Numerical Dominance and Phylotype Diversity of Marine *Rhodobacter* Species during Early Colonization of Submerged Surfaces in Coastal Marine Waters as Determined by 16S Ribosomal DNA Sequence Analysis and Fluorescence In Situ Hybridization

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Early stages of surface colonization in coastal marine waters appear to be dominated by the marine *Rhodobacter* group of the α subdivision of the division *Proteobacteria* (α -*Proteobacteria*). However, the quantitative contribution of this group to primary surface colonization has not been determined. In this study, glass microscope slides were incubated in a salt marsh tidal creek for 3 or 6 days. Colonizing bacteria on the slides were examined by fluorescence in situ hybridization by employing DNA probes targeting 16S or 23S rRNA to identify specific phylogenetic groups. Confocal laser scanning microscopy was then used to quantify and track the dynamics of bacterial primary colonists during the early stages of surface colonization and growth. More than 60% of the surface-colonizing bacteria detectable by fluorescence staining (Yo-Pro-1) could also be detected with the *Bacteria* domain probe EUB338. *Archaea* were not detected on the surfaces and did not appear to participate in surface colonization. Of the three subdivisions of the *Proteobacteria* examined, the α -*Proteobacteria* were the most abundant surface-colonizing organisms. More than 28% of the total bacterial cells and more than 40% of the cells detected by EUB338 on the surfaces were affiliated with the marine *Rhodobacter* group. Bacterial abundance increased significantly on the surfaces during short-term incubation, mainly due to the growth of the marine *Rhodobacter* group organisms. These results demonstrated the quantitative importance of the marine *Rhodobacter* group in colonization of surfaces in salt marsh waters and confirmed that at least during the early stages of colonization, this group dominated the surface-colonizing bacterial assemblage.

The development of a biofilm community on a submerged surface is thought to proceed via an ordered sequence of steps (7, 10, 47), which starts with initial interactions of primary colonists with the surface and proceeds through surface attachment and subsequent colonization by other organisms. Bacterial primary colonists play critical roles in shaping the structure and function of the mature biofilm through establishment of the initial surface-colonizing assemblage, through modification of surface physiochemical properties that make the surface more (or less) suitable for recruitment of later colonists, and through production of adhesive exopolymers. The functions of the primary colonists during the formation of dental plaque are fairly well understood (29). However, the role(s) of primary colonists of surfaces in other environments, including marine environments, is largely unknown.

Recent studies have shown that one of the major groups of the α subdivision of the division *Proteobacteria* (α -*Proteobacteria*), the marine *Rhodobacter* group (12), plays a number of important roles in coastal marine ecosystems. These organisms are numerically important bacterioplankton in southeastern United States estuaries and other coastal environments (19, 36,

44) and are active participants in the carbon and sulfur cycles (22, 23). They are particularly important in the degradation and mineralization of vascular plant (mainly *Spartina alterniflora*)-derived lignocellulose materials and lignin-derived aromatic compounds (6, 21). The marine *Rhodobacter* group organisms were also the most common primary colonists on a variety of artificial surfaces incubated in a southeastern United States salt marsh tidal creek (14). However, the dominance of this group was assessed by constructing and screening 16S ribosomal DNA (rDNA) libraries (14). These methods are problematic for quantitative analysis because of their potential biases (48). Fluorescence in situ hybridization (FISH) with DNA oligonucleotide probes targeting the 16S and/or 23S rRNA (4), coupled with confocal laser scanning microscopy (CLSM) (8, 31), is better suited for determining bacterial community composition and dynamics on surfaces.

In this study, we used FISH and CLSM to quantitatively examine primary surface colonists. Our goals were to identify and to quantify the primary colonists, particularly members of the marine *Rhodobacter* group. We also extended our examination of the diversity of *Rhodobacter* group organisms on freshly colonized surfaces in salt marsh tidal creeks.

