

Molecular Evidence for the Evolution of Metal Homeostasis Genes by Lateral Gene Transfer in Bacteria from the Deep Terrestrial Subsurface

J. M. Coombs and T. Barkay*

*Department of Biochemistry and Microbiology, Cook College, Rutgers University,
New Brunswick, New Jersey 08901*

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Lateral gene transfer (LGT) plays a vital role in increasing the genetic diversity of microorganisms and promoting the spread of fitness-enhancing phenotypes throughout microbial communities. To date, LGT has been investigated in surface soils, natural waters, and biofilm communities but not in the deep terrestrial subsurface. Here we used a combination of molecular analyses to investigate the role of LGT in the evolution of metal homeostasis in lead-resistant subsurface bacteria. A nested PCR approach was employed to obtain DNA sequences encoding P_{IB} -type ATPases, which are proteins that transport toxic or essential soft metals such as Zn(II), Cd(II), and Pb(II) through the cell wall. Phylogenetic incongruities between a 16S rRNA gene tree and a tree based on 48 P_{IB} -type ATPase amplicons and sequences available for complete bacterial genomes revealed an ancient transfer from a member of the β subclass of the *Proteobacteria* (β -proteobacterium) that may have predated the diversification of the genus *Pseudomonas*. Four additional phylogenetic incongruities indicate that LGT has occurred among groups of β - and γ -proteobacteria. Two of these transfers appeared to be recent, as indicated by an unusual G+C content of the P_{IB} -type ATPase amplicons. This finding provides evidence that LGT plays a distinct role in the evolution of metal homeostasis in deep subsurface bacteria, and it shows that molecular evolutionary approaches may be used for investigation of this process in microbial communities in specific environments.

The role of lateral gene transfer (LGT) in the evolution of microorganisms becomes more and more apparent with every new microbial genome that is sequenced and annotated (54, 58), which has led many workers to question our basic concepts of microbial speciation (18, 23). The prevalence of laterally transferred genes clearly suggests that this mode of evolution is advantageous to microbial life, possibly by providing the means for genetic innovation in the absence of frequent sexual recombination (37). This variation is likely to increase metabolic diversity, and consequently competence, in environments subject to frequent change (13, 61). The possibility that LGT is an important force in shaping the structure and function of microbial communities in their natural habitats is suggested by (i) the fact that abundant and diverse plasmids (12, 73), viruses (8, 89), insertion sequences (70), transposons (59, 78), integrons (49), and other elements that contribute to genomic plasticity are commonly isolated from environmental samples and strains; (ii) the fact that natural competence is common among microbes (11) and occurs in soils (17, 50) and natural water (88); (iii) the fact that LGT has been demonstrated in model ecosystems (i.e., microcosms) (52, 62, 75, 80) and in intact environmental samples (19); and (iv) the fact that seeding soils with bacteria carrying conjugal catabolic plasmids results in transfer of the plasmids to indigenous soil microbes and the stimulation of plasmid-specified activities as detected by enhanced degradation of specific substrates (14, 48).

In contrast to the large body of research that has explored gene transfer in microbial communities of topsoils and natural waters, little is known about this phenomenon in the deep subsurface. Nevertheless, the deep subsurface is the habitat for a large proportion of the world biomass (86), and major lineages of *Bacteria* and *Archaea* have been detected in aquifer sediments and vadose zone samples. Microbial communities of the deep subsurface are constrained by the scarcity of growth substrates (60), water availability, and spatial separation (77), which result in low metabolic rates (28) and the dominance of chemolithoautotrophic metabolism (10, 77). Because population densities (52) and metabolic activities (72) stimulate LGT, this process may be limited in the deep subsurface. Nevertheless, anecdotal evidence indicates that LGT affects the genetic and metabolic diversities of subsurface microbial communities. Plasmids of various sizes were observed in aerobic heterotrophs that were isolated from deep Savannah River sediments (22); the molecular structure of some of these plasmids suggested a common evolutionary origin (29), and conjugal transfer of catabolic plasmids was monitored in subsurface microcosms (71). Here we employed approaches and tools derived from molecular evolution studies (9, 54, 61) to examine evolution by LGT in subsurface microbial communities; in this study we focused on metal cation homeostasis genes encoding the P_{IB} -type ATPases, which are known for their broad distribution in the bacterial world (2).

P_{IB} -type ATPases are involved in fundamental cellular processes, such as the transport of essential micronutrients, delivery of ion cofactors to specific proteins, and extrusion of toxic ions from the cytoplasm (3). These transporters prevent the overaccumulation of essential but highly reactive ions, such as

* Corresponding author. Mailing address: Department of Biochemistry and Microbiology, 76 Lipman Dr., New Brunswick, NJ 08901. Phone: (732) 932-9763, ext. 333. Fax: (732) 932-8965. E-mail: barkay@aesop.rutgers.edu.

TABLE 1. Plasmids and bacterial strains used as controls in this study

Strain or plasmid	Comments	Classification	Phenotype	Reference(s)
<i>Arthrobacter</i> sp. strain VN23-1		High G+C content, gram positive	Pb ^r	Jerke and Nakatsu, personal communication
<i>Arthrobacter</i> sp. strain KJ2	Plasmid-cured derivative of VN23-1	High G+C content, gram positive	Pb ^s	Jerke and Nakatsu, personal communication
<i>E. coli</i> W3110		γ -Proteobacteria	Pb ^r	64
<i>E. coli</i> znt4 Δ	zntA deletion mutant of W3110	γ -Proteobacteria	Pb ^s	C. Rensing, personal communication
<i>R. metallidurans</i> CH34		β -Proteobacteria	Pb ^r	7, 51
<i>R. metallidurans</i> AE104	Plasmid-cured derivative of CH34	β -Proteobacteria	Pb ^s	51
<i>S. aureus</i> K10		Low G+C content, gram positive	Pb ^r	39
<i>S. aureus</i> K10S	Plasmid-cured derivative of K10	Low G+C content, gram positive	Pb ^s	39
pB23F	Carries cloned Pb ^r gene from VN23-1	Plasmid	Pb ^r	Jerke and Nakatsu, personal communication
pCGR2	Carries cloned Pb ^r gene from <i>E. coli</i>	Plasmid	Pb ^r	64

Cu(I), Zn(II), or Co(II) (5, 63, 66), and further protect the cell by removing toxic ions, such as Ag(I), Cd(II), and Pb(II), from the cytoplasm (53, 65, 74). P_{IB}-type ATPases specific for monovalent Cu(I) and Ag(I) are found in all three domains of life, while divalent ion-specific Zn(II)-Co(II)-Cd(II)-Pb(II)-transporting P_{IB}-type ATPases are common in prokaryotes but have not been observed in animals and fungi (3).

