Microbial Ecology

Isolation and Characterization of Four Gram-Positive Nickel-Tolerant Microorganisms from Contaminated Sediments

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Abstract

Microbial communities from riparian sediments contaminated with high levels of Ni and U were examined for metal-tolerant microorganisms. Isolation of four aerobic Ni-tolerant, Gram-positive heterotrophic bacteria indicated selection pressure from Ni. These isolates were identified as *Arthrobacter oxydans* NR-1, *Streptomyces galbus* NR-2, *Streptomyces aureofaciens* NR-3, and *Kitasatospora cystarginea* NR-4 based on partial 16S rDNA sequences. A functional gene microarray containing gene probes for functions associated with biogeochemical cycling, metal homeostasis, and organic contaminant degradation showed little overlap among the four isolates. Fifteen of the genes were detected in all four isolates with only two of these related to metal resistance, specifically to tellurium. Each of the four isolates also displayed resistance to at least one of six antibiotics tested, with resistance to kanamycin, gentamycin, and ciprofloxacin observed in at least two of the isolates. Further characterization of *S. aureofaciens* NR-3 and *K. cystarginea* NR-4 demonstrated that both isolates expressed Ni tolerance constitutively. In addition, both were able to grow in higher concentrations of Ni at pH 6 as compared with pH 7 (42.6 and 8.5 mM Ni at pH 6 and 7, respectively). Tolerance to Cd, Co, and Zn was also examined in these two isolates; a similar pH-dependent metal tolerance was observed when grown with Co and Zn. Neither isolate was tolerant to Cd. These findings suggest that Ni is exerting a selection pressure at this site for metal-resistant actinomycetes.

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Introduction

Microorganisms tolerant to metals are often isolated from areas of high metal loading, suggesting that metal tolerance or resistance is an adaptive response to excessive metal exposure [47]. Nickel (Ni)-tolerant microorganisms, including species of *Cupriavidus* (*Alcaligenes*), *Burkholderia*, *Arthrobacter*, *Rhodococcus*, and *Streptomyces*, have been isolated from naturally Ni-rich soils associated with Ni-hyperaccumulating plants [20, 30, 39, 48]. Similarly, neocaledonian soils with naturally high concentrations of Ni, as well as acidic, nonneocaledonian soils were shown to have the actinomycete group emerge after spiking with Ni [18]. Two of the most-studied Ni-resistant microorganisms, *Cupriavidus metallidurans* CH34 and 31A, were isolated from a decantation tank at a zinc factory [31] and a metal-contaminated industrial site [47], respectively. Ni has been found at more than half of the 1674 national priority list sites and is listed as a Comprehensive Environmental Response, Compensation, and Liability Act priority hazardous substance (ranked 55 out of 799) [16]. Therefore, microbial tolerance to Ni may be essential to ecosystem function at many of these sites.

Ni is also encountered as an environmental contaminant at material production facilities within the Department of Energy (DOE) nuclear weapons complex, such as the Savannah River Site (SRS, Aiken, SC), where metal-clad uranium (U) targets were fabricated for plutonium pro-

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duction [38]. On the SRS, wastewater from metal plating and fabrication processes was directly discharged to Tims Branch, a second-order stream, from 1954 to 1982 [36]. As a result, significant quantities of U (depleted and natural) and Ni accumulated in riparian sediments, with a large fraction being deposited in Steed Pond, a pre-SRS farm pond [8, 44]. Sediment concentrations of U and Ni at this site commonly exceed 1000 mg kg^{-1} [8]. Following failure of its spillway in the mid-1980s, Steed Pond was transformed into a wetland subject to intermittent flooding.

Because co-contaminant metals commonly occur at waste sites, like the SRS, we have been studying the impact of co-contaminant metals on microorganisms to understand the broader implications of mixed waste scenarios on biogeochemical processes [53]. Here, we report the isolation of four aerobic Gram-positive microorganisms as the primary Ni-tolerant microorganisms isolated from riparian sediments contaminated with U and Ni. All four isolates were phylogenetically assigned to the actinomycete group and were characterized with respect to metal tolerance and antibiotic resistance patterns. In addition, the presence of genes involved in metal homeostasis, biogeochemical cycling, and organic contaminant degradation was determined for the four isolates using a functional gene microarray.

Materials and Methods

Sediment Sampling. Sediment cores were collected from Steed Pond and an uncontaminated pond basin located on Boggy Gut, another SRS stream not affected by site activities. Steed Pond is a radiological controlled area, which constrained the number of cores collected and the manner in which they were collected and preserved. A stainless-steel hand corer lined with removable plastic sleeves (Wildlife Supply, Co., Saginaw, MI) was used to collect intact sediment cores, which were immediately sealed and stored on ice. Subsamples from the interior of each core were transferred to sterile 50 mL polypropylene tubes and stored at 4°C. Sediment samples from 13 different cores comprising 46 distinct core zones (depths) were initially screened for sediment properties and microbial diversity. In this article, we present results from a representative subset of these samples, including two cores from contaminated sediments from Steed Pond and a core from an uncontaminated sediment from Boggy Gut. Sediment core samples, segregated by zone, for Steed Pond exhibited a pH range between 4.3 and 5.8 and contained between 4.9 and 3200 mg kg^{-1} Ni and between 2.1 and 4900 mg kg^{-1} U. Boggy Gut sediment samples exhibited a pH range between 4.2 and 5.4 and had between 3.8 and 10 mg kg^{-1} Ni and between 1.2 and 3.3 mg kg^{-1} U.

Preparation of Solutions and Media. All glassware was acid washed in 25% HCl and thoroughly rinsed with distilled water before use in experiments. Solutions were prepared from reagent-grade chemicals and 18 MΩ distilled–deionized (DI) water. Metal solutions were prepared using $CoCl_2 \cdot 6H_2O$, $ZnCl_2$ (Fisher Scientific, Suwanee, GA), $Ni(NO_3)_2 \cdot 6H_2O$ (J.T. Baker, Phillipsburg, NJ), and $UO_2(NO_3)_2 \cdot 6H_2O$ (Alfa Aesar, Ward Hill, MA). Stock solutions were prepared using DI water and filter sterilized (0.2 μm cellulose nitrate analytical filter; Nalge Nunc, Rochester, NY).

The growth medium used in these experiments was designed to minimize metal complexation with medium components. A mineral medium [45] was modified to reduce the inorganic phosphate concentration to minimize metal complexation with medium components (referred to as 4M) as previously described [53]. A nonphosphate containing buffer [2-(*N*-morpholino) ethanesulfonic acid (MES; 100 mM; J.T. Baker)] and 3 mM β-glycerophosphate (Sigma, St. Louis, MO), an organic phosphate source [19], were used. Lactate and glucose (10 g L^{-1} each) were used as carbon sources. For plate cultures, the medium was solidified with 15 g L^{-1} of agar (Fisher Scientific). Filter-sterilized U and Ni solutions were added to the medium before solidification of agar.

