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Full Length Research Paper

Isolation and characterization of a novel *Micrococcus* strain for bioremediation of strontium in radioactive residues

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There has been increased interest in the isolation of bacteria with a potential role in bioremediation from extreme environments such as phosphate mines containing various metals and radionuclides. This paper describes the isolation and characterization of a novel *Micrococcus* species from a phosphate mining region in the South of Tunisia, designated as strain BRM7. Colonies of bacterial strain BRM7 produced on Tryptone-Glucose-Yeast Extract (TGY) agar plates were yellow, smooth, circular and 0.5–1.5 mm in diameter. Cells of strain BRM7 were Gram-positive cocci, with a diameter of 0.7–1.0 µm. The most abundant cellular fatty acids of strain BRM7 were found to be anteiso-C15: 0 (49.42%) and iso-C15: 0 (32.63%). On the basis of its morphological attributes, biochemical characteristics, and partial sequencing of 16S rRNA gene (rDNA), the strain BRM7 belongs to the genus *Micrococcus* with 99% identity to *Micrococcus luteus*. Strain BRM7 grew well in tryptone, glucose and yeast extract (TGY) medium, and tolerated (1) high salt concentrations (up to 20%), (2) a wide range of pH (5.0–12.0), and (3) high temperatures (up to 45°C). The bacterial isolate *Micrococus* sp. BRM7 showed a high tolerance to strontium (Sr, D_{10} (dose for 90% reduction in Colony Forming Units (CFUs)) = 350 mM) with a similar tolerance curve to *Cupriavidus metallidurans* CH34, best known for its high tolerance to a wide range of heavy metals. Interestingly, *Micrococus* sp. BRM7 has an ionizing-radiation (IR) resistance D_{10} (~800 Gy) four times higher than that of *C. metallidurans*. Immobilization into alginate beads indicated that *Micrococus* sp. BRM7 cells have the potential to adsorb 17 and 34% of Sr following an incubation time of 3 and 24 h, respectively. Overall, the results of this study suggest that BRM7 can be valorized to bioremediate Sr in radioactive residues like phosphogypsum (PG), an industrial concentrator of this toxic metal.

Key words: Bioremediation, immobilization, ionizing radiation, *Micrococcus*, phosphate, strontium.

INTRODUCTION

The increasing pollution of toxic metals, metalloids, radionuclides, and organometal(loid)s in the environment has boosted a few decades research towards the better understanding of the impact of toxic metals on microorganisms, and *vice versa*, the biotechnological potential of microorganisms for metal removal and/or recovery from the biosphere (Beveridge et al., 1997). Presently, there is a growing interest in microbiologically mediated remediation because it promises to be simpler, cheaper, and more environment-friendly than the more commonly used non-biological options (Lovley, 2003). Particularly, microorganisms able to survive well in high concentrations of heavy metals and ionizing radiation are of great interest as bioremediation agents for radioactive

residues (Barkay and Schaefer, 2001). Among the most important natural sources of heavy metals and radionuclides there are phosphate mines and their industrial residues like PG resulting from processing phosphate rocks. These rocks, and subsequently PG, vary considerably in their content of heavy metals and radionuclides depending on the geographical area from which they were mined. In Tunisia, Sr is the most abundant among trace elements in phosphate mines and PG (1100 mg Kg^{-1}) ((Sattouf et al., 2007; Ajam et al., 2009) and references therein). In addition, the Gulf of Gabès between Sfax, Gabès, and the Kerkennah Islands, in Tunisia, is one of the most polluted places in all of the Mediterranean Sea. This is a result of several factors including the massive production (~10 million tones per year) and accumulation of PG piles. When it rains or when the wind blows, PG gets spread around the general vicinity of these warehouses. This can lead to many health problems ((Ajam et al., 2009) and references therein), unless toxic chemicals were cleaned up using biological options (Barkay and Schaefer, 2001).

