A survey on bacteria inhabiting the sea surface microlayer of coastal ecosystems

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Summary

Bacterial populations inhabiting the sea surface microlayer from two contrasted Mediterranean coastal stations (polluted vs. oligotrophic) were examined by culturig and genetic fingerprinting methods and were compared with those of underlying waters (50 cm depth), for a period of two years. More than 30 samples were examined and 487 strains were isolated and screened. Proteobacteria were consistently more abundant in the collection from the pristine environment whereas Gram-positive bacteria (i.e., *Actinobacteria* and *Firmicutes*) were more abundant in the polluted site. Cythophaga-Flavobacter–Bacteroides (CFB) ranged from 8% to 16% of total strains. Overall, 22.5% of the strains showed a 16S rRNA gene sequence similarity only at the genus level with previously reported bacterial species and around 10.5% of the strains showed similarities in 16S rRNA sequence below 93% with reported species. The CFB group contained the highest proportion of unknown species, but these also included *Alpha*- and *Gammaproteobacteria*. Such low similarity values showed that we were able to culture new marine genera and possibly new families, indicating that the sea-surface layer is a poorly understood microbial environment and may represent a natural source of new microorganisms. Genetic fingerprinting showed, however, no consistent differences between the predominant bacterial assemblages from surface microlayer and underlying waters, suggesting that the presence of a stable and abundant neustonic bacterial community is not a common trait of coastal marine environments.

Introduction

The marine air-water interface, located between the atmosphere and the hydrosphere, form a specific life environment in the ocean with peculiar and characteristic physico-chemical processes (Liss et Duce 1997). Through this interface a dynamic exchange of gases, water and organic and inorganic compounds occur. Dissolved substances, particles and microorganisms are deposited at this interface by simple diffusion, rising bubbles, convection and upwelling of underlying waters (UW). At the same time, the sea surface microlayer (SML) is a sink for atmospheric deposition (Garrett 1967, Norkrans 1980).

Microorganisms in the surface microlayer are submitted to a combination of both favourable and detrimental factors. Favourable factors are high concentrations of inorganic and organic nutrients, while detrimental factors are intense solar radiation in the ultraviolet and visible spectra, high concentrations of heavy metals and organic pollutants, temperature fluctuations and salinity changes. Therefore, the surface microlayer has often been considered as an extreme environment for microorganisms that may contain unusual species and taxa (Maki 2002). This fact led to the thinking that a specific near-surface complex assemblage of bacteria, the bacterioneuston, would have a wide distribution in the world ocean (Tsyban 1971). In addition, different reports suggest that microbial concentration exceeds that of underlying waters by orders of magnitude (Macintyre 1974, Hardy 1982), but despite their abundance and expected widespread distributions, bacterial communities thriving at the surface microlayer are poorly characterized (Norkrans 1980).

Isolation and cultivation techniques remain essential to understand the physiology and ecology of marine bacteria. Previous cultivation efforts on bacteria from the surface microlayer have collected strains belonging to Proteobacteria and Actinobacteria, including mostly misidentified genera such as *Pseudomonas*, *Bacterium*, *Chromobacterium*, *Aeromonas* and *Micrococcus* (Sieburth 1971, Tsyban 1971, Carty et Colwell 1975, Fehon et Oliver 1979),

which have also been found in these early studies in various marine water samples (Zobell 1946, Sieburth 1968, Fehon et Oliver 1979). Evidence of unusual neustonic bacterial genera inhabiting the surface microlayer is therefore not available so far. This may be due to the well-known limitations for bacterial identification during the 1970s and 1980s and the fact that often less than 0.1% of total bacteria cells form colony on standard culture media (Amann *et al.* 1995, Pinhassi *et al.* 1997). The application of DNA-based techniques (Woese 1987) leading to the rapid identification of isolated strains makes it easier to survey the culturable bacteria inhabiting the surface microlayer. In addition, high-resolution biological and chemical techniques are now available to study the sea surface microlayer at the microscale, preserving its structure (Agogué *et al.* 2004 , Monzikoff *et al.* 2004).

The present study represents an effort to obtain a collection of bacterial strains from coastal surface seawater, and was undertaken within the European Union project AIRWIN. We focused on two coastal sites in the Mediterranean Sea, including an oligotrophic site (Bay of Banyuls-sur-Mer, France) and a heavily humanimpacted station (off Barcelona, Spain). Overall, 487 bacterial strains isolated on solid media were screened by RFLP (restriction fragment length polymorphism) and identified by 16S rRNA gene sequencing. Surface microlayer bacteria are good candidates for specific adaptations such as resistance to high solar radiation (Agogué *et al.* in press) or pharmacological applications. Moreover, a PCR fingerprinting method SSCP (single-stranded-conformational polymorphism) was used to quickly compare the general structure of the predominant bacterial assemblages from surface microlayer and underlying waters. In a series of companion papers, a comparison of several surface microlayer-sampling devices has been described (Agogué *et al.* 2004 , Monzikoff *et al.* 2004), the surface microlayer microbial food webs are presented (Joux *et al.* submitted) and the microbial heterotrophic activity is analyzed (Obernosterer *et al.* in press).

Materials and methods

Field sites and sample collection

Samples were collected at two coastal stations in the NW Mediterranean Sea: the Bay of Banyuls-sur-Mer (France) and off the Olympic Harbour in Barcelona (Spain). The Bay of Banyuls-sur-Mer is oligotrophic (Médernach *et al.* 2001, Grémare *et al.* 2003). The Barcelona site is moderately eutrophic and heavily impacted by the urban sewage sludge outfall (Valls *et al.* 1990, Bayona *et al.* 1991, Chalaux *et al.* 1994). Samples were collected early in the morning around 4-5 h during four field campaigns (March 2001, September 2001, March 2002 and June-July 2002).

The surface microlayer (SML) was collected with different types of devices as described in detail by Agogué et al. (Agogué *et al.* 2004). The different samplers differed in the thickness of the surface microlayer sampled and in the volume of SML water collected per unit of time (see details in Agogué *et al.* 2004). The bacterial strain collection was mostly obtained from both the metal screen and the glass plate samplers, but also from the nylon screen, the Harvey_ roller and after sampling with two types of membranes (Teflon and polycarbonate) (Hühnerfuss 1981a, b, Agogué *et al.* 2004). We did not detect differences in the bacterial species recovered from SML with the different samplers used. Samples from underlying waters (UW) were collected for direct comparison with each surface microlayer sample by submerging a polycarbonate bottle and opening it at a depth of 0.5 m.

Enumeration of total bacteria

Total heterotrophic bacteria were counted by flow cytométrie. Subsamples of 3 ml were fixed with formaldehyde (2% final concentration) in cryotubes and were left at room temperature for 10-15 min, frozen in liquid nitrogen and stored at –80°C until analysis. Bacterial cells were stained with a nucleic acid dye (SYBR-Green I; final concentration 0.01% [vol/vol] of the commercial solution; Molecular Probes) and stored for at least 15 min in the dark at room

temperature before counting. The samples were run through a flow cytometer (FACScan, Becton Dickinson) equipped with a 488 nm, 15 mW Argon laser. Stained cells were discriminated and counted according to their right angle light scatter and green fluorescence measured at 530/30 nm (Marie *et al.* 1997, 2000). Other biological components were also evaluated and have been presented elsewhere (Agogué *et al.* 2004, Joux *et al.* submitted).

