Letters to the Editor

Effect of Sample Storage Conditions on Culture-Independent Bacterial Community Measures in Cystic Fibrosis Sputum Specimens[▽]

Culture-independent approaches to studying the microbiota of the airways of cystic fibrosis (CF) patients have employed analysis of the bacterial 16S rRNA gene through Sanger sequencing of clone libraries (5, 6, 12), terminal restriction fragment length polymorphism analysis (8, 11), microarray hybridization (3, 7), or pyrosequencing (2, 5) and have revealed the presence of far more complex bacterial communities than has been appreciated with culture-based methods. Expectorated sputum has been used in many of these studies and, when collected properly, is estimated to contain a relatively low abundance of bacteria from the oropharynx (9). However, sputum samples typically require transportation and/or storage before culture-independent analysis. We sought to assess the effects of sputum sample storage conditions on bacterial community measures obtained by using pyrosequencing analysis.

A sputum specimen, obtained with the approval of the University of Michigan Institutional Review Board during the course of routine care of a 23-year-old man with CF, was stored at 4°C for 2 h and then divided into multiple 0.5-ml aliquots. Aliquots were either immediately frozen at −80°C or stored at room temperature (RT) (~25°C), 4°C, or −20°C for 1, 2, or 4 weeks before being transferred to −80°C. To isolate DNA, sputum aliquots were thawed on ice, treated with Sputolysin (EMD Chemicals, San Diego, CA), mechanically disrupted, and processed on an automated nucleic acid purification platform (MagNA Pure compact system; Roche Diagnostics, Indianapolis, IN). Bar-coded pyrosequencing of the 16S rRNA V3-V5 region was performed using protocols developed for the Human Microbiome Project (http://www.hmpdacc.org/tools_protocols/tools_protocols.php). The software package mothur V1.19 was used to process sequences and calculate alpha and beta diversities (http://www.mothur.org/wiki/Costello_stool_analysis) (10).

Pyrosequencing with the Roche 454 Titanium platform produced an average of 4,988 sequences (range, 2,625 to 7,241) and 15 operational taxonomic units (OTUs) (range, 10 to 20) per specimen. To compare bacterial community structures, Bray-Curtis distances were calculated using normalized and $\log_2$-transformed abundance data for each OTU from individual samples and assessed with principal coordinate analysis (PCoA) (4). Based on nonparametric analysis of molecular variance (NP-AMOVA), the communities in the six samples stored at RT were significantly different ($P < 0.001$) (1) from the 17 samples stored at other temperatures, which showed insignificant variation (Fig. 1A). For each of the temperatures studied, community structure was not influenced by storage time ($P = 0.65$, NP-AMOVA). By two-way analysis of variance (ANOVA), community diversity and richness were not significantly different between samples from different storage temperatures or durations ($P > 0.05$). However, sequences representing *Pseudomonas aeruginosa* increased significantly ($P < 0.05$), while sequences representing *Gemella haemolysans*, *Rothia dentocariosa*, and *Lactococcus lactis* decreased significantly ($P < 0.05$), in samples stored at RT compared to those stored at all other temperatures, irrespective of storage duration. Despite the variation in community structure observed in
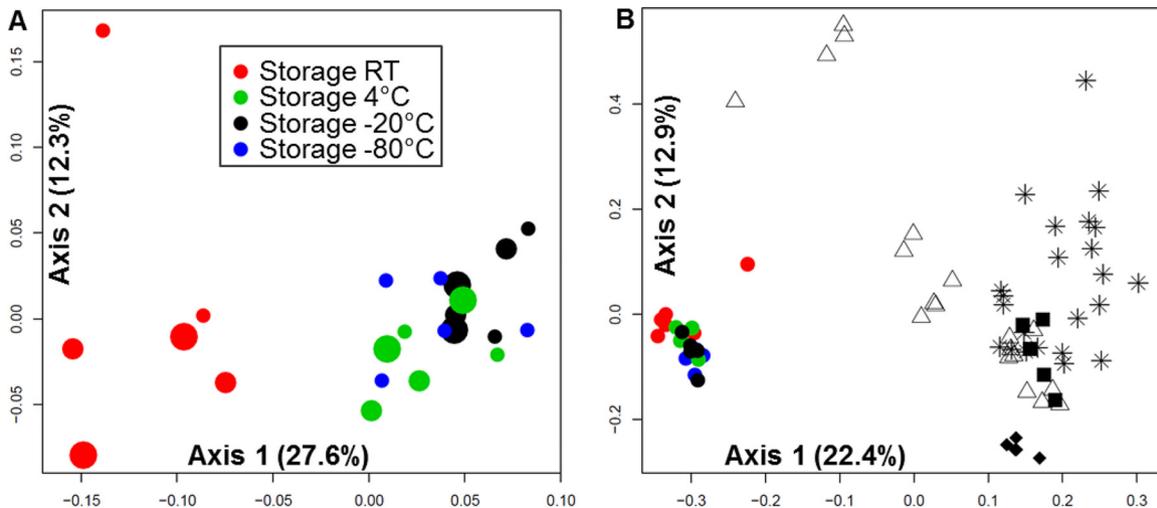
FIG. 1. Principal coordinate analysis (PCoA) of sputum bacterial community structures using $\log_2$-transformed Bray-Curtis distances. Each symbol represents a sputum sample. The distance between symbols on the ordination plot indicates the relative similarity in their community structures. (A) PCoA of the 23 sputum storage aliquots. Two aliquots each were stored at RT (red), 4°C (green), or −20°C (black) for each of the storage times indicated by small (1 week), medium (2 weeks), or large (4 weeks) circles. Blue circles represent samples stored immediately at −80°C ($n = 5$). (B) PCoA of 77 sputum samples, including the 23 storage test aliquots (colored circles in lower left), the 5 interrun control samples (solid black squares), and the 44 serial sputum samples from two other CF patients (stars and open triangles). For the interrun controls, a single specimen was analyzed in five separate pyrosequencing runs using the same barcoded primer. For the intrarun controls, five aliquots from a single sample were analyzed on the same pyrosequencing run.

