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Microbial community composition of anoxic marine sediments in the Bay of Cádiz (Spain)

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Summary. The composition of the microbial community inhabiting the anoxic coastal sediments of the Bay of Cádiz (southern Spain) was investigated using a molecular approach consisting of PCR cloning and denaturing gradient gel electrophoresis (DGGE), based on 16S rRNA sequences. The total cell count was 1–5 × 10^8 cells/g sediment and, as determined by catalyzed reporter deposition–fluorescent in situ hybridization (CARD-FISH), the proportion of *Bacteria* to *Archaea* was about 70:30. The analysis of 16S-rRNA gene sequences revealed a wide spectrum of microorganisms, which could be grouped into 111 operational taxonomic units (OTUs). Many of the OTUs showed high phylogenetic similarity to microorganisms living in marine sediments of diverse geographic origin. The phylogenetic groups that were predominantly detected were Firmicutes, Deltaproteobacteria, and Gammaproteobacteria, accounting for 23, 15, and 14% of the clones, respectively. Diversity in the domain *Archaea* was significantly lower than in the domain *Bacteria*. The majority of the archaeal OTUs belonged to the Crenarchaeota phylum. Since most of the sequences could not be identified precisely at the genus/species level, the functional roles of the microorganisms in the ecosystem could not be inferred. However, seven OTUs affiliated with the Delta- and Epsilonproteobacteria were identified down to the genus level, with all of the identified genera known to occur in sulfate-rich marine environments. [**Int Microbiol** 2011; 14(3):143-154]

Keywords: microbial community composition · anoxic marine sediments · 16S rDNA gene library

Introduction

Prokaryotic organisms (*Bacteria* and *Archaea*) that live in marine sediments participate in a variety of biochemical pathways involving both inorganic and organic compounds. Analyses of the microbial community composition of many sediments in different parts of the world have shown that the predominant phylogenetic groups in these habitats are often highly similar. Various studies have determined the presence of Gamma- and Deltaproteobacteria, Flavobacteria, and Planctomycetes in sediments from the North Sea and Arctic Sea [7,24,25,36–38] as well as from the Antarctic Sea [3–5,45]. In sediments from Tokyo Bay, Japan, the predominant bacterial groups are Deltaproteobacteria, Gammaproteobacteria, Epsilonproteobacteria, Gram-positive bacteria, and Verrucomicrobia [43]. In one of the first molecular studies

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of marine sediments from Puget Sound (near Seattle, USA), the analysis of a clone library showed similar phylogenetic affiliations [11]. Recent studies examining sediments from the Mediterranean Sea [14,31–33,40,18] also described an abundance of members of the Deltaproteobacteria and Gammaproteobacteria, as well as Betaproteobacteria, Planctomycetes, Acidobacteria, Bacteroidetes, and Firmicutes.

Although these phylogenetic groups are prominently represented in most surveys, and sequences retrieved from very distant habitats often show similarities of over 90% (at the class/phylum level), when examined more closely the clone libraries represent a high level of biodiversity, including many unique ribotypes [3,5]. Similarly, comparisons of the composition of various clone libraries based on sequences obtained from the Mediterranean Sea showed that microbial communities from different regions are highly distinct [32]. Only 14% of the complete set of sequences had a similarity of 92% or higher between libraries. Significantly distinct community compositions were also found between two stromatolites in Shark Bay, Western Australia [29]. These examples show that (i) many of the same phylogenetic groups are detected in sediments independent of their geographic location, and (ii) the level of microbial diversity within each set of sampled sequences can be very high.

The purpose of this study was to determine the microbial community composition of the anoxic sediment in the Sancti Petri Channel of the Bay of Cádiz (south of Spain). Since only a fraction of the microorganisms inhabiting soils and sediments are readily culturable, this study made use of a culture-independent approach. Based on PCR and the cloning of total genomic community DNA, eight clone libraries of near full-length 16S rDNA sequences were constructed, allowing a detailed phylogenetic analysis. DGGE was employed as a complementary technique to characterize the microbiota, and CARD-FISH to quantify the *Bacteria* and *Archaea* domains.

Materials and methods

Sampling sites and procedure. Sediment samples were taken at the Sancti Petri Channel (Cádiz, Spain, 36° 28.48′ N, 6° 10.71′ W), a coastal marine area partially polluted by the spillage of untreated municipal sewage (SP16). The physicochemical properties and nutrient profiles of the sediment have been published elsewhere [23]. Samples were taken in 2002, 2003, and 2004. Shortly after sampling in 2002, a domestic wastewater treatment plant began operation, and the sediment site no longer received severely contaminated wastewater input [22]. This allowed us to compare the effect of human contamination on the microbiota of the sediments. To compare the polluted sampling zone SP16 to an area that had not been exposed to domestic sewage, a beach-like zone (SP02) was also analyzed. All the samples were extracted by a diver using PVC cores 50 cm in length and 6 cm in diameter. The cores were sealed and immediately transferred to the laboratory, where they were cut into slices of defined thickness, corresponding to depths of 0–2, 6–8, 8–10, 12–14, and 18–20 cm. Sediment samples for genomic DNA extraction were stored at –20ºC until use. Samples to be used for in situ hybridization were fixed for 4 h in 4% formaldehyde, washed twice with PBS, and stored in PBS:ethanol (1:1, v:v).

Genomic DNA extraction from sediment samples. Sediment samples (~ 0.6 g wet weight) from the cores of layers 0–2 (layer A), 6–8 (layer B), 12–14 (layer C), and 18–20 cm (layer D) were washed with PBS to eliminate excess salt. The samples were lysed by mechanical disruption in a bead beater (FastPrep FP120, Qbiogene), exposing the samples to five cycles of cell rupture (intensity level 6; 20 s per cycle) with intermittent incubation on ice. Microbial community DNA was extracted using the FastDNA SPIN Kit for Soil (Qbiogene). DNA integrity and yield were examined on 0.8% agarose gels with ethidium bromide staining (0.5 mg/l).