Several P_{IB}-type ATPases are associated with mobile genetic elements. A plasmid location has been reported for *Lactococcus lactis* (56), *Staphylococcus aureus* (53), *Ralstonia metallidurans* (7), and *Arthrobacter* spp. (K. Jerke and C. Nakatsu, personal communication). Transposons carrying P_{IB}-type ATPases were described for *Listeria monocytogenes* (34) and *S. aureus* (Chikramane and Dubin, 1993, unpublished data), and in *Pseudomonas aeruginosa* the locus is a part of a gene island (31). LGT of a P_{IB}-type ATPase has previously been documented in *Stenotrophomonas maltophilia* (1) and *Streptococcus thermophilus* (67). To date, the locus has not been considered with respect to LGT and the genetic diversity of natural communities and their responses to metal stress.

The objective of this study was to use phylogenetic analyses to examine the role of LGT in the evolution of metal homeostasis in subsurface bacteria. We obtained PCR amplicons of zntA/cadA/copA-like genes from a group of previously described lead-resistant (Pb^r) bacteria from the deep terrestrial subsurface (6). Phylogenetic analyses of the DNA and deduced amino acid sequences of these amplicons revealed that 4 of 48 subsurface zntA/cadA/copA-like loci may have evolved by LGT. Transfer among *Comamonas* spp. and between members of the β subclass of the class *Proteobacteria* (β -proteobacteria) and γ -proteobacteria were noted, indicating that LGT, while not widespread, has played a distinct role in the evolution of metal resistance in deep subsurface bacteria.

MATERIALS AND METHODS

Bacterial strains. The sources and the cultivation and storage conditions for 105 Pb^r subsurface strains obtained from the Subsurface Microbial Culture Collection (Tallahassee, Fla.) were described previously (6). Pairs of Pb^r bacteria and lead-sensitive (Pb^s) mutants of these bacteria used here to optimize amplification of zntA/cadA/pbrA-like genes are listed in Table 1. These strains, as well as a deletion mutant of *Escherichia coli* W3110 and host strains carrying zntA/cadA/pbrA-like genes on plasmids (Table 1), were grown as described previously (6).

DNA preparation. Bacterial strains were removed from glycerol stocks and cultured on Luria-Bertani agar, pH 6.8 (containing [per liter] 10 g of tryptone, 5 g

of peptone, 5 g of NaCl, and 15 g of agar). Isolated colonies were transferred to 5 ml of Luria-Bertani broth and incubated at 37°C overnight. One-milliliter samples of each overnight culture were centrifuged and resuspended in 1 ml of phosphate-buffered saline (containing [per liter] 8 g of NaCl, 0.2 g of KCl, 1.15 g of Na₂HPO₄ · 7H₂O, and 0.2 g of KH₂PO₄). The cells were washed three times in phosphate-buffered saline and then resuspended in 100 μ l of cold 1:10 Tris-EDTA (10 mM Tris-Cl [pH 7.4], 1 mM EDTA [pH 8.0]). The cells were subjected to three freeze-thaw cycles at -75 and 65°C and then dialyzed for 2 to 3 h by depositing them onto 0.025- μ m-pore-size nitrocellulose filters (Millipore, Bedford, Mass.) floating on Tris-EDTA. The lysates were stored at -20°C or used immediately. When other cell components interfered with PCR, 1 to 5 ml of a culture was centrifuged and used for extraction of chromosomal DNA (Puregene kit; Gentra, Minneapolis, Minn.). Purified DNA samples were quantitated with a spectrophotometer (260 and 280 nm; Ultrospec 3000; Amersham Biosciences, Piscataway, N.J.) and stored at -20°C until they were used.

PCR of 16S rDNA genes. Amplification of the 16S ribosomal DNA (rDNA) gene from genomic DNA was carried out by using 50 pmol of primer fD1 (30), 50 pmol of primer rP2 (85), 5 μ l of crude cell lysate or 50 to 250 ng of purified DNA, each deoxynucleoside triphosphate at a concentration of 0.2 mM, 5 μ l of 5 \times PCR buffer (Promega Corporation, Madison, Wis.), 0.02 U of *Taq* polymerase (Promega), and 1.5 mM (final concentration) MgCl₂. The PCR conditions included a 10-min hot start step at 94°C, followed by 30 cycles of 94°C for 1.5 min, 55°C for 1.5 min, and 72°C for 2 min (GeneAmp PCR System 9700; Applied Biosystems, Foster City, Calif.). After cycling, PCR mixtures were incubated for 7 min at 72°C and then kept at 4°C. PCR products were electrophoresed on a 0.75% agarose Tris-acetate-EDTA (0.04 M Tris-acetate and 0.001 M EDTA) gel at 70 V, stained in a solution containing 0.4 μ g of ethidium bromide per ml, and destained in double-distilled H₂O. DNA bands that were approximately 1.5 kb long (as determined with λ HindIII markers [Invitrogen, Carlsbad, Calif.]) were excised from the gel and purified by using a Nucleotrap gel purification kit (Clontech, Palo Alto, Calif.) before storage at -20°C.

