

## Acetogenic and Sulfate-Reducing Bacteria Inhabiting the Rhizoplane and Deep Cortex Cells of the Sea Grass *Halodule wrightii*†

KIRSTEN KÜSEL,<sup>1</sup> HOLLY C. PINKART,<sup>1‡</sup> HAROLD L. DRAKE,<sup>2</sup> AND RICHARD DEVEREUX<sup>1\*</sup>

Gulf Ecology Division, U.S. EPA/National Health and Environmental Effects Research Laboratory,  
Gulf Breeze, Florida 32561,<sup>1</sup> and Department of Ecological Microbiology, BITOEK,  
University of Bayreuth, 95440 Bayreuth, Germany<sup>2</sup>

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Recent declines in sea grass distribution underscore the importance of understanding microbial community structure-function relationships in sea grass rhizospheres that might affect the viability of these plants. Phospholipid fatty acid analyses showed that sulfate-reducing bacteria and clostridia were enriched in sediments colonized by the sea grasses *Halodule wrightii* and *Thalassia testudinum* compared to an adjacent unvegetated sediment. Most-probable-number analyses found that in contrast to butyrate-producing clostridia, acetogens and acetate-utilizing sulfate reducers were enriched by an order of magnitude in rhizosphere sediments. Although sea grass roots are oxygenated in the daytime, colorimetric root incubation studies demonstrated that acetogenic O-demethylation and sulfidogenic iron precipitation activities were tightly associated with washed, sediment-free *H. wrightii* roots. This suggests that the associated anaerobes are able to tolerate exposure to oxygen. To localize and quantify the anaerobic microbial colonization, root thin sections were hybridized with newly developed <sup>33</sup>P-labeled probes that targeted (i) low-G+C-content gram-positive bacteria, (ii) cluster I species of clostridia, (iii) species of *Acetobacterium*, and (iv) species of *Desulfovibrio*. Microautoradiography revealed intercellular colonization of the roots by *Acetobacterium* and *Desulfovibrio* species. Acetogenic bacteria occurred mostly in the rhizoplane and outermost cortex cell layers, and high numbers of sulfate reducers were detected on all epidermal cells and inward, colonizing some 60% of the deepest cortex cells. Approximately 30% of epidermal cells were colonized by bacteria that hybridized with an archaeal probe, strongly suggesting the presence of methanogens. Obligate anaerobes within the roots might contribute to the vitality of sea grasses and other aquatic plants and to the biogeochemistry of the surrounding sediment.

Sea grasses are centrally important to the productivity of estuarine ecosystems. They provide a link between sediment and water nutrient cycles, stabilize sediments, and offer protective habitats for invertebrates, fish, and their larvae (24, 52). Despite loss of sea grasses to disease and coastal development, little attention has been given to microorganisms that might affect their viability (24, 43, 49). Although sea grass roots were once considered to serve primarily as anchors, it is now known that in certain species they are important for obtaining nutrients (25, 57). In particular, upwards of 50% of the plant nitrogen requirement may be fulfilled by nitrogen fixation in the rhizosphere (15, 16). Sea grass roots exude easily degradable organic carbon compounds that may chemotactically attract microorganisms from the surrounding sediment (57). Most rhizosphere bacteria are thought to live either near the root tips due to the excretion of mucilage or in the rhizoplane, which is defined as the root surface and outermost cell layers (13).

Sea grasses are adapted to being rooted in highly reduced, anoxic sediments where high rates of sulfate reduction might yield toxic levels of sulfide (7, 8, 57). During the day, oxygen produced by leaf photosynthesis is transported to the roots via mass flow or diffusion, generating an oxygen gradient around

the roots. At night, the roots enter a near-dormant state of anaerobic fermentative metabolism and excrete phytotoxins like ethanol (36, 52). Thus, strict anaerobes in the sea grass rhizosphere could experience periods of elevated oxygen tension. While rates of anaerobic microbial processes are known to be high in sea grass bed sediments (8, 9, 31), little is known about the colonization of aquatic plant roots by anaerobic bacteria or their potential occurrence in deeper root cell layers.

The ability to detect bacteria in situ with rRNA probes (5) makes it possible to design and optimize oligonucleotide probes that target anaerobes enriched in sea grass rhizospheres and to localize and quantify the distribution of anaerobic bacteria associated with sea grass roots on a microhabitat scale. The objectives of this study were therefore to examine (i) the extent that sea grass rhizosphere sediments are enriched with anaerobic bacteria and (ii) the colonization of sea grass roots by the anaerobes.

