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BACTERIAL REDUCTION OF SOLUBLE URANIUM: THE FIRST STEP OF *IN SITU* IMMOBILIZATION OF URANIUM

LARRY L. BARTON AND KEKA CHOUDHURY
Department of Biology, The University of New Mexico, Albuquerque, New Mexico

BRUCE M. THOMSON AND KAREN STEENHOUDT
Department of Civil Engineering, The University of New Mexico, Albuquerque, New Mexico

ARMAND R. GROFFMAN
Jacobs Engineering Group, Inc. Albuquerque, New Mexico

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The mobility of uranium in groundwater is a problem of considerable magnitude. One approach would be to control the distribution of uranium by converting the water-soluble uranium ion to one that is less soluble. This study focuses on the use of *Desulfovibrio gigas, D. baculatus, D. vulgaris, D. desulfuricans, Pseudomonas putida,* a denitrifying *Pseudomonas* strain and mixed cultures from sludge or uranium mill tailing sites for the bioconversion of uranyl, U(VI), to uraninite, U(IV). In general, 82% to 92% of U(VI) was reduced in pure cultures, while 45% to 99% of added uranium was transformed by diverse bacteria present in groundwater. The oxyanions of selenium and vanadium had little effect on the uranium reduction by bacteria, while arsenic and molybdenum at 1.0 mM inhibited reduction of uranium. The product of uranium metabolism was U collected in needle-like crystals. A model is proposed for *in situ* bioremediation of uranium in groundwater at uranium mill tailing sites.

INTRODUCTION

Milling of uranium ores is accomplished by crushing the host rock, leaching it in strongly acidic or alkaline solutions to dissolve uraniferous materials, and recovering soluble uranium from solution. The process produces large volumes of acidic or

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alkaline tailings which are disposed of as a slurry to tailings piles, most of which are not lined. Leachates from these tailings contain high concentrations of several metals that form soluble oxyanions under oxidizing conditions. Concern has been expressed for uranium compounds as well as for arsenate, selenate, molybdate and vanadate found in groundwater near uranium milling activities[1]. Due to acidification and neutralization of uranium ores, the concentration of sulfate and/or nitrate in the groundwaters adjacent to milling activities may exceed allowable levels[2,3]. Remediation of groundwater problems at 24 abandoned tailings piles across the United States is a major problem facing the U.S. Department of Energy's Uranium Mill Tailings Remedial Action (UMTRA) project[4]. It has been estimated that tailings in the United States attributed to abandoned mills contain more than 25 million metric tons, while currently active mills are generating 170 million metric tons of contaminated material. Similarly, uranium mill sites throughout the world have produced vast quantities of tailings that are contaminating groundwaters. The mobility of uranium in groundwater involves soluble uranyl, U(VI), compounds. In acidic environments, UO_2^{2+} occurs, while in alkaline carbonate waters, $UO_2(CO_3)_3^{4-}$ is present. Under reducing conditions, uranium maybe found as uraninite (UO_2), coffinite ($USiO_4$), or other insoluble forms[5].

The use of bacteria in remediation of sites contaminated with heavy metals is receiving considerable attention[6] and a recent review has outlined the potential of bacteria for remediation of uranium-contaminated waters[7]. The reduction of U(VI) to U(IV) has been reported for several species of anaerobic bacteria including *Gleobacter metallireducens*[8], *Shewanella putrefaciens*[9], *Desulfovibrio desulfuricans*[9] and *Clostridium* spp.[7]. This laboratory study was initiated to investigate the parameters of transformation of U(VI) to U(IV) by anaerobic bacteria that would be important for uranium mill tailings. We report that U(VI) reduction to U(IV) can be accomplished by various anaerobic bacteria including strains of sulfate-reducing and heterotrophic bacteria. A model is presented for the application of anaerobic bacteria in bioremediation of groundwater contaminated with soluble uranium.

