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The potential of subterranean microbes in facilitating actinide migration at the Grimsel Test Site and Waste Isolation Pilot Plant

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Summary. Microorganisms may affect the long-term stability and mobility of radionuclides disposed of in deep geological formations. Of particular concern is the association of radionuclides with subterranean microbial cells and their subsequent transport as biocolloids. We characterized the total microbial populations in two groundwater samples: one from the Culebra dolomite member of the Rustler Formation at the Waste Isolation Pilot Plant (WIPP), NM, and the other from the granitic formation at the Grimsel Test Site (GTS), Switzerland. Culebra groundwater (ionic strength 2.8 M, pH 7) contained $1.51 \pm 1.08 \times 10^5$ cells ml^{-1}, with a mean cell length of 0.75 ± 0.04 μm and width of 0.58 ± 0.02 μm. In contrast, low ionic-strength GTS groundwater (0.001 M, pH 10) contained $3.97 \pm 0.37 \times 10^3$ cells ml^{-1}, with a mean cell length of 1.50 ± 0.14 μm and width of 0.37 ± 0.01 μm. Adding appropriate electron donors and acceptors to the groundwaters facilitated the growth of aerobic, denitrifying, fermentative, and acetogenic microorganisms. Uranium biosorption was studied in two isolates from these groundwaters, as well as several pure cultures from saline and non-saline environments. Halophilic and non-halophilic bacteria exhibited differences in the amount of U associated with the cells. Plutonium uptake by *Acetobacterium* sp. isolated from GTS varied from 30–145 pg of Pu mg^{-1} dry weight of cells.

Introduction

A significant portion of nuclear wastes is expected to be stored in deep geological formations such as the salt deposits at the Waste Isolation Pilot Plant (WIPP) repository and deep subsurface granite formations [1]. Groundwater is expected to be the principal medium for the migration of radionuclides from the repositories. Bacteria exist in the groundwaters of the repository environments investigated: Grimsel, Switzerland; Stripa Mine, Sweden; WIPP and Yucca Mountain, USA [2–6]. We have only limited information on their influence on the mobilization or immobilization of radionuclides in near-field and far-field environments [6–8]. Microbes may directly or indirectly affect the transport and retardation of dissolved actinides [9].

Colloidal transport of radionuclides is recognized as a potential mechanism of migration and includes mineral fragments, humic substances, intrinsic colloids, and microorganisms [10–13]. Microorganisms fall within the colloidal size range of 1 nm to 1 μm, and can be transported with bulk liquid flow [14]. In addition, biomass accumulated on subsurface materials (biofilms) can generate biocolloids via detachment. Microbial surfaces consist of peptidoglycan/techoic acid layers with an overall negative charge due to hydrophilic anionic functional groups, such as phosphate, carboxylate, and hydroxyl moieties, giving bacteria considerable ability to bind actinides [15–18]. Free-living bacteria are mobile suspended particles that may have a radionuclide-sorbing capacity higher than that of the surrounding mineral phases [19]. In this paper, we report the microbial populations in the groundwater of the WIPP far-field (Culebra dolomite, WIPP, NM) and the Grimsel Test Site (Switzerland) near-field, and the ability of selected microorganisms to sorb uranium and plutonium thus forming potential colloidal transport agents for radionuclides.

Materials and methods

Collection of groundwater samples from Culebra H19B Hydropad and Grimsel Site

The WIPP is a US Department of Energy (DOE) facility located 23 km northeast of Carlsbad, NM, licensed to demonstrate the safe, permanent disposal of transuranic waste. The WIPP facility is constructed 658 m below land surface in a bedded salt formation. One potential far-field transport pathway for actinides is the Culebra dolomite member of the Rustler Formation. The Culebra is 14 m thick and lies about 200 m below land surface. The Grimsel Test Site (GTS) is a facility operated by the Swiss National Cooperative for the Disposal of Radioactive Waste (NAGRA). The GTS is ∼ 450 m below land surface in a granitic formation of the Swiss Alps. The GTS site serves as a research facility for the European radioactive-waste disposal concept.

