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ORIGINAL PAPER

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Isolation and identification of bacteria from spent nuclear fuel pools

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Abstract The aim of the present research was to isolate and identify bacteria from spent nuclear fuel pools of a Spanish nuclear power plant. Water samples were collected and inoculated onto different culture media to isolate the highest number of species. 16S rDNA fragments from colonies growing on solid media were amplified and analysed by denaturing gradient gel electrophoresis. Sequencing revealed the presence of 21 different bacteria belonging to several phylogenetic groups (α, β, and γ-Proteobacteria, Actinomycetales, *Flavobacterium*, and the *Bacillus*/*Staphylococcus* group). The isolation of these microorganisms in this particular environment (oligotrophic and radioactive) is highly interesting because of the possibility of their being used for the bioremediation of radionuclide-contaminated waters.

Keywords Spent nuclear fuel pool · Radioactive water · Oligotrophic water · 16S rDNA · Denaturing gradient gel electrophoresis

Introduction

Although nuclear power plants (NPPs) were designed to last several years, the possibility of increasing their lifespan still further was projected some time ago. The main problem is disposal of spent nuclear fuel (SNF). SNF must be kept underwater in nuclear pool racks where it is cooled before being sent for final storage [38]. The water filling the SNF pools needs to be treated by filtration and demineralisation to avoid accumulation of salts and the risk of corrosion. Nevertheless, the ultrapure and radioactive character of the water does not prevent microbial growth [6, 40, 41]. Moreover, algae, fungi, yeasts and bacteria have been found in the water covering the damaged core reactor at the Three Mile Island NPP [2], and microbial growth has also been observed in nuclear fuel waste disposal containers [44], as well as in soil samples, around the Chernobyl NPP [39].

In unfavourable environments, biofilm formation is a survival strategy for bacterial communities [5]. Microorganisms within biofilms form synergistic communities conducting combined actions that individual species cannot perform. Biofilm formation allows adherence to surfaces leading to biofouling and/or microbiologically influenced corrosion (MIC) [48]. MIC has already been described in power plants [28]. The nuclear industry began to devote particular attention to this phenomenon following publication of the INPO Significant Event Report SER 73-84 [18] and NRC Information Notice IN 85-30 [17] in the 1980s. Since then, many case histories from nuclear installations have been described [28], but none was related to nuclear pools. SNF pools are, in most cases, made of concrete clad in austenitic stainless steel due to its higher resistance to corrosion, but MIC has been documented in this metal [16]. However, due to the lack of information about surface/oligotrophic microbiology, coupled with the belief that gamma radiation from fuel within the containers would effectively sterilise storage environments, the potential for problems in this area has been neglected [29].

Biofilm formation also provides potential resistance to adverse conditions such as the toxic effect of radionuclides [41]. It is well known that microbial cells can interact with radionuclides in different ways such as bioaccumulation, biosorption and bioprecipitation [30]. These interactions could be used for the improvement of

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bioremediation systems to remove or reduce radionuclides at contaminated sites [32]. Thus, the identification of microorganisms able to grow in radioactive sites could be of great interest.

In this investigation, the presence of microorganisms in the water of SNF pools has been studied, and bacteria able to grow in such extreme environmental conditions have been isolated and identified.

Materials and methods

Sampling and water samples

This study was conducted in the SNF pools (east and west pools in both sides of the transference pool) of the Cofrentes NPP in Valencia, Spain. The pools have thick concrete walls clad in austenitic stainless steel. They are 12–15 m long ×7–8 m wide ×12–13 m deep. The water filling them is treated in a closed-loop system including filtration and demineralisation that ensures the high purity of the water.

From June 1999 to March 2001, six water samples were collected from the nuclear pools a few centimetres below the surface of the water, using 2 L sterile disposable bottles and following the recommendations of the "as low as reasonably achievable" (ALARA) principle to avoid possible radiological contamination [8].

Water analysis

Physical/chemical analysis of the water is carried out once a week in the NPP as a routine test for radiological security control. Conductivity, pH, temperature and total organic carbon (TOC) were analysed according to AWWA-APHA-WEF [1]. The concentrations of nitrates, sulphates, and chlorides were measured by gradient ion chromatography (Model DX500, Dionex IC, Sunnyvale, Calif.) using a microbore configuration, conductivity detection, and Peaknet 5.1 software (Dionex).

Following the recommendations of Debertin and Helmer [10], a radiochemical analysis was carried out by a Canberra gamma-spectrometer (Canberra Industries, Meriden, Conn.) consisting of a High Purity p-type coaxial Germanium detector with 40.6% efficiency, Canberra-associated electronic equipment, and a Canberra Genie2k gamma spectrometry software package.