METHODS AND MATERIALS

Bacterial Cultures

Sulfate-reducing bacteria used in this study included *D. desulfuricans* NCIB 8307, *D. baculatus* strain Norway 4 NCIB 8310, *D. gigas* NCIB 9332, and *D. vulgaris* NCIB 8303. Also tested for uranium transformation were the nitrate-reducing strains of *Pseudomonas* sp. ATCC 13867 and *P. putida* ATCC 15070. For a mixed culture of anaerobic bacteria, a sludge sample was obtained from the Albuquerque Municipal Sewage Treatment Facility.

Media and Cultivation

The anaerobic cultures were grown in 16 mm × 125 mm test tubes fitted with a rubber septum and screw cap. For growth of *Desulfovibrio* and bacteria in the sludge inoculum, a lactate-sulfate medium[10] was employed. Nitrate Broth (Difco Co.,

Detroit, MI) was used to grow the denitrifying bacteria. Two different concentration of nitrate or sulfate were used as electron acceptors in these experiments. In the media employing high concentrations of electron acceptor, 3.0 g/L of sodium sulfate or 1.0 g/l of sodium nitrate was added. To prepare media containing low levels of electron acceptor, 0.3 g/L of sodium sulfate or 0.1 g/l of sodium nitrate was used. Sodium salts of arsenate, molybdate, selenate, and vanadate were sterilized separately and added aseptically to the growth media as indicated. A 10 mM solution of uranyl acetate was autoclaved and alliquots were dispensed into the media or culture. To initiate growth, inoculations were with 0.1 ml of a bacterial culture in stationary phase. Incubation was at 35°C. Before inoculation, each tube was flushed with purified N_2 to replace oxygen in the media. To facilitate anaerobic transfer, 1 ml sterile syringes fitted with 26-gauge needles were used.

Characteristics of Bacteria from Groundwater

Groundwater samples were obtained from UMTRA project sites located in Bowman, North Dakota; Tuba City, Arizona; Falls City, Texas; and Cannonsberg, Pennsylvania. Samples were refrigerated and transported to the laboratory within a day of collection. Plate Count Agar (Difco Co, Detroit, MI) was used to cultivate heterotrophic bacteria and Nitrate Agar (Difco Co.) was used to grow nitrate-respiring bacteria. Anaerobic cultivation was achieved using the Gas Pak System (BBL, Baltimore, MD). Agar plates were inoculated directly with 0.1 ml of water sample or with a bacteriological filter (Millipore Co., Bedford, MA) through which 10 ml of groundwater had been filtered. Procedures used were previously described[11].

To demonstrate the presence of sulfate-reducing bacteria or nitrate-reducing bacteria, 10 ml of groundwater was added to 50 ml of sterile fluid media. Incubation was in Gas Pak Systems at 30°C for 7 days. The formation of H_2S in lactate-sulfate medium[10] was the criteria for the presence of sulfate-reducing bacteria in the inoculum. The production of gas in an inverted tube placed in the 25 mm × 150 mm test tube containing defined media[12] enriched with lg/L of sodium nitrate was used to indicate the presence of nitrate-reducing bacteria.

Bacteria present in the groundwater samples were tested to determine if U(VI) could be removed from solution as the organism grew. Groundwater, 50 ml, from each of the UMTRA sites was placed in sterile 25 mm × 150 mm screw cap test tubes and a solution of sodium phosphate, adjusted to pH 7.0, was added to each tube to give a final concentration of 1.0 g/L. A sterile solution of ammonium chloride was added to each sample to give a final concentration of 1.0 g/L. Four sterile solutions of carbon sources (glucose, acetate, lactate and ethanol) were added to each groundwater sample enriched with ammonium and phosphate to give a final concentration of 0.1% carbon source. To each tube was added U(VI) to give a concentration of 1 mM. These cultures were incubated at 20°C for 30 days before the culture fluid was tested for U(VI).

Chemical Measurements

Bacterial growth was determined by following cell protein using the Folin reagent[13]. To prepare the cells for protein measurements, 1 ml of culture was placed in a 1.6 ml

microfuge tube and cells were collected by centrifugation for 3 min at 35,000 rpm. Bacterial cells were resuspended in physiological saline (0.85% NaCl) and collected by centrifugation. This washing of cells was conducted twice before cells were dissolved in 0.2 ml of 10% NaOH and protein was determined.