PCR. Near full-length bacterial 16S rDNA for clone library construction was PCR-amplified using the primer set 27F/1492R [20]. A shorter fragment was amplified for DGGE using primer set 341F/518R [27]. For the archaeal community, the primer combination 21F/958R was used to construct a clone library [10], and the primer set 344F/518R [27,41] for DGGE. AGC-tag (5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3′) was attached to the 5′ end of primer 518R to prevent complete dissociation in the gel. Each reaction (50 µl) contained 1× PCR buffer (Promega), 0.5 µM of each primer, 0.25 mM of each dNTP (dATP, dCTP, dGTP, and dTTP), 1.5–3.0 mM $MgCl_2$, and 1 U of Taq polymerase (Promega). PCR product size and yield were estimated by agarose gel electrophoresis using a set of molecular weight markers (ϕ-29/*Hin*dIII).

DG-DGGE. Five layers of sediment were sampled (0–2, 6–8, 8–10, 12–14, and 18–20 cm). The PCR products were resolved on a DCode Mutation Detection System (Bio-Rad) in double-gradient (DG) gels. A denaturing gradient of 30–60% urea/formamide (with 100% defined as 7 M urea/40% formamide) was superimposed on a porous gradient of 8–12% polyacrylamide. Electrophoresis conditions were 60ºC, 200 V, and a running time of 5 h. The gels were run in 0.5× TAE buffer (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, pH 7.4), stained in 0.5 mg ethidium bromide/l, and photographed on a UV trans-illuminator (Fotodyne, Hartland, WI, USA). For sequencing, bands were excised from the gel using a scalpel and the DNA then eluted by incubating the acrylamide blocks at 50ºC for 1 h. The supernatant was used for PCR reamplification of the bands with primer pairs 341F/518R (*Bacteria*) and 344F/518R (*Archaea*). The resulting products were sequenced on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Cluster analysis of the DGGE banding patterns was performed with the PHYLIP software package (PHYLogeny Inference Package, Joe Felsenstein, http://evolution.genetics.washington.edu/phylip.html), applying the neighbor-joining method. Dendrograms were generated and edited with MEGA software (Molecular Evolutionary Genetics Analysis) [19].

Clone library construction. Four bacterial and four archaeal gene libraries were constructed with samples from sediment depth layers A–D (described above). The sediment cores were those taken at SP16 in the year 2002. 16S rDNA was PCR-amplified and the products cloned into the TOPO-TA vector (Invitrogen). Competent One Shot *E. coli* cells (Invitrogen) were transformed with the vector-16S rDNA constructs and grown on LB (Luria-Bertani) agar plates with ampicillin (50 µg/ml), using β-galactosidase blue/white screening. Positive clones were regrown overnight in 5 ml LB medium containing ampicillin, followed by extraction of plasmid DNA by alkaline lysis (plasmid miniprep) [39]. After the amplified ribosomal DNA restriction analysis (ARDRA) of 770 clones, approximately 300 clones were sequenced with the primers M13F and M13R (vec-

tor sites near the polylinker). Chimeras, vectors, and short sequences were removed, yielding a total of 170 bacterial and 22 archaeal sequences for phylogenetic analysis.

Phylogenetic analysis. Sequencing chromatograms were checked and edited with the programs Chromas (Technelysium) and Genedoc [28]. Comparative analysis of the sequences was done using the BLAST routine from NCBI (National Center for Biotechnology Information) employing the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/) [1] and the tools Classifier and Sequence Match from the Ribosomal Database Project (RDP) at Michigan State University (http://rdp.cme.msu.edu/). The clone library was examined for possible chimeric sequences using the informatics tool available on the website of the Center for Microbial Ecology [Michigan State University, USA, http://rdp8.cme.msu.edu/cgis/chimera.cgi?su=SSU].

Clone sequences affiliated with the same phylum and class, as determined by BLAST comparison and the Classifier function of RDP, were grouped for the construction of independent phylogenetic trees together with sequences retrieved from the database corresponding to related microorganisms and representative members of the analyzed phylogenetic group. These sets of sequences were aligned with the ClustalX program [42]. Phylogenetic trees were calculated using the PHYLIP software package, applying the maximum-likelihood method. The resulting trees were visualized and edited with MEGA.

CARD-FISH. Aliquots of fixed sediment samples were diluted in 1× PBS, sonicated briefly with an UP50H Ultrasonic Processor (Hielscher) at 40% maximum amplitude (100 μm) and 0.5 cycle setting (acoustic power: 300 W/cm^2), vortexed, filtered onto a 0.2-μm pore size GTTP polycarbonate membrane (Millipore), and embedded in agarose. These samples were hybridized [30], counterstained with 4′,6′-diamidino-2-phenylindole (DAPI), and counted on an epifluorescence microscope (Axioskop, Zeiss) to determine total cell numbers. For each sample, between 800 and 1000 cells (as determined by DAPI staining) were counted. The probes used for domain specific hybridization were EUB338-HRP for *Bacteria* and ARC915-HRP for *Archaea* [35].

Nucleotide sequence accession numbers. Clone sequences were deposited in the GenBank database under the accession numbers GQ249466–GQ249661.

Results

DGGE. Comparisons of the bacterial community fingerprints over a period of three years and among the five different depth layers in the sediment revealed a similar composition of the dominant microorganisms in nearly all samples (Fig. 1). Cluster analysis of the DGGE band patterns showed that, with the exception of two samples from the deepest sediment layer (18–20 cm, 2002 and 2004), differences between samples were low, ranging between 0.4 and 3%, throughout the different depth layers of the sediment and over the three years of observation (Fig. 3A). Most of the sequences retrieved were equally distributed among the Gamma-, Delta-, and Epsilonproteobacteria. Two other amplicons were affiliated with Bacteroidetes and Firmicutes.

