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Identification of Culturable Oligotrophic Bacteria within Naturally Occurring Bacterioplankton Communities of the Ligurian Sea by 16S rRNA Sequencing and Probing

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ABSTRACT

Typical marine bacteria (i.e., obligately oligotrophic) that were numerically dominant members of naturally occurring marine communities were identified by cloning and sequencing the amplified 16S rRNA genes obtained from dilution cultures of the original samples. The data reported here refer to two different habitats of a marine pelagic environment (28 miles offshore, in the northwestern Mediterranean Sea). The samples were taken from the water column at two representative layers, i.e., the 30-m depth, corresponding to the chlorophyll maximum layer, and the 1800-m depth, representative of a deep, oligotrophic environment. Three major lineages were found in the 16S rDNA clone libraries prepared from the two samples, two of which could be assigned to the *Vibrio* and the *Rhodobacter* groups. The third lineage was a distant relative of the genus *Flavobacterium,* but it was not closely related to any marine isolate. Six oligonucleotide probes, either complementary to the conserved sequence domains or selectively hybridizing to the clone sequences, were designed for use as hybridization group-specific and strain-specific probes. A single-mismatch discrimination between certain probes and nontarget sequences was demonstrated by detecting the probes' specificity at different hybridization and washing conditions. The screening of the clone libraries with the obtained probes revealed that neither the 30-m sample higher dilution nor the 1800-m one were pure cultures. While some representatives of the *Vibrio* group were found in both the surface and the deep sample, the members of the *Flavobacterium* and *Rhodobacter* lineages were detected only in the deep and the euphotic layers, respectively. We suggest an approach for analyzing autochthonous marine bacteria able to grow in unamended seawater.

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Introduction

Since bacteria respond quickly to biotic and abiotic changes in their environment, the vertical distribution of physical and chemical parameters such as salinity, oxygen content, and particulate matter concentration in the marine water column may be the basis for a pronounced stratification of the prominent microbial populations [31, 32]. Therefore, the knowledge of the active population within the total microbial marine communities may give important information about the different seawater habitats. The molecular approaches based on small subunit rRNA gene sequence analysis have resulted in new estimates of the phylogenetic diversity of the naturally occurring microbial assemblages and have shown many new phylogenetic lineages [2, 5, 10, 12, 13]. For the vast majority of isolates, however, little is known about their abundance in the natural microbial communities from which they were isolated [15], and the few works on such a subject are oriented toward the analysis of cultivable bacteria from coastal marine environments [14, 28].

This study focuses on the indigenous oligotrophic marine bacteria obtained from complex communities of a pelagic station by using culture conditions as close as possible to the natural environments. According to previous work [4], we used dilution series of the natural samples in unamended 0.2-μm-filtered and subsequently autoclaved seawater to enrich the representative heterotrophic marine bacteria to the extent of extinction. The method was applied to two different seawater samples collected at two representative depths of the water column (i.e., the chlorophyll maximum layer and one deep, strongly oligotrophic layer). The diversity in the last positive dilutions of both samples were first monitored by restriction fragment length polymorphism (RFLP) analysis of the amplified cloned 16S rDNA PCR products. One to three representatives per each RFLP phylotype of bacteria were then identified by direct sequencing and analysis of the 16S ribosomal DNAs and the sequence information was used to design strain- and group-specific oligonucleotide probes that we empirically checked for specificity. The methods used in the analysis are applicable to other natural microbial communities. We demonstrate a protocol to identify numerically abundant oligotrophic bacteria within the natural populations in seawater samples. Minor constituents of the communities may not be detected using the presented method.

Materials and Methods

Seawater Samples

During the DYFABAC 07 cruise (15 to 21 May 1996), aboard the *Tethys II* research vessel, water samples were collected with 6 L Niskin bottles attached to a CTD (conductivity–temperature–depth) rosette from two representative depths (30 and 1800 m) of an oligotrophic [30], pelagic station (DYFAMED: 43° 25′ N and 07° 52′ E), in the northwestern Mediterranean Sea, 28 miles from the coast of Nice. The sampling bottles were acid cleaned (10% HCl) and rinsed with distilled water before use. Samples were transferred into sterile polycarbonate flasks and processed on board the research vessel. To disrupt the naturally occurring bacterial aggregates we sonicated samples for 5 min in a Bransonic 221 ultrasonic cleaner (48 kHz, 50 W) before further processing.