samples stored at RT, the overall variation within the test samples was comparable to that found within five interrun and five intrarun control samples (Fig. 1B).

To put these results in the context of CF lung bacterial community dynamics, communities from the storage aliquots were compared to two sets of sputum samples serially collected from two adult CF patients during the course of 9 and 8 years, respectively. PCoA using $\log_2$-transformed Bray-Curtis distances indicated that the bacterial communities from the CF patient samples were widely distributed throughout the ordination plot relative to the storage test samples and control samples (Fig. 1B).

In summary, while sputum samples stored at RT showed greater variation in community structure than samples stored at the other temperatures tested, the overall variation within the storage test samples was comparable to that found within the intra- and interrun control samples. Importantly, these variations were insignificant relative to those found among samples serially obtained from CF patients during prolonged periods of observation, suggesting that storage at any of these temperatures, at least for the durations of time included in this study, does not significantly affect pyrosequencing-based measures of community structure. The variation observed in intra- and interrun controls illustrates the limits of reproducibility of pyrosequencing-based bacterial community profiling. This limitation warrants further study and must be taken into account in studies designed to assess the fine-scale dynamics of the CF lung microbiota.

REFERENCES

1. **Anderson, M. J.** 2001. A new method for non-parametric multivariate analysis of variance. Austral Ecol. **26:**32–46.
2. **Armougom, F., et al.** 2009. Microbial diversity in the sputum of a cystic fibrosis patient studied with 16S rDNA pyrosequencing. Eur. J. Clin. Microbiol. Infect. Dis. **28:**1151–1154.
3. **Cox, M. J., et al.** 2010. Airway microbiota and pathogen abundance in age-stratified cystic fibrosis patients. PLoS One **5:**e11044.
4. **Dethlefsen, L., and D. A. Relman.** 2011. Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. Proc. Natl. Acad. Sci. U. S. A. **108**(Suppl. 1)**:**4554–4561.
5. **Guss, A. M., et al.** 2011. Phylogenetic and metabolic diversity of bacteria associated with cystic fibrosis. ISME J. **5:**20–29.
6. **Harris, J. K., et al.** 2007. Molecular identification of bacteria in bronchoalveolar lavage fluid from children with cystic fibrosis. Proc. Natl. Acad. Sci. U. S. A. **104:**20529–20533.
7. **Klepac-Ceraj, V., et al.** 2010. Relationship between cystic fibrosis respiratory tract bacterial communities and age, genotype, antibiotics and Pseudomonas aeruginosa. Environ. Microbiol. **12:**1293–1303.
8. **Rogers, G. B., et al.** 2004. Characterization of bacterial community diversity in cystic fibrosis lung infections by use of 16s ribosomal DNA terminal restriction fragment length polymorphism profiling. J. Clin. Microbiol. **42:**5176–5183.
9. **Rogers, G. B., et al.** 2006. Use of 16S rRNA gene profiling by terminal restriction fragment length polymorphism analysis to compare bacterial communities in sputum and mouthwash samples from patients with cystic fibrosis. J. Clin. Microbiol. **44:**2601–2604.
10. **Schloss, P. D., et al.** 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl. Environ. Microbiol. **75:**7537–7541.
11. **Sibley, C. D., et al.** 2008. A polymicrobial perspective of pulmonary infections exposes an enigmatic pathogen in cystic fibrosis patients. Proc. Natl. Acad. Sci. U. S. A. **105:**15070–15075.
12. **van der Gast, C. J., et al.** 2011. Partitioning core and satellite taxa from within cystic fibrosis lung bacterial communities. ISME J. **5:**780–791.

Jiangchao Zhao
Department of Pediatrics and Communicable Disease
University of Michigan Medical School
Ann Arbor, Michigan

Jun Li
Department of Human Genetics
University of Michigan Medical School
Ann Arbor, Michigan

Patrick D. Schloss
Department of Microbiology and Immunology
University of Michigan Medical School
Ann Arbor, Michigan

Linda M. Kalikin
Tracy A. Raymond
Department of Pediatrics and Communicable Disease
University of Michigan Medical School
Ann Arbor, Michigan

Joseph F. Petrosino
Human Genome Sequencing Center
Department of Molecular Virology and Microbiology
Baylor College of Medicine
Houston, Texas

Vincent B. Young
Department of Internal Medicine
University of Michigan Medical School
Ann Arbor, Michigan

John J. LiPuma*
Department of Pediatrics and Communicable Disease
University of Michigan Medical School
8323 MSRB III
1150 W. Medical Center Dr.
Ann Arbor, Michigan 48109-5646

*Phone: (734) 615-5909
Fax: (734) 764-4279
E-mail: jlipuma@umich.edu