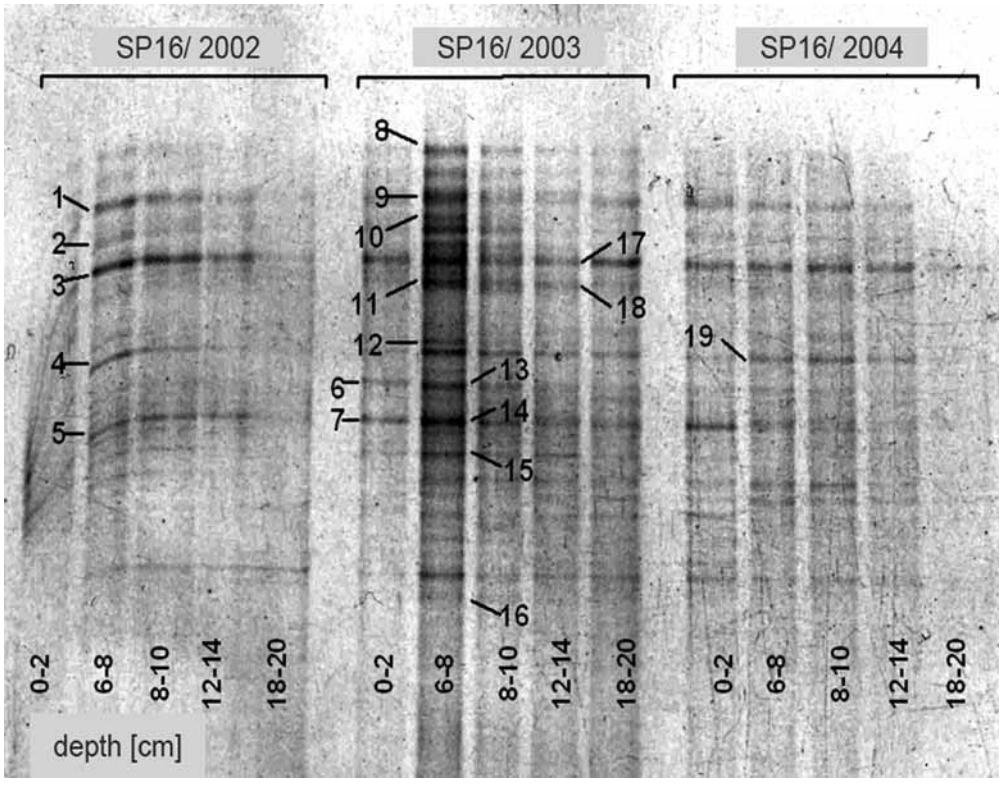

Fig. 1. DGGE fingerprints of bacterial amplicons. Samples from three years (2002, 2003, and 2004) and five depth layers (0–2, 6–8, 8–10, 12–14, and 18–20 cm) were analyzed. Numbered bands were excised and sequenced.

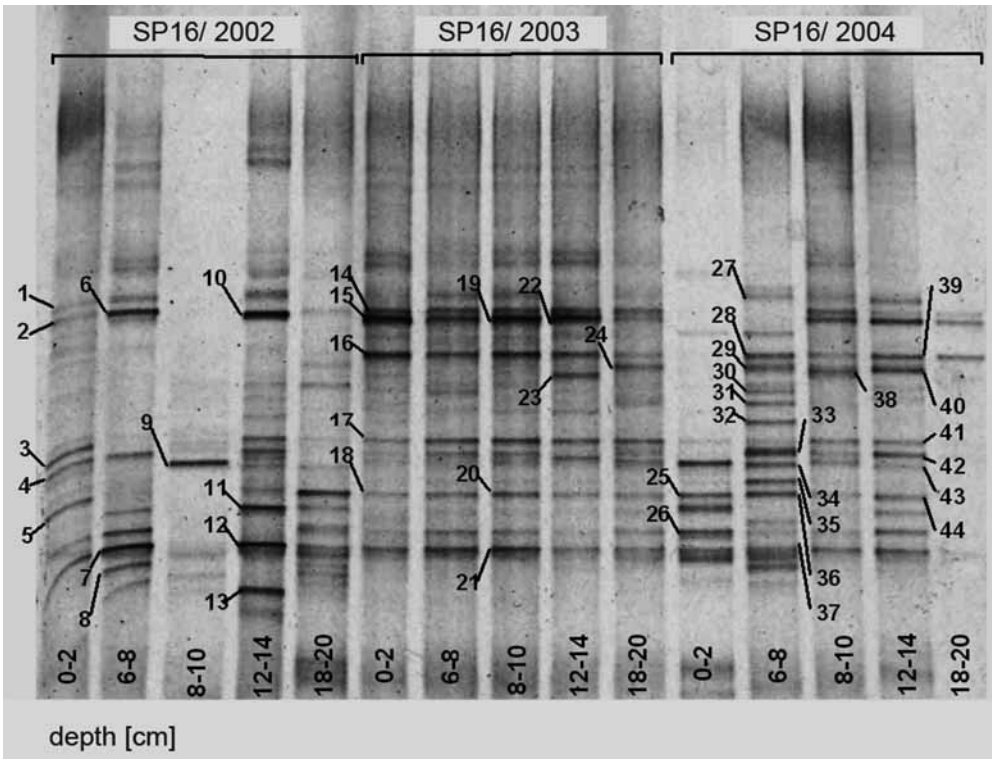

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Fig. 2. DGGE fingerprints generated for *Archaea* with domain-specific primers. The sediment samples were the same as those used for bacterial analysis. Numbered bands were excised, reamplified, and sequenced.

