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High phylogenetic diversity in a marine-snow-associated bacterial assemblage

Johannes Rath[1,*], Ke Ying Wu[2], Gerhard J. Herndl[3], Edward F. DeLong[4]

[1]Molecular Biology Laboratory, Institute of Zoology, University of Vienna, Althanstr. 14, A-1090 Vienna, Austria
[2]Department of Ecology, Evolution and Marine Biology, University of California, Santa Barbara, California 95616, USA
[3]Department of Biological Oceanography, Netherlands Institute for Sea Research (NIOZ), PO Box 59, 1790 AB Den Burg, Texel, The Netherlands
[4]Monterey Bay Aquarium Research Institute, PO Box 628, 7700 Sandholdt Rd, Moss Landing, California, USA

ABSTRACT: Large (>500 µm), suspended particles of organic and inorganic material, often referred to as 'marine snow', are considered important in the vertical transport and biogeochemical transformation of particulate organic carbon in the marine environment. Previous work has indicated that bacterial species inhabiting marine snow particles may differ greatly from those commonly found living free in the surrounding water column. To further characterize marine-snow-attached bacterial populations, we sampled marine snow by SCUBA diving during periods of intense macroaggregate formation in the northern Adriatic Sea in August 1991. Small subunit ribosomal DNA (rDNA) fragments of bacteria were amplified from extracted nucleic acids by the polymerase chain reaction (PCR). The diversity of the recovered rDNA clones was initially assessed by comparing restriction fragment length polymorphisms (RFLPs) of individual clones. Ninety-five bacterial clones examined yielded 90 different RFLP patterns, representing an estimated sampling coverage of only 5.3%. Sequence analysis of 39 randomly chosen clones was used to assess the general phylogenetic affiliation of individual clones. Bacterial phyla represented in the library included affiliates of the Planctomyces, the Gram-positive bacteria, the Cytophaga-Flavobacteria-Bacteroides (CFB) lineage, and the alpha-, gamma-, delta-, and epsilon-subdivisions of the Proteobacteria. The results suggest that bacterial colonization of suspended marine macroaggregates can result in diverse and complex assemblages, with specific phyla, such as the CFB, being commonly associated with marine particles. Futhermore, this particle-attached bacterial assemblage was similar to other bacterial assemblages found in marine sediments and terrestrial soils, with respect to the nature of the associated phylogenetic groups.

KEY WORDS: Diversity · Bacteria · Particles · Adriatic Sea

INTRODUCTION

The occurrence and ecological importance of macroscopic organic aggregates (marine snow) in the pelagic environment has been demonstrated in a variety of different coastal and open ocean environments. Marine snow has been found to be enriched in nutrients, trace metals, microbial biomass and production when compared to the surrounding water (Alldredge & Silver 1988, Caron et al. 1986, Herndl et al. 1993). However, biomass specific activities (e.g. ectoenzymatic activity per cell, specific bacterial production) in particle-attached bacterial communities are often similar to or sometimes lower than those estimated for free-living bacteria. Little is known about the specific bacterial species inhabiting such aggregates (Caron et al. 1986). Potential formation of methane (Karl & Tilbrook 1994), nitrogen fixation by cyanobacteria (Paerl & Pruffert 1987), sulfide production (Shanks & Reeder 1993), and the finding that organic particles may sometimes exhibit distinct oxygen and pH gradients (Alldredge & Cohen 1987, Paerl 1984) all suggest that particles may be suitable sites for anaerobic processes in an otherwise oxygenated water column (Sieburth 1991). In addition, comparative phylogenetic evidence based on small subunit ribosomal DNA (SSU rDNA) sequences (DeLong et al. 1993) and low molecular weight RNAs

*E-mail: johannes.rath@univie.ac.at

(Bidle & Fletcher 1995) of aggregate-associated and free-living bacteria indicated that particle-attached bacterial assemblages may differ in composition from those inhabiting the surrounding water column. These data also suggest potential differences in the metabolic capacities of particle-attached versus free-living marine bacterial assemblages. The above observations suggest that there are substantial microbiological and functional differences between particle-attached and free-living bacteria.

Intense formation of large marine snow particles, which occurred in the northern Adriatic Sea in summer 1991, has been described in a series of publications which addressed a variety of aspects on the chemical and biological properties and formation of marine snow in the Adriatic (Bochdansky & Herndl 1992a, b, Herndl 1992, Kaltenböck & Herndl 1992, Müller-Niklas et al. 1994, Rath & Herndl 1994). Here we describe the phylogenetic diversity of bacteria associated with marine snow as determined by cloning and sequencing of SSU rDNAs derived from the complex assemblage.

METHODS

Sample collection. Marine snow samples were collected in the Gulf of Trieste (northern Adriatic Sea) about 2 km off the Laboratorio di Biologia Marina, Aurisina (Italy), on 11 August 1991, during a period of intense marine snow formation. Samples were collected by SCUBA divers using 780 ml hand-made plexiglass syringes (rinsed with 0.1 N HCl and distilled water prior to sampling) at depths between 1 and 15 m. Aggregate size varied between 5 mm and several centimeters. On the boat, marine snow was transferred to a cooling box and held at 4°C. Within 1 h, the samples were centrifuged at 13000 × *g* for 20 min at 4°C. The resulting pellet was divided into 1 g aliquots (wet weight), transferred into Eppendorf vials and stored frozen at –80°C.