### MATERIALS AND METHODS

**Field site and sampling.** The sampling site for the study was near Big Sabine Point in Santa Rosa Sound, located in northwestern Florida. This sound is relatively unimpacted by pollution and contains a sea grass bed dominated by *Halodule wrightii* interspersed with *Thalassium testudinum*. Sediments and sea grasses were obtained in clear plastic cores (cellulose-acetate-butyrate; Wildlife Supply Co., Saginaw, Mich.; inner diameter, 4.7 cm) from a depth of about 1 m below the surface of the overlying brackish water. Sea grasses rooted to a maximum depth of approximately 10 to 12 cm; sediment from this zone was considered rhizosphere sediment due to the dense network of roots. Unvegetated sediment samples were collected from a barren site immediately adjacent to the sea grass bed. The pH of the sediments was approximately 7.2.

**Phospholipid fatty acid (PLFA) analysis.** Four replicate sediment cores from each site were obtained in late June and mid-October 1997, placed in a -70°C freezer for storage within an hour of collection, and sectioned by depth just prior to analysis (within 1 month of collection). Total lipids were extracted by using a modified Bligh-Dyer extraction procedure (59). The phospholipid and plasmalo-

\* Corresponding author. Mailing address: Gulf Ecology Division, U.S. EPA/NHEERL, 1 Sabine Island Dr., Gulf Breeze, FL 32561. Phone: (850) 934-9346. Fax: (850) 934-9346. E-mail: [devereux.richard@epamail.epa.gov](mailto:devereux.richard@epamail.epa.gov).

† Contribution no. 1064 from the Gulf Ecology Division, U.S. EPA/NHEERL.

‡ Present address: Department of Biological Sciences, Central Washington University, Ellensburg, WA 98926.

TABLE 1. 16S rRNA oligonucleotide probes and target groups

Probe	Sequence (5'-3')	Target site <sup>a</sup>	Positive control	Wash temp (°C)	Targeted bacteria
LGC	TCACGCGGCGTTGCTC	399	<i>B. subtilis</i>	51	Low-G+C-content gram-positive organisms <sup>b</sup>
Clost I	TTCTTCCTAATCTCTACGCA	864	<i>C. magnum</i>	53	88 clostridia of cluster I, 2 clostridia of cluster II
AW	GGCTATTCCCTTCCATAGGG	228	<i>A. woodii</i>	52	4 <i>Acetobacterium</i> species, <i>E. limosum</i>
DSV	TGGGCCGTGTTNCAGT	344	<i>D. desulfuricans</i>	46	13 <i>Desulfovibrio</i> species

<sup>a</sup> According to numbering of the *Escherichia coli* 16S rRNA.

<sup>b</sup> The LGC probe targets 104 *Bacillus* species, 45 *Staphylococcus* species, 41 *Mycoplasmata* species, 37 *Lactobacillus* species, 33 *Streptococcus* species, 25 *Paenibacillus* species, 15 *Brevibacillus* species, 11 *Sporolactobacillus* species, and also some other genera in low numbers.

gen fractions were obtained through fractionation of the total lipid extract by solid-phase extraction (45). The fractionated components were methylated by using a mild alkaline methanolysis followed by a mild acid methanolysis (59). The resulting fatty acid methyl esters and plasmalogens were separated, quantified, and identified by gas chromatography-mass spectrometry (58).

**Enumeration of sediment microflora.** Anaerobic bacteria were enumerated by the most-probable-number (MPN) technique in selective media (39), using a 10-fold dilution series with three replicate tubes per dilution. Anoxic media were prepared by using the modified Hungate technique (32). All substrates and inhibitors were added from anoxic sterile stock solutions.

Acetogens were cultivated in an undefined, reduced, bicarbonate-buffered medium (21) supplemented with NaCl (20 g liter<sup>-1</sup>) and either vanillate (6 mM) plus Ti(III)-nitriacetate solution (0.25 mM) (42) or H<sub>2</sub> (30 mM). Bromoethanesulfonic acid was added to a final concentration of 15 mM to inhibit methanogenic bacteria. The gas phase was 100% CO<sub>2</sub>, and the final pH was approximately 6.9. Acetogenic activity with vanillate was visualized by a colorimetric reaction, based on the capacity of acetogens to O demethylate methoxylated aromatic compounds via the acetyl coenzyme A pathway, described by Harriott and Frazer (29). Protocatechuate and catechol, the O-demethylated products of vanillate, form a yellow complex with Ti(III) (41).

To cultivate clostridia, an undefined, reduced medium that contained the following components (in grams per liter) was prepared: NaCl, 20.0; K<sub>2</sub>HPO<sub>4</sub>, 5.0; MgCl<sub>2</sub> · 6H<sub>2</sub>O, 0.2; FeCl<sub>3</sub> · 6H<sub>2</sub>O, 0.2; methylene blue, 0.002; yeast extract, 1.0; L-arginine-HCl, 0.5; L-lysine-HCl, 0.5; cellulose, 7.0; Na<sub>2</sub>S · 9H<sub>2</sub>O (as the reducing agent), 0.5; and the redox indicator resazurin, 0.001. A mineral solution containing MnCl<sub>2</sub> · 4H<sub>2</sub>O (10.0 mM), CaCl<sub>2</sub> · 2H<sub>2</sub>O (30.0 mM), CoCl<sub>2</sub> · 6H<sub>2</sub>O (5.0 mM), and Na<sub>2</sub>MoO<sub>4</sub> · 2H<sub>2</sub>O (5.0 mM) was added at 0.5 ml per liter. The gas phase was 100% N<sub>2</sub>, and the pH after autoclaving was approximately 6.9. To selectively enrich for spore-forming clostridia, 10 g (wet weight) of sediment was treated with 10 ml of ethanol (95%) for 1 h on a shaker (35) at room temperature prior to dilution.