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Groundwater from the Culebra H19B hydropad was allowed to run until clear of sediment and then collected into precleaned (acid-washed and rinsed) and sterile (autoclaved at 121 °C, 20 psi) 160-ml serum bottles containing 2% v/v formalin. Precleaned sterile 1 L glass bottles with polypropylene caps and teflon liners were used to collect large volumes (10 L) of unpreserved groundwater. Groundwater from the GTS was collected from borehole BOMI 86.004. Water flowed through a stainless-steel extension on the existing borehole sample port for 10 minutes at a free-flow rate of $\sim 300\ \mathrm{ml\ min^{-1}}$. Preserved and unpreserved water samples were collected. The groundwater samples were packed on ice, and shipped to BNL via overnight delivery. Upon receipt, the samples were stored at 4 ± 1 °C until further use.

Enumeration of total bacteria and their size determination

Formalin preserved samples were brought to room temperature and an aliquot was stained with the DNA-specific fluorochrome 4′6-diamidino-2-phenylindole (DAPI, Sigma Chemical Co.) [20]. The bacteria in the samples were counted and 50 cells were measured at $1250 - 2000\times$ magnification using a Zeiss Axiophot microscope equipped for epifluorescence microscopy and image analysis [6].

Effect of adding nutrients on the growth of bacteria

To study the growth capacity of the Culebra and GTS bacteria, unpreserved groundwater was amended with three different nutrient media designed to stimulate the growth of (i) general heterotrophs, (ii) fermenters, and (iii) denitrifiers. The media contained the following ingredients ($\mathrm{g\,l^{-1}}$ DI H_2O): (i) peptone, 5, yeast extract, 1, ferric citrate, 0.1, Na_2SO_4, 3.24, $CaCl_2$, 1.8, KCl, 0.55; (ii) glucose, 5, NH_4Cl, 1, glycerol phosphate, 0.3, $MgSO_4 \cdot 7H_2O$, 0.2, $CaCl_2 \cdot 2H_2O$, 0.5, peptone, 0.1, and yeast extract, 0.1; and (iii) sodium succinate, 5, K_2HPO_4, 0.25, KNO_3, 1. The pH of the media were adjusted to 7.0, filter-sterilized (0.2 μm), and added to 10 ml of groundwater to give a 1 : 100 dilution. All manipulations were carried out under anaerobic conditions in a N_2 atmosphere. Samples were incubated at 30 ± 2 °C and examined by epifluorescence microscopy after 1 and 12 days.

In addition the populations of iron-reducing and sulfate-reducing bacteria were examined in GTS groundwater samples only using the following media ($\mathrm{g\,l^{-1}}$ DI H_2O): sodium acetate, 6.8, NH_4Cl, 1.5, $NaH_2PO_4 \cdot H_2O$, 0.6, ferric pyrophosphate, 1.5 (for iron-reducing bacteria); sodium lactate, 3, Na_2SO_4, 2.25, KH_2PO_4, 0.5, NH_4Cl, 1, $CaCl_2 \cdot 2H_2O$, 0.06, $MgSO_4 \cdot 7H_2O$, 0.06, $FeSO_4 \cdot 7H_2O$, 0.002, sodium citrate, 0.3, yeast extract, 1 (for sulfate-reducing bacteria). The samples were incubated at room temperature at the pH of the groundwater (9.9).

Identification of cultures

The microbial population in the GTS groundwater, as received, was analyzed by polymerase chain reaction (PCR) amplification of 16S rDNA gene fragments, then sequenced by denaturing gradient gel electrophoresis (DGGE) [21, 22]. In addition, using this technique, two isolates – one from GTS groundwater grown in fermenter medium, and one in sulfate-reducer medium – were identified using the BLASTN facility of the National Center for Biotechnology Information.