Some species of *Micrococcus* carry out biosorption of Sr (Faison et al., 1990). Indeed, these species belong to a group of bacterial strains that are particularly well adapted to environments contaminated with elevated levels of toxic metals, and that are potentially useful for bioremediation applications ((Young et al., 2010) and references therein). The genus *Micrococcus* now includes five species: *M. luteus*, *Micrococcus lylae*, *Micrococcus antarcticus*, *Micrococcus endophyticus*, and *Micrococcus flavus*. *M. luteus* (NCTC2665, DSM 20030^T, "Fleming strain") is the type species of the genus *Micrococcus*. With one of the smallest actinobacterial genomes (2,501,097 bp) sequenced to date, *M. luteus* possesses unusual abilities to remediate co-contaminated sites with organic and metal pollutants through a whole repertoire of functions which deal with these pollutants (Sandrin and Maier, 2003; Young et al., 2010). In addition, *M. luteus* can degrade hydrocarbons and olefinic compounds (Zhuang et al., 2003), use biphenyl as a carbon source, and metabolize dibutylphthalate by a pathway which has at least five regulatory units (Eaton and Ribbons, 1982).

The aim of this study was to isolate a novel *M. luteus* species which is capable to bioremediate Sr from radioactive residues. Following a primary screening molecular studies were carried out to characterize and identify a bacterial isolate designated *Micrococcus* sp. BRM7. In comparison with reference strains, the adsorption potential and tolerance to Sr of BRM7, and its highest IR resistance D_{10} compared to *C. metallidurans* indicated that it could be used as a novel biocatalyst for co-contaminated residues including PG.

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MATERIALS AND METHODS

Study area and sampling

Surface soil samples were collected from phosphate mine in Metlaoui region in the Prefecture of Gafsa (Tunisia) (Figure 1). Samples were collected from the soil surface (0-5 cm). Sampling was carried out in sterile screw-capped glass bottles and transported to the laboratory. Transport and storage of the samples were done at a controlled temperature of 4°C.

Isolation and biochemical characterization of bacteria

Samples (0.5 g) were suspended in 10 ml of 0.9% NaCl (saline), vortexed thoroughly, and allowed to settle. Appropriate dilutions were plated on solidified TGY media (Bacto Tryptone 1%; Yeast extract 0.5%; glucose 0.1%, and Bacto agar 1.5%) in triplicate and incubated in the dark at 30°C for 5 days (Shukla et al., 2007; Fredrickson et al., 2008). Yellow isolates obtained in this manner were subsequently cultured in TGY broth (30°C, 100 rpm) to a mid-log phase and preserved by freezing at –80°C in 40% glycerol (Fredrickson et al., 2008). Gram-positive cocci were identified to species using API-Staph (bioMérieux).

Determination of optimal growth temperature, osmotic pressure and pH

The isolate was used to examine its temperature tolerance by inoculating equal volume of overnight grown culture in TGY broth and incubating at temperatures ranging from 30 to 60°C for 24 h. Cell growth was determined by measuring absorbance at 600 nm. The cultures obtained on TGY medium were further screened for their salt tolerance. Media supplemented with various concentrations of NaCl (ranging from 1 to 50%) were used for inoculation. Incubation was carried at 30°C, 120 rpm for 24 h. The growth was measured turbidimetrically at 600 nm (Shukla et al., 2007). High salt-tolerant culture was further studied for pH tolerance in TGY broth adjusted to pH 5.0–12.0. Incubation was carried out at 30°C at 150 rpm for 24 h and cell growth determined by measuring absorbance at 600 nm (Shukla et al., 2007).

PCR amplification of 16S rDNA and bioinformatics analyses

The genomic DNA was isolated using the DNeasy Blood and Tissue Kit (Qiagen). The PCR assay was performed using Applied Biosystems model 9700 with 1 µl of DNA extract in a total volume of 50 µl as recommended (Polz and Cavanaugh, 1998) to reduce bias in amplification, and to obtain a final DNA concentration equal to 100 ng/µl. The PCR master mixture contained 5 µl of 10X PCR reaction buffer, 4 µl of 25 mM $MgCl_2$, 5 µl of 20 mM dNTPs, 5 µl of 10 µM of each oligonucleotide primers UP1-F/5′ (5′ AGAGTTTGATCCTGGCTCAG 3′), UP1-R/5′ (5′ GTTACCTTGTTACGACTT 3′), 0.24 µl of Taq DNA polymerase (5 U/µl) and 24.76 µl of molecular grade sterile distilled water. Initially, denaturation accomplished at 95°C for 5 min. Thirty cycles of amplification consisted of denaturation at 95°C for 30 s, annealing at 56°C for 30 s and extension at 72°C for 1 min 30 s. A final extension phase at 72°C for 15 min was performed. The PCR product was purified by the EZ-10 Spin Column PCR Purification Kit. The amplicons were detected by electrophoresing the sample on 1.0% agarose gel. The sequences were determined by cycle

