Journal of Industrial Microbiology (1996) 16, 189–196

Biosorption of cadmium, cobalt, nickel, and strontium by a *Bacillus simplex* strain isolated from the vadose zone

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A subsurface Gram-positive, endospore-forming, filamentous bacterium, designated ZAN-044, was isolated from a depth of 96.2 m in the vadose zone of the Hanford Site in Washington State. A phylogenetic analysis of the 16S rRNA gene sequence of strain ZAN-044 revealed it to be 99.5% similar to *Bacillus simplex* strain DSM 1321, indicating that they may be members of the same species. *B. simplex* ZAN-044 was studied along with *Bacillus subtilis* 168, and *Escherichia coli* K-12 (AB264), two well-characterized metal-sorbing bacteria, for the binding of Cd^{2+}, Co^{2+}, Ni^{2+}, and Sr^{2+}. There was rapid (less than 1 h) uptake of 1 μM metal by the three bacteria in the order Cd > Ni ≥ Co >Sr. Binding followed a saturation isotherm at cation concentrations from 0.1 μM to 1 mM. Cation binding was pH-dependent, with less binding at low pH. *B. simplex* ZAN-044 bound more metal than *B. subtilis* or *E. coli*, demonstrating that subsurface microorganisms can remove significant quantities of metals from solution and may be able to influence radionuclide and metal transport in the subsurface.

Keywords: biosorption; subsurface bacterium; vadose; cadmium; cobalt; nickel; strontium

Introduction

Radioactive wastes result from a variety of activities including the commercial nuclear fuel cycle, defense related activities, institutions (universities, hospitals, and research facilities), industrial use of isotopes, and mining and milling of uranium ore [30]. Liquid wastes that contained transuranics, other radionuclides, and metals were disposed on-site in the early days of operation of US Department of Energy (DOE) sites. Some of the radioactive waste was disposed in tanks for interim storage, but a large volume was disposed to the ground surface, landfills, ponds, and other holding areas, which has resulted in subsurface contamination [33]. It is currently very difficult to estimate either the volume of soil and sediment impacted by these disposal practices or the amount of radionuclides and metals released [30]. There is concern over the transport of radionuclides and heavy metals in the subsurface and their potential for contaminating domestic ground water supplies.

One mechanism by which microorganisms may alter radionuclide and metal mobility is through biosorption, the binding of metals to cells, which does not require metabolic activity [29]. Binding of cationic metals occurs mainly by attraction to the negatively-charged sites on the cell walls [26]. Biosorption is influenced by the type and form of the metal as well as by the microbial ligand responsible for sequestering the metal [26].

The ability of Gram-positive and Gram-negative bacterial walls to bind metals has been studied extensively [2–7,10,11,23,37]. The peptidoglycan layer of the cell wall is responsible for most of the metal-binding capacity of the Gram-positive bacterium *Bacillus subtilis* [7]. The Gram-negative bacterium *Escherichia coli* also utilizes the peptidoglycan for metal uptake by a two-step deposition method [5]. These studies used well-characterized microbial strains. There is currently limited information on the ability of subsurface microorganisms to bind metals and on how geochemical conditions influence this process.

The objective of our study was to determine the binding of Cd, Co, Ni, and Sr by a spore-forming bacterium isolated from the vadose zone. This study investigated metal binding by washed, exponential-phase cells in well-defined aqueous systems as a function of time, metal concentration, and pH. The microorganism used in this study was designated ZAN-044 and was isolated from the Hanford site. *B. subtilis* 168 and *E. coli* K-12 were also used in this study as a basis for comparison with isolate ZAN-044 because they have been well characterized as to cell wall structure and metal biosorption [3,5–7,11,21–23,27,29]. Cd, Co, Ni, and Sr were selected because they would be present as divalent metal ions in our experimental systems and because they have different chemical properties. Cd and Ni usually are toxic to microorganisms, although Ni is an essential micronutrient for many microorganisms [26]. Also, ^{60}Co and ^{90}Sr are radionuclides of concern at DOE sites because of their quantities [33] and migration in the subsurface [17].

Materials and methods

ZAN-044 isolation and bacterial culture conditions

The bacterium ZAN-044 was isolated from sediments obtained from a depth of 96.2 m within the vadose zone of the Hanford site in Washington State as described by Fredrickson *et al* [18]. Isolate ZAN-044 biosorbed more Cd, Co, Ni, and Sr than 59 other vadose zone isolates from this borehole (data not shown). The well-characterized *B. subtilis* 168 and *E. coli* K-12 (AB264) (courtesy of Dr T

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Received 1 September 1995; accepted 26 December 1995

Beveridge, University of Guelph, Ont, Canada) were also included.

Bacteria were stored in 50% glycerol at −80°C and were cultured in 10% PTYG broth (pH 7) containing the following per liter: peptone, 0.5 g; tryptone, 0.5 g; yeast extract, 1 g; glucose, 1 g; $MgSO_4 \cdot 7H_2O$, 0.6 g; $CaCl_2 \cdot 2H_2O$, 0.07 g [1].