MetPLATE™ Assay. A metal-specific toxicity assay, MetPLATE™ (Department of Environmental Engineering, University of Florida), was used to evaluate toxic concentrations of U and Ni. MetPLATE™ is a colorimetric assay used to evaluate metal toxicity by monitoring β-galactosidase activity in *Escherichia coli* [4, 5]. The addition of a chromogenic substrate yields a direct measure of enzyme activity and therefore metal toxicity. A toxicity endpoint (percentage inhibition) is determined as the relative difference between control and sample responses. The initial pH of U and Ni solutions was adjusted to 4.5–5 with 1 M HCl and 1 M NaOH. Assays were conducted according to the instructions of the manufacturer, except for the substitution of 0.15 M 3-*N*-morpholinopropanesulfonic acid (pH 7.5) buffer solution for the supplied phosphate buffer [5]. All exposures, sample blanks (deionized water), negative control (deionized water), and positive control (supplied with the kit) were performed in triplicate. Samples were read on a 96-well plate reader at 575 nm (FLUOstar, BMG Labtech, Durham, NC).

Isolation of Metal-Tolerant Microorganisms. Metal-tolerant microorganisms were isolated from sediments by the spread plate method. Sediment samples (1 g) were suspended in 9 mL of saline solution (0.9 g L^{-1} of NaCl in DI water) and vortexed for 1–2 min at room temperature. Aliquots (100 μL) of successive dilutions (10^{-5}–10^{-7}) were spread onto agar plates amended with increasing concentrations of metal (0, 0.04, 0.4, 4.2, and 21.0 mM U or 0, 0.2, 1.7, 17.0, and 85.2 mM Ni) at pH 5, 6, and 7.

These concentrations were chosen based on concentrations observed in the literature with respect to Ni and U resistance. Due to the limited amount of sediment, each dilution was analyzed in duplicate. Plates were incubated aerobically at 30°C in the dark for 200 h, and then colony-forming units (CFU) were counted.

Phylogenetic Characterization of Ni-Tolerant Microorganisms. Four morphologically distinct aerobic microorganisms from the 85.2 mM Ni agar plates were isolated at pH 5 and 6 in pure culture for identification and further study. Isolates were stored at −80°C in equal parts of 4M (pH 6) and 50% glycerol until needed. Total genomic DNA was extracted using the method of Wilson [55]. Cell pellets from 1.5 mL of culture were suspended in 556 μL TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and treated with 11 μL lysozyme (50 mg mL^{-1}; Sigma) for 30 min at 37°C. The suspension was then incubated with 30 μL 10% sodium dodecylsulfate (SDS) and 3 μL of proteinase K (20 mg mL^{-1}; Fisher Scientific) for 1 h at 37°C. After incubation, 100 μL of 5 M NaCl was added and mixed thoroughly, and 80 μL of 10% cetyl trimethyl ammonium bromide in 0.7 M NaCl was added and further incubated at 65°C for 10 min. The DNA was sequentially extracted at room temperature using equal volumes of chloroform/isoamyl alcohol (24:1), phenol/chloroform/isoamyl alcohol (25:24:1), and chloroform/isoamyl alcohol (24:1). The aqueous phase was recovered at each step. Ice-cold isopropanol (0.6 volumes) was added and the solution was incubated at −20°C for at least 2 h to precipitate the DNA. The DNA was pelleted (10 min, 12,000 rpm, 4°C) and then rinsed with ice-cold 70% ethanol for 30 min and centrifuged (10 min, 12,000 rpm, 4°C). The supernatant was removed and the pellet was allowed to air dry before dissolution in 50 μL TE buffer.

The 16S rRNA genes of the isolates were amplified by PCR using a primer set targeting a 351 bp region of the gene [11]. The PCR protocol was that of Muyzer *et al.* [32]. PCR products were purified using Wizard® PCR Preps DNA purification system (Promega Corporation, Madison, WI) and sequenced at the Biotechnology Resource Laboratory at the Medical University of South Carolina (Charleston, SC) using an ABI 377 DNA Sequencer (Applied Biosystems, Foster City, CA). Sequences were analyzed using the Similarity Rank Function of the Ribosomal Database Project II to determine best match identification [26]. Sequences were then aligned using the ClustalW multiple alignment algorithm within BioEdit [15]. Additional reference sequences were obtained from the GenBank database (http://www.ncbi.nlm.nih.gov). Phylogenetic analysis was performed with 329 bp of sequence using PAUP* software, version 4.0b10 [51]. Phylogenetic trees were constructed using the neighbor-joining and maximum-likelihood methods with bootstrap analysis ($n = 1000$). The partial rRNA gene sequences of the isolates were deposited in the GenBank database under accession numbers DQ459361–DQ459364.

Determination of Antibiotic Resistance. For antibiotic resistance experiments, bacterial stocks were maintained at −80°C in Luria–Bertani (LB) broth. These stocks were thawed, inoculated into 3 mL 4M (pH 7), and incubated at 30°C with shaking (100 rpm) for 48–72 h. Cultures were serially diluted and aliquots (250 μL) were plated onto 4M agar plates (pH 7) and incubated at room temperature for 7 days. Ten colonies were randomly picked and transferred with sterile toothpicks to an antibiotic-free control plate and incubated at room temperature for 5 days. The colonies were then replica plated using sterile velveteen squares and a replica-plating block. The block was first plated onto an antibiotic-free plate to remove excess cells. The antibiotic plates were then inoculated in a random order followed by an antibiotic-free plate to confirm that cells were successfully transferred. The six antibiotics tested were 30 mg L^{-1} gentamycin, 30 mg L^{-1} tetracycline, 30 mg L^{-1} ciprofloxacin, 30 mg L^{-1} chloramphenicol, 100 mg L^{-1} kanamycin, and 100 mg L^{-1} streptomycin. Plates were incubated at room temperature for 11 days, and colonies were counted at 4, 7, and 11 days. Sensitivity and resistance were determined based on the number of colonies that grew. Growth of less than two colonies was considered sensitive, growth of more than eight colonies was considered resistant, and growth of three to seven colonies was considered intermediate.

DNA Microarray for Metal Homeostasis Genes. The isolates were grown in 4M (pH 6) amended with 4.3 mM Ni. DNA was extracted as described above. Aliquots of DNA (1500 ng) were transferred to fresh tubes, and the volume was increased to 10 μL by the addition of DI water. The DNA samples were added to a prelabeling mix [60 μL 3 mg mL^{-1} random primers (8mers), 0.9 μL spermidine, 49.1 μL DI water] and heated at 99.9°C for 10 min. Samples were then immediately chilled on ice. The chilled samples were added to a labeling master mix [7.5 μL 2.5 mM dTTP, 5 mM dACG-TP, 3 μL Cy5-dUTP (1 mM; Amersham, Piscataway, NJ), 2.1 μL RecA (490 ng μL^{-1}), 6 μL Klenow, and 41.4 μL DI water]. The samples were incubated at 37°C in a thermocycler for 6 h. After the addition of Cy5, samples were protected from the light as much as possible. Labeled DNA was cleaned using a QIAquick purification kit (Qiagen, Valencia, CA) per the instructions of the manufacturer and then dried down in a SpeedVac (45°C, 45 min; ThermoSavant, Milford, MA).