Nucleic acid extraction, purification and polymerase chain reaction (PCR) amplification. Extraction and purification of the nucleic acids were performed by gentle enzymatic and detergent lysis, phenol:chloroform extraction and CsCl equilibrium density gradient centrifugation as previously described (DeLong 1992, DeLong et al. 1993). Approximately 50 mg wet weight of marine snow was extracted. Nucleic acid concentrations were estimated by Hoechst dye binding conferred fluorescence (Hoefer TK), following the manufacturer's recommendations. Ribosomal DNA was amplified from purified DNA using GeneAMP Kit reagents (Perkin Elmer Cetus, Norwalk, CT, USA) as recommended by the manufacturer. Reaction mixtures (100 µl) contained 2 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 200 µM deoxynucleotide triphosphate, 0.5 units *Thermus aquaticus* DNA polymerase, 0.2 µM of each oligonucleotide primer and 100 ng DNA template. Thermal cycling was performed on a Perkin Elmer Thermal Cycler, with denaturation at 95°C for 1.5 min, annealing at 55°C for 1.5 min and extension at 72°C for 1.5 min for a total of 30 cycles.

The primers used for amplification of bacterial SSU rDNA were (Lane 1991):

27F: AGA GTT TGA TCC TGG CTC AG
1492R: GGT TAC CTT GTT ACG ACT T

Ribosomal DNA cloning, sequencing, and phylogenetic analysis. Amplified 16S SSU rDNA fragments from 3 independent reactions were pooled and sequentially extracted using equal volumes of phenol:chloroform (1:1) followed by a chloroform:isoamyl alcohol (24:1) extraction. The DNA products in the aqueous extract were ethanol precipitated and resuspended in 1:10 volume of sterile water. The purified amplification products were cloned using a commercial 'T-vector' cloning system (Invitrogen Corp, San Diego, CA, USA) following the manufacturer's recommendations. Plasmid minipreparation was performed by alkaline lysis (Sambrook et al. 1989). Insert-containing clones were identified by the size of the recombinant plasmid and by PCR amplification of the insert using M13F and M13R primers. Restriction fragment length polymorphisms (RFLPs) of the amplified inserts were used to identify unique rDNA clones. The PCR amplification conditions were identical to those described above, with the following exceptions: plasmid DNA was added to a final concentration of 1 ng μl^{-1}, annealing temperature was 40°C and a total of 40 cycles was performed. Restriction digests were performed following the manufacturer's recommendations using *Hae*III (BRL, Gaithersberg, MD, USA). Fragments were separated by agarose gel electrophoresis using a 3% (w/v) wide range agarose (Sigma, St. Louis, USA).

Sequencing was performed using a commercially available sequencing kit and Sequenase 2.0 (USB, Cleveland, OH, USA). Universal rDNA sequencing primers (Lane 1991) and M13 forward and M13 reverse primers were used. Sequences were aligned in the mutilple sequence editor, Genetic Data Environment (GDE 2.2, Smith et al. 1994), and the SSU rDNA database available from the ribosomal database project (RDP, Maidak et al. 1994).

Phylogenetic distance analyses were performed in GDE using the algorithm of DeSoete (1983). Similarity searches were performed using the program 'SIMILARITY_RANK' on the RDP server (http://rdpgopher.life.uiuc.edu) (Maidak et al. 1994). Full length sequences were also screened for potential chimeras

using 'Check_Chimera' (Maidak et al. 1994). Sequences have been submitted to GenBank and were assigned the following accession numbers:
AF030782, ADRIATIC15; AF030783, ADRIATIC79; AF030784, ADRIATIC84; AF030785, ADRIATIC87; AF030786, ADRIATIC83; AF030787, ADRIATIC80; AF030788, ADRIATIC74; AF030790, ADRIATIC92; AF030791, ADRIATIC97; AF030792, ADRIATIC95; AF030793, ADRIATIC64; AF030794, ADRIATICR19; AF030796, ADRIATIC6; AF030797, ADRIATIC54; AF030798, ADRIATIC31; AF030799, ADRIATIC69; AF030800, ADRIATIC86; AF030801, ADRIATIC81; AF030802, ADRIATIC77; AF030803, ADRIATIC100; AF030804, ADRIATIC53; AF030805, ADRIATIC60; AF030806, ADRIATIC72; AF030807, ADRIATIC99, AF030808, ADRIATIC89; AF030809, ADRIATICR24; AF030810, ADRIATIC52; AF030811, ADRIATIC61; AF030812, ADRIATIC76; AF030813, ADRIATIC90; AF030814, ADRIATICR27; AF030815, ADRIATICR16; AF030816, ADRIATIC33; AF030817, ADRIATICR20; AF030818, ADRIATIC7; AF030819, ADRIATICR25; AF030820, ADRIATIC65; AF030821, ADRIATIC50; AF030822, ADRIATIC91.

