Prokaryotic functional gene diversity in the sunlit ocean: stumbling in the dark

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Abstract

Prokaryotes are extremely abundant in the ocean where they drive biogeochemical cycles. The recent development and application of *-omics* techniques has provided an astonishing amount of information revealing the existence of a vast diversity of functional genes and a large heterogeneity within each gene. The big challenge for microbial ecologists is now to understand the ecological relevance of this variability for ecosystem functioning, a question that remains largely understudied. This brief review highlights some of the latest advances in the study of the diversity of biogeochemically relevant functional genes in the sunlit ocean.

Introduction

The ocean is the largest ecosystem on Earth. Prokaryotes are the most abundant cells in the ocean (ca. $\sim 10^{29}$, [1]) where they are the engines driving nutrient cycles and energy flow. In the last two decades, molecular studies based on rRNA genes have unveiled that the phylogenetic diversity of marine microorganisms is immensely larger than the few thousand species formally described [2]. After the initial surveys of ribosomal genes, the study of protein-coding genes followed by the development of *meta'omics* approaches has opened a window to the exploration of marine microbial diversity at the functional level. In fact, the first large-scale ocean metagenomic survey identified novel protein families and added a tremendous diversity to known protein families [3].

Hence, the study of functional diversity has improved our view of the roles that marine microbes play in global biogeochemical cycles.

Some clear examples are the discovery of novel metabolisms, such as proteorhodopsin-driven photoheterotrophy [4], or the role that urea plays in nitrification by marine archaea [5]. These studies have shown that prokaryotes play more functions than previously thought and that there is large sequence diversity for each gene [6-9]. But, what are the ecological implications of this variability? Addressing this important question is a major challenge in marine microbial ecology.

The tremendous functional diversity of marine prokaryotes occurs at different levels of complexity, such as variability in gene content, differences in operon structure, heterogeneity in gene sequence, or existence of different proteins which drive redundant functions. Furthermore, the presence of functional metabolic genes in viral genomes, such as those reported to be involved in photosynthesis, carbon or phosphorus metabolism, have added another dimension to this complexity [10]. Most of the studies conducted so far have focused on genes that confer an ecological advantage to prokaryotic populations, such as nitrogen fixation or photoheterotrophy. In this review, we summarize the current knowledge in prokaryotic (mostly bacterial) functional gene diversity in relation to the four major marine biogeochemical cycles (carbon, phosphorus, sulfur and nitrogen) (Figure 1). We focus on the sunlit ocean where most of the studies have been conducted, and discuss the potential relevance of genetic diversity for ecosystem functioning.

Biogeochemically relevant genes in the sunlit ocean

Carbon cycle. The paradigm of the ocean carbon flow depicting phototrophs as the only autochthonous producers of organic carbon (C) and heterotrophic bacteria as the consumers fell apart in the last decade. Nowadays we know that mixotrophy, the ability of an organism to use both light and organic matter to obtain energy, seems to be the rule rather than the exception in sunlit marine microbial communities. Since the discovery of a proteorhodopsin (PR) gene in a metagenomic fragment almost 15 years ago [4], we now know that up to 80% of bacteria inhabiting surface waters can harbor this gene [11]. Proteorhodopsins display large genetic diversity [12] that translates for example in different spectral tuning, likely as an adaptation to absorb the prevalent light wavelengths (blue or green) found in different marine waters. While this proton pump has been demonstrated to enhance growth under light in certain marine Bacteroidetes [13], no direct effect on growth was observed in *Pelagibacter*, in which, however, PR increased long-time survival during starvation [14], as was also observed in a *Vibrio* isolate [15]. Recently, new types of evolutionarily distinct rhodopsins have been uncovered which translocate either Na^+ or Cl^- [16, 17]. Interestingly, multiple rhodopsin types, each with different ion specificities, can be found within a single strain of marine Bacteroidetes [17]. However, the ecological implications of containing as many as three functionally different rhodopsins are unknown. Rhodopsin is indeed a neat example in which gene diversity likely results in diverse physiological and ecological functions. Yet, we appear to know only a small fraction of the functional significance of the diversity of this gene.

