Can Germs Help?

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biological diversity and the importance of microbes

— most genetic diversity in the tree of life occurs among microbes
— this diversity exists in polymicrobial communities, and is difficult to access (dark matter)
  — < 1% of microbes have been grown in the lab
— microbes run Earth’s biogeochemical cycles, and outnumber human cells in the body
  — much of the human microbiome remains uncultivated
— cultivation-independent techniques like metagenomics and single-cell approaches are needed to study microbial systems
— work on microbes in the lung has focused on pathogens cultured from states of disease
— cystic fibrosis (CF) is a common autosomal recessive disorder (30,000 patients in US)
  — caused by mutations in the gene encoding the CF transmembrane conductance regulator (CFTR)
  — among the manifestations of CF, mucociliary clearance is disrupted, leading to chronic lung infection
    with microbial biofilms
— recently, metagenomic studies identified complex microbial communities in the CF lung
  — what is the health impact of these organisms?
Bacteria in lungs of CF patients by age

Germs Found in the Lungs of People with CF by Age, 2012

*P. aeruginosa includes people with MDR-PA.
**MDR-PA is multi-drug resistant Pseudomonas aeruginosa (P. aeruginosa).
\( S. \text{ aureus} \) includes people with MRSA.
\( MRSA \) is methicillin-resistant Staphylococcus aureus (S. aureus).

CFF Registry, 2012
Paradigms of Pulmonary Infection

conventional paradigm of microbial ecology in CF

healthy

CF-early life/stable

CF-later life/exacerbated

pathogenesis
Paradigms of Pulmonary Infection

Does a healthy pulmonary microbiome exist?
- clinical cultures for pathogens often negative, (although reports of bacterial rRNA sequences exist)
- Human Microbiome Project: no pulmonary sampling

- Are we missing the big picture in microbial ecology of the lung?

“. . . but virtually nothing is known about the microbiome of the lung. Indeed, the presence of a lung microbiome in normal individuals has yet to be established conclusively.”
- abstract of a recently funded NIH R01
Lung Study: subject and sample characteristics

Deeply probe pulmonary microbial population using 16S ribosomal gene sequencing
— 454 DNA pyrosequencing of the V4 region, \( 10^3 \) – \( 10^5 \) sequences per subject
— each 16S read serves as a phylogenetic ‘name tag’ for the source organism

Induced sputum collected from 9 healthy control individuals (CT) & 16 CF patients

<table>
<thead>
<tr>
<th>Table 1. Summary of CF subject characteristics.</th>
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<tbody>
<tr>
<td><strong>Age in years (mean ± SD)</strong></td>
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<td><strong>CFTR genotype Δ/Δ</strong></td>
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<td>Δ/other</td>
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<tr>
<td>Other/Other</td>
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<tr>
<td><strong>Sweat Chloride (mmol/L)</strong></td>
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— Methodology:
— DNA extraction: bead beating, enzymatic digestion, DNeasy
— prep PCR with barcoded 454 fusion primers (454A-barcode-515F, 454B-1391R)
— quantification by the ultrasensitive and accurate digital PCR method*
— classified quality-filtered sequences using Ribosomal Database Project tool

CF study: Blainey, Milla, Cornfield, & Quake, Science Translational Medicine 2012
Phylum-level classification

- CF cohort not dominated by cultured pathogens (mostly Proteobacteria)
  - (Pseudomonas, Haemophilus, Burkholderia)
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- CF cohort not dominated by cultured pathogens (mostly Proteobacteria)
  - (Pseudomonas, Haemophilus, Burkholderia)
- healthy individuals harbor a complex, endemic, pulmonary microbiome
- control cohort appears richer, more even
- control enriched for Bacteriodetes ($p = 0.04^*$)
- CF enriched for Actinobacteria ($p = 0.01^*$)

*p-values are Bonferroni-corrected for multiple testing

Blainey, Milla, Cornfield, & Quake, Science Translational Medicine 2012
Cohort analysis: Phylum level

- Higher diversity at phylum level in healthy control cohort
- Greater abundance of ‘dark matter’ (uncultivated organisms) in healthy control cohort
  - uncultured phyla are enriched TM7 ($p = 0.01^*$), and SR-1 ($p < 0.001^*$)

* $p$-values are Bonferroni-corrected for multiple testing

personal pulmonary microbiomes

- Both cohorts show strong inter-individual variability—how to look for characteristic patterns?
  - PCA transforms high-dimensional data to a new set of coordinates
    - allows representation of the dataset variation in a smaller number of dimensions
    - reveals hidden or composite control variables

100 bootstraps of 1000 sequences are plotted for each subject to sample error distribution.

principal components analysis (PCA) of microbial family abundances (96% seqs classified with confidence >80%)

- CF cluster holds in PC1-PC3
- microbial ‘signature’ of CF microbiome identified, despite inter-individual variation
- healthy subjects are more diverse on an individual bases, AND more distinct from one another

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100 bootstraps of 1000 sequences are plotted for each sample to show statistical placement margins

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dark matter index = 1
families with cultured ‘CF pathogens’
CF-associated families lacking known CF pathogens

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Table 1. Summary of CF subject characteristics.

| Characteristic                            | Mean ± SD       | Percentage (%) of Predicted
|-------------------------------------------|-----------------|----------------------------
| Age in years (mean ± SD)                  | 28.14 ± 7.23    | 85.65 ± 8.9                |
| Gender                                    | Male: Female: 10:6 |                           |
| FVC (mean ± SD) Liters                    | 3.88 ± 0.60     | 71.89 ± 12.70              |
| FEV1 (mean ± SD) Liters                   | 2.72 ± 0.54     |                            |
| CFTR genotype                              | Δ/Δ Δ/other Other/Other | 7:4:2                     |
| Sweat Chloride (mmol/L)                    | 97.40 ± 29.29   |                            |
| Chronic Antibiotic Therapy (not mutually exclusive) |                 |                            |
| Oral Azithromycin                          | 9/15            |                            |
| Inhaled Tobramycin                         | 8/15            |                            |
| Inhaled Colistin                           | 5/15            |                            |
| Oral Dicloxacillin                         | 1/15            |                            |
| Oral Levofloxacin                          | 1/15            |                            |
| No Antibiotic                              | 3/15            |                            |

CF pulmonary microbiome/clinical correlates

• microbial diversity declines over life course of CF patients (Lynch et al, Pone, 2010)
• within our adult cohort, phylum-level diversity correlates significantly with inflammation, decreased diversity, increased inflammation
  – not with pathogens, antibiotic treatment status, or patient age
  – this association suggests a possible link between microbial ecology and etiology of CF
  ⇒ use as diagnostic/prognostic tool?
• healthy pulmonary microbiome more diverse than that CF pulmonary microbiomes
Part I: conclusions

• contrary to conventional wisdom, each of us has a complex & personal pulmonary microbiome
  – biological dark matter is abundant in healthy and CF lungs
• a focused microbial ‘signature’ of CF exists, characterized by a patterns of microbial diversity
  – new targets for antimicrobials
  – new candidate taxa for probiotic therapy
• microbial diversity is associated with inflammation in our adult cohort
future work: investigating *Rothia*-*Pseudomonas* interaction

- *Rothia* species seem to be overlooked players in CF (abundant & disease-associated)
- significant negative correlation between *Rothia* and *Pseudomonas* ($r = -0.56$)
- cultured *Rothia* and *Pseudomonas* species are known to form biofilms
  - parse competition between these organisms using microscopy and RNA-sequencing