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Authors

Tringe, Susannah Green

Rubin, Edward M.

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Metagenomics: DNA sequencing of environmental samples

Susannah Green Tringe and Edward M. Rubin*

DOE Joint Genome Institute, 2800 Mitchell Drive, Walnut Creek, CA 94598, USA

*To whom correspondence should be addressed: emrubin@lbl.gov

While genomics has classically focused on pure, easy-to-obtain samples, such as microbes that grow readily in culture or large animals and plants, these organisms represent but a fraction of the living or once living organisms of interest. Many species are difficult to study in isolation, because they fail to grow in laboratory culture, depend on other organisms for critical processes, or have become extinct. DNA sequence-based methods circumvent these obstacles, as DNA can be directly isolated from live or dead cells in a variety of contexts, and have led to the emergence of a new field referred to as metagenomics.

Complete genome sequences have been obtained from hundreds of organisms. In the well-studied, easily manipulated organisms targeted by early genome projects, genotypic and phenotypic data could be compared and genome-based hypotheses tested by experiment. Comparative genomics allowed experiment-based annotations to be transferred to novel genomes, and quickly gained prominence as a valuable tool for understanding both genes and genomes¹. DNA is universal, and protocols for its purification are well established; though some optimization is usually required for DNA extraction from novel organisms, the effort involved is generally much less than that required to develop techniques for genetic manipulation. As a result, the focus of some genomic sequencing has changed dramatically, such that DNA sequence is used to predict features and behaviors of otherwise poorly understood organisms as well as to understand the genetic basis of characterized traits.

Barriers to genome sequencing range from the lack of sufficient material for the construction of sequencing libraries to the cost of sequencing. Improvements in cloning

and sequencing technologies have consistently decreased the amount of starting material needed for library construction, making DNA sequencing feasible for a variety of organisms that are otherwise difficult to study. Meanwhile, the progressive reduction in the cost of high-throughput sequencing has made feasible the sequencing of libraries constructed from mixtures of organisms, even those “contaminated” with genomes other than that of the targeted organism². This has opened the door to sequence-based studies of organisms and environments previously thought inaccessible, including obligate pathogens and symbionts, which cannot survive outside their hosts; environmental microbes, most of which cannot be grown in pure culture; and ancient organisms whose only record is fossilized remains. DNA for these studies is extracted directly from the organisms in their natural habitat, such as host tissue or soil, and cloned into sequencing vectors. The resulting libraries contain genome fragments from a heterogeneous mix of species, strains and subpopulations. Thus the sequence data from these libraries harbor a wealth of information on community dynamics, such as species interactions and selective processes.

This article focuses on the insights that have emerged from DNA sequencing of naturally occurring populations and communities. The first section describes methodological advances that have enabled the sequencing of natural populations, the second section gives examples of studies that have used these techniques and the third section suggests future directions these studies may take.

Environmental nucleic acid analysis

Natural samples contain DNA in a variety of packages, including free DNA, virus particles, and prokaryotic and eukaryotic cells. These can be suspended in water, bound to a solid matrix like soil, or encased in a biofilm or tissue. Extraction methods must be chosen carefully based on the medium and the DNA population of interest.

Aquatic samples must be concentrated, typically by impact or tangential flow filtration, and may also be prefiltered to remove large cells or debris³. The choice of filter sizes is a critical one, as cells that are smaller or larger than the size fraction targeted will be invisible to further analysis. Thus filtration protocols can be chosen to enrich for eukaryotic cells, prokaryotic cells, or viral particles^{4,5}. Cells in soils and sediments are less easily concentrated than aquatic samples and often contain enzyme inhibitors, such as humic acids, that must be removed prior to amplification or cloning. Solid-matrix DNA isolation is either direct, in which cells are lysed within the sample material, or indirect, in which cells are separated from noncellular material prior to lysis. In either case, contaminants that tend to copurify with DNA from samples high in organic matter can be removed by methods such as agarose gel electrophoresis or column chromatography^{6,7}. Direct isolation may also capture DNA from virus particles or free DNA from dead cells; when these noncellular DNAs are the intended target, they can be directly solubilized and concentrated without lysis of cells in the sample^{8,9}.

The techniques used to lyse cells may also affect the composition of environmental DNA libraries, as the harsh lysis methods necessary to extract DNA from every organism will cause degradation of the DNA from some organisms⁷. Hard-to-lyse cells, such as **Gram positive bacteria**, may therefore be under- or over represented in environmental DNA preparations¹⁰. Often the desire for complete lysis must be balanced with the need

for high-quality DNA, especially when preparing high molecular weight DNA for large-insert libraries^{11,12}.

Once DNA has been obtained, it can be directly cloned into small-insert vectors for high-throughput sequencing (see, for example, <http://www.jgi.doe.gov/sequencing/protocols/>). Alternatively, it can be cloned into large-insert libraries and screened for clones with activities or genes of interest, which are then subcloned and sequenced.

