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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,

50 Mm Tris–HCl, 0.75 M sucrose) and stored at −70 °C. Microbial biomass from the experiments was collected by filtering 0.5–1 L of water onto 0.2 μm pore size polycarbonate filters (Millipore) at <200 mmHg, and filters were stored in lysis buffer at −70 °C. Nucleic acids were extracted by a standard protocol using phenol/chloroform (details in [56]).

Origin of the sequences used in the phylogenetic comparison

(1) Bacterial isolates. A total of 625 bacterial colonies were collected from the above-mentioned sources, from which 136 isolates were selected from different sampling occasions or experiments for 16S rRNA gene sequencing. The selection was undertaken based on differences in colony morphology, in an attempt to retrieve a wide diversity of bacteria that formed colonies on standard culture media. In the case of very small colonies without distinct features, the selection was carried out at random. Selected isolates were intentionally collected in different years, seasons and from native seawater and enrichments, in order to allow the capture of bacteria with different temporal occurrence and different growth preferences (while still having the capacity to form colonies on rich media). This approach was based on our experience that, in general, and with a standard sampling effort, it was possible to retrieve bacteria belonging to 20–30 different taxa when sampling natural seawater on a specific occasion. The isolates were obtained by plating 100 μL of undiluted, or 10×, 100× and 1000× diluted seawater of untreated or experimental samples, in triplicates, onto modified Zobell agar plates (i.e. 5 g peptone, 1 g yeast extract and 15 g agar per liter medium [1 L was composed of 750 mL seawater from the sampling location and 250 mL Milli-Q water]). Agar plates were incubated at *in situ* temperature (between 11 and 25 °C, see Fig. 1) in the dark for 10–15 days and the targeted bacteria were isolated as described above. The 16S rRNA gene sequences of the 136 bacterial isolates were amplified by means of PCR using Taq polymerase (Boehringer–Mannheim) from DNA preparations and using bacterial 16S rRNA gene primers 27f and 1492r. Isolates were sequenced with internal primer 358f, which yielded sequence lengths of approximately 700–850 base pairs. Accession numbers of the isolate sequences are given in Supplementary Table S1.

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.syapm.2013.11.005.

(2) DGGE bands. DGGE bands from both environmental samples and enrichment cultures (whole seawater enrichments and dilution cultures) were also retrieved. The DGGE fingerprints were performed as previously described [54,56]. The 16S rRNA gene fragments (approximately 550 base pairs in length) were amplified with the *Bacteria* specific primer 358f that is complementary to position 341–358 (with the GC-clamp underlined; 5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG) and universal primer 907rm complimentary to position 927–907 (5′-CCGTCAATTCA/CTTTGAGTTT). The PCR products were loaded into a 6% polyacrylamide gel with a DNA-denaturant gradient ranging from 40% to 80%. The gel was run at 100 V for 16 h at 60 °C in 1× TAE running buffer. The DGGE bands were excised, reamplified, and verified by a second DGGE. Bands were sequenced using primer 358f without the GC-clamp with the Big Dye Terminator Cycle-Sequencing Kit (Perkin Elmer Corporation) and an ABI PRISM model 377 (v3.3) automated sequencer. These sequences have been reported in already published studies [2,3,46,48] and have GenBank accession numbers AY573520–AY573530, DQ473559–DQ473568, DQ778276–DQ778298, EF441555–EF441565, and EF441568-EF441570.

(3) Clone libraries. Clone libraries were constructed from *in situ* samples collected on 3 March, 14 May, 14 July, 4 August and 21 October 2003 [3]. Briefly, 16S rRNA gene sequences were amplified from total community DNA by PCR with universal primers 27f (5′-AGAGTTTGATCMTGGCTCAG) and 1492r (5′-GTTTACCTTGTTACGACTT), and the resulting amplicons were cloned using the TOPO-TA cloning kit. PCR amplicons were digested with the restriction enzyme HaeIII (Invitrogen), and the RFLP patterns were compared in order to identify unique clones. Clones were sequenced with internal primer 358f (5′-CCTACGGGAGGCAGCAG), which yielded sequences of approximately 700–850 base pairs.

(4) Additional sequences from other sites. For comparison, eight DGGE sequences from neighboring Banyuls Bay (ca. 150 km away) were included [55]. The isolate sequences obtained were additionally compared to the bacterial diversity in 16 samples collected on a transect from Blanes Bay to deep oceanic waters of the NW Mediterranean performed in September 2007. These samples were analyzed by 454 pyrosequencing through the International Census of Marine Microbes Initiative, and data from these analyses have been published previously [9,52].

Phylogenetic analysis

The 16S rRNA partial gene sequences of the isolates from this study were compared to sequences in GenBank (NCBI) using BLAST [5]. Comparison of BLAST results using different regions of the sequences showed that none of the sequences were chimeras. Phylogenetic analyses were performed using the parsimony methods implemented in the ARB sequence analysis package by which sequences were grouped into major phylogenetic clusters. Phylogenetic trees were constructed from sequences corresponding approximately to nucleotide positions 420–770 (*Escherichia coli* numbering). Sequences of close relatives found in GenBank after the BLAST process were included in the trees as references in order to delineate and name different clusters of sequences. The 16S rRNA gene sequence GenBank accession numbers of the isolates from this study were DQ294291, DQ403809, DQ403810, DQ481462, DQ481463, DQ681131–DQ681197, and EU253571–EU253601 (of which only the first five have been reported previously), and they are specified in Supplementary Table S1.

Comparison of sequence diversity

Rarefaction analysis was carried out using the online rarefaction calculator software (http://www2.biology.ualberta.ca/jbrzusto/rarefact.php). All clones with the same RFLP pattern were assumed to belong to the same OTU, and all OTUs were used for the analysis by considering the frequency of their patterns. Previous work in our laboratory had found this to be a very reasonable assumption [34]. In the first clone library, all clones were sequenced and it was found that clones with the same RFLP pattern shared a sequence identity of 98.9% (details not shown). In the context of this study, “sequence identity” was used to denote the percentage identity over the part of the 16S rRNA gene sequence investigated. For clustering sequence analysis, the software Clusterer was used [28]. This allowed grouping of sequences into percentage identity clusters (100%, 99%, etc.) by the nearest-neighbor approach (i.e. sequences were added to clusters if there was at least one sequence that was within a set identity threshold). These clusters based on a 99% identity cut-off served as the basic units for statistical extrapolation for comparison of sequence composition between the major phylogenetic groups, and between molecular and isolation approaches.

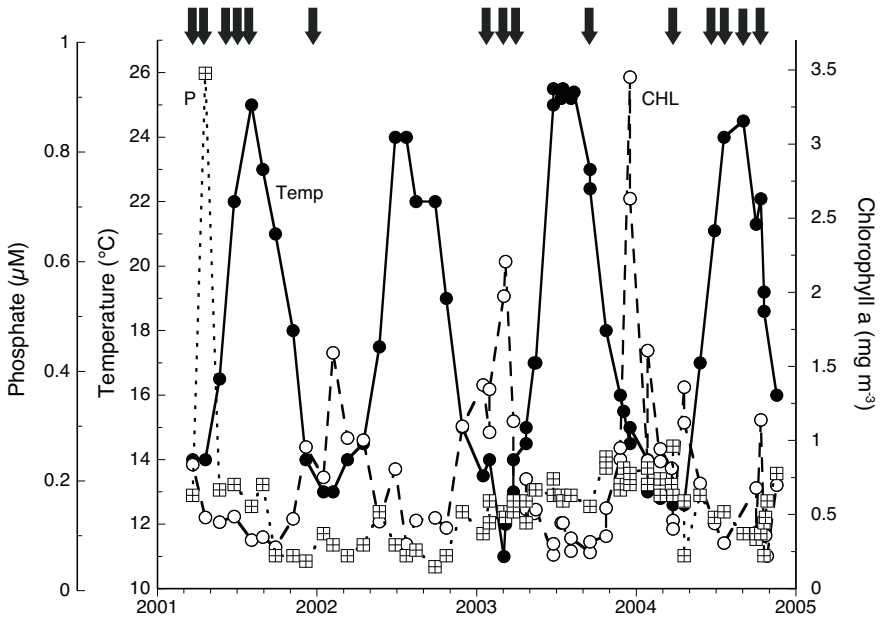

Fig. 1. Seasonal variations in chlorophyll *a* concentration, temperature and phosphate concentration (μM) in Blanes Bay during the period 2001–2004. The arrows indicate the samples from which isolates were obtained.