Isolate ZAN-044 was grown at room temperature (about 23°C), *B. subtilis* at 30°C and *E. coli* at 37°C with shaking (140 rpm) to late exponential phase as determined by measuring optical density (OD) at 600 nm. *B. subtilis* and isolate ZAN-044 did not form spores.

Cells were harvested by centrifugation at 22000 × *g* for 15 min at 4°C and were washed twice in buffer (0.001 M piperazine-N, N′-bis (2-ethanesulfonic acid) (PIPES) with 0.01 M KNO_3) at pH 6. Cells of isolate ZAN-044 were resuspended in pH 6 buffer to an OD of 1.0 at 600 nm. This OD corresponded to approximately 1.5×10^8 colony forming units (CFU) ml^{-1} or 400 $\mu g\ ml^{-1}$ dry wt. *B. subtilis* cells were resuspended to an OD of ~ 0.8 (4.0×10^8 CFU ml^{-1} or 130 $\mu g\ ml^{-1}$ dry wt) and *E. coli* to an OD of ~ 0.6 (2.5×10^8 CFU ml^{-1} or 160 $\mu g\ ml^{-1}$ dry wt).

Endospore formation by ZAN-044

Endospores were tentatively identified in cells of isolate ZAN-044 after 7 days of growth on 10% PTYG plates via phase contrast microscopy. To verify endospore formation, exponential phase cells or spores were exposed to 80°C for 15 min, 95% ethanol for 1 h, or radiation from a ^{60}Co source at 0.25 Mrads. Cells were serially diluted in sterile 0.85% saline and plated onto 10% PTYG to determine percent survival in comparison to unexposed controls.

Physiological tests and FAME/MIDI analysis

Isolate ZAN-044 was screened for Gram reaction using Difco Gram stain reagents (Difco Laboratories, Detroit, MI, USA) with *B. subtilis* 168 and *E. coli* AB264 as controls. Several nutritional characteristics were determined using API Rapid NFT test strips (API Analytical Products Division of Sherwood Medical, Plainview, NY, USA).

The organism's fatty acid methyl esters (FAMEs) were analyzed with the MIDI system (Microbial Identification System, Microbial ID Inc, Newark, DE, USA). Saponification, methylation, purification, and analysis were conducted in a single tube; the methods are described by Miller and Berger [28] and in the Microbial Identification System Manual. FAME profiles for isolate ZAN-044 were screened against the MIDI TSBA and Clin libraries (Rev 3.60).

16S ribosomal DNA sequencing and phylogenetic analysis

DNA extraction, PCR amplification of the 16S rRNA gene, and automated sequencing were performed as described previously [8]. Sequencing primers were A and C [24], G and H [32] (courtesy of RH Reeves and JY Reeves, Florida State University, Tallahassee, FL, USA), and a reverse primer corresponding to *E. coli* positions 357–342 [9,36]. A 1096-base contiguous sequence corresponding to *E. coli* positions 281–1375 was analyzed as described below. The GenBank data base accession number for the assembled 16S rDNA sequence for isolate ZAN-044 is U39933.

The 16S rDNA sequence for strain ZAN-044 was hand-aligned to various 16S rDNA or rRNA sequences for selected species of eubacteria from the Ribosomal Database Project ([25]; RDP Release 5.0, updated 17 May 1995) and GenBank and/or the European Molecular Biology Laboratory (EMBL) database. All of the aligned sets of sequences were analyzed with parsimony (Phylogenetic Analysis Using Parsimony, Macintosh Version 3.1.1; [35]) and distance matrix methods (PHYLIP package of computer programs Version 3.5c; [14]). Consensus phylogenetic trees for each alignment were then produced [13]. Distances were calculated [20] and phylogenies were estimated with the FITCH option (which makes use of Fitch-Margoliash criterion [15] and some related least squares criteria).

Metal binding as a function of concentration and time

The radioactive tracers included $^{109}CdCl_2$ in 0.1 M HCl, 9.74×10^4 MBq mg^{-1}, 99% purity (Isotope Products Laboratories, Burbank, CA, USA); $^{57}CoCl_2$ in 0.1 M HCl, 3.1×10^5 MBq mg^{-1}, 99.7% purity (ICN, Irvine, CA, USA); $^{63}NiCl_2$ in 0.5 M HCl, 514 MBq mg^{-1}, 99% purity (Dupont, Wilmington, DE, USA); and $^{85}SrCl_2$ in 0.5 M HCl, 461.4 MBq mg^{-1}, 99% purity (Dupont). The metal salts used were $Cd(NO_3)_2 \cdot 4H_2O$, $Co(NO_3)_2 \cdot 6H_2O$, $Ni(NO_3)_2 \cdot 6H_2O$, or $Sr(NO_3)_2$ dissolved in 0.32 M HNO_3 (Ultrex, JT Baker Inc, Phillipsburg, NJ, USA). A combination of radioactive and stable metal salts were combined to achieve final concentrations of 0.2, 2, 20, 200, and 2000 μM metal at 1.67×10^{-3} MBq ml^{-1} in buffer at pH 6.

