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Attached Bacterial Populations Shared by Four Species of Aquatic Angiosperms[▽]

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Symbiotic relationships between microbes and plants are common and well studied in terrestrial ecosystems, but little is known about such relationships in aquatic environments. We compared the phylogenetic diversities of leaf- and root-attached bacteria from four species of aquatic angiosperms using denaturing gradient gel electrophoresis (DGGE) and DNA sequencing of PCR-amplified 16S rRNA genes. Plants were collected from three beds in Chesapeake Bay at sites characterized as freshwater (*Vallisneria americana*), brackish (*Potomogeton perfoliatus* and *Stuckenia pectinata*), and marine (*Zostera marina*). DGGE analyses showed that bacterial communities were very similar for replicate samples of leaves from canopy-forming plants *S. pectinata* and *P. perfoliatus* and less similar for replicate samples of leaves from meadow-forming plants *Z. marina* and *V. americana* and of roots of all species. In contrast, bacterial communities differed greatly among plant species and between leaves and roots. DNA sequencing identified 154 bacterial phylotypes, most of which were restricted to single plant species. However, 12 phylotypes were found on more than one plant species, and several of these phylotypes were abundant in clone libraries and represented the darkest bands in DGGE banding patterns. Root-attached phylotypes included relatives of sulfur-oxidizing *Gammaproteobacteria* and sulfate-reducing *Deltaproteobacteria*. Leaf-attached phylotypes included relatives of polymer-degrading *Bacteroidetes* and phototrophic *Alphaproteobacteria*. Also, leaves and roots of three plant species hosted relatives of methylotrophic *Betaproteobacteria* belonging to the family *Methylophilaceae*. These results suggest that aquatic angiosperms host specialized communities of bacteria on their surfaces, including several broadly distributed and potentially mutualistic bacterial populations.

Most plants form intimate relationships with microorganisms, ranging from mutualistic to parasitic. Plant-bacterium and plant-fungus symbioses in the terrestrial environment are extremely common and have been well studied. Leguminous plants host symbiotic nitrogen-fixing bacteria in their roots, while most angiosperms (flowering plants) and members of the Pinaceae (pine family) have root-associated symbiotic mycorrhizal fungi that facilitate plant uptake of soil nutrients (2). Plant leaves also host complex assemblages of bacteria (76), some of which produce compounds that promote plant growth (17, 29, 59), including the plant growth-regulating auxin indole-3-acetic acid, a compound involved in plant development (37, 43).

In contrast, very little is known about microbes associated with aquatic plants. Surfaces of aquatic plant roots and rhizomes host active sulfate-reducing and nitrogen-fixing bacterial communities (18, 54). Similarly, leaves of aquatic plants support very active bacterial communities that are thought to be influenced by plant primary production (24, 68). Most research on aquatic plant-bacterium interactions, however, describes broader relationships with general sediment bacteria in the rhizosphere. These sediments contain active bacterial communities that consume molecules excreted by plant roots, including organic molecules (amino acids, sugars, and organic acids) and gasses (O_2, N_2, and CO_2) (8, 28, 49, 57). In return, microbial processes, including organic matter mineralization (64), phosphorous solubilization (75), and nitrogen fixation, provide nutrients for the plants. The final process, nitrogen fixation, is the only well-studied aquatic plant-bacterium interaction in which bacteria are directly attached to the plant surface (11, 44). These diazotrophic bacteria grow on both the roots and the leaves of aquatic plants and potentially provide as much as 50% of the nitrogen demand of the plant (9). However, aside from diazotrophs, research on symbiotic aquatic plant-associated organisms lags far behind parallel research on terrestrial plants.

A few studies using molecular techniques describe the diversity of bacteria in plant-colonized sediments (5, 12, 13), but very few studies describe the diversity of organisms attached to aquatic plant surfaces. One pair of studies revealed broad microbial diversity associated with the seagrass *Halophila stipulacea* in the northern Gulf of Elat (71, 72), and another study identified epiphytic bacterial communities on three seagrass species from the East African coast (70). One recent study identified bacteria attached to roots of *Zostera marina* (33), and another identified methanotrophic bacteria associated with aquatic plants growing in methanogenic sediments (66). Most of these studies suggested that plant-attached bacterial communities are different than the bacterial communities in surrounding environments (e.g., sediments and water column), and such studies are beginning to reveal what may be a unique assemblage of aquatic plant-associated microorganisms.

The goals of this study were to describe variability in the composition of bacterial communities attached to surfaces of leaves (phylloplane) and roots (rhizoplane) of four aquatic angiosperms in the Chesapeake Bay and to identify common

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TABLE 1. Plant species, sampling dates, and sampling locations

Plant species	Common name	Sampling date	Latitude	Longitude	Water column salinity	Site description
Z. marina	Eelgrass	18 May 2005	37°50′37″N	75°59′15″W	13	Northeast side of Tangier Island
P. perfoliatus	Redhead grass	14 June 2005	39°01′53″N	76°31′31″W	7	North bank of Severn River
S. pectinata	Sago pond weed	27 June 2005	39°01′53″N	76°31′31″W	7	North bank of Severn River
V. americana	Wild celery	26 July 2005	39°23′20″N	76°02′27″W	1	Mouth of Sassafrass River

and potentially mutualistic bacterial populations. These plants inhabit environments ranging from freshwater (*Vallisneria americana*) to brackish water (*Potomogeton perfoliatus* and *Stuckenia pectinata*) to marine water (*Z. marina*). We found evidence of plant-specific bacterial communities on leaves and roots and observed that most bacterial phylotypes were restricted to single plant species. However, among 154 bacterial phylotypes identified by DNA sequencing, we found 12 phylotypes that were associated with more than one plant species and determined that many of these phylotypes were abundant in clone libraries and denaturing gradient gel electrophoresis (DGGE) banding patterns.

MATERIALS AND METHODS

For each plant species, 10 individual plants were collected by hand randomly from ~100-m^2 areas in different regions of Chesapeake Bay (Table 1) and dissected with presterilized equipment. Leaves and roots were triple rinsed by vigorous shaking with filter-sterilized seawater (pore size, 0.2 μm) in sterile 50-ml tubes, frozen in tubes on dry ice, and stored at −80°C for later DNA extraction. A separate set of whole-plant samples was collected and stored in seawater for plant tissue culture.

Following each field collection, tissue cultures were established using standard techniques (37, 48). Briefly, meristems of the different plant species were dissected, surface sterilized, and allowed to grow under controlled axenic conditions. *P. perfoliatus*, *S. pectinata*, and *V. americana* became established in tissue culture, but *Z. marina* did not. Leaves and roots of axenically grown plants were dissected and frozen at −80°C.

DNA was extracted from plant material using an UltraClean fecal DNA kit (MoBio) by following the manufacturer's instructions, except that bead tubes containing plant material were gently shaken with a titer plate shaker (LabLine) rather than a vortex mixer so that the leaves and roots remained intact during bead beating.