Biosorption of uranium

Uranium uptake was examined in a pure culture isolated from the Culebra groundwater amended with succinate and nitrate (denitrifying culture "CDn"), and in an *Acetobacterium* sp. isolated from GTS groundwater (cultured in sulfate-reducer medium described above). For comparison, we studied the following halophilic and non-halophilic type strains: *Halobacterium halobium* (American Type Culture Collection (ATCC) 43214, *Haloanaerobium praevalens* ATCC 33744, a *Halomonas* sp. (WIPP-1A) isolated from the WIPP repository [6], *Pseudomonas fluorescens* ATCC 5524, and *Bacillus subtillis* ATCC 27370 (from Dr. T. Beveridge, U. of Guelph, Canada). The cultures were grown in appropriate media at 30 ± 2 °C to late log phase, the cells harvested by centrifugation at $5000 \times g$, and washed $2\times$ with the appropriate NaCl electrolyte at pH 5 (see Table 4 for NaCl concentration). Cells were resuspended in electrolyte (pH 5.0) to an optical density (OD) of $0.4 - 1.6$ as measured at 600 nm, and purged with nitrogen. One milliliter of a stock solution of uranyl nitrate (8 mM $UO_2(NO_3)_2 \cdot 6H_2O$), pH 5.0, was added to each aliquot of cells to give a final concentration of 0.2 mM U ($50\ \mu\mathrm{g\,ml^{-1}}$). The pH was maintained at 5.0 ± 0.2 using a Mettler DL-53 automated titrator. Cells plus uranium were in contact at room temperature in a nitrogen atmosphere for 90 minutes, after which 10 ml was filtered through a 0.45 μm syringe filter into a plastic vial and acidified with 0.1 ml of conc. HCl. Uranium was analyzed by ICP-AES (Varian Instrument Corp.). One milliliter of the sample was preserved with 10% v/v formalin for bacterial-cell counts. The dry weight of the cells was determined after drying at 90 ± 2 °C overnight. A cell-free brine solution at pH 5 containing 0.2 mM uranyl nitrate in the appropriate electrolyte was incubated for 90 minutes as a control for chemisorption to the incubation vessel, filters, and precipitation. Analysis of uranium after 0.45 μm filtration showed that all of the added U remained in solution.

Biosorption of plutonium

A ^{241}Pu-nitrate standard (Isotope Products Laboratories (Burbank, CA, USA), isotopic purity = 99%) was diluted to give a stock solution of 1.8×10^{-8} M (0.4 μCi/ml). Plutonium-241 was used so that very low concentrations (10^{-10} M) could be detected by β-liquid scintillation counting (LSC) using a Packard model A2550; interference from the α-decay of the daughter, ^{241}Am, was eliminated by pulse-shape analysis. The oxidation state of Pu was not determined, however, at these low concentrations, at near neutral pH, and in the absence of alpha-radiolysis it is likely that Pu was in the pentavalent state. Plutonium uptake by *Acetobacterium* sp. (OD 0.4) was examined at a concentration of 1 to 15×10^{-10} M in 0.1% w/v NaCl, with a 15–20 minute equilibration time between additions. All manipulations were done inside a gloved box using a Mettler DL-55 automated

titrator. One milliliter aliquots were removed after each equilibration period and reserved as-is or filtered (0.2 μm nylon syringe filter (Whatman Autovial)) into a pre-weighed 22 mL glass vial. Samples were counted by LSC with 15 mL of Ultima Gold XR scintillation cocktail and 4 ml water. Plutonium was added to a cell-free brine solution to determine chemisorption.

Results

Table 1 presents the chemical composition of Culebra and GTS groundwater. Culebra groundwater had an ionic strength of 2.8 M, pH 7, while GTS water was 0.0012 M, pH 9.95. Table 2 shows the total number of bacteria, and the length, width, and volume of individual cells in Culebra and GTS groundwaters. Culebra groundwater contained $1.51 \pm 1.08 \times 10^5$ cells ml^{-1} and many large clumps of inorganic material with a mean length of 3.68 ± 0.25 μm and a mean width of 2.22 ± 0.14 μm. This material is evident as large irregular-shaped flocs (Fig. 1, Panel A); the small rod- and coccoid-shaped forms in Panel A are the bacterial cells with a mean length of 0.75 ± 0.04 μm and a width of 0.58 ± 0.02 μm.

The growth of the bacterial population in the Culebra groundwater is given in Table 3. The number of bacteria in unamended samples incubated aerobically or anaerobically did not increase. Adding nutrients to the groundwater incubated under aerobic or anaerobic conditions increased their numbers in 24 hours by two orders-of-magnitude, as shown in Fig. 1 (B-D).

Table 1. Chemical composition of Culebra and GTS groundwater [12, 23].

Constituents[a]	Culebra GW (Sample H17)	GTS GW (Sample B)
Na^+	2300	0.31
K^+	28	0.012
Ca^{2+}	42	0.18
Mg^{2+}	74	< 0.001
Cl^-	2500	0.023
HCO_3^-	1	0.12
SO_4^{2-}	77	0.11
Ionic strength (M)	2.8	0.0012[b]
Total cations (meq l^{-1})	2549	0.99
Total anions (meq l^{-1})	2675	1.0
Temp. (°C)	n/a	11.4
pH	7	9.95