PCR primer design and specificity range. Novel primers for a two-step nested PCR approach were developed to obtain zntA/cadA/pbrA-like amplicons. Multiple primer sets were designed by using eight bacterial sequences whose gene products have a documented role in metal homeostasis of Zn(II), Cd(II), and/or Pb(II) (*E. coli* zntA [5], *S. aureus* cadA [53], *L. monocytogenes* cadA [35], *Pseudomonas putida* cadA [36], *L. lactis* cadA [41], *Synechocystis* sp. strain PCC6803 ziaA [79], *R. metallidurans* pbrA [7], and *Helicobacter pylori* cadA [26]) and sequences homologous to these loci in whole bacterial genomes (*Chlamydomonas reinhardtii* [accession no. AE001363], *Halobacterium* sp. strain NRC-1 [AE004437], *Mycobacterium tuberculosis* [AL123456], *Synechocystis* sp. strain PCC6803 [BA000022], *R. metallidurans* contig 649 [AAAI01000310], *R. metallidurans* contig 691 [AAAI01000352], *Ralstonia solanacearum* megaplasmid [NC_003296], *P. aeruginosa* PA1549, PA2435, PA3690, and PA3920 [NC_002516], *Pseudomonas syringae* PSPT00570, PSPT05532, PSPT05279, and PSPT01996 [NC_004578], *Pseudomonas fluorescens* Pflu0176 [NZ_AAAT02000118], *P. fluorescens* Pflu4370 [NZ_AAAT02000008], *P. fluorescens* Pflu2473 [NZ_AAAT02000032], and *P. fluorescens* PFO-1 [NZ_AAAT00000000]). Deduced amino acid sequences were aligned with MegAlign (DNASoft, Inc.), and the alignments were used to detect conserved regions in the encoding DNA. Sequences encoding the phosphatase and the ATP-binding domains were used as forward and reverse PCR primer binding sites, respectively, for the first PCR step. The transmembrane metal-binding domain (forward) and the ATP-binding

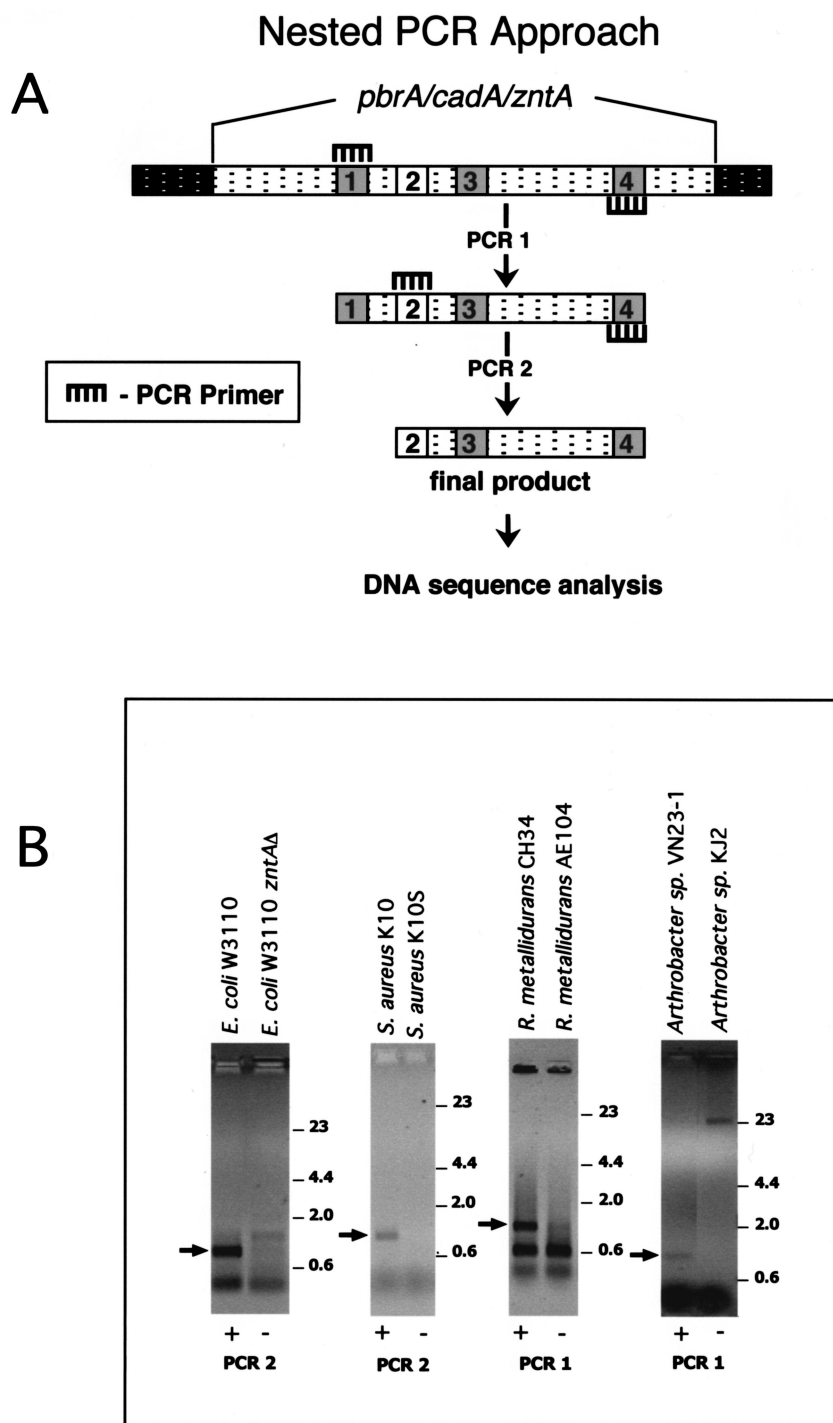


FIG. 1. (A) Approach for obtaining *zntA/cadA/pbrA*-like amplicons from bacterial genomic DNA preparations. The gray boxes indicate conserved sequences found in all P-type ATPases, and the white boxes indicate domains found only in heavy metal-transporting (P_{IB} -type) ATPases. 1, phosphatase domain; 2, transmembrane metal-binding domain; 3, phosphorylation domain; 4, ATP-binding domain. Primer sequences are shown in Table 2. (B) Amplification of characterized *zntA/cadA/pbrA* genes of control strains by the nested PCR approach. The arrows indicate amplification products of the expected size. Differences between positive and negative control strains were evident in either the first or second nested PCR, labeled PCR 1 and PCR 2, respectively. Plus and minus signs indicate $Pb(II)^r$ and $Pb(II)^s$, respectively.

