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Mechanism of uranium (VI) removal by two anaerobic bacterial communities

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ABSTRACT

The mechanism of uranium (VI) removal by two anaerobic bacterial consortia, recovered from an uncontaminated site (consortium A) and other from an uranium mine (consortium U), was investigated. The highest efficiency of U (VI) removal by both consortia (97%) occurred at room temperature and at pH 7.2. Furthermore, it was found that U (VI) removal by consortium A occurred by enzymatic reduction and bioaccumulation, while the enzymatic process was the only mechanism involved in metal removal by consortium U. FTIR analysis suggested that after U (VI) reduction, U (IV) could be bound to carboxyl, phosphate and amide groups of bacterial cells. Phylogenetic analysis of 16S rRNA showed that community A was mainly composed by bacteria closely related to *Sporotalea* genus and *Rhodocyclaceae* family, while community U was mainly composed by bacteria related to *Clostridium* genus and *Rhodocyclaceae* family.

1. Introduction

Uranium mining and mineral processing for production of nuclear power have resulted in the generation of significant amounts of radioactive wastes with severe impact on environment [1,2]. Radionuclides like uranium are of particular concern due to their high toxicity and long half lives. Uranium exists as U (VI) in the form of divalent oxocomplex (UO_2^{2+}) in oxic aqueous systems [3].

The conventional remediation processes of wastes containing uranium are highly expensive and ineffective particularly at low metal concentrations [4]. Thus, the search of novel technologies is encouraged. Recently, bioremediation strategies based on the use of microorganisms are considered a potential alternative and an economically attractive strategy when compared with the traditional techniques [1].

During the last two decades, many researchers have discovered that different groups of microorganisms, such as bacteria [5,6], yeasts and fungi [5], have the ability to remove uranium from aqueous media. This ability was also observed for *Cystoseria indica*, a brown algae [7]. Although several studies described the ability of metals removal by diverse bacteria, reports focused on the mechanism of uranium removal were only recently available. Bacteria have been shown ability for uranium removal by several mechanisms such as adsorption [7,8] and accumulation inside the cells [2]. In addition, some bacteria have showed the ability to reduce uranium (VI) [6,9]. After reduction, the highly soluble and mobile U (VI) is converted to highly insoluble U (IV), which can be separated from aqueous solutions [3,6].

Although mixed bacterial cultures were frequently used in bioremediation strategies, only few studies about the mechanisms of metals removal by consortia are reported [10,11], and at our knowledge none of them focus uranium removal. The advantages of employing mixed cultures as opposed to pure cultures in bioremediation applications are widely demonstrated [12,13]. Those advantages over pure cultures include greater stability and increased metabolic capabilities, which can be linked to the effects of synergistic interactions among members of the bacterial communities [12,13].

Taking into account the diversity of microorganisms it is of great importance to characterize metal resistant bacterial communities with ability for uranium removal, as well as to identify the mechanism involved in metal removal. Therefore, the mechanism involved in uranium (VI) removal from aqueous solution by two anaerobic bacterial consortia (one from an uncontaminated site and other from an uranium mine) was investigated for the first time. Moreover, the molecular identification of the two consortia was also performed. The identification of the bacterial community,

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as well the clarification of the process of metal–bacteria interaction can contribute to the development of an effective bioremediation strategy for uranium removal.

2. Materials and methods

2.1. Microorganisms and growth conditions

The bacterial communities used in the present study were selected from previous studies [14]. These consortia were obtained from sediments from the uranium mining area of Urgeiriça (sample U) and from soil from Monchique thermal place (sample A). Urgeiriça mine is located in the north Portugal and Monchique thermal place is located in the south Portugal. Stock cultures were maintained in modified MTM medium [15], which contains 1 g/L NH_4Cl, 0.06 g/L $CaCl_2{\cdot}6H_2O$, 0.05 g/L yeast extract, 1 g/L $MgSO_4{\cdot}7H_2O$, 2 g/L Na_2SO_4, 5 g/L sodium lactate and 20 mg/L of uranium (VI), as uranyl acetate dehydrate. This medium was optimized in order to avoid chemical removal of uranium. The bacterial consortia were sub-cultured every 4 weeks using 10% (v/v) of inoculum and incubated at room temperature ($21 \pm 1\,^{\circ}C$).

2.2. Uranium (VI) bio-removal experiments

The studies of U (VI) bio-removal were performed in 35 mL glass bottles, in anaerobic conditions, using the MTM growth medium previously described, containing 22 mg/L of uranium (VI). Each set of experiments was carried out in duplicate. The medium was purged with nitrogen gas to achieve an anaerobic environment prior to inoculation. After inoculation, oxygen diffusion was eliminated by adding 5 mL of sterile liquid paraffin. The bottles were sealed with butyl rubber stoppers and aluminium crimp seals and incubated.

2.2.1. U (VI) removal by live cells in different conditions

The effect of pH and temperature on uranium (VI) removal was tested for both bacterial consortia in order to found the optimal conditions for uranium (VI) removal. The pH of the medium was adjusted to 4.6, 6.2 and 7.2 with 1 M NaOH or HNO_3 and incubation was performed at room temperature ($21 \pm 1\,^{\circ}C$). For uranium (VI) bio-removal experiments at different temperatures, medium with pH 7.2 was used. Temperatures of 4 °C, 21 °C and 37 °C were tested.