A comprehensive 50mer functional gene array, constructed using the methods of Li *et al.* [23] and Liebich *et al.* [24], was used to examine the isolates for the presence of 25,098 functional gene probes, among which, 4546 probes are from metal homeostasis genes

(He *et al.*, unpublished data). Microarray slides (Super-Amine substrates, ArrayIt, Sunnyvale, CA) were spotted with 50mer probes as described by Rhee *et al.* [37] and cross-linked at 600 mJ (Stratagene UV Stratalinker 1800, La Jolla, CA). Slides were incubated at 50°C for 30–60 min in prehybridization solution (50% formamide, 5× SSC buffer, 0.1% SDS, 0.1 mg mL^{-1} bovine serum albumin), washed three times in distilled water and once with isopropanol, and dried by centrifugation (500×*g*, 2 min).

Before hybridization, all items (slides, lifter slips, hybridization chambers, pipette tips) were heated to 60°C. Microarray slides were preassembled with lifter slips. To maintain the temperature of all items, setup was done on a 60°C heatblock. Hybridization mix (38.4 μL; 50% formamide, 5× SSC, 0.1% SDS, 0.1 μg μL^{-1} Herring sperm DNA, 8.25 μM spemidine) was added to the labeled genomic target DNA and incubated at 95°C for 5 min then maintained at 60°C. Two wells within the hybridization chamber were filled with preheated 5× SSC (15 μL). Just before hybridization, 1.6 μL of RecA protein was added to the hybridization solution. The entire sample was then pipetted onto the surface of the slide at the edge of the lifter slip, allowing capillary action to draw the solution between the lifter and the slide. Once the solution reached the end of the lifter, the hybridization chamber was sealed and immediately placed into a 50°C water bath and incubated overnight.

After incubation, the slides were washed for 5 min in prewarmed (50°C) wash buffer I (1× SSC, 0.1% SDS). This step was repeated using room temperature wash buffer I. The arrays were washed twice (10 min each) in room temperature wash buffer II (0.1× SSC, 0.1% SDS) and then five times (1 min each) in wash buffer III (0.1× SSC) with gentle shaking. The arrays were dried by centrifugation (500×*g*, 2 min), imaged (ScanArray Express Microarray Scanner, Perkin Elmer, Boston, MA), and analyzed using the Imagene software (6.0 premium ed, Biodiscovery, El Segundo, CA). A signal-to-noise ratio of >2 was considered a positive signal. A positive signal in at least two of the triplicate arrays was required before a probe was considered positive.

Determination of Metal Tolerance in Streptomyces aureofaciens NR-3 and Kitasatospora cystarginea NR-4. The influence of Ni, Co, Cd, or Zn on growth of *S. aureofaciens* NR-3 and *K. cystarginea* NR-4 at pH 5, 6, and 7 was determined. Growth in the absence of metals was used as a control. For inoculum, freezer stocks of each isolate were plated onto LB agar plates and incubated at 30°C for 72 h to check for purity. Colonies were transferred into 25 mL 4M (pH 6, 0.85 mM Ni) and incubated in the dark at 30°C with shaking (200 rpm) for 48 h. Aliquots (2 mL) were transferred into 50 mL 4M (pH 6) amended with 0.85 mM Ni at 30°C with shaking (200 rpm) for 48–72 h. Cultures were centrifuged (6000 rpm, 10 min) and resuspended in 4M (pH 6) to an optical density of 0.4 at 660 nm for use as inoculum. One milliliter of the inoculum was added to 125 mL flasks containing 24 mL 4M, (pH 5, 6, or 7) amended with increasing concentrations of each metal (8.5, 17.0, 42.6, 68.1, and 85.2 mM Ni; 0.85, 1.7, 4.2, 8.5, 17.0 mM Co; 0.89, 2.2, 4.4, 8.9 mM Cd). Growth was monitored by measuring total protein [6]. Growth inhibition with metals was determined by comparison to metal-free controls.

Regulation of Ni Tolerance in S. aureofaciens NR-3 and K. cystarginea NR-4. *Streptomyces aureofaciens* NR-3 and *K. cystarginea* NR-4 were examined to determine if Ni tolerance was inducible or constitutively expressed. Inoculum for each isolate was obtained as described above in 4M (pH 6) with either 0.85 mM Ni (induced) or 0 mM Ni (uninduced). Erlenmeyer flasks (125 mL) with Teflon-lined screw caps containing 24 mL of 4M (pH 5, 6, or 7) amended with 0, 0.85, or 8.5 mM Ni were inoculated with 1 mL of induced or uninduced culture. Cultures were incubated with shaking (200 rpm) at 30°C in the dark. Growth was monitored by measuring total protein [6].

Results and Discussion

Isolation and Characterization of Metal-Tolerant Microorganisms. The Tims Branch watershed, including Steed Pond, has been contaminated with significant concentrations of Ni and U along with smaller quantities of other metals (Cr, Cu, Zn, Cd, and Pb) for over four decades [8]. The history of metal contamination at the site and the relatively undisturbed sediments provide an ideal location to examine the adaptation of microorganisms within a metal-contaminated environment. Sediment samples from within Steed Pond and the control site, Boggy Gut, were examined for aerobic heterotrophic microorganisms tolerant to Ni and U (Table 1). At pH 7, using LB agar plates that did not contain Ni or U, 0.62×10^6 CFU g^{-1} sediment were isolated from contaminated sediments, whereas 1.045×10^6 CFU g^{-1} sediment were isolated from uncontaminated sediments. This suggests that the presence of metals exerts selection pressure, which resulted in fewer numbers of culturable microorganisms.

Although microorganisms were isolated from naturally acidic sediments (e.g., pH 5.0–5.5), more colonies formed on pH 6 and 7 plates compared with pH 5, regardless of the presence or absence of metals (Table 1). This could result from a number of reasons. For example, carbon sources not provided by 4M may be required for these isolates to grow at pH 5 [50]. Alternatively, microorganisms may occupy microenvironments having higher pH values than the bulk sediment. The pH of sediment cores from Steed Pond varied with depth and sampling site (data not shown). Additionally, differences in pH may result in altered growth behavior. For

