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Microbial Diversity and Resistance to Copper in Metal-Contaminated Lake Sediment

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ABSTRACT

Contamination of habitats with heavy metals has become a worldwide problem. We describe herein the analysis of lake sediment contaminated with high concentrations of copper as a consequence of mine milling disposal over a 100-year period. Copper concentrations in the sediment were found to vary with depth and ranged from 200 to 5500 ppm. Analysis of the microbial community with T-RFLP identified a minimum of 20 operational taxonomic units (OTU). T-RFLP analysis along a depth profile detected as many as nine shared OTUs across 15 centimeters, suggesting a conservation of community structure over this range. Only two genera, *Arthrobacter* and *Ralstonia*, were detected among 50 aerobic copper-resistant isolates cultivated on R2A, one of which (*Ralstonia* sp.) was characterized by the sequestration of copper, identified by electron diffraction scanning, in growing colonies. Scanning electron microscopy showed changes to the outer envelope of the cells when grown in the presence of copper. The copper-resistant *Ralstonia* isolates were also resistant to Ni, Cd, and Zn, showing two patterns of phenotypic resistant to these three metals in which either resistance to Zn or Ni was expressed in an isolate but never both.

Introduction

The contamination of soils, sediments, and waterways with metals has become an increasingly important issue [5, 29, 31]. Nowhere has such contamination been more problematic than at the primary sources for industrially important metals, the mine. Metal mining has been conducted for over two millennia with increasingly tragic results as human populations and the demands for metals increase [1, 14, 24, 28]. A typical example of the impact of metal contamination is Torch Lake, a 2700-acre lake located in Houghton County of the upper peninsula of Michigan. Over the course of 100 years, 1868 through 1968, copper-mining by-products were dumped into the lake, filling up at least 20% of its volume. Although copper mining ceased in the late 1960s, an additional discharge of 27,000 gallons of cupric ammonium carbonate into Torch Lake from a leaching plant took place in 1972. Lake sediments contain up to 5500 ppm copper [8].

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In subsequent years, discoloration of several acres of Torch Lake bottom and fish abnormalities were observed as well as measurements of high concentrations of heavy metals in lake sediments. This prompted an EPA-directed partial removal of waste drums and soil from the western shore of the Torch Lake to an offsite hazardous waste landfill between 1988 and 1989. The EPA remedy for the contaminated lake sediment (1994) is one of "No-Action," relying on "ongoing natural sedimentation and detoxification" [7].

The contaminated sediments of Torch Lake, which in some places is 30 feet deep, have become an extreme environment for the development and evolution of microbial communities. Not only is copper present in high concentrations, but other metals such as arsenic, chromium, and lead have been detected in the sediments along with organics and explosive residues. In the present work, we report on the structure of the microbial community from two samples of Torch Lake sediment using terminal restriction fragment length polymorphism of 16S rRNA. In addition, we have isolated and partially characterized bacterial populations with resistance to high levels of Cu and other metals from this community. The T-RFLP data identified at least 20 phylotypes or OTUs, and at least two genera capable of growth under aerobic conditions in the presence of high concentrations of copper (~800 ppm). One genus was of particular interest in that its apparent mechanism of copper resistance involved active sequestration of copper to a level visibly detectable in the colony.

Methods

Sampling

Sediment cores were collected from two locations in Torch Lake at water depths of 89 and 105 feet during the summer of 1999. An Oceans Instruments Multi-corer aboard the RV/*Mud Puppy* was used to retrieve four replicate cores at each site. One core was dedicated to metal analysis including copper, one for ^{210}Pb dating, one for the microbial analysis, and one for archival purposes. Each core was sectioned at 0.5-cm intervals near the surface and at 1-cm intervals toward the bottom. The average length of the cores was 0.6 m. The gross physical attributes of each sediment section was described, stored on ice in the field, and frozen at −20°C in the laboratory until analysis.

Geochemical Measurements

Sediments were lyophilized after collection and metals extracted via microwave, nitric acid digestion [11]. A standard reference material (SRM 2704, Buffalo River sediment) was also digested and analyzed for quality-control purposes. Extraction fluids were filtered through a 0.4-μm Nuclepore filter that had been acid washed. Metal concentrations in the extraction fluids were quantified using inductively coupled plasma mass spectroscopy with hexapole technology (Micromass Platform). Elemental concentrations that were in the high mg/L range were quantified using flame atomic absorption spectroscopy (PerkinElmer Model 5100PC). Elements analyzed included Cu, Zn, Ni, Cd, Cr, Co, Pb, and Fe.

DNA Isolation

DNA was extracted from sediments using the Mega soil DNA extraction kit from MO-BIO (MO-BIO Laboratories Inc., CA, USA) according to the recommendations of the manufacturer. Approximately 5 g of sediment was extracted and the DNA was precipitated and washed in 70% ethanol. The same kit was used for DNA isolation from pure cultures. The concentration of the purified DNA was determined on a diode-array spectrophotometer (Hewlett Packard) and the size of the DNA was checked on 1% agarose gels. DNA preparations were stored at −20°C until analysis.

PCR Amplification of the 16S rDNA for Sequencing

The SSU rRNA gene was PCR amplified from genomic DNA extracted from isolates using universal primers for the Bacterial domain. PCR reactions (100 μL) contained 200 ng of template DNA, 1× PCR buffer (PerkinElmer), 0.25 μM concentration of each of the four deoxynucleoside triphosphates (Gibco BRL), 2.5 mM $MgCl_2$ (PerkinElmer), 0.2 μM of forward and reverse primer, 2 units of *Taq* polymerase (PerkinElmer Amplitaq), and 8 ng of bovine serum albumin (Boehringer Mannheim, Germany). The primers used for the amplification were 27-Forward (5′-AGAGTTTGAT CCTGGCTCAG) and 1492-Reverse (5′-GGTTACCTTGTTACGACTT). A 2400 GeneAmp PCR system (PerkinElmer) thermocycler was used to incubate reactions through an initial 3-min denaturation step at 94°C, followed by 30 cycles of denaturation at 94°C for 1 min, primer annealing at 60°C for 0.45 min, and primer extension at 72°C for 2 min. The annealing temperature was selected as that temperature that consistently yielded a single PCR product of the expected size. The PCR product was purified using Microcon purification columns (Millipore Corporation 100,000 MW cutoff) according to manufacturer's recommendations.

