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Bacterial Diversity among Small-Subunit rRNA Gene Clones and Cellular Isolates from the Same Seawater Sample

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Numerous investigations applying the cloning and sequencing of rRNA genes (rDNAs) to the study of marine bacterioplankton diversity have shown that the sequences of genes cloned directly from environmental DNA do not correspond to the genes of cultured marine taxa. These results have been interpreted as support for the hypothesis that the most abundant heterotrophic marine bacterioplankton species are not readily culturable by commonly used methods. However, an alternative explanation is that marine bacterioplankton can be easily cultured but are not well represented in sequence databases. To further examine this question, we compared the small-subunit (SSU) rDNAs of 127 cellular clones isolated from a water sample collected off the Oregon coast to 58 bacterial SSU rDNAs cloned from environmental DNAs from the same water sample. The results revealed little overlap between partial SSU rDNA sequences from the cellular clones and the environmental clone library. An exception was the SSU rDNA sequence recovered from a cellular clone belonging to the *Pseudomonas* subgroup of the γ subclass of the class *Proteobacteria*, which was related to a single gene cloned directly from the same water sample (OCS181) (similarity, 94.6%). In addition, partial SSU rDNA sequences from three of the cultured strains matched a novel rDNA clone related to the γ subclass of the *Proteobacteria* found previously in an environmental clone library from marine aggregates (AGG53) (similarity, 94.3 to 99.6%). Our results support the hypothesis that many of the most abundant bacterioplankton species are not readily culturable by standard methods but also show that heterotrophic bacterioplankton that are culturable on media with high organic contents include many strains for which SSU rDNA sequences are not available in sequence databases.

The cloning of rRNA genes (rDNAs) from natural ecosystems precipitated a fundamental shift in microbial ecology away from the study of cultured strains and towards molecular approaches that emphasized the importance of in situ diversity. This transition began earlier with the discovery that, in many ecosystems, bacterial numbers estimated by epifluorescence direct counts are orders of magnitude higher than those estimated by CFU counts (16, 20). This discrepancy has been referred to as the great plate count anomaly (31).

Marine microbiologists were quick to recognize the significance of the anomalously low plate counts for heterotrophic bacterioplankton. Faced with uncertainty about the relevance of cultured species, at an early stage they moved away from cultivation-based techniques and towards a model that treated marine bacterioplankton as a functional unit with indistinguishable components, sometimes known as the microbial black box. Later, this view was reinforced by experience with autotrophic bacterioplankton (cyanobacteria and prochlorophytes), which showed that very abundant species could be difficult to detect and cultivate without the development of specialized techniques (18, 23, 34). Subsequently, the discovery that the abundance of culturable species estimated by plate counts is increased by manipulation and confinement of seawater samples (8) led to the formulation of two nonexclusive hypotheses that could be applied generally to many ecosystems: (i) bacterial communities are composed of known species that are capable of forming colonies on agar plates but do so with low efficiency, and (ii) bacterial communities are composed of unknown species that cannot easily be grown on common microbiological media (14). The actual explanations for the failure of cells to form colonies may be as diverse as the species in consideration, but these two alternative hypotheses capture the essence of the debate.

Most of the evidence supporting both hypotheses was gathered after the introduction of molecular biology techniques to aquatic microbial ecology. Hypothesis two was proposed after the cloning and sequencing of rDNAs from environmental DNA samples showed that the most common genes recovered belong to undescribed species and, in some cases, novel groups of bacteria (5, 10, 12, 29). Since most of the marine bacteria that have been described in the systematic literature are represented in rRNA databases (15), these comparisons led to the conclusion that many marine bacterioplankton species were unknown because they are difficult to culture (14). However, the same results could be attributed not to a failure to culture bacteria because of physiological differences but instead to the incompleteness of systematic descriptions and small-subunit (SSU) rDNA sequence databases for cultured marine bacteria. Rehnstam et al. (27) studied bacteria, cultured on Zobell's marine medium, from seawater collected from Scripps pier. After partially sequencing 16S rRNAs from these isolates, they developed strain-specific oligonucleotide probes and hybridized them to genomic DNA isolated from water samples collected from the same area. They concluded that the cultured strains were indeed significant members of the bacterioplankton community.

In this work, we report the results of a study in which *Hae*III restriction fragment length polymorphism (RFLP) patterns and partial gene sequences were used to compare the SSU rDNAs of bacterial isolates to bacterial SSU rDNAs cloned

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TABLE 1. Composition of marine R2A agar medium

Compound	g/liter of 75% seawater
Yeast extract	0.5
Proteose Peptone (Difco)	0.5
Casamino Acids	0.5
Dextrose	0.5
Soluble starch	0.5
Sodium pyruvate	0.3
Agar	15

directly from the same sample of Oregon coastal seawater. The results show that there is little overlap between SSU rDNA clones and cultured strains from the same water sample. In addition, we found that many of the cultured strains could not be identified by SSU rDNA sequence comparisons.