Table 1. Replicate heterotrophic plate counts of sediment bacteria growing on Ni- and U-amended agar plates

	pH 5		*pH 6*		*pH 7*	
	Rep 1	*Rep2*	*Rep 1*	*Rep2*	*Rep 1*	*Rep2*
Uncontaminated sediment						
0 mM	0.18	0.19	1.41	1.38	1.04	1.05
Uranium						
0.04 mM U	0.09	0.04	0.37	0.44	0.66	0.62
0.42 mM U	0.02	0.02	0.04	0.05	0.31	0.19
4.2 mM U	0	0	0.01	0.01	0.01	0
21 mM U	0	0	0	0	0	0
Nickel						
0.17 mM Ni	0.14	0.12	1.13	1.14	0.96	0.98
1.7 mM Ni	0.09	0.11	0.83	0.79	0.33	0.40
17 mM Ni	0.02	0.02	0.20	0.22	0.09	0.07
85.2 mM Ni	0	0	0	0	0	0
Contaminated sediment						
0 mM	0.04	0.02	0.44	0.46	0.60	0.64
Uranium						
0.04 mM U	0.04	0.07	0.15	0.12	0.35	0.40
0.42 mM U	0.05	0.03	0.06	0.22	0.05	0.13
4.2 mM U	0	0	0	0	0.01	0
21 mM U	0	0	0	0	0	0
Nickel						
0.17 mM Ni	0.03	0.05	0.54	0.68	0.60	0.67
1.7 mM Ni	0.08	0.05	0.43	0.48	0.56	0.41
17 mM Ni	0.03	0.04	0.20	0.21	0.11	0.14
85.2 mM Ni	0.20	0.21	0.14	0.17	0.01	0.01

Counts are expressed as number of CFU per gram of sediment$\times 10^6$. Results are from duplicate plates.

example, at pH 4.3, *Bacillus polymyxa* cells have been shown to form elongated chains but not increase in CFUs over a 25-day period; however, at pH 4.7, an increase in CFUs did occur over the same time period [9]. Accordingly, the Ni-tolerant isolates may exhibit different growth behavior in their native riparian sediments than under laboratory conditions.

Microorganisms were able to grow at higher levels of Ni than U on metal-amended agar plates. Colonies were observed on the 85.2 mM Ni plates (0.205, 0.155, and 0.01×10^6 CFU g^{-1} sediment at pH 5, 6, and 7, respectively), whereas no colonies were detected on the 21.0 mM U plates. Morphologically distinct colonies from the 85.2 mM Ni-amended agar plates at pH 5 (designated NR-3) and pH 6 (NR-1, 2, and 4) were isolated, and a 351 bp region of the 16S rDNA gene was sequenced. These isolates were identified as the Gram-positive microorganisms [similarity (Sab) values in parentheses] *Arthrobacter oxydans* NR-1 (0.996), *Streptomyces galbus* NR-2 (0.992), *S. aureofaciens* NR-3 (1.000), and *K. cystarginea* NR-4 (0.964). Phylogenetic trees were created based on the 16S rDNA gene of the Ni-tolerant isolates. Trees were produced with similar topologies using both neighbor joining and maximum likelihood, so the maximum-likelihood tree is presented with bootstrap values transferred from the neighbor-joining tree (Fig. 1).

All four isolates from Steed Pond are members of the order *Actinomycetales*. Several studies have shown that actinomycetes are often isolated from sites with increased concentrations of Ni, either naturally occurring or from anthropogenic activities. *Streptomyces* spp. has been shown to be dominant members of the microbial community in soils naturally rich in Ni [18, 30]. Idris *et al.* [20] isolated 11 strains of actinomycetes that were tolerant to 5–12 mM Ni from sediments around a Ni-hyperaccumulating plant. Two Ni-tolerant *Arthrobacter* strains were also isolated from the soils around Ni-hyperaccumulating plants [39, 48]. Similarly, Ni spiking has been shown to select for the actinomycete group in sediments [18]. Tolerant *Arthrobacter* spp. have been isolated from metal-contaminated industrial sites [27]. Seven strains of *Streptomyces* were isolated from soil at a former U mine contaminated with up to 17 mM Ni and were tolerant to 2–10 mM Ni; U tolerance was not examined [1]. In this same study, a transporter homologous to NixA and HoxN, which transport Ni^{2+} into the cell [17], was found in two of the *Streptomyces* sp. These investigations demonstrated that actinomycetes are often

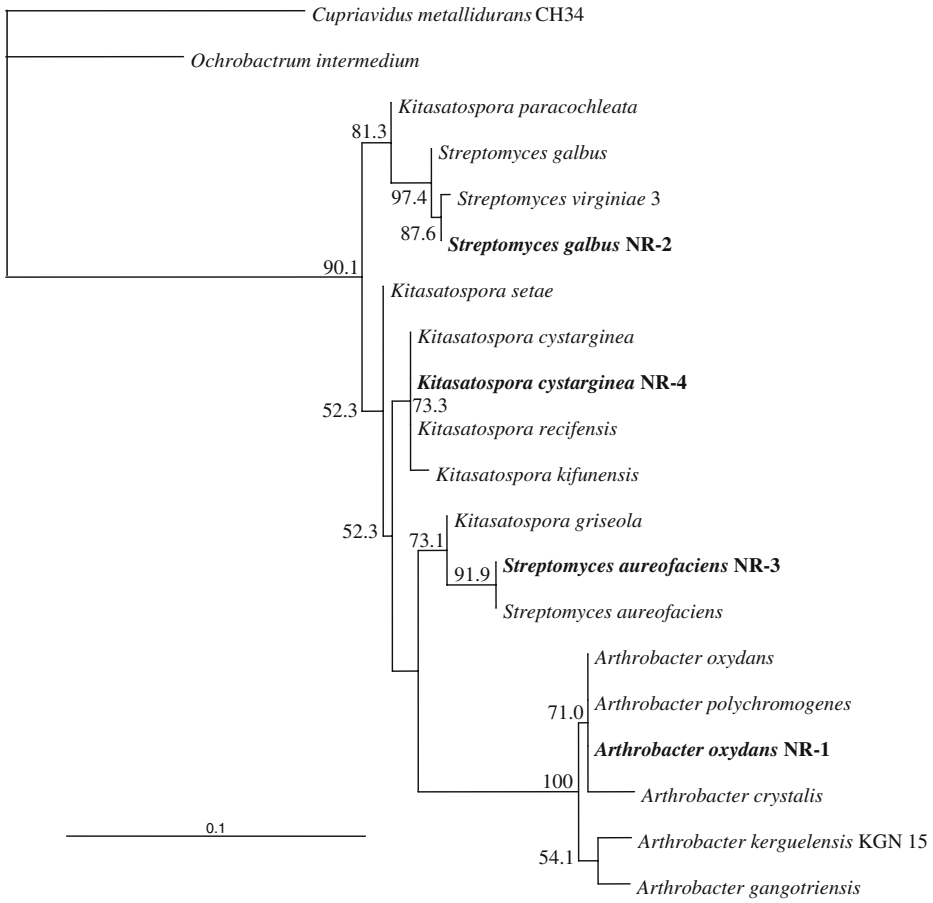

Figure 1. Phylogenetic tree of the Gram-positive isolates from Steed Pond based on partial 16S rRNA sequences. Sequences from the isolates were compared with other closely related Gram-positive microorganisms. Maximum-likelihood tree with bootstrap values from neighbor-joining tree. Isolates from Steed Pond are in bold.

selected for in Ni-contaminated soils and sediments. However, little is known about the actual mechanism(s) of Ni resistance/tolerance in this group.

