The status of the microbial census: an update

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Abstract

Abstract goes here.

Importance

Importance goes here.

Introduction

The effort to quantify the number of different organisms in a system remains fundamental to understanding ecology (**???**). At the scale of microorganisms, small physical sizes, morphological ambiguity, and highly variable population sizes complicate this process. Furthermore, creating standards for delimiting what makes one microbe "different" from another has been contentious (**???**). In spite of these challenges, we continue to peel back the curtain on the microbial world with the aid of more and more informative, if still limited, technologies like cultivation, 16S rRNA gene surveys, single cell technologies, and metagenomics.

Generating a comprehensive understanding of any system with a single gene may seem a fool's errand, yet we have learned a considerable amount regarding the diversity, dynamics, and natural history of microorganisms using the venerable 16S rRNA gene. In 1983, the full-length 16S rRNA gene sequence of *Escherichia coli* (accession J01695) was deposited into NCBI's GenBank making it the first of what is now more than 10 million 16S rRNA gene sequences to be deposited into the database (1). 16S rRNA gene accessions represent nearly one-third of all sequences deposited in GenBank, making it the best-represented gene. As Sanger sequencing has given way to so-called "next generation sequencing" technologies, hundreds of millions of 16S rRNA gene sequences have been deposited into the NCBI's Sequence Read Archive. The expansion in sequencing throughput and increased access to sequencing technology has allowed for more environments to be sequenced at a deeper coverage, resulting in the identification of novel taxa. The ability to obtain sequence data from microorganisms without cultivation has radically altered our perspective of their role in nearly every environment from deep ocean sediment cores (e.g. accession AY436526) to the International Space Station (e.g. accession DQ497748).

Previously, Schloss and Handelsman (2) assigned the then available 56,215 partial 16S rRNA gene sequences to operational taxonomic units (OTUs) that were available in the Ribosomal Database Project and concluded that the sampling methods of the time were insufficient to identify the previously estimated 10^7 to 10^9 different species (3). That census called for a broader and deeper characterization of all environments. Refreshingly, this challenge was largely met. There have been major investments in studying the Earth's microbiome using 16S rRNA gene sequencing through

initiatives such as the Human Microbiome Project (**???**), the Earth Microbiome Project (**???**), and the International Census of Marine Microorganisms. But most importantly, the original census was performed on the cusp of radical developments in sequencing technologies. That advancement has moved the generation of sequencing throughput from large sequencing centers to individual investigators and leveraged their diverse interests to expand the representation of organisms and environments represented in public databases.

Disconcertingly, the increase in sequencing volume has come at the cost of sequence length. The commonly used MiSeq-based sequencing platform from Illumina is extensively leveraged to sequence the approximately 250 bp V4 hypervariable region of the 16S rRNA gene; other schemes have used different parts of the gene that are generally shorter than 500 bp. Youssef et al. (2009) demonstrated high variability between the number of OTUs that different hypervariable sites estimated compared to full-length 16S rRNA gene sequences, and also that sample location influenced this behavior. Thus, it remains unclear to what degree richness estimates from short read technology over or underestimate the numbers from full-length sequences. We likely lack the references necessary to adequately classify the novel biodiversity we are sampling when we generate 100-times the sequence data from a community than we did using full-length sequencing.

Here we update the status of the microbial census with nearly or completely full-length 16S rRNA gene sequences. In the 13 years since the collection of data for Schloss and Handelsman's analysis, the number of full-length sequences has grown exponentially, despite the overwhelming contemporary focus by most researchers on short-read technologies. This update to the census allows us to evaluate the relative sampling thoroughness for different environments and clades, and make an argument for the continued need to collection full-length sequence data from many systems that have a long history of study. Although there has been a robust growth in the number of full-length sequences deposited to GenBank since its creation in 1983, the rate of growth has stalled over the past 5 years and the deposits have been dominated by a handful of research groups studying a limited number of environments. As researchers consider coalescing into a Unified Microbiome Initiative (5), it will be important to balance the need for mechanism-based studies with the need to generate full-length reference sequences from a diversity of environments. In this, continued technological advances, such as the reconstruction of nearly full-length sequences from

metagenomics with EMIRGE (Miller 2011) and innovations in culturing (references needed: iChip, Epstein dormancy, Thrash 2015; Gifford 2014), will be vital.