Direct Bacterial Counts

To determine the total number of bacteria in the natural samples, 50 ml of each natural sample were preserved with 0.2 μm-pore-size-filtered buffered formalin (2% final concentration). Within 4 h after sampling, cells were collected onto a 25-mm black polycarbonate Nuclepore membrane (0.2-μm pore size) and stained with 4′,6′-diamidino-2-phenylindole (DAPI) [27]. Counts were made by epifluorescence microscopy as previously described [4].

Viable Bacteria Counts and Dilution Cultures

An overview of the analysis procedure for the most abundant microbial populations, described below, is illustrated in a flowchart in Fig. 1. A most-probable-number (MPN) technique was developed to enumerate the dominant bacteria among the natural populations. Triplicates of decimal dilution series of the natural samples were prepared in aged seawater collected during a previous cruise on the DYFAMED sampling station, at 30 m and 1800 m of depth, respectively. The 0.2 μm pore-size filtered seawater was autoclaved (121°C for 15 min) and then filtered again through fired Gelman A/E glass filters to eliminate the precipitated salts. Growth of the resulting cultures was checked 3, 4, and 8 weeks after inoculation by epifluorescence microscopy analysis, as described previously [4]. Tubes were scored as positive when the number of cells per field appeared greater than 4, i.e., the average background determined by counting the cells into the culture tubes prior to incubation. The endpoint of bacterial growth in the tubes was characterized by a bacterial density of $(5.05 \pm 3.9) \times 10^5$ cells per ml. No further changes in the bacterial numbers appeared between 4 and 8 weeks of incubation (data not shown). In order to collect a high amount of bacterial biomass, the positive tubes from the higher MPN dilutions served as source for a second inoculum into 200 ml of filtered and autoclaved seawater. Specifically, the 200-ml subcultures were obtained from the 10^{-3} dilution of the surface sample (SRF) and both the 10^{-3} and the 10^{-4} dilutions of the deep sample

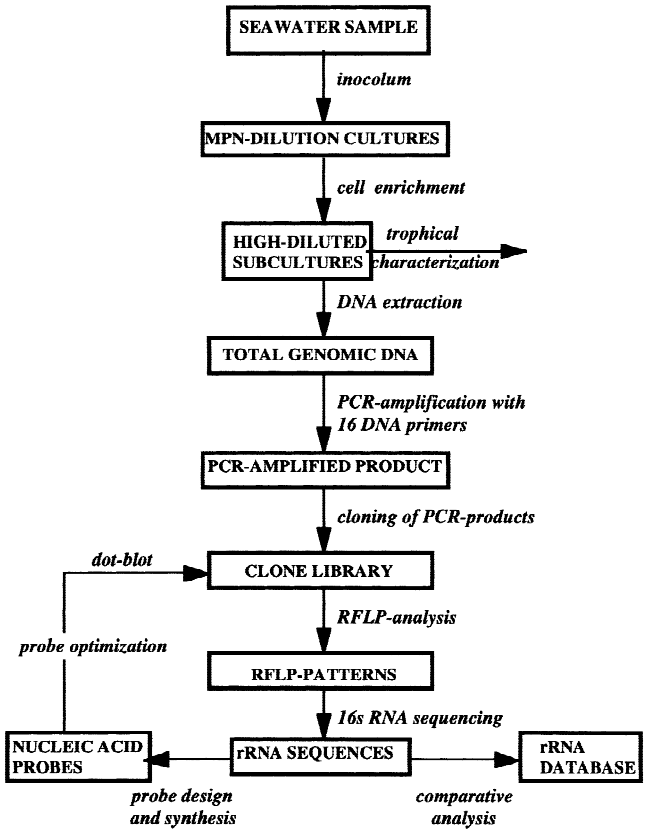

Fig. 1. Flowchart of the dilution approach and the molecular analysis.

(DPT1 and DPT2). The subcultures were originally inoculated with an average cell density of 10^2 to 10^3 cells flask^{-1}. Dilution cultures and the subcultures were incubated in the dark at 14°C (the characteristic temperature of the water column at sampling time ranged from 13 to 14°C). Growth in the subcultures was monitored, by epifluorescence microscopy analysis [4], once every week during 2 months of incubation. For any subculture, the observation frequency resulted in detection of changes in bacterial numbers after 4 weeks of incubation, when the number of bacterial cells in each subculture shifted to $(2.87 \pm 0.5) \times 10^5$ cells per ml. The subcultures did not show any further changes in the bacterial densities until the last observation. All subcultures were used to inoculate two different media representative of nutrient-rich (2216 Difco marine broth) and oligotrophic conditions (1:10 diluted Akagi medium [1], final concentration of the organic carbon: 1.8 mg L^{-1}). The ability of bacteria to grow under the different trophic conditions was checked by the technique of aggregate-forming units (AFU), which combines the membrane culture procedure with the epifluorescence microscopy counts [4]. Briefly, after sonication, 1 ml of each subculture was filtered in duplicate through 25-mm, 0.2-μm pore-size, black polycarbonate membranes (Nucleopore). The membranes with the associated cells were incubated on the different media and observed after 1, 2, 5, and 14 days of incubation by counting the DAPI-stained bacterial aggregates (AFU).