Compared to the bacterial domain, the archaeal fingerprints had a higher number of total bands (DGGE *Archaea*: 39, DGGE *Bacteria*: 27) and a greater variability between samples (Fig. 2). This can be observed in the length of the interconnecting branches in the corresponding dendrogram, showing differences of 4 to 8% between many samples (Fig. 3B). Forty-four bands were excised from the gel, reamplified, and sequenced successfully. All sequences were affiliated with the phylum Euryarchaeota. Furthermore, most of the sequences were related to environmental clones encountered in saline marine habitats, anoxic coastal sediments, subsurface waters, springs, or salt lakes, and matched microorganisms from diverse geographic locations such as China, Turkey, South Africa, and Mexico. Some of the sequences could be phylogenetically narrowed down to the order level, including those of Halobacteriales. The nearest described relatives to the DGGE bands are *Natrinema* sp., *Haladaptatus paucihalophilus*, and *Haloterrigena limicola*, with similarities to our clones in the range of 90–98%. Due to the heterogeneity of the band patterns from the 2002 and 2004 samplings, it was difficult to determine the predominant bands in the gel. However, considering the narrow phylogenetic spectrum, with all amplicons belonging to the phylum Euryarchaeota and probably to the class Halobacteria, the level of diversity may have been lower than in *Bacteria*.

As we did not expect to detect only sequences related to Euryarchaeota, we analyzed the literature describing the use of primers 344F and 518R for archaeal DGGE amplification. Two studies described a bias towards Euryarchaeota detection that was attributed to a mismatch in primer 518R [2,44]. We therefore aligned the sequence of 344F to that of several of the clones from our archaeal library and found a *Crenarchaeota*-incompatible mismatch in this primer as well (T by C at position 347).

Clone libraries. Sequences that showed a similarity of over 97% were grouped together, resulting in 111 different OTUs. Their distribution in the major phylogenetic lineages is shown in Fig. 4. The phylogenetic tree for *Bacteria* is provided in Fig. 5.

The taxonomic affiliation of the cloned sequences following comparative analysis of the 16S rRNA gene revealed a high degree of microbial diversity in the analyzed sediment, as reflected by the presence of microorganisms falling into eight different phyla of the domain *Bacteria*. The most numerous group of detected *Bacteria* belonged to the phylum Proteobacteria. With the exception of Betaproteobacteria, members of all described classes of this phylum were encountered, with Gamma- and Deltaproteobacteria representing 14 and 15%, respectively, of the total OTUs. Firmi-

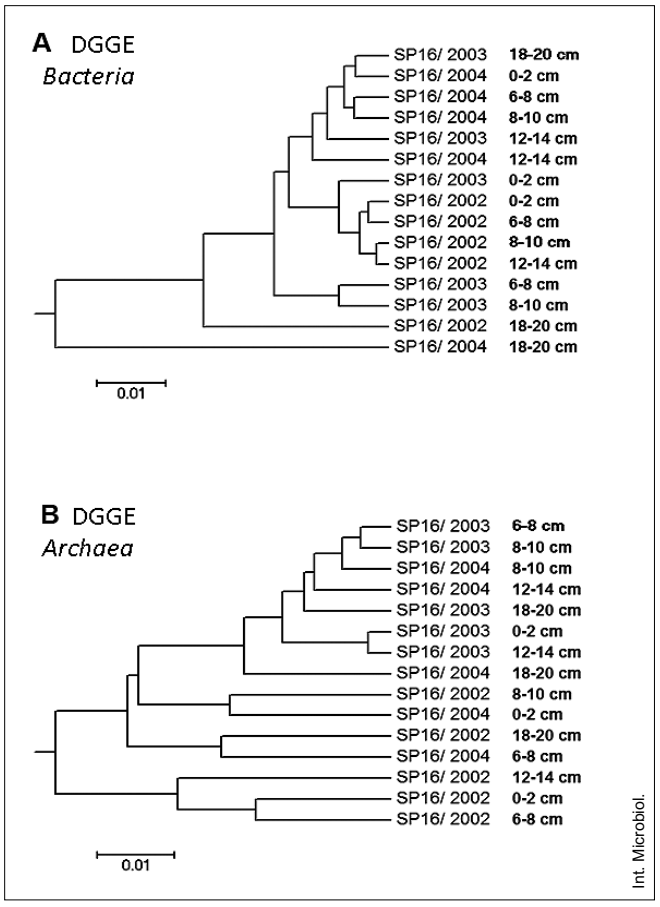

Fig. 3. Cluster analysis of DGGE banding patterns for domains *Bacteria* (**A**), and *Archaea* (**B**). (Bar: 1% distance.)

cutes, comprising 23% of the phylotypes, was the second most abundant phylum. The other phylogenetic groups were less abundant, representing 3–10% of the total OTUs. Microorganisms of the phyla Planctomycetes and Verrucomicrobia were grouped together for analysis [12].

Alphaproteobacteria. OTU 01 was affiliated with the genera *Sphingomonas/Sphingopysis*, OTU 02 was a member of the order Rhizobiales, and most clones comprising OTUs 03–08 clustered into the family Rhodobacteraceae. In this group, various microorganisms were identified with the genus *Loktanella* (OTU 03) and *Roseobacter/Sulfitobacter* (OTUs 04–07).

Gammaproteobacteria. Most of the clones could not be assigned to described species but showed high sequence similarity to uncultured microorganisms retrieved from aquatic environments. OTUs 09–13, all of them retrieved from the most superficial sediment layer, were related to the Chromatiaceae family. Only the OTUs 09 (*Thiorhodococcus*), 22 (*Haliea*), 23 (*Klebsiella/Enterobacter*), and 24 (*Stenotrophomonas*) could be assigned to the genus level.