Phylogenetic relationships were inferred using the suite of programs available in PHYLIP (version 3.5; Felsenstein 1989) on a Sun Sparc 10 workstation. Bootstrapped data sets were generated using 'seqboot', and evolutionary distances calculated from the resulting data sets with 'DNAdist', using the Kimura 2 parameter model, assuming a transition/transversion ratio of 2.0. Neighbor joining analyses on the evolutionary distance data sets were performed using 'neighbor', and the results of 100 bootstrap replications compiled using 'consensus'. Phylogenetic trees were graphically visualized using 'treetool', which was obtained by anonymous ftp from the RDP (Maidak et al. 1994).

RESULTS

To initially assess the diversity of recovered rDNA clones, RFLPs in reamplified SSU rDNA fragments were compared (DeLong et al. 1993, Moyer et al. 1996). Various restriction enzymes (*Hae*III, *Eco*RI, *Pst*I) were tested. As expected, enzymes with 6 bp recognition sequences revealed only a few differences between clones. Restriction enzymes with 4 bp recognition sequences were therefore used to roughly assess clonal diversity. Ninety-five clones of the bacterial rDNA library were characterized by RFLP analysis using *Hae*III, which cleaves the tetrameric recognition sequence GGCC. The fragment patterns of individual clones were distinct and readily dinstinguishable. Of the 95 characterized clones, 90 different distinguishable RFLP types were identified. One RFLP type was represented 3 times, and 3 patterns were identified twice in the library. Estimated percent coverage, defined as $[1-(n/N)] \times 100$, where n is the number of unique clones detected in a subsample of size N, approximates the probability that *all* species present in a given sample are represented at least once in the subsample (Good 1953, Mullins et al. 1995). RFLP analysis of the 95 clones in the Adriatic Sea marine snow library indicated that the coverage was approximately 5.3%.

Partial sequences (approximately 200 bp, positions 240 to 440, *E. coli* numbering) were obtained for 41 randomly chosen clones to initially determine the approximate phylogenetic affiliation of individual clones. Initially, the SIMILARITY-RANK option from the RDP was used to identify the most similar sequences available in the unaligned database. Two clones with identical *Hae*III restriction fragment patterns were analyzed to assess their sequence similarity. Clones ADRIATIC77 and ADRIATIC100, which have identical *Hae*III restriction patterns, also had identical rDNA sequences over the 200 bp region analyzed. Sequence data derived from clones containing unique *Hae*III restriction patterns indicated that the rDNA clones analyzed were derived from a wide spectrum of phylogenetically diverse Bacteria. Although high resolution phylogenetic analyses are best perfomed with full length rDNA sequences, approximate phylogenetic placement is possible (and analytically advantageous for screening large numbers of clones) using shorter regions of rDNA sequence (Schmidt et al. 1991). Phylogenetic analyses of shorter sequence regions (Table 1, Figs. 1 & 2a, b) revealed that δ-Proteobacteria (23.1%,

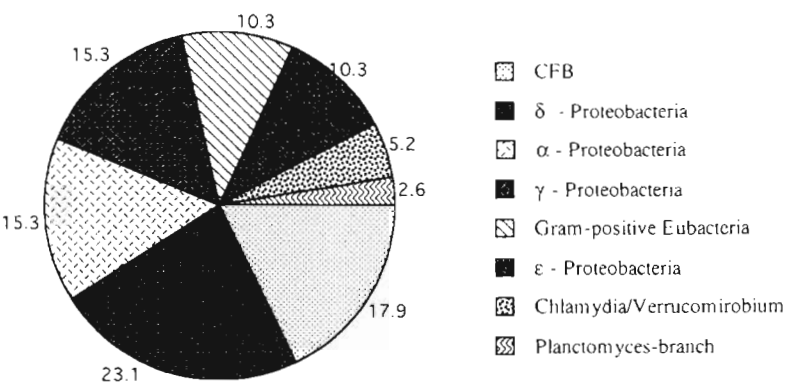


Fig. 1. Recovery of Adriatic Sea macroaggregate-associated bacterial ribosomal DNA clones belonging to different phylogenetic groups. The relative percentage of clones affiliated with each group, out of a total of 39 clones sequenced, is indicated

n = 9) represented the predominant group in the Adriatic marine snow rDNA library, followed by the Cytophaga-Flavobacteria-Bacteroides (CFB) branch (17.9%, n = 7). All subdivisions of the Proteobacteria, except for the β-Proteobacteria subdivision, were represented in the aggregate-attached bacterial rDNA library (Table 1, Figs. 2b & 3). One Adriatic rDNA clone out of 39 sequenced was affiliated with Planctomyces and close relatives (Figs. 2a & 3). The other clones were affiliated with the Verrucomicrobium branch, or the Gram-positive bacteria (Figs. 1 & 2, Table 1).