DNA sequence-based insights into “inaccessible” organisms

The first forays into sequencing of natural samples aimed to characterize the genomes of organisms that occur in tight association with one or more additional species, and therefore cannot be easily studied in isolation. Here, the challenge is to extract the relevant sequence from a mixed-species library, which may contain only a small fraction of clones from the target species. A variety of pre- and post-sequencing “sifting” techniques have enabled the genomic characterization of organisms that cannot be cultivated, such as obligate pathogens and symbionts, and even long-extinct species.

16S rRNA: a launch pad for novel prokaryotic genomes

The genomic study of natural communities has been largely driven by interest in the ~99% of microbes that are not easily isolated in culture. These species are identified by their 16S/18S small subunit rRNA genes, which are commonly used as phylogenetic markers because every cellular organism contains the gene, and virtually all gene variants

can be amplified by standard sets of degenerate primers (Box 1). Several investigators have used rRNA genes as a starting point to explore the genomes of uncultivated microbes via large-insert clone sequencing. One such “**phylogenetic anchoring**” study led to the discovery of proteorhodopsin, a type of light-harvesting protein, in oceanic bacteria – a surprise not only because these microbes were previously believed to depend on organic matter, not light, as an energy source, but because rhodopsin-like proteins had never been seen in the bacterial domain¹³⁻¹⁵. Others have provided glimpses of the genomes of several additional uncultivated prokaryotes, including crenarchaeota^{11,16-18} and Acidobacteria¹⁰ from multiple habitats. In some cases, these sequences have provided evidence for unexpected functions¹¹ or **horizontal gene transfers**¹⁹.

Host-associated bacteria: genomic insights into pathogenesis and symbiosis

Though discussion of uncultivated microbes most often brings environmental organisms to mind, the several uncultivated microbes whose genomes have already been sequenced are largely obligate pathogens or symbionts²⁰⁻³². The amenability of these host-associated microbes to physical separation makes them well-suited to this approach (See Table 1), in contrast to organisms residing in complex environmental communities. The first complete genome of an uncultured bacterium, the syphilis spirochete *Treponema pallidum*, was released in 1998 – a landmark in genome sequencing²⁰. While the bacterial origin of syphilis was recognized a century ago, the infectious agent has never been isolated in continuous culture. DNA for sequencing the intracellular pathogen was obtained from the testes of infected rabbits - some 400 of them - by a series of lysis and centrifugation steps that eventually resulted in an essentially pure bacterial

preparation (Table 1). Sequence analysis immediately identified potential contributors to virulence, and aided the development of DNA-based diagnostics³³. A year and a half of painstaking growth in coculture with human fibroblasts was necessary to obtain sufficient DNA to sequence the genome of the Whipple's disease bacterium *Tropheryma whipplei*, which revealed deficiencies that suggested an explanation for the failure to propagate in **axenic** culture. Based on these genomic insights, Renesto *et al.* then used a standard tissue culture medium, supplemented with amino acids implicated by the sequence analysis, to successfully cultivate *T. whipplei* in the absence of host cells, shortening their doubling time by an order of magnitude³⁴. This is one of many cases in which DNA sequence information has been used to improve culture techniques, diagnostics and therapies for fastidious organisms³⁵⁻³⁷.

Several genomes of obligate intracellular symbionts, primarily from insect hosts, that could not be grown by conventional means have also been obtained by various separation and purification methods (Table 1). The first was *Buchnera aphidicola*²⁴, an *E. coli* relative that provides nutrients to supplement its aphid host's restricted diet of plant sap. Bacteriomes – specialized symbiont-harboring organs - from 2000 aphids were isolated by dissection prior to crushing and filtration, resulting in virtually pure *Buchnera* cells for DNA isolation. Symbionts of tsetse flies, fruit flies, carpenter ants, a nematode and two other aphid species have since had their genomes sequenced, as has one uncultured plant pathogen^{25-29,31,32}. In each project, techniques such as dissection, differential lysis and pulsed-field gel electrophoresis, often in combination, have helped enrich for prokaryotic material (Table 1); where reported, between 5% and 47% of the sequences were host-derived²⁷⁻²⁹. Another essentially complete symbiont genome recently emerged as a

byproduct of a metazoan genome project, as the sequencing libraries were constructed from symbiont-harboring whole embryos³⁰.

Paleogenomics

Evolutionary biology depends heavily on DNA sequence data to reconstruct evolutionary pathways, but these molecular trees are limited to the modern species at the ends of the branches whose DNA is readily available. Phylogenetic placement and hypothesized phenotypes of the organisms at the branching nodes, or the branches that terminate before the modern era, are based primarily upon morphological examination of fossilized specimens. The ability to sequence genomes from ancient organisms would offer a “genomic time machine” to study these poorly characterized species.

