14 should only grow if provided with diffusible
13 metabolites from the lower phase. After 60 h growth, turbidity was noticeable in the lower phase
14 and a yellow-green pigment characteristic of *P. aeruginosa* was observed throughout the co-
15 culture tube (Figure 2A). By contrast, in the absence of oral anaerobes, little pigment was
16 produced. Colony counts were also determined for *P. aeruginosa*, *Achromobacter xylosoxidans*,
17 and *Staphylococcus aureus* grown in the presence and absence of mucin-enriched bacteria
18 (Figure 2b). For each pathogen, significant differences (>10-fold; p<0.01) in growth were
19 observed in the presence of mucin fermenters relative to tubes in which oral anaerobes were
20 omitted. These data support the hypothesis that oral-derived microbiota can serve as primary
21 mucin degrading organisms in the CF airways, in turn liberating metabolites that can support the
22 growth of opportunistic pathogens.

1 **Oral-associated bacteria are present in sputum and are enriched in anaerobic mucin**
2 **fermentation cultures.**

3 To determine if there exists a fraction of the CF lung microbiota that has the ability to
4 degrade and ferment mucins, we performed enrichment experiments on sputum from 14 stable,
5 non-exacerbating CF patients. To do so, a small fraction of sputum was used to inoculate an
6 anaerobic culture with mucins supplied as the sole carbon source. Following growth, genomic
7 DNA was isolated from the initial sputum samples and corresponding enrichment cultures
8 followed by 16S rRNA gene sequencing to identify mucin-fermenting taxa. Consistent with
9 previous studies, sputum (prior to enrichment) harbored a diverse microbiota (Figure 3a) that
10 was highly variable between patients. As expected, *Pseudomonas sp.* made up the highest
11 percentage of sequence reads with a per-patient average of 31.3% across the cohort. Notably,
12 taxa previously characterized for their mucin-degrading activity (*Prevotella*, *Veillonella*,
13 *Streptococcus*, and *Fusobacterium*) also made up 35.1% of the population (11.4%, 9.7%, 9.9%,
14 and 4.1%, respectively).

15 Post-enrichment, communities were predominated by oral-associated fermenting
16 organisms (Figure 3b). On average, enrichment communities were composed of 73% of taxa
17 known to have mucin degradation ability (27.4% *Prevotella*, 19.2% *Veillonella*, 10.7%
18 *Streptococcus*, 8.4% *Fusobacterium*) with all other genera present at 4% or below.
19 *Lachnoanaerobaculum* and *Prevotella* were significantly enriched on mucins while *Neisseria*,
20 *Staphylococcus*, and *Pseudomonas* were selected against ($p < 0.05$). The high composition of
21 mucin-fermenting taxa in the initial sputum samples indicates a suitable niche space exists in the
22 CF lung for oral-associated mucin fermenting anaerobes. The enrichment results demonstrate

1 that these taxa found within sputum retain their mucin fermentation capacity during growth in
2 the lower airways, and could potentially cross-feed lung pathogens *in vivo*.

3 **Mucin fermenting taxa are present within explanted CF lungs.**

4 The presence of oral flora in the lower airways and their contribution to CF lung disease
5 progression is controversial due to the possibility of contamination from the route of
6 sampling^{14,15}. To address this concern, we sampled mucus directly from multiple lobes of three
7 explanted pairs of lungs derived from late-stage CF patients (Supplementary Table 1). Not
8 surprisingly, each lobe from all three patients harbored an abundance of *Pseudomonas* sp., which
9 is characteristic of late-stage lung disease^{14,20,21}. Yet, two patients also contained a notable
10 proportion of mucin-fermenting taxa identified in our enrichment experiments. For example, the
11 left lingula (left middle lung) from patient A contained relatively diverse microbiota, of which
12 approximately 4.7% were *Prevotella*, *Streptococcus*, and *Veillonella*. Patient B harbored known
13 mucin-fermenting taxa (~7.9% of sequence reads) in the lower left lung. Patient C was
14 dominated (>99%) by *Pseudomonas*, but even in this patient, it is noteworthy that mucin-
15 fermenting anaerobes were associated with several regions of the airways. Together, these data
16 support that a proportion of oral-associated anaerobes in our sputum samples were derived from
17 the lower airways.

18 **Sputum-derived fermenting organisms produce short-chain fatty acids and amino acids** 19 **when grown on mucin.**

20 To study the cross-feeding ability of mucin fermenters in further detail, we characterized
21 the metabolites that were generated during anaerobic mucin enrichment. To do so, we used
22 targeted high performance liquid chromatography (HPLC) for the quantification of organic acids
23 produced in mixed acid fermentation (acetate, butyrate, citrate, formate, isobutyrate, isovalerate,

1 ketobutyrate, ketoisovalerate, lactate, 2-methylbutyrate, propionate, succinate and valerate), and
2 gas chromatography for the quantification of amino acids. Mucin-enriched sputum cultures
3 generated high amounts of acetate (30.2 +/- 9.7 mM) and propionate (15.4 +/- 3.8 mM), while
4 only one enrichment sample containing an abundance of *Streptococcus sp.* (#7025, see Figure
5 3a) produced a detectable concentration of lactate (7.8 mM) (Figure 4a). No other SCFAs
6 assayed were found in the mucin-enrichment supernatants. Not surprisingly, free amino acids
7 were also present in each sample (1.37 +/- 1.32 mM total) (Figure 4b), suggestive of their
8 liberation from the mucin polypeptide backbone. The abundance of amino acids correlates well
9 with previous studies of sputum composition where they were found to be present in appreciable
10 quantities²². These data demonstrate that acetate, propionate, and amino acids are byproducts of
11 mucin fermentation by sputum-derived anaerobes and suggest that they may be bioavailable
12 carbon sources that can cross-feed pathogens *in vivo*.

13 **Cystic fibrosis sputum contains short-chain fatty acids and *Pseudomonas aeruginosa***
14 **responds to propionate *in vivo*.**

15 16S rRNA gene analyses demonstrated the presence of a fermentative bacterial
16 community in the CF airways. To provide further evidence of fermentative activity *in vivo*, we
17 used two complementary techniques: mass spectrometry and quantitative reverse transcription
18 PCR (qRT-PCR). First, using gas chromatography mass spectrometry (GC-MS), acetate and
19 propionate were quantified in paired saliva/sputum samples collected from 9 stable CF patients.
20 These data revealed no significant differences for either SCFA between the oral cavity and lung
21 (Supplementary Figure 2). Given the presence of fermenting taxa and SCFA previously
22 identified in saliva²³, this result was not unexpected. To circumvent the effect of SCFA
23 contaminants from the mouth during sampling, an oral rinse was first performed, prior to saliva

1 and immediate sputum collection (see Methods). Using this sampling strategy on 7 additional
2 patients, acetate concentrations were significantly higher in sputum relative to saliva (5.9 +/- 1.8
3 mM versus 2.4 +/- 1.8, $p < 0.01$) (Figure 5a), consistent with metabolites generated during
4 enrichment culture (see Figure 4A) and indicative of a fermentative bacterial community within
5 the CF lung. Propionate, on the other hand, showed no significant difference ($p = 0.89$) between
6 paired samples (Figure 5b). This result was surprising given the high levels of propionate
7 generated during *in vitro* mucin enrichments. Because of this disparity, we hypothesized that
8 propionate, over acetate, is preferentially utilized by members of the CF lung community in a
9 cross-feeding relationship.