MATERIALS AND METHODS

Sequence analyses of 16S rDNA clone libraries. In our previous study (14), partial 16S rDNA sequences (length, approximately 585 bp) and their phylogenetic affiliations were determined for 26 unique clones selected from 12 16S

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TABLE 1. DNA oligonucleotide probes used in this study

Probe	Specificity	Probe sequence (5'-3')	Target site (rRNA, positions) ^a	Label	% Formamide ^b	Reference
EUB338	Domain <i>Bacteria</i>	GCTGCTCCCGTAGGAGT	16S, 338–355	Cy3 or Cy5	20 or 35	3
ALF968	α - <i>Proteobacteria</i>	GGTAAGGTTCTGCGCGTT	16S, 968–986	Cy3	35	18
Rb	<i>Rhodobacter</i> group	GTCAGTATCGAGCCAGTGAG	16S, 735–754	Cy3	35	17
R1	<i>Ruegeria atlanticum</i> cluster	TGCTACTGTCATTATCATACA	16S, 444–490	Cy3	35	This study
R2	<i>Sagittula stellata</i> cluster	CCGAATTGCATGCAACCCG	16S, 835–853	Cy3	35	This study
R3	<i>Ruegeria algicola</i> cluster	CTAGACCAGGAGTTTGG	16S, 637–656	Cy3	20	This study
GAM42a	γ - <i>Proteobacteria</i>	GCCTTCCCACATCGTTT	23S, 1027–1043	Cy5	35	34a
SRB385	Some δ - <i>Proteobacteria</i>	CGGCGTCGCTGCGTCAGG	16S, 385–402	Cy5	35	3
ARCH915	Domain <i>Archaea</i>	GTGCTCCCCGCCAATTAAT	16S, 915–935	Cy5	20	43a
NON8	Negative control	AGAGTTTGATCCTGGCTCA	None	Cy5	20	This study

^a 16S or 23S rRNA positions based on *Escherichia coli* numbering.

^b Percentage of formamide in the in situ hybridization buffer.

rDNA clone libraries (a total of 136 unique clones) derived from primary colonists from a variety of artificial surfaces having different physicochemical properties. Two of these clone libraries, BR-24 and BR-72 [obtained after 24 and 72 h of incubation of plates coated with the bis(2-hydroxyethyl ether) of tetrabromobisphenol, designated surface type BR], were selected for further examination through additional sequencing and rarefaction analyses (25, 27, 43). In this study, 29 additional unique clones from the BR libraries were sequenced in order to expand our database of primary colonist sequences. The methods used for sequencing and sequence analysis have been described previously (14). Rarefaction curves were produced by using an analytical approximation algorithm (27), and 95% confidence intervals were estimated (25) by using aRarefactWin (<http://www.uga.edu/P5strata/software/index.html>). National Center for Biotechnology Information GenBank and Ribosomal Database Project (RDP) II database 16S rDNA sequences most similar to our cloned sequences were used for phylogenetic reconstruction, in which both distance and maximum-parsimony methods were used.

Slide incubation in the field. Since our previous work showed that the major types of primary colonists were very similar for a broad range of artificial surfaces (14), we used glass microscope slides as the model surface in this study. Pre-cleaned Gold Seal glass microscope slides having two etched 10-mm-diameter rings (Fisher, Pittsburgh, Pa.) were incubated at Oyster Landing in the North Inlet salt marsh system near Georgetown, S.C. (33°20'N, 79°11'W) at a constant depth of 1 m below the water surface starting on 4 March 2000 (spring) and 17 July 2000 (summer). The average water temperatures during incubation were 19 and 33°C for the spring and summer, respectively, and the average salinities were 29 and 35 ppt, respectively. Replicate slides were recovered after 3 or 6 days of incubation. Microscopic examination indicated that slides incubated in the spring for less than 24 h contained too few bacterial cells for accurate quantification (data not shown).

Slide sample fixation. Upon recovery, slides were fixed with 4% paraformaldehyde in 1× phosphate-buffered saline (7 mM Na₂HPO₄–3 mM NaH₂PO₄ [pH 7.2] containing 130 mM NaCl) for at least 4 h at 4°C. The fixed slides were washed once with 1× phosphate-buffered saline for 30 min, air dried, dehydrated in an ascending ethanol series (50, 80, and 100% ethanol, 3 min each), and then air dried at room temperature. The slides were stored in sealed petri dishes at 4°C until they were used.

Probe selection, design, and optimization. The DNA oligonucleotide probes employed for FISH were selected based on the types of sequences recovered from the 16S rDNA libraries (14) and are shown in Table 1. We used well-established probes for the *Bacteria*, *Archaea*, α -*Proteobacteria*, γ -*Proteobacteria*, δ -*Proteobacteria*, and *Rhodobacter* group and, in addition, air new probes, R1, R2, and R3, that were designed to target three clusters of 16S rDNA sequences frequently recovered from members of the marine *Rhodobacter* group. The probes were evaluated by using the RDP II Check-Probe program (33) and were also checked by using the National Center for Biotechnology Information GenBank database and the advanced BLAST search program (2). Probe R1 targets sequences found in the *Ruegeria atlanticum* subgroup, probe R2 targets sequences found in the *Sagittula stellata* subgroup, and probe R3 targets sequences found in the *Ruegeria algicola* subgroup. The subgroups targeted by probes R1 to R3 do not include all members of the marine *Rhodobacter* group.