Actinomycetes are ecologically important and abundant members of the soil microbial community. *Arthrobacter* and *Arthrobacter*-like species account for 14–83% of the culturable microbial community in different types of soils [14]. *Streptomyces* are among the dominant members of the strawberry rhizosphere bacterial community as determined by denaturing gradient gel electrophoresis [43]. As a result of their ability to utilize a wide variety of carbon sources [29], including organic contaminants such as aromatic compounds [54], actinomycetes play an important role in organic matter decomposition, degrading the more recalcitrant components (e.g., lignin) as well as promoting soil structure [21].

Relative Toxicity of Nickel and Uranium. Although the bulk sediment concentrations represent the contamination level of a site, microorganisms within the sediments are not exposed to the total concentration of metals but rather to the bioavailable fraction [25]. As the most chemically labile fraction, metal concentrations within the porewater would be the most readily available to microorganisms. However, this concentration may not reflect the actual bioavailability of the Ni and U as other factors, including pH, metal speciation, and organic content will influence bioavailability [12]. The simulated porewater concentration of Ni and U in Steed Pond sediments (operationally determined by a 1:1 sediment–water equilibration) ranged from 2 to 53.5 μM Ni and from 0.12 to 1.7 μM U, respectively [44]. Greater concentrations of Ni compared with U in simulated porewater as well as the higher concentration of labile Ni in sediments compared with U suggest that Ni is more bioavailable than U in these sediments [44]. Consistent with this, the isolation of microorganisms capable of growing at higher concentrations of Ni compared with U suggests that Ni is exerting a stronger selection pressure than U in these contaminated sediments.

To further evaluate the relative toxicity of Ni and U at concentrations more relevant to those observed in the sediment porewater, the MetPLATE™ toxicity assay was used as a reference. A 27.5% inhibition of β-galactosidase activity was observed at 42 μM U, whereas no inhibition was noted at 13 μM U (data not shown). A 13.2%, inhibition was observed at 54 μM Ni, whereas no inhibition was found at 17 μM Ni (data not shown).

Although U is intrinsically more toxic based on the MetPLATE™ assay, Ni toxicity was observed at concentrations representative of Steed Pond sediment porewater (2–53.5 μM Ni) [44]. U toxicity was not observed until concentrations exceeded those of the Steed Pond sediment porewater (0.12–1.7 μM U) by a factor of ≥40 [44]. These findings also suggest that, at the Ni and U concentrations typical of Steed Pond porewater, Ni would be exerting a greater selection pressure.

Nickel Tolerance in S. aureofaciens NR-3 and K. cystarginea NR-4. *Streptomyces aureofaciens* NR-3 and *K. cystarginea* NR-4 were chosen to further characterize Ni tolerance over the pH range 5–7 because preliminary experiments indicated consistent growth in the 4M medium. Because the mechanism(s) of Ni resistance is unknown in these two isolates, the term tolerance is employed because resistance implies that a specific mechanism has been identified [33, 53]. Both *S. aureofaciens* NR-3 and *K. cystarginea* NR-4 showed minimal growth at pH 5 (<50 mg L^{-1} increase in protein concentration), although NR-3 was initially isolated on pH 5 plates. Both isolates grew at higher Ni concentrations at pH 6 as compared with pH 7 (Fig. 2, data not shown). At pH 6, growth was observed for *S. aureofaciens* NR-3 at 42.6 mM Ni after 46 h with negligible inhibition compared with growth in the absence of Ni. At pH 7 and 8.5 mM Ni, growth was inhibited by 65% after 77 h as compared with growth of the Ni-free control; no growth was observed at 42.6 mM Ni. Growth of *K. cystarginea* NR-4 at pH 6 and 17.0 mM Ni was comparable with growth in the absence of Ni, whereas a 9% growth inhibition was observed at 42.6 mM Ni after 76.5 h. At pH 7, there was 7.5% growth inhibition at 8.5 mM Ni, and no growth was observed at higher Ni concentrations (>17.04 mM Ni). Growth of microorganisms at >1 mM Ni has been used as a benchmark in determining whether a microorganism is resistant/tolerant [33]. Both isolates exhibited Ni tolerance at both pH 6 and 7, with increased tolerance observed at pH 6.

Increased Ni toxicity with an increase of pH over the pH range of 5–7 has been observed in Gram-negative Ni-tolerant *Burkholderia vietnamiensis* $PR1_{301}$ but was not observed in the Gram-negative Ni-resistant *Cupriavidis metallidurans* CH34 and 31A using the same medium as in this study [53]. Conversely, previous studies have reported increased Ni toxicity to microorganisms with decreased pH [2, 3]. Growth of the actinomycete *Nocardia rhodochrous* was completely inhibited by 0.43 mM Ni at pH 5.5–6.5, whereas growth was observed for this same concentration at pH 7–8.5 [2]. Chemical speciation of Ni in 4M over the concentration range 0.85–17.0 mM Ni, as calculated by the thermodynamic chemical speciation model MINTEQA2, revealed identical patterns in complex species distribution between pH 5 and 6, whereas at pH 7 and Ni concentrations >3.41 mM, precipitation of $NiOH_2$ was predicted [53]. Therefore, the observed changes in Ni toxicity to *B. vietnamiensis*

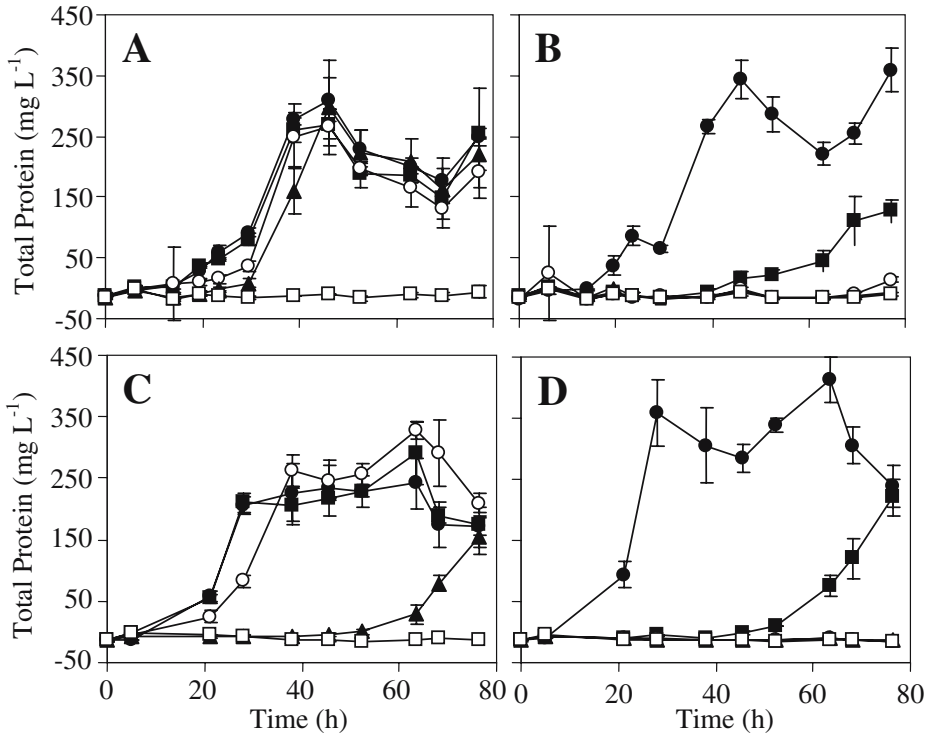

Figure 2. *Streptomyces aureofaciens* NR-3 (A and B) and *K. cystarginea* NR-4 (C and D) grown in 4M in the presence of 0 (•), 8.5 (▪), 17.0 (○), 42.1 (▲), and 85.2 (□) mM Ni at pH 6 (A and C) and 7 (B and D). Error bars are standard deviation of triplicate samples.

