Community structure of marine bacterioplankton: patterns, networks, and relationships to function

Jed A. Fuhrman*, Joshua A. Steele

Department of Biological Sciences and Wrigley Institute for Environmental Studies, University of Southern California, Los Angeles, California 90089-0371, USA

ABSTRACT: Major challenges in marine microbial ecology include determining patterns of community structure and structure–function relationships. Progress has occurred by a combination of molecular and classical techniques. An example for this is work on the marine Crenarchaeota, extremely abundant organisms that we now think play important roles both as autotrophic ammonium oxidizers and as heterotrophs. Another involves the recently discovered Proteorhodopsins, pigments that are potentially important in solar energy capture, yet often do not seem to confer a clear growth benefit to organisms that possess them. Proteorhodopsins occur in numerous phylogenetic groups and may have multiple roles, complicating the linking of these proteins to a well-defined function. Community fingerprinting is a powerful tool for examining community structure and for examining spatial and temporal distribution patterns. Using fingerprinting, we found that ocean communities tend to occur in patches with horizontal dimensions of several km, while samples 10s of km or more apart can be quite different. Temporal analyses such as the San Pedro Ocean Time Series (SPOT) show that near-surface communities are somewhat similar from month to month, then change significantly over several months, and finally return to a similar community annually. Near-surface communities can also be predicted from environmental parameters like temperature, salinity, nutrients, and chlorophyll. Time series data show co-occurrence of organisms (resembling a succession of communities) under particular conditions, or conversely can show which organisms are significantly negatively correlated, indicative perhaps of competition, resource partitioning, or allelopathy. These relationships are visualized here as mathematical interaction networks to map out the 'niche space' of microbial taxa and their correlations, displaying complex ecological interactions. Observations indicate that ecological relatedness does not necessarily closely follow phylogenetic relatedness.

KEY WORDS: Bacteria · Archaea · ARISA · Proteorhodopsin · Patchiness · Time series · Networks

INTRODUCTION

About 2 decades ago, Olsen et al. (1986) and Pace et al. (1986) introduced the concept of studying the composition of natural microbial communities by sequencing genes cloned directly from biomass, rather than cultures. This started the molecular revolution in microbial ecology, leading to the discovery of previously unknown groups of microorganisms and information about where they live. We can now accurately identitify organisms that were previously considered little dots in the microscope, and say how they are distributed in space and time. Yet one of the biggest challenges is still to determine not only what organisms are present, but what each particular kind of organism is doing and how it interacts and fits into the functioning of the ecosystem. In a sense, we are asking about the natural history of the various microbes, analogous to the intuitive understanding that people have had with animals and plants for millennia, by simply observing what these organisms were doing in nature. With microbes it is, of course, not so simple. Yet this basic understanding is the first step in creating conceptual and predictive models of how the system works as a whole, broken down to include the constituent microorganisms. Linking community structure with

*Email: fuhrman@usc.edu

ecological function is therefore an important, but elusive goal. This review summarizes key approaches, successes, and ongoing challenges in this field. It includes studies that address the problem directly, by linking particular functions to particular organisms, and indirectly, by examining the statistical relationships among organisms and environmental parameters measured over various spatial and temporal scales. Both approaches have their advantages, and statistical investigations even present the possibility of revealing the network of microbial relationships within marine ecosystems. To examine the direct links, 2 case studies were used: one focusing on the functions of a recently discovered group of organisms, the marine Crenarchaea, and the other focusing on a newly discovered protein, proteorhodopsin, which has the potential to significantly impact energy budgets in marine environments.

CASE STUDY 1: MARINE ARCHAEA IN THE NITROGEN CYCLE

In the 1970s, Woese & Fox (1977) and Woese et al. (1978) made the startling discovery that a group of organisms originally called 'Archaebacteria' (later renamed Archaea) are fundamentally different from Bacteria, both genetically and physiologically. At that time, all known Archaea were 'extremophiles', living only in places like hot springs (extreme thermoacidophiles), saturating salt concentrations (extreme halophiles) and strictly anaerobic conditions (methanogens). Archaea were not expected to be found in 'ordinary', non-extreme environments. So when the rRNA cloning and sequencing method was applied to ocean midwater samples (100 and 500 m water depths) for the first time, using 'universal' PCR primers, it was a complete surprise that many of the sequences obtained originated from Archaea and not Bacteria (Fuhrman et al. 1992). In that study, 5 out of 7 random clones from 500 m depth and 2 out of 10 from 1 of 3 samples taken at 100 m water depth were found to belong to the Crenarchaeota, the group previously assumed to contain only extreme thermoacidopiles. Shortly afterwards, related Crenarchaeota (named Marine Group I), plus members of the Euryarchaeota (named Marine Group II), were detected in shallow coastal waters by Archaea-specific PCR, and reported to represent <2% of the prokaryotic community in that environment (Delong 1992). Fuhrman et al. (1992) suggested that the high proportion of archaeal clones from 500 m water depth pointed to a high potential abundance and resulting impact of this group on deep sea metabolism, but it was uncertain if relative clone abundance was suitable to draw such conclusions. Numerous nucleic acid hybridization studies indicated high proportions and dynamism of Archaea in deeper waters and the Antarctic (e.g. Massana et al. 1997, Murray et al. 1998), but quantitative hybridization has its own uncertainties regarding the measurement of cellular abundance. The high abundance was confirmed most convincingly by direct cell counts via fluorescent *in situ* hybridization (FISH). Such studies reported that Archaea are remarkably abundant, particularly in ocean midwaters (usually depths below 100 m), represent typically 20 to 40% of the cells visible by epifluorescence at such depths (often exceeding bacterial abundance), and are highly dynamic (Fuhrman & Ouverney 1998, DeLong et al. 1999, Karner et al. 2001, Herndl et al. 2005, Teira et al. 2006a). So Archaea are not just some rare type of cell, but rather constitute a major part of the prokaryotic community, especially in the deep sea and some colder, shallower waters. The number of marine Archaea globally has been calculated to total about 10^{28} cells, a mind-boggling quantity. Lined up like beads on a string, these Archaea would stretch longer than the entire Milky Way galaxy (to borrow a comparison from Suttle 2007)! So the question arises — just what are these Archaea doing? In other words, how does one link their identity to their function?

