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The phylogenetic and ecological context of cultured and whole genome-sequenced planktonic bacteria from the coastal NW Mediterranean Sea

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ABSTRACT

Microbial isolates are useful models for physiological and ecological studies and can also be used to reassemble genomes from metagenomic analyses. However, the phylogenetic diversity that can be found among cultured marine bacteria may vary significantly depending on the isolation. Therefore, this study describes a set of 136 bacterial isolates obtained by traditional isolation techniques from the Blanes Bay Microbial Observatory, of which seven strains have had the whole genome sequenced. The complete set was compared to a series of environmental sequences obtained by culture-independent techniques (60 DGGE sequences and 303 clone library sequences) previously obtained by molecular methods. In this way, each isolate was placed in both its "ecological" (time of year, nutrient limitation, chlorophyll and temperature values) context or setting, and its "phylogenetic" landscape (i.e. similar organisms that were found by culture-independent techniques, when they were relevant, and when they appeared). Nearly all isolates belonged to the *Gammaproteobacteria*, *Alphaproteobacteria*, or the *Bacteroidetes* (70, 40 and 20 isolates, respectively). Rarefaction analyses showed similar diversity patterns for sequences from isolates and molecular approaches, except for *Alphaproteobacteria* where cultivation retrieved a higher diversity per unit effort. Approximately 30% of the environmental clones and isolates formed microdiversity clusters constrained at 99% 16S rRNA gene sequence identity, but the pattern was different in *Bacteroidetes* (less microdiversity) than in the other main groups. Seventeen cases (12.5%) of nearly complete (98–100%) rRNA sequence identity between isolates and environmental sequences were found: nine in the *Alphaproteobacteria*, five in the *Gammaproteobacteria*, and three in the *Bacteroidetes*, indicating that cultivation could be used to obtain at least some organisms representative of the various taxa detected by molecular methods. Collectively, these results illustrated the largely unexplored potential of culturing on standard media for complementing the study of microbial diversity by culture-independent techniques and for obtaining phylogenetically distinct model organisms from natural seawater.

Introduction

The use of molecular methods to retrieve small subunit rRNA sequences from natural environments revolutionized microbial ecology [41]. About 20 years later, environmental sequences have significantly expanded the known diversity of *Bacteria* [18], *Archaea* [17] and *Eukarya* (e.g. [33]). Current high-throughput technologies easily provide estimates of the magnitude of bacterial diversity while identifying the most abundant organisms in each environment (e.g. [31]). In addition, and with the recent development of novel culturing protocols which combine the use of very dilute low-carbon media with strategies of dilution-to-extinction, the isolation of organisms that are known to form dominant populations in the oligotrophic ocean has begun to be successful [19,38,62]. Laboratory experiments with these isolates are generating novel knowledge on the physiology, metabolism and ecology of marine bacteria [19,67].

With traditional culturing methods it is also possible to isolate new microorganisms from the natural environment (i.e. [11]). In fact, the expanding literature describing novel marine

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bacterial taxa is evidence of a continuous and determined effort to explore the bacterial diversity in the sea. However, since the advent of molecular analyses, comparatively few studies in microbial ecology have comprehensively investigated the phylogenetic diversity of marine bacteria that can be maintained in the laboratory by standard culturing methodologies [12,13,24,32,44,47,49,57,60,62,70]. The well-founded reason for this is that, although standard culturing on rich media retrieves bacteria that belong to the same major groups of bacteria as the uncultured majority (e.g. *Proteobacteria* and *Bacteroidetes* [24]), there is only very limited overlap with uncultured microbes at the species, genus, and even family levels [62]. In spite of this, the advent of genomics in marine microbiology has promoted the experimental exploration of bacterial isolates from different environments, which has resulted in descriptions of diverse adaptations of marine bacteria to their environment (e.g. [20]). Although easily cultured marine bacteria are in many aspects different from the uncultured majority of the ocean, for instance, in the extent of genome streamlining [63], comparative genomics reveals that cultured bacteria encompass a substantial range of physiological adaptations to different growth conditions and therefore represent a wide variety of life strategies (e.g. [30,43,69]). Analysis of the diversity of cultured marine bacteria can therefore be helpful for selecting relevant raw material for experiments aimed at understanding the different ecological roles of marine bacteria.

The easily cultured bacteria typically form part of the rare biosphere [42], which is the large collection of microorganisms present in ecosystems at very low abundances [58]. This low abundance possibly protects them from viral lysis and predation, and allows their persistence for very long periods of time with little or no activity [42]. It has been suggested that when conditions change, rare bacteria can quickly multiply and become part of the abundant taxa where they potentially participate actively in bulk carbon cycling and energy flow [42,64]. Isolation in pure culture remains one of the windows into the characterization of a fraction of this rare biosphere. Even though many rare bacteria never become abundant they can still exert important environmental impacts, for example, if they are specialized in processing specific substrate types (e.g. [65]) or if they are potential pathogens such as *Vibrio* species. Moreover, experiments with isolates have the potential to uncover mechanisms underlying specific ecological processes, as shown by the work carried out using a large culture collection of marine vibrios as model microorganisms that revealed details concerning the evolution of resource use [7].