Bacterial communities attached to plant leaves and roots were characterized and compared by performing PCR-DGGE analyses of the 16S rRNA gene using previously described laboratory and statistical techniques (16, 51), with the following modifications. The touchdown PCR conditions consisted of an initial 5 min of incubation at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 65 to 55°C (with the temperature reduced by 1°C per cycle for 10 cycles plus 20 cycles at 55°C), and 1 min at 72°C followed by 1 h at 72°C. Temperature reductions during PCR were done at a rate of 0.3°C per s was used. PCR products were separated into bands by electrophoresis (CBS Scientific) for 24 h at 75 V on acrylamide (8%) gels prepared with 30% acrylamide/bisacrylamide (37.5:1; Bio-Rad) and 0.5× TAE buffer (1× TAE buffer is 40 mmol $liter^{-1}$ Tris [pH 8.0], 20 mmol $liter^{-1}$ acetic acid, and 1 mmol $liter^{-1}$ EDTA) and containing 35 to 50% denaturant (urea and formamide) gradients. Gel images were assembled from images collected for a range of exposure times, but no modifications were made within the banding pattern of each sample. DGGE bands were scored when the density was at least 5% that of the darkest band in the sample pattern.

DNA extracted from axenically grown plants (*P. perfoliatus*, *S. pectinata*, and *V. americana*) was also analyzed to identify the DGGE band representing the 16S rRNA gene from the chloroplasts of each plant. This was done so that bands representing chloroplasts could be omitted from the DGGE analysis of bacterial communities. The chloroplast band for *Z. marina* was identified by DNA sequencing of DGGE bands (see below).

Dominant bacterial populations from one plant of each species (root and leaf) were identified using two different techniques, DGGE band sequencing and PCR-clone library sequencing. 16S rRNA genes were PCR amplified using

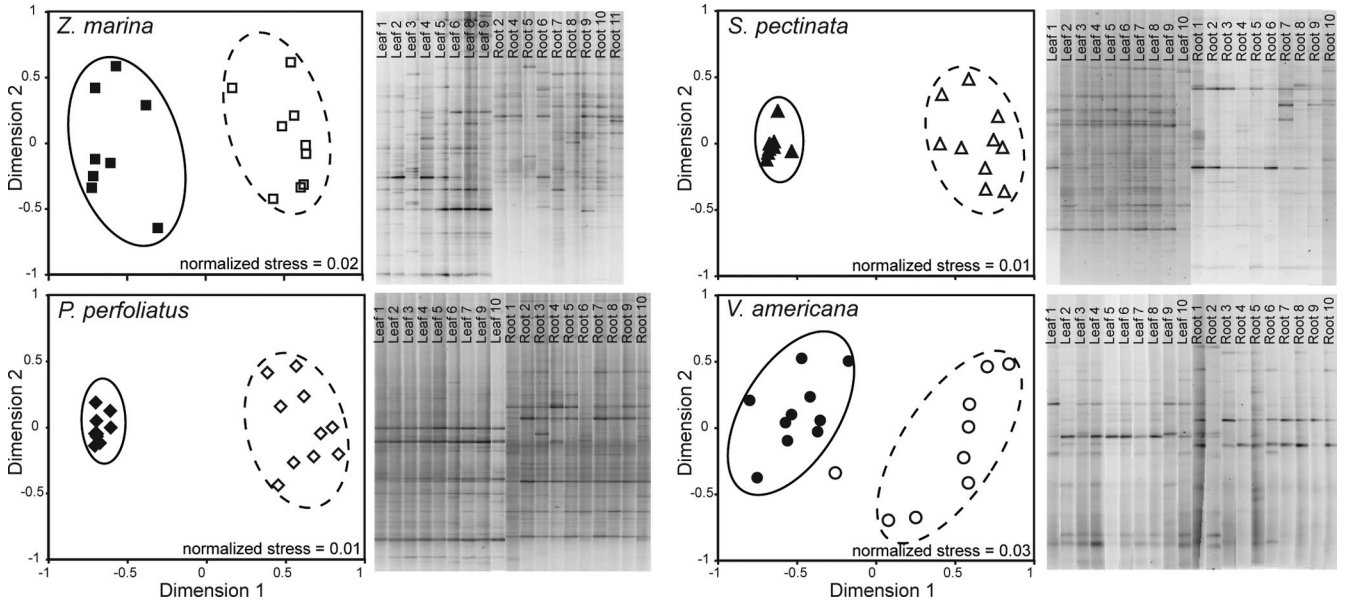


FIG. 1. DGGE banding patterns and cluster analyses (multidimensional scaling) of pairwise similarity values (Dice) calculated for each plant species based on the presence or absence of each DGGE band. Filled symbols indicate leaf samples, and open symbols indicate root samples.

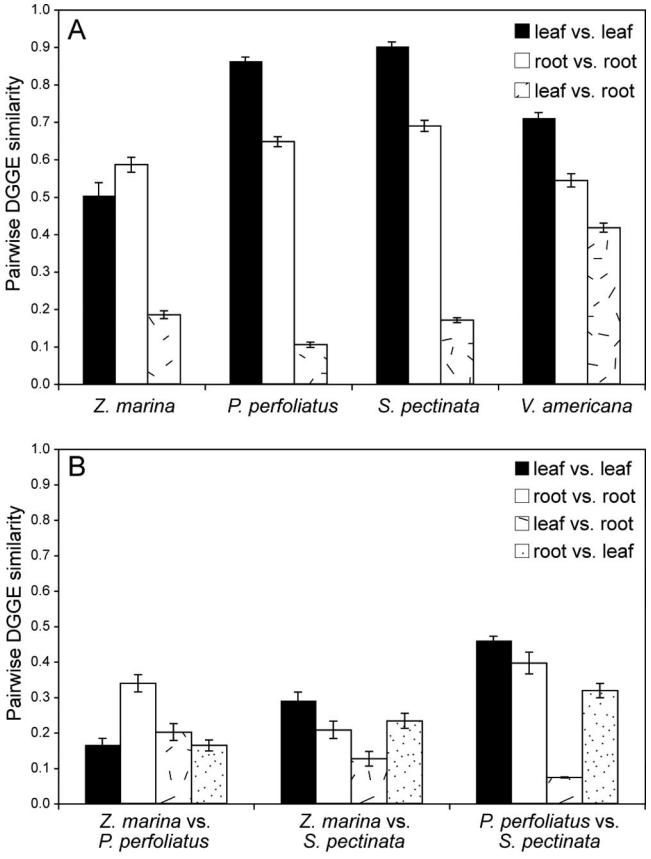


FIG. 2. Average DGGE similarity values (Dice) for pairwise comparisons of DGGE banding patterns within (A) and between (B) plant species. Plants are listed in order from low salinity to high salinity from left to right. Error bars indicate standard errors.