Bacterial cells obtained previously were harvested by centrifugation at 4000 rpm for 10 min, washed with MTM medium, inoculated (10%, v/v) in the bottles containing MTM medium with 22 mg/L U (VI) and incubated at the previously described conditions. For each experimental set an abiotic control (without bacteria) was carried out.

2.2.2. U (VI) removal by heat-killed cells

Bacterial cells (30 mL) collected after 20 days of incubation were harvested by centrifugation at 4000 rpm for 10 min and washed with MTM medium. The cells were killed by autoclaving (121 °C, 30 min) and added to bottles contained MTM medium with 22 mg/L U (VI) (pH 7.2).

2.2.3. U (VI) removal by extracellular metabolic products

U (VI) was added to 30 mL of cell-free medium obtained from the bacterial cultures after 20 days of growth. The medium was filtered with a 0.2 μm hydrophilic polyestersulfone membrane (Machererey-Nagel) to remove cells and purged with nitrogen gas.

2.3. TEM-EDS, FTIR and XRD analyses

The precipitates generated during the bio-removal process were characterized by X-ray powder diffraction (XRD). Transmission electron microscopy coupled with an energy dispersive spectrometer (TEM-EDS) and Fourier transform infrared (FTIR) spectroscopy were also used to assess possible metal–bacterial cells interactions. Samples were dried at 60 °C [7] and used for XRD using Bruker powder diffractometer (model D8 Advanced) using Cu-Kα radiation. The diffraction pattern was recorded from 3° to 60° (2θ) with a step length of 0.05° and time per step 20.0 s. TEM-EDS (Hitachi H8100) was used to establish the localization of the metal precipitates in the cells and the elemental characterization of the metal deposits. Samples of fresh bacterial cells exposed to uranium (VI) were prepared for TEM by fixation with glutaraldehyde 3% followed by dehydration and embedding in Epon-Araldite [16]. Thin sections (79–90 nm) stained with uranyl acetate and lead citrate were used for morphological studies and sections without staining for detection of electron-dense precipitates. For FTIR spectroscopic analysis, samples of bacterial cultures exposed and not exposed to uranium (VI) were dried and blended with KBr. The FTIR spectra were recorded within the range 400–4000 cm^{-1} in Bruker, Tensor 27 FTIR spectrophotometer.

2.4. Analytical methods

Periodically, samples from cultures were collected using a syringe. Optical density at 600 nm (OD600) and uranium (VI) were measured in each sample. Dissolved uranium was measured after centrifugation of samples at 4000 rpm for 5 min and quantified using the method described by Martins et al. [14].

2.5. Molecular characterization

2.5.1. Extraction of DNA, PCR amplification and cloning of 16S rRNA gene

Total genomic DNA was extracted after harvesting cells by centrifugation at 4000 rpm for 10 min. DNA extraction was carried out as described by Martins et al. [17].

Amplification of full-length 16S rRNA gene was performed using the primer pair 8F (5′-AGA GTT TGA TCC TGG CTC AG-3′)/1492R (5′-GGT TAC CTT GTT ACG ACT T-3′) [18]. The primers were purchased from Thermo Fisher Scientific. The reaction mixture used for PCR amplification contained 31.75 μL of sterilized MiliQ water, 1 μL of each primer (10 pmol/μL), 1 μL of dNTP's (10 mM), 4 μL of $MgCl_2$ (25 mM), 10 μL of 5 × Go Taq® buffer (Promega, Madison, USA), 0.25 μL of GoTaq® DNA polymerase (Promega, Madison, USA), and 1 μL of DNA. PCR amplification was performed in a thermocycler (T1, Biometra, USA). Thermal cycling was carried out by using an initial denaturation step of 94 °C for 3 min, followed by 30 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 2 min and completed with an extension period of 5 min at 72 °C. The PCR products were analyzed by electrophoresis, in 1% (w/v) agarose gel and TAE buffer. The band with the proper size range (approximately 1.4 Kb) was excised and purified with E.Z.N.A.™ Gel Extraction Kit (Omega).

The purified products were ligated into the cloning vector pGEM®-T Easy according to the manufacturer's instructions (Promega, Madison, USA), followed by transformation into *Escherichia coli* DH5α competent host cells. The white colonies were screened for inserts by amplification with a vector-specific primer set (Sp6 and T7). Thermal cycling was carried out by using an initial denaturation step of 94 °C for 3 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min and completed with an extension period of 5 min at 72 °C. The PCR products were analyzed by electrophoresis, in 1% (w/v) agarose gel and TAE buffer and the clones containing expected DNA insert were saved at −20 °C.

2.5.2. Restriction fragment length polymorphism analysis (RFLP) of 16S rRNA gene

RFLP analysis of the previously amplified 16S rRNA gene was performed using the restriction enzymes *Hha*I and *Msp*I (Promega) to search for similar rRNA gene clones. Fragments of the digested PCR products were separated in a 2% (w/v) TAE agarose gel. A representative clone from each digestion pattern was selected for sequencing. The 16S rRNA gene inserted in plasmids was amplified using the primers Sp6 and T7, according to the conditions described above. PCR products were purified using the Jetquick PCR Purification (Genomed GmbH, Lohner, Germany) and sequenced by CCMAR (Centro de Ciências do Mar, Universidade do Algarve). Sequences obtained in this study have the following accession numbers: GU255481–GU255507.

