

NOTE

Metal resistance among aerobic chemoheterotrophic bacteria from the deep terrestrial subsurface

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Abstract: The metal resistance of 350 subsurface bacterial strains from two U.S. Department of Energy facilities, the Savannah River Site (SRS), South Carolina, and the Hanford site, Washington, was determined to assess the effect of metal toxicity on microorganisms in the deep terrestrial subsurface. Resistance was measured by growth inhibition around discs containing optimized amounts of Hg(II), Pb(II), and Cr(VI). A broad range of resistance levels was observed, with some strains of *Arthrobacter* spp. demonstrating exceptional tolerance. A higher level of resistance to Hg(II) and Pb(II) ($P < 0.05$) and a higher occurrence of multiple resistances suggested that metals more effectively influenced microbial evolution in subsurface sediments of the SRS than in those of the Hanford site. Common resistance to heavy metals suggests that toxic metals are unlikely to inhibit bioremediation in deep subsurface environments that are contaminated with mixed wastes.

Key words: deep subsurface, metal resistance, mercury, chromium, lead.

Résumé : Nous avons vérifié la résistance aux métaux de 350 souches de bactéries isolées de la couche souterraine dans deux installations du U.S. Department of Energy, soit celle de Savannah River Site (SRS) et celle de Hanford, Washington. Le but était de mesurer l'effet de la toxicité des métaux sur les microorganismes présents dans la couche terrestre profonde. La résistance a été évaluée d'après l'inhibition de la croissance autour de disques contenant des concentrations optimales de Hg(II), Pb(II) et de Cr(VI). Un large spectre de niveaux de résistance a été observé et certaines souches d'*Arthrobacter* spp. ont présenté une tolérance exceptionnelle. Des niveaux de résistance plus élevés à Hg(II) et à Pb(II) ($P < 0.05$) et une fréquence plus élevée de résistance multiple ont été observés chez les souches provenant du SRS comparativement à celles de Hanford, ce qui laisse croire que les métaux influencent de façon préférentielle l'évolution des populations microbiennes du SRS plutôt que celles de Hanford. La résistance commune aux métaux lourds suggère que les métaux toxiques sont probablement peu capables d'inhiber la bioremédiation dans les zones profondes de la couche souterraine qui sont contaminées par des mélanges de déchets.

Mots clés : couche profonde, résistance aux métaux, mercure, chrome, plomb.

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The production of nuclear weapons and fuel for nuclear power plants has resulted in the contamination of vast areas of the terrestrial subsurface with mixtures of radionuclides, metals, anions, and a variety of organic solvents and chelating agents. In the United States alone, the Department of Energy (DOE) is entrusted with 3000 inactive waste sites where leakage from underground storage facilities and past surface disposal of wastes is threatening the quality of groundwater resources (Riley et al. 1992). Bioremediation may be the only feasible approach for the treatment of such vastly contaminated environments (Cox et al. 2000; McCullough et

al. 1999). Promising bioremediation approaches aim at (i) the immobilization of metals and radionuclides as insoluble complexes in aquifers and the vadose zone (Cooper et al. 2000; Gadd 2000; Wang 2000) and at (ii) the degradation of chelating agents to retard mobilization (Thomas et al. 1998; VanBriesen et al. 2000; Witschel et al. 1997). The biodegradation of organic contaminants by microbes in deep subsurface (Kim et al. 1996) and shallow aquifers (Broholm and Arvin 2000; Skubal et al. 2001) has been demonstrated. However, bioremediation in the subsurface requires metabolically active microbial communities. The vadose zone and

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Table 1. Metal-resistant and -sensitive strains used as references in this study.