The first direct measures of archaeal activity were made by a combination of autoradiography and FISH (Substrate Tracking AutoRadiographic FISH: STARFISH), and showed that about 60% of the Archaea from 200 m depth in the NW Mediterranean Sea and Monterey Bay (California) took up a mixture of tritiated L-amino acids, added to seawater samples at nM concentrations (Ouverney & Fuhrman 2000). Recent similar studies with improved sensitivity and covering extensive areas (and a wide range of water depths) in the North Atlantic showed that the Archaea (Eury- and Cren-) take up both D- and L- amino acids (Teira et al. 2004, 2006b, Herndl et al. 2005). These studies suggest that Crenarchaea have heterotrophic capabilities and in fact compete successfully with Bacteria for the extremely low concentrations of dissolved amino acids in these waters. However, other evidence, initially from geochemical studies, suggested a very different situation. Because Archaea have unique lipids that can be extracted and studied individually, investigation of the properties of particular kinds of Archaea, using chemical characteristics including isotopic content, is possible. Pearson et al. (2001) reported that the ^{14}C-content of archaeal isoprenoids from Santa Monica Basin (California) surface sediments indicated that the Archaea incorporated inorganic carbon chemoautotrophically below the euphotic zone. Kuypers et al. (2001) examined fossil marine archaeal lipids and concluded, based upon ^{13}C-content, that

Archaea were living chemoautotrophically during a Cretaceous ocean anoxic event. Direct measurements on live marine Archaea, grown in coastal mesocosms under controlled conditions (15°C, dark), provided additional evidence: Wuchter et al. (2003) showed that ^{13}C-bicarbonate was incorporated into archaeal lipids. Herndl et al. (2005) reported significant ^{14}C bicarbonate incorporation into Archaea in deep sea field samples. Together, these studies have made it clear that the Archaea have a significant chemoautotrophic capability.

This raises the obvious question about what kind of chemoautotrophy is involved. Interestingly, the initial answer to this did not come from a study focusing on Archaea, but from the shotgun metagenomic survey of the Sargasso Sea by Venter et al. (2004). In that study, the entire microbial community (0.1 to 0.8 µm) from near the sea surface was collected by filtration and the DNA extracted, sheared, cloned, and >6 billion bases of DNA sequence generated and assembled (only a small fraction overlapped sufficiently to assemble). Venter et al. (2004, p. 73) reported '…because it has been believed that only members of the bacterial domain were capable of oceanic nitrification, it is interesting to note that an ammonium monooxygenase gene was found on an archaeal-associated scaffold within our data set.' In other words, their assembler put together several DNA fragments that included genes of archaeal origin, and among these genes was ammonia monooxygenase, the enzyme that catalyzes the first, critical step in oxidation of ammonia to nitrite. This was an incredibly exciting discovery because until that time, ammonia oxidation was thought to be carried out only by members of the domain Bacteria. However, the evidence from the Venter et al. (2004) study alone was not fully conclusive, because it was possible that the gene might have actually been for methane oxidation (the 2 genes are related), or that the scaffold might have been some sort of chimera — and as Carl Sagan was famous for saying in his television series, Cosmos, 'Extraordinary claims require extraordinary proof.' But such evidence developed quickly. The result was confirmed by more conclusive data, most notably the sequence of a large-insert metagenomic clone from soil that included a crenarchaeal 16S rRNA gene, the ammonia monooxygenase (*amoA*) gene, plus some other related genes like nitrite reductase (Schleper et al. 2005). Yet even that was not as convincing as the isolation of a Marine Group I crenarcheon, later named *Nitrosopumilus maritimus*, from the rocky substrate of a marine aquarium, where it grew autotrophically by the oxidation of ammonia to nitrite (Konneke et al. 2005). Thus, some Archaea are indeed capable of ammonia oxidation. Further confirmation of this capability, together with other valuable information about autotrophic and mixotrophic functionalities, has come from the metagenomic analysis of *Cenarchaeum symbiosum*, an uncultivated marine archaeon that lives symbiotically within a sponge (Hallam et al. 2006).

What about the evidence on amino acid uptake in nature? Are the Archaea mostly autotrophs or mostly heterotrophs? Ingalls et al. (2006) evaluated the natural ^{14}C-content of specific planktonic marine archaeal lipids in order to estimate autotrophic and heterotrophic contributions to their metabolism. Getting enough material for the analysis required filtering an astonishing 200 000 l of seawater from 670 m water depth off Hawaii through a 0.2 µm pore size filter. The calculations required a model and several assumptions that were reasonable, but not certain. The authors concluded that 83 % of the crenarchaeal carbon was derived from bicarbonate at the sampling depth, and the remaining 17 % from 'modern', recently fixed carbon originating from the euphotic zone. This result applies specifically to the 670 m depth, and probably cannot be generalized (see below). It also does not say whether most of the Marine Group I Crenarchaea are pure autotrophs, with only some heterotrophs in the group, or if all them have some heterotrophic capability.

A different kind of analysis also addressed the question whether all Marine Group I Crenarchaea are ammonia oxidizers. Wuchter et al. (2006) and Herfort et al. (2007) compared crenarchaeal counts with *amoA* gene copy numbers by QPCR, and found that they were highly correlated. Their data suggested 2.8 times as many *amoA* genes as crenarchaeal 16S genes in this group, or 2.5 *amoA* copies per cell counted by CARD FISH. So if the data are accurate and all the cells have the gene, it suggests 2 to 3 copies per cell (assuming a single 16S gene per cell). It is possible that only some cells have the gene, but with a higher number of copies.