MATERIALS AND METHODS

Water samples. Subsurface (10-m) water samples were collected in Niskin bottles at a station located 8 km west of the mouth of Yaquina Bay, Oreg. (44°39.1′N, 124°10.6′W). The water was prescreened through 10-μm Nitex mesh and transported in autoclaved polyethylene carboys to the laboratory for filtration and plating.

Isolation of cellular clones. Subsamples (100 μl each) were spread onto 20 marine R2A agar plates and incubated at 15°C in the dark in a constant-temperature incubator. Marine R2A is a complex medium with the same composition as R2A medium (26) except that inorganic salts are not added and 75% seawater is used instead of distilled water. The carbon content of this medium is lower than that of marine agar but is still orders of magnitude higher than the carbon content of natural seawater (Table 1). All colonies appearing on 10 plates over a period of 3 weeks were characterized by several criteria related to colony morphology and streaked for isolation onto R2A plates. Subsequently, all isolated cellular clones were grown in R2A broth, and a 1-ml aliquot was frozen for storage in 10% dimethyl sulfoxide at −80°C. After the first 3 weeks, only colonies that had a previously unseen morphology were picked from the 20 plates, up to a period of 3 months. After 12 months of storage at −80°C, all cellular clones were inoculated into R2A broth for further experiments. Cellular clones were assigned the prefix R2A.

Cell counts. Direct counts were performed by epifluorescence microscopy with DAPI (4′,6-diamidino-2-phenylindole) to stain cells according to the protocol described in reference 33, except that the samples were fixed with 1% formaldehyde. Most-probable numbers were estimated from the number of CFU appearing in 10 R2A agar plates in a period of 3 months.

SSU rDNA clone library. Picoplankton from a 16-liter subsample were collected by filtration onto 0.2-μm-pore-size polysulfone filters (Supor-200; Gelman Inc., Ann Arbor, Mich.). Total cellular nucleic acids were isolated from the picoplankton sample by lysis with proteinase K and sodium dodecyl sulfate, followed by phenol-chloroform extraction as previously described (13). SSU rDNAs were amplified from total genomic DNA by PCR using two general bacterial SSU rDNA primers (27F, 5′-AGA GTT TGA TCM TGG CTC AG-3′; 1522R, 5′-AAG GAG GTG ATC CAN CCR CA-3′) (11) in a PTC100 thermal cycler (MJ Research, Watertown, Mass.). In a final volume of 100 μl, the reaction mixture contained 0.2 μM each amplification primer, 200 μM each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 5.0% acetamide, 10 ng of template, and 2.5 U of *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.), which was added after a precycling in which the reaction mixture was heated to 95°C for 5 min and held at 80°C. *Pfu* DNA polymerase was used for DNA amplification, because it exhibits proofreading activity and therefore can replicate DNA with higher fidelity than *Taq* DNA polymerase. The amplification conditions were 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with extension of the elongation step by 5 s per cycle for 35 cycles. Following the final cycle, the reaction was extended at 72°C for 10 min. The amplification products from six reactions were visualized by electrophoresis through a 1.0% SeaKem (FMC, Rockland, Maine) agarose gel in 1× TAE (40 mM Tris-acetate, 1 mM EDTA) containing ethidium bromide (0.50 $\mu g \cdot ml^{-1}$). For each reaction, the band corresponding to the correctly sized product (1.5 kb) was cut out of the gel and purified (24). The resulting products were pooled, quantified with a UV160U spectrophotometer (Shimazu Co., Kyoto, Japan), and visualized as described above. The amplified rDNA was inserted into the *Sma*I restriction site of the phagemid vector pBluescript KSII− (Stratagene) by blunt-end ligation as previously described (12), with the following modifications: the ligation reaction mixture, consisting of 260 ng of insert, 50 ng of vector, and 1 U of T4 DNA ligase in ligation buffer (10 mM Tris-HCl [pH 8.3], 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.4 mM ATP), was incubated at 16°C overnight. The ligation product was diluted 2:3 with TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) and used to transform competent *Escherichia coli* XL1-blue cells (Stratagene). Transformants were subsequently plated onto LB agar plates (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% Bacto-agar) containing the antibiotic ampicillin (200 $\mu g \cdot ml^{-1}$), spread evenly with 40 μl of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (40 $mg \cdot ml^{-1}$) and 100 μl of IPTG (isopropyl-β-D-thiogalactopyranoside) (100 mM), and grown at 37°C overnight. Positive (white-colony-morphotype) transformants were streaked for isolation. For long-term storage, stab cultures of all were made in LB top agar (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.7% Bacto-agar) and kept at 4°C. Clones were also stored in 7.0% dimethyl sulfoxide at −80°C. Gene clones were assigned the prefix OCS.