Five hundred microliters of the cell suspensions in buffer at pH 6 were added to 2-ml polypropylene Cube Tubes (DBM Scientific, CA, USA). Five hundred microliters of metal solution were added to the tubes, resulting in a final metal concentration of 0.1, 1, 10, 100, or 1000 μM. There were two replicate samples for each metal concentration. Controls containing no cells were also included. The tubes were placed on an orbital shaker (140 rpm) at room temperature. The 200-μl samples were filtered through a 0.2-μm pore size 96-well plate Millipore filtering system (Millipore Multiscreen Assay System, MA, USA) attached to a vacuum. The filtered cells were washed twice with 200 μl of pH 6 buffer. After drying them, the filters containing the cells were punched out into scintillation vials with a Millipore filter punch. Five hundred microliters of water were added to each scintillation vial containing a filter and they were placed on an orbital shaker (140 rpm) for 30 min. Five milliliters of scintillation cocktail (Ultima Gold, Packard, CT, USA) were added to each vial and the vials were analyzed in a Beckman LS 9800 scintillation counter. To determine cell dry weight, 3 ml of the cell suspension was filtered through a pre-weighed 0.2-μm pore size cellulose nitrate filter and washed once with an equal volume of water. Control filters with buffer only were also included. The filters were air dried at room temperature for at least 48 h and weighed to calculate metal bound by the cells on a dry weight basis.

Metal binding as a function of pH

The radionuclides and metal salts were used to investigate metal binding by cells from pH 4 to 9. Radioactive metal

salts at a final concentration of 2 μM in buffer were adjusted with 1 M KOH or 1 M HNO_3. The cell suspensions in buffer were adjusted to pH 4, 5, 6, 7, 8 or 9. The cell suspension and metal solution were mixed for a final metal concentration of 1 μM. The tubes were placed on an orbital shaker and 200 μl was filtered at 2 and 4 h as described above except the filtered cells were washed twice with their respective buffer. The dry weight of cells was determined for each pH suspension.

Metabolic inhibitors

To investigate whether metal binding isolate ZAN-044 was an active or passive process, viable cells in buffer at pH 6 or heat-treated cells (80°C for 15 min) were exposed to 1 μM metals and the metabolic inhibitors 1 mM sodium azide or 0.1 mM dinitrophenol added. Cells were filtered after 2 h.

Remobilization of metals

Cells in buffer at pH 6 were exposed to 1 μM metals. Following the 2-h sampling, an equal volume of either buffer at pH 6 or buffer at pH 6 containing 50 μM EDTA was added. The tubes were processed as described above.

Results

Isolation and identification of the organism

Isolate ZAN-044 grew as linked rods with a filamentous-like growth pattern (Figure 1a). Colonies on 10% PTYG plates were about 1 mm diameter, round, mucoid, convex, and white-cream colored. After 5–7 days of growth on 10% PTYG plates, endospore formation was evident via phase-contrast microscopy (Figure 1b). Endospores were resistant to 80°C heat for 10 min, 95% ethanol for 60 min, and 0.25 Mrads of ^{60}Co radiation, while vegetative cells were not. Cells stained Gram-positive when grown $\leq$ 24 h. In API NFT tests isolate ZAN-044 was negative for nitrate reduction, tryptophan utilization, glucose fermentation, arginine dihydrolase, urease, esculin hydrolysis, β-galactosidase, and caprate utilization; isolate ZAN-044 was oxidase- and gelatinase-positive and was positive for the utilization of D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetyl-glucosamine, maltose, D-gluconate, adipate, L-malate, citrate, and phenylacetate. Analysis by MIDI indicated the organism was most related to *Bacillus maroccanus* (data not shown) of those organisms in the MIDI database. However, its cellular morphology was distinct from that described for *B. megaterium*. To more competely locate the phylogenetic position of ZAN-044, we analyzed the 16S ribosomal gene sequence.