Epifluorescence microscopy

Water (100 ml) was filtered through 0.22 μm-pore-size isopore membrane filter (GTBP 02500, Millipore, Bedford, Mass.). The filters were stained with the LIVE/DEAD *Bac*Light Viability Kit (L-7012) (Molecular Probes, Eugene, Ore.), which is composed of SYTO 9 as a viability marker and propidium iodine (PI) as a membrane-compromised cell marker. Each filter was covered with a 1:1 staining solution with final concentrations of SYTO 9 and PI of 3.41 μM and 6.01 μM, respectively. The filters were incubated for 15 min in the dark at room temperature and were then washed with deionised sterile water. The samples were examined by epifluorescence microscopy with a magnification of 100 times immersion oil objective, using 100 times immersion oil [Immersol 518N, n_e = 1.518 (23°C); Axioskop 2, Zeiss, Germany]. SYTO 9 and PI could be observed together by using a filter block (Zeiss 487709: excitation 450–490 nm, FT 510 nm, LP 515 nm).

Cultivation and DNA extraction

To isolate bacteria from the water in the pools, 100 mL aliquots of water were filtered through 0.45 μm-pore-size and 0.22 μm-pore-size membrane filters (HAWP 04700 and GVWP 04700, Millipore). The filters were then placed onto different culture media in order to isolate the highest number of species. The culture media used were: nutrient agar (NA, Oxoid CM3); brain heart infusion, [BHI, Oxoid CM225, supplemented with agar (UPS) purissimum, Panreac]; tryptone soya broth [TSB, Oxoid CM 129, supplemented with agar (UPS), purissimum, Panreac]; STC [starch from potato soluble for analysis, Panreac, 10 g L^{-1} , casein hydrolysate (acid), Oxoid, 1 g L^{-1} , potassium phosphate monobasic, KH_2PO_4, Panreac, 0.5 g L^{-1} , agar (UPS), Panreac, 20 g L^{-1} , pH 7.2], and R2 medium oligotrophic agar (DMT 215; Microkit Iberica, Barcelona, Spain). Filters were incubated at 30°C until colonies appeared. The colonies growing on the solid media were picked and purified several times by restreaking on the same culture medium. Genomic DNA of the isolated microorganisms was then extracted using PrepMan Ultra (PE Applied Biosystems, Foster City, Calif.) according to the manufacturer's protocol.

Polymerase chain reaction amplification and denaturing gradient gel electrophoresis analysis

Polymerase chain reaction (PCR) amplification of 16S rDNA fragments corresponding to nucleotides 5–531 in the *Escherichia coli* sequence, was performed using the bacterial universal primers 5F (containing a 40-base GC-clamp at the 5′ end) and 531R as previously described [6]. To increase the specificity of amplification a "touchdown" PCR was performed. The annealing temperature was lowered from 65 to 55°C over 20 cycles and then another 10 cycles were performed at 55°C. The mixture was preincubated for 5 min at 94°C. The temperature cycles were at follows: 45 s at 94°C, 30 s at the appropriate annealing temperature, and 45 s at 72°C. Following the final cycle, the reaction was extended for 7 min at 72°C. PCR was carried out in a GeneAmp PCR System 2400 (Perkin-Elmer, Norwalk, Conn.). All PCR products were checked by electrophoresis in 1% (w/v)

agarose gels in 1× TBE buffer with ethidium bromide (0.5 μg mL^{-1}) before being subjected to further analysis.

Amplified fragments from bacteria were analysed by denaturing gradient gel electrophoresis (DGGE). It was carried out in 6% polyacrylamide gels (37.5:1, acrylamide: bisacrylamide) in 1× TAE buffer containing a linear chemical gradient ranging from 30% to 50% denaturant [100% denaturant contains 7 M urea and 40% (v/v) formamide]. Electrophoresis was performed at 60°C for 6 h at 120 V on a DcodeTM Universal Mutation Detection System (Bio-Rad, ON, Canada). Following electrophoresis, the gel was incubated for 10 min in an ethidium bromide solution (0.5 μg mL^{-1}) and visualised in an image analyser (Kodak Image Station 440CF) using a shortcut with KDS1D 3.0.2 software. DGGE allowed differentiation of microorganisms based on a nucleotide sequence of 500 bp and was used as a pre-screening technique to select microorganisms for sequencing.

Sequencing

Identification of bacteria presenting different migration patterns in DGGE was accomplished by sequencing the complete 16S rDNA gene (first water sample) or a 500 bp fragment (all subsequent samples). Two bacterial sequencing kits, the MicroSeq Fullgene 16S rDNA and the MicroSeq 500 16S rDNA (PE Applied Biosystems) were used. Sequences were resolved in an ABI PRISM 310 Genetic Analyser following the manufacturer's instructions. The sequences obtained were compared directly to all known sequences deposited in the NCBI (National Center of Biotechnology Information) databases using the basic local alignment search tool Blastn.

Sequence alignments were performed using the ClustalX software Version 1.81 [46]. Phylogenetic and molecular evolutionary analyses were conducted using MEGA (molecular evolutionary genetics analysis) version 2.1 [26]. Phylogenetic trees were constructed using the neighbour-joining method with the Jukes-Cantor model [21]. A total of 1,000 bootstrapped replicate resampling data sets were generated. The *E. coli* 16S rDNA sequence was used as outgroup.

