Microbacterium isolates from the vicinity of a radioactive waste depository and their interactions with uranium

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Abstract

Three oligotrophic bacterial strains were cultured from the ground water of the deep-well monitoring site S15 of the Siberian radioactive waste depository Tomsk-7, Russia. They were affiliated with *Actinobacteria* from the genus *Microbacterium*. The almost fully sequenced 16S rRNA genes of two of the isolates, S15-M2 and S15-M5, were identical to those of cultured representatives of the species *Microbacterium oxydans*. The third isolate, S15-M4, shared 99.8% of 16S rRNA gene identity with them. The latter isolate possessed a distinct cell morphology as well as carbon source utilization pattern from the *M. oxydans* strains S15-M2 and S15-M5. The three isolates tolerated equal amounts of uranium, lead, copper, silver and chromium but they differed in their tolerance of cadmium and nickel. The cells of all three strains accumulated high amounts of uranium, i.e. up to 240 mg U $(g\,dry\,biomass)^{-1}$ in the case of *M. oxydans* S15-M2. X-ray absorption spectroscopy (XAS) analysis showed that this strain precipitated U(VI) at pH 4.5 as a meta-autunite-like phase. At pH 2, the uranium formed complexes with organically bound phosphate groups on the cell surface. The results of the XAS studies were consistent with those obtained by transmission electron microscopy (TEM) and energy dispersive X-ray analysis (EDX).

Introduction

Radioactive waste has been stored underground for decades with the primary objective of permanently isolating the disposed waste from the biosphere (Stroes-Gascoyne & West, 1996; Christofi & Philip, 1997; Bachofen *et al.*, 1998). A number of countries are involved in research programs that examine the option of permanent storage of radioactive waste for the future (Pedersen, 1996; Bachofen *et al.*, 1998). The main concern about this method of disposal is the possibility of radioactive waste escaping and migrating into sediments and ground water. Due to this concern, multidisciplinary studies have been undertaken in order to evaluate the impact of such waste on subsurface environments and their potential remediation (Stroes-Gascoyne & West, 1996; Anderson & Pedersen, 2003; Wickham *et al.*, 2003; Nazina *et al.*, 2004). It has been demonstrated that these extreme habitats harbor viable and metabolically active microorganisms. These microorganisms exploit a wide range of redox reactions to maintain viability and thus influence geochemical conditions (Lovley & Chapelle, 1995; Pedersen, 1996; Ehrlich, 1998; Pedersen, 2000; Nedelkova *et al.*, 2005).

A number of studies have shown that indigenous microorganisms isolated from heavy-metal-contaminated habitats (Selenska-Pobell *et al.*, 1999, 2001; McLean & Beveridge, 2001; Merroun & Selenska-Pobell, 2001; Selenska-Pobell, 2002; Shelobolina *et al.*, 2004) or from other extreme environments (Duxbury & Bicknell, 1983; Benyehuda *et al.*, 2003), as well as some from nonextreme ones (Ben Omar *et al.*, 1997; Merroun *et al.*, 2001) effectively interact with toxic metals and radionuclides via direct and indirect mechanisms. These interactions can cause mobilization and/or immobilization of the metals (Francis, 1998; Banaszak *et al.*, 1999; Lloyd & Lovley, 2001; Selenska-Pobell, 2002). They encompass biotransformations such as the oxidation or reduction of metals (Lovley, 1993; Lack *et al.*, 2002; Shelobolina *et al.*, 2004; Beller, 2005), biosorption by cell surface polymers (Selenska-Pobell *et al.*, 1999; Vieira & Volesky, 2000; Merroun *et al.*, 2005), uptake of metals into the cells (McLean & Beveridge, 2001; Merroun *et al.*, 2002; Francis *et al.*, 2004; Suzuki & Banfield, 2004), induction of metal precipitation and generation of minerals (Macaskie *et al.*, 1992; Douglas & Beveridge, 1998; Renninger *et al.*, 2004) or alteration of metal speciation caused by microbially induced redox changes in the environment (Bosecker, 1997).

The above described microbial activities strongly influence the fate of toxic metals both in and outside the habitat where the radioactive waste was deposited (Banaszak *et al.*, 1999).

Investigation of the microorganism–metal interactions provides insight into the potential of microorganisms to alter toxicity of radionuclides and heavy metals, and to influence their behavior in the environment. Understanding the underlying mechanisms of these interactions is important for the development of bioremediation strategies (Finneran *et al.*, 2002; Raff *et al.*, 2003). The optimization of such strategies requires extensive studies on the speciation of toxic metals and their localization in microorganisms. The speciation and bonding environment of uranium in bacteria has been examined by a variety of spectroscopic techniques such as Fourier-transformed infrared (FTIR) and time-resolved laser-induced fluorescence spectroscopy (TRLFS; Merroun *et al.*, 2003a). Synchrotron-based techniques have been used to determine the oxidation state of uranium by X-ray absorption near-edge spectroscopy (XANES) and the molecular structure of the uranium complexes by extended X-ray absorption fine structure (EXAFS) formed by various microorganisms (Kelly *et al.*, 2002; Merroun *et al.*, 2003b, 2005; Francis *et al.*, 2004). In addition, using transmission electron microscopy (TEM) and energy dispersive X-ray analysis (EDX) techniques the metal deposits can be localized and identified (McLean & Beveridge, 2001; Merroun *et al.*, 2003b; van Hullebusch *et al.*, 2003).

The aim of this work was to characterize three oligotrophic bacterial strains recovered from the water of the subsurface monitoring well S15 which is located in close proximity to the radioactive waste disposal site Tomsk-7 in Siberia, Russia. Here we specifically investigate the interactions between these isolates and uranium and other toxic metals. Our results will assist in the risk assessment of the studied Siberian subsurface environment where storage of radioactive waste in repositories is still practiced.