domain (reverse) were selected as sites for the development of primers for the second nested PCR step (Fig. 1A). For primer design we utilized the most frequently occurring base at each position and included degeneracies for those that contained two or more base pairs in nearly equal proportions. Primer sequences were tested against the nontarget P-type ATPases encoded

by *kdpB* (P_{IA} type), *mgtA* (P_{IIB} type), and *copA* (P_{IB} type) and were rejected if the level of match was the same as or greater than the worst level of *zntA/cadA/pbrA* match. KdpB, MgtA, and CopA are the bacterial P-type ATPases that have the highest degrees of homology to ZntA/CadA/PbrA (57). Base pair substitutions in the final primer sequence were made only in

TABLE 2. PCR primers used to obtain *zntA/cadA/pbrA*-like amplicons

PCR primer type	Primer	Primer sequence	Sequence(s) that primer design was based on	Targets amplified
Reverse	84JC	5' GGAGCATCGTTAATDCRCDCC 3'	Sequences of Zn(II)-, Cd(II)-, and Pb(II)-specific ATPases	All
Forward, first reaction	79JC	5' TGACTGGCGAATCGGTBCCBG 3'	Sequences of Zn(II)-, Cd(II)-, and Pb(II)-specific ATPases	For control, <i>E. coli</i> W3110, <i>S. aureus</i> K10; for subsurface, <i>Bacillus</i> , <i>Acinetobacter</i> , <i>Pseudomonas</i> spp.
	87JC	5' TCASCGGCGARAGCSTGCCSGT 3'	<i>zntA</i> -like sequences from complete β -proteobacterial genomes	For control, <i>R. metallidurans</i> CH34; for subsurface, <i>Comamonas</i> spp.
	101JC	5' CDATCACGGGCGAAAGCCTGC 3'	<i>zntA</i> -like sequences from complete pseudomonad genomes	For subsurface, <i>Pseudomonas</i> spp.
	102JC	5' CGATTACGGGCGAGTCAGTGC 3'	<i>zntA</i> -like sequences from complete pseudomonad genomes	For subsurface, <i>Pseudomonas</i> , <i>Ralstonia</i> spp.
	132JC	5' CTAAGTGGCGAATCAGTCCC 3'	<i>pbrA</i> from <i>Arthrobacter</i> sp. strain VN23-1	For control, <i>Arthrobacter</i> sp. strain VN23-1; for subsurface, <i>Arthrobacter</i> spp.
Forward, second reaction	81JC	5' GGATGTCCTTGTGCTYTART 3'	Sequences of Zn(II)-, Cd(II)-, and Pb(II)-specific ATPases	For control, <i>E. coli</i> W3110, <i>S. aureus</i> K10; for subsurface, <i>Bacillus</i> , <i>Acinetobacter</i> , <i>pseudomonas</i> spp.
	88JC	5' CGTSGTGATYTCSACSCC 3'	<i>zntA</i> -like sequences from complete β -proteobacterial genomes	For control, <i>R. metallidurans</i> CH34; for subsurface, <i>Comamonas</i> spp.
	103JC	5' CGATTACGGGCGAGTCAGTGC 3'	<i>zntA</i> -like sequences from complete pseudomonad genomes	For subsurface, <i>Pseudomonas</i> , <i>Ralstonia</i> spp.
	133JC	5' CCCTCACCTTGTGCTYCTGG 3'	<i>pbrA</i> from <i>Arthrobacter</i> sp. strain VN23-1	For control, <i>Arthrobacter</i> sp. strain VN23-1; for subsurface, <i>Arthrobacter</i> spp.

cases in which severe secondary structure formation was predicted (VectorNTI Suite; Informax, Frederick, Md.).

One reverse primer, 84JC, was used in combinations with five first-reaction forward primers and four second-reaction forward primers to obtain *zntA/cadA/pbrA* PCR amplicons (Table 2). Combinations with primers 79JC and 81JC were used to obtain PCR products from *E. coli* W3110 and *S. aureus* K10 (Fig. 1B) and one product each from subsurface strains of *Bacillus*, *Acinetobacter*, and *Pseudomonas* spp. Forward primers 87JC and 88JC amplified *zntA/cadA/pbrA*-like genes of 25 of 31 Pb^r subsurface *Comamonas* spp. and the control β -proteobacterium *R. metallidurans* CH34. Forward primers 101JC, 102JC, and 103JC, designed by aligning *ZntA/CadA/PbrA*-like amino acid sequences from complete pseudomonad genomes, were used with 17 of 27 Pb^r subsurface *Pseudomonas* spp. Finally, amplicons from 2 of 15 subsurface *Arthrobacter* spp. were obtained by using primers 132JC and 133JC, which were designed to target a known *pbrA* sequence of *Arthrobacter* sp. strain VN23-1 (Jerke and Nakatsu, personal communication).

Nested PCR optimization. The initial conditions for nested PCR were established by using purified plasmid pCGR2 (a gift from C. Rensing) carrying the *E. coli zntA* gene. With the exception of the primers (Table 2), the nested PCR mixture was identical to that used for the 16S rDNA PCR described above. The first and second PCRs were performed for 30 cycles, with a 94°C melting step for 0.5 min, 59 and 49°C annealing steps for 0.5 min for the first and second reactions, respectively, and a 72°C elongation step for 1.5 min. After each PCR, the product was electrophoresed in a 0.75% agarose gel and gel purified (Clontech). The identity of the *zntA*-like gene product was verified by restriction analysis (by using enzymes and buffers supplied by Invitrogen) and DNA sequencing (see below).