10 If SCFAs were simultaneously produced and consumed by the airway bacterial
11 community, we would expect the concentration of these metabolites to remain low, and genes
12 required for their catabolism to be highly expressed. To address this possibility, we used
13 qRTPCR to measure *in vivo* bacterial gene expression. Genomic content suggests that
14 *Pseudomonas* has committed pathways for both acetate and propionate catabolism (encoded by
15 *acsA/B* and *prpBCD*, respectively). Therefore, as a proxy of the use of mucin-derived
16 metabolites by *P. aeruginosa*, we targeted the *in vivo* expression of *acsA* (encoding acetyl-coA
17 synthetase) and *prpD* (methylcitrate lyase) relative to their expression levels under controlled
18 conditions. *In vitro*, *acsA* and *prpD* were differentially expressed by PA14 in the presence of
19 acetate (5.8-fold higher, $p = 0.02$) and propionate (11.4-fold, $p < 0.01$), respectively, relative to
20 growth on glucose alone (Figure 6). When both SCFAs were present, *prpD* was expressed
21 significantly higher than *acsA* ($p < 0.01$), which were both upregulated relative to the glucose
22 control (2.4 and 4.8-fold for *acsA* and *prpD*, respectively). When compared to *in vitro* cultures,
23 analysis of sputum revealed that *prpD* (5.4-fold, $p = 0.02$) but not *acsA* (no change, $p = 0.98$) was

1 upregulated. These data are indicative that propionate is generated in the airways of CF patients
2 and that *P. aeruginosa* both senses and catabolizes propionate *in vivo*, providing further evidence
3 that mucin-associated fermentative metabolisms contribute to the metabolic landscape of the CF
4 lung.

5 **DISCUSSION**

6
7 Metabolite constituents of expectorated sputum are known to support bacterial growth *in*
8 *vitro*^{22,24,25} but the nutritional role of intact mucins has not been characterized. Previous studies
9 reported that mucins support the carbon demands of *P. aeruginosa in vitro*^{26,27}; however, these
10 studies included autoclaved preparations of commercial PGM that contain low molecular mass
11 impurities. In fact, when PGM was filtered and dialyzed in our study (leaving only intact, large
12 glycoproteins), *P. aeruginosa* inefficiently utilized mucins as a carbon source on their own.
13 Rather, in the presence of purified mucin, fermentative oral-associated bacteria were required to
14 stimulate pathogen growth. Moreover, we revealed that *in vitro* mucin fermentation generated
15 high concentrations of SCFAs and amino acids, which were both abundant (acetate) and utilized
16 by *P. aeruginosa* (propionate) in patient sputum. Together, these data provide strong evidence
17 for a central role of mucin fermentation in the carbon flux of lower airway disease and define a
18 pathogenic role for oral-associated anaerobes in the progression of CF-associated airway disease.

19 *In vitro*, degradation of O-linked glycans and the polypeptide backbone of mucin by
20 sputum-derived anaerobes generated an abundance of SCFAs and amino acids. Given the known
21 substrate utilization profile of *P. aeruginosa*¹⁸, these compounds also likely sustained pathogen
22 growth within the co-culture experiment. Consistent with recent studies^{32,33}, SCFAs were also
23 found in sputum across our CF patient cohort. Because SCFAs serve as a reliable biomarker of
24 fermentative metabolism³⁴, their universal presence suggests that bacterial fermentation is a

1 ubiquitous metabolic activity in the CF airways. More specifically, our data suggest that the
2 fermentation of mucins is a likely origin for metabolites that have been shown to support growth
3 of *Pseudomonas*²² in expectorated sputum. Moreover, the high levels of *prpD* expression
4 confirms that *P. aeruginosa* is both sensing and utilizing propionate *in vivo*, suggesting that the
5 lower-than-expected concentrations of this metabolite in sputum can be explained by its
6 consumption as opposed to a lack of production within the airway environment.

7 Our data create a compelling model for the role of oral anaerobes in the development of
8 CF lung disease, whereby pathogens cannot become established until mucin fermenters have
9 colonized the lower airways. In CF, impaired mucociliary clearance and defective
10 immunological responses increase the likelihood of oral anaerobe colonization and the
11 degradation of respiratory mucins. In turn, anaerobes left uninhibited can condition the lung
12 environment into a niche that is suitable for pathogens to thrive. Several lines of clinical
13 evidence support this model: (i) pediatric patients have asymptomatic primary colonization by
14 oral anaerobes^{35,36} prior to the establishment of chronic *P. aeruginosa* infections, (ii) routine
15 administration of broad-spectrum antibiotics is an effective therapy to delay the onset of chronic
16 colonization by *P. aeruginosa* and reduce the frequency of acute exacerbations^{37,38}, and (iii) the
17 *in vitro* antibiotic susceptibility of lung pathogens does not correlate with clinical outcomes³⁹. In
18 the latter instance, we suspect that antibiotics do not solely target the pathogen, but rather disrupt
19 the complex metabolic interactions that supply them with substrates for growth and stimulate
20 their pathogenicity.

21 This work raises the question of the importance of mucin-degrading anaerobes in late
22 stages of CF disease when bacterial diversity often declines and becomes predominated by *P.*
23 *aeruginosa*⁴⁰. In severe disease states, it is known that chronic airway infections can lead to a

1 'leaky' lung epithelium that allows bioavailable metabolites to reach the epithelial surface⁴¹.
2 Additionally, persistent *P. aeruginosa* infections incite a neutrophil-dominant inflammatory
3 response that is associated with increased concentrations of host-derived proteases⁴². Human
4 neutrophil elastase, for example, is capable of cleaving glycoproteins into free amino acids, and
5 has been implicated in the progression of lung disease⁴³. Moreover, neutrophilic burst may also
6 contribute to the bioavailable nutrient pool, potentially diminishing the need for anaerobic
7 fermenters as *P. aeruginosa* populations colonize the airway niche and incite a heightened
8 immune response. We are currently investigating the temporal dynamics of both host and
9 microbiota mucin degradation across the spectrum of disease severity.

10 Altogether, this study underscores the importance of defining the underlying microbial
11 ecological dynamics associated with airway disease. In particular, our data warrant further
12 studies of targeted therapies towards fermentative organisms and their metabolisms that may
13 contribute to the establishment and progression of chronic CF lung infections. In a broader
14 context, the presence of both oral anaerobes and known pathogens in other, mucus rich
15 environments – chronic obstructive pulmonary disease, chronic sinusitis, ventilator-associated
16 pneumonias – suggests that mucin fermentation and metabolic cross-feeding may be a
17 widespread disease phenomenon. A more detailed understanding of these metabolic interactions
18 are vital to the development of effective therapeutic strategies and improved clinical outcomes.

19 **Methods**

20
21 **Bacterial strains and culture conditions.** *P. aeruginosa* PA14 and *Staphylococcus aureus* MN8
22 were obtained from D.K. Newman (California Institute of Technology). Clinical strains of *P.*
23 *aeruginosa* (CI46, CI47) and *A. xylosoxidans* MN001⁴⁴ were isolated from patients enrolled in
24 this study. Strains were routinely cultured in Luria Bertani medium or a minimal mucin medium

1 containing 60mM KH₂PO₄ (pH 7.4), 90mM NaCl, 1mM MgSO₄, 10 g L⁻¹ (or 15 g L⁻¹ where
2 specified) porcine gastric mucin (Sigma), and a trace minerals mix⁴⁵. During preparation, mucin
3 was autoclaved, dialyzed using a 13 kDa molecular weight cutoff membrane, clarified by
4 centrifugation, followed by passage through a 0.45 µm Millipore filter to sterilize soluble intact
5 glycoproteins. Glucose and NH₄Cl were supplemented at 13mM and 60mM, respectively, where
6 specified. MEM essential and non-essential amino acid mixes (Sigma) were added at a final
7 concentration of 0.5X the recommended concentration. For enrichment growth, ~100 µL of
8 sputum was used to inoculate minimal mucin medium under anoxia (95% N₂, 5% CO₂) and the
9 remainder was frozen at -80°C. Following 48h of incubation at 37°C, 100 µL of culture was used
10 to inoculate a second anaerobic culture tube and allowed to grow for 48h. Genomic DNA was
11 isolated from both the initial sputum sample and enrichment cultures, and bacterial community
12 composition was determined using 16S rRNA gene sequencing.