Enumeration and isolation of culturable heterotrophic bacteria

Samples for culturable heterotrophic bacteria were processed within 4 h after sampling. Serial dilutions (1:10 and 1:100 using filter-sterilized seawater) were prepared and 100 µl of each dilution was plated on 3 replicate plates on Marine Agar 2216 (Difco). Colony forming units were counted after incubation at 25°C in the dark after 2 weeks. Previous tests with low nutrient media did not offer higher culturability for the surface microalyer samples. Colonies were selected, picked and purified according to differences in colour and shape. We assigned a triple code for each strain, for example the code 14III/A01/020 correspond to a strain number 20 isolated on March 14 (14III), the year 2001 (A01).

Molecular characterization of the strains

For DNA extraction, bacterial cells were lysed either by thermal shock (the colony was resuspended in 500 µl of MilliQ sterile water and heated for 3 min at 94°C) or by chemical treatment. For chemical treatment, bacterial colonies were diluted in 500 µl lysis buffer (EDTA 40 mM, Tris 50 mM, pH 8, 750 mM saccharose) and incubated with lysozyme (final concentration, 1 mg ml^{-1}) at 37°C for 45 min with gentle agitation. Sodium dodecyl sulfate (final concentration, 0.5% [w/v]) and proteinase K (final concentration 0.1 mg ml^{-1}) were added, and the samples were incubated at 55°C for 1 h. DNA was extracted with an equal volume of phenol– chloroform–isoamyl alcohol (25:24:1 [v/v/v]) and chloroform–isoamyl alcohol (24:1 [v/v]). DNA was precipitated with 2 vol of isopropanol and recovered

by centrifugation, washed with 70% cold ethanol (-20°C), air dried and diluted in 50 µl of sterile water. The 16S rRNA gene was amplified by PCR using the primer sets, SAdir (5'-AGAGTTTGATCATGGCTCAGA-3'; *Escherichia coli* 16S rRNA gene positions 8–27, forward) and S17 Rev (5'-GTTACCTTGTTACGACTT-3'; *E. coli* 16S rRNA gene positions 1491–1508, reverse) (numbering according to Brosius *et al*. (1978) at 48°C annealing temperature. RFLP (restriction fragment length polymorphism) pattern analysis was performed by digesting 16S rRNA PCR products with the restriction endonuclease *Hin*6I (Eurogentec) at 37°C over night, and run in 2% agarose gels. Complete 16S rRNA gene products representing unique RFLP patterns were sequenced (Genome Express). Nucleotide sequences have been deposited in GenBank database under Accession Nos. AY576689–AY576777 and AY612746–AY612779.2.5.

Phylogenetic analysis

About 1300 bp of the 16S rRNA gene were used in a BLAST search (Altschul *et al.* 1990), and the phylogenetic position of putative new ‘‘species’’ (i.e., strains with less than 97% similarity in the 16S rRNA gene as compared with already described species) was investigated. A multiple sequence file was produced, including the new sequences and their closest relatives given by the BLAST query (http://www.ncbi.nlm.nih.gov). Alignments were performed using the CLUSTAL W program (Thompson *et al.* 1994) and were then manually refined using SEAVIEW (Galtier *et al.* 1996). Phylogenetic trees were constructed by the neighbor-joining (Saitou et Nei 1987) and maximum-likelihood (Felsentein 1981) methods using the PHYLO-WIN software (Galtier *et al.* 1996), and graphic representation of the resulting trees was made using NJPLOT software (Perrière *et al.* 1996). Regions of ambiguous alignment were omitted from subsequent analyses. Evolutionary distance values were calculated using Kimura 2-state parameters and used in neighbor-joining analyses. Bootstrap values were determined according to Felsentein (1985)

SSCP fingerprinting

Natural assemblages from SML and UW were quickly compared by SSCP fingerprinting. High volume water samples (20-50 l) were collected and pooled together from the surface microlayer using the metal screen sampler previously washed with bleach, and were transferred to the laboratory within 1-3 h interval. There, a 1 l sample was filtered through 0.2 µm pore size polycarbonate filters using a vacuum pump and the filters were stored at -80°C. Filters were thawed and resuspended in 900 µl of lysis buffer and digested as reported above (Dumestre *et al.* 2002). DNA was then extracted with an equal volume of phenol–chloroform–isoamyl alcohol (25:24:1 [v/v/v]) and was precipitated by addition of one-tenth of the volume of 3 M sodium acetate and two volumes of cold isopropanol, followed by incubation for 3 h at -20°C (Casamayor *et al.* 2000). The DNA was recovered by centrifugation at 10,000g for 30 min and the pellet was washed with cold ethanol (70%), dried and resuspended in 20 µl of sterile MQ water. For PCR amplification, the bacterial primers W49 dir (*E. coli* position 331; 5'-ACG-GTC-CAGACT-CCT-ACG-GG-3') and W34 rev (*E. coli* position 533; 5'-TTA-CCG-CGG-CTG-CTG-GCA-C-3'; 5'-labelled with fluoresceine phosphoramidite TET, Applied Biosystems, Courtaboeuf, France) were used as reported (Servais *et al.* 2003). For SSCP fingerprinting analysis, PCR products were heat-denatured at 94 °C for 5 min and immediately placed on ice for at least 10 min. A fluorescently labeled internal size marker (Genescan-400 Rox, Applied Biosystems) was added. The size standard contained a different fluorophore and permitted, after computing correction, a reliable comparison of patterns from many samples. SSCP electrophoresis was carried out as previously described (Servais *et al.* 2003), using the ABI 310 Genetic Analyzer equipped for capillary electrophoresis (Applied Biosystems). Computer files were analyzed with the 310 Genescan Analysis software (Applied Biosystems).

Results

Total counts and percentage of culturability

Total averaged bacterial counts for surface microlayer and underlying waters at the Barcelona sampling point were $1.43x10^6$ and $1.30.10^6$ cells ml^{-1}, respectively (n = 10) (Table 1). At the Banyuls-sur-Mer site, total counts were $0.97x10^6$ and $0.93x10^6$ cells ml^{-1} for SML and UW, respectively (n = 13). Thus, higher cell concentrations were found in Barcelona than in Banyuls-sur-Mer and slightly higher, statistically non-significant numbers in the surface microlayer samples compared to the underlying water samples. Viable counts on marine agar show higher percentage culturability vs. total counts in the surface microlayer than in underlying waters (Table 1). In Barcelona waters, culturable bacteria represented 4.16% and 2.51%, respectively. In Banyuls-sur-Mer waters, the percentages were 1.58% and 0.34%, respectively. Therefore, the percentage of culturable bacteria was higher in Barcelona and in the surface microlayer at both sites.