Figure 1. Location of the Metlaoui mine in Tunisia. The arrow shows the location of the Metlaoui mine.

sequencing using the Taq Dye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems), and underwent fragment separation in an ABI Prism 3130 DNA sequencing as previously described (Gtari et al., 2004). Alignments of 16S rDNA gene sequences were performed with the CLUSTAL_X program, version 1.64b (Thompson et al., 1997). A neighbour-joining phylogenetic tree was constructed based on evolutionary distances that were calculated with the Kimura two-parameter model. Alignment positions with insertions or deletions were excluded from the calculations.

Analysis of fatty acids

Extraction and analysis of fatty acids (Miller, 1982) of BRM7 were performed at the Deutsche Sammlung von Mikroorganismen und

Zellkulturen (DSMZ, Braunschweig, Germany) using the Sherlock Microbial Identification System (MIDI, Inc.).

Resistance to ionizing radiation (IR)

Yellow colonies obtained after primary screening were grown in TGY broth for 18 h. The cells were pelleted down, washed, and resuspended in 0.9% NaCl, exposed at a dose rate 16.07 Gy/min for doses below 2 kGy in a ^{60}Co irradiator Gamma chamber (CNSTN, Sidi Thabet, Tunisia). The survival was checked by streaking the culture on TGY plates and incubating the plates at 30°C for 48–72 h.

Toxicity of strontium (Sr)

Three replicates of the inoculums from the isolated bacteria were prepared by mixing 0.5 ml of a stationary bacterial culture with 49.5 ml of double concentrated Tris-salt mineral medium (MM284 medium) (Mergeay et al., 1985) supplemented with yeast extract. In 96-well plates, 100 µl of these inoculum suspensions was mixed with 100 µl double concentrated medium containing Sr concentrations ranging from 0 (control), 40, 80, 120, 160, 200, 240, 300 to 360 mM; so giving final concentrations of 0 (control), 20, 40, 60, 80, 100, 120, 150 to 180 mM in normal MM284 medium. The test microplates were incubated at 30°C on an orbital shaker at 180 rpm. Bacterial growth was measured in terms of optical density at 630 nm after 48 h using the Bio-Tek® Gen5 Date Analysis Softeware.

Cell immobilization and biosorption studies

BRM7 and CH34 cells were collected from cultures in liquid MM284 medium MM284 medium: supplemented with yeast extract, in mid-exponential growth phase (~5 ×10^8 CFU/ml). 17.5 ml of cells were diluted in 87.5 ml of sterile distilled water and incubated 10 min under stirring. 3.063 g of sodium alginate ($NaC_6H_7O_6$) were added to the final volume (100 ml). The mixture was incubated under stirring until it becomes viscous. Using a sterile syringe, the volume was transferred and mixed to 100 ml of calcium chloride ($CaCl_2$, 0.12 M). Once the cells were immobilized, the alginate beads were conserved in 100 ml of $CaCl_2$ (5 mM) (Wuyep et al., 2007).

Measurement of strontium (Sr) by inductively coupled plasma atomic emission spectroscopy (ICP-AES)

Immobilized cells of BRM7 and CH34 were incubated in presence or absence of 180 mM $SrCl_2$ under agitation (120 rpm) during 24 h. Volumes of supernatant were collected from each solution for chemical analysis. These supernatant samples were filtered through a 45 µm filter, conserved in a 0.01 M HCl solution, and analyzed by ICP-AES, a standard analytical technique used for the detection of trace metals.

Scanning electron microscopy (SEM) and energy dispersive X-ray analysis (EDX)

Bacterial pellet of BRM7 was collected from cultures growing in the presence of 60 mM Sr. Small droplets of the dried pellet at 50°C were placed on aluminum stubs. These stubs were mounted on a cold stage and imaged at 2.08°C in an environmental SEM (Philips XL30, Institut National de Recherche et d'Analyses Physico-chimiques, Technôpole Sidi-Thabet, Tunisia), which was equipped with a field emission gun and EDX spectrometer. The instrument was operated in the wet imaging mode (that is, 100% relative humidity) at 20 kV.