$PR1_{301}$ as a function of pH was not explained by changes in chemical speciation [53].

Although examples of Ni-resistant actinomycetes can be found in the literature, isolates NR-3 and NR-4 grew at higher concentrations of Ni than reported previously for other actinomycetes [1, 20, 27]. It should be noted that direct comparisons between studies are complicated by differences in medium used in various studies, as well as differences in metal concentration and length of previous metal exposure. The medium used in this study was selected to minimize metal complexation with medium components [53]. Additionally, the actinomycetes examined in this study were isolated from riparian sediments, which had been contaminated with Ni and U for over four decades [8]. The number of tolerant microorganisms isolated is often related to the concentration of Ni in the surrounding environment [18, 27, 39]. The length of time over which the isolates were exposed may have allowed them to develop mechanisms to reduce the toxicity of higher concentrations of Ni.

Regulation of Ni resistance in S. aureofaciens NR-3 and K. cystarginea NR-4. To determine if Ni resistance in *S. aureofaciens* NR-3 and *K. cystarginea* NR-4 is constitutively expressed or induced by the presence of Ni, both isolates were grown in the absence of Ni (uninduced) or with 0.85 mM Ni (induced) and then exposed to higher concentrations of Ni (data not shown). No difference in growth was observed when either isolate was induced at 0.85 mM Ni as compared with growth of uninduced cultures, demonstrating that Ni tolerance was constitutively expressed. Margesin and Schinner [27] found that of four *Arthrobacter* sp. isolated from a soils and sediments surrounding a Ni/Cr recycling plant, three displayed inducible Ni resistance when pregrown at higher concentrations of Ni (4, 8 or 20 mM Ni, depending on level of resistance) while the fourth isolate displayed constitutive Ni resistance.

Resistance of S. aureofaciens NR-3 and K. cystarginea NR-4 to Cadmium, Cobalt, and Zinc. Nickel-resistant microorganisms are often tolerant to other metals as well, either because the Ni resistance mechanism confers resistance to other metals or because the resistance determinants are linked. Stoppel and Schlegel [48] found that tolerance to Co, Zn, and Cd was frequently observed with Ni tolerance for a variety of microorganisms, including strains of *Cupriavidus* (*Alcaligenes*) spp., *Burkholderia* spp., *Acinetobacter* sp., *Pseudomonas* spp., and *Arthrobacter* spp., isolated from sites containing high concentrations of Ni and/or other metals. For example, two *Arthrobacter* spp. isolated from naturally Ni-enriched soil around a Ni-hyperaccumulating plant were found to be resistant to Ni and Co but not other divalent cations [48]. Seven *Streptomyces* spp. isolated from a U mine were also resistant to Co (2–20 mM), Zn (20–50 mM), Cr(III) (2–5 mM), and some of the *Streptomyces* sp. were also resistant to Cd (2 mM) and Cu (2–5 mM) as determined by an agar diffusion assay [1].

Growth of *S. aureofaciens* NR-3 was observed at 4.2 and 1.7 mM Co at pH 6 and 7, respectively (data not shown). *K. cystarginea* NR-4 grew at 8.5 and 1.7 mM Co at pH 6 and 7, respectively (data not shown). Zn tolerance was also observed in *S. aureofaciens* NR-3 and *K. cystarginea* NR-4, with greater tolerance at pH 6 compared with pH 7 (Fig. 3). Growth of *S. aureofaciens* was observed at 7.6 mM Zn at pH 6 after a 70-h lag phase and at 0.76 mM Zn at pH 7. Growth of *K. cystarginea* NR-4 was observed at 15.3 mM Zn at pH 6 and at 3.8 mM Zn at pH 7. Growth of both isolates was completely inhibited at 0.89 mM Cd ($Cd(NO_3)_2{\cdot}4H_2O$; data not shown).

The level of Co and Zn tolerance observed in NR-3 and NR-4 was less than that observed in previously published studies as discussed above. However, decreased Co tolerance as compared with Ni was observed in four different *Cupriavidus* (*Alcaligenes*) strains [41]. This lower level of tolerance for Co and Zn contrasts the higher level of Ni tolerance observed in these same organisms relative to previous studies. Except for U, far greater quantities of Ni were released into the Tims Branch corridor than other metals (e.g., Zn, Cu, Cd, Cr, and Pb), resulting in lower sediment concentrations of these metals as compared with Ni [36, 44]. The resulting higher concentrations of Ni in sediments as compared with other divalent cations may have provided greater selection pressures for Ni tolerance than for other metals.

Efflux of divalent cationic metals is the most commonly observed mechanism of resistance in prokaryotes [33]. However, much less is known about the mechanisms of metal resistance in Gram-positive bacteria. As discussed previously, Ni-tolerant actinomycetes have been isolated, but to the best of our knowledge, no specific mechanisms have been identified. Thus, whereas tolerance to Ni, Co, and Zn was observed in *S. aureofaciens* NR-3 and *K. cystarginea* NR-4, the mechanism(s) of resistance/tolerance remains unknown.

Correlation of Antibiotic Resistance with Metal Resistance. An increasing number of studies have demonstrated that metal resistance/tolerance is often associated with antibiotic resistance due to physical linkage of the genes [28, 46, 49]. Metal-resistant strains are more likely to be resistant to antibiotics than metal-sensitive strains [56]. Of the six antibiotics tested, *S. galbus* NR-2 was only resistant to one (ciprofloxacin), whereas *K. cystarginea* NR-4 was resistant to gentamycin and kanamycin. *A. oxydans* NR-1 was also resistant to gentamycin and kanamycin but showed an intermediate resistance to ciprofloxacin, whereas *S. aureofacians* NR-3