Strain	Genotype;phenotype	Resistance* (mm) (N)	Reference and (or) comments
<i>Arthrobacter</i> sp. Cr15	Cr ^r	0±0.3 (22)	Nakatsu 2002
<i>Arthrobacter histidinolorovans</i> ATCC 11442	Cr ^s	12.7±1.3 (22)	Cr-sensitive <i>Arthrobacter</i> strain
<i>Ralstonia metallidurans</i> CH34(pMOL28)	chr ^r ;Cr ^r	4.3±1.6 (20)	Nies and Silver 1989; Peitzsch et al. 1998
<i>Ralstonia metallidurans</i> AE104	chr ^r ;Cr ^s	6.9±1.4 (20)	Sensitive cured derivative of CH34(pMOL28)
<i>Bacillus cereus</i> 5	mer ^r ;Hg ^r	10±2.8 (16)	Izaki 1981; Belliveau and Trevors 1990
<i>Bacillus cereus</i> ATCC 14579	Hg ^s	18.9±4.1 (16)	Hg-sensitive <i>B. cereus</i> strain
<i>Pseudomonas stutzeri</i> OX	mer ^r ;Hg ^r	4.7±0.8 (22)	Reniero et al. 1995, 1998
<i>Pseudomonas stutzeri</i> OX1	mer ^r ;Hg ^s	11.7±1.1 (22)	Sensitive cured derivative of OX
<i>Staphylococcus aureus</i> K10	Pb ^r	1.7±1.5 (12)	Levinson et al. 1996
<i>Staphylococcus aureus</i> K10S	Pb ^s	8.7±4.1 (12)	Pb-sensitive mutant of K10
<i>Escherichia coli</i> W3110	znt ^r ;Pb ^r	4.4±0.5 (27)	Rensing et al. 1998
<i>Escherichia coli</i> RW3110	znt ^r ;Pb ^s	11.8±1.3 (27)	Pb-sensitive mutant of W3110

*As determined by the zone of inhibition around paper discs containing 2 µmol Cr(VI), 50 nmol Hg(II), and 500 nmol Pb(II). See Materials and methods for details. Values are the averages ± one standard deviation for the indicated number of determinations (N).

groundwater aquifers are less than hospitable environments for microbial life because of a scarcity of nutrients and often of water as well (Amy 1997). Moreover, the presence of toxic metals and radionuclides in mixed waste sites may further inhibit microbial activities and thus bioremediation.

The promise of bioremediation in the subsurface has motivated research on (i) the structure and distribution of microbial communities in this environment (Ringelberg et al. 1997), (ii) the microbial reduction of radionuclides and metals (Fredrickson et al. 2000; Lovley 1993), (iii) the degradation of organic contaminants (Kim et al. 1996) and chelating agents (Bohuslavek et al. 2001), and (iv) the mobility of microbes in porous media (Balkwill et al. 1998; Clement et al. 1999). Furthermore, the possible effect of radiation on these activities has been addressed by extensive studies on radiation resistance, most notably in *Deinococcus radiodurans* (Daly and Minton 1995), including the construction and testing of recombinant strains that degrade organic contaminants and transform metals (Brim et al. 2000). Conspicuously scarce in this research are studies that focus on the resistance of subsurface microbial communities to metal stress. Because exposure to metals inhibits soil microbial activities and biomass production (Giller et al. 1998), resistance might be critical for microbial activities in the presence of toxic concentrations of metals in the deep subsurface. Here we report the preliminary characterization of resistance to three metals that are of concern in contaminated subsurface sediments: Hg(II), Pb(II), and Cr(VI) (Riley et al. 1992).

Bacterial strains and growth conditions

Bacterial cultures were shipped on slants from the Subsurface Microbial Culture Collection (SMCC), Tallahassee, Fla., to Rutgers University, New Brunswick, N.J. Strains originated from two sources: saturated Atlantic Coastal Plain sediments collected at the DOE's Savannah River Site (SRS) (borehole P24, 261 strains) and saturated unconsolidated sediments of the Ringold formation in the DOE's Hanford Site (borehole YB-02, 89 strains). The strains were originally isolated by plating dilutions of soils in phosphate buffer on rich media followed by aerobic incubation