Phylogenetic diversity of the bacterial strains

Overall, 487 isolates were isolated from both sites and both layers, yielding 124 different ribotypes after analysis of RFLP patterns. We did not detect differences in the bacterial species recovered from SML with the different samplers used. All the strains could be clustered in 5 phylogenetic bacterial groups (Table 2): Alpha- and gamma subdivisions of the Proteobacteria, CFB (Cytophaga–Flavobacter–Bacteroides), Actinobacteria (high G+C Gram-positive) and Firmicutes (low G+C Gram-positive). Proteobacteria were more abundant in the Banyuls-sur-Mer collection, whereas Gram-positive (i.e., Actinobacteria and Firmicutes) were more abundant in the Barcelona sampling point. The group of Gammaproteobacteria was the predominant cultured group in all the samples, except for the SML of Barcelona, where Actinobacteria were more abundant (Table 2).

Similarity values for the 16S rRNA gene sequences from the culture collection with previously reported species or clones in data bases are presented in Table 3. Strains with sequence similarities P97% were considered to be representatives of the same species, similarities between 93% and 97% were considered to be representatives of the same genus and sequence similarities <93% would represent differences above genus (Hagstrom *et al.* 2000). Overall, 22.5% of our isolates showed a 16S rRNA gene sequence similarity at the genus level with previously reported bacterial species and around 10.5% showed similarities below 93% (Table 3, considering both surface microlayer and underlying water strains). Therefore, we found consistent novelty at the genus level within our strain collection. The CFB group appeared to contain the highest proportion of unknown species (i.e., 7 out of 15 strains showed a sequence similarity less than 97% with cultured species), followed by *Alpha-* and *Gammaproteobacteria*.

We also compared the cultured strains in our collection with the closest environmental clone known from databases (Table 3). Obviously, the percentage of strains with sequence similarities >97% to already reported 16S rRNA gene sequences, was higher. Nevertheless, nine strains showed similarities below 97% and three other strains (two surface microlayer *Gammaproteobacteria* isolated in Barcelona and one underlying water *Gammaproteobacteria* isolated in Banyuls-sur-Mer) had similarities lower than 93% with known environmental clones.

Phylogenetic trees were constructed with those surface microlayer- and underlying water strains that were not closely related to already known species (<97% similarity in the 16S rRNA gene). Only two Actinobacteria strains were putative new species (the *Nocardioidaceae* 14III/A01/020 and the *Microbacteriaceae* 18III/A01/077) (Fig. 1). Conversely, seven strains related to CFB showed low similarities with previously described species (Fig. 2): five strains (14III/A01/006, 14III/A01/012, 18III/A01/061, 18III/A01/068 and 12IX/A01/169) belonged to *Flavobacteriaceae* and two (5IX/A01/134 and

13IX/A01/164) to *Flexibacteraceae*. Among these, four strains were isolated from the surface microlayer and three from underlying waters.

Finally, the *Alpha*- and *Gammaproteobacteria* were grouped on the same tree (Fig. 3). For *Gammaproteobacteria* (Table 3), we included 10 sequences out of 12 (two strains were very similar to the others). Most of *Gammaproteobacteria* (i.e., strains 14III/A01/015, 18III/A01/067, 26III/A02/220, 3IV/A02/222, 3X/A02/235, 17X/A02/237 and 17X/A02/240) clustered into the genera *Vibrio, Pseudoalteromonas*, *Pseudomonas* and *Alteromonas*. For *Alphaproteobacteria*, seven putative new species were found and most of them (except 3X/A02/232) belonged to Rhodobacteriales, mainly the genera *Roseobacter* and *Erythrobacter*.

Putative neustonic isolates

To explore whether some strains were specific to the air–seawater interface, we included in a table the 41 isolates that were exclusively isolated from the surface microlayer either of Barcelona or Banyuls-sur-Mer stations (Table 4). Initially, we considered these strains as potential neustonic bacteria. Most of the isolates showed ≥97 % similarity in the 16S rRNA gene, with either already known bacterial species (71% of the isolated strains) or environmental clones (95% of the strains). Environmental clones had diverse marine origins ranging from deep-sea sediments or hydrothermal vents to Arctic sea ice. However, we also found two potentially ‘‘neustonic’’ *Gammaproteobacteria* strains isolated from Barcelona (5IX/A01/131 and 14III/A01/031), which were poorly related to any previously described sequence (less than 93%). Most of the Gram-positive strains were closely related to environmental clones found in diverse ecosystems, mainly from soils and polluted areas. Therefore, only a few unusual bacterial taxa were collected from the surface microlayer isolates and most of the strains were closely related to bacteria of widespread distribution.

General structure of bacterial communities

To explore whether bacterial communities inhabiting both the surface microlayer and underlying waters were consistently different or not, we targeted the whole bacterial assemblage by SSCP genetic fingerprinting. We examined several vertical profiles from Banyuls-sur-Mer (n = 18) and Barcelona (n = 12) over two years. Overall, the number of SSCP peaks recovered from both surface microlayer and underlying waters was within the same range (18–24 predominant peaks). For all the samples collected in the Banyuls-sur-Mer station, we found very similar SSCP profiles for the surface microlayer and underlying waters (e.g., March 27, 2002 in Fig. 4). In contrast, differences in SSCP fingerprints between surface microlayer- and underlying water samples were often found at the Barcelona station (7 samples out of 12, Table 5). In those cases, only a few additional (2 or 3) peaks were found in the SML, but these peaks were highly significant, representing more than 30% of the total surface of the SSCP profile (e.g., September 7, 2001 in Fig. 4). Unfortunately, SSCP peaks cannot be directly recovered and used for 16S rRNA gene sequencing. Interestingly, such differences between surface microlayer and underlying waters profiles were not reproducible from one day to another. For instance, the Barcelona station was visited along three consecutive days during March 2001, September 2001, March 2002 and June 2002, and well-developed specific surface microlayer bacterial assemblages were only found in a few occasions (14 March 2001, 7 September 2001, 20 and 21 March 2002; Table 5). As an example, Fig. 4 shows the bacterial community structure found for two consecutive days (6 and 7 September 2001): on the first day, only one minor peak was specific for the surface microlayer, whereas in the following days, several major peaks appeared.

Discussion

Range of culturability

Bacterial culturability in oligotrophic to mesotrophic marine habitats generally range from 0.001% to 1%, whereas this percentage can increase in nutrient-rich environments (e.g., activated sludge) (Amann *et al.* 1995). The percentages reported in this study greatly varied (from 0.34% up to 14.5% of total cells counts in Barcelona), but the mean value was consistently lower at the oligotrophic site (Banyuls-sur-mer), in agreement with this general trend. In addition, at both stations culturability was higher for the surface microlayer. This may be easily explained by the accumulation of organic matter at this interface (POC enrichment: 1.8 [n = 5] and 3.2 [n = 7] for Banyuls-sur-Mer and Barcelona sites, respectively) (Joux *et al.* submitted). We tested different media at the beginning of our survey, including low-nutrient media such as R2A and using several samples from the surface microlayer. We observed that the largest diversity (in terms of conspicuous morphotypes and ribotypes after RFLP profiles) was consistently recovered with the Zobell medium. Other reports have shown biochemical and bacterial activities to increase in the surface microlayer when compared to underlying waters (Sieburth 1971, Carlucci *et al.* 1985, Mudryk *et al.* 1991). This would explain why bacterial species isolated from the surface microlayer represent a higher fraction of total bacteria than in underlying waters.