13 **Patient cohort and sample collection.** Forty-eight participants with CF were recruited at the
14 University of Minnesota Adult CF Center and informed consent was obtained for all subjects.
15 For enrichment cultures, sputum was expectorated into 50ml conical tubes, placed on ice, and
16 processed within three hours. Sputum used for qRTPCR analysis was placed in RNALater
17 (Sigma) immediately following expectoration, and stored at -80°C for further processing. For
18 metabolite analysis (n=9), paired saliva and sputum samples were also collected in conical tubes
19 and immediately stored at -80°C. For a second set of samples, subjects (n=7) first performed an
20 oral rinse, followed by saliva then sputum collection less than 30 seconds afterwards. To obtain
21 clinical isolates of *Pseudomonas sp.*, sputum aliquots were cultured on Pseudomonas Isolation
22 Agar (Oxoid) for 72 hours at 37°C. Colonies were screened using PCR⁴⁶. Isolates positively
23 identified as *P. aeruginosa* were stored in 15% glycerol and frozen at -80°C.

1 Lung tissue was obtained from three late-stage CF patients undergoing double lung
2 transplantation. Upon extraction, explanted lung parenchyma was separated from the main
3 bronchi and dissected into six lobes (right upper, right middle, right lower, left upper, left
4 lingula, left lower) by the UMN BioNet Tissue Procurement Facility. Mucus was extracted via
5 syringe from the conducting airways of each lobe, and frozen at -80°C for subsequent 16S rRNA
6 gene profiling. These studies were approved by the Institutional Review Board at the University
7 of Minnesota (UMN IRB nos. 1401M47262 and 1404M49426).

8 **DNA extraction and 16S community analysis.** Lung mucus, sputum and enrichment cultures
9 were thawed to room temperature, and 500 μ L of each sample was used for genomic DNA
10 extraction using the PowerSoil DNA isolation kit (MoBio, Carlsbad, CA). Purified DNA was
11 submitted to the University of Minnesota Genomics Center (UMGC) for 16S library preparation
12 using a two-step PCR protocol. Briefly, the primary amplification step was performed using
13 KAPA HiFi Polymerase (Kapa Biosystems, Woburn, MA) and primers specific for the region
14 flanking 16S rRNA V4 region that included adapter tails for adding indices in a secondary
15 amplification step (Supplementary Table 2). Amplicons were diluted 1:100, and a subsequent
16 PCR reaction was performed to add indices and Illumina flow cell adapters. For each sample, a
17 pair of 8 base bar codes were included to distinguish between samples. Indexing primers are
18 listed in Supplementary Table 2. Amplicons were quantified using PicoGreen (Life Technologies,
19 Carlsbad, CA), normalized, pooled, and \sim 1 μ g of material was concentrated to 10 μ l using 1.8x
20 AMPureXP beads (Beckman Coulter, Brea, CA). The pooled sample was size selected at 427bp
21 +/- 20% on a Caliper XT DNA 750 chip (Caliper Life Science, Hopkinton, MA). Amplicons
22 were cleaned using AMPureXP beads and analyzed using an Agilent Bioanalyzer Chip (Agilent
23 Technologies, Santa Clara, CA). The final pooled sample was then diluted to 2 nM, 10 μ l was

1 denatured using 0.2N NaOH, diluted to 8pM in HT1 buffer, spiked with 15% phiX, heat
2 denatured for 2 min, and sequenced using a MiSeq 600 cycle v3 kit (Illumina, San Diego, CA).
3 A defined mock community and reagent blanks were also sequenced as controls.

4 Raw sequence reads were obtained from UMGC and analyzed using a QIIME⁴⁷ pipeline
5 developed by the UMGC. Briefly, read pairs were stitched together and 16S amplicon primers
6 were removed using PandaSeq (version 2.7)⁴⁸. Fastq files were merged and sequence IDs
7 converted to QIIME format using a custom perl script. Chimeric sequences were detected using
8 the QIIME (version 1.8.0) script identify_chimeric_seq.py function, using the usearch61 method.
9 Open reference OUT picking was performed using the pick_open_reference_otus.py script, using
10 the usearch61 method and the Greengenes 13_8 16S rRNA reference database⁴⁹ clustered at 97%
11 similarity. MetaStats was used to detect differentially abundant features of the mucin-enriched
12 bacterial community⁵⁰. All fastq files are available online at the NIH Sequence Read Archive
13 (<http://www.ncbi.nlm.nih.gov/bioproject>) under BioProject accession number SRP067035.

14 ***In vitro* cross-feeding.** A saliva-derived mucin-fermenting bacterial community (Supplementary
15 Figure 1) was used to inoculate 2 mL of mucin (15g/L) minimal medium in molten 1% agar at
16 50°C in a glass culture tube under anoxic conditions (Fig. 2A). Upon solidification, an additional
17 2 mL of molten minimal medium agar (without mucin) was inoculated with a 1/10000 dilution of
18 an overnight culture of *P. aeruginosa* (or other strains where specified). Samples were then
19 poured over the mucin fermenting community. Tubes without mucin-fermenters were used as
20 controls. After solidification, co-cultures were placed at 37°C for 65 hours. Agar plugs were then
21 removed from the upper phase, sectioned and normalized by weight, followed by
22 homogenization by pipetting in 1 mL of phosphate buffered saline. Colony forming units were
23 determined by serial dilution and plating on LB agar.

1 **Metabolite quantification.** Targeted quantification of mucin fermentation byproducts (acetate,
2 butyrate, citrate, formate, isobutyrate, isovalerate, ketobutyrate, ketoisovalerate, lactate, 2-
3 methylbutyrate, propionate, succinate and valerate) was performed via high performance liquid
4 chromatography (GC-MS). The system consisted of a Shimadzu SCL-10A system controller,
5 LC-10AT liquid chromatograph, SIL-10AF autoinjector, SPD-10A UV-Vis detector, and CTO-
6 10A column oven. Separation of compounds was performed with an Aminex HPX-87H guard
7 column and an HPX-87H cation-exchange column (Bio-Rad [Hercules, CA]). The mobile phase
8 consisted of 0.05 N H₂SO₄, set at a flow rate of 0.5 ml min⁻¹. The column was maintained at
9 50°C and the injection volume was 50 µl. Amino acid and metabolite (acetate and propionate)
10 quantification from enrichment supernatants were performed by Millis Scientific, Inc. (Baltimore,
11 MD) using liquid chromatography-mass spectrometry and gas chromatography-mass
12 spectrometry (GC-MS). Samples for amino acid quantification were spiked with 1 µl of
13 uniformly labeled amino acids (Cambridge Isotope Labs) and derivatized using AccQ-Tag
14 reagent (Waters Corp.) for 10 min at 50°C. A Waters Micromass Quatro LC-MS interfaced with
15 a Waters Atlantis dC18 (3 µm 2.1x100 mm) column was used. Reverse-phase LC was used for
16 separation (mobile phases A:10mM ammonium formate in 0.5% formic acid, B:methanol) with a
17 constant flow rate (0.2 ml/min) and a column temperature of 40°C. Electrospray ionization was
18 used to generate ions in the positive mode and multiple reaction monitoring was used to quantify
19 amino acids. Samples (~100 µl) for acetate and propionate quantification were first diluted (150
20 µl water), spiked with internal standards (10 µl of 1000ppm acetate [¹³C₂] and 1000 ppm
21 propionate [¹³C₁]) and acidified using 2 µl of 12N HCl. After equilibration at 60°C for 2h,
22 carboxen/ polydimethylsiloxane solid phase microextraction (SPME) fiber was used to adsorb
23 the headspace at 60°C for 30min. Acids were then desorbed into the gas chromatograph inlet for

1 2 min. A 30 m x 0.32 mm ID DB-624 column attached to a Thermo Electron Trace gas
2 chromatograph with helium carrier gas (2.0 ml/min) was used for separation of analytes. A
3 Waters Micromass Quatro GC mass spectrometer was used for detection and quantification of
4 target ions. Significance between sputum and saliva samples were determined by paired
5 Student's t-test.

