The Rare Bacterial Biosphere

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Abstract

All communities are dominated by a few species that account for most of the biomass and carbon cycling. On the other hand, a large number of species are represented by only a few individuals. In the case of bacteria, these rare species were until recently invisible. Owing to their low numbers, conventional molecular techniques could not retrieve them. Isolation in pure culture was the only way to identify some of them, but current culturing techniques are unable to isolate most of the bacteria in nature. The recent development of fast and cheap high-throughput sequencing has begun to allow access to the rare species. In the case of bacteria, the exploration of this rare biosphere has several points of interest. First, it will eventually produce a reasonable estimate of the total number of bacterial taxa in the oceans; right now, we do not even know the right order of magnitude. Second, it will answer the question of whether "everything is everywhere." Third, it will require hypothesizing and testing the ecological mechanisms that allow subsistence of many species in low numbers. And fourth, it will open an avenue of research into the immense reserve of genes with potential applications hidden in the rare biosphere.

INTRODUCTION

All communities comprise a large number of species. A few of these species are very abundant, others are moderately abundant, and a large number are represented by very few individuals. To better understand and analyze this fact, ecologists have developed a series of tools: collectors curves (**Figure 1**), rank-abundance curves (**Figure 2**), species-area relationships, and so on. Why do these relationship have that particular shape? Is it a trivial consequence of some mathematical rule? Or does it reflect some important property of how communities are assembled? Do differences among curves reveal something important about the functionality of different communities?

These questions have intrigued ecologists for over a century and are still a matter of intense research (Magurran 2004, McGill et al. 2007). Particularly intriguing are the questions of how and why so many species become rare, considering the evolutionary disadvantages of having small population sizes (Rabinowitz 1981, Magurran & Henderson 2003). The most obvious disadvantage is the difficulty of finding a mate. Effectively, this creates a lower viable limit of abundance for species below which potential partners will not meet and, therefore, the population becomes destined to extinction. Bacteria, however, do not need to find a partner to reproduce. Under favorable conditions, a single cell can grow exponentially and fill the ecosystem with its replicas. Thus, the number of rare species among bacteria may be very large.

This intuition is behind the famous Baas Becking statement that "everything is everywhere, but the environment selects" (de Wit & Bouvier 2006). In principle, because of their small size and large numbers, microorganisms will be easily distributed widely and thus will be present everywhere (Finlay 2002). Their local abundance will depend on the conditions of the particular ecosystem, but many different species will be present at very low abundance. This fascinating world of rare species recently received the name of rare biosphere (Sogin et al. 2006). Since the term was coined, the number of papers citing it has increased exponentially. At a recent International Society for Microbial Ecology (ISME) meeting, it was one of the topics used to group communications, and a growing number of scientists are using the concept as a framework to place their work. I believe there are two things that make the concept attractive: first, the realization that, in the case of microbes, being rare has very interesting ecological and evolutionary consequences (Pedrós-Alió 2006, 2007), and second, the appearance of novel sequencing technologies that allow the effective experimental study of these rare bacteria (Sogin et al. 2006).

METHODOLOGICAL ISSUES

In a previous review (Pedrós-Alió 2006), I contrasted the standard molecular methods with the culturing approach. Although isolation in pure culture can retrieve both rare and abundant bacteria from an ecosystem, most of the members of the community are difficult to culture. Conventional molecular methods, on the other hand, are very good at retrieving the abundant members of the community, but cannot find the rare ones. The only possible way to access these least abundant microbes using molecular methods is to increase the number of sequences by several orders of magnitude.

In the past few years, a revolution in sequencing technology is making this increase possible (see **Figure 3**). Fingerprinting techniques retrieve the 10–50 most abundant taxa (Muyzer et al. 1993, Casamayor et al. 2000). Usually, these more abundant taxa represent more than 0.1%–1% of the total cell counts. Conventional clone libraries can go up to 100 taxa if a large number of clones (approximately 1,000) are examined (Acinas et al. 2004, Hong et al. 2006, Pommier et al. 2007), and metagenomics approaches can further increase this number, reaching a few thousand taxa (Venter et al. 2004, DeLong et al. 2006, Rusch et al. 2007). The advantage of the latter