DNA Extraction

DNA was extracted from the 200-ml subcultures of the diluted bacterial samples by the method of Fuhrman et al. [9]. Briefly, bacteria from the seawater subculture (approximately 6×10^7 cells) were collected on 47-mm diameter, 0.2-μm pore-size Nuclepore filters which were cut into small strips, divided in two 1.5-ml conical-bottom centrifuge Eppendorf tubes, and vortexed in 1 ml of resuspension mixture (10 mM Tris hydrochloride [pH 8.3], 1 mM EDTA, 100 mM NaCl). A 0.1 volume of 10% sodium dodecylsulfate (SDS) was added and the tubes were placed in a boiling water bath for 2 min. Lysate was poured into polypropylene centrifuge tubes. A second and a third extraction were carried out with fresh phenol–chloroform–isoamyl alcohol (25:24:1, v/v), and chloroform–isoamyl alcohol (24:1, v/v), as described by Gonzalez et al. [15]. The DNA was precipitated in one volume of isopropanol overnight at −70°C, washed once with cold 70% ethanol, collected by centrifugation, and resuspended in double-distilled water.

PCR Amplification, Cloning, and RFLP Analysis of the 16S rRNA Gene

The 16S ribosomal RNA coding regions of the extracted DNA were amplified and cloned using the PCR-Direct cloning system (Clontech Corp.). Such a system provides a method for directional cloning of PCR products into the specific cloning vector pDIRECT without the need for either ligation or restriction-enzyme digestion. A pair of universal primers for 16S rRNA gene [18], 16F27 (S-D-Bact-0027-a-S-20) and 16R1492 (S-*-Proc-1492-a-A-22), were modified to have PCR-Direct sequences (12 and 13 nucleotides long) at their respective 5′ ends and were used to amplify the nearly complete 16S rRNA gene. The forward primer, $_{\text{pdirect}}$16F27: 5′-**CTCGCTCGCCCA**AGAGTTTGAT-CMTGGCTCAG-3′, corresponds to positions 8–27 of *Escherichia coli* 16S rDNA, whereas the reverse primer, $_{\text{pdirect}}$16R1492: 5′-**CTGGTTCGGCCCA**TACG-GYTACCTTGTTACGACTT, corresponds to the complement of positions 1492–1515 of *E. coli.* Amplifications were carried out with a thermal cycler (Perkin-Elmer GeneAmp 9600), in volumes of 100 μl containing 100 ng of target DNA, 200 mM each of dATP, dCTP, dGTP, and dTTP (Pharmacia Corp.), 0.6 mM of each of the appropriate primers, 10 μl of 10× AmpliTaq buffer (final concentrations: 20 mM Tris-HCl [pH 8.3], 2 mM $MgCl_2$, 25 mM KCl, 0.05% [v/v] Tween 20, 0.1 mg ml^{-1} gelatine), and 60 μl of double-distilled water (MilliQ). After the initial denaturing step (94°C, 5 min), 2.5 U (0.5 μl) AmpliTaq polymerase (Perkin-Elmer Cetus) was added to the reaction mixture. The cycler was programmed to carry out 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and elongation at 72°C for 2 min, followed by a final amplification step at 72°C for 10 min. The amplified DNA fragments were separated from free PCR primers by electrophoresis in 1.5% (w/v) FMC Seakem agarose gel, purified by the JETsorb DNA extraction kit (Genomed Corp.), and redissolved in 100 μl of sterile MilliQ water. PCR products were directionally cloned into the linearized pDIRECT plasmids, containing, at the two 5′ ends, differ-

Table 1. Designed clone- and group-specific oligonucleotide probes correspond to the hypervariable or group-specific regions of the 16S rRNA from analyzed environmental clones

