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Shifts in Rhizoplane Communities of Aquatic Plants after Cadmium Exposure

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In this study we present the comparative molecular analysis of bacterial communities of the aquatic plant *Lemna minor* from a contaminated site (RCP) and from a laboratory culture (EPA), as well as each of these with the addition of cadmium. Plants were identified as *L. minor* by analysis of the *rpl*16 chloroplast region. Comparative bacterial community studies were based on the analyses of 16S rRNA clone libraries, each containing about 100 clones from the root surfaces of plants. Bacterial communities were compared at three phylogenetic levels of resolution. At the level of bacterial divisions, differences in diversity index scores between treatments, with and without cadmium within the same plant type (EPA or RCP), were small, indicating that cadmium had little effect. When we compared genera within the most dominant group, the β-proteobacteria, differences between unamended and cadmium-amended libraries were much larger. Bacterial diversity increased upon cadmium addition for both EPA and RCP libraries. Analyses of diversity at the phylotype level showed parallel shifts to more even communities upon cadmium addition; that is, percentage changes in diversity indices due to cadmium addition were the same for either plant type, indicating that contamination history might be independent of disturbance-induced diversity shifts. At finer phylogenetic levels of resolution, the effects of cadmium addition on bacterial communities were very noticeable. This study is a first step in understanding the role of aquatic plant-associated microbial communities in phytoremediation of heavy metals.

Effluent from industrial sources and urban runoff often contains toxic concentrations of heavy metals such as cadmium, copper, zinc, nickel, and lead that pollute surface waters. The heavy metal cadmium is well studied in aquatic environments because it is a particularly toxic heavy metal. Cadmium has no known function in biological systems, and even small amounts can be toxic to organisms. While concentrations in natural fresh waters are typically around 0.01 μg/liter, concentrations in impacted waters can be above several μg/liter (45). The U.S. Environmental Protection Agency has set the drinking water limit for Cd at 5 μg/liter (46) and has classified Cd as a priority pollutant.

Phytoremediation, the use of plants to remove pollutants from the environment, is an emerging technology that may be used to remediate aquatic systems. Many studies have focused on terrestrial plants and environments (5, 49), but this technology could be applied to the remediation of surface waters (38). Plants that can accumulate metals at higher concentrations than those found in the environment (for Cd, at least 100 mg/kg in dry plant tissues) are termed hyperaccumulators (4). Hyperaccumulation may also be based on the bioconcentration factor, the amount of metal taken up by the plant compared to the amount in the outside solution (52).

Lemna minor, a small aquatic floating angiosperm, has been well studied for its ability to remove metals from surface waters. This plant rarely flowers in nature and most often grows clonally, doubling every 2 to 3 days under optimal conditions (28). *L. minor* has been shown to accumulate as much as 1,300 times more Cd than concentrations present in the surrounding water or as much as 14,000 mg/kg Cd (48, 52), suggesting that this plant is a hyperaccumulator of Cd. Consequently, a number of studies in the past two decades have focused on the ability of *L. minor* to remove Cd from surface waters for phytoremediation (10, 18). This plant may be a good choice for remediation projects because, due to its rapid growth rate and ease of harvest, it can serve as a harvestable accumulator, keeping the metal from continuous reintroduction into the ecosystem (10).

Several studies of phytoremediation in terrestrial systems have shown that rhizosphere bacteria may contribute to plant metal tolerance and increase metal uptake. Metal-tolerant bacteria associated with *Brassica juncea* increased heavy metal uptake in the plants, possibly due to increased root biomass stimulated by bacteria (37). Several possible mechanisms for how bacteria might increase plant metal tolerance and uptake include bacterial stimulation of the production of metal transport proteins in plants, bacterial increase of metal bioavailability to plants, and increase in root biomass by bacteria (12). Others have related the basis of this plant-microbe interaction to bacterial metal resistance, since the bioavailability of metals could be altered by bacterial expression of resistance systems (47). Some bacteria expressing Cd resistance produce more extracellular polymeric substances, which binds the metal, perhaps making the microenvironment around the plant less toxic (13).

In aquatic systems, there have not been studies on the effects of rhizosphere or rhizoplane (root surface) bacteria on metal uptake in plants. While the definitions of the rhizosphere may differ from terrestrial to aquatic systems due to high diffusion

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rates of root exudates in water, Coler and Gunner observed a rhizosphere effect in the aquatic environment, showing that microbial activity around the roots of *Lemna minor* was greater than that around inert surfaces (8). From the 1960s to the 1980s, several studies of *Lemna* and associated bacteria were conducted. These included microscopic observations and enumeration of bacteria on plant surfaces as well as several culture-dependent studies (for review, see reference 27).

Despite the increased interest in *L. minor* for removal of metals from aquatic environments, there have been few recent studies of microbial communities associated with the plant, especially in relation to heavy metal uptake or sensitivity. This study is the first to address microbial communities associated with *L. minor* in the presence of a heavy metal. Because plant-associated microbial communities have previously been shown to be involved in controlling plant uptake of metals from their surrounding environment, it is important to address the influence of Cd exposure on the indigenous bacterial communities of plants from aquatic environments. This investigation was based on the hypothesis that plant metal uptake is influenced by the microbial communities associated with aquatic plants.