It is remarkable how rapidly we have learned about marine Archaea, as shown by accelerating discoveries in the past few years (Fig. 1). But what do all these data mean for understanding the activities of marine Crenarchaeota? While it is not possible to be sure when comparing different studies, a parsimonious interpretation of the data suggests that, in the top several hundred meters of the sea which have been studied most extensively, the large majority of Marine Group I Crenarchaea probably possess the *amoA* gene and are capable of ammonia oxidation, and also have the ability to take up and incorporate dissolved L- and D-amino acids. We speculate that these processes occur either simultaneously or closely spaced in time in the same cell. Interestingly, G. J. Herndl et al. (pers. comm.) have noted that, as depth increases from a few

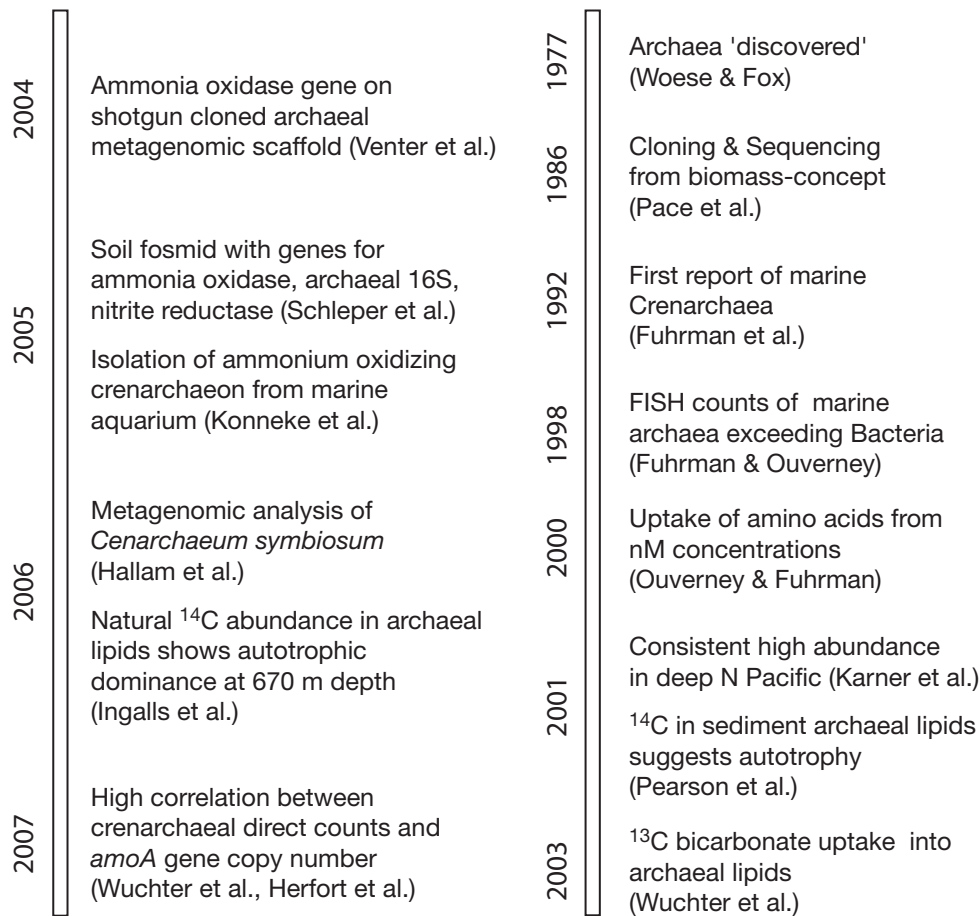| 2004 | Ammonia oxidase gene on shotgun cloned archaeal metagenomic scaffold (Venter et al.) | 1977 | Archaea 'discovered' (Woese & Fox) |
2005	Soil fosmid with genes for ammonia oxidase, archaeal 16S, nitrite reductase (Schleper et al.)	1986	Cloning & Sequencing from biomass-concept (Pace et al.)
2005	Isolation of ammonium oxidizing crenarchaeon from marine aquarium (Konneke et al.)	1992	First report of marine Crenarchaea (Fuhrman et al.)
2006	Metagenomic analysis of *Cenarchaeum symbiosum* (Hallam et al.)	1998	FISH counts of marine archaea exceeding Bacteria (Fuhrman & Ouverney)
2006	Natural ^{14}C abundance in archaeal lipids shows autotrophic dominance at 670 m depth (Ingalls et al.)	2000	Uptake of amino acids from nM concentrations (Ouverney & Fuhrman)
2007	High correlation between crenarchaeal direct counts and *amoA* gene copy number (Wuchter et al., Herfort et al.)	2001	Consistent high abundance in deep N Pacific (Karner et al.)
		2001	^{14}C in sediment archaeal lipids suggests autotrophy (Pearson et al.)
		2003	^{13}C bicarbonate uptake into archaeal lipids (Wuchter et al.)

Fig. 1. Marine Crenarchaeota. Timeline for selected discoveries starting at the upper right corner

hundred meters to 3000 m, the presence of the *amoA* gene drops much faster than the number of crenarchaeal 16S rRNA genes, with the ratio dropping from ~2 to <0.1. The implication of this observation is that most or all Crenarchaea occurring in the top few hundred meters of the ocean may have the potential to be ammonium oxidizers, but only a small portion of those occurring deeper have this capability. This seems plausible, considering that a large part of ammonium gets metabolized at shallower depths and only small amounts reach the deep sea. However, the same argument could be made for organic carbon, so the explanation is not fully clear. It is possible that the presence or absence of the *amoA* gene in the Crenarchaea is related to the C:N ratio of sinking organic matter, which typically increases with depth, suggesting relatively less N as depth increases (Honjo & Manganini 1993); but some reports indicate that C and N degrade in sinking material at similar rates (Hernes et al. 2001). In any event, we expect that the relative extent of heterotrophy vs. autotrophy probably depends on environmental conditions, notably the avail-

ability of ammonium (and oxygen) compared to usable organic compounds. In environments with chronic shortages of ammonium (e.g. as encountered by plankton at the deepest depths), the ammonium oxidation pathway genes might be considered unnecessary baggage that can only rarely, if ever, be used, and thus are easily lost. In contrast, the heterotrophic pathways are more generally usable, so are less likely lost. Overall, it seems that even though Crenarchaea are probably the major ammonia oxidizers in the world (see review by Francis et al. 2007), there may be many places where they do not possess the capability.