6 **RNA isolation, purification, and qRTPCR.**

7 To quantify *P. aeruginosa* gene expression *in vivo*, sputum (n=17) was expectorated into
8 RNAlater and immediately frozen to preserve the gene expression profile. Frozen sputum was
9 thawed in TriZol (Life Technologies), homogenized using ceramic beads and purified according
10 to the manufacturer's protocol. RNA was concentrated using the Clean & Concentrator kit
11 (Zymo) and de-salted using Turbo DNA-free (Life Technologies). Bacterial RNA was enriched
12 using the MicrobEnrich kit (Life Technologies), and purity was confirmed using Qubit
13 (LifeTechnologies) spectrophotometry. qRTPCR was performed as previously described⁵¹.
14 Briefly, DNA was reverse transcribed from 1 µg of total RNA using the iScript cDNA synthesis
15 kit (BioRad). cDNA was then used a template for quantitative PCR on an iQ5 thermocycler
16 (BioRad) using iTaq Universal SYBR Green Super Mix (BioRad). Triplicate measurements were
17 made on each sputum sample. For control cultures, *P. aeruginosa* was grown in 4-
18 morpholinepropanesulfonic acid (MOPS) minimal medium to an OD600 of ~0.6 supplemented
19 with glucose (12mM), acetate (20mM), propionate (20mM), or acetate + propionate (20mM
20 each) where specified. After growth, cells were harvested by centrifugation, frozen at -80°C, and
21 RNA was extracted as described above. Primer pairs are listed in Supplementary Table 2. For all
22 primer sets, the following cycling parameters were used: 94°C for 3 min followed by 40 cycles
23 of 94°C for 60s, 55°C for 45s, and 72°C for 60s. Primer efficiencies were tested for *clpX* (91.6%),
24 *acsA* (91.6%) and *prpD* (96.8%). Relative RNA values were calculated from the Ct values

1 reported and the experimental primer efficiencies, and were normalized to the expression of *clpX*.
2 *clpX* was compared to *oprI* values to ensure constitutive expression levels. Significance between
3 treatments was determined by two-tailed unpaired Student's t-test.

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9
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11
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20 **Author Contributions**

21
22 J.M.F. performed all experiments except for Illumina library generation and sequencing. D.N.
23 and J.M.D. recruited, consented, and collect samples/tissue from human subjects. R.C.H. and
24 J.M.F. analysed and processed the data. R.C.H. and J.M.F. wrote the manuscript. R.C.H.
25 supervised the project.

26

1 **Competing Interests**

2 The authors declare no competing financial interests.

3

4 **Figure Legends**

5 **Figure 1. *P. aeruginosa* growth on mucin.** (A) *P. aeruginosa* PA14 grown with mucin and
6 glucose (+/- amino acid supplement). Data are presented as mean values from four independent
7 measurements. (B) *P. aeruginosa* clinical isolates (CI46 and CI47) and PA14 grown with mucin
8 and glucose (+/- complete amino acids supplement) for 40hr. Data are shown as mean +/- SEM
9 of endpoint change in optical density (600nm)(n=4).

10 **Figure 2. Oral-derived mucin fermenters support pathogen growth using mucin as the sole**
11 **carbon source.** (A) Saliva-derived bacteria were grown anaerobically in 1% agar with mucins
12 and overlaid with an immobilized *P. aeruginosa* culture in an agar gel without mucins. After
13 60hr of growth, co-cultures (inset) containing mucin fermenting anaerobes (+) were pigmented,
14 whereas tubes lacking the fermenting inoculum (-) were not. Pigment is indicative of *P.*
15 *aeruginosa* growth. (B) Co-culture tubes containing PA14, CI46, CI47, *S. aureus*, and *A.*
16 *xylosoxidans* overlays in the presence (□) and absence (□) of mucin fermenters. All strains
17 showed significant ($p<0.01$) growth differences when cultured with a fermenting community.
18 Data are presented as mean values +/- SEM from three biological replicates.

19 **Figure 3. Mucin enrichment of sputum-derived bacterial communities.** Taxonomic analysis
20 of bacterial communities in (a) sputum and (b) mucin enrichment cultures. A total of 12
21 enrichment cultures were obtained from 14 cultures seeded with sputum; #12003 and #12021 did
22 not grow in enrichment conditions, accounting for the disparity between sample numbers Oral-
23 associated anaerobes (cross-hatching) capable of mucin fermentation are significantly enriched

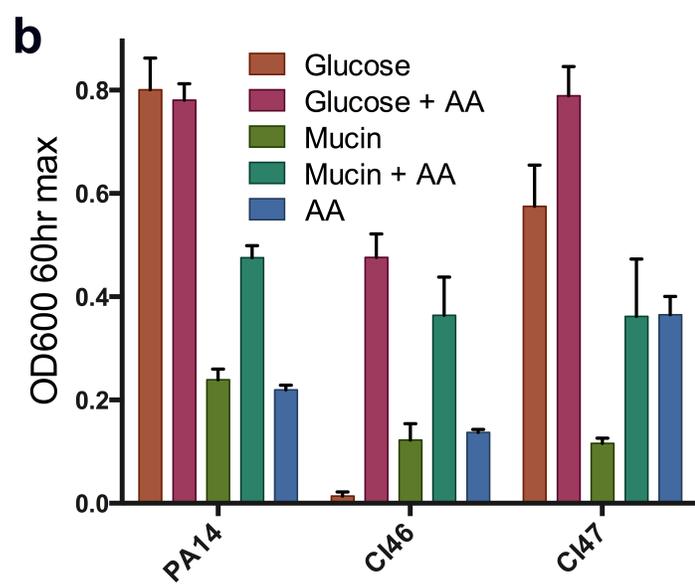
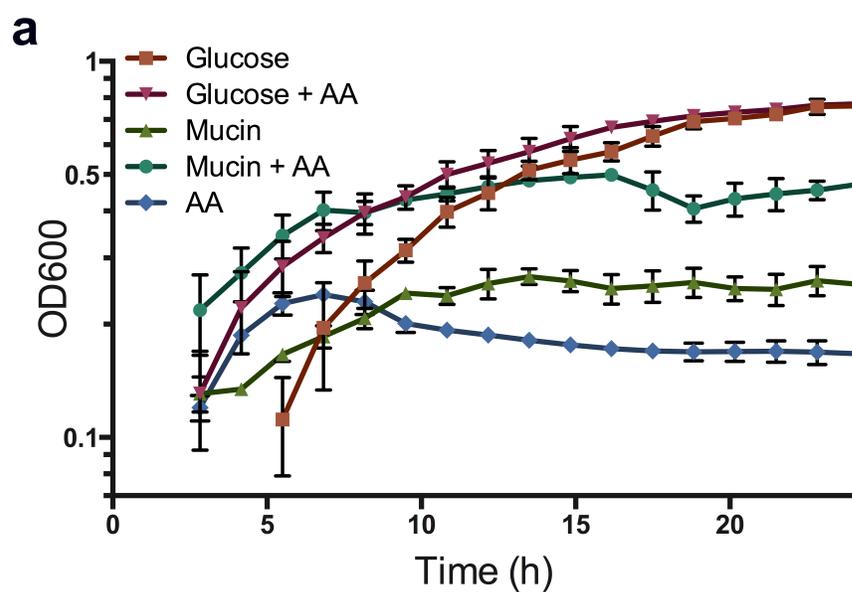
1 ($p < 0.05$) during growth on mucins under anoxic conditions. Data shown are genus-level
2 abundances.

