INTRODUCTION
Both basic and applied science issues drive our interests in the microbiology of the deep terrestrial subsurface. As an environment that is disconnected from the Earth’s surface, the deep subsurface is less subject to variations in temperature and light and, in unsaturated zones, to intense gradients across interfaces created at the microscale level. These characteristics dictate an average growth rate that is very slow, up to thousands of years per cell division (Kieft & Brockman, 2001), and an ecosystem where change occurs over very long time scales (Fredrickson & Onstott, 2001). Thus, the subsurface is one of the most extreme environments on Earth, and identifying what limits life in the subsurface has value as a model for life on other planets (Chapelle et al., 2002; Nealson & Cox, 2002). The inadvertent release of contaminants from industrial processing plants and storage tanks, as well as the possibility of permanently depositing nuclear wastes deep below the Earth’s surface (Pedersen, 2001), raise questions about how microbial activities might exacerbate or mitigate contamination problems in the subsurface.

The terrestrial subsurface is the habitat for diverse microbial communities that, together with the oceanic subsurface, may be the habitat for the largest proportion of Earth’s biomass (Whitman et al., 1998). As subsurfaces are characterized by a range of physical and chemical properties, from fully aerated sedimentary shallow aquifers to deep igneous rocks devoid of oxygen and elevated in temperatures, their microbial communities are equally varied (Fredrickson & Fletcher, 2001). Microbial life in the subsurface is greatly constrained by temperature, pressure, limited space and availability of water and scarce resources of electron donors, acceptors and micro-
nutrients, challenging microbial life to its limit (Colwell, 2001). Nevertheless, studies over the last 30 years have revealed metabolically and phylogenetically diverse microbial communities in the subsurface (Amy et al., 1992; Balkwill et al., 1997).

**Microbial biomass and diversity in the subsurface**

Microbial biomass has been estimated by direct and viable counts and by the quantification of total phospholipid fatty acids (PLFA) (Kieft et al., 1997; Ringelberg et al., 1997). Biomass estimates show variability that corresponds to the heterogeneity of the geological strata that were sampled. Direct counts range from $10^7$ cells (g soil)$^{-1}$ in the sediments of the Atlantic coastal plain in North America, Rainier Mesa in Nevada and Witwatersrand Basin in South Africa to $10^4$ cells g$^{-1}$ in deep sediments of the western USA, and groundwater samples rarely contain more the $10^4$ cells ml$^{-1}$ (summarized by Fredrickson & Onstott, 2001). Thus, the microbial biomass of both the soil and the aqueous subsurface is orders of magnitude lower than those of the corresponding surface environments.

Representatives belonging to the major prokaryotic lineages have been detected in the subsurface, as revealed by culturing methods (Balkwill et al., 1997) and by molecular signatures of both PLFA and 16S rRNA clone libraries (Chandler et al., 1998; Feris et al., 2004). Interesting unique observations emerge, however, when community structure and diversity are examined within the context of the unique spatial properties of the subsurface. For example, in the vadose zone, the area located between the top soil and the water table, water availability is a limiting resource not so much due to desiccation (water potentials of $>-0.1$ MPa, sufficient for hydration, are common), but mostly because water is trapped in small spaces, creating discontinuous environments limiting transport of microbes, nutrients and toxicants (Kieft & Brockman, 2001). This spatial discontinuity of microbial niches determines microbial distribution and diversity patterns and limits microbial interactions to microniches. For example, Takai et al. (2003b) demonstrated a varied distribution of methanogens in the transition from low-sulfate and organic- and methane-rich shale to high-sulfate and methane- and organic-poor sandstone, thus relating community structure to geochemical gradients and lithologies. Zhou et al. (2002) reported a much higher diversity in surface relative to subsurface soils, but both had a high degree of species evenness rather than species dominance, suggesting non-competitive diversity patterns. The authors proposed that, in soils typified by discontinuity, microbial growth is limited by lack of diffusion of essential substrates rather than by competition (Zhou et al., 2002, 2004).

