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# Depth-specific distribution of *Bacteroidetes* in the oligotrophic Eastern Mediterranean Sea

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ABSTRACT: Representatives of the phylum *Bacteroidetes* are repeatedly reported to be abundant members of oceanic bacterioplankton, probably because of their ability to degrade complex organic matter. 16S rDNA analysis was performed in order to address the importance of this phylum in a highly oligotrophic region such as the Eastern Mediterranean Sea and to investigate its distribution patterns and community composition. A new antisense primer was designed for PCR and used in combination with the general eubacterial sense primer 27F to specifically target *Bacteroidetes* representatives. Data were correlated with water depth and water mass properties. Denaturant gradient gel electrophoresis (DGGE) analysis and sequencing of environmental clone libraries revealed specific distribution patterns. A major fraction of the sequences was assigned to the AGG58 cluster, a branch of yet uncultured members of the *Bacteroidetes* lineage. Our results demonstrate a considerable diversity within the *Bacteroidetes* lineage in the oligotrophic Eastern Mediterranean Sea. Differing water mass properties are considered to be of major relevance for the spatial distribution with depth; several environmental clone sequence clusters could be specifically assigned to a defined water mass or to the deep waters of investigated locations. Depth-specific distribution of *Bacteroidetes* is demonstrated for the first time by the results of this study.

KEY WORDS: *Bacteroidetes* · Depth specific distribution · Eastern Mediterranean Sea · Bacterial diversity



## INTRODUCTION

The phylum *Bacteroidetes* is a widely distributed group of chemoheterotrophic bacteria in marine habitats and is abundant within marine bacterioplankton (Bowman et al. 1997, Glöckner et al. 1999, Cottrell & Kirchman 2000, Fry 2000, Kirchman 2002). It is a major eubacterial group and can be divided into different lineages based on 16S ribosomal DNA sequences. Major branches are the *Cytophaga–Flavobacterium* lineage, the *Bacteroides* lineage as well as the families *Sphingobacteriaceae*, *Flexibacteraceae* and *Crenotrichaceae* (Kirchman 2002). A new family within the phylum, the *Cryomorphaceae*, has recently been proposed (Bowman et al. 2003), phylogenetically branching between the families *Flavobacteriaceae* and *Bacteroidaceae*. The type genus of this new family is the genus *Cryomorpha*, type species *C. ignava*, originally isolated from marine and marine-derived habitats in Antarctica (Bowman et al. 2003).

Representatives of *Bacteroidetes* were found to comprise 14% of bacterial isolates from a marine habitat (Uphoff et al. 2001), but the majority of *Bacteroidetes* sequences detected in environmental samples by molecular methods remains as yet uncultured (DeLong et al. 1993, Suzuki et al. 1997, Eilers et al. 2000).

Members of the *Bacteroidetes* represented 10 to 40% of total bacterial numbers (DAPI counts) in ocean waters (Glöckner et al. 1999, Abell & Bowman 2005a) as observed by fluorescence *in situ* hybridisation (FISH). In lakes, a growth rate 2-fold faster than that of other bacterial groups was observed for the *Cytophaga–Flavobacterium* lineage (Jürgens et al. 1999), providing a possible explanation for the high abundances of this group in aquatic systems.

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In contrast to the data obtained by FISH, only few *Bacteroidetes* sequences have been found in clone libraries from open-ocean environments using general eubacterial primers (5 to 20%; Kirchman 2002, Kirchman et al. 2003). *Bacteroidetes* sequences are often underrepresented in clone libraries and consequently in DNA sequence databases (O'Sullivan et al. 2004), because general eubacterial primers are likely to discriminate against this phylum (Suzuki et al. 2001).

Although data from FISH suggest that this phylum represents an important part of bacterial communities in ocean waters, little is known of its ecological function and specific distribution (Reichenbach 1991, Hagström 2000, Hagström et al. 2000, Kirchman 2002). Representatives of the *Bacteroidetes* and especially the *Flavobacterium* lineage are known to be degraders of complex organic matter. In recent years, the distribution of marine representatives of *Bacteroidetes* has attracted increasing attention, mainly due to their supposed essential role in vertical energy and carbon flow (Kirchman 2002, Bowman et al. 2003). Abell & Bowman (2005a) hypothesized an opportunistic response of *Flavobacteria* to the organic carbon present. As major decomposers, uptake and degradation (especially of high molecular weight, HMW) of organic matter is assumed for this bacterial group (Cottrell & Kirchman 2000, Kirchman 2002). Pinhassi et al. (2004) further emphasized the particular importance of *Bacteroidetes* in the processing of organic matter during algal blooms.

Crump et al. (1999) detected a high proportion of *Bacteroidetes* in the particle-attached fraction of an estuarine system, underlining the importance of this bacterial phylum especially in the degradation of marine snow aggregates. DeLong et al. (1993) found that particle-attached bacterial assemblages shared no identical rRNA types with free-living assemblages and suggested major compositional differences between the 2 assemblages. DeLong et al. (1993) also retrieved the first sequence of the so-called 'AGG58' cluster of the *Bacteroidetes* (O'Sullivan et al. 2004) from marine snow in the Santa Barbara Channel, USA. The AGG58 cluster is a coherent branch with closest similarity to the *Flavobacteria* lineage, now incorporated into the new family *Cryomorphaceae* (O'Sullivan et al. 2004, Abell & Bowman 2005b). The AGG58 cluster comprises sequences from such various environments as the Santa Barbara Channel (DeLong et al. 1993), the Delaware Estuary (Kirchman et al. 2003), the Arctic Ocean (Bano & Hollibaugh 2002) and coastal seawater off North Carolina (Rappé et al. 1997). It is therefore considered to be cosmopolitan (O'Sullivan et al. 2004). The different branches of the cluster showed changes in dominance during the course of a phytoplankton bloom, leading to the assumption that different members of the cluster are specialized to different ecological conditions (O'Sullivan et al. 2004). O'Sullivan et al. (2004) found comparably low similarity values of *Bacteroidetes* clones to GenBank sequences and concluded that the phylogenetic diversity of this group is not as well investigated as that for the proteobacteria.

A specific prokaryotic community is considered to inhabit the deep Mediterranean Sea (Zaballos et al. 2006). Zaballos et al. (2006) found substantial differences in the bacterial community composition compared to other ocean environments. In general, the ubiquitous alpha-bacterial SAR-11 cluster contributed a major fraction of the total bacterial community of open ocean environments, whereas in the Ionian Basin (Eastern Mediterranean Sea), *Gammaproteobacteria* dominated the bacterioplankton assemblage (Zaballos et al. 2006). *Bacteroidetes* were the most heterogenous group retrieved in clone libraries of seawater samples from the Greenland Sea and the Ionian Sea, indicating a high diversity within this group in these waters.

To date, depth-specific distribution has been reported for several bacterial phyla, e.g. the green non-sulfur bacteria (Gordon & Giovannoni 1996), the ubiquitous SAR11 cluster (Field et al. 1997) and *Deltaproteobacteria* (Wright et al. 1997). Lee & Fuhrman (1991) reported a varying bacterial community composition with depth, but the phylum *Bacteroidetes* has never been the focus of studies dealing with depth-specific distribution. However, with changing availability of HMW organic matter with depth, a depth-specific distribution particularly of *Bacteroidetes* representatives as major degraders seems likely. Seritti et al. (2003) reported a water-mass-specific distribution of dissolved organic carbon (DOC) in the Eastern Mediterranean Sea. *Bacteroidetes* as major mineralizers of particulate organic carbon (POC) may contribute a major fraction of DOC, further indicating a possible depth- or even water-mass-specific distribution of this phylum.

The Mediterranean Sea can be primarily characterized by unusually high deep water temperatures (>13°C, CIESM 2003) and extremely low nutrient concentrations (Krom et al. 1991). The Eastern Mediterranean is divided into 2 main sub-basins, the Levantine Basin and the Ionian Basin (Theocharis et al. 1993). Characteristic surface water masses of the Eastern Mediterranean Sea are the Levantine Surface Water (LSW, origin: Levantine Basin) and the Modified Atlantic Water (MAW, origin: Atlantic Ocean). Intermediate water masses are the Levantine Intermediate Water (LIW, main origin: Rhodes cyclonic gyre; Theocharis et al. 1999) and the newly formed Cretan Intermediate Water (CIW, origin: Aegean Sea; Schlitzer et al. 1991, Manca et al. 2003). Deep water circulation has changed over the last decade: the Adriatic Sea as primary source of Eastern Mediterranean Deep Water ($EMDW_{Adr}$; Kress et al. 2003) has been replaced by the

Aegean Sea (Roether et al. 1996, Lascaratos et al. 1999, water mass designation $EMDW_{Aeg}$; Kress et al. 2003). This phenomenon was termed the Eastern Mediterranean Transient (EMT).

Well-defined water mass properties, distinguished by different physical properties (e.g. temperature, salinity), offer the possibility to analyze the variation of microbial communities within the different water masses. Because of the special importance of *Bacteroidetes* in marine systems, the present study focused on this lineage by using a combined molecular approach of DGGE and 16S rDNA clone libraries. Community variations of this important bacterial phylum were investigated as a function of water mass and depth.

## MATERIALS AND METHODS

Samples were collected in the Eastern Mediterranean Sea during the RV 'Meteor' Cruise 44, Leg 4 in April and May 1999. Samples from 7 stations (Stn 219: SW-Cyprus, Levantine Basin; Stn 236: Levantine Basin; Stns 254, 265: M40/3D-Ierapetra Basin, Cretan Passage; Stn 291: Cretan Passage; Stns 293, 295: Ionian Basin) were analyzed by DGGE. The M40/3D sample was collected 1 yr earlier in January 1998. Fig. 1 shows the geographical locations of the stations. At each station at least 12 different depths covering different water masses were sampled by a rosette sampler (24 Niskin bottles) attached to a CTD probe. For collection of bacterial community DNA, 5 l of water were filtered through polycarbonate filters (Millipore) with 0.2 µm pore size. Filters were stored at –20°C until use. Stns 219, 254 and 295 were selected for comparison of different examples of water mass distribution. Characteristic physical properties (e.g. temperature and salinity) of the water masses at these stations are shown in Table 1. Total numbers of prokaryotes were determined using the acridine orange direct count method and a Zeiss axioplan epifluorescence microscope.

Filters used for DNA extraction were treated with lysozyme solution (20 mg $ml^{-1}$ lysozyme; 20 mM Tris-HCl, pH 8.0; 2 mM EDTA; 1.2% Triton) and incubated at 37°C for at least 30 min. DNA extraction was performed using a QIAamp DNA mini kit (Qiagen) following the manufacturer's instructions.