3 **Figure 4. Mucin-enrichment metabolite profiling.** (a) short chain fatty acids (n=8) and (b)
4 amino acids (n=10) were quantified in mucin enrichment cultures. Arginine and cysteine were
5 undetectable due to their co-elution with other metabolites.

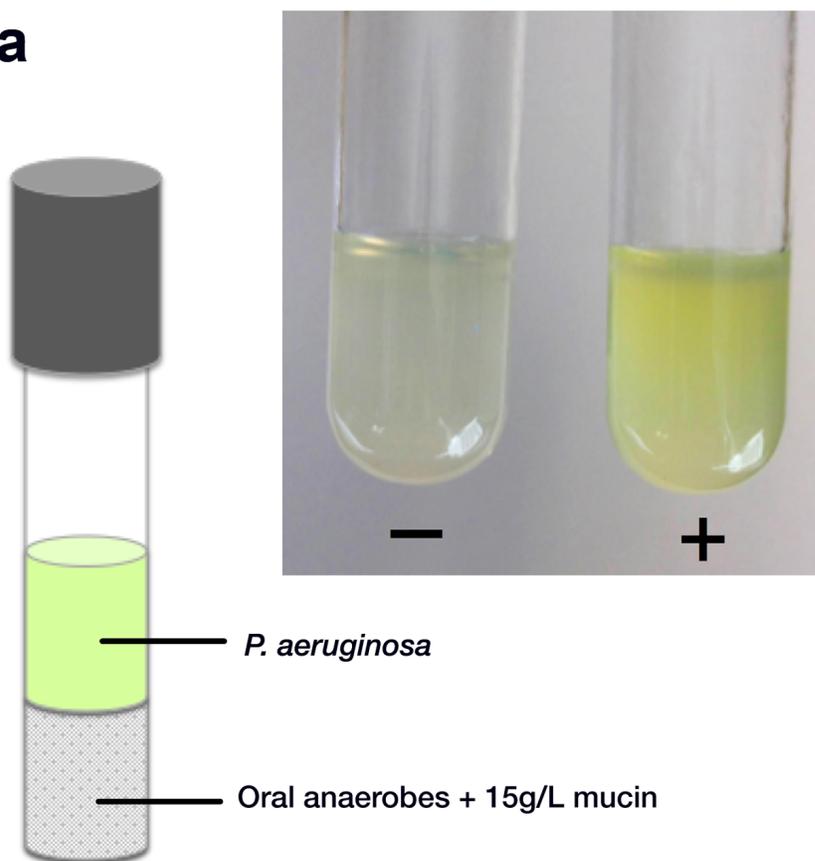
6 **Figure 5. Evidence for mucin-derived short chain fatty acid production *in vivo*.** Direct
7 measurements of (a) acetate and (b) propionate in paired sputum and saliva samples from CF
8 patients. Acetate concentrations were significantly higher in sputum ($p = 0.006$, **).

9 **Figure 6.** *In vitro* expression (left) of *acsA* (▲) and *prpD* (■) are upregulated in response to
10 acetate and propionate, respectively. *prpD* is preferentially expressed in the presence of both
11 acetate and propionate. Data shown are biological triplicate measurements for PA14. *In vivo*
12 (right), *prpD* is upregulated (5.4 fold \pm 3.1) while *acsA* is not (1.0 fold \pm 1.3 SD) ($n=17$). $p \leq$
13 0.01, **; $p \leq 0.001$, ***; $p \leq 0.0001$, ****.