Analysis of 16S rRNA gene sequences

Parsimony and distance matrix analyses of a set of aligned sequences for representative species in the 15 major taxonomic groups of eubacteria in the RDP [25] assigned isolate ZAN-044 to the Gram-positive division. Analysis of alignments including representative species from the major subdivisions of Gram-positive bacteria placed the organism in the *Bacillus-Lactobacillus-Streptococcus* subdivision and indicated that it is most closely related to the '*Bacillus megaterium* group'. The sequence from isolate ZAN-044 was then aligned to sequences from the '*Bacillus megaterium* group' and other groups in the *Bacillus-Lactobacillus-Streptococcus* subdivision. The phylogenetic tree obtained by distance matrix analysis of the final sequence alignment is shown in Figure 2. Isolate ZAN-044 clustered with and appeared to be quite closely related to *Bacillus simplex*, *B. maroccanus*, and *B. macroides*. The next most closely related species was *B. psychrosaccharolyticus*. Virtually identical results were obtained by parsimony analysis of the sequence alignment (not shown). Similarities (over the 1069-base segment of sequences compared) to organisms in the *Bacillus-Lactobacillus-Streptococcus* subdivision ranged from 89.2% to 99.5%, the most similar species being *B. simplex* (the two sequences differed by only five bases). These results indicate that isolate ZAN-044 and *B. simplex* are possibly members of the same species. *B. simplex* ATCC 49097 grown in TSB (trypticase soy broth) was visually indistinguishable from *B. simplex* ZAN-044 (Figure 1) by phase contrast microscopy (not shown).

Biosorption of Cd, Co, Ni, and Sr as a function of time and concentration

Figure 3 shows the binding of Cd^{2+}, Co^{2+}, Ni^{2+} and Sr^{2+} by *B. simplex* ZAN-044, *B. subtilis* and *E. coli*. The four metals were rapidly bound by all three strains although the amounts varied. The amount of metals bound to *B. simplex* ZAN-044 at 2 h was 61, 67, 87, and 54% of the amount at 4 h for Ni^{2+}, Co^{2+}, Cd^{2+} and Sr^{2+}, respectively. We chose 2- and 4-h sampling times for the remainder of the study based on these results and also because a previous study of metal sorption by *B. subtilis* and *E. coli* used a 2-h sampling [29].

The relative amounts of cations bound by the three strains was Cd > Ni $\geq$ Co > Sr. *B. simplex* ZAN-044 bound the greatest amount of metal per unit biomass among the three strains. Binding of Cd^{2+}, Co^{2+}, Ni^{2+} and Sr^{2+} at pH 6 by *B. simplex* ZAN-044, *B. subtilis*, and *E. coli* was, in general, linear from 0.01 to 100 μM when expressed on a log-log plot (data not shown). At 1000 μM metal, biosorption was no longer linear. A Freundlich isotherm was used to model metal binding by the three strains after 2 h (Table 1). Results from the 4-h samplings were similar. In all cases the K values for *B. simplex* ZAN-044 were higher than for *B. subtilis* and *E. coli* (Table 1). The sorption of Co and Ni by *B. simplex* ZAN-044 was ~ 2-fold higher than *E. coli* and *B. subtilis*. The sorption of Cd by the organism was ~ 3-fold higher than *B. subtilis* and ~ 2-fold higher than *E. coli*. The sorption of Sr by *B. simplex* ZAN-044 was > 1000-fold higher than for *B. subtilis* or *E. coli*, which bound only trace quantities.

Influence of pH on Cd, Co, Ni, and Sr binding

The maximum binding of Cd^{2+}, Co^{2+}, Ni^{2+} and Sr^{2} by *B. simplex* ZAN-044 was found at pH 9, with the lowest adsorption of these four metals at pH 4 (Figure 4). Results from the 2- and 4-h samplings were very similar, so only the 2-h data are shown. The organism did not bind metals well at pH 4, but binding increased as a function of pH. Binding of Ni^{2+}, Co^{2+} and Sr^{2+} at pH 5 was 3 to 4 times higher than that of *B. subtilis* and *E. coli*. Binding of Cd^{2+} at pH 5 was 2-fold higher than for *B. subtilis* and *E. coli*.