(Balkwill 1989; Balkwill et al. 1997; Kieft et al. 1995), thus selecting for aerobic chemoheterotrophs. The identity of most strains was previously determined by hybridization with group-specific 16S rDNA-targeted probes, by relating restriction patterns to those of a sequenced strain or by 16S rDNA gene sequence data. The SRS group of isolates included eight α-, 69 β- (58 of which were *Comamonas* spp.), and 80 γ-Proteobacteria (49 *Acinetobacter* spp. and 31 *Pseudomonas* spp.), and 104 high GC Gram-positive bacteria (of which 95 were *Arthrobacter* spp.). The Hanford group of isolates was more diverse, with five α-Proteobacteria (two *Caulobacter* spp. and one strain each of *Sphingomonas* sp., *Rhodobacter* sp., and *Blastobacter* sp.), six β-Proteobacteria (four *Variovorax* spp., a *Telluria* sp., and a *Rhodocyclus* sp.), four γ-Proteobacteria (three *Pseudomonas* spp. and an *Acinetobacter* sp.), 37 high mol% GC Gram-positive bacteria (21 *Arthrobacter* spp.), and 14 low mol% GC Gram-positive bacteria (mostly *Bacillus* spp. and *Staphylococcus* spp.). The identity of the remaining 21 Hanford strains was unknown.

Upon receipt, the bacteria were streaked on peptone-tryptone-yeast-glucose (PTYG) medium ((in g/L) glucose (10), yeast extract (10), peptone (5), tryptone (5), MgSO₄·7H₂O (0.6), CaCl₂·2H₂O (0.07), and Bacto agar (20)). Following growth, a single colony was inoculated into 3 mL PTYG broth, and 500 µL of an overnight culture was transferred to a 1.5-mL sterile vial containing 500 µL of sterile glycerol. Vials were placed in a -80°C freezer. The SMCC strains were routinely grown at 28°C.

Reference strains (Table 1) were grown in PTYG with the exception of *Ralstonia metallidurans* strains, which were grown in a Tris-buffered minimal salt medium supplemented with 2 g/L sodium gluconate (Mergeay et al. 1985). With the exception of *Escherichia coli* and *Staphylococcus aureus*, which were grown at 37°C, all reference strains were grown at 28°C.

Disc inhibition assays for the determination of metal resistance

Assays were performed as described by Barkay et al.

(1990) except that test strains were streaked as a line from the plate center to its edge rather than applied as a lawn. For each metal, the medium-test metal combination that distinguished resistance from sensitive responses was selected by preliminary assays using reference strains. These consisted of strains carrying characterized metal resistance determinants and their sensitive mutants or phylogenetically related strains. The reported differences for each pair of resistant and sensitive strains (Table 1) were highly significant ($P < 0.001$). One-percent PTYG media and filter discs impregnated with 500 nmol $(C_2H_3O_2)_2Pb \cdot 3H_2O$ (Sigma–Aldrich Corp., St. Louis, Mo.) or 50 nmol $HgCl_2$ (Sigma–Aldrich Corp.) were selected as optimal assay conditions for Pb(II) and Hg(II) resistances, respectively. Cr(VI) resistance and sensitivity were distinguished with disks containing 2 μ mol CrK_2O_4 (Fluka Chemical Corp., Ronkonkoma, N.Y.) placed on full strength PTYG. Fresh filter-sterilized metal stock solutions in distilled water were prepared monthly at 2 M, 50 mM, and 2 M for Cr(VI), Hg(II), and Pb(II), respectively. Mercury stocks were acidified to 0.1 N with HCl. Stocks of Hg(II) and Cr(VI) were stored at 4°C and the Pb(II) stock was stored at room temperature. At least two replicate determinations were obtained for each SMCC strain for each metal.

Data analysis

Variables analyzed included (i) metal resistance, i.e., the size of the zone of inhibition, and (ii) site of isolation. The populations were further divided between Gram-positive and Gram-negative organisms, and comparisons were made within Gram-stain designations and not between them. Mean comparisons between organisms resistant to metals from an individual site or from both sites combined were tested by ANOVA. Pair-wise multiple comparison procedures were analyzed by Dunn's method. A P value of 0.05 was considered significant. A paired t test was used to compare the zones of inhibition of resistant and sensitive reference strains (Table 1). All statistical analyses were performed using Jandel SigmaStat (SPSS Science, Chicago, Ill.) and Microsoft Excel (Redmond, Calif.) statistical software.