To substantiate preliminary phylogenetic placement based on partial sequence analysis, 10 clones were selected for more extensive analysis. Analyses of nearly complete rDNA sequences (Fig. 3) generally confirmed the phylogenetic placement within the various bacterial phyla inferred from partial sequences (Figs. 2 & 3). Analyses of nearly complete sequences also verified a close affiliation of the rDNA fragments of clones ADRIATIC76 and ADRIATICR16 with marine sulfate-reducing bacteria (SRB) of the δ-Proteobacterial subdivision (Devereux & Mundfrom 1994), the highest similarity being shared between these 2 SRB-like sequences and *Desulforhopalus vaculolatus*. The close phylogenetic relationship of cloned rDNA fragment ADRIATIC87 to SSU rDNA from Gram-positive bacteria, and the high similarity of ADRIATIC74 to SSU rDNA from members of the Planctomycetales, was also supported in full length sequence analyses (Fig. 3).

DISCUSSION

PCR-based studies of phylogenetic diversity are subject to their own inherent errors, biases and artifacts, as is any methodology applied to study complex environments or diverse microbial consortia (Liesack et al. 1991, Reysenbach et al. 1992, Amann et al. 1995, Suzuki & Giovannoni 1996, Wang & Wang 1996). Biases and artifacts include the potential creation of chimeric molecules during amplification (Liesack et al. 1991, Robinson-Cox et al. 1995, Wang & Wang 1996), overrepresenation of specific groups as a function of increasing PCR cycle number (Suzuki & Giovannoni 1996), and underrepresentation due to potential primer mismatches. Such potential artifacts suggest caution in the interpretation of PCR-based data sets, particularly in the description of 'novel' taxa, or with regard to assumptions about the quantitative representation of specific taxa based on PCR-product recoveries. Despite these potential problems, the utility of the PCR for microbial diversity studies is still apparent. For instance, PCR-based analyses of oceanic picoplankton (Fuhrman et al. 1992, Giovannoni et al. 1990) have been supported in parallel studies using genomic shotgun clone libraries (not involving PCR biases) from similar assemblages (Schmidt et al. 1991). In addition, the independent recovery of near-identical rDNA sequences (98 to 99% similarity) from as-yet uncultivated bacterial (Giovannoni et al. 1990, Schmidt et al.

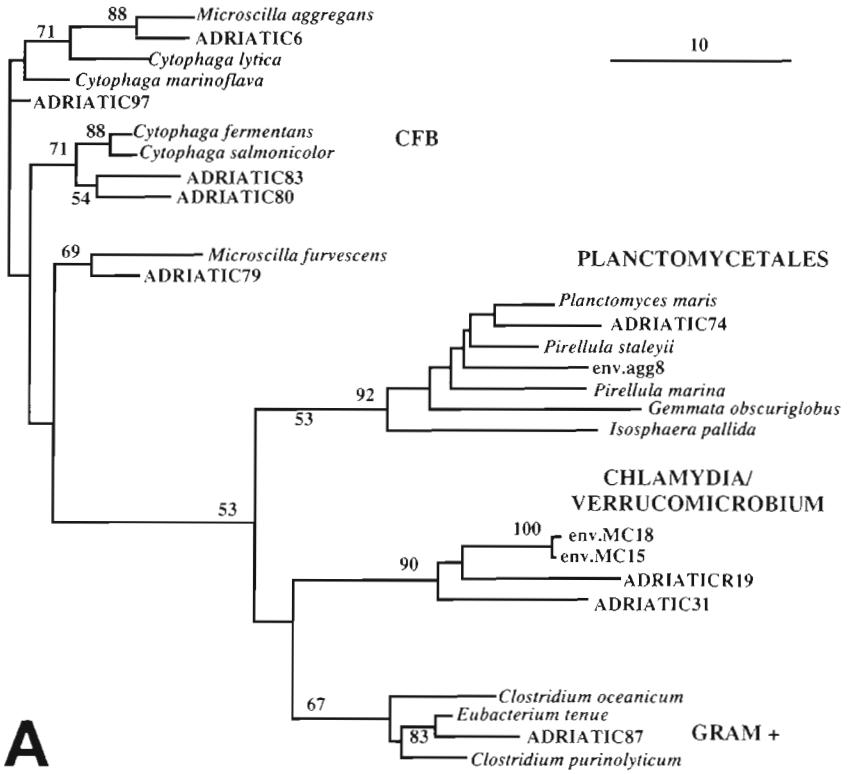
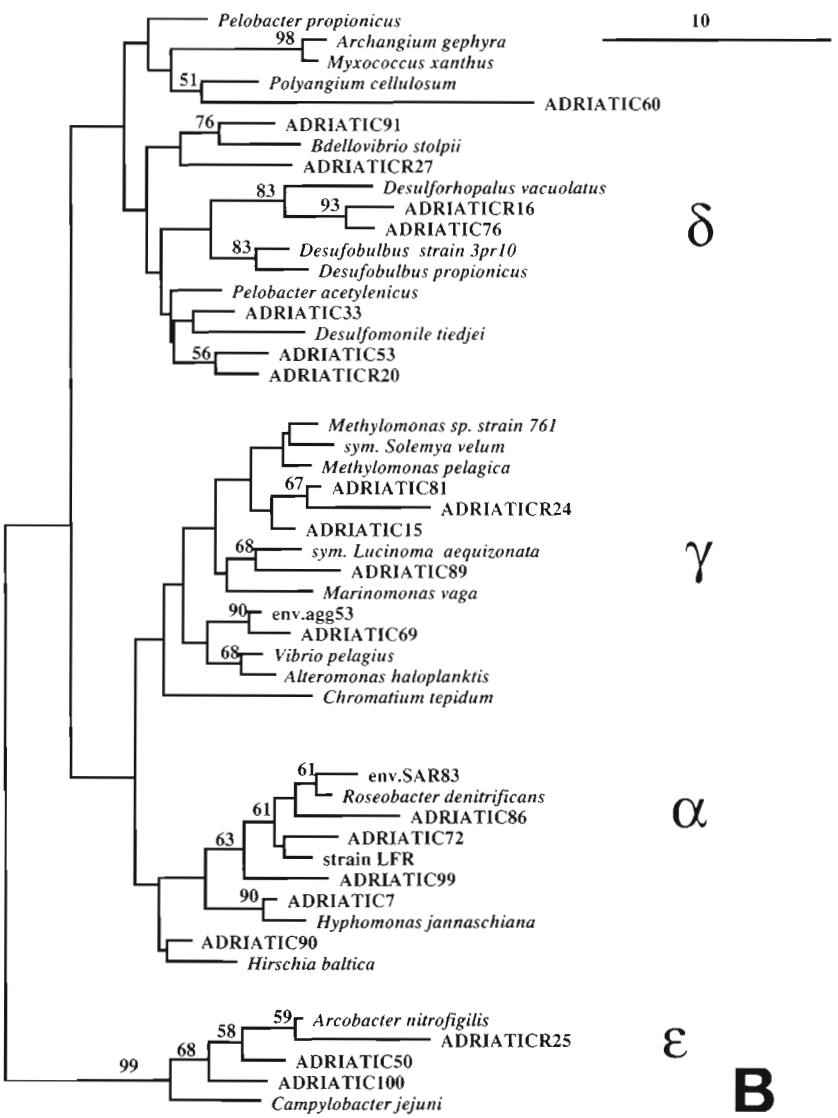