2.5.3. Phylogenetic analysis

For phylogenetic analysis, sequence alignments were made with Clustal X [19] and visually corrected. The Bayesian Markov chain Monte Carlo (MCMC) method of phylogenetic inference [20] was applied to estimate phylogenetic relationships using MrBayes software [21]. This method allows the estimation of the a *posteriori* probability that groups of taxa are monophyletic given the DNA alignment (i.e., the probability that corresponding bipartitions of the species set are present in the true unrooted tree including the given species). This Bayesian approach was repeated several times, using random starting trees and default starting values for the model parameters to test the reproducibility of the results.

3. Results

3.1. Effect of pH and temperature on U (VI) removal by bacterial communities

The influence of pH and temperature on the efficiency of U (VI) removal by bacterial communities from Monchique thermal place (A) and from Urgeiriça mine (U) was studied (Fig. 1). Considering pH, it was observed that the metal removal and bacterial growth were affected by this factor (Fig. 1a). For both consortia, the U (VI) removal efficiency increased with pH increase. The best performance was observed at pH 7.2. At this pH value, both consortia achieved 97% of U (VI) removal after 16 days of incubation, while at pH 6.2 only 70% and 77% of U (VI) was removed by consortia A and U, respectively. At pH 4.6 no relevant removal of U (VI) was observed. The decrease of U (VI) in the abiotic sets was 16% at pH 4.6 and 22% at pH 6.2 and pH 7.2 on the end of the experiments (data not shown). Bacterial growth was not affected by pH in the same way as metal removal, as the growth of both cultures at pH 6.2 was higher than at pH 7.2.

The effect of temperature incubation on U (VI) removal by bacterial consortia A and U is shown in Fig. 1b. Both cultures presented the highest U (VI) removal rate (97% in 16 days) at room temperature (±21 °C). At 37 °C only about 50% U (VI) removal was achieved within the same period of time for both mixed cultures, but no relevant bacterial growth was detected. At 4 °C no obvious U (VI) removal was observed, though bacterial growth was observed in this case. Although the lag phase was higher, bacterial growth achieved similar OD_{600} values at 4 °C and 21 °C at the end of the experiment. The results of U (VI) decrease in the abiotic sets were 22% at room temperature and 29% at 4 °C and 37 °C at the end of the experiments.

3.2. U (VI) bio-removal experiments

Fig. 2 shows uranium (VI) removal by live cells, by heat-killed cells and by extracellular metabolic products. The highest U (VI) removal from the medium was observed with live cells: 97% of U (VI) was removed by both consortia in 16 days. However, no relevant uranium (VI) removal was observed with heat-killed cells, comparatively to the abiotic sets: 27% for inoculum A and 11% for inoculum U, while 22% was achieved in the abiotic solutions. U (VI) removal by extracellular metabolic products was not detected.

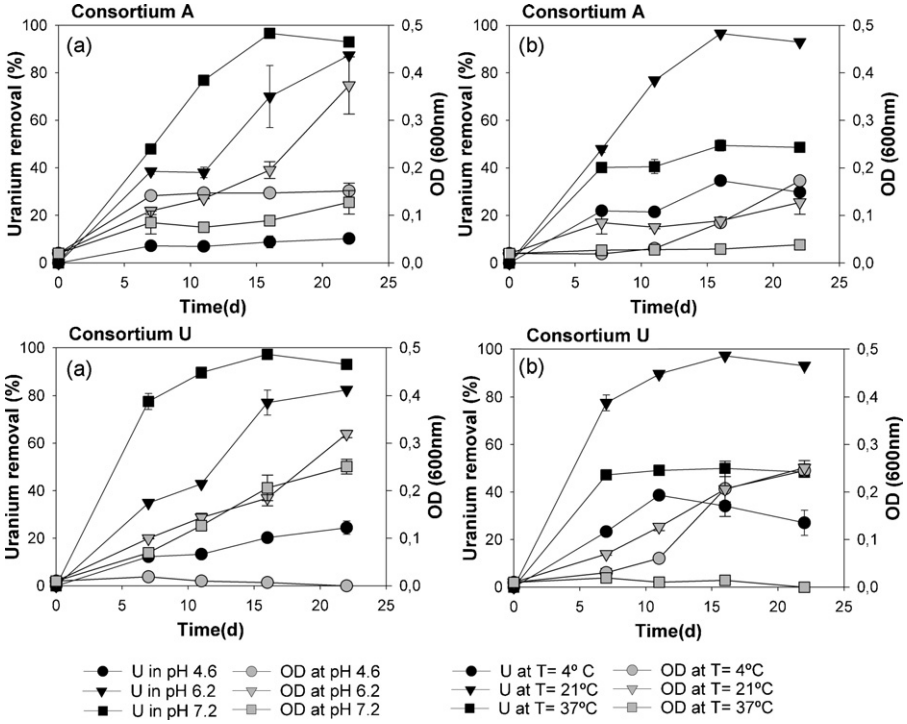

Fig. 1. Effect of pH (a) and incubation temperature (b) on uranium removal and growth of bacterial consortia from Monchique thermal place (A) and from Urgeiriça mine (U). Data are the average of duplicates and error bars indicate the standard deviations of the average values.