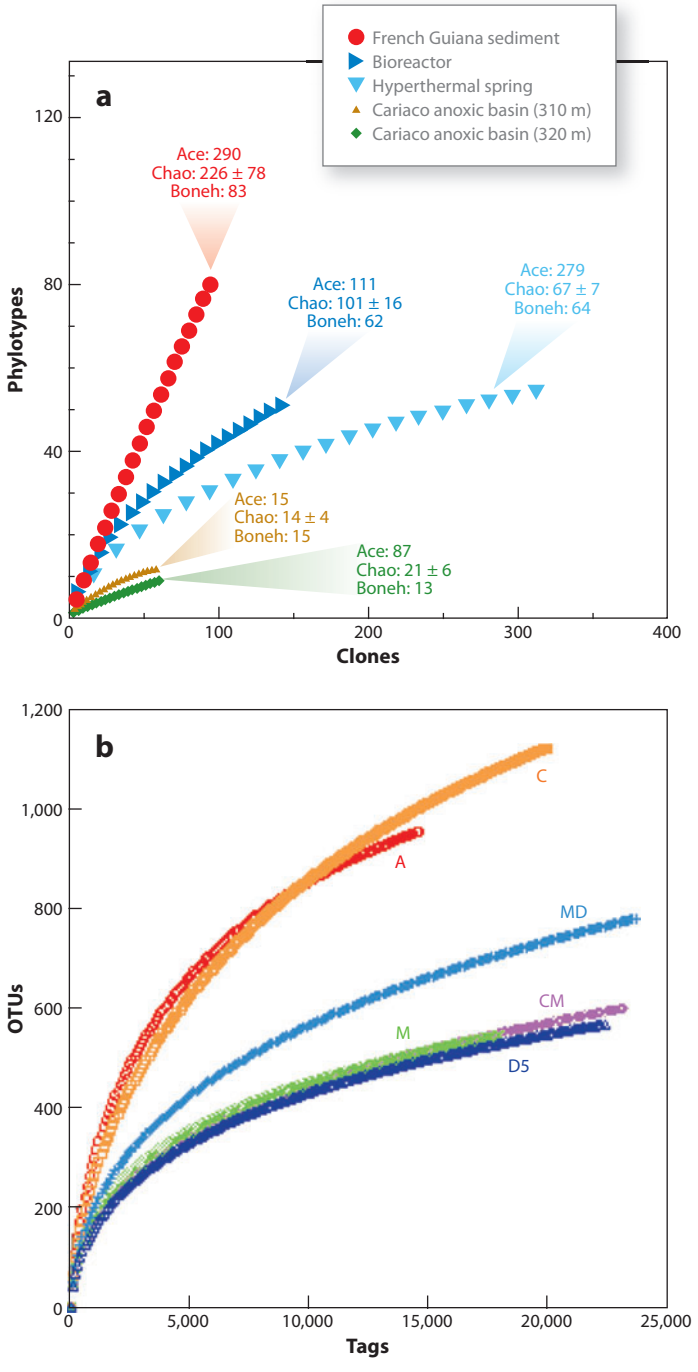

Figure 1

Collectors curves for bacteria obtained from (*a*) conventional clone libraries and (*b*) 454 pyrosequencing data. OTU, operational taxonomic unit; A, C, CM, M, MD, and D5 denote the stations sampled along a surface transect in the original study, from the shore (A) to the open Mediterranean Sea (D5). Panel *a* adapted from Kemp & Aller 2004; panel *b* adapted from Pommier et al. 2010.

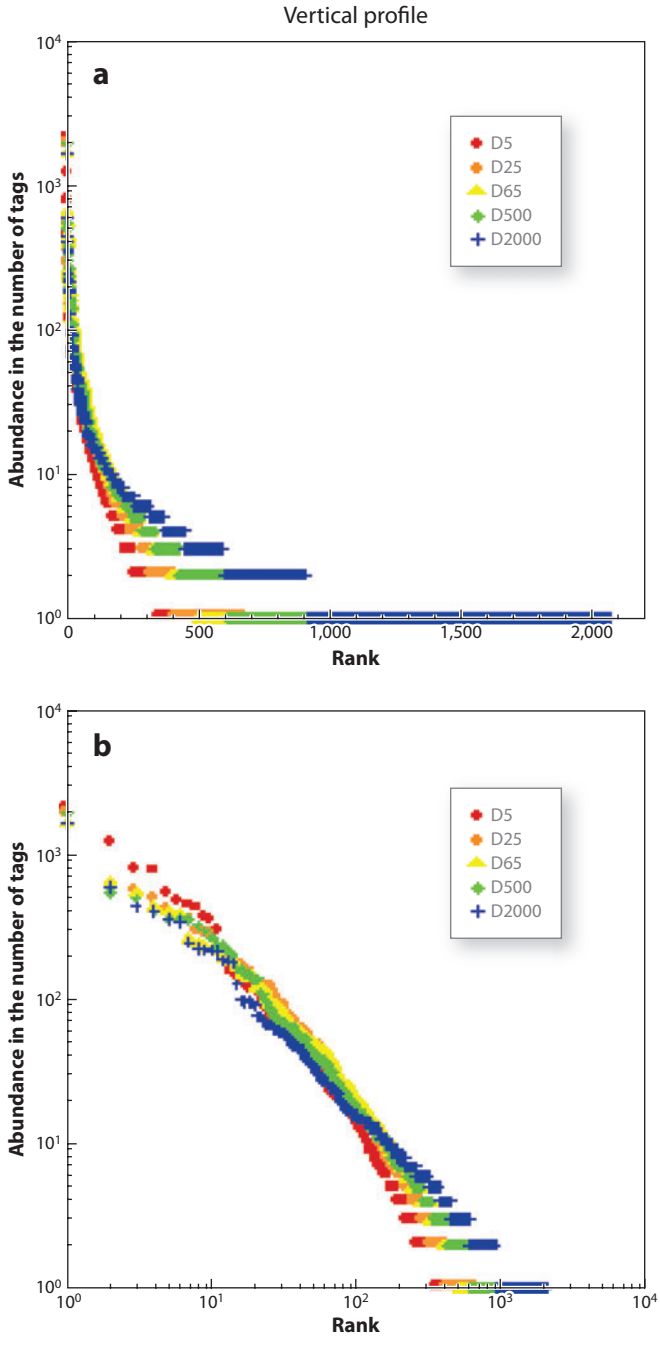

Figure 2

Rank-abundance curves for bacteria obtained with 454 pyrosequencing, shown with (*a*) a conventional semilog plot and (*b*) a clearer log-log plot. Colors denote different sampling depths from station D in the original study. Adapted from Pommier et al. 2010.

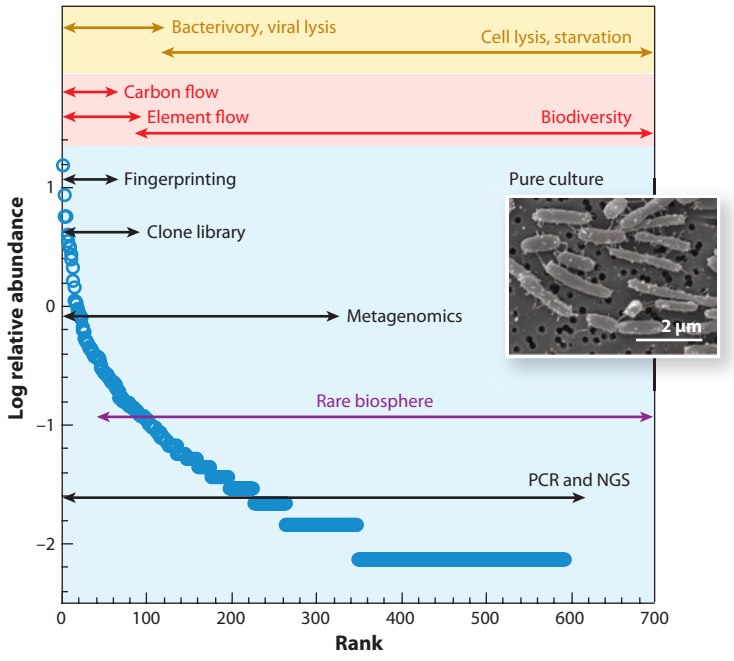

Figure 3

Rank-abundance curve for bacteria, showing the loss factors relevant in each part of the curve (*gold*), the ecological relevance (*red*), and the depth of sampling that different molecular techniques allow (*black*). The extent of the rare biosphere is shown in purple. (*Inset*) The scanning electron microscopy picture of the *Dokdonia* MED152 strain is shown to stress that sequences are only an indicator of the organisms and that isolation in pure culture is the ultimate objective to study bacterial diversity. Abbreviations: NGS, next-generation sequencing; PCR, polymerase chain reaction.

approach is that it does not involve a polymerase chain reaction (PCR), so that PCR's biases are avoided and the relative abundances of different reads are likely closer to their abundance in natural samples. On the other hand, most reads do not belong to the small subunit (SSU) ribosomal DNA (rDNA). These other genes are essential to study functions in the ecosystem but are not adequate to study diversity. Thus, an enormous number of sequences are needed to extract the taxonomically relevant ones.