Paired sets of reference strains of Pb^r organisms and their Pb^s derivatives were used to optimize amplification conditions for the target sequence from genomic DNA. PCR products were obtained from *E. coli* W3110, *S. aureus* K10, *R. metallidurans* CH34, and *Arthrobacter* sp. strain VN23-1 (Fig. 1B). Digestion with restriction enzymes (Invitrogen) and DNA sequencing (see below) verified the identities of these products. For most of the PCRs we utilized the conditions described above; the only exceptions were reactions with that the *Arthrobacter*-specific primers included an annealing temperature of 55°C in the first PCR and 1 mM MgCl₂ in both the first and second PCRs. The PCR conditions used for the subsurface isolates were identical to those used for the related control strains with the corresponding primer sets (Table 2).

Sequencing and sequence analysis. Sequencing was performed directly with *zntA/cadA/pbrA*-like and 16S rDNA PCR products with a BigDye version 3 reagent mixture (Applied Biosystems). In sequencing reactions for the former products we utilized the 84JC, 81JC, 88JC, 103JC, and 133JC primers, with additional primer walking as needed. Of the 16S rDNA PCR products we utilized the fD1 and 519r primers (30, 85). The sequencing reactions were performed

with an ABI 3100 genetic analyzer (Applied Biosystems) at the Biotechnology Center for Agriculture and the Environment, Cook Campus, Rutgers University. Sequence data were compiled in Contig Express (VectorNTI Suite; Informax), translated into amino acid sequences in the case of P_{1B}-type ATPase amplicons, and aligned with ClustalX (81) by using default program settings, followed by verification by eye. Construction of unrooted trees was performed by using the parsimony and distance functions of PAUP*, version 4.0 beta 10 (Sinaur and Associates, Sunderland, Mass.). Bootstrap analyses were performed for all completed trees.

The G+C content and codon usage of each *zntA/cadA/pbrA*-like amplicon were calculated by using the countcodon program available at the Codon Usage Database website (46). The G+C content of each subsurface amplicon was then compared to the G+C contents of all other organisms belonging to the same genus listed in the database. The ranges given below were verified by using values published in *The Prokaryotes* (27, 69, 87) when available. Indel analysis was performed visually by observing alignments created in BioEdit (25) by using Clustal W (82). A gap was labeled as an indel only if it was the same length in the organisms in which it was present.

Nucleotide sequence accession numbers. The nucleotide sequences of the 48 subsurface *zntA/cadA/pbrA*-like amplicons (average length, 750 bp) have been deposited in the GenBank database under accession numbers AY463172 to AY463192. The nucleotide sequences of the partial 16S rDNA genes (average length, 500 bp) have been deposited in the GenBank database under accession numbers AY463193 to AY463212.

RESULTS

Amplification of *zntA/cadA/pbrA*-like genes from deep subsurface isolates. Resistance to Pb(II) in *E. coli* (65), *R. metallidurans* (7), *S. aureus* (53, 65), and possibly *Arthrobacter* (Jerke and Nakatsu, personal communication) is mediated by a P_{1B}-type ATPase-based efflux pump. In *E. coli*, substrates for this pump also include Cd(II) and Zn(II) (65). In a previous study, 105 of 261 deep subsurface bacterial strains were found to be Pb^r (6). To investigate if Pb^r evolved by LGT in subsurface strains, we developed a nested PCR approach (Fig. 1A) that specifically amplified *zntA/cadA/pbrA*-like amplicons for subsequent sequencing and phylogenetic analysis. The specificity of the approach for *zntA/cadA/pbrA*-like genes was tested by using three different categories of templates: (i) purified plasmids

carrying a *zntA* gene (pCGR2) or a *pbrA*-like gene (pB23F) (Table 1), which tested the ability of the *zntA/cadA/pbrA*-like primers to recognize the target; (ii) positive controls consisting of genomic DNA containing *zntA*, *cadA*, *pbrA*, or, in the case of *Arthrobacter* sp. strain VN23-1 and *Staphylococcus* sp. strain K10, *zntA/cadA/pbrA*-like genes, which tested the ability of the primers to recognize *zntA/cadA/pbrA*-like genes in the presence of excess heterologous DNA; and (iii) negative controls consisting of genomic DNA from deletion mutants or cured strains in which the target genes are not present, which tested the ability of the primers to distinguish *zntA/cadA/pbrA*-like genes from genes encoding other P_{IB}-type ATPases (*copA*/*silA*-like genes) and phylogenetically related bacterial P_{IA}- and P_{IIIB}-type ATPases (*kdpB*- and *mgtA*-like genes, respectively) (57) that may be present. Using four sets of paired positive and negative control strains belonging to β - and γ -proteobacteria and the low- and high-G+C-content gram-positive bacteria, we were able to specifically obtain *zntA/cadA/pbrA*-like products (Fig. 1B). These products included the product of *Staphylococcus* sp. strain K10, in which the mechanism of Pb^r has not been characterized previously (38), suggesting that resistance in this strain is mediated by an efflux pump, as it is in *Staphylococcus* plasmid pI258 (53).