PCR for 16S rDNA cloning and for DGGE analysis was performed with 'ready-to-go' PCR beads (Amersham Pharmacia Biotech) in a Techne Progene thermocycler. A new PCR antisense primer, Cyt1020R: CATTTAAGCCTTGGTAAGG, *Escherichia coli* Positions 978 to 995, *Flavobacterium aquatile* Positions 964 to 982, was designed for the specific amplification of *Bacteroidetes* sequences. This primer was used in combination with the general eubacterial primer 27F (Brosius et al. 1978). The performance of the new primer for amplification of *Bacteroidetes* sequences is evaluated in the 'Results'. PCR was performed using the following protocol: an initial denaturation step (94°C for 2 min) followed by 15 touchdown cycles with denaturation at 94°C for 30 s, a decreasing annealing temperature from 65 to 50°C and elongation at 72°C for 40 s. Touchdown cycles were followed by 20 cycles at 94°C (denaturation 30 s), 50°C annealing for 30 s and elongation at 72°C for 40 s. Final annealing was performed at 42°C for 60 s and final elongation at 72°C for 5 min.

Specific amplification of *Bacteroidetes* sequences for DGGE was assured by using a nested PCR protocol. The first amplification was performed with the newly

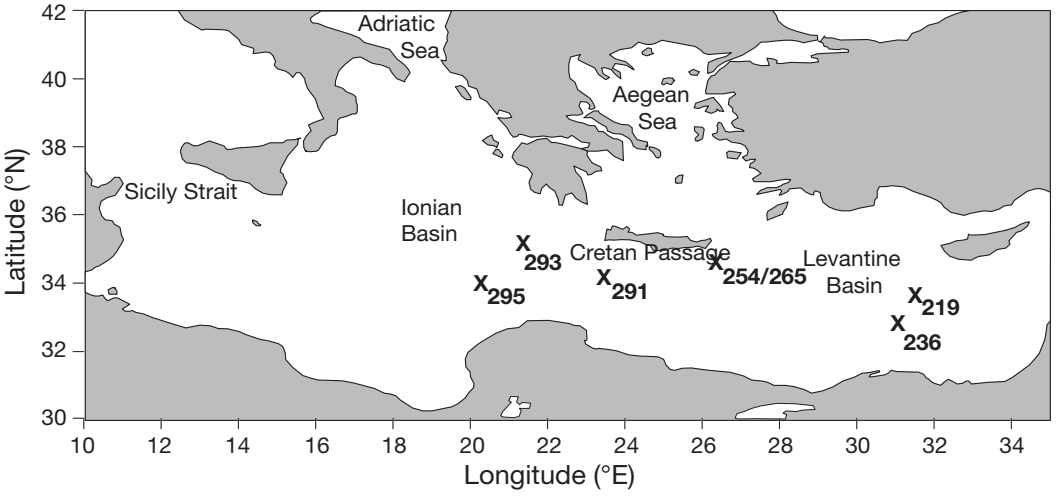


Fig. 1. Locations of stations for cloning experiments and DGGE: Stn 219, SW-Cyprus (Levantine Basin, 34° 07′ 96″ N, 31° 55′ 85″ E); Stn 254, Ierapetra (Cretan Passage, 34° 25′ 51″ N, 26° 05′ 11″ E); Stn 295, Ionian (Ionian Basin, 34° 25′ 00″ N, 20° 20′ 15″ E); Additional DGGE experiments (data not presented) were made at Stns 236 (Levantine Basin), 265 (Cretan Passage), 291, 293 (Ionian Basin)

Table 1. Temperature (*T*), Salinity (*S*), geographic locations and sampling depths of main stations. Samples used for clone library construction are underlined. LSW: Levantine Surface Water; LIW: Levantine Intermediate Water; CIW: Cretan Intermediate Water; EMDW: Eastern Mediterranean Deep Water (Adr = Adriatic, Aeg = Aegean Sea); MAW: Modified Atlantic Water

| Depth (m) | *S* (psu) | *T* (°C) | Water mass |
|---|---|---|---|
| **Stn 219 (SW-Cyprus, Levantine Basin)** | | | |
| 10 | 38.9185 | 17.4885 | LSW |
| 100 | 38.9317 | 16.9976 | LSW |
| 200 | 38.9322 | 16.4295 | LSW |
| 250 | 39.0276 | 15.9164 | LIW |
| 300 | 39.0193 | 15.2973 | LIW |
| 500 | 38.8401 | 14.0528 | LIW/CIW |
| 750 | 38.7761 | 13.8060 | CIW |
| 1000 | 38.7456 | 13.7198 | CIW/ $EMDW_{Adr}$ |
| 1300 | 38.7326 | 13.7129 | $EMDW_{Aeg}$ |
| 2000 | 38.7822 | 13.9345 | $EMDW_{Aeg}$ |
| 2300 | 38.8082 | 14.0503 | $EMDW_{Aeg}$ |
| 2500 | 38.8197 | 14.1143 | $EMDW_{Aeg}$ |
| **Stn 254 (Ierapetra; Cretan Passage)** | | | |
| 10 | 38.5366 | 17.2863 | MAW |
| 50 | 38.5941 | 16.5287 | MAW |
| 100 | 38.7563 | 16.0934 | LIW |
| 200 | 39.0226 | 15.2898 | MAW |
| 500 | 38.8122 | 13.9362 | LIW |
| 1000 | 38.7567 | 13.7573 | CIW |
| 1500 | 38.7811 | 13.8821 | $EMDW_{Adr}$ |
| 2000 | 38.8222 | 14.0646 | $EMDW_{Aeg}$ |
| 2500 | 38.8404 | 14.1757 | $EMDW_{Aeg}$ |
| 3000 | 38.8407 | 14.2481 | $EMDW_{Aeg}$ |
| 4000 | 38.8718 | 14.5007 | $EMDW_{Aeg}$ |
| 4250 | 38.8739 | 14.5541 | $EMDW_{Aeg}$ |
| **Stn 295 (Ionian Basin)** | | | |
| 10 | 38.4052 | 17.8304 | MAW |
| 30 | 38.5154 | 16.2233 | MAW |
| 50 | 38.5828 | 15.6473 | MAW |
| 100 | 38.5998 | 14.6357 | MAW/LIW |
| 200 | 38.8654 | 14.3537 | LIW |
| 300 | 38.8465 | 14.1192 | LIW |
| 400 | 38.8039 | 13.9166 | LIW |
| 500 | 38.7779 | 13.8046 | LIW |
| 900 | 38.7429 | 13.6954 | CIW |
| 1500 | 38.7313 | 13.7099 | $EMDW_{Adr}$ |
| 2000 | 38.7116 | 13.7022 | $EMDW_{Adr}$ |
| 2500 | 38.6920 | 13.7071 | $EMDW_{Adr}$ |
| 2650 | 38.6918 | 13.7277 | $EMDW_{Adr}$ |
| 2700 | 38.6943 | 13.7439 | $EMDW_{Adr}$ |

designed antisense primer Cyt1020R in combination with 27F. In the second PCR for DGGE, Primers 342GC-F and 534R (Muyzer et al. 1993) were used. PCR conditions for both reactions were selected as described above.

A general eubacterial amplification and control 16S rDNA clone library was created using Primers 27F and 1387R (Marchesi et al. 1998). PCR conditions were selected as described above for the amplification of *Bacteroidetes* sequences in order to check the specificity of the new antisense primer Cyt-1020-R.

DGGE was performed with a CBS Scientific DGGE-2001 system. DGGE gels contained a denaturing gradient from 40 to 64% (100% defined as 7 M urea and 10 M formamide according to Abrams & Stanton 1992) and an acrylamide (37.5:1 v/v) gradient from 6 to 8% (Petri & Imhoff 2001). Electrophoresis was run for 14 h at 80 V.

For sequencing of DGGE bands, selected DGGE bands were excised and reamplified. Reamplification of the excised fragments was performed using a primer specifically designed to bind within the GC-clamp (GC-Clamp-M F: GGGGGCACGGGGGGC; Younis 2001). Thus, exclusive amplification of the excised fragment could be assured. For reamplification, the primer GC/M was combined with Primer 534R with the following PCR conditions: 50°C annealing temperature for 40 s, elongation at 72°C for 40 s and denaturation at 94°C for 30 s for 25 cycles. Cluster analysis of DGGE gel band patterns and ANOSIM was performed using PAST software (Hammer et al. 2001, available at: http://palaeo-electronica.org/2001–1/past/issue1–01.htm).

For cloning experiments, DNA was purified with the QIAquick PCR purification kit (Qiagen) following the manufacturer's instructions. Cloning was realized with the TOPO TA cloning kit for Sequencing (Invitrogen). For screening, 50 colonies per depth were chosen randomly. Screening PCR after cloning experiments was performed with Primers M13F and M13R (Yanisch-Peron et al. 1985) using 55°C annealing temperature for 30 s, 72°C elongation for 90 s and 94°C denaturation for 30 s for 25 cycles. Fragments with correct insert length (1000 nucleotides, nt) were chosen for sequencing.

The 'big dye terminator cycle sequencing ready reaction kit' (Applied Biosystems) was used for cycle sequencing following the manufacturer's instructions. Primer 534R was applied for cycle sequencing. Afterwards, DNA was purified using a 2-step ethanol precipitation protocol; 80 µl of 80% ethanol (Merck) were applied to each reaction in the first step. After 30 min precipitation time the reaction was centrifuged for 20 min at 4°C and 13500 rpm (16300 × *g*) (Rotor No. 12454) in a Sigma 3k30 centrifuge. Supernatant was removed and 250 µl of 70% ethanol were added, followed by an additional centrifugation step for 10 min at 4°C and 13500 rpm (16300 × *g*). Supernatant was removed again, remaining ethanol was evaporated and 20 µl template suppression reagent (Applied Biosystems) were added. For sequence analysis an ABI 310 genetic analyzer (Perkin Elmer Applied Biosystems) was used. Sequences obtained from DGGE bands comprised 140 to 190 nt, sequences derived from cloning experiments comprised 200 to 500 nt. The entire insert length of several DNA fragments was sequenced (approx. 1000 nt). Sequences were edited

by Chromas (Version 1.45) and compared with the NCBI database using BLAST (Altschul et al. 1997; see also www.ncbi.nlm.nih.gov/blast/Blast.cgi?CMD=Web&LAYOUT=TwoWindows&AUTO_FORMAT=Semiauto&PAGE=Nucleotides&NCBI_GI=yes&FILTER=L&HITLIST_SIZE=100&SHOW_OVERVIEW=yes&AUTO_FORMAT=yes&SHOW_LINKOUT=yes) to obtain first estimates on closest phylogenetic relatives.

All sequences were aligned to the ARB database (see http://www.arb-home.de) (Ludwig et al. 2004) using the integrated aligner function. The 8 closest phylogenetic relatives according to BLAST were added to the ARB database when not already present. Alignments were refined manually and aligned sequences were added to the ARB tree (quick add marked function, parsimony). Based on the affiliation to the ARB backbone tree, 63 environmental clone sequences were selected for further phylogenetic calculations with PHYML (Guindon & Gascuel 2003). Maximum likelihood analysis was performed assuming nucleotide substitution according to the general time reversible (GTR) model. For primer design, a type-strain alignment of major *Bacteroidetes* representatives was calculated using default parameters of the ClustalX program (Thompson et al. 1994).

16S rDNA sequences determined during this study were deposited in the EMBL Nucleotide Sequence Database and were assigned Accession Nos. AM411213–AM411295, AM411296–AM411358, AM411613, AM398930–AM398941, AJ577829, AJ635360–AJ635362.