Materials and Methods

Sequence data curation. The July 19, 2015 release of the ARB-formatted SILVA small subunit (SSU) reference database (SSU Ref v.123) was downloaded from http://www.arb-silva.de/fileadmin/silva_databases/release_123/ARB_files/SSURef_123_SILVA_19_07_15_opt.arb.tgz (**???**). This release is based on the EMBL-EBI/ENA Release 123, which was released in March 2015. The SILVA curators identify potential SSU sequences using keyword searches and sequence-based search using RNAmmer (http://www.arb-silva.de/documentation/release-123/). The SILVA curators then screened the 7,168,241 resulting sequences based on a minimum length criteria (<300 nt), number of ambiguous base calls (>2%), length of sequence homopolymers (>2%), presence of vector contamination (>2%), low alignment quality value (<75), and likelihood of being chimeric (Pintail value < 50). Of the remaining sequences, the bacterial reference set retained those bacterial sequences longer than 1,200 nt and the archaeal reference set retained those archaeal sequences longer than 900 nt. The aligned 1,515,024 bacterial and 59,240 archaeal sequences were exported from the database using ARB along with the complete set of metadata. Additional sequence data was included from single-cell genomes available on the Integrated Microbial Genomes (IMG) system (**???**), many of which were recently obtained via the GEBA-MDM effort in Rinke et al. (**???**). "SCGC" was searched on the IMG database March 12, 2015 to download the bacterial (N=249) and archaeal (N=46) 16S rRNA gene sequences and their associated metadata. Further, sequences generated from amplicon and shotgun metagenomic data using the EMIRGE program were also included. These were obtained from XXXXXXXXXX. The IMG and EMIRGE sequences were aligned against the respective SILVA-based reference using mothur (**???**). The aligned bacterial and archaeal sequence sets were pooled and processed in parallel in mothur. Using mothur, sequences were further screened to remove any sequence with more than 2 ambiguous base calls and trimmed to overlap the same alignment coordinates. The sequences in the resulting bacterial dataset overlapped bases 113 through 1344 of an *E. coli* reference sequence (V00348) and had a

median length of 1,227 nt. The sequences in the resulting archaeal dataset overlapped positions 362 to 937 of a *Sulfolobus solfataricus* reference sequence (X03235) and had a median length of 580 nt. The archaeal sequences were considerably shorter than their initial length because it was necessary to find a common overlapping region across the sequences. The final datasets contained 1,412,681 bacterial and 53,618 archaeal 16S rRNA gene sequences. Sequences were assigned to OTUs using the average neighbor clustering algorithm (**???**).

Metadata curation. The metadata that was contained within the SSU Ref database was used to expand our analysis beyond a basic count of sequences and the number of OTUs in each domain. The environmental origins of the 16S rRNA gene sequences were manually classified using seven broad "coarse" categories, and further refined to facilitate additional analyses with twenty-six more specific "fine" categories (Table 1). These were assigned based on manual curation of the "isolation_source" category within the ARB database associated with each of the sequences (Table 1). For source definitions that were not identifiable by online searches, educated guesses were made or they were placed into the coarse "Other" category. There were 150,310 bacterial and 2,590 archaeal sequences where an "isolation_source" term was not collected. We ascertained whether a sequence came from a cultured organism by including those sequences that had data in their "strain" or "isolate" fields within the database and excluded any sequences that had "Unc" as part of their database name as this is a convention in the database that represents sequences from uncultured organisms. Complete tables containing the ARB-provided metadata, taxonomic information, OTU assignment, and our environmental categorizations are available at (**???**).

Calculating coverage. Sequencing coverage ($C_{Sequence}$) was quantified by two methods. The first was to use Good's coverage according to

$$C_{Sequence} = 1 - \frac{n_1}{N_t}$$

where n_1 is the number of OTUs represented by only one sequence and N_t is the total number of sequences (**???**). Although Good's coverage provides information about the success of the sequencing effort in sampling the most abundant organisms in a community, it does not directly

provide information about the success of the sequencing effort in recovering previously unobserved OTUs. To quantify the ability of sequencing to identifying novel OTUs or, in other words, to quantify the "distance" in the peak of the rarefaction curves to their hypothetical asymptote, we defined "OTU coverage" (C_{OTU}) as

$$C_{OTU} = 1 - \frac{n_1}{S_t}$$

where S_t is the total number of OTUs. Whereas Good's coverage estimates the probability that a new sequence will have already been seen, OTU coverage estimates the probability that a new OTU will match an existing one. It is therefore an extension of Good's coverage in that it quantifies the probability that, for any given set of sequences clustered into an OTU, that OTU will have already been seen. Thus, high Good's coverage means that any new sequence is unlikely to be novel, and high OTU coverage means that any new OTU is unlikely to be novel.