Finally, the next-generation sequencing (NGS) approaches have reached past the 10,000 mark (Sogin et al. 2006, Huber et al. 2007, Caporaso et al. 2011). One of these approaches was selected for the International Census of Marine Microbes (ICoMM) initiative (Amaral-Zettler et al. 2010), and will be discussed in more detail below. The protocol introduces a PCR step with universal primers against SSU rDNA before the massive parallel sequencing. Thus, all sequences will be taxonomically relevant at the cost of introducing the usual PCR biases. We still do not know how far we are from characterizing the whole rare biosphere, but these improvements in technology promise to eventually explore the whole biodiversity of microorganisms.

A different kind of methodological consideration concerns the limitations of the NGS methods. All of these methods provide extremely large numbers of very short to moderately long fragments. Usually, the larger the number of sequences provided, the shorter the reads. To cite two extremes as examples, Illumina produces thousands of millions of reads in parallel, but their length is very

short [approximately 75 base pairs (bp)], whereas 454 pyrosequencing "only" generates one or a few million reads, but their length may currently reach 400 bp.

To determine the identity of the reads, length is the limitation. One would need the whole SSU ribosomal RNA (rRNA) sequence to properly identify a read. Even this long sequence does not guarantee identification to the species level; studies have repeatedly shown that SSU rRNA underestimates species diversity (Rosselló-Mora & Amann 2001, Orsini et al. 2004), although it at least provides all the information available from this molecule. Obviously, 75 bp is an extremely short sequence, and many times it will be possible to identify the read only as a bacterium, or as a flavobacterium, which is clearly not satisfactory. For this reason, 454 pyrosequencing has been favored for diversity studies (Rothberg & Leamon 2008). The length of reads currently provided (400 bp) approaches the length of denaturing gradient gel electrophoresis (DGGE) sequences (approximately 550 bp) and begins to be reasonably good. Undoubtedly, the lengths of the reads provided by the different technologies will keep increasing.

This technology does have its own problems, however. The main one is the sequencing errors. The error rate of the technique is very low and is not a problem for metagenomic or genomic studies. But when trying to determine the taxon richness of an environmental sample, one wants to know whether that unique read that has appeared only once in the data set is the result of a sequencing error (and thus must be discarded) or corresponds to a true different taxon that is so rare in the sample that it has appeared only once (and thus must be added to the taxon count). This problem has generated a lively debate and has stimulated the development of different processing methods to try to minimize the number of erroneous reads (Huse et al. 2007, 2008, 2010; Huber et al. 2009; Quince et al. 2009, 2011; Reeder & Knight 2009, 2010; Engelbrektson et al. 2010; Kunin et al. 2010). There are efforts to try to implement pipelines incorporating these methods that will allow users to process their sequences online (Schloss et al. 2009, Sun et al. 2009, Caporaso et al. 2010, Quince et al. 2011).

If the purpose of the study is not to determine the taxon richness of the sample, then one can simply eliminate the reads appearing only once, or twice, or a few times. The number of sequences remaining is still so large that diversity studies or comparisons among communities can be carried out without problems (see, for example, Kirchman et al. 2010). One option that most researchers take is to do some kind of clustering, frequently clustering together reads that differ by less than 3% of the sequence in an operational taxonomic unit (OTU) (for example, Galand et al. 2009). Many of the reads appearing only once in the data set will likely correspond to abundant taxa with one or a few badly called bases. Clustering will incorporate them to the correct OTU and alleviate the problem (Huse et al. 2010).

The final methodological question is how far we are from identifying all the microbial taxa living in the oceans. Early work based on conventional clone libraries has estimated that the number of bacterial taxa in the oceans would be a few thousand (Hagström et al. 2002). Working from some theoretical assumptions, Curtis et al. (2002) came up with an estimate of 1 million bacterial taxa for the whole ocean. Using a different line of reasoning, Dykhuizen (1998) estimated 100 million bacterial taxa for the whole planet. Obviously, the uncertainty of these estimates shows our current ignorance. Experimentally, the number of taxa found depends on two items: the number of sequences obtained and the technique being used. As more and more sequences are obtained from a sample, the number of new taxa increases. At a certain point the rate of increase starts to abate, and eventually the collectors curve reaches a plateau; at this point all the taxa in the sample have been retrieved. In reality, collectors curves for microbes rarely reach a plateau, indicating that many taxa are still unknown (**Figure 1**). Different statistical indices have been developed to estimate what this plateau would be if enough sequences were retrieved. Interestingly, the technique used has an influence on this estimate. Thus, through the use of conventional clone

libraries, these indices provide estimates approximately one order of magnitude lower than the same indices calculated with 454 pyrosequencing data (compare **Figures 1*a*** and **1*b***). It is wise to take all estimates with a grain of salt.

This question has another element to consider, which is the number of different marine areas that need to be sampled in order to retrieve all the taxa in the oceans. If it were true that everything is everywhere, then it would be enough to sample from the pier next to the lab and just keep sequencing 454 runs until the collectors curve saturated. If that assumption is not true, however, then we need to sample all the different ecosystems in the oceans. Two major efforts to sample the oceans have been the Global Ocean Sampling (GOS) expeditions (**http://www.jcvi.org/cms/research/projects/gos/overview**) and the ICoMM initiative (**http://icomm.mbl.edu**). The GOS expeditions sampled surface waters around the Atlantic, Pacific, and Indian Oceans as well as, more recently, the Southern Ocean and interior seas such as the Baltic and the Mediterranean. The ICoMM initiative put together samples from approximately 50 different research groups and processed them with the same protocol.