Rarefaction analysis of *Bacteroidetes*-specific cloning experiments was performed using the freeware program aRarefactWin (Holland 2003, available at University of Georgia Stratigraphy Laboratory, www.uga.edu/strata/software/) and regression analysis was made using SigmaPlot Version 6.00 (SPSS).

## RESULTS

The stations investigated displayed different hydrographical conditions with regard to water mass distribution, especially in respect to the contribution of different water masses to the deep water (Fig. 2). At Stn 219 (Levantine Basin), there was only slight $EMDW_{Adr}$ contribution to the deep water. At Stn 254 (Ierapetra Basin), $EMDW_{Adr}$ was underlain by $EMDW_{Aeg}$ below 1500 m. At Stn 295 (Ionian Basin), deep water was exclusively provided by $EMDW_{Adr}$. With regard to surface waters, at Stn 219 LSW was present, whereas at the other stations MAW was the prevailing surface water mass.

The ultra-oligotrophic situation in the Eastern Mediterranean Sea is well demonstrated in the nutrient profiles and total prokaryote analysis (in Fig. 2a,c). Phosphate and nitrate as important nutrients for biological processes were either not detectable (phosphate, surface waters of Stn 219) or were present at extremely low concentrations at Stn 295 (phosphate: 0.03 to 0.04 µM, maximum nitrate concentrations 5.8 µM at 1000 m depth). Total numbers of prokaryotes were $2.5 \times 10^5$ cells $ml^{-1}$ in surface waters of Stn 219 and decreased by about 1 order of magnitude at 2500 m depth (Fig. 2a). This also applied to Stns 295 ($2.15 \times 10^5$ cells $ml^{-1}$ at 10 m depth, $1.0 \times 10^4$ cells $ml^{-1}$ at 2700 m) and 254 ($4.37 \times 10^5$ cells $ml^{-1}$ at 10 m depth, $3.8 \times 10^4$ cells $ml^{-1}$ at 2000 m). Maximum total prokaryote abundance corresponded to the chlorophyll maxima at 100 to 150 m depth, as detected by the fluorometer connected to the CTD (Fig. 2b).

The analysis of clone libraries amplified with the general eubacterial primer set 27F and 1387R resulted in 16S rDNA sequences from the *Alpha-*, *Beta-*, *Gamma-* and *Deltaproteobacteria*, the *Planctomycetes*, the *Chaldithrix* group, the *Acidobacteria*, the *Nitrospina* group, the *Cyanobacteria* and the *Thermomicrobia*, but no *Bacteroidetes* sequence. Thus, the results from previous studies, suggesting that general eubacterial primers are not suitable for detecting and analyzing environmental *Bacteroidetes* communities, were confirmed. In order to specifically amplify representatives of the *Bacteroidetes* from environmental samples, the 27F/Cyt1020R primer set was subsequently used for 16S rDNA clone libraries. The RDPII probe match (see http://rdp.cme.msu.edu/probematch/search.jsp) and BLAST search confirmed the specificity of the Cyt1020R primer for the *Bacteroidetes*. When applied for amplification of *Bacteroidetes* sequences from environmental samples, this primer set also showed a good performance: 83 of a total of 88 DGGE band sequences and 64 of 71 16S rDNA clone sequences were assigned to the *Bacteroidetes* by the ARB software. A few exceptions were found within the *Actinobacteria*, *Chloroflexaceae*, *Verrucomicrobia*, *Thermomicrobia* and the OD1-OP11-WS6-TM7 group.

The horizontal and vertical variability of the *Bacteroidetes* community in the Eastern Mediterranean Sea was investigated using DGGE. The band patterns of DGGE gels provided a first insight into the diversity of the *Bacteroidetes* lineage in different depths and water masses at Stns 219, 236, 254, 265, 291, 293, and 295 (DGGE results not shown for Stns 236, 265, 291, 293, M40/3D). DGGE showed major and characteristic bands in the different water masses of the investigated stations; examples are shown for Stns 219 (SW Cyprus, Levantine Basin), 254 (Ierapetra, Cretan Passage) and 295 (Ionian Basin) in Fig. 3a–c. Although some bands were present in samples throughout the water column, several bands were depth-specific. For example, Bands 254-1, -2, -3 and -9 characterized the MAW at

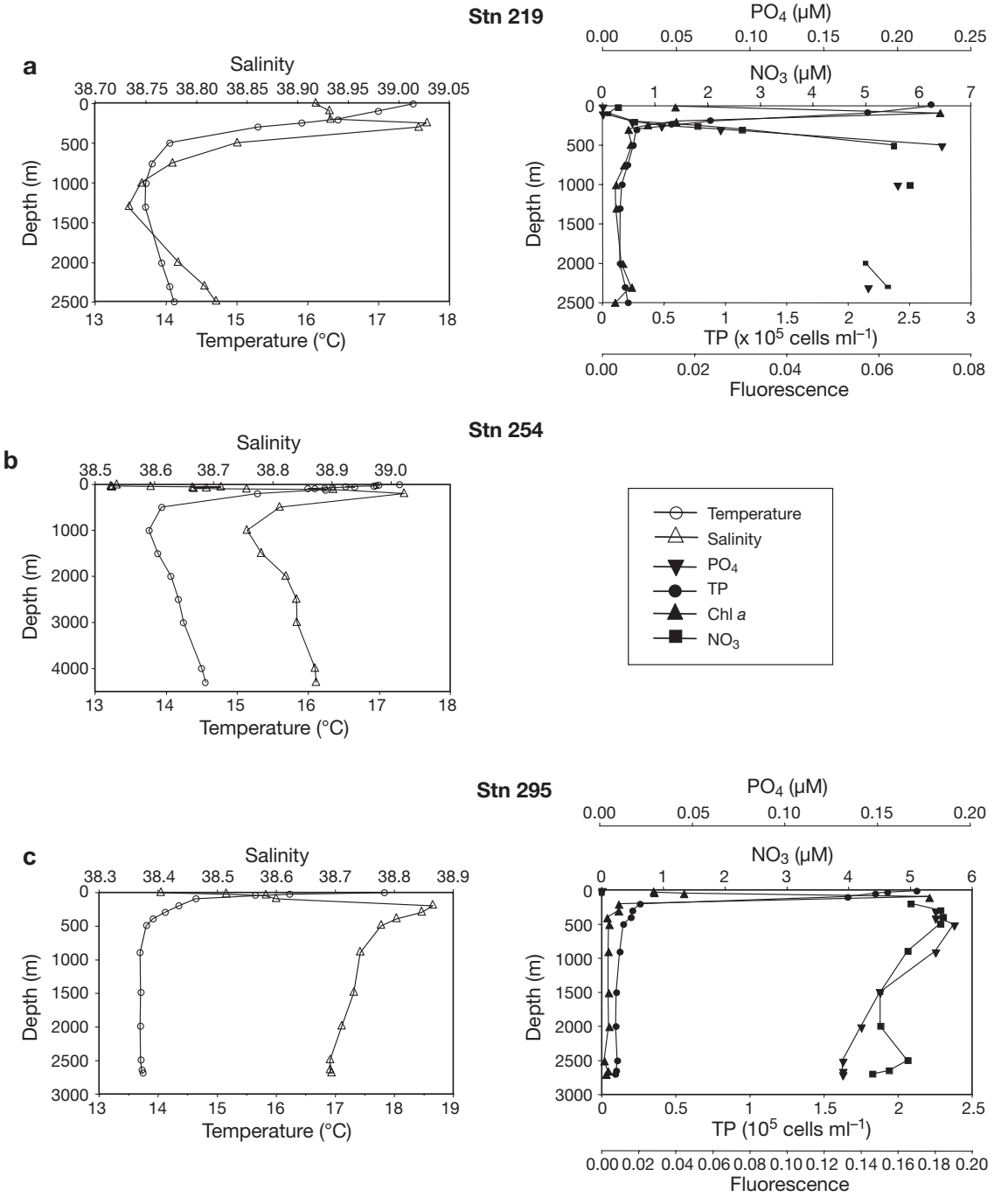


Fig. 2. Depth profiles of temperature and salinity, $PO_4$, $NO_3$ and chlorophyll *a* including counts of total prokaryotes (TP) at Stns 219, 254 and 295. No nutrient and TP data were available for Stn 254 (Ierapetra basin)

Stn 254 and Bands 295-1, -3, -4, -5, -6 at Stn 295, respectively. Bands 219-1, -2, -3, -4, -5 were characteristic of surface and intermediate waters at Stn 219. Band 219-6 was typical for the LIW at Stn 219, as were Bands 254-4 and 295-2, -7, -8, -9, -10 at the respective stations. Deep water masses ($EMDW_{Adr}$ and $EMDW_{Aeg}$) were characterized by Bands 219-7, -9, 254-10 and 295-11, -12, -13, -14. To infer *Bacteroidetes* sequence distribution with depth along physical water mass gradients, a cluster analysis of the DGGE bands was performed in combination with an ANOSIM based on a presence/absence matrix (Fig. 3d–f). Cluster analysis of all 3 stations investigated resulted in distinct clusters primarily according to water mass distribution. Especially at Stn 295, the individual water masses were clearly distinguished in the DGGE pro-