Using genomic DNA as a template, we obtained *zntA/cadA/pbrA*-like amplicons from 48 of the 105 Pb^r subsurface strains. 16S rDNA amplicons were amplified from all genomic DNA samples, suggesting that any PCR failures with our primers were due to either the absence of the target gene or the presence of genes with divergent sequences in the regions targeted by the PCR primers. Multiple *zntA/cadA/pbrA*-like amplicons were found in at least four subsurface isolates, all *Pseudomonas* spp. (data not shown). As the complete genome sequences of *Pseudomonas alcaligenes* (76) and *P. fluorescens* (unfinished genome; accession no. NZ_AAAT00000000) also contain multiple *zntA/cadA/pbrA*-like sequences, this may be a general feature of pseudomonads. However, our primers were strongly biased for a specific *zntA/cadA/pbrA*-like locus because we were able to obtain readable DNA sequence from mixed populations of PCR products. Nevertheless, sequences for the four *Pseudomonas* spp. strains were not included in the phylogenetic analysis. The bias of the primers for a specific locus was also evident in the recovery of a single *zntA/cadA/pbrA*-like amplicon from *R. metallidurans* CH34, which was identified as *pbrA* from plasmid pMOL30 (44), although a minor *zntA/cadA/pbrA*-like amplicon was obtained from cured *R. metallidurans* AE104 (Fig. 1B). The sequence of the AE104 amplicon indicates that it most likely originated from a gene in contig 649 that was recently identified as a chromosomal *cadA* locus (43). This locus exhibits only 62% identity to the pMOL30-encoded *pbrA* gene.

Phylogenetic analysis of *zntA/cadA/pbrA*-like genes of deep subsurface isolates. Evolution by LGT may be identified by examining the congruency between gene phylogenies (33). A neighbor-joining tree was created by using the amino acid sequences deduced from the *zntA/cadA/pbrA*-like amplicons (Fig. 2B). Heuristic analysis of the same sequences yielded a tree with the same branching order, although the tree lacked bootstrap support for several of the internal nodes (data not shown). A neighbor-joining tree was also created for a 500-bp section of the 16S rDNA genes from the same strains (Fig. 2A). Some

organisms yielded amplicons from both *zntA/cadA/pbrA*-like and 16S rDNA that were >99% identical, and in these cases selected representatives were chosen for inclusion in the final trees.

The grouping that is immediately noticeable (Fig. 2B) is the large cluster of *Pseudomonas* (γ -proteobacterial) amplicons that group within the β -proteobacteria (neighbor-joining bootstrap support at the basal node of the clade, 84). One of two *zntA/cadA/pbrA*-like sequences from the completed genome of *P. aeruginosa* and a single *zntA/cadA/pbrA*-like gene from the genomes of both *P. fluorescens* and *P. syringae* (data not shown) also clustered with this group. The most parsimonious explanation for this incongruency is a single ancient transfer event involving a β -proteobacterium that happened prior to the divergence of the pseudomonads.

In four instances the 16S rDNA tree and the *zntA/cadA/pbrA*-like tree of the subsurface strains were incongruent (Fig. 2). (i) *Comamonas* sp. strain B0173 contained a 16S rDNA gene that grouped in the tight cluster of *Comamonas* spp. genes with nearly identical sequences (Fig. 2A) (bootstrap value for the basal node of the clade, 100) and a *zntA/cadA/pbrA*-like gene that branched outside most of the β -proteobacterial cluster (Fig. 2B) (bootstrap value, 68). The sequence did not cluster closely with any known *zntA/cadA/pbrA*-like sequences, and thus the origin of the gene could not be deduced. (ii) The *zntA/cadA/pbrA*-like gene of *Comamonas* sp. strain B0669 clustered with the large group of *Comamonas* spp. *zntA/cadA/pbrA*-like genes (bootstrap value for the basal node of the clade, 100), yet the 16S rDNA gene was more closely related to the *Comamonas* sp. strain B0329 gene on a separate *Comamonas* spp. branch (bootstrap value, 98). Thus, strain B0669 may have acquired its *zntA/cadA/pbrA*-like gene from an organism resembling the organisms found in the larger *Comamonas* cluster. (iii) The 16S rDNA gene of *Acinetobacter* sp. strain B0064 grouped with the γ -proteobacterial genes (bootstrap value, 84), and its *zntA/cadA/pbrA*-like gene clustered with genes from members of the β -proteobacterial group (bootstrap value, 89), suggesting that there was a transfer from a β -proteobacterium to a γ -proteobacterium. (iv) *Ralstonia* sp. strain B0665 contained a *zntA/cadA/pbrA*-like gene that grouped very closely with the cluster of *Pseudomonas* spp. loci (bootstrap value, 100) within the β -proteobacterial clade (see above), suggesting that there was a possible transfer from this source. Thus, an analysis of the incongruencies between the *zntA/cadA/pbrA*-like and 16S rDNA gene sequences suggested that 4 of 48 *zntA/cadA/pbrA*-like loci in subsurface bacteria evolved by LGT.

Unusual sequence features of subsurface *zntA/cadA/pbrA*-like amplicons. Transferred genes ameliorate to the new host's transcriptional and translational machinery over time (32). Consequently, unusual G+C contents may provide evidence only for recent LGT events. Therefore, the subsurface *zntA/cadA/pbrA*-like amplicons were examined for unusual DNA base compositions that might indicate recent transfer. *Comamonas* sp. strain B0173 contained a *zntA/cadA/pbrA*-like gene with a G+C content lower than those of other known *Comamonas* strains (60 mol% instead of 61 to 67 mol% [87]), and *Acinetobacter* sp. strain B0064 had a G+C content higher than those of other *Acinetobacter* spp. (65 mol% rather than 31 to 63 mol%). Evolution by LGT of the *zntA/cadA/pbrA*-like genes of these two strains was also suggested by the incongruence anal-

