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Phylogenetic Analysis of a Natural Marine Bacterioplankton Population by rRNA Gene Cloning and Sequencing

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The identification of the prokaryotic species which constitute marine bacterioplankton communities has been a long-standing problem in marine microbiology. To address this question, we used the polymerase chain reaction to construct and analyze a library of 51 small-subunit (16S) rRNA genes cloned from Sargasso Sea bacterioplankton genomic DNA. Oligonucleotides complementary to conserved regions in the 16S rDNAs of eubacteria were used to direct the synthesis of polymerase chain reaction products, which were then cloned by blunt-end ligation into the phagemid vector pBluescript. Restriction fragment length polymorphisms and hybridizations to oligonucleotide probes for the SAR11 and marine *Synechococcus* phylogenetic groups indicated the presence of at least seven classes of genes. The sequences of five unique rDNAs were determined completely. In addition to 16S rRNA genes from the marine *Synechococcus* cluster and the previously identified but uncultivated microbial group, the SAR11 cluster [S. J. Giovannoni, T. B. Britschgi, C. L. Moyer, and K. G. Field, Nature (London) 345:60–63], two new gene classes were observed. Phylogenetic comparisons indicated that these belonged to unknown species of α- and γ-proteobacteria. The data confirm the earlier conclusion that a majority of planktonic bacteria are new species previously unrecognized by bacteriologists.

Within the past decade, it has become evident that bacterioplankton contribute significantly to biomass and biogeochemical activity in planktonic systems (4, 36). Until recently, progress in identifying the microbial species which constitute these communities was slow, because the majority of the organisms present (as measured by direct counts) cannot be recovered in cultures (5, 14, 16). The discovery a decade ago that unicellular cyanobacteria are widely distributed in the open ocean (34) and the more recent observation of abundant marine prochlorophytes (3) have underscored the uncertainty of our knowledge about bacterioplankton community structure.

Molecular approaches are now providing genetic markers for the dominant bacterial species in natural microbial populations (6, 21, 33). The immediate objectives of these studies are twofold: (i) to identify species, both known and novel, by reference to sequence data bases; and (ii) to construct species-specific probes which can be used in quantitative ecological studies (7, 29).

Previously, we reported rRNA genetic markers and quantitative hybridization data which demonstrated that a novel lineage belonging to the α-proteobacteria was abundant in the Sargasso Sea; we also identified novel lineages which were very closely related to cultivated marine *Synechococcus* spp. (6). In a study of a hot-spring ecosystem, Ward and coworkers examined cloned fragments of 16S rRNA cDNAs and found numerous novel lineages but no matches to genes from cultivated hot-spring bacteria (33). These results suggested that natural ecosystems in general may include species which are unknown to microbiologists.

Although any gene may be used as a genetic marker, rRNA genes offer distinct advantages. The extensive use of 16S rRNAs for studies of microbial systematics and evolution has resulted in large computer data bases, such as the RNA Data Base Project, which encompass the phylogenetic diversity found within culture collections. rRNA genes are highly conserved and therefore can be used to examine distant phylogenetic relationships with accuracy. The presence of large numbers of ribosomes within cells and the regulation of their biosyntheses in proportion to cellular growth rates make these molecules ideally suited for ecological studies with nucleic acid probes.

Here we describe the partial analysis of a 16S rDNA library prepared from photic zone Sargasso Sea bacterioplankton using a general approach for cloning eubacterial 16S rDNAs. The Sargasso Sea, a central oceanic gyre, typifies the oligotrophic conditions of the open ocean, providing an example of a microbial community adapted to extremely low-nutrient conditions. The results extend our previous observations of high genetic diversity among closely related lineages within this microbial community and provide rDNA genetic markers for two novel eubacterial lineages.

