BioMetals **14:** 171–179, 2001.

Interactions of three eco-types of *Acidithiobacillus ferrooxidans* with U(VI)

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Received 5 March 2001; accepted 13 March 2001

Key words: Acidithiobacillus ferrooxidans, uranium accumulation, U resistance

Abstract

The interaction of uranium with cells of three recently described eco-types of *Acidithiobacillus ferrooxidans* recovered from uranium mining wastes was studied. The uranium sorption studies demonstrated that the strains from these types possess different capabilities to accumulate and tolerate uranium. The amount of uranium biosorbed by all *A. ferrooxidans* strains increased with considerable concentrations. We have found that the representatives of type II accumulate significantly higher amounts of uranium in comparison to the other *A. ferrooxidans* strains. The investigations of the tolerance to uranium showed that the types I and III are resistant to 8 and 9 mM of uranium respectively, whereas the type II does not tolerate more than 2 mM of uranium. The recovery of the accumulated uranium by desorption was investigated using various desorbing agents as sodium carbonate, sodium citrate and EDTA at different concentrations. Sodium carbonate was the most efficient desorbing agent, removing 97% of the uranium sorbed from the cells of *A. ferrooxidans* type III, and 88.33 and 88.50% from the cells of the types I and II, respectively.

Introduction

The mobilization of heavy metals in the environment due to industrial activities is of serious concern because of their toxicity to humans and other forms of life. Metals can be divided into four major categories on the basis of chemical properties, physiological effect on life, and their applications: (i) toxic heavy metals, (ii) strategic metals, (iii) precious metals, and (iv) radionuclides (Volesky & Holan 1995). Removal of toxic heavy metals and radionuclides from industrial waste waters is essential from the standpoint of environmental pollution control. Uranium is an example of these metals, and is considered one of the most seriously threatening heavy metals mainly because of its high toxicity, not so much radioactivity. Various ionic forms are possible, and it may be used as a substrate for anaerobic respiration (Lovely *et al.* 1991). No other biological beneficial actions of this radioactive element are known. Uranium contamination poses a threat in some surface and groundwater. Activities associated with the nuclear industry, mining and wastewater treatment have brought excessive amounts of uranium into the environment (Macaskie *et al.* 1997; Yang & Volesky 1999)

Uranium and other actinides may be present initially as soluble or insoluble forms and, after disposal, may be converted from one to the other by micro-organisms. Under appropriate conditions, actinides can be mobilized or immobilized by direct (enzymatic) or indirect (nonenzymatic) microbial actions. These include: (i) oxidation-reduction reactions, (ii) changes in pH and Eh, (iii) chelation, or production of specific sequestering agents, (iv) formation of stable minerals, (v) biodegradation of actinides-organic complexes, and (vi) biosorption by biomass and biopolymers (Francis 1998). Biosorption processes are essentially chemical ones whereby the biomass acts as a surface upon which metals bind by ligand interactions or by ion exchange. Living and dead micro-organisms possess abundant functional groups, such as carboxyl, hydroxyl and phosphate on their surface that bind

metal ions. In Gram-negative bacteria, lipopolysaccharide (LPS) layer can be highly anionic and extends beyond the outer membrane proteins; this layer has been implicated as the major source of metal binding in this group of bacteria (Beveridge & Koval 1981; Ferris & Beveridge 1984). For instance, Ferris & Beveridge (1986) demonstrated that the phosphoryl residues of the polar head of phospholipids and LPS in the outer membrane were the most probable binding sites for metal cations in the *Escherichia coli* K-12, whereas in *Pseudomonas aeruginosa* the phosphoryl groups in the core-lipid A region of the LPS are mainly involved in metal binding (Langley & Beveridge 1999). In contrast, in Gram-positive bacteria, the main metal binding capacity is generated by the thick peptidoglycan layer. Numerous studies have examined the metal ion-cell wall interactions of Gram-positive bacteria (particularly members of the genus *Bacillus*). The sites responsible for metal binding in these organisms are probably the carboxyl sites within the peptidoglycan, as well as the phosphoryl groups of the teichoic and/or teichuronic acids, and other secondary polymers (Doyle *et al.* 1980).

