Metallomics

RSC Publishing

PAPER

View Article Online
View Journal | View Issue

Cite this: *Metallomics*, 2013, **5**, 390

Aerobic uranium immobilization by *Rhodanobacter* A2-61 through formation of intracellular uranium–phosphate complexes

Tânia Sousa,[a] Ana-Paula Chung,[a] Alcides Pereira,[ab] Ana Paula Piedade[c] and Paula V. Morais*[ad]

Received 9th November 2012,
Accepted 20th February 2013

DOI: 10.1039/c3mt00052d

www.rsc.org/metallomics

Severe environmental problems arise from old uranium mines, which continue to discharge uranium (U) *via* acid mine drainage water, resulting in soil, subsoil and groundwater contamination. Bioremediation of U contaminated environments has been attempted, but most of the conceptual models propose U removal by cell suspensions of anaerobic bacteria. In this study, strain *Rhodanobacter* A2-61, isolated from Urgeiriça Mine, Portugal, was shown to resist up to 2 mM of U(VI). The conditions used (low nutrient content and pH 5) potentiated the interaction of the toxic uranyl ion with the tested strain. The strain was able to remove approximately 120 μM of U(VI) when grown aerobically in the presence of 500 μM U. Under these conditions, this strain was also able to lower the phosphate concentration in the medium and increased its capacity to take up inorganic phosphate, accumulating up to 0.52 μmol phosphate per optical density unit of the medium at 600 nm, after 24 hours, corresponding approximately to the late log phase of the bacterial culture. Microscopically dense intracellular structures with nanometer size were visible. The extent of U inside the cells was quantified by LS counting. EDS analysis of heated cells showed the presence of complexes composed of phosphate and uranium, suggesting the simultaneous precipitation of U and phosphate within the cells. XRD analysis of the cells containing the U–phosphate complexes suggested the presence of a meta-autunite-like mineral structure. SEM identified, in pyrolyzed cells, crystalline nanoparticles with shape in the tetragonal system characteristic of the meta-autunite-like mineral structures. U removal has been reported previously but mainly by cell suspensions and through release of phosphate. The innovative *Rhodanobacter* A2-61 can actively grow aerobically, in the presence of U, and can efficiently remove U(VI) from the environment, accumulating it in a structural form consistent with that of the mineral meta-autunite inside the cell, corresponding to effective metal immobilization. This work supports previous findings that U bioremediation could be achieved *via* the biomineralization of U(VI) in phosphate minerals.

Introduction

Former uranium mines are a source of environmental contamination, since the leaching of acid water resulting from mining activity can transfer heavy metals and radioisotopes to the surrounding environmental compartments.[1,2] U in the environment occurs mainly as 3 different isotopes (^{238}U, ^{235}U and ^{234}U), all of them being radioactive; however, it is its chemical toxicity that is of greatest ecological risk.[2] U at contaminated sites has two major oxidation states stable in aqueous media: U(VI), the most common oxidation state of the uranyl ions, $(UO_2)^{2+}$, which is highly soluble and mobile; and U(IV), which is extremely insoluble and usually precipitates as uraninite, UO_2. U(VI) is therefore considered to be more toxic since it is highly mobile in the environmental systems.[2,3]

Existing physical/chemical methods that treat U-contaminated groundwater, such as anion exchange, lime softening, conventional and activated alumina coagulation, and pump-and-treat, are expensive.[4] An alternative to these technologies is the use of indigenous subsurface bacteria for immobilizing U in contaminated groundwater and soil, in a process defined as bioremediation.[1,5,6] Bioremediation of metals, including U, is distinctly different from the biodegradation of toxic organic

[a] *IMAR-CMA, 3004-517 Coimbra, Portugal*
[b] *Department of Earth Sciences, FCTUC, University of Coimbra, 3001-401 Coimbra, Portugal*
[c] *CEMUC – Department of Mechanical Engineering, FCTUC – Pinhal de Marrocos, 3030-788 Coimbra, Portugal*
[d] *Department of Life Sciences, FCTUC, University of Coimbra, 3001-401 Coimbra, Portugal. E-mail: pvmorais@ci.uc.pt; Fax: +351 239855789; Tel: +351 239824024*

substances since they cannot be chemically or biologically degraded into innocuous compounds and their remediation depends on a method of containment that decreases bioavailability and/or biological access.[6–8] Microorganisms can use different mechanisms to immobilize U: (1) biosorption, by establishing nonspecific interactions between the U and the extracellular surfaces of microorganisms;[9–11] (2) bioaccumulation of U within the cells;[9,12,13] (3) oxidation–reduction processes which transform soluble U forms, U(VI), into more stable forms, U(IV);[7,14–16] and (4) biomineralization by U precipitation as minerals.[13,17–19] Among the previous mechanisms, under aerobic conditions, biomineralization is the process that can be more feasible since it implies the immobilization of U(VI) soluble species with enzymatically-generated ligands like phosphate and sulfides. Under these conditions, biomineralization leads to precipitation of U(VI) as uranyl-phosphate minerals like HUO_2PO_4, autunite $[Ca(UO_2)_2(PO_4)_2]$, chernikovite $[H_2(UO_2)_2(PO_4)_2]$ and uramphite $[NH_4(UO_2)(PO_4)\cdot 3H_2O]$.[1,17,19–21]