Sulfate-reducing bacteria were enumerated in a defined, reduced saltwater medium (18) with either acetate (5 mM) or lactate (5 mM) as the electron donor. The gas phase was N<sub>2</sub>-CO<sub>2</sub> (90:10; vol/vol), and the final pH was approximately 7.2.

Tubes were incubated at 30°C for 4 months and inspected weekly. Those that contained H<sub>2</sub> as a substrate were incubated horizontally. Growth was determined visually and verified microscopically. MPN tubes were scored positive based on consumption of substrates or formation of products; uninoculated MPN tubes were used as controls. MPN values were calculated from standard tables and were within 95% certainty (1).

**Root incubation studies.** *H. wrightii* was obtained by coring and brought to the laboratory within 1 h of collection. The cores were released into a sterile glass dish, and the roots were carefully separated from the sediment. Healthy roots, white in appearance and free of lesions, were excised with a clean razor blade and washed twice in sterile phosphate-buffered saline (120 mM sodium phosphate, 0.85% NaCl, pH 7.2) to remove all sediment particles. Five to eight roots (2 to 3 cm long) were transferred to sterile serum bottles containing either (i) 50 ml of the acetogen medium supplemented with vanillate (6 mM) and Ti(III)-nitriacetate solution (0.25 mM) but lacking yeast extract or (ii) 50 ml of filter-sterilized, anoxic seawater under an N<sub>2</sub> gas phase supplemented with acetate, lactate, malate, and ethanol (1 mM each), electron donors typically used by sulfate-reducing bacteria.

**Oligonucleotide probes.** Oligonucleotide probes were designed by examining 16S rRNA sequences obtained from the Ribosomal Database Project (40) and aligned with the CLUSTAL W program (55). The intended probe specificity was verified with the CHECK\_PROBE utility from the Ribosomal Database Project. Oligonucleotide probes were developed and optimized for (i) cluster I of the clostridia (Clost I) (19), (ii) species of *Acetobacterium* and *Eubacterium limosum* (AW), (iii) low-G+C-content gram-positive bacteria (LGC), and (iv) species of *Desulfovibrio* (DSV) (Table 1).

Optimal wash temperatures were determined by hybridizing positive and negative control rRNAs fixed to membranes with <sup>33</sup>P-labeled probes and washing the probe off at increasing temperatures (61). Nucleic acids used in these experiments were obtained from *Desulfovibrio desulfuricans* ATCC 27774, *Acetobac-*

*terium woodii* ATCC 29683, *Bacillus subtilis* 6051 (kindly provided by Fred J. Genthner), and *Clostridium magnum* ATCC 49199. Preparation of nucleic acids, membranes, and hybridization buffers and conditions used were as described by Raskin et al. (48). The hybridization temperature was 40°C.

Additional probes were used for the detection of bacteria (Eub) (5), α-subdivision bacteria (Alfa) (5), archaea (Arch) (48), and sulfate-reducing bacteria (SRB 385) (3). Previously detailed procedures (4) were used to obtain purified oligonucleotides conjugated with the fluorescent dye Cy3, Cy5, fluorescein, or rhodamine (Amersham Life Science, Pittsburgh, Pa., and Research Organics, Cleveland, Ohio) or labeled with <sup>33</sup>P by using [γ-<sup>33</sup>P]ATP (NEN Life Science Products, Boston, Mass.) and polynucleotide kinase (Boehringer Mannheim, Indianapolis, Ind.).

**In situ hybridization studies.** Microbial colonization on the roots incubated in serum bottles for sulfate reduction was examined microscopically by staining intact or sliced roots with 4',6-diamidino-2-phenylindole (DAPI) (Sigma Chemical Co., St. Louis, Mo.) (46) followed by hybridizing with rhodamine-labeled Eub or SRB 385 probe (6, 30). Colonization of freshly collected *H. wrightii* roots was determined by using root thin sections that were hybridized with <sup>33</sup>P-labeled probes and examined with microautoradiography (47). The freshly collected roots were washed twice in sterile phosphate-buffered saline and then fixed overnight in a fresh preparation of 4% paraformaldehyde in 10 mM phosphate buffer, pH 7.2. Fixed roots were transferred to 50% ethanol until sectioned. The roots were embedded in paraffin, and 8-μm sections were fixed on slides (47). The sections were deparaffinized with xylene and then dehydrated through a graded ethanol series. Probe was added to the sections and sealed beneath a coverslip. Hybridization and washing of the root sections were performed essentially as previously described (4).