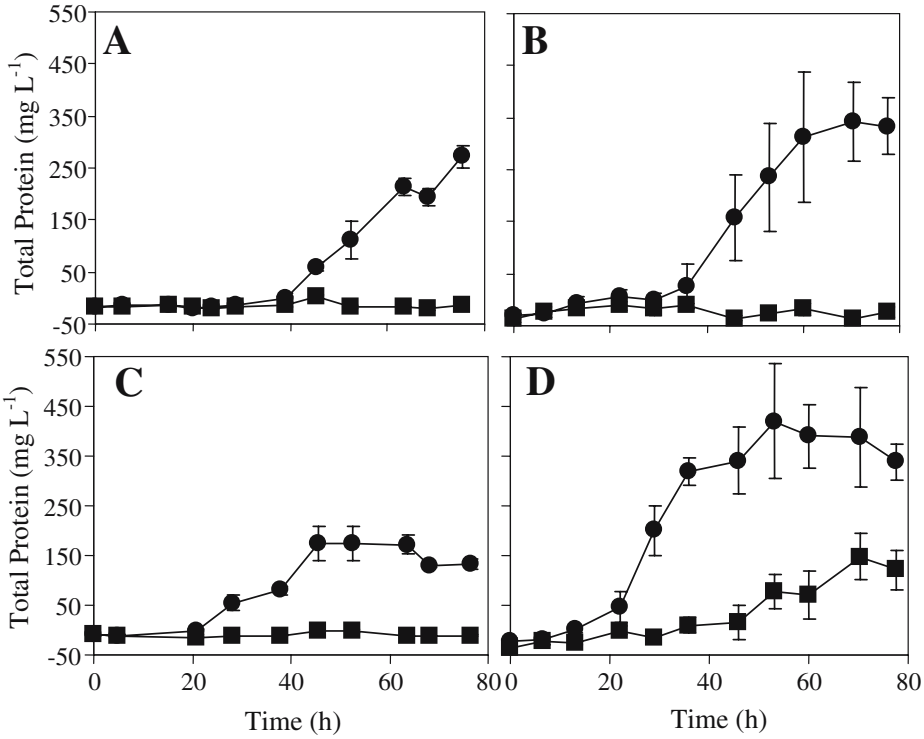

Figure 3. *Streptomyces aureofaciens* NR-3 (A and B) and *K. cystarginea* NR-4 (C and D) grown in 4M amended with 4.2 mM Co (A and C) and 3.8 mM Zn (B and D) at pH 6 (•) and 7 (▪). Error bars are standard deviation of triplicates samples.

displayed only intermediate resistance to kanamycin and was susceptible to the other five antibiotics. Although this is a very small sample of the microbial community at this site and only a limited number of antibiotics was tested, these results suggest a diversity of antibiotic resistance patterns between these closely related isolates. Stepanauskas *et al.* [46] found that Ni resistance was associated with resistance to kanamycin, gentamycin, tetracycline, ciprofloxacin, and streptomycin in microorganisms isolated from coal combustion ash settling basins. The Ni-tolerant isolates examined in this study each showed resistance to at least one of these antibiotics. However, despite their isolation from the same sediments and their similarity in Ni, Co, and Zn tolerance, different patterns of antibiotic resistance were observed for each isolate.

Genes involved in metal resistance or other beneficial functions (e.g., antibiotic resistance) can be housed on mobile genetic elements that may be passed to other bacterial species through horizontal gene transfer. Warren *et al.* [54] found evidence of four genomic islands in the actinomycete *Rhodococcus* sp., with one of the islands containing putative metal transport genes. These genetic elements can contain groups of genes and gene cassettes that aide in the survival of microorganisms under various unfavorable conditions and would most likely be found in environments with large diverse bacterial populations [7].

Genes Involved in Metal Resistance. A functional gene microarray designed to examine microbial DNA for the presence of genes associated with biogeochemical cycling of C, N, S, organic contaminant degradation, and metal homeostasis [He *et al.*, unpublished data; 37] was utilized to examine the four Ni-resistant Gram-positive isolates. *S. galbus* NR-2 had the highest number of positive genes detected (360 total) and *A. oxydans* NR-1 the least (66) (Table 2). These probes and hybridization conditions were designed to be as specific as possible; therefore, a similar gene from a different genus or species may not be detected. The array contained 414 genes from *Streptomyces*, 97 from *Arthrobacter*, and no genes specific to *Kitasatospora*. In spite of the relatively limited number of probes on the array specific to the genera of our isolates, we were able to detect genes from a wide variety of Gram-negative and Gram-positive microorganisms in these actinomycete isolates.

Several metal resistance genes were detected in the isolates, including those associated with Al, As(III), Cd(II), Cr(VI), Cu(II), Hg(II), Ni(II), Te, and Zn(II) (Table 3). We observed that *S. aureofaciens* NR-3 and *K. cystarginea* NR-4 were resistant to Ni, Co, and Zn based on growth in the presence of these metals (Figs. 2 and 3). Three of the isolates, *A. oxydans* NR-1, *S. aureofaciens* NR-3, and *K. cystarginea* NR-4, were positive for *nreB*, a gene that codes for an efflux transporter that is believed to confer resistance only to Ni [13, 40]. The *nre* determinant has a broad host range [52] but confers a low level of resistance (3 mM Ni) both in the host microorganism and in transconjugants [40]. *S. aureofaciens*

NR-3 and *K. cystarginea* NR-4 displayed a much higher level of Ni resistance in this study (42.6 and 8.5 mM Ni at pH 6 and 7, respectively), suggesting that other mechanisms of Ni resistance may be present in addition to *nreB*.

A portion of the *czc* operon (*czcA*) was detected in *S. galbus* NR-2 and *K. cystarginea* NR-4. This operon encodes an efflux transporter that confers resistance to Cd^{2+}, Zn^{2+}, and Co^{2+}, but not Ni^{2+} [34, 35]. However, the CzcA designation is based on a 127-amino-acid sequence and a subsequent BLAST search of this sequence did not match with the Czc protein. An additional gene associated with Cd resistance was also found in *S. galbus* NR-2, *S. aureofaciens* NR-3, and *K. cystarginea* NR-4. Both of the Cd-related genes were hypothetical or predicted resistance proteins; thus, they may not function as predicted.

Kitasatospora cystarginea NR-4 was also positive for another Zn^{2+} exporter (ZiaA, gi16329893; data not shown). This transporter was not detected in the other isolates. The presence of an additional transporter could explain the higher level of Zn resistance in *K. cystarginea* NR-4 as compared with *S. aureofaciens* NR-3 (Fig. 3). Interestingly, although the degree of resistance appeared similar among the four isolates, only two metal resistance genes (for Te resistance) were detected in all of the isolates. Little is known regarding resistance to Te [42]. Because relatively little sequence information is known for metal resistance genes in Gram-positive organisms, most of the metal resistance probes on the microarray were designed based on genes from Gram-negative microorganisms. Therefore, other novel resistance genes may have been present in the isolated organisms but were not detected.

In addition to metal-resistance genes, the array also contained genes for metalloproteins and genes involved in metal uptake. The presence of Ni^{2+} uptake and Ni containing metalloproteins suggests that other efflux transporters, not identified by the array, may be present to maintain homeostasis. All four isolates were positive for Ni-containing urease or a component of the urease enzyme system (UreH, UreE, UreG; data not shown). In addition, *S. aureofaciens* was also positive for the Ni^{2+} uptake transporter nikB, gi26990058 (data not shown), but the other isolates were not. A Ni^{2+} transporter homologous to NixA and HoxN was found in two *Streptomyces* sp. isolated from a Ni-contaminated U mine [1]. Although not all of the isolates were positive for the Ni-efflux genes contained on the array, the presence of Ni-containing enzymes suggests that both Ni-influx and Ni-efflux transporters would be present to maintain homeostasis.