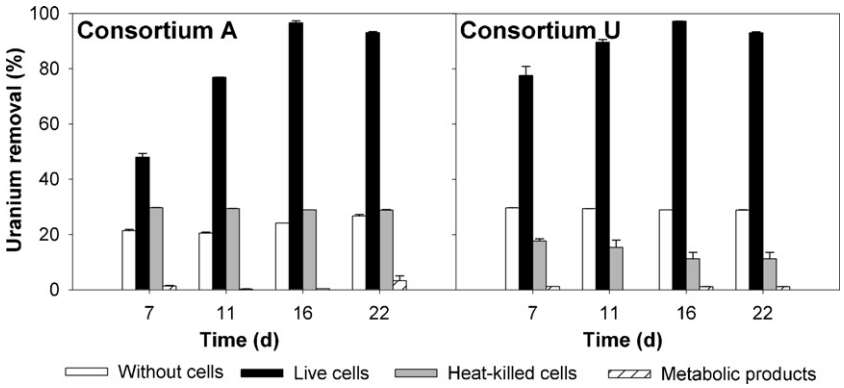

Fig. 2. Uranium removal from medium with 22 mg/L uranium by cells (live and heat-killed), and by extracellular metabolic products (pH 7.2; 21 °C). Data are the average of duplicates and error bars indicate the standard deviations of the average values.

3.3. X-ray powder diffraction analysis (XRD)

The black precipitate generated during the bio-removal experiments was composed by amorphous and crystallized material. Characterization of the mineral phase by XRD gave a spectrum that in accordance with PDF2 database is consistent with the presence of uranium oxide as U_3O_7 (Fig. 3).

3.4. Cellular localization of uranium

To establish the distribution and localization of uranium deposits in the cells, thin sections of cells exposed to uranium (VI) were viewed using TEM (Fig. 4). The cells of consortia A and U exhibited dense precipitates mainly within the periplasmic region (Fig. 4a, b, f–h). Fibrillar precipitates were observed in the capsule of some cells of consortium A (Fig. 4c and d) and round precipitates were also occasionally present in the cytoplasm of the cells of this consortium (Fig. 4e). Using EDS coupled to TEM for the elemental characterization of the metal deposits it was possible to confirm that the dense precipitates observed contained uranium (Fig. 4i). EDS spectrum also showed the presence of other elements such as P, Cu, Cl and Si. Some elements observed in the spectrum like Cu, Cl and Si, could be originated from external sources, since were also present in background areas and therefore represent elements from the supporting grid.

3.5. FTIR spectroscopy

FTIR spectral analysis of control (metal-free) and uranium loaded cells allows some characteristic peaks to be assigned to the main functional groups present in the bacterial cells and to their role in metal binding process. Correspondences of the IR frequencies in the present study were based on known data from the literature [1,2,8,22–26].

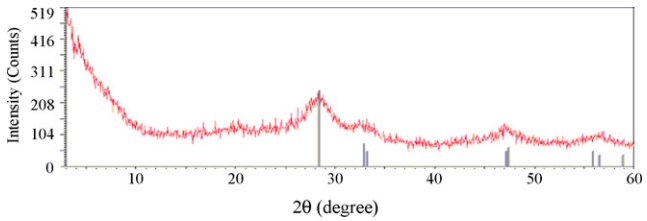

Fig. 3. X-ray diffraction spectrum of the precipitate formed during uranium bio-removal experiments. The vertical lines indicate the characteristic X-ray diffraction of U_3O_7.

The FTIR spectra from 400 to 4000 cm^{-1} of control cells (metal-free) and metal loaded cells are shown in Fig. 5. The spectra of control and metal loaded cells of consortia A and U showed a broad band between 3700 and 3000 cm^{-1} with a maximum around 3400 cm^{-1}. Bands corresponding to the N–H bond of amino groups, along with the O–H of the hydroxyl groups, usually lay in the region between 3800 and 3200 cm^{-1}.

All four spectra showed the presence of two peaks between 3000 and 2900 cm^{-1} which can be attributed to the asymmetric stretching of C–H bond of the $-CH_2$ groups combined with that of the $-CH_3$ groups.

The strong broad band between 1700 and 1470 cm^{-1} can be assigned to amide groups. The C=O stretching of amide (referred to as amide I) was observed at 1634 (consortium A) and 1647 cm^{-1} (consortium U) while the N–H bending (coupled to C–N stretching and referred to as amide II) appeared at 1566 cm^{-1} in both consortia. The spectrum of metal loaded cells (Fig. 5b) showed a shift of these bands to 1651 and 1541 cm^{-1} in consortium A and to 1641 and 1543 cm^{-1} in consortium U. Furthermore, a change in the relative intensities of the above bands was observed in cells of culture A.

In all spectra a peak around 1460 cm^{-1} was observed, which is characteristic of the scissoring motion of $-CH_2$ groups.

The peaks within 1400–1200 cm^{-1} are due to COO^- symmetric stretching, non-ionized carboxylic groups and P=O stretching of the C–PO_3^{2-} moiety. The peaks observed at 1298 or 1317 cm^{-1} in consortia A and U, respectively, are corresponding to stretching of non-ionized carboxylic groups (C–OH). The C–OH bands were shifted to 1321 (consortium A) and 1325 cm^{-1} (consortia U) after cell exposure to uranium (Fig. 5b). The peaks at 1234 and 1225 cm^{-1} observed in uranium loaded cells of consortia A and U, respectively, correspond to P=O stretching of the C–PO_3^{2-} moiety.

A complex band was observed in the range 1200–950 cm^{-1}, which corresponds to C–O, C–C and C–H bonds in carbohydrates and alcohols, along with the symmetric and asymmetric stretching bands of PO_2^- and $P(OH)_2$ from phosphates. Two peaks at 1165 and at 1113 cm^{-1} were observed in control cells A (Fig. 5a) and one peak at 1113 cm^{-1} in control cells U (Fig. 5a). In the spectra of metal loaded cells a broadening of the last band (at 1113 cm^{-1}) was evident, which results in the appearance of a new maximum at 1067 cm^{-1} in both spectra.