**Microscopy.** An Optiphot microscope (Nippon Kogaku K.K., Tokyo, Japan), equipped with an EF-D episcopic fluorescence attachment, was used for epifluorescence and phase-contrast microscopy. Confocal laser scanning microscopy (CLSM) was performed with a Bio-Rad (Richmond, Calif.) model 1000 instrument.

**Analytical techniques.** Headspace gases were measured with Hewlett-Packard Co. (Palo Alto, Calif.) 5980 series II gas chromatographs (38). Gas values included the total amounts in both the liquid and gas phases. In this study, no distinction was made between CO<sub>2</sub> and its carbonate forms. Concentrations were corrected for the changing liquid-to-gas phase volume ratio due to liquid samplings (0.7 ml). Aliphatic acids and aromatic compounds were determined with Hewlett-Packard 1090 series II high-performance liquid chromatographs (38). Sulfate was determined turbidimetrically following precipitation with barium (53). The sediment pH was measured with an Ingold (Hightstown, N.J.) U457-S7/110 combination pH electrode.

## RESULTS

**PLFA analysis.** PLFA analyses have been used to monitor shifts in microbial communities, to detect specific groups of microorganisms, and to correlate biogeochemical activities with specific populations (11, 58). Analysis of PLFA biomarkers in samples taken from within the sea grass bed at Big Sabine Point revealed that gram-negative sulfate-reducing bacteria were approximately 11% of the bacterial biomass in the upper root zone of the rhizosphere sediment, compared to 6% in the same zone of the adjacent unvegetated sediment (Table 2). These values correspond to calculated densities of 3.3 × 10<sup>8</sup> and 2.4 × 10<sup>8</sup> sulfate-reducing bacteria g (dry weight) of sediment<sup>-1</sup>, respectively (58). Biomasses of bacteria and sulfate-reducing bacteria decreased slightly with increasing sediment depth (Table 2).

Biomarkers indicative of sulfate reducers belonged primarily

to the genera *Desulfovibrio* and *Desulfobulbus* (34, 54). *Desulfovibrio* species possess a characteristic iso-branched monounsaturated series (iso-pentadecenoic acid [i15:1], iso-heptadecenoic acid [i17:1], and iso-nonadecenoic acid [i19:1]) that is unique to the genus. *Desulfobulbus* species contain the unique monounsaturated heptadecenoic fatty acid (17:1 $\omega$ 6). Relatively little of the methyleneheptadecanoic acids (cy18:0) that are unique for *Desulfobacter* species were detected.

A prominent feature of many clostridia (especially the butyric acid group) is the presence of plasmenyl analogues of phospholipids, known as plasmalogens (42). Acid methanolysis of plasmalogens releases dimethyl acetals, which are readily detected via mass spectrometry. A large amount of saturated acyl chains ranging in length from 12 to 18 carbons was detected (Table 2). These biomarkers (i) were indicative of clostridia, (ii) were found only in upper root zones of the rhizosphere sediment (Table 2), and (iii) yielded calculated densities of approximately  $1.7 \times 10^7$  and  $1.0 \times 10^7$  clostridia g (dry weight) of sediment<sup>-1</sup> in the upper and middle root zones, respectively.

**Enumeration of sediment microflora.** Since the lipid compositions of many acetogens and clostridia have not been characterized, an MPN approach with selective media was used to further evaluate the contributions of clostridia, acetogens, and sulfate reducers to the microbial communities in these sediments. Densities of butyrate-producing clostridia or their spores did not significantly differ between sediments obtained from the upper zones (0 to 10 cm) of the sea grass bed or the unvegetated site (Table 3). The main products from fermentation of the organic carbon and nitrogen mixture by cultures arising with the lowest rhizosphere sediment dilution were acetate, propionate, and butyrate (18.4, 3.5, and 8.2 mM, respectively). Similarly, in the lowest dilutions of the unvegetated sediment, the concentrations of acetate, propionate, and butyrate were approximately 16.0, 4.5, and 7.9 mM, respectively. Butyrate concentrations in cultures obtained with the lowest dilutions of ethanol-pretreated rhizosphere and unvegetated sediments were enhanced to 18.8 and 13.1 mM, respectively, confirming that spore-forming butyrate-producing clostridia were selected by the ethanol pretreatment (35).

In contrast to the case for butyrate producers, acetogens were enriched by an order of magnitude in the rhizosphere sediment (Table 3). Consumption of H<sub>2</sub> indicated by negative pressures in MPN tubes was confirmed by gas chromatography analyses. Larger amounts of acetate were produced in H<sub>2</sub>-utilizing dilutions than in sediment dilutions in medium lacking added substrate. Methane was not detected in any of these tubes. Likewise, O-demethylating acetogens were enriched in the rhizosphere sediments, although present in low numbers. Protocatechuate was detected as the main aromatic product formed from vanillate in the MPN tubes. The ring recovery approximated 68%, indicating a partial utilization of the aromatic ring.