Cultures of *Desulfovibrio desulfuricans*, *Desulfovibrio vulgaris*, *Geobacter metallireducens*, *Shewanella putrefaciens* MR1, and *Deinococcus radiodurans*, among others, have been demonstrated to reduce U(VI) to U(IV).[12] Cultures of *Pseudomonas aeruginosa*,[22] *Bacillus subtilis*,[10] and *Chryseomonas* MGF48[23] can immobilize U by cellular uptake. Cultures of *Citrobacter* sp., *Bacillus sphaericus*,[24] and *Acinetobacter johnsonii*[25] have been demonstrated to remove U from water using a phosphate release mechanism. Natural immobilization of U as U(VI) phosphates has been demonstrated to occur extensively at the Koongarra deposit in Australia.[26]

In order to evaluate the potential of the implementation of a remediation strategy through bio-immobilization of U in contaminated groundwater that runoff from a shaft in the former underground uranium mine of Urgeiriça (Central Portugal[27,28]), a microbial community diversity study was conducted. *Rhodanobacter* A2-61 was isolated and shown to resist U(VI). The objective of the present work was to study the U transformations by *Rhodanobacter* A2-61 under aerobic conditions. The understanding of the mechanisms involved is necessary for the future use of this strain as a bioremediation tool. Results showed that the variation of inorganic phosphate during bacterial growth with U was related to aerobic U immobilization inside the cells. Different spectroscopic techniques were used to understand the U removal process and to show U biomineralization by this *Rhodanobacter* strain A2-61.

Materials and methods

Microorganism and growth conditions

The bacterial strain *Rhodanobacter* A2-61 was isolated from U-contaminated wastewater collected in the mining area of Urgeiriça in R2A medium at pH 6.0, consisting of 0.5 g l^{-1} yeast extract, 0.5 g l^{-1} proteose peptone, 0.5 g l^{-1} casein hydrolysate, 0.5 g l^{-1} glucose, 0.5 g l^{-1} starch, 0.3 g l^{-1} di-potassium phosphate, 0.024 g l^{-1} magnesium sulfate, 0.3 g l^{-1} sodium pyruvate, and 15 g l^{-1} agar (Oxoid). To study the ability of the strain to remove U from the medium, several media were tested in order to select the medium supporting the higher strain growth with the lower chemical unspecific U removal. After testing several media: (1) Minimal Medium (MM) consisting of 40 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), 0.0793 g l^{-1} $CaCl_2\cdot 2H_2O$, 0.008 g l^{-1} NaCl, 0.103 g l^{-1} KNO_3, 0.698 g l^{-1} $NaNO_3$, 0.1 g l^{-1} $MgSO_4\cdot 7H_2O$, 0.1 g l^{-1} nitriloacetic acid (NTA), amended with 0.05% (v/v) NH_4Cl, 0.5% (v/v) glucose and pH adjusted to 5 after autoclaving, which was then divided into MMA (enriched with 0.05% yeast extract) and MMB (enriched with 0.1% yeast extract); (2) LB 1/10 consisting of 1.0 g l^{-1} tryptone, 0.5 g l^{-1} yeast extract and 0.5 g l^{-1} NaCl, pH adjusted to 5; (3) the modified Nutrient Broth (NB, Merck) (diluted 1/10) consisting of 0.5 g l^{-1} peptone and 0.3 g l^{-1} meat extract, amended with 0.01% (v/v) glucose after autoclaving, and pH adjusted to 5 with HCl 1.0 M was used. For analytical assays, this strain was grown in duplicate, under aerobic conditions, in 50 ml of the modified NB medium supplemented with 0.5 mM U(VI) as uranyl acetate dehydrate ($(CH_3COO)_2UO_2\cdot 2H_2O$) (Merck), for 48 h at 26 °C. Non-inoculated medium with U and inoculated medium without U were used as controls. At appropriate intervals, samples were harvested to determine the O.D. at 600 nm, U(VI) concentration and phosphate. The viability of the cells was assessed through plating in R2A medium incubated for 48 h at the temperature of 26 °C.

Biochemical characterization and resistance to other metal ions

The enzymatic characterization of the strain was performed by APIZYM profiling (BioMerieux, API ZYM Test System, S.A., La Balme-les-Grottes, France) according to the manufacturer's instructions.

The strain was also tested for its ability to grow in the presence of different concentrations of the following metals: $(CH_3COO)_2UO_2\cdot 2H_2O$ (U(VI)); $Na_2HAsO_4\cdot 7H_2O$ (As(V)); $NaAsO_2$ (As(III)); $Na_2Cr_2O_7\cdot 2H_2O$ (Cr(VI)); Na_2CrO_4 (Cr(VI)); $KSb(OH)_6$ (Sb(VI)); Na_2SeO_3 (Se(IV)), all from Sigma-Aldrich, and $ZnSO_4$ (Zn(II)); $CoCl_2\cdot 6H_2O$ (Co(II)); $CuSO_4\cdot 5H_2O$ (Cu(II)) from Merck. Growth was evaluated in R2A at 26 °C after 2, 5, 7 and 10 days.