Fig. 2. Phylogenetic analysis of partial sequences (168 nucleotide positions) of small subunit ribosomal DNA (SSU rDNA) amplified and cloned from macroaggregate-associated bacteria from the Adriatic Sea (ADRIATIC) affiliated (A) with the CFB, Gram-positive, Verrucomicrobium and Planctomyces groups and (B) (facing page) with the Proteobacteria. The trees were inferred by neighbor joining analyses using PHYLIP (version 3.5; Felsenstein 1989). Distances were calculated with 'DNAdist' using the Kimura 2 parameter model assuming a transistion/transversion ratio of 2.0. Neighbor joining analysis was conducted on 100 bootstrap replicates with random taxon addition. Values represent the percentage of bootstrap replicates greater than 50% which support the associated node in the majority rule consensus tree. Scale bar represents the number of fixed mutations per 100 nucleotide residues. *Thermosipho africanus*, *Fervidobacterium islandicum* and *Geotoga subterranea* were used as outgroups. Scale bar represents the number of fixed mutations per 100 nucleotide residues

1991, DeLong et al. 1993, Fuhrman et al. 1993, Mullins et al. 1995) and archaeal (DeLong 1992, Fuhrman et al. 1992, DeLong et al. 1994) phylotypes indicates that many of the novel sequences recovered by these techniques are genuine and not the result of amplification artifacts. Indeed, rDNA sequences reported for newly cultivated marine oxygenic photoautotrophs (*Prochlorococcus marinus*) were highly similar to those described in prior mixed population PCR analyses (Schmidt et al. 1991, Urbach et al. 1992, Mullins et al. 1995). Additionally, phylogenetic hypotheses which originated from analysis of PCR amplified SSU rDNA have been subsequently supported by analyses of highly conserved, rDNA-linked protein encoding genes identified by 'chromosome walking' (Stein et al. 1996).

The formation of marine snow in the northern Adriatic Sea has been observed and recorded for over a century (Degobbis et al. 1995). A massive formation in 1991 was investigated to study the course of various microbial, nutritional and chemical characteristics of the aggregates and the aggregate-attached communities (Bochdansky & Herndl 1992, Kaltenböck & Herndl 1992, Herndl et al. 1993, Müller-Niklas et al. 1994, Rath & Herndl 1994). 'Aged' stages of marine snow which we used in this study have been described as 'gel-like aggregates' (Müller-Niklas et al. 1994) or 'clouds' (Kaltenböck & Herndl 1992). These aggregates accumulated at the pycnocline and are characterized by a massive increase in volume-specific dry mass. Also typical for these particles is the enrichment of high molecular weight organic material and an enormous enrichment in the bacterial biomass (Müller-Niklas et al. 1994). In contrast low molecular weight dissolved organic matter (i.e. free amino acids) was not found to be enriched in these aggregates.