Results

The identity of the 136 bacterial cultures representing 84 species isolated and analyzed from Blanes Bay has not been reported in previous studies. Their origin, closest environmental sequence, and closest cultured relative are detailed in Supplementary Table S1. Environmental conditions at the BBMO during the 3.5 year period when the isolates were retrieved are presented in Fig. 1. The 16S rRNA gene sequence diversity of the isolates was investigated by BLAST, multiple alignment and phylogenetic analyses, in conjunction with sequences from previously published studies at the same site retrieved from both natural seawater samples (331 sequences – 162 operational taxonomic units – OTUs – obtained from 16S rRNA gene clone libraries and DGGE – denaturing gradient gel electrophoreses [3,56]) and from incubation experiments (32 sequences obtained from DGGE analyses [2,48]) (see Table 1 for summary).

An example of a DGGE gel analysis from an enrichment experiment (conducted in 2002; unpublished data) is shown in Fig. 2. In this experiment, the response of the natural bacterial community to phosphate, ATP and DNA (i.e. different forms of phosphorous in this environment where bacterial growth is typically P-limited) was determined. DGGE band B90 was seen to increase in intensity in the three treatments with P-rich compounds, but was not found in the unenriched control. This band was identical to isolate MED128, a member of the genus *Nereida*, which had been isolated 1 year earlier.

Sequence analysis showed that the number of OTUs was relatively similar for isolates and clones (Fig. 3A), although the initial number of clones was larger. Moreover, this occurred even though all isolates were obtained on ZoBell medium, with no particular efforts to provide a variety of culture conditions. The increase in number of OTUs as a function of increasing cut-off levels for delineating OTUs for either the clones, the isolates or for both together showed distinct discontinuities around 86% and 97%. It was noted that the former was slightly lower than sequence identity levels within families, while the latter corresponded to the once assumed "species" level (e.g. [53]), although this threshold is now considered to be closer to 98.5% [59]. Both the *Gammaproteobacteria* and *Alphaproteobacteria* showed the inflection point at approximately 97% identity, with the number of OTUs nearly doubling or more when increasing from 97% to 100% sequence identity for delineating an OTU (Fig. 3B). In contrast, the *Bacteroidetes* showed a much more uniform increase of OTUs with increases in percentage identity (Fig. 3B). This indicated a larger microdiversity in the *Proteobacteria* than in the *Bacteroidetes*.

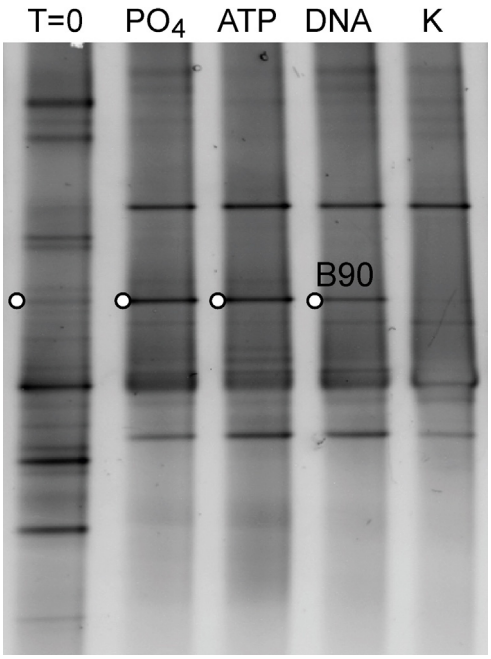

Fig. 2. DGGE gel showing resulting band patterns from the enrichment experiment with P-containing nutrients. The *in situ* sample (labeled T = 0) showed a larger number of bands than the enrichments. Enrichments consisted of inorganic P (PO_4), adenosine triphosphate (ATP) and deoxyribonucleic acid (DNA) compared to a control (K). One band, *Alphaproteobacteria* phylotype B90, indicated by white circles, strongly increased in intensity with P enrichment. This phylotype was also present *in situ*, but in a much lower relative abundance. Notably, phylotype B90 was identical to the *Roseobacter* clade isolate *Nereida* sp. M128 (see Supplementary Table S1).

Table 1
Number of characterized isolates, clones, and DGGE bands belonging to different taxonomic groups retrieved from Blanes Bay.

	Isolates		Clones		DGGE bands	
	Total	Unique	Total	Unique	*In situ*	Experiments
Alphaproteobacteria	40	20	219[a]	80	16	17
Betaproteobacteria	2	2	1	1	0	0
Gammaproteobacteria	70	39	50	26	4	2
Deltaproteobacteria	0	0	0	0	1	0
Epsilonproteobacteria	0	0	1	1	0	0
Bacteroidetes	20	19	18	14	7	13
Firmicutes	3	3	0	0	0	0
Actinobacteria	1	1	4	3	0	0
Verrucomicrobia	0	0	10	9	0	0
All	136	84	303	134	28	32

[a] 95 belonged to the SAR11 clade.

Rarefaction curve analysis showed that a substantially larger number of distinct OTUs were obtained for the *Alphaproteobacteria* isolates than for clones, while for *Gammaproteobacteria* and *Bacteroidetes* similar estimates of OTU numbers were obtained from both isolates and clones (Fig. 3C). The rarefaction curves also indicated that *Bacteroidetes* was the most diverse taxonomic group at the BBMO. Moreover, both isolation and the cloning efforts were far from covering the actual diversity of the site, which, based on the 454 pyrosequencing of the of 16S tags with 20,000 tags per sample, is estimated to be in the order of 2000 OTUs per sample [52].

Identity of the cultures

To visualize tentative phylogenetic relationships between isolates, phylotypes deduced from 16S rRNA clones, DGGE sequences, and reference species, phylogenetic trees were constructed using the parsimony methods implemented in ARB for the *Alphaproteobacteria*, *Gammaproteobacteria* and *Bacteroidetes* (Figs. 4–6). The word "tentative" denoted that partial sequences, such as those used here for constructing the tree, should not be used to infer strict phylogenetic relationships; these trees primarily serve to visualize an otherwise complex dataset. Nevertheless, sequence alignment using ClustalW and neighbor-joining for tree construction with the software MegAlign in the DNASTAR package (version 7.0) yielded similar branching patterns (trees not shown). In the phylogenetic trees, some sequence clusters were numbered in order to aid in navigating the data set. This is further clarified in Table 2, where the variable percentage identities between sequences in each cluster are presented 94–98% for *Proteobacteria*, but much wider for the highly divergent *Bacteroidetes* phylum.

(a) Alphaproteobacteria. Phylogenetic analysis of the *Alphaproteobacteria* revealed that a substantial fraction of the isolates belonged to different clusters within the *Roseobacter* clade (clusters Alpha-1 to -4, Fig. 4). In addition, isolates were found belonging to *Erythrobacteraceae* (cluster Alpha-5), *Hyphomonadaceae* and *Aurantimonadaceae*. Clusters Alpha-1 (related to clone NAC11-7) and Alpha-4 (related to clone CHAB-1-5) included only sequences obtained by culture-independent techniques from *in situ* samples, and showed within-group similarities of >95% and >96%, respectively (Fig. 4 and Table 2). On the other hand, cluster Alpha-2 included only sequences from isolates and P-enrichment experiments (Mes9 and B-260) but none were retrieved directly from the *in situ* samples. In addition, six isolates represented bacterial groups/genera that were not detected by molecular techniques (MED588, MED464, MED441, MED624, MED623 and MED463).

Two clusters containing both isolates and environmental sequences were found (Alpha-3 and -5, Fig. 4). In cluster Alpha-3 the sequences showed within-group similarities of >97% and belonged to the genus *Nereida*. Isolate MED128 was 99.8% similar to clone sequence SPR23 and 99.6% similar to DGGE bands B90 and B94, obtained in P, ATP and DNA addition experiments. Cluster Alpha-5 consisted of the genus *Erythrobacter*, with four isolates, two clone library sequences, and one *in situ* DGGE band. *Erythrobacter* isolate MED442 was 99.8% similar to the clone sequence AUT4 and was identical to DGGE band 27. Apart from these clusters, the *Roseobacter* clade isolate MED483 was 99.8% similar to clone SPR41. Thus, isolates MED128, MED442, and MED483 seemed to be good candidates for investigating pure cultures representative of marine bacterioplankton.

(b) Gammaproteobacteria. *Gammaproteobacteria* accounted for half of the isolated bacteria. This was the most diverse group in terms of richness, and most isolates fell into five major clusters (Table 1 and Fig. 5). In cluster Gamma-1 (*Alteromonas*) there were several coincidences of isolates with sequences from *in situ* clone libraries. This cluster consisted of nine isolates from different experiments with combined enrichments of inorganic P and C sources, such as glycerol, glucose and dimethylsulfoniopropionate

Table 2
Number of sequences of different origins from each cluster and the percentage identity between sequences within each cluster. The sequences not closely related to any cluster are not represented in this table.