Probe	Sequence (5′–3′)	*E. coli* 16S rRNA position	Specificity	*T* (°C)[a] prehyb/hyb	*T* (°C)[a] washing
Clone specific					
SRF3	CTCAAGACTACCAGTATTAG	806–787	Clone SRF3	43	37
DPT1.1	AAATCCTCCGAAGATTCAAT	47–28	Clones DPT1.1, DPT1.2 and DPT2.1	41	37
DPT1.3a	GGTTCTTCCTCTGTAAAAGC	502–483	*Flavobacterium salegans,* clones DPT1.3 and DPT1.4	45	37
DPT1.3b	CTGGCAACTAACCACAGGGG	1128–1109	*Flavobacterium salegans,* clones DPT1.3 and DPT1.4	46	37
Group specific					
G Rb	GTCAGTATCGAGCCAGTGAG	645–626	Group *Rhodobacter*	49	42
G V	AGGCCACAACCTCCAAGTAG	841–822	Group *Vibrio*	49	42

[a] The optimal prehybridization (prehyb), hybridization (hyb), and washing conditions for screening of the clone libraries were determined empirically (see Material and Methods).

ent PCR-Direct single-stranded tails (11 and 12 nucleotides long, respectively) not complementary to each other and complementary to the PCR-Direct sequences incorporated into the primers. Following the suggestion of the Clontech protocol, the transformation of *E. coli* DH5α competent cells was made by thermal shock. A clone library of 96 plasmids was prepared, for both the 30-m and the 1800-m samples. The diversity of the recovered PCR-amplified 16S rDNA fragments in the library was examined by restriction fragment length polymorphism (RFLP) analysis. For such a purpose, the plasmid DNA was isolated by the QIAwell 8 Plasmid kit (QIAGEN Corp.) and digested with the restriction enzyme *Rsa*I (New England BioLabs Inc.) that recognizes the GTAC-sequence (positions 1135 and 2904 in the pDIRECT vector). Separation of the digested fragments was performed by electrophoresis in a 1.5% agarose gel (FMC Seakem Corp.) followed by ethidium bromide staining.

Sequencing and Phylogenetic Analysis

One to three clones for each RFLP pattern (OTU: operational taxonomic units) were sequenced. Sequencing of cloned 16S rDNA PCR fragments was performed with an Applied Biosystems 373A DNA sequencer (Perkin-Elmer Applied Biosystems) according to the protocols of the manufacturer. Sequence data were aligned initially with 16S rRNA and rDNA sequences using the electronic mail servers at the Ribosomal Database Project (RDP) [21] and FASTA searches a protein or DNA sequence data bank version 3.0t76 [27]. Phylogenetic relationships were estimated using the Phylogeny Inference Package (PHYLIP version 3.4). Jukes–Cantor evolutionary distances [16] were calculated using the DNADIST program, and dendrograms depicting phylogenetic relationships were derived using the FITCH program (Fitch–Margoliash method, version 3.572c) with random order input of sequences, and the global rearrangement option [8]. All sequences were submitted to the RDP program CHECK_CHIMERA [19]. The returned S_ab values revealed database relatives closer to the submitted full-length sequences (S_ab values ranging from 0.761 to 0.919) than to each of their component fragments. Therefore, the absence of chimeric PCR products was assumed.

Nucleotide Sequence Accession Numbers

The 16S rDNA sequences determined in this study have been deposited in the EMBL Nucleotide Sequence DataBase under accession numbers clone DPT1.1, AJ002566; clone DPT1.2, AJ002567; clone DPT1.3, AJ002569; clone DPT1.4, AJ002570; clone DPT2.1, AJ002568; clone SRF1, AJ002563; clone SRF2, AJ002564; clone SRF3, AJ002565.

Oligonucleotide Probe Synthesis and Labeling

Six oligonucleotide probes, 20 nucleotides long, were designed to complement conserved regions of the *Rhodobacter* and *Vibrio* 16S rRNA domains (G Rb, G V) and variable regions specific for the analyzed clones (SRF3, DPT1.1, DPT1.3a, DPT1.3b) (Table 1). The probes were evaluated with the RDP program CHECK_PROBE [19] and synthesized by Gibco BRL (Germany). The DIG Oligonucleotide 3′-End Labeling Kit (Boehringer Mannheim, Germany) was used to label the 3′ end of the oligonucleotide probes with DIG-ddUTP (digoxigenin-11-ddUTP) following the protocol of manufacturer.