Taxonomic groups of culturable bacteria from both sites

The dominant bacterial groups found in the surface microlayer- and underlying water isolates collected at Banyuls-sur-Mer site were similar to those commonlyfound in seawater samples (Benlloch *et al.* 1995, Bowman *et al.* 1997, Pinhassi *et al.* 1997, Eilers *et al.* 2000, 2001). Around one half of the bacteria isolated from the Banyuls-sur-Mer site belonged to *Gammaproteobacteria,* and a significant proportion of the strains belonged to *Alphaproteobacteria* and CFB. *Gammaproteobacteria* represent a large phylogenetic group of

cosmopolitan species generally well-represented in culture collections (Bowman *et al.* 1997, Pinhassi *et al.* 1997, Suzuki *et al.* 1997, Eilers *et al.* 2000). The high proportion of *Gammaproteobacteria* in collections can be partly biased, since the isolation procedure may favour bacterial strains able to rapidly grow on nutrient-rich media. Similarly, the *Cytophaga–Flavobacteria* cluster is characterized by mainly aerobic species, especially effective at degrading complex polymers (Kirchman, 2002) and able to develop conspicuous colonies on solid media. The same types of *Gammaproteobacteria* and CFB were found in both surface microlayer and underlying waters, suggesting that culturable surface microlayer species are not different from underlying water species with the growth medium used, and were in agreement with SSCP fingerprinting profiles. Overall, the molecular fingerprints indicated that in most cases the same bacterial populations dominated surface microlayer and underlying waters. The fact that similar SSCP profiles were found suggests that, at least for predominant bacterial populations (above 1% of total bacteria), there was no evidence for unusual neustonic species that do not occur in underlying waters. This is congruent with the hypothesis that most bacteria in the surface microlayer are planktonic cells that accumulate from a passive flotation process, although these cells may potentially grow in the surface microlayer. We cannot discard, however, differences for minor populations that could escape detection limits of the SSCP fingerprinting. The Barcelona station seemed to behave quite different and the dominant cultured groups included species commonly found in wastewater and polluted coastal areas (Hugenholtz *et al.* 1998) in both surface microlayer and underlying waters. The Gram-positive species isolated at Barcelona are abundant in activated sludge (Kampfer *et al.* 1996, Snaidr *et al.* 1997) and were probably released from the sewage outfall. Interestingly, we found Actinobacteria, (i.e., the high G+C Gram-positive group) (Stackebrandt *et al.* 1997) to be more abundant in the surface microlayer than in underlying waters. Actinobacteria are known to degrade toxic compounds such as hydrocarbons, pesticides and heavy metals in soil and aquatic environments (Kashner *et al.* 1994, De

Schrijver et De Mot 1999, Gremion *et al.* 2003, Zhuang *et al.* 2003). Such compounds have been already found to accumulate in the surface microlayer at the Barcelona station (Valls *et al.* 1990, Bayona *et al.* 1991) and, therefore, these strains would probably be well-adapted to handle high concentrations of these toxic substances in the surface microlayer. Duration of the surface microlayer and mixing with water from the deeper layer seems to be similar for both stations. Romano and Garabetian (1996) showed, after an extensive study carried out in the north-western Mediterranean coast, that when the wind speed is below 4-5 m/s slicks are large and frequent, whereas when the wind increases slicks are smaller and rare. The slicks were maintained under natural conditions as far as the wind speed was low. Most of the days selected for sampling in our study had wind speed below this threshold. In Banyuls, most of the days wind speed was below 4 m/s, but we did not detect significant differences between surface microlayer- and underlying water bacterial assemblages. In Barcelona, we did detect differences, although wind data were in the same range than in Banyuls (see Table 5).

Phylogenetic richness of the culture collection

The phylogenetic analyses revealed high genetic diversity among the isolated bacteria, with 16S rRNA gene sequence similarities ranging from 88% to 100% to already known species (Table 3). Particularly, around 25% of the isolated strains showed sequence similarities lower than 97% to previously reported species, 10% of them having sequence similarities lower than 93%. Such low similarity values indicate that we were able to bring into culture new marine genera or even new families (Devereux *et al.* 1990). Most of these strains were CFB (i.e., 47%), whereas Proteobacteria generally showed higher similarity values to already known species. These observations agreed with others reports on the prevalence of novel marine bacteria among the culturable bacterial fraction (Bowman *et al.* 1997, Pinhassi *et al.* 1997, Suzuki *et al.* 1997, Hagstrom *et al.* 2000) that may have a great interest for future research. Although, we initially expected that these new culturable bacterial taxa might be the dominant

bacterial components in the unexplored surface microlayer habitat, these potential ‘‘new’’ bacterial genera were not specific for the surface microlayer. This reinforces the idea that the sea-surface layer from 0 to 100 cm is still a poorly known microbial environment that represents a natural source of new microorganisms.

Bacterioneuston specificity

Although some strains were only found in the surface microlayer, the 16S rRNA gene sequence analysis indicated that they were closely related to sequences retrieved from different marine environments and different water depths. In addition, the apparent higher occurrence of Actinobacteria in the surface microlayer at both stations should be interpreted with caution because of (i) the low number of strains obtained (especially in the case of Banyuls-sur-Mer, with only three isolates) and (ii) the influence of the treated-wastewater outfall in the Barcelona sampling station. Norkrans (1980) explained the dominance of *Bacillus* species in the surface microlayer by the fact that spore-forming species coming from the air are more resistant to desiccation and to solar radiation than the bubble-transported marine species. For Carty and Colwell (1975), bacteria belonging to *Bacillus* and *Micrococcus* genera were also found in abundance in air and may have a terrestrial origin. Although terrestrial origin cannot be excluded in coastal areas, these species cannot be considered as neustonic bacteria. From this field study and considering these two coastal sites, we hypothesize that most of the bacteria living in the sea surface microlayer are planktonic and may accumulate via a flotation process when they attach to particles and/or bubbles coming from the water column. A fraction of these bacteria may also accumulate via atmospheric deposition. As a consequence, viable cells collected from the surface microlayer may be those that have developed mechanisms to resist intense solar radiations and/or high concentrations of toxic compounds. We cannot disregard, however, the existence of resistance-forms in the surface microlayer, such as spores, that could grow under laboratory

conditions. From this field experiment we can suggests that specific neustonic bacterial species are scarce rather than abundant. However, the existence of specific neustonic populations at the marine surface microlayer cannot be excluded, as some adapted species have already been described in freshwater environments (Glöckner *et al.* 1998, Paddies *et al.* 2004). If this is the case for marine environments, they do not seem to be strongly enriched in the marine neuston of coastal areas.