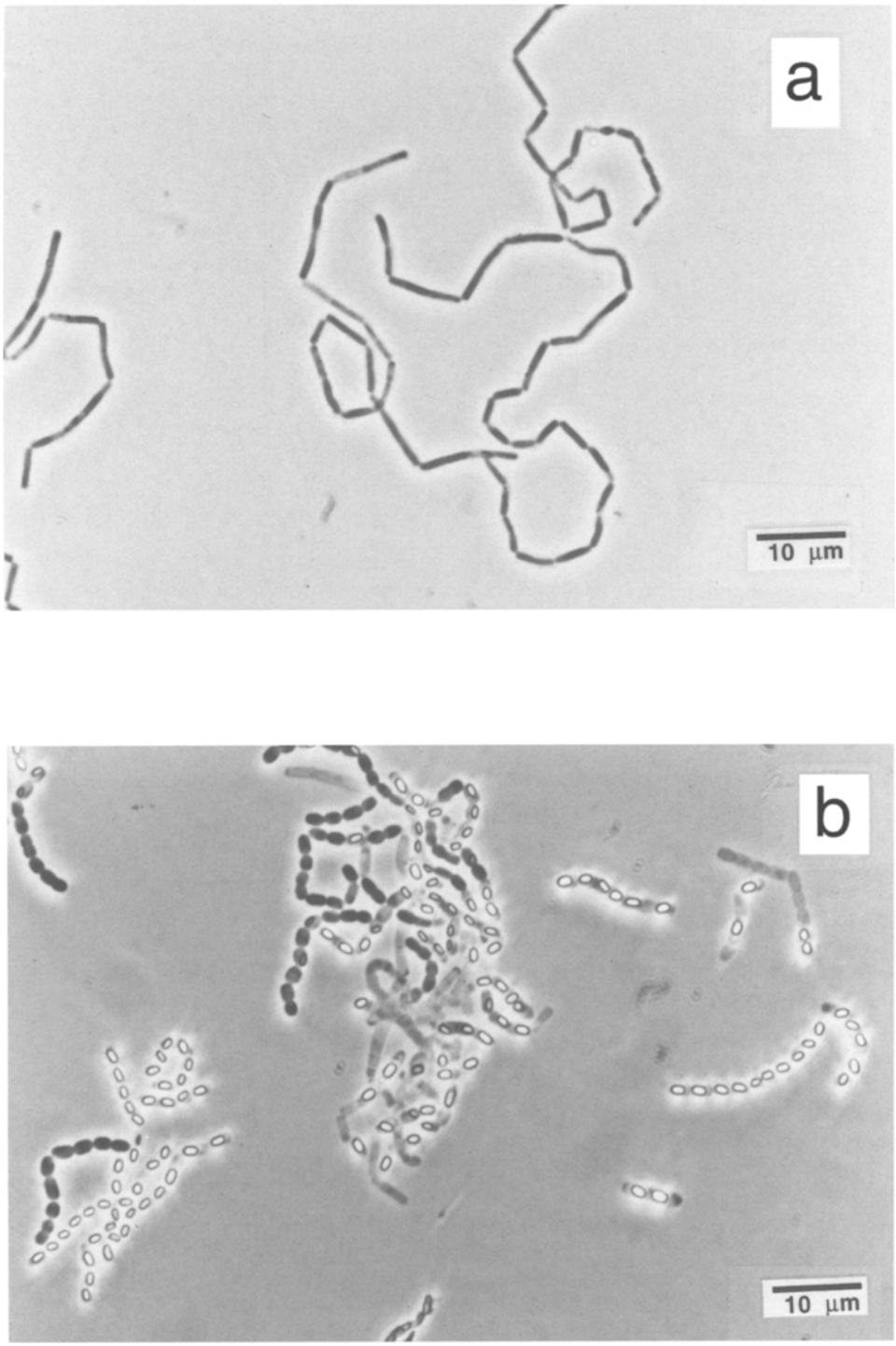

Figure 1 Vegetative cells (a) and endospores (b) of isolate ZAN-044 viewed with phase contrast microscopy.

Active/passive uptake of metals

Uptake of divalent metal ions by microorganisms in our studies was most likely a passive binding process, since the cells were washed twice with buffer and no exogenous nutrients were present during our assays. The addition of the metabolic inhibitors 1 mM sodium azide or 0.1 mM dinitrophenol or the energy source 10 μg ml^{-1} glucose did not change the amount of metal bound by *B. simplex* ZAN-044 at either pH 6 or 7. This also suggested a passive process for metal binding in our studies. Finally, heat-killed cells removed more Cd, Co, and Ni from solution than vegetative cells (Table 2), further suggesting a passive binding process for metal removal from solution. Heat-killed cells removed significantly less Sr than vegetative cells.

Mobilization of metals with EDTA

Greater than 70% of the Cd, Co, and Ni associated with *B. simplex* ZAN-044 was released into solution after a 1-min

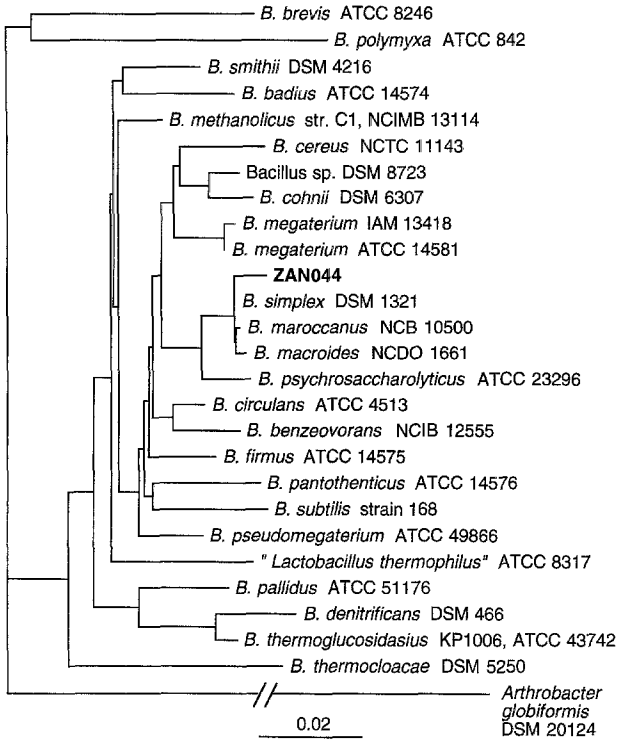

Figure 2 Phylogenetic tree for isolate ZAN-044 and 26 species of eubacteria, based on 16S rDNA distance matrix analysis. *Arthrobacter globiformis* was used as the outgroup; its branch was approximately 1.5 times longer than shown in the figure. The following regions were not included in the analysis because the alignment was ambiguous or there were gaps in some of the sequences: *E. coli* positions 1–280, 462–470, 841–845, 1025–1036, and 1376+. (1069 bases were retained for analysis.) Scale bar indicates two substitutions per 100 nucleotides.