DGGE-PCR primers as described above and run on a denaturing gradient gel. Samples of DNA fragments from DGGE bands were obtained with sterile pipette tips, and the DNA was PCR amplified using the same protocol and run on another DGGE gel along with the original natural samples in order to identify the appropriate bands from the reamplification reactions. This procedure was repeated until each reamplification reaction produced only one strong band. Samples of the bands were removed, and the DNA was PCR amplified with M13-linked primers (primers M13f-357f [TGTAAAACGACGGCCAGTCTAC GGGAGGCAGCAG] and M13r-519r [GGAAACAGCTATGACCATGACCG CGGCTGCTGGCAC]), purified, and sequenced bidirectionally with M13 primers using an ABI-3100 automated DNA sequencer.

For PCR-clone library analysis, DNA samples were PCR amplified with general bacterial primers 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-GGCTACCTTGTTACGACTT-3′) in 50-μl reaction mixtures under the following conditions: 4 min at 94°C, followed by 27 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 2 min and then 5 min at 72°C. PCR products were then diluted 1:10 in fresh PCR cocktail in 10 separate tubes and subjected to three more PCR cycles. PCR products were combined and purified with a MoBio PCR purification kit, ligated into TOPO-XL plasmids (Invitrogen), and used to transform TOP-10 chemically competent cells by following manufacturer's instructions. Forty-eight clones from each library were selected at random and grown overnight at 37°C in 1 ml of SB broth (33 g tryptone, 20 g yeast extract, 2.5 g NaCl, 1 liter water; autoclaved) in 96-well deep-well plates. Cells were centrifuged for 20 min at 3,000 × *g*, and plasmids were extracted as follows. Pellets were resuspended in 50 μl of GTE solution (50 mM glucose, 25 mM Tris buffer [pH 8.0], 10 mM EDTA; autoclaved). Then 100 μl of an NaOH-sodium dodecyl sulfate solution (0.2 M NaOH, 1% [wt/vol] sodium dodecyl sulfate; filter sterilized) and 50 μl of KAc solution (29.5 ml glacial acetic acid, KOH pellets added to bring the pH to 4.8, H_2O to bring the volume to 100 ml; prepared on ice and filter sterilized) were added, and each preparation was shaken for 2 min and centrifuged for 15 min at 1,200 × *g*. One-hundred-microliter portions of the supernatants were transferred to sterile 96-well shallow-well plates, 75 μl of 100% isopropanol was added to each well, and the plates were sealed, shaken, incubated for 30 min at −20°C, and centrifuged for 15 min at 1,200 × *g*. The supernatants were poured and blotted off, the pellets were washed once with 150 μl of 70% ethanol and once with 150 μl of 95% ethanol and were dried in a roto-evaporator and resuspended in 100 μl of Tris buffer (10 mM, pH 8.0). Plasmid inserts were sequenced bidirectionally with primers 27f and 907r (5′-C CGTCAATTCCTTTRAGTTT3′).

DNA sequences from DGGE bands and clone libraries were assembled, quality checked, aligned using the ARB sequence alignment program (www.arb-home.de), screened for chimera formation using BELLEROPHON (30), CHIMERA_CHECK (14), and visual inspection of secondary structure base pair matches, and analyzed with the Basic Local Alignment Search Tool (BLAST) (www.ncbi.nlm.nih.gov) to determine the closest relatives and to group sequences by major phylogenetic taxa. Fifteen chimeras were identified and excluded from further analysis.

Aligned clone sequences (670 bp) were exported from ARB after applying a 50% base pair frequency filter to remove nonhomologous sequences. Phylogenetic distances were calculated with DNADIST using the Jukes-Cantor model, and sequences were assigned to operational taxonomic units (OTUs) based on 97% sequence similarity using DOTUR (61). In order to place DGGE band sequences into these OTUs, DGGE band sequences and clone sequences were exported from ARB after applying 50% base pair frequency filters constructed for each major bacterial phylum or subphylum. This was done to account for variability in sequence length of the DGGE band sequences in the different phyla (121 to 158 bp was analyzed, depending on the phylum). DGGE band sequences were assigned to OTUs based on 99% sequence similarity.

Nucleotide sequence accession numbers. The DNA sequences have been deposited in the GenBank database under accession numbers EU542029 to EU542414.

RESULTS

Bacterial communities attached to the surfaces of aquatic angiosperms were similar for replicates of each plant species, suggesting that there was a native, adapted, plant-associated microflora (Fig. 1). Replicate *P. perfoliatus* and *S. pectinata* plants hosted very similar, ubiquitous communities of leaf-attached bacteria (average levels of similarity, 86 and 90%, respectively) (Fig. 2A), whereas replicate *Z. marina* and *V. americana* plants hosted less similar leaf-attached communities (average levels of similarity, 50 and 71%, respectively). Root-attached communities were also less similar among replicates for each plant species (average levels of similarity ranged from 54 to 69%). In contrast, leaf- and root-attached bacterial communities were very different from one another for most plant species, and the levels of similarity ranged from 11 to 42% (Fig. 1 and 2A).

TABLE 2. Numbers of DGGE bands

Species	Surface	No. of plants analyzed	Total no. of DGGE bands	No. of ubiquitous DGGE bands	No. of bands found in <5 replicates
Z. marina	Leaf	8	63	8	45
	Root	9	63	4	42
P. perfoliatus	Leaf	9	34	15	6
	Root	10	58	11	33
S. pectinata	Leaf	10	30	14	4
	Root	10	40	6	23
V. americana	Leaf	10	28	4	17
	Root	10	28	2	19

TABLE 3. Phylogenetic information for bacterial populations identified by sequencing DNA from DGGE bands, including closest database matches and closest cultivated relatives based on MEGABLAST analysis[a]