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Table 1. Cell counts and bacterial culturability (in percentage vs. total cells count) in the sea surface microlayer (SML) sampled by the metal screen and underlying waters (UW) from Barcelona and Banyuls-sur-Mer sites, respectively

	Barcelona		Banyuls sur Mer	
	SML	UW	SML	UW
n	10	10	13	13
Total counts[a] (10^6 cells ml^{-1})	1,43 ± 0,43	1,30 ± 0,38	0,97 ± 0,16	0,93 ± 0,14
CFU / total counts (%)	4,16	2,51	1,58	0,34
% CFU range	0,3 - 14,5	0,001 - 7,6	0,1 - 7,3	0,05 - 1,42

[a] mean ± standard deviation
n number of samples

Table 2. Relative contribution (%) of taxonomic groups of culturable fraction of the sea surface microlayer (SML) and underlying waters (UW) bacterial communities from Banyuls-sur-Mer and Barcelona.

	Barcelona		Banyuls-sur-Mer	
	SML	UW	SML	UW
Alphaproteobacteria	19	18	19	24
Gammaproteobacteria	24	35	53	47
CFB group	8	12	13	16
Total Gram-negative	51	65	85	87
Actinobacteria	27	6	6	3
Firmicutes	22	29	9	10
Total Gram-positive	49	35	15	13

Table 3. Sequence similarity in the 16S rRNA gene of isolated strains to the closest matching relative in the data bank (the closest species on the left and the closest clone on the right).

Taxonomic group			n[c]	Most closest sp. [a] Known sp. ≥ 97 %	Most closest sp. [a] New putative sp. 93 % - 97 %	Most closest sp. [a] New putative gen. < 93 %	Most closest clone [b] Known sp. ≥ 97 %	Most closest clone [b] New putative sp. 93 % - 97 %	Most closest clone [b] New putative gen. < 93 %
Bayuls-sur-Mer site									
Gram -	Gamma	SML	17	14 (82%)	2 (12%)	1 (6%)	17	0	0
		UW	17	12 (71%)	2 (12%)	3 (18%)	16 (94%)	0	1 (6%)
	Alpha	SML	6	4 (67%)	1 (17%)	1 (17%)	6	0	0
		UW	10	9 (90%)	0	1 (10%)	9 (90%)	1 (10%)	0
	CFB	SML	4	1	2	1	4	0	0
		UW	6	5 (83%)	0	1 (17%)	5 (83%)	1 (17%)	0
Gram +	Actinobacteria	SML	2	2	0	0	2	0	0
		UW	1	0	1	0	0	1	0
	FLGC	SML	3	3	0	0	3	0	0
		UW	4	4	0	0	4	0	0
Barcelona site									
Gram -	Gamma	SML	9	5 (56%)	0	4 (44%)	7 (78%)	0	2 (22%)
		UW	6	6	0	0	6	0	0
	Alpha	SML	7	5 (71%)	2 (29%)	0	7	0	0
		UW	3	1	2		2	1	0
	CFB	SML	3	2	1	0	3	0	0
		UW	2	0	1	1	0	2	0
Gram +	Actinobacteria	SML	10	9 (90%)	1 (10%)	0	10	0	0
		UW	1	1	0	0	1	0	0
	FLGC	SML	8	8	0	0	8	0	0
		UW	5	5	0	0	5	0	0
All groups (for both sites)		SML	69	53 (77%)	9 (13%)	7 (10%)	67 (97%)	0	2 (3%)
		UW	55	43 (78%)	6 (11%)	6 (11%)	48 (87%)	6 (11%)	1 (2%)

Strains were arranged according to putative new genus (< 93 % sequence similarity in the 16S rRNA gene to database), putative new species within previously characterized genus (93% to 97%) and previously characterized species (> 97%)

[a] Sequence similarity values to previously reported species

[b] Sequence similarity values to previously reported environmental clones

[c] Number of isolates

Table 4. List of strains exclusively isolated from the SML in Banyuls-sur-Mer and Barcelona stations

Code	Acc. N°	Closest species (% homology)	Closest clone acc. N° (% homology)	Origin of closest clone
Gamma proteobacteria				
Banyuls - sur - Mer isolates				
3X/A02/235	AY576769	*Pseudomonas pseudoalcaligenes* (90%)	AF468388 (98%)	Arctic sea ice
17X/A02/240	AY576773	*Microbulbifer maritimus* (96%)	AF500211 (99%)	?
18III/A01/067	AY576718	*Pseudomonas anguilliseptica* (96%)	AJ293824 (98%)	Arctic seawater
18III/A01/066	AY576717	*Pseudomonas putida* (98%)	AJ297355 (98%)	freshwater fishfarm
27III/A02/219	AY576760	*Marinobacter litoralis* (99%)	AF479689 (99%)	East Sea in Korea
18III/A01/058	AY576713	*Pseudoalteromonas agarivorans* (99%)	AY028200 (99%)	Diatom detritus
17X/A02/242a	AY576775	*Alcanivorax venustensis* (100%)	AF328762 (100%)	Mediterranean Sea
Barcelona isolates				
5IX/A01/131	AY576729	*Marinobacter stanieri* (90%)	AF228694 (91%)	marine environment
14III/A01/031	AY576709	*Oleispira antarctica* (91%)	AF353237 (91%)	Arctic Ocean
14III/A01/015	AY612750	*Pseudoalteromonas agarivorans* (92%)	AY028198 (98%)	diatom detritus
7IX/A01/156	AY576745	*Alteromonas infernus* (98%)	AB015135 (99%)	deep-sea hydrothermal vent
6IX/A01/151	AY576743	*Enterobacter sakazakii* (99%)	AB004746 (99%)	clinical specimens
7IX/A01/137	AY576733	*Pseudoalteromonas mariniglutinosa* (99%)	AJ551143 (99%)	deep sea sediments
14III/A01/025	AY576705	*Pseudoxanthomonas broegbernensis* (99%)	AF273082 (99%)	diverse environments
Alpha proteobacteria				
Banyuls - sur - Mer isolates				
3X/A02/236	AY576770	*Ruegeria atlantica* (95%)	AJ391187 (97%)	Adriatic Sea
18III/A01/069	AY576720	*Paracoccus aminophilus* (98%)	D32239 (98%)	soil
18III/A01/070	AY576721	*Paracoccus marcusii* (99%)	AJ532681 (99%)	uranium mining waste piles
Barcelona isolates				
5IX/A01/143	AY576739	*Jannaschia helgolandensis* (95%)	AB018689 (99%)	marine environments
5IX/A01/132	AY615725	*Salipiger muscesens* (97%)	AY162092 (99%)	Sargasso Sea
14III/A01/023	AY576704	*Agrobacterium tumefaciens* (99%)	AY221181 (99%)	soil
5IX/A01/139	AY576735	*Sulfitobacter pontiacus* (99%)	AJ298355 (99%)	Mediterranean sea
Cytophaga/Flavobacterium/Bacteroides group				
Banyuls - sur - Mer isolates				
12IX/A01/169	AY576752	*Psychroflexus tropicus* (91%)	U85882 (97%)	Antarctic sea ice
18III/A01/068	AY576719	*Salegentibacter salegens* (95%)	AY319330 (99%)	Southern Ocean
18III/A01/061	AY576714	*Algibacter lectus* (96%)	AF359540 (97%)	dinoflagellates
Barcelona isolates				
14III/A01/010	AY576693	*Maribacter sedimenticola* (97%)	AF367849 (99%)	sea water
7IX/A01/155	AY576744	*Muricauda ruestringensis* (97%)	AY445076 (98%)	East Sea in Korea
Actinobacteria				
Banyuls - sur - Mer isolate				
17X/A02/245	AY576777	*Arthrobacter agilis* (99%)	X80748 (99%)	water, soil and human skin
Barcelona isolates				
14III/A01/020	AY576701	*Nocardioides jensenii* (96%)	AJ316318 (99%)	mural painting environments
14III/A01/014	AY576697	*Corynebacterium ammoniagenes* (97%)	X84440 (97%)	piggery waste
14III/A01/017	AY576699	*Brachybacterium arcticum* (99%)	AF468445 (100%)	Arctic sea ice
14III/A01/011	AY576694	*Microbacterium kitamiense* (99%)	AB042082 (99%)	plant-nematode
6IX/A01/148	AY576742	*Microbacterium esteraromaticum* (99%)	AB099658 (99%)	activated sludge
14III/A01/022	AY576703	*Micrococcus luteus* (99%)	AJ441006 (99%)	mats from Antarctic lakes
14III/A01/028	AY576707	*Arthrobacter nitroguajacolicus* (100%)	AJ512504 (100%)	forest soil
14III/A01/021	AY576702	*Dietzia maris* (100%)	Y18883 (100%)	soil, skin and intestine of carp
Firmicutes Low GC content				
Banyuls - sur - Mer isolate				
5IX/A01/142b	AY576738	*Paenibacillus glucanolyticus* (99%)	AB073189 (99%)	soil
13IX/A01/167	AY576750	*Planococcus rifietoensis* (99%)	AJ493659 (99%)	mat from a sulfurous spring
Barcelona isolates				