RFLP patterns. (i) Cellular clones. Based on their colony morphology, 60 cellular clones were selected for characterization by *Hae*III RFLP analysis of their SSU rDNAs. Briefly, the cellular clones were grown in R2A broth and harvested by centrifugation (5 min at 620 × *g*), and their genomic DNA was extracted by the CTAB (cetyl trimethyl ammonium bromide) (1). The SSU rDNA of each of the clones was amplified from 10 to 100 ng of genomic DNA by the PCR. The reaction mixtures contained 0.5 μM bacterial primers 27F and 1522R, 0.2 mM premixed deoxynucleoside triphosphates, 1.5 mM $MgCl_2$, and 2.5 U of *Taq* DNA polymerase (Promega, Madison, Wis.) in a final volume of 100 μl. All PCRs except for the one used to amplify environmental DNA for the construction of the clone library were performed with *Taq* DNA polymerase since all templates originated from a single cellular clone, and so we had no concern about preferential amplification biases. The DNA polymerase was added after a precycling step in which the reaction mixture was heated to 95°C for 1 min and held at 80°C. The amplification conditions were 1 min at 94°C, 1 min at 55°C, and 3 min at 72°C for 35 cycles. The PCR products were purified on Qiaquick-spin columns (Qiagen, Chatsworth, Calif.), and their concentrations were measured spectrophotometrically as described above. For the determination of RFLPs, 700 ng of purified PCR product from each cellular clone was digested with 5 U of the restriction endonuclease *Hae*III (Promega) for 5 h. The reactions were stopped by the addition of EDTA to 50 mM. The restriction fragments were resolved by gel electrophoresis in 3% NuSieve low-melting-point agarose (FMC) in TAE and stained with ethidium bromide (0.5 $\mu g \cdot ml^{-1}$). Photographs of the gels were digitized with an HP ScanJetIIcx scanner (Hewlett Packard Co., San Diego, Calif.). The images were processed by using Adobe Photoshop (Adobe Systems Inc., Mountain View, Calif.).

(ii) Gene clones. All gene clones were characterized by *Hae*III RFLPs. Plasmids bearing complete inserts, isolated by alkaline lysis, were used as templates in the amplification of environmental clone SSU rDNAs by the PCR. PCR conditions were the same as those employed above for the cellular clones, with the following exceptions. For the precycling step, the gene clones were held at 95°C for 5 min instead of 1 min. Also, each reaction mixture contained approximately 20 ng of template plasmid DNA. Since all of the PCRs yielded similar amounts of product, 7 μl of nonpurified PCR products was digested with 3 U of the restriction endonuclease *Hae*III for 2 h. The restriction fragments were resolved by gel electrophoresis as described above.

Sequencing. (i) Cellular clones. Sequences were determined for all combinations of RFLP pattern and colony morphology that were unique. An exception was the most common colony morphotype (colony type I), which presented slight variations in colony morphology, not all of which were sequenced. rDNA amplicons from 32 separate cellular clones were sequenced bidirectionally by using the primers 27F and 338R by the fluorescent dideoxy termination reaction (Applied Biosystems Inc., Foster City, Calif.). The fluorescently labeled fragments were resolved by polyacrylamide gel electrophoresis in a model 373A automated DNA sequencer (Applied Biosystems Inc.).

(ii) Gene clones. All gene clones that had unique RFLP patterns were chosen for sequencing. Briefly, template plasmid DNAs were prepared from overnight cultures by alkaline lysis using a Prep-A-Gene plasmid purification kit (Bio-Rad, Richmond, Calif.) or a Qiaprep spin plasmid kit (Qiagen) and quantified spectrophotometrically. Plasmid DNAs were visualized by electrophoresis through a 1.0% SeaKem (FMC) agarose gel as described above. Plasmid DNAs were sequenced bidirectionally with conserved primers as described above, with at least 500 bases of sequence data on the 5′ end of each SSU rDNA molecule being obtained.

Phylogenetic analyses. Unaligned sequences were submitted to the SIMILARITY_RANK (Simrank) program of the Ribosomal Database Project (RDP) to obtain a preliminary list of closest phylogenetic neighbors (21). Simrank results are expressed as S_{AB} values, the number of shared oligomers of seven bases divided by either the number of unique oligomers in the submitted sequence or the database sequence, whichever is lower. Sequences were then sorted according to phylum and subphylum affiliation and were manually aligned with the DNA sequence editor GDE version 2.1 (supplied by Steve Smith). The consensus sequences were resubmitted to Simrank by employing the MY_DATABASE option. This allowed us to append the SSU rDNA sequences available through the RDP database with all cellular and gene clone sequences obtained from this study. The results were used to sort the sequences into sets of related phylogeny, from which similarity values between the cellular and gene clones and their closest relatives were calculated.