MATERIALS AND METHODS

Collection and amplification of rDNA. A surface (2-m) bacterioplankton population was collected from hydrostation S (32°4′N, 64°23′W) in the Sargasso Sea as previously described (8). The polymerase chain reaction (24) was used to produce double-stranded rDNA from the mixed-microbial-population genomic DNA prepared from the plankton sample. The amplification primers were complementary to conserved regions in the 5′ and 3′ regions of the 16S rRNA genes. The amplification primers were the universal 1406R (ACGGGCGGTGTGTRC) and eubacterial 68F (TNANAC ATGCAAGTCGAKCG) (17). The reaction conditions were as follows: 1 μg of template DNA, 10 μl of 10× reaction buffer (500 mM KCl, 100 mM Tris HCl [pH 9.0 at 25°C], 15 mM $MgCl_2$, 0.1% gelatin, 1% Triton X-100), 1 U of *Taq* DNA polymerase (Promega Biological Research Products, Madison, Wis.), 1 μM (each) primer, 200 μM (each) dATP, dCTP, dGTP, and dTTP in a 100-μl total volume; 1 min at 94°C, 1 min at 60°C, 4 min at 72°C; 30 cycles. Polymerase chain

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reaction-amplified genes were detected by electrophoresis in a 0.75% agarose gel.

Construction of the clone library. Amplification products were made blunt ended by the addition of 2 U of the large fragment of DNA polymerase I (Bethesda Research Laboratories, Gaithersburg, Md.) and 10 mM $MgCl_2$ to the polymerase chain reaction mixture and incubation at room temperature for 15 min. After phenol extraction, the rDNA was inserted into the *Sma*I restriction site of pBluescript KS II− (Stratagene, La Jolla, Calif.) by blunt-end ligation. The ligation reaction mixture, which contained 260 ng of insert, 15 ng of vector, and 1 U of T4 ligase in ligation buffer (0.01 M Tris HCl [pH 8.3], 0.01 M $MgCl_2$, 0.01 M dithiothreitol, 0.4 mM ATP), was incubated at 16°C overnight. The resulting ligation products were diluted 2:3 with TE buffer (10 mM Tris HCl, 1 mM EDTA [pH 8]) and used to transform competent *Escherichia coli* DH5α(F′) cells (Bethesda Research Laboratories). One hundred white colonies were picked from YT plates (0.8% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% agar) and stored at 4°C on YT agar slants containing 75 μg of kanamycin (Sigma, St. Louis, Mo.) per ml.

Plasmid restriction analysis. Plasmid DNA was prepared from the clones by a rapid boiling method (13). The plasmids were cut with restriction endonucleases *Bam*HI and *Pst*I (Sigma), producing a variety of fragmented rDNAs, which were chromatographically separated by agarose gel electrophoresis and visualized with ethidium bromide.

Oligonucleotide probe hybridization. The following oligonucleotide probes were hybridized to the plasmid DNAs: ATAAGTCTTTACGGAGAAAGCTT (SAR11 F), CAGCM GCCGCGGTAATWC(536 F [17]), and TGAATTTCGCCTG AGGATGAG (SYNF). The SAR11 F and SYN F probes complement conserved regions in the 16S rDNAs of the SAR11 and marine *Synechococcus* clusters, respectively (6). T4 polynucleotide kinase (Promega Biological Research Products) was used to label the 5′ terminus of each of the oligonucleotide probes (0.5 μg) with 50 μCi of [γ-^{32}P]dATP (New England Nuclear Corp., Boston, Mass.) as described previously (27). Dot blots were prepared from plasmid DNAs with a dot blot Minifold (Minifold I SRC 096/0; Schleicher & Schuell, Keene, N.H.). One hundred nanograms of each of the plasmid DNAs was dissolved in 400 μl of denaturation solution (0.5 M NaOH, 1.5 M NaCl), applied to Zeta-probe membranes (Bio-Rad Laboratories, Richmond, Calif.), dried at 80°C, and UV cross-linked (200 J/m^2). The membranes were prehybridized for 10 min at room temperature in 25 ml of hybridization buffer (1 mM EDTA, 7% sodium dodecyl sulfate, 0.5 M sodium phosphate buffer [pH 7.2]) without labelled probe. The solution was replaced with fresh buffer containing 20 μCi of γ-^{32}P-labelled DNA probe, and the membranes were incubated at room temperature for 4 to 18 h. Following hybridization, the membranes were washed in two changes of wash buffer (1 mM EDTA, 40 mM sodium phosphate buffer [pH 7.2], 1% sodium dodecyl sulfate) at room temperature for 10 min each. A final wash for 10 min at 37°C was done in 1 mM EDTA–40 mM sodium phosphate buffer (pH 7.2)–5% sodium dodecyl sulfate.

DNA sequencing and phylogenetic analysis. Single-stranded DNA templates for sequencing were prepared by infection with R408 helper phage (Stratagene) (1). Five of the cloned rRNA genes were sequenced by standard dideoxynucleotide chain-terminating methods (25) with universal primers (17) and Sequenase (United States Biochemical Co., Cleveland, Ohio). The phylogenetic relationships among the 16S rRNA gene sequences were determined by a distance matrix method (15, 20).