6IX/A01/147	AY612762	*Bacillus firmus* (99%)	AF348731 (99%)	selenium-contaminated hypersaline pond
9IX/A01/138	AY576734	*Staphylococcus pasteuri* (99%)	AJ276810 (99%)	biodeteriorated wall paintings
14III/A01/027	AY576706	*Staphylococcus aureus* (99%)	L37597 (99%)	human
5IX/A01/135	AY576731	*Bacillus cereus* (100%)	Y15466 (100%)	intestinal symbionts of animals

Table 5. Similarity values (Jaccard coefficient) for the absence/presence of data from the genetic fingerprintings carried out in the surface microlayer (SML) and underlying waters (UW) of the Barcelona coastal station

Site	Date	SML vs. UW	Wind speed (m/s)
Barcelona	March 13, 2001	1.00	5-8*
	March 14, 2001	0.58	2-4
	March 15, 2001	1.00	2-3
	September 5, 2001	1.00	4-6*
	September 6, 2001	0.90	2-4
	September 7, 2001	0.60	2-3
	March 19, 2002	1.00	<2
	March 20, 2002	0.77	<2
	March 21, 2002	0.60	<2
	June 25, 2002	1.00	<2
	June 26, 2002	0.90	<2
	June 27, 2002	0.90	<2

No significant differences were detected for Banyuls-sur-Mer coastal station (see Results).
* Sampling was carried out inside the sheltered harbour.

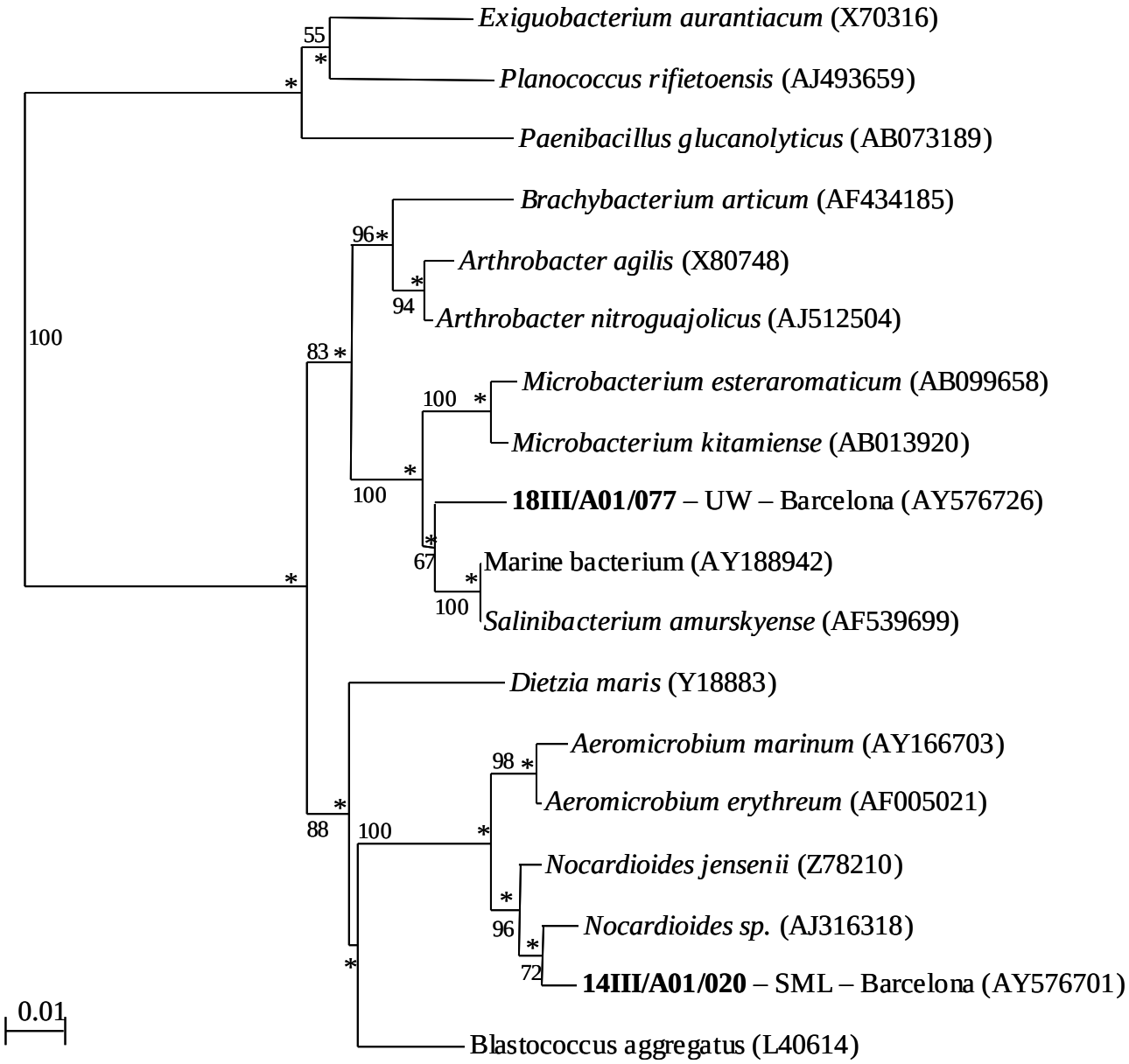

Fig. 1. Phylogenetic tree constructed using almost complete 16S rDNA gene sequences of isolated strains and related species of Gram-positive bacteria. The tree was built with the neighbor-joining method by using the Kimura distance. Values at nodes indicate bootstrap percentages for 500 replicates. Values less than 50 % are not reported. The scale bar indicate Kimura distance of 0.01. Asterisk (*) indicates branches that were also found using the Maximum Likelihood method.