RESULTS

Morphological and biochemical characterization of BRM7

Following plating on TGY medium, an isolate, with a yellow pigmentation on TGY agar plates, was obtained. Good growth of BRM7 was observed on TGY plates incubated for 72 h at 30°C. Cells of strain BRM7 were Gram-positive spherical cocci, with a diameter range of 0.7–1.0 µm. Flagella were not observed. Colonies were yellow, smooth, circular and 0.5–1.5 mm in diameter after 3 days cultivation on TGY agar. It was non-motile and non-spore forming. Cells of strain BRM7 were positive for catalase and alkaline phosphatase but negative for urease. Acid was not produced from D-glucose, D-fructose, D-mannose, maltose, lactose, D-trehalose, D-mannitol, xylitol, D-melibiose, raffinose, xylose, saccharose, a-methyl-D-glucoside, and N-acetyl-glucosamine. Reduction of nitrates to nitrites by BRM7 was negative. Strain BRM7 was aerobic and grew over the temperature range of 30–45°C, pH range of 5.0–12.0, and tolerated high salt concentrations (up to 20%). Optimal growth was observed at 30°C and pH 7.

16S rDNA and bioinformatics analyses

16S rRNA gene of strain BRM7 was amplified, sequenced, and submitted to NCBI (1273 bp, accession no. GU451721.1). The BLAST search of the nearly complete 16S rRNA gene sequence of the isolate was carried out. The isolate matched best with the genus *Micrococcus* and showed 99% similarity to *M. luteus*. 16S rDNA gene sequence analysis indicated that strain BRM7 was phylogenetically related to members of the genus *Micrococcus*. The phylogenetic tree (Figure 2) also indicated that strain BRM7 clustered with *Micrococcus* species and this cluster was strongly supported (100%). *Micrococcus* sp. BRM7 was publicly deposited under DSMZ GmbH Number DSM 24578, under Japan Collection of Microorganisms Number JCM 17588, and under Belgian Coordinated Collections of Microorganisms (BCCM/LMG) Number LMG 26301.

Resistance to ionizing radiation (IR)

Micrococcus sp. BRM7 has an IR resistance D_{10} (~800 Gy) four times higher than that of *C. metallidurans*, but it is extremely IR sensitive compared to *D. radiodurans* (Figure 3).

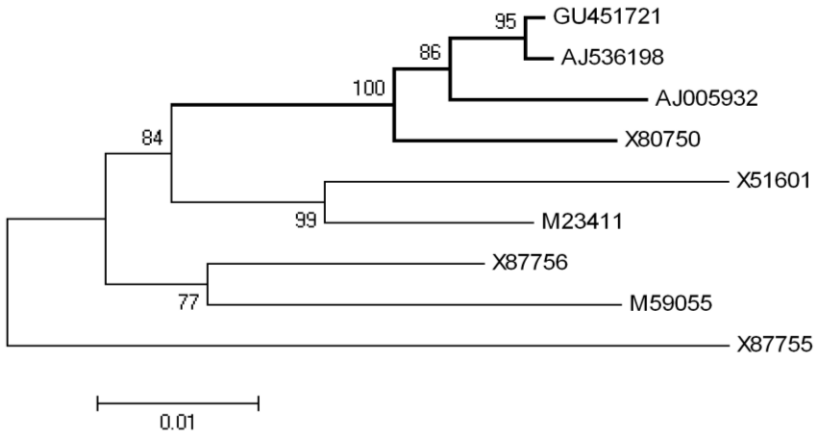

Figure 2. Phylogenetic tree constructed with the neighbour-joining method according to 16S rDNA gene sequence evolutionary distance among *Micrococcus* sp. BRM7 and the type strains of recognized members of the genus *Micrococcus* and type species of the family *Micrococcaceae*. GenBank accession numbers and microorganisms are from top to bottom: GU451721 - *Micrococcus* sp. strain BRM7 ; AJ536198 - *Micrococcus luteus* DSM 20030^T ; AJ005932 - *Micrococcus psychrophilum* JCM 11467^T; X80750 - *Micrococcus lylae* DSM 20315^T; M23411 - *Arthrobacter globiformis* DSM 20124^T; X51601 - *Renibacterium salmoninarum* ATCC 33209^T; M59055 - *Rothia dentocariosa* ATCC 17931^T; X87756 - *Kocuria rosea* DSM 20447^T; X87755 - *Kytococcus sedentarius* DSM 20547^T. *Kytococcus sedentarius* DSM 20547^T was used as an outgroup. Subtree of *Micrococcus* species is indicated in bold. Numbers represent confidence levels from 100 replicates bootstrap samplings. Bar, evolutionary distance (K_{nuc}) of 0.01.