Nucleotide sequence accession numbers. The determined nucleotide sequences will appear in the EMBL Nucleotide Databases under the accession numbers M63810, M63811, M63812, M63813, and M63814.

RESULTS

Restriction fragment length analysis and probe hybridization results. The oligonucleotide primers used to amplify the genes from the natural population were complementary to regions conserved in a broad range of eubacterial phyla, with the exception of the phylum *Planctomycetales*. Thus, it was anticipated that the clone library would contain an array of genes nearly as diverse as that of open-ocean bacterioplankton populations. Restriction fragment length polymorphisms (RFLPs) indicated at least five categories of unique clones. Of the 100 colonies chosen (which were ampicillin resistant but defective in β-galactosidase synthesis), 87 contained an insertion. Of these, 51 contained the entire 1.3-kb amplified rDNA. The SAR83 group, comprising nine clones, had a *Pst*I site at position 1100 (*E. coli* numeration; 2), resulting in 1- and 0.3-kb fragments. The SAR92 group (three clones) had an internal restriction site at position 1189 (*E. coli* numeration; 2). The SAR175 group, with three clones, produced 1.1- and 0.2-kb fragments. Finally, a single clone, SAR132, had an RFLP pattern which indicated a 0.7-kb fragment and possibly two 0.35-kb fragments. The remaining 36 clones contained no internal restriction sites; therefore, we attempted to group them by testing their hybridization to oligonucleotide probes.

Figure 1 illustrates the results of the DNA hybridization experiments. Nine clones reacted with the SAR11 F probe (SAR11 cluster). Two clones which contained full-length insertions, SAR100 and SAR152, reacted with the SYN F probe. Subsequent sequencing studies demonstrated that SAR139 is a member of the marine *Synechococcus* cluster and that it has the target sequence of the SYN F probe. Therefore, the failure of this clone to hybridize to the SYN F probe in Fig. 1 is probably the result of experimental error.

The results of the preliminary grouping by RFLP analysis and probe hybridization studies are summarized in Fig. 2. Twenty-four of the 51 clones did not react with either probe, nor did they contain any internal restriction sites. They remain uncharacterized.

Primary sequence and secondary structure analyses. Two clones from the marine *Synechococcus* cluster (SAR100 and SAR139), one clone from the SAR11 cluster (SAR95), and two clones of unknown evolutionary origin (SAR83 and SAR92) were sequenced completely.

A secondary structural model for SAR100 which indicates the sites of sequence variation among clones from the marine *Synechococcus* cluster (SAR6, SAR7, SAR100, and SAR139) is shown in Fig. 3. Signature nucleotides at positions 799 (U), 1207 (C), and 1233 (A) in SAR100 and SAR139 indicated that these clones belonged to the oxygenic phototroph phylogenetic group (37). Previous work demonstrated close sequence similarities among SAR6, SAR7, and 16S rRNAs from cultivated marine *Synechococcus* spp. Similarities among SAR100, SAR139, SAR6, and SAR7 ranged from 0.971 to 0.996 (6). SAR100 and SAR139 differ at only six positions, which are localized in variable regions but include no examples of covariance between base pairs. SAR100 and SAR139 differ from their closest relative, SAR7, at 25 positions, including three pairs of compensatory