Band	Plant	Source	Closest BLAST match			Closest cultivated relative		
			Phylum or subphylum	Accession no.	% Identity	Taxon	Accession no.	% Identity
SAV01Z01	*Z. marina*	Leaf	*Bacteroidetes*	AY828430	99	Marine bacterium SCRIPPS_413	AF359548	98
SAV01Z02	*Z. marina*	Leaf	*Bacteroidetes*	AF235111	96	*Cytophaga* sp. strain RP8	EU375128	96
SAV01Z03	*Z. marina*	Leaf	*Bacteroidetes*	AY678493	92	*Lewinella agarilytica*	AM286229	92
SAV01Z04	*Z. marina*	Leaf	*Gammaproteobacteria*	AF448465	97	*Methylotenera mobila*	DQ287786	96
SAV01Z05	*Z. marina*	Leaf	Plastid	EF036309	100	Chloroplast of *Yucca schidigera*	EU016691	100
SAV01Z06	*Z. marina*	Leaf	*Alphaproteobacteria*	DQ659412	100	*Rhodobacteraceae* sp. strain BS110	DQ659412	100
SAV01Z07	*Z. marina*	Leaf	*Alphaproteobacteria*	DQ436521	100	*Thalassobacter* sp. strain GA2-M15	EU342372	100
SAV01Z08	*Z. marina*	Leaf	*Alphaproteobacteria*	DQ103624	100	*Roseobacter* sp. strain CSQ-2	EF512125	99
SAV02Z02	*Z. marina*	Root	*Epsilonproteobacteria*	EF028999	100	*Arcobacter nitrofigilis*	EU106662	100
SAV02Z03	*Z. marina*	Root	*Gammaproteobacteria*	DQ234105	96	Dibenzofuran-degrading bacterium DBF-MAK	AB086228	93
SAV02Z04	*Z. marina*	Root	*Gammaproteobacteria*	AY711846	100	Gammaproteobacterium SP5	AM884361	95
SAV02Z05	*Z. marina*	Root	*Gammaproteobacteria*	DQ462284	97	*Oceanospirillaceae* bacterium JAMM 1548	AB330882	96
SAV02Z06	*Z. marina*	Root	*Gammaproteobacteria*	EF028933	100	Gammaproteobacterium M41	AM156910	96
SAV03Z01	*P. perfoliatus*	Leaf	Plastid, diatom	DQ906722	96	Chloroplast of *Phaeodactylum tricornutum*	EF067920	96
SAV03Z02	*P. perfoliatus*	Leaf	*Betaproteobacteria*	AY678527	94	Bacterium HTCC4045	EF628482	93
SAV03Z03	*P. perfoliatus*	Leaf	*Alphaproteobacteria*	AM157616	99	*Porphyrobacter tepidarius*	AY568467	99
SAV03Z04	*P. perfoliatus*	Leaf	*Betaproteobacteria*	DQ064982	99	Betaproteobacterium IMCC1762	DQ664233	96
SAV03Z05	*P. perfoliatus*	Leaf	Plastid	EF036309	100	Chloroplast of *Yucca schidigera*	EU016691	100
SAV03Z06	*P. perfoliatus*	Leaf	*Alphaproteobacteria*	AB220125	100	*Sphingomonadaceae* bacterium PB208	AB220125	100
SAV03Z07	*P. perfoliatus*	Leaf	*Alphaproteobacteria*	EF029025	100	*Rhodobacteraceae* sp. strain DG1295	DQ486507	100
SAV03Z08	*P. perfoliatus*	Leaf	*Bacteroidetes*	DQ229334	90	*Lewinella persicus*	EU371935	84
SAV04Z01	*P. perfoliatus*	Root	*Gammaproteobacteria*	AF165907	97	Endosymbiont of *Lamellibrachia satsuma*	AB073120	97
SAV04Z02	*P. perfoliatus*	Root	*Gammaproteobacteria*	AY129090	97	Endosymbiont of *Lamellibrachia barhami*	AY129113	97
SAV04Z03	*P. perfoliatus*	Root	*Bacteroidetes*	AF141549	98	Symbiont b-Z43 of *Tuber borchii*	AF233292	90
SAV04Z04	*P. perfoliatus*	Root	*Betaproteobacteria*	AB240458	97	*Azoarcus* sp. strain CC-11	AB033745	96
SAV04Z05	*P. perfoliatus*	Root	*Betaproteobacteria*	AB264575	94	*Azoarcus anaerobius*	Y14701	93
SAV04Z06	*P. perfoliatus*	Root	Plastid	EF036309	100	Chloroplast of *Yucca schidigera*	EU016691	100
SAV04Z07	*P. perfoliatus*	Root	*Betaproteobacteria*	DQ988325	100	*Hydrogenophaga* sp. strain BAC306	EU130968	100
SAV04Z08	*P. perfoliatus*	Root	*Betaproteobacteria*	AB179685	99	*Methyloversatilis universalis*	DQ923115	93
SAV05Z01	*S. pectinata*	Leaf	*Bacteroidetes*	DQ154800	90	*Cytophaga* sp. strain RP8	EU375128	86
SAV05Z02	*S. pectinata*	Leaf	*Bacteroidetes*	AY171331	93	*Cytophaga* sp. strain I-545	AB073573	86
SAV05Z03	*S. pectinata*	Leaf	*Cyanobacteria*	DQ269094	97	*Aphanocapsa* sp. strain 0MI27S1	DQ264196	96
SAV05Z05	*S. pectinata*	Leaf	*Cyanobacteria*	DQ072927	98	*Phormidium* sp. strain LMECYA 214	EU078511	96
SAV05Z06	*S. pectinata*	Leaf	*Betaproteobacteria*	DQ064982	99	Betaproteobacterium IMCC1762	DQ664233	96
SAV05Z07	*S. pectinata*	Leaf	*Bacteroidetes*	AY711389	92	*Bacteroidetes* sp. strain K3	AM749788	88
SAV05Z08	*S. pectinata*	Leaf	*Alphaproteobacteria*	AJ298351	99	*Rhodobacteraceae* sp. strain DG1295	DQ486507	99
SAV06Z01	*S. pectinata*	Root	*Spirochaeta*	DQ521098	94	*Spirochaeta isovalerica*	M88720	94
SAV06Z02	*S. pectinata*	Root	*Bacteroidetes*	DQ456238	96	*Bacteroides uniformis*	EU136680	94
SAV06Z03	*S. pectinata*	Root	*Gammaproteobacteria*	AF165907	96	Endosymbiont of *Lamellibrachia satsuma*	AB073120	96
SAV06Z04	*S. pectinata*	Root	*Bacteroidetes*	EF028993	94	*Marinilabilia salmonicolor*	M62422	94
SAV06Z06	*S. pectinata*	Root	*Deltaproteobacteria*	AM418397	91	*Desulfovibrio pangongensis*	AM418397	91
SAV06Z07	*S. pectinata*	Root	*Betaproteobacteria*	AB265944	99	*Methyloversatilis universalis*	DQ923115	92
SAV06Z08	*S. pectinata*	Root	*Betaproteobacteria*	AB265944	99	*Methyloversatilis universalis*	DQ923115	92
SAV07Z01	*V. americana*	Leaf	*Betaproteobacteria*	AJ318162	96	Bacterium HTCC4045	EF628482	93
SAV07Z02	*V. americana*	Leaf	*Betaproteobacteria*	DQ315717	98	*Leptothrix ginsengisoli*	AB271046	89