Phylogenetic trees were calculated with the neighbor-joining algorithm (28) by using the program NEIGHBOR of the PHYLIP version 3.5 software (7). Evolutionary distances were calculated by using the program DNADIST and the Kimura 2-parameter model for nucleotide change, with a transition/transversion

TABLE 2. Distribution of R2A cellular clones classified by colony morphotype

Colony type	No. of isolates	RFLP type	Representative isolate[a]	Nearest RDP-determined phylogenetic neighbor(s)	Similarity (%)
I	33	1	R2A30[b]	*Azospirillum* sp. strain DSM1727	100.0
II	5	13	R2A5	*Flexibacter maritimus*	87.8
III	11	1	R2A30	*Azospirillum* sp. strain DSM1727	100.0
IV	8	10	R2A10	*Flexibacter maritimus*	86.7
V	6	1	R2A30	*Azospirillum* sp. strain DSM1727	100.0
V		2	R2A9	*Vibrio fischeri*	91.1
V		20	R2A37	Environmental clone AGG53	94.3
V		21	R2A62	Environmental clones OCS19 and OM42	95.6
V		29	R2A84	Sargasso Sea isolate S34	95.6
VI	4	3	R2A15	*Aeromonas salmonicida* subsp. *masoucida* 1-1-1	93.8
VI		4	R2A85	Environmental clone AGG53	96.0
VI		5	R2A88	Unknown, unnamed purple bacterium[c]	94.4
VI		20	R2A81	Environmental clone AGG53	99.6
VII	2	5	R2A113	Unknown, unnamed purple bacterium[c]	99.6
VIII	1	18	R2A44	*Oceanospirillum multiglobuliferum*	93.5
IX	20	6	R2A54	*Arthrobacter globiformis*	89.4
IX		26	R2A135	*Blastobacter* sp. strain BF14	96.0
IX		28	R2A170	Citrus disease-associated bacterium strain UW103/A31	90.9
X	15	7	R2A153	*Sphingomonas* sp. strain SYK6	96.6
XI	2	8	R2A63	*Blastobacter* sp. strain BF14	95.1
XII	5	9	R2A103	*Capnocytophaga gingivalis*	83.7
XIII	1	26	R2A166	*Blastobacter* sp. strain BF14	96.6
XIV	4	11	R2A148	*Oceanospirillum multiglobuliferum*	94.8
XV	1	19	R2A86	*Alteromonas haloplanktis*	95.2
XVI	1	12	R2A180	*Staphylococcus equorum*	98.0
XVII	1	22	R2A173	*Oceanospirillum beijerinckii*	87.4
XVIII	3	14	R2A57	Environmental clones OCS19 and OM42	94.4
XVIII		24	R2A117	Environmental clone OM64	95.1
XVIII		16	R2A114	Environmental clone SBR2045[d]	95.2
XIX	1	15		ND[e]	
XX	1	18		ND[e]	
XXI	1	1	R2A30	*Azospirillum* sp. strain DSM1727	100.0
XXII	1	23	R2A130	*Hyphomicrobium*-like organism strain US-353	87.3
XXIII	1	17	R2A161	*Bacillus firmus*	97.4
XXIV	1	31	R2A163	Environmental clone SBR2045[d]	93.6
XXV	1	30	R2A28	*Bacillus* sp. strain DSM8716	89.1
XXVI	1	25	R2A132	*Flavobacterium gondwanense*	87.4
XXVII	1	27	R2A160	*Micrococcus luteus*	94.1

[a] Only includes those clones for which a GenBank accession number was obtained.
[b] R2A30 is the representative cellular clone for all colony types with RFLP type 1.
[c] GenBank accession number Z25522.
[d] GenBank accession number X84583.
[e] ND, no rDNA sequence data available.

ratio of 2.0 (19). To check the consistency of the resulting tree, random resampling of the sequences (bootstrapping) was performed, and a tree representing a consensus of 100 trees was obtained (6). Similarities were calculated from partial sequences by considering all available overlapping regions, with the exclusion of ambiguous nucleotides.

Nucleotide sequence accession numbers. The following nucleotide sequences were filed in GenBank under the accession numbers indicated: R2A5, U78932; R2A9, U78920; R2A10, U78933; R2A15, U78921; R2A28, U78936; R2A30, U78922; R2A37, U78923; R2A44, U78924; R2A54, U78939; R2A57, U78909; R2A62, U78910; R2A63, U78911; R2A81, U78925; R2A84, U78912; R2A85, U78926; R2A86, U78927; R2A88, U78928; R2A103, U78924; R2A113, U78929; R2A114, U78913; R2A117, U78914; R2A130, U78915; R2A132, U78935; R2A135, U78916; R2A148, U78930; R2A153, U78917; R2A160, U78940; R2A161, U78938; R2A163, U78918; R2A166, U78919; R2A170, U78941; R2A173, U78931; R2A180, U78937; OCS19, U78942; OCS84, U78943; OCS116, U78944; OCS122, U78945; OCS181, U78946; S34, U87407.

RESULTS

Cell counts estimated from CFU on marine R2A medium were orders of magnitude lower than those estimated from DAPI direct counts. The number of bacteria estimated from the number of CFU was 72 ± 17 ml^{-1}, and the number of bacteria estimated by DAPI direct counts was $2.07 \cdot 10^6$ ml^{-1}. Similar results, not reported here, were obtained with other media, including Zobell's marine agar.

