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# A renaissance for the pioneering 16S rRNA gene

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Culture-independent molecular surveys using the 16S rRNA gene have become a mainstay for characterizing microbial community structure over the past quarter century. More recently this approach has been overshadowed by metagenomics, which provides a global overview of a community's functional potential rather than just an inventory of its inhabitants. However, the pioneering 16S rRNA gene is making a comeback in its own right thanks to a number of methodological advancements including higher resolution (more sequences), analysis of multiple related samples (e.g. spatial and temporal series) and improved metadata, and use of metadata. The standard conclusion that microbial ecosystems are remarkably complex and diverse is now being replaced by detailed insights into microbial ecology and evolution based only on this one historically important marker gene.

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### Introduction

16S ribosomal RNA (16S for short) holds a special place in the study of microbial evolution and ecology. By virtue of a number of uncommon properties (ubiquity, extreme sequence conservation, and a domain structure with variable evolutionary rates [1]) it spearheaded two revolutions in these fields. First, it radically changed our view of evolution from a five kingdom to three domain paradigm by providing an objective phylogenetic framework in which to classify cellular life [1], and second, through the cloning and sequencing of 16S genes directly from the environment using conserved broad-specificity PCR primers (16S surveys), it demonstrated that microbial diversity is far more extensive than we ever imagined from culture-based studies [2].

Despite this impressive pedigree, 16S has been overshadowed in recent years by the application of high

throughput shotgun sequencing to environmental DNAs (metagenomics) [3–5]. Metagenomic sequencing randomly samples all genes present in a habitat rather than just 16S, thereby providing clues to the functional capacity of a community rather than just its phylogenetic composition. ‘Classical’ community composition profiling by 16S is now often used as a preliminary step before metagenomic analysis and can be of great value in guiding decisions regarding sequencing technology to be used (454 vs. Sanger, shotgun vs. large-insert clones) and amount of sequencing necessary. However, 16S is re-emerging as a stand-alone molecular tool owing to a confluence of methodological advancements.

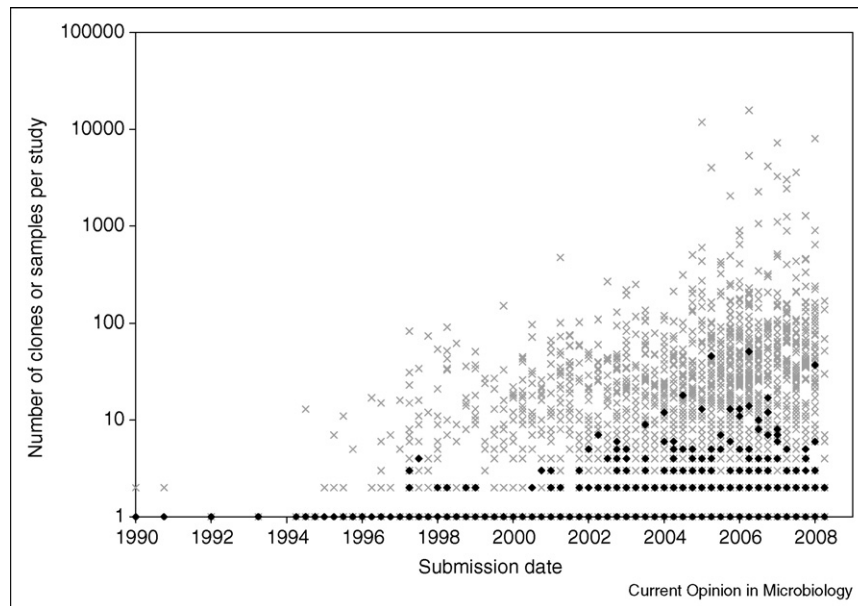
### Data generation

16S data are being generated at an unprecedented rate owing to new and improved sequencing technologies that dramatically increase throughput and decrease cost. These include lower Sanger sequencing costs as well as inexpensive 454 pyrosequencing and the PhyloChip, a custom microarray for 16S surveys [6]. The flood of 16S data stemming from these advances has in most cases continued to reveal that most diversity estimates, even those based on culture-independent methodologies, fall far short of reality.

Whereas the typical 16S survey by traditional PCR clone sequencing a decade ago might have included a few dozen sequences, many today encompass thousands [7–9]. Indeed, there has been a near-exponential increase in the size of the largest surveys (Figure 1), though these numbers are probably underestimates as many studies only deposit unique phylotypes in the database rather than every clone sequenced. Today's 16S surveys also typically encompass multiple samples, even dozens, rather than targeting a single habitat (Figure 1) [7,9–11]. Despite the expense of the clone-and-sequence approach, it remains the ‘gold standard’ for identifying novel lineages as only full-length or near full-length sequences are adequate for accurate phylogenetic tree building. Such studies continue to expand the known ‘tree of life’ at a steady pace and provide a valuable reference base for the high-throughput technologies discussed below.