CASE STUDY 2: PROTEORHODOPSIN

One of the most interesting and unexpected early discoveries from metagenomics was the presence of proteorhodopsin (PR) genes in marine planktonic Bacteria (Beja et al. 2000). This was unexpected, because rhodopsin-like proteins had formerly only been known from eukaryotes or rare halophilic Archaea that in-

habit hypersaline niches. In eukaryotes, rhodopsins function as light sensors (e.g. in the human eye), and in halophilic Archaea these proteins function as light-driven transporters or photosensory pigments. Rhodopsin-like pigments are 7-helix trans-membrane apoproteins (opsins), capable of binding all-*trans*-retinal (vitamin A aldehyde) inside the membrane-embedded helices (opsin proteins bound to retinal are called rhodopsins) (Spudich et al. 2000). Upon illumination, the bound retinal molecule in rhodopsins undergoes light-induced isomerization, inducing conformational changes in the protein backbone and causing translocation of a proton (or other ion, like chloride) across the membrane, or signaling to transducer proteins.

When the initial PR gene found by Beja et al. (2000) was cloned and expressed in *Escherichia coli*, after providing the organism with exogenous retinal, the PR started pumping protons from the inside to the outside of the cell when illuminated. Subsequent studies have shown that this proton gradient can be used to phosphorylate ADP to ATP in such clones (Martinez et al. 2007). Another important observation is that the wavelengths of light that are maximally absorbed by PR can be 'tuned' by the alteration of key amino acids (Man et al. 2003), permitting the protein to optimize energy capture in environments dominated by green or blue light. Shallow or coastal waters tend to be greenish, while deeper areas are dominated by blue light. As a consequence, marine PRs include many near-surface forms that absorb maximally in the green, and deep-euphotic-zone forms that absorb maximally in the blue range of the light spectrum (Man et al. 2003). Furthermore, the 'Sorcerer II' near-surface ocean metagenomic survey, covering a transect from Halifax through the Panama Canal to the Eastern Tropical Pacific, reported over 2600 PR genes that were dominated by green-absorbing types in nearshore waters and dominated by blue-absorbing types in offshore ('blue') waters (Rusch et al. 2007). PR has been found in members of many of the most common marine bacterial groups, including the ubiquitous SAR11 and SAR86 surface clades (Beja et al. 2000, Giovannoni et al. 2005), marine Euryarchaeota (Frigaard et al. 2006), Planctomycetes (McCarren & DeLong 2007), Betaproteobacteria (McCarren & DeLong 2007), the predominately coastal SAR92 clade (Stingl et al. 2007), the marine *Roseobacter* group (Moran & Miller 2007), and at least 3 representatives from the widespread class Flavobacteria within the phylum Bacteroidetes (*Dokdonia* sp., *Polaribacter* sp. and *Leeuwenhoekiella blandensis*) (Gomez-Consarnau et al. 2007). Taken together, these clades typically comprise 50% or more of the total bacteria in marine surface waters (Giovannoni & Rappe 2000). PR is also present in minor prokaryotic components of the marine community (Venter et al. 2004, Rusch et al. 2007). The phylogenetically widespread distribution, even across domains, suggests that horizontal gene transfer of this gene is common (Frigaard et al. 2006), and implies that the gene confers a significant advantage to the cell. However, it also suggests that the gene is not diagnostic of a particular clade, and we do not know which members of which clades possess it or express it. PR abundance estimates from individual metagenomic samples collected from various ocean waters have ranged from as little as 2% to more than 100% (perhaps implying the presence of more than one PR gene per cell) of the estimated total number of Bacteria plus Archaea (Sabehi et al. 2005, Rusch et al. 2007, Fuhrman et al. 2008), and conservative quantitative PCR analysis suggests that about half of the Bacteria and Archaea in the Sargasso Sea and 23% elsewhere in the North Atlantic possess the gene (Campbell et al. 2007).

These observations all raise the intriguing prospect that considerable solar energy might be harvested by PR, potentially allowing cells to grow more efficiently, and thus having an important role in global ecosystem models and energy budgets. At this time, however, direct evidence for a broad energetic role in most Bacteria or Archaea is lacking.

One might expect that cells that benefit energetically from PR would grow faster in the light compared to the dark. Interestingly, of the 4 examined pure cultures that express PR tested to date, only one shows faster growth in the light. The 3 that do not (*Pelagibacter ubique* in the alphaproteobacterial SAR11 cluster, HTCC2207 in the gammaproteobacterial SAR92 clade, and HTCC2181 in the betaproteobacterial OM43 cluster) grow in low-nutrient, filtered seawater at the same rate and with the same yield whether cultured in the light or dark, even though the cells are thought to be limited by the availability of carbon (Giovannoni et al. 2005, Stingl et al. 2007, Giovannoni et al. 2008). The culture that is reported to grow faster in the light is *Dokodonia* (in the Flavobacteria), and that organism actually showed no growth under dark conditions (grown in unenriched filtered seawater), but grew reasonably well in the light. When grown in artificial seawater, the light:dark yield ratio was 1 for very low (0.06 mM) and high (242 mM) organic carbon media, but up to 4 at intermediate levels of organic carbon (0.14 to 1.1 mM) (Gomez-Consarnau et al. 2007).