Since metal ion species are generally more readily soluble in acidic environments, acidogenic microbial metabolic activities may contribute to the introduction of metals into groundwater from contaminated soils. In these environments, highly contaminated with heavy metals, microbial populations have developed different tolerance mechanisms (Cunningham & Lundie 1993). Some of them involve extracellular precipitation with microbially produced oxalate, phosphate, or sulfide (Macaskie & Dean 1984) or binding to microbial organic molecules such as lipopolysaccharides (Langley & Beveridge 1999). In bacteria, a wide range of efflux pumps, predominantly P-type ATPases, have been shown to mediate metal detoxification (Silver & Phung 1996) as well.

The uranium ores contain significant amounts of sulfidic minerals which, together with Fe^{2+} (and to a lesser extent other metals), represent an oxidisable energy source for a large group of bacteria, collectively termed acidophilic lithotrophs. This group is exemplified to the thiobacilli; a group of Gram-negative rod-shaped bacteria which are able to oxidise sulphide minerals, elemental sulfur, ferrous iron, and one of them (*A. ferrooxidans*) in presence of uranium minerals, also U(IV) to U(VI) (DiSpirito & Tuovinen 1982; Ehrlich 1997). *A. ferrooxidans* has been used industrially in metal leaching from ores (Rawlings & Silver 1995). Moreover, this bacterium is able to accumulate uranium (DiSpirito *et al.* 1983).

Recently, three different eco-types of *A. ferrooxidans* were recovered from different sites and depths of two uranium mining wastes (Flemming *et al.* 2000; Selenska-Pobell *et al.* 2000, 2001; Selenska-Pobell 2001). The objective of this work was to investigate whether these eco-types differ in their capability to accumulate and tolerate uranium.

Material and methods

Bacterial strains used

The bacterial strains used in this work are listed in Table 1.

A. ferrooxidans S1, 2, 6 were recovered from a soil sample from the surface of the uranium waste mine piles near the town of Johanngeorgenstadt, Germany. *A. ferrooxidans* S3, 4, 5 were cultured from a soil sample drawn from a depth of 3 m below the land surface of the same pile. The latter possesses three times higher amounts of uranium and was also significantly more polluted with other toxic metals in comparison to the surface samples (Kutschke *et al.* 2000; Selenska-Pobell 2001). *A. ferrooxidans* K2 was cultured from a highly contaminated with uranium sample from an oxic sediment of the uranium depository site, Deponie B1, near the river Weiße Elster, Germany (Flemming *et al.* 2000; Selenska-Pobell *et al.* 2001). Strains W1, D2, D6, D7, R1, N2 and F1 were kindly provided by Leo Leduc (Leduc *et al.* 1997).

A. ferrooxidans strains were cultured in 9K liquid medium (Silverman & Lundgren 1959) containing 3 g of $(NH_4)_2SO_4$, 0.5 g of K_2HPO_4, 44.2 g of $FeSO_4$. $7H_2O$, 0.5 g of $MgSO_4$. $7H_2O$ and 0.1 g of KCl and 0.014 g of $Ca(NO_3)_2$. $4H_2O$ per liter. Solid medium 2:2 described by Peng *et al.* (1994) was used to determine the Minimum Inhibitory Concentrations of uranium for the growth of the strains studied.

Amplified Ribosomal DNA Restriction Enzyme Analysis (ARDREA) of the A. ferrooxidans strains

The typing of the strains studied was performed using ARDREA. For this species-specific 16S rDNA PCR amplicons were generated and digested with the *Rsa*I endonuclease as described by Selenska-Pobell *et al.* (2000, 2001).

Table 1. Bacterial strains used.