The absorption peaks at 893 cm^{-1} (control cells A and U) and 895 cm^{-1} in metal loaded cells may be attributed to substituted ethylenic system CH=CH groups.

In the spectra of metal loaded cells (Fig. 5b) new bands at 916 and 841 cm^{-1} were observed in cells A, and at 926 and 841 cm^{-1} in cells U.

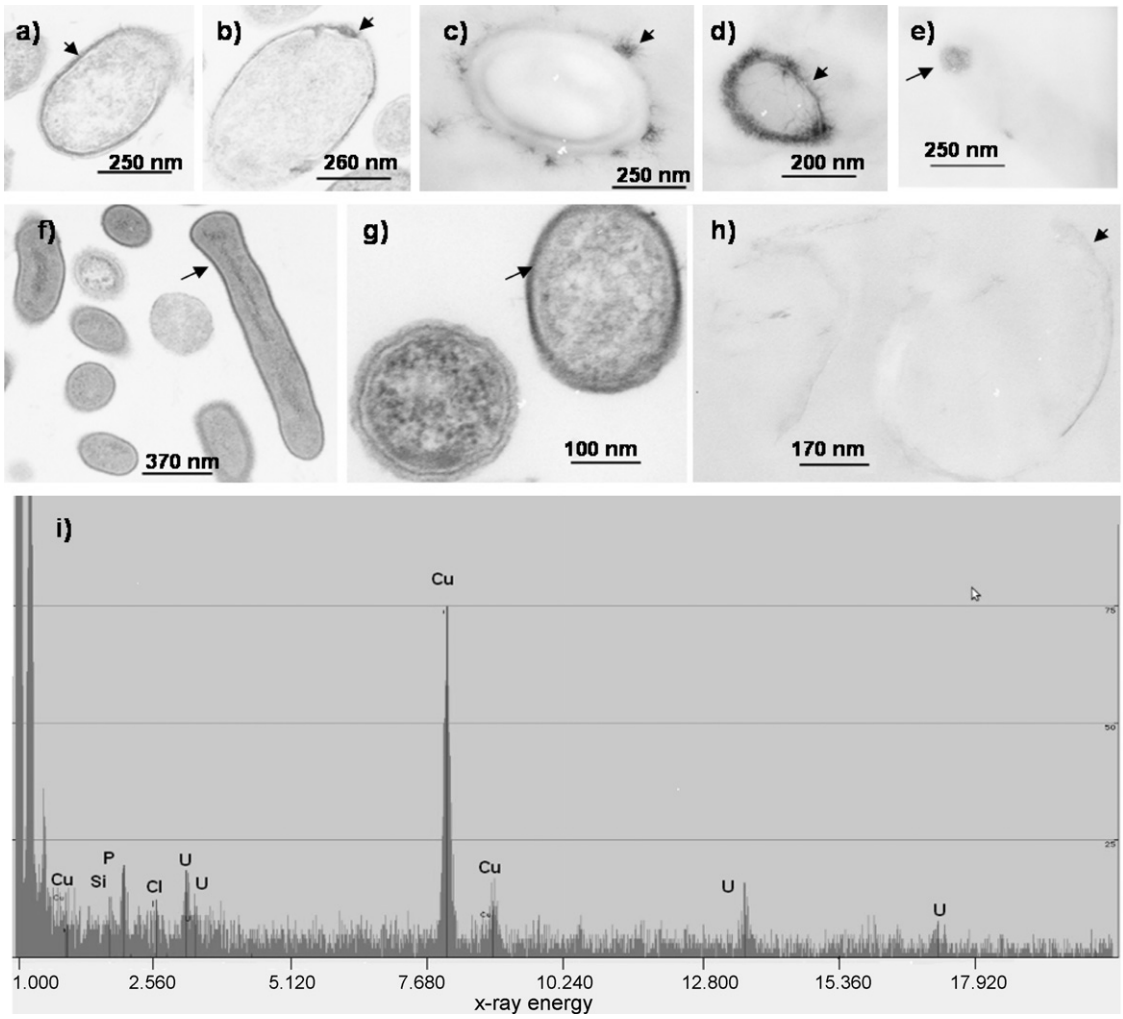

Fig. 4. TEM of thin sections (70–90 nm) of bacterial cells of inoculum A (a–e) and of inoculum U (f–h) after 20 days of growth with 22 mg/L U (VI): cells sections stained with uranyl acetate and lead citrate (a, b, f and g) and cells without staining (c, d, e and h). (→) Dense precipitates. EDS spectrum of precipitate (i).

A broad band between 700 and 400 cm^{-1}, with a maximum at 619 cm^{-1} was due to O–H bending. A change in this band shape in the metal loaded cells was observed. In metal loaded cells A, a band at 548 cm^{-1} and a band at 552 cm^{-1} in cells U were also observed.