Continued on following page

TABLE 3—*Continued*

Band	Plant	Source	Closest BLAST match			Closest cultivated relative		
			Phylum or subphylum	Accession no.	% Identity	Taxon	Accession no.	% Identity
SAV07Z03	*V. americana*	Leaf	Plastid, diatom	DQ906722	98	Chloroplast of *Phaeodactylum tricornutum*	EF067920	97
SAV07Z04	*V. americana*	Leaf	*Betaproteobacteria*	AF448465	99	*Methylotenera mobila*	DQ287786	98
SAV07Z05	*V. americana*	Leaf	*Alphaproteobacteria*	EF016501	100	*Novosphingobium pentaromativorans*	EU167958	100
SAV07Z06	*V. americana*	Leaf	*Bacteroidetes*	EF686991	93	*Salegentibacter* sp. strain BH206	EF520007	91
SAV07Z07	*V. americana*	Leaf	Plastid	EF036277	96	Chloroplast of *Croomia pauciflora*	DQ629458	96
SAV07Z08	*V. americana*	Leaf	*Betaproteobacteria*	AY144233	96	*Leptothrix mobilis*	X97071	96
SAV08Z01	*V. americana*	Root	*Bacteroidetes*	AM159414	95	Bacterium HTCC4101	EF628490	87
SAV08Z03	*V. americana*	Root	*Betaproteobacteria*	EF107794	97	*Methylophilus* sp. strain u33	EU375653	95
SAV08Z04	*V. americana*	Root	*Betaproteobacteria*	EF107794	97	*Methylophilus* sp. strain u33	EU375653	95
SAV08Z05	*V. americana*	Root	*Alphaproteobacteria*	EF016501	100	*Novosphingobium pentaromativorans*	EU167958	100
SAV08Z06	*V. americana*	Root	Plastid, diatom	AY711816	100	Chloroplast of *Asterionella glacialis*	AJ319828	99
SAV08Z07	*V. americana*	Root	Plastid	EF036277	96	Chloroplast of *Croomia pauciflora*	DQ629458	96
SAV08Z08	*V. americana*	Root	*Alphaproteobacteria*	EF027003	100	*Agrobacterium tumefaciens* strain CCBAU	EU256457	100

[a] See www.ncbi.nlm.nih.gov.

Different plant species hosted different bacterial communities, as demonstrated by very low DGGE similarity values for comparisons of leaf- and root-attached communities of different plant species (Fig. 2B). However, these similarity values were somewhat higher for communities attached to *P. perfoliatus* and *S. pectinata* collected from the same bed.

For each plant species and surface type, most bacterial populations represented by DGGE bands could be categorized as ubiquitous (present on all replicates) or rare (present on fewer than one-half of the replicates). Table 2 shows that most populations detected with DGGE were not present on all replicate plants. In fact, for most plants, fewer than 20% of the DGGE bands were found on all replicates. However, the bands found on all replicates were generally the darkest bands in the patterns, suggesting that ubiquitous bacterial populations dominated the attached bacterial communities.

DNA sequencing of DGGE bands revealed a broad diversity of bacteria attached to plant leaves and roots (Table 3 and Fig. 3). The communities were dominated by *Proteobacteria* and included members of the *Bacteroidetes*, *Spirochaeta*, and *Cyanobacteria*, and some populations were related to water column and sediment bacteria also found in other estuaries. Two DGGE bands from *Z. marina* roots (SAV02Z02 and SAV02Z05) and two DGGE bands from *Z. marina* leaves (SAV01Z07 and SAV01Z08) were closely related to organisms associated with *Z. marina* roots in a study conducted in Denmark (33), suggesting that there is a cosmopolitan *Z. marina*-associated microbial community. *P. perfoliatus* and *S. pectinata* collected from the same bed shared bacterial taxa belonging to the *Deltaproteobacteria*, the *Burkholderiales* and *Methylophilales* orders of the *Betaproteobacteria*, and the unicellular algae. *V. americana* hosted bacteria related to organisms from freshwater systems. Also, the locations of single bands containing plant plastid DNA sequences within the complex DGGE banding patterns were confirmed for three of the four species (all species but *Z. marina*) through DGGE analysis of DNA extracted from axenic plant tissue cultures.

DNA sequencing of PCR-based clone libraries provided another comparison of bacterial communities. Analysis of the DNA sequences demonstrated that there was great variability in diversity between roots and leaves and among plant species (Fig. 4). *Z. marina* leaves were dominated by typical marine *Alphaproteobacteria* that were rare or absent elsewhere, while *Z. marina* roots hosted a diverse microbial assemblage. *P. perfoliatus* leaves and roots were dominated by several types of *Betaproteobacteria*, many of which were also found on roots of other plant species in this study. *S. pectinata* leaves were dominated by several groups of *Bacteroidetes*, and the roots were dominated by organisms related to sulfate-reducing *Deltaproteobacteria*. These *Deltaproteobacteria* were also found on the roots of *P. perfoliatus* and *V. americana*. The communities on *P. perfoliatus* and *S. pectinata* shared only a few OTUs even though they were collected from the same bed in the Severn River. *V. americana* leaves were dominated by *Bacteroidetes*, but these organisms were not closely related to those that dominated *S. pectinata* leaves. However, many of the *Betaproteobacteria* on *V. americana* leaves were similar to those attached to *P. perfoliatus* leaves. *V. americana* roots were completely dominated by one large group of *Bacteroidetes* that were not related to organisms from other plants.

Bacterial 16S rRNA gene sequences collected from all plants clustered into 152 OTUs, 23 of which included both clone library sequences and DGGE band sequences. Most of these OTUs were associated with single plant species, but 12 OTUs included sequences from multiple plant species, comprising a total of 78 clone sequences (24%) and 13 DGGE bands (26%) (Fig. 5).

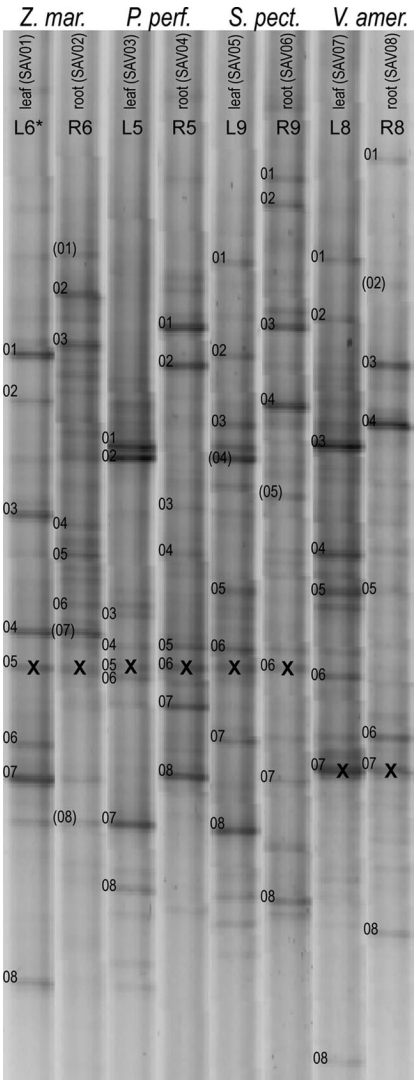


FIG. 3. DGGE banding patterns for leaf and root samples of four plant species. The numbered DGGE bands were selected for DNA sequencing. The numbers in parentheses indicate bands that were not successfully sequenced. An X indicates a band for a sequence from a plant chloroplast 16S rRNA gene.