a: mM except where otherwise noted
b: [29].
n/a = not analyzed

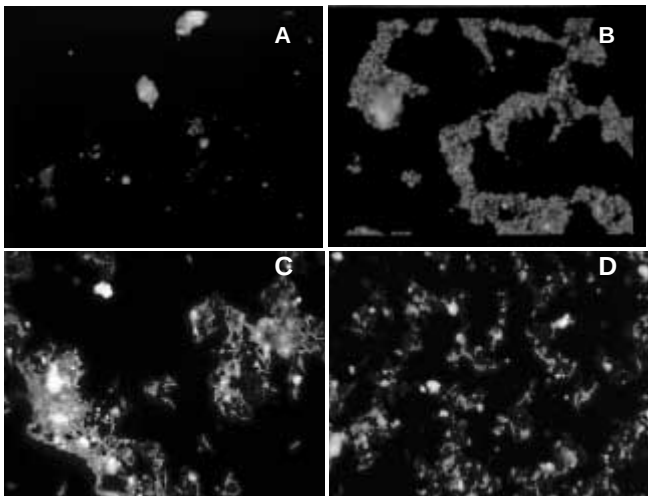

Fig. 1. Epifluorescence micrographs of (**a**) Culebra groundwater without nutrient amendment, (**b**) after 288 hours incubation with the addition of glucose and ammonium chloride, (**c**) after 288 hours incubation with the addition of succinate and nitrate and (**d**) peptone and yeast extract (1250× magnification).

Table 3. Growth of bacteria in Culebra groundwater in response to various nutrient additions.

Treatment	Hours[a]	
	24	288
	Number of cells ml^{-1}	
Anaerobic:		
Unamended	$6.82 \pm 1.33 \times 10^{4}$[b]	$1.19 \pm 0.19 \times 10^5$
Heterotroph Medium	$1.66 \pm 0.13 \times 10^7$	$1.43 \pm 0.08 \times 10^7$
Denitrifier Medium	$3.58 \pm 0.22 \times 10^7$	$4.37 \pm 0.61 \times 10^7$
Fermenter Medium	$1.87 \pm 0.11 \times 10^7$	$6.62 \pm 0.53 \times 10^7$
Aerobic:		
Unamended	$6.39 \pm 1.84 \times 10^4$	n/a
Heterotroph Medium	$3.98 \pm 0.18 \times 10^6$	$9.85 \pm 0.93 \times 10^6$

a: In all cases, the initial number of cells ml^{-1} was $1.51 \pm 1.08 \times 10^5$.
b: Mean ± standard error of the mean.
n/a = not analyzed

In contrast, the GTS sample contained $3.97 \pm 0.32 \times 10^3$ cells ml^{-1}, and most had an elongated morphology. Adding nutrient increased the bacterial population within 6 days. Fig. 2 shows the unamended sample (Panel A), the elongated rod-shaped bacteria in the groundwater (Panel B), and a sample amended with glucose and ammonium chloride at 6 days (Panel C). A *Clostridium* sp. (Fig. 2, Panel D), able to ferment glucose under anaerobic conditions, and an *Acetobacterium* sp., capable of using HCO_3^- and H_2 for

Table 2. Enumeration of the total bacterial population and measurements of individual cell size and volume.

Sample	Total number of cells ml^{-1}[a]	Length (μm)	Width (μm)	Volume (μm^3)	Mean cell shape[b]
Culebra	$1.51 \pm 1.08 \times 10^5$	0.75 ± 0.04	0.58 ± 0.02	0.21 ± 0.01	0.77
Grimsel	$3.97 \pm 0.37 \times 10^3$	1.50 ± 0.14	0.37 ± 0.01	0.19 ± 0.03	0.24

a: Mean ± standard error of the mean of 50 measurements.
b: Cell shape = width/length.

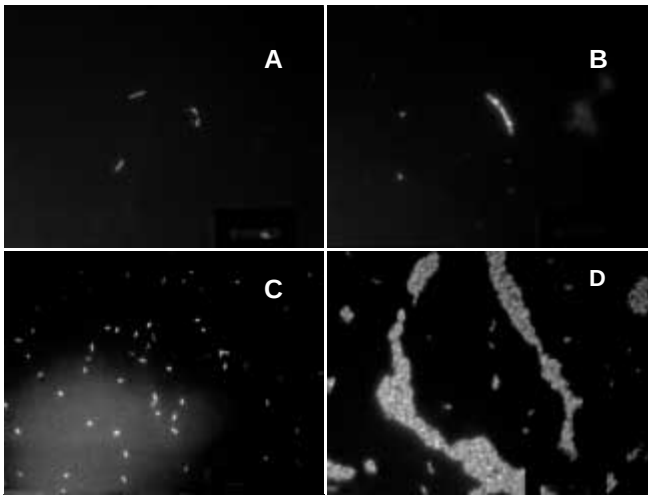

Fig. 2. Epifluorescence micrographs of the Grimsel groundwater as received (**a**) 1250× magnification (**b**) 2000×. (**c**) Sample amended with succinate and nitrate incubated anaerobically for 6 days, and (**d**) a fermentative anaerobic culture (*Clostridium* sp.) isolated from the groundwater (1250× magnification).