In this study, we have used 16S rRNA analysis to describe bacterial communities associated with the roots of *Lemna minor* plants from an environment with low-level surface water pollution as well as from the sensitive toxicity test strain of *L. minor* used by the U.S. Environmental Protection Agency. We compared bacterial communities based on types of plants or Cd concentrations in water and also determined how bacterial communities on the plant surfaces change when plants are exposed to Cd.

MATERIALS AND METHODS

Plant collections and culture. Samples of *Lemna minor* were collected from Rice City Pond (RCP), an impoundment of the Blackstone River near Uxbridge, Massachusetts. Sediments from this site have Cd concentrations in the mg/kg range and variable surface water concentrations in the range of 1 to 100 μg/liter (30, 41; G. Lanza, personal communication). Historically, mill wastes contributed to the heavy metal pollution at this site; today, contamination originates mostly from urban runoff (41). Plants were kept in culture with water from Rice City Pond. In addition, a Cd-sensitive toxicity test strain of *Lemna minor*, obtained from the U.S. Environmental Protection Agency, Cincinnati, OH (EPA), was grown in culture containing autoclaved distilled water amended with a nutrient solution defined by the American Society for Testing and Materials (here termed EPA water) (2). Both plant types were grown under constant conditions (20°C, 16 h light, 8 h dark).

Plant identification. Because the genus *Lemna* has few distinguishing characteristics, we identified plants isolated from RCP as *L. minor* by molecular analysis of the chloroplast *rpl*16 intron region (22, 31), one marker that has been previously used for plant classification. DNA was extracted from plants using the Wizard Genomic DNA purification kit (Promega, Madison, WI) followed by PCR with primers F71 (22) and R622 (31). PCR amplifications were prepared with 1× buffer, 0.25 mM each deoxynucleoside triphosphate, 1 U/10 μl *Taq* polymerase (all from Promega, Madison, WI), 1.5 mM $MgCl_2$ (Sigma, St. Louis, MO), 0.5 μM each primer (Integrated DNA Technologies, Coralville, IA), and 40 ng DNA template in a final volume of 30 μl. Reactions were performed in a PTC-200 Peltier thermal cycler (MJ Research, Waltham, MA) and consisted of 35 cycles of 95°C for 45 seconds, 52°C for 45 seconds, and 72°C for 45 seconds, with an initial denaturation at 95°C for 5 min and a final extension at 72°C for 5 min (31). PCR products were purified with the QIAquick PCR purification kit (QIAGEN, Valencia, CA), quantified by comparison to PCR ladder V (PGC Scientifics, Frederick, MD) using a digital imaging system with LabWorks software (UVP, Upland, CA), and directly sequenced.

Sequencing reactions were prepared using the ABI Big Dye 3.1 kit (Applied Biosystems, Foster City, CA). Dye terminator cycle sequencing reactions were prepared according to manufacturer's instructions with the primer F71 and were sequenced with a model 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA). Sequences were compared to those previously submitted to GenBank by Les et al. (31) and were aligned with those in the database using the Clustal interface in the software package BioEdit (17). BioEdit was also used to create similarity matrices for *Lemnaceae* sequences and phylogenetic trees were created with the software MEGA 2 (26).

Experimental setup. EPA plants and RCP plants were used for 14-day treatment experiments and were grown either with or without 100 μg/liter $CdSO_4$. EPA plants were grown in EPA water and RCP plants were grown in RCP water. One hundred μg/liter was chosen as a test concentration based on literature reports and our previous test series that showed this as the highest sublethal concentration of Cd to both plant types over two weeks (21; data not shown). Experiments were run for 14 days because previous studies showed that longer periods of incubation may lead to metal desorption from plant tissue (10, 18).

Total DNA extraction. After 14-day treatments, about 20 plant roots per sample were harvested. Plants were rinsed in sterile distilled water and roots were separated from fronds. Roots from each sample were pooled in 0.1 M potassium phosphate buffer at pH 7 and were shaken for 5 min to remove bacteria from the root surface. Roots were removed from buffer and cell material was collected by centrifugation. The protocol for mini preparation of genomic DNA from bacteria followed that from the work of Ausubel et al. (3), with the following modifications: lysozyme (300 μg/ml) was added followed by sodium dodecyl sulfate (0.5%) to facilitate cell lysis, and RNase A (25 μg/ml) was added prior to proteinase K addition to remove any coextracted RNA.

PCR amplification and cloning of 16S rRNA genes. DNA extraction was followed by PCR using bacterium-specific primers 338F (1) (the complement of EUB338) and 1492R (29) (*E. coli* numbering). Reaction mixtures were prepared with PCR buffer, $MgCl_2$, deoxynucleoside triphosphates, primers, *Taq* polymerase, and DNA template in 30-μl volumes at the same concentrations described above. Amplification used 30 cycles of 95°C for 30 seconds, 56°C for 30 seconds, and 72°C for 30 seconds with an initial denaturation at 95°C for 5 min and a final extension at 72°C for 5 min. In order to minimize PCR bias in subsequent cloning steps, three separate reactions were run for each sample and then pooled together before DNA quantification. Quantification of PCR products was performed as previously described.