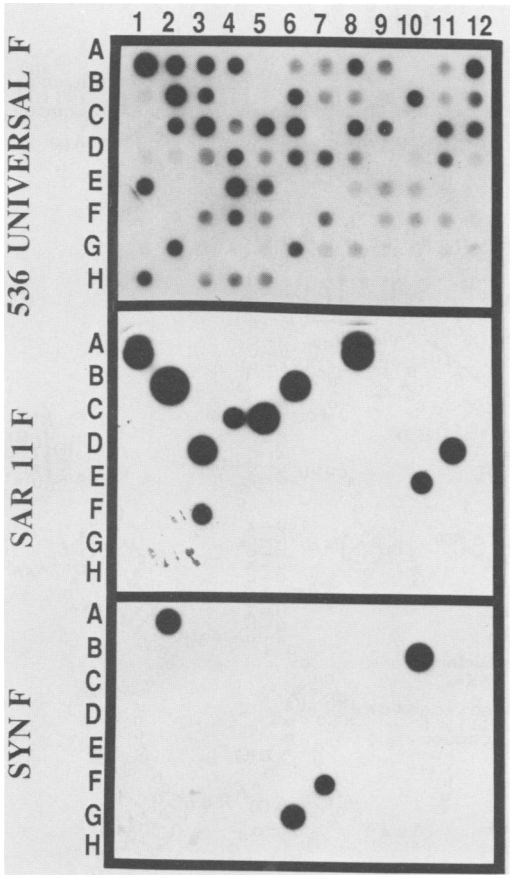

FIG. 1. Hybridization of oligonucleotide probes for the SAR11 cluster (SAR11 F) and the marine *Synechococcus* cluster (SYN F) to 87 recombinant plasmid DNAs containing putative 16S rDNAs. The blot was washed and reprobed three times. The 536 F universal probe served as a positive control for rDNA gene insert concentration. Wells A1 and A2 contain positive-control single-stranded DNAs from 16S rDNA clones SAR1 (SAR11 cluster) and SAR6 (*Synechococcus* cluster), respectively (6). The unknown Sargasso Sea rDNAs were dot blotted in the wells as follows: row A, SAR80, SAR81, SAR82, SAR83, SAR84, SAR85, SAR86, SAR87, SAR88, and SAR89; row B, SAR90, SAR91, SAR92, SAR93, SAR94, SAR95, SAR96, SAR97, SAR99, SAR100, SAR101, and SAR102; row C, SAR103, SAR104, SAR105, SAR106, SAR107, SAR108, SAR109, SAR111, SAR113, SAR115, SAR116, and SAR 117; row D, SAR118, SAR119, SAR120, SAR121, SAR122, SAR123, SAR124, SAR125, SAR126, SAR127, SAR128, and SAR129; row E, SAR130, SAR131, SAR132, SAR134, SAR135, SAR136, SAR137, SAR138, SAR139, SAR140, SAR141, and SAR142; row F, SAR143, SAR146, SAR148, SAR149, SAR150, SAR151, SAR152, SAR153, SAR154, SAR156, SAR157, and SAR158; row G, SAR159, SAR160, SAR161, SAR162, SAR163, SAR164, SAR165, SAR166, SAR167, SAR168, SAR169, and SAR170; and row H, SAR172, SAR173, SAR175, SAR176, SAR178, and SAR179.

changes in bases which are believed to be paired across helices (positions 154/167, 156/165, and 1003/1037 [9]). The majority of the differences are localized in hypervariable domains (e.g., positions 998 to 1043 and 1247 to 1290).

The sequence of SAR95, which hybridized to the SAR11 F probe, was compared with those of the SAR1 and SAR11 16S rRNA genes cloned and sequenced previously (6). SAR95 differed from SAR1 at six positions, including one case of covariance in a Watson-Crick base pair (position

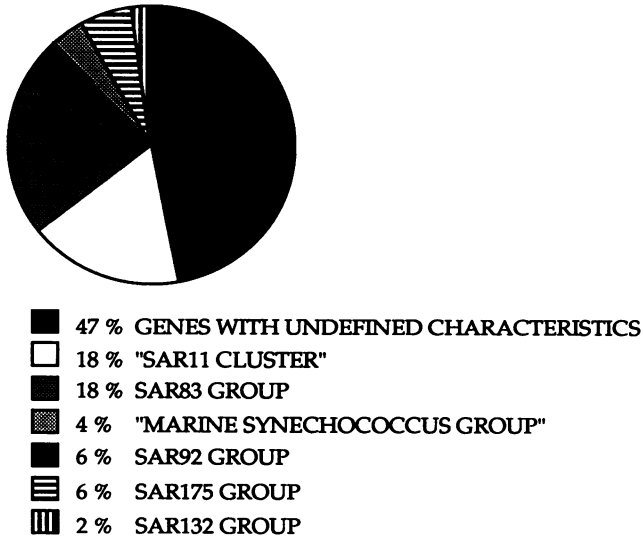

FIG. 2. Summary of RFLP analysis and probe hybridization results. Seven unique classes of clones were identified. Of the 51 clones which contained the entire 1,350-bp rDNA gene, 24 had no internal *Bam*HI or *Pst*I restriction sites and did not hybridize to the phylogenetic group-specific probes.