Because of its relevance and dimensions, the ICoMM initiative merits special attention. This was one of the projects within the Census of Marine Life (CoML), whose aim was to determine the diversity of life in the oceans considering three different aspects: an inventory of the known diversity, an estimate of the unknown diversity, and an estimate of that part of the unknown that was knowable in the short run (Yarincik & O'Dor 2005). If these objectives were ambitious in the case of marine animals, the challenge in the case of microorganisms was formidable. The knowable was only a few thousand phylotypes of bacteria, a few hundred phylotypes of archaea, and approximately 200,000 morphospecies of protists (microbial eukaryotes). The available techniques were insufficient, and the perception was that a large diversity remained in the unknown and, worse, was unknowable. Sogin et al. (2006) demonstrated the potential of PCR and NGS to do the job of mapping the microbial diversity in the oceans. Based on this approach, funds were secured to carry out a global study engaging microbial ecologists from around the world to analyze the diversity of a very large set of samples. Five years later, the legacy of this initiative is one of the most robust data sets of marine microbial diversity (Amaral-Zettler et al. 2010).

There has been considerable discussion about its shortcomings. As mentioned earlier, the initial PCR to obtain only SSU rDNA will introduce the usual PCR biases. Second, the 454 technology available at the time was able to provide only short reads, of approximately 100 bp. This limitation forced the choice of the V6 region of the rRNA as target. This region is hypervariable, thus providing maximal phylogenetic information for a short fragment, and it is flanked by conserved regions against which appropriate 20-bp universal primers could be designed. The short, taxonomically informative fragments obtained, however, were only approximately 60 bp; therefore, taxonomic assignment was many times impossible. Many sequences could be identified only to class level, or even to domain level in some cases. Third, the 454 technology generated millions of sequences, but many were singletons or doubletons, and an unknown proportion were likely sequencing errors. This last point was critical for the objective of determining diversity: It was essential to know whether a given sequence was an artifact or a new taxon.

Despite these limitations, it can safely be said that the field has changed dramatically thanks to the ICoMM initiative. There is no comparable data set, with over 18 million sequences from around the world's oceans, obtained with exactly the same protocols and curated with the same bioinformatics pipeline. A summary of the first results can be found in Amaral-Zettler et al. (2010) as well as at the ICoMM Web site and the VAMPS (Visualization and Analysis of Microbial Population Structures; **http://vamps.mbl.edu**) and MICROBIS (Microbial Oceanic Biogeographic Information System; **http://icomm.mbl.edu/microbis**) database sites. Several papers have been published analyzing particular data subsets, but the information contained in the global data set

will take years to extract. Some examples can be found in Agogué et al. (2011), Galand et al. (2010), and Gilbert et al. (2009). Despite the methodological difficulties mentioned above, a conservative estimate of the number of bacterial taxa in a liter of seawater is 20,000 (Amaral-Zettler et al. 2010). At the moment, it is not possible to extrapolate to the whole ocean. ICoMM has opened an avenue of research into both the abundant and the rare biospheres that has now taken several paths, with different NGS technologies, different sections of the SSU rDNA, and different bioinformatics pipelines (Caporaso et al. 2010).

Both the GOS cruises and ICoMM have increased our knowledge of marine microbial diversity enormously. Yet the sampled points are a minuscule percent of the oceans. Currently, at least two circumnavigation programs are collecting samples for diversity studies. Tara Oceans (**http://oceans.taraexpeditions.org**) is a three-year tour around the world collecting samples from the surface to 200 m, including samples from all the major ocean basins. Malaspina 2010 completed its circumnavigation in July 2011, collecting samples from the deep oceans (Laursen 2011). Large programs such as these will help saturate the collectors curves for marine microorganisms in a few years (Pedrós-Alió 2011). Scientists reviewing these issues in five years will likely be able to provide, for the first time, a reasonable estimate of the total number of microbial taxa in the oceans.

THEORY AND EVIDENCE

This section contains some of the ideas presented in Pedrós-Alió (2006), modified by the findings and discussions that have taken place in the past few years. To minimize repetition, the essential characteristics expected from rare and abundant taxa are summarized in **Table 1** and **Figure 3**. In the remaining text, I will illustrate the different aspects with examples from the recent literature.

Active Bacteria

The abundance of any given bacterium is the result of a balance between its growth rate and its loss factors. For planktonic bacteria, the main loss factors are predation and viral lysis. Very close to the surface, solar radiation—and particularly UV radiation—can also cause cell death. Finally, there are at least two physiological mechanisms that, under certain circumstances, cause the death of the cell. One of them is programmed cell death (Chaloupka & Vinter 2010); the relevance of this in natural bacterioplankton communities is unknown. The other is prolonged starvation; if this state persists long enough, the cell will run out of reserves, will not be able to carry out the basic metabolism of maintenance, and will die.

All bacterial taxa must grow at some point in time and at some place in space. Otherwise, the different loss mechanisms would make them eventually disappear. Growth is indispensable to compensate for losses. All the taxa found in the rare biosphere must therefore have been growing in the past, currently be growing at a different place, or both. Therefore, one objective of studying the rare biosphere is to determine where in time and/or space its members grow.

One reason to be a member of the rare biosphere is that present environmental conditions are unfavorable for growth. However, favorable conditions existed in the past and will likely appear again in the future. The most obvious instance of this situation is the seasonal cycle. For example, the same dominant populations have been found year after year on the Pacific coast (Fuhrman et al. 2006). These populations dominated the assemblage during a period of time and then declined in the abundant biosphere, so that they became undetectable by the fingerprinting technique used. Presumably, NGS would allow all the populations to be found year-round.

Table 1 Characteristics expected of abundant and rare bacteria

Property	Abundant bacteria	Rare bacteria
Contribution to ecosystem		
Abundance	≥1%	≤0.1%
Biomass	Major	Minor
Biodiversity	Minor	Major
Carbon flow	Major	Minor
Nutrient cycling	Major	Significant in some cases
Growth rate	High	Low
Losses and resistance		
Predation	High	Low
Predation resistance	Probably not	Passive (small size, palatability)
Viruses	High	Absent
Virus resistance	Some	No (betaproteobacteria?)
UV radiation	Intense at surface	Intense at surface
UV pigments and repair	Variable	Variable
Starvation	No	Yes
Starvation resistance stages	No	Some
Detectability		
Fingerprinting	Yes	No
Clone libraries	Yes	0–10
Metagenomics	Yes	100–1,000
PCR and NGS	Yes	Up to 10,000
Culturing	Some	Some

Abbreviations: NGS, next-generation sequencing; PCR, polymerase chain reaction.