Data analysis. Our analysis made use of ARB (OS X v.6.0) (6), mothur (v.1.37.0) (7), and R (v.3.2.2) (8). Within R we utilized knitr (v.1.10.5) (9) and openxlsx (v. 2.4.0) (10) packages. A reproducible version of this manuscript including data extraction and processing is available at https://www.github.com/SchlossLab/Schloss_Census2_mBio_2015.

Results and Discussion

The status of the bacterial and archaeal census. To assess the field's progress in characterizing the biodiversity of bacteria and archaea we assigned each 16S rRNA gene sequence to OTUs using distance threshold varying between 0 and 20%. Although it is not possible to link a specific taxonomic level (e.g. species, genus, family, etc.) to a specific distance threshold, we selected distances of 0, 3, 5, 10, and 20% because they are widely regarded as representing the range of genetic diversity of the 16S rRNA gene within each domain. By rarefaction, it was clear that the ongoing sampling efforts have started to saturate the number of current OTUs. After sampling 1,412,681 near full-length bacterial 16S rRNA gene sequences we have

identified 239,622, 95,726, 54,268, 14,883, and 973 OTUs at the respective thresholds (Figure results/figures/domain_rarefaction.pdf A, Table 1). Using only the OTUs generated using a 3% threshold, we calculated a 95.7% Good's coverage (percent of sequences belonging to OTUs that have been observed more than once), but only 36.6% OTU coverage (percent of the OTUs that have been observed more than once). Paralleling the bacterial results, after sampling 53,618 archaeal 16S rRNA gene sequences we have identified 7,543, 4,208, 2,351, 815, and 112 OTUs (Figure results/figures/domain_rarefaction.pdf B, Table 1). Using only the OTUs generated using a 3% threshold, we calculated a 95.2% Good's coverage, but only 38.8% OTU coverage. These results indicate that regardless of the domain, continued sampling with the current strategies for generating full-length sequences will largely reveal OTUs that have already been observed, even though a large fraction of OTUs have only been sampled once. Furthermore, considering more than 63.4% of the OTUs have only been observed once, it is likely that an even larger number of OTUs have yet to be sampled for both domains.

The status of the bacterial census. One explanation for the large number of OTUs that have only been observed once is that with the the broad adoption of highly parallelized sequencing platforms that generate short sequence reads, the rate of full-length sequence generation has declined. In fact, since 2009 the number of new bacterial sequences generated has slowed two an average of 191,390 sequences per year (Figure results/figures/time_course_figure.pdf A). Although this is still an impressive number of sequences, since 2007 the number of new bacterial OTUs has plateaued at an average of 9,647 new OTUs per year (Figure results/figures/time_course_figure.pdf B). Given the expense of generating full-length sequences using the Sanger sequencing technology and the transition to other platforms at that time, we expected that the large number of sequences were being deposited by a handful of large projects. Indeed, when we counted the number of submissions responsible for depositing 50% of the sequences, we found that with the exception of 2006 and 2013, eight or fewer studies were responsible for depositing the majority of the full-length sequences each year since 2005 (Figure results/figures/time_course_figure.pdf C). Between 2009 and 2012, 904,013 total sequences were submitted and 6 submissions from 5 studies were responsible for depositing 548,274 (60.6% of all sequences). These studies generated sequences from the human gastrointestinal tract (11), human skin (12, 13), murine skin (14), and hypersaline microbial

mats (15). The heavy zoonotic focus is reflected in the rarefaction curve for this category (Figure results/figures/domain_rarefaction.pdf C). In contrast to recent years, between 1995 and 2006, an average of 39.8 studies were responsible for submitting more than half of the sequences each year. Although these deep surveys represent significant contributions to our knowledge of bacterial biogeography, their small number and lack of environmental diversity is indicative of the broader problems in advancing the bacterial census.