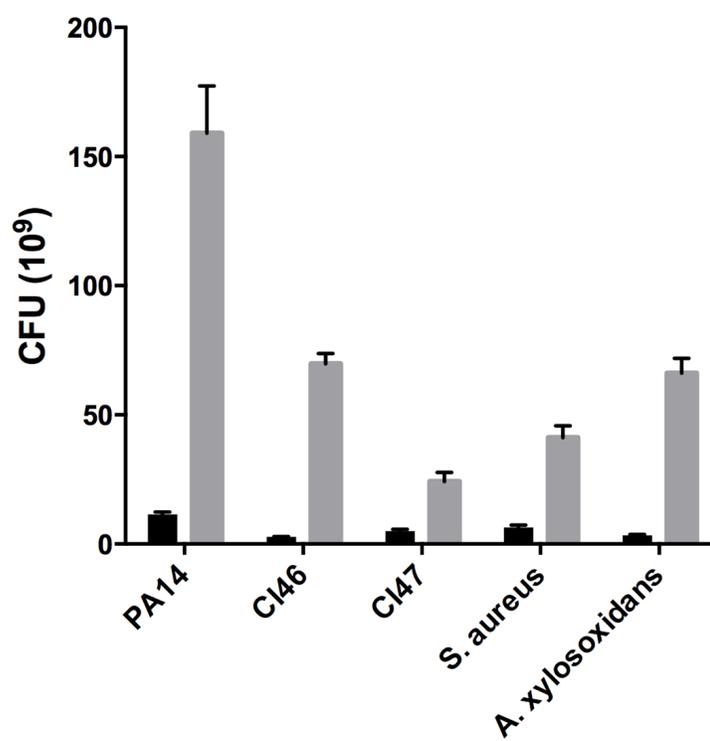
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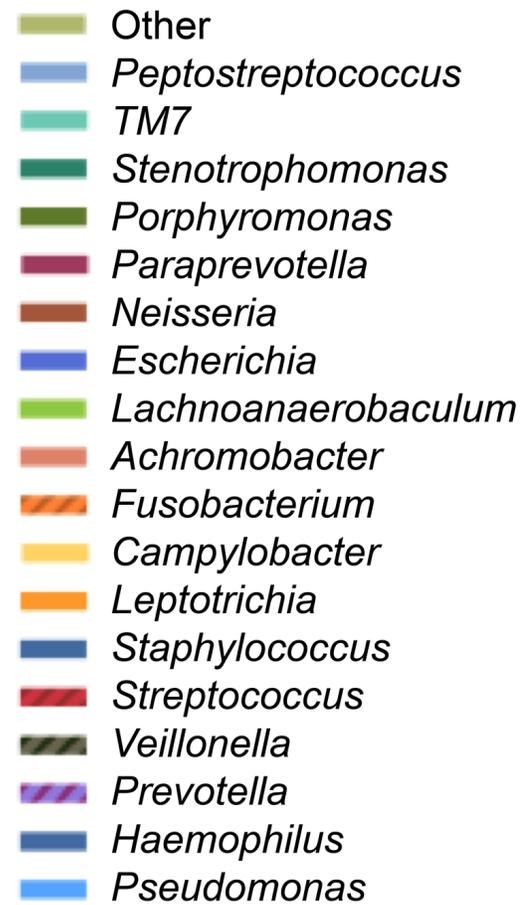
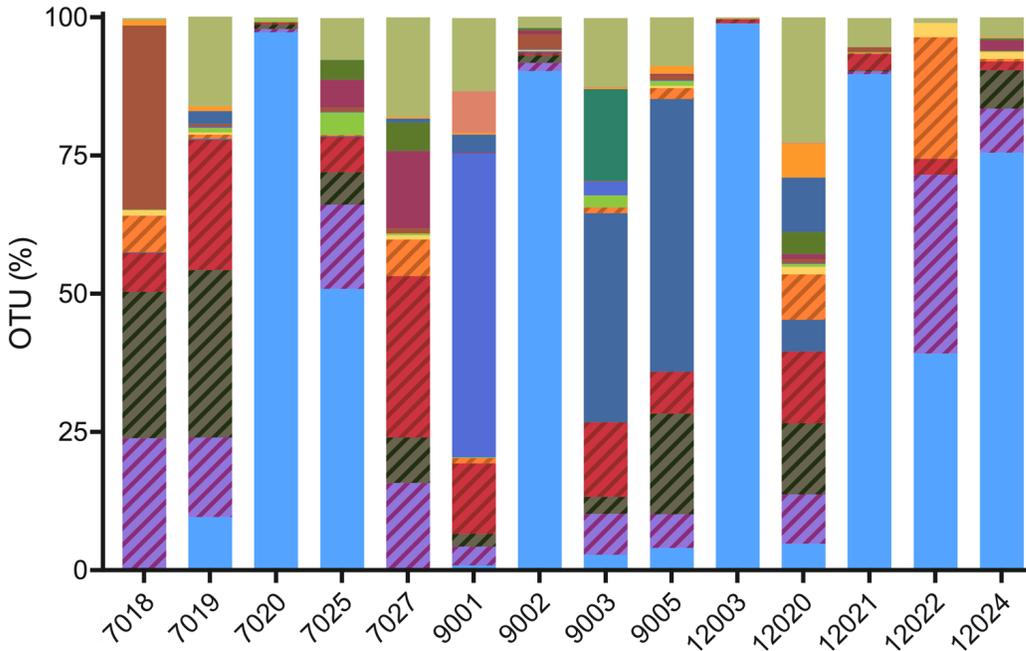
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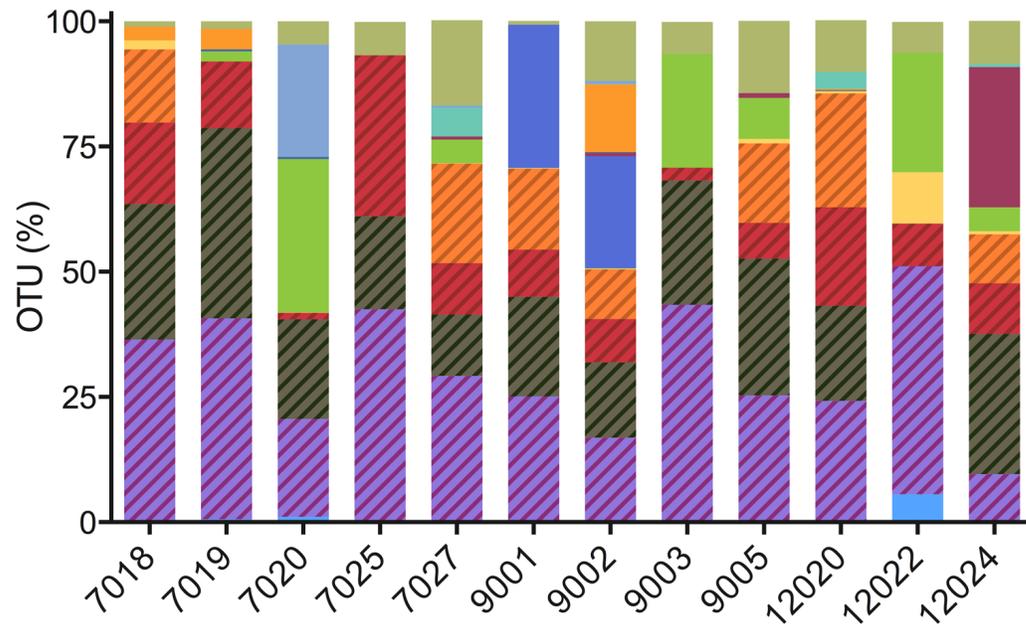
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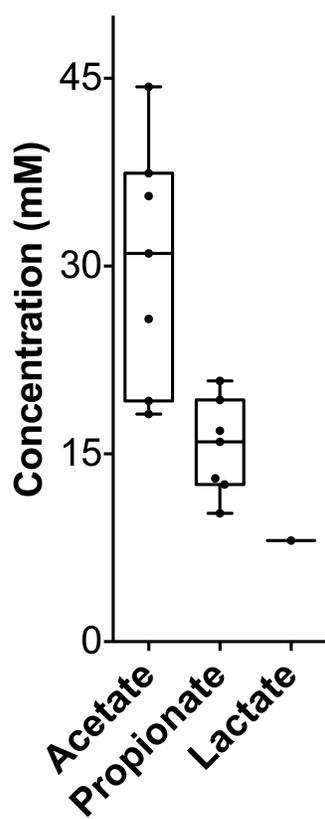
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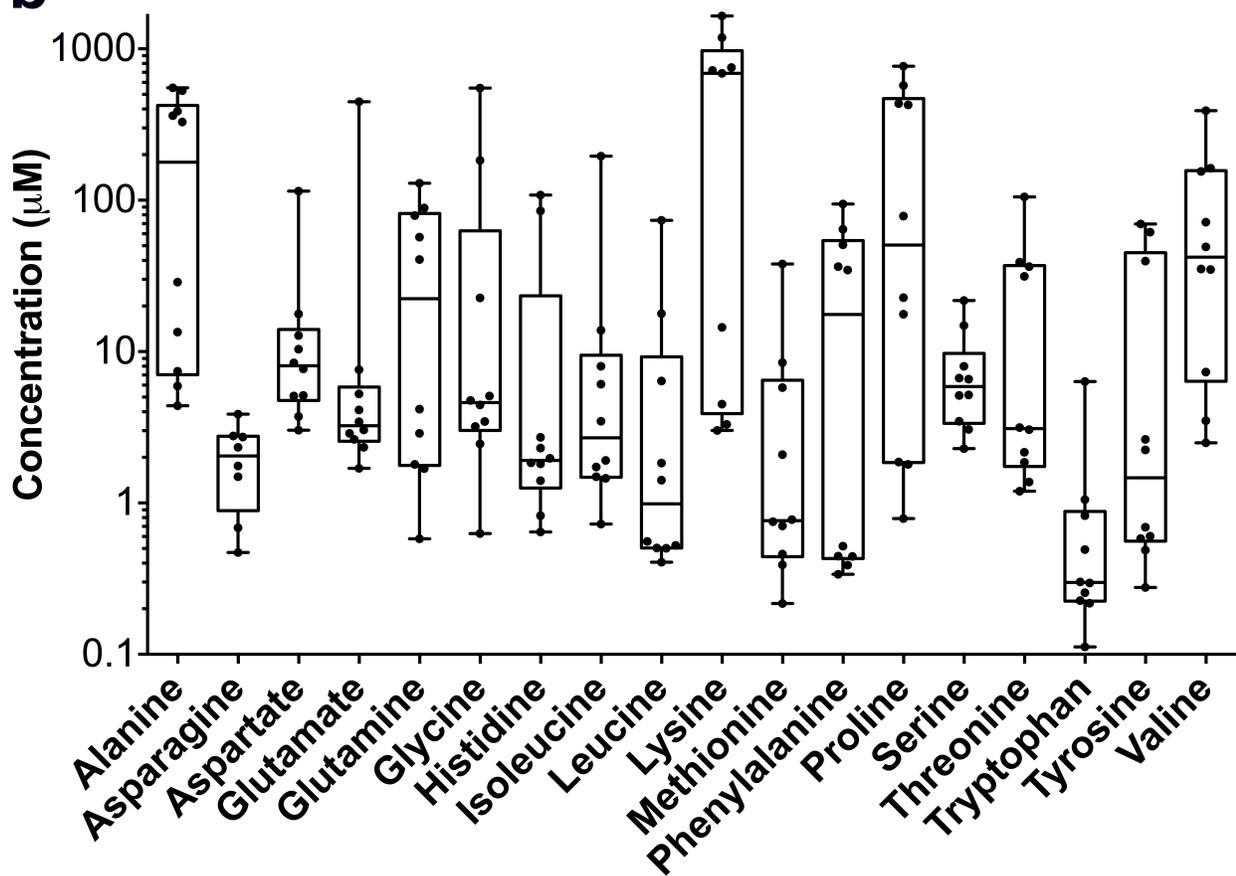
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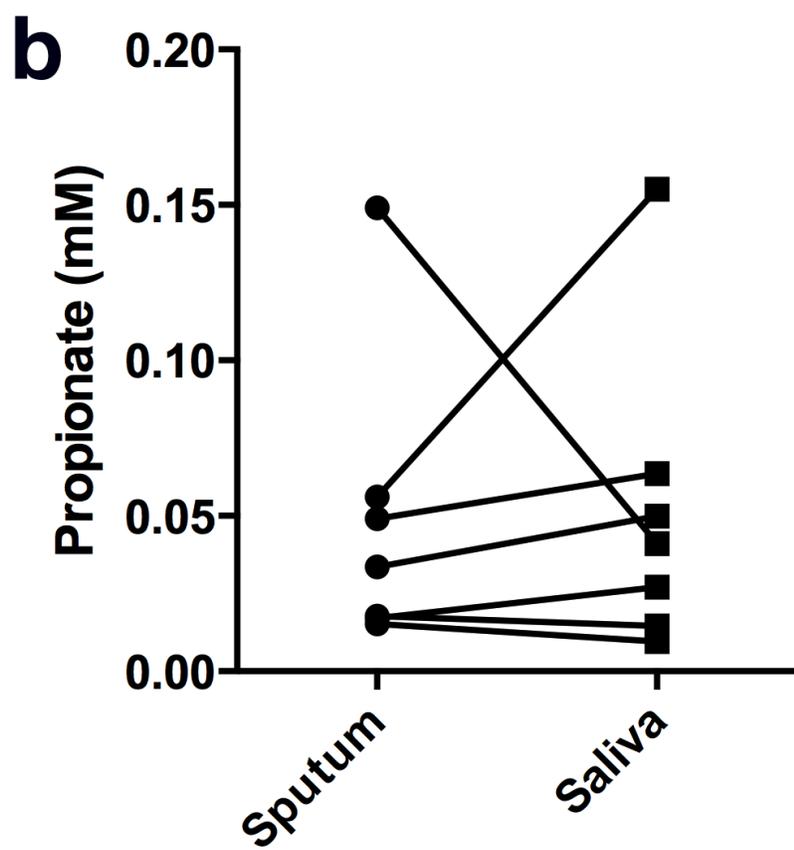
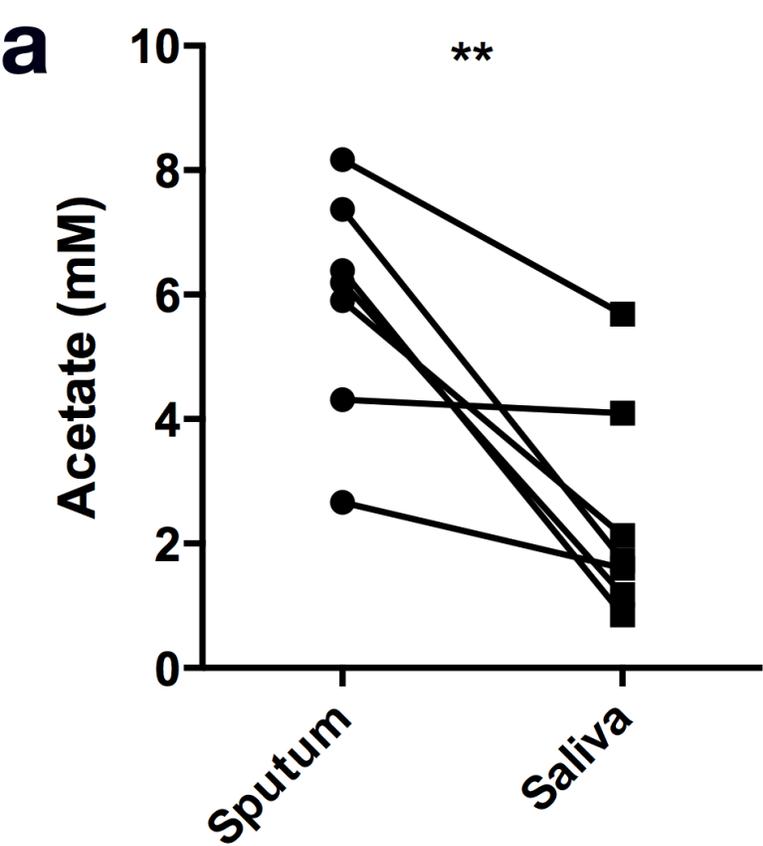