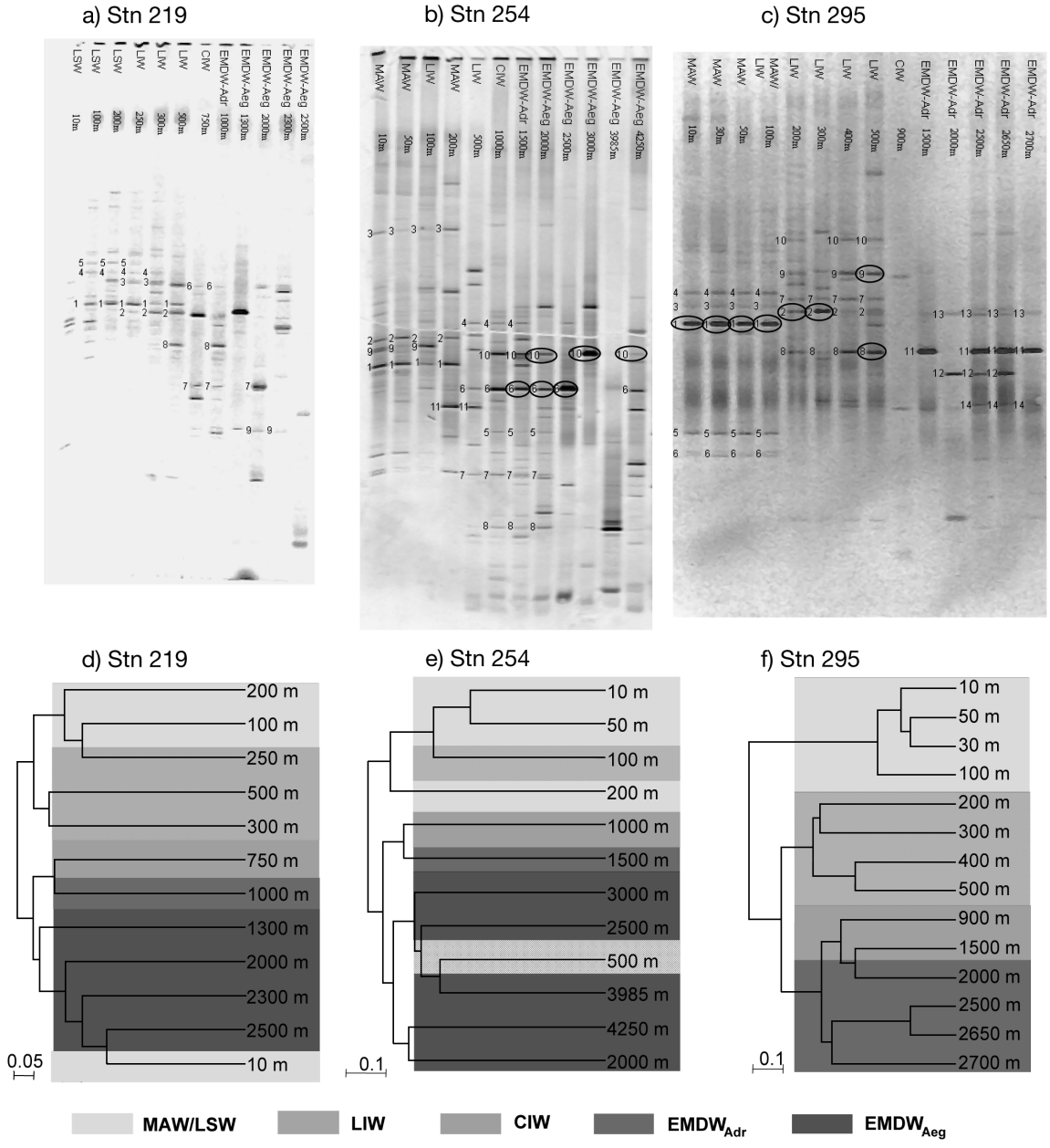


Fig. 3. (a–c) 16S rDNA DGGE depth profiles at Stns 219 (Levantine Basin), 254 (Ierapetra, Cretan Passage) and 295 (Ionian Basin); numbers inside gel bands indicate bands with depth-specific distribution. Circles mark bands which were excised and sequenced (boldface in Table 2). (d–f) Cluster analysis (algorithm: paired group; similarity measure: Euclidean) of DGGE gels from Stns 219, 254 and 295 based on a presence/absence matrix. Grey shading depicts water masses. MAW: Modified Atlantic Water, LSW: Levantine Surface Water, LIW: Levantine Intermediate Water, CIW: Cretan Intermediate Water, EMDW$_{Adr}$: Eastern Mediterranean Deep Water of Adriatic origin, EMDW$_{Aeg}$: Eastern Mediterranean Deep Water of Aegean origin

file. In general, this also applied to Stns 219 and 254. However, the 10 m sample clustered within the EMDW$_{Aeg}$ at Stn 219, and at Stn 254 a sample from LIW (500 m) clustered with EMDW$_{Aeg}$. ANOSIM confirmed these results (Bray-Curtis distance: Stn 295, R = 0.72; Stn 254, R = 0.65; Stn 219, R = 0.43).

For identification of the bacteria represented by the DGGE bands, DNA sequences of a total of 88 excised bands were determined. Table 2 shows the EMBL accession numbers and the phylogenetic affiliation of band sequences according to BLAST. Unfortunately, no sequences could be obtained from excised bands of Stn 219. The majority of sequences from DGGE bands (83) clustered within the AGG58 lineage of the *Bacteroidetes* phylum and were assigned to 16S rDNA sequences from different environments such as the Delaware Estuary, the

Columbia River Estuary, the Arctic and Southern Oceans and the English Channel off Plymouth. Within the AGG58 cluster, sequences from DGGE bands derived from the Mediterranean Sea clustered separately (Fig. 4).

The investigation of *Bacteroidetes* communities within the Mediterranean Sea based on sequences from DGGE bands was supported by 16S rDNA sequence analysis from clone libraries of selected samples. A total of 71 16S rDNA clone sequences was obtained from 3 clone libraries using the primer set 27F/1020CytR for PCR amplification. 16S rDNA cloning experiments were performed with 2 samples from different water masses at Stn 219 (LIW-300 m and $EMDW_{Aeg}$-2300 m) and 1 sample from Stn 254

Table 2. DGGE band sequence names, corresponding EMBL Accession Nos., closest relatives, and phylogenetic affiliation (Phyl. affil) of closest relatives according to BLAST search (Overl: overlap). Corresponding band numbers from DGGE gels in Fig. 3a to c in boldface

| DGGE band | Acc. No. | Length (nt) | Closest relative | Acc. No. closest relative | Overl (nt) | Identity (%) | Phyl. affil |
|---|---|---|---|---|---|---|---|
| 254-D-$EMDW_{Adr}$-19 | AM411224 | 159 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 157 | 98 | *Bacteroidetes* |
| **Band 254-6, 1500 m** | | | | | | | |
| 254-D-$EMDW_{Adr}$-22 | AM411225 | 160 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 155 | 96 | *Bacteroidetes* |
| **Band 254-10, 2000 m** | | | | | | | |
| 254-D-$EMDW_{Aeg}$-24 | AM411227 | 165 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 162 | 98 | *Bacteroidetes* |
| **Band 254-6, 2000 m** | | | | | | | |
| 254-D-$EMDW_{Aeg}$-27 | AM411229 | 153 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 149 | 96 | *Bacteroidetes* |
| **Band 254-6, 2500 m** | | | | | | | |
| 254-D-$EMDW_{Aeg}$-28 | AM411232 | 142 | Uncultured bacterium BAx5 16S ribosomal RNA gene | AF087086 | 123 | 87 | *Bacteroidetes* |
| 254-D-$EMDW_{Aeg}$-29 | AM411228 | 155 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 149 | 96 | *Bacteroidetes* |
| **Band 254-10, 3000 m** | | | | | | | |
| 254-D-$EMDW_{Aeg}$-30 | AM398940 | 136 | Sponge bacterium Zo24 | AY948380 | 133 | 97 | *Actinobacteria* |
| 254-D- $EMDW_{Adr}$-33 | AM411226 | 160 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 154 | 95 | *Bacteroidetes* |
| 254-D-$EMDW_{Aeg}$-34 | AM411230 | 150 | Marine bacterium KMM 6048 16S ribosomal RNA gene | AY753911 | 144 | 95 | *Bacteroidetes* |
| **Band 254-10, 4250 m** | | | | | | | |
| 254-D-$EMDW_{Aeg}$-35 | AM411231 | 143 | Uncultured bacterium gene for 16S rRNA, clone: AL-5 | AB232535 | 139 | 96 | *Bacteroidetes* |
| 254-D-$EMDW_{Aeg}$-36 | AM411233 | 149 | *Flavobacterium* sp. CI33 16S ribosomal RNA gene | DQ530100 | 141 | 93 | *Bacteroidetes* |
| 254-D-1[a] | AM411238 | 148 | Uncultured *Bacteroidetes* bacterium clone FFW55 | AY828423 | 143 | 95 | *Bacteroidetes* |
| 254-D-2[a] | AM411236 | 148 | Uncultured *Bacteroidetes* bacterium clone FFW55 | AY828423 | 145 | 96 | *Bacteroidetes* |
| 254-D-3[a] | AM411237 | 150 | Uncultured *Bacteroidetes* bacterium clone FFW55 | AY828423 | 150 | 98 | *Bacteroidetes* |
| 254-D-6[a] | AM411246 | 130 | Uncultured *Bacteroidetes* bacterium clone FFW55 | AY828423 | 119 | 91 | *Bacteroidetes* |
| 254-D-10[a] | AM411242 | 152 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 139 | 91 | *Bacteroidetes* |
| 254-D-12[a] | AM411235 | 159 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 156 | 97 | *Bacteroidetes* |
| 254-D-13[a] | AM411247 | 133 | Uncultured *Flavobacteriaceae* bacterium clone ESP200-K10-6 | DQ810543 | 124 | 93 | *Bacteroidetes* |
| 254-D-14[a] | AM411244 | 140 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 128 | 90 | *Bacteroidetes* |
| 254-D-15[a] | AM411240 | 156 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 149 | 95 | *Bacteroidetes* |
| 254-D-16[a] | AM411234 | 157 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 153 | 96 | *Bacteroidetes* |
| 254-D-17[a] | AM411239 | 144 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 138 | 95 | *Bacteroidetes* |
| 254-D-18[a] | AM411241 | 158 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 151 | 95 | *Bacteroidetes* |
| 254-D-19[a] | AM411245 | 126 | Uncultured *Bacteroidetes* bacterium clone PLY-P1-17 | AY354711 | 122 | 96 | *Bacteroidetes* |
| 254-D-20[a] | AM411243 | 150 | Marine bacterium KMM 6048 | AY753911 | 143 | 95 | *Bacteroidetes* |
| M40/3D-D-1[b] | AM411250 | 136 | Uncultured *Bacteroidetes* bacterium clone FFW55 | AY828423 | 134 | 97 | *Bacteroidetes* |
| M40/3D-D-11[b] | AM411248 | 140 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 135 | 96 | *Bacteroidetes* |
| M40/3D-D-12[b] | AM411249 | 158 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 143 | 90 | *Bacteroidetes* |
| M40/3D-D-14[b] | AM411251 | 57 | Uncultured bacterium clone SIMO-1962 | AY711328 | 52 | 91 | *Bacteroidetes* |
| 295-D-MAW-1 | AM411284 | 151 | Uncultured *Bacteroidetes* bacterium clone FFW55 | AY828423 | 148 | 98 | *Bacteroidetes* |
| **Band 295-1, 10 m** | | | | | | | |
| 295-D-MAW-2 | AM411282 | 158 | Uncultured *Bacteroidetes* bacterium clone FFW55 | AY828423 | 155 | 98 | *Bacteroidetes* |
| **Band 295-1, 30 m** | | | | | | | |
| 295-D-MAW-3 | AM411281 | 158 | Uncultured *Bacteroidetes* bacterium clone FFW55 | AY828423 | 155 | 98 | *Bacteroidetes* |
| **Band 295-1, 50 m** | | | | | | | |
| 295-D-LIW-4 | AM411285 | 157 | Uncultured *Bacteroidetes* bacterium clone FFW55 | AY828423 | 155 | 98 | *Bacteroidetes* |
| **Band 295-1, 100 m** | | | | | | | |
| 295-D-LIW-5 | AM411294 | 161 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 156 | 96 | *Bacteroidetes* |
| **Band 295-2, 200 m** | | | | | | | |
| 295-D-LIW-6 | AM411283 | 158 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 154 | 97 | *Bacteroidetes* |
| **Band 295-2, 300 m** | | | | | | | |
| 295-D-LIW-7 | AM411293 | 156 | Uncultured *Bacteroidetes* bacterium clone SBI04_186 | DQ186942 | 145 | 92 | *Bacteroidetes* |
| 295-D-LIW-8 | AM411289 | 153 | Uncultured *Bacteroidetes* bacterium clone FFP61 | AY830006 | 146 | 95 | *Bacteroidetes* |
| 295-D-LIW-9 | AM411292 | 157 | Uncultured *Bacteroidetes* bacterium clone FFP61 | AY830006 | 148 | 94 | *Bacteroidetes* |