Deltaproteobacteria. All but one of the sequenced clones related to this class could be assigned to families of sulfate-reducing bacteria: OTUs 26–29 to Desulfuromonadaceae, OTUs 32–36 to Desulfobacteraceae, and OTUs 37–41 to Desulfobulbaceae. Various clones were identified

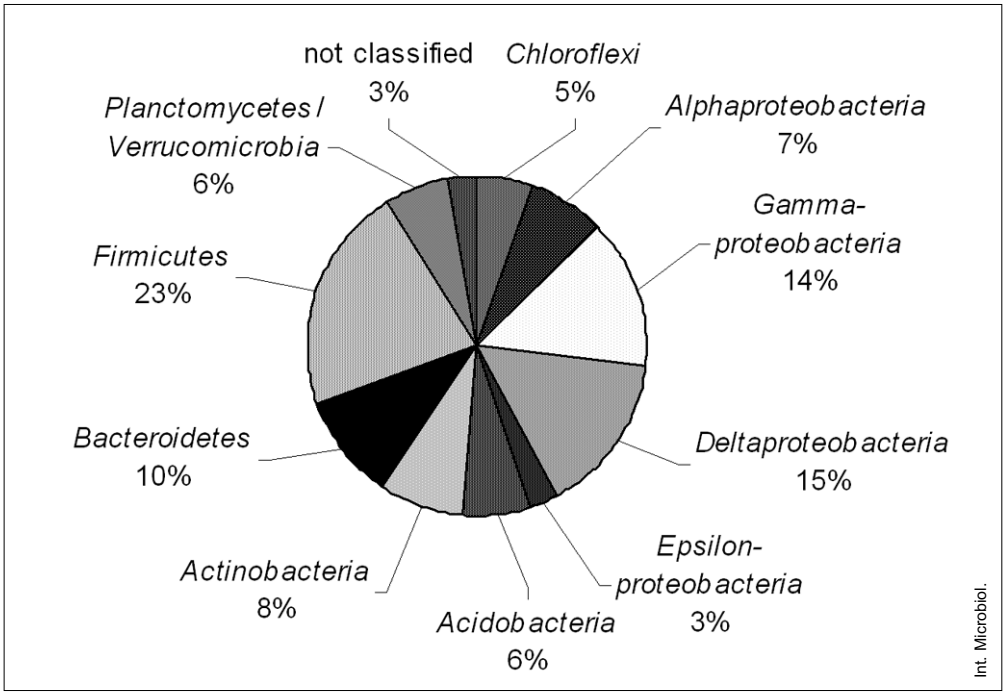

Fig. 4. Percent distribution of bacterial OTUs over the phylogenetic groups.

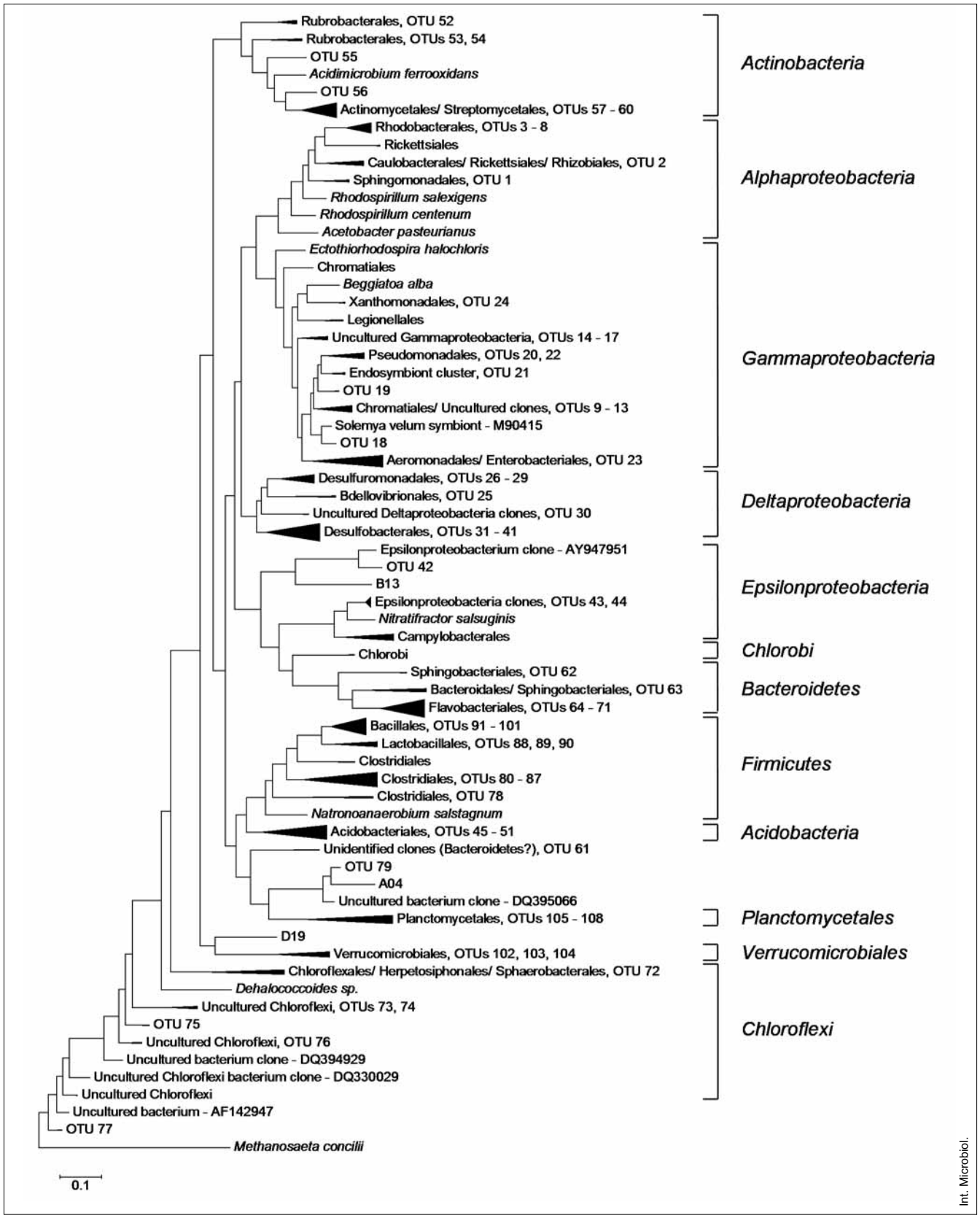

Fig. 5. Phylogenetic tree for the domain *Bacteria*.