Cloning of mixed community 16S rRNA gene fragments was performed using the pGEM T-easy vector (Promega, Madison, WI). Ligation into the vector at an insert to vector ratio of 1:1 and transformation of *E. coli* JM109 cells followed manufacturer's instructions. One hundred positive clones were chosen randomly for community analysis. We designed PCR primers specific for the vector sites flanking the insert, pGEMF (5′-GCAAGGCGATTAAGTTGGG-3′) and pGEMR (5′-ATGACCATGATTACGCCAAG-3′), to amplify 16S rRNA gene products from the vector of individual clone colonies. Products inserted randomly into the vector in a forward (sequence beginning with 338F) or reverse (sequence beginning with 1492R) direction relative to the forward vector primer site.

Sequencing and phylogenetic analysis. PCR products from clone libraries were sequenced as previously described using the forward vector primer pGEMF. Sequences were first edited manually using the program SeqEd (Genetics Computer Group, Madison, WI) and were searched against the Ribosomal Database Project (RDP II) (7) to determine nearest matches. Sequences were divided into two groups, based on whether orientation in the vector was in the forward or reverse direction. Multiple sequence alignments were created using ClustalX (43) or the Clustal interface within the software package BioEdit. BioEdit was used to create similarity matrices for all sequences and to determine phylotypes (based on ≥97% similarity). Phylogenetic trees were created with MEGA 2. Possible chimeras were detected using CHIMERA_CHECK in the RDP II and also with the software Bellerophon (20). Partial sequences of ≈600 bp in length, from positions 338 to 940 or ~940 to 1492 (*E. coli* numbering) were deposited in GenBank with the accession numbers AY707525-AY707602 (forward sequences) and AY803199-AY803235 (reverse sequences).

Diversity indices and statistics. Diversity profiles, or relative abundance patterns, of the community based on sequences were created (35). The Shannon-Weiner index (H), Simpson's reciprocal index (1/D), richness (S), the number of phylotypes, evenness ($E_H = H/\ln S$), and the distribution of phylotype abundance were calculated at the division, genus, and phylotype levels. The Shannon-Weiner index [$H = -\Sigma(pi)\ (\ln pi)$] and Simpson's reciprocal index, 1/D, where D equals $\Sigma(pi)^2$ and where pi is the proportion of phylotypes (or groups) i relative to the total number of phylotypes, both take richness and evenness into account (32).

Further analyses of libraries at the phylotype level included nucleotide diversity and $\theta\pi$ using the software package Arlequin (39). θ_π is the average sequence divergence or an estimate of the total genetic variation in a sample. Dividing θ_π

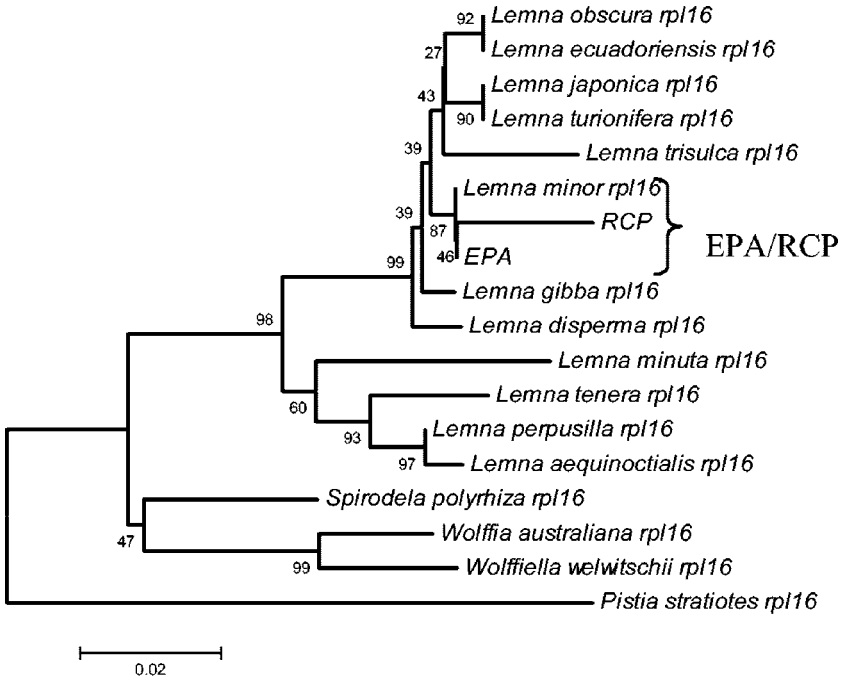

FIG. 1. Phylogenetic identification of the plants used in this study. Phylogenetic analysis of the *rpl*16 chloroplast intron region was used for identification of members of the family *Lemnaceae*. EPA plants and RCP plants group with the *Lemna minor rpl*16 gene, while other species of *Lemna* form separate clusters. The *rpl*16 region from *Pistia stratiotes* was used as an outgroup. The phylogenetic tree was constructed using neighbor-joining analysis with 1,000 bootstrap replicates. Bootstrap values are indicated at nodes. The scale bar indicates 0.02 nucleotide substitution.

over the length of sequences equals the average nucleotide diversity, the probability that randomly chosen bases differ at a given position (33). LIBSHUFF was used to determine whether clone libraries from plants of various metal treatments (with and without Cd) were significantly different ($P < 0.05$) from one another by comparing coverage curves of libraries to each other first in an X/Y fashion and then the reverse, Y/X (40). Rarefaction curves were created using the software aRarefactWin (19) to determine whether environments were exhaustively sampled. The software EstimateS 6 was used to calculate S_{ACE} and S_{Chao1} (9), coverage estimators that determine the number of probable phylotypes in the environment compared to the numbers observed.