**Microbial metabolism in the subsurface**

Diverse modes of microbial metabolism exist in the subsurface. Heterotrophic metabolism is supported in aquifers recharged by surface water containing soluble...
organic carbon, where the consumption of limited oxygen leads to anaerobic conditions and the dominance of anaerobic respiratory pathways. Organic carbon accreted during the slow process of sediment formation is another energy source in the subsurface (Krumholz et al., 1997; Colwell, 2001), and microbial degradation of natural petroleum reserves is an example of heterotrophic anaerobic metabolism with far-reaching consequences for oil quality and quantity (Aitken et al., 2004; Head et al., 2003).

Dissimilatory iron reduction is a dominant respiratory pathway in anoxic aquifers (Lovley et al., 2004). Iron reducers representing common (Petrie et al., 2003) and novel (Coates et al., 2001) members of the Geobacteraceae are often detected in such environments. Furthermore, the novel iron reducer Rhodoferax ferrireducens, the first non-phototrophic species of its genus, was isolated from subsurface sediment (Finneran et al., 2003). Iron reducers are important targets for bioremediation efforts in the subsurface because they can use other electron acceptors, among them uranium (Lovley et al., 2004) and vanadium (Ortiz-Bernad et al., 2004), inducing the formation of insoluble precipitates. These activities, stimulated in situ by the injection of readily oxidizable substrates such as acetate (Anderson et al., 2003) or ethanol (North et al., 2004) into contaminated aquifers, result in the precipitation of metal and radionuclide contaminants. Invariably, such treatments stimulate growth of Geobacteraceae in the treated subsurface communities (Anderson et al., 2003; North et al., 2004).

Sulfate reduction is another common respiratory pathway in the subsurface (Wong et al., 2004), driven by sulfate in groundwater and possibly by the activity of pyrite-oxidizing bacteria (Ulrich et al., 1998). Sulfide that accumulates during sulfate reduction may complex with metals and radionuclides (Neal et al., 2004) and retard their mobility in the subsurface, thus making stimulation of sulfate-reducing bacteria another strategy for bioremediation in the subsurface.

Chemolithoautotrophic metabolism is a major mode of microbial metabolism in the subsurface, supporting vast communities of methanogens (Chapelle et al., 2002) and possibly acetogens (Pedersen, 2001) in environments with very low levels of organic substrates, such as deep aquifers or igneous rocks. Pedersen (2001) has proposed the hydrogen-driven biosphere hypothesis to explain microbial life in the latter. According to this hypothesis, hydrogen and carbon dioxide drive methanogens and acetogens, which then support the activities of acetoclastic methanogens and acetate-utilizing iron and sulfate reducers, resulting in the formation of organic polymers that, upon their degradation, are converted to hydrogen and carbon dioxide. A continuous source of hydrogen in the subsurface is required to support this hypothesis. The issue of whether hydrogen can (Stevens & McKinley, 1995; Freund et al., 2002) or cannot (Anderson...
et al., 1998) be produced in the subsurface by the reaction of water with minerals is presently undecided. The prevalence and diversity of chemolithoautotrophic metabolism among subsurface microbes is also highlighted by the isolation of a thermophilic hydrogen- and sulfur-utilizing chemolithoautotroph from a thermal aquifer, *Sulfurhydrogenibium subterraneum*, belonging to the order *Aquificales* (Takai et al., 2003a). Hydrogen-driven chemoautotrophy by aerobes may be an important, as-yet unexplored, process in vadose zones where little organic matter is available for heterotrophic processes. This lack of information may reflect the fact that the focus of subsurface microbiology research has been on anaerobic processes in groundwater aquifers because of the higher biomass and metabolic rates relative to those in the vadose zone.

In this chapter, we address the issue of the interactions of subsurface micro-organisms with heavy metals and how they are affected by the exchange of genetic material. Thus, we touch on the issues of metal homeostasis and genetic diversity in subsurface microbiology, two topics barely investigated to date.