The ability of *Rhodanobacter* A2-61 to reduce nitrate and to denitrify was tested in NB medium supplemented with 0.1% KNO_3 and 0.17% agar. The presence of nitrite (pink coloration) was evaluated after 48 h and 5 days incubation in 100 μl of growth medium, after the addition of 1 ml of solution A (0.6 g of *N*,*N*-dimethyl-1-naphthylamine (Sigma) in 100 ml of 5N acetic acid (Merck)) and 1 ml of solution B (0.8 g of sulfanilic acid (Sigma) in 100 ml of 5N acetic acid).[29]

Uranium quantification

In all assays, to follow U(VI) removal from the medium, aliquot samples (2 ml) were removed periodically during the assays and centrifuged at 16 000 × *g* for 20 min (Sorvall Legend Micro 17 centrifuge, Thermo Scientific). U(VI) removal was evaluated in the supernatants and in the pellets obtained after centrifugation. The U(VI) in the pellets was obtained by washing the pellet twice with 1 M $NaHCO_3$ buffer, pH 8.3 (Merck), ensuring that all complexed U(VI) would be solubilized.[30] In non-inoculated samples, the amount of U(VI) quantified in the pellet was

considered to result from inorganic precipitation due to aerobic shaking of the medium. In inoculated samples, the pellets included the inorganically precipitated U(VI) and also the cells. Therefore, the U(VI) quantified was considered the result of the inorganic precipitation plus the U(VI) adsorbed to the cells. U(VI) was measured by the colorimetric procedure of Arsenazo III (0.1%, w/v) (1,8-dihydroxynaphthalene3,6-disulphonic acid-2,7-bisSTA(azo2)-phenylarsonic acid, Sigma-Aldrich) and quantified spectrophotometrically (Jenway 6405 UV/Vis Spectrophotometer) at 652 nm.[31]

The U(VI) internalized (taken up) by the cells was not quantifiable with the strategies described above. Therefore, U inside the cells was measured by liquid scintillation counting (LS counting).[32] Shortly, 2 ml aliquots from the different assays were centrifuged. The pellets were first removed from complexed U(VI) as described above, and then digested in MOPS buffer (20 mM MOPS, pH 7.4; 0.1% Triton X-100) at 37 °C for 45 min. From the digested pellets, and also from the supernatants, 100 μl were added to 5 ml of an aqueous scintillation cocktail (Universol) in a 5 ml polyethylene plastic vial.

The counting of the decay of the U isotopes was performed using an ultralow level spectrometer, Quantulus 1200, from Perkin-Elmer, using an alpha–beta discriminator and controlling the quench effect through the external standard method. The background was evaluated by counting, under the same conditions and time, a blank vial filled with the same scintillation cocktail. The average count for the blank was 0.723 cpm (counts per minute). A spike of a natural U standard, supplied by Ciemat (Spain), was used to calibrate the window of the multichannel analyzer as well as the alpha–beta discriminator. Under these experimental conditions the measured total count is directly proportional to the activity of the U isotopes (U-238 and U-234[33]).

Phosphate quantification

The soluble Pi was determined in the supernatants obtained as described above. The intracellular inorganic phosphate was determined in the cells washed twice with NaCl 0.85% (VWR), resuspended in a solution (5 : 1) of MilliQ H_2O : concentrated HCl (Merck) and heated at 120 °C for 45 min. Soluble and intracellular phosphate were measured based on the reaction of Pi with ammonium molybdate and ascorbic acid.[34] Quantification was performed by mixing 300 μl of the samples with 700 μl of a solution (1 : 6) of ascorbic acid (Sigma-Aldrich) 10% : reagent B (0.42 g $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ in 2.86 ml of concentrated H_2SO_4 and MilliQ H_2O up to 100 ml), incubated at 45 °C for 20 min and quantified spectrophotometrically at 820 nm.

Differential gradient cell separation

Cells (1 g) of strain A2-61 grown in the presence of 0.5 mM U(VI) were washed twice in 1 M $NaHCO_3$ buffer, pH 8.3, ensuring that all U(VI) adsorbed to the cells would be removed. Washed cells were separated in a sucrose gradient, generated using sucrose (Panreac) solutions at concentrations of 100%, 85%, 70%, 45% and 30% (w/v) centrifuged at 3200 × *g*, for 30 min (Eppendorf Centrifuge 5810R). The cells in the sucrose fraction of 45% were selected for further studies since, when observed under an Optical Microscope (OM), they were intact and showed dense structures.

Solid-state methods of biomass examination

Cells grown in the presence of U(VI), collected from the sucrose gradient centrifugation (45% sucrose), and cells grown in the absence of U, were pyrolysed in a controlled muffle furnace at 700 °C for 2 hours. The solids obtained were examined by Scanning Electron Microscopy (SEM) using an Electron Microprobe JEOL-JXA860, and analyzed using the software SAMX MAXVIEW. The identity of crystalline metal deposits was confirmed using X-ray powder Diffraction (XRD) and Energy Dispersive X-ray Spectroscopy (EDS). EDS analysis was accomplished using an Oxford Instruments Inca X-Sight, with an accelerating voltage of 15 kV and a beam current of 20 nÅ, and the SAMX IDFIX software. XRD patterns were examined in a Philips Analytical X-ray diffractometer, over a range of 2–60 (2Θ), using a Cu-Kα radiation at 40 kV/20 mA, and the crystalline phases were identified using the PDF-2 database of the International Center for Diffraction Data. The interpretation of the results was performed through comparison with a standard spectrum obtained from pure concentrates.

Statistical analysis

The significance of the differences observed between experiments inoculated and non-inoculated with *Rhodanobacter* A2-61 was evaluated with analysis of variance (ANOVA) considering: (1) the amount of U(VI) in the supernatant and (2) the amount of U(VI) complexed (obtained pelleted after centrifugation). The statistical analysis was performed using GraphPadPrism v5.0 for windows, GraphPad software, San Diego, California, USA, www.graphpad.com. All differences were considered to be statistically significant for $P < 0.05$.