($EMDW_{Aeg}$-2000 m depth). Affiliation of selected whole insert 16S rDNA clones (approx. 1000 nt from 219-C-EMDW-Aeg-15, 219-C-LIW-40 and 2119-C-LIW-49) was identical to that of the 5′ terminal fragments comprising approx. 400 nt. Thus, short fragments were determined for the majority of the environmental clones. Sequences from environmental clones were mainly assigned to the *Bacteroidetes* (Table 3) and branched almost exclusively within the AGG58 cluster (Fig. 4). Closest relatives were sampled from various environments: the English Channel off Plymouth, surface waters near Sapelo Island, the Columbia River Estuary, the Southern Ocean, the Sargasso Sea, the Pacific Ocean off San Pedro (California), from ridge flank crustal flu-

Table 2 (continued)

| DGGE band | Acc. No. | Length (nt) | Closest relative | Acc. No. closest relative | Overl (nt) | Identity (%) | Phyl. affil |
|---|---|---|---|---|---|---|---|
| **Band 295-9, 500 m** | | | | | | | |
| 295-D-LIW-10 | AM411291 | 125 | Uncultured *Flavobacteriaceae* bacterium clone ESP200-K10-6 | DQ810543 | 111 | 88 | *Bacteroidetes* |
| 295-D-LIW-11 | AM411286 | 165 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 161 | 97 | *Bacteroidetes* |
| **Band 295-8, 500 m** | | | | | | | |
| 295-D-CIW-12 | AM398930 | 147 | Uncultured *Chloroflexaceae* group bacterium Arctic95A-18 | AF355054 | 140 | 95 | *Chloroflexi* |
| 295-D-$EMDW_{Adr}$-13 | AM411279 | 160 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 157 | 98 | *Bacteroidetes* |
| 295-D-$EMDW_{Adr}$-14 | AM411288 | 154 | Marine bacterium KMM 6048 | AY753911 | 147 | 95 | *Bacteroidetes* |
| 295-D-$EMDW_{Adr}$-15 | AM411287 | 157 | Marine bacterium KMM 6048 | AY753911 | 155 | 98 | *Bacteroidetes* |
| 295-D-$EMDW_{Adr}$-16 | AM398931 | 135 | Uncultured Chloroflexi bact. isolate DGGE gel band SuluC14 | DQ273982 | 133 | 98 | *Chloroflexi* |
| 295-D-$EMDW_{Adr}$-17 | AM411290 | 161 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 150 | 93 | *Bacteroidetes* |
| 295-D-$EMDW_{Adr}$-18 | AM411215 | 129 | Uncultured *Flavobacteriaceae* bacterium clone ESP450-K6II-56 | DQ810695 | 116 | 89 | *Bacteroidetes* |
| 295-D-$EMDW_{Adr}$-19 | AM411295 | 153 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 150 | 98 | *Bacteroidetes* |
| 295-D-$EMDW_{Adr}$-20 | AM411280 | 71 | Uncultured *Bacteroidetes* bacterium clone FFW55 | AY828423 | 66 | 92 | *Bacteroidetes* |
| 236-D-LSW-1 | AM411253 | 152 | Uncultured *Bacteroidetes* bacterium clone FFW5 | AY828423 | 148 | 97 | *Bacteroidetes* |
| 236-D-LSW-2 | AM411259 | 134 | Uncultured *Bacteroidetes* bacterium clone FFW55 | AY828423 | 132 | 98 | *Bacteroidetes* |
| 236-D-LSW-3 | AM411252 | 150 | Uncultured *Bacteroidetes* bacterium clone FFW55 | AY828423 | 147 | 98 | *Bacteroidetes* |
| 236-D-LIW-5 | AM411255 | 160 | Uncultured *Bacteroidetes* bacterium clone PLY-P1-17 | AY354711 | 156 | 97 | *Bacteroidetes* |
| 236-D-LIW-6 | AM411256 | 152 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 141 | 92 | *Bacteroidetes* |
| 236-D-LIW-7 | AM411258 | 153 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 147 | 96 | *Bacteroidetes* |
| 236-D-LIW-8 | AM411260 | 174 | Uncultured bacterium clone E53-156 | DQ639403 | 169 | 97 | *Bacteroidetes* |
| 236-D-LIW-9 | AM411265 | 146 | Uncultured bacterium clone CTD005-74B-02 | AY704387 | 145 | 99 | *Bacteroidetes* |
| 236-D-$EMDW_{Adr}$-13 | AM411263 | 135 | *Flavobacterium* columnare | AY747592 | 133 | 98 | *Bacteroidetes* |
| 236-D-$EMDW_{Aeg}$-17 | AM411257 | 148 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 136 | 91 | *Bacteroidetes* |
| 236-D-$EMDW_{Aeg}$-18 | AM411264 | 108 | *Flavobacteriaceae* bacterium LZXC41 | DQ659086 | 107 | 99 | *Bacteroidetes* |
| 236-D-$EMDW_{Aeg}$-20 | AM411254 | 163 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 160 | 98 | *Bacteroidetes* |
| 236-D-$EMDW_{Aeg}$-21 | AM411261 | 157 | Uncultured bacterium clone SG2-42 | AY135906 | 152 | 96 | *Bacteroidetes* |
| 236-D-$EMDW_{Aeg}$-22 | AM411262 | 157 | *Chryseobacterium* sp. RHA2-9 | DQ673672 | 154 | 98 | *Bacteroidetes* |
| 291-D-LIW-2 | AM411217 | 165 | Uncultured *Bacteroidetes* bacterium clone FFW55 | AY828423 | 160 | 96 | *Bacteroidetes* |
| 291-D-LIW-3 | AM411223 | 100 | Uncultured *Cytophagales* bacterium clone CD2C7 | AY038572 | 94 | 94 | *Bacteroidetes* |
| 291-D-LIW-4 | AM411214 | 152 | Uncultured *Bacteroidetes* bacterium clone FFP61 | AY830006 | 147 | 96 | *Bacteroidetes* |
| 291-D-LIW-5 | AM411213 | 169 | Uncultured bacterium clone WLB16-196 | DQ015861 | 150 | 88 | *Bacteroidetes* |
| 291-D-LIW-6 | AM411219 | 167 | Uncultured *Cytophaga* sp. | AJ635360 | 161 | 96 | *Bacteroidetes* |
| 291-D-LIW-7 | AM411218 | 165 | Uncultured bacterium clone A714014 | AY907801 | 161 | 97 | *Bacteroidetes* |
| 291-D-CIW-8 | AM411216 | 145 | Uncultured bacterium clone E53-156 | DQ639403 | 138 | 95 | *Bacteroidetes* |
| 291-D-$EMDW_{Aeg}$-11 | AM411222 | 118 | Uncultured *Flavobacteriaceae* bacterium clone ESP200-K10-6 | DQ810543 | 112 | 94 | *Bacteroidetes* |
| 291-D-$EMDW_{Aeg}$-13 | AM411220 | 154 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 141 | 91 | *Bacteroidetes* |
| 291-D-$EMDW_{Aeg}$-14 | AM411221 | 132 | Uncultured *Flavobacteriaceae* bacterium clone ESP200-K10-6 | DQ810543 | 132 | 100 | *Bacteroidetes* |
| 293-D-MAW-2 | AM411266 | 159 | Uncultured *Bacteroidetes* bacterium clone FFW55 | AY828423 | 158 | 99 | *Bacteroidetes* |
| 293-D-MAW-3 | AM411272 | 140 | Uncultured *Bacteroidetes* bacterium clone ECS-R21 | DQ656214 | 137 | 97 | *Bacteroidetes* |
| 293-D-MAW-4 | AM411275 | 154 | uncultured *Bacteroidetes* bacterium MTAE19 | AJ619057 | 144 | 93 | *Bacteroidetes* |
| 293-D-MAW-5 | AM411273 | 157 | Uncultured *Bacteroidetes* bacterium clone ECS-R21 | DQ656214 | 149 | 94 | *Bacteroidetes* |
| 293-D-LIW-7 | AM411271 | 146 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 144 | 98 | *Bacteroidetes* |
| 293-D-CIW-10 | AM398932 | 142 | *Arthrobacter* sp. SMB28 | DQ868689 | 134 | 94 | *Actinobacteria* |
| 293-D-CDW-11 | AM411276 | 144 | uncultured *Bacteroidetes* bacterium MTAE19 | AJ619057 | 132 | 91 | *Bacteroidetes* |
| 293-D-CDW-12 | AM411269 | 164 | Uncultured *Bacteroidetes* bacterium clone SC001B77 | AY807715 | 164 | 100 | *Bacteroidetes* |
| 293-D-CDW-17 | AM411270 | 160 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 156 | 97 | *Bacteroidetes* |
| 293-D-CDW-18 | AM411277 | 144 | Uncultured bacterium clone LC1537B-77 | DQ272585 | 131 | 90 | *Bacteroidetes* |
| 293-D-BRINE-19 | AM411267 | 152 | Uncultured bacterium clone E53-156 | DQ639403 | 151 | 99 | *Bacteroidetes* |
| 293-D-BRINE-20 | AM411268 | 157 | *Capnocytophaga* sp. AHN9528 | DQ012324 | 154 | 98 | *Bacteroidetes* |
| 293-D-BRINE-21 | AM411278 | 136 | Uncultured bacterium clone LC1537B-77 | DQ272585 | 126 | 92 | *Bacteroidetes* |
| 293-D-BRINE-23 | AM398941 | 155 | *Brevibacterium linens* AC825 | AY017070 | 154 | 99 | *Actinobacteria* |
| 293-D-BRINE-25 | AM411274 | 165 | *Flavobacterium* sp. B11 | AJ518814 | 168 | 98 | *Bacteroidetes* |

[a]No corresponding water mass recorded; [b]sample from cruise M40/3, no corresponding water mass recorded