However, many other options are possible. Some bacteria may be adapted to use only a few specific substrates that are present only at certain times, or to episodic situations of high nutrient abundance. One example was studied by Teira et al. (2007). These authors incubated natural bacterial assemblages in microcosms with different amendments of polycyclic aromatic hydrocarbons (PAHs). These additions were designed to mimic an oil spill. The abundance of different bacterial groups was followed by CARD-FISH (catalyzed reporter deposition–fluorescence in situ hybridization) as the PAHs were degraded by the natural assemblage. One of the populations followed was that of *Cycloclasticus*, a hydrocarbon-degrading gammaproteobacterium. This bacterium was not detectable by FISH in the original assemblage, but it became detectable and even formed a peak of abundance during the experiments. Once the PAHs had disappeared, the *Cycloclasticus* population disappeared again (**Figure 4**). Further, the size of the peak was proportional to the initial concentration of PAHs added, and the increase in bacteria coincided exactly with the decrease in PAHs. This provides a clear example of a member of the rare biosphere that, when conditions become favorable, grows, becomes an abundant member of the community while the resource in which it is specialized is consumed, and then is culled down by loss processes back to the rare biosphere.

This kind of situation likely occurs constantly for many different bacteria. The rank-abundance distribution must be a dynamic one, with members of the abundant part decreasing toward the rare biosphere and vice versa, as environmental conditions change both regularly (as with seasons) and episodically (as with heavy rains or storms). Evidence of this dynamism is provided by comparing

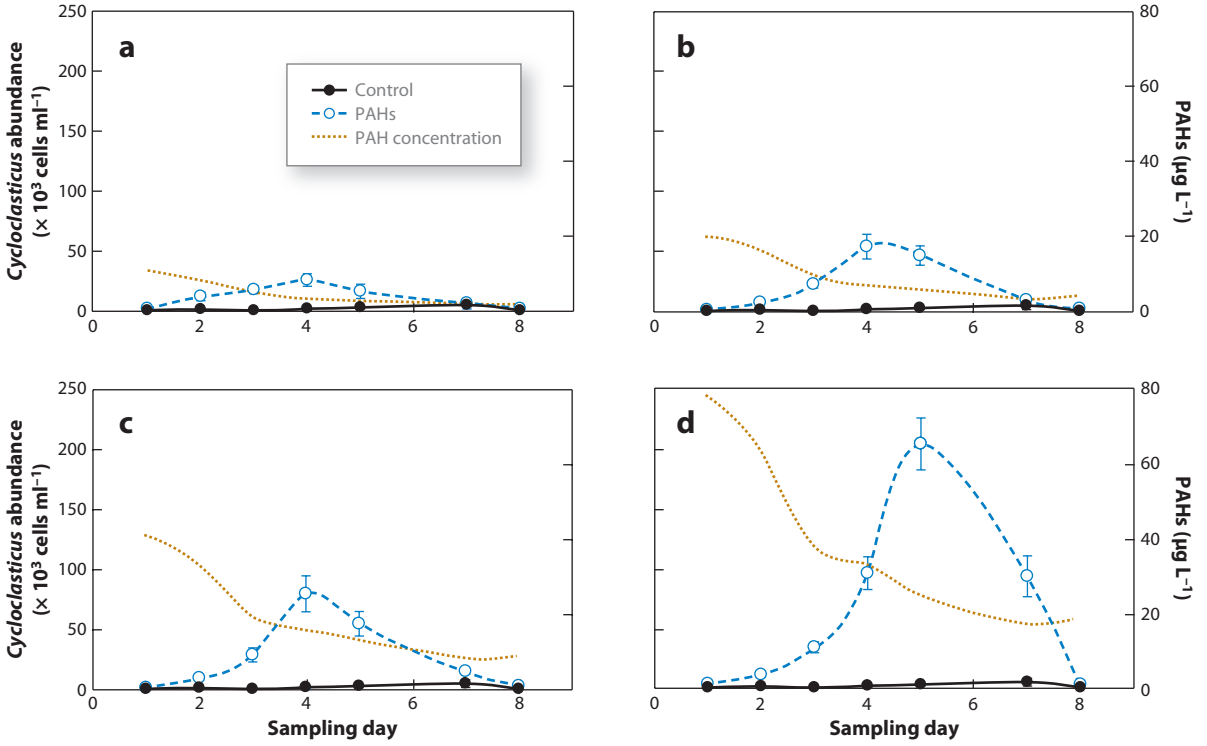

Figure 4

Microcosm experiments in which different amounts of polycyclic aromatic hydrocarbons (PAHs) were added and the abundance of *Cycloclasticus* was followed in microcosms amended with (*a*) 10, (*b*) 20, (*c*) 40, and (*d*) 80 mg of PAHs per liter. Counts with fluorescence in situ hybridization (FISH) are shown in the microcosms amended with PAHs (*blue*) and in controls without amendments (*black*). Adapted from Teira et al. 2007.

the abundance of different OTUs in terms of DNA with that same abundance in terms of rRNA. Because the number of ribosomes per cell is generally proportional to growth rate, the proportion between tags from RNA and tags from DNA will be an indication of how active a particular OTU is. Jones & Lennon (2010) published a comparison of the two in some freshwater lakes (**Figure 5**), and Campbell et al. (2011) showed the same relationship in a study of Chesapeake Bay during three seasons. The latter study is particularly revealing. Over half of the taxa cycled between being abundant and rare, and 6% always remained rare. In general, growth rates declined as the abundance of an OTU increased, leading the authors to think that the community was dominated by defense specialists rather than growth specialists.

What determines whether a particular taxon may be abundant? In principle, bacteria that are growing actively should be abundant, whereas bacteria that are growing slowly or not growing should be rare. Different SAR11 clades are the most abundant heterotrophic bacteria in the oceans (Morris et al. 2002). In the ICoMM data set, Amaral-Zettler et al. (2010) found that two particular SAR11 phylotypes were the two most abundant ones in the global ocean. Although doubling times of the SAR11 clade member *Pelagibacter ubique* in the lab are of the order of 1–2 days (Rappé et al. 2002), their growth rates in the sea are comparable to, or larger than, those of the global bacterial assemblage (Malmstrom et al. 2005). Thus, SAR11 seems to confirm that abundant taxa grow actively.