Results

Biochemical characterization and resistance to other metal ions

APIZYM characterization of the strain revealed strong enzymatic activity for both alkaline and acidic phosphatases. The strain also showed activity for esterase lipase C8, leucinearylamidase, valinearylamidase, α-chymotrypsin, naphthol-AS-BI-phosphohydrolase, α-and β-glucosidase and *N*-acetyl-β-glucosaminidase.

The strain was able to grow in the presence of 1 mM of Cu(II) and Se(IV), 2 mM of U(VI), 10 mM of Zn(II) and Sb(V), and 200 mM of As(V), after 10 days of growth in R2A at 26 °C. The strain was not able to reduce nitrate or to denitrify.

Uranium resistance and U(VI) removal

Strain *Rhodanobacter* A2-61 growth curve in modified NB (1/10) with U(VI) showed that the presence of U(VI) did not reduce the maximum $O.D_{600nm}$ achieved after 24 h growth (0.25 O.D.) although the strain growth started earlier in the presence of U(VI) (Fig. 1). In U amended medium, the solubilization of the U acetate salt could make available the acetate as an additional carbon source to the strain growth. The maximum

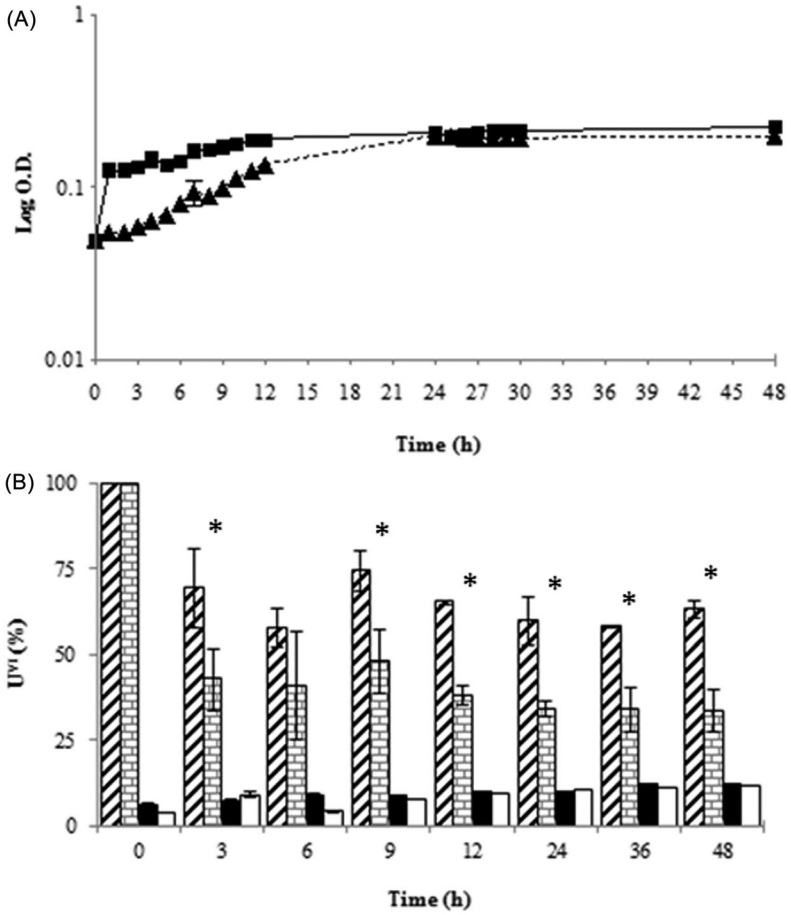

Fig. 1 (A) Growth curves of *Rhodanobacter* A2-61 grown in modified NB (1/10) in the absence (—▲—) and in the presence (—■—) of 0.5 mM U. (B) U(VI) variation in percentage, in A2-61 inoculated (U(VI) soluble in the medium (▤) and U(VI) inorganic precipitated and adsorbed to the cells (▭)) and in non-inoculated medium (U(VI) soluble in the medium (▨) and U(VI) in inorganic precipitates (■)). (*) symbols above bars indicate treatments with significant differences ($P < 0.05$) between soluble U(VI) from inoculated and non-inoculated samples.

cell concentration achieved was limited by a low nutrient content of the medium and not by the presence of U. Strain A2-61 maintained its cellular viability after 48 hours of incubation, as determined by plating in R2A medium at 26 °C.

Spontaneous U(VI) precipitation occurred immediately after addition of 0.5 mM U(VI) to the medium, leaving soluble in the medium 0.3 mM U(VI). This concentration of soluble U(VI) was regarded as the baseline concentration (100% initial concentration) for all assays performed with this medium (Fig. 1). Furthermore, the soluble U(VI) in cell free controls, incubated aerobically, decreased 40% after 3 h incubation, but remained stable after that decrease. From these, 10% were detected as precipitated U(VI) complexes. In the experiments where *Rhodanobacter* A2-61 was inoculated, the total amount of U(VI) in the medium decreased 69%, which was found to be a value significantly higher ($P < 0.05$) than the U(VI) decreased in the non-inoculated medium. Therefore, the U(VI) removal by the cells corresponded to an additional removal of 29% of the bioavailable U which corresponds to 120 μM U(VI). There was no significant difference between the amount of U(VI) found precipitated, in the presence or in the absence of cells ($P > 0.05$). The maximum U(VI) removal from the medium was achieved after 24 h incubation. Further incubation time (up to 48 h) did not result in increased U(VI) removal.