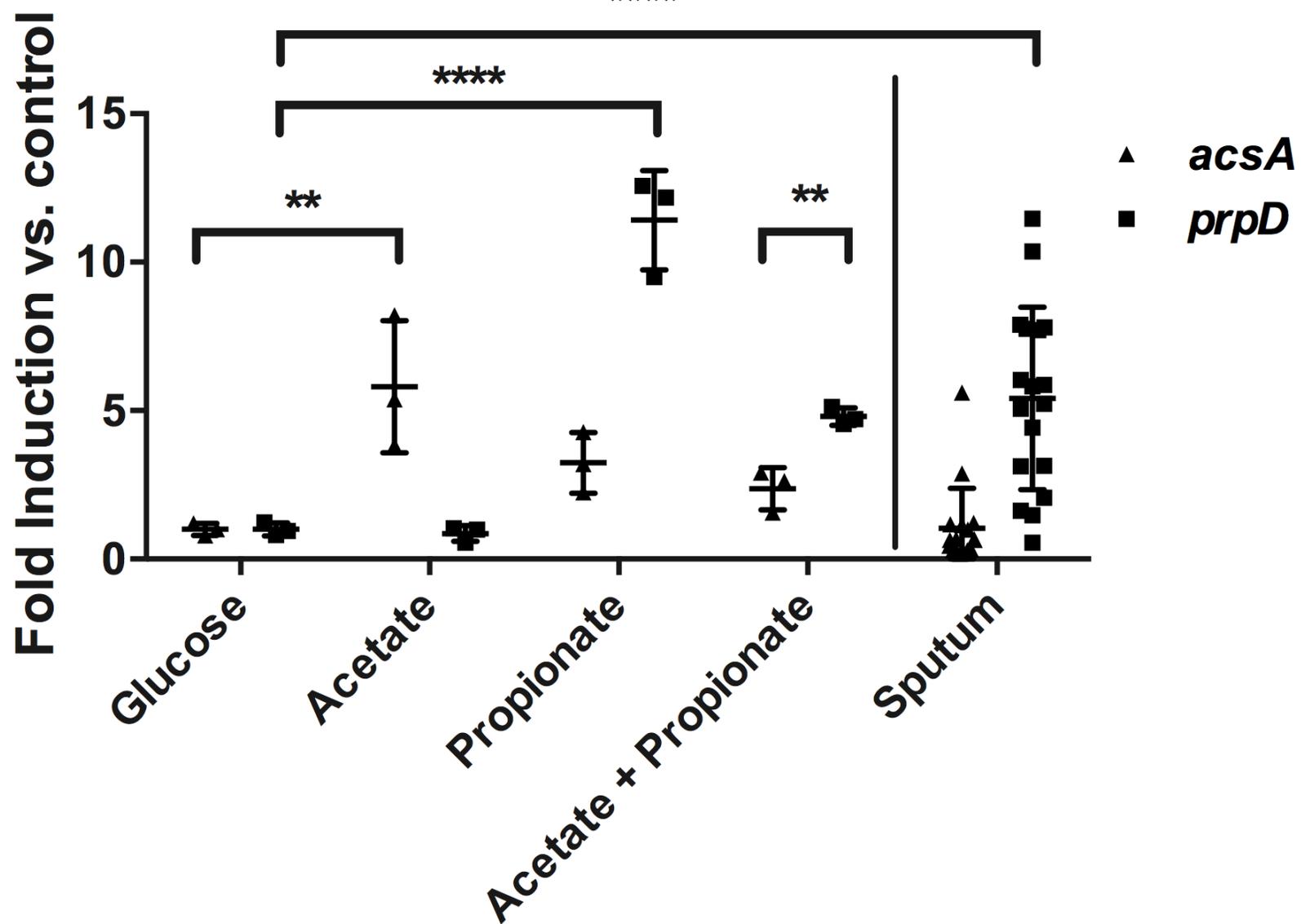
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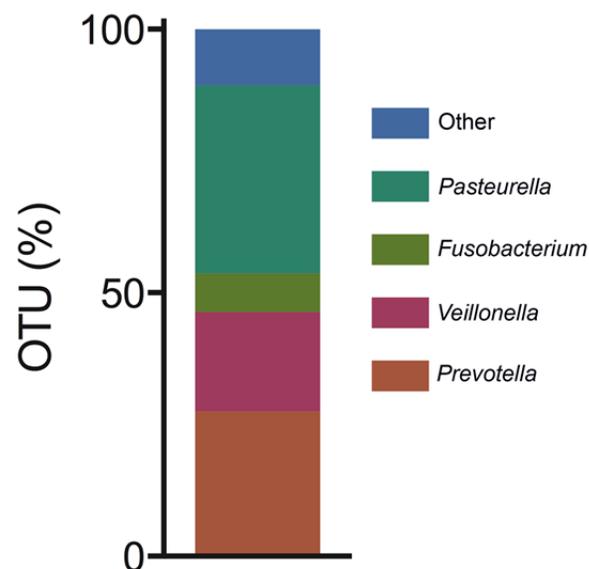