Genus-specific levels of metal resistance

Disc inhibition assays showed a wide range of responses to Hg(II), Pb(II), and Cr(VI) (Fig. 1). Because Gram-negative and Gram-positive microbes fundamentally differ from each other in their interactions with metals (Duxbury and Bicknell 1983; Giller et al. 1998), the data are considered separately for these two groups. The broadest range of response was to Hg(II) with zones of inhibition ranging from 0, observed for two *Arthrobacter* spp. from the SRS group, to 37 mm. Gram-negative strains had a slightly narrower range of response to Hg(II) than did Gram-positive ones, 1–30 mm. While none of the tested bacteria had zones of inhibition larger than 17 mm around discs containing either 500 nmol Pb(II) or 2 μ mol Cr(VI), a large number, most notably among Gram-positive strains, had no zones of inhibition at all (Fig. 1). With the exception of *Arthrobacter* spp., where some strains were exceptionally metal tolerant (see below), no relationship of the response to the generic affiliation of the test strains could be discerned. The results of an

earlier study on resistance to Cr(III), Cu(II), and Hg(II) among SRS strains (Fredrickson et al. 1988) are not comparable to data presented here because of different experimental approaches and methods. The variable response of strains belonging to the same generic group was not surprising because metal resistance is often specified by plasmids and transposons (Mergeay 1991; Mindlin et al. 2001; Silver and Phung 1996) whose copy number in a given genome may vary with the specific plasmid replicon and the interaction of plasmid-specified functions with those of the host.

Among *Arthrobacter* spp., 37% of 95 SRS strains and 38% of 21 Hanford strains were fully resistant to Cr(VI), similar to the response of *Arthrobacter* sp. Cr15 (Table 1), a soil isolate (Nakatsu 2002) that served as a reference strain. Likewise, many *Arthrobacter* spp. (20% and 48% of SRS and Hanford strains, respectively) were not inhibited by Pb(II). Furthermore, eight SRS *Arthrobacter* spp. exhibited a higher level of Hg(II) resistance than did *Bacillus cereus* 5 (Table 1), a resistant reference soil bacterium (Izaki 1981), with two of these strains having no zone of inhibition (Fig. 1, Hg(II)). Others have also reported the high levels of metal tolerance among soil *Arthrobacter* spp. (Roane and Pepper 1999; Trajanovska et al. 1997). Thus, the genus *Arthrobacter* may be exceptionally resistant to toxic heavy metals.

Though little research to date has focused on metal resistance in *Arthrobacter*, anecdotal evidence suggests that in this genus this phenotype may be specified by novel mechanisms. Resistance to Cd(II) by a soil isolate, *Arthrobacter* sp. D9, was due to extracellular binding, while genes encoding for Cd(II) efflux, the documented resistance mechanism in bacteria (Silver and Phung 1996), were not detected in its genome (Roane et al. 2001). Aluminum resistance in *Arthrobacter* sp. from acidic soil (Jo et al. 1997) and Cr(VI)-resistance in strain Cr15 (Nakatsu 2002) were possibly due to the production of extracellular soluble substances. Frequent occurrence of high levels of metal resistance among *Arthrobacter* spp., which may be specified by novel mechanisms, and *Arthrobacter*'s ubiquity in contaminated soils should stimulate research on *Arthrobacter*–metal interactions. Such research would be enhanced by the availability of tools for the genetic manipulation of strains belonging to this genus (Gartemann and Eichenlaub 2001; Margesin and Schinner 1997).