Nucleotide sequence accession numbers. Sequences were submitted to GenBank with the accession numbers AY714543 to AY714544, AY707525 to AY707602, and AY803199 to AY803235.

RESULTS

Plant identification. Phylogenetic analysis of the *rpl*16 chloroplast intron region of EPA and RCP plants revealed that RCP plants and EPA plants clustered well with *Lemna minor* (Fig. 1). Based on a similarity matrix of all *Lemnaceae rpl*16 sequences used in the construction of the phylogenetic tree, the nearest match for both EPA and RCP plants, with similarity >98%, was *L. minor*. Based on the region analyzed, EPA plants are identical to the strain in the database and RCP plants are slightly less closely related but still within the *Lemna minor* cluster.

Bacterial community analysis. 16S rRNA gene analysis was completed for clone libraries of bacteria from roots of EPA plants, EPA plants in Cd, RCP plants, and RCP plants in Cd. Eight putative chimeras out of 365 sequences were detected and removed from the analysis. Bacterial communities were compared at the level of bacterial divisions (Fig. 2) down to levels of genera (Fig. 3a to d), and phylotypes. Results from forward and reverse analyses were combined where possible. Where results were not combined (LIBSHUFF analysis, genetic diversity, and phylogenetic tree construction) (Fig. 4), forward and reverse data sets were both analyzed separately. Results were found to be similar, and only the results of forward analyses are shown.

Bacterial diversity at the division level. The dominant bacterial division in all libraries was the β-proteobacteria, with cyanobacteria, α-proteobacteria, γ-proteobacteria and sphingobacteria also represented in all libraries. Shannon-Weiner and Simpson's reciprocal indices calculated at the division level showed that shifts in diversity between Cd treatments were rather small, less than 10% for the Shannon index and about 15% for the Simpson's index (Table 1).

Bacterial diversity at the genus level. When comparing genera within the β-proteobacteria, an increase in diversity was observed with Cd addition in both the EPA and RCP clone libraries. The Shannon and Simpson's reciprocal indices showed an increase in diversity for Cd-amended libraries over unamended libraries for both RCP and EPA plants, at the same percentages (Table 1). Cd addition also caused an increase in evenness, or abundance distribution of genera. For the EPA plants, there was a shift from clear dominance by *Sterolibacterium* (70%) in the unamended library to a more even community distribution in the presence of Cd. While *Sterolibacterium* decreased to only 19% of the total community, *Hydrogenophaga* rose to 27% and *Acidovorax* appeared at 12%. There was also an increase in the abundance of β-proteobacteria that matched most closely with unclassified environmental clones, mostly belonging to the order *Burkholderiales* (Fig.

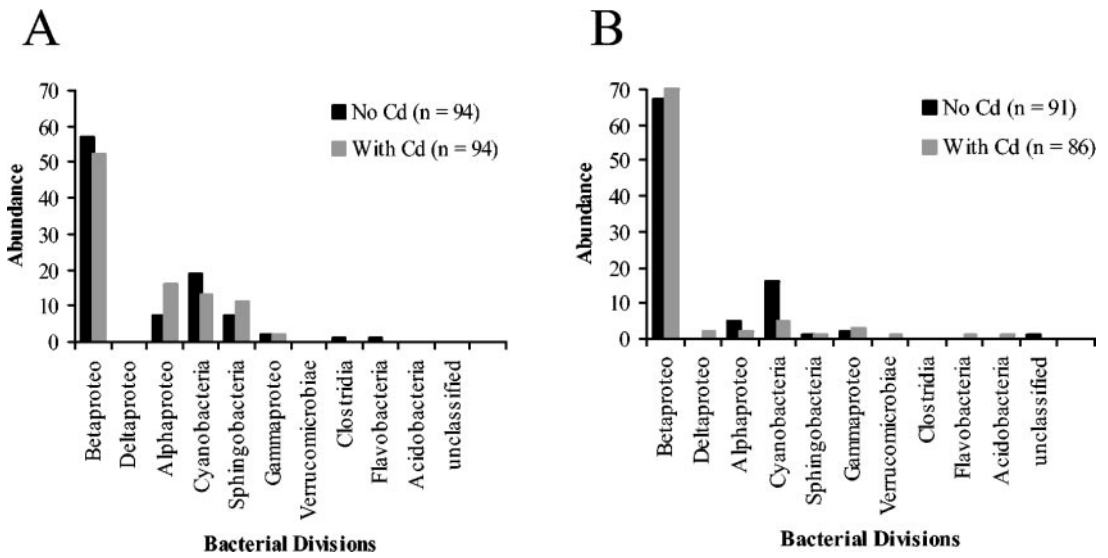

FIG. 2. Abundance of clones from bacterial divisions present in clone libraries of (A) EPA plants (black bars) or EPA plants plus Cd (gray bars). RCP clone libraries are shown in panel B for RCP plants (black bars) or RCP plants plus Cd (gray bars). The number of clones used for each clone library analysis is indicated.