Materials and methods

Site description and sample collection

The S15 monitoring well is located *c.* 2 km from the radioactive waste injection site Tomsk-7 and penetrates into an aquifer at 290–324 m below ground, which corresponds to one of the radioactive disposal horizons (Wickham *et al.*, 2003). The water from the well was described as oligotrophic with insignificant content of organic matter. The dominant ions were HCO_3^-, Na^+, Ca^{2+} and Mg^{2+}. Only naturally occurring radionuclides were found. At the time of collection (July 2002) the water had the following characteristics: temperature 14 °C, pH 6.7 and Eh −95 mV. Sampling from the well was performed after pumping out the underground water using an electric pump (Wickham *et al.*, 2003). Three parallel water samples, with a volume of 1 L each, were poured into sterile glass vessels filled with nitrogen gas in order to keep them isolated from the atmosphere. The biomass from each of the samples was concentrated via sequential filtration using a glass fiber filter with a pore size of 1.2 μm and two nitrocellulose filters with pore sizes of 0.45 and 0.22 μm. The filters were kept frozen at −20 °C for further analyses. The biomass collected on the filters of two of the samples was used for microbial diversity characterization by molecular approaches based on 16S rRNA and RubisCO genes (Nedelkova, 2005; Nedelkova *et al.*, 2005; Nedelkova & Selenska-Pobell, 2006). The three bacterial isolates studied in this work were recovered from the biomass collected on the 0.22-μm filter from the third of the above-mentioned water samples.

Bacterial strains, isolation and cultivation conditions

An oligotrophic R2A medium (Reasoner & Geldreich, 1985) was used to isolate and cultivate the S15 strains. Pieces of the 0.22-μm filter (*c.* 1 cm^2) were incubated in an Erlenmeyer flask in 50 mL of R2A medium at 25 °C and shaken at 110 r.p.m. After 3 days of incubation, serial dilutions from 10^{-1} to 10^{-5} were made and 10-μL aliquots plated on solid R2A agar medium. Colonies with different morphology and colors were picked from the 10^{-4} and 10^{-5} dilutions and transferred again to liquid R2A medium. In order to isolate pure bacterial strains, the cultures were subjected to two additional cultivation cycles, each including incubation in liquid followed by solid R2A medium. Bacterial growth in the liquid medium was monitored by measuring optical densities at 600 nm using a Pharmacia Biotech spectrometer Ultrospec 1000 (Amersham, Bioscience, Freiburg, Germany). The isolates were stored in glycerol stocks at −80 °C.

Molecular characterization of the isolates

DNA extraction was performed from 2 mL bacterial cultures collected at the mid-exponential growth phase using the NucleoBond® AXG 100 Kit (Macherey-Nagel, Düren, Germany) according to the instructions of the manufacturer. The 16S rRNA gene fragments were amplified using the primer pair $16S_{7-27:F}$ (5′-AAGAGTTTGATYMTGGCTCAG-3′) and $16S_{1492-1513:R}$ (5′-TACGGYTACCTTGTTACGACTT-3′), *Escherichia coli* numbering. The 16S rRNA gene products were sequenced on an ABI PRISM 310 Genetic Analyser (Perkin Elmer, Foster City, California, USA). The sequences were compared with those available in the GenBanks by BLAST (Basic Local Alignment Search Tool) analysis. The multiple sequence alignment program CLUSTAL W (Thompson *et al.*, 1994) was used for sequence alignment. Phylogenetic trees were generated based on the results of the neighbor-joining algorithm with distance analysis using

Jukes-Cantor corrections according to the PHYLIP v.3.5c package (Felsenstein, 1993). The almost complete 16S rRNA gene sequences of the isolates S15-M2 and S15-M5 (*E. coli* positions 37–1542) and S15-M4 (*E. coli* positions 31–1542) are available in the public gene libraries under accession numbers AM234158, AM234159 and AM234160.

Physiological analysis

The substrate utilization patterns were determined in duplicate. Cells grown to mid-exponential growth phase were harvested by centrifugation at 8600 ***g*** for 10 min at 4 °C and washed three times with 0.9% NaCl to remove residual substrates. For each growth test, 200-μL aliquots of the cell suspension were inoculated in inorganic basal medium (Hahn *et al.*, 2003), which was distributed in Cellstar® 24-well plates (Greiner Bio-one GmbH, Frickenhausen, Germany). The wells with an end volume of 2 mL were supplemented with different substrates to the required final concentrations. After 4 weeks of incubation at room temperature and shaking at 110 r.p.m., growth was assessed by determining culture turbidity.

To test for growth on different carbon sources, sterile stock solutions of glucose, fructose, L- and D-arabinose, D-sorbitol, sucrose and maltose were added to the inorganic basal medium at a final concentration of 5 mM. The ability to utilize potential electron donors was analyzed by adding the following compounds to the medium: sodium acetate (5 mM), sodium pyruvate (5 mM), citric acid (2 mM), maleate (5 mM), Na-L-lactic acid (5 mM), tartaric acid (2 mM) and nicotinic acid (2 mM). Growth with different alcohols such as methanol (2 mM), ethanol (5 mM) and 2-propanol (5 mM) was also tested. In addition, L-arginine (2 mM), L-glutamine (2 mM) and Tween 80 (0.001%, v/v) were tested.

Scanning electron microscopy (SEM)

Morphological examination of the bacterial cells was performed using a low voltage scanning electron microscope, LEO 982 Gemini (LEO, Elektronenmikroskopie GmbH, Oberkochen, Germany). Cell suspensions were fixed in 4% glutaraldehyde solution in 25 mM KH_2PO_4 buffer (pH 7) for 24 h at 4 °C. Cells were harvested by centrifugation (8600 ***g***, 5 min, 4 °C) and dehydrated with increasing ethanol concentrations (from 10% to 99%). The dehydrated samples were fixed on a carbon-tape-covered aluminum sample holder, followed by shadow casting with carbon (MED 010, Baltec AG, Lichtenstein).