PCR Amplification of the 16S rDNA for TRFLP

For TRFLP analysis, labeled forward (Hex-27F) and reverse (Fam-1492R) primers (Operon Technologies) were used in two separate PCR amplifications with an unlabeled primer partner (27F or 1492R) to provide coverage of at least 80% of 16S rDNA. PCR conditions were the same as described above. Three sepa-

rate 100-μL PCR reactions were performed for each labeled amplification of a community. This ensured sufficient concentration of product and avoided template-founder effects that can occur with variation in pipetting community DNA when constructing the PCR mix. After amplification, the reactions were combined and concentrated.

T-RFLP

Forward and reverse labeled PCR products derived from each sediment sample were purified and combined. Approximately 300 ng of product were digested for 3 hours at 37°C with either *Hha*I, *Msp*I, or *Rsa*I (Gibco BRL) restriction enzymes in 15-μL reaction mixtures. The reaction mixtures contained 1.5 μL of 10× restriction enzyme buffer (Gibco BRL), 1.2 μL of restriction enzyme (Gibco BRL), 8–10 μL of DNA template, and ultrapure water to a final volume of 15 μL. Reactions were inactivated by incubation at 65°C for 5 min and stored at −20°C until electrophoresis. Digestions were tested for completeness with a pure culture control (*Ralstonia eutropha*). T-RFLP digests were run on an ABI automated sequencer (model 377) in a 6% denaturing acrylamide gel. The gel was scanned using ABI Genescan software (PerkinElmer). Distribution of peaks from collected profiles was performed with ABI Genotyper software (Genotyper 2.5, PerkinElmer). Terminal fragments smaller than 50 bases or larger than 600 bases were deleted from analysis, the former because of interference from unincorporated labeled primer and the latter because of sizing inaccuracies for such large fragments. Finally, a level of 50 fluorescence units was imposed as a minimum threshold value for all peaks in the selected size range. Profiles were visually inspected and aligned based on relative peak distribution. In total, seven samples, four from the four sampling depths of site 1 and three from the three sampling depths of site 2, were digested with each enzyme. For each enzyme, duplicates were run as a means of confirming the reproducibility of the method.

Isolation of Copper-Resistant Strains

Two to four grams of sediment was vortexed vigorously in a tube with 2 mL of water. Large particulate matter was allowed to settle and 0.5 mL of the supernatant was serially diluted in 1/3 strength Trypticase Soy broth (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) and plated onto R2A and R2A supplemented with copper (200 mg/L $CuSO_4$) in triplicate. Plates were incubated at 30°C and counted after 48–72 h. Based on colony morphology, a diverse collection of 76 (10–12 from each of the seven samples) copper-resistant strains were clonally purified on $CuSO_4$-supplemented R2A agar. A subset of 14 strains from the four sampled depths of site 1 were selected for further analysis. Robust growth on copper-supplemented media and isolates representative of the diversity of colony morphology were used to select these strains.

Levels of Resistance to Cu and Other Metals

The level of Cu resistance of the strains was determined by patching part of a colony from the 200 mg/L $CuSO_4$ plates onto plates with increasing concentrations of $CuSO_4$. Using a similar procedure, copper-resistant isolates were tested for resistance to zinc (200 and 500 mg/L), nickel (1 mM and 2 mM), and cadmium (50, 150, and 300 mg/L) on R2A plates. These concentrations are considered selective for highly resistant strains [6, 27, 29].

Light Microscopy

Light microscopy to determine the cell morphology of the copper-resistant isolates was performed with a Zeiss Axiophot phase-contrast microscope. A small fraction of a colony from a plate incubated for 48–72 h at 30°C was diluted in a solution of India ink (1:5 India ink:water) and covered with a coverslip. Cell morphology was determined at 1000× with phase contrast optics.

Scanning Electron Microscopy

Scanning electron microscopy was performed on two isolates (12F and 12J) grown on unsupplemented R2A agar and R2A supplemented with $CuSO_4$ at 200 mg/L. Incubation was at 30°C for 48 h. The cells were removed from the top surface of colonies, fixed in 4% glutaraldehyde, mounted on polylysine-coated coverslips, dehydrated in an ethanol series (25%, 50%, 75%, and 95%), critical-point dried, and sputter coated with gold. At each step the cells were treated as gently as possible to preserve the integrity of the outer cellular architecture. The mounted cells were viewed on a JEOL 6400 Scanning Electron Microscope at magnifications of 7500, 15,000, and 33,000. Images were captured and stored electronically. At least 10 fields at 7500× were viewed for each isolate to obtain a representative view of morphology. The average dimensions of each isolate were computed from measurements of at least 15 well-isolated cells at 15,000×.

Energy Dispersive Spectroscopy (EDS)

EDS was conducted on a JEOL Scanning Microscope fitted with a Noran EDS detector. Identification of elements was with Noran Vantage 1.2.1 software. Cells were prepared in two ways. Using the same cells grown for standard scanning electron microscopy, in either the presence or absence of copper, a small amount of cells was removed from the top of the colony and suspended in 100 μL of distilled water. Two different support surfaces were used to check for background signals derived from the support surface. Approximately 20–40 μL of the cell suspension was placed on either a glass coverslip, or a carbon planchet, and allowed to air dry. The glass coverslips were carbon-coated in a Ladd Vacuum Evaporator and then scanned. The carbon planchets were scanned without coating.