DISCUSSION

It is becoming evident that aquatic angiosperms host unique and potentially mutualistic assemblages of microorganisms on their surfaces. Recent research on several aquatic angiosperms demonstrated that root-attached bacteria are different than rhizosphere sediment bacteria (33, 41, 56), and leaf-attached bacteria are unlike typical bacterioplankton (70, 72). A number of different microbial metabolic processes are elevated on plant surfaces, including sulfide oxidation (42), methane oxidation (66), iron reduction (34), sulfate reduction, and nitrogen fixation (54), all of which are influenced by plant activity and some of which are arguably beneficial to the plant. Moreover, aquatic angiosperms produce antimicrobial agents, including zosteric acid, that limit bacterial and fungal colonization of plant surfaces and allow only certain microbes to become established (7, 25, 32, 53). Our study shows that four different species of aquatic angiosperms in the Chesapeake Bay each host species-specific microbial communities attached to root and leaf surfaces and that they share several of the same types of bacteria despite growing under a broad range of environmental conditions.

Leaf-attached bacterial communities and root-attached bacterial communities were very different from one another for most plant species (Fig. 2), which is not surprising given the obvious differences in environmental conditions faced by the microbes (3). Leaf communities included *Cyanobacteria* and relatives of phototrophic *Alphaproteobacteria* in the *Rhodobacteraceae*, as well as close relatives of aerobic organisms, including the alphaproteobacterium *Loktanella vestfoldensis* and the *Bacteroidetes* species *Fluviicola taffensis* and *Leadbetterella byssophilla*. Root communities included relatives of obligately anaerobic organisms in the *Deltaproteobacteria*, *Spirochaeta*, and *Clostridiales.* However, root- and leaf-attached communities on *V. americana* plants were more similar based on DGGE banding patterns (average level of similarity, 42%), and although most clones clustered into leaf- and root-specific clusters, two large clusters of root-attached clones included clones from *V. americana* leaves.

Attached bacterial communities were always more similar within a plant species than across plant species, suggesting that each plant species hosts a unique microbial community. Uku et al. (70) compared leaf-attached bacterial communities from

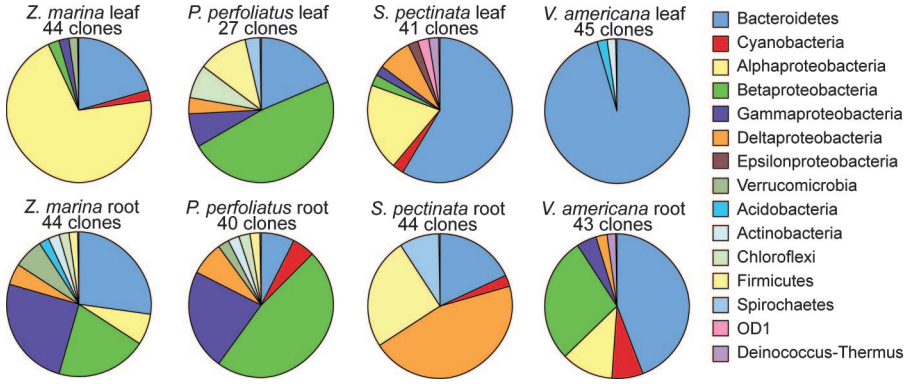
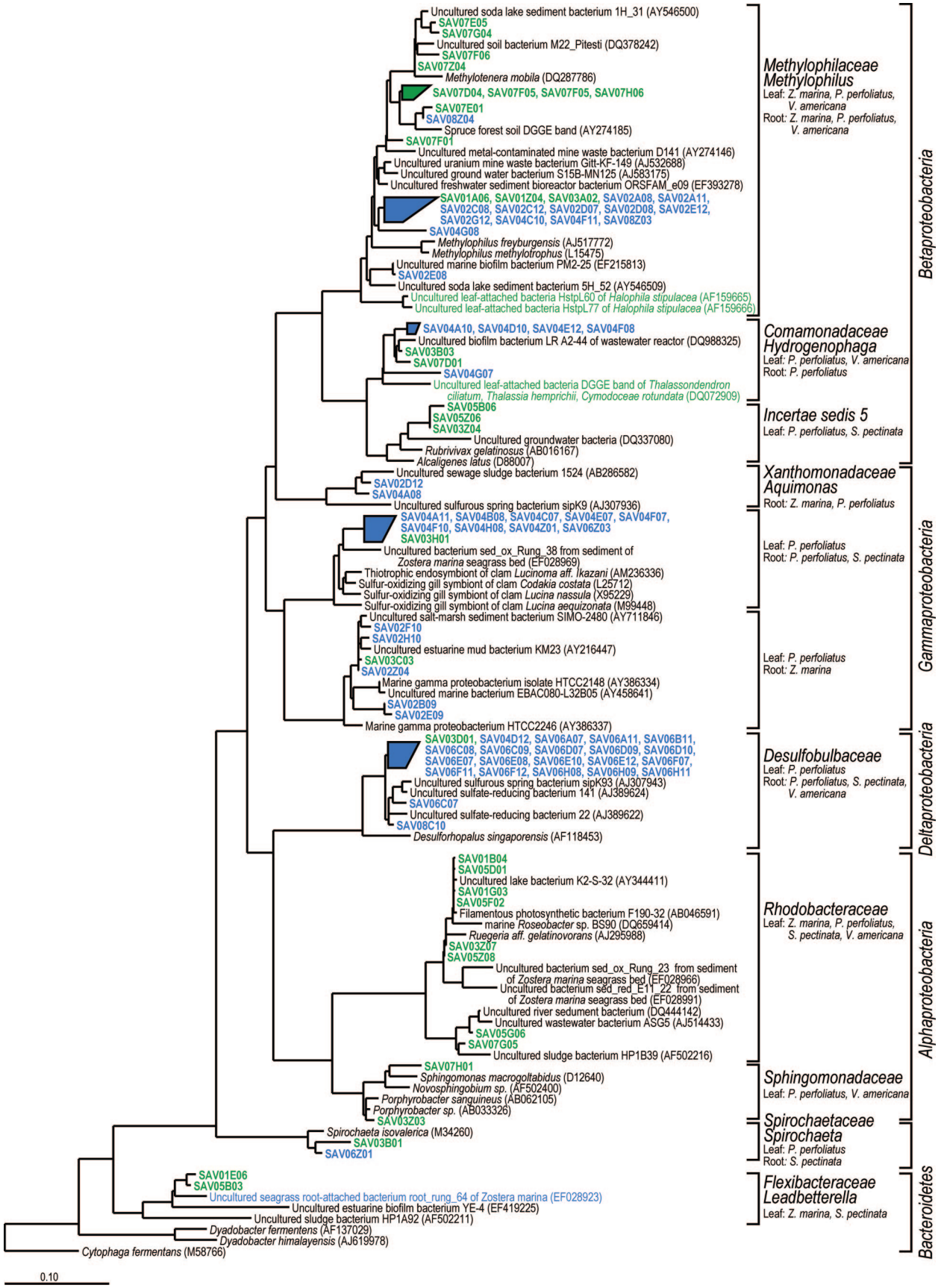


FIG. 4. Pie diagrams showing the major phylogenetic taxa of bacteria represented in PCR-based clone libraries of 16S rRNA genes from leaf and root samples.