The in situ hybridization conditions used for probes EUB338, ARCH915, ALF968, GAM42a, and SRB385 were those used in previous studies (Table 1). To optimize the in situ hybridization conditions for probes Rb and R1 to R3, we

employed six different combinations of hybridization and wash conditions. The concentrations of formamide in the hybridization buffers tested were 20, 30, 35, 49, 50, and 60%, and the corresponding concentrations of NaCl in the wash buffers were 225, 102, 88, 62.4, 31.2, and 15.6 mM, respectively. The positive control bacteria *Ruegeria gelatinovorans* B6 (= ATCC 25655), *R. algicola* FF3 (= ATCC 51440), and *S. stellata* E-37 (= ATCC 700073) and the negative control bacteria *Rhizobium leguminosarum* biovar trifolii TA1, *Rhizobium leguminosarum* biovar viciae USDA 2370, and *Sinorhizobium meliloti* USDA 1025 were used to test probe Rb. The positive control bacterium *R. gelatinovorans* and the negative control bacteria *R. algicola* and *S. stellata* were used to test probe R1. The positive control bacterium *S. stellata* and the negative control bacteria *R. algicola* and *R. gelatinovorans* were used to test probe R2. As there was no matching sequence from any formally described bacterium for probe R3, no positive control bacterium was used to test this probe. *R. algicola* was used as the negative control bacterium for R3 because probe R3 had one mismatch with the corresponding 16S rDNA target sequence of *R. algicola*. The final hybridization conditions for R3 were based both on probe testing and on the theoretical melting temperature of the duplex of probe R3 and its target 16S rRNA sequence (46). Positive and negative control bacteria were spotted onto gelatin-coated [0.1% gelatin, 0.01% KCr(SO₄)₂] Teflon-coated microscope slides and checked for each hybridization-wash combination. The optimal formamide concentrations for in situ hybridization with probes Rb and R1 to R3 are shown in Table 1. A negative control probe to test nonspecific probe binding, NON8, was designed for this study based on a *Bacteria* domain-specific sequence (26) (Table 1). The frequently used negative control probe NON338 (45) could not be used with EUB338 for our in situ triple-staining technique (see below).

FISH and bacterial cell staining. Probes were synthesized with a primary amino group at the 5' end. The indocarbocyanine fluorescent dye Cy3 or Cy5 phosphoramidite (Amersham Pharmacia, Piscataway, N.J.) was covalently bound to the amino group of the oligonucleotide probe by using a model 8800 Plus nucleic acid synthesizer (PerSeptive Biosystems, Framingham, Mass.). High-performance liquid chromatography-purified stock preparations of oligonucleotide-dye conjugates were stored at –70°C in sterile double-distilled water. The DNA concentrations in working solutions were adjusted to 50 ng/μl in TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM Na₂EDTA) (35).

An in situ triple-staining technique was employed for FISH. Eighteen microliters of hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl [pH 7.2], 5 mM EDTA, 0.01% sodium dodecyl sulfate, 20 or 35% formamide) was added to each window of the microscope slides, which had been incubated in the field as described above. After the slides were prewarmed at 46°C for 30 min, all subsequent steps were performed in the dark. One microliter of probe EUB338 (Cy5 or Cy3 conjugated) and 1 μl of a (sub)group-specific or negative control probe (Cy3 or Cy5 conjugated) were added to bring the final reaction volume to 20 μl and the final probe concentration to 2.5 ng of DNA/μl for each of the two probes. The slides were placed in sealed petri dishes (39) along with a piece of absorbent paper soaked with 4 ml of hybridization buffer that was added to prevent drying and to provide a formamide-saturated atmosphere (1, 9, 34). The petri dishes were incubated for at least 2 h at 46°C in a shaking water bath incubator. After this, the hybridization buffer was drawn off with a piece of absorbent paper placed at the edge of each window on the slides. The slides were subsequently transferred to 100 ml of prewarmed wash buffer (20 mM Tris-HCl [pH 7.2]–0.01% sodium dodecyl sulfate–10 mM EDTA containing 225 and 88 mM NaCl for hybridization buffer formamide concentrations of 20 and 35%, respectively)

and incubated for 1 h at 48°C. For CLSM analysis, the slides were rinsed with sterile double-distilled water, air dried, counterstained with a general nucleic acid dye, Yo-Pro-1 (0.5 μ M; Molecular Probes Inc., Eugene, Oreg.), for 10 to 20 min at room temperature, and mounted in an antifading glycerol medium containing 0.2% Dabco (1,4-diazobicyclo[2.2.2]octane; Aldrich, Milwaukee, Wis.) (38). Two replicate microscope slides from the same field incubation batch were randomly selected for FISH for each probe combination. One of the two windows of each slide was randomly selected for image collection.