Heavy metal solutions

One molar stock solutions of $Cu(NO_3)_2 \cdot 3H_2O$, $Ni(NO_3)_2 \cdot 6H_2O$, $Cr(NO_3)_3 \cdot 9H_2O$, $Cd(NO_3)_2 \cdot 4H_2O$, and $Pb(NO_3)_2$ and 0.1-M stock solution of $UO_2(NO_3)_2 \cdot 6H_2O$ were prepared by dissolving appropriate quantities of the metal salts in 0.1 M $NaClO_4$. A 1-M stock solution of $AgNO_3$ was prepared by dissolving the appropriate quantity of metal in 0.1 M HNO_3. The metal solutions were sterilized by filtration through 0.22-μm nitrocellulose filters.

Heavy metal tolerance

The minimal inhibitory concentrations (MICs) of seven heavy metals were determined in duplicate. Cells were grown to mid-exponential phase in low phosphate medium (LPM; Rossbach *et al.*, 2000), washed twice with 0.9% NaCl, and 10 μL of the cell suspension was transferred to LPM agar. The LPM agar contained increasing metal concentrations in twofold increments as follows: for copper, nickel and uranium, increasing concentrations from 0.25 to 16 mM; for chromium, from 0.5 to 16 mM; for cadmium and silver, from 0.03 to 1 mM; for lead, from 0.06 to 2 mM. After spreading the inoculum, the plates were incubated at room temperature for 1 week. The MIC was defined as the lowest concentration of each of the metals at which complete inhibition of colony formation was observed (Rossbach *et al.*, 2000).

Uranium sorption

Bacterial cells grown to mid-exponential phase were harvested by centrifugation at 8600 ***g*** for 20 min at 4 °C and washed twice with 0.1 M $NaClO_4$ to remove the growth medium ingredients. The washed cells (between 0.2 and 0.3 mg mL^{-1} dry weight) were resuspended and shaken for 48 h in 10 mL of solutions containing different concentrations of uranium (from 2.75 to 159 mg L^{-1}) at pH values of 2, 3 and 4.5. After contact with uranium solutions, the cells were harvested and 1 mL of the supernatant was collected for metal analysis using an ELAN 9000 inductively coupled plasma–mass spectrometer (ICP-MS; Perkin-Elmer, Shelton, CT, USA). Accumulation of metal ions (q) by biomass was calculated from a metal biomass balance yielding (Volesky, 1990): q (mg U g^{-1} dry biomass) $= V(C_i - C_f)/m$, where V is the sample volume (L), C_i and C_f are the initial and the final metal concentrations (mg L^{-1}), respectively, and m is the amount of dry biomass (g). Three replicates were prepared for each concentration. In addition, experimental control samples without biomass were treated identically. For determining biomass dry weight, the cell pellets were dried at 70 °C for 48 h and their weight determined using a Sartorius LA 120 S balance (Göttingen, Germany; ± 0.1 mg).

To determine the time course of uranium sorption the bacterial cells were treated as described above. The washed cells were resuspended and shaken in triplicate for 30 min, 2, 16, 24, 48 and 72 h at 30 °C in 10 mL uranium solution (23 mg L^{-1}, pH 4.5). Cells were then harvested and the metal

content of the supernatants analyzed by ICP-MS as described above.

Preparation of samples for X-ray absorption spectroscopy (XAS) analysis

Bacterial cells grown to mid-exponential phase were harvested by centrifugation at 8600 ***g*** for 20 min at 4 °C and washed twice with 0.1 M $NaClO_4$. Washed cells were resuspended and shaken for 48 h in 10 mL of uranium solution (119 mg L^{-1}) at pH values of 2, 3 and 4.5. We used 0.1 M $NaClO_4$ as a background electrolyte.

After contact with the uranium solution, cells were harvested and washed with 0.1 M $NaClO_4$. The pelleted samples were dried in a vacuum incubator at 30 °C for 24 h and powdered.

XAS measurements

Uranium L_{III}-edge X-ray absorption spectra were collected at the Rossendorf Beamline at the European Synchrotron Radiation Facility (ESRF), Grenoble (France; Matz *et al.*, 1999) using a Si(111) double-crystal monochromator, and Pt-coated mirrors for focusing and rejection of higher harmonics. Data were collected in fluorescence mode using a 13-element Ge detector (Canberra). The energy was calibrated by measuring the Y K-edge transmission spectrum of an yttrium foil and defining the first inflection point as 17 038 eV. The biological/uranyl samples were measured as dry samples. Eight spectra for each sample were recorded. The EXAFS oscillations were isolated from the raw, averaged data by removal of the pre-edge background, approximated by a first-order polynomial, followed by μ_0 removal via spline-fitting techniques and normalization using a Victoreen function. Dead-time correction was applied. The ionization energy for the U L_{III} electron niveau, E_0, was arbitrarily defined as 17 185 eV for all averaged spectra. The EXAFS spectra were analyzed according to standard procedures using the program EXAFSPAK (George & Pickering, 1995). The theoretical phase and amplitude functions used in data analysis were calculated with FEFF8 (Ankudinov *et al.*, 1998) using the model that contains fragments of two molecules, meta-autunite and uranyl triacetate (Merroun *et al.*, 2005). All fits included the four-legged multiple scattering (MS) path of the uranyl group, $U–O_{ax}–U–O_{ax}$. The coordination number (N) of this MS path was linked to N of the single-scattering (SS) path $U–O_{ax}$. The radial distance (R) and Debye–Waller factor (σ^2) of the MS path were linked at twice the R and σ^2 of the SS path $U–O_{ax}$, respectively (Hudson *et al.*, 1996). During the fitting procedure, N of the $U–O_{ax}$ SS path was held constant at two. The amplitude reduction factor S_0^2 was held constant at 1.0 for the FEFF8 calculation and EXAFS fits. The shift in threshold energy, ΔE_0, was varied as a global parameter in the fits.