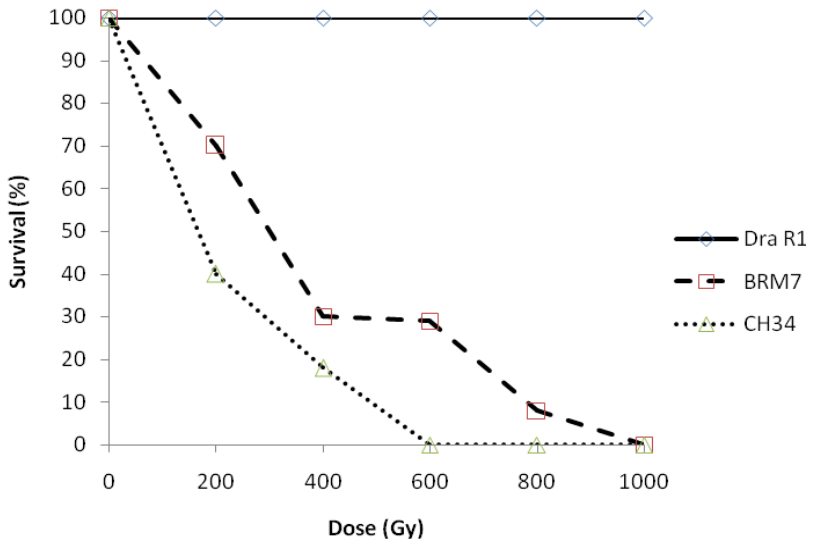

Figure 3. Resistance of *Micrococcus* sp. BRM7 and reference strains to acute gamma-radiation exposure. Strain BRM7 is represented by open squares. *Deinococcus radiodurans* R_1 (open lozenges) and *Cupriavidus metallidurans* CH34 (open triangles) were used as reference strains. Values are means of three independent experiments.

Tolerance to strontium (Sr) and its adsorption by *Micrococcus* sp. BRM7

Sr D_{10} of BRM7 was approximately 350 mM (Figure 4). During immobilization experiments, BRM7 cells have the potential to adsorb Sr and reached 17 and 34% of fixed Sr after 3 and 24 h, respectively. The most intense peak identified with EDX corresponds to Sr, with no other

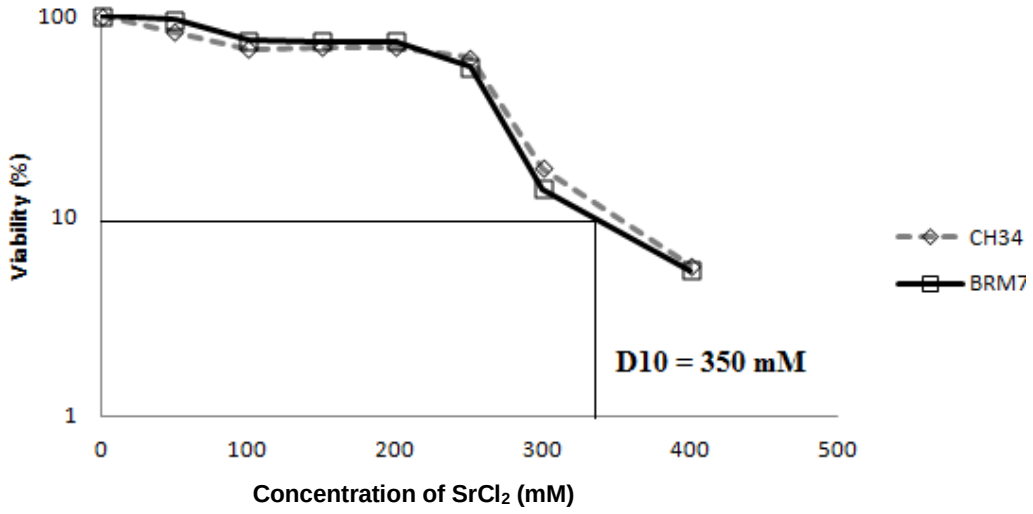

Figure 4. Tolerance of *Micrococcus* sp. BRM7 and *Cupriavidus metallidurans* CH34 to strontium. Strain BRM7 is represented by a dark line. The reference strain *C. metallidurans* CH34 is represented by an interrupted line. Values are means of three independent experiments.