Image collection and analyses. An MRC 1024 Multiphoton microscope (Bio-Rad, Hercules, Calif.) equipped with a krypton-argon laser and an Eclipse TE 300 inverted microscope (Nikon, Melville, N.Y.) was used to collect digital images of slide colonization. The samples were examined by using the excitation and emission lines of the krypton-argon laser as follows: blue (excitation at 488 nm and emission at 540 $\pm$ 15 nm) for Yo-Pro-1, yellow (excitation at 568 nm and emission at 598 $\pm$ 20 nm) for Cy3-conjugated probes, and red (excitation at 647 nm and emission at 680 $\pm$ 16 nm) for Cy5-conjugated probes.

Images were collected by scanning 60 randomly selected microscope fields (30 fields from each of the two replicate windows). For each microscopic field (136 by 136 μ m; area, 18,496 μ m²) a series of thin optical sections was collected by scanning the z dimension at 2- μ m depth intervals. This was especially important for summer 6-day samples, as the thickness of some samples was 40 μ m. Each image was collected by using the LaserSharp program (version 3.2; Bio-Rad) with the Kalman filter and three running average scans. In order to minimize the effect of cross-excitation and photobleaching and to optimize image quality, sequential collection was used (31); i.e., the Cy5-conjugated probe signals were collected first, followed by the Cy3-conjugated probe signals and finally the Yo-Pro-1 stain signals, for each field.

Digital images were compiled by using the Projection and Merge routines of the Confocal Assistant 4.02 software package (<ftp://ftp.genetics.bio-rad.com/Public/confocal/cas>), and cell counts were determined by using the Scion Image beta 4.0.2 software package (Scion Corporation [<http://www.scioncorp.com>]; based on NIH Image for MacIntosh [<http://rsb.info.nih.gov/nih-image>]), with manual correction. Image thresholds were adjusted sizes for bacteria to define the boundaries of cells and the upper and lower size cutoffs for image objects with reasonable (35). This facilitated determination of cell numbers by using the Analyze Particles routine of Scion Image. Only the signals that were detectable with both Yo-Pro-1 and EUB338 were counted as bacteria for each probe combination (except ARCH915) or as false signals for the negative control probe NON8; in this way false signals caused by autofluorescence could be effectively eliminated.

Statistic analysis. FISH data were analyzed by two-way analysis of variance (MINITAB, release 13.31; Minitab Inc., State College, Pa.) with an α value of 0.05 to determine the short-term and seasonal dynamics of bacteria belonging to the phylogenetic groups or subgroups studied. Both the absolute cell numbers and the abundances of these groups and subgroups relative to Yo-Pro-1 and EUB338 cell counts were analyzed.

Nucleotide sequence accession numbers. The 16S rDNA sequences determined in this study have been deposited in the GenBank database under accession numbers AF367380 to AF367404.

RESULTS

Rarefaction analysis of the two BR libraries. Rarefaction analysis was used to estimate the coverage of the two BR libraries (Fig. 1). The rarefaction curves did not reach saturation for either library, indicating that more clones were needed to reveal the full diversity of the bacteria on the submerged surfaces. This finding is consistent with our original diversity estimates based on the percent coverage method (14). However, many of the sequences were closely related to each other (see below).

Phylogenetic analyses of primary colonists on BR plates. Of the 29 clones sequenced in this study, 15 were affiliated with the α -Proteobacteria, 7 were affiliated with the γ -Proteobacteria, 2 were affiliated with the δ -Proteobacteria, 1 was affiliated with the gram-positive bacteria, and 4 were apparently chimeras. Only one clone in the two BR clone libraries exhibited an exact (100%) sequence match with a known bacterium; clone D060 was identical to the 16S rDNA of *Methylobacterium extorquens*

FIG. 1. Rarefaction curves for the different amplified rDNA restriction analysis (ARDRA) patterns (unique clones) of the two libraries derived from the BR surfaces (see Materials and Methods). The standard deviations of the expected values are indicated by error bars.

in the DNA sequence region examined. If sequences recovered from the BR libraries in the previous study (14) were included, the α -Proteobacteria accounted for 72.1% (31 of 43 unique clones, excluding chimeras) of the primary colonist sequences.