Uncultured soda lake sediment bacterium 1H_31 (AY546500)
SAV07E05
SAV07G04
Uncultured soil bacterium M22_Pitesti (DQ378242)
SAV07F06
SAV07Z04
Methylotenera mobila (DQ287786)
SAV07D04, SAV07F05, SAV07F05, SAV07H06
SAV07E01
SAV08Z04
Spruce forest soil DGGE band (AY274185)
SAV07F01
Uncultured metal-contaminated mine waste bacterium D141 (AY274146)
Uncultured uranium mine waste bacterium Gitt-KF-149 (AJ532688)
Uncultured ground water bacterium S15B-MN125 (AJ583175)
Uncultured freshwater sediment bioreactor bacterium ORSFAM_e09 (EF393278)
SAV01A06, SAV01Z04, SAV03A02, SAV02A08, SAV02A11, SAV02C08, SAV02C12, SAV02D07, SAV02D08, SAV02E12, SAV02G12, SAV04C10, SAV04F11, SAV08Z03
SAV04G08
Methylophilus freyburgensis (AJ517772)
Methylophilus methylotrophus (L15475)
Uncultured marine biofilm bacterium PM2-25 (EF215813)
SAV02E08
Uncultured soda lake sediment bacterium 5H_52 (AY546509)
Uncultured leaf-attached bacteria HstpL60 of Halophila stipulacea (AF159665)
Uncultured leaf-attached bacteria HstpL77 of Halophila stipulacea (AF159666)
Methylophilaceae
Methylophilus
Leaf: Z. marina, P. perfoliatus, V. americana
Root: Z. marina, P. perfoliatus, V. americana
SAV04A10, SAV04D10, SAV04E12, SAV04F08
Uncultured biofilm bacterium LR A2-44 of wastewater reactor (DQ988325)
SAV03B03
SAV07D01
SAV04G07
Uncultured leaf-attached bacteria DGGE band of Thalassondendron ciliatum, Thalassia hemprichii, Cymodoceae rotundata (DQ072909)
Comamonadaceae
Hydrogenophaga
Leaf: P. perfoliatus, V. americana
Root: P. perfoliatus
SAV05B06
SAV05Z06
SAV03Z04
Uncultured groundwater bacteria (DQ337080)
Rubrivivax gelatinosus (AB016167)
Alcaligenes latus (D88007)
Incertae sedis 5
Leaf: P. perfoliatus, S. pectinata
Betaproteobacteria
Uncultured sewage sludge bacterium 1524 (AB286582)
SAV02D12
SAV04A08
Uncultured sulfurous spring bacterium sipK9 (AJ307936)
Xanthomonadaceae
Aquimonas
Root: Z. marina, P. perfoliatus
SAV04A11, SAV04B08, SAV04C07, SAV04E07, SAV04F07, SAV04F10, SAV04H08, SAV04Z01, SAV06Z03
SAV03H01
Uncultured bacterium sed_ox_Rung_38 from sediment of Zostera marina seagrass bed (EF028969)
Thiotrophic endosymbiont of clam Lucinoma aff. Ikazani (AM236336)
Sulfur-oxidizing gill symbiont of clam Codakia costata (L25712)
Sulfur-oxidizing gill symbiont of clam Lucina nassula (X95229)
Sulfur-oxidizing gill symbiont of clam Lucina aequizonata (M99448)
Leaf: P. perfoliatus
Root: P. perfoliatus, S. pectinata
Uncultured salt-marsh sediment bacterium SIMO-2480 (AY711846)
SAV02F10
SAV02H10
Uncultured estuarine mud bacterium KM23 (AY216447)
SAV03C03
SAV02Z04
Marine gamma proteobacterium isolate HTCC2148 (AY386334)
Uncultured marine bacterium EBAC080-L32B05 (AY458641)
SAV02B09
SAV02E09
Marine gamma proteobacterium HTCC2246 (AY386337)
Leaf: P. perfoliatus
Root: Z. marina
Gammaproteobacteria
SAV03D01, SAV04D12, SAV06A07, SAV06A11, SAV06B11, SAV06C08, SAV06C09, SAV06D07, SAV06D09, SAV06D10, SAV06E07, SAV06E08, SAV06E10, SAV06E12, SAV06F07, SAV06F11, SAV06F12, SAV06H08, SAV06H09, SAV06H11
Uncultured sulfurous spring bacterium sipK93 (AJ307943)
Uncultured sulfate-reducing bacterium 141 (AJ389624)
SAV06C07
Uncultured sulfate-reducing bacterium 22 (AJ389622)
SAV08C10
Desulforhopalus singaporensis (AF118453)
Desulfobulbaceae
Leaf: P. perfoliatus
Root: P. perfoliatus, S. pectinata, V. americana
Deltaproteobacteria
SAV01B04
SAV05D01
Uncultured lake bacterium K2-S-32 (AY344411)
SAV01G03
SAV05F02
Filamentous photosynthetic bacterium F190-32 (AB046591)
marine Roseobacter sp. BS90 (DQ659414)
Ruegeria aff. gelatinovorans (AJ295988)
SAV03Z07
SAV05Z08
Uncultured bacterium sed_ox_Rung_23 from sediment of Zostera marina seagrass bed (EF028966)
Uncultured bacterium sed_red_E11_22 from sediment of Zostera marina seagrass bed (EF028991)
Uncultured river sedument bacterium (DQ444142)
Uncultured wastewater bacterium ASG5 (AJ514433)
SAV05G06
SAV07G05
Uncultured sludge bacterium HP1B39 (AF502216)
Rhodobacteraceae
Leaf: Z. marina, P. perfoliatus, S. pectinata, V. americana
SAV07H01
Sphingomonas macrogoltabidus (D12640)
Novosphingobium sp. (AF502400)
Porphyrobacter sanguineus (AB062105)
Porphyrobacter sp. (AB033326)
SAV03Z03
Sphingomonadaceae
Leaf: P. perfoliatus, V. americana
Spirochaeta isovalerica (M34260)
SAV03B01
SAV06Z01
Spirochaetaceae
Spirochaeta
Leaf: P. perfoliatus
Root: S. pectinata
Alphaproteobacteria
SAV01E06
SAV05B03
Uncultured seagrass root-attached bacterium root_rung_64 of Zostera marina (EF028923)
Uncultured estuarine biofilm bacterium YE-4 (EF419225)
Uncultured sludge bacterium HP1A92 (AF502211)
Dyadobacter fermentens (AF137029)
Dyadobacter himalayensis (AJ619978)
Flexibacteraceae
Leadbetterella
Leaf: Z. marina, S. pectinata
Cytophaga fermentans (M58766)
Bacteroidetes
0.10