TEM and EDX

After incubation with uranium solution (119 mg L^{-1}) at pH 2, 4.5 cells were fixed in 2.5% glutaraldehyde in 0.1-M cacodylate buffer (pH 7.2) for 2 h at 4 °C and washed three times with cacodylate buffer. Cell pellets were fixed for 1 h at 4 °C in 1% OsO_4 in cacodylate buffer before being dehydrated with ethanol and embedded in Spurr resin. The samples were thin sectioned (0.25 μm) using a diamond knife on a Reichert Ultracut S ultramicrotome, and the sections were supported on copper grids and coated with carbon. EDX analysis was performed at 200 kV using a spot size of 70 Å, and a live counting time of 200 s. For selected area electron diffraction, we used a Philips CM 200 electron microscope (Philips, Eindhoven, the Netherlands) in the diffraction mode with a camera length of 1000 mm and an exposure time of between 15 and 20 s. Bacterial cell slides were examined with the high-resolution transmission Philips CM 200 at an acceleration voltage of 200 kV under standard operating conditions with the liquid nitrogen anticontaminator in place.

Results

Phylogenetic affiliation of the S15 bacterial isolates

The phylogenetic affiliation of the three bacterial isolates recovered from the ground-water monitoring well S15 of the Siberian radioactive depository Tomsk-7 was determined on their 16S rRNA gene analysis. As evident from the results presented in Table 1, the three isolates were affiliated to the actinobacterial genus *Microbacterium*. The rRNA gene sequence of the strain S15-M2 was identical with those of three *M. oxydans* strains CV71a, AC44 and SW366-KB-3. The strain S15-M5 was affiliated, also with a 100% of 16S rRNA gene identity, with another *M. oxydans* strain, AC94. The *M. oxydans* strains, CV71a, AC44 and AC94 were recently isolated from alkaline ground waters (Tiago *et al.*, 2004), while the strain SW366-KB-3 was recovered in our

Table 1. Affiliation of the strains studied based on the 16S rRNA gene analysis

Strain	Closest relative (accession No.)	Identity (%)
S15-M2 (AM234158)	*M. oxydans* CV71a (AJ717358)	100
	M. oxydans AC44 (AJ717357)	100
	M. oxydans SW366-KB-3 (AM234157)	100
S15-M5 (AM234159)	*M. oxydans* AC94 (AJ717356)	100
S15-M4 (AM234160)	*Microbacterium* sp. AS-44 (AJ391205)	100
	M. oxydans AC44 (AJ717357)	99.8
	M. oxydans CV71a (AJ717358)	99.8
	M. oxydans AC94 (AJ717356)	99.8

	E. coli 191	*E. coli* 412	*E. coli* 613	*E. coli* 627
S15-M2	GTGACCGCAT	GGACGACG	CGGAGGCTCAACCTCCG	
S15-M5	GTGATCGCAT	GGATGACG	CGGAGGCTCAACCTCCG	
S15-M4	GTGACCGCAT	GGATGACG	CCGAGGCTCAACCTCGG	

Fig. 1. Variable positions found in the 16S rRNA gene sequences of the three studied *Microbacterium* isolates S15-M2, S15-M5 and S15-M4.

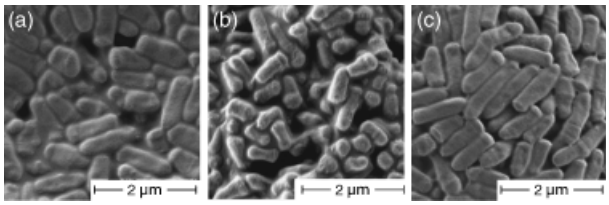

Fig. 2. Scanning electron micrographs of the strains: (a) S15-M2, (b) S15-M4 and (c) S15-M5.

laboratory from a heavy-metal-contaminated water sample from flooded uranium mill tailings in Germany (Heilig, 2004). The 16S rRNA gene sequence of the isolate S15-M4 was identical to that of the marine isolate *Microbacterium* sp. AS-44 and shared 99.8% of identity with the other mentioned *M. oxydans* strains (Table 1).

The almost identical 16S rRNA gene sequences of the three S15 isolates possessed few, but very characteristic, variations (Fig. 1). In the case of the *M. oxydans* strains S15-M2 and S15-M5 there were only two mismatches, at positions 191 and 412 of the gene. The same 16S rRNA gene positions were variable in the *Microbacterium* sp. strain S15-M4, one matching with S15-M2 at position 191 and one with S15-M5 at position 412. The 16S rRNA gene sequence of the strain *Microbacterium* sp. S15-M4 possessed two additional differences at positions 613 and 627 (Fig. 1).

Morphological and physiological characterization of the microbacterial S15 isolates

The scanning electron micrographs (Fig. 2) demonstrate that the cells of the *Microbacterium* isolate S15-M4 were morphologically different and fairly smaller than those of the isolates S15-M2 and S15-M5.

The strain S15-M4 utilized five of the tested substrates, namely glucose, fructose, L-arabinose, maltose and sucrose (Table 2). In addition, weak growth was observed in the presence of sodium acetate, sodium pyruvate and L-arginine, as the sole source of carbon. In contrast, the two *M. oxydans* strains, S15-M2 and S15-M5, utilized fewer of the mentioned substrates. As shown in Table 2, like S15-M4, they both grew well in the presence of sucrose and weakly on sodium pyruvate, but only the *M. oxydans* S15A-M2 strain utilized L-arabinose similarly to S15-M4.

Table 2. Substrate utilization patterns of *Microbacterium oxydans* S15-M2 and S15-M5 and *Microbacterium* sp. S15-M4

Substrate	*M. oxydans* S15-M2	*Microbacterium* sp. S15-M4	*M. oxydans* S15-M5
Glucose	–*	+†	–
Fructose	–	+	–
L-Arabinose	+	+	–
D-Arabinose	–	–	–
Maltose	–	+	–
Sucrose	+	+	+
D-Sorbitol	–	–	–
Sodium acetate	–	w	–
Sodium pyruvate	w‡	w	w
Citric acid	–	–	–
Maleate	–	–	–
Na-L-Lactic acid	–	–	–
Tartaric acid	–	–	–
Nicotinic acid	–	–	–
Methanol	–	–	–
Ethanol	–	–	–
2-Propanol	–	–	–
Tween-80	–	–	–
L-Arginine	–	w	–
L-Glutamine	–	–	–

*No growth.
†Growth.
‡Weak growth.