When an animal dies, its tissues are quickly exploited as an organic nutrient source by a variety of creatures, particularly single-celled microbes. Rarely, conditions are such that the carcass escapes total decomposition and parts, particularly bone, remain preserved; however, the DNA contained therein is not only damaged and fragmented but mixed with the genomes of the abundant opportunistic microbes that have invaded the tissue. Nonetheless, gentle and rigorously sterile DNA isolation procedures have allowed the generation of verifiable mitochondrial and nuclear sequence from materials such as bones, teeth, and coprolites (fossilized fecal material) dating to as long as 50,000 years ago^{38,39}. These studies, relying on PCR-amplified mitochondrial sequence, have been used to resolve phylogenetic relationships between extinct and modern animals⁴⁰. Mitochondria are present in more than 1000 copies per cell and are therefore relatively easily amplified; the single-copy nuclear genome, which could offer far more phenotypic

information, have remained minimally explored due to technical hurdles^{41,42}. Low-cost high-throughput sequencing, coupled with a **metagenomic** approach, now provides a means to access the nuclear genomes of extinct organisms without amplification. This was recently applied to the analysis of cave bear, *Ursus spelaeus*, a relative of modern brown and black bears that lived in caves throughout Europe in the late Pleistocene but became extinct tens of thousands of years ago. The investigators exploited a metagenomic strategy to demonstrate the presence of verifiable cave bear sequence in libraries created by directly cloning DNA extracted from 40,000-year-old bones⁴³. A library construction protocol that involved neither lysis nor shearing enabled the cloning of end-repaired ancient DNA isolated from cave bear bone and tooth samples. While cave bear sequence constituted a mere 1-5% of the libraries described by Noonan *et al.*, these sequences were readily identified by their high sequence identity to a related carnivore, dog, whose genome is fully sequenced⁴⁴ (Figure 1). Roughly 27 kb of putative cave bear sequence was obtained, and PCR amplification of orthologous sequences from modern black, brown and polar bears verified their origin and allowed the reconstruction of a phylogenetic tree congruent with that based on mitochondrial sequences. Modern human contamination from laboratory personnel, a surprisingly low 0.05% of clones, was easily identified as this proof-of-principle study focused on a species which is readily distinguishable from modern human.

These techniques open up the possibility of genome projects targeting extinct species and could revolutionize paleobiology. Our closest hominid relatives, the Neanderthals, diverged from modern humans roughly 500,000 years ago but survived until the late Pleistocene, and numerous Neanderthal remains of ages comparable to the

sequenced cave bear samples have been found. By providing sequence from another hominid, the Neanderthal genome could define human-specific sequences and expand knowledge of the biology of both *Homo sapiens* and Neanderthals

High-throughput shotgun sequencing of environmental samples

Environmental samples are many times more complex than single organisms, as they may contain tens, hundreds or even thousands of distinct species, and were therefore until recently widely considered unsuitable for high-throughput sequencing. Similar concerns once accompanied the application of **whole-genome shotgun** (WGS) sequencing to large genomes, as it was thought that assembly of WGS reads into chromosomes and genomes would prove too computationally complex. Yet WGS has proven to be the most efficient and effective approach to generating complete genomes both large and small, largely due to computational advances. In the case of environmental genomics, analysis tools have once again risen to the task, enabling the simultaneous study of whole ensembles of genomes via high throughput sequencing. A new perspective, in which genes and genomes are viewed as subunits of a larger whole, is changing the way in which we understand evolution and adaptation.

The first large-scale environmental shotgun sequencing project interrogated the organisms making up an acid mine biofilm⁴⁵. Acid mine drainage is an environmentally devastating consequence of commercial mining which results from the production of sulfuric acid when pyrite (FeS_2) is exposed to air and water during mining operations. Microorganisms have long been recognized as important players in this process, as the

rate-limiting step of ferric (Fe^{3+}) ion regeneration is slow under sterile conditions but can be greatly accelerated by microbes that derive energy from the reaction (chemolithotrophs)⁴⁶. Microbial communities flourish under these seemingly hostile conditions, forming extensive underwater streamers and floating biofilms anchored in pyritic sediments, but are typically of relatively low diversity as few organisms can tolerate the extreme acidity.

To address the physiology of the uncultivated microbes in the mine, Tyson *et al.* built a short insert genomic library from biofilm DNA and generated 76.2 million base pairs of sequence from the resident bacteria and archaea⁴⁵. From this, they assembled near-complete genomes for two community members and partial genomes for three more, enabling metabolic reconstruction to assess the role of each individual organism. Interestingly, one organism, an uncultivated *Leptospirillum* group III, was the only member of this community that possessed the genes for nitrogen fixation. As this process is essential in such a nutrient-limited environment, this low-abundance species appears to be a linchpin for the whole community and, theoretically, a potential biological target for cleanup efforts.

Another study reported the metagenomic sequencing of the surface water microbial community of the Sargasso Sea, a body of low-nutrient water in the North Atlantic⁴. Planktonic microbes were collected from multiple locations and extracted DNA was used to construct seven independent libraries, from which a total of more than 1.6 Gb of DNA sequence was generated. Reflecting the unexpected complexity of the Sargasso Sea planktonic community, just 3% of this sequence was covered at 3X depth or more – even though this quantity of raw sequence would be sufficient to complete as

many as 50 prokaryotic genomes at 8X depth. More than 1.2 million genes were found to have significant similarity to database entries. Although less than a third could be assigned tentative cellular roles, some functions stood out, including numerous rhodopsin-related genes and genes involved in phosphorus uptake and metabolism, consistent with the need to efficiently utilize the plentiful sunlight and limited phosphate available in this environment⁴⁷. Most of the predicted genes in the Sargasso Sea data could not, however, be definitively linked to particular phylogenetic groups, much less individual species. These data have since been mined for a variety of genes including iron-sulfur proteins, chitinases, proteorhodopsins, and electron transport proteins⁴⁸⁻⁵¹. Each of these studies has identified genes highly divergent from known family members, highlighting the novelty of environmental sequences as compared to genome sequences of cultured isolates.