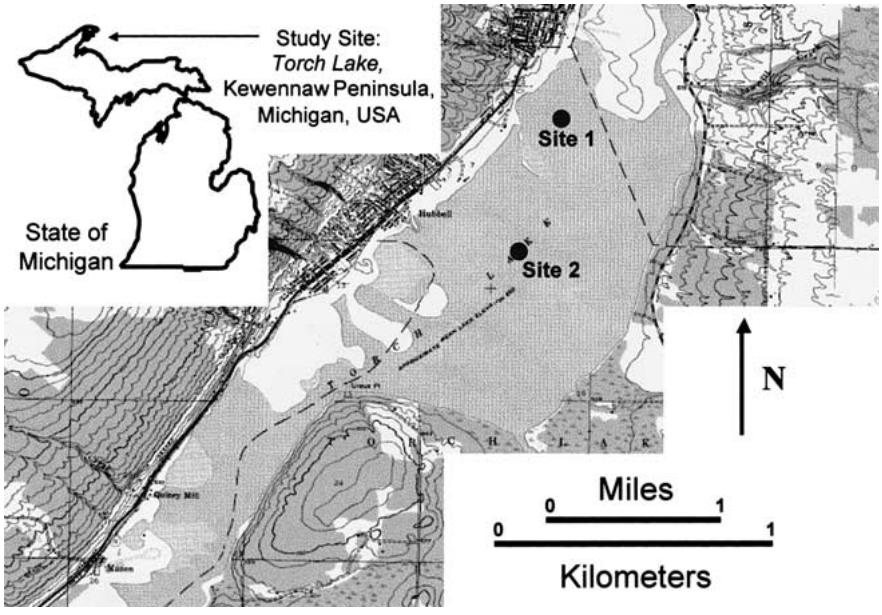

Fig. 1. Map of Torch Lake (Upper Peninsula, Michigan) and location of sampling sites. Sediment surfaces were at water depths of 89 feet and 105 feet for sites 1 and 2, respectively.

Phylogenetic Analysis

Sequencing of the 16S-rDNA gene was performed with three primers, 357Forward (5′-TACGGGAGGCAGCAG), 522Reverse (5′-GWATTACCGCGGCKGCTG), and 1057Forward (5′-ATAGCGCTGRTTCATYTC), in the Sequencing Facility of Michigan State University. Contig assembly and editing were performed with Sequencer software. Alignment and initial phylogenetic analysis were made in ARB [16] using the Ribosomal Database (RDP release 8.0) [18]. Final phylogenic analyses using neighbor joining, parsimony, and maximum likelihood were performed in PAUP* [32]. The GenBank accession numbers for these sequences are AY191843-AY191856

Results

Metal Concentrations in Torch Lake Sediment

The location of Torch Lake and the two sampling sites are shown in Fig. 1. The U.S. EPA began investigating Torch Lake in 1984 and shortly thereafter issued two records of decision (RODs) [7]. The lake sediment received more than 200 million tons of mine tailings and several recent releases of copper-contaminated leachates. As a consequence, the lake sediment has relatively high concentrations of copper as well as other contaminants. Vertical concentration profiles of copper are presented in Fig. 2 for the two sampling sites at Torch Lake. At site 1, copper concentrations in the cap layer (top 10 cm of sediment) and surface sediments are around 2000 ppm. With depth, concentrations increase to approximately 5500 ppm and then decrease to around 1500 ppm. At site 2, sediment concentrations below 20 cm are around 800 ppm. From 20 cm to 10 cm (base of the cap layer), concentrations increase to 2400. Concentrations then decrease to 2200 ppm in the surface sediments. Additional metal concentrations are also shown in Fig. 2. Most of the noncopper metal distributions paralleled the copper distribution. The notable exceptions were at site 2 where the peak concentrations of nickel, chromium, and cobalt partitioned at somewhat lower depths. Iron concentrations are not presented in the figure but vary irregularly from 20,981 ppm through 88,717 ppm with average concentrations of 36,772 ppm and 36,749 ppm for sites 1 and 2, respectively.

Sediment Description

In general, the top (approximately 10 cm thick) layers of the lake sediments were brown in color with various layers of gray, purple, and red material. These top sediments, referred to as the cap layer, were relatively firm, grading from typical lake bottom sediments that are watery near the sediment–water interface to thicker clayey material at about 10 cm. The porosity of these sediments was greater than 90%. Below the cap layer, the color of the sediment changed to pink. The sediments were extremely fine grained and loose with porosities around 80%. These sediments were not homogenous as they contained alternating layers of more firm material and of more sandy material. The thickness of these layers was on the order of centimeters.