599/639), one G-to-A transition resulting in an A · U base pair in place of a G · U base pair in a stem (position 237), one transition in a variable region (position 263), and a transversion resulting in an A · G base pair (position 1191) separated by 1 base from a transition changing a G · U base pair to an A · U base pair (position 1189).

The SAR92 group did not hybridize to the two group-specific probes but was unique on the basis of its *Bam*HI and *Pst*I RFLPs. The sequence similarities, secondary structural features, and signature nucleotides indicate that SAR92 is a deeply branching lineage of the γ-proteobacteria (Fig. 4). The SAR92 sequence was not specifically related to those of *Vibrio* or *Oceanospirillum* spp. Thus, SAR92 apparently represents a previously unrecognized bacterioplankton species.

The SAR83 group also did not hybridize to the two group-specific probes but was unique on the basis of its *Bam*HI and *Pst*I RFLPs. The highest similarity observed between SAR83 and a known 16S rRNA gene was to *Erythrobacter longus* Och114 (similarity, 0.93).

DISCUSSION

The preliminary analysis of the clone library of bacterioplankton 16S rDNAs yielded striking results. RFLPs and hybridizations to two phylogenetically specific oligonucleotide probes indicated a minimum of seven unique classes of genes. Two of these belonged to previously identified phylogenetic groups—the SAR11 cluster and the marine *Synechococcus* cluster. Two additional gene classes, each representing a unique species of proteobacteria, were identified by sequencing.

A close examination of the sequences of the cloned genes demonstrated that they precisely matched universal secondary structural models for 16S rRNAs; both base pairing and conserved elements of the primary sequence were conserved (9). Furthermore, phylogenetically conserved signature nucleotides and structural motifs for the α, γ, and oxygenic