The results of phylogenetic analyses performed by the distance and parsimony methods were very consistent for the sequence affiliations within the α -Proteobacteria (Fig. 2). A substantial number of the α -Proteobacteria sequences (12 sequences) were most closely related to the marine *Rhodobacter* group, and the levels of similarity to known sequences ranged from 94.9 to 99.1%. If the sequences from our previous study (14) were included, the marine *Rhodobacter* group accounted for 67.4% (29 of 43 unique clones) of the primary colonists recovered from the BR plates. The levels of sequence similarity for the cloned sequences ranged from 90.1 to 100%, indicating that diverse, but closely related *Rhodobacter* phylotypes occurred on the BR surfaces.

The seven cloned sequences affiliated with the γ -Proteobacteria were related (range of levels of similarity, 93.6 to 95.1%) to several important marine bacterial groups, including the genera *Pseudomonas*, *Alteromonas*, *Pseudoalteromonas*, and *Oceanospirillum*, as well as to cultivated and uncultivated bacterial symbionts associated with marine invertebrates and algae. The results obtained by the distance and parsimony methods were also very consistent for the sequence affiliations within the γ -Proteobacteria (Fig. 2). The γ -Proteobacteria sequences accounted for 16.3% of the total primary colonist sequences recovered from the BR plates in both the previous study (14) and this study.

Two sequences recovered from the BR plates were affiliated with the δ -Proteobacteria (Fig. 2). The levels of sequence similarity to the most similar known sequences in the GenBank and RDP II databases were 89.4% for clone D062 and 99.3% for clone D070. A single clone (D064) recovered from the BR plates was affiliated with the high-G+C-content gram-positive bacterial group (Fig. 2); it was only 89.5% similar to the most similar previously described sequence.

Total cell counts on the glass surfaces. The total cell counts for the glass surface samples were determined by using the nucleic acid stain Yo-Pro-1 and are shown in Table 2. Due to the heterogeneous spatial distribution of the bacteria on the

FIG. 2. Phylogenetic tree showing the affiliations of the cloned, nonchimeric sequences recovered from the two BR 16S rDNA libraries. Twenty-five of the sequences were from this study and are indicated by boldface type. The tree was constructed by using the maximum-parsimony method. The distances represent the numbers of nucleotide substitutions per homologous sequence site. Nodes labeled with asterisks were supported by bootstrap values greater than 70% (based on 100 bootstrap resamplings) in both maximum-parsimony and neighbor-joining analyses. Nodes labeled with exclamation points were supported by bootstrap values greater than 70% in either maximum-parsimony or neighbor-joining analysis. The sequence clusters targeted by the three probes designed for this study, probes R1, R2, and R3, are also shown.

TABLE 2. Yo-Pro-1 total cell counts

Incubation	Total cell count (10^3 mm^{-2}) (mean $\pm$ SD)
Spring, 3 days	2.62 $\pm$ 1.10
Spring, 6 days	9.37 $\pm$ 3.35
Summer, 3 days	5.52 $\pm$ 2.04
Summer, 6 days	29.45 $\pm$ 5.36

surfaces, the cell numbers were highly variable even within the same batch of slides incubated in the same season and collected on the same date. The morphologies of the bacteria stained by Yo-Pro-1 were diverse and included cocci, rods, spirilla, filaments, cell chains, and colonies of various sizes. Statistically significant (two-way analysis of variance, $P < 0.05$) increases in absolute cell numbers for short-term incubations were observed for both spring and summer surface samples. The cell numbers for the summer samples were significantly higher than the cell numbers for the spring samples for both 3- and 6-day incubations.

Domain-specific FISH. When probe EUB338 was used, bacteria accounted for (63.1 ± 14.6) to (82.1 ± 4.4)% of the total cell counts determined with Yo-Pro-1 (Fig. 3). The types of bacterial morphology detected by EUB338 on the glass slides

included the same major types observed when Yo-Pro-1 was used. The absolute cell numbers and the relative levels of EUB338-detectable cells were significantly higher for 6-day incubations than for 3-day incubations during both the spring and the summer. No *Archaea* were detected with probe ARCH915 in any sample. The numbers of false signals due to autofluorescence or nonspecific probe binding, as determined with negative control probe NON8, were very low (Fig. 3 and 4). Results obtained with slides containing neither Cy3-conjugated nor Cy5-conjugated probes showed that the false signals were due mainly to autofluorescence of phototrophs (cyanobacteria or microalgae) and inorganic particles and that nonspecific probe binding made only a small contribution.