Uranium in the culture fluid following centrifugation of the growth media was quantitated using a colorimetric procedure that quantitated the benzyhydroxamic acid-uranyl complex produced at pH 6.2[14].

Electron Microscopy

Samples were removed from bacterial cultures that had reached stationary growth phase and were subjected to centrifugation for 3 min in a microfuge. The pellet was resuspended in 0.1 M HEPES buffer (pH = 7.6) containing 2.5% glutaraldehyde. The unstained specimen was embedded in a low-viscosity plastic resin[15] and sectioned. Observations were with a JOEL 2000EX scanning transmission electron microscope using 100 keV beam voltage. Analysis of crystals was by energy dispersive X-ray (EDX) microanalysis using a Tracor Northern spectrometer system attached to the microscope.

RESULTS AND DISCUSSION

Reduction of Uranium by Desulfovibrio and Pseudomonas

All strains of *Desulfovibrio* tested grew readily in the presence of U(VI), with highest levels of growth in the cultures with high levels of sulfate (Tables I and II, Figure 1). While arsenate at 1 mM frequently inhibited growth, molybdate at 1 mM stimulated the growth response of a few cultures. Nitrate-reducing bacteria also grew in the presence of various metal oxyanions including uranyl acetate. In a couple of the sludge samples, the growth (protein production/ml media) appeared to be greater with uranium. Future studies should pursue this observation to determine if bacteria can be isolated that can grow with the use of U(VI) as the final electron acceptor. With sludge-inoculated tubes, sulfate additions would encourage the growth of sulfate-reducing bacteria, while methanogens would be selected in tubes receiving no sulfate[16].

Soluble uranium was reduced in all test tubes with bacterial growth; however, the quantity varied with the strain (Tables III and IV). In general, the amount of uranium reduced by a culture in stationary phase was greater than when uranium was added at the time of inoculation (Table IV). While the use of stationary phase cells could provide interesting kinetics, these studies were not pursued because *in situ* bioremediation would have uranium already present in the groundwater. The effect of heavy-metal oxyanions on uranium reduction varied with the cultures; however, the following generalizations can be made: (i) arsenate at 1.0 mM, but not 0.1 mM, inhibited the reduction of U(VI); (ii) molybdate at 1.0 M, but not 0.1 mM, inhibited the reduction of U(VI); and (iii) selenate at 1.0 mM or vanadate at 0.1 mM had little effect on U(VI) reduction. With sludge as the inoculum, the growth in the

TABLE I

Growth response of bacteria in media supplemented with metal anions and uranium

Addition		Protein (mg/ml) levels in the following cultures			
		D. desulfuricans	*D. vulgaris*	*D. gigas*	*P. denitrificans*
Media with High Concentration of Electron Acceptor					
Arsenate	1.0 mM	0.29	0.40	0.02	0.08
	0.1 mM	0.28	0.68	0.28	0.08
Selenate	1.0 mM	0.32	0.06	0.07	0.06
	0.1 mM	0.20	0.02	0.11	0.04
Molybdate	1.0 mM	0.56	0.03	0.39	0.05
	0.1 mM	0.48	0.58	0.01	0.04
Vanadate	0.1 mM	0.18	-	0.24	0.05
Media with Low Concentration of Electron Acceptor					
Arsenate	1.0 mM	0.08	0.10	0.17	0.03
	0.1 mM	0.07	0.13	0.06	0.05
Selenate	1.0 mM	0.07	0.08	0.05	0.05
	0.1 mM	0.06	0.02	0.04	0.04
Molybdate	1.0 mM	0.05	0.03	0.02	0.07
	0.1 mM	0.07	0.06	0.02	0.32
Vanadate	0.1 mM	0.09	-	0.04	0.11

TABLE II

Growth of *Pseudomonas putida* and a mixture of bacteria in media containing additions of heavy metals