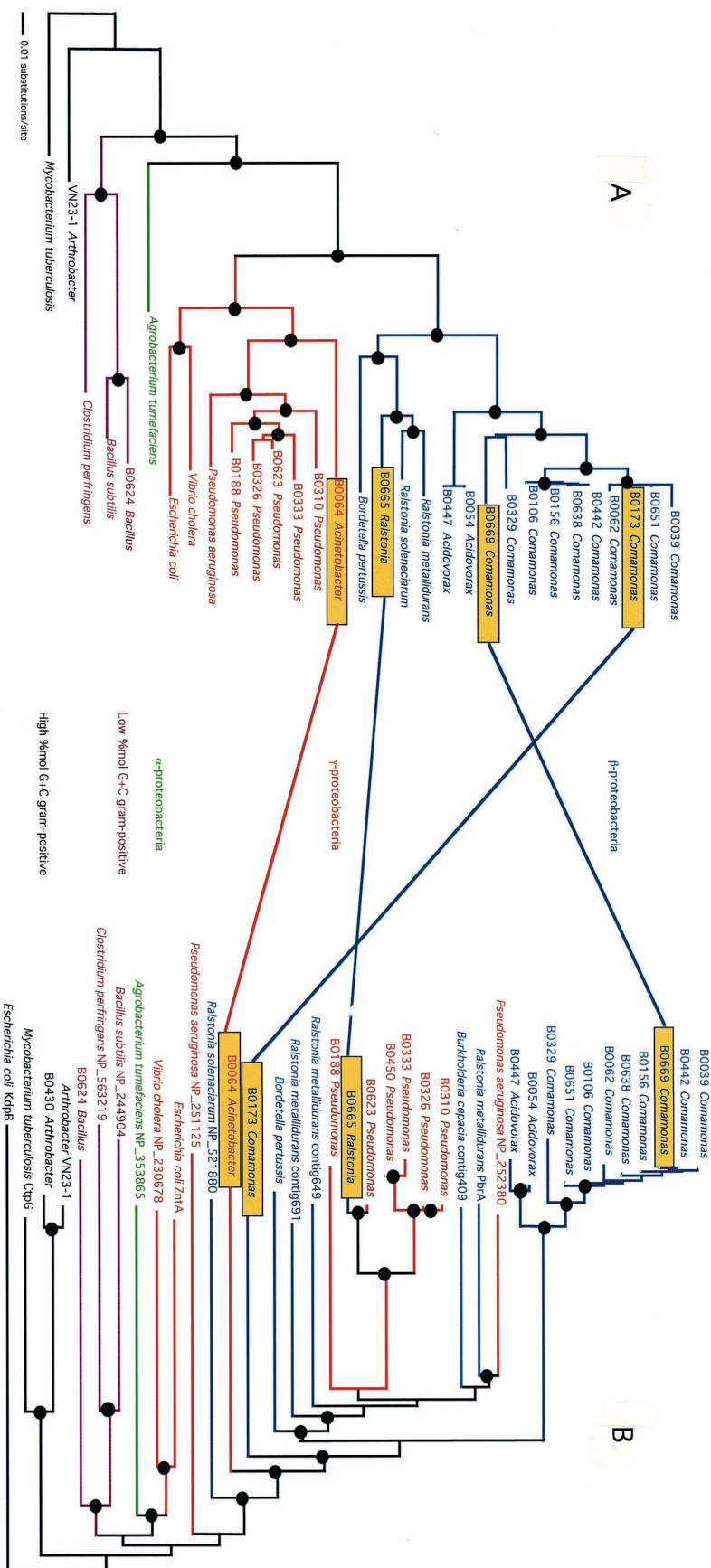


FIG. 2. Neighbor-joining analysis of 16S rDNA (A) and *znlA/cadA/phrA*-like (B) sequences amplified from subsurface isolates or obtained from completed genomes. Accession numbers are given for completed genomes of *Ralstonia metallidurans*, *Ralstonia solanacearum*, *Bordetella pertussis*, *Pseudomonas aeruginosa*, *Vibrio cholerae*, *Escherichia coli*, *Agrobacterium tumefaciens*, *Bacillus subtilis*, *Clostridium perfringens*, and *Mycobacterium tuberculosis*. The colors of the type and the lines correspond to the organisms' phylogenetic groups. A solid circle at a node indicates that the level of bootstrap support is >50%. The strain designations in boxes and lines connecting entries in boxes in the two neighbor-joining trees indicate incongruencies in the phylogenies of the 16S rDNA and the *znlA/cadA/phrA*-like loci of the corresponding subsurface strains.

ysis (see above). The G+C content of *Pseudomonas* sp. strain B0188's *zntA/cadA/pbrA*-like gene (68 mol%) was at the very high end of the range reported for pseudomonads (46), exceeded only by the G+C contents of three open reading frames from *Pseudomonas marginata* with a combined G+C content of 70.6 mol% (46). The G+C contents of the remaining subsurface *zntA/cadA/pbrA*-like loci fell within the known range for the genomes of the organisms most closely related to their subsurface hosts.

Codon bias (45, 90) and shared insertions and deletions (indels) (24) are additional indications of evolution by LGT. The former could not be used here, most likely because of the small size of the deduced amino acid sequences (250 amino acids) obtained from the *zntA/cadA/pbrA*-like amplicons. However, two *zntA/cadA/pbrA*-like amplicons, one from *Comamonas* sp. strain B0329 and one from *Bacillus* sp. strain B0624, contained unique indels on the large cytoplasmic loop connecting transmembrane helices 6 and 7 (63, 83; data not shown). The 4-bp indel of *Bacillus* sp. strain B0624 was located within a variable region following the phosphorylation domain, and the 1-bp indel of *Comamonas* sp. strain B0329 was located close to the ATP-binding domain.

DISCUSSION

In this paper we describe one of the first documented accounts of the application of molecular evolutionary approaches to the study of microbial LGT in a specific environment, and this study was the first thorough examination of this process as it relates to the evolution of metal homeostasis genes in deep subsurface bacteria. To date, previous retrospective studies have had limited application to the study of LGT as a process that shapes the genetic diversity of natural communities. Rather, this issue has been addressed either by demonstrating transfer in microcosms (4, 15) or by examining the potential for LGT by isolation, identification, and characterization of mobile genetic elements obtained from environmental strains (12) or exogenously from the environment (70). The time scale for the acquisition of new genetic material by LGT, estimated to occur at a rate of 31 kb per 10^6 years in *E. coli* (32), may mean that microcosm studies have little relevance. Indeed, in many cases, LGT could be detected only following nutrient amendment (15), by inoculation of unrealistically high numbers of donor and recipient strains (4), or in the presence of selection for phenotypes encoded by the transferred genes (81). Observing LGT in microcosm studies simulating deep subsurface environments may be particularly difficult, as low population densities and metabolic rates may require prohibitively long incubation times before transformants and transconjugants are obtained. Thus, retrospective molecular evolutionary approaches, which can trace specific LGT events that have occurred over long time spans, may be the best approach to study LGT in subsurface environments.