While acid mines and the Sargasso Sea represent relatively nutrient-poor environments, a recent study by Tringe *et al.*⁵² explored two different nutrient-rich environments: agricultural soil and deep sea whale skeletons, a.k.a. “whale falls,” which sustain thriving communities of micro- and macro-organisms as they decompose⁵³. The combination of these environments with the previously sequenced samples spans a wide range of environmental variables such as temperature, pH and illumination, providing a rich testing ground for comparative analysis. Just as comparative genomics forms the foundation for most genome annotation efforts, it was reasoned that patterns of gene abundance among environments would enhance understanding of both the environments and the gene products.

Genomic sequencing of complex, nutrient-rich samples did not result in assembled genomes - indeed, it was estimated that several billion bases of sequence would need to be generated from a complex environment like soil before genomes would begin to assemble - but did identify gene families important for survival in the environments sampled. In this gene-centric approach, each sequence obtained was termed an Environmental Gene Tag (EGT), because it contained a snippet of sequence potentially encoding a protein adaptive for that environment (Box 2). Predicted genes on the EGTs from each sample were compared with each other and with sequences from previous environmental sequencing projects^{4,45}. A number of characterized and uncharacterized orthologous groups, functional modules or biochemical processes emerged that were unevenly distributed across the samples⁵². This provided an EGT “fingerprint” of each environment and demonstrated that similar environments, such as two whale skeletons 8000 miles apart on the ocean floor, have similar gene content.

Analysis of functions overrepresented in particular niches provided unique insights into the demands placed on organisms living there. One of the most significant disparities in gene distribution to emerge from this analysis was the overabundance of rhodopsin-like proteins in the Sargasso Sea as compared to non-illuminated environments. Similarly, as might be predicted in hindsight, numerous homologs of cellobiose phosphorylase, an enzyme involved in the breakdown of plant material, were found in the soil sample, taken near a silage bunker, but not in the other samples. A preponderance of sodium transport and osmoregulation proteins in all the marine samples, both surface and deep sea, was consistent with the high sodium content of seawater. The soil sample, by contrast, contained far more potassium transporters, and

biochemical analysis revealed that potassium ions outnumbered sodium in the sample seven to one. Overall, variations in gene distribution were most evident in transporters and metabolic enzymes - those molecules most involved in interacting with, and presumably adapting to, the environment. The many uncharacterized orthologous groups exhibiting highly skewed distributions across samples may function in niche adaptation and therefore make promising candidates for future investigations. With these comparative tools in hand, researchers can now investigate the factors that influence microbial colonization or the changes that occur in environments under stress, without the constraints on diversity created by the need to assemble genomes.

Future directions

The goals of metagenomic projects vary considerably, from characterizing one particular species to understanding the dynamics of a whole community. While the “difficult to access” genome projects described herein might seem to share little in common with environmental projects examining complex communities, many of the methods and challenges overlap. These two previously separate fields are rapidly converging in the several metagenomic projects now targeting either individual members of free-living communities, such as marine Crenarchaeota⁵⁴, or entire communities of symbiotic organisms, such as the syntrophic consortium inhabiting the marine oligochaete *Olavius olgarvensis*⁵⁵ (For information on these and other ongoing projects at the DOE Joint Genome Institute, see <<http://www.jgi.doe.gov/sequencing/cspseqplans.html>>). A “second human genome

project” has even been proposed to sequence the genomes of the human-associated microbiota⁵⁶.

This review has described many innovations that have improved our ability to study “inaccessible” genomes. However, the current methods of DNA isolation, library construction, sequence assembly and bioinformatic analysis are all still optimized for single-genome analysis and will likely need to be modified for application to metagenomic projects.

DNA isolation and library construction

The methods used to isolate DNA from mixed samples and construct libraries substantially affect the results obtained, as cells differ in their sensitivity to lysis and DNA “cloneability” varies widely. For environmental samples, particular effort has been devoted to obtaining DNA representative of all organisms present, to best study the community as a whole. These representative libraries are effective tools for community overviews and for characterizing the dominant activities in an environment⁵². However, when complete genomes are desired, representative libraries are an inefficient means of sequencing non-dominant community members; one organism in the Sargasso Sea study, for example, was sequenced at 21X coverage⁴.