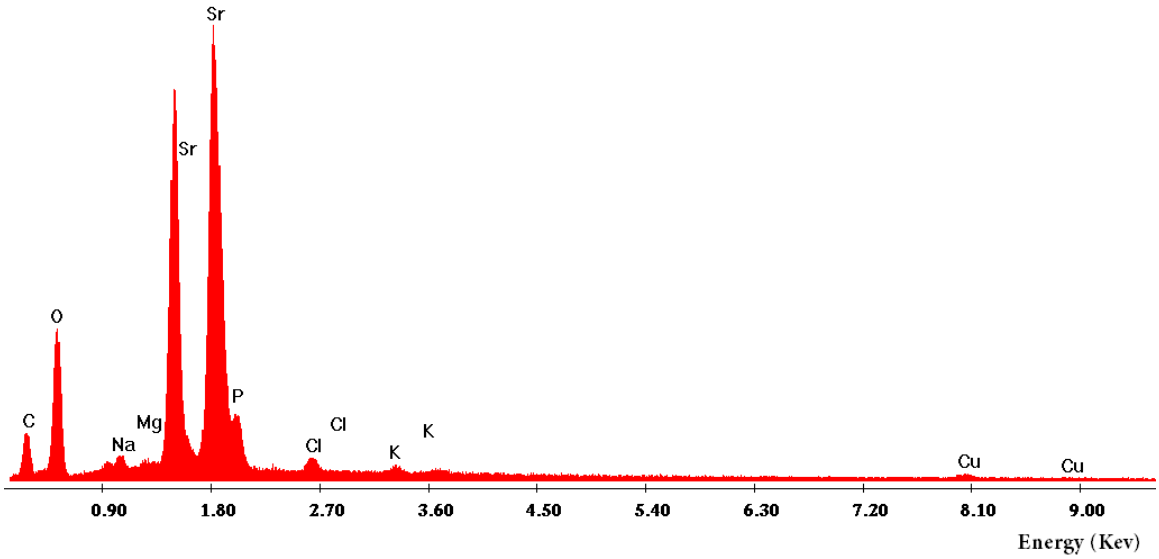

Figure 5. Elemental analysis of the dried pellet of *Micrococcus* sp. BRM7 obtained from a culture with strontium (60 mM $SrCl_2$). EDX analysis showed a semi-quantitative spectrum of the composition of BRM7 membrane (See the section Results).

elements detected at similar levels of abundance (Figure 5).

DISCUSSION

The genus *Micrococcus* seems to be well suited for long-term survival in extreme environment which may give them an important role in bioremediation (Greenblatt et al., 2004). especially, *M. luteus* is potentially useful forbioremediation of Sr (Faison et al., 1990), particularly in Tunisian phosphate and PG mines where it represents the component the most abundant among trace elements ((Ajam et al., 2009) and references therein). Samples of soil from a phosphate mine in the South of Tunisia were used to isolate and characterize *Micrococcus* species with a bioremediation potential. As suggested from the restricted distribution of genes concerned with pigment production (Young et al., 2010), our primary screening was based on yellow pigmentation for the identification of

Table 1. Residual strontium in the medium after contact with immobilized *Micrococcus* sp. BRM7 and reference strain *Cupriavidus metallidurans* CH34.

Hours (h)	Residual Sr concentration (%) in the medium		
	A	B	C
0	100	100	100
3	95.34	83.78	69.84
24	96.03	66.41	52.12

A: Beads of alginate + 180 mM of $SrCl_2$ (without cells), B: Beads of alginate containing BRM7 cells + 180 mM of $SrCl_2$, C: Beads of alginate containing CH34 cells + 180 mM of $SrCl_2$.

M. luteus. Besides its yellow pigmentation, the most abundant cellular fatty acids of strain BRM7 were anteiso-C15 : 0 (49.42%) and iso-C15 : 0 (32.63%), which were also found to be the dominant cellular fatty acids of other members of the genus *Micrococcus* (Wieser et al., 2002).