	%	Isolates	Clones	DGGE *in situ*	DGGE experiments
Alphaproteobacteria					
Cluster 1 (NAC11-7)	>95	–	2	2	3
Cluster 2 (*Phaeobacter*)	>98	2	–	–	2
Cluster 3 (*Nereida*)	>97	2	1	–	2
Cluster 4 (CHAB-1-5)	>96	–	2	5	2
Cluster 5 (*Erythrobacter*)	>97	4	2	1	–
Gammaproteobacteria					
Cluster 1 (*Alteromonas*)	>97	9	2	4	1
Cluster 2 (*Pseudoalteromonas*)	>97	4	–	–	–
Cluster 2 (*Vibrio*)	>94	10	–	–	–
Cluster 4 (*Marinomonas*)	>94	7	–	–	–
Cluster 5 (*Marinobacter*)	>96	3	–	–	–
Bacteroidetes					
Cluster 1 (*Dokdonia*, *Gramella*)	>89	9	1	1	–
Cluster 2 (ZD0403 and CF6)	>88	–	6	2	1
Cluster 3 (*Polaribacter*)	>94	6	–	2	4
Cluster 4 (AGG58)	>85	–	4	1	5

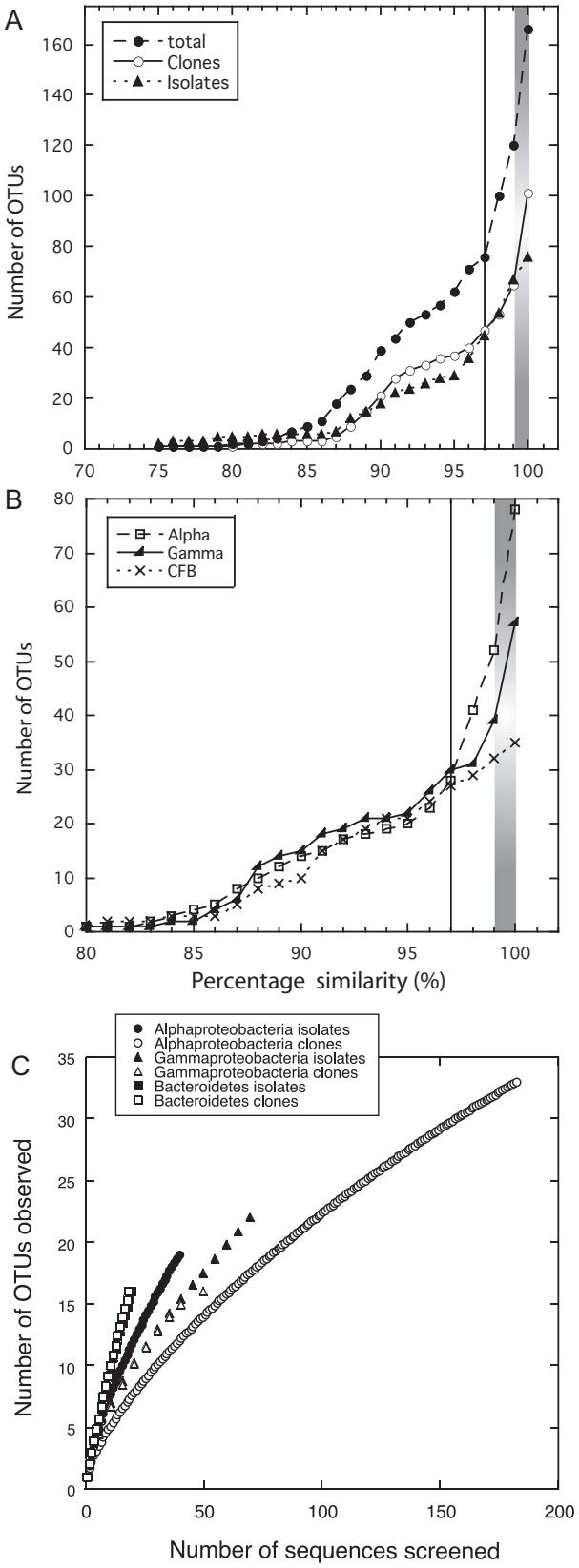

Fig. 3. (A) Number of OTUs obtained as a function of the percentage identity cut-off chosen to group sequences, with the three major phylogenetic groups of bacteria combined as retrieved by culturing, cloning or both together. (B) Total number of OTUs obtained at each cut-off level using clone libraries and isolation techniques combined for each of the three main groups. (C) Rarefaction analysis of all sequences divided into the three main groups analyzed (*Alphaproteobacteria*, *Gammaproteobacteria* and *Bacteroidetes*).

(DMSP). In this cluster, three isolates (MED111, MED185 and MED292) were found that were identical or nearly identical (>99%) to the DGGE bands (labeled as AY and BY) obtained in a mesocosm experiment carried out in 1996 in Banyuls, approximately 150 km northeast of Blanes Bay [55]. Furthermore, isolate MED169 was identical to DGGE band B145 from an experiment (isolate MED604 was 99.8% similar to this sequence). All the sequences from this *Alteromonas* group were distantly related to clone JUL4, which represented 30% of the clones in the July clone library.

Three clusters were formed only by isolates: Gamma-2 (*Pseudoalteromonas*), Gamma-3 (*Vibrio*), and Gamma-4 (*Marinomonas*). Among the *Vibrio* sequences there was a substantial phylogenetic diversity (within-cluster identity of 94%, Table 2). Most vibrios were isolated from C and P enrichment experiments. In contrast, the *Pseudoalteromonas* isolates were obtained from unenriched seawater samples from different seasons (March, June and November). *Marinomonas* isolates were primarily obtained from enrichments with ATP.

Outside the major clusters, isolate MED266 (*Spongiispira*), from unenriched seawater cultures, was 99.3% similar to clone JUL18 that was found in three copies in the July clone library. Isolates affiliated with the genus *Marinobacter* (cluster Gamma-5) were retrieved from enrichments with three different carbon sources (glucose, pyruvate or DMSP) in combination with phosphate. Bacteria belonging to six genera, which had not been detected by molecular techniques, were detected by isolation: *Pseudoalteromonas*, *Vibrio*, *Halomonas*, *Marinomonas*, *Oleispira* and *Marinobacter*.

(c) Bacteroidetes. Most of the *Bacteroidetes* sequences belonged to the *Flavobacteriaceae* family (Fig. 6 and Table 2) and most isolates belonged to clusters Bacteroidetes-1 and -3. Cluster Bacteroidetes-1 contained five different subclusters (roughly corresponding to genera): *Gramella*, *Sufflavibacter*, *Dokdonia* (including *Krokinobacter*), *Salegentibacter* and *Leeuwenhoekiella* (Fig. 5). The latter two genera had not been found in Blanes by molecular techniques. In this cluster, one coincidence between isolates and environmental sequences was found: the genome-sequenced isolate *Dokdonia* sp. MED134 [23] was nearly identical (99%) to a DGGE band from Banyuls. In cluster Bacteroidetes-3 there were three subclusters: sequences related to *Polaribacter*, including isolates and DGGE sequences from P-enrichment experiments and mesocosm algal blooms – this cluster contained the genome-sequenced isolate *Polaribacter* sp. MED152 [22]; sequences related to *Tenacibaculum* with a similar origin; and isolate MED341 related to two environmental sequences found in winter samples in two separate years obtained by DGGE (BL98-5 and BL03-7).

Cluster Bacteroidetes-2 included a large number of sequences obtained by molecular techniques, and they affiliated to Delaware cluster 2 and North Sea sequences CF6 and ZD0403 [26,71]. Cluster Bacteroidetes-4 was also composed of uncultivated phylotypes and largely corresponded to the AGG58 clade [40]. Isolates MED466 and the MED571, belonging to the genus *Microscilla*, had not been found before in Blanes Bay by cultivation-independent techniques.