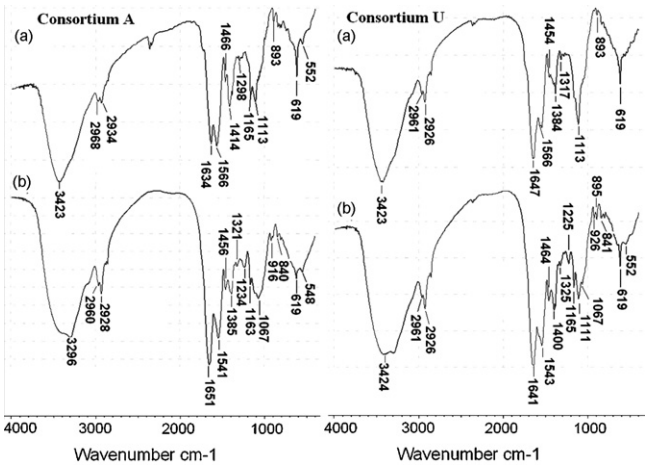

Fig. 5. FTIR spectra of bacterial cells of consortia obtained from Monchique thermal place (A) and from Urgeiriça mine (U): control cells (a) and metal loaded cells (b).

3.6. Phylogenetic analysis

All recombinant colonies (87) were recovered and approximately 1.4 kb fragment of bacterial 16S rRNA gene was amplified and used for RFLP analysis. Sixteen RFLP groups were originated from mixed culture A and thirteen from mixed culture U. Phylogenetic analysis of the representative clones allowed the identification of the corresponding sequences (Fig. 6).

Most of clones sequences (59%) from community A were closely related to *Sporotalea*, while majority of clones from community U were affiliated to *Clostridium* (53%). Clones with sequences closely related to *Clostridium* were also present in community A (2%). Both communities included members of *Rhodocyclaceae* family. Phylogenetic analysis also showed that sequences of 26% of clones from community A and 32% of clones from community U were closely related to *Rhodocyclus* or *Propionivibrio* and bacteria affiliated to *Brevundimonas* were also present in both communities (9% and 3% of clones from consortia A and U, respectively). Additionally, community A included bacteria closely related to *Pelosinus* (4% of clones). Bacteria affiliated to *Sphaerochaeta* (3%) and *Anaerofilo pentosovarans* (9%) were also detected in community U.

4. Discussion

In previous studies [14] were discovered three bacterial communities with ability for uranium (VI) removal: one was recovered

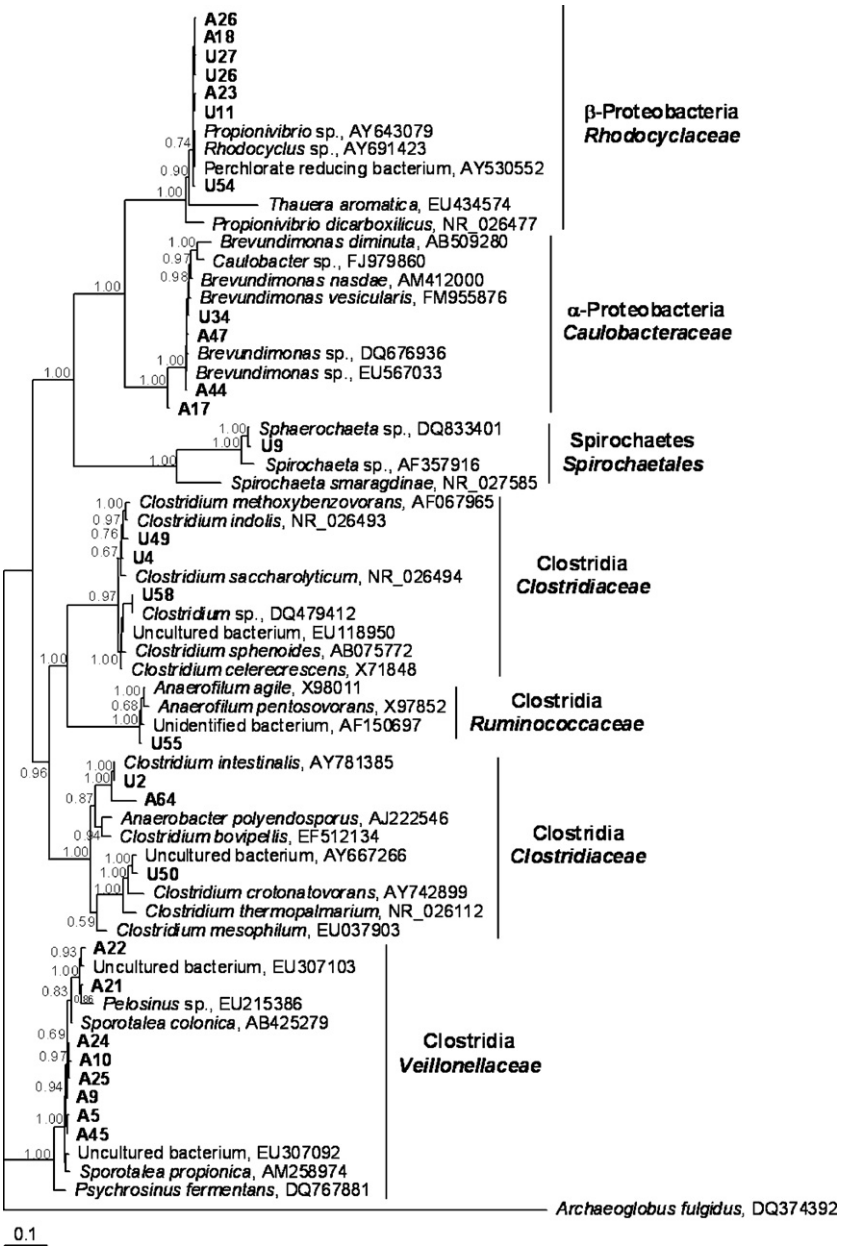

Fig. 6. Phylogenetic tree obtained with 16S rRNA sequences (1300 nucleotide positions), corresponding to the clones representative of each restriction profile and to the most closely related ones retrieved from BLAST search. Phylogeny was inferred using the Bayesian Markov Chain Monte Carlo analysis of aligned 16S rRNA fragments. *Archaeoglobus fulgidus*, a species from Archaea Domain was included to root the tree. Probability values associated to each node are showed. Access numbers of GenBank sequences are indicated in the figure and names in bold face correspond to sequences determined in this work. The main bacterial groups detected in bacterial consortia are also indicated.