Group-specific FISH. Probes targeting three major phylogenetic subdivisions (α -, γ -, and δ -*Proteobacteria*) important in marine systems were used to determine the affiliations of bacteria on the glass surfaces more precisely. Many of the cells on the surfaces could be detected with probe ALF968. The α -*Proteobacteria* accounted for (34.1 ± 15.1) to (54.6 ± 16.6)% of the total cells (Fig. 3) and (48.7 ± 19.0) to (66.1 ± 16.7)% of the EUB338-detectable cells (Fig. 4). The absolute cell numbers and relative levels of α -*Proteobacteria* were significantly greater after 6 days of incubation than after 3 days of incubation. Both the absolute cell numbers and the relative levels were significantly higher in the spring than in the summer.

FIG. 3. Representations of bacterial groups determined with group- and subgroup-specific FISH probes relative to total cell counts determined by using Yo-Pro-1. d, days.

FIG. 4. Representations of bacterial groups determined with group- and subgroup-specific FISH probes relative to the bacterial counts determined by using probe EUB338. d, days.

The γ -*Proteobacteria* accounted for (3.8 ± 3.0) to $(16.6 \pm 7.3)\%$ of the total cells detected with Yo-Pro-1 (Fig. 3) and (4.6 ± 3.9) to $(22.2 \pm 10.5)\%$ of the EUB338-detectable cells (Fig. 4). The absolute cell numbers and the relative levels of the γ -*Proteobacteria* decreased significantly during short-term incubation. The absolute cell numbers and the relative levels of the γ -*Proteobacteria* on glass surfaces were significantly higher in the summer than in the spring.

The δ -*Proteobacteria* identified with probe SRB385 accounted for (4.0 ± 3.4) to $(6.3 \pm 4.0)\%$ of the total bacterial cells detected with Yo-Pro-1 (Fig. 3) and (5.2 ± 4.4) to $(9.5 \pm 6.7)\%$ of the EUB338-detectable cells (Fig. 4). Both the absolute cell numbers of δ -*Proteobacteria* and the levels compared to the levels of EUB338-detectable cells decreased slightly during short-term incubation. Because probe SRB385 does not detect all members of the δ -*Proteobacteria* (3), the numbers of δ -*Proteobacteria* cells may have been underestimated.

α -*Proteobacteria* subgroup-specific FISH. Probe Rb was used to determine the abundance of the bacteria in the marine *Rhodobacter* group. The *Rhodobacter* group accounted for (27.7 ± 18.6) to $(50.1 \pm 22.0)\%$ of the total cell counts determined with Yo-Pro-1 (Fig. 3) and (42.7 ± 14.6) to $(60.0 \pm 23.4)\%$ of the EUB338-detectable cells (Fig. 4). The absolute numbers of Rb-detectable cells were significantly higher after 6 days of incubation than after 3 days of incubation, as were the levels of Rb-detectable cells relative to the total cell counts and to the EUB338-detectable cell counts. The levels of *Rhodobacter* cells relative to the EUB338-detectable cell

counts were significantly lower in the summer than in the spring.

Probes R1, R2, and R3, which targeted three clusters of marine *Rhodobacter*-like 16S rRNA sequences defined in our previous study (14), were used in an attempt to further determine the affiliations of the primary colonists. However, the number of bacteria detected by these probes was less than one-half the number detected by probe Rb.

The R1-targeted bacteria accounted for (1.3 ± 1.5) to $(6.0 \pm 3.3)\%$ of the total cell counts (Fig. 3) and (1.9 ± 2.2) to $(8.2 \pm 5.4)\%$ of the EUB338 cell counts (Fig. 4). The absolute cell numbers and the relative levels of R1-targeted bacteria were significantly lower after 6 days of incubation than after 3 days of incubation. The absolute cell numbers and relative levels of R1-targeted bacteria were significantly higher during the summer than during the spring.

The R2-targeted bacteria accounted for (4.31 ± 3.84) to $(6.8 \pm 2.9)\%$ of the total cell counts (Fig. 3) and (5.5 ± 3.8) to $(9.2 \pm 4.0)\%$ of the EUB338-detectable cells (Fig. 4). The absolute numbers of R2-targeted bacterial cells were significantly lower after 6 days of incubation than after 3 days of incubation in the summer, and the relative level decreased during short-term incubation in both the spring and the summer.