95 Similarly, aerobic anoxygenic phototrophs (AAPs) can derive a portion of their
96 energy requirements harvesting light using bacteriochlorophyll *a* (BChl *a*). Marine
97 AAPs contain diverse *pufM* gene sequences (the phylogenetic marker for AAPs)
98 as well as *puf* operon structures that delimit several phylogroups distributed
99 across the *Alpha*- and *Gammaproteobacteria*. Recent experimental work with
100 isolates has shown that while light is the main factor controlling the regulation of
101 Bchl *a* in representatives of the *Alphaproteobacteria*, this is not the case in the
102 *Gammaproteobacteria* [18]. Likewise, evidences that distinct phylogroups have
103 preferences to live under different environmental conditions [8,19,20] suggest
104 that different AAPs posses diverse strategies to adapt to a changing environment
105 and may have different roles in the functioning of the ecosystem. However, a
106 direct linkage between *puf* diversity and its relevance in ecosystem function has
107 not yet been established.

108 CO lithoheterotrophy has also received attention as a potential form of obtaining
109 energy for bacterial metabolism [21]. CO is formed through the photochemical
110 degradation of organic matter in sunlit waters and can be oxidized by means of
111 the carbon monoxide dehydrogenase encoded by the *cox* genes [22]. These
112 genes are structured in operons that form two phylogenetically distinct groups, in
113 which the gene order also diverges. Some bacterial species contain one operon
114 from each phylogenetic group, whereas others can contain multiple operons of
115 the same group. The active site configuration of the two phylogenetic groups also
116 differs. Although the significance of these distinctions has not yet been resolved,
117 it is believed that they may affect substrate specificity and activity [22].

118 Nevertheless, although CO oxidation mediated by bacterioplankton is
119 biogeochemically significant, the energy obtained through this process seems to
120 have a negligible effect on bacterial metabolism [23].

121 Besides their ability to obtain energy from light, some heterotrophic bacteria may
122 obtain additional carbon by fixing CO₂ through RuBisCO [24], or anaplerotic
123 pathways [25]. Nevertheless, mixotrophy is not restricted to heterotrophic
124 prokaryotes. It was recently shown that *Prochlorococcus* cells use a sugar
125 transporter gene to take up glucose to tune their carbon metabolism [26] and
126 other evidences of organic matter incorporation by cyanobacteria exist [e.g., 27].

127 Yet, a major challenge to our comprehension of the global carbon cycle is to
128 understand bacterial processing of dissolved organic carbon (DOC). DOC is
129 composed by thousands of compounds, many of them uncharacterized, and
130 utilized by a diverse community of heterotrophic bacteria with varying enzymatic
131 capabilities and ecological strategies for carbon metabolism [28]. The number of
132 studies dealing with specific genes encoding for these enzymatic activities is
133 notably limited, with the exception of the genes involved in chitin degradation
134 [29]. Currently, *meta'omics* allows depicting the diverse enzymes involved in
135 DOC utilization [30]. However, the functional annotation of the genes encoding
136 these enzymes is frequently based on distant homology to proteins characterized
137 in non-marine isolates, which may translate into a misinterpretation of their
138 function. Besides the utilization of complex DOC compounds, genes encoding
139 pathways for demethylation and C1 oxidation have been identified in SAR11 and
140 other marine bacteria like *Roseobacter* or methylotrophs, supporting the

hypothesis that C1 oxidation might be a mechanism by which dissolved organic carbon is significantly recycled to CO₂ in the upper ocean [31].