Colony morphology was used to sort the cellular clones into categories, because a majority of the isolates could be described by a few recognizable colony morphotypes. Of 127 cellular clones examined, 27 colony morphotypes were identified. *Hae*III RFLPs of SSU rDNAs were determined for 60 cellular clones, with resampling of colony morphologies that were either very abundant or else nondescript in appearance. Our reasoning was that variety was more likely to be overlooked among the nondescript colonies. In general, each colony morphotype was associated with a unique RFLP pattern, though variation in RFLP patterns was found within 4 of the 27 colony morphotypes (colony types V, VI, IX, and XVIII [Table 2]). In three cases (RFLP types 1, 20, and 26 [Table 2]), cellular clones with different colony morphologies were found to have identical RFLP patterns.

A comparison of the *Hae*III RFLP patterns of the SSU rDNAs from the cellular and gene clones showed little overlap. The gene clone library consisted of 116 full-length SSU rDNA clones, of which 54 were of plastid origin, 2 were chimeras, and

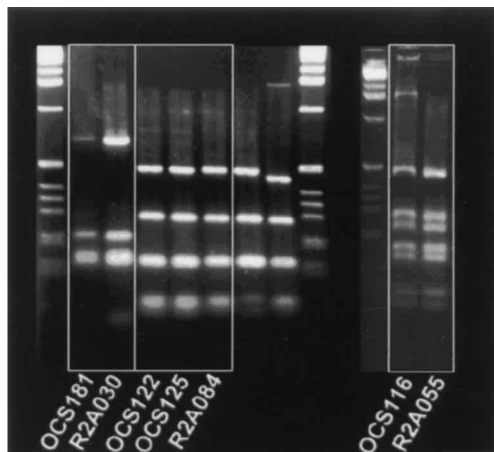

FIG. 1. *Hae*III RFLP patterns common to cellular isolate and environmental gene clone SSU rDNAs. Cellular clones: R2A030 (RFLP type 1), R2A084 (RFLP type 29), and R2A055 (RFLP type 7). Gene (environmental) clones: OCS181 (RFLP type 35), OCS122 (RFLP type 14), OCS125 (RFLP type 14), and OCS116 (RFLP type 37). The composite picture was generated by using Adobe Photoshop 3.0 (Adobe Systems Inc.).

2 were not identified. The *Hae*III RFLP patterns of the remaining 58 bacterial gene clones fell into 25 RFLP types, 13 of which were unique to single clones. Three RFLP patterns were common to both cellular and gene clones (Fig. 1; Table 3). Sequence comparisons showed that the cellular and gene clones with similar RFLP patterns were phylogenetically related in two of these cases (Table 3).

Sequence comparisons supported the same conclusion drawn from comparisons of the *Hae*III RFLP patterns—that there was little overlap in the species present in the two sets of SSU rDNAs. Although RFLP comparisons were determined for a larger number of cellular clones ($n = 60$), comparisons of 5′-terminal gene sequences from 32 cellular clones provided greater resolution of genetic differences as well as phylogenetic identification. Sequencing showed that the cellular clone collection was composed, in order of abundance, of members of the γ and α subdivisions of the class *Proteobacteria*, members of the cytophaga-flavobacter-bacteroides line of descent, and members of the high- and low-G+C-content gram-positive phyla.

The most common cellular clones, RFLP type 1, accounted for 80% of the γ-*Proteobacteria* cellular clones (Fig. 2A). The sequences of the SSU rDNAs of these isolates were identical to those of the published gene sequence of *Azospirillum* sp. strain DSM1727, a member of the *Pseudomonas* subgroup of the γ-*Proteobacteria*. As previously mentioned, cellular clone RFLP type 1 was one of the three cellular clone types with a *Hae*III RFLP pattern matching that of a gene clone. The second-most-abundant group of γ-*Proteobacteria* cellular clones (RFLP types 4 and 20) were related to the *Colwellia* assemblage and the *Alteromonas* group and were most similar (94.3 to 99.6%) to the environmental rDNA clone AGG53, isolated from marine phytodetrital aggregates by DeLong et al. (Fig. 3; Table 2) (5).

The most abundant α-*Proteobacteria* cellular clones were members of the *Sphingomonas* and *Roseobacter* groups (Fig. 2A), while the majority of the clones affiliated with the cytophaga-flavobacter-bacteroides line of descent were members of the *Vesiculatum* and the *Cytophaga lytica* subgroups. Finally, the cellular clones belonging to the gram-positive phyla were members of the *Arthrobacter* (high-G+C-content) and *Bacillus* (low-G+C-content) groups.

The gene clones were, in order of abundance, members of the γ, α, and β subdivisions of the class *Proteobacteria* and members of the high-G+C-content gram-positive phylum. All but one of the γ-*Proteobacteria* clones in the clone library were members of the SAR86 cluster (22) or closely related to sulfur-oxidizing symbionts (Fig. 2B; Fig. 3). The remaining γ-*Proteobacteria* gene clone (OCS181) was a member of the *Pseudomonas* subgroup. OCS181 had a *Hae*III RFLP pattern identical to that of the cellular clone RFLP type 1 (Fig. 1) and was 94.6% similar in rDNA sequence to the cellular clone R2A30 (Table 2). Proteobacteria gene clones of the α subdivision were members of the SAR11 cluster (12) or SAR116 cluster (22) or were related to the SAR83 cluster within the *Rhodobacter* group (Fig. 2B) (2). The high-G+C-content gram-positive gene clones were members of the BDA1-5 cluster (10, 25).