several aquatic plants in two separate beds and found that microbial diversity was linked to plant species rather than study site. Similar results were obtained for rhizosphere soil microbial communities of some terrestrial plants (15, 40). However, in other studies the terrestrial plant-associated microbial diversity was influenced more by soil type (63), plot typology, and local vegetation profile (55) than by individual plant species. Sediment characteristics and other environmental conditions (e.g., salinity) were clearly different for our three sampling locations and may have been responsible for the variability in attached bacterial communities for some plant species. However, *P. perfoliatus* and *S. pectinata* were collected from the same bed, and their associated bacterial communities were more similar within species than between species (Fig. 2). This might have resulted from a community shift during the 13 days between the sampling dates for the two plant species, but it may also be explained by the presence of plant-species-specific microbial communities.

Bed morphology appeared to influence the heterogeneity of leaf-attached bacterial communities. DGGE similarity values for replicates of leaf-attached communities of *P. perfoliatus* and *S. pectinata* were much higher than those for *Z. marina* and *V. americana* (Fig. 2). *P. perfoliatus* and *S. pectinata* are canopy-forming species, meaning that they grow all the way to the water surface. These dense beds greatly slow water flow over and through the bed and create a very-low-energy environment in which leaf-attached communities may develop. In contrast, *Z. marina* and *V. americana* are meadow-forming species that reach the water surface only when they are reproductive, and, although they reduce the hydrodynamic energy of their environment, their leaves are always exposed to overlying currents and waves. Additionally, our sampling sites for these meadow-forming species were more exposed to wind and waves than the sampling sites for the canopy-forming species. Such conditions allow more water and suspended material to move over and through the beds and may result in greater heterogeneity in leaf-attached bacterial communities.

Despite differences in environmental conditions, bed morphology, overall bacterial community composition, and sample collection date, we identified 12 bacterial OTUs that appeared on more than one plant species. We hypothesized that these ubiquitous populations comprise unique, adapted, and potentially mutualistic communities of plant-attached bacteria. Rare populations may have been part of these adapted communities but may also have been sediment or planktonic bacterial populations that randomly associated with the dominant plant-attached bacterial communities. Bacterial groups associated with more than one plant species were members of the *Proteobacteria*, *Spirochaetes*, and *Bacteroidetes* (Fig. 5). For many of these groups, attachment to plant surfaces seems to be appropriate based on the physiology and metabolism of cultivated relatives. *Bacteroidetes* related to uncultivated bacteria from *Z. marina* roots (33) were found attached to leaves of *Z. marina* and *S. pectinata*. This bacterial phylum includes many organisms capable of producing a wide array of enzymes for polymer degradation and are commonly found attached to surfaces (58).

Deltaproteobacteria related to the *Desulfurobulbaceae* were found on roots of *P. perfoliatus*, *S. pectinata*, and *V. americana*. These organisms are obligately anaerobic sulfate reducers and are found in salt marsh rhizospheres (6, 35) and other marine sediments. Sulfate-reducing bacteria are known colonizers of aquatic plant roots (4, 19, 41) and are thought to be responsible for most nitrogen fixation in seagrass and salt marsh sediments (10, 73).

Gammaproteobacteria related to sulfur-oxidizing isolates and to uncultivated bacteria from seagrass sediments (33) were identified on roots of *P. perfoliatus* and *S. pectinata*. Sulfur-oxidizing bacteria have been isolated from seagrass roots (41) and may contribute to chemical and plant-mediated detoxification of sulfides produced by sulfate-reducing bacteria (38).

Populations of *Roseobacter* sp. were identified on the leaves of *Z. marina*, *P. perfoliatus*, and *S. pectinata*. *Roseobacter* sp. is thought to use a photoheterotrophic metabolism in which heterotrophy is supplemented with a light-harvesting system to produce ATP (62, 67), an ability appropriate for life attached to a plant leaf. This genus of *Alphaproteobacteria* includes cultivated organisms capable of metabolizing organic sulfur compounds, such as the common algal osmolyte dimethylsulfoniopropionate (22). Organic osmolytes in seagrasses include organic acids, soluble carbohydrates, and amino acids but apparently do not include sulfur-containing compounds (26, 50, 69, 77).

Populations belonging to the order *Methylophilales* were found on the leaves or roots of *Z. marina*, *P. perfoliatus*, and *V. americana*. Cultivated organisms belonging to this order of *Betaproteobacteria* metabolize single-carbon organic molecules, such as methanol and methylamine, and some are capable of denitrification (21). Distantly related methylotrophic bacteria belonging to the *Alphaproteobacteria*, such as *Methylobacterium extorquens*, are well-known plant-growth-promoting microorganisms for terrestrial plants (1, 27, 46, 59) and are thought to consume methanol released by plants as a by-product of metabolism (45, 52). These bacteria express regulator proteins involved in epiphytic growth (23) and are also capable of producing plant hormones, such as cytokinins, including *trans*-zeatin (31, 39). It is not known whether methylotrophic *Betaproteobacteria* play a similar role when they are attached to aquatic angiosperms.

A global effort is under way to restore coastal and estuarine ecosystems, and a major part of this effort is the reestablishment of aquatic angiosperm beds. Aquatic angiosperms have

FIG. 5. Bacterial rRNA gene sequences found on more than one plant species arranged in a neighbor-joining tree calculated with the Jukes-Cantor model (*Escherichia coli* positions 104 to 894; 666 bp), aligned using ARB, and subjected to a 50% base pair frequency filter in which gaps were treated as valid base pairs. Short sequences were added to the neighbor-joining tree with ARB_Parsimony. Phylum, subphylum, family and genus assignments were made with Naive Bayesian rRNA Classifier v. 2.0 of the Ribosomal Database Project using an 80% confidence threshold (70a). Green indicates leaf-attached bacteria, and red indicates root-attached bacteria. Sequences used in this study are indicated by bold type (see Table 3).

several physiological traits, such as oxygenation of the rhizosphere (60) and production of antimicrobial agents (7), that influence the composition of attached microbial communities and may encourage the growth of mutualistic microbial populations. Such mutualistic relationships are common in terrestrial plants and have been exploited for the development of plant-growth-promoting microorganisms and biocontrol agents to improve crop growth (36, 47, 74) and to aid terrestrial plant restoration (20, 65). A similar approach could be used for aquatic angiosperm restoration efforts. Application of these naturally occurring microbes during propagation and transplantation of aquatic angiosperms may improve the success of aquatic angiosperm restoration.

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