Additions		Protein levels (mg/ml) in cultures with uranium (VI) additions made as indicated			
		P. putida with U(VI) added before inoculation	*P. putida* with U(VI) added after 14 days	sludge with U(VI) added before inoculation	sludge with U(VI) added after 14 days
Media with High Concentration of Electron Acceptor					
Arsenate	1.0 mM	0.08	-	0.09	0.04
	0.1 mM	0.07	0.07	0.20	0.04
Selenate	1.0 mM	0.10	0.11	0.03	0.05
	0.1 mM	0.10	0.03	0.12	0.04
Molybdate	1.0 mM	0.13	0.11	0.35	0.06
	0.1 mM	0.07	0.06	0.09	0.05
Vanadate	0.1 mM	0.10	0.02	0.15	-
Media with Low Concentration of Electron Acceptor					
Arsenate	1.0 mM	0.06	0.06	0.28	0.09
	0.1 mM	0.11	0.10	0.09	0.02
Selenate	1.0 mM	0.12	0.18	0.04	-
	0.1 mM	0.06	0.06	0.13	0.02
Molybdate	1.0 mM	0.07	0.12	0.39	0.17
	0.1 mM	0.17	0.04	0.14	0.03
Vanadate	0.1 mM	0.07	0.09	0.09	0.07

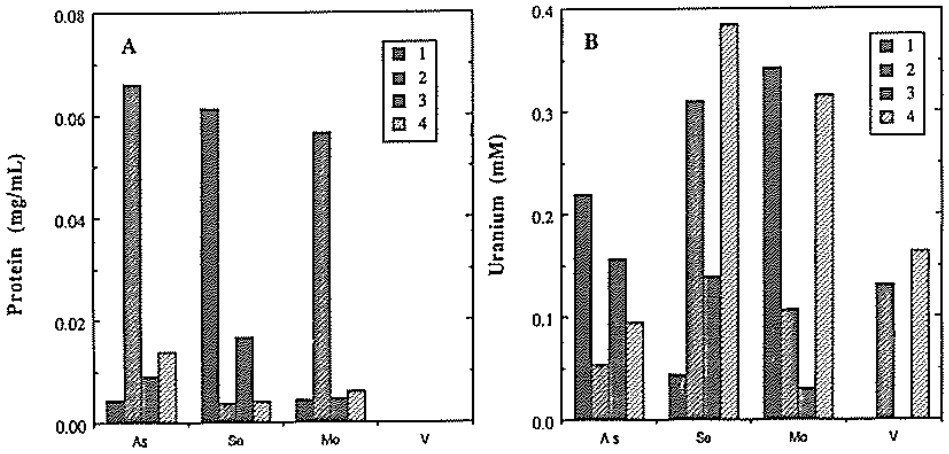

FIGURE 1 Growth and reduction of uranium by *Desulfovibrio vulgaris*. A: Uranium, arsenate, selenate, molybdate and vanadate were added at the time of inoculation B: The initial concentration of uranium in the growth medium was 1.0mM and the amount remaining after 14 days is indicated. 1 and 2 = 10 mM Sulfate; 3 and 4 = 1 mM Sulfate; 1 and 3 = 1 mM metal ion; 2 and 4 = 0.1 mM metal ion.

presence of sulfate at 3.0 g/L accounted for greater levels of U(VI) reduced than when 0.3 g/L sulfate was added.

In an earlier paper[17], it was reported that uranium reduction was accomplished by bacteria when uranium was added as a carbonate complex. From our research it is apparent that uranium reduction is not dependent on the organization of the soluble uranyl ion (Table V).

Bacteria from the sludge inoculum had greater protein levels when uranium was added at the time of inoculation. This observation could be attributed to certain bacteria growing with uranium as an electron acceptor or to the requirement that uranium reduction is an inducible process in mixed cultures. With *P. putida* growing in nitrate at 1.0 g/L and sludge growing in 3.0 g/L sodium sulfate, greater levels of U(VI) were removed when uranium was added to stationary phase cultures. Perhaps this reflects a distinction of coupling between early and secondary metabolic processes[18] in these bacteria.

Bacteria in Groundwater

Groundwater from the various UMTRA sites contained a myriad of bacteria. Numbers of heterotrophic aerobic bacteria ranged from 1×10^3 to 7.5×10^4/100 ml (Figure 1), while heterotrophic anaerobic bacteria were generally present in comparable numbers (Table VI). Although the bacterial numbers were not determined, all ground waters contained sufficient sulfate-reducing and nitrate-reducing bacteria that at least one viable organism was present in 10 ml of water.