The status of the archaeal census. The depth of sequencing being done to advance the archaeal census has been 26-times less than that of the bacterial census (Table 1). The annual number of sequences submitted has largely paralleled that of the bacterial census with a plateau starting in 2009 and an average of 7,079.2 sequences each year since then. The number of new archaeal OTUs represented by these sequences began to slow in 2005 with an average of 359 new OTUs per year. With the exception of 2012 and 2014, the number of submissions responsible for more than 50% of the archaeal sequences submitted per year has varied between 2 and 11 submissions per year. The clear bias towards sequencing bacterial 16S rRNA genes has limited the ability to more fully characterize the biodiversity of the archaea, which is clearly reflected in the relatively meager sampling effort across habitats, compared to bacteria (Figure results/figures/domain_rarefaction D),

The ability to sample microbial life is taxonomically skewed (meh.) The Firmicutes, Proteobacteria, Actinobacteria, and Bacteroidetes represent 89.1% of the bacterial sequences and the Euryarchaeota and Thaumarchaeota 86.4% of the archaeal sequences. We sought to understand how the representation of individual phyla has changed relative to the state of the census in 2006. We used 2006 as a reference point for calibrating the dynamics of the bacterial and archaeal censuses since that was the year that the first highly parallelized 16S rRNA gene sequence dataset was published and ushered in a radical change in how microbial communities are studied (16). In 2006 there were 62 bacterial and 18 phyla. Since then there have been 4 new bacterial (CKC4, OC31, S2R-29, and SBYG-2791) and 2 new archaeal candidate phyla (Ancient Archaeal Group and TVG8AR30). Relative to the overall sequencing trends before and after 2006, several phyla stand out for being over and underrepresented in sequence submissions (Figure results/figures/phylum_effort.pdf). Among the bacterial phyla with at least 1,000 sequences, Atribacteria and Kazan-3B-09 were sequenced 4-fold more often while Deinococcus-Thermus and

Tenericutes were sequenced 2-fold less often than would have been expected since 2006. Among the archaeal phyla with at least 1,000 sequences, the Thaumarchaeota were sequenced 2.0-fold more often and the Crenarchaeota were sequenced 6.7-fold less often than expected. Together, these results demonstrate a change in the phylum-level lineages represented in the census from before and after 2006.

Focusing the census by environment We were able to assign 89.3 and 94.5% of the sequences to one of seven broad environmental categories based on the metadata that accompanied the SILVA database. Across these broad categories there was wide variation in the number of sequences that have been sampled. Among bacterial sequences, the three best represented groups were from zoonotic (N=799,542), aquatic (N=226,070), and built environment (N=106,723) sources. Among the archaeal sequences the three best represented groups were the same, but ordered differently: aquatic (N=34,434), built environment (N=7,019), and zoonotic (N=5,597) (Figure 1C,D)). For both domains, soil samples were the fourth most represented category (bacteria: 73,804; archaea: 2,521). The orders of these categories was surprising considering soil and aquatic environments harbor the most microbial biomass and biodiversity (17). In spite of wide variation in sequencing depth and coverage (Table 1), the interquartile range across the fine-level categories for the bacterial OTU coverage only varied between 31.3 to 36.6 (median coverage=33.8%). The interquartile range in the OTU coverage by environment for the archaeal data was 38.5 to51.7 (median coverage=41.9%). The archaeal coverage was higher than that of the bacterial OTU coverage for all categories except the food-associated, plant surface, and other invertebrate categories. Across all categories, the bacterial and archaeal sequencing data represented a limited number of phyla (Figure results/figures/category_phylum_heatmap.pdf). Among the bacterial data, the fine-scale categories were dominated by Proteobacteria (N=22), Firmicutes (N=4), Actinobacteria (N=1), and Bacteroidetes (N=1) and among the archaeal data, they were dominated by Euryarchaeota (N=17), Thaumarchaeota (N=10), and Aenigmarchaeota (N=1). Regardless, there were clear phylum-level signatures that differentiated the various categories. Within each of the bacterial and archaeal phyla, there was considerable variation in the relative abundance of each across the categories confirming that taxonomic signatures exist to differentiate different environments even at a broad taxonomic level.