**THE INTERACTIONS OF SUBSURFACE BACTERIA WITH HEAVY METALS**

The contamination of the deep subsurface with mixtures of radionuclides, metals and organic solvents that may leach into groundwater aquifers is one of the most detrimental legacies of the Cold War. Immobilization of these contaminants may be the only feasible approach to solving this problem, and micro-organisms that convert inorganic contaminants to less soluble precipitates play a prominent role in *in situ* immobilization strategies for the subsurface (NABIR, 2001). As these strategies depend on the activity of microbes, the presence of a mixture of toxicants may result in the inhibition of reactions essential for immobilization. It is for that reason that strains of *Deinococcus* spp. with high levels of resistance to ionizing and gamma radiation have been engineered with the ability to degrade organic contaminants and withstand metal toxicity (Brim et al., 2000, 2003), and that the toxicity of metals and actinides to bacteria with a potential in bioremediation has been evaluated (Reed et al., 1999; Ruggiero et al., 2005).

A study to determine the level of metal resistance among subsurface aerobic heterotrophic bacteria was initiated, reasoning that these microbes play a role in facilitating microbial metabolism in the subsurface (Benyehuda et al., 2003). The microbes tested were from the subsurface microbial culture collection (SMCC) that is maintained at Florida State University, USA, and included 261 isolates from the Savannah River Site (SRS) in South Carolina, USA (borehole P24) and 89 strains from the Hanford site in Washington state (borehole YB-02). The SRS isolates belonged to
the α-, β- and γ-proteobacteria, as well as to the high-G+C Gram-positive group. The Hanford strains contained representatives of these taxonomic groups, as well as the low-G+C Gram-positive group. Resistance to Pb(II), Hg(II) and Cr(VI) was determined by disk-inhibition assays on solid growth media and by comparison with the response of well-characterized reference resistant and sensitive strains (Benyehuda et al., 2003). Results were analysed for the relationship of metal resistance to the properties of the tested microbial communities and the environments from which they were isolated. The major findings were:

(i) Resistance to Pb(II) and Cr(VI) was common among subsurface strains from SRS and Hanford sediments, while fewer, mostly Gram-positive strains, were resistant to Hg(II).

(ii) With the exception of a high level of metal tolerance among Arthrobacter spp., there was no relationship between the phylogeny of the microbes and their metal-resistance patterns. This is not surprising, as metal resistance is often specified by mobile elements such as plasmids and transposons (Silver & Phung, 1996; Kholodii et al., 2002). Some subsurface Arthrobacter isolates proved to be exceptionally resistant to Cr(VI) and Hg(II). Other researchers have also reported high levels of metal tolerance among soil Arthrobacter spp. (Roane, 1999; Megharaj et al., 2003) and the high abundance of this genus in soils impacted by mixed-waste contamination (Fredrickson et al., 2004). Thus, further investigation of Arthrobacter–metal interactions is highly warranted.

(iii) Resistances to Hg(II) and Pb(II) were more common in the SRS collection than in the Hanford collection (ANOVA; P<0.05) and multiple metal resistance was also higher for the SRS, with 33 % of all strains resistant to more than one metal in this group compared with 23 % for the Hanford group (Fig. 1). Thus, toxic metals influenced the evolution of resistance more effectively in the SRS community than in the Hanford community. Varying geological and geochemical factors may explain this difference. For example, metal toxicity is mitigated by low redox potential and high clay and organic matter (Collins & Stotzky, 1989; Giller et al., 1998), and the clay content of Hanford sediment is higher than in the more sandy SRS sediment. These results illustrate that, in complex environments, microbe–metal interactions are greatly impacted by environmental factors, most likely by controlling bioavailability and thus metal toxicity. For more details of this study, see Benyehuda et al. (2003).