Probe Optimization and Specificity Study

We evaluated the abilities of the synthetic probes to form stable hybrids with filter-bound, isolated, target 16S rDNA obtained from the clone libraries. Tests were performed by hybridizing the non-radioactively labeled probes to dot-blots of various target and non-target 16S rDNA immobilized on QIABRANE nylon membranes (QIAGEN, Germany). Following exposure and development of X-ray film, the T_d (temperature of dissociation) values of target DNA–oligonucleotide duplex structures were empirically determined by densitometric measurement of each spot. Individual hy-

bridized dots were cut from the membrane and washed for 30 min in 1× SSC–0.1% SDS at each temperature point (from 36 to 58°C at 2°C intervals). The probe specificity was tested by using five different combinations of hybridization and washing conditions. The optimal stringent hybridization and washing temperatures were defined by the empirically determined T_d and according to the formula of Lathe [20] modified by Stahl and Amann [34]. Membranes were prehybridized in hybridization buffer, at the stringent hybridization temperature for two hours, before addition of 5 pmol of DIG-labelled probe. Hybridizations were performed by varying the main protocol suggested by Boehringer (Boehringer Mannheim). Approximately 20 ml of hybridization buffer [5× SSC (0.75 M sodium chloride, 75 mM sodium citrate trisodium salt, pH 7.0), 2% blocking reagent (Boehringer Mannheim), 0.1% *N*-lauroylsarcosine, 0.02% SDS] was added per 100 cm^2 of membrane. With the hybridization temperature held constant, the effect of 20% and 30% formamide in the hybridization buffer was evaluated. The hybridization mixtures were incubated overnight at the chosen stringent temperature. After hybridization, membranes were washed twice for 5 min in posthybridization washing solution (2× SSC, 0.1% SDS) at room temperature, and then were washed twice for 15 min in 40 ml per 100 cm^2 of membrane of washing solution (0.1× SSC, 0.1% SDS). Three different washing temperatures (T_d, T_d − 10°C, and T_d + 10°C) were checked for membranes which hybridized without formamide. The membranes treated with 20% or 30% formamide hybridization buffer were washed only at lower temperatures. Detection of hybridization by chemiluminescence was carried out using the DIG Luminescent Detection kit for nucleic acids provided by Boehringer Mannheim.

Screening of the Clone Libraries

The results of the dot-blot tests previously described were used to develop the probe-specific protocols for the screening of the clone libraries (Table 1). For this, the transformed cells were lysed and their DNA was immobilized onto QIABRANE nylon membranes (QIAGEN) following the classical method described in Maniatis et al. [22]. The screening was made by dot-blotting the denaturated plasmids with two DIG-labeled probes specific for the conserved regions of the *Rhodobacter* and *Vibrio* groups (G Rb and G V) and with four DIG-labeled probes complementary to the hypervariable regions of the analyzed clones (SRF3, DPT1.1, DPT1.3a, DPT1.3b).

Results

Total and Viable Counts

The total bacterial densities were 1.98×10^6 bacteria per ml in the surface sample and 5.3×10^4 bacteria per ml in the depth sample. The viable bacterial density, as determined by MPN counts, remained below 12.9 cells per ml, which was, according to de Man [7], the upper limit of the 95% confidence interval for the highest MPN count (30 m depth). Considering that, for a given set of cells in an incubation tube, the viability is the ratio of the number of viable cells to the total number of cells originally present [6], the viability of the bacteria contained in the SRF, DPT1, and DPT2 subcultures, obtained from the high diluted MPN cultures, was identical. The trophic tests indicated that bacterial populations from the subcultures were not able to grow on nutrient-rich conditions (2216 Difco marine agar). In oligotrophic conditions (10-fold-diluted Akagi medium), AFU appeared after 5 days of incubation. At the end of incubation the oligotrophic bacterial counts were 3.4×10^4 AFU per ml from SRF subculture, and 1.6×10^3 AFU per ml from the DPT1 subculture (10% and 0.7% of the total number of bacteria in the source cultures, respectively). The bacteria from the DPT2 subculture did not show any growth in both the checked media.

DYFAMED Surface and Deep Water 16S rDNA Clones

Three libraries, 96 clones each, were created using the DNA extracted from the SRF, DPT1, DPT2 subcultures. A partial analysis of 20 randomly picked clones from each of the clone libraries was made by RFLP.

The RFLP patterns of the subcultures clustered at least in four categories of unique clones (OTUs) (Fig. 2). Clones from the DPT1 subculture fell in two major OTUs, namely A (17 clones) and B (3 clones), while the all clones from DPT2 subculture were grouped in category A. The clones from SRF subculture clustered in two other OTUs, C (14 clones) and D (6 clones), which were not present in the deep-water subcultures. Eight clones were selected for further analyses as representative of each of these OTUs. Particularly, the clones DPT1.1, DPT1.2, and DPT2.1 were representative of category A, and DPT1.3 and DPT1.4 of category B, while the clones SRF1, SRF2, and SRF3 were chosen for analysis of categories C and D, respectively.