from a soil sample of an uncontaminated site (Monchique thermal place), while the other two were obtained from sediments collected in uranium mine site (Urgeiriça). Thus, in the present work, the mechanism of uranium (VI) removal from aqueous solutions by two of these cultures (one from an uncontaminated site and other from an uranium mine) was investigated for the first time.

The U (VI) removal was tested with live and heated-killed cells, as well with extracellular metabolic products. U (VI) removal by both communities was only observed in the presence of live cells. The lack of metal removal by extracellular products and heat-killed cells suggests that only viable cells can be responsible for uranium (VI) removal from the solution. A slight decrease in the uranium (VI) concentration in the abiotic solutions was also observed. This fact, already mentioned in the literature, is reported as related to the adsorption of the metal to the glass material [8]. This explanation was supported by the fact that no decrease of U (VI) was observed when plastic material was used (data not shown), even in the sets with extracellular metabolic products. The solution with extracellular metabolic products may contain other substances that can also be adsorb to the glass bottles, thus, competing with the metal to the adsorption sites.

X-ray diffraction analysis of the precipitate formed during the bio-removal experiments showed that it was mainly composed by uranium oxide as U_3O_7. The presence of U_3O_7 indicates that the mineral phase was composed by a mixture of uranium (VI) and (IV). This result can be explained by the slight oxidation of uranium (IV) due to oxygen exposition. The presence of U (IV) was also consistent with the black colour of the precipitate formed. Typically, U (VI) has an orange/yellow colour, while the precipitate of UO_2 shows a black/brown colour [27,28]. The presence of U (IV) in the precip-

itate, together with the fact that uranium (VI) was only removed from solution in the presence of live cells, suggests a mechanism of enzymatic reduction, where U (VI) is converted to insoluble U (IV).

Many microorganisms affiliated with genera *Desulfovibrio*, *Geobacter*, *Tolumonas*, *Clostridium*, *Arthrobacter*, *Dechlomonas*, *Shewanella* and *Pseudomonas* can reduce U (VI) to U (IV) [6,9,29]. *Clostridium* species are considered one of the major players in uranium (VI) reduction [9,18] and bacteria affiliated to this genus were present in both consortia. Bacteria affiliated with *Pelosinus* were also found in community A and are reported as capable of reducing Fe (III) [30]. Considering that dissimilatory Fe (III)-reducing bacteria have already been reported as having the ability for U (VI) reduction [3], U (VI) reduction by *Pelosinus* cannot be excluded. Regarding *Rhodocyclaceae* members, they have been reported in uranium contaminated mines [18], and also in microbial populations stimulated for uranium removal [29], but to date the knowledge about their role on uranium removal is limited.

The maximum U (VI) removal by consortia A and U occurred at room temperature (±21 °C) and with pH 7.2. However, at 4 °C bacterial growth was observed without significant uranium (VI) reduction. This result can be explained by the presence of different species in the consortia, which probably have different optimal growth conditions. Thus, this temperature (4 °C) may promote the growth of metal resistant bacteria, but without ability for uranium (VI) removal. Other explanation is that, since the enzymatic process is the dominant mechanism for uranium (VI) removal, at 4 °C the culture can grow but the enzymes responsible for metal reduction cannot be expressed. It was reported that U (VI) reduction by dissimilatory Fe (III)-reducing bacteria (GS-15) [3] and Cr (VI) reduction by *Enterobacter cloacae* [31] were repressed at 4 °C.

It was also observed that uranium (VI) reduction increased with the increase of pH. The increase of pH can promote the optimal conditions for the expression of the enzymes responsible for uranium (VI) reduction. The influence of pH in metal reduction was also observed in previous studies concerning uranium (VI) reduction by *Clostridium* sp. [9] and platinum (IV) reduction by a sulfate-reducing bacteria consortium [11].

Due the insoluble nature of U (IV), the site of its deposition in cell should give an indication of the reductase location. Hence, TEM was used to establish the distribution and localization of uranium deposits in the cells. TEM images showed the presence of dense precipitates mainly within the periplasmic region of cells of both consortia. This observation is consistent with other studies that reported the existence of reductases in the periplasmic region, in the outer membrane, or in both [6,11]. Fibrillar precipitates were also observed in the capsule of some cells of consortium A and round precipitates were also occasionally present in the cytoplasm. Ohnuki et al. [32] have reported the presence of fibrillar precipitates contained uranium in cells of *Saccharomyces cerevisiae*. The presence of uranium precipitates in the cytoplasm of some cells of inoculum A can be explained by the ability of some bacteria of this consortium to accumulate uranium inside the cells. Since the mixed culture was composed by diverse species of bacteria, the occurrence of more than one mechanism of uranium removal was expected.