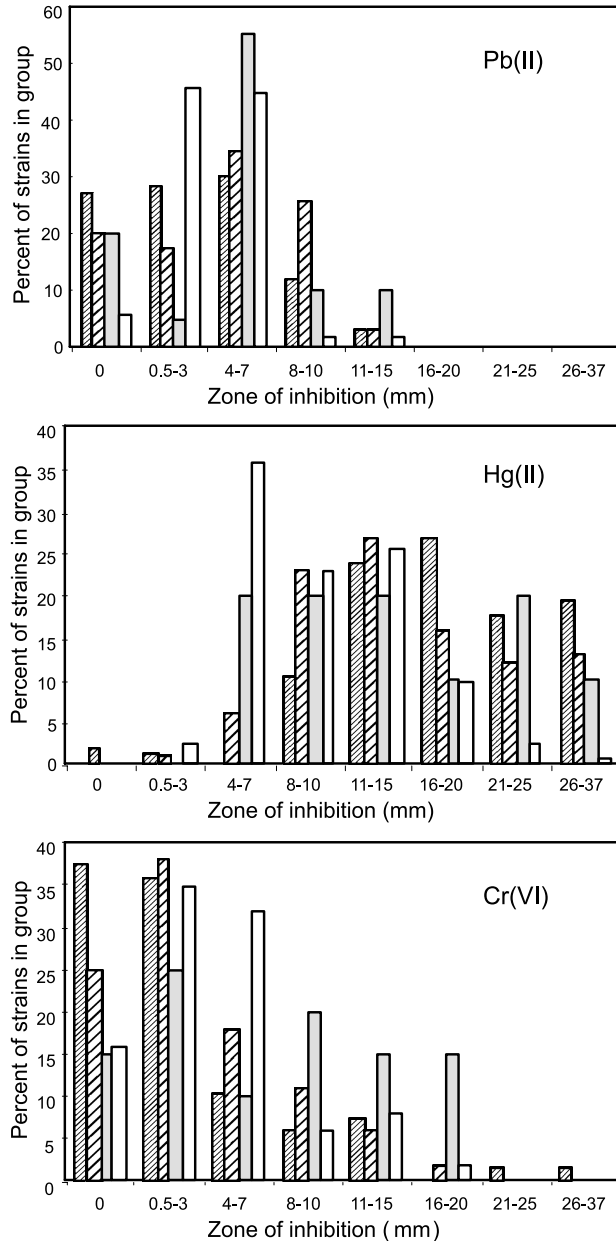
Site-specific levels of metal resistance

Resistances to Hg(II) and Pb(II) were more common among SRS strains than Hanford strains. The size of the zone of inhibition around discs with 50 nmol Hg(II) was significantly higher for both Gram-positive ($P = 0.001$) and Gram-negative ($P = 0.007$) SRS strains than for Hanford strains (Fig. 1). Lead resistance levels among Gram-negative strains showed a similar trend with significantly higher levels for SRS strains than Hanford strains ($P = 0.015$).

Incidence of multiple metal resistances

The number of strains with more than one resistance, defined here as a zone of inhibition similar to or smaller than that of a reference resistant strain, was higher in the SRS group of isolates (33% of all strains) than the Hanford group (23%) (Fig. 2). Resistance to three metals was similarly

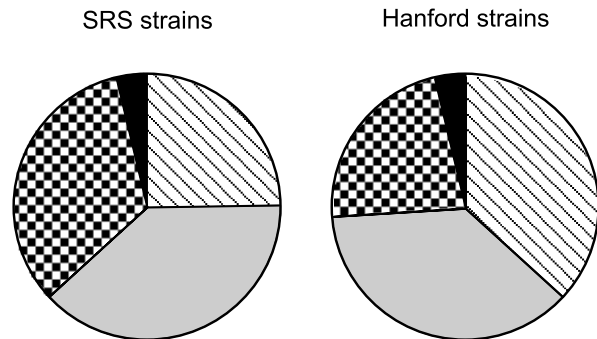
Fig. 1. Levels of metal resistance among four groups of subsurface strains. Percent of strains among Gram-positive Hanford strains (▨), Gram-positive Savannah River Site (SRS) strains (▩), Gram-negative Hanford strains (□), and Gram-negative SRS strains (◻) with the corresponding zones of inhibition.



common in both groups of isolates, with 3.5% of the strains in the SRS group and 3.6% in the Hanford group.