As with marine snow samples collected in the Santa Barbara Channel (CA, USA; DeLong et al. 1993), clones affiliated with the γ-Proteobacteria, CFB, and Planctomyces groups were detected in the Adriatic marine snow bacterial rDNA library (Table 1, Figs. 1–3). Additionally, several SSU rDNA fragments recovered in Adriatic Sea marine snow were highly similar to SSU rDNA sequences from bacteria with known surface-associated life histories (e.g. ADRIATIC7 with *Hyphomonas jannaschii*; ADRIATIC6, 54, 79, 80, 83, 92, and 97 with members of the Cytophaga-Flavobacteria group). In contrast to marine snow samples from other studies, however, the phylogenetic diversity was much greater in the bacterial assemblages in Adriatic marine snow samples (DeLong et al. 1993, Fowler & DeLong in unpubl.). Some of the differences between the different marine snow samples may reflect differences in successional stage (age) of the marine snow and the types of algal species which initially form the aggregates' structural and chemical matrices. The interior of the Adriatic marine snow was highly anaerobic, apparently due to the high nutrient levels and associated high metabolic (respiratory) activity, as well as the substantial diffusive barrier provided by the large gel-like organic matrix.

A nocturnal increase and diurnal decrease of sulfide observed at

Table 1 Nearest neighbors and inferred phylogenetic group of 39 partially sequenced clones. CFB: Cytophaga-Flavobacteria-Bacteroides. S_{ab}: similarity coefficient used by the RDP

Clone	Presumptive phylogenetic group	Nearest neighbor[a]	S_{ab} coefficient	% similarity
ADRIATIC6	CFB	*Microscilla aggregans* subsp. *catalatica*	0.530	89.5
ADRIATIC7	α-Proteobacteria	*Hyphomonas* sp. str. MHS3	0.767	97.0
ADRIATIC15	γ-Proteobacteria	Symbiont of *Solemya*	0.809	88.3
ADRIATIC31	Chlamydia/Verrucomicrobium	*Verrumicrobium spinosum*	0.464	84.7
ADRIATIC33	δ-Proteobacteria	*Desulfosarcina variabilis 2*	0.540	83.9
ADRIATIC50	ε-Proteobacteria	*Arcobacter* spp. 1	0.654	90.4
ADRIATIC52	α-Proteobacteria	*Paracoccus alkaliphilus*	0.642	90.2
ADRIATIC53	δ-Proteobacteria	*Bdellovibrio stolpii* str. UKi2	0.649	81.3
ADRIATIC54	CFB	*Myroides odoratus*	0.729	91.3
ADRIATIC60	δ-Proteobacteria	*Polyangium cellulosum*	0.461	83.7
ADRIATIC61	γ-Proteobacteria	*Vibrio nigripulchrituda*	0.543	87.5
ADRIATIC64	Gram-positive Eubacteria	*Propionibacterium acnes*	0.834	91.9
ADRIATIC65	δ-Proteobacteria	*Desulfuromonas palmitatus*	0.553	84.1
ADRIATIC69	γ-Proteobacteria	*Alteromonas macleodii* 2	0.751	95.1
ADRIATIC72	α-Proteobacteria	DMSP degrading bacterium str. LFR	0.800	95.6
ADRIATIC74	Planctomyces branch	*Pirellula* sp. str. Schlesner 1	0.731	90.3
ADRIATIC76	δ-Proteobacteria	*Desulforhopalus vacuolatus*	0.754	95.2
ADRIATIC77	ε-Proteobacteria	*Arcobacter nitrofigilis*	0.640	93.0
ADRIATIC79	CFB	*Microscilla furvescens* str. TV-2	0.632	91.0
ADRIATIC80	CFB	*Porphyromonas endodontalis*	0.678	90.3
ADRIATIC81	γ-Proteobacteria	*Methylomonas* sp. str. 761	0.698	90.9
ADRIATIC83	CFB	*Cytophaga fermentans*	0.580	90.4
ADRIATIC84	Gram-positive Eubacteria	*Heliobacterium chlorum* 2	0.417	71.9
ADRIATIC86	α-Proteobacteria	*Roseobacter litoralis*	0.698	94.9
ADRIATIC87	Gram-positive Eubacteria	*Eubacterium tenue*	0.613	90.0
ADRIATIC89	γ-Proteobacteria	Symbiont of *Lucinoma aequizonata* gill	0.627	87.5
ADRIATIC90	α-Proteobacteria	*Bartonella quintana*	0.720	96.3
ADRIATIC91	δ-Protoebacteria	*Bdellovibrio stolpii* str. UKi2	0.460	85.2
ADRIATIC92	CFB	*Flexibacter filiformis* str. FXe1	0.478	86.8
ADRIATIC95	Gram-positive Eubacteria	*Heliobacterium chlorum* 2	0.367	79.1
ADRIATIC97	CFB	*Flavobacterium salegens*	0.763	91.2
ADRIATIC99	α-Proteobacteria	*Roseobacter algicola*	0.894	99.1
ADRIATIC100	ε-Proteobacteria	*Arcobacter nitrofigilis*	0.659	93.5
ADRIATICR16	δ-Proteobacteria	*Desulforhopalus vacuolatus*	0.749	93.4
ADRIATICR19	Chlamydia/Verrucomicrobium	*Verrumicrobium spinosum*	0.481	79.7
ADRIATICR20	δ-Proteobacteria	*Bdellovibrio stolpii* str. UKi2	0.668	88.6
ADRIATICR24	γ-Proteobacteria	*Methylomonas* sp. str. 761	0.707	86.3
ADRIATICR25	ε-Proteobacteria	*Arcobacter nitrofigilis*	0.554	89.9
ADRIATICR27	δ-Proteobacteria	*Syntrophus gentianae*	0.716	89.9