Reduction of Uranium by Groundwater

Bacteria in groundwater rapidly grew with each of the four carbon sources. A blue-green color was observed in the culture tubes after a few days and persisted for several weeks before the color disappeared. Greatest putative production of

TABLE III
Removal of uranium by bacteria growing in metal-supplemented media

Additions		$\mu g\ UO_2^{2-}$/mg cell protein			
		D. desulfuricans	*D. vulgaris*	*D. gigas*	*P. denitrificans*
Media with High Concentration of Electron Acceptor					
Arsenate	1.0 mM	8.91	6.75	10.8	0.32
	0.1 mM	86.7	19.8	33.8	4.50
Selenate	1.0 mM	42.2	27.0	366.0	4.50
	0.1 mM	40.5	13.5	-	675.0
Molybdate	1.0 mM	0.48	9.00	0.70	5.40
	0.1 mM	52.8	0.46	18.0	472.0
Vanadate	0.1 mM	45.0	-	61.8	4.91
Media with Low Concentration of Electron Acceptor					
Arsenate	1.0 mM	3.7	2.70	-	9.00
	0.1 mM	115.0	20.7	180.0	5.40
Selenate	1.0 mM	96.4	1.50	162.0	4.91
	0.1 mM	427.0	13.5	-	6.00
Molybdate	1.0 mM	5.40	540.0	234.0	3.86
	0.1 mM	3.86	4.50	270.0	0.84
Vanadate	0.1 mM	75.0	-	202.0	2.45

TABLE IV
Uranium removal by growing cultures and non-growing cultures of bacteria

Additions		$\mu g\ UO_2^{2-}$ removed/mg cell protein with additions made as indicated			
		P. putida with U(VI) added before inoculation	*P. putida* with U(VI) added after 14 days	sludge with U(VI) added before inoculation	sludge with U(VI) added after 14 days
Media with High Concentration of Electron Acceptor					
Arsenate	1.0 mM	15.9	-	254.0	193.0
	0.1 mM	72.0	180.0	114.0	337.0
Selenate	1.0 mM	2.50	196.0	495.0	285.0
	0.1 mM	2.64	540.0	129.0	-
Molybdate	1.0 mM	50.0	2.45	11.7	98.2
	0.1 mM	3.86	393.0	127.0	534.0
Vanadate	0.1 mM	2.25	105.0	21.6	-
Media with Low Concentration of Electron Acceptor					
Arsenate	1.0 mM	4.50	-	18.9	254.0
	0.1 mM	241.0	2.70	79.4	-
Selenate	1.0 mM	2.25	112.0	6.75	-
	0.1 mM	4.35	49.1	100.0	-
Molybdate	1.0 mM	3.97	64.8	6.83	47.6
	0.1 mM	1.54	6.75	42.4	-
Vanadate	0.1 mM	3.96	3.18	2.84	207.0

TABLE V
Removal of U(VI) by different bacterial culture

Strain	Uranium (VI) removed (%)
Mixed culture (sludge)	92.4
D. desulfuricans	91.1
D. gigas	90.5
P. putida	84.7
D. baculatus (Norway 4)	84.1
Pseudomonas sp.	84.0
D. vulgaris	82.2

TABLE VI
Anaerobic bacteria present in groundwater from mill tailing sites

Location	Heterotrophic bacteria (number/100 ml)	Sulfate-reducing bacteria (present in 10 ml)	Nitrate-reducing bacteria (present in 10 ml)
Arizona 901	6,000	+	+
Texas 881	18,140	+	+
North Dakota 516	11,000	+	not tested
Pennsylvania 504	34,040	+	+

sidero-phores occurred in lactate-supplemented media and lowest levels of siderophore were found in glucose-supplemented water. This color was characteristic of sidero-phores produced by *Pseudomonas* sp.[12]. Siderophore production would be expected because the groundwater bottles were not sealed and iron had collected on the bottom of the bottles as a rust-brown precipitate.