The cultured census In the 2004 bacterial census there was great concern that although culture-independent methods were significantly enhancing our knowledge of microbial life, there were numerous bacterial phyla with no or only a few cultured representatives. To update this assessment, we identified those sequences that came from cultured and uncultured organisms. Overall, 18.6% of bacterial sequences and 6.8% of archaeal sequences have come from isolated organisms. Comparing the fraction of sequences deposited during and before 2006 from isolates to those collected after 2006, we found that culturing rates lag by 2.5 and 2.4-fold for bacteria and archaea, respectively. Among the 67 bacterial phyla, 20 have no cultured representatives and 20 of the 10 archaeal phyla have cultured representatives. This lag is likely due to the differences in throughput of culture-dependent and -independent approaches. Of the phyla with at least one cultured representative, the median percentage of sequences coming from a culture was only 2.8% for the bacterial phyla and 1.7% for the archaeal phyla (Figure results/figures/phylum_effort.pdf). So, even though many phyla have cultured representatives, there is still a skew in the representation of most phyla found in cultivation efforts. Considering the possibility that large culture-independent sequencing efforts may only be re-sequencing organisms that already exist in culture, we asked what percentage of OTUs had at least one cultured representative. We found that 13.0% of the 95,734 bacterial OTUs and 9.1% of the 4,205 archeael OTUs had at least one cultured representative (Figure results/figures/venn_otu_by_method.pdf.pdf). Comparing the percentage of sequences with cultured representatives to the percentage of OTUs containing a sequence from a cultured representative revealed strong cultivation biases for several phyla associated with model/biomedically relevant organisms (Fig. results/figures/phylum_cultured.pdf). The Tenericutes, Proteobacteria, Firmicutes, and Spirochaeatae all had considerably higher percentages of sequences generated by cultivated representatives than would be expected based on the number of cultured organisms represented by OTUs. This likely reflects the extremely high number of cultivated *Mycobacterium tuberculosis*, *Escherichia coli*, *Bacillus*, *Streptococcus*, *Lactobacillus*, *Staphylococcus*, *Borrelia*, and others. Conversely, clades such as the Actinobacteria, Fusobacteria, and Bacteroidetes had a lower percentage of sequences belonging to cultivated representatives than would be expected based on the percentage of OTUs that have sequences from cultured organisms, indicating that the cultivation efforts in these clades are relatively efficient with regards to available diversity. Regardless of these observations, the majority of OTUs from any phylum

remain uncultivated, to say nothing of the diversity of organisms that may be encapsulated within the 97% sequence identity cutoff.

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- Would be nice if we could comment somewhere on whether or not the EMIRGE/SAG sequences are more or less "efficient" at uncovering new OTUs. I.e., does incorporating EMIRGE/SAG sequences increase the OTU coverage at a greater rate, per new sequence, than those created by other methods?

New technologies to access novel biodiversity Assembly of metagenomic and single cell shotgun sequence data offers the hope of identifying large fragments of genomic data from as yet uncultured organisms. To test the ability of single cell technologies to expand our knowledge of microbial diversity beyond that of the 16S rRNA gene and pure cultures, we compared the overlap of OTUs found by the three methods (Figure results/figures/venn_otu_by_method.pdf). Utilizing the 16S rRNA gene sequences extracted from the single-cell genomes available on the Integrated Microbial Genomes (IMG) system (**???**), we identified 295 bacterial and 68 archaeal sequences that met our criteria, which were assigned to 102 and 24 bacterial and archaeal OTUs, respectively. Interestingly, only 9.8 and 25% of the bacterial and archaeal OTUs, respectively, that the single-cell 16S rRNA gene sequences belonged to had not been observed by cultivation or PCR-based efforts. For both domains, the fractions of the single-cell 16S rRNA gene sequences that were recovered by cultivation and PCR were similar. Furthermore, among the bacteria 54.9% of the single-cell OTUs were previously observed by both cultivation and PCR-based methods. Although the majority of single-cell genome projects have originated from a single study (**???**), 27.5 and 41.7 of the bacterial and archaeal OTUs, respectively, were from previously uncultured organisms. This represents an encouraging avenue to expanding our knowledge of bacterial diversity beyond the 16S rRNA gene.