In addition to these studies of pure cultures, there have also been mesocosm studies of the effects of light on the growth of various marine bacteria in natural seawater, following the different phylotypes of bacteria individually by using Amplified Ribosomal Intergenic Spacer Analysis (ARISA), a genetic fingerprinting method. (Schwalbach et al. 2005) studied bacteria in samples from meso- and oligotrophic areas of the

eastern Pacific in 20 l mesocosms under light or dark conditions for 5 to 10 d. They found that most members of groups reported to possess PR (e.g. SAR11, SAR86, Bacteroidetes) actually showed more net growth under dark conditions, with only a few members growing more under light conditions. The only phylogenetic group consistently growing better in the light were the Cyanobacteria (no surprise). These results are consistent with the work on pure cultures mentioned above (Giovannoni et al. 2005, 2008, Stingl et al. 2007, Gomez-Consarnau et al. 2007), where different PR-containing taxa showed different light/dark responses, with the majority not having a positive response of growth to light.

Although many marine PRs can act as light-driven proton pumps, there is still uncertainty about when, or even if, this provides substantial energy to most organisms that possess and express the gene. Most marine PR genes that have been examined closely have proton-pumping characteristics, which includes the amino acid residues thought to be important for this function, and a 'fast' photocycle time, which refers to the time it takes the protein to return to its original state after its light-induced conformational change (Sabehi et al. 2005). But several marine PR-like genes in metagenomic datasets lack key amino acids in the retinal binding pocket, similar to known sensory rhodopsins, and some also have what appear to be a sensory transducing gene adjacent to the PR gene (Spudich 2006). It should be noted that some archaeal sensory rhodopsins, which do not pump protons in their native cell, can pump protons when expressed in the bacterium *Escherichia coli* without their corresponding transducing molecules (Spudich 1994). Therefore, one must be careful in interpreting heterologous expression studies as proof of function. It is probably also important to bear this in mind for proteins besides PR. In any case, we cannot rule out a sensory role for many marine PRs.

Note that with PR as a potential energy source, one would expect a growth benefit only if cells are energy limited and not limited by nutrients like P, N, or Fe. Yet such nutrients are thought to limit bacterial growth (even heterotrophic bacteria) in many parts of the sea (Pomeroy et al. 1995, Pakulski et al. 1996, Cotner et al. 1997, Thingstad et al. 1998, Kirchman et al. 2000). Consequently, the advantages — if any — of PR in such places still need to be explored.

So which organisms can we say for sure possess PR, and what function(s) do we link to it? There is no clear answer to either question. PR appears widespread in the SAR11 cluster and distributed widely in other clades. The answer about functions quite possibly lies in multiple strategies in different organisms. It appears that some organisms, like *Dokodonia*, use PR to pro-

vide considerable energy for growth (Gomez-Consarnau et al. 2007), and these organisms might sometimes rely on PR and light to express net growth (analogously to the way that cyanobacteria rely on chlorophyll and light for growth, even if carbon fixation may not be involved in *Dokodonia*). Other organisms, probably including members of the SAR11 cluster, probably do not rely on PR and light for growth, and may not even show a clear growth benefit under many typical ocean conditions. It is possible that, in these cells, PR provides a valuable energetic benefit mostly under extremely low organic carbon conditions, permitting the cell to keep up critical functions like DNA repair, and thus survive when otherwise they may not. It is also possible that, in other cells, PR is largely regulatory or sensory, detecting when the cell is in the euphotic zone during daytime and switching functions most suitable for those conditions on or off. One might even imagine that some viruses could use light sensory functions to delay the assembly and release of virions from cells until the onset of darkness, because light damages viral DNA but such damage is repaired as long as the viral DNA is in the bacterial host. Releasing virions in the evening would give them maximal time in the dark to diffuse to new hosts without suffering light damage. PR may have other functions that have not yet been revealed, and it is possible that PR serves multiple functions in some organisms. There is certainly much to learn about the marine PRs.

USING DISTRIBUTION PATTERNS TO LINK IDENTITY WITH FUNCTIONS

While it is difficult to link most complex functions with particular microorganisms directly, an alternative approach is to examine the distributions of various organisms and environmental parameters over space and time, and to correlate subsequently which functions go with which organisms. The first step is simply to look for patterns in distributions, to see the appropriate scales of variability and learn what a particular sample might represent. For example, is a 1 l sample representative of a cubic meter, or a region hundreds of meters or several kilometers wide? Similarly, how quickly do communities change — over hours, days or weeks? Such questions have been investigated in our lab primarily with community fingerprinting approaches, mostly ARISA. Scales of variability often can, by themselves, hint at factors controlling community composition.

It makes sense to first briefly describe what ARISA fingerprinting can demonstrate. We have found that ARISA can distinguish taxa (actually operational taxonomic units, or OTUs) with 16S rRNA sequence similarity of about 98 % or less, similar to what many con-

sider near the 'species' level for Bacteria (Brown & Fuhrman. 2005). We have also found that our highly standardized version of the method is generally quantitative, especially when comparing how the amount of a particular OTU changes between different samples (as opposed to comparing the amount of one OTU vs. another). To do this, we compared flow cytometric and ARISA-based estimates of the abundance of *Prochlorococcus* at our San Pedro Ocean Time Series (SPOT) station monthly over 3 yr. The linear regression of these variables had an r^2 value of 0.86, indicating that the ARISA provided an excellent measure of *Prochlorococcus* abundance (Brown et al. 2005). While the method is not perfect and might miss certain taxa that do not have linked 16S and 23S rRNA genes (e.g. most Planctomycetes and close relatives), it provides a reasonably comprehensive picture of the bacterial community in most marine samples.