growth were isolated from glucose/ammonium nitrate- and lactate/sulfate-amended groundwater, respectively.

Table 4 summarizes uranium biosorption by the isolates from Culebra and GTS, and by various halophiles and non-halophiles. At pH 5.0, the CDn isolate ($0.90 \pm 0.02 \times 10^8$ cells ml^{-1}) removed 32% of the added uranium (180 ± 10 mg U g^{-1} of dry cells), and *Acetobacterium* sp. ($3.55 \pm 0.11 \times 10^8$ cells ml^{-1}) sorbed 21% (70 ± 2 mg U g^{-1} of dry cells). Other cultures sorbed more uranium, specifically *H. halobium* which removed 90% of the added U. In general more U was removed by microorganisms from saline media (e.g. 0.15 pg U cell^{-1} (CDn) vs. 0.02 pg U cell^{-1} (*P. fluorescens*))(Table 4). Determining the amount of Pu taken up by *Acetobacterium* sp. ($1.28 \pm 0.12 \times 10^8$ cells ml^{-1}) was problematic due to significant filterable Pu ($\sim$ 80%) (Table 5); the amount was an apparent function of the Pu added. The maximum amount removed was 145 pg Pu mg^{-1} dry cells. No decrease in uptake with increasing Pu concentration was observed, therefore, the cells can probably sorb more Pu (surface sorption sites were not saturated).

Discussion

The size of the microbial cells in both the Culebra and GTS groundwater falls within the boundaries set for colloids. Studies of the effect of bacterial-cell shape and size on transport through columns packed with clean quartz sand (0.75 mm grain size) showed that the mean cell length was greater in column's influent (1 μm) vs. effluent ($\sim$ 0.5–0.7 μm); however, cell shape was postulated to be the most important factor governing transport (cells eluted from a quartz sand column were more spherical than those suspended in the influent) [25]. We observed that Culebra cells were spherical, while those in the Grimsel sample were elongated and appear almost stalked (they may have been attached to rock surfaces). Both groundwaters can be considered oligotrophic (low-nutrient) environments; the cells' sizes most likely reflect this condition. The length of starved cells of *Shewanella alga* BrY in culture decreased from $\sim$ 2.25 μm to $\sim$ 1.0 μm in

Table 4. Uranium biosorption by groundwater and selected bacterial isolates.

Isolate	Number of cells ml^{-1}	Uranium added μg ml^{-1}	Uranium removed from Sol'n by cells[a] μg ml^{-1}	Uranium association per cell[b] pg cell^{-1}
WIPP:				
Culebra denitrifier CDn (5% w/v NaCl)[c]	$0.90 \pm 0.02 \times 10^8$	42.9 ± 1.5	13.9 ± 0.5 (32%)	0.15
Halomonas sp. (WIPP-1A) (20% w/v NaCl)	$2.2 \pm 0.3 \times 10^8$	29.4 ± 2.1	24.8 ± 3.2 (79%)	0.11
Grimsel:				
Acetobacterium sp. (0.1% w/v NaCl)	$3.55 \pm 0.11 \times 10^8$	56.8 ± 0.5	12.0 ± 0.8 (21%)	0.03
Haloanaerobium praevalens (ATCC 33744) (13% w/v NaCl)	$4.8 \pm 0.2 \times 10^8$	46.4 ± 2.0	33.9 ± 1.5 (73%)	0.07
Halobacterium halobium (ATCC 43214) (25% w/v NaCl)	$0.75 \pm 0.01 \times 10^8$	48.0 ± 2.7	43.2 ± 2.4 (90%)	0.57
Bacillus subtilis (ATCC 27370) (0.6% w/v NaCl)	$8.6 \pm 0.5 \times 10^8$	32.1	28.0 (87%)	0.03
Pseudomonas fluorescens (ATCC 55241) (0.6% w/v NaCl)	$17 \pm 6 \times 10^8$	32.1	27.7 (86%)	0.02

a: Number in parenthesis is percent of uranium biosorbed.
b: Uranium association per cell = uranium removed from solution by cells/number of cells per ml.
c: Concentration of NaCl required for growth and in electrolyte used for biosorption experiments.