3). In the case of RCP plants, *Leptothrix* clearly dominated at 73% in the unamended library, but in the presence of Cd, there was a decrease of *Leptothrix* to 23%. Also, the relative abundance of *Rhodoferax* more than tripled from 10% without Cd to 36% with Cd, while *Zoogloea* appeared only in the RCP plus Cd library at 20% (Fig. 3).

In EPA libraries, the shift from dominance by *Sterolibacterium* caused a sharp decrease in the order *Rhodocyclales* and an increase in the order *Burkholderiales* upon Cd addition. In RCP libraries members of the order *Burkholderiales* dominated libraries plus or without Cd. *Rhodocyclales* were absent from the RCP without Cd library, but the appearance of *Zoogloea* added *Rhodocyclales* to the RCP plus Cd library (Table 2).

Bacterial diversity at the phylotype level. At the phylotype level, Shannon and Simpson's reciprocal indices also showed an increase in bacterial diversity from unamended to Cd-amended libraries. Nucleotide diversity and θ_π increased following the addition of Cd, but the relative increase was smaller than that seen with other diversity indices (Table 1).

LIBSHUFF analysis showed that RCP plants without Cd compared to RCP plants with Cd had significantly different bacterial communities in both X/Y and Y/X comparisons (P =

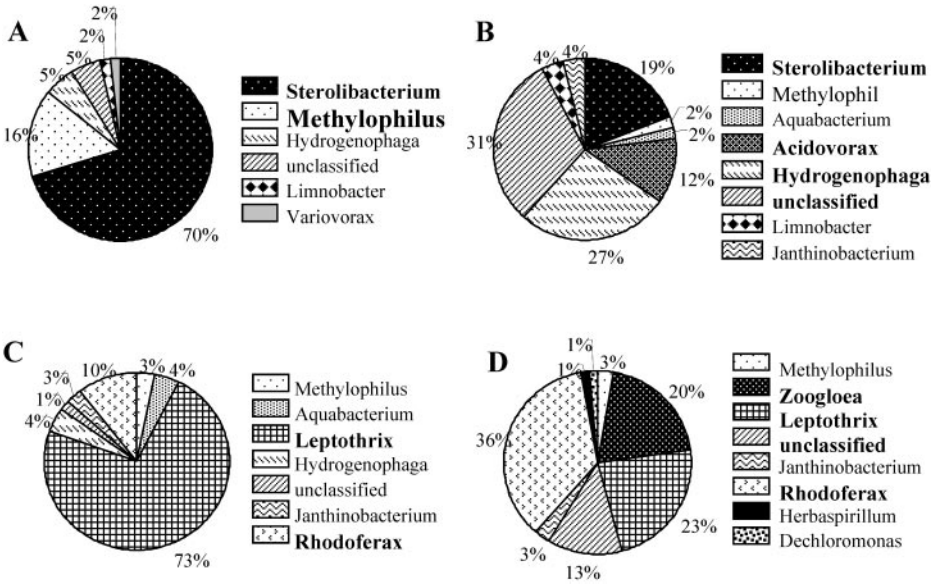

FIG. 3. Relative abundance of genera within the dominant group, β-proteobacteria, in plant-associated communities. The influence of cadmium addition is shown for (A) EPA plants in EPA water, (B) EPA plants in EPA water amended with Cd, (C) RCP plants in RCP water, and (D) RCP plants in RCP water amended with Cd. Genera are assigned based on closest RDP II matches. Genera listed in bold are dominant groups within each library. Percentages are based on the number of clones (n) of β-proteobacteria in each library.