Acetate-utilizing sulfate reducers were highly enriched in the rhizosphere sediment compared to the unvegetated sediment (Table 3) and represented a substantial part of the cell numbers calculated from the PLFA biomarkers (Table 2).

**Root incubation studies.** Acetogenic and sulfate-reducing activities were tightly bound to the rhizoplane when washed *H. wrightii* roots were incubated in serum bottles containing a medium to stimulate acetogens or sulfate reducers. During 4 weeks of incubation, no growth appeared in the selective medium for acetogens. However, roots turned yellow prior to coloration of the medium, indicating that vanillate was O-demethylated to protocatechuate, which was confirmed by high-performance liquid chromatography analyses. Acetate was

TABLE 2. PLFA biomarkers and calculated cell numbers at different depths in rhizosphere and unvegetated sediments<sup>a</sup>

Biomarker type	pmol g (dry wt) <sup>-1</sup> [cell no. <sup>b</sup> g (dry wt) <sup>-1</sup> ] in:								
	Rhizosphere sediment		Unvegetated sediment		Rhizosphere sediment		Unvegetated sediment		
	0-6 cm	6-12 cm	12-18 cm	0-6 cm	6-12 cm	12-18 cm	0-6 cm	12-18 cm	
Total bacterial biomass <sup>c</sup>	( $1.2 \pm 0.2$ ) $\times 10^5$ [( $3.0 \pm 0.4$ ) $\times 10^9$ ]	( $6.3 \pm 0.7$ ) $\times 10^4$ [( $1.6 \pm 0.2$ ) $\times 10^9$ ]	( $3.2 \pm 0.4$ ) $\times 10^4$ [( $8.0 \pm 0.1$ ) $\times 10^8$ ]	( $1.5 \pm 0.1$ ) $\times 10^5$ [( $3.8 \pm 0.1$ ) $\times 10^9$ ]	( $3.3 \pm 0.6$ ) $\times 10^4$ [( $8.3 \pm 1.7$ ) $\times 10^8$ ]	( $1.3 \pm 0.3$ ) $\times 10^4$ [( $3.3 \pm 0.7$ ) $\times 10^8$ ]	( $6.8 \pm 1.2$ ) $\times 10^2$ [( $1.7 \pm 0.3$ ) $\times 10^7$ ]	( $4.1 \pm 0.2$ ) $\times 10^2$ [( $1.0 \pm 0.1$ ) $\times 10^7$ ]	ND <sup>f</sup>
Sulfate reducers <sup>d</sup>	( $1.3 \pm 0.4$ ) $\times 10^4$ [( $3.3 \pm 0.9$ ) $\times 10^8$ ]	( $5.8 \pm 2.2$ ) $\times 10^3$ [( $1.5 \pm 0.6$ ) $\times 10^8$ ]	( $2.5 \pm 0.4$ ) $\times 10^3$ [( $6.3 \pm 1.1$ ) $\times 10^7$ ]	( $9.4 \pm 2.4$ ) $\times 10^3$ [( $2.4 \pm 0.6$ ) $\times 10^8$ ]	( $2.9 \pm 0.8$ ) $\times 10^3$ [( $7.3 \pm 2.0$ ) $\times 10^7$ ]	( $9.4 \pm 2.0$ ) $\times 10^2$ [( $2.3 \pm 0.5$ ) $\times 10^7$ ]	ND	ND	ND
Clostridia <sup>e</sup>	( $6.8 \pm 1.2$ ) $\times 10^2$ [( $1.7 \pm 0.3$ ) $\times 10^7$ ]	( $4.1 \pm 0.2$ ) $\times 10^2$ [( $1.0 \pm 0.1$ ) $\times 10^7$ ]	ND <sup>f</sup>	ND	ND	ND	ND	ND	ND

<sup>a</sup> All data are averages and standard deviations from triplicate experiments.

<sup>b</sup> Obtained with the conversion factor from White et al. (58).

<sup>c</sup> The biomarkers used were total monounsaturated and saturated PLFAs of 18 carbons or fewer in length.

<sup>d</sup> The biomarkers used included 10mcl6, cy18( $\omega$ 7,8), i17:1 $\omega$ 7c, i15:1 $\omega$ 7c, and 17:1 $\omega$ 6c.

<sup>e</sup> The biomarkers used were plasmalogen-derived dimethyl acetals, primarily saturated 16:0 and 18:0 and minor amounts of 13:0 and 15:0.

<sup>f</sup> ND, not detected.