[a]The closest matching sequence from a cultivated and characterized strain was identified using SIMILARITY_RANK (Maidek et al. 1994). The analysis is based on approximately 200 bp, positions 240 to 440, *E. coli* numbering. (In some cases, higher similarities were found with environmental rDNA clones, or uncharacterized strains.) Unrestricted pairwise sequence similarities were calculated using the General Data Environment software package (Smith et al. 1994)

marine thermoclines (Cutter & Krahforst 1988) might indicate that a sulfidogenic bacterial consortium may be active in some marine snow habitats. Anaerobic processes on particles accumulating at the pycnocline—the 'transfer of benthic processes to the pycnocline'—was proposed by Sieburth (1991), and prompted the formulation of the 'false benthos' concept. Recently, sulfide production in marine snow has been directly observed in both laboratory-generated and field-collected particles (Shanks & Reeder 1993). The close phylogenetic affiliation of several marine-snow-associated bacterial rDNAs (Table 1, Fig 2) to other isolated or cloned phylotypes occurring in terrestrial soil (Borneman et al. 1996, Liesack & Stackebrandt 1992) or marine sediment communities (Devereux & Mundfrom 1994) supports the hypothesis that pelagic particles may, under suitable conditions, form a 'false benthos' sensu Sieburth (1991). In turn, sediment bacterial communties may be influenced by such particle-attached bacteria, since the sinking particles ultimately may give rise to the sediments themselves (Novitsky 1990).

Not suprisingly, our results differ from those of a recent study of freshwater 'lake snow' (Weiss et al. 1996), in which an apparent large proportion of attached bacteria was reported to be affiliated with the β-Proteobacteria. In contrast, no β-Proteobacteria-like SSU rDNA sequences were detected on Adriatic marine snow in this study, nor have they been detected in previous (DeLong et al. 1993) or ongoing surveys (Fowler & DeLong unpubl.) of marine macroaggregate-associated bacteria. The manifold differences existing between marine and freshwater systems may explain the differences in the microbial community. Trace nutrient composition and concentration, phytoplankton species from which the macroaggregates are derived, salinity, annual temperature variation, available terminal electron acceptors, specific carbon and energy sources, and associated zooplankton species are all expected to be quite different in marine systems. Moreover, the high ion concentration in marine systems facilitates cationic bridging of polysaccharide fibrils (Decho 1990), enhancing the formation of marine snow. Additionally, there are many well-documented examples of bacterial species which have specifically evolved in marine habitats and are not commonly found in freshwater habitats. Therefore, differences in the phylogenetic composition of bacterial assemblages associated with marine versus freshwater macroaggregates are not surprising.