ARISA fingerprints indicate that, in the places studied to date, bacterial communities occur in coherent spatial patches of horizontal extents ranging from a few km to about 50 km at a given depth (Fig. 2). This preliminary conclusion is based primarily on studies in oligotrophic surface waters. In the oligotrophic open Pacific, we found that the similarity of communities from 2 stations (7 replicate 20 l samples each), collected as the ship drifted over a distance of about 2 km, was 85 to 95 %

within stations and 75 % between stations (Fig. 2A and Hewson et al. 2006). For this study, similarity was compared using the Whittaker Index, which takes into account identity and proportions of all OTU. We also noted that in both Pacific and Atlantic surface waters, the similarities of community composition of stations >50 km apart were low, typically about 40 %, and this similarity did not vary much whether the stations were 50 km, 250 km, or even 1000 km apart (Fig. 2B and Hewson et al. 2006). Our interpretation of these data was that, within a few km, there is a coherent patch with a particular community composition, but once one leaves that patch, the community is quite different. This suggests that factors with spatial scales <10 km or so appear to be most important in the development and maintenance of particular microbial communities. This is similar to the spatial extent of typical mesoscale eddies of chlorophyll as seen from satellites, so the controlling factors may relate to physical mesoscale eddies, the equivalent of the 'weather' in the sea. While these patches are apparently set up and maintained by physical factors, biological interactions within each eddy seem to lead to unique combinations of dominant microorganisms. It will be interesting to see if these conclusions, which are based upon relatively few data, will hold up to broader studies.

The relationship between distance and community similarity described above, where communities jumped from being very similar in nearby samples to being very different at distances above 50 km, is typical of open ocean samples (Hewson et al. 2006). However, similarity at one location appeared to decrease nearly linearly with distance. That occurred when several samples were compared across the shallow and narrow Torres Strait between Australia and Papua New Guinea, separating the Arafura Sea (Indian Ocean) from the Coral Sea (Pacific Ocean). Hewson et al. (2006) concluded that this may be due to the observation of a 'mixing curve' between 'endpoint' Indian and Pacific Ocean communities. It is not yet known to what extent the differences between these communities represent different functionalities, and what those differences might be.

Temporal variation has also been examined on scales from days to years. Lee & Fuhrman (1990), using DNA/DNA hybridization of whole communities, found that communities collected 1 wk apart from Long Island Sound, New York, were 92 to 94 % similar to
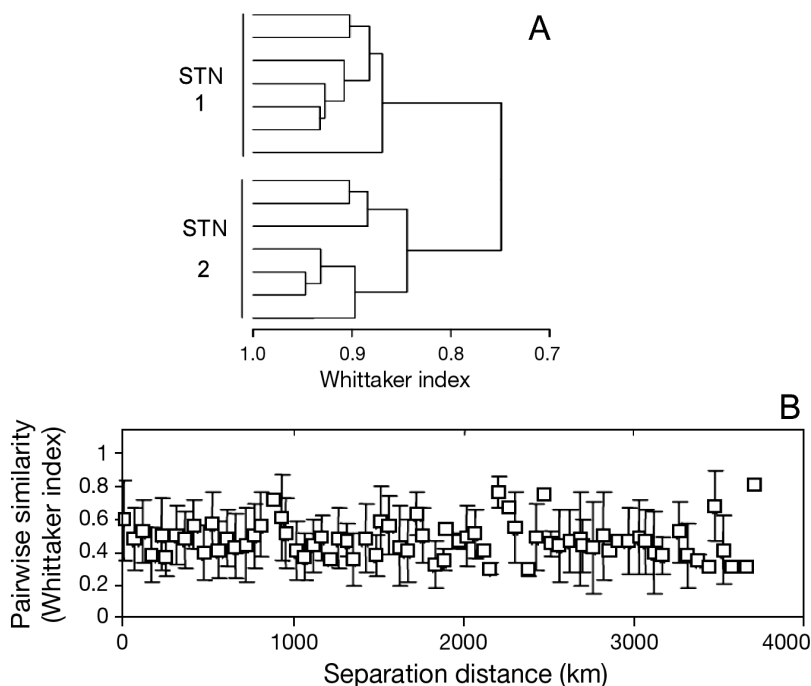

Fig. 2. (A) ARISA-based community similarities among 7 replicate near-surface 20 l samples from 2 stations in the central North Pacific Ocean. Similarity was measured using the Whittaker Index. (B) ARISA-based similarity, plotted as a function of distance between sampling locations, also using the Whittaker Index. Modified from Hewson et al. (2006)

each other, while samples collected 7 mo apart were only 33 to 57% similar. Samples from the same location collected 2 wk apart were only about 40% similar (Lee & Fuhrman 1991). The authors also reported that, of open ocean samples collected from 25 m water depth at one geographic location over 3 consecutive days, those from Days 1 and 2 were >90% similar to each other, while samples collected on the third day were only 50 to 70% similar to those collected on the other 2 days, respectively. Part of the difficulty in interpreting these studies (and others described above) is the separation of spatial from temporal changes, because patches can drift with currents and no ship can simultaneously sample over a large region.

To examine temporal variability, Hewson et al. (2006) performed 7 drifter studies of 24 to 360 h duration in the oligotrophic Gulf of Mexico, the North Pacific and the West Tropical Atlantic, and analyzed the samples using ARISA. The similarity indices between assemblages collected over time in the same drifter changed on average by a Sorensen index of $0.12 \, d^{-1}$ (comparing the presence/absence of, OTU and a Whittaker index of $0.17 \, d^{-1}$ (comparing proportions in various OTUs) per fingerprint across all surface drifter studies. We found similar or smaller daily changes in community composition in Southern California coastal waters sampled at one geographic location over a period of about 1 wk, or when following drifters over a period of a few days (J. A. Steele & J. A. Fuhrman unpubl.). Overall, these results suggest that, most of the time, there are modest day-to-day changes in community composition.