Isolates versus environmental sequences – inclusion of 16S tag sequences from 454 pyrosequencing

The similarities between isolates and the most closely related sequences detected by molecular methods are presented in Table 3. In this case, we chose to add correspondences to the 16S tag sequences from 454 pyrosequencing that were obtained in September 2007 (cf. [52]). Since these 16S tags were short (average 60 base pairs), only those pairs of sequences that had 100% identity were considered similar. Sequences from cultures obtained from one season of the year tended to cluster together with environmental sequences from the same time of year for the *Alphaproteobacteria* and *Bacteroidetes*. However, no seasonal

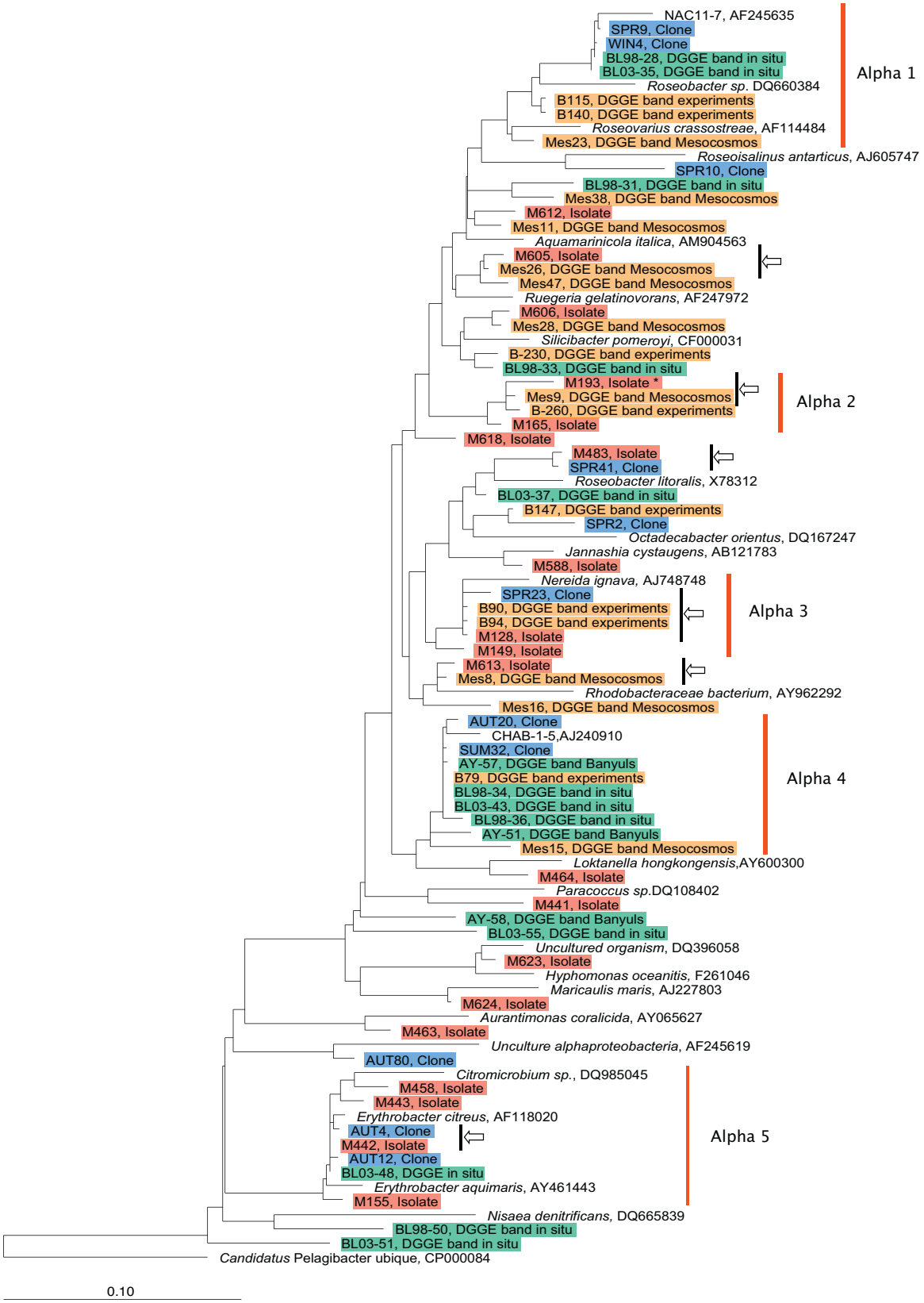

Fig. 4. Maximum parsimony tree illustrating the relationships between the partial 16S rRNA gene sequences from Blanes Bay for the *Alphaproteobacteria*. Colors indicate the origin of the sequences: bacteria isolated on solid media (red), DGGE bands from experiments (orange), DGGE bands from *in situ* samples (green), and clones retrieved from *in situ* samples (blue). Clusters are indicated by vertical red lines but note that the level of within-cluster sequence identity differs between clusters (see Table 2 for details). Identities between isolates and sequences, defined as >99% sequence identity, are marked by white arrows (see Table 3 for detail of the identities). The isolates marked with asterisks have been genome sequenced. SAR11 sequences detected in 16S rRNA gene clone libraries are not displayed in the tree.

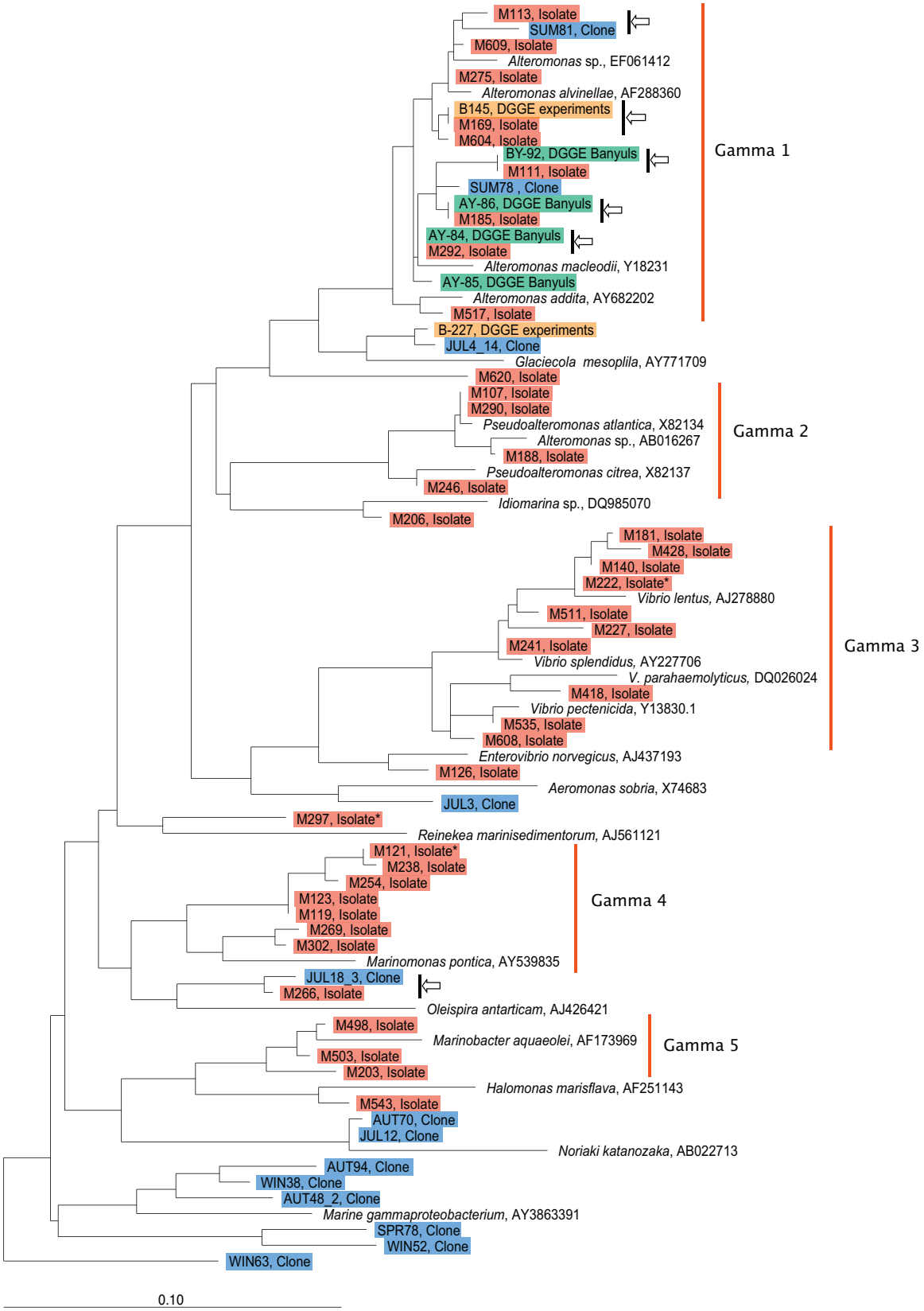

Fig. 5. Figure legend as Fig. 4 but for the *Gammaproteobacteria*.

effect was detected for *Gammaproteobacteria*. Three of the genome-sequenced isolates could not be associated to any environmental sequence, and one (M217) was only close to a 16S tag. The genome-sequenced isolates MED134, MED152, and MED193 were similar to DGGE bands and 16S tag 454 sequences, but not to clones. Various other isolates (8) were similar to 16S tags, others (7) to DGGE bands, and a few (M128, M266, M442 and M483) were similar to clones.