A number of techniques have been used to normalize or enrich environmental libraries for a variety of applications, based on generic properties like cell size or DNA composition. Filtration is one that has already been mentioned as a means of separating cells based on size, particularly for separating prokaryotes from eukaryotes; it has also been used to separate multicellular consortia from individual cells⁵⁷. Separation of DNA

on bisbenzimidazole gradients allows fractionation based on GC content, exploiting the change in buoyant density that occurs when bisbenzimidazole binds to adenine and thymidine⁵⁸. Other techniques that have been applied to host-associated microbes include differential centrifugation⁵⁹, **density gradients**^{25,57}, **differential lysis**²⁰, **pulsed-field electrophoresis**³² and selective use of restriction enzymes⁶⁰.

In some cases, a particular organism or group of organisms in a community is of interest; for example, those that carry out a particular metabolic process or are members of an uncharacterized phylogenetic group. Successful targeting of these organisms could significantly reduce the amount of sequence needed for genome coverage and simplify assembly. Stable isotope probing (SIP) holds promise as a means to obtain DNA from organisms capable of metabolizing a particular substrate, and may serve as a valuable method for community fractionation⁶¹. **Flow cytometry** is a highly specific method to isolate organisms based on viability⁶², membrane properties⁶³, surface protein expression⁶⁴, or SSU rRNA sequence⁶⁵. Finally, **affinity purification** might hold promise for separating out some groups^{66,67} based on cell wall characteristics or extracellular markers. Building libraries from such enriched DNA will greatly improve sequencing efficiency as compared to whole-community libraries.

Whole genome amplification via **isothermal strand displacement** could dramatically open up the possibilities for sequencing unculturable organisms by significantly reducing the amount of starting material required for library construction. DNA from prokaryotic and eukaryotic cells has been amplified by this technique and used for a variety of PCR- and hybridization-based genomic analyses⁶⁸. Encouraging results were recently reported for a metagenomic sample, where PCR results from

amplified and unamplified DNA were comparable⁶⁹. Short-insert shotgun sequencing libraries have also been constructed from whole-genome-amplified samples^{70,71}; however, a high rate of sequencing artifacts has thus far precluded genome assemblies based on these libraries (P. Richardson, personal communication).

Library construction is a potentially major source of bias, as some genome segments are uncloneable and/or lethal to *E. coli*. New, highly parallel non-Sanger sequencing technologies already being marketed, such as **pyrosequencing**, obviate the need for libraries of any sort⁷². By eliminating this major source of bias while decreasing time, effort and expense, they could have a major impact on the field; however, this will require surmounting key obstacles such as short read lengths.

Data analysis

One of the most pressing issues in metagenomics is genome assembly, which is critical for some types of genomic analysis. The most basic obstacle to assembly is simply the cost of achieving sufficient sequence coverage of a single microbe in a community that may contain hundreds of species; however, given the dropping cost of sequencing, this may soon be less of a problem. Another concern is how assembly algorithms will perform when confronted with mixed data from multiple species. Fortunately, experience suggests that cross-species assemblies are not a common occurrence⁴⁵, except in the case of highly conserved genes such as rRNA⁷³. Perhaps the most serious challenge in assembling genomes from metagenomic data is population heterogeneity, in the form of sequence polymorphisms and genomic rearrangements.

Assembly algorithms appear to be fairly robust to sequence polymorphisms^{28,45,74}, though very high polymorphism can interfere with proper assembly especially in complex genomes⁷⁵. Genomic rearrangements, however, may require serious rethinking of the meaning and purpose of genome assembly⁷⁶. It is not yet clear what level of heterogeneity is “typical”: in the limited set of communities that have been explored, some populations are virtually clonal, some exhibit high polymorphism, and some contain extensive insertions, deletions, and translocations^{4,45,57}. It will be interesting to see whether heterogeneity correlates with features like growth rate, competition or community stability.

Once sequences have been generated, be they whole genomes, large scaffolds, or individual reads, we often want to assign them to phylogenetic groups. For closed or nearly closed genomes scaffold assignment is straightforward, because functional genes are directly linked to phylogenetic markers like 16S rRNA. But even under optimal conditions each genome may be divided into multiple scaffolds, and many sequences, particularly those from low-abundance community members, will remain in small **contigs** or unassembled reads lacking obvious marker genes. The simplest method of taxonomic assignment, best BLAST hit, should be used with caution: it is only reliable when close relatives are available for comparison, and is essentially useless when no relatives have been fully sequenced⁷⁷. Other features that have been used to “bin” scaffolds or contigs into taxonomic groups include GC content and oligonucleotide frequency, coverage depth, and similarity to sequenced genomes^{4,43,45,78}.

Another field that is in its infancy is gene calling in metagenomic data because the data is fragmented, heterogeneous, and abundant. Homology-based methods are very

accurate but not very sensitive, particularly for genomes that lack sequenced relatives, and will always miss novel genes, which are potentially the most interesting. *Ab initio* methods can predict novel genes, but training is optimally performed on complete genomes and false positive rates may be high even for assembled genomes⁷⁹. One method for circumventing this problem is to use a sampling of sequenced genomes as a training set, preferably of a similar phylogenetic range as the species in the sample⁵², but improvements could almost certainly be made and this is an important area for future work. Further validation of potential novel genes can be obtained through sequence clustering: predicted proteins that have homologs within the dataset are likely to be valid⁴.