Table 5. Plutonium uptake by *Acetobacterium* sp. isolated from Grimsel groundwater.

^{241}Pu concentration [M]	^{241}Pu Filter retentate (control)[a] [M]	^{241}Pu Filter retentate (cells)[a] [M]	^{241}Pu biosorbed [M]	^{241}Pu association (ag Pu cell^{-1})[b]
$1.4 \pm 0.2 \times 10^{-10}$	1.1×10^{-10} (78%)[c]	1.3×10^{-10} (92%)	0.2×10^{-10} (14%)	1.5
$2.6 \pm 0.4 \times 10^{-10}$	1.8×10^{-10} (69%)	2.1×10^{-10} (81%)	0.3×10^{-10} (12%)	2.3
$4.8 \pm 0.6 \times 10^{-10}$	3.8×10^{-10} (79%)	n/d	n/d	n/d
$15 \pm 2 \times 10^{-10}$	11×10^{-10} (73%)	12×10^{-10} (80%)	1.0×10^{-10} (7%)	7.5

a: Standard error of the mean $< 0.1 \times 10^{-10}$ M, Biosorbed = cell retentate − control retentate.
b: 6.67 mg cells tested, $1.28 \pm 0.12 \times 10^{8}$ cells ml^{-1}.
c: Numbers in parentheses are the amount (% of total) of plutonium retained or associated with biomass.
n/d = not detected

9 weeks [26]. We believe that because the samples were collected from free-flowing groundwater, the cell sizes reported are the best measurement of the population that will be transported through the groundwaters at these two sites.

Bacteria in both groundwaters grew readily when nutrients were added. Previous studies of the microbial population (3×10^4 cells ml^{-1}) in GTS groundwater identified aerobic- and anaerobic-heterotrophs, including denitrifying bacteria and iron-reducing bacteria [23]. Microbial populations in deep granitic aquifers, 70 m to 1240 m, similar to that at GTS have been studied at two sites in Sweden: the Stripa research mine, and the Aspo hard rock laboratory [3, 4]. Microbes that use hydrogen as an energy source and carbon dioxide as their sole carbon source (homoacetogens and autotrophic methanogens) were metabolically active down to 450 m. They were thought to be important due to the lack of dissolved organic carbon at such depths, and to their unique metabolic ability to thrive in the deep hydrogen-driven biosphere. The *Acetobacterium* sp. described here isolated from the GTS grows mixotrophically, using either H_2 or organic acids, such as formate, similar to other members of this genera [24].

Uranium biosorption by bacteria ranges from 6 to 450 mg U g^{-1} dry weight at pH 5 [27]. The quantity of U sorbed by CDn, *Acetobacterium* sp., and other isolates varied; at pH 5, the carboxylate moieties at the cell surface probably are deprotonated, and are responsible for sorption of soluble cationic uranyl species. At a higher pH, uranium exists as an organic complex or inorganic uranyl-carbonate complex, the anionic forms of which do not biosorb. The reasons for the differences in U uptake are not known; there may be marked differences in the characteristics of the cell-surfaces' functional groups of the isolates due to the extreme differences in ionic strength of the medium. We observed that the cell surfaces of halophilic bacteria appear to contain a greater density of anionic sites than common freshwater microorganisms (Gillow *et al.* unpublished results). Uptake of Pu was determined at very low concentrations of the actinide. Only a fraction of the Pu added was sorbed by *Acetobacterium* sp. at pH 5.0, consistent with studies of growing cultures of halophiles at pHs 6 and 8 in which only 2.5 to 9% of the Pu was associated with biomass [6]. Kudo *et al.* [28] examined the uptake of Pu (10^{-17} M) by sulfate-reducing bacteria and found that living cells sorbed more than dead cells. This study shows that the near- and far-field repository environments investigated harbor viable microbial populations capable of actinide uptake. Given the small size of the cells, once sorbed the actinides may be transported as colloids. A quantitative assessment of the sorptive species at a bacterial cell's surface, coupled with more thorough understanding of the speciation, bioavailability, and stability of biosorbed actinides, at various pHs and ionic strengths will be useful in determining the potential role of microbes in colloidal transport of actinides.

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