Strain name	Type	Origin
A. ferrooxidans A1	Type I	Canada (Leduc *et al.* 1997)
A. ferrooxidans W1		//
A. ferrooxidans 23270		ATCC[a], coal mine, USA
A. ferrooxidans 21834		ATCC[a], coal mine, Japan
A. ferrooxidans 19859		ATCC[a], copper mine, Canada
A. ferrooxidans S3		Uranium mine, Germany (Kutschke *et al.* 2000)
A. ferrooxidans S4		//
A. ferrooxidans S5		//
A. ferrooxidans 33020	Type II	ATCC[a], uranium mine, Japan
A. ferrooxidans S1		Uranium mine, Germany (Kutschke *et al.* 2000)
A. ferrooxidans S2		//
A. ferrooxidans S6		//
A. ferrooxidans R1		Uranium mine, Canada (Leduc *et al.* 1997)
A. ferrooxidans N2		//
A. ferrooxidans K2	Type III	Uranium depository site, Deponie B1, Germany (Flemming *et al.* 2000)
A. ferrooxidans D2		Uranium mine, Canada (Leduc *et al.* 1997)
A. ferrooxidans D6		//
A. ferrooxidans D7		//
A. ferrooxidans F1		Nickel Mine, Canada (Leduc *et al.* 1997)

[a]American type culture collection

Uranium solutions

Stock solutions of uranium were prepared by dissolving appropriate quantities of $UO_2(NO_3)_2$. $5H_2O$ in 0.01 M perchloric acid ($HClO_4$). These solutions were diluted with 0.01 M $HClO_4$ to obtain the desired concentrations of the metal.

Preparation of samples for uranium biosorption

Bacterial cells grown to the mid-exponential phase (optical density at 600 nm, 0.4) were acidified with sulphuric acid to pH 1.3 to dissolve the Fe(III) precipitate which was produced during the growth. The cells were harvested by centrifugation (12,500 rpm for 30 min at 4 °C) and washed three times with 0.1 N sulphuric acid to remove the disturbing ingredients of the growth medium. Then the pellets were washed 2 times with 10 ml perchloric acid (0.01 M) using centrifugation at 12,500 rpm for 10 min for each wash. The washed cells were resuspended in solutions possessing different concentrations of uranium (ranging from 5 to 25 mg l) adjusted to pH values 1.5 and 4. The bacterial concentrations used were between 0.10 and 0.14 g dry weight l. After 48 h of shaking, the cells were removed from the solution by centrifugation and the supernatant was collected and used for metal analysis. The cell pellets were dried at 70 °C for 24 h, their dry weights were determined. The dried samples were then acid hydrolyzed for analysis by Inductive Coupled Plasma-Mass Spectroscopy (ICP-MS). During these analyses a mass balance of metals was carried out by analysing all the supernatants.

The amount of uranium removed by the cells was determined on a dry-weight basis. Uptake of metal ions (q) was calculated from a metal mass balance yielding (Volesky 1990): q (mmol metal g dry biomass) $= V(C_i - C_f)/mM$ where V is the sample volume (l), C_i and C_f are the initial and field metal concentrations (mg l), respectively, m is the amount of dry biomass (g) and M is the relative molecular mass of the metal.

Three replicates consisting of three samples each were prepared, in addition to control which one consisted of cells which were not treated with any metal. Experimental control samples with no biomass added were treated identically as blanks.

Uranium desorption

0.25 ml portions of cell suspensions (optical density at 600 nm, 0.4) were contacted in 1.25 ml of 25 mg l uranyl nitrate for 48 h at room temperature and shaking at 110 rpm. After the contact with the metal, the cells were centrifuged at 11,000 rpm for 10 min, the cell pellets were then washed three times in 1.5 ml of ultrapure water with centrifugation at 11,000 rpm for 10 min for each wash. The cell pellets, resuspended in 1.5 ml of one of the following desorbing agents EDTA/Tris pH 7.2; sodium carbonate; sodium citrate, at 2 different concentrations (0.1 and 0.5 M) were again incubated for 2 h. As in the previous case, separation of the supernatant fluid from the biomass was achieved through centrifugation at 12,500 rpm for 10 min. The uranium concentration in supernatants was measured by ICP-MS. Finally, the cell pellets were dried at 70 °C for 24 h, and their dry weights were determined.