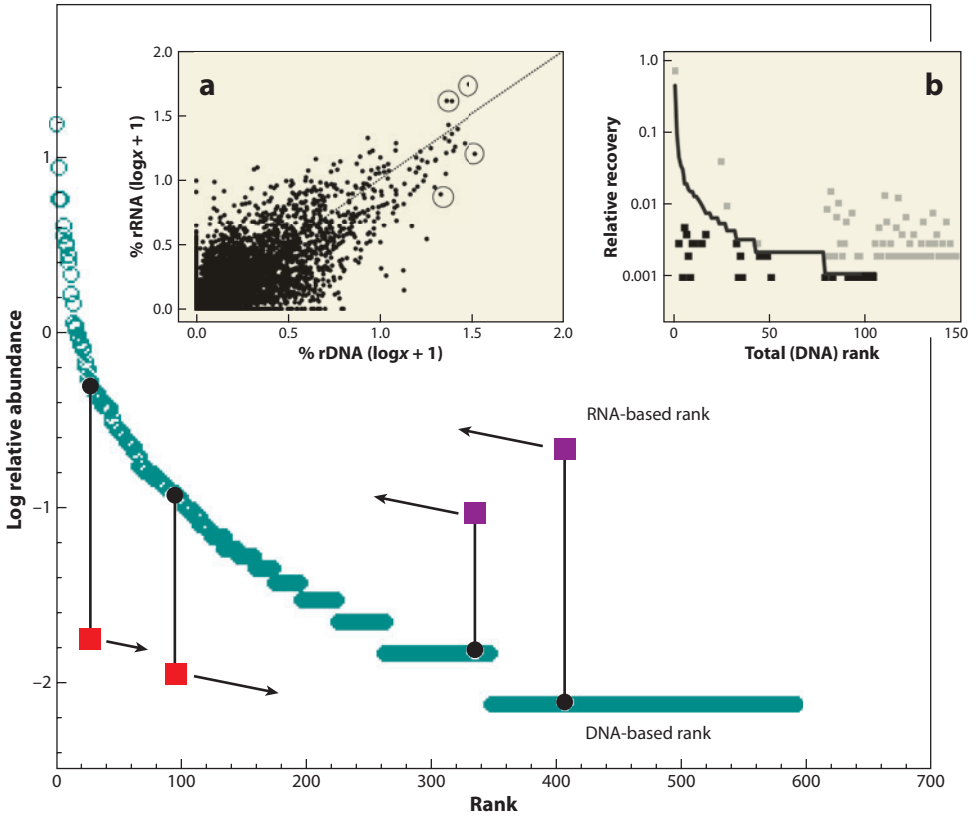

Figure 5

The same rank-abundance curve shown in **Figure 3**. Most operational taxonomic units (OTUs) are similarly represented in both DNA- and RNA-based estimates of their abundance, as indicated in inset panel *a* (adapted from Campbell et al. 2011). However, some OTUs are clearly above or below the 1:1 line. In inset panel *b* (adapted from Jones & Lennon 2010), the OTUs have been ranked according to their DNA-based estimates of abundance (curve). Some OTUs (*black squares*) were underrepresented in RNA-based estimates, while others (*gray squares*) were overrepresented. As examples, four OTUs are shown in the main panel, ranked according to their DNA-based estimates of abundance (*black circles*); the corresponding RNA-based estimates are also shown for four OTUs as examples (*squares*). The two OTUs overrepresented in RNA (*purple squares*) likely correspond to populations that have started to grow actively but have not yet reached a large abundance. Eventually, their ranks in the DNA curve will move to the left. The two OTUs underrepresented in RNA (*red squares*) likely correspond to populations that are not growing anymore and are being culled down by loss factors. Eventually, they will move to the right. The *Cycloclasticus* shown in **Figure 4** would appear like the purple OTU at the beginning of the microcosm experiments and like the red OTUs when its substrate was exhausted.

Showing the opposite, that the rare bacteria are not growing actively or are dormant, is more complicated experimentally. That at least some bacteria are dormant in the sea is proven, for example, by the presence of *Desulfosporosinus* spores in Arctic sediments (see below). But determining the extent of this situation is difficult. Jones & Lennon (2010) discussed this possibility and developed a model of the effects of dormancy on the diversity of natural bacterioplankton communities.

This does not exclude the possibility that some rare bacteria may be actively growing. This intriguing scenario was investigated by Bouvier & del Giorgio (2007). They incubated natural samples of bacterioplankton in bottles with virus-free seawater and with virus-containing seawater,

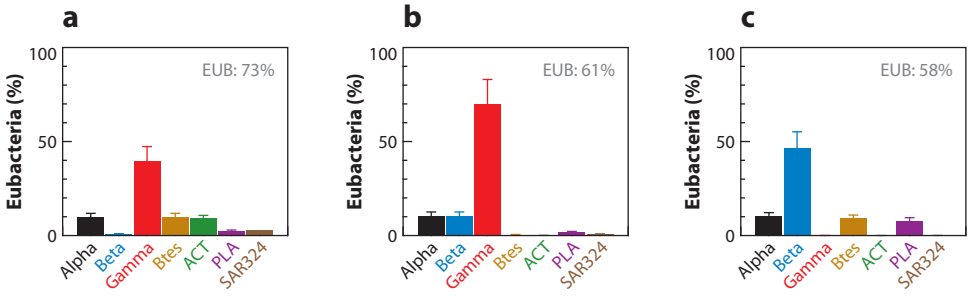

Figure 6

(*a*) In situ bacterioplankton community composition in station 15 (shelf) region. (*b*) Bacterioplankton phylogenetic composition after 70 h of incubation in the presence of autochthonous viruses at ambient densities. (*c*) Bacterioplankton phylogenetic composition after 70 h of incubation in virus-depleted water (<5% of ambient density). Data are expressed as percentage of total bacteria counts. The number (as percentage) that appears at the top right of each graph represents the proportion of cells detected by the bacteria probes relative to the total number of cells in that sample. Error bars represent an average error determined for a subset of replicated samples. Abbreviations: alpha, alphaproteobacteria; beta, betaproteobacteria; gamma, gammaproteobacteria; Btes, bacteroidetes; ACT, actinobacteria; PLA, planctomycetales; SAR324, SAR324 lineage. Adapted from Bouvier & del Giorgio 2007.

and followed the composition of the bacterial assemblage using FISH. In the presence of viruses, gammaproteobacteria grew in the incubations, whereas alphaproteobacteria and bacteroidetes decreased. When the samples were incubated with virus-depleted water, however, betaproteobacteria became dominant in shelf and slope waters, whereas actinobacteria dominated the open ocean samples off the coast of Oregon (**Figure 6**). This suggests that these other groups were growing actively in the samples but did not become sufficiently abundant to be detected by FISH because of their high susceptibility to viral attack. Thus, some members of the rare biosphere may actually be quite active. Their contribution to carbon flow, however, will remain relatively small because their biomass is negligible compared with that of the abundant members of the assemblage.