Bacterial cells were also analyzed by energy dispersive spectrometer (EDS) coupled to TEM, allowing the confirmation of uranium presence in the dense precipitates. EDS could identify not only the elements present in the precipitate but also those of the microbial cells, such as phosphorous, which is an essential element in the bacterial cell wall [2].

The presence of dense uranium precipitates around bacterial cells, specially in consortium A, can be explain by the fact that bacteria are excellent nucleation sites for mineral formations [11], due to the electronegative surface of functional groups such as carboxyl, hydroxyl, phosphoryl and amide groups [8,25]. Therefore, after U (VI) reduction, the U (IV) ions could be bound to functional groups of bacterial cell surface. FTIR spectroscopy was applied to determine the functional groups of the bacterial cells that can be involved in U binding. According to Jiang et al. [25], the FTIR spectra of Gram-positive and Gram-negative bacteria are similar. This is in accordance with the fact that no significant differences in the spectra of both consortia were observed.

The amide I absorption peak was mainly accounted by 3_{10}-helical secondary structure of proteins, although amino sugars (with N-acetyl/glucuronamide groups) from cell associated polysaccharides could also show an absorbance band in this region [1,2]. The shift of the peaks of the amide groups after uranium exposition indicates a possible interaction of metal with cellular proteins. Furthermore, a change in the relative intensities of these bands was observed in consortium A. These changes in peak positions and relative intensities, most probably reflect some alteration in the secondary structure of cellular proteins from the predominant 3_{10}-helix to other possible conformation as a result of radionuclide sequestration [2]. The shift of the C–OH bands of carboxylic groups (from 1298 to 1321 cm^{-1} in consortium A and from 1317 to 1325 cm^{-1} in consortium U) after uranium exposure and reduction could reflect the involvement of these groups in metal binding. In fact, the strong role of carboxylic groups in radionuclide binding after sorption of U and Th by *Pseudomonas* sp. has already been reported by Kazy and co-workers [2]. Furthermore, the intense peak at 1234 and 1225 cm^{-1} observed in the uranium loaded samples A and U, respectively, is probably a result of uranium binding to the phosphate [2]. In the spectra of metal loaded cells, modifications were observed in the complex band in the range 1200–950 cm^{-1}, corresponding to C–O, C–C and C–H bonds in carbohydrates and alcohols, along with the symmetric and asymmetric stretching bands of PO_2^- and $P(OH)_2$ from phosphates [1,25]. The above groups, mostly belonging to various cellular components like phospholipids, nucleic acids, peptidoglycan, cell associated polysaccharides and peptides, are able to complex different metals [1,24]. Following metal exposition, a broadening of the band at 1113 cm^{-1} and a new peak at 1067 cm^{-1} were observed. Both changes may be attributed to U–O asymmetric stretching in U (IV) oxides [26] formed during the process. The change of band shape between 700 and 400 cm^{-1} observed in the metal loaded biomass comparatively with the control, could also be assigned to the presence of U–O bonds [2].

The band observed at 916 (consortium A) or at 926 cm^{-1} (consortium U) in metal loaded cells may be ascribed to asymmetric stretching of U=O bonds, either in remaining UO_2^{2+} or in U (VI) complexes formed with bacteria functional groups. The peak at 840 cm^{-1} can correspond to symmetric stretching of the last one [2,22]. It was observed that the first band was stronger in a non-freshly prepared sample (data not shown) suggesting that oxygen exposition promotes metal oxidation, which results in an increment of the peak around 920 cm^{-1}. Finally, the band at 548 (consortium A) or 552 cm^{-1} (consortium U) can be attributed to symmetric stretching of weekly bonded oxygen ligands ($U–O_{ligand}$), both in U (IV) and U (VI) [2,22]. The overall spectral analysis indicates that carboxyl, amide and phosphate groups of bacterial cells are the dominant functional groups involved in bacteria–metal interaction. The involvement of phosphate groups is also in concordance with the presence of phosphorous in the EDS spectrum.

Uranium (VI) was only removed from solution in the presence of live cells and the presence of U (IV) in the precipitate was observed. These results together with the presence of dense precipitates mainly within the periplasmic region of cells of both consortia suggest a mechanism of enzymatic reduction by both consortia. Moreover, the presence of uranium precipitates in the cytoplasm of some cells of inoculum A suggests that this consortium could accumulate uranium inside the cells. Therefore, the present work

showed that U (VI) removal by consortium A was carried by two mechanisms: enzymatic reduction and bioaccumulation, while the enzymatic process was the only mechanism involved in U (VI) removal by consortium U. The results also suggest that probably the process can be divided in two steps: first the enzymatic reduction of U (VI) to U (IV) occurs and then U (IV) binds to carboxyl, phosphate and amide groups of bacterial cells. These results are in accordance to those reported for plutonium removal by *Bacillus subtilis* [33].

5. Conclusion

The present work demonstrated the ability of two anaerobic bacterial communities for U (VI) removal. Both communities were composed by several species of bacteria, a large number of them never reported as U (VI) reducing bacteria or even metal resistant. This is the case of *Sporotalea* sp., *Rhodocyclaceae* members, *A. pentosovarans*, *Brevundimonas* sp., *Pelosinus* sp. and *Sphaerochaeta* sp. This result is a relevant finding, encouraging the exploitation of microorganisms with new abilities that can be useful for bioremediation purposes. Uranium is not known to be an essential component for biologic function and is toxic. Therefore, the discovery that the mechanism of U (VI) removal by these cultures occurs mainly through an enzymatic reduction opens a new research field for understanding the enzymes responsible for metal reduction.

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