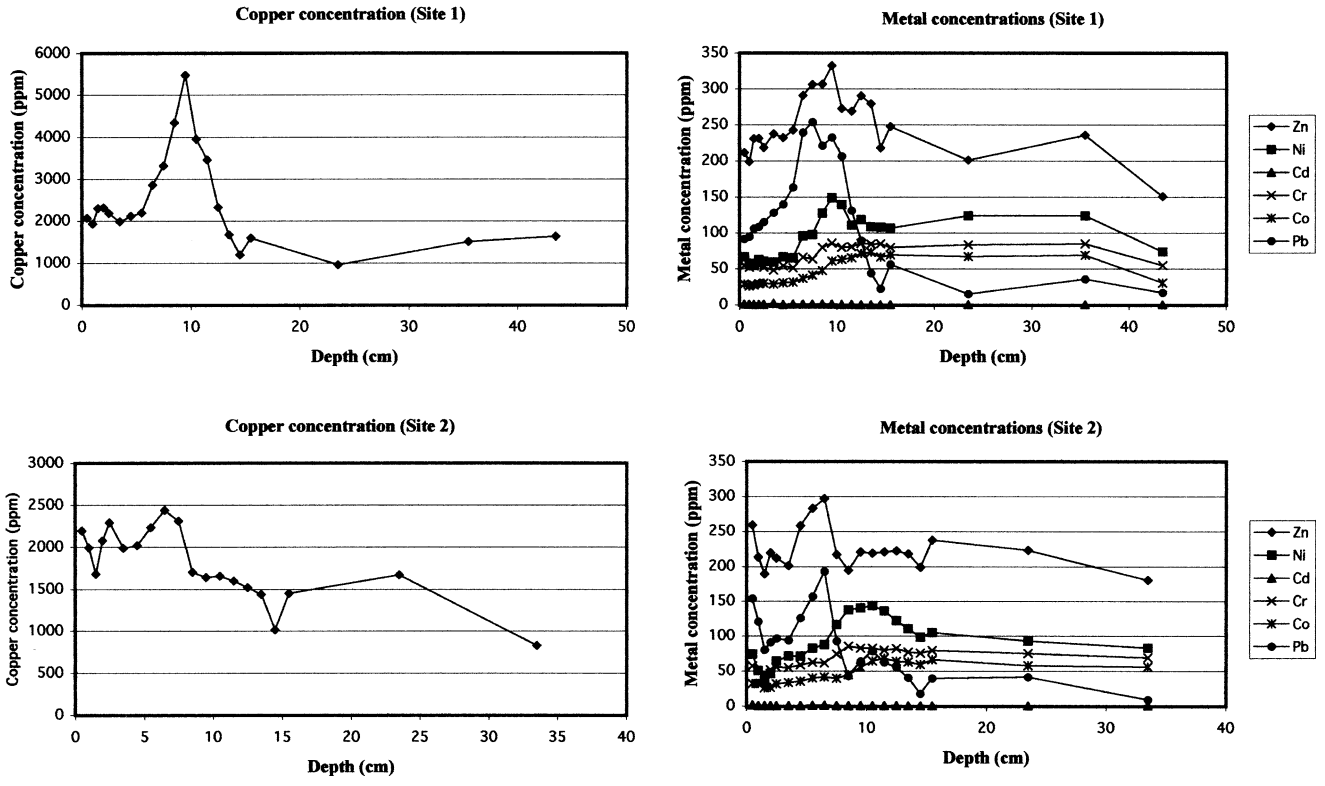

Fig. 2. Vertical metal concentrations in sediments from Torch Lake. Left panels show copper concentrations at the two sites. Right panels show concentrations of Zn, Ni, Cd, Cr, Co, and Pb.

Microbial Community Analysis

T-RFLP of 16S rDNA was used to assess the number and distribution of operational taxonomic units within the bacterial community of Torch Lake sediment. Distribution of restriction fragments from *Msp* I digestions are presented in Fig. 3 and a summary of all T-RFLP profiles is presented in Table 1. Figure 3 includes both the 5′-labeled (27F-HEX) and the 3′-labeled (1492-FAM) profiles from the *Msp* I digestions. As can be seen, differences in the profiles were detected over the 15 cm of depth surveyed. Moreover, there were from 4 to 20 fragments detected, depending upon the sample and restriction enzyme. The maximum number of terminal fragments detected was 20 for the *Hha* I digestion (sample 1.4, 3′-label), 19 for the *Msp* I digestion (sample 1.1, 3′-label) and 14 for the *Rsa* I digestion (sample 1.2, 5′-label). From the TRFLP profiles (Fig. 3 and Table 1), it is apparent that there were fragments found in all samples (pandemic), as well as fragments found in a select subset of samples (endemic). For example, the results for the 3′-labeled amplifications indicated that there were six, four, and nine terminal fragments found in all four depths of site 1 for the *Hha* I, *Msp* I, and *Rsa* I digestions, respectively. For the three depths of site 2, seven, seven, and nine 3′-terminal fragments were found at all depths for *Hha* I, *Msp* I, and *Rsa* I digestions, respectively. This suggests the possibility that a significant fraction (~50%) of the community was conserved throughout the 15 cm of sampled depth. A similar comparison between the two sampling sites suggests that at least a third of the community was in common. Terminal fragment sizes consistent with the presence of the copper resistant isolates described below were detected in most samples, as indicated in Fig. 3.

Isolation and Phenotypic Characteristics of Copper-Resistant Isolates

Copper-resistant bacterial strains were isolated from site 1 by plating cells washed from the substratum directly onto R2A agar supplemented with 200 μg/mL $CuSO_4$, as described above. The surface layers contained 4.5 times as many cells/gram as the deeper layers. Moreover, the copper resistant fraction of the surface community represented only 1/100th of the cultivable community. Below the surface, the viable cell count dropped and the fraction of the community comprised of copper resistant populations increased to 30–75% of the cultivable cells. Viable