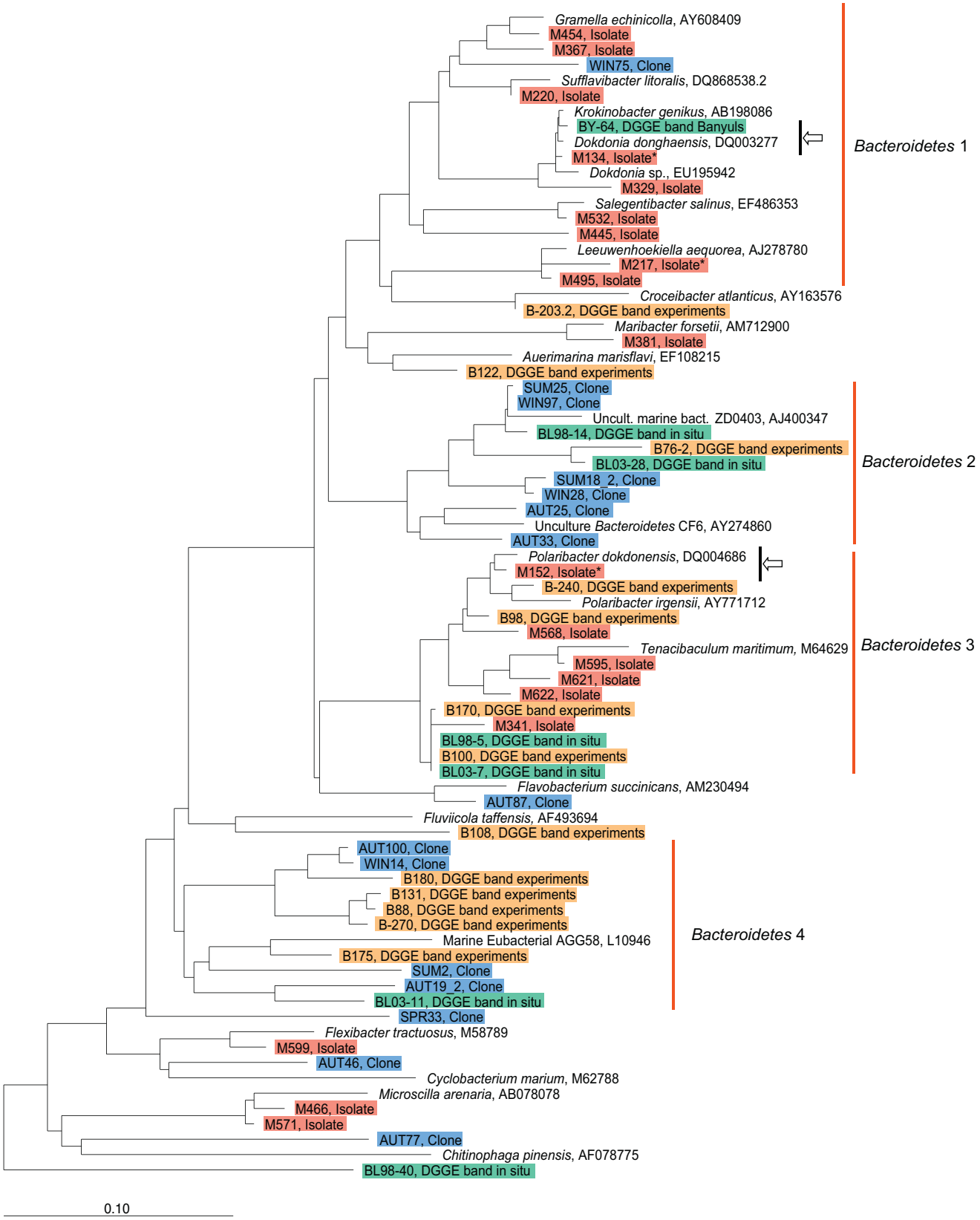

Fig. 6. Figure legend as Fig. 4 but for the *Bacteroidetes*.

Discussion

In contrast to molecular surveys of bacterial diversity, a rather limited number of microbial ecology studies have investigated the diversity of marine bacteria that can be cultured on solid media, or have compared this diversity with that obtained independently of cultivation (e.g. [11,12,24,62]). Furthermore, the variety of marine environments for which information exists on the diversity of

Table 3
Isolates and the most similar sequences retrieved by molecular methods.

Isolate	Similar seq. name	Seq. type	Seq. origin	Identity (%)
Coincidences (99–100%)				
M105		454-16S tag	Summer 2007	100
M111	Banyuls BY-92	DGGE band	June 2001	99
M128	SPR23	Clones	Spring 2003	99
	D90, D94	DGGE bands	June 2001	99
[a]M134	Banyuls BY-64	DGGE band	June 2001	99
		454-16S tag	Summer 2007	100
[a]M152	B-240	DGGE band	May 2001	97
		454-16S tag	Summer 2007	100
M169	B145	DGGE band	May 2001	99
M177		454-16S tag	Summer 2007	100[b]
M185	Banyuls AY-86	DGGE band	June 2001	100
[a]M193	Mes 9	DGGE band	Oct 2004	100
	BL206	DGGE band	July 2007	100
		454-16S tag	Summer 2007	100[b]
[a]M217		454-16S tag	Summer 2007	100[c]
M266	JUL18_3	Clones	Summer 2003	99
292	Banyuls AY-84	DGGE band	June 2001	99
M442	AUT4	Clones	Autumn 2003	99
		454-16S tag	Summer 2007	100
M456		454-16S tag	Summer 2007	100
M463		454-16S tag	Summer 2007	100
M483	SPR41	Clones	Spring 2003	99
		454-16S tag	Summer 2007	100
M495		454-16S tag	Summer 2007	100[c]
M504		454-16S tag	Summer 2007	100
M513		454-16S tag	Summer 2007	100
M588		454-16S tag	Summer 2007	100
M605	Mes26	DGGE band	October 2004	99
M613	Mes8	DGGE band	October 2004	99
≥5% different (96–98%)				
M113	SUM81	Clone	Summer 2003	98
M568	B98	DGGE band	April 2001	98
M606	Mes28	DGGE band	October 2004	98
M612	Mes11	DGGE band	October 2004	96
Not found but genome-sequenced				
[a]M121	*Marinomonas*	Different seasons		
[a]M222	*Vibrio*	Different seasons		
[a]M297	*Reinekea*	Autumn		

[a] Genome-sequenced isolates.
[b] These two organisms shared 100% of their V3 sequence targeted by the 454 primers.
[c] These two organisms shared 100% of their V3 sequence targeted by the 454 primers.

cultured bacteria is rather limited. However, substantial advances have been made in recent years in culturing part of the "uncultured" diversity. Most notably, cultivation of members of the SAR11 clade has allowed their taxonomic status, as well as some of their physiological and ecological features, to be characterized (e.g. [61]). Nevertheless, it would be desirable to be able to investigate as many different model organisms as possible, whether they are abundant in molecular surveys and can be simultaneously retrieved in culture, or whether they are rarer representatives of major groups widespread in the sea. Considering the limited attention given to cultured bacterial isolates, disclosing patterns of diversity obtained by culturing as well as molecular approaches could be rewarding.

Both culture-dependent and -independent methods indicated that *Alphaproteobacteria*, *Gammaproteobacteria* and *Bacteroidetes* were the predominant groups in the waters of the Blanes Bay Microbial Observatory. Together, these three groups accounted for 96% of the isolates, 95% of the clones, and 96% of the DGGE bands (Table 1). A seasonal study carried out at the same sampling site by FISH, indicated that, on average, *Alphaproteobacteria*, *Gammaproteobacteria* and *Bacteroidetes* accounted for 30%, 6%, and 11% of the DAPI counts, respectively (and 75% of the EUB+ count [3]). A recent study focused on pyrosequencing (454) 16S tags, including the BBMO site as well as more oceanic samples and particle-attached bacteria, found 48% *Alphaproteobacteria*, 25% *Gammaproteobacteria* and 4% Bacteroidetes [9]. If FISH is taken to be the most quantitative technique for identifying bacteria in marine waters, *Gammaproteobacteria* were overrepresented in the three sets of sequences considered in this study, particularly in the culture collection, where they represented 51% of the isolates, as well as in clones (17%) and DGGE bands (14%, Table 1). *Alphaproteobacteria*, although often overrepresented in molecular surveys [3,8], showed roughly the same importance in the culture collection (30%) as in FISH, but were overrepresented with the two molecular approaches (72% of the clones and 57% of the DGGE bands). Finally, the *Bacteroidetes* were represented in percentages not too different from FISH by the three approaches (15% in cultures, 6% in clone libraries, and 25% in DGGE bands). Among the least represented groups, *Betaproteobacteria* and *Actinobacteria* were recovered by both culturing and molecular approaches, while *Deltaproteobacteria*, *Epsilonproteobacteria*, and *Verrucomicrobia* were only found by molecular techniques, potentially reflecting that the latter taxa do not typically grow on rich standard media.