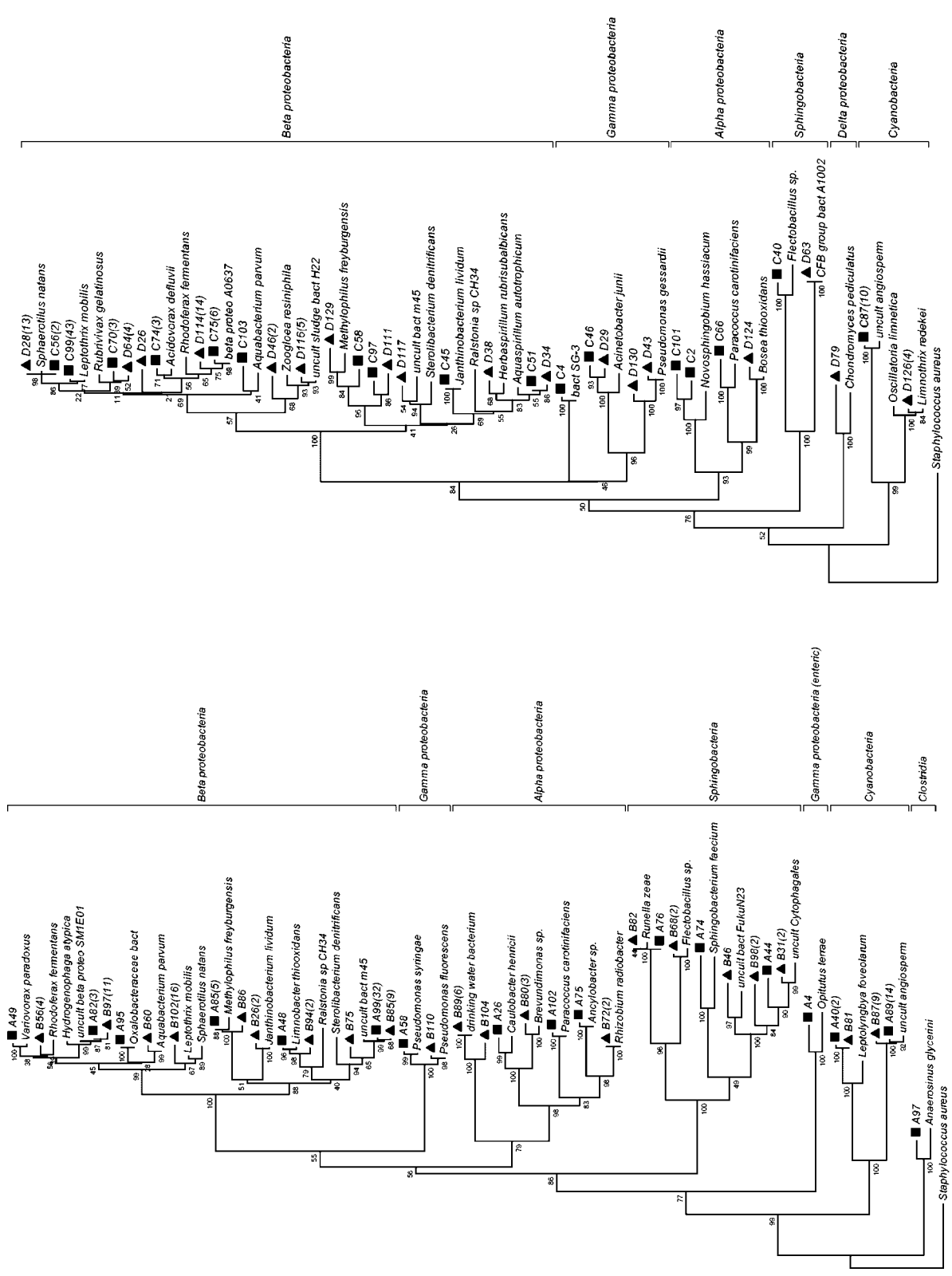

FIG. 4. Phylogenetic relationships of bacteria detected on EPA plants (EPA libraries A and B, left) and on RCP plants (RCP libraries C and D, right) inferred from 16S rRNA sequence analysis. Trees were constructed using the neighbor-joining algorithm with 1,000 bootstrap replicates. For each tree, ■ represents no Cd added and ▲ represents the addition of Cd. Numbers in parentheses represent the number of clones represented by each phylotype (≥97% similarity). *Staphylococcus aureus* is included as an outgroup for each tree. The scale bar represents nucleotide substitutions.

TABLE 1. Diversity indices for clone libraries at the division level, genus level (within β-proteobacteria), and phylotype level

Level	Treatment	Shannon (H)	Simpson's (1/D)	Richness (S)	Evenness (E_H)	Nucleotide diversity	Total genetic variation (θ_π)
Division	A (EPA plants)	1.19	2.41	7	0.61		
	B (EPA plants + Cd)	1.24	2.77	5	0.77		
	C (RCP plants)	0.88	1.80	6	0.49		
	D (RCP plants + Cd)	0.83	1.50	9	0.38		
	% change[a]						
	A→B	+4	+13	−29	+20		
	C→D	−6	−17	+33	−23		
	A→C	−26	−25	−14	−20		
	B→D	−33	−46	+44	−51		
Genus	A (EPA plants)	0.99	1.94	6	0.55		
	B (EPA plants + Cd)	1.68	4.86	8	0.81		
	C (RCP plants)	0.97	1.78	7	0.50		
	D (RCP plants + Cd)	1.62	4.4	8	0.78		
	% change						
	A→B	+41	+60	+25	+32		
	C→D	+40	+60	+13	+36		
	A→C	−2	−8	+14	−10		
	B→D	−4	−9	0	−4		
Phylotype	A (EPA plants)	2.19	4.71	24	0.69	0.174	107.85
	B (EPA plants + Cd)	2.80	13.41	26	0.86	0.183	113.37
	C (RCP plants)	1.97	3.93	20	0.66	0.108	66.70
	D (RCP plants + Cd)	2.66	10.66	26	0.82	0.127	78.32
	% change						
	A→B	+22	+65	+8	+20	+5	+5
	C→D	+26	+63	+23	+19	+15	+15
	A→C	−10	−17	−17	−4	−38	−38
	B→D	−5	−21	0	−5	−31	−31

[a] Percent change in diversity index scores from unamended to Cd-amended or from EPA to RCP plants.

0.001). When coverage curves of EPA plants grown without Cd were compared to EPA plants plus Cd (X/Y), the difference was not significant ($P = 0.205$); however, when EPA plants plus Cd were compared to EPA plants without Cd (Y/X), there was a significant difference ($P = 0.001$), indicating that the RCP and RCP plus Cd libraries have fewer common genera than the EPA and EPA plus Cd libraries. The EPA without Cd library contains most sequences found in the EPA plus Cd library, but the EPA plus Cd library contains new sequences not found in the EPA without Cd library.