The R3-targeted bacteria accounted for (3.4 ± 2.4) to $(6.3 \pm 2.2)\%$ of the total cell counts (Fig. 3) and (4.2 ± 3.4) to $(8.9 \pm 6.6)\%$ of the EUB338 cell counts (Fig. 4). Both the absolute cell numbers and the relative levels decreased significantly during short-term incubation in the summer.

DISCUSSION

Although two-phase rRNA analysis, also called full-cycle rRNA analysis, has been recommended for bacterial community analysis in situ (4), few environmental samples have been investigated using this approach (42). This study is the first study in which key groups of bacterial primary colonists of a submerged artificial surface in a coastal ecosystem were quantified by using a combination of 16S rDNA sequence analysis, FISH, CLSM, and digital imaging and analysis techniques. Previous data obtained by 16S rDNA library construction and screening revealed the compositional dominance of the assemblage of primary colonists on submerged surfaces by bacteria belonging to the marine *Rhodobacter* group (14). However, PCR biases might obscure the contributions of some lineages (11), resulting in an inaccurate assessment of the abundance of members of the marine *Rhodobacter* group on freshly colonized surfaces. This study was undertaken to extend and to quantitatively assess our previous findings.

In order to more thoroughly assess the diversity of the marine *Rhodobacter*-like phylotypes, we first analyzed additional clones in our previously constructed libraries. The new 16S rDNA sequence data support our previous findings, although more sequences from organisms not belonging to the marine *Rhodobacter* group were recovered. In addition to the compositional dominance observed for this group, the diversity of organisms belonging to the primary surface-colonizing marine *Rhodobacter* group was also impressive. For the BR plates, 29 of the 43 unique clones were from members of the marine *Rhodobacter* group, and their levels of sequence similarity ranged from 90 to 100% (the differences detected in clones D005, D009, and D099 were outside the 16S rDNA segment sequenced). Numerous diverse 16S rDNA sequences of members of the marine *Rhodobacter* group have been recovered from various marine ecosystems (12, 14, 16, 17, 19, 20, 22–24, 36, 41, 44), and this is clearly a significant and heterogeneous group of organisms.

In order to quantitatively assess the numerical dominance of the marine *Rhodobacter* group organisms on freshly colonized slides, FISH was employed, and the quantitative results were collected by using CLSM imaging and image analysis. Due to the significant spatial heterogeneity of bacterial cells on submerged surfaces, highly variable values for both absolute cell numbers and relative levels were obtained. Sixty replicate images were obtained, and a relatively large area of the glass surface was sampled ($60 \times 18,496 \mu\text{m}^2 \approx 1.11 \text{ mm}^2$) for each treatment (probe). Korber et al. (30) indicated that a minimum area of $10^5 \mu\text{m}^2$ is required for statistically valid determinations of cell numbers in highly heterogeneous samples, such as intact biofilm samples. Our total sampling area on the glass surfaces was 10 times larger than this minimum requirement. The high standard deviations obtained in our analyses were typical for extremely patchy cell distributions, as would be expected for submerged surfaces and undisturbed biofilm samples (35).

More than 63% of the total bacterial cells on the glass slides could be detected by using the *Bacteria* domain probe EUB338. The detection rates of EUB338 in this study were typical, as numerous FISH studies in which EUB338 was used produced similar or lower detection rates for natural marine

and freshwater systems (18, 35). Failure to detect some cells by FISH has been attributed to incomplete detection of *Bacteria* with EUB338 (13) and/or to the fact that not all bacteria in natural systems are readily detectable (due to low numbers of ribosomes [37], for example). However, other factors could contribute to the failure of FISH to detect some bacterial cells, including poor penetration of the probes into some cells (4).

The finding that no *Archaea* could be detected on the slides with probe ARCH915 was consistent with results obtained with river biofilms (35). On our glass slides, ARCH915 stained only a few particles that were also stained by EUB338, as revealed by the triple-staining technique. The cross-detection of the putative bacterial cells by both the *Bacteria* probe and the *Archaea* probe suggested that the false-positive signals obtained with ARCH915 were due to nonspecific binding of ARCH915 to *Bacteria* or due to autofluorescence by phototrophs or by inorganic particles. This finding emphasized the importance of the triple-staining technique, as false-positive and false-negative signals are among the most serious and frequently encountered problems for bacterial FISH identification and community analyses (40). Inadequate detection of *Archaea* with ARCH915 due to incomplete detection of the *Archaea* by this probe, or to poor probe penetration, might also contribute to low detection of *Archaea* (18), or *Archaea* simply may not have participated in surface colonization or the early stages of biofilm formation.