Phosphorus cycle. Phosphorus (P) availability is one of the dominant selective forces driving niche partitioning and ecotype divergence in oceanic prokaryote populations [32]. Inorganic phosphate (P_i) is the preferred source of P for prokaryotes, and they have evolved mechanisms for monitoring and responding to fluctuations of this essential nutrient. The Pho regulon integrates the sensing of P_i availability with co-regulation of genes involved in P_i scavenging (like *pstS*) or the use of alternative sources of P, such as phosphoesters (through *phoA*, *phoX*, *phoD* and other phosphatase genes), phosphonates (*phn* genes) and phosphite (*ptx* genes) [33,34]. Recent studies show that bacteria inhabiting permanent P-depleted areas of the ocean contain a higher number and diversity of Pho regulon genes, thereby increasing their ability to obtain P [32]. A recent study provides a remarkable example among the ubiquitous SAR11, which despite their streamlined genomes display striking differences in their P-related gene content. An isolate from the P-rich northeast Pacific Ocean is only able to grow on phosphate, whereas a strain isolated from the P-deprived Sargasso Sea can utilize a broad range of alternative compounds for P nutrition [35]. Similar results have been observed in *Prochlorococcus* strains pointing out how the environment shapes the genetic diversity of different bacterial populations [32,36].

Sulfur cycle. The marine sulfur cycle has been focus of intense research in the last years due to the potential role of dimethylsulphide (DMS) in regulating

climate. Dimethylsulphoniopropionate (DMSP) is released by some phytoplankton cells and is either demethylated to methylmercaptopropionate (MMPA), or cleaved to DMS by DMSP lyases [37,38]. DMS is a volatile compound that is later photo-oxidized to sulfate aerosols that form cloud condensation nuclei, initiating cloud cover over the oceans which leads to increases in albedo. As for the other essential nutrients, the combination of classical techniques and -omics has been crucial for the advancement on the knowledge of sulfur cycling in the ocean. Metagenomic data revealed that the key gene in the demethylation process (*dmdA*) is diverse among different taxa and can be up to two orders of magnitude more abundant than DMSP lyases [38]. Indeed, the demethylation pathway provides reduced carbon for energy or biomass and reduced sulfur that can be incorporated into S-containing aminoacids or oxidized for energy. Hence, this pathway seems ecologically more advantageous in oligotrophic environments than the cleavage pathway. Nevertheless the DMSP lyases are a wide group of enzymes that seem to catalyze the same reaction, whereas *dmdA* is the only known demethylase [37].

Nitrogen cycle. Traditionally, *Trichodesmium* and other heterocyst-forming cyanobacteria were thought to be the predominant N₂-fixing microorganisms in the ocean [39]. However we currently know that unicellular cyanobacteria such as *Croscosphaera watsonii* [40] or the prymnesiophyte symbiont UCYN-A [41], together with heterotrophic bacteria [6] dominate the diazotrophic community in ocean surface waters. Furthermore, ammonia-oxidizing archaea were discovered a decade ago [12] and are now known to be responsible for most of the

nitrification in the upper ocean [42]. These findings have arisen interest towards genes involved in N cycling with several studies that have explored the diversity of *nifH* (involved in nitrogen fixation) and *amoA* (involved in ammonia oxidation) among other genes. Some of these studies have allowed linking gene diversity with functional significance. For example, a recent study showed that two distinct types of archaeal *amoA* genes have different biogeographies, one operating at medium, and one at low ammonia concentrations [43]. In addition, recent metagenomic analyses have shown that Thaumarchaeota contain genes involved in urea transport and degradation (*ureA*, *ureB*, *ureC* genes) and that these organisms may fuel nitrification in oceanic polar regions [5].

Towards understanding functional diversity

Rapid technological development has enabled the description of the functional diversity of ocean microbes. Yet, the astonishing amount of genetic information generated is not accompanied by a significant improvement in our understanding of its functional significance and therefore, many relevant ecological questions remain unsolved. Here, we discuss some of these questions.

What is the significance of the genetic variability for well-studied genes?