We have found that month-to-month variation at our SPOT site provides particularly valuable information on relationships among organisms and environmental parameters like temperature and nutrients. The monthly patterns of community composition in near-surface (5 m water depth) samples were studied over 4 yr using a statistical technique called discriminant function analysis (DFA), linked with autocorrelation

and multiple regression (Fuhrman et al. 2006). DFA was performed to evaluate whether it was possible to 'predict' the month based on the bacterial community composition, and in fact several subsets of the bacterial community, collectively including most of the taxa, did this very well with 80 to 100% accuracy. Autocorrelation was high at a 1 mo lag, showing significant similarities from month to month in community composition. With a 3 mo lag, there was little or no autocorrelation, and by 5 mo lag, the autocorrelation was negative, indicating a shift to a very different community in the 'opposite' season (Fig. 3). Most interestingly, the autocorrelation coefficient returned to a significant positive value with a lag of 10 or 11 mo, and varied sinusoidally with a period of a year. This indicates that the community predictably returned to a similar composition during the same season in consecutive years. Multiple regression showed that the community composition could often be predicted well ($r^2 \approx 0.7$) from parameters like temperature, salinity, inorganic nutrients, chlorophyll, bacterial and viral abundances. The predictability indicates that different bacteria indeed have different niches, with limited redundancy, otherwise different combinations of bacteria would appear under the same conditions and prediction would be difficult. Different subsets of the community could be predicted from different combinations of parameters, allowing us to start evaluating which bacteria thrive under which conditions.

NETWORKS

The statistical relationships among microorganisms, and between microbes and other parameters, permit us to differentiate the organisms in terms of their preferred conditions, and also to assign microbes into groupings that tend to co-occur and those that do not occur together (non-randomly). The information to elucidate these groupings is available from simple corre-
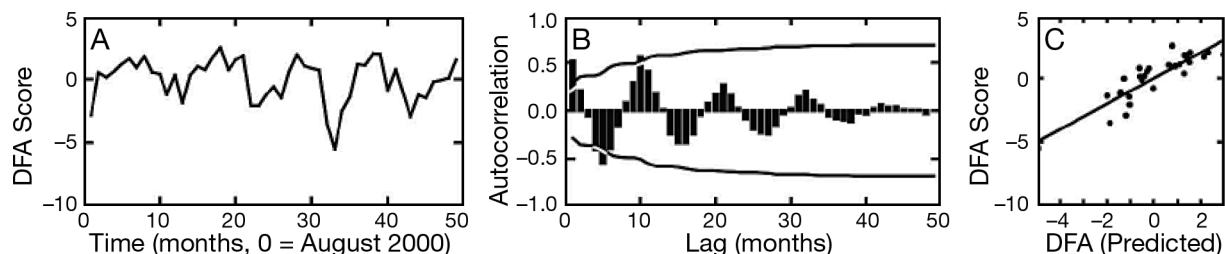

Fig. 3. Bacterial community composition at the San Pedro Ocean Time Series (SPOT) site at 5 m water depth: results of Discriminant Function Analysis (DFA), Time Series Analysis (Autocorrelation), and Multiple Regression Analysis for a 4 yr time series. (A) First discriminant function (DFA1, a measure of bacterial community composition) over time; (B) autocorrelation of DFA1 with various time lags, pairwise comparisons among all possible pairs of samples; line represents the $p < 0.05$ significance level. (C) Actual DFA1 vs. predicted DFA1 using multiple regression from parameters including temperature, salinity, nutrients, chlorophyll, viral and bacterial abundance; $r^2 = 0.7$. Modified from Fuhrman et al. (2006)

lations between organisms as individual OTU change over time, so again we can use our time series data to help characterize properties of particular taxa. We have found that it is particularly valuable to look not only for correlations of co-occurrence at each time point, but also for correlations that may be lagged in time — i.e. when one organism tends to follow another, or tends to decline after another one increases. Ruan et al. (2006) developed a mathematical method called Local Similarity Analysis (LSA) to permit evaluation of such time-lagged relationships, and showed, using examples from SPOT, how LSA can detect significant relationships that would be missed if time lags are ignored. Furthermore, by examining the pairwise relationships revealed by LSA, it is possible to draw an interaction diagram, or network diagram, that indicates the positive and negative mathematical relationships among microorganisms and between microbes and environmental parameters (Ruan et al. 2006). In a sense, this is a diagram of the 'niche space' of the various organisms, and we believe that it provides a very powerful tool for the examination of the 'natural history' of microbes in their complex wild habitats.

Additional examples for these mathematical relationships among bacteria and between bacteria and environmental parameters beyond those described by Ruan et al. (2006) are shown in Fig. 4. The figure is based on data collected as described by Fuhrman et al. (2006), with 36 samples from the chlorophyll maximum depth (average 32 m), collected approximately monthly between September 2000 and March 2004. Mathematical analytical methods are described in Ruan et al. (2006), and images were generated using Cytoscape (www. cytoscape.org). In these bacterial community network interaction diagrams, a few selected bacterial target groups are presented as central nodes, and only their nearest neighbors in the network are shown. These nearest neighbors are organisms or parameters with a direct or time-lagged significant positive or negative correlation (p < 0.05, by permutation tests) with the individuals of the target group. Some interesting relationships are apparent. First note that there are 10 different SAR11 types, each with unique combinations of relationships to other organisms and parameters (Fig. 4A). This suggests that there are probably at least 10 different 'ecological species' of SAR11 at our study site; an observation that contrasts with a few broadly defined types of SAR11 that have been reported to respond differently to different environmental conditions elsewhere (e.g. Mor-
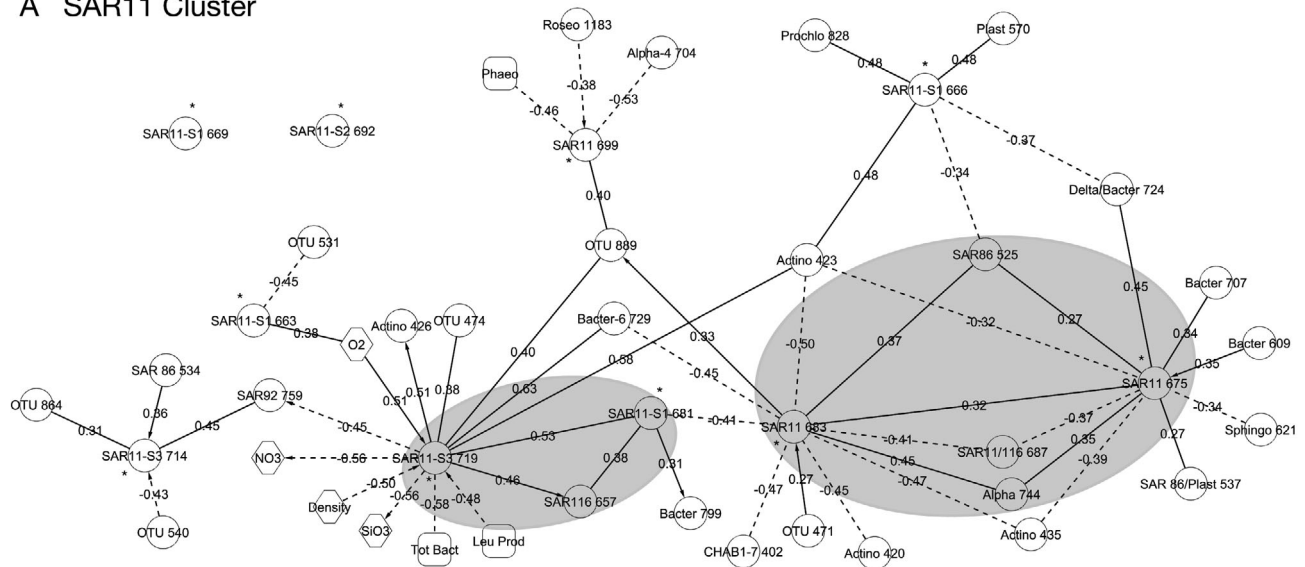