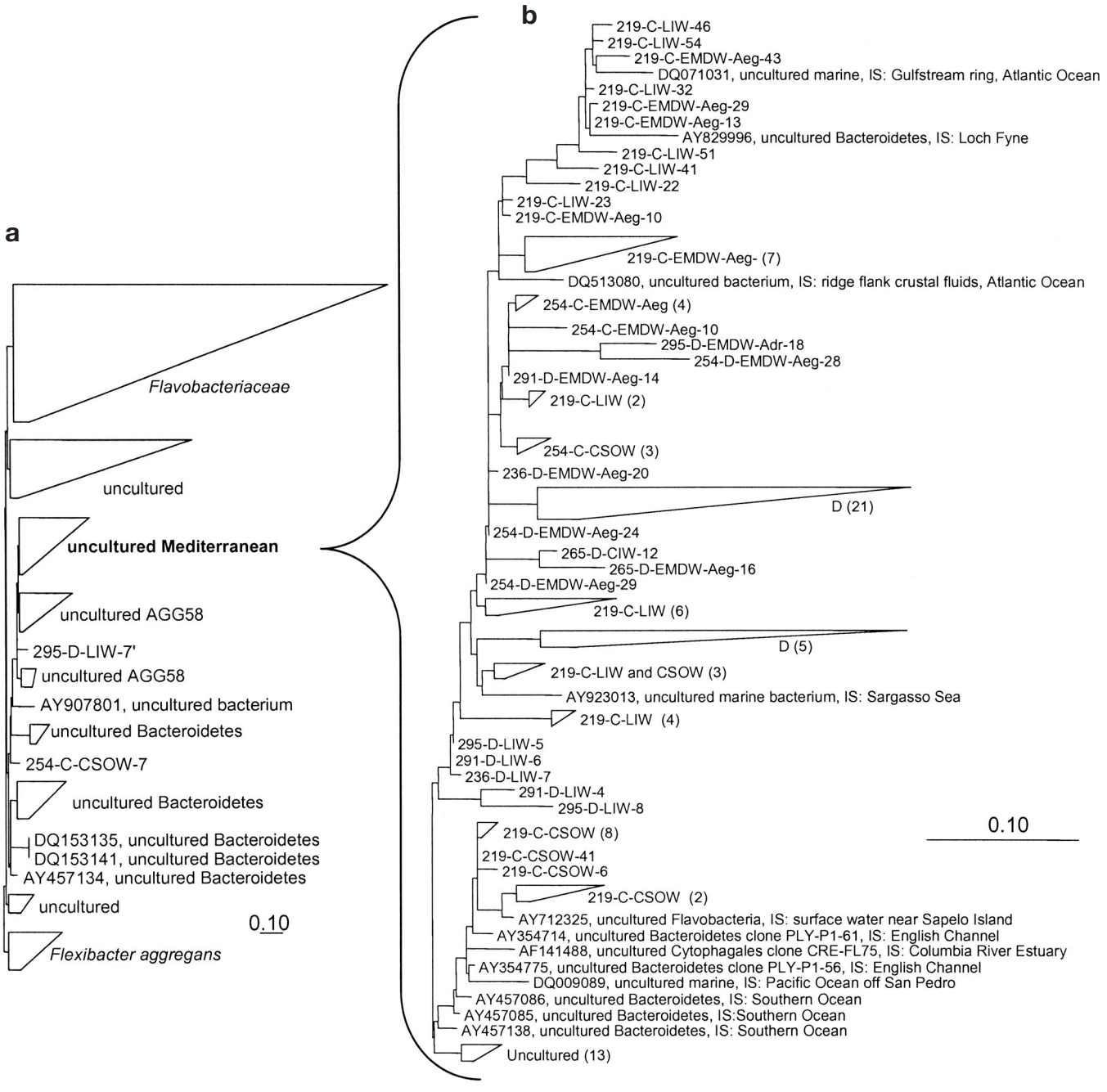


Fig. 4. (a) Phylogenetic 16S rDNA tree (parsimony, exported from ARB) showing position of 'Mediterranean' cluster (boldface) in *Bacteroidetes* phylum. (b) High-resolution phylogenetic 16S rDNA tree (parsimony, exported from ARB) of 'Mediterranean' cluster showing majority of sequences obtained from environmental clones and DGGE bands during this study as well as reference sequences from closest relatives; 219/236/254/265/M40-3D/291/293/295: isolation source/station; C/D: origin of sequence (environmental **c**lones/**D**GGE); LIW/EMDW-Adr/EMDW-Aeg: corresponding water masses; numbers in parentheses: no. of sequences within wedges; In reference sequences: IS: isolation source; other abbreviations as in Fig. 3 key

ids, the Gulf Stream, Loch Fyne (Scotland) after nutrient addition, and from the Delaware River Estuary. Because of the limited number of clones analyzed from each water mass (219-LIW: 29, 219-EMDW$_{Aeg}$: 26, 254-EMDW$_{Aeg}$: 16) analytical rarefaction analysis combined with a regression analysis was performed in order to infer the total *Bacteroidetes* diversity covered by clone libraries. For rarefaction, it was assumed that 1 OTU is formed by sequences presenting a similarity equal to or greater than 99% (Fig. 5). Results from rarefaction/regression analysis showed a coverage of approximately half the expected number of OTUs in the respective

Table 3. Environmental clone sequence names, corresponding EMBL Accession Nos., closest relatives, and phylogenetic affiliation (Phyl. affil) of closest relatives according to BLAST search (Overl: overlap). 219-C-LIW: n = 29, 219-C-EMDW$_{Aeg}$: n = 26, 254-C- EMDW$_{Aeg}$: n = 16

| Clone | Acc. No. | Length (nt) | Closest relative | Acc. No. Closest relative | Overl (nt) | Identity (%) | Phyl. affil |
|---|---|---|---|---|---|---|---|
| 219-C-LIW-1 | AM411296 | 373 | Uncultured marine bacterium clone SPOTSFEB02_70m25 | DQ009434 | 361 | 96 | *Bacteroidetes* |
| 219-C-LIW-2 | AM411298 | 357 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 337 | 94 | *Bacteroidetes* |
| 219-C-LIW-3 | AM411313 | 414 | Uncultured bacterium clone A714014 | AY907801 | 397 | 95 | *Bacteroidetes* |
| 219-C-LIW-21 | AM411321 | 157 | Uncultured *Bacteroidetes* bacterium clone SBI04_174 | DQ186940 | 156 | 99 | *Bacteroidetes* |
| 219-C-LIW-22 | AM411312 | 323 | Uncultured *Bacteroidetes* bacterium clone FFW19 | AY830011 | 301 | 93 | *Bacteroidetes* |
| 219-C-LIW-23 | AM411613 | 454 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 448 | 98 | *Bacteroidetes* |
| 219-C-LIW-25 | AM411301 | 293 | Uncultured marine bacterium clone D92_53 | AY923013 | 277 | 94 | *Bacteroidetes* |
| 219-C-LIW-26 | AM411310 | 268 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 262 | 97 | *Bacteroidetes* |
| 219-C-LIW-29 | AM411299 | 337 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 331 | 98 | *Bacteroidetes* |
| 219-C-LIW-30 | AM411297 | 398 | Uncultured marine bacterium clone D92_53 | AY923013 | 372 | 93 | *Bacteroidetes* |
| 219-C-LIW-31 | AM411306 | 353 | Uncultured *Bacteroidetes* bacterium clone FFW80 | AY828437 | 338 | 95 | *Bacteroidetes* |
| 219-C-LIW-32 | AM411320 | 176 | Uncultured *Bacteroidetes* bacterium clone FFP21 | AY829996 | 175 | 99 | *Bacteroidetes* |
| 219-C-LIW-34 | AM411303 | 399 | Uncultured marine bacterium clone SPOTSFEB02_70m25 | DQ009434 | 369 | 97 | *Bacteroidetes* |
| 219-C-LIW-36 | AM411311 | 346 | Uncultured *Bacteroidetes* bacterium clone FFW80 | AY828437 | 335 | 96 | *Bacteroidetes* |
| 219-C-LIW-37 | AM411302 | 399 | Uncultured *Bacteroidetes* bacterium clone JL-ETNP-Z67 | AY726974 | 389 | 97 | *Bacteroidetes* |
| 219-C-LIW-38 | AM411300 | 455 | Uncultured marine bacterium clone D92_53 | AY923013 | 426 | 93 | *Bacteroidetes* |
| 219-C-LIW-39 | AM411305 | 432 | Uncultured *Cytophagales* Arctic97A-17 | AF354617 | 425 | 98 | *Bacteroidetes* |
| 219-C-LIW-40 | AM411308 | 444 | Uncultured marine bacterium clone Chl1.45 | DQ071058 | 418 | 94 | *Bacteroidetes* |
| 219-C-LIW-41 | AM411314 | 419 | Uncultured marine bacterium clone D92_53 | AY923013 | 394 | 94 | *Bacteroidetes* |
| 219-C-LIW-43 | AM411315 | 342 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 312 | 91 | *Bacteroidetes* |
| 29-C-LIW-44 | AM411319 | 139 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 134 | 96 | *Bacteroidetes* |
| 219-C-LIW-45 | AM411317 | 228 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 223 | 97 | *Bacteroidetes* |
| 219-C-LIW-46 | AM411307 | 394 | Uncultured marine bacterium clone Chl1.10 | DQ071031 | 377 | 95 | *Bacteroidetes* |
| 219-C-LIW-47 | AM411322 | 376 | Uncultured bacterium clone HF200_E5_P1 | DQ300888 | 371 | 98 | *Bacteroidetes* |
| 219-C-LIW-49 | AM411309 | 404 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 392 | 97 | *Bacteroidetes* |
| 219-C-LIW-52 | AM398933 | 435 | Unidentified eubacterium clone SAR307 | U20798 | 428 | 98 | *Chloroflexi* |
| 219-C-LIW-54 | AM411316 | 291 | Uncultured marine bacterium clone Chl1.10 | DQ071031 | 279 | 95 | *Bacteroidetes* |
| 219-C-LIW-57 | AM411304 | 365 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 359 | 98 | *Bacteroidetes* |
| 219-C-LIW-59 | AM411318 | 331 | Uncultured marine bacterium clone Chl1.10 | DQ071031 | 312 | 94 | *Bacteroidetes* |
| 219-C-EMDW$_{Aeg}$-1 | AM411347 | 137 | Uncultured *Bacteroidetes* bacterium clone SBI04_174 | DQ186940 | 137 | 100 | *Bacteroidetes* |
| 219-C-EMDW$_{Aeg}$-2 | AM411348 | 113 | Uncultured Flavobacteria bacterium clone SIMO-788 | AY712325 | 107 | 94 | *Bacteroidetes* |
| 219-C-EMDW$_{Aeg}$-3 | AM411340 | 325 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 316 | 97 | *Bacteroidetes* |
| 219-C-EMDW$_{Aeg}$-4 | AM411342 | 376 | Uncultured marine bacterium clone SPOTSAPR01_5m159 | DQ009089 | 372 | 98 | *Bacteroidetes* |
| 219-C-EMDW$_{Aeg}$-6 | AM411331 | 466 | Uncultured marine bacterium clone SPOTSAPR01_5m159 | DQ009089 | 457 | 98 | *Bacteroidetes* |
| 219-C-EMDW$_{Aeg}$-7 | AM411341 | 417 | Uncultured marine bacterium clone SPOTSAPR01_5m159 | DQ009089 | 408 | 97 | *Bacteroidetes* |
| 219-C-EMDW$_{Aeg}$-10 | AM411345 | 165 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 160 | 96 | *Bacteroidetes* |
| 219-C-EMDW$_{Aeg}$-11 | AM411343 | 397 | Uncultured marine bacterium clone SPOTSAPR01_5m159 | DQ009089 | 391 | 98 | *Bacteroidetes* |
| 219-C-EMDW$_{Aeg}$-12 | AM411326 | 445 | Uncultured *Flavobacteria* bacterium clone SIMO-788 | AY712325 | 442 | 99 | *Bacteroidetes* |
| 219-C-EMDW$_{Aeg}$-13 | AM411346 | 197 | Uncultured *Bacteroidetes* bacterium clone FFP21 | AY829996 | 196 | 99 | *Bacteroidetes* |
| 219-C-EMDW$_{Aeg}$-14 | AM411334 | 364 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 355 | 97 | *Bacteroidetes* |
| 219-C-EMDW$_{Aeg}$-15 | AM411335 | 470 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 460 | 97 | *Bacteroidetes* |
| 219-C-EMDW$_{Aeg}$-19 | AM411332 | 396 | Uncultured marine bacterium clone SPOTSAPR01_5m159 | DQ009089 | 389 | 98 | *Bacteroidetes* |
| 219-C-EMDW$_{Aeg}$-20 | AM411327 | 455 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 447 | 98 | *Bacteroidetes* |
| 219-C-EMDW$_{Aeg}$-21 | AM411325 | 459 | Uncultured marine bacterium clone SPOTSAPR01_5m159 | DQ009089 | 454 | 98 | *Bacteroidetes* |
| 219-C-EMDW$_{Aeg}$-22 | AM411339 | 371 | Uncultured marine bacterium clone SPOTSAPR01_5m159 | DQ009089 | 366 | 98 | *Bacteroidetes* |
| 219-C-EMDW$_{Aeg}$-23 | AM411328 | 433 | Uncultured *Bacteroidetes* bacterium clone JL-ETNP-Z67 | AY726974 | 426 | 98 | *Bacteroidees* |
| 219-C-EMDW$_{Aeg}$-28 | AM411324 | 387 | Uncultured marine bacterium clone SPOTSAPR01_5m159 | DQ009089 | 384 | 99 | *Bacteroidetes* |
| 219-EMDW$_{Aeg}$-29 | AM411329 | 407 | Uncultured *Bacteroidetes* bacterium clone FFP21 | AY829996 | 390 | 95 | *Bacteroidetes* |
| 219-C-EMDW$_{Aeg}$-30 | AM411323 | 442 | Uncultured *Bacteroidetes* bacterium clone FFP74 | AY828436 | 438 | 99 | *Bacteroidetes* |
| 219-C-EMDW$_{Aeg}$-39 | AM411330 | 339 | Uncultured bacterium clone FS266-67B-03 | DQ513081 | 333 | 98 | *Bacteroidetes* |
| 219-C-EMDW$_{Aeg}$-41 | AM411344 | 336 | Uncultured marine bacterium clone SPOTSAPR01_5m159 | DQ009089 | 331 | 98 | *Bacteroidetes* |
| 219-C-EMDW$_{Aeg}$-42 | AM411336 | 401 | Uncultured *Flavobacteria* bacterium clone SIMO-788 | AY712325 | 399 | 99 | *Bacteroidetes* |
| 219-C-EMDW$_{Aeg}$-43 | AM411337 | 375 | Uncultured marine bacterium clone Chl1.10 | DQ071031 | 359 | 95 | *Bacteroidetes* |
| 219-C-EMDW$_{Aeg}$-44 | AM411338 | 322 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 316 | 98 | *Bacteroidetes* |
| 219-C-EMDW$_{Aeg}$-45 | AM411333 | 473 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 465 | 98 | *Bacteroidetes* |
| 254-C-EMDW$_{Aeg}$-2 | AM411351 | 375 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 369 | 98 | *Bacteroidetes* |
| 254-C-EMDW$_{Aeg}$-3 | AM398934 | 437 | Uncultured green non-sulfur bacterium clone MBMPE46 | AJ567560 | 422 | 96 | *Chloroflexi* |
| 254-C-EMDW$_{Aeg}$-6 | AM398937 | 257 | Uncultured *Verrucomicrobiales* Sva0821 | AJ297461 | 245 | 95 | *Verrucomicrobia* |
| 254-C-EMDW$_{Aeg}$-7 | AM411358 | 148 | Uncultured *Cytophagaceae* bacterium clone 1-13 | AY094494 | 143 | 96 | *Bacteroidetes* |
| 254-C-EMDW$_{Aeg}$-8 | AM398939 | 254 | Uncultured bacterium clone HF500_B6_P1 | DQ300691 | 246 | 96 | *Bacteroidetes* |
| 254-C-EMDW$_{Aeg}$-9 | AM411352 | 418 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 417 | 99 | *Bacteroidetes* |
| 254-C-EMDW$_{Aeg}$-10 | AM411349 | 457 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 451 | 98 | *Bacteroidetes* |
| 254-C-EMDW$_{Aeg}$-12 | AM398935 | 359 | Unidentified eubacterium clone SAR307 | U20798 | 358 | 99 | *Chloroflexi* |
| 254-C-EMDW$_{Aeg}$-13 | AM411354 | 398 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 395 | 99 | *Bacteroidetes* |
| 254-C-EMDW$_{Aeg}$-15 | AM398936 | 282 | Uncultured bacterium clone:Mb-NB06 | AB193900 | 260 | 92 | *Bacteroidetes* |
| 254-C-EMDW$_{Aeg}$-16 | AM411356 | 375 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 366 | 97 | *Bacteroidetes* |
| 254-C-EMDW$_{Aeg}$-17 | AM398938 | 244 | Uncultured bacterium clone:Mb-NB06 | AB193900 | 229 | 93 | *Bacteroidetes* |
| 254-C-EMDW$_{Aeg}$-19 | AM411355 | 386 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 380 | 98 | *Bacteroidetes* |
| 254-C-EMDW$_{Aeg}$-20 | AM411350 | 189 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 188 | 99 | *Bacteroidetes* |
| 254-C-EMDW$_{Aeg}$-22 | AM411357 | 360 | *Flavobacteriaceae* bacterium 'BSE RB 01' | AY259513 | 357 | 99 | *Bacteroidetes* |
| 254-C-EMDW$_{Aeg}$-23 | AM411353 | 386 | Uncultured bacterium clone FS266-38B-03 | DQ513080 | 386 | 100 | *Bacteroidetes* |