This study, as well as others addressing the issue of survival and activities of microbes in mixed-waste-contaminated subsurfaces, has focused on aerobic bacteria. However, current in situ immobilization efforts target the activities of metal- and radionuclide-reducing anaerobic bacteria (Anderson et al., 2003; Istok et al., 2004). The few who
have examined the response of anaerobes to metal toxicity reported conflicting results. *Desulfovibrio desulfuricans* G20, a model organism for the immobilization of metals as sulfides, was susceptible to micromolar concentrations of Cu(II), Zn(II) and Pb(II) when a medium designed to minimize metal complexation was used (Sani *et al.*, 2003). Mixed cultures of sulfate reducers were inhibited by Cr(VI) (Smith & Gadd, 2000) and Cu(II) and Zn(II) (Utgikar *et al.*, 2003). Likewise, *Shewanella* spp., studied for their role in immobilizing metals and radionuclides by reducing them to insoluble forms, were affected by U(VI) (Wade & DiChristina, 2000) and Cr(VI) (Viamajala *et al.*, 2004). These observations clearly suggest that susceptibility to metals and thus the acquisition of metal resistance by microbes in the subsurface is critical to the success of bioremediation in environments contaminated by mixed wastes.

**THE EVOLUTION OF METAL HOMEOSTASIS GENES BY HORIZONTAL GENE TRANSFER (HGT) IN SUBSURFACE MICROBIAL COMMUNITIES**

Gene transfer among microbes in the subsurface environment

Genes encoding resistance to heavy metals are often transferred among microorganisms in much the same way that antibiotic-resistance genes travel through microbial populations, by horizontal gene transfer (HGT) mechanisms. Like antibiotics, which are frequently produced by soil organisms, heavy metals are compounds that subsurface organisms are likely to encounter as part of their environment.

![Fig. 1. Multi-resistance to Pb(II), Hg(II) and Cr(VI) among bacteria from the subsurface. The proportion of strains resistant to none (white segments), one (pale grey), two (dark grey) or all three (black) of the test metals among strains from the SRS and the Hanford sites are shown. Redrawn with permission from Benyehuda *et al.* (2003).](image-url)
Bioavailable heavy metals are produced naturally by the geochemical weathering of ores and mobilized with the movement of groundwater. Anthropogenic contamination, however, may increase the concentrations of toxic metals in a given ecological niche manyfold. The presence of resistance genes on mobile genetic elements within the subsurface community is therefore a distinct advantage. The occurrence of HGT in topsoils, natural waters and in association with the internal and external surfaces of plants and animals is well recognized. However, HGT has barely been examined in the deep subsurface. Because population densities (Normander et al., 1998; Licht et al., 1999) and active metabolism (Smets et al., 1993) stimulate HGT, while most deep subsurface environments are notorious for low population densities and metabolic rates (Balkwill, 1989; Kieft & Brockman, 2001), the deep subsurface may be the least conducive environment for genetic exchange. To examine HGT and its role in the evolution of metal resistance in the subsurface, we have looked for evidence of the horizontal inheritance of genes encoding $P_{\text{IB}}$-type ATPases in bacteria from subsurface sediments of the SRS (Coombs & Barkay, 2004).

$P_{\text{IB}}$-type ATPases and their roles in metal homeostasis and HGT

$P_{\text{IB}}$-type ATPases are membrane-associated ion pumps that are responsible for maintaining metal homeostasis by mediating the transport of heavy metals using the energy generated by the hydrolysis of ATP. Those that are specific for monovalent cations [Cu(I) and/or Ag(I)] are found in the three domains of life, while those specific to divalent cations [Zn(II), Cd(II) and/or Pb(II)] are only found among the prokaryotes. These metal pumps can function in either the import of essential ions or the export of ions that have reached harmful levels in the cell cytoplasm, depending on the orientation of the protein in the membrane (Rosen, 2002).