The sequences of their 16S rRNA genes fell into only 2 of the 12 major phylogenetic groups of the Bacteria domain [35]: the *Proteobacteria* and the *Flavobacterium–Cytophaga–Bacteroides* (FCB) group (Fig. 3). Particularly, three of the inspected sequences, DPT1.1, DPT1.2, and DPT2.1, belonged to the γ-subclass of the *Proteobacteria* and were in a tight phylogenetic cluster with the marine *Vibrio.* The three bacterial clones SRF1, SRF2, and SRF3 were assigned to the *Rhodobacter* lineage of the α-subclass of the *Proteobacteria,* and the two remaining clones, DPT1.3 and DPT1.4, diverged to the FCB group. None of the clone sequences was an exact match to any sequences represented in the RDP and EMBL data bases.

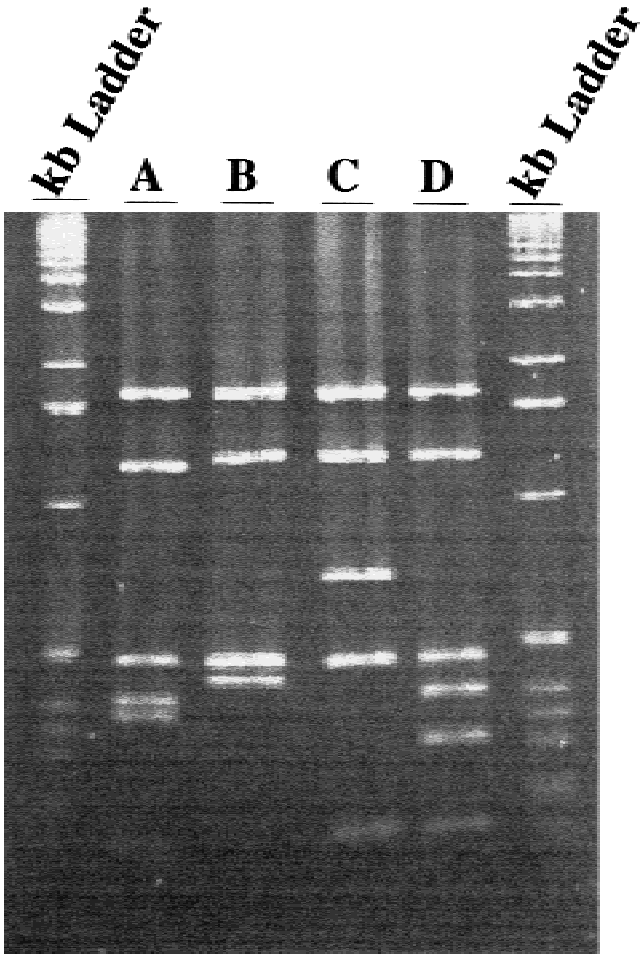

Fig. 2. The major RFLP patterns of the environmental clones from DPT1, DPT2, and SRF subcultures obtained by digestion with *Rsa* I restrictase.

According to the full-length comparative analysis of the 16S rRNA sequences, all of the characterized sequences exhibited signature sequences exclusively characteristic of the phylogenetic groups to which they belonged.

16S rRNA Probe Development and Application

It was possible to identify highly specific and conserved regions in all of the eight analyzed clones compared with the sequences available from the RDP and EMBL data banks. Six oligonucleotide probes were developed to complement either the hypervariable clone-specific regions or conserved group-specific region of the analysed clones (Table 1). For clone-specific probes, the results of analyses using the CHECK_PROBE program indicated that there were a minimum of two or three mismatches with any known 16S rRNA sequence. The exceptions were the probes DPT1.3a and DPT1.3b, which also showed complementation to the 16S rRNA sequence of *Flavobacterium salegens* DSM5424. The G Rb and G V oligonucleotide probes revealed high specificity to the corresponding groups (Table 1).

For SRF3, DPT1.3a, G Rb, and G V probes complemented by their target sequences, experimental T_d values and the theoretical estimates of the T_d agreed to within about 1 to 3°C. In contrast, the measured T_d of DPT1.1 probe from its target sequence was 6°C above the theoretical value, while the experimental values for DPT1.3b were considerably lower than the estimated one (around 5°C). Because of the high specificity of the probes, the hybridizations with the target 16S rDNA, obtained by combining the stringent hybridization and wash conditions, were giving a detectable signal in the X-ray film. Since the results suggested that the resolution of stringent hybridizations followed by low stringent wash conditions was always sufficient to discriminate a single mismatch (data not shown), we selected rather low washing temperatures for the screening of the clone libraries (Table 1). The presence of formamide in the hybridization solutions drastically reduced the probe binding to the targeted nucleic acid, and in only two cases (DPT1.1, G V) was the sensitivity of probes sufficient to give a detectable signal.