TABLE 3. MPN values of microbial anaerobic populations in rhizosphere and unvegetated sediments<sup>a</sup>

Metabolic type	MPN <sup>b</sup> (range) in:	
	Rhizosphere sediment	Unvegetated sediment
Lactate-utilizing sulfate reducers	$4.0 \times 10^6$ ( $8.6 \times 10^5$ – $1.9 \times 10^7$ )	$4.0 \times 10^6$ ( $8.6 \times 10^5$ – $1.9 \times 10^7$ )
Acetate-utilizing sulfate reducers	$2.3 \times 10^7$ ( $4.9 \times 10^6$ – $1.1 \times 10^8$ )	$4.0 \times 10^5$ ( $8.6 \times 10^4$ – $1.9 \times 10^6$ )
H <sub>2</sub> -utilizing acetogens	$1.5 \times 10^5$ ( $3.2 \times 10^4$ – $7.0 \times 10^6$ )	$9.0 \times 10^3$ ( $1.9 \times 10^3$ – $4.2 \times 10^4$ )
Vanillate-utilizing acetogens	$9.0 \times 10^4$ ( $1.9 \times 10^4$ – $4.2 \times 10^5$ )	$2.3 \times 10^2$ ( $4.9 \times 10^1$ – $1.1 \times 10^3$ )
Butyrate producers	$2.3 \times 10^4$ ( $4.9 \times 10^3$ – $1.1 \times 10^5$ )	$9.0 \times 10^3$ ( $1.9 \times 10^3$ – $4.2 \times 10^4$ )
Spores of butyrate producers	$9.0 \times 10^2$ ( $1.9 \times 10^2$ – $4.2 \times 10^3$ )	$2.3 \times 10^3$ ( $4.9 \times 10^2$ – $1.1 \times 10^4$ )

<sup>a</sup> MPN dilutions were incubated in three replicates at 30°C for 4 months.

<sup>b</sup> MPN per gram (wet weight) of sediment.

formed during the incubation in a 4.2:3 substrate-to-acetate ratio, indicative of *O*-methyl group-dependent acetogenesis (21). Controls lacking vanillate did not show a color reaction.

Roots incubated in the seawater medium for sulfate-reducing bacteria blackened within 3 days of incubation, indicating sulfidogenesis and the subsequent formation of iron sulfides. No blackening or growth appeared in the medium.

**In situ hybridization studies.** As a first approach to examine microbial colonization, the blackened roots were stained with DAPI and then hybridized with fluorescent-dye-labeled oligonucleotide probes. Epifluorescence observation of DAPI-stained bacteria showed colonization of both the root surface and deeper root cells by a variety of morphologically distinct types. Bacteria were most abundant in the blackened parts of the roots. Most of the DAPI-stained bacteria also showed hybridization with the rhodamine-labeled Eub probe, a generalized probe capable of detecting practically all bacteria. A large proportion (about 50%) of DAPI-stained bacteria also hybridized with the rhodamine-labeled SRB 385 probe. However, quantification was not possible due to strong autofluorescence of the root material. The autofluorescence could not be readily overcome even by using CLSM in combination with the different fluorescent labels.

These problems were circumvented by using <sup>33</sup>P-labeled probes and detecting hybridization with microautoradiography and light microscopy. Hybridization of root thin sections prepared with freshly sampled *H. wrightii* roots showed that bacteria occupied the intercellular root spaces and resided predominantly in the rhizoplane (Table 4). The rhizoplane, here considered the epidermal root cells, was colonized by bacteria that hybridized with all of the probes used. All rhizoplane cells were colonized by *Desulfovibrio* species (probe DSV), and a significant portion were colonized by clostridia (Clost I), *Acetobacterium* species (AW),  $\alpha$ -subdivision proteobacteria (Alfa), and archaea (Arch). Bacteria identified as acetogens formed large clusters, whereas *Desulfovibrio* species occurred singly and were more evenly distributed (Fig. 1).

Clostridial, acetogen, and archaeal colonization extended into the cortex and was greater than that of  $\alpha$ -subdivision proteobacteria. High numbers of bacteria inside the root hybridized with the *Desulfovibrio* probe (DSV) and were detected on the majority (61%) of the deepest cortex cells (Table 4). Fewer than 10% of the deepest cortex cells were colonized by gram-positive low-G+C-content bacteria and *Acetobacterium* species.

## DISCUSSION

Sulfate reduction is the dominant terminal electron-accepting process in sea grass bed sediments (8). It was therefore expected that biomarkers for sulfate-reducing bacteria would be abundant in rhizosphere sediments. However, based on the habitats from which most clostridial species have been isolated, the abundance of biomarkers from clostridia in marine rhizosphere sediments was unexpected. The large phylogenetic group of gram-positive clostridia contains numerous physiological types, including the acetogenic bacteria (19). Although many clostridia and acetogens have not been characterized with respect to lipid composition, all clostridia examined to date contain plasmalogens, including the butyric acid group of clostridia from cluster I and the acetogen *E. limosum* (42). Thus, while the PLFA analysis could not specifically identify acetogens, the biogeochemical implications of acetogenic bacteria in sea grass bed sediments was intriguing and was pursued by enumeration studies with selective media. The results clearly showed that acetogens were enriched and comprised a significant fraction of the bacterial community in the rhizosphere sediment, in contrast to butyrate-producing clostridia, which were not enriched. MPNs of *O*-demethylating acetogens were less than those of H<sub>2</sub>-utilizing acetogens, suggesting that H<sub>2</sub> is an important electron donor in the sea grass rhizosphere.