exposure to 50 μM EDTA (data not shown). The amount of Cd, Co, and Ni released into solution increased as a function of time to 90–97% at 4 h (Table 3). The time required to mobilize Sr from *B. simplex* ZAN-044 by EDTA was longer than for Cd, Co, or Ni. For the Sr treatment, the control cells released Sr into solution without EDTA being present. Thus when the data were calculated based on the control, little increase in Sr mobilization was found with the addition of EDTA. As the EDTA concentration increased, a greater amount of all four metals was released from cells into solution (Table 3).

Discussion

This is one of the first studies that investigated metal biosorption by a subsurface microorganism. The 16S RNA gene sequence analysis indicated that strain ZAN-044 was closely related to *B. simplex* (DSM 1321; ATCC 49097) (Figure 2). *B. simplex* (DSM 1321, ATCC 49097) was isolated from soil [19] and isolate ZAN-044 was isolated from the vadose zone at 96.2 m below the soil surface of the Hanford Site. Fredrickson *et al* [18] suggested that sediments of the depth where *B. simplex* ZAN-044 was isolated do not currently receive moisture from artificial or natural recharge. They suggested that the origin of the microorganisms present was either the sediments at the time of their deposition or transport from the surface approximately 13000 years ago during the last major proglacial flood.

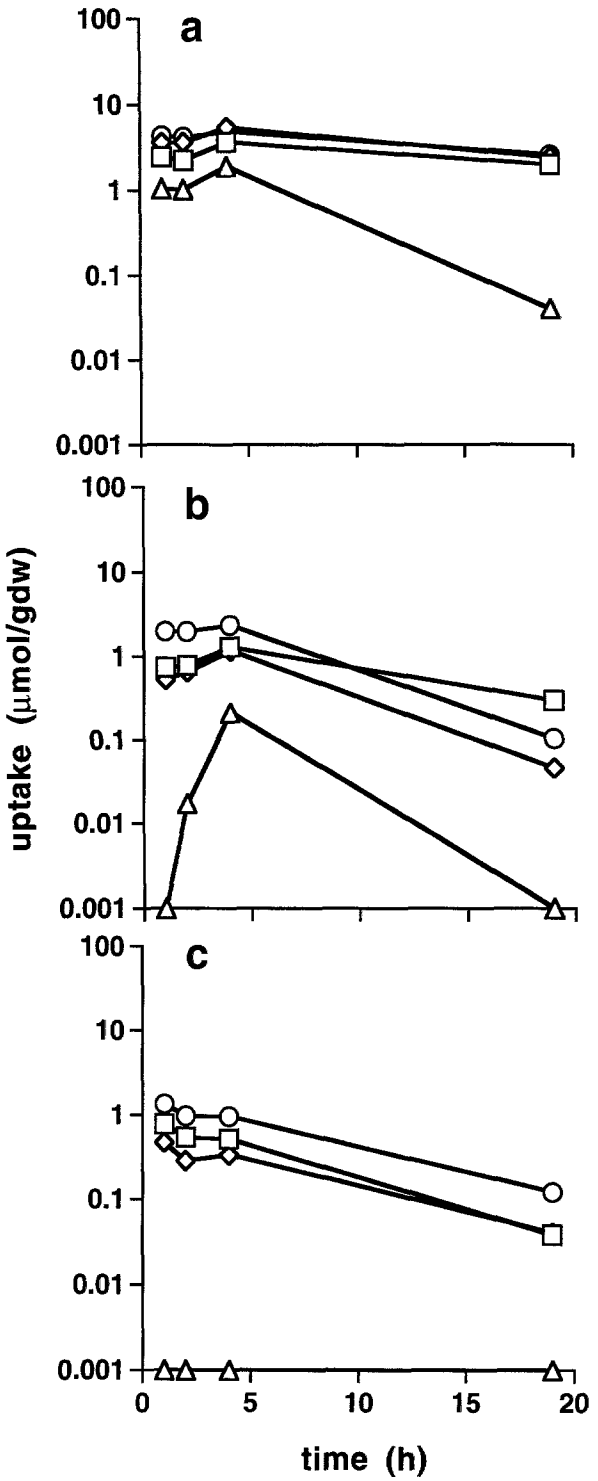

Figure 3 Biosorption of 1 μM Cd (○), Co (◇), Ni (□), and Sr (△) by *B. simplex* ZAN-044 (a), *B. subtilis* (b), and *E. coli* (c) as a function of time.