The widespread availability of 454 pyrosequencing, a technology roughly an order of magnitude less expensive than Sanger sequencing in terms of cost per base, has changed the landscape of genomics [12]. The first commercially available pyrosequencers generated reads of just 100 bp on average, but could produce 20 Mbp of data in a single run. This first generation of pyrosequencing was

Figure 1



Increases in the number of full-length (>1200 nt) Sanger-sequenced 16S clones (gray Xs) and samples (black diamonds) per study since 1990. Note logarithmic scale on Y-axis. Data were obtained from the greengenes database [38] and parsed such that each entry for a given study was assumed to be an independent clone, and each unique entry in the 'isolation source' field was assumed to be an independent sample. No attempt was made to further manually curate the clone or sample counts on the basis of information in publications or manual examination of clone names or other information, beyond spot-checking the accuracy of the parsing scripts. Submission dates were converted to numeric format by taking the submission year and adding zero for submissions in January through March, 0.25 for April–June, 0.5 for July–September, and 0.75 for October–December.

termed GS20 and is already an historical footnote. To adapt pyrosequencing technology for 16S analysis, Sogin *et al.* PCR-amplified the short V6 variable region of the bacterial 16S rRNA gene from eight distinct environments using universal primers and ran them separately within a single 454 run [13<sup>••</sup>]. This single run generated a total of ~118 000 sequence tags (16S pyrotags), more than any Sanger-based study to date. A follow-up study, also using GS20 technology, generated more than 900 000 bacterial and archaeal 16S pyrotags [14].

Second generation pyrosequencing technology (454-FLX) produces average read lengths of more than 200 bp and yields ~100 Mb per run, and the third generation of pyrosequencing (titanium) has recently appeared on the scene producing ~500 Mb per run and average read lengths >400 bp. These enhancements will continue to improve the throughput and resolution of 16S pyrotag investigations [15]. Barcoding, in which sequences from particular samples can be identified by unique sequences incorporated into the amplification primers, has enabled multiplexing of samples within runs and has further enhanced the usefulness of this approach [16<sup>•</sup>,17].

Another major development in 16S analysis is not directly dependent on DNA sequencing but involves a high-density microarray of phylogenetically specific probes called the PhyloChip [6]. Designing such a microarray

is non-trivial owing to the highly conserved nature of the 16S rRNA gene; however, DeSantis *et al.* have been able to use such an array to accurately differentiate among phylotypes in diverse environmental samples, documenting not only the vast majority of taxa identified by traditional cloning and sequencing but also groups not seen in clone libraries that were subsequently confirmed by taxon-specific PCR [6]. Advantages of the PhyloChip are low cost and high speed (facilitated by dedicated software, PhyloTrac, to analyze the output) and drawbacks include only being able to identify phylotypes targeted on the chip and an inability to determine phylotype abundance distribution in the one sample (although individual phylotypes can be tracked quantitatively across samples).

### Analytical tools

With the ample data produced by these new technologies has come unprecedented statistical power in discerning similarities and differences among communities. The unidimensional diversity indices and total operational taxonomic unit (OTU) estimates commonly used in single-sample studies have given way to tools designed to directly compare the communities found in different samples. Some of these are aimed primarily at discerning overall phylogenetic similarities, while others assess the structure of the communities as well (e.g. abundance information).

Once sequences have been grouped into OTUs on the basis of some set of similarity criteria (e.g. using DOTUR [18]), similarity indices such as Bray-Curtis can be calculated to estimate the relatedness of different communities. Regression techniques can then be applied to isolate variables that contribute significantly to community composition, as well as correlate the abundances of specific phylogenetic groups with environmental factors [19].

A recent technique more precisely tailored to 16S sequence analysis is UniFrac, a program designed to determine the fraction of unique branch lengths within a phylogenetic tree (comprising sequences from multiple samples) that is attributable to a particular sample [20]. Once this is determined, principal coordinate analysis (PCoA) can be used to identify specific environmental variables that drive differences among communities [21]. One key advantage of this approach is that it circumvents the controversial and often arbitrary process of assigning sequences to OTUs and deals entirely with tree-based metrics. Thus differences at the species or genus level receive less weight than those at the phylum level, but are still considered in the overall analysis. This method has been applied to data from a spectrum of environments and a variety of studies, often leading to new biological insights [20,21]. However, the original implementation of UniFrac takes only unique sequences into account, and thus is insensitive to changes in abundance that may be important to understanding community responses to environmental variation.

A later version of UniFrac, called weighted UniFrac, deals with this weakness by assigning weights to branches of the tree on the basis of the abundance of specific phylotypes. Comparison of the two methods revealed that they measure very different characteristics of the communities, and thus should really be considered complementary approaches rather than different implementations of the same algorithm [22].

A recent rRNA-based study uses a new set of metrics, the phylogenetic species variability (PSV) and phylogenetic species evenness (PSE), to separate out the effects of environmental selection versus interspecies competition [23] (discussed in more detail in the next section). These metrics summarize the relatedness of species within communities such that PSV is equal to one if the members of a community are unrelated and approaches zero if all of the members are closely related. PSE incorporates abundance information in addition to prevalence, such that PSE decreases both when community members are closely related and when members are unevenly represented [23,24\*,25]. Permutation tests can then be used to indicate whether species in the communities are underdispersed, such that closely related species tend to co-occur, or overdispersed, such that closely related

species tend to occur exclusively from one another. Underdispersion may indicate that environmental filtering is an important force in generating community structure and supports the use of additional tools to correlate environmental variables with species composition.