Rapid blackening of *H. wrightii* roots in the seawater medium was indicative of rhizosphere-associated sulfate-reducing activity, as reported in a recent study with *Zostera marina* (8).

TABLE 4. Distribution of microbes within *H. wrightii* roots determined by in situ hybridization<sup>a</sup>

Probe	% of root cells colonized (no. of microbes cell <sup>-1</sup> [mean $\pm$ SD]) for:			
	Epidermal cells	1–3 cells inside cortex	4–9 cells inside cortex	Stele
Eub	100 (74.7 $\pm$ 38.5)	80.0 (42.6 $\pm$ 22.0)	0 (0)	6.0 (6.1 $\pm$ 1.0)
Alfa	44.4 (16.6 $\pm$ 7.1)	3.4 (9.5 $\pm$ 3.2)	0 (0)	0 (0)
DSV	100 (47.9 $\pm$ 32.4)	94.4 (15.2 $\pm$ 6.1)	61.1 (46.9 $\pm$ 80.0)	0 (0)
LGC	93.3 (27.1 $\pm$ 13.8)	42.2 (29.4 $\pm$ 28.1)	8.8 (51.2 $\pm$ 88.7)	0 (0)
Clost I	61.1 (12.5 $\pm$ 7.4)	20.0 (11.4 $\pm$ 6.8)	0 (0)	0 (0)
AW	62.2 (14.1 $\pm$ 5.7)	24.4 (14.7 $\pm$ 6.8)	2.2 (46.0 $\pm$ 23.5)	0 (0)
Arch	32.2 (9.4 $\pm$ 3.8)	23.3 (13.3 $\pm$ 6.1)	0 (0)	0 (0)

<sup>a</sup> The cortex in the sections examined was on average 12 cell layers thick. Data represent observations of ca. 30 plant cells in each of the sections from different roots.



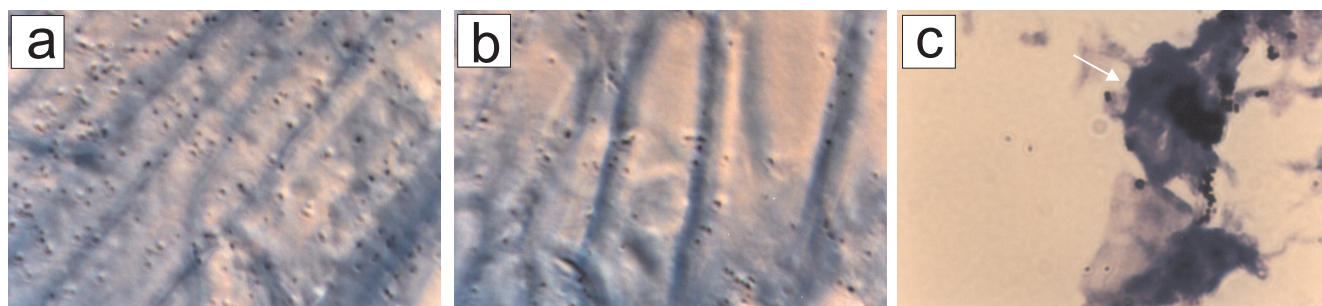


FIG. 1. Phase-contrast micrographs of bacteria visualized by microautoradiography following in situ hybridization of *H. wrightii* longitudinal root sections with  $^{33}\text{P}$ -labeled rRNA probes. Magnification,  $\times 1,000$ . Bacteria that retained probe appear black due to the deposition of silver grains from the photographic emulsion. (a) EUB probe for virtually all bacteria; (b) DSV probe for the sulfate-reducing *Desulfovibrio* species; (c) AW probe for the acetogens *E. limosum* and *Acetobacterium*. Cells hybridizing with the AW probe were found in dense clusters (arrow) on the outer root surface.

The presence of viable sulfate reducers in the blackened portions of roots was verified by in situ hybridization with the fluorescent-dye-labeled SRB 385 probe. However, root metabolism in an anoxic in vitro assay is clearly very different from in situ root metabolism. In addition, the observed distribution of root-inhabiting sulfate reducers might have been influenced by the use of excised roots, since bacteria can colonize both the ecto- and the endorhizosphere upon the death of epidermal and cortical cells (13).