Waste management

All materials used in this research as well as all waste products generated were treated as radioactive waste.

Results

Water analysis

The conductivity and pH of the water were about 1.06 $\mu S\ cm^{-1}$ and 5.67, respectively. The temperature was between 25 and 30°C. The concentration of sulphates, nitrates, and chlorides were about 0.59, 0.57, and 0.85 ppb, respectively. Occasional variations were observed for conductivity and pH due to maintenance carried out in the nuclear pools. TOC values ranged from 0.01 to 0.45 ppm indicating the oligotrophic character of the water.

The radiochemical analysis of the water by gamma spectrometry is shown in Fig. 1. The high peak matches with the refuelling period. The most common radionuclides found in the water were ^{60}Co, ^{137}Cs, ^{134}Cs, ^{54}Mn, and ^{65}Zn. The presence of these radionuclides is usual in SNF pools of NPPs operating with a boiling water reactor (BWR).

Epifluorescence microscopy

Epifluorescence microscopy studies revealed the presence of different sized cocci, rod-shaped, and filamentous microorganisms in the nuclear pool water; however, fungi were not observed by microscopy. The microorganisms were mostly viable, and the average number of total microbial cells (stained with SYTO 9) was approximately 4.4×10^3 cells mL^{-1}. Similar values have been found in other SNF pools [48] and seem to represent a normal value for SNF storage waters [40].

Culturable microorganisms

The number of culturable microorganisms isolated in this research ranged from 0.41 to 1.77 colony forming units (cfu) mL^{-1}. More colonies were isolated on STC and NA than on the other culture media used. In all cases, culturable microorganisms were about 1% of the total microorganisms counted by epifluorescence microscopy. The remaining microorganisms are assumed to be viable but non-culturable (VBNC) [7].

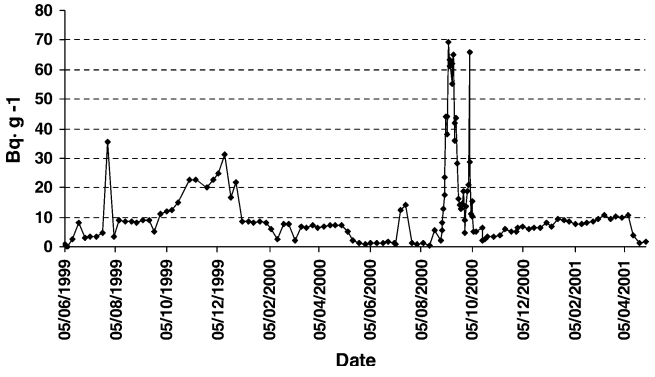


Fig. 1 Radiochemical analysis of pool water by gamma spectrometry (Bq g^{-1}). A *peak* can be observed between August and October 2000, following the refuelling period conducted in the nuclear power plant (NPP) in August. Radiation level increases observed in August 1999 and October–November 1999 come from water movement in the spent nuclear fuel (SNF) pool due to maintenance work at the NPP

It has been demonstrated that bacteria are capable of developing a survival strategy, i.e. a VBNC state, when faced with adverse environmental conditions. This state allows for changes in bacterial morphology, size, and cell structure, as well as in composition. In this research, a higher number of bacteria were isolated using 0.22 μm-pore-size membrane filters than with 0.45 μm-pore-size filters, which can be attributed to a bacterial size reduction due to adverse conditions. Isolation of only a small fraction of the total consortium of microorganisms from environmental samples is quite usual, and the limitation of culture techniques in their identification from the environment is acknowledged here. However, the use of different culture media has permitted us to isolate a variety of bacteria. Molecular microbiology techniques allow us to study the structure and composition of microbial communities and to identify VBNC microorganisms [47]. At present, such techniques have not been used inside NPPs due to radiation at the sampling site and the limitations imposed by the Nuclear Security Council on manipulating samples contaminated with radionuclides.

Identification of microorganisms

From the filters cultured on different solid media, 115 microorganisms were isolated. Their DNA was extracted and analysed by PCR-DGGE as described above. On this basis, 25 different bacteria were detected after comparing their migration patterns. Some of these can be observed in Fig. 2. By sequencing two or three co-migrating bands, it was determined that the same band patterns corresponded to the same microorganisms. Therefore, PCR-DGGE could be used as a pre-screening technique to select microorganisms to be sequenced. In order to identify these microorganisms, a 500 bp fragment of the 16S rDNA or the complete gene (1,500 bp) was sequenced and compared with those deposited in the NCBI databases. Isolated microorganisms belonging to different phylogenetic groups were found in the water of the SNF pools. The most abundant were those belonging to the β-Proteobacteria subclass, followed by the α-Proteobacteria subclass. Bacteria from the γ-Proteobacteria, Actinomycetales, *Flavobacterium*, and *Bacillus*/*Staphylococcus* groups were also isolated. A phylogenetic tree was constructed by the neighbour-joining method based on the sequence of