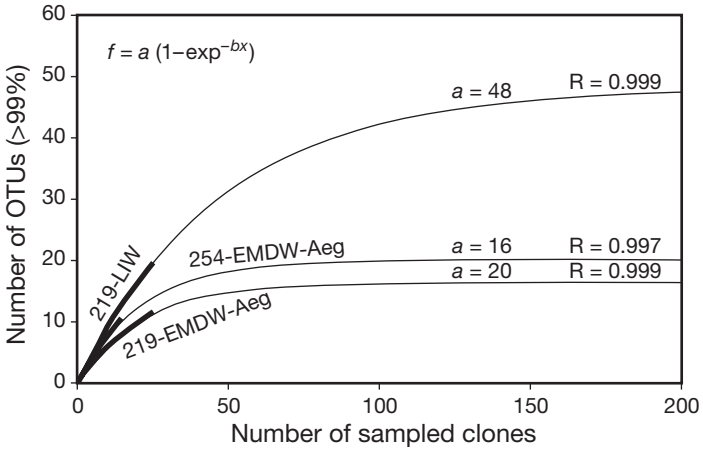


Fig. 5. Rarefaction/regression analysis based on data from 16S rDNA environmental clones; bold portion of regression lines indicates retrieved proportion of total assumed *Bacteroidetes* diversity. *a*: assumed number of OTUs; R: correlation coefficient. Rarefaction equation used was $f = a\,(1 - \exp^{-bx})$. Abbreviations as in Fig. 3 key

water masses by the clone libraries (219-LIW: obtained 20, expected 48, R = 0.999; 219-$EMDW_{Aeg}$: obtained 12, expected 20, R = 0.999; 254-$EMDW_{Aeg}$: obtained 9, expected 16, R = 0.997).

Altogether, 3 sequence sub-clusters originating exclusively from the Mediterranean Sea and showing characteristic association with a specific water mass were resolved (LIW-1, LIW-2, $EMDW_{Aeg}$-2, Fig. 6) within the AGG58 cluster. In Sub-clusters LIW-1 and -2 only sequences from LIW were present. Sub-cluster $EMDW_{Aeg}$-1 contained sequences from Stn 219 (2300 m) together with some reference sequences from the Columbia River Estuary, the English Channel and the Pacific Ocean. In contrast, Sub-cluster $EMDW_{Aeg}$-2 contained exclusively environmental clone sequences from $EMDW_{Aeg}$ of Stns 219 and 254.

## DISCUSSION

In recent years, the phylum *Bacteroidetes* has been shown to be of major importance in the marine realm, especially for carbon conversion. Consequently, it is considered to be one of the most abundant bacterial phyla in seawater. The oligotrophic character of the Eastern Mediterranean Sea and its well known water mass distribution offer possibly unique conditions for bacteria of this phylum, and analysis of the *Bacteroidetes* community in these waters is therefore of special interest. A molecular approach, providing detailed information on the microbial community structure, was performed in the present study in order to assess diversity and distribution of *Bacteroidetes*. Studies using FISH for the detection of *Bacteroidetes* have demonstrated high abundances of this bacterial group in various marine environments (Glöckner et al. 1999, Abell & Bowman 2005a). Several studies further emphasized the under-representation of this bacterial lineage in PCR-based studies when general 16S rRNA gene-targeted eubacterial primers are used (Bowman et al. 1997, Pinhassi et al. 1997, Glöckner et al. 1999, Cottrell & Kirchman 2000, Eilers et al. 2000). After comparison of *Bacteroidetes* type strain sequences from GenBank, the newly designed primer Cyt-1020R was successfully applied as antisense primer for amplification of *Bacteroidetes* sequences from environmental samples. As shown by sequencing of DGGE bands and environmental clones, Cyt-1020R appears to be especially suitable for amplifying representatives of the yet uncultured AGG58 cluster within the new family *Cryomorphaceae* (Bowman et al. 2003). While this study was in progress, other oligonucleotides targeting the *Bacteroidetes* phylum have been described. In contrast to the present study, these specific sense primers were combined with general eubacterial antisense primers (O'Sullivan et al. 2002, Kirchman et al. 2003). All primer sets amplified a DNA fragment comprising approx. 1000 nt. Thus, both approaches, i.e. the combination of a general sense primer with a specific antisense primer (as in this study), and the combination of a specific sense primer with a general antisense primer as in the studies of O'Sullivan et al. (2002) and Kirchman et al. (2003) appear to be useful for specifically amplifying *Bacteroidetes* sequences from environmental samples.

Using the *Bacteroidetes*-specific primer combination 27F/Cyt-1020R, we obtained molecular evidence for AGG58 cluster representatives even in the oligotrophic Eastern Mediterranean Sea. Our data confirmed the results of O'Sullivan et al. (2004), who stated that the AGG58 cluster is ubiquitous.