Active members of the rare biosphere may indeed contribute significantly to the cycling of particular elements such as nitrogen or sulfur. For example, *Desulfosporosinus* constituted only 0.006% of the total cell count in a peat; however, owing to its high cell-specific sulfate reduction rates, it could contribute most of the sulfate reduction in the peat (Pester et al. 2010). In enrichment cultures with sulfate added, this bacterium grew to form up to 3.1% of the total cell count, showing its potential to became an important member of the community under more favorable conditions. Apparently, this bacterium is able to persist actively at very low concentrations. If the high sulfate reduction rates allow it to grow, however, some loss mechanism must be invoked for the concentrations to remain stable and low. At any rate, this is a good example that members of the rare biosphere are not necessarily inactive or dormant and that their contribution to the cycling of some elements may be important. Their contribution to carbon flow, however, is logically small compared with fermenters and methanogens in the same environment.

Members of the Rare Biosphere That Do Not Grow

Even if some of the rare bacteria are actively growing, it seems reasonable to assume that a large fraction of the rare biosphere is made up of nongrowing cells. In the following sections I examine some cases for which there is experimental evidence, but there certainly must be several other possibilities as well.

Dispersal without resistance stages: on the way to extinction. Many members of the rare biosphere likely arrived from elsewhere because of the ease of dispersal of microorganisms. However, the new environment is not adequate for their growth, and thus they will be present only if the input from outside the ecosystem balances the slow loss mechanisms acting on this low-abundance bacteria. An example is the bacteria transported with dust storms from deserts to other areas. Dust was collected from the Saharan desert in Mauritania and from high mountain lakes in the Pyrenees (Hervás & Casamayor 2009, Hervás et al. 2009). Autoclaved lake water was then inoculated with these dusts from different origins, and the bacteria growing were followed. Identical *Acinetobacter* sequences were found in both enrichments, indicating that the same bacterium had been able to travel between Mauritania and the Pyrenees. Identical sequences were retrieved from another enrichment with dust collected in a high mountain lake in Sierra Nevada (southern Spain) and in a clone library from snow in the Pyrenees. Thus, a bacterium commonly found in soils but not in freshwaters was found to have traveled between the Saharan desert and European high mountain lakes. These experiments do not reveal the direction of the dispersal. However, the fact that storms frequently carry dust from Africa to Europe and not the other way around suggests that the dispersal was toward the north. Because the bacteria grew in enrichment experiments, they were obviously alive after the long trip exposed to solar radiation, dryness, and any other offensive environmental factors.

In a second set of experiments, Saharan dust was used as an inoculum in filtered lake water. If inorganic nutrients or dust were added, bacteria grew in this medium. However, these bacteria were shown to be autochthonous to the lake—that is, the dust and the nutrients provided the necessary carbon and element sources for the bacteria to grow. The dust bacteria were not able to compete and did not grow. When the water was autoclaved (not just filtered), the *Acinetobacter* from the dust grew in the enrichments. This shows that the *Acinetobacter* were alive but were not able to compete with the autochthonous microbiota of the lake. The intermittent but relatively frequent inputs of dust bacteria with storms appear to be enough to maintain the *Acinetobacter* cells in the lake at a low abundance even though they cannot grow in the lake—not because of the physicochemical environment but as a consequence of the competition from the local bacteria. This is a great example of how some members of the rare biosphere may be recruited from other environments thanks to the ease of dispersal of bacteria.

This *Acinetobacter* sequence was not found in clone libraries from either the neuston or the plankton. However, when a specific FISH probe was developed for this phylotype, it could be found in both environments in fairly low amounts (between 1% and 3% of the total bacterial count). An interesting finding was that the amount of *Acinetobacter* found in the neuston decreased with the time elapsed between a Saharan dust event and sampling, whereas no relationship was found in the water column. The most plausible interpretation of these data is that *Acinetobacter* were carried with dust from the Mauritanian Sahara to Europe and were deposited in high mountain lakes (as well as all over the place, of course). These bacteria were alive (as demonstrated in the enrichment experiments), reached the neuston, and were transferred to the plankton, where they remained throughout the year in relatively low abundance.

Dispersal and colonization of the seed bank with spores. Thermophilic bacteria had been regularly isolated from cold (between −2°C and +4°C) sediments around Svalbard. In particular, a spore-forming, sulfate-reducing bacterium, *Desulfotomaculum* spp., was isolated. This bacterium was not able to grow at temperatures below 26°C, and therefore it was not possible that the organisms had grown in situ. This was another example of "everything is everywhere, but the environment selects." Furthermore, the sea bottom experienced a sedimentation rate of 0.19 cm per year and, thus, to remain present in the surface sediment without the possibility of growth,

these thermophilic bacteria had to be imported at that precise rate to compensate for losses due to burial. It had to come from somewhere.

Hubert et al. (2009) identified several potential sources of thermophilic anaerobic bacteria in the vicinity of Svalbard: deeply buried warm sediments, sometimes containing petroleum or gas, could provide the organisms through cold-water seeps known to exist around west Svalbard. Other potential sources could be the fluids from the nearby spreading midocean ridge. In fact, Hubert et al. (2009) constructed clone libraries of the bacteria in sediments incubated at 50°C (to kill the autochthonous psychrophiles and stimulate sporulation of the thermophiles) and found that most clones were relatives of clones precisely from these kinds of warm environments. Thanks to the possession of spores, these thermophilic bacteria can travel through the cold ocean and seed the cold sediments in a large area, extending along at least 1,000 km of the Svalbard coast. They are not able to grow, but they remain viable and, therefore, can be isolated under conditions appropriate for germination of the spores. An additional point is that these thermophilic sulfate-reducing bacteria could not be amplified from the natural cold sediments by PCR with specific primers. Either the spore walls were impermeable to the probes or the abundance was too low for PCR.