To understand the ecological roles of a specific organism requires either that ecophysiological response experiments with isolates are carried out (e.g. [21]), or that potential function is deduced from the spatiotemporal distribution of that particular taxon in relation to that of environmental variables [56,64]. One way of doing this is by describing the "environmental context" (in what season, with what type of nutrient regime, etc.) from which an isolate was obtained. Similarly, potentially relevant information can be obtained by defining the "phylogenetic context" of the isolate. In other words, which organisms detected by molecular or culturing techniques are similar to the target ones, and under which conditions they have been retrieved. Even if the isolates do not appear in culture-independent molecular surveys, close relatives might have appeared under some ecological circumstances, thus providing information that can be used to interpret genomic data and metabolic capacities (i.e. [27,62]).

The aim of the present study was twofold: (i) to describe the identity and diversity of the isolates retrieved with limited effort at the group level (i.e. phylum, class) from a coastal marine environment, and (ii) to compare these isolates to sequences previously obtained by molecular techniques (mainly DGGEs and clone libraries) at the same location and during the same period of time. Information is presented on 136 isolates obtained during a 3 year period from a well-studied site, the Blanes Bay Microbial Observatory. Seven isolates from this collection had their whole genome sequenced by the Moore Foundation Marine Microbiology Initiative, and several have been found to have interesting ecological features [21,22]. This exercise was undertaken in order to try to uncover, for example, isolates that responded to specific environmental conditions, and did not appear in molecular surveys or, conversely, isolates that were representative of dominant groups according to the molecular surveys and could be useful to aid genome interpretation.

Materials and methods

Study area and sampling

The study was carried out at the Blanes Bay Microbial Observatory (BBMO) in the NW Mediterranean, approximately 70 km north of Barcelona. Water was sampled monthly approximately 1 km offshore (41°40′ N, 2°48′ E) and immediately filtered through a 200 μm mesh. Seawater was kept in 25 L polycarbonate carboys and transported to the laboratory under dim light, where it was processed further within 2 h. Sampling was undertaken in parallel with carbon and sulfur cycling studies during the period 1998–2004 (e.g. see [4,68]).

Origin of bacterial isolates and community DNA

Samples for bacterial isolates and community DNA were obtained from the following sources: (1) Natural seawater – seawater collected as described above; (2) whole water enrichments – the effect of nutrient additions on the growth of heterotrophic bacteria was examined in unfiltered, whole water samples [46]. Briefly, nutrients were added to 250 mL samples in final concentrations of 40 μM C (as glucose), 2 μM N (NH_4Cl), and 0.6 μM P (NaH_2PO_4), singly and in all possible combinations. Samples were collected after incubation for 24 h at *in situ* temperature in the dark; (3) dilution cultures – several times during the period 2001–2004, 1.9 L of seawater were used as growth media after filtration through 0.2 μm pore size Sterivex™ units (Durapore–Millipore) using a peristaltic pump at pressures of <200 mmHg (for details, see [46]). Inocula consisted of 100 mL seawater prepared by gravity filtration through 0.8 μm pore size filters (Nuclepore™). The dilution cultures were either unamended controls or were enriched with C and P, alone or in combination. The substrates added were glucose, dimethylsulfoniopropionate (DMSP), pyruvate, glycerol, acetate, amino acids, inorganic P, ATP, or DNA; all at a final concentration of 20 μM C for carbon compounds or 0.6 μM P for P-containing compounds. Cultures were incubated in 2 L polycarbonate bottles (Nalgene) at *in situ* temperature (15–22 °C) in the dark. The samples were collected when bacteria reached the stationary phase (i.e. 2–5 days after inoculation); (4) mesocosm experiments – two mesocosm (20–100 L) experiments reported in detail in Pinhassi et al. [48] and Allers et al. [2] provided additional sequences. For these experiments, surface seawater was collected from Blanes Bay and transferred to the laboratory. Bacterial growth and diversity were monitored in mesocosms enriched with N and P and/or C for approximately 1 week.

Collection of community DNA

To collect microbial biomass, between 5 and 15 L of seawater were filtered through a 5 μm pore size Durapore™ filter (Millipore) and a 0.2 μm pore size Sterivex™ filter (Durapore, Millipore) in succession using a peristaltic pump. The 0.2 μm pore size Sterivex™ unit was filled with 1.8 mL of lysis buffer (40 mM EDTA,