As seen in Figure 2, all water samples removed significant quantities of U(VI). All of the carbon sources accounted for growth that removed 60% to 99% of soluble uranium. A conclusion that can be drawn from this is that bacteria capable of uranium metabolism exist in groundwater near UMTRA sites and that when appropriate nutrients are added, bacterial growth can remove soluble uranium. In these aerobic cultures there was no growth evidence of sulfate-reducing or nitrate-reducing bacteria. Thus, we conclude tht heterotrophic bacteria are also capable of reducing soluble U(VI). Clearly, this is an area that should be pursued.

Microscopic Analysis of the U Product

Examination of thin sections of material from a *Pseudomonas* sp. culture revealed several needle-like crystals (Figure 3). EDX analysis indicated that the crystals contained uranium. This provides information that, at least in the *Pseudomonas* sp. culture, U(VI)

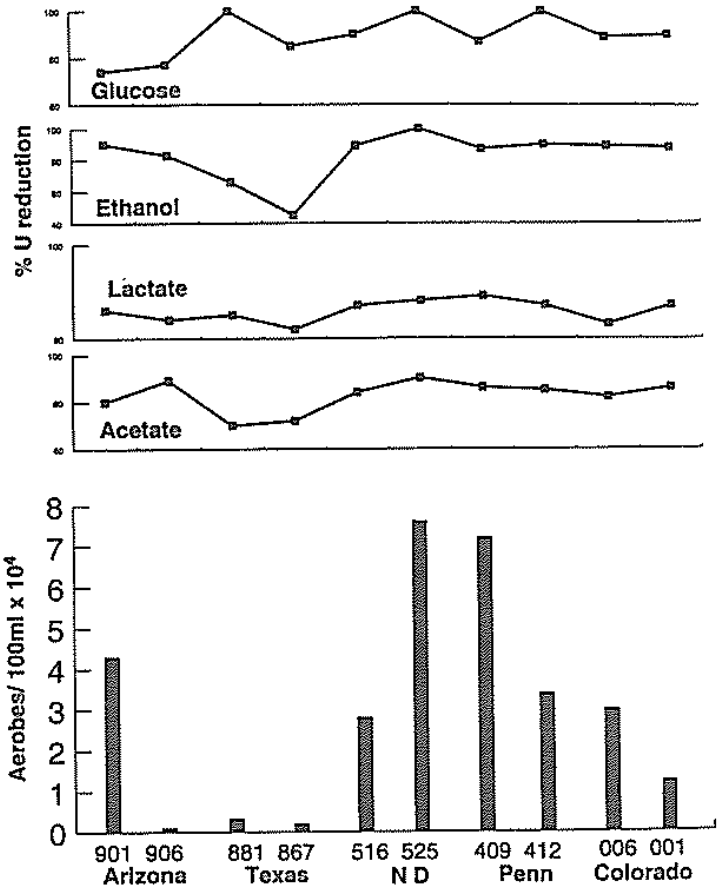

FIGURE 2 Aerobic bacteria present in groundwater at UMTRA sites and removal of U(VI) by these aerobes.

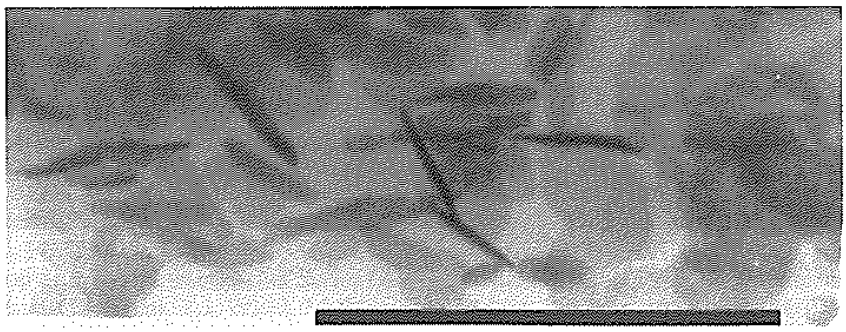

FIGURE 3 Uranium Crystals produced by *Pesudomonas* sp. growing in 1 mM urany acetate. Bar = 200 nM.

is transformed to U-containing crystals. In previous reports[9,17], insoluble uranium compounds have been reported to be produced by D. *desulfuricans* ATCC 29577. It will be important in future research to determine if crystals of uranium are formed by aerobic bacteria that remove U(VI), as shown in Figure 2.