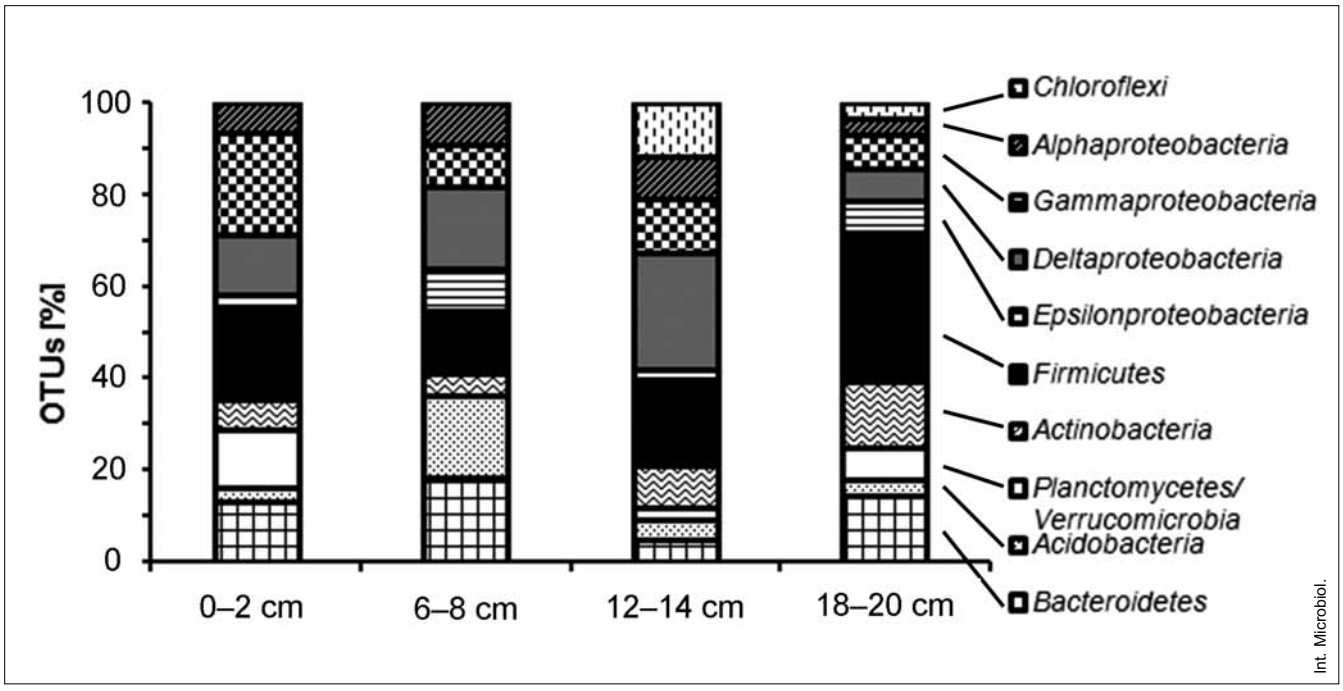

Fig. 6. Relative abundance of OTUs belonging to the different phylogenetic lineages throughout the four sediment depth layers.

further as related to the genera *Desulfosarcina, Desulfocapsa*/*Desulfotalea*, *Desulfuromonas*/*Pelobacter*, and *Desulforhopalus*. Furthermore, all the retrieved sequences were related to sulfate-reducing bacteria, known to play a key role in marine sediments. Only OTU 25 was phylogenetically distant from the remaining set of clones, as it was related to the genus *Anaeromyxobacter* (order Myxococcales).

Epsilonproteobacteria. OTU 43 could be assigned to the genus *Sulfurovum*. Our clones in this class were closely related to clone Milano-WF213 [14], retrieved from the deep-sea sediment in the Eastern Mediterranean Sea. OTU42 could not be assigned to a described genus. Sequences with a similarity of 92% have been retrieved from a deep phreatic sinkhole (FJ485585) and from bacterioplankton communities (AY947951) [9].

Firmicutes. After the Proteobacteria, Firmicutes were the most highly represented group in our study, comprising 24 affiliated taxonomic units. The sequenced clones were distributed evenly over the two most relevant environmental subgroups, classes Bacilli and Clostridia. Fifteen OTUs could be identified at the genus level, including members of *Clostridium*, *Sporacetigenium*, *Ruminococcus*, *Enterococcus*, *Streptococcus*, and *Bacillus*.

Actinobacteria. Nine OTUs were assigned to the phylum Actinobacteria, covering the orders Actinomycetales and Rubrobacterales. Five OTUs were further identified as belonging to the genera *Propionibacterium*, *Jiangella*, *Conexibacter*, *Rubrobacter*, and *Blastococcus*. Most of the clones were retrieved from the two deepest layers.

Bacteroidetes. Two of the three major subgroups of this phylum were represented in the clone library, namely, the classes Flavobacteria and Sphingobacteria, whereas members of the third subgroup, Bacteroidetes, were not detected. Several sequences affiliated with the genera *Flavobacterium*, *Gaetbulibacter*, and *Psychroserpens* belonged to the family Flavobacteriaceae.

Chloroflexi. With the exception of one clone, related to the genus *Sphaerobacter* (OTU 72), the other Chloroflexi (OTUs 73–77), all retrieved from layer D, could be assigned to the family Anaerolineaceae, a strictly anaerobic non-photosynthetic filamentous bacteria found in anoxic environments rich in organic matter.