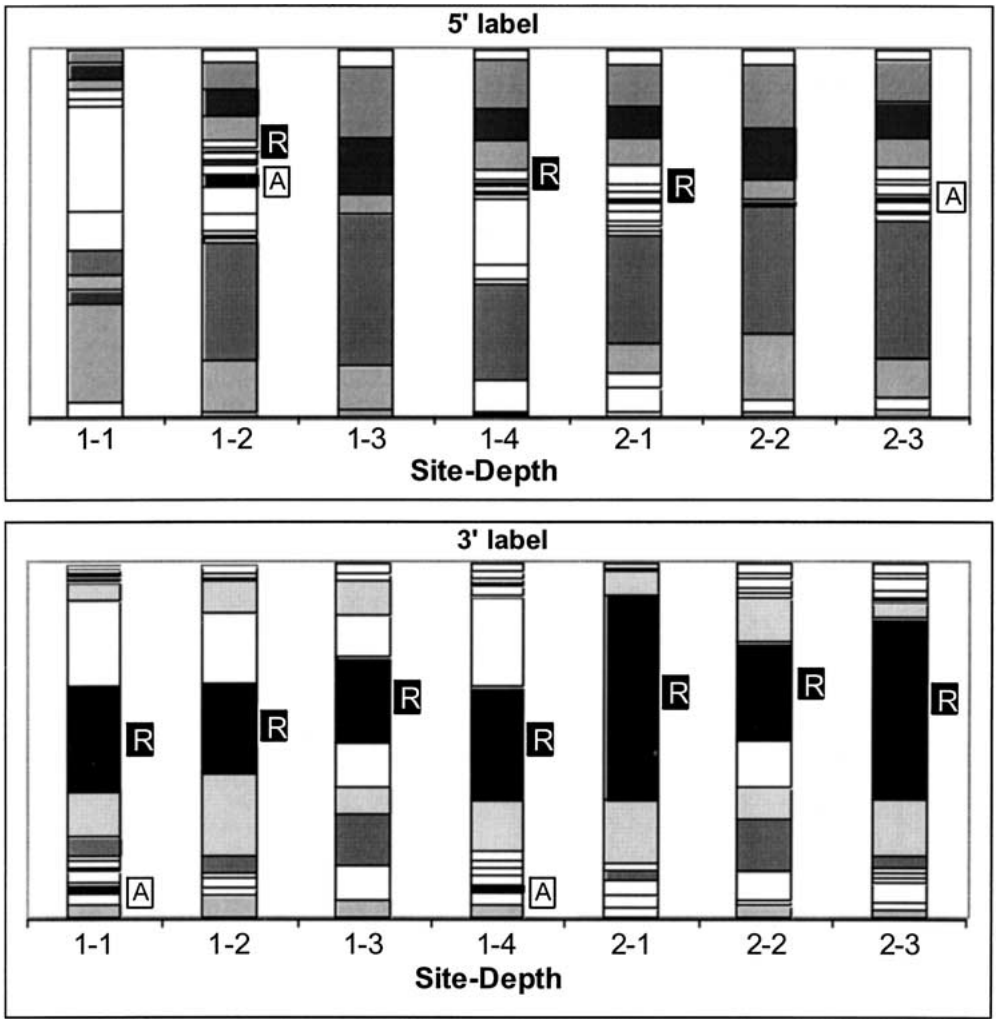

Fig. 3. Terminal restriction fragments detected for Msp I digestions of PCR amplified 16S rDNAs. Fragments that were found in more than one sample have the same shading in bars representative of the sites. Fragments detected in only one or two samples are white (non-pandemics). The amplitude of the peak corresponds to the size of the shaded segment. The predicted fragment sizes of the *Ralstonia* (R) and *Arthrobacter* (A) isolates are indicated.

cell counts on R2A media were 0.69×10^6, 1.52×10^5, 1.56×10^5, and 0.98×10^5 at depths of 1 cm, 6 cm, 12 cm, and 15 cm respectively. Viable counts of copper resistant organisms at these respective depths were 6.9×10^4, 0.38×10^5, 0.94×10^5, and 0.73×10^5, respectively.

Fourteen cultivable copper resistant strains from site 1 were characterized more fully. We focused on site 1 so that four different depths could be surveyed (only three depths were available for site 2). The colony morphology and metal resistance patterns of these isolates are described in Table 2. Isolates from the topmost sediment, 11I and 11J, were moderately resistant to copper whereas isolates from deeper layers showed higher levels of copper resistance. The isolates with high-resistance phenotypes (+++) developed colonies with an intense green hue when cultivated on 800 mg $CuSO_4$/L R2A after approximately 48 h at 30°C.

In addition to copper, the 14 isolates were tested for resistance to zinc, cadmium, and nickel. Similar to the copper resistant patterns, isolates from deeper layers were more resistant to these metals. Colony growth on nickel plates was, in general, slower than on the other metals. Three patterns of metal resistance were detected. The first group showed only limited resistance to Zn, Cd, and Ni (11I, 11J, 12E). This group was phylogenetically identified as *Arthrobacter* sp. (see below). The second group was resistant to Zn and Cd (12B, 12I, 12J, 13A, 13H, 13I, 14I). The third group was resistant to Ni and had low-level resistance to Cd, but lacked resistance to Zn (12D, 12F, 14C, 14H). The second and third groups (based on resistance patterns) were most closely related to *Ralstonia* sp. (see Fig. 6, below).

Energy Dispersive Spectroscopy

The green coloration of the colonies grown on copper supplemented plates suggested the possibility that the cells sequestered copper. To test this, isolates 12F and 12J,

Table 1. Terminal restriction fragment distribution patterns[a]

	Number of terminal restriction fragments								
	*Hha*I			*Msp*I			*Rsa*I		
Sample	Total	Shared within sites	Shared between sites	Total	Shared within sites	Shared between sites	Total	Shared within sites	Shared between sites
5′ end									
1.1	9	3	3	12	5	5	9	3	3
1.2	9			17			14		
1.3	5			7			4		
1.4	16			16			7		
2.1	15	4		18	5		8	3	
2.2	8			9			5		
2.3	17			15			7		
3′ end									
1.1	14	6	8	19	4	8	13	9	9
1.2	13			13			11		
1.3	8			10			11		
1.4	20			16			13		
2.1	13	7		10	7		11	9	
2.2	11			13			12		
2.3	17			16			12		

[a] The total number of terminal fragments detected in T-RFLP profiles and the number of fragments detected in all samples (pandemic) within one sampling site, and between the two sampling sites.

which had high levels of copper and nickel resistance and showed a demonstrable ability to produce green colonies, were scanned with energy dispersive spectroscopy (EDS). As described above, cells were removed from the surface of colonies growing on either R2A alone or R2A supplemented with copper and carefully prepared for the SEM. The results for 12F are presented in Fig. 4 (scans of 12J were identical). EDS scans on these cells showed three characteristic copper peaks associated with cells grown in the presence of copper. No Cu peaks were detected from the same strain when grown in the absence of copper. In addition, the cells had elevated levels of phosphorus and oxygen when grown in the presence of Cu. No differences were seen between samples prepared on the glass coverslips or the carbon planchets.