By using the three *Proteobacteria* subdivision-specific probes, ALF968, GAM42a, and SRB385, the phylogenetic affiliations of the majority (75.9 to 89.1%) of the bacteria detectable with the EUB338 probe could be determined more precisely. This indicates that bacteria belonging to other phylogenetic groups that were not examined in this study were not very important numerically on the surfaces, at least during the early stages of bacterial colonization and biofilm formation. Significant increases in cell numbers with incubation time both in the spring and in the summer were mostly due to α -*Proteobacteria* ($y = 0.5048x - 201.9$ [$R^2 = 0.9936$; absolute numbers of EUB338- and ALF968-detected bacterial cells]) and particularly the marine *Rhodobacter* group ($y = 0.3775x + 130.67$ [$R^2 = 0.9802$; absolute numbers of EUB338- and Rb-detected bacterial cells]) and $y = 0.7502x + 270.24$ [$R^2 = 0.9931$; absolute cell numbers of ALF968- and Rb-detected bacterial cells]), further indicating that groups that were not examined in this study were not numerically important in primary surface colonization.

While it is clear that methodological biases could obscure the contributions of some taxa to primary surface colonization (11), FISH results obtained with probe Rb strongly supported our previous finding that members of the *Rhodobacter* group are dominant in primary surface colonist communities. More than 43% of the EUB338-detectable bacteria on the surfaces were affiliated with the *Rhodobacter* group. The contribution of the marine *Rhodobacter* group to surface colonization was also clear from the presence of colonies consisting of tens to hundreds of Rb-detectable bacteria that were visible in the CLSM images, especially in the spring 6-day and summer 3- and 6-day samples. In contrast, members of the other bacterial groups, such as the γ -*Proteobacteria* and the δ -*Proteobacteria*, never exhibited similar abundance or formed extensive colonies on slides. The finding that the γ -*Proteobacteria* group was not a dominant bacterial group on our surfaces (14; this study) is

consistent with data obtained previously for both freshwater and marine systems (5, 28, 32, 35).

The failure of the three sequence cluster-specific probes which we designed, R1, R2, and R3, to detect the majority of the bacteria belonging to the marine *Rhodobacter* group on glass surfaces should not be considered surprising. We recovered a substantial diversity of marine *Rhodobacter*-like sequence types from our clone libraries in both the previous study (14) and the present study, and the three probes matched only a subset of the sequences which we recovered. The high yields for clones belonging to the R1 and R3 sequence groups from the 16S rDNA clone libraries could have been due in part to PCR biases, as some 16S rDNA sequences from α -*Proteobacteria* appear to be subject to preferential amplification (11). Low in situ accessibility of the probe target site of the 16S rRNAs for probe R3 might be another factor that contributes to the low detection rate of R3. This would result in a lower hybridization rate for the 16S rRNA sequence targeted by R3 and hence weaker fluorescence (15). Although the 16S rDNA sequences recovered from surfaces having a wide variety of physicochemical properties were remarkably consistent from surface to surface (14), the surface used might also play a role in the selection of certain bacteria for surface colonization and growth. For example, pure cultures of the marine bacterium *S. stellata*, which was closely related to our R2-targeted sequences, could not attach to glass surfaces, although they could attach to cellulose or lignocellulose particles (21, 22). Apparently, a common *Rhodobacter* strain(s) on the glass surfaces escaped detection by our *Rhodobacter* sequence cluster probes. This result also demonstrates, indirectly, the substantial genotypic diversity of the marine *Rhodobacter* group on submerged surfaces.

Using 16S rDNA clone library construction, sequencing and sequence analysis, and 16S or 23S rRNA probing with CLSM visualization and quantitative image analysis, we demonstrated the numerical dominance and the genotype diversity of the marine *Rhodobacter* group during early stages of colonization of submerged surfaces in a coastal marine salt marsh system. It is clear that organisms belonging to the marine *Rhodobacter* group are highly successful colonists of submerged surfaces in salt marsh waters and, as demonstrated by increases in numbers during short incubations, capable of proliferation on these surfaces. The extent of participation of these organisms in the development of biofilms and biofouling communities and their exact roles after colonization await further investigation.

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