While the different methods overlapped well at the phylum/class level, there was limited overlap at the genus/species level. In fact, 12 out of the unique 84 bacterial isolates (Table 3) had 16S rRNA gene sequences that coincided (>99%) with a clone or DGGE sequence. This number increased to 22 coincidences if the comparison to 16S tag sequences (considering only reads with 100% sequence identity to isolates) were also included. All in all, from 14% (DGGEs) to 26% (clones) of the isolates, also including 16S tags, were detected in molecular surveys, and we are not aware of similar comparisons in the literature.

By combining different methodologies for determining bacterial diversity a substantial increase was recorded in the detected diversity. In total, 194 different 16S rRNA gene sequences were found by culture-independent techniques (not considering the plastid and cyanobacterial sequences) and isolation resulted in an increase of 75 sequence types/OTUs detected in Blanes Bay to a total of 269 OTUs (i.e. >25% increase). Thus, the cultured bacteria contributed to describing the ecosystem biodiversity by adding information to the diversity determined by PCR-based molecular techniques (see seed bank discussion below). Notably, a large part of the cultured bacteria belonged to major phylogenetic lineages (at least 13 different ones) that remained undetected by culture-independent techniques. Our findings indicated that the phylogenetic diversity found by culturing techniques could complement and widen the perception of the diversity present at a particular location of interest [11].

Interestingly, there were substantial differences in the diversity of clones and isolates on different specific sampling occasions. This was likely to be a consequence of the seasonal changes in the structure of the marine bacterioplankton community in Blanes Bay. At a large group level, it appeared that the changes throughout the year were relatively limited [3,56], while within each of the groups there was a pronounced internal succession of closely related phylotypes [10,56]. Table 3 shows how isolates from the spring season clustered together with sequences obtained at the same time of the year retrieved by molecular methods in the *Alphaproteobacteria* and *Bacteroidetes* phyla. This was consistent with a detailed study of the seasonality in the Blanes Bay *Bacteroidetes* showing that the phylotypes appearing in summer were very different from those appearing in winter [10]. However, this was not seen in *Gammaproteobacteria* where isolates seemed to have less seasonal preferences. These dynamics had consequences for the number of coincidences found between isolates and molecularly-retrieved sequences. For example, if the spring clone library had not been carried out the coincidences in two of the *Roseobacter* clusters (1 and 2) would have been missed, and without the autumn library

the coincidence with *Erythrobacter* would also have been missed. Therefore, it is relevant to note that an increased sampling effort resulted both in a higher total diversity detected, as well as an increase in the number of coincidences between isolates and *in situ* sequences.

The cases where isolates and *in situ* sequences (from clones or DGGE bands) shared identical or nearly identical 16S rRNA gene sequences provided good candidates for laboratory studies of environmentally relevant microorganisms, although it remains uncertain as to what degree the behavior of bacteria in pure culture is representative of the behavior in their complex natural environment. Irrespective of their absolute abundances or activities on any particular sampling occasion, it was noted that a growing number of studies on cultured bacteria (both cultured and previously "uncultured") have recently contributed significantly to a further understanding of bacteria in the sea. This is largely thanks to genome sequencing that provides testable hypothesis about their metabolism, ecology and evolution [15,20,37,51]. Seven of the bacteria isolated in this study have been whole-genome sequenced and this genomic information has been used in different recent studies. For example, *Phaeobacter* sp. MED193 has been featured in a study on the diversity and evolution of gene transfer agents in marine *Alphaproteobacteria* [29], and *Reinekea blandensis* MED297 [47] was included in a recent analysis of bacterial group II introns [36]. Three sequenced genomes belonged to the *Bacteroidetes*: we recently showed that *Dokdonia* sp. MED134 and *Polaribacter* sp. MED152 contained proteorhodopsin and that light could stimulate growth of the former bacterium, and anaplerotic CO_2-fixation in the latter [21,22]. MED217 has been formally described as a new species [45] and its genome features the first mercuric reductase found in the *Bacteroidetes* phylum [39]. Knowledge of the "ecological and phylogenetic environment" of cultured bacteria (e.g. at what times of the year the organism appears, whether it does so also in the PCR-retrieved sequence pool, or with/without nutrient additions, etc.), as carried out in this study, provides the framework needed to exploit the genomic and physiological information provided by a study of the isolates. As an example, the MED152 genome carried genes for particle attachment, gliding motility and polymer degradation [22] and it could thus be predicted to appear attached to particles. Indeed, MED152 coincided with a 16S tag (Table 3) that was found associated with particles in the Crespo et al.'s study [9]. Such features help to hypothesize and interpret how the genetics and physiology may translate into ecological benefits and fitness trade-offs in the natural environment.

In this study, we reasoned that enrichment cultures would be a good source of isolates with potential ecological relevance. By incubating seawater samples with different carbon sources and/or nutrients we intended to retrieve bacteria that would respond to nutrient inputs in nature. It is a frequent observation that novel bands appear in DGGE gels in response to experimental manipulations (e.g. Fig. 2 and [35]). Therefore, it was speculated that these bands would correspond to bacteria that are not very abundant but are relatively easy to isolate in pure culture. In fact, some coincidences were found between isolates and DGGE bands that appeared in response to enrichment experiments (Figs. 2 and 4–6). It is envisaged that these enrichment experiments will provide an opportunity for bacteria in the seed bank to grow and become abundant members of the bacterial community [42], hopefully mimicking episodic enrichments in nature (see e.g. [65]). The band that appeared in the gel of Fig. 2 in response to P additions can be seen in the lane corresponding to the natural samples before the start of the experiment. However, this band was too faint for successful sequencing and, thus, would have remained unidentified by the conventional molecular techniques used, although under appropriate conditions it became one of the dominant members of the assemblage. This suggested that MED128 is normally a member of the rare biosphere, but may become important when episodes of P enrichment occur. The presence of these bacterial types in the environment would be primarily controlled by bottom-up mechanisms. The recurrence of the same bacterial populations in a given environment year after year in the same season, which has been observed often [16], can be traced potentially to the dependence of most phylotypes on physical and chemical forcing in the environment.

Microdiversity clusters, defined as clusters constrained at 99% 16S rRNA gene sequence identity in the sense of Acinas et al. [1], are often observed in environmental clone libraries [1,14,51]. However, with the exception of the pioneering work on *Vibrionaceae* isolates [25,66], we are not aware of any reports on microdiversity of larger collections of marine bacterial isolates. It was observed that a high fraction of the total diversity for both environmental clones and isolates formed microdiversity clusters. In fact, 30% of the total observed OTUs (by both approaches) were grouped in microdiversity clusters (Fig. 3A). However, it should be noted that the sampling strategy for the isolates to analyze primarily bacteria that showed differences in colony morphology was, in effect, designed to detect more distantly related bacteria rather than those that were potentially closely related (i.e. forming microdiverse clusters). Thus, our findings should represent conservative estimates of the microdiversity among the cultured bacteria.

Interestingly, the microdiversity was not equally distributed among all taxonomic groups, since the observed microdiversity was mostly due to the *Gammaproteobacteria* in the case of isolates and to the *Alphaproteobacteria* in the case of clones, while *Bacteroidetes* exhibited the lowest microdiversity frequency of all groups (Fig. 3B). This low microdiversity could be the effect of a lower number of *Bacteroidetes* sequences compared to the 223 and 116 sequences from *Alphaproteobacteria* and *Gammaproteobacteria*, respectively. However, the low microdiversity of *Bacteroidetes* found in our study has also been a characteristic observed in other studies [1,51]. The ecological significance of the relatively high abundance of very divergent lineages for *Bacteroidetes* remains unresolved.

Previous studies have shown that microdiversity clusters could represent important units of evolutionary differentiation, such as ecotypes in natural populations of bacteria [6,50,66]. The finding of a wide distribution of microdiversity clusters among isolate and clone sequences alike, suggested that similar evolutionary processes act on cultured and still uncultured bacteria. Thus, marine bacterial isolates could serve as models to investigate how microdiversity clusters arise and provide indications of the ecological significance of such genetic variability.

Conclusion

The analysis of the diversity of bacteria cultured from seawater in the NW Mediterranean Sea revealed some relevant findings, some of which were expected although others were unexpected. First, an increase was shown in the bacterial diversity detected by combining culturing and molecular approaches. Second, there were striking similarities in patterns of microdiversity within both cultured and uncultured clusters of major bacterial groups. Third, isolates representative of taxa that were amenable to ecophysiological response experiments were identified. Fourth, indications were obtained that a substantial diversity could be found among cultured bacteria that were part of a microbial "seed bank". Moreover, since several of the isolates have been whole-genome sequenced, the current analysis also provided an example of the phylogenetic and ecological context necessary to make the most out of the genome sequences.