The amount of total U detected by LS counting, considered 100%, equaled the sum of the counts obtained from the medium and the counts obtained from the digested pellets (Fig. 2). The amount of total U, during incubation, in the control assay (without cells) did not change in the medium nor precipitated. Comparatively, the total U precipitated in the inoculated assay varied from 0.153 to 0.335 counts per 100 μl of digested sample, corresponding to a duplication of the total amount of U immobilized by the cells in 24 hours.

Variation of the phosphate concentrations in growth assays

Soluble phosphate was detected in the medium only in the absence of U. The soluble phosphate concentration in the medium varied from 123 to 50 μM during strain growth in the absence of U(VI) (Fig. 3). In the presence of U, immediately after inoculation, the amount of intracellular phosphate in strain A2-61 was very low (0.08 μmol per O.D.) but after 6 h of growth, the concentration increased up to 0.42 μmol per O.D. and

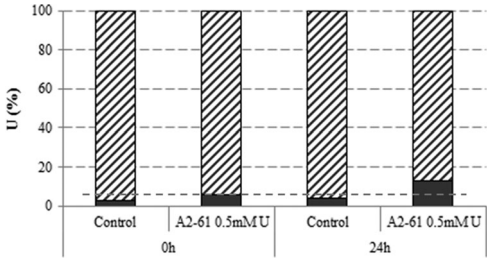

Fig. 2 Percentage of total U detected by liquid scintillation counting in non-inoculated and in *Rhodanobacter* A2-61 inoculated samples, in the presence of 0.5 mM U. U in the medium (▨) and U in 100 µl of digested pellets obtained from 2 ml samples (■).

remained constant during growth. In the absence of U, intracellular phosphate in strain A2-61 was almost undetectable (0.09 µmol per O.D.) and remained as such during all incubation periods (Fig. 3).

Scanning electron microscopy and energy dispersive X-ray spectroscopy analysis

Microscopic observations (magnification ×1000) of cells from strain A2-61 grown in medium with U(VI) (Fig. 4) showed dense precipitates inside the cells, suggesting the intracellular accumulation of U. A SEM image of the pyrolysed cells (heat treatment at 700 °C) detected the presence of crystalline nanoparticles with shape in the tetragonal system characteristic of the meta-autunite-like mineral structures (Fig. 5). EDS analysis of this cell material identified the presence of the chemical elements U, O and P, suggesting the presence of U-phosphate compounds (Fig. 5). The analysis of the background was negative for these elements.

X-ray powder diffraction analysis

XRD was used to identify and characterize the chemical nature of U accumulated by *Rhodanobacter* A2-61 under aerobic conditions (Fig. 6). XRD patterns obtained from cells of strain A2-61 grown in the presence of U showed distinct reproducible

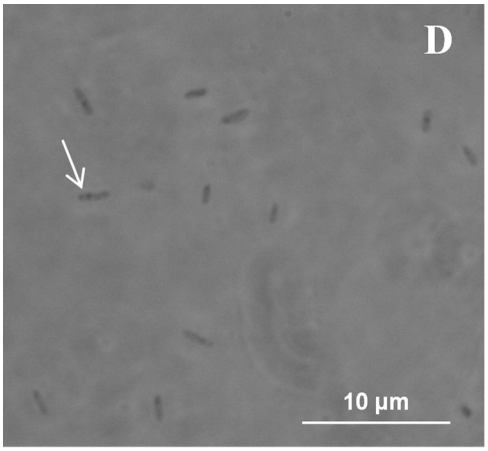

Fig. 4 Light microscopy observation of *Rhodanobacter* A2-61 after growth with 0.5 mM U for 24 h (1000× total magnification). Arrow indicates the presence of dense precipitates within the bacterial cells.

patterns typical for characterized bio-minerals. The XRD pattern revealed distinct peaks at 2Θ which were 10.46, 16.99, 19.98, 20.80, 21.16, 25.20, 27.42 and 29.72 corresponding to *d*-spacing values of 8.45 Å, 5.21 Å, 4.44 Å, 4.27 Å, 4.20 Å, 3.53 Å, 3.25 Å and 3.00 Å, respectively. A comparison of these *d*-spacing values with data files of known compounds indicates the presence of U-phosphate compounds: uranyl phosphate $[UO_2(PO_4)_2]$, uranyl phosphate hydrate $[(UO_2)_3(PO_4)_2{\cdot}H_2O]$ or meta-autunite $[Ca(UO_2)_2(PO_4)_2{\cdot}4(H_2O)]$. Data obtained from XRD analysis of inoculated medium without U confirmed the amorphous nature of this sample.

Discussion

The strain A2-61 is a species from the genus *Rhodanobacter*. Species from this genus were already found in an uranium- and nitrate-contaminated soil and demonstrated to be useful as denitrification tools[35] however strain A2-61 was not able to reduce nitrate. During this study, the conditions used limited