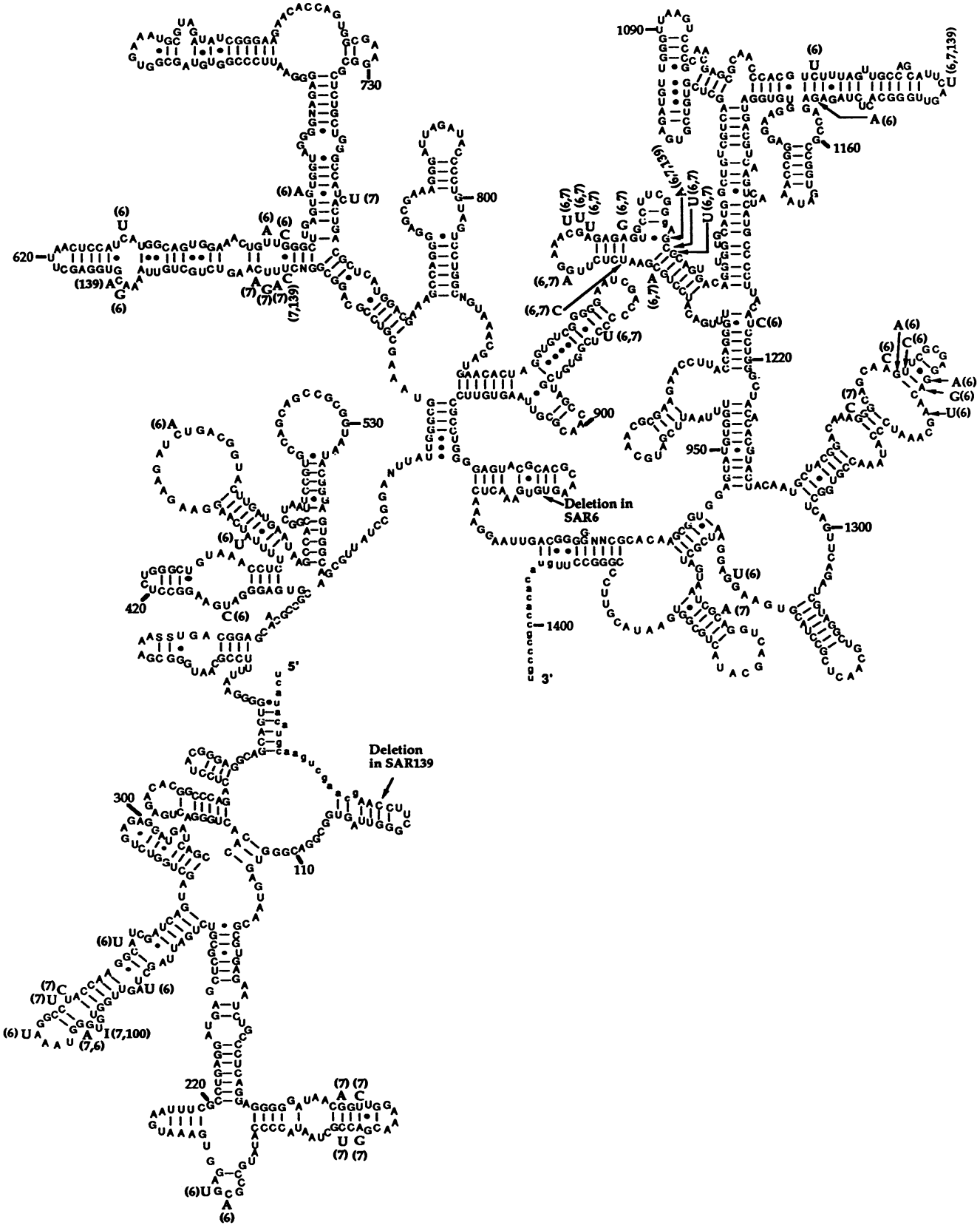

FIG. 3. Secondary structural model of clone SAR100 16S rDNA. The variations in clones SAR6, SAR7, and SAR139 are noted by the numbers in parentheses (6). Lowercase nucleotides at either end denote amplification primers; within the sequence they indicate uncertain positions.

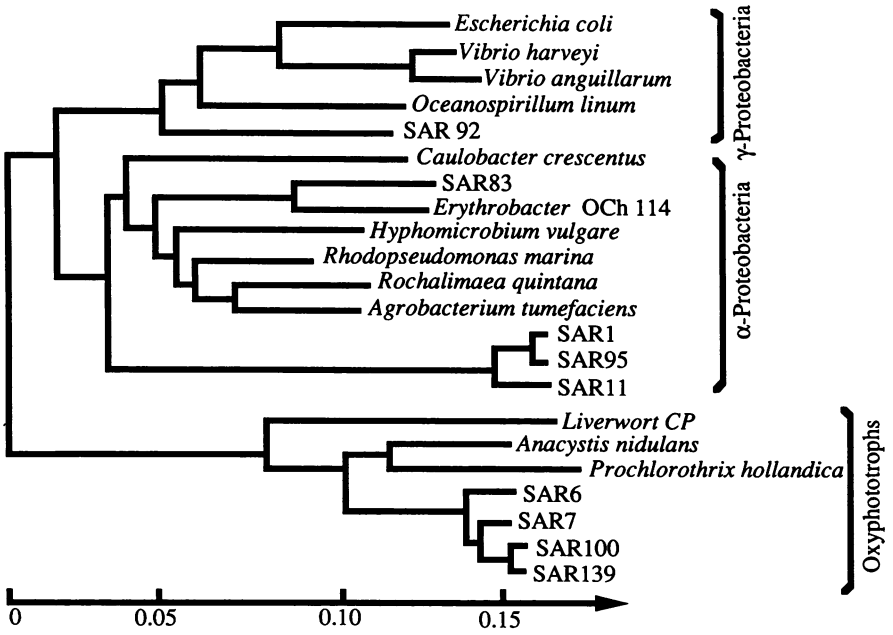

FIG. 4. Phylogenetic tree showing relationships of the rDNA clones from the Sargasso Sea to representative, cultivated species (2, 6, 18, 30, 31, 32, 35, 40). Positions of uncertain homology in regions containing insertions and deletions were omitted from the analysis. Evolutionary distances were calculated by the method of Jukes and Cantor (15), which corrects for the effects of superimposed mutations. The phylogenetic tree was determined by a distance matrix method (20). The tree was rooted with the sequence of *Bacillus subtilis* (38). Sequence data not referenced were provided by C. R. Woese and R. Rossen.

phototroph phyla were conserved in the corresponding genes, which could also be identified by overall primary sequence similarities. The oxygenic phototroph genes cloned from the bacterioplankton population were similar to the 16S rRNA genes of cultured marine *Synechococcus* spp. isolated from the same oceanic region (similarity, ≥0.96; 33). Moreover, the cloned oxygenic phototroph genes contained all of the eubacterial and cyanobacterial signature nucleotides described by Woese (37). The differences among the genes were localized in hypervariable regions of the molecule and were confirmed by compensatory base changes across helices.