Uranium tolerance

Minimum Inhibitory Concentration (MIC) of uranium was determined in triplicate for each strain of *A. ferrooxidans* by transferring 0.1 ml of cells (optical density at 600 nm, 0.4) washed with basal salt solution of solid medium 2:2 (0.45% of $(NH_4)_2SO_4$, 0.015% of KCl, 0.075% of $MgSO_4$. $7H_2O$, pH 4.6) in 2:2 solid medium containing different concentrations of uranium (ranging from 0.01 to 12 mM). After spreading, the plates were inverted and incubated at room temperature. The MIC was defined as the lowest concentration at which complete inhibition of colony formation was observed (Rossbach *et al.* 2000).

Results

Uranium biosorption by the three different types of A. ferrooxidans

Recently analysing a large number of soil samples collected from two uranium mining waste piles, Selenska-Pobell *et al.* (2000, 2001) have found that the natural *A. ferrooxidans* populations consist of three ecological types which are inhomogeneously distributed in the wastes. The three 16S rDNA types of the species found in the uranium wastes are presented in Figure 1.

The same authors have demonstrated that in highly polluted soil samples representatives of the type I (see Figure 1) are predominant, whereas the type II was found mainly in less polluted sites close to the surface. The original strain of type III, namely *A. ferrooxidans* K2 was cultured from a very polluted with uranium sediment sample from a uranium depository site (Flemming *et al.* 2000).

In order to clear how the three types of *A. ferrooxidans* are interacting with uranium we have performed uranium biosorption with the representatives of the three types given in the Table 1.

In Figure 2 the amounts are shown of uranium accumulated by the three types of *A. ferrooxidans* at different metal concentrations (5, 10, 15 and 25 mg l) and at pH values of 1.5 and 4. Significant differences among the three eco-types for uranium removal from the medium were found at concentration 25 mg l and pH 1.5. At these conditions 21.54, 30 and 44.62 mgU g dry weight were accumulated by *A. ferrooxidans* type I, type III and II, respectively. Similarly, at the same concentration and pH 4, the type II accumulates more uranium (53.25 mg g of dry biomass) than do the type I and III, which under these conditions, accumulate approximately similar amounts of uranium (up to 33 and 30 mg g of dry biomass, respectively).

The capacity of the three eco-types to accumulate uranium was a function of the metal concentration. In the concentration range of 5 to 25 mg l, the amount of uranium accumulated increases with the increase of the metal concentration at pH 1.5 and 4.

The biosorption of uranium was influenced by the pH of the uranium solution. When the pH was reduced from 4 to 1.5 a decrease of the capability of the strains to bind uranium was observed. At uranium concentration of 25 mg l, for instance, the ability of types I and II to bind uranium decreased from 33.81 to 21.54; from 44.62 to 33.81, respectively, whereas in the case of type III the decrease was insignificant, from 33 to 30 mg g dry weight, respectively.

Uranium desorption

In order to determine how strong the uranium was bound by the eco-types of *A. ferrooxidans*, desorption experiments were performed using sodium carbonate, sodium citrate and EDTA as desorbing agents. The results shown in Figure 3 demonstrate that sodium carbonate was the most efficient desorbing agent tested. At concentration 0.5 M, this strong agent recuperate 97, 88.50 and 88.33% of the uranium sorbed from the cells of *A. ferrooxidans* types III, II and I, respectively. Sodium citrate, was the second best metal desorbing