Several $P_{\text{IB}}$-type ATPases have been shown to be associated with mobile genetic elements, from Gram-positive organisms such as Lactococcus lactis (O’Sullivan et al., 2001), Staphylococcus aureus (Nuñifora et al., 1989) and Arthrobacter spp. (K. Jerke and C. Nakatsu, personal communication) and from Gram-negative organisms such as Ralstonia metallidurans (Borremans et al., 2001) and Stenotrophomonas maltophilia (Alonso et al., 2000). However, the occurrence of HGT and its effects on the evolution of this locus within a specific microbial community had not previously been examined. We have targeted the genes encoding $P_{\text{IB}}$-type ATPases for a study on the role of HGT in the evolution of metal homeostasis among subsurface bacteria because of the importance of metal ion homeostasis for survival in the harsh environment of the metal-contaminated subsurface. Isolates from the SMCC were selected for study because of the large number of Pb(II)-resistant bacteria in the SRS community (Benyehuda et al., 2003).
Evolution of P_{IB}-type ATPases by HGT in a subsurface microbial community

We have used a retrospective approach, based on the recognition of genomic indicators for evolution by HGT. We reasoned that, because HGT is estimated to occur at rates of 31 kb per million years (Lawrence & Ochman, 1997) and subsurface micro-organisms reproduce very slowly, prospective approaches to HGT detection, such as microcosm incubations, may be of little relevance to subsurface communities. However, because genomic data may be interpreted in different ways, calling into question the validity of all molecular signatures of HGT (Eisen, 2000), we employed multiple methods in combination while determining whether or not a gene encoding a P_{IB}-type ATPase was horizontally transferred. These methods included (i) examining the congruence of the P_{IB}-type ATPase phylogeny with that of the 16S rRNA gene, (ii) looking for unusual sequence composition (G+C content) of the P_{IB}-type ATPase when compared with that of the host genome and (iii) looking for shared indels (insertion/deletion events) among P_{IB}-type ATPase genes from different organisms.

To obtain P_{IB}-type ATPase genes (zntA/cadA/pbrA-like genes) from the SRS aerobic heterotrophs, a nested PCR approach was developed. Novel primer sets for PCR amplification were designed by aligning conserved domains in zntA/cadA/pbrA-like genes that were available in databases. The first PCR targeted the phosphatase domain and the ATP-binding domain and the second reaction used conserved sequences in the transmembrane metal-binding domain and the ATP-binding domain. Using nine PCR primer pairs, amplification products of zntA/cadA/pbrA-like genes from 48 of 105 Pb(II)-resistant subsurface strains were obtained and sequenced. These sequences and the DNA sequences of 16S rRNA coding genes of the corresponding hosts were then used to determine whether HGT has contributed to the evolution of zntA/cadA/pbrA-like genes among the subsurface bacteria. For more details about this approach, see Coombs & Barkay (2004). Phylogenetic incongruence using both parsimony (heuristic) and distance (neighbour-joining) methods indicated that, in four of the isolates, zntA/cadA/pbrA-like genes evolved by HGT (Fig. 2), and three of these were supported by unusual sequence composition, i.e. G+C content and the presence of indels. All transfers were among the β- and/or γ-proteobacteria, which were the predominant groups among the Pb(II)-resistant subsurface bacteria from which sequence data were obtained. Two of these transfers were to Comamonas spp., and comparison of clustering patterns between the zntA/cadA/pbrA and the 16S rRNA trees suggested that, in one case (in strain BO669), transfer could have occurred from another Comamonas sp., and in the other (in strain BO173), the origin could not be clearly identified (Fig. 2). A third HGT event, the acquisition of a Pseudomonas-like P_{IB}-type ATPase by Ralstonia sp. B0665, was not supported by additional sequence features, but the phylogenetic evidence as indicated by the bootstrap support value was very strong. Finally, a P_{IB}-type
Fig. 2. Phylogenetic evidence for HGT among metal-resistant subsurface strains from the SRS site. Neighbour-joining trees of the deduced amino acid sequence of the zntA/cadA/pbrA-like gene (a) and the nucleotide sequence of the 16S rRNA gene (b) are shown. Incongruencies between the positions of sequences from the same organism are indicated by slanted lines connecting boxed branches in the two trees. Names of subsurface strains are in bold. Filled circles indicate bootstrap-supported nodes. Reproduced with permission from Coombs & Barkay (2004).
ATPase from \textit{Acinetobacter} sp. strain B0064 grouped phylogenetically within a \(\beta\)-proteobacterial clade. The G+C content supported the phylogenetic evidence, indicating that this could have been a recent gene acquisition by the \textit{Acinetobacter} strain.