There were 80 culturable microorganisms g^{-1} recovered from the vadose zone sediment sample directly above 96 m [18]. Viable counts for the sediment at 96 m were lower; in fact, viable microorganisms were detected only as colonies growing directly from sediment sprinkled onto 10% PTYG plates (JK Fredrickson, Pacific Northwest National Laboratory, Richland, WA, USA, personal communication). Thus,

Table 1 Freundlich isotherms[a] for the binding of cadmium, cobalt, nickel, and strontium by *B. simplex* ZAN-044, *B. subtilis*, and *E. coli*[b]

Metal	Bacterium	K	n	r^2
Cd	ZAN-044	10.57	0.705	0.976
	B. subtilis	3.81	0.609	0.949
	E. coli	5.05	0.703	0.986
Co	ZAN-044	4.56	0.749	0.992
	B. subtilis	2.12	0.813	0.961
	E. coli	2.58	0.796	0.986
Ni	ZAN-044	6.47	0.678	0.988
	B. subtilis	2.94	0.744	0.952
	E. coli	3.28	0.747	0.977
Sr	ZAN-044	0.69	0.903	0.994
	B. subtilis	0.002	−0.120	0.067
	E. coli	0.003	−0.187	0.067

[a]($\log_{10}S = \log_{10}K + n\log_{10}C$) where the parameters include: S, the quantity of metal adsorbed in μmol g^{-1} dw; C, the equilibrium solution concentration in μmol L^{-1}; n, the slope of the line; and K, the concentration of metal associated with the cell in μmol metal g^{-1} dw when the equilibrium solution concentration is 1 μM.
[b]Metal concentrations from 0.1 to 100 μM were used. Binding assays were conducted for 2 h.

the number of culturable bacteria in this sediment was very low. In sediments from this borehole at the Hanford site, 21 out of 43 microbial isolates assayed from the vadose zone were spore-formers, while none of 24 isolates assayed from the saturated zone was a spore former (data not shown). This indicates that spore formation is important in the long-term survival of microorganisms in the vadose zone at this arid site.

Isolate ZAN-044 was not the only *B. simplex*-like isolate

Table 2 Binding of Cd, Co, Ni, or Sr by live and heat-killed[a] cells of *B. simplex* ZAN-044[b]

Treatment	Metal biosorbed (μmol g^{-1} dw)			
	Cd	Co	Ni	Sr
Live cells	14.21[b]	7.91a[c]	7.56a	3.04
Heat-killed cells	15.87[b]	10.17	13.49	1.19

[a]Cells were heated at 80°C for 15 min.
[b]Binding assays were conducted for 2 h.
[c]Means followed by the same letter are not significantly different ($P \leq 0.05$; $n = 2$).

Table 3 Influence of EDTA concentration on the mobilization of Cd, Co, Ni, and Sr bound by *B. simplex* ZAN-044[a]

Metal	Percent of control in presence of EDTA at (μm)		
	50	200	500
Cd	90.0	95.1	95.7
Co	97.6	99.6	99.8
Ni	96.6	99.8	99.9
Sr	57.9	86.2	100

[a]Mobilization assays were conducted for 4 h and results are presented as a percent of the control without *B. simplex* ZAN-044 present.