Heavy metal tolerance

The MICs of copper (8 mM), chromium (16 mM), lead (2 mM), silver (0.06 mM) and uranium (4 mM) were identical for all three *Microbacterium* strains studied (data not shown). The strain S15-M4 tolerated lower concentrations of nickel (2 mM) in comparison to the strains S15-M2 and S15-M5 (4 mM). The MIC of Cd for the strain S15-M2 (2 mM) was four times higher than that found for the strain S15-M5. This tolerance value (2 mM) was unusually high in comparison to the MIC for other *Microbacterium* strains studied in our laboratory (Heilig, 2004), as well to that of other bacterial strains isolated from heavy-metal-contaminated sites (Brottka, 2003). The MIC of cadmium for S15-M4 was of an intermediate level (1 mM).

Uranium biosorption kinetics

The time course of U(VI) sorption at pH 4.5 and the initial uranium concentration of 23 mg L^{-1} by *M. oxydans* S15-M2 showed that in the first 2 h the sorption of U(VI) took place very rapidly and afterwards gradually slowed to maximum sorption [77.63 ± 0.5 mg U (g dry biomass)$^{-1}$]. Equilibrium was reached after a contact time of 48 h. These results indicate that the accumulation of U by this strain is probably

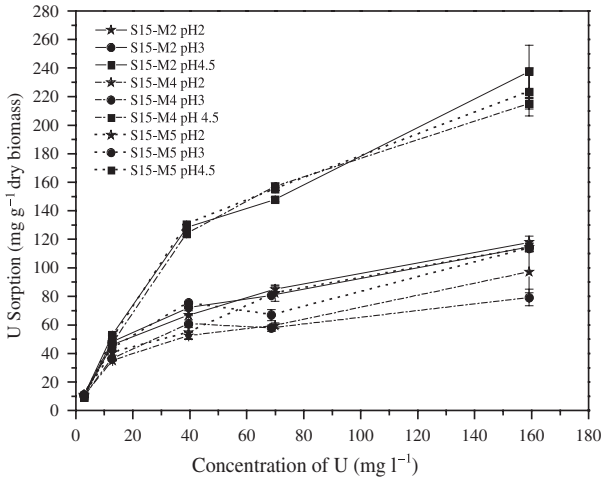

Fig. 3. Effect of the pH and metal concentration on the biosorption of U(VI) by the strains S15-M2, S15-M5 and S15-M4.

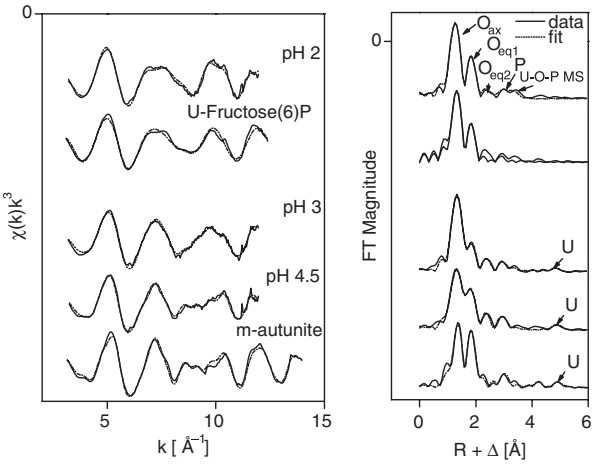

Fig. 4. Uranium L_{III}-edge k^3-weighted EXAFS spectra (left) and the corresponding Fourier transforms (right) of the uranium complexes formed by the cells of *Microbacterium oxydans* S15-M2 at pH values 2, 3 and 4.5, as well as of the reference compounds (meta-autunite and U-Fructose(6)Phosphate).

a two-phase process. The biosorption kinetics of U(VI) by the strains S15-M4 and S15-M5 were similar (data not shown).

Effect of pH and metal concentration on uranium sorption

The accumulation of uranium by the three *Microbacterium* isolates was dependent on the initial uranium concentration and the pH of the metal solution. As shown in Fig. 3, the amount of the uranium bound by the strains studied increased with increasing metal concentration (from 2.75 to 159 mg L^{-1}) at pH 2, 3 and 4.5. At pH 4.5 all strains were able to accumulate higher amounts of uranium [up to 240 ± 18 mg (g dry biomass)$^{-1}$ in the case of *M. oxydans* S15-M2] than at pH 2 and 3. At these pH values, all three strains accumulated almost identical amounts of uranium and their uranium-binding capacities were comparable under studied conditions to those of the above-mentioned isolate *M. oxydans* SW366-KB-3 (Table 1) recovered from uranium mining waste water in Germany (Heilig, 2004).

XAS analysis

XANES analysis (data not shown) demonstrated that the oxidation state of uranium species bound to the cells was unchanged, indicating that the bacteria do not reduce U(VI).

Information on the local environment of uranium atoms in the uranium-treated bacterial samples was provided by analysis of EXAFS data. Uranium L_{III}-edge EXAFS spectra of the uranium species formed at pH 2, 3 and 4.5 by the cells of *M. oxydans* S15-M2 and their corresponding Fourier transforms (FTs) are presented in Fig. 4. The FTs represent a pseudoradial distribution function of the uranium near-neighbor environment. The FT peaks appeared at lower *R*-values ($R+\Delta$) relative to the true near-neighbor distances *R* as a result of the EXAFS phase shift. This shift depends on the scattering phase function ϕ of the electron wave and initiates a shift in the interatomic distance of from 0.2 to 0.5 Å.