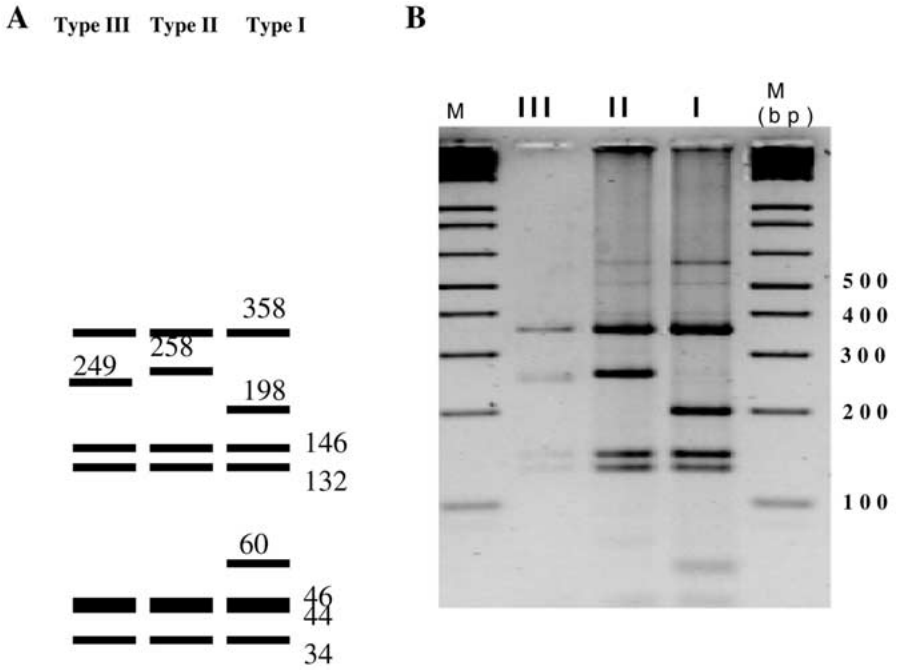

Fig. 1. Discrimination of the three *A. ferrooxidans* eco-types based on the 16S-ARDREA. A) Schema of the *Rsa*I profiles of the type I, II and III drawn on the basis of the sequence analysis of the 16S rDNA. B) *Rsa*I -16S-ARDREA of the following strains: *A. ferrooxidans* W1 (type I), *A. ferrooxidans* ATCC 33020 (type II), and *A. ferrooxidans* D2 (type III).

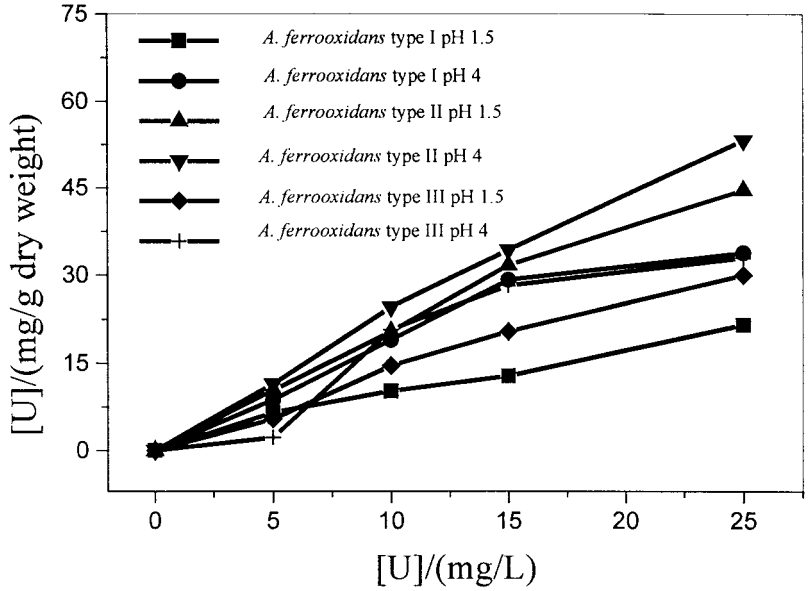

Fig. 2. Biosorption of uranium by the three types of *A. ferrooxidans*.