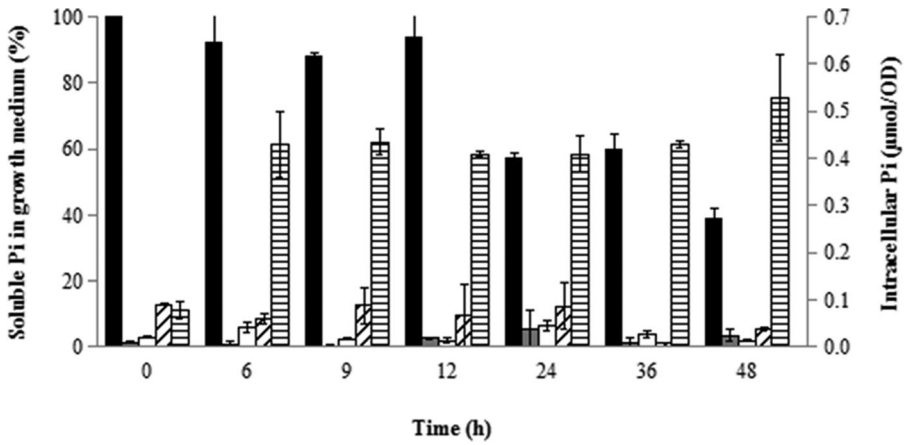

Fig. 3 Phosphate variation in the growth medium and intracellular structure during *Rhodanobacter* A2-61 growth, in the presence and in the absence of 0.5 mM U. Soluble inorganic phosphate in A2-61 inoculated medium in the absence of U (■). Soluble inorganic phosphate in non-inoculated (▬) and in A2-61 inoculated medium (▭) in the presence of U. Intracellular inorganic phosphate in A2-61 cells incubated with (▤) and without (▨) U.

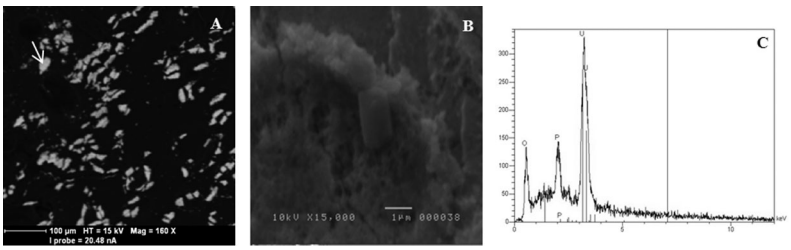

Fig. 5 SEM and EDS analyses of *Rhodanobacter* A2-61 cells grown with 0.5 mM U for 24 h, after heat treatment at 700 °C. SEM detected (A) crystalline nanoparticles (160× magnification) (B) with shape in the tetragonal system characteristic of the meta-autunite-like mineral structures (15 000× magnification); (C) EDS analysis of cells (arrow) showing the presence of U, O and P.

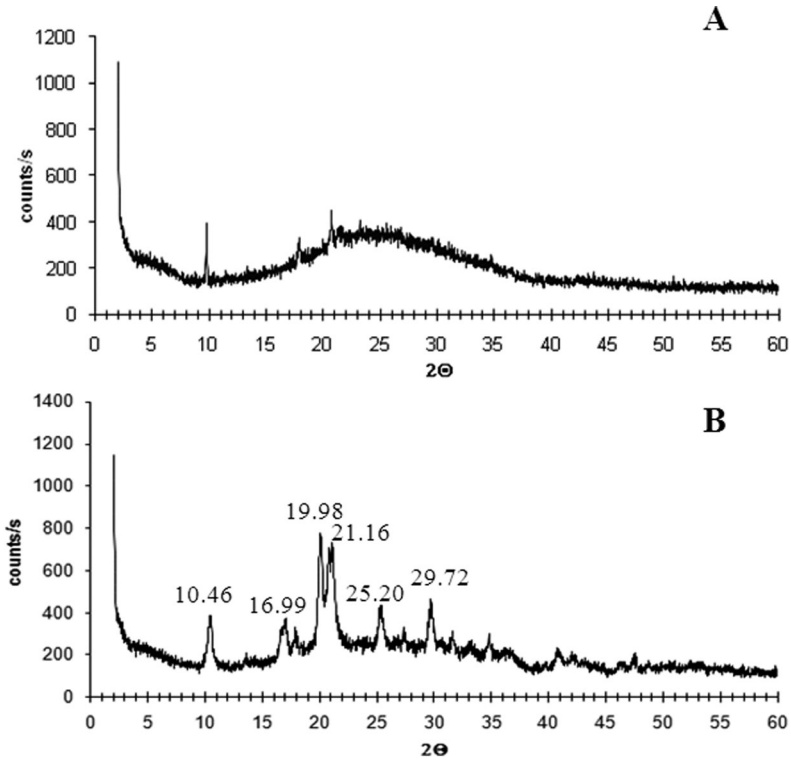

Fig. 6 XRD analysis of *Rhodanobacter* A2-61 cells after heat treatment at 700 °C: (A) grown in the absence or (B) in the presence of 0.5 mM U for 24 h, following sucrose gradient separation (fraction of 45%). The peaks obtained are included in the fingerprint of uranium–phosphate compounds.

the strain growth as a result of the low nutrient content and low pH (pH 5) of the medium. These conditions were chosen to ensure the presence of the uranyl ion (UO_2^{2+}), thus potentiating the interaction of the most soluble toxic form of U with the tested strain.[2] Also, a very diluted medium was used, since richer nutrient conditions are known to have higher non-specific abiotic interactions with UO_2^{2+}. These abiotic interactions are related to the presence of high quantities of organic ligands and/or phosphates and/or sulfates that limit U exposure to the tested strain.[36] In addition, the maximum growth of strain A2-61 in the presence or in the absence of U was similar, reaching the same O.D. after 24 h, indicating that *Rhodanobacter* A2-61 is resistant up to 500 μM U(VI) under aerobic conditions. In the presence of U, the strain showed a very rapid increase in cell number which may be a consequence of the presence of acetate. This additional, immediately usable, carbon source follows the solubilization of the uranium acetate, added to the medium as a uranium source. The increase in cell number during the remaining incubation period, after this immediate increase, was lower than in the absence of U.