Acidobacteria. Seven OTUs were associated with this phylum, although all of the clones were phylogenetically situated at considerable distances from the cultured members of

Table 1. Total cell counts and hybridization rates with specific probes for *Bacteria* and *Archaea*. Hybridization values are given as the percentage of the total bacteria (DAPI-stained) counts

				Hybridization (%)	
Sampling point	Year	Depth (cm)	Total cells ($\times 10^8$/g sediment)	EUB338	ARC915
SP02	2002	0–2	2.0 ± 0.3	60.7 ± 14.6	ND
		15–17	1.4 ± 0.4	37.0 ± 12.6	ND
		27–30	0.7 ± 0.2	20.2 ± 15.4	ND
SP16	2002	0–2	5.0 ± 2.0	58.7 ± 13.6	25.8 ± 10.2
		6–8	3.3 ± 1.0	52.0 ± 13.6	18.7 ± 10.3
		12–14	5.5 ± 2.1	45.5 ± 9.0	9.9 ± 6.2
		18–20	1.0 ± 0.1	55.8 ± 21.4	11.4 ± 13.2
		38–40	1.6 ± 0.1	NC	NC
SP16	2003	0–1	2.5 ± 0.7	81.1 ± 15.1	33.4 ± 7.9
		15–16	2.9 ± 0.9	63.7 ± 13.4	16.9 ± 6.0
SP16	2004	0–2	1.2 ± 0.2	61.0 ± 16.1	20.7 ± 16.9
		6–8	1.3 ± 0.4	33.1 ± 20.6	7.2 ± 12.8
		12–14	0.3 ± 0.1	44.7 ± 25.9	9.8 ± 14.0
		18–20	0.4 ± 0.1	53.0 ± 18.2	19.2 ± 18.0
		25	0.7 ± 0.5	NC	NC

ND: Not detected or <0.5% of the total DAPI stained cells. NC: Not counted.

this group. Five of the OTUs were related to the uncultured "genus Gp21" and the other two OTUs to uncultured genera Gp21 and Gp22.

Verrucomicrobia and Planctomycetes. Three OTUs were affiliated with the phylum Verrucomicrobia, clustering in the family Verrucomicrobiacea. Four OTUs belong to the phylum Planctomycetes. OTUs 107–108 were associated with the genera *Pirellula/Rhodopirellula*. None of the Planctomycetes-affiliated clones were related to the anammox bacteria.

Relative distribution of the major phylogenetic groups in the four different depth layers of the sediment. As shown in Fig. 6, there was an almost constant degree of biodiversity, illustrated by the distribution of the OTUs belonging to the different phylogenetic lineages over the four sediment layers (A, B, C, and D). The majority of the detected groups were present in every layer from the surface to the deepest zone (18–20 cm). The proportions, however, differed along the vertical profile. For example, in the surface sediment, the majority of the OTUs were related to Gammaproteobacteria and Firmicutes (two groups present in all samples), whereas the largest share of the taxonomic units in layers B and C was associated with the Deltaproteobacteria class. In contrast, in the deepest layer, one third of the OTUs were members of Firmicutes; only a small fraction was affiliated with the Gamma- and Deltaproteobacteria.

Domain *Archaea*. Using BLAST, the sequenced amplicons clustered into 17 different OTUs: 14 were assigned to the phylum Crenarchaeota and three to the phylum Euryarchaeota. All Crenarchaeota OTUs showed similarity to uncultured environmental clones of diverse origin (such as soils, marine habitats, and thermal springs) and were distantly related (70–80% according to the Classifier function of RDP) to the class Thermoprotei. With respect to the Euryarchaeota, one of the three OTUs encountered was related to a group of uncultured clones from a hypersaline environment (OTU 15). The phylogenetic affiliation of OTUs 16 and 17 could be established more precisely, with OTU 16 closely related to the family Halobacteriaceae, a group of *Archaea* also living in high-salinity habitats, and OTU 17 corresponding to the genus *Methanococcoides*, which is frequently encountered in marine sediments [34,41].

Total cell counts and CARD-FISH. Table 1 shows the numbers of DAPI-stained cells per gram of sediment (dry weight). Sampling zones SP16 and SP02 differed significantly with respect to the samples collected in 2002, as the biomass of the contaminated sediment SP16 was twice as high as that of SP02, reflected by the total cell numbers. The cell numbers also decreased with depth in the SP16 samples. The notable decrease in the total cell numbers of the samples collected between 2002 and 2004 was consistent with the start of operation of a wastewater treatment plant for effluents of the town of San Fernando. The high standard deviations of almost all counts were due to the pronounced heterogeneity of the subsamples, in which aggregates, microcolonies, and blank areas were observed. The number of cells that hybridized with the Bacteria-specific probe EUB338 tended to be 2–5 times higher than the number that hybridized with ARC915, targeting *Archaea*, except for the SP02 sediments in which either no *Archaea* were detected or the hybridization rates were below 0.5%.

Discussion

The high degree of bacterial diversity present in the Sancti Petri Channel sediments was reflected by the clone library and DGGE band patterns. Our cloning approach led to the detection of members of 11 major phylogenetic lineages, with the phylum Proteobacteria (mostly the Delta and Gamma classes) represented by the highest number of unique OTUs (39%), followed by Firmicutes (23%). Bacteroidetes, Actinobacteria, Acidobacteria, Planctomycetes/Verrucomicrobia, and Chloroflexi were detected in minor proportions, comprising 3–10% of the total phylotypes. The predominance of one or several of these groups has been described in marine sediments subjected to similar climatic conditions [3,8,12,32] and in more extreme habitats such as the Antarctic Sea [5]. All the genera of the sulfate-reducers identified thus far occur in anoxic marine or brackish sediments [6]. Their abundance in our study was expected, given the reducing conditions and high sulfate content in the Sancti Petri Channel. Indeed, the presence of an efficient sulfate-reducing community in the Sancti Petri sediments, as assessed by microcosm tests, had been previously reported by our group [21]. The cloning approach used in the present work revealed many subpopulations of Epsilonproteobacteria. Of the 23 clones, 22 were contained in a single OTU, distributed throughout the sampled depth layers, and showed 97–100% nucleotide sequence similarity, suggesting a phylogenetic diversity of the genus *Sulfurovum* in our samples. The type species, *Sulfurovum lithotrophicum*, is a mesophilic, microaerobic sulfur-oxidizing bacterium isolated from the sediment of a hydrothermal system [16]. Most of the *Sulfurovum*-related sequences reported have been retrieved from deep sea sediments [13,15,25]. Sequences affiliated with *Sulfurovum* were also found in an anoxic reactor treating sulfide wastewaters. In all cases, the sequences are related to ecosystems/environments that contain reduced-sulfur compounds. Chemolithoautotrophic marine Epsilonproteobacteria have been detected in diverse sulfide-rich environments: sulfide structures at hydrothermal fields [17], sulfidic waters of pelagic redoxclines, where they represent the major portion of chemoautotrophic *Bacteria* [11,13], and sulfur mats, where a numerical dominance of Epsilonproteobacteria was described [25]. These Epsilonproteobacteria (like the genera *Sulfurovum* and *Sulfurimonas*) are sulfur oxidizers and likely play an important role in the marine sulfur cycle and as anaerobic or microaerophilic dark CO_2-fixing microorganisms [11].