Thus, both individual (Fig. 1) and multiple (Fig. 2) resistance patterns clearly show a higher metal tolerance among SRS strains than Hanford strains, suggesting that toxic metals more effectively influenced the evolution of resistance in the SRS community than in the Hanford community. This conclusion is supported by the large difference in the phylogenetic composition between the two collections, the most

Fig. 2. Multi-resistance to metals among subsurface strains. The proportion of strains resistant to 0 (▨), 1 (□), 2 (▩), and 3 (■) of the test metals in the two groups of isolates.



striking of which is a lower number of genera and a higher frequency of Gram-negative bacteria in the SRS collection than the Hanford collection. For strains tested here, 156 of 261 SRS strains (60%) compared with 22 of 89 (25%) Hanford strains were Gram negative. Several studies have documented a reduced diversity and a prominence of Gram-negative bacteria in metal-contaminated soils and among metal-resistant aerobic chemoheterotrophic soil isolates (Barkay et al. 1985; Duxbury 1986; Duxbury and Bicknell 1983; Giller et al. 1998). To explain these observations, Duxbury and Bicknell (1983) suggested that the Gram-negative cell wall is a more efficient barrier to toxic metals than the Gram-positive one.

As both boreholes P24 and YB-02 were extracted from locations upstream from known sources of contamination (Balkwill 1989; Fredrickson et al. 1995), factors intrinsic to the SRS and Hanford sites must be responsible for the observed differences in metal tolerance. These factors could be a natural enrichment in heavy metals in the former and the presence of physical-chemical factors that reduce the bioavailability, and thus toxicity, of metals in the latter. To the best of our knowledge, the soils of P24 and YB-02 were not analyzed for their mineral composition, so the possible role of the relative enrichment in heavy metals remains unknown.

The effect of physical-chemical parameters on metal availability is complex and is a function not only of their interactions with the metals but also of these parameters with each other. However, studies on the toxicity of metals to both pure cultures (Collins and Stotzky 1989) and soil microbial processes (Giller et al. 1998) consistently show that toxicity is mitigated by low redox and high clay and organic matter contents, as well as high cation and anion concentrations. pH, a factor that clearly controls metal speciation (Simkiss and Taylor 1995), either increases or decreases toxicity, depending on the metal and microbe analyzed (Collins and Stotzky 1989). The clay content of Hanford soils was between 8 and 41%, with most samples at >20% (Kieft et al. 1995). In contrast, SRS samples, noted for their coarse texture with sequences rich in clay and silt (Sargent and Fliermans 1989), ranged between 3 and 80% clay, but the majority of the samples had a clay content of <20% (Fliermans and Balkwill 1989). Charged clay particles limit metal availability by sequestration (Collins and Stotzky

1989) and their higher concentration in Hanford subsurface soils might be one reason for the lower levels of metal resistance in the Hanford isolates relative to the SRS isolates.

Results presented here show that resistance to three metals that are of concern in subsurface contaminated sites (Riley et al. 1992) is common among chemoheterotrophic bacteria isolated from unimpacted sediments. Thus, subsurface microbial communities should adapt and sustain their activities in sediments contaminated by high concentrations of heavy metals. Whether resistance of individual strains affect the resilience of the subsurface microbial community to metal stress remains to be determined, since this study only addressed resistance of isolates that may or may not represent the soil community at large. The high frequency of metal resistance raises the question of how these resistances evolved in the deep subsurface. Metal resistance in surface soil microbiota often evolves by lateral gene transfer (Mergeay et al. 1985; Osborn et al. 1997; Silver and Phung 1996). Yet, gene transfer in the soil environment depends on metabolic rates and population densities (Kroer et al. 1998; Normander et al. 1998; Sørensen and Jensen 1998), while the density of microbes in the subsurface is orders of magnitude lower than in surface soils (Balkwill 1989; Kieft et al. 1995), and their metabolic rates are extremely low. Initial results from ongoing research in our laboratory suggest that lateral transfer of metal-resistance genes occurs in some of the subsurface bacteria described here.

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