Screening of the Clone Libraries

The frequencies of group- and clone-specific probes in the clone libraries are presented in Table 2. Of SRF clones, 92.2% hybridized with the *Rhodobacter* group-specific probe (G Rb) while no sequences complementary to this probe were found in the DPT1 and DPT2 clone libraries. The *Vibrio* group-specific probe (G V) covered 95.8% of the DPT1 clone library, all clones of the DPT2 library, and only 7.8% of the SRF library. The percentage of specific sequences within the three clone libraries was highly variable. The probe SRF3 hybridized only with 3.7% of the total amount of SRF clones. The probes DPT1.3a and DPT1.3b covered 4.2% of clones from the DPT1 library, and they did not hybridize with the clones from the two remaining libraries. The DPT1.1 specific sequence was highly represented in the DPT1 and DPT2 clone libraries with frequencies of 43.8 and 71.9%, respectively. Such results revealed that neither the 30-m sample higher positive dilution nor the 1800-m ones were pure cultures, as already observed from the RFLP analysis of the isolated plasmids.

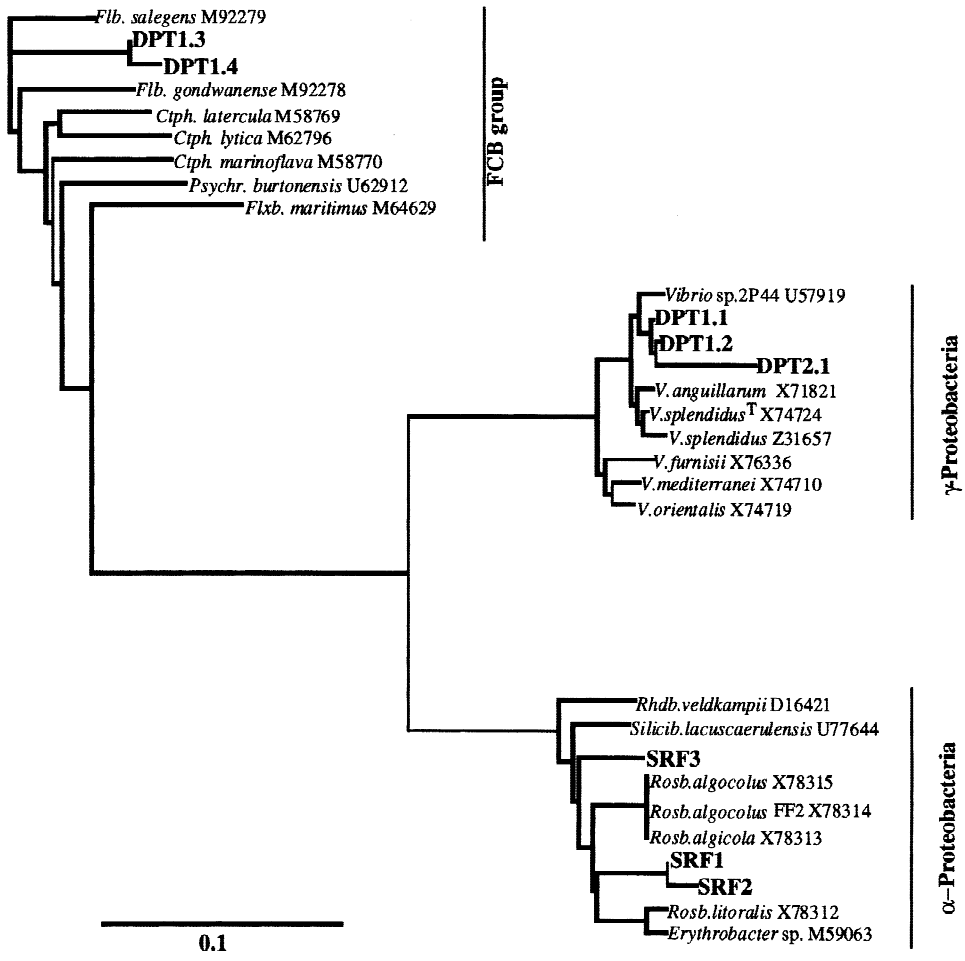

Fig. 3. Phylogenetic tree illustrating the relationship among 16S rDNA sequences of analyzed clones (boldface) and bacteria from the EMBL sequence data bank. Abbreviations used: *Ctph., Cytophaga; Flb., Flavobacterium; Flxb., Flexibacter; Psychr., Psychroserpens; Rhdb., Rhodobacter; Rosb., Roseobacter; Silicib., Silicibacter; V., Vibrio.*