These results indicate that HGT has occurred, albeit at low frequencies, during the evolution of metal homeostasis genes among subsurface bacteria. Other observations also support these conclusions (Table 1). Plasmid-borne genes for metal resistance and the degradation of hydrocarbons have been obtained from subsurface isolates, and phylogenetic analyses have suggested transfer of hydrocarbon-degradation genes in a shallow aquifer contaminated with coal tar (Herrick \textit{et al.}, 1997). The demonstration of conjugation in microcosms simulating low-nutrient subsurface soils (Smets \textit{et al.}, 2003) suggests that HGT can affect the evolution and genetic diversity of subsurface soil communities.

Here, we used the primary DNA sequences of \textit{zntA/cadA/pbrA}-like genes to deduce the evolutionary pathway of an environmentally important function, metal homeostasis,
among subsurface bacteria. While it is tempting to conclude from this study that HGT occurs in the subsurface, such a conclusion is impossible without clear evidence that the studied strains evolved in the subsurface. Without such evidence, the alternative possibility that transfer occurred prior to deposition in the subsurface cannot be ruled out. Evidence for evolution in situ currently exists for a collection of *Arthrobacter* spp. from the Yakima Barricade, where a coherence exists between the 16S rRNA- and *recA*-based phylogenies and the geological strata from which the strains originated, suggesting a long-term evolution, possibly as long as 8 million years, in the subsurface (van Waasbergen *et al.*, 2000). Examining microbial communities from this and other similar environments may reveal HGT and other processes that affect genetic diversity as they have occurred in the subsurface.

**Evidence for HGT of *P*~*IB*~-type ATPases in complete prokaryotic genomes**

In order to evaluate the observed frequency of HGT among subsurface microbes, we analysed genes encoding *P*~*IB*~-type ATPases of 188 bacterial and 22 archaeal genomes. As a clear phylogenetic distinction between *P*~*IB*~-type ATPases specifying mono- and divalent pumps does not currently exist, our analysis encompassed all *zntA* and *copA*-like sequences. Only *P*~*IB*~-type ATPases loci that exactly matched the amino acid sequence of the phosphatase and the transmembrane metal-binding domains of enzymes with documented activity were included in the collection. The resulting collections of 311 *P*~*IB*~-type ATPases and the 16S rRNA genes of the corresponding genomes were subjected to phylogenetic analysis and cases of incongruence between the two phylogenies were identified. When incongruence was detected, supportive evidence was sought by examining sequence composition as described above. In addition, sequences proximal, i.e. within 5 kbp, were examined for the presence of regulatory genes that might have been co-transferred with the *P*~*IB*~-type ATPase genes. When found, these were subjected to the incongruence test as above to determine their phylogenetic affiliation (Coombs & Barkay, 2005).

Twelve instances of phylogenetic incongruence were detected, six of which were transfers across subclasses within the *Proteobacteria* (Table 2). This is not surprising, because our collection of *P*~*IB*~-type ATPases was dominated by proteobacteria. However, the remaining transfer events were across a longer phylogenetic distance. In two cases, *zntA* loci from low-G+C Gram-positive bacteria were found in the genomes of the γ-proteobacteria *Stenotrophomonas maltophilia* and *Legionella pneumophila* and a transfer of an ε-proteobacterial *copA* to *Ureaplasma parvum*, a low-G+C Gram-positive, was also noted. These findings highlight an extensive involvement of Gram-positive bacteria in gene exchange. Evidence that *zntA* and *copA* were transferred to *Deinococcus radiodurans* from α-proteobacteria emerged from the
Table 2. HGT of the \textit{zntA} and \textit{copA} genes based on evidence from complete microbial genomes

When the genome contained more than one \textit{zntA} or \textit{copA} gene, the locus number, as designated in the annotated genome, is included (e.g. \textit{D. radiodurans} \textit{ZntA} locus 1).