Gene annotation is also a challenge for metagenomic projects, as the amount of data generated is likely to be large for manual annotation. Fortunately, there are several high-quality automated annotation tools for complete microbial genomes, such as ERGO < <http://ergo.integratedgenomics.com/ERGO/login.cgi> >⁸⁰, GenDb < <https://www.cebitec.uni-bielefeld.de/software/gendb/cgi-bin/login.cgi> >⁸¹ and PRIAM < <http://bioinfo.genopole-toulouse.prd.fr/priam/> >⁸². In general, these can be adapted with minimal effort to metagenomic data sets; accuracy, however, is always a concern as no automated methods can fully replace manual annotation. The greatest improvements in accuracy are likely to result from the further production of high-quality complete genomes, particularly in phylogenetic groups, such as Chloroflexi and Acidobacteria, that are well-represented in the environment but poorly represented in sequence databases⁸³. Such high-quality genome data will provide better substrates for homology searches.

Conclusions

Genome sequencing has made invaluable contributions to evolutionary biology, medicine, and agricultural science and is rapidly being adapted to studies of organisms in their natural habitats. Such studies offer a number of unique benefits beyond those of traditional genomic studies of clonal laboratory strains.

The most obvious benefit of sequencing DNA from natural samples is the ability to access a much wider range of genomes. Many organisms fail to “reproduce in captivity” and therefore cannot be subjected to laboratory manipulation and genomic study. These include not only exotic groups (e.g. Nanoarchaeota), but many close relatives of cultivable microbes. Others species are extinct, and therefore cannot provide clean material for DNA isolation – most notably, ancient hominids such as the Neanderthals which may soon be the target of their own “human genome project.”

A less immediately apparent advantage of this technique is the ability to capture the genomic diversity within a natural population. While DNA sequence from a clonal strain is easier to generate and assemble, an individual genome represents a single snapshot of the population from which it derives. Both clonal strain sequencing and environmental studies reveal that there can be substantial variation in gene content, gene order and nucleotide sequence even within populations thought of as a single species⁸⁴⁻⁸⁶. Sequence from natural samples reflects this variation and reveals the prevalence of specific subgroups.

By offering access to genomes of hard-to-study organisms, environmental genomics and its offshoots have advanced our understanding of species interrelationships, environmental niche adaptation, and human evolutionary history. Technologies now

under development will continue to lower the barriers to genome sequencing, allowing the study of ever scarcer and more complex samples and vastly expanding the range of species on the genomics radar.

Definitions:

Metagenomics: the genomic analysis of assemblages of organisms. Meta- is used to indicate a collection of similar items, as in meta-analysis⁸⁷; genomics is the study of genomes.

Gram positive bacteria: Members of the Actinobacteria and Firmicutes phyla, which have a single membrane and a thick cell wall made of cross-linked peptidoglycan and therefore can be stained with the Gram staining procedure.

Phylogenetic anchoring: A technique that involves screening large-insert libraries made from environmental DNA for clones containing phylogenetic marker genes, and sequencing those clones in their entirety.

Horizontal gene transfer: The transfer of genetic material from one species to another.

Axenic: A pure culture of a single species of microorganism.

Metagenomic: A term used to describe techniques that characterize the genomes of whole communities of organisms rather than individual species.

whole-genome shotgun: An approach to genomic sequencing that involves breaking the DNA up into small pieces and cloning them into vectors, then sequencing clones at random.

Biofilm: A layered aggregate of microorganisms.

density gradient: A solution in which the concentration of the solute is lowest at the top and gradually becomes more dense as it gets deeper.

differential lysis: A technique that uses conditions that will only lyse certain cells so that the DNA from those cells can be isolated from other cells in a community.

pulsed-field electrophoresis: The use of pulsed electric fields of alternating polarity to separate large fragments of DNA.

Flow cytometry: A technique that measures the fluorescence of individual cells as they pass through a laser beam in an individual stream.

fluorescence in situ hybridization (FISH): A technique that uses fluorescently labeled DNA probes that hybridize to cellular DNA or RNA to label individual cells that can be examined under a microscope.

affinity purification: A means of purifying cells or molecules based on specific binding to a protein or other molecule that has been immobilized on a solid substrate like beads or a column.

Isothermal strand displacement: A DNA amplification technique using rolling circle amplification with phi29 DNA polymerase to generate large quantities of DNA without thermal cycling.

Pyrosequencing: a DNA sequencing technique that relies on detection of pyrophosphate release upon nucleotide incorporation rather than chain termination with dideoxynucleotides.

Contig: a continuous stretch of DNA sequence assembled from multiple independent sequencing reads.

Methanogens: a group of hydrogen-consuming Archaea that generate methane by reduction of carbon dioxide.