Caveat Emptor

Recent data suggests that a considerable short-read diversity of microorganisms may be missing based on biases in existing 16S rRNA gene primers (Furhman, 2015). Furthermore, Brown et al. (2015) have recently used metagenomic assemblies to show evidence for introns in the 16S rRNA genes of organisms within the so-called “Candidate Phyla Radiation” (CPR- Saccharibacteria (TM7), Peregrinibacteria, Berkelbacteria (ACD58), WWE3 Microgenomates (OP11), Parcubacteria (OD1), et al.), that would preclude detection with standard cultivation-independent microbial surveys. Furthermore, many of these CPR organisms are very small and frequently pass through 0.2 µm filters (Luef, 2015). Thus, for many environments, the estimates for the census must be considered as lower bounds.

Conclusions

It is clear that considerable biodiversity has been discovered since the first census in 2004. However, much of it has been biased towards particular phyla and environments. Nevertheless, novel technologies such as single-cell genomics and algorithms to recover full-length sequences from shotgun metagenomic data have demonstrated promise in circumventing previous limitations in identifying new OTUs. Additional technologies coming on line that can provide full-length sequences, such as PacBio and potentially Nanopore, will likely provide considerable value. Additionally, as researchers attempt to answer questions about the physiology and biochemistry of the organisms identified in the massive deluge of cultivation-independent sequence data, a renewed emphasis on cultivation is beginning. Focused innovation to overcome the challenges of isolating new microorganisms will also contribute to improving out understanding of extant diversity.

The first 16S sequence was published in 1978, not deposited until 1983. A bit of an allegory for our time.

What are the most significant improvements since 2004, and where are we still lacking the most data?

Renewed call for cultivation

Figure results/figures/domain_rarefaction.pdf. Number of OTUs sampled among bacterial and archaeal 16S rRNA gene sequences for different OTU definitions and level of sequencing effort. Rarefaction curves for different OTU definitions of Bacteria (A) and Archaea (B). Rarefaction curves for the coarse environments in Table 1 for Bacteria (C) and Archaea (D). The number of bacterial and archaeal OTUs observed among the longest sequences in the SILVA database continues to grow at a rate too slow to ever reach estimates of 10^6 to 10^{11} bacterial species.

Figure results/figures/time_course_figure.pdf. Progression of the microbial census since the first full-length 16S rRNA gene sequence was deposited into GenBank in 1983.* The number of bacterial and archaeal 16S rRNA gene sequences deposited (A) and the new OTUs they represent (B) has increased exponentially until the last several years when the rate of change has plateaued. For both bacterial and archaeal sequences, the number of studies that are responsible for depositing more than 50% of the sequences each year has been relatively small (C).

Figure results/figures/category_phylum_heatmap.pdf. Heatmap depicting the relative abundance of the most common bacterial and archaeal phyla across different environments. Each environmental category exhibited a phylum-level signature although the bacterial census was dominated by sequences from the Firmicutes, Proteobacteria, Actinobacteria, and Bacteroidetes and the archaeal census was dominated by sequences from the Euryarchaeota and Thaumarchaeota. The ten most abundant phyla across all environmental categories are shown. The data for all bacterial and archaeal phyla are available in Supplemental Tables 2 and 3, respectively.

Figure results/figures/phylum_effort.pdf. Relative rate of sequence deposition for each bacterial and archaeal phylum before and after 2006 relative to the sequencing of all bacteria. The figure shows the relative rates for those phyla with at least 1,000 sequences and the x-axis is on a log2 scale. The data for all bacterial and archaeal phyla are available in Supplemental Tables 4 and 5, respectively.

Figure results/figures/phylum_cultured.pdf. The rate that sequences and OTUs are generated from bacterial and archaeal cultures relative to all sequences and OTUs by

phlum. Phyla with greater than 1,000 sequences are listed by domain. Open circles indicate the percentage of sequences in the database that match cultured organisms. Closed circles indicate the percentage of OTUs in this analysis that contain sequences belonging to a cultured organism.

Figure results/figures/venn_otu_by_method.pdf. The number of OTUs that were found by either cultivation, PCR, single-cell genomics or multiple methods for bacterial and archaeal sequences.

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