<table>
<thead>
<tr>
<th>Genome</th>
<th>Lineage of likely donor*</th>
<th>HGT supported by:</th>
<th>Unusual G+C content†</th>
<th>Proximal regulatory gene‡</th>
<th>Shared indel</th>
</tr>
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<td>\textit{ZntA}</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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</tr>
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</tr>
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</table>

*As revealed by the incongruent phylogenetic clustering of the gene relative to that of the 16S rRNA gene (see text).
†Unusual G+C content is defined here as differing by more than one standard deviation from the mean for the genome.
‡Proximal is defined here as a gene that is within 5 kb of a gene encoding a ZntA or a CopA. The phylogenetic affiliation of the regulatory gene is indicated in parentheses.
incongruence analysis and separate locations of these two loci in the genomes of both D. radiodurans and α-proteobacteria suggested that two independent transfer events were involved. A single transfer event of copA between closely related euryarchaeota was detected in the genome of Pyrobaculum aerophilum (Table 2) and, most excitingly, a zntA of cyanobacterial origin was present in the genome of the archaeon ‘Pyrococcus abyssi’ (Fig. 3). Supportive evidence confirming HGT was available for six of the transfers that were revealed by incongruent phylogenies (Table 2) and in three cases these included the presence of a gene with homology to regulatory elements that are known to control expression of zntA/cadA/pbrA loci. Phylogenetic analysis of these regulatory genes showed that they likely shared an origin with the zntA or copA genes they accompanied. This latter criterion also confirmed the cross-domain transfer between archaea and cyanobacteria.

Thus, it seems that, as we have found with the subsurface strains, the evolution of genes encoding P\textsubscript{IB}-type ATPases in sequenced genomes has been subjected to HGT but that their inheritance has mostly proceeded vertically. This is in contrast to the well-documented dominance of HGT in the evolution of other traits that enhance fitness to toxicants, such as resistance to mercury (Kholodii et al., 2002) and antibiotics. As P\textsubscript{IB}-type ATPases mediate metal homeostasis, they may be considered more essential...
to core metabolism than phenotypes that are exclusively involved in detoxification, thus enhancing stable genomic inheritance. The frequency of transfer detected among the microbial genomes, 12 of 311, was slightly lower than that among the subsurface bacteria, 4 of 48 (see above). This difference was most likely due to differences in the composition of the datasets. The genome study encompassed a broader phylogenetic range, and therefore we were able to detect transfers across large phylogenetic distances, whereas the subsurface study detected transfer among more closely related organisms. However, it is possible that the frequency of HGT among the subsurface strains was underestimated. The more closely related microbes are phylogenetically, the more likely they are to exchange genetic material (Lawrence & Ochman, 1997), but the less likely are the transfer events to leave a detectable molecular footprint in the new host genome (Eisen, 2000).

**HGT gene microarray**

DNA and expression microarrays are a powerful tool in biological research, and applications to the study of microbial community structure (Small et al., 2001) and function (Taroncher-Oldenburg et al., 2003; Rhee et al., 2004) have been documented. Zhou and his collaborators have identified three types of microarrays in microbial ecology (Zhou & Thompson, 2002). Phylogenetic oligonucleotide arrays (POA) consist of probes homologous to 16S rRNA genes and are used to study community composition and its response to environmental change. Functional gene arrays (FGA) are designed to evaluate the metabolic potential of a community by probing for genes that specify major biogeochemical reactions, including those essential for biodegradation and bioremediation. Community genome arrays (CGA) target genes of pure isolates from a specific environment. Our discovery of molecular signatures indicative of HGT in the genomes of subsurface bacteria (Coombs & Barkay, 2004) prompted us to develop a fourth type of microarray, possibly a variation on the CGA, the horizontal gene transfer array (HGT array). This array was designed to answer the question: ‘what are the genetic elements that transfer metal-resistance genes among subsurface bacteria?’