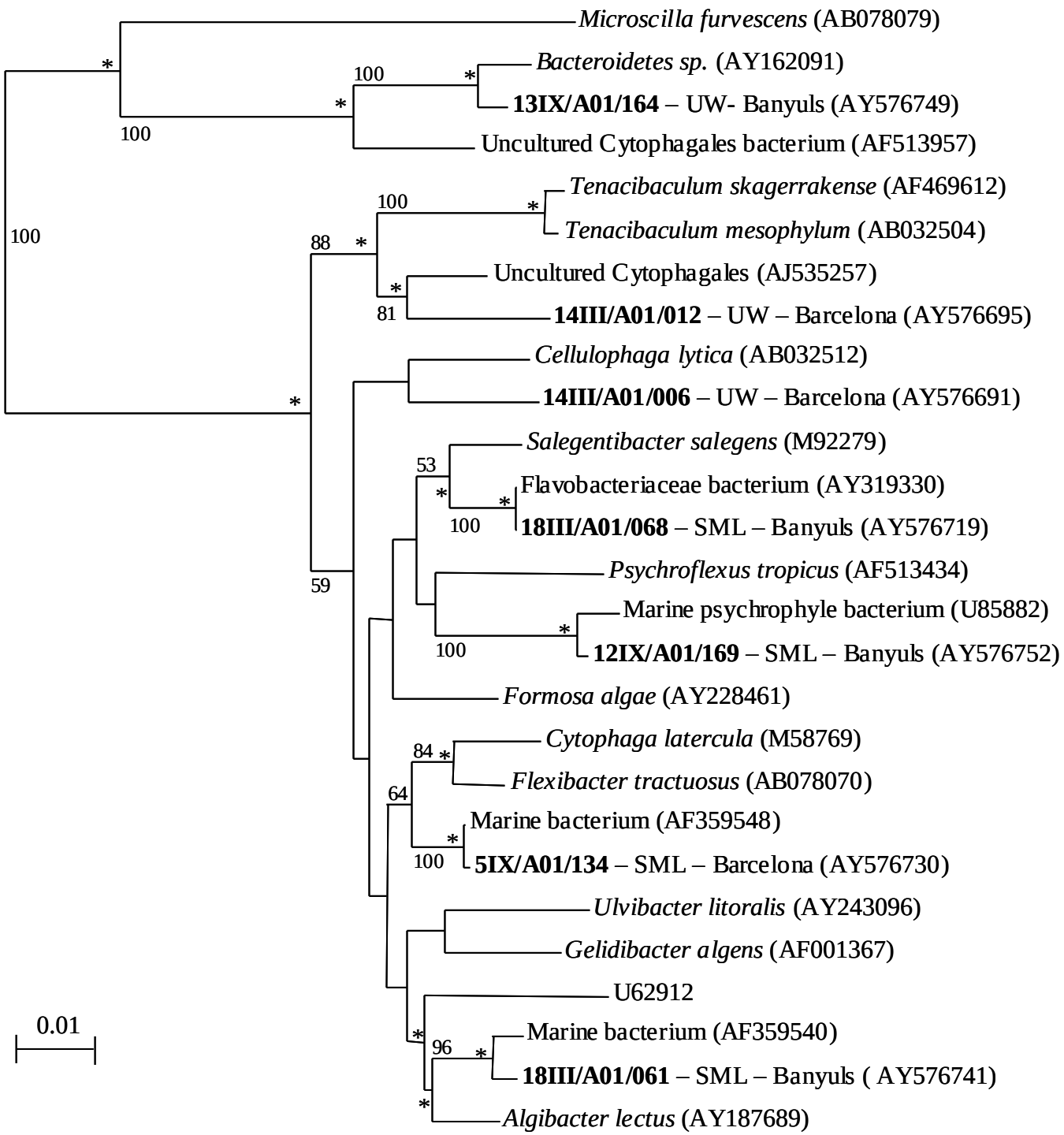

Fig. 2. Phylogenetic tree constructed using almost complete 16S rDNA gene sequences of isolated strains and related species of CFB group. The tree was built with the neighbor-joining method by using the Kimura distance. Values at nodes indicate bootstrap percentages for 500 replicates. Values less than 50 % are not reported. Scale bar indicate a Kimura distance of 0.01. Asterik (*) indicate branches that were also found using the Maximum Likelihood method.