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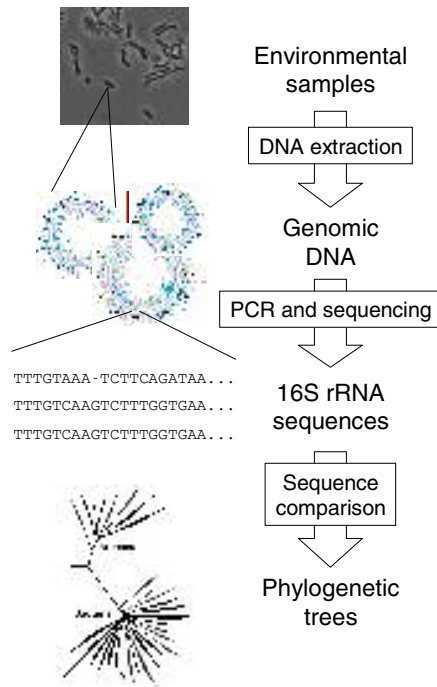
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Online links:

ARB: <http://www.arb-home.de/>

Ribosomal Database Project: <http://rdp.cme.msu.edu/index.jsp>

Box 1: 16S rRNA analysis of microbial communities



In the 1980s environmental microbiologists realized that only a small fraction of the microscopically observable organisms in a sample were capable of colony formation. Pioneering experiments by Norman Pace and colleagues revealed, through ribosomal RNA (rRNA) sequencing, that these “unculturable” microbes represented novel species often only distantly related to known, cultured lineages^{14,88,89}. An rRNA sequence can serve as a unique molecular “bar code” to identify an organism and place it in an evolutionary context, providing a first glimpse into the broad diversity invisible to culture-based approaches. The labor-intensive methods initially used, such as direct sequencing of isolated 5S rRNA or screening of genomic libraries prior to sequencing, were eventually supplanted by PCR-based methods. This is because well-conserved sequences that participate in secondary structure formation can be targeted for amplification by “universal” primers to generate clone libraries^{90,91}.

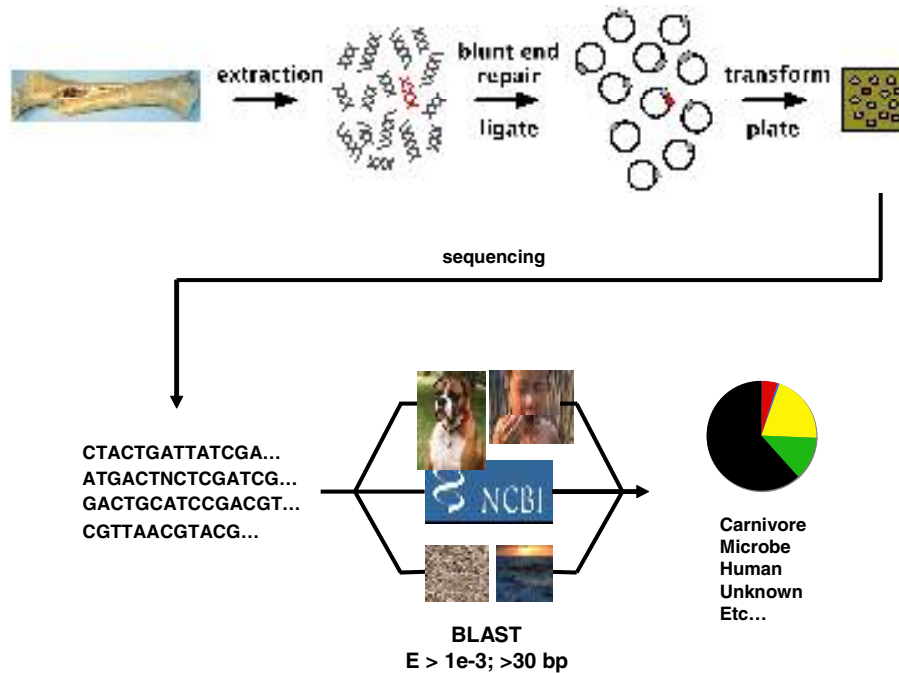
In these studies, DNA is extracted directly from an environmental sample such as ocean water, soil, or a biofilm, and the 16S genes of the community microbes are then amplified from the mixed genomic DNA using PCR (for review, see⁹² or⁹³). The PCR products are cloned into vectors and sequenced, producing rRNA “signatures” for the microbes that were present in the sample. Comparison of these sequences against databases of 16S ribosomal RNA genes allows them to be phylogenetically classified. The frequencies of particular SSU rRNA clone sequences provide a rough preliminary estimate of the community structure, as sequences from dominant community members should be more abundant. In some cases, the presence of SSU rRNA sequences from specialized clades such as **methanogens** can suggest functional activities as well. The downside, however, is that even species that are closely related based on SSU rRNA

sequence can have very different lifestyles, and the phylogenetic position of organisms with no cultured close relatives frequently offers little to no insight into their phenotypic characteristics.

16S rRNA genes have been amplified, cloned and sequenced from thousands of distinct environmental niches, yet these surveys routinely continue to identify unique new bacterial and archaeal taxa. Tools (such as ARB, <<http://www.arb-home.de/>>, and EstimateS <<http://purl.oclc.org/estimates>>) and databases (such as the Ribosomal Database Project, <<http://rdp.cme.msu.edu/index.jsp>>) have been developed to manage and analyze this flood of data.