Rarefaction curves were used to determine whether the level of sampling effort was sufficient to represent the possible number of phylotypes in samples, determined by asymptotic leveling off of curves. Coverage estimators provide an estimate of the number of phylotypes expected in the sample source and allow us to determine how well the sample represents that environment. For all samples, rarefaction curves began to level off only asymptotically, meaning that the full level of sampling effort had not been reached (Fig. 5). Coverage estimators suggest that ≈45 to 75% of the diversity present in our samples was covered, with the highest level of coverage occurring in the EPA plus Cd library (Table 3).

In order to exhaustively sample and fully cover all of the diversity from aquatic and other environments, extremely large

TABLE 2. Relative abundance of orders of bacteria within the β-proteobacteria

Treatment	Order	Order %[a]
A	*Burkholderiales*	14
	Methylophilales	16
	Rhodocyclales	70
B	*Burkholderiales*	79
	Methylophilales	2
	Rhodocyclales	19
C	*Burkholderiales*	97
	Methylophilales	3
D	*Burkholderiales*	76
	Methylophilales	3
	Rhodocyclales	21

[a] Percentage of order out of total β-proteobacteria in a given library.

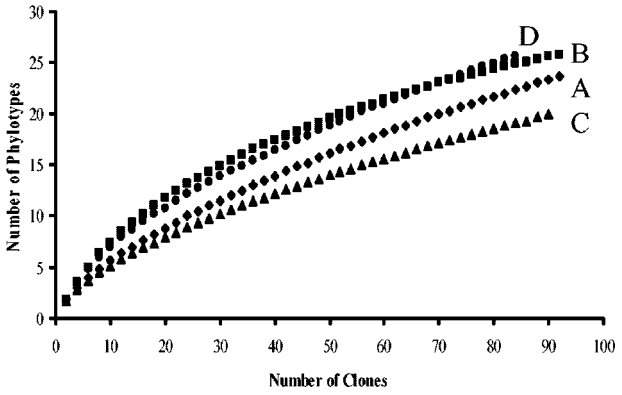

FIG. 5. Rarefaction curves for clone libraries from EPA (♦), EPA plus Cd (■), RCP (▲), and RCP plus Cd (●). Phylotypes were determined by ≥97% similarity.

TABLE 3. Estimators of phylotype abundance in clone libraries

Treatment	Richness observed[a]	S_{ACE}	S_{Chao1}	% Coverage[b]
A	24 (94)	63.5 ± 3.9	50.7 ± 29.6	42
B	26 (94)	38.6 ± 1.6	31.7 ± 5.9	74
C	20 (92)	46.5 ± 2.9	42.7 ± 33.4	45
D	26 (86)	70.2 ± 4.6	43.7 ± 4.6	46

[a] Numbers in parentheses indicate the number of 16S rRNA clones used in analyses. Richness is the number of phylotypes observed.

[b] Percent coverage is the ratio of the number of phylotypes observed over the average number of phylotypes estimated (based on the average of estimates from S_{ACE} and S_{Chao1}).

(>1,000 clones) libraries may need to be constructed, which for many studies is not feasible. Coverage estimators can give unstable, biased estimates of phylotype richness if libraries are too small; however, if estimates are stable and unbiased, valid comparisons can be made (23). We determined that all of our libraries were most likely large enough to produce stable coverage estimates by using a program available at http://www.aslo.org/lomethods/free/2004/0114a.html (24).

Comparisons between plant groups (EPA versus RCP). While the main focus of this study was to study shifts in plant-associated bacterial communities with and without added Cd, there were some noteworthy patterns that appeared when looking across plant ecotypes (EPA compared to RCP). Data shown here compared plants in their own types of water (EPA plants grown in EPA water, or RCP plants grown in RCP water) with the addition of Cd as the only added stress to either system.

Within the β-proteobacteria, while distinct differences between genera from EPA or RCP were evident, such as the dominance of *Sterolibacterium* in EPA libraries and *Leptothrix* in RCP libraries, Shannon and Simpson's index scores showed that there was little difference in diversity between EPA and RCP libraries. Interestingly, these scores showed the same percentage increase between unamended and Cd-amended libraries for both EPA and RCP plants (Table 1).

Analysis at the phylotype level showed that the RCP libraries had less diverse microbial communities than their EPA counterparts. As at the genus level, parallel shifts in diversity were observed from unamended to Cd-amended libraries for EPA and RCP. The difference in diversity scores between EPA and RCP plants was in most cases less than half of the change in diversity observed between unamended and Cd-amended treatments within the same plant groups (Table 1). The exceptions were the genetic diversity measures, nucleotide diversity and $\theta\pi$. These parameters showed changes above 30% between EPA and RCP plant groups.

DISCUSSION

Based on chloroplast intron analyses, we determined that the field isolate of *Lemna* from Rice City Pond was part of the species *L. minor*. Our comparisons, therefore, were made with plants from the same species, although there is slight genetic variation between the field isolate and other strains. Other factors besides genetic variation may influence plant-associated bacterial communities, such as environment. The type of water in which the plants are grown may provide different nutrients to plants, causing different growth patterns. Because the two types of plants in our study had been originally cultured in different types of water, we chose to focus on the effect of additional Cd, as the effects of placing the plants in new types of water might result in additional stress to the plants and associated microbes.