Light and Electron Microscopy

Light microscopy revealed that cells from the 14 isolates were small rods with no distinct characteristics detectable. Scanning electron microscopy of 12F and 12J and 12J (Fig. 5) confirmed the general morphology detected under the light

Table 2. Colony morphology and metal resistance of copper-resistant strains

Isolate	Phylogeny	Cu[a]	Zn[a]	Ni[a]	Cd[a]	Colony Morphology[b]
11I	*Arthrobacter*	+[b]	−	−	+	Green—wrinkled—round
11J	*Arthrobacter*	+	−	+	+	Bright yellow—round
12B	*Ralstonia*	+++	+++	−	++	Bright yellow—irregular
12D	*Ralstonia*	+++	−	++	+	Dull green—round
12E	*Arthrobacter*	++	−	−	+	Bright green—round
12F	*Ralstonia*	+++	−	++	+	Dull green—round
12I	*Ralstonia*	+++	+++	−	+	Bright green—round
12J	*Ralstonia*	+++	+++	−	+++	Dull green—irregular
13A	*Ralstonia*	++	+++	−	+++	Dull green—irregular
13H	*Ralstonia*	++	+++	−	+++	Dull green—round
13I	*Ralstonia*	+	+++	−	+++	Bright green—round
14C	*Ralstonia*	++	−	+++	+	Dull green—round
14H	*Ralstonia*	++	−	+++	+	Bright green—round
14I	*Ralstonia*	++	+++	−	+++	Bright green—round

[a] A qualitative level of resistance was, determined by measuring colony size after 48 h of growth on supplemented R2A agar: −, Sensitive; +, low resistance; ++, moderate resistance; +++, high resistance.
[b] Colony morphology on 200 mg/L $CuSO_4$-supplemented plates.

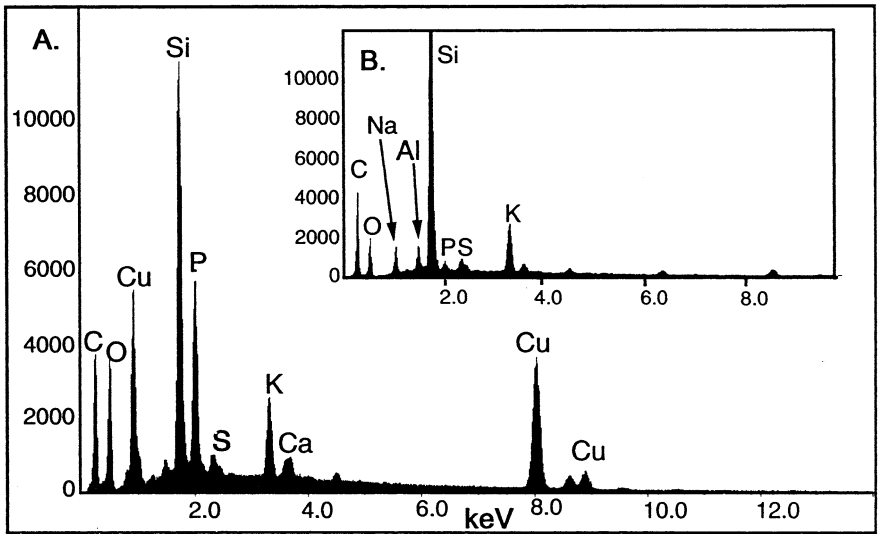

Fig. 4. Electron diffraction scanning of isolate 12F (*Ralstonia* spp.) grown on copper-supplemented R2A agar or copper-free R2A agar (insert). Elements were identified with Noran Vantage 1.2.1 software.

microscope (1 μm × 0.4 μm). Differences in the outer cell envelope could be detected between cells grown on copper-free agar (panels A, C) and cells grown on copper-supplemented agar (panels B, D). The cell membrane of the former appeared smooth and without the many surface irregularities and blebs apparent on cells grown in the presence of copper.

Phylogenetic Analysis

To determine the phylogeny of the copper resistant isolates, 16S rDNA was amplified and sequenced from all 14 isolates. The isolates fell into two phylogenetic groups. Isolates from the upper regions (11I, 11J, and 12E) of the sediment were most closely related to the *Arthrobacter* spp. subdivision as shown in Fig. 6, bottom. All of the remaining isolates were closely related to *Ralstonia pickettii* strains (Fig. 6, top). Based on 1100 aligned bases, the *Ralstonia* and *Arthrobacter* isolates were at least 98% identical or better, in pairwise comparisons within their respective groups (data not shown).

Discussion

Torch Lake Sediment

Highly contaminated sites provide extreme environments for microbial communities. As contaminants, metals are

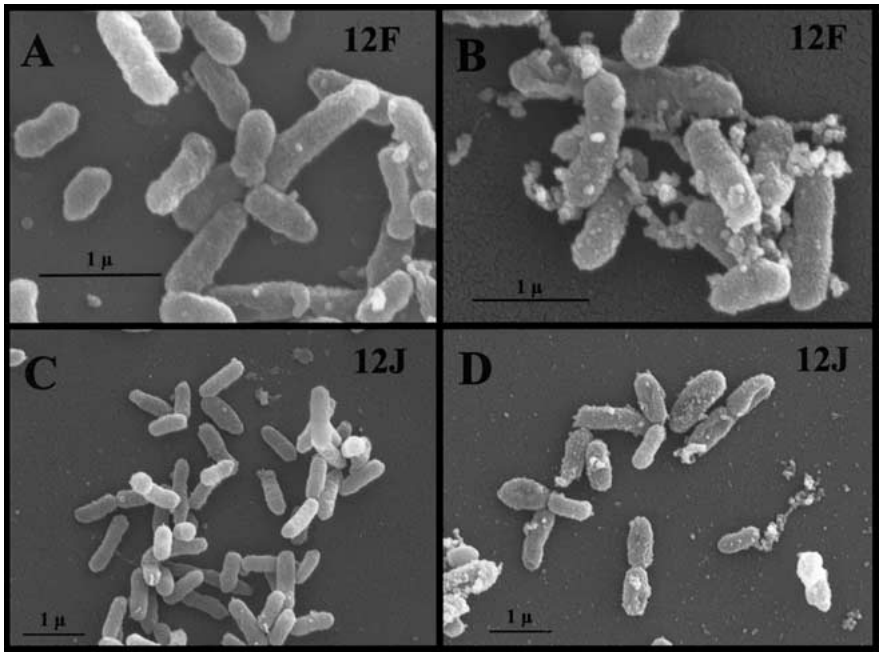

Fig. 5. Scanning electron microscopy of isolates 12F (panels A; B) and 12J (panels C; D) grown on copper-free agar (panels A; C) or copper-supplemented R2A agar (panels B; D).