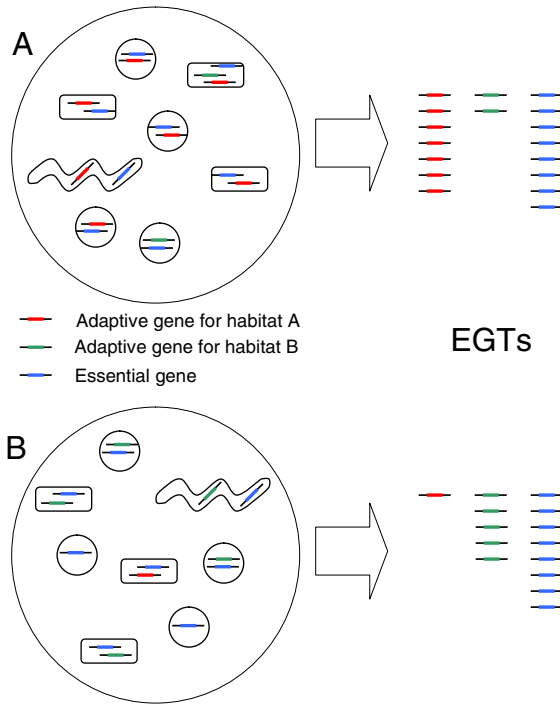
Table: Assembled genomes of uncultivated microbes				
Genome	Size	Host / Habitat	Separation technique	Reference
<i>Treponema pallidum</i>	1.1 Mb	Human, rabbit	Dissection, differential lysis	20
<i>Rickettsia prowazekii</i>	1.1 Mb	Human, chicken	Differential centrifugation	21
<i>Mycobacterium leprae</i>	3.3 Mb	Human, armadillo	Gradient centrifugation	22
<i>Tropheryma whipplei</i>	0.9 Mb	Human	Differential centrifugation	23
<i>Buchnera aphidicola</i> sp. APS	0.6 Mb	Aphid (A. pisum)	Dissection, differential lysis, filtration	24
<i>Buchnera aphidicola</i> sp. Sg	0.6 Mb	Aphid (S. graminum)	Gradient centrifugation	25
<i>Wigglesworthia glossinidia brevipalpis</i>	0.7 Mb	Tsetse fly (G. brevipalpis)	Dissection, differential lysis	26
<i>Blochmannia floridanus</i>	0.7 Mb	Carpenter ants	Differential lysis	27
<i>Buchnera aphidicola</i> sp. BBp	0.6 Mb	Aphid (B. pistaciae)	Differential lysis, filtration	28
<i>Wolbachia pipientis</i> wMel	1.27 Mb	Fly (D. melanogaster)	Differential lysis, pulsed-field electrophoresis	29
<i>Wolbachia pipientis</i> wAna	1.4 Mb	Fly (D. ananassae)	None	30
<i>Wolbachia pipientis</i> wBm	1.1 Mb	Parasitic nematode worm (B. malayi)	BAC library screening	31
<i>Phytoplasma asteris</i> , line OY-M	0.9 Mb	Plants and leafhoppers	Differential lysis, pulsed-field electrophoresis	32
<i>Nanoarchaeum equitans</i>	0.5 Mb	Ignicoccus sp. coculture	Differential centrifugation	59
<i>Ferroplasma acidarmanus</i> type II	1.8 Mb	Acid mine biofilm	None	45
<i>Leptospirillum</i> sp. Group II	2.2 Mb	Acid mine biofilm	None	45
<i>Burkholderia</i> sp.	~8.8 Mb	Sargasso Sea	Filtration	4
<i>Shewanella</i> sp.	~5 Mb	Sargasso Sea	Filtration	4
<i>Shewanella</i> sp.	~5 Mb	Sargasso Sea	Filtration	4

Figure 1: Ancient DNA sequencing



Genomic sequence of extinct organisms can be obtained from the DNA in ancient remains such as bone. Bones are first milled into powder, then immersed in a solution to extract the DNA. The damaged ends of the DNA molecules are then repaired enzymatically and cloned into a sequencing vector. The clones are then sequenced according to standard protocols, and probable species of origin determined by BLAST. In the study by Noonan *et al.*⁴³, up to 5% of the clones found their closest match in the dog genome, a carnivore closely related to bears. Only a few (~0.05%) of the reads were of human origin, while 10-20% only had significant matches to environmental sequences.

Box 2: Environmental Gene Tags



Each organism in a community has a unique set of genes in its genome; the combined genomes of all the community members make up the metagenome. Essential genes are present in each individual genome, regardless of environment, and will thus occur frequently in the metagenome. Among nonessential genes, those that are adaptive for a particular niche will appear in the genomes of many organisms in that environment, while those that are not adaptive may appear at low abundances.

Environmental Gene Tags (EGTs) are short sequences from the DNA of microbial communities that contain fragments of functional genes. Each EGT derives from a different member of the community, but genes that are important for survival and adaptation will be present in many genomes (possibly in more than one copy) and will therefore appear repeatedly in the EGT data. When the gene abundances in the EGT data are compared between environments, genes that are adaptive in only one context are more abundant in that environment.