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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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Table S1 -Supplementary information- Identity of the bacteria isolated from Blanes Bay in the period 2001-2004. The sequences had different origins and came from different years, different seasons, and also from different types of enrichment experiments The percentage sequence identity to other isolates or sequences in GenBank is indicated based on approximately 500 base pairs. Accession numbers are shown for the sequences included in GenBank. The date of isolation is in dd/mm/yy. Origins: *in situ* - from untreated seawater samples; ATP - from ATP-amended seawater cultures; P - from inorganic P-amended seawater cultures; Control - seawater cultures without enrichments; etc. - when there was more than one GenBank accession number for a given isolate type, only the one without parenthesis is used in the text.

Acc#	Name	Date isol.	Origin	Most similar organism(s)	Identity (%)	Lineage	Family
• DQ681131	M107	16/03/01	Control	*Pseudoalteromonas agarivorans* AJ417594	99.8	Gamma	*Pseudoalteromonadaceae*
(DQ681165)	M271	13/11/01	Control	M107 DQ681131	100		
(EU253571)	M306	13/11/01	P	M107 DQ681131	100		
• DQ681132	M111	16/03/01	Control	*Alteromonas* sp. AY576689	100	Gamma	*Alteromonadaceae*
• DQ681133	M113	16/03/01	Control	*Alteromonas alvinellae* AF288360	98.7	Gamma	*Alteromonadaceae*
				Alteromonas sp. AJ391191	99.8		
• DQ681134	M114	16/03/01	Control	*Thalassobacillus devorans* AJ717299	99.8	Firmicutes	*Bacillaceaea*
• DQ681136	M119	20/03/01	ATP	*Marinomonas dokdonensis* DQ011527.1	95.7	Gamma	*Marinomonas*
(DQ681135)	M117	16/03/01	Control	M119 DQ681136	99		
• DQ403809	M121	20/03/01	ATP	*Marinomonas dokdonensis* DQ011526	95	Gamma	*Marinomonas*
				Marine bacterium AY028196	98.9		
	M234	25/06/01	DMSP/P				
	M252	25/06/01	ATP				
• DQ681137	M123	20/03/01	ATP	*Marinomonas pontica* AY539835	95.5	Gamma	*Halomonadaceae*

	M221	23/05/01	ATP				
	M236	25/06/01	DMSP/P				
• DQ681138	M126	20/03/01	ATP	*Enterovibrio norvegicus* AJ437193	99.8	Gamma	*Vibrionaceae*
				Vibrio sp. AJ316207	100		
• DQ681139	M128-M130	20/03/01	Control	*Nereida ignava* AJ748748	99.4	Alpha	*Rhodobacteraceae*
				Alphaproteobacterium AY612764	99.8		
	M156-M157	20/03/01	*In situ*				
	M479	22/03/04	Control				
	M584-M585	29/06/04	*In situ*				
	M592	29/06/04	*In situ*				
	M625	11/10/04	Control				
(DQ681140)	M132	20/03/01	Control	M128 DQ681139	100		
• DQ481462	M134	20/03/01	Control	*Dokdonia donghaensis* DQ003277	99.4	CFB	*Flavobacteriaceae*
				Krokinobacter genikus AB198086	100		
• DQ681142	M140	20/03/01	Glu/P	*Vibrio* sp. AF242274	99.6	Gamma	*Vibrionaceae*
				Vibrio sp. DQ219366	100		
• DQ681143	M146	20/03/01	P	*Thalassobacillus devorans* AJ114484	99.8	Firmicutes	*Bacillaceae*
				Halobacillus sp. AY553123	100		
• DQ681144	M149	20/03/01	P	*Roseovarius crassostreae* AF114484	98.1	Alpha	*Rhodobacteraceae*
				Uncultured alphaproteobacterium DQ376156	100		
• DQ481463	M152	20/03/01	P	*Polaribacter dokdonensis* DQ004686	99.5	CFB	*Flavobacteriaceae*
				Marine bacterium AY745862	99.9		
• DQ681145	M155	20/03/01	*In situ*	*Erythrobacter litoralis* AF465836	99.0	Alpha	*Erythrobacteraceae*

• DQ681146	M165	15/05/01	P	*Phaeobacter inhibens* AY177712	98.4	Alpha	*Rhodobacteraceae*
				Roseobacter sp. AY576690	99.8		
• DQ681147	M169	15/05/01	P	*Alteromonas alvinellae* AF288360	99.1	Gamma	*Alteromonadaecae*
				Uncultured marine bacterium DQ071162	100		
	M209	23/05/01	Glycerol				
	M480	22/03/04	*In situ*				
	M496	22/03/04	DMSP				
• DQ681148	M181	15/05/01	P	*Vibrio tasmaniensis* AJ514912	99.1	Gamma	*Vibrionaceae*
				Vibrio sp. AF022409			
• DQ681149	M185	15/05/01	P	*Alteromonas addita* AY682202	99.6	Gamma	*Alteromonadaceae*
				Alteromonas sp. ASP294361	100		
	M188			*Pseudoalteromonas* sp. EU440056			
• DQ681150	M193	15/05/01	P	*Phaeobacter inhibens* AY177712	97	Alpha	*Rhodobacteraceae*
M177-M163-M192-M194		15/05/01	P				
• DQ681151	M203	23/05/01	Pyruvate	*Marinobacter aquaeolei* AJ000726	100	Gamma	*Alteromonadaceae*
				M. hydrocarbonoclasticus AY669169	100		
• DQ681152	M206	23/05/01	*In situ*	*Idiomarina seosinensis* AY635468	99.2	Gamma	*Idiomarinaceae*
				Marinobacter sp. DQ270761	99.9		
• DQ681154	M216	23/05/01	P	*Planococcus citreus* AF500008	99.6	Firmicutes	*Planococcaceae*
				Planococcus sp. DQ177334	99.9		
• DQ294291	M217	23/05/01	P	*Leeuwenhoekiella accommodimaris* AJ780980	97	CFB	*Flavobacteriaceae*
				Cytophaga sp. AY745817	99.6		
• DQ681155	M219	23/05/01	P	*Kocuria polaris* AJ278868	99.7	Acinobact.	*Micrococcaceae*

				Kocuria rosea DQ176452	100		
• DQ681156	M220	23/05/01	P	*Salegentibacter mishustinae* AY576653	94.1	CFB	*Flavobacteriaceae*
				Uncultured organism DQ396362	99.9		
• DQ681157	M222	25/06/01	Glu/P	*Vibrio* sp. AF242274	100	Gamma	*Vibrionaceae*
				Vibrio sp. DQ492722	100		
	M226	25/06/01	Glu/P				
• DQ681158	M227	25/06/01	Glu/P	*Vibrio gigantis* AJ582807	97.8	Gamma	*Vibrionaceae*
				Marine bacterium AY028201	98.5		
	M230	25/06/01	Glu/P				
• DQ681159	M238	25/06/01	DMSP	*Marinomonas blandensis* DQ403809	99.8	Gamma	*Oceanospirillales*
• DQ681160	M241	25/06/01	Glu/P	*Vibrio gigantis* AJ582807	100	Gamma	*Vibrionaceae*
	M285	13/11/01	Control				
	M300	13/11/01	P				
(DQ681141)	M137	20/03/01					
(EU253572)	M310	28/01/03	P	M241 DQ681160	99		
(EU253575)	M344	04/03/03	P	M241 DQ681160	99		
(EU253591)	M509	22/03/04	Amino acids	M241 DQ681160	98		
• DQ681161	M246	25/06/01	DNA	*Pseudoalteromonas flavipulchra* AF297958	99	Gamma	*Pseudoalteromonadaceae*
• DQ681162	M254	25/06/01	ATP	Marine bacterium AY028196	99.2	Gamma	*Marinomonas*
				Marinomonas pontica AY539835	95.2		
• DQ681163	M266	13/11/01	Control	*Saccharophagus degradans* AF055269	93.6	Gamma	*Oceanospirillales*
				Oleispira antarcticam AJ426421	92		
M299-M294-M279		13/11/01	Control				
M278-M268		13/11/01	Control				