Table 2. Minimum Inhibitory Concentrations (MICs) of uranium for the growth of *A. ferrooxidans* types.

Strains	Uranium (mM)	
	Tolerated	MICs
A. ferrooxidans type I	8	9
A. ferrooxidans type II	2	4
A. ferrooxidans type III	9	10

agent and it removed uranium from *A. ferrooxidans* type II (68%) more effectively than from type III (53.92%). EDTA could remove only between 10 and 30% of the accumulated uranium. The ultra pure water (data not shown) could remove only between 3 to 5% of the accumulated uranium.

Uranium tolerance

In Table 2 the minimum inhibitory concentrations of uranium are shown for the growth of the *A. ferrooxidans* eco-types. The strains belonging to type III and I showed a higher tolerance to uranium, and they grew at concentrations of this metal up to 9 and 8 mM, respectively. Representatives of the type II, in contrast, are more sensitive to uranium (do not tolerate more than 2 mM of uranium) as compared to the representatives of types I and III.

Discussion

In nature, noticeable microbial interaction with metals frequently manifests itself through metal immobilization or mobilization (Ehrlich 1996). Metal immobilization may be through cellular sequestration and accumulation, or through extra-cellular precipitation. Metal mobilization results from dissolution of insoluble metal-containing phases. Bioleaching of metals from ores is a practical example (Ehrlich & Brierley 1990). *A. ferrooxidans* is considered to be the most important micro-organism in commercial bioleaching (Rawlings *et al.* 1999). This bacterium plays a significant role in the solubilization of uranium from ores, in mill tailings and coal wastes. Furthermore, *A. ferrooxidans* accumulates uranium (DiSpirito *et al.* 1983; Merroun & Selenska-Pobell 2000) which can be transported and released elsewhere by remineralization.

The 16S rRNA genes of a large number of *A. ferrooxidans* reference and uranium waste pile isolates possess specific signatures which distinguish three types within this species (Flemming *et al.* 2000; Selenska-Pobell *et al.* 2001). In the current study, we have found that the strains from the above mentioned types possess different capability to accumulate uranium, these differences are significant particularly at concentration of the solution of 25 mg l and pH 1.5. It was reported that differences in metal accumulation specificity and efficiency exist not only between kinds of micro-organisms but also between strains of the same species. Strains of *Saccharomyces cerevisiae* obtained from breweries exhibit wide variation in biosorption capacities for metal sorption (Simmons *et al.* 1995). In addition, metal accumulation by bacteria has been shown to be profoundly influenced by pH, buffer type, ionic strength, incubation time (Nakajima & Sakaguchi 1986) and the growth conditions. Different culture media as well as growth conditions are known to affect growth characteristics and metal sequestering abilities of microbial cultures (Volesky & May-Phillips 1995). However, in the present study the biosorption experiments were performed under identical growth conditions (culture media, temperature, age culture etc.). Thus, it seems that the accumulation of uranium by *A. ferrooxidans* is type specific.

In addition, as the solution pH increased from 1.5 to 4, (higher pH values could not be tested because of the formation of metal hydroxide precipitates) an increase of U(VI) accumulation was observed for all three types studied. Metal sorption by microorganisms is affected by the pH solution; the lower metal uptake at highly acidic pH could be attributed to competition for metal binding sites between metal and hydrogen (H^+) and hydronium (H_3O^+) ions (Gadd 1988), while the increase in pH favours metal sorption mainly because of the elevated levels of negatively charged groups (Luef *et al.* 1991).

Uranium biosorption studies carried out at varying initial metal concentrations (from 5 to 25 mg l) revealed that uranium is sorbed linearly with increasing concentration. This behaviour agrees with the findings of DiSpirito *et al.* (1983) for other *A. ferrooxidans* strains. Observed enhancement in metal sorption could be due to increase in electrostatic interactions (relative to covalent interactions), involving sites of progressively lower affinity for metal ions (Al-Asheh & Duvnjak 1995).