In all assays inoculated with *Rhodanobacter* A2-61, soluble U(VI) in the medium decreased over time reaching a total removal of ~70%. An immediate initial decrease of U(VI) was also observed in non-inoculated assays most likely due to the reaction of U with the medium compounds, as it was reported before.[21,36] In the presence of strain A2-61, besides the immediate initial decrease, an additional removal of 25–29% of soluble U(VI) was attained after 24 hours of incubation, comparatively to the non-inoculated controls. The amount of total U detected inside the cells by LS counting showed that this additional removal was most likely due to the presence of the cells. The U concentration inside the cells is more than

duplicated, corresponding to approximately 30% of the bioavailable U taken up by the cells.

Bacteria are reported to be effective biosorbents for U. Rapid removal of U(VI) from solution has been described to occur in the presence of different bacterial species,[10,37,38] but the mechanism of U removal that has been postulated for those bacteria is the adsorption to the cell wall. In our case, the removal of U(VI) by growing cells of strain A2-61 could not be related to the 10% of U(VI) measured in the U-precipitate complexes since a similar amount was detected in non-inoculated assays. Furthermore, U complexes could be quantified (LS counting) and observed (SEM, EDS) inside strain A2-61 cells. Therefore, these findings exclude the non-specific absorption as the bioremediation process used by strain A2-61.

In the assays, in the presence of U, intracellular phosphate concentrations of *Rhodanobacter* strain A2-61 were initially very low, near the detection limit, tending to increase with time. Under these conditions, phosphate was not detected in the medium. In the absence of U, the opposite occurred: the strain almost did not accumulate phosphate and phosphate was detected in the medium. It has been demonstrated that some bacteria are capable of generating sufficient phosphate, liberating it into solution, which is then coupled to biologically induced precipitation of heavy metals or radionuclides.[17–19] Several studies reported the role of phosphatase enzymes in the release of phosphates that precipitate U.[18,20] Evidence of active phosphatase enzymes in *Rhodanobacter* A2-61 was obtained during strain characterization. Therefore, it is probable that phosphatase activity in strain A2-61 could be involved in phosphate release to be used in U removal. In the case of strain A2-61, O.M. observation, SEM and EDS analysis showed the presence of precipitated U-phosphate complexes inside the cells, suggesting a different precipitation strategy: instead of the release of phosphate into solution, phosphate was accumulated inside the cells and complexed with U. Additionally, XRD analysis of the cells supports the idea of intracellular U(VI) immobilization through the formation of U-phosphate complexes such as uranyl phosphate $[UO_2(PO_4)_2]$, uranyl phosphate hydrate $[(UO_2)_3(PO_4)_2{\cdot}H_2O]$ or meta-autunite $[Ca(UO_2)_2(PO_4)_2{\cdot}4(H_2O)]$. SEM identified crystalline nanoparticles with shape in the tetragonal system characteristic of the meta-autunite-like mineral structure. Under anoxic conditions, U(IV) is stable in the form of the mineral uraninite (UO_2(s)), but upon exposure to oxidizing conditions, uraninite is prone to oxidation.[39] The susceptibility of uraninite to oxidation makes it potentially unstable in subsurface environments.[40] Therefore, the capacity of strain A2-61 to perform the U complexation under aerobic conditions to meta-autunite-like mineral structure represents an advantage in the processes of bioremediation, since meta-autunite-like mineral has been proved to be stable for long periods of time, over a wide range of pH.[19]

In conclusion, U bioremediation, especially under aerobic environments where U bioreduction strategies are limited and restricted due to an excess of oxygen,[1,7] may now be attempted based on *Rhodanobacter* strain A2-61, an environmental strain resistant to different metals besides U. The strain proved to, under aerobic conditions, efficiently remove U from the environment, accumulating it inside the cells, in a structural form consistent with that of the mineral meta-autunite. This work supports previous findings that U bioremediation could be achieved *via* the biomineralization of U(VI) in phosphate minerals.

Acknowledgements

The SEM and EDS analyses were performed at Centre of Earth Resources from the University of St. Andrews, Scotland. We thank Dr Ed Stephens and Mr Donald Hurd for their technical support in the SEM and EDS analyses. A. P. Chung is funded by a Post-Doc grant (SFRH/BPD/34799/2007). Tânia Sousa is funded by a scholarship of the project (PTDC/BIA-MIC/114958/2009). Research was funded by Fundação para a Ciência e a Tecnologia (PTDC/MAR/109057/2008).