M319-M324-M326-M327		28/01/03	Control				
• DQ681164	M269	13/11/01	Control	*Marinomonas dokdonensis* DQ011527 Marine bacterium AY028204	97.6	Gamma	*Marinomonas*
• DQ681166	M275	13/11/01	Control	*Alteromonas alvinellae* AF288360 Gammaproteobacterium AB010855	99.6	Gamma	*Alteromonadaceae*
(DQ681153)	M212 M614	23/05/01 11/10/04	Glycerol P	M275 DQ681166	99		
• DQ681167	M290	13/11/01	*In situ*	*Pseudoalteromonas agarivorans* AJ417594 *Pseudoalteromonas* sp. AY781155	99.6 99.8	Gamma	*Pseudoalteromonaceae*
• DQ681168	M292	13/11/01	*In situ*	*Alteromonas addita* AY682202 Uncultured *Alteromonas* sp. AY664213	99.6 99.6	Gamma	*Alteromonadaceae*
(EU253590)	M506	22/03/04	DMSP	M292 DQ681168	99		
• DQ403810	M297	13/11/01	*In situ*	*Reinekea marinisedimentorum* AJ561121 Uncultured bacterium AY172302	94.8 96.1	Gamma	*Reinekea*
• DQ681169	M302	13/11/01	P	*Marinomonas dokdonensis* DQ011527 Uncultured *Marinomonas* sp. DQ421663	98.3 98.4	Gamma	*Marinomonas*
• EU253573	M329	04/03/03	*In situ*	*Krokinobacter diaferitkos* AB198089	96	CFB	*Flavobacteraceae*
• EU253574.1	M341			*Tenacibaculum* sp. EU021293	95	CFB	*Flavobacteraceae*
• EU253577	M367	13/05/03	*In situ*	*Gramella echinicola* AY608409	97	CFB	*Flavobacteraceae*
• EU253579	M381	13/05/03	Control	Marine bacterium AF359541 *Maribacter forsetii* subsp. Forsetii AM71900	99 97	CFB	*Flavobacteraceae*

• EU253580	M418	16/09/03	P	Gammaproteobacterium AF384142	99	Gamma	*Vibrionaceae*
• EU253581	M428	16/09/03	P	Gammaproteobacterium AF384142	99	Gamma	*Vibrionaceae*
• EU253582	M441	16/09/03	Control	*Paracoccus* sp. AB264129	99	Alpha	*Rhodobacteraceae*
• DQ681170	M442	16/09/03	Control	*Erythrobacter citreus* AF118020	100	Alpha	*Erythrobacteraceae*
	M537	25/05/04	*In situ*				
(EU253576)	M365	13/05/03	*In situ*	M442 DQ681170	100		
(EU253578)	M380	13/05/03	*In situ*	M442 DQ681170	99		
(DQ681172)	M456	22/03/04	*In situ*	M442 DQ681170	99.4		
(EU253594)	M525	25/05/04	*In situ*	M442 DQ681170	100		
(DQ681181)	M539	25/05/04	*In situ*	M442 DQ681170	96		
• EU253583	M443	16/09/03	Control	Unidentified bacterium DQ985889 *Erythrobacter* sp. AY646157	98	Alpha	*Erythrobacteraceae*
• EU253584	M445	16/09/03	*In situ*	*Salegentibacter* sp. EF520007	97	CFB	*Flavobacteraceae*
• EU253585	M447	16/09/03	*In situ*	*Palleronia marisminoris* AY926462	96	Alpha	*Rhodobacteraceae*
• DQ681171	M454	22/03/04	*In situ*	*Gramella echinicola* AY608409	99.9	CFB	*Flavobacteriaceae*
• DQ681173	M458	22/03/04	*In situ*	*Erythrobacter aquimaris* AY461443	98.1	Alpha	*Erythrobacteraceae*
(EU253595)	M531	25/05/04	*In situ*	M458 DQ681173	100		
• DQ681174	M463	22/03/04	*In situ*	*Aurantimonas coralicida* AY065627	100	Alpha	*Aurantimonadaceae*
• EU253587	M464	22/03/04	*In situ*	Unidentified bacterium DQ985896 *Methylarcula* sp. AJ534207	99 98	Alpha	*Rhodobacteraceae*

• EU253588	M466	22/03/04	*In situ*	*Microscilla arenaria* AB078078	98	CFB	*Flammeovirgaceae*
• DQ681175	M483-M485	22/03/04	Control	*Antarctobacter heliothermus* RS21916	97.3	Alpha	*Rhodobacteraceae*
				Uncultured *Roseobacter* AF245634	97.9		
• DQ681176	M495	22/03/04	DMSP	*Leeuwenhoekiella aequorea* AJ780980	99.6	CFB	*Flavobacteriaceae*
• DQ681177	M498	22/03/04	Glucose	*Marinobacter flavimaris* AY517632	99.5	Gamma	*Alteromonadaceae*
				Uncultured Arctic sea ice bt. AY165592	99.9		
• DQ681178	M503	22/03/04	DMSP	*Marinobacter flavimaris* AY517632	97.0	Gamma	*Alteromonadaceae*
				Uncultured *Marinobacter* sp. AY687537	100		
• EU253589	M504	22/03/04	DMSP	*Limnobacter thiooxidans* AJ289885	99.9	Beta	*Burkholderiaceae*
• EU253592	M511	22/03/04	Amino acids	*Vibrio* sp. DQ923444	100	Gamma	*Vibrionaceae*
				Vibrio splendidus AJ874364	100		
• EU253593	M513	22/03/03	Acetate	*Limnobacter thiooxidans* AJ289885	99.9	Beta	*Burkholderiaceae*
• DQ681179	M517	22/03/03	DMSP	*Alteromonas addita* AY682202	99.9	Gamma	*Alteromonadaceae*
(EU253598)	M555	25/05/04	*In situ*	M517 DQ681179	97		
• EU253596	M532	25/05/04	*In situ*	*Salegentibacter slinus* EF486353	99	CFB	*Flavobacteriaceae*
• DQ681180	M535	25/05/04	*In situ*	*Vibrio pectenicida* Y13830	99.7	Gamma	*Vibrionaceae*
(EU253597)	M536	25/05/04	*In situ*	M535 DQ681180	100		
• DQ681182	M543	25/05/04	*In situ*	*Halomonas marisflava* AF251143	99.3	Gamma	*Halomonadaceae*

• EU253599	M568	25/05/04	*In situ*	*Polaribacter dokdonensis* DQ481463 Marine bacterium AF359539	96 97	CFB	*Flavobacteriaceae*
• EU253600	M571	25/05/04	*In situ*	*Microscilla arenaria* AB078078	99	CFB	*Flammeovirgaceae*
• DQ681183	M588	29/06/04	*In situ*	*Thalassobacter stenotrophicus* AB121782	100	Alpha	*Rhodobacteraceae*
• DQ681184	M595	29/06/04	*In situ*	*Tenacibaculum skagerrakense* AF469612 *Cytophaga* sp. AB073587	97.2 100	CFB	*Flavobacteriaceae*
• EU253601	M599 M602	19/07/04 11/10/04	*In situ* Glu/P	*Microscilla sericea* AB078081	99	CFB	*Flexibacteriaceae*
• DQ681185	M604	11/10/04	P	Uncultured *Alteromonas* sp. AY664213	100	Gamma	*Alteromonadaceae*
• DQ681186	M605	11/10/04	Glu/P	*Thalassobius mediterraneus* AJ878874	99.3	Alpha	*Rhodobacteraceae*
• DQ681187	M606	11/10/04	Glu/P	*Ruegeria atlantica* D88527 *Roseobacter* sp. AY745859	97.9 100	Alpha	*Rhodobacteraceae*
• DQ681188	M608	11/10/04	Glu/P	*Vibrio probioticus* AJ345063 *Vibrio* sp. AJ316168	99.1 99.6	Gamma	*Vibrionaceae*
• DQ681189	M609	11/10/04	P	*Alteromonas alvinellae* AF288360	99.6	Gamma	*Alteromonadaceae*
• DQ681190	M612	11/10/04	P	*Thalassobius gelatinovorus* D88523 Uncultured bacterium DQ117434	97.5 99.9	Alpha	*Rhodobacteraceae*
• DQ681191	M613	11/10/04		*Thalassobius aestuarii* AY442178 Uncultured alphaproteobacterium AY663968	97.9 100	Alpha	*Rhodobacteraceae*

• DQ681192	M618	11/10/04	Glu/P	*Phaeobacter inhibens* AY177712 Uncultured *Roseobacter* sp. AY663966	97.6 98	Alpha	*Rhodobacteraceae*
• DQ681193	M620	11/10/04	Glucose	*Glaciecola mesophila* AJ488501 Uncultured marine eubacterium AF159672	92.8 97.3	Gamma	*Alteromonadaceae*
• DQ681194	M621	11/10/04	Glucose	*Tenacibaculum skagerrakense* AF469612	98.2	CFB	*Flavobacteriaceae*
• DQ681195	M622	11/10/04	Glucose	*Tenacibaculum skagerrakense* AF469612	98.8	CFB	*Flavobacteriaceae*
• DQ681196	M623	11/10/04	Glucose	*Hyphomonas oceanitis* AF082797 Marine bacterium AF359546	99.1 100	Alpha	*Hyphomonadaceae*
• DQ681197	M624	11/10/04	Glucose	*Maricaulis* sp. AJ301665 *Maricaulis maris* AJ301665	99.7 99.9	Alpha	*Hyphomonadaceae*