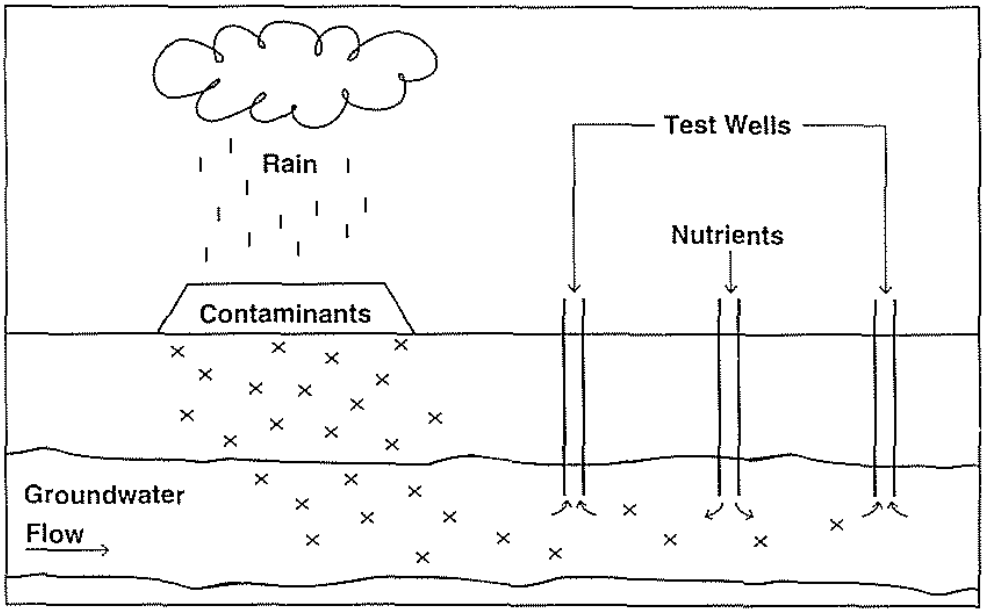

FIGURE 4 Scheme indicating *in situ* bioremediation of uranium-contaminated groundwater.

Model for *In Situ* Bioremediation

There is a growing amount of information to support the hypothesis that bacteria can transform soluble uranium to insoluble UO_2[2]. The production of uranium crystals by bacteria presents the possibility of *in situ* immobilization of soluble uranium. A model of this proposed activity is given in Figure 4. Through selective addition of nutrients, bacteria in the groundwater will grow and their metabolic activities will result in removal of soluble uranium from the environment. Since uranium mill tailing groundwaters commonly contain selenate or selenite, the proliferation of sulfate-reducing bacteria could result in formation of elemental selenium[19,20]. If surface water near uranium mill tailings is to be remediated, a modification of the system proposed for bioremediation of waters containing lead or selenium could employed[21]. Obviously there are many details that must be addressed before this bioremediation can become a reality; however, this initial phase dealing with bacterial metabolism of U(VI) contributes information to support this *in situ* approach.

CONCLUSIONS

The amount of soluble uranium in groundwater may reflect the activity of bacteria that are present. In this research it was demonstrated that various strains of sulfate-reducing and nitrate-reducing bacteria are capable of reducing the amount of soluble uranium in aqueous environments. From these experiments it did not appear that cultures of *Desulfovibrio* or *Pseudomonas* were able to couple uranium metabolism to cell growth. The presence of arsenate, molybdate at 1 mM slightly inhibited reduction of U(VI), while selenate or vanadate had little effect on uranium

reduction by bacteria. Heterotrophic bacteria in groundwater from UMTRA sites also removed U(VI) from solution, presumably through transformation activities. Crystals of complexed uranium would appear to be markedly less mobile than U(VI) and may be an appropriate form for *in situ* bioremediation of uranium at milling sites.

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