The other major microbial lineage, the domain *Archaea*, was much less abundantly represented by the number of different OTUs; indeed, the quantitative CARD-FISH analysis yielded a 70:30 ratio of *Bacteria* to *Archaea*. The inability to precisely identify the sequences retrieved from the sediment samples was notable: only one OTU could be identified at the genus level (*Methanococcoides*) and one other OTU was determined to be related to the order Halobacteriales. The low number of different ARDRA patterns suggests a lower diversity within the *Archaea* in the analyzed sediments of the Bay of Cádiz. A lower abundance of archaeal microorganisms is commonly encountered in marine sediments [3,26]. Although the DGGE patterns suggested a higher diversity of *Archaea* than *Bacteria*, the sequences derived from the different bands were similar, indicative of a low level of diversity.

Regarding the low archaeal diversity detected by DGGE, it is noteworthy that primer set 344F/518R produced a distorted picture of the archaeal community by introducing a bias towards templates affiliated with the phylum Euryarchaeota, thus excluding members of Crenarchaeota from the experiment. A similar phenomenon was reported in two publications [2,44], in which the probable cause was suggested to be mismatches in the sequence of the reverse primer 518R. Accordingly, we revised and aligned the 344F sequence, which includes a nucleotide signature that is only compatible with euryarchaeal species; however, the mismatch was located at the 5′ end of the primer and was therefore unlikely to interfere significantly with the results.

As evidenced by DAPI staining, the total number of microorganisms decreased with the depth of the analyzed sediments. These findings concur with data from studies of other marine environments [19,32]. The phylogenetic diversity of the clones was homogeneous throughout the sediment column when the OTUs were clustered into phylogenetic groups at the class or phylum level, with the exception of the phylum Chloroflexi, whose members appeared exclusively in the clone library sets of the two deepest levels (12–14 and 18–20 cm). A homogeneous microbial community composition was also observed in analyses of the DGGE band patterns of the bacterial fraction of the microorganisms. However, the DGGE results did not necessarily illustrate an identical community composition, because many of the assigned OTUs were exclusive to one of the four depth layers of the SP sediment. In contrast, the archaeal patterns varied significantly between distinct depth layers in two out of the three analyzed sample sets. Thus, DGGE may not be the ideal tool to assess microbial diversity in environmental samples, since probably only a minor fraction of the microbial community can be resolved by this method. Instead, whole 16S rRNA gene cloning is in such cases the more adequate strategy to approach the true level of diversity in any given environmental sample.

Although shortly after the first sampling a domestic wastewater treatment plant located nearby began operation, the level of bacterial diversity detected by DGGE was equally high in sample sets from 2002 and 2004. It therefore appears that exposure of the sediment to untreated wastewater effluents did not significantly influence the microbial community in terms of diversity. However, the reduction of these wastewater effluents may have led to the observed decrease in population size (total cell numbers) over the three years (Table 1).

A problem that often arises when working with clone sequence databases clearly occurred for the sequences affiliated with Acidobacteria, Chloroflexi, many Gammaproteobacteria, and Crenarchaeota. These clones were similar only to other environmental sequences and did not cluster in the vicinity of any described species, precluding a precise classification of these microorganisms. When the closest related organisms are not described, it is difficult or impossible to infer a metabolic or functional role for a particular phylotype within a given ecosystem. Despite these challenges, the detailed documentation that accompanies most database entries provides some degree of insight into the possible characteristics of a microorganism. In our study, we identified various clones whose 16S rDNA sequences coincided 99% with clones encountered in similar habitats, as published in other surveys. The high proportion of OTUs close to described sulfate-reducing Deltaproteobacteria allowed us to deduce their function in the studied sediments.

The Bay of Cádiz sediments showed a high level of bacterial and a lesser degree of archaeal diversity while the quantitative ratio between the two domains was 70:30 (*Bacteria*:*Archaea*). Total cell numbers decreased with increasing sediment depth. Since a mismatch in the 518R primer likely resulted in the exclusion of the Crenarchaeota from the archaeal DGGE, this primer should be avoided in future surveys of a complete archaeal community. A cluster of Epsilonproteobacteria exhibiting a high level of microdiversity, probably on sub-species level, was encountered in the sediments. Our analysis provided evidence of a phylogenetically diverse microbial community whose close relatives are encountered in many similar habitats of diverse geographic origin. The massive presence of Deltaproteobacteria could be plausibly linked to sulfate-reducing activity; however molecular methodologies did not yield clear information about the ecological functions of the other detected microorganisms. Our strategy of employing three different 16S rRNA-dependent techniques (PCR cloning, PCR-DGGE, and CARD-FISH) to describe the microbial community structure of the Bay of Cádiz sediments resulted in a more comprehensive picture than obtained by a single approach. However, in environmental microbial community studies aimed at inferring the ecological and metabolic functions of its members, culture-independent methods should be combined with the classical microbiological strategy of isolating and growing microorganisms in pure cultures, as this approach will greatly benefit and complement modern molecular methodologies.

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