Each of these approaches has its strengths and weaknesses, and no tool can address each individual situation [26]. But the currently available tool kit of experimental and analytical approaches allows a wide variety of experimental hypotheses to be tested.

### Case studies

In combination, these experimental and analytical developments are converting 16S surveys from 'fishing expeditions' to hypothesis-driven studies. The ability to take multiple samples over time, space, or other metrics and deeply interrogate each has enabled an entirely new class of studies, in both the environmental and medical arenas, in which 16S presence and abundance are correlated with specific factors.

A recent novel application of 16S rRNA sequencing to medical diagnosis and treatment investigated the microbial diversity of the lung in intubated patients and the effects of antibiotic therapy [27]. It found that while the lung remained sterile during brief intubation, patients inevitably became colonized during long-term intubation. Intriguingly, though, patients who were culture-positive for *Pseudomonas aeruginosa*, a primary agent in ventilator associated pneumonia (VAP), were often colonized with a spectrum of other pathogenic and non-pathogenic bacterial species. Paradoxically, when patients were treated with antibiotics targeted to the *Pseudomonas* strains found by culture, the diversity of the flanking populations decreased and *Pseudomonas* became more dominant, potentially as a result of biofilm formation [28]. This finding held true whether the diversity was examined via cloning and sequencing or by PhyloChip, and the decreased diversity correlated with poorer patient outcome in patients with active infections [27]. This study provided an excellent example of the usefulness of PhyloChip analysis in communities dominated by a single member, as far greater diversity was revealed by microarray than could feasibly have been sampled by traditional PCR clone library. The availability of multiple samples from individual patients, taken over the course of therapy, greatly increased the ability to correlate community composition with therapeutic intervention and patient outcome.

Ribosomal RNA sequencing has also been used to study spatial variability in similar environments, both for complete microbial communities and for specific components of those communities. In one study of freshwater lakes, Newton *et al.* [23] investigated the prevalence and abundance of members of the acI lineage of Actinobacteria in

18 different locations. Using the PSV and PSE metrics (described above) they concluded that the 11 distinct acI lineages observed were significantly underdispersed. However, the pattern of dispersion displayed little dependence on distance and rather correlated with environmental variables such as pH, indicating the strong effects of environmental filtering on this set of populations [23].

One field in which the relative roles of evolutionary history and environmental selection have been difficult to sort out is the study of mammalian gut microbiota. A number of studies have revealed strong similarities among the gut communities of diverse mammals, but it has been unclear whether this was the result of similarities among the habitats and the nature of the host-microbe symbiosis, or simply the legacy of descent from a common ancestor whose gut community was already established. Ley *et al.* recently tackled this question in a study systematically characterizing the fecal communities of 59 distinct mammalian species, from diverse phylogenetic lineages and with widely varying lifestyles, as well as numerous humans [29<sup>\*</sup>]. In total, the study encompassed more than 20 000 sequences from 106 samples, including some previously published data. Using UniFrac and PCoA, they found that host phylogeny had a dominant effect on community composition, while diet had a strong secondary role.

### Some remaining challenges

The increased statistical power that comes with more data as well as the many tools available to correlate environmental variables with 16S and other molecular data has highlighted the need for accurate, standardized and accessible metadata (i.e. non-sequence data associated with the samples being analyzed such as biogeochemical data). Coordinated efforts are now underway to address this need, such as the Genomics Standards Consortium [30,31,32<sup>\*</sup>].

Almost all of the 16S sequence data in the public repositories to date are the products of PCR amplification of the 16S rRNA gene using 15–25 nucleotide primers broadly targeting bacteria or archaea. However, such primers are known to miss some organisms owing to target mismatches [33,34] and the recent application of short (10 nucleotide) ‘miniprimers’ suggests that a considerable amount of diversity may be overlooked in environmental samples using standard 16S primers [35]. Pyrosequencing of cDNAs prepared from environmental RNAs may be the way of the future. This approach not only bypasses any potential primer bias, but simultaneously also provides a community profile of all three domains of life and functional information in the form of expressed messenger RNAs [36<sup>\*\*</sup>].

A good quality reference taxonomy based on phylogenetic inference of full-length 16S sequences is required to classify pyrotag and PhyloChip data. Unfortunately, such

a reference tree remains somewhat elusive owing to the rate of data accumulation (Figure 1) and difficulties associated with producing and managing trees with hundreds of thousands of taxa. The problem is particularly acute for environmental sequences that are mostly unclassified in the public databases. The issue is currently being addressed through dedicated 16S databases [37–39] and tools developed to handle large sequence datasets [40,41] (<http://www.microbesonline.org/fasttree/>). It is important to note, however, that a number of the new analytical tools can provide biological insights through correlative analyses without the need to classify the underlying 16S data [20,24<sup>\*</sup>].

### Conclusion

A continuing central role for 16S rRNA in microbial ecology and evolution looks certain thanks to methodological advances in the field.

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