Discussion

The aim of the work was to calibrate a method for studying the representative indigenous marine bacteria able to grow in unamended filtrates of seawater. According to Schut et al. [33], extinction dilution is the only remedy to obtain liquid cultures of dominant autochthonous organisms from seawater samples. In fact, the dilution culture medium usually contains only the autochthonous organic compounds from seawater as carbon and energy source [3], and therefore it favors the growth of the most abundant, rather than the most nutrient-tolerant, bacteria [6]. The highest positive dilution of both the 30-m and the 1800-m samples contained bacteria that did not tolerate high nutrient concentrations such as marine broth [26]. The RFLP analysis of the isolated plasmids from the obtained 16S rDNA clone libraries was used to sort the clones into groups and to facilitate the choice of clones to be sequenced. The obtained patterns revealed the presence of different genotypes in each of the analyzed subcultures. The dilution tubes containing pure cultures may have been not detected and the tubes scored as negative if the contained microorganisms did not divide a sufficient number of times to produce enough growth for observation. According to Button et al. [6], the content of mixed cultures would be larger than the content of the pure

Table 2. Percentage of positive clones from clone libraries obtained by hybridization with the different oligonucleotide probes

Bacterial group	Probe	Clone library (% of positive clones)		
		SRF	DPT1	DPT2
α-Proteobacteria	SRF3	3.7	0.0	0.0
	G Rb	92.2	0.0	0.0
γ-Proteobacteria	DPT1.1	0.0	43.8	71.9
	G V	7.8	95.8	100.0
FCB group	DPT1.3a	0.0	4.2	0.0
	DPT1.3b	0.0	4.2	0.0

one if synergistic influences increase viability of the cells. The clones characterized by the same RFLP pattern showed closely related 16S rDNA sequences and they were significantly affiliated in the phylogenetic analysis. The results obtained from the clone library screening with the designed probes indicate that, at the time of the sampling, the *Rhodobacter* cluster genes were located near the chlorophyll maximum of the water column, whereas the *FCB* genes were located in the deep layer. According to previous studies in different oceans [14, 25], such results suggest that α-*Proteobacteria* are best adapted to the conditions of the euphotic zone when the water column is highly stratified. Particularly, the SRF1 and SRF2 clones were closely related to some *Roseobacter* spp. that had been isolated from the surfaces of unicellular algae (i.e., the dinoflagellate *Prorocentrum lima*) [17]. Despite the general oligotrophic conditions, some eutrophication events usually occur in the euphotic layers of the sampled area in springtime [23, 24, 30]. The high frequency of dinoflagellate bloom events in the DYFAMED station surroundings could support speculation about some spatial relations between SRF1 and SRF2 populations and these protists. The *Vibrio*-related genes, more abundant at the deep layer, were also found at the surface layer, suggesting a wide distribution of these bacteria in the water column. The DPT1.1, DPT1.2, and DPT2.1 16S rDNA genes were tightly related with a PAH-degrading marine *Vibrio* sp. strain 2P44 [11]. Because of their oligotrophic behavior and their phylogenetic affiliation with marine autochthonous species, all the bacterial populations obtained from the highly diluted samples should have had a marine origin. The 60 random clones analyzed by RFLP were sufficient to give an overview of the level of diversity within created clone libraries. In fact, clones from the three analyzed libraries were totally covered by the combination of G V, G Rb, DPT1.3a, and DPT1.3b probes. Particularly, the SRF and DPT1 clone libraries were composed of two major taxonomical lineages, whereas the DPT2 clone library consisted only of members of group *Vibrio*. The results of the presented work provide a set of 16S rRNA genetic markers specific for typical marine bacteria. Preliminary application of G Rb and G V fluorescently labeled probes for *in situ* hybridization of natural samples revealed that the identified marine bacteria accounted for up to 32% of total bacterial counts in the source environment (data not shown, manuscript in preparation). The approach used here, previous RFLP analysis and subsequent sequence analysis of 16S rRNA amplified genes obtained from highly diluted seawater samples, seems to be a suitable way to identify the representative oligotrophic bacteria among the seawater natural populations.

Acknowledgments

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