Here we used phylogenetic incongruity, unusual G+C contents, and the presence of indels to trace the probable evolutionary path of metal homeostasis genes in 48 representative isolates belonging to four different groups of common soil bacteria: high- and low-G+C-content gram-positive bacteria and γ - and β -proteobacteria. Table 3 summarizes our findings. For two of the strains, *Acinetobacter* sp. strain B0064

TABLE 3. Evidence for evolution of *zntA/cadA/pbrA*-like genes by LGT in subsurface strains

Subsurface strain	Evidence for LGT from:		
	Phylogenetic congruency	G+C content	Indel congruency
<i>Acinetobacter</i> sp. strain B0064	Yes	Yes	No
<i>Comamonas</i> sp. strain B0173	Yes	Yes	No
<i>Pseudomonas</i> sp. strain B0188	Maybe ^a	Yes	No
<i>Ralstonia</i> sp. strain B0665	Yes	No	No
<i>Comamonas</i> sp. strain B0669	Yes	No	No

^a Phylogenetic position of the *zntA/cadA/pbrA* amplicon was not supported by the bootstrap value (see text).

and *Comamonas* sp. strain B0173, evidence of LGT determined by phylogenetic incongruity was supported by unusual G+C contents, suggesting that there was relatively recent transfer of the *zntA/cadA/pbrA*-like locus. As determined by this reasoning, *Pseudomonas* sp. strain B0188 may also have acquired its *zntA/cadA/pbrA*-like locus through LGT, since the G+C content of the amplicon does not fall within the known range for *Pseudomonas* spp. The use of sequence composition alone as a marker for LGT is controversial since it has been shown that G+C contents of genes can vary greatly depending upon the position in the genome (68), amelioration through time (32), and level of expression. Since the phylogenetic position of B0188 is not supported by bootstrap analysis (Fig. 2B), we have not ruled out the possibility that chromosome positioning and/or a higher-than-usual mutation rate, rather than LGT, affected both the G+C content of the gene and its position in the phylogenetic tree. In the case of *Ralstonia* sp. strain B0665 and in the case of *Comamonas* sp. strain B0669, phylogenetic evidence was not supported by the G+C content of the *zntA/cadA/pbrA*-like amplicon (Table 3). These cases may represent transfers from unidentified organisms having similar G+C contents or ancient transfer events.

Because microbes are transported into and within the deep subsurface (21), it is not possible to determine whether the transfer events detected here occurred prior to or following the deposition of the strains in this environment. At least in the case of *Acinetobacter* sp. strain B0064 and in the case of *Comamonas* sp. strain B0173, in which transfer appears to have occurred relatively recently, LGT may have occurred in the deep subsurface. Unequivocal evidence for the occurrence of transfer in the subsurface might be obtained by studying populations known to have evolved in this environment for extended periods of time. Such an opportunity may be presented by the discovery of coherence between the 16S rRNA- and *recA*-based phylogenies of *Arthrobacter* spp. and the geological strata from which the strains were isolated in the Yakima Barricade at the U.S. Department of Energy's Hanford Site, a finding interpreted to indicate spatial separation and long-term evolution in the deep subsurface (84).

The collection of bacteria examined here represented closely related phylogenetic groups. The more closely related microbes are phylogenetically, the more likely they are to exchange genetic material (23) but the less likely they are to have a detectable molecular footprint of this transfer in their genomes (20). Many of our isolates belong to the same genus or similar genera; therefore, our study may have underestimated

the magnitude of LGT of the *zntA/cadA/pbrA*-like locus. To obtain a more accurate picture of LGT in a given environment, enrichments and selection methods could be used to obtain isolates representing a broad phylogenetic distance. Targeting this diversity would create its own problems, however, since it would entail a greater degree of diversification in the primary DNA sequence of the gene of interest. Even with the diversity of primers designed for the current collection of isolates (Table 2), amplicons were obtained from only 48 of 105 P^b subsurface strains. Most likely the diversity of our PCR primer did not match that of the broadly distributed metal homeostasis genes in the bacterial world (2). This possibility, easily explored by Southern hybridization with *zntA/cadA/pbrA*-specific probes and relaxed stringency to encompass a greater diversity, was not pursued in the present work because our primary goal was to obtain DNA sequences for phylogenetic analysis. As demonstrated by our approach to the design of the β -proteobacterium-specific primers 87JC and 88JC (Table 2), the challenge of primer and probe design may be lessened as more microbial genomes are sequenced. Furthermore, with the advances in metagenome applications in microbial ecology and the creation of databases linking 16S rDNA genes and functional genes in uncultured microbes (16, 40), studying LGT among uncultured members of the microbial community may be possible one day.

The evolution of metal homeostasis genes by LGT in subsurface bacteria may have ramifications for bioremediation in mixed-waste-contaminated subsurface environments, where the presence of toxic metals may inhibit bioremediation of metals, radionuclides, and organic contaminants. As conjugal transfer of catabolic plasmids from donor strains to soil bacteria enhanced degradation of organic contaminants in topsoils (14, 48) and stimulation of bacterially induced metal immobilization (47, 55) and biodegradation (42) is now practiced in the subsurface, manipulating the genetic potential of subsurface communities to enhance bioremediation may become feasible.

In summary, in this study we used approaches and tools from molecular evolution to examine the role of LGT in the evolution of an ecologically important phenotype in a subsurface microbial community. The integration of molecular evolution and microbial ecology as applied to organisms within a specific environment should expand our understanding of the processes that shape the genetic diversity of microbial communities in their natural habitats.

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