References

1 M. L. Merroun and S. Selenska-Pobell, *J. Contam. Hydrol.*, 2008, **102**, 285–295.
2 M. Gavrilescu, L. V. Pavel and I. Cretescu, *J. Hazard. Mater.*, 2009, **163**, 475–510.
3 A. Meinrath, P. Schneider and G. Meinrath, *J. Environ. Radioact.*, 2003, **64**, 175–193.
4 J. R. Spear, L. A. Figueroa and B. D. Honeyman, *Environ. Sci. Technol.*, 1999, **33**, 2667–2675.
5 T. Borch, R. Kretzschmar, A. Kappler, P. Van Cappellen, M. Ginder-Vogel, A. Voegelin and K. Campbell, *Environ. Sci. Technol.*, 2010, **44**, 15–23.
6 M. Vidali, *Pure Appl. Chem.*, 2001, **73**, 1163–1172.
7 J. D. Wall and L. R. Krumholz, *Annu. Rev. Microbiol.*, 2006, **60**, 149–166.
8 N. K. Srivastava and C. B. Majumder, *J. Hazard. Mater.*, 2008, **151**, 1–8.
9 R. Gupta, P. Ahuja, S. Khan, R. K. Saxena and H. Mohapatra, *Curr. Sci.*, 2000, **78**, 967–973.
10 D. A. Fowle, J. B. Fein and A. M. Martin, *Environ. Sci. Technol.*, 2000, **34**, 3737–3741.
11 M. Nedelkova, M. L. Merroun, A. Rossberg, C. Hennig and S. Selenska-Pobell, *FEMS Microbiol. Ecol.*, 2007, **59**, 694–705.
12 V. Sivaswamy, M. I. Boyanov, B. M. Peyton, S. Viamajala, R. Gerlach, W. A. Apel, R. K. Sani, A. Dohnalkova, K. M. Kemner and T. Borch, *Biotechnol. Bioeng.*, 2011, **108**, 264–276.
13 S. Choudhary and P. Sar, *J. Hazard. Mater.*, 2011, **186**, 336–343.
14 D. R. Lovley, E. J. P. Phillips, Y. A. Gorby and E. R. Landa, *Nature*, 1991, **350**, 413–416.
15 D. R. Lovley and E. J. P. Phillips, *Environ. Sci. Technol.*, 1992, **26**, 2228–2234.
16 R. M. Gargarello, D. Di Gregorio, H. Huck, J. F. Niello and G. Curutchet, *Hydrometallurgy*, 2010, **104**, 529–532.
17 R. J. Martinez, M. J. Beazley, M. Taillefert, A. K. Arakaki, J. Skolnick and P. A. Sobecky, *Environ. Microbiol.*, 2007, **9**, 3122–3133.

18 M. J. Beazley, R. J. Martinez, P. A. Sobecky, S. M. Webb and M. Taillefert, *Environ. Sci. Technol.*, 2007, **41**, 5701–5707.
19 M. J. Beazley, R. J. Martinez, P. A. Sobecky, S. M. Webb and M. Taillefert, *Geomicrobiol. J.*, 2009, **26**, 431–441.
20 L. E. Macaskie, K. M. Bonthrone, P. Yong and D. T. Goddard, *Microbiology*, 2000, **146**, 1855–1867.
21 S. Y. Lee, M. H. Baik and J. W. Choi, *Environ. Sci. Technol.*, 2010, **44**, 8409–8414.
22 G. W. Strandberg, S. E. Shumate and J. R. Parrot Jr, *Appl. Environ. Microbiol.*, 1981, **41**, 237–245.
23 F. Malekzadeh, A. Farazmand, H. Ghafourian, M. Shahamat, M. Levin and R. R. Colwell, *World J. Microbiol. Biotechnol.*, 2002, **18**, 295–300.
24 R. Knopp, P. J. Panak, L. A. Wray, N. S. Renninger, J. D. Keasling and H. Nitsche, *Chem.–Eur. J.*, 2003, **9**, 2812–2818.
25 C. D. Boswell, R. E. Dick and L. E. Macaskie, *Microbiology*, 1999, **145**(7), 1711–1720.
26 P. Duerden, *Alligator rivers analogue project, 1st Annual Report 1989–1990, Australian Nuclear Science and Technology Organization (ANSTO)*, 1990.
27 A. J. S. C. Pereira, J. M. M. Dias, L. J. P. F. Neves and J. M. G. Nero, in *Proceedings of XI International Congress of the International Radiation Protection Association*, 2004, p. 10.
28 A. J. S. C. Pereira, L. J. P. F. Neves, J. M. M. Dias and S. V. T. Barbosa, in *Proceedings of International Workshop on Environmental Contamination from Uranium Production Facilities and Remediation Measures – International Atomic Energy Agency*, 2005, pp. 181–191.
29 R. M. Smibert and N. R. Krieg, in *Manual of Methods for General Bacteriology*, ed. P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg and G. B. Phillips, Washington, DC, USA, 1981, p. 419.
30 D. A. Elias, J. M. Senko and L. R. Krumholz, *J. Microbiol. Methods*, 2003, **53**, 343–353.
31 M. Martins, M. L. Faleiro, S. Chaves, R. Tenreiro and M. C. Costa, *Sci. Total Environ.*, 2010, **408**, 2621–2628.
32 D. S. Muller, P. Houpert, J. Cambar and M.-H. Hengé-Napoli, *Toxicol. Sci.*, 2008, **101**, 254–262.
33 *Handbook of Radioactivity Analysis*, ed. M. F. L'Annunziata, Academic Press, 2nd edn, 2003.
34 B. N. Ames, *Methods Enzymol.*, 1966, **8**, 115–118.
35 S. J. Green, O. Prakash, T. M. Gihring, D. M. Akob, P. Jasrotia, P. M. Jardine, D. B. Watson, S. D. Brown, A. V. Palumbo and J. E. Kostka, *Appl. Environ. Microbiol.*, 2010, **76**, 3244–3254.
36 Y. Suzuki and J. F. Banfield, *Geomicrobiol. J.*, 2004, **21**, 113–121.
37 R. Bencheikh-Latmani, J. O. Leckie and J. R. Bargar, *Environ. Sci. Technol.*, 2003, **37**, 3555–3559.
38 A. L. N'Guessan, H. A. Vrionis, C. T. Resch, P. E. Long and D. R. Lovley, *Environ. Sci. Technol.*, 2008, **42**, 2999–3004.
39 D. Langmuir, *Geochim. Cosmochim. Acta*, 1978, **42**, 547–569.
40 M. Fayek, T. M. Harrison, M. Grove and C. D. Coath, *Int. Geol. Rev.*, 2000, **42**, 163–171.