Fig. 4. (Above and following page.) Bacterial community network interaction diagrams based on San Pedro Ocean Time Series (SPOT) data, analyzed by Local Similarity Analysis (LSA). (*) Central nodes (members of the target group for each figure, i.e. SAR11 cluster in A). Only nearest neighbors, i.e. parameters that correlated significantly (p < 0.05, by permutation tests) with central nodes, are shown. Circles: bacterial OTU, hexagons: abiotic environmental parameters, squares: biotic environmental parameters, (—) positive correlations, (----) negative correlations, arrows indicate a 1 mo time lagged correlation, with the arrow pointing from the earlier to the later parameter. Numbers on lines are LSA correlation values, normalized to a −1 to +1 scale. Grey-shaded areas show where 2 members of the target group correlate positively with each other and share multiple positive correlations to other bacteria, as discussed in the text. Actino: Actinobacteria; Alpha, Delta, Gamma: types of Proteobacteria; Altero: Alteromonas; Bacter: Bacteroidetes; Flavo: Flavobacteria; Leu Prod: leucine-estimated bacterial production; Plast: plastid; Phaeo: phaeopigments; Roseo: Roseobacteria; SAR11 S1, S2, S3: SAR11 cluster surface 1, 2 and 3 subclades; Sphingo: Sphingobacteria; Tot Bact or Tot Vir: total bacteria or virus counts; Temp: temperature; Sal: salinity; Verruco: Verrucobacteria

Fig. 4 (continued)

ris et al. 2005). Interestingly, the 2 most strongly correlated members of the SAR11 cluster also happen to be distantly related to each other phylogenetically, with SAR11 719 belonging to surface cluster 3 and SAR11 681 belonging to surface cluster 1 (see Brown et al. 2005 for phylogenetic tree). Thus, even within a 'narrow' group, ecological relatedness does not necessarily follow phylogenetic relatedness. It can also be seen that some OTUs correlate primarily with other bacteria, while others correlate substantially with different elements of their environment. This may suggest that certain organisms are more influenced by their biotic environment, while others are influenced by their abiotic environment (e.g. physical or chemical forcing). Note that Fig. 4 shows many positive and negative correlations. Positive correlations may represent common preferred conditions or perhaps cooperative activities like cross-feeding, while negative correlations may represent competition for limiting resources, resource partitioning, or perhaps active negative interactions like targeted allelopathy (Long & Azam 2001). Note that many interactions involve a time lag, and it is likely that careful examinations of these temporal transitions can provide ecological insights as well as fodder for focused experimental design.

Each node and its nearest neighbors that are positively correlated constitute a group of organisms that tend to co-occur over time, like subsets of the ecological community that rise and fall together in abundance. Note that, in most cases, these community subsets include only a single member of the target group for that figure, i.e. 2 members of the same phylogenetic group or cluster are only in some instances directly connected by solid lines, but most are not. To us this suggests that, even though there may be physiological similarities among members of the same cluster, the different members tend to co-occur with different organisms. However, some of these subsets include members of the same cluster as well as a few other organisms that are consistently correlated with both group-mates. These are shown in Fig. 4 with grey shading, and include Actinobacteria 423 and 426, SAR11 683 and 675, SAR11 681 and 719, and *Roseobacter* 1183 and 989. These subsets may represent very similar organisms that share aspects of their niche, or alternatively might somehow complement or cross-feed each other. Note that some members of these groups (e.g. SAR11 675 and 666) are connected to several members of the same OTU, but with opposite correlations, suggesting possible exclusion mechanism(s) or perhaps niche partitioning.

This analysis is only a start, but we think it is a very promising approach to examining microbial interactions in nature. We are currently expanding this approach to include protists and Archaea in the interaction networks, yielding a remarkably comprehensive picture of microbial interactions (J. A. Steele et al. unpubl.). We believe that, as environmental microbial databases grow, this sort of analysis will provide a very powerful tool for the examination of complex interactions in nature.

In summary, the combination of molecular fingerprinting techniques and extensive marine sampling permits the evaluation of diversity patterns that have, until now, been undetectable. We have started to investigate what might be considered the natural history of planktonic marine microorganisms, hopefully permitting a more complete understanding of their roles and interactions.

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