The amount of uranium bound to the biomass increases in the order *A. ferrooxidans* type I, type III and type II. Interestingly, the strains belonging to the types I and III are resistant to 8 and 9 mM of uranium, respectively, whereas those of the type II do

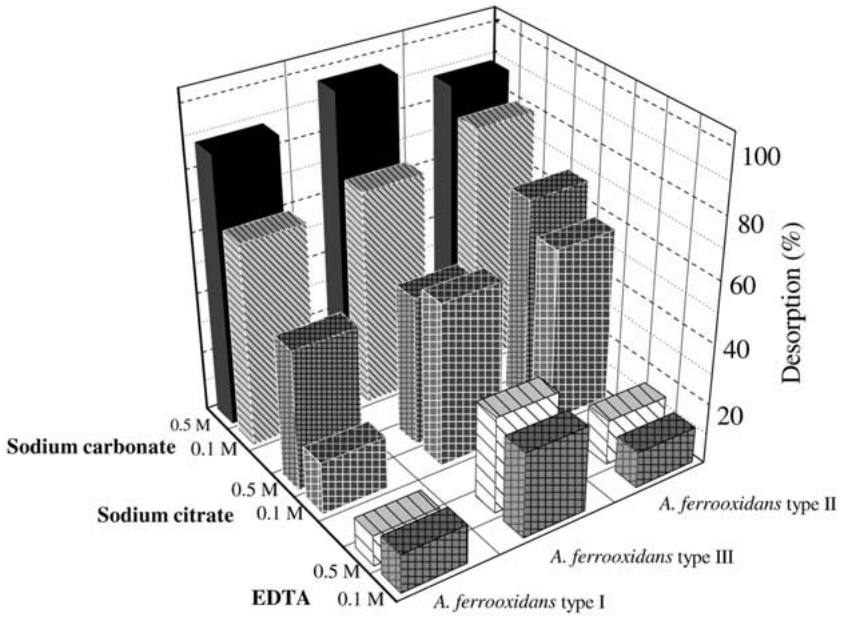

Fig. 3. Desorption of uranium accumulated by the three types of *A. ferrooxidans*.

not tolerate more than 2 mM of uranium. On the basis of the results presented one may speculate that the strains of the type I and III are more resistant to uranium, probably because they possess a mechanism which limits the uranium binding below the lethal amounts. Such physiological differences (tolerance to uranium) between the different *A. ferrooxidans* types could explain their heterogeneous distribution in the uranium mining waste piles: The group of isolates belonging to types I and III, were predominant in more contaminated samples from greater depths, while the representatives of the type II were distributed in less contaminated area preferably close to the surface. In uranium mining waste piles, all *A. ferrooxidans* types are present together, by increasing the concentration of uranium and other heavy metals, adaptation of the type I and III in these environments occurs by natural selection, consequently the later two types outgrow its competitors (representatives of the type II) and dominate the population. There are many reports on the adaptation and dominance of microbial population in acidic environments (Bond *et al.* 2000; Fortin *et al.* 2000). These reports has tended to focus on the competition between mesophilic iron-oxidising chemolithotrophs (*A. ferrooxidans* and *Leptospirillum ferrooxidans*) for ferrous iron and mineral oxidation (Norris *et al.* 1988). Because of its greater affinity for ferrous iron, tolerance of very low pH (< 1.8) and greater tolerance of ferric iron, *L. ferrooxidans* tends to be more effective when leaching ores (e.g. gold concentrates) which are rich in pyrite, or in environments where ferrous iron concentration and/or pH are low. In contrast, the faster growth rate of *A. ferrooxidans* generally results in this iron-oxidiser dominating situations (such as enrichment cultures used frequently to isolate iron-oxidising acidophiles) where ferrous iron concentrations are relatively high and/or pH is greater than 2 (Johnson 1998).