When multiple genes of a given type were sequenced, as with the SAR11 and oxygenic phototroph groups, a pattern of sequence variation which indicated the presence of clusters of very closely related genes emerged. We reported this phenomenon earlier and further confirm it with the additional sequences presented here (SAR95, SAR100, and SAR139). Three approaches indicated that the sources of this variation were biological, rather than artifactual. First, in all cases compensatory changes were found in bases paired across helices. Second, identical primary sequences and variations were observed when SAR11 cluster genes amplified from natural population Sargasso Sea DNA with specific primers (SAR11 F and 1406 R) were sequenced directly (unpublished data). Third, the distributions of the variations within the sequences were inconsistent with the explanation that chimeric gene artifacts had occurred. For example, there were no genes containing ambiguous combinations of signature nucleotides, tracts of unpaired bases in helical regions, or mosaic patterns of similarity in dot plots. A noteworthy exception occurred in the SAR11 cluster, in which all members exhibited an inversion on the order of a very highly conserved base pair between positions 570 and 880 (G · C to C · G). We note with caution that some classes of chimeric gene artifacts, particularly those involving multiple exchanges among very closely related genes, would be impossible to detect.

There are several alternative explanations for the variability within the sequence clusters. Microheterogeneities in 16S rDNA gene families may account for some of the variability, particularly among very closely related genes (e.g., SAR1 and SAR95; SAR100 and SAR139). There currently are no documented cases of microheterogeneities in 16S rDNA gene families, although many investigators informally report their occurrence. It is likely that some of the sequence variability among the more distantly related sequences is a consequence of the divergence and speciation of cellular lineages. Wood and Townsend reported high genetic diversity among marine *Synechococcus* isolates on the basis of RFLP data from four loci (39). The clone model of bacterial population structure, which states that natural populations are mixtures of independent clonal cell lines (26), predicts that genetic diversity will be high in bacterial populations with large effective population sizes. We estimate that the actual marine *Synechococcus* population size is ca. 10^{26}. Viral predation may also play a role by promoting the divergence of lineages through disruptive selection (22).

The two novel eubacterial 16S rRNA lineages described here, SAR83 and SAR92, could both be identified as proteobacteria. SAR92, a member of the γ-proteobacteria, formed no closer specific relationships. Since the γ-proteobacteria are diverse in metabolic capacity, an ecological role for SAR92 could not be deduced from the observed phylogenetic relationships.

The specific phylogenetic relationship between SAR83 and the marine species *E. longus* Och114 suggests the intriguing possibility that these organisms might also share

common metabolic features. *Erythrobacter* spp. are aerobic photoheterotrophs. They are metabolically capable of photophosphorylation (11) but require organic carbon and oxygen for growth (19). Because of a higher growth efficiency in the presence of light and oxygen, in low-nutrient environments these organisms may use organic carbon more efficiently than respiratory heterotrophs do (12, 28). Several lines of evidence add further credibility to the theory that α-proteobacteria related to *Erythrobacter* spp. may be active in the open ocean. Marine sediments contain the carotenoid spheroidenone, which has been found only in photoheterotrophic bacteria of the α-purple subgroup (23). Furthermore, the standing crop of heterotrophic bacterioplankton is too large to be explained from dissolved organic carbon estimates with a respiratory conversion factor of 0.5 to 0.6 (10) but could be explained better with the higher growth efficiencies associated with photoheterotrophy.

Future studies with phylogenetically specific probes will be needed to understand the physiology and ecological role of the novel microbial lineages indicated by this study. Culture collections may contain strains which are closely related to the lineages we describe but whose role in oceanic ecosystems has not been previously established. Furthermore, the abundance of clones within the library may not reflect their numerical abundance in the open-ocean ecosystem, since the polymerase chain reaction may bias the representation of clones within libraries. Quantitative data on abundance can be obtained by independent nucleic acid hybridization experiments. This approach was used previously to establish the abundance of the SAR11 cluster in the open ocean (6).

The construction of the clone library described here has allowed us to investigate genetic diversity within a highly oligotrophic, open-ocean bacterioplankton community. The results support the widespread view that microbial ecosystems contain novel, uncultivated species. A broader view of prokaryotic diversity within this community will be gained from the complete analysis of this library, which is the focus of our continuing efforts.

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