Simrank comparisons were used to identify nearest phylogenetic neighbors. For the majority of cellular clones, the nearest phylogenetic neighbors formed monophyletic groups related by high S_{ab} values, thus permitting unambiguous identification. However, in some cases, the Simrank results were ambiguous because they were either (i) a polyphyletic list of organisms with low S_{ab} values relative to that of the submitted sequence (i.e., R2A55, cellular clone RFLP type 7), or (ii) a list of uncultured environmental clones with high S_{ab} values relative to that of the submitted sequence (i.e., R2A57, cellular clone RFLP type 14). Both cases suggest that the cellular clones could represent new species.

In general, phylogenetic analyses of partial rDNA sequences supported the results obtained by Simrank by showing that, although most of the SSU rDNA sequences from cellular clones were phylogenetically related to clades of cultured species, some were instead closely related to environmental SSU rDNA clones. In particular, many of the cellular clones belonging to the *Rhodobacter* group were related to environmental gene clones recovered from seawater (Fig. 4). Also, cellular clones belonging to the *Colwellia* assemblage and the *Alteromonas* group were closely related to the environmental clone AGG53 (Fig. 3) (5).

DISCUSSION

The single most important observation reported in this work is that the genes recovered by cloning techniques and the genes

TABLE 3. Summary of Oregon coast clones with overlapping *Hae*III RFLP patterns or rDNA sequence

Cellular clone collection		Gene clone library		Same RFLP type[a]	Same RDP group
RFLP type(s)	Phylogenetic affiliation	RFLP type(s)	Phylogenetic affiliation		
1	*Pseudomonas* subgroup	35	*Pseudomonas* subgroup	+	+
29	*Roseobacter* group	14	*Roseobacter* group	+	+
7	*Sphingomonas* subgroup	37	*Rhodopseudomonas marina* subgroup	+	−
14, 16, 21, 24, 29, 31, 42	*Roseobacter* group	21, 12, 14	*Roseobacter* group	−	+

[a] Clones were determined to belong (+) or not to belong (−) to the same RFLP type or RDP group.