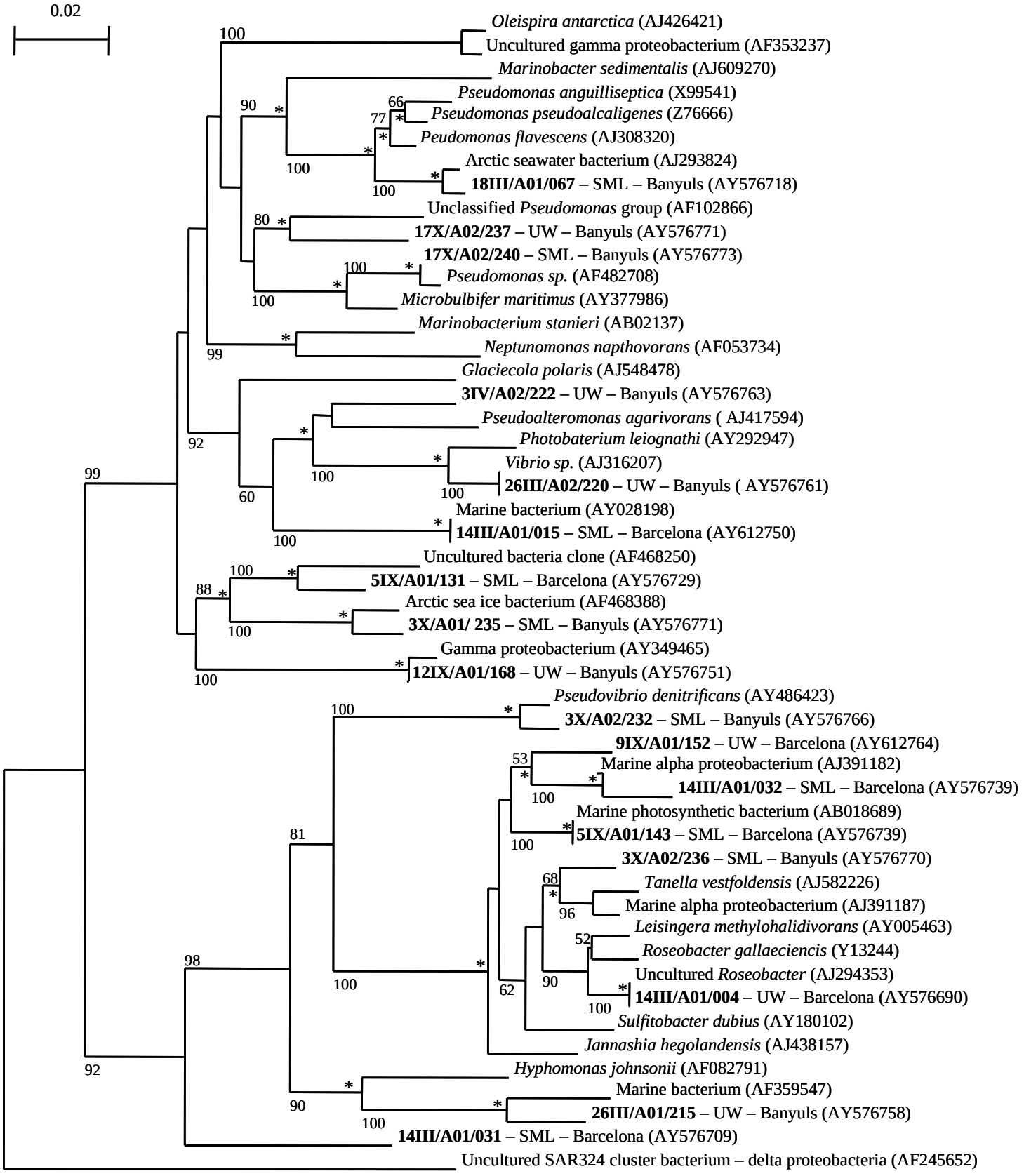

Fig. 3. Phylogenetic tree constructed using almost complete 16S rDNA gene sequences of isolated strains and related species of Proteobacteria. The tree was built with the neighbor-joining method by using the Kimura distance. Values at nodes indicate bootstrap percentages for 500 replicates. Values less than 50 % are not reported. Scale bar indicate a Kimura distance of 0.02. Asterik (*) indicate branches that were also found using the Maximum Likelihood method.

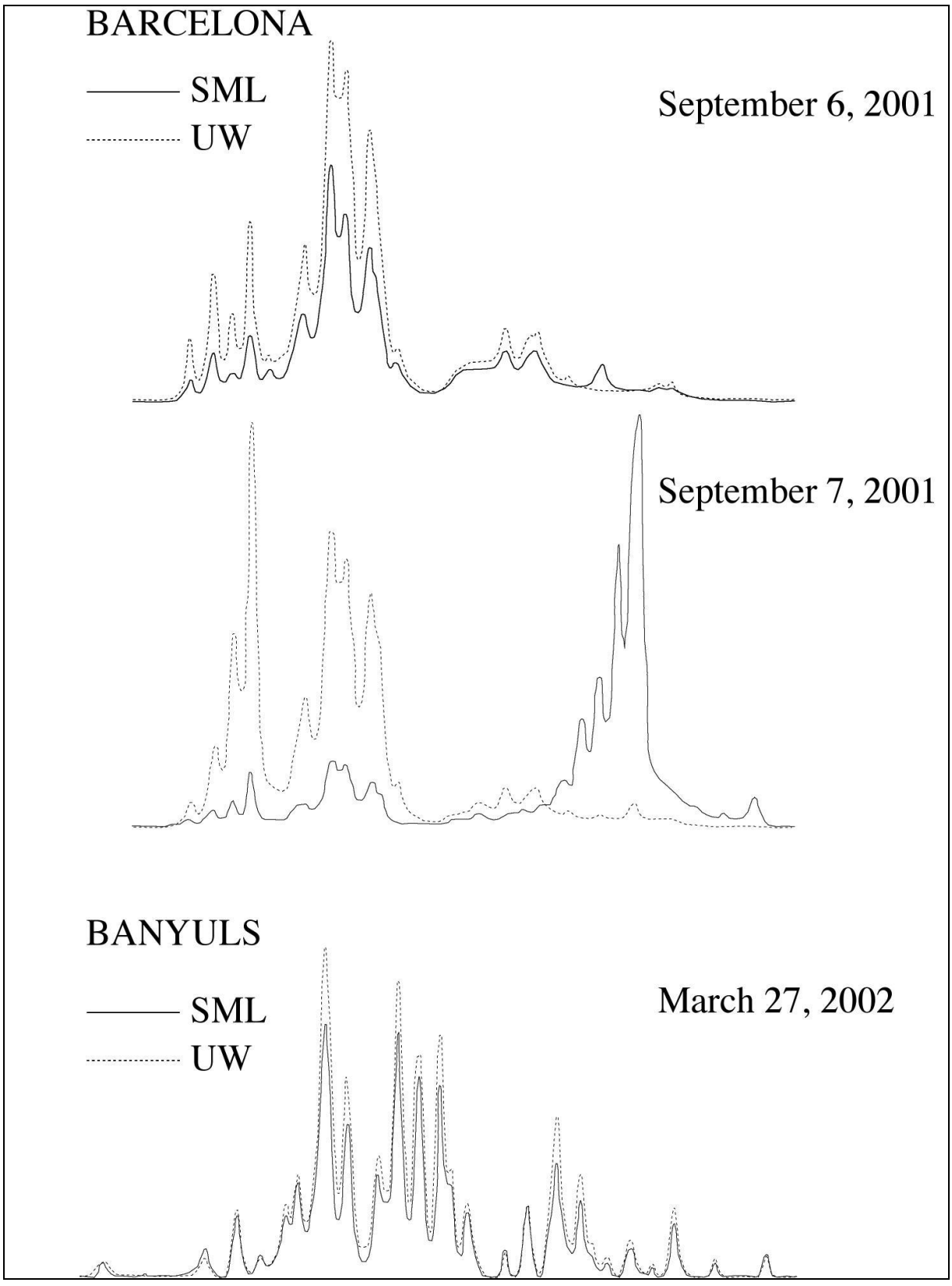

Fig. 4. Comparison between surface microlayer (SML) and underlying water (UW) bacterial community structure using SSCP fingerprints of 16S rRNA genes from Barcelona (6 and 7 September, 2002) and Banyuls-sur-Mer (27 March 2002) sampling stations

Agogué, H., E. O. Casamayor, F. Joux, I. Obernosterer, C. Dupuy, F. Lantoine, P. Catala, M. G. Weinbauer, T. Rheinthaler, G. J. Herndl, et P. Lebaron. 2004. Comparison of samplers for the biological characterization of the sea surface microlayer. Limnol. Oceanogr. Methods. 2:213-225

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