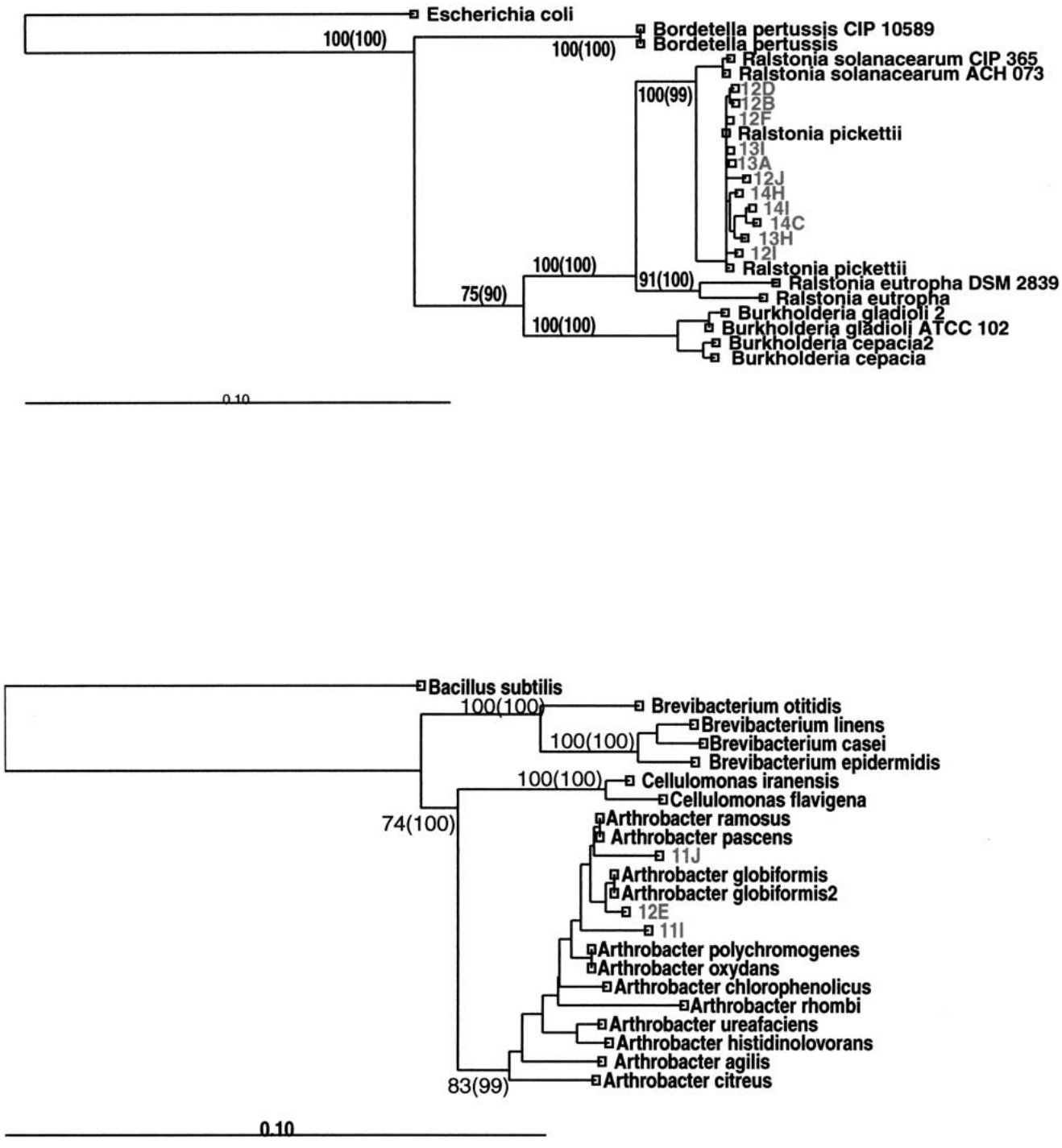

Fig. 6. Phylogenetic relationships of Torch Lake isolates based on maximum likelihood analysis of 16S rRNA sequence. Sequences were aligned in ARB against the Ribosomal Database Project 16S rRNA alignment and a sequence mask was constructed, specific to each dataset, that eliminated ambiguous reads (Ns), gaps, and regions of uncertain alignment. A total of at least 1100 aligned characters was included in the final phylogenetic analysis. Confidence estimates from 500 bootstrapped replicates of parsimony and neighbor joining are indicated at the nodes (neighbor joining in parentheses).

particularly onerous because their fate is limited to reversible redox transformations [5, 31]. Remediation options are limited as well, frequently reduced to labor-intensive reclamations or "no action" policies dependent on natural processes to maintain the metal in the least toxic and immobile form. Torch Lake, MI, is an example of a contaminated site with 100-year history, currently serving as a repository for copper milling waste (200

million tons of stamp sands). The resulting lake sediment contains very high levels of copper with moderately elevated levels of other metals (e.g., Zn, Ni, Co). Factors influencing the concentrations of the other metals in Torch Lake have not been identified but may be related to mining activities, atmospheric deposition, and postdepositional remobilization processes. Our measurements identified very high copper concentrations in the sediments of site 1 at depths between 7 and 10 cm. Using sediment chronologies obtained from ^{210}Pb data [8], these sediments were deposited around 1972. Thus, the high concentrations of copper in these sediments were consistent with a documented spill of cupric ammonium carbonate in this area of the lake around 1972 [33]. The levels of Fe ranging from 20,981 ppm through 88,717 ppm are not appreciably higher than other lake sediments in Michigan.

The Microbial Community

Sampling at Torch Lake was unusually difficult because of the fine silty nature of the lake sediment. This physical structure made the extraction of intact cores difficult and labor intensive. Because the coverage of the lake sediment was so limited, we cannot draw any broad conclusions regarding the microbial ecology of the habitat. The two sites sampled were taken from previously identified milling disposal sites, regions that appeared typical of the contaminated lake bottom with respect to gross physical attributes. The copper concentration profiles for the two sites revealed the expected high levels of contamination ranging from 200 to 5500 ppm.