The HGT array includes 158 oligonucleotide (70-mer) probes specific for genes that encode replication/incompatibility (inc/rep) loci in 86 broad-host-range (BHR) plasmids belonging to 13 distinct plasmid groups and 100 probes for metal-resistance genes. The linkage of metal resistance on specific plasmids is suggested by positive signals obtained following hybridization of Cy3- or Cy5-labelled plasmid DNA extracted from subsurface isolates with the array. The emerging patterns classify plasmids according to their incompatibility groupings and linkage with metal-resistance genes.
Analysis of the four SRS subsurface isolates that were shown to have inherited Pb(II) resistance by HGT (Fig. 2) indicated that at least two of them carried multiple small plasmids. Plasmid DNA extracts of these strains and of additional metal-resistant isolates from contaminated subsurface sediments in Oak Ridge, TN, USA, were hybridized with the array following optimization and testing with 26 exact-match reference plasmids (J. Coombs and T. Barkay, in preparation). Of these, a plasmid extract from *Comamonas* sp. B0173, a strain that inherited its *zntA/cadA/pbrA* gene by transfer from an unknown donor (Coombs & Barkay, 2004), hybridized to two probes homologous to *rep/inc* loci of plasmids belonging to IncP1β and to a P$_{IB}$-type ATPase probe (Fig. 4). This finding indicates that inheritance of Pb(II) resistance in strain B0173 likely occurred by conjugal transfer of an IncP1β BHR Pb(II)-resistance plasmid. Array studies with a plasmid extract from the other SRS isolate are currently in progress.

The three strains from the contaminated sediments in Oak Ridge, TN, are a part of a large collection of Gram-positive bacteria where metal-resistance patterns correlated well with plasmid carriage (Patty Sobecky, personal communication). Strain *Bacillus* sp. U26 hybridized to *rep/inc* probes from a group of characterized *Bacillus* loci and hybridized weakly to probes for arsenic-resistance genes. Interestingly, plasmid DNA from another *Bacillus* strain, strain V6, hybridized to *rep/inc* probes homologous to
those of plasmids that had been described previously in both Gram-positive and
-negative bacteria. In addition, the plasmid preparation hybridized to arsenic-resistance
probes. These results suggest either that two plasmids exist in strain V6 or that a single
arsenic-resistance plasmid has two different origins of replication, and imply that strain
V6 carries plasmids of a broader diversity than has previously been described in other
Gram-positive bacteria. Although our dataset is currently very small, it emphasizes that
interesting, previously uncharacterized metal-resistance plasmids exist in subsurface
soil bacteria.

CONCLUSIONS
The studies reported here focused on two issues that are critical to the activities of
microbial communities in the subsurface, metal homeostasis and HGT. Critical,
because both of these are considerations that affect strategies for controlling the
transport of metals and radionuclides in contaminated subsurface environments.
Results showed a high frequency of resistance to divalent cations and a modest, yet
significant, inheritance of a gene encoding metal homeostasis by HGT. This frequency
of HGT of metal homeostasis genes was similar to that in the microbial world at large
as suggested by the analysis of sequenced microbial genomes. While these results
suggest that HGT may have contributed to the survival of microbes in the harsh
subsurface environment, they could not determine whether transfer occurred in sittu.
This question could only be addressed by examining signatures of HGT in the genomes
of subsurface microbes from communities whose evolution in the subsurface can be
documented unequivocally, enabling the histories of microbial speciation to be related
to geological processes. This opportunity may exist in certain ecological niches within
the subsurface, which are permanently isolated from the surface and where microbial
migration is restricted by the discontinuity of niches that support life.

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REFERENCES
contains a cluster of genes from gram-positive bacteria involved in antibiotic and