Quantitative fit results (see Table 3) indicate that the adsorbed U(VI) has the common linear *trans*-dioxo

Table 3. Structural parameters of the uranium complexes formed by *Microbacterium oxydans* S15-M2 at different pH values and reference compounds

Sample	Shell	*N**	*R* (Å)†	σ^2 (Å^2)‡	Δ*E* (eV)
pH 2	U–O_{ax}	2§	1.75	0.0022	−14.1
	U–O_{eq1}	5.3 (5)	2.33	0.010	
	U–O_{eq2}	0.6 (1)	2.85	0.0030§	
	U–P	3.1 (3)	3.62	0.0040§	
	U–O_{eq1}–P (MS)	6.2	3.76	0.0040§	
pH 3	U–O_{ax}	2§	1.77	0.0021	−13.54
	U–O_{eq1}	4.0 (3)	2.27	0.0082	
	U–O_{eq2}	0.8 (1)	2.86	0.0030§	
	U–P	1.8 (2)	3.59	0.0040§	
	U–O_{eq1}–P (MS)	3.6	3.71	0.0040§	
	U–U	1.9 (6)	5.19	0.0080§	
pH 4.5	U–O_{ax}	2§	1.77	0.0046	−14.87
	U–O_{eq1}	4.3 (2)	2.28	0.0062	
	U–O_{eq2}	1.0 (2)	2.90	0.0030§	
	U—P	3.4 (3)	3.59	0.0040§	
	U–O_{eq1}–P (MS)	6.8	3.70	0.0040§	
	U–U	2.4 (5)	5.20	0.0080§	

*Errors in coordination numbers are ±25%, and standard deviations, as estimated by EXAFSPAK are given in parentheses.
†Errors in distance are ±0.02 Å.
‡Debye–Waller factor.
§Value fixed for calculation.

structure: two axial oxygens at about 1.75–1.77 Å, and an equatorial shell of 4–5 oxygens at 2.27–2.33 Å. The U–O_{eq1} bond distance is within the range of previously reported values for phosphate bound to uranyl (Hennig *et al.*, 2001; Merroun *et al.*, 2002, 2003b, 2005).

The FT spectra of *M. oxydans* samples contain an FT peak at about $R+\Delta \sim 2.3$ Å, after correcting for the scattering phase shift. This distance is typical for carbonate groups coordinated to U(VI) in a bidentate fashion (Coda *et al.*, 1981). Indeed, carbon atoms at $R = 2.85$–2.91 Å provide a good fit to the 2.3 Å FT peak. However, previous investigations (data not shown) excluded the implication of carboxyl groups in the coordination of uranium. In addition, oxygen atoms provide a good fit to the residual EXAFS spectrum corresponding to this shell. Thus, this shell is interpreted a contribution from oxygen neighbors (O_{eq2}). The fourth FT peak, which appears at $R+\Delta \sim 3$ Å (radial distance $R = 3.59$–3.62 Å) is a result of the back scattering from phosphorus atoms. This distance is typical for a monodentate coordination of U(VI) by phosphate (Hennig *et al.*, 2001; Merroun *et al.*, 2002, 2003b, 2005).

For the studied *M. oxydans* S15-M2 strain, at pH 3 and 4.5, an FT shell corresponding to a distance of $R = 5.19$–5.20 Å can be related to a U–U contribution. The EXAFS spectra of the *M. oxydans* samples at this pH value are similar to that of m-autunite (Hennig *et al.*, 2001) with regard to the U–O_{eq}, U–P and U–U distances, suggesting that a similar inorganic uranyl phosphate phase was precipitated by the bacterial cells at pH 3 and 4.5, probably because of the release of inorganic phosphates by the cells as resulting from microbial activity. At pH 2, however, the U–U peak is absent, indicating a lower site symmetry around uranium. All known U(VI) complexes with organic phosphates show a $N_{Oeq} \geq 5$ and are related to a longer U–O_{eq} distance, e.g. 2.32 Å in the case of fructose 6-phosphate (F6P) used as reference in Fig. 4. Interestingly, we have found that EXAFS spectra of the samples at this pH have high similarities to those of organic phosphate ligands complexed with U such as fructose 6-phosphate (Koban *et al.*, 2004).

TEM

As shown in Fig. 5a, the cells of the three strains studied accumulated at pH 4.5 uranium at their cell surface (cytoplasmic membranes, peptidoglycan) and also extracellularly as precipitates. At pH 2, however, only small amounts of uranium were bound to the surfaces of the bacterial cells and there was no extracellular precipitation of uranium (Fig. 6a). These results support those obtained with EXAFS spectroscopy (see above).

EDX analysis provides elemental information via the analysis of X-ray emissions caused by a high-energy electron beam. The EDX spectra derived from the uranium deposits (Figs 5b and 6b) show that they are composed of oxygen (O), phosphorus (P) and uranium (U). The copper (Cu) peak resulted from the copper grid used to support the specimen. The lead (Pb) peak originated from the lead citrate solution which was used to improve the contrast of the uranium-treated thin cell sections. The presence of the silicon (Si) peak can be attributed to impurities in the culture medium and/or from the glass material of the flasks in which the cells were grown.

Discussion

The present study describes three oligotrophic bacterial isolates recovered from a groundwater sample, collected from the S15 deep monitoring well of the Siberian radioactive waste depository Tomsk-7, and characterizes their interactions with uranium and other heavy metals. The strains were affiliated with members of the actinobacterial genus *Microbacterium*. This result is concurrent with the observations of Wickham *et al.* (2003), based on fatty acid analysis, that the *Actinobacteria* were highly abundant in other samples collected previously from the S15 monitoring well. Actinobacterial representatives were isolated from different subsurface habitats (Boivin-Jahns *et al.*, 1995; Chandler *et al.*, 1997; Miteva *et al.*, 2004; Tiago *et al.*, 2004; Fields *et al.*, 2005). Interestingly, the most common isolates from radioactive-waste-contaminated sediments were also members of *Actinobacteria* (Fredrickson *et al.*, 2004).