Several genes conferring the ability to degrade a variety of carbon sources, including organic contaminants, were present in each of the isolates (data not shown). These included chitinases, cellulases, mannanases, hydrolases, oxygenases, and isomerases involved in organic pollutant degradation. This is not unexpected as actinomycetes are known for their ability to utilize a wide range of carbon sources [29]. For example, the actinomycete *Rhodococcus* sp. contains an operon presumed to be involved in the degradation of aromatic compounds [54]. All four of the NR isolates were positive for a form of oxygenase. Some oxygenases have been shown to be involved in the cometabolic degradation of trichloroethylene (TCE), an organic contaminant at the SRS [10, 22], where the four actinomycetes were isolated. Although the ability of these isolates to degrade TCE has not been examined, the presence of various oxygenases suggests that these isolates may be able to degrade TCE. All four of the isolates possessed genes involved in the degradation of a range of organic contaminants.

This study and others over the past decade have demonstrated that actinomycetes are capable of growing at high concentrations of Ni and other metals. However, most research on metal resistance has focused on Gram-negative organisms, whereas relatively little is known about the distribution, resistance mechanisms, and genetics in actinomycetes and other Gram-positive microorganisms. We isolated four Ni-resistant actinomycetes from metal-contaminated riparian sediments, suggesting that the high Ni loading may have selected for these organisms. Further examination of two of the isolates demonstrated resistance

Table 2. Number of microarray genes present in each of the isolates

Gene category	*Total*[a]	*A. oxydans NR-1*	*S. galbus NR-2*	*S. aureofaciens NR-3*	*K. cystarginea NR-4*	*All*[b]	*Two or more*[c]
Metal homeostasis	4546	15	86	20	33	2	35
Carbon degradation	2808	9	46	9	16	2	19
Carbon fixation	1018	2	9	0	3	0	3
Organic contaminant degradation	8028	23	130	30	42	8	46
Sulfur reduction	2615	2	7	1	1	0	2
Methane oxidation/ reduction	773	2	11	0	1	1	4
Nitrogen cycling	5310	13	71	20	24	2	29

[a]Total number of gene probes on the array.
[b]Number of positive genes found in all four isolates.
[c]Number of positive genes found in at least two of the isolates.

Table 3. Microarray genes related to metal homeostasis present in at least two of the Gram-positive isolates

Gene ID	*Gene description*	*Organism*
23041758[a,b]	Cystathionine beta-lyase family protein involved in aluminum resistance	*Trichodesmium erythraeum* IMS101
23011662[a,c]	Arsenite efflux pump ACR3 and related permeases	*Magnetospirillum magnetotacticum*
15792511[b,d]	Putative arsenical pump membrane protein	*Campylobacter jejuni* subsp. *jejuni* NCTC 11168
32470154[a,d]	Hypothetical protein, similar to arsB	*Salmonella typhimurium*
17548775[a,c]	Probable chromate resistance signal peptide protein	*Ralstonia solanacearum* GMI1000
23023727[a,b,c]	Predicted permease, cadmium resistance protein	*Leuconostoc mesenteroides* subsp. *mesenteroides* ATCC 8293
34557205[a,b]	Hypothetical protein WS0807, similar to czcA	*Wolinella succinogenes* DSM 1740
23012809[c,d]	Chromate transport protein ChrA	*M. magnetotacticum*
33865857[a,b,d]	Putative chromate transport protein, CHR family	*Synechococcus* sp. WH 8102
15807403[b,d]	Chromate transport protein	*Deinococcus radiodurans* R1
23123941[a,b,c]	Chromate transport protein ChrA	*Nostoc punctiforme*
687689[a,b]	Similar to ChrA from *A. eutrophus*, Swiss-Prot accession number P17551	*Synechococcus* sp.
23137197[a,b,c]	Chromate transport protein ChrA	*Cytophaga hutchinsonii*
16080666[b,c,d]	hypothetical protein, ywrA, similar to chromate transport protein	*B. subtilis* subsp.*subtilis* str. 168
151188[a,b]	CopA peptide	*P. syringae*
38347977[a,b]	Copper resistance protein	*Serratia marcescens*
10047068[b,c]	copF Cu-ATPase	*C. metallidurans*
23003052[a,b]	Uncharacterized protein involved in copper resistance	*Lactobacillus gasseri*
37520133[a,b]	Mercuric reductase	*Gloeobacter violaceus* PCC 7421
16331903[a,b,c]	Nickel resistance, nreB	*Synechocystis* sp. PCC 6803
22988087[b,d]	Membrane protein TerC, possibly involved in tellurium resistance	*Burkholderia fungorum*
23003390[a,b]	Membrane protein TerC	*L. gasseri*
23055848[a,b,c,d]	Membrane protein TerC	*Geobacter metallireducens*
32035774[a,b]	Tellurite resistance protein and related permeases	*Actinobacillus pleuropneumoniae* serovar 1 str. 4074
23057746[a,b,c]	Tellurite resistance protein and related permeases	*P. fluorescens* PfO-1
23472386[a,b,c,d]	Tellurite resistance protein and related permeases	*P. syringae* pv. *syringae* B728a
16077361[a,b]	Hypothetical protein BSU02920, similar to tellurium resistance protein	*B. subtilis* subsp. *subtilis* str. 168
23054496[b,d]	ABC-type transport system involved in cytochrome *c* biogenesis, permease component	*G. metallireducens*
23055461[a,b,c]	ABC-type transport system involved in cytochrome *c* biogenesis, permease component	*G. metallireducens*
23055477[b,c]	Cytochrome *c*, monoheme and diheme variants	*G. metallireducens*
24376080[a,b]	Cytochrome *c* oxidase, subunit I	*Shewanella oneidensis* MR-1
39997978[a,b]	Cytochrome *c* family protein	*G. sulfurreducens* PCA
32475631[b,c]	Similar to PbrT-putative c-type cytochrome	*Pirellula* sp. 1
22956535[b,c,d]	Hypothetical protein Rsph020175	*Rhodobacter sphaeroides*

[a]Gene present in *S. galbus*.
[b]Gene present in *K. cystarginea*.
[c]Gene present in *S. aureofaciens*.
[d]Gene present in *A. oxydans*.
S. galbus /NR-2.
K. cystarginea/ NR-4.
S. aureofaciens/ NR-3.
A. oxydans/ NR-1.

to Co and Zn. All four isolates were resistant to several antibiotics, which are often associated with Ni tolerance. In addition, the isolates contained genes involved in the degradation of numerous organic contaminants. Because actinomycetes are key members of the soil community, it is important to understand the implications of metal contamination on this group of organisms. Further work is underway to determine the mechanism(s) and confirm the specific genes behind the observed divalent metal resistance.

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