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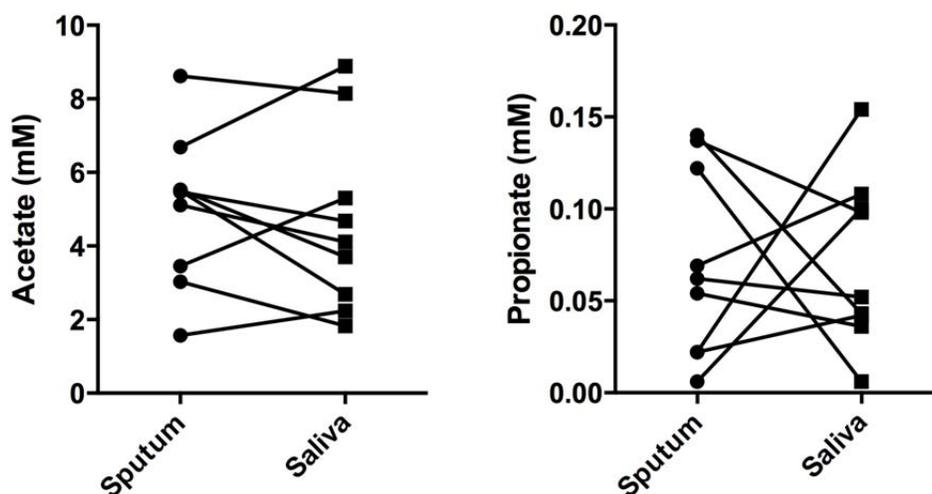








Supplementary Figure 1. Taxonomic composition of the mucin-enriched, saliva-derived bacterial community used in this study.



Supplementary Figure 2. Direct measurements of (a) acetate and (b) propionate in paired sputum and saliva samples between CF patients (n=9) without an oral rinse between sampling sites. There were no significant difference between saliva and sputum for either SCFA ($p=0.51$ and 0.98 , respectively).

Supplementary Table 1. Relative abundance of bacterial OTUs in explanted CF lungs.

Patient A

Genus^a	Right Upper	Right Middle	Right Lower	Left Upper	Left Lingula	Left Lower
<i>Pseudomonas</i>	98.87%	98.53%	97.49%	98.31%	82.75%	96.90%
<i>Streptococcus</i>	0.01%	0.05%	0.14%	0.09%	1.96%	0.03%
<i>Veillonella</i>	-	0.04%	0.10%	-	0.39%	0.45%
<i>Prevotella</i>	-	0.07%	0.12%	0.03%	2.35%	0.48%
<i>Haemophilus</i>	-	-	-	0.01%	3.14%	-
<i>Staphylococcus</i>	0.02%	0.04%	0.06%	0.08%	0.20%	0.15%
<i>Fusobacterium</i>	-	-	0.12%	0.01%	-	-

Patient B

Genus^a	Right Upper	Right Middle	Right Lower	Left Upper	Left Lingula	Left Lower
<i>Pseudomonas</i>	99.75%	99.32%	99.26%	99.88%	99.34%	86.46%
<i>Streptococcus</i>	-	0.04%	-	0.02%	-	1.61%
<i>Veillonella</i>	-	0.06%	0.09%	-	0.02%	3.83%
<i>Prevotella</i>	-	0.01%	0.02%	-	0.01%	1.45%
<i>Haemophilus</i>	0.03%	-	0.01%	-	-	-
<i>Staphylococcus</i>	0.03%	0.01%	0.02%	-	0.04%	0.31%
<i>Fusobacterium</i>	-	-	0.01%	-	-	0.99%

Patient C

Genus^a	Right Upper	Right Middle	Right Lower	Left Upper	Left Lingula	Left Lower
<i>Pseudomonas</i>	99.89%	99.87%	99.88%	99.82%	99.86%	99.86
<i>Streptococcus</i>	-	0.01%	-	0.01%	-	-
<i>Veillonella</i>	0.01%	-	-	0.05%	-	0.01%
<i>Prevotella</i>	0.01%	-	-	0.02%	-	-
<i>Haemophilus</i>	-	-	-	-	-	-
<i>Staphylococcus</i>	0.01%	-	-	-	-	-
<i>Fusobacterium</i>	-	-	-	-	-	-

^a Shown are the three most abundant known lung pathogens (*Pseudomonas*, *Haemophilus*, *Staphylococcus*) and the percentage of known mucin-degrading anaerobes (*Prevotella*, *Streptococcus*, *Veillonella* and *Fusobacterium*)

Supplementary Table 2. Oligonucleotide primers used in this study.

qRT-PCR oligonucleotide primers			
Target Gene	Primer	Sequence (5'-3')	Primer Efficiency %
<i>clpX</i>	<i>clpX</i> F	CCTGTGCAATGACATCATCC	91.6
	<i>clpX</i> R	TTCTTGTCACGCTGGTTGAG	
<i>oprI</i>	<i>oprI</i> F	TGCGATCACCACCTTCTACT	
	<i>oprI</i> R	GCCCAGAGCCATGTTGTAC	
<i>prpD</i>	<i>prpD</i> F	GCAGTGGGGTTTCTACGATG	96.8
	<i>prpD</i> R	CTTGAACAGCACGTTTTCCA	
<i>acsA</i>	<i>acsA</i> F	TGTTCCGAGGGCGTACCGAACT	91.6
	<i>acsA</i> R	AGCAGACGCAGGCTGGAA	
16S gene sequencing indexing primers			
Amplification Step	Primer	Sequence (5'-3')	
Primary	V3F Nextera	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCTACGGGAGGCAG CAG	
	V5R Nextera	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCGTCAATTCMT TTRAGT	
Indexing	Index F	AATGATACGGCGACCACCGAGATCTACACXXXXXXXXTCGTCGGCA GCGTC ^a	
	Index R	GATXXXXXXXXGTCTCGTGGGCTCGG ^a	

^a X marks the position of 8bp indices