This natural physiological variability among isolates of *A. ferrooxidans* is very important because it allows for natural selection to occur in the leaching environment (Tuovinen & Kelly 1974; Leduc & Ferroni 1993), those strains which are better adapted to the environmental conditions will survive and proliferate. It is probable, therefore, that the levels of uranium resistance reported in the present work could be explained by an acquired tolerance to uranium as result of natural selection during the leaching process, as suggested by Tuovinen & Kelly (1974).

For the use of micro-organisms as biosorbent, it is important to determine whether bound metals could desorb. In the present work, we demonstrated that the sodium carbonate is able to remove up to 97, 88.50 and 88.33% of the uranium sorbed from the cells of *A. ferrooxidans* type III, II and I, respectively. Similar results were reported about uranium by Marqués

et al. (1991), and Gonzalez-Muñoz *et al.* (1997) working with *Pseudomonas* sp. and *Myxococcus xanthus*, respectively. They found that the sodium carbonate recuperate 98 and 81%, respectively, of the uranium biosorbed. The desorption of uranium by sodium carbonate treatment may be a result of the alkalisation of the medium. Although an effect of the carbonate ion itself resulting in the formation of uranyl carbonate complexes, which might be stronger than the 'bacterial uranyl' ones cannot be ruled out. Approximately 12% of the bound metal ions were not removed from the cells of *A. ferrooxidans* type I and II by this treatment. It is not excluded that in these cases part of the uranium was complexed at sites with higher stability constants than those of the uranyl carbonate.

The treatment of cells with EDTA had no pronounced effect on the desorption of uranium (the uranium elution was between 10 and 30%). Panak *et al.* (1999) found that EDTA could recuperate only between 11 and 41% of the uranium accumulated by *A. ferrooxidans* 33020 and *A. ferrooxidans* 19859. Shuttleworth & Unz (1993) working on the sorption of Ni, Cu and Zn by filamentous bacterium *Thiothrix* strain A1, found that approximately 20% of the bound metal ions were not removed from *Thiothrix* A1 by the strong chelating agent EDTA; this nonextractable metal could have diffused into the interior of the cells. Alternative explanations of the observed phenomenon in *A. ferrooxidans* types include: (i) complexation of uranium at sites with higher stability constants than EDTA and (ii) the presence of metal-binding sites which are inaccessible to EDTA. It seems unlikely, however, that there were surface-binding sites with higher affinity for metals than EDTA, because there was no evidence of any bacterial metal uptake from performed metal-EDTA complexes.

Extended X-ray Absorption Fine Structure (EXAFS) spectroscopy and Infrared (IR) spectroscopy analysis (Merroun *et al.* 2000a, b, c) indicated that phosphorus or/and sulfur containing residues from the cell surfaces of *A. ferrooxidans* are involved in the interaction with uranium. Furthermore, no structural differences were observed for the uranium complexes formed at the cells of these types. However, the EXAFS spectra are indicating a formation of uranium complexes which are different from those formed by Bacilli (Hennig *et al.* 2001). Further studies in these complexes are currently under analysis using Time-Resolved Laser-Induced Fluorescence Spectroscopy (TRLFS) and Nuclear Resonance Magnetic (NMR) spectroscopy.

Conclusions

The different types of *A. ferrooxidans*, recently described in our laboratory interact with uranium and tolerate this radionuclide in a type-specific way. As the various types of the above mentioned bacterial isolates interact with uranium in different ways, a monitoring of their distribution in the polluted environments may be useful for modelling the process of migration of this hazardous radionuclide in nature. In addition, such strains might be suitable for in situ bioremediation of the uranium wastes from where they are recovered, because they are well adapted to these extremely complex environments.

Acknowledgements

This work was supported by grant 7531.50-03-FZR/607 from the Sächsisches Staatsministerium für Wissenschaft und Kunst, Dresden, Germany. We thank Leo Leduc for providing us some of the strains and W. Wiesener and U. Schaefer for the ICP-MS-measurements.

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