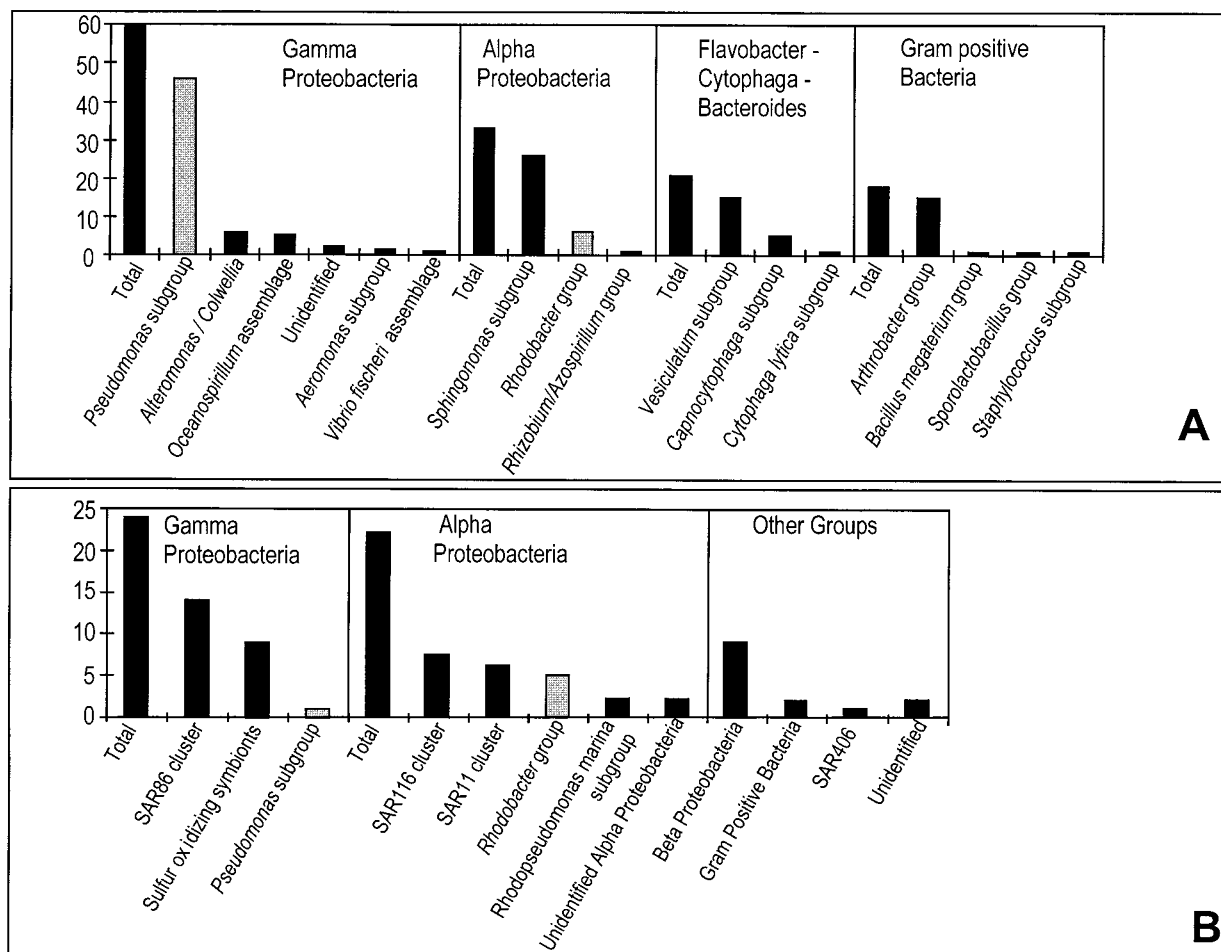

FIG. 2. Taxonomic grouping of Oregon coast cellular clones (A) and gene clones (B), obtained by a comparison of partial (5′-end) SSU rDNA sequences to sequences available through the RDP by using the program SIMILARITY_RANK. Cellular clone and environmental clone groups with identical *Hae*III RFLP patterns are indicated by shaded bars; otherwise, the clones are represented by solid bars. Values on the *y* axis are numbers of clones.

recovered from bacteria isolated in cultures from the same water sample were different. Explanations that attribute this difference to biases in molecular techniques seem unlikely, since investigations of bacterioplankton diversity by different gene cloning methods have led to the identification of many of the same phylogenetic lineages, regardless of the water mass investigated or the method used for gene cloning (5, 10, 12, 25, 29).

Although the main conclusion of this study is that many abundant bacterial species in seawater are not easily cultured, this fact tells only a small part of the story concerning the relationship between cultured bacteria and the distributions of bacterioplankton species in nature. The observation that many of the cellular clones could not be identified by comparisons of their SSU rDNAs to those in sequence databases indicates that a significant fraction of the bacterioplankton that can be grown on organically rich media are not represented in the databases. This result suggests that microbial cultivation has not yet been employed exhaustively for determining the taxonomic identities and distributions of marine bacteria. However, an alternative explanation is that the unidentified isolates are among the ca. 26% of systematically described marine bacterial species that are not yet represented in rDNA sequence databases (15). In either case, the results clearly point to a need for further investigations of the systematics of the marine heterotrophic bacteria which easily can be cultivated on common microbiological media that have high organic carbon contents.

The application of innovative culturing methods, such as seawater culture, that more accurately recreate the physical and chemical conditions found in the ocean, are likely to lead to the cultivation of microbial strains that cannot be grown on organically rich media such as the marine R2A medium we employed in this study (3, 30). Nature harbors microbial species with diverse growth requirements; no single cultivation medium could be expected to culture a majority of these species. Seawater culture has yielded isolates of heterotrophic bacteria, oligotrophs, that cannot be grown on organically rich marine media (3, 17, 30). However, so far there have been no reports of seawater culture methods being used successfully to culture microbial species corresponding to the 16S rDNAs recovered most frequently from seawater by molecular techniques. In light of these observations, it seems appropriate to regard bacteria as occupying a physiological continuum which may render them more or less amenable to cultivation. Thus, the terms culturable and unculturable, although convenient, have meaning relevant only to particular, defined experimental conditions.

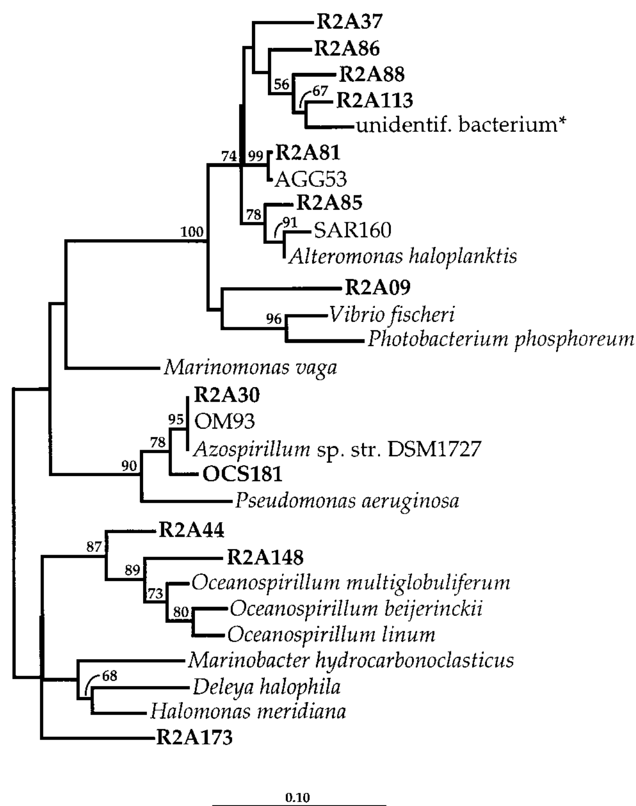

FIG. 3. Phylogenetic tree generated by the neighbor-joining method from a mask of ca. 200 nucleotide positions, showing the relationships between Oregon coast cellular clones and gene clones related to the γ-*Proteobacteria*. Bootstrap values (n = 100 replicates) are indicated for each of the branches. OM93, environmental rDNA clone from the eastern continental shelf of the United States (25). *, GenBank accession number Z25522. The scale bar indicates the number of substitutions per sequence position.