CLSM has been used to overcome the strong autofluorescence of root material hybridized with fluorescent probes and to localize indigenous microorganisms in situ on the rhizoplane (6, 28). This advanced microscopic technique permitted detection of sulfate reducers on the *H. wrightii* root; however, it was not immediately applicable to quantify bacteria with either intact or sectioned roots. Hybridization of root thin sections with  $^{33}\text{P}$ -labeled rRNA probes followed by microautoradiography enabled detection and quantification, but of course this method lacks many of the capabilities available with CLSM, such as multicolor imaging with differently labeled probes or three-dimensional sectioning (2, 6).

Nonetheless, the use of radioactive probes (i) confirmed colonization of the sea grass rhizoplane with sulfate-reducing bacteria, as described by Blaabjerg and Finster (8); (ii) detected sulfate-reducing bacteria, acetogens, and methanogens within the cortex; and (iii) provided a means with which to quantify bacterial colonization on a per-root-cell basis (Table 4). To our knowledge, this is the first observation of what are considered to be strictly anaerobic bacteria as endorhizobacteria, i.e., living in deep root cell layers. Furthermore, the distribution of anaerobes suggests the successive utilization of electron acceptors across a sharp oxygen gradient emanating from the center of the root.

*Desulfovibrio* species were associated with almost every epidermal cell and with about 60% of the cells deeper inside the cortex. Although sulfate-reducing bacteria are described as strict anaerobes, they occur in oxic surface layers of aquatic habitats, are active in microbial mats at oxygen tensions near saturation (14, 27), and can even respire oxygen (20). Recently it was demonstrated that exposure of sea grass roots to oxygen does not affect root surface-associated sulfate-reducing activity (8). Thus, the colonization of the *H. wrightii* rhizoplane by sulfate-reducing bacteria was not as surprising as was their occurrence in the deeper root cell layers. The in situ activity of these endorhizobacterial sulfate reducers is not known. Earlier, microbially produced sulfide found within the roots of *Spartina alterniflora* was considered to have originated from the sediment pore water (17). If root-inhabiting bacteria reduce

sulfate to sulfide, a reoxidation of sulfide via the microbial reduction of oxygen or nitrate might be a potential mechanism by which the plant tolerates endorhizobacterial sulfate-reducing activity.

About 60% of the root surface cells, and a smaller amount of the outermost cortex cells, had bacteria that hybridized with the clostridial Clost I and acetogen AW probes. Colonization of sea grass roots by acetogenic bacteria could be even greater, since the AW probe targeted only a limited number of known acetogens. Use of the archaeal probe revealed low but significant numbers of root-associated archaea. Recently, in situ hybridization with fluorescent probes detected methanogens on the surfaces of rice roots (28). Temporary exposure of rice roots to oxygen did not affect the activity of root-associated methanogens in anoxic in vitro assays (26, 33), and it is known that methanogens can survive oxygen stress for several hours (23). Thus, the rhizoplanes of aquatic plants appear to be common habitats for certain methanogenic bacteria.

Acetogens have not been previously reported to be associated with the roots of aquatic plants. Little is known about the activity of acetogenic bacteria under oxic conditions. Acetogens can be isolated or enriched from oxic forest soils (37, 38) and from termite guts that have steep oxygen gradients (12), and acetogens can tolerate periods of oxygenation in soil (56). Enumeration studies indicated that the sea grass root environment might be a preferred habitat for acetogens compared to the unvegetated sediment. The relatively low growth rates, low substrate affinities, and low energy yields of acetogens on certain substrates (e.g.,  $\text{H}_2\text{-CO}_2$ ) suggest that they are less competitive than general fermenters, sulfate reducers, and methanogens (50). However, the ability of acetogens to simultaneously utilize various substrates and terminal electron acceptors might compensate for such bioenergetic disadvantages (22). Since acetate is the main product of acetogens, it is noteworthy that biomarkers commonly associated with acetate-utilizing *Desulfobacter* species were detected only in very small amounts in the rhizosphere sediment. Although a possible trophic link between sulfate-reducing bacteria and acetogens in the endorhizosphere remains unclear, low-G+C-content gram-positive acetate-utilizing sulfate reducers might be involved (11).

The intercellular colonization of root cells might afford acetogens and sulfate reducers access to substrates that could facilitate an energy-intensive process like nitrogen fixation, a beneficial plant-microbe interaction. In fact, certain acetogens and sulfate reducers fix molecular nitrogen (10, 50, 51), and some  $\text{N}_2$ -fixing species of clostridia and sulfate reducers can be enriched from both sea grass roots (60) and rhizosphere sed-

iment (44). The observation that anaerobic bacteria colonize the endorhizospheres of submerged aquatic plants should foster interest in resolving (i) the in situ activity of these bacteria, (ii) the beneficial or harmful effects of these root-microbe interactions, and (iii) the occurrence of anaerobes in deeper root cell layers of other plants, such as rice, that are rooted in anoxic soils.

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