Hubert et al. (2009) estimated the in situ abundance at 10^5 spores cm^{-3} (0.01% of the total bacterial count). Interestingly, the sulfate reducers did not come alone. Through different incubation experiments, the authors could show that a whole community of thermophilic fermenters were also present in the sediments. It is unlikely that the temperature of the Arctic sediments will ever reach the hot temperatures required by the thermophiles to germinate and grow. However, the presence of such a complex assemblage shows that bacterial communities have a large seed bank of genetic resources. No wonder that bacterial communities are so versatile in responding to changes in the environment.

Transport between favorable environments. Another group of taxa from the rare biosphere is that of specialists of particular environments. One example is all the bacteria living in symbiosis with marine animals, which include pathogenic, commensal, or mutualistic bacteria living in the digestive tract; mutualistic bioluminescent bacteria in light organs; fish pathogens; chemoautotrophic bacteria in the gills of deep vent worms; and many others. In their specialized habitat, these taxa are extremely abundant. Eventually, when their symbionts die, they need to move to new colonizable habitats, and this will force them to travel through the open water, where they will be totally incapable of growing. As they travel away from the source, their concentration will decrease both by simple dilution and by the progressive death of the nongrowing cells. Therefore, they will form part of the rare biosphere.

The case of *Vibrio fischeri* and the bobtailed squid (*Euprymna scolopes*) has been particularly well studied (McFall-Ngai 2008). This squid has light organs with a concentration of 10^{10} *V. fischeri* cells ml^{-1}. This bacterium is bioluminescent. The light organ is in the ventral surface of the squid and, thus, the diffuse light mimics the light coming from the moon or the stars. Thus, the squid uses the light emitted by the bacteria to camouflage itself at night, when it feeds actively in the water column. The squid actually nurtures its bacterial population. By regulating the amount of oxygen provided to the bacteria, the squid is able to determine how much light is produced. The squid also has two ways of controlling how many bacteria it has. For the bacteria to bioluminesce, it has to provide them with carbon and nutrient sources. This would cause a never-ending growth of the bacteria unless a mechanism existed to get rid of the excess cells. In effect, every day at dawn, before retiring to rest hidden in the sediments, the squid vents approximately 80% of the bacteria in the light organ into the environment (Lee & Ruby 1994). The remaining bacteria will grow back during the day, so that at night a sufficient number of bioluminescent cells will be available

again. Obviously, this daily venting has clear consequences for the rare biosphere. Lee & Ruby (1994) demonstrated that the concentration of *V. fischeri* was more than an order of magnitude higher in localities with the squid than in other areas without the squid. Moreover, these authors found that the concentration of the bacterium decreased along a transect away from the location where the squid were abundant.

Jones et al. (2007) determined the abundance of *V. fischeri* in waters around Hawaii and found that the concentrations were one order of magnitude higher in bays where the squid was present than in bays where it was absent. The abundances varied between 600 ml^{-1} in bays with low abundance of the squid and 86,000 ml^{-1} in bays with high abundance of the squid. Thus, *V. fischeri* accounted for 0.1% of the bacterial assemblage in bays with squid and 0.001% in those without squid. The highest abundances were found in bays with presence of the squid and with restricted communication with the sea. Bays open to sea currents did not show such large concentrations. Jones et al. (2007) also counted *V. fischeri* in waters around Australia at dawn and dusk. Not surprisingly, they found the concentrations to be significantly higher at dawn than at dusk in Botany Bay, where the squid (another species, *Euprymna tasmanica*) was abundant, but concentrations were equally low at dusk and dawn in bays without the squid. The dilution and mixing had erased the diel signal at a distance from the source of the bacteria. The final point of this example is that juvenile squid need to acquire an inoculum of this bacterium from the environment. They have a sophisticated mechanism to recognize the correct bacterium and then modify the morphology of the light organ to pull the inoculum inside. If there were no *V. fischeri* cells in the environment, even if at very low concentrations, the juvenile squid would not be able to acquire their symbionts.

In a study of sponge-associated bacteria, Webster et al. (2010) detected almost 3,000 OTUs per sponge (at a 95% level of sequence similarity). These OTUs belonged to 23 different bacterial phyla. The most abundantly represented groups were Proteobacteria, Acidobacteria, Actinobacteria, and Chlorobi. Webster et al. (2010) separately analyzed the 33 bacterial clusters thought to be sponge specific. All the sponge-specific clusters that were abundant in the sponges (accounting for more than 1% of the cells in the sponge) could also be found in the water column, albeit at very low concentrations. Of those that were less abundant (less than 0.1%), 75% could not be found in the water column, whereas 25% were found. This suggests that finding them in the water column is a question of the depth of sampling. If more sequences had been retrieved from the water column, these other clusters would likely have also been found. At any rate, several sponge-specific clusters were also members of the rare biosphere in the water column. These two examples show that symbionts of two very different animals groups, such as mollusks and sponges, are members of the rare biosphere. Because virtually all marine animals have bacterial symbionts, the number of cases such as these must be enormous.

This type of membership in the rare biosphere is by no means limited to symbionts. Bacteria specialized in living attached to surfaces, for example, need to colonize new particles, and the only way to do this is by liberating cells into the medium and letting them disperse across the water column until by chance they may find a new colonizable particle (Pedrós-Alió & Brock 1983). González et al. (2008) proposed such a mechanism for bacteroidetes possessing proteorhodopsin.

CONCLUSION

The concept of a rare biosphere has proven to be a useful framework for comparing different studies carried out with different techniques and perspectives. Most of its associated ideas can be tested experimentally with NGS technologies. Therefore, we can expect the increase in studies of bacterial diversity to continue in the next few years. Hopefully, these studies will carry out not only descriptions of the diversity, but also experiments to test the mechanisms underlying

the existence of the rare biosphere (those described in this review or not). The massive number of new sequences expected from different projects will help to saturate the collectors curve and, therefore, to come up with a realistic estimate of the total number of bacterial taxa in the ocean. These will be two major milestones in understanding the diversity of life on the planet.

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