Community analysis with T-RFLP provides a sensitive and efficient method for measuring diversity and relative changes across habitat areas [17, 19]. Moreover, putative phylogenetic associations can be made by reference to a database [20]. The T-RFLP analyses of the microbial communities from these sites indicated a reasonably complex community with up to 20 phylotypes detected. Different patterns of terminal fragments were detected at different depths, but the overall diversity, as measured by total number of terminal fragments detected, did not change appreciably over 15 cm of sediment depth. Moreover, based on T-RFLP profiles, up to 50% of the community was conserved over this depth. Copper-resistant bacteria represented a significant fraction of the cultivable community under aerobic conditions. At the surface of the sediment, the resistant strains accounted for approximately 1% of the community whereas at lower, presumably less aerobic depths, the resistant populations represented from 30 to 75% of the cultivable community. Somewhat surprisingly, all of the resistant isolates were from two genera, *Arthrobacter* and *Ralstonia*. Additional diversity may reside in the anaerobic sector of the community. *Arthrobacter* were isolated from only the top layers of the sediment while *Ralstonia* spp. were the only aerobically cultivated copper-resistant strains isolated below 6 cm. However, the total number of isolates fully characterized from each depth was small (~5); hence, few conclusions regarding the relative distribution of populations are possible. The detection of these two genera at these two sites is consistent with past observations of other investigators regarding the ecology of *Arthrobacter* and *Ralstonia*. *Arthrobacter*, while among the most abundant soil bacteria, have also been isolated from cave silt as psychrophiles and psychrotrophs [10], glacial silt [21], and subsurface oil brine [12]. *Arthrobacter* are clearly a robust and widely distributed genus of bacteria [13]. *Ralstonia* isolates are well known for metal resistances. Indeed, a number of strains have been identified that are resistant to several heavy metals [2, 23]. All of the *Ralstonia* spp. isolated from Torch Lake were most closely related to *R. pickettii* within the *R. solanacearum* subgroup.

Assuming a resolution of ±2 bases in the T-RFLP analysis, terminal fragments consistent with the presence of *Arthrobacter* were detected in approximately 36% (15/42) of the cases (seven communities, three enzymes, and terminal fragments derived from forward and reverse labeled primers). The predicted terminal fragments for *Ralstonia* spp. (also presented in Table 1), on the other hand, were found in 81% (34/42) of the analyses. Although extrapolation of T-RFLP profiles back to the community is risky, these data were roughly consistent with the numbers obtained in the isolation of Cu-resistant strains that indicated a spotty distribution of *Arthrobacler* and a more broadly distributed *Ralslonia*. Moreover, given high similarity of the rDNA sequences (≥98.8% similarity) from the *Ralstonia* spp. isolated throughout the sediment core, the isolates appear to be a single population. It is worth noting that, based on estimates of phylogenetic diversity with T-RFLP and the limited phylogenetic diversity detected among the aerobic copper resistant isolates (two genera), much of the phylogenetic diversity, and perhaps the physiological diversity of metal detoxification, of the sediment remains unexplored.

Characterization of Cu-Resistant Isolates

Because of the different multiple resistance patterns of the *Ralstonia* isolates and their apparent ability to accumulate copper from growth media, we conducted additional investigations on representative isolates to confirm these observations. All of the *Ralstonia* spp. isolated from Torch Lake sediments carried resistance to one or more heavy metals in addition to Cu resistance. The two patterns detected were Zn and Cd resistance together, or Ni resistance and a low-level resistance to Cd. Resistance to Zn and Ni were not detected in the same strain. The EDS scans of *Ralstonia* spp. were consistent with the visual observation that colonies grown on copper-containing medium accumulated copper. All EDS scans of cells grown in the presence of copper had three diagnostic copper peaks whereas cells grown in the absence of copper showed no such peaks. Cells grown in the presence of copper also had increases in the amplitudes of peaks derived from oxygen and phosphorus, suggesting the possibility that phosphates may be involved in copper sequestration. Differences at the level of the cell surface could also be distinguished with scanning electron microscopy. The level of resistance to copper (800–1200 μg/mL) was among the highest recorded for bacteria [6, 30].

Copper resistance in bacteria is documented [3, 4, 15, 23, 25, 26] but not well characterized at the molecular level. In gram-positive *Enterococcus hirae*, two structural genes code for P-type ATPases responsible for uptake (CopA) and efflux and detoxification (CopB) [25, 26]. In the gram-negatives that have been investigated, four gene products have been invoked as participating in copper resistance, although the mechanism is ill described. In *Escherichia coli*, two of the proteins bind copper in the periplasmic space (CopA and CopB) and two of the proteins promote the intake of copper (CopC and CopD) [3, 4, 23]. The specific nature of copper binding has not been determined nor has the fate of copper after efflux from the cell. Homologous proteins have been identified in *Pseudomonas*, but the phenotypic consequences of the resistance mechanism are apparently different inasmuch as colonies of *Pseudomonas* turn green when grown with copper whereas *E. coli* does not [9, 22]. Resistance in the *Ralslonia* spp. from Torch Lake appears similar to the phenotype expressed in *Pseudomonas*. Indeed, the "Pseudomonas pickettii" described previously by Gilotra and Srivastava [9] may be a *Ralstonia pickettii*.

Summary

Our investigations of copper-contaminated sediment revealed a microbial community with at least 20 identifiable phylotypes. Direct isolation of copper-resistant strains from four sediment depths detected only two genera, *Arthrobacter* and *Ralstonia*. Both genera exhibited resistance to at least 200 μg/mL of $CuSO_4$. However, the *Ralstonia* were resistant up to 1200 μg/mL of $CuSO_4$ and produced green colonies when grown with $CuSO_4$, suggesting copper sequestration as a mechanism of resistance. Current investigations are continuing in several directions. New isolates from two phylogenetic groups other than *Arthrobacter* and *Ralslonia* from Torch Lake sediment have copper resistance and, more interestingly, apparent copper-sequestering representatives (N. Isaacs, T.L. Marsh, unpublished). In addition, genetic evidence has revealed significant genomic plasticity in the 11 *Ralstonia* isolates. The possible ecological significance of this plasticity as well as the mechanism(s) are being investigated.

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