The intermediate disturbance hypothesis states that when a disturbance (such as Cd addition) is neither too high nor too low, communities will contain the most species (14, 44). While the intermediate disturbance hypothesis has been applied to few microbial systems (11), it may offer an explanation for the increases in diversity upon the addition of heavy metal. Giller et al. (15) describe how stable environments may contain high numbers of competitive species, and adding moderate stress may decrease competitive exclusion. As stress increases, there will be an increase of species until the stress becomes too great, at which point diversity will decrease. Organisms that are adapted to the stress (tolerance to higher concentrations of Cd, for instance) will survive. In our study, we saw that upon addition of 100 μg/liter $CdSO_4$, a low-level contamination concentration, there was an increase in diversity at finer phylogenetic levels after two weeks. This could be tested with a range of Cd concentrations. We may expect that at higher concentrations of Cd, we will see an increase in the number of metal-tolerant bacteria.

Several genera within the β-proteobacteria are known for high tolerance to heavy metals (47). *Leptothrix*, found in the RCP libraries, and its close relative *Sphaerotilus* are able to adsorb heavy metal cations to their outer sheaths (36). This adsorption could keep levels of Cd entering the plant at lower, less toxic concentrations.

The *Burkholderiales*, the order within the β-proteobacteria containing known metal-resistant bacteria such as *Ralstonia* (7, 16), dominated the EPA plus Cd, RCP, and RCP plus Cd libraries, while the EPA library was dominated by the *Rhodocyclales*. As the EPA library was the least exposed to Cd, we may expect that there are fewer Cd-tolerant bacteria present here. While there are no reports of Cd tolerance in *Rhodoferax*, *Hydrogenophaga*, *Sterolibacterium*, or *Methylophilus*, these *Burkholderiales* genera did increase in relative abundance upon Cd addition. In RCP plants, when Cd was added, the genus *Zoogloea* appeared in the clone library. *Zoogloea*, while not of the order *Burkholderiales* but of the order *Rhodocyclales*, is also known for its ability to bind heavy metals in its polysaccharide matrix and has been proposed as a bioremediation agent itself (25).

At the division level, comparisons between Cd-amended and unamended libraries within the same plant type showed very similar diversity index scores, and larger changes were observed when comparing between plant types than between Cd treatments for the same plant type. This indicated that Cd had little influence on community diversity at the division level. Diversity scores were lower for RCP plants than EPA plants, and there was less evenness in the RCP libraries due to higher dominance of β-proteobacteria.

Diversity index calculations for genera within the β-proteobacteria showed that at this level, the largest changes in diversity were related to Cd addition, as opposed to plant type. Community shifts between unamended and Cd-amended libraries for both EPA and RCP plants followed the same pro-

portional changes, showing that while the genera within communities of the two plant types were different, the addition of Cd caused a parallel shift in the overall diversity of genera detected.

The dominant member of the EPA library was most closely related to *Sterolibacterium denitrificans*, which is a cholesterol-oxidizing bacterium (42). Other members of this genus that cluster well with those found in the EPA samples include uncultured clones found in biofilms on oxygen transfer membranes, or in drinking water systems (6, 50). It is reasonable to expect *Sterolibacterium* to be associated with plant roots since certain root exudates are sterol compounds. *Leptothrix* was dominant in the RCP libraries and has previously been reported in waters of *Lemna*-covered ponds (27).

Phylotype analyses, while showing similar results to those seen at the genus level, also allowed comparisons of genetic diversity and comparison of libraries using LIBSHUFF. Genetic diversity parameters revealed that libraries within plant types with or without Cd were closely related, while those from different plant types were dissimilar. Coupled with diversity index data, it is apparent that despite compositional differences, Cd causes the communities to shift similarly. RCP plants are exposed to low concentrations of Cd both at the site and in laboratory cultures when grown with RCP water, as opposed to EPA plants, which have been grown in EPA water without any Cd input. Because changes in diversity with Cd addition were the same for either plant type, history of preexposure may be independent from the observed disturbance-induced diversity shifts.

Molecular analyses give more description of the diversity of a particular environment since culture-based studies may detect only a small fraction of the bacteria present. Despite biases associated with molecular studies (51), these currently offer the most complete picture of community composition and thus are an ideal starting point for community studies. Combining molecular analyses with culturing methods will allow for a deeper understanding of community composition and function. Future isolation of bacteria from *Lemna* roots will allow us to characterize directly metal tolerance or resistance of plant-associated bacteria (L. M. Stout, M. T. Rothfeder, and K. Nüsslein, Abstr. 103rd Gen. Meet. Am. Soc. Microbiol., abstr. N276, 2003) and their potential effects on plant metal uptake or plant metal tolerance. Studies of Cd resistance systems, such as sequestration, efflux pump mechanisms, or adsorption to extracellular polymeric substances may elucidate how the plant-microbe interaction might support plant heavy metal tolerance.

To our knowledge, this is the first detailed molecular study of the bacterial communities associated with *Lemna minor* and the first report to show *Lemna* community changes in response to the addition of a heavy metal. In relation to plants and heavy metals in general, this is one of the first studies describing bacterial communities associated with aquatic plants. Investigating the response of the microbial community to heavy metal amendments will lead to a better understanding of the major factors that link microbial activity to the metal toxicity and uptake behavior of plants. Because aquatic plants may be used in phytoremediation projects, knowledge of what types of bacteria are present and whether these bacteria may facilitate metal uptake will be important for controlling and improving phytoremediation efficiency.

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