The taxonomy of strain BRM7 was also confirmed on the basis of comparative analysis of the 16S rDNA sequences. Using BLAST search, BRM7 showed 99% similarity to *M. luteus*. Phylogenetic results indicated in Figure 2 are supported by the currently accepted phylogenetic tree of the actinobacteria. Indeed, *Micrococcus* clusters with *Arthrobacter* and *Renibacterium* (Stackebrandt et al., 1995). Some other coccoid actinobacteria originally are also called *Micrococcus*, but reclassified into four new genera (*Kocuria*, *Nesterenkonia*, *Kytococcus*, and *Dermacoccus*), which are more distant relatives (Stackebrandt et al., 1995). In Figure 2, *Kytococcus* was chosen as an out group organism.

C. metallidurans CH34 (Janssen et al., 2010) and *Deinococcus radiodurans* R_1 (Cox and Battista, 2005) were used as reference strains for bioremediation of non radioactive and radioactive residues. Initially, the bioremediation potential of *Micrococcus* sp. BRM7 was evaluated, compared to *C. metallidurans*, based on its resistance to IR, because this resistance reflects an enhanced capacity for scavenging reactive molecular species, protecting proteins, repairing DNA, and tolerating desiccation ((Daly, 2010) and references therein). Our results indicating that the D_{10} of BRM7 was between those of reference strains *D. radiodurans* (~15 kGy) (Cox and Battista, 2005) and *C. metallidurans* (Janssen et al., 2010) — (~800 Gy) four times higher than that of *C. metallidurans* (Figure 3) — suggest that BRM7, compared to *C. metallidurans*, is a good candidate as a biocatalyst for co-contaminated residues, particularly radioactive ones. Yet, the mechanisms of IR resistance ((Confalonieri and Sommer, 2011) and references therein) in *Micrococcus* sp. BRM7 need further investigation. For instance, the question of whether orthologs belonging to BRM7 of the five transcripts of *D. radiodurans* (*ddrA*, DR_0423; *ddrB*, DR_0070; *ddrC*, DR_0003; *ddrD*, DR_0326; *pprA*, DR_A0346) that are most highly induced following IR and recovery from desiccation are present and also up-regulated in *Micrococcus* sp. BRM7 remains to be answered ((Sghaier et al., 2008) and references therein).

Interestingly, like *C. metallidurans*, *Micrococcus* sp. BRM7 cells have a similar potential to tolerate Sr (D_{10} ≈ 350 mM) (Figure 4). To study the Sr adsorption efficiency of *Micrococcus* sp. BRM7, cells were immobilized in beads of alginate and grown in contact with high concentration of $SrCl_2$. Only 4% of Sr was fixed by the alginate indicating that this matrix is inert and no significant interference occurred between the alginate and Sr during the incubation. Immobilization experiments indicated that *Micrococus* sp. BRM7 cells have the potential to adsorb 34% of Sr following an incubation time of 24 h. IR sensitive *C. metallidurans* (D_{10} ≈ 200 Gy) showed an accumulation of Sr that reached about 50% after 24 h (Table 1). SEM with EDX spectrometry of the bacterial membrane surface of *Micrococcus* sp. BRM7 showed that the membrane contains a significant fraction of Sr indicating that this metal is adsorbed on the surface membrane. Also, EDX results indicate that the bacterial membrane (mainly carbone (C), oxygen (O), and phosphorous (P) peaks) of *Micrococcus* sp. BRM7 has the potential to adsorb Sr (Figure 5). Also, these results suggest that *Micrococcus* sp. BRM7 might be using the process of passive adsorption, a fast process limited by the saturation of binding sites on the membrane.

Conclusion

The results made in the present study suggest that the newly isolated bacterium *Micrococcus* sp. BRM7 is more suitable than *C. metallidurans* for the bioremediation of radioactive residues like PG. The choice of *Micrococcus* sp. BRM7 as a biocatalyst for co-contaminated residues is supported by previous papers indicating that *M. luteus* cells possess a biosorption capacity for U and Th equal to 38.8 and 77.0 mg/g, respectively (Nakajima and Tsuruta, 2004; Wang and Chen, 2009). Indeed, since Sr is an end product of U decay, it could be also a major component of atomic energy waste management. This idea is realistic since *M. luteus* species were also isolated from irradiated waste samples (Fredrickson et al., 2004), and they were shown to have numerous adaptations for

survival in extreme nutrient-poor environments (Greenblatt et al., 2004). In the future, the bioremediation potential of *Micrococcus* sp. BRM7 has to be further investigated to evaluate its utility in the field.

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