A third point to emerge from the comparisons we present is that the gene sequences of culturable microbes recovered from seawater in some cases are very similar but not identical to the rDNA sequences of closely related species. The significance of this variation is difficult to determine at this time. There is no exact relationship between SSU rDNA sequence similarity and taxonomic divisions. Furthermore, there is little comprehensive information available on rDNA variability within taxonomic groups, such as among strains of a species defined by phenotypic criteria (4). Gene clusters are observed commonly among 16S rDNA clones retrieved from environmental nucleic acids; the issue of physiological variability among the closely related strains represented by these sequence clusters may be of considerable significance to microbial ecologists interested in investigating microbial distribution with gene probes (4, 9).

Our investigation uncovered some cultured isolates that are closely related to microbial species that previously had been described only from environmental clone libraries (e.g., isolate R2A81 and clone AGG53 [similarity, 99.6%]). Interestingly, these isolates, which correspond to 16S rDNAs recovered from marine aggregates, flocculate in suspension. These strains, which remain unidentified at a systematic level, may be of use as laboratory models for investigating the mechanisms of marine microaggregate formation.

Despite the challenges of culturing some recalcitrant species, the isolation and identification of representative marine bacterioplankton strains remains an indispensable source of physiological and genetic information about marine microbes. Phylogenetic trees can be highly informative; nonetheless, many metabolic pathways in bacteria are polyphyletically distributed, thereby limiting inferences about the biogeochemical impact of bacterioplankton based on rDNA sequence comparisons alone. Partial genomic DNA sequences retrieved from nature offer one means of obtaining more information about uncultured species. In a recent study, this approach was used to study group I marine crenarchaeotes (32).

It was not the aim of this study to develop innovative culturing techniques. Instead, this study was based on the application of those methods used most routinely in marine microbiology to recover bacterial species from seawater. Nonetheless, among the bacterial isolates recovered were many that appeared to constitute new microbial species by the criterion of SSU rRNA comparisons. In this light, we might regard genetic investigations of microbial diversity as successful not just because they employ novel methods, but also because they have invited renewed scrutiny of a fertile problem.

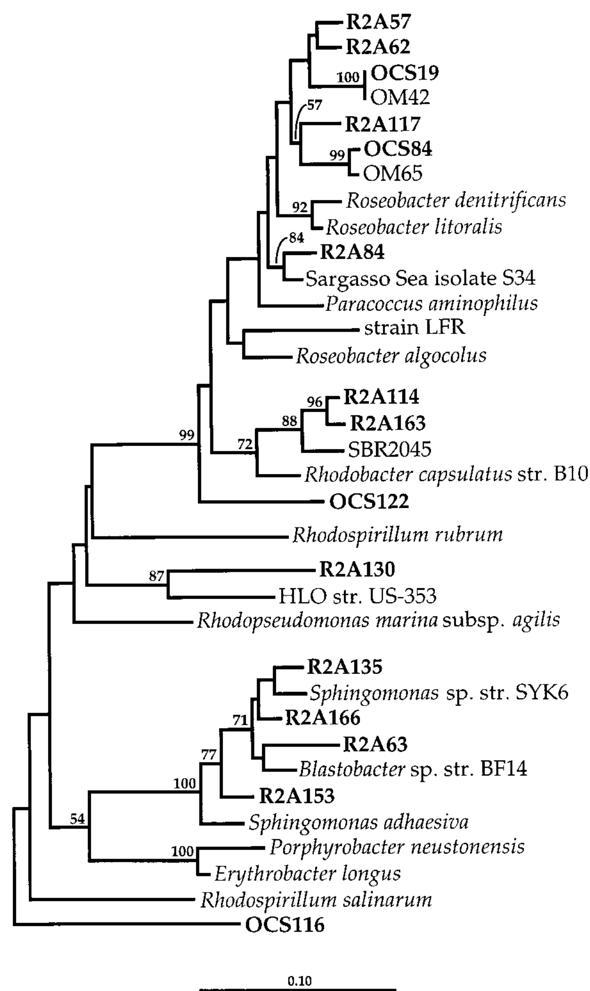

FIG. 4. Phylogenetic tree generated by the neighbor-joining method from a mask of ca. 200 nucleotide positions, showing the phylogenetic relationships among Oregon coast cellular and gene clones within the α subdivision of the *Proteobacteria*. Bootstrap values (n = 100 replicates) are indicated for each of the branches. Clones with the prefix OM are environmental rDNA clones from the eastern continental shelf of the United States (25). The scale bar indicates the number of substitutions per sequence position.

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