The three *Microbacterium* strains S15-M2, S15-M4 and S15-M5 possessed two 16S rRNA gene stretches with characteristic exchangeability between C or T in the variable regions I and II of the gene at positions 191 and 412, respectively. The exchangeability at position 412 was demonstrated earlier in other closely related actinobacterial isolates cultured from different freshwater habitats (Hahn *et al.*, 2003). The 16S rRNA gene of the strain S15-M4 possessed two additional compensatory exchanges between G and C at positions 613 and 627 of its 16S rRNA gene. This strain had distinct physiological requirements and also slightly different cell morphology then the strains S15-M2 and S15-M5. The latter features might be an indication of the presence of microdiverse *M. oxydans* subpopulations able to rapidly adapt to changing conditions in the studied environment, as demonstrated for other bacterial species (Fuhrman and Campbell, 1998; Moore *et al.*, 1998; Selenska-Pobell, 2002). However, an intragenomic heterogeneity between the 16S ribosomal RNA (rrn) operons of the same strain can not be excluded because our analyses were based on PCR and sequencing and not on the whole genome sequence analysis. However, it was demonstrated that bacteria possessing different rrn operons with unique 16S rRNA

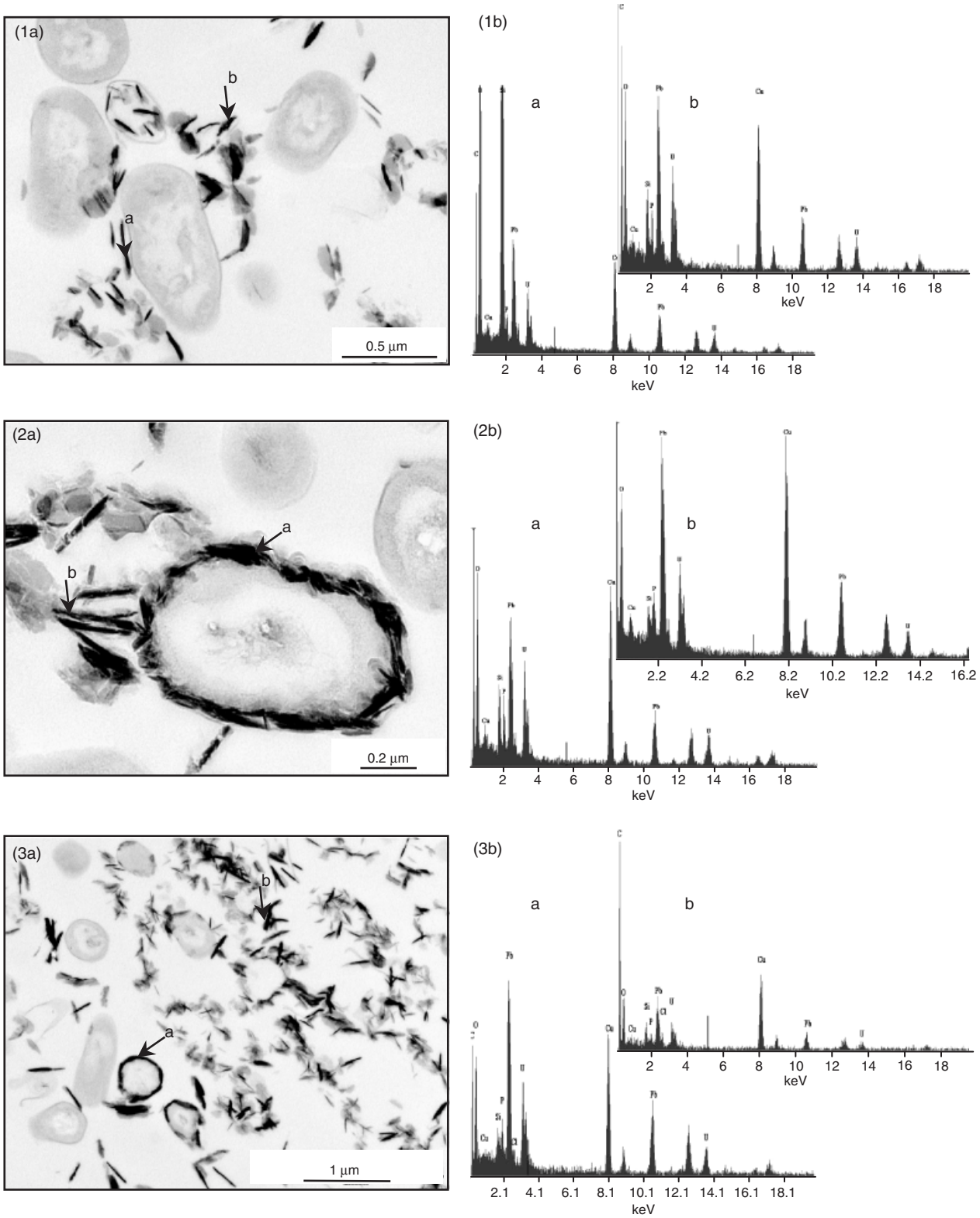

Fig. 5. Transmission electron micrographs (a), coupled with energy dispersive X-ray spectra (b), of a thin section of: (1a) S15-M2; (2a) S15-M4 and (3a) S15-M5 treated with U(VI) at pH 4.5. The metal accumulated is localized on the cell surface (a) and in the extracellular space (b).