Molecular techniques have unveiled the diversity of the key players in marine biogeochemical cycles and how genetic diversity is structured in relation to environmental variables. For example, differential effects of abiotic factors on AAP bacteria harboring different *pufM* sequences suggest that not all phylogroups are functionally and ecologically equal [8]. Likewise, experimental

data have shown that the abundance of different sequence clusters of the demethylation gene *dmdA* linked to specific marine taxa including *Roseobacter*- and SAR11-like bacteria, correlate with certain environmental characteristics [38]. A global study of *nifH*-harboring bacterioplankton has revealed specific biogeography for different taxa [6]. Additionally, studies on diversity of enzymes degrading organic compounds show that there is a whole suit of different enzymes carrying out the same function but with very different kinetics [44], probably reflecting the heterogeneity of substrate concentration at the microscale [45]. Microbes may also overcome limitation of enzyme cofactors by swapping out one metal for another in the same enzyme [46] or synthesizing different metalloenzymes for the same purpose. For example, calcium based phosphatases like PhoX and PhoD seem to be prevalent in oligotrophic waters, as opposed to the classical zinc-binding form of the enzyme (PhoA) [33,47]. Nevertheless, perhaps the best-known example of the significance of genetic variability is the case of the rhodopsins, which as seen above, display large genetic diversity that translates into various physiological and ecological functions.

What is the function of the astounding number of hypothetical proteins? –Omics datasets contain thousands of sequences encoding hypothetical proteins whose characterization requires tedious experimental work. While the genetic engineering approach is rarely conducted in marine studies, the results obtained so far stress its value. Heterologous expression was used to prove that proteorhodopsins are light-induced proton pumps [4] and through mutagenesis

we learned more about their differential spectral tuning [48]. Functional screening of fosmid libraries allowed the identification of a phosphonatase pathway as well as a novel pair of genes that allow utilization of 2-aminoethylphosphonate, alternative sources of P for marine microorganisms [49]. These approaches have led to major advances in our understanding of functional diversity and thus, more effort should be put in applying these techniques to advance in our knowledge of the functional capabilities within microbial populations.

What genes are actually expressed and what environmental variables regulate gene expression? Metatranscriptomic studies provide insights into which genes are transcribed, and therefore likely expressed, in the environment. Comparative expression studies have evidenced for example that coexisting heterotrophic bacterial populations exhibit diel oscillations in their expression patterns, resembling those shown by their photosynthetic counterparts [50], or that bacterioplankton increase the transcription of genes involved in cell aggregation when accompanying a phytoplankton bloom [51]. Studies combining both metagenomics and metatranscriptomics are essential to better understand what genes are expressed under which circumstances, but these studies are only starting to appear [52]. Furthermore, current metatranscriptomic studies recover only a very small percentage of the total pool of transcripts (~0.000001%, [52]). Significant gains in the analysis of biogeochemically informative gene expression patterns require a greater sequencing investment. Initiatives like the global circumnavigation expedition TARA Oceans [53] are presently applying massively high-throughput sequencing to describe the functional profiles of the world's

oceans and promise to greatly advance our understanding of ecosystem functioning. Yet, to understand what factors drive the observed trends, a precise characterization of the environment is necessary, something that is not always conducted.

What mechanisms drive the observed functional genetic diversity? Comparative gene and genome analyses have revealed the ecological and evolutionary forces that influence genome content. Genome streamlining seems to be a successful adaptation to live under permanent low nutrient concentration, as seen in SAR11, *Prochlorococcus* [54] and in wide array of marine single amplified genomes [55]. However, even those reduced genomes display a significant variability in gene content among closely related populations. As mentioned above, one of the dominant selective forces driving niche partitioning and ecotype divergence within these populations is nutrient availability [32]. Indeed, many of the genes involved in the efficient acquisition of nutrients are horizontally transferred and often found in genomic islands [32]. For example, certain microdiverse lineages of uncultured *Prochlorococcus* harbor a genomic island that contains the genes necessary to assimilate nitrite and nitrate, as opposed to their cultured representatives that rely on ammonia [56]. Likewise, alkaline phosphatases (*phoA*, *phoX* and *phoD*) are not found in SAR11 isolates, but are present in uncultured representatives of this ubiquitous clade [47,57]. Phosphatases are needed for the utilization of a major component of the dissolved organic P pool, and have broad substrate specificity. Therefore, the acquisition of just one phosphatase gene enables the cell to access a wide spectrum of P compounds,