DGGE analyses resulted in the detection of several characteristic bands for distinct water masses, and cluster analysis/ANOSIM of DGGE gels confirmed the observed depth-specific distribution of *Bacteroidetes* sequences. At Stn 295 in the Ionian Basin especially, samples from different water masses clearly fell into separate clusters. In contrast to Stns 219 in the Levantine Basin and Stn 254 in the Cretan Passage, Stn 295 is the only location unaffected by the EMT (B. Manca pers. comm.). Thus, in regard to water mass distribution, Stn 295 can be considered comparably stable. In contrast, at Stn 254 in the Cretan Passage, the 500 m sample (LIW) falls within the $EMDW_{Aeg}$ cluster, possibly due to substantial mixing processes caused by the EMT. The deep waters at this station were especially subject to substantial changes, and perhaps a stable *Bacteroidetes* community was not yet established in

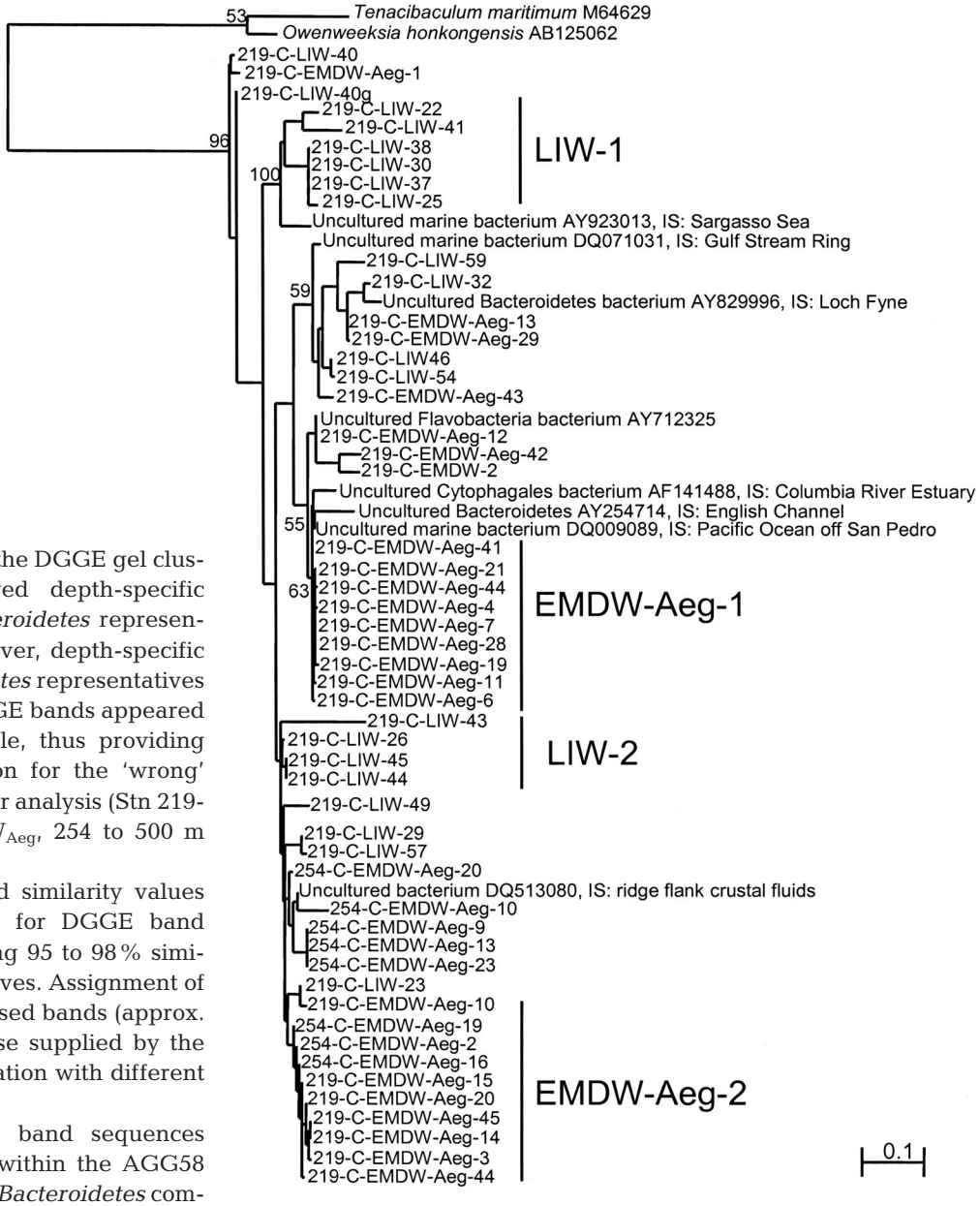


Fig. 6. Maximum likelihood 16S rDNA phylogenetic tree (PHYML, evolutionary model: GTR) based on 51 partial sequences (approx. 400 nt) of environmental clones from 'Mediterranean' cluster and their closest relatives. Numbers on nodes indicate bootstrap values (100 replicates). Vertical lines mark water mass specific sub-clusters. LIW-1 and -2: sequences from 219-LIW; EMDW-Aeg-1: sequences from 219-$EMDW_{Aeg}$ and reference sequences; EMDW-Aeg-2: sequences from 219-$EMDW_{Aeg}$ and 254-$EMDW_{Aeg}$. IS: Isolation source; other abbreviations as in Fig. 3 key

this water mass. In general, the DGGE gel cluster analysis clearly showed depth-specific distribution of certain *Bacteroidetes* representatives at all stations. However, depth-specific distribution of all *Bacteroidetes* representatives seems unlikely. Certain DGGE bands appeared throughout the depth profile, thus providing another possible explanation for the 'wrong' affiliation achieved by cluster analysis (Stn 219-10 m affiliated with $EMDW_{Aeg}$, 254 to 500 m affiliated with $EMDW_{Aeg}$).

A BLAST search revealed similarity values ranging from 87 to 100% for DGGE band sequences, with most sharing 95 to 98% similarity with their closest relatives. Assignment of 16S rDNA sequences of excised bands (approx. 190 nt) to the large database supplied by the ARB project permitted affiliation with different bacterial groups.

The majority of excised band sequences formed a separate lineage within the AGG58 cluster, indicating a specific *Bacteroidetes* community. Sequences from clone libraries confirmed the data obtained by DGGE band sequencing. Environmental clone sequences and DGGE band sequences were assigned to a separate branch within the AGG58 cluster, indicating that both separation techniques resolved a comparable range of sequence variation in the samples. The total diversity of *Bacteroidetes* can be assumed to be even higher than revealed by our experiments as shown by the results of rarefaction/regression analysis.

Similarity values for environmental clones ranged from 91 to 100%, with most environmental clones showing similarities of 96 to 98% to their closest relatives. With regard to the *Bacteroidetes* phylum, the similarity values were exceptionally high, but since the database is being constantly enlarged due to growing interest in this bacterial phylum, especially in the marine realm, such high values are to be expected.

The environmental clone sequences from the Eastern Mediterranean Sea were closely related to AGG58 Branch 2 clones from the study by O'Sullivan et al. (2004). Thus, AGG58 Branch 2 together with sequences from the Mediterranean Sea and sequences obtained from other marine or marine-related locations (e.g the Sargasso Sea, Arctic sea ice) may reflect *Bacteroidetes* representatives specialized or adapted to extremely oligotrophic marine waters. O'Sullivan et al. (2004) reported a ubiquitous distribution of

the AGG58 cluster but hypothesized that different members of the cluster were specialized for different ecological conditions, supporting this assumption.

The sequences from environmental clones also confirmed the hypothesis of a depth-specific distribution of certain *Bacteroidetes* representatives within the Eastern Mediterranean Sea. Environmental clone sequence Sub-clusters LIW-1 and -2 were specific for intermediate water layers (Stn 219), and Sub-cluster $EMDW_{Aeg}$-2 (Stn 219: Clones 3, 14, 20, 44, 45; Stn 254: Clones 2, 10, 16, 19) for deep waters (Fig. 6). To date, depth-specific distribution has been reported for several bacterial groups (e.g. green non-sulfur bacteria, SAR 11 cluster and *Deltaproteobacteria*), but to our knowledge, this study presents the first molecular evidence for depth-specific distribution of *Bacteroidetes* representatives.

Two major properties of the water column may be responsible for the observed depth-specific distribution: depth itself (increasing hydrostatic pressure) and the water mass boundaries, primarily defined through physical variations. The specific effects of the water mass properties and depth (hydrostatic pressure) upon bacterial community structure cannot be easily distinguished.

Although hydrostatic pressure is considered to be of relevance for the spatial distribution of bacteria within the water column (Lee & Fuhrman 1991, Gordon & Giovannoni 1996, Acinas et al. 1997, 1999, Field et al. 1997, Wright et al. 1997, Pinhassi et al. 2000), it can be assumed to have but minor influence on the bacterial community composition in surface and intermediate waters. Specific adaptation to high hydrostatic pressure may explain the high similarity of *Bacteroidetes* communities in deep waters but seems unlikely for intermediate waters.

Different characteristics of water masses may contribute to the different *Bacteroidetes* communities at different depths. Besides differences in temperature and salinity, one outstanding characteristic is the content of organic matter (DOC and POC). Seritti et al. (2003) found a direct correlation between DOC concentration and salinity in different water masses of the Ionian Sea. They observed higher DOC values in the newer water masses CIW and $EMDW_{Aeg}$ than in the older water masses LIW and $EMDW_{Adr}$. Furthermore, DOC values were correlated with microbial activities, as measured by oxygen consumption (Seritti et al. 2003). The major contribution of *Bacteroidetes* representatives to nutrient and carbon recycling (Suzuki et al. 2001, Kirchman et al. 2003) and different DOC concentrations in different water masses (Seritti et al. 2003) can be explained by the water-mass-specific distribution of *Bacteroidetes* sequences found in this study. In the Eastern Mediterranean Sea, production is mainly due to recycling of nutrients (Kress et al. 2003). Because of their special physiological capabilities, members of the *Bacteroidetes* would have an important function in these waters, despite the highly oligotrophic nature of the region. In the Eastern Mediterranean Sea, regenerated production mainly relies on microbial turnover (primary production limited by phosphorus; Krom et al. 1991, Malanotte-Rizzoli et al. 2003). In spring and early summer, degradation of organic material produced during the spring phytoplankton bloom may cause high abundances of *Bacteroidetes* in intermediate and deep water masses. Differing preferences with respect to the amount and composition of organic matter may be the reason for depth-specific distribution of these bacteria. The amount and quality of organic matter can be assumed to change with depth due to the ongoing degradation and conversion in upper layers. Because the amount of organic matter (at least in regard to DOC; Seritti et al. 2003) has also been observed to differ between water masses, different *Bacteroidetes* representatives (as possible producers of DOC) may develop in different water mass bodies.

Zaballos et al. (2006) proposed the presence of a unique prokaryotic community within the Mediterranean Sea, and data obtained during the present study for the *Bacteroidetes* confirmed this. *Bacteroidetes* sequences from DGGE bands as well as from environmental clones were positioned within the ubiquitous AGG58 cluster, but formed a coherent lineage within the cluster together with but a few reference sequences from other habitats (Fig. 4).

Although many bacterial taxa are known to be globally distributed, a specific distribution along physico–chemical gradients (e.g. water mass boundaries) were demonstrated for the *Bacteroidetes* phylum in the present study. Thus, the aerobic water column within the ocean may represent a heterogeneous habitat, specifically inhabited by different bacterial taxa.

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