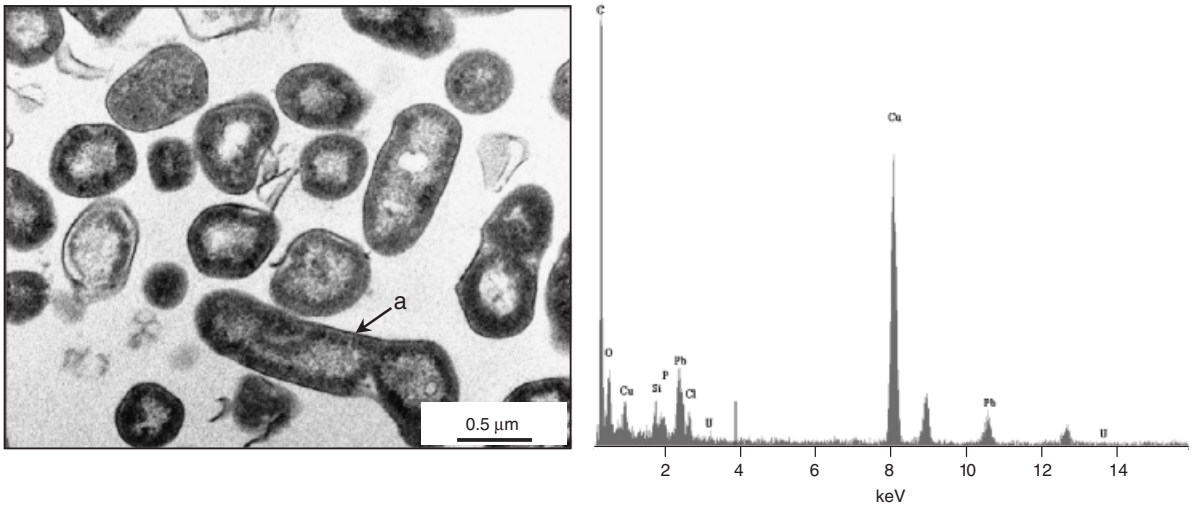

Fig. 6. Transmission electron micrographs (a), coupled with the energy dispersive X-ray spectrum (b), of a thin section of the strain S15-M4 treated with uranium at pH 2. The metal accumulated is localized on the cell surface (a).

signatures may express them individually in links to particular environmental conditions such as different temperatures, controlling in this way the growth rate of the cells (Prüß *et al.*, 1999). The 16S rRNA microdiversity/heterogeneity found in the studied *Microbacterium* isolates indicates that their populations are capable of fast adaptation to different changes in their milieu.

Interestingly, all the three isolates sorbed relatively high and very similar amounts of uranium as the strain *M. oxydans* SW366-KB-3, recovered from heavily uranium-polluted water of a flooded uranium mill tailings in Germany (Heilig, 2004). This is in accordance with our suggestion that the *Microbacterium* populations at the S15 site are flexible, able to adapt rapidly to stress conditions and can sustain changes in their habitat induced by the introduction of heavy metals.

The amount of U accumulated by the three *Microbacterium* isolates increased with increasing pH value from 2 to 4.5. EXAFS studies demonstrated that at pH 2 uranium formed complexes with organically bound phosphate at the cell surface while at pH 4.5 the radionuclide was precipitated with inorganic phosphate as meta-autunite-like phase. At pH 3, both organic and inorganic phosphate uranyl species occurred together, dominating the inorganic phase. To our knowledge, there are only a few spectroscopic studies on the effect of pH on the speciation and association of U to bacterial cells. Kelly *et al.* (2002) used EXAFS spectroscopy to study the effect of this environmental parameter on the complexation of U with the soil bacterium *B. subtilis*. The authors found that at pH 1.67, U is bound to the phosphate groups of the cell surfaces, in agreement with our studies at pH 2. However, at the higher pH value of 4.5 the complexation of U by the cells of *M. oxydans* S15-M2 strain differed significantly from those by the *Bacillus* cells. As previously described by Kelly *et al.* (2002) and Merroun *et al.* (2005), in the cases of *B. subtilis* and *B. sphaericus*, U was coordinated by both phosphate and carboxylic groups at pH 4.8 and 4.5, respectively. In the present work we demonstrate that the involvement of the carboxylic groups in the complexation of U at pH 4.5 can be excluded.

At pH 2 the uranium was exclusively bound to organophosphate groups, while at pH 3 both the organic and inorganic phosphates were involved in the complexation of uranium. This indicates that the number of available organic phosphate groups could have decreased, and the number of inorganic phosphate groups increased with a pH change from 2 to 4.5.

TEM analyses, which demonstrated the cellular localization of the accumulated uranium in the studied strains, supported the findings from the EXAFS studies. The three strains were able to precipitate uranium in the bulk phase as well as on the cell surface at pH 4.5. The precipitation of U as a mineral phase could be associated with the activity of nonspecific microbial acidic phosphatase (Rossolini *et al.*, 1998), which in the presence of U at pH 4.5 and 3 causes release of inorganic phosphates precipitating this radionuclide as a meta-autunite-like phase. Precipitation of uranium and other heavy metals, through the use of membrane-bound acidic phosphatase, was reported for *Citrobacter* sp. (Macaskie *et al.*, 1992; Boswell *et al.*, 2001). However, we can not exclude that the activity of the enzyme occurs at the three studied pH values resulting in the precipitation of U as meta-autunite, which is insoluble at pH 4.5, less soluble at pH 3 but soluble at pH 2.

Another possible mechanism of the uranyl phosphate precipitation on the microbial cell membrane via the

controlled metabolism of polyphosphate bodies has been described in *P. aeruginosa* (Renninger *et al*., 2004). The controlled polyphosphate metabolism can be used to sequester large quantities of phosphate in the form of intracellular polyphosphate, degrade the polyphosphate, secrete the resulting phosphate from the cell and precipitate uranyl phosphate on the cell wall (Renninger *et al*., 2004). However, our TEM studies indicate that the cells of the studied strains do not accumulate U intracellularly at levels high enough to be detected.

It has been suggested that cell-surface associated mineralization results in limitations of nutrient transport and disruption of the proton motive force (Southam, 2000). Therefore, the extracellular precipitation of uranium by the *Microbacterium* strains could be a good survival strategy, especially in oligotrophic environments such as the S15 monitoring site. The ability of the studied *Microbacterium* strains to immobilize high amounts of uranium as extracellular meta-autunite phase is important in the context of the radionuclide mobility. It has been shown that stable assemblages of U(VI) as meta-autunite, were responsible for uranium retention for hundreds of years, within slightly acidic oxidizing bedrock aquifer (Jerden & Sinha, 2003).

In this study, oligotrophic actinobacterial strains from the genus *Microbacterium* were isolated from the S15 subsurface monitoring well of the Siberian radioactive waste depository Tomsk-7 and characterized. Our findings indicate the presence of rapidly adapting bacterial populations in the Siberian subsurface habitat around the radioactive waste depository site Tomsk-7, which can influence the migration of uranium and other heavy metals.

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