which can be very advantageous in oligotrophic environments. Other examples of horizontally transferred genes are those involved in the adaptation to different light regimes or in obtaining additional energy from light [59]. Moreover, genomic islands are enriched in genes encoding hypothetical proteins which could play a role in increasing bacterial fitness under changing environmental conditions [60], but determining their function is necessary to confirm this hypothesis. Thus, despite not being the only mechanism (e.g. mutation), horizontal gene transfer is largely responsible for the observed diversity.

In summary, although several key questions remain to a large extent unanswered, the *-omics* approach provide a framework for evaluating functional gene diversity in an environmental context while at the same time is a powerful tool to formulate hypotheses on microbially mediated processes. What the field is currently lacking is taking a step further to experimentally test *-omics* emerged hypotheses. Function-driven metagenomics and combination of *-omics* with classical microbiology techniques are necessary to obtain a more comprehensive view of the functioning of the very complex marine microbial communities, a relevant matter for marine ecology.

Acknowledgments

The author's work is partially supported by grants DEVOTES (FP7-ENV-2012-308392) and MICRO-3B (FP7-OCEAN-2011-287589) funded by European Union, grants HOTMIX (CTM2011-30010/MAR), DOREMI (CTM2012-34294) and

PANGENOMICS (CGL2011-26848) funded by the Spanish Government, and grant XTM (2014SGR/1179) funded by the Catalan Government.

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Figure legends

Figure 1. Simplified view of major biogeochemical cycles in the sunlit ocean. Solid lines indicate prokaryotic mediated processes and the key functional genes involved in the processes are shown in boxes. Asterisks denote groups of genes with a related function. Briefly, phytoplankton fix carbon through photosynthesis, incorporate inorganic nutrients and release dissolved organic matter, which includes dissolved organic carbon (DOC), dissolved organic phosphorus (DOP), and dissolved organic nitrogen (DON). Certain phytoplankton species also release dimethylsulphoniopropionate (DMSP). Zooplankton also excretes dissolved organic matter, including urea. Prokaryotes, especially heterotrophic bacteria but also certain cyanobacteria, use the organic matter released by phyto- and zooplankton, and compete with phytoplankton for the inorganic nutrients. DMSP can be either cleaved by different DMSP lyases (encoded by *ddd* genes) to DMS and eventually released to the atmosphere, or demethylated by *dmdA* and used by prokaryotes as a reduced sulfur source. DOC is metabolized by a diverse number of enzymes, such as glucosidases, sulfatases or chitinases, but only the chitinases genes are well characterised. DOP is mainly composed of phosphoesters and phosphonates, which to be used require the action of alkaline phosphatases (*phoX*, *phoA*, *phoD*) and phosphonates genes (*phn* genes). The key enzymes for the utilization of DON are aminopeptidases, but the molecular basis of these enzymes is still largely unknown. Some heterotrophic bacteria are able to obtain additional carbon by fixing CO₂ through RuBisCO (*rbcL*) or alternative pathways (see main text for details). Other heterotrophic bacterial cells can obtain additional energy from sunlight by means of the proteorhodopsin or the *puf* operon, or from the oxidation of carbon monoxide (CO). Prokaryotes can obtain nitrogen from ammonia (NH₄⁺) or nitrate (NO₃⁻), and some cyanobacteria and heterotrophic bacteria are capable of fixing N₂ by means of nitrogenases (*nif*). The oxidation of ammonia in the sunlit ocean is mainly performed by archaea by means of the *amoA* gene, and the degradation of urea (*ure* genes) plays a major role in fuelling this process. Viruses lyse prokaryotic and eukaryotic cells, releasing DOM, particulate organic matter and nutrients. Viruses also play a crucial role in the exchange of genetic information among marine prokaryotes.