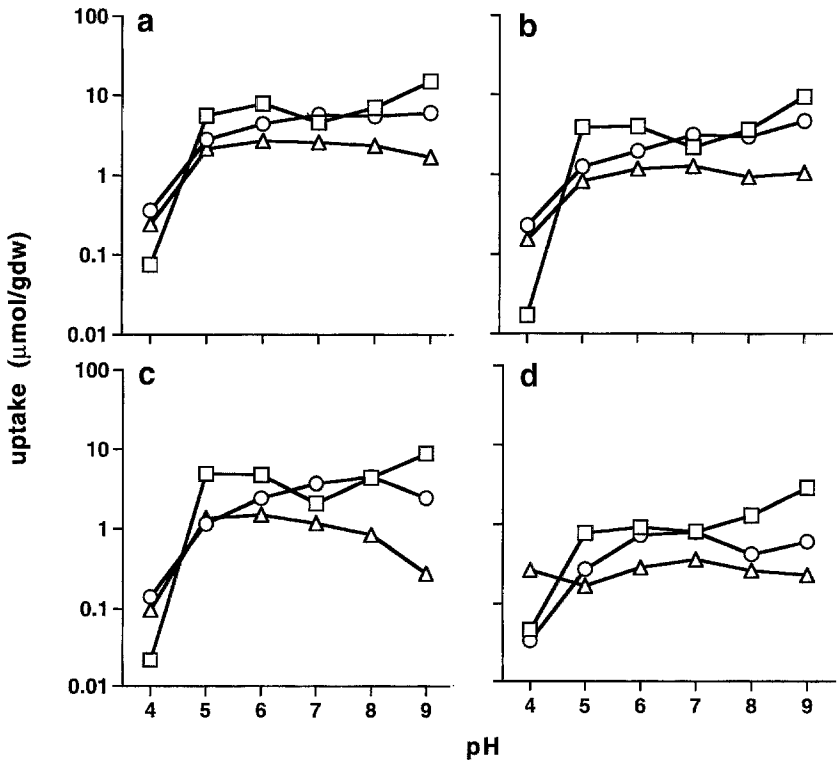

Figure 4 Biosorption of 1 μM Cd (a), Co (b), Ni (c), and Sr (d) by *B. simplex* ZAN-044 (□), *B. subtilis* (○) and *E. coli* (△) as a function of pH.

from this borehole. Isolate ZAN-032, isolated from the same depth as *B. simplex* ZAN-044 (JK Fredrickson, personal communication), had all but 4 16S rDNA bases identical to *B. simplex* ZAN-044 (over 472 bases compared), and thus the two organisms are very closely related. These two isolates were from the Ringold formation—fluvial-lacustrine deposits ranging in age from 4 to 10.5 million years [18]. A second borehole approximately 14.5 km south of the one where *B. simplex* ZAN-044 and isolate ZAN-032 were isolated (EM Murphy, Pacific Northwest National Laboratory, Richland, WA, personal communication) also contained two *B. simplex* ZAN-044-like isolates at 2.7 and 4.6 m depths (data not shown). These two isolates were 99.3 and 99.6% similar to *B. simplex* ZAN-044 (over 1096 bases) and are most likely members of the same species. This demonstrates that *B. simplex* ZAN-044-like organisms are spatially distributed vertically as well as horizontally in the Hanford vadose zone.

All three microorganisms studied here had a relative affinity for Cd > Ni ≥ Co > Sr (Table 2). There was no apparent relationship between binding of the various metals to bacteria and their ionic radii or Gibbs free energy of hydration as was also found by Beveridge *et al* [6] with *B. subtilis*.

The concentration of biomass used in our studies (130–400 μg dry wt ml^{-1}) exhibited linear uptake of metals to a 100 μM concentration. Above this concentration, saturation of the metal-binding sites occurred. Scott and Palmer [34] compared the effect of Cd concentration on Cd binding by five different bacteria and concluded that three of the five reached external adsorption saturation as the concentration approached 200 μM. Premuzic *et al* [31] noted that there is a limiting factor to the amount of metal that can be taken up regardless of the concentration of the metal in solution and they concluded that metal uptake was not a simple adsorptive process but was also affected by the chemical organization of the cell wall.

The binding of Sr to cell walls of *Micrococcus luteus* is a passive process [12]. *M. luteus* showed a high affinity for Sr^{2+}. Of the three microorganisms we studied, only *B. simplex* ZAN-044 demonstrated significant binding of Sr^{2+}.

Binding of metals by the three microorganisms generally increased as pH increased (Figure 4). Premuzic *et al* [31] and Cotoras *et al* [10] found this for Pb binding to *E. coli* (not the K-12 strain) and Cd binding to *B. subtilis*, respectively. With *B. simplex* ZAN-044, neutral to alkaline pH values seem to be the optimum for metal uptake, indicating variably charged protonation sites (eg carboxylate, phosphate, amino groups) were responsible for metal uptake. *B. simplex* ZAN-044 was isolated from a sediment of pH~8. *B. simplex* ZAN-044-like microorganisms should therefore sorb metals efficiently at the native sediment pH in the subsurface of the Hanford Site if their growth could be stimulated.

For the use of microorganisms as biosorbents, it is important to determine whether bound metals could desorb. In analyzing the four metals in our study, we determined that the desorption of metals from *B. simplex* ZAN-044 by EDTA was rapid and could be enhanced as the concentration of EDTA was increased (Table 3). The desorption of metals from *B. subtilis* [16] and *M. luteus* [12] by EDTA has also been shown.

In summary, *B. simplex* ZAN-044 bound more metal than *B. subtilis* or *E. coli*, demonstrating that subsurface microorganisms can remove significant quantities of metals from solution. Bacteria closely related to *B. simplex* ZAN-044 appear to be common in the Hanford Site vadose zone based on phylogenetic analysis of other isolates obtained from various vadose samples from the site. A strategy for immobilizing radionuclude and metal contaminants might be to stimulate *in situ* growth of *B. simplex* ZAN-044-like bacteria. The increased biomass could then biosorb metals and radionuclides associated with contaminated water as it percolates through the vadose zone, limiting contamination of groundwater.

Acknowledgements

This research was supported by the Subsurface Science Program, Office of Health and Environmental Research, US Department of Energy (DOE). The continued support of Dr Frank Wobber is greatly appreciated. Pacific Northwest Laboratory is operated for the DOE by Battelle Memorial Institute under Contract DE-AC06-76RLO 1830.

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