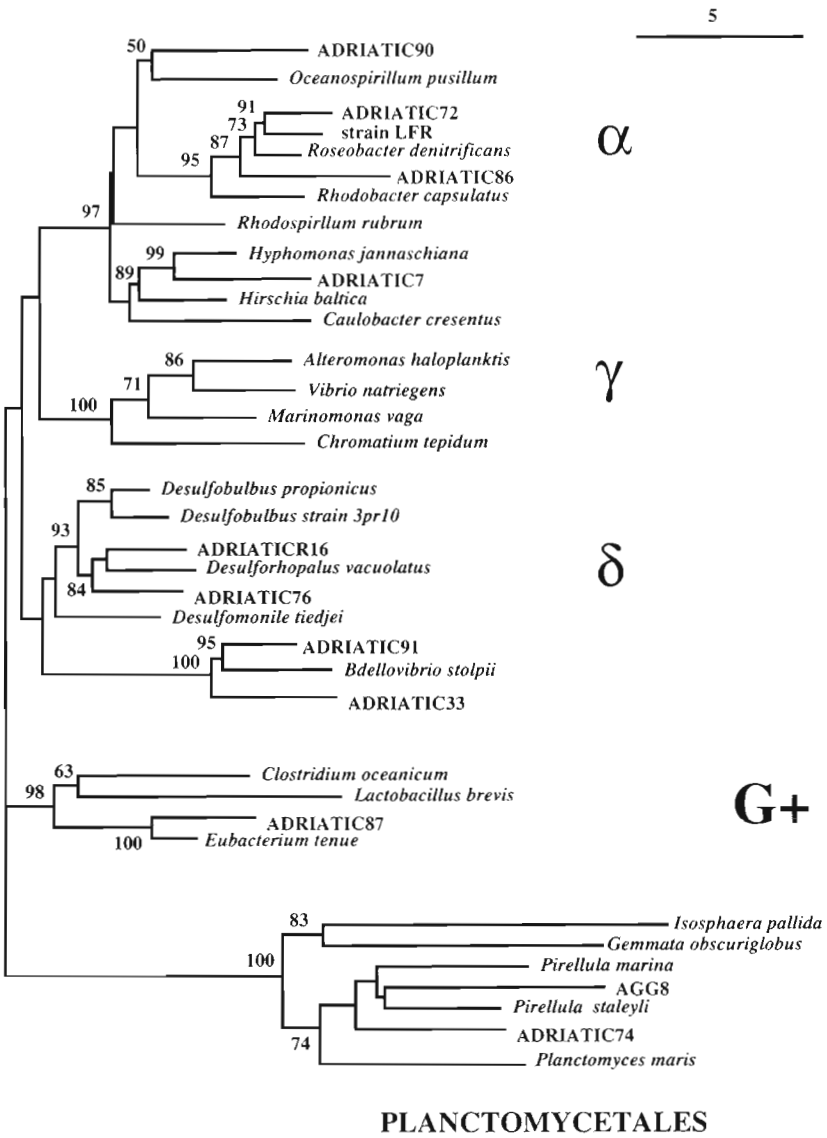


Fig. 3. Phylogenetic analysis of the nearly complete sequences of SSU rDNA amplified and cloned from macroaggregate-associated bacteria in the Adriatic Sea. The tree was inferred by neighbor joining analyses with PHYLIP (Felsenstein 1989) using 1051 nucleotide positions and neighbor joining boostrap analysis as described in Fig. 2. Values represent the precentage of bootstrap replicates greater than 50% which support the associated node in the majority rule consensus tree. Scale bar represents the number of fixed mutations per 100 nucleotide residues. *Thermosipho africanus*, *Fervidobacterium islandicum* and *Geotoga subterranea* were used as outgroups

It is likely that complex trophodynamics (e.g. syntrophic interactions) occur along the complex physical, chemical and biological gradients in marine snow (Alldredge & Silver 1988, Sieburth 1991, DeLong et al. 1993, Shanks & Reeder 1993). Steep redox-gradients may develop in the particles (Alldredge & Cohen 1987), and their role in habitat structuring has been discussed by Paerl (1984). Physical and chemical gradients, which are presumably favorable for the development of highly diverse microbial assemblages, may form in the complex matrix of marine snow. The phylogenetic diversity found in some other rDNA clone libraries, obtained from a variety of bacterial assemblages using similar procedures, appears somewhat lower than that observed in our Adriatic marine snow library, as judged by the greater coverage obtained for similar numbers of clones (Fuhrman et al. 1992, DeLong et al. 1993, Moyer et al. 1994, Höfle & Brettar 1995, Mullins et al. 1995). The low coverage (5.3%) represented in our library may reflect relatively high phylogenetic diversity which developed in this partic-

ular particle-attached bacterial community. Similarly high levels of bacterial diversity have also been observed or postulated for soil ecosystems (Torsvik et al. 1990, Borneman et al. 1996), habitats which represent similar spatially structured microhabitats with associated complex physical and chemical 'microgradients'

Assessment of the bulk activity of highly diverse and complex assemblages associated with marine snow may be extremely difficult, since commonly employed radiolabeling techniques (thymidine or leucine incorporation) may not be adequate to measure the particle-associated metabolic diversity. This may be relevant to our study, since the CFB lineage appears to be a frequently recovered group in marine, particle-attached assemblages, and various *Flavobacteria* isolates have been shown to be unable to incorporate thymidine (Davis 1989). Additionally, sulfate-reducing bacteria are not always capable of efficiently incorporating exogenous thymidine (Winding 1992). Diverse bacterial assemblages such as those revealed by our phylogenetic analysis are likely to exhibit markedly different substrate preferences and metabolic activities, and their non-uniform response to added radiotracers may result in underestimation of bacterial production in specific habitats such as sediments and marine snow (Painting et al. 1989).

The above discussion is based solely on diversity inferred from the analyses of SSU rDNA fragments recovered from subsamples of marine snow. Little is known, however, about associated variability of physiological or functional characteristics such as the diversity and substrate range of particle-associated extracellular hydrolytic isozymes (Rath & Herndl 1994). The results reported here are best viewed as a continuing survey of phylogenetic diversity in marine bacterial assemblages associated with marine macroaggregates. Some of our results were similar to those seen in a previous study of marine snow off the Californian coast, while other features of the Adriatic marine snow assemblage were remarkably different. Although there are strong indications that aggregate-attached communities differ significantly from free-living bacterial communities (DeLong et al. 1993, Bidle & Fletcher 1995), there is currently no information available on the composition of the free-living bacteria in the investigated area and the co-occurrence of bacteria on aggregates in the free-living community can therefore not be ruled out. Nevertheless, surveys of the bacterial inhabitants found on diverse marine macroaggregates provide some of the necessary information for correlating patterns of the bacterial community structure and diversity with the natural history, *in situ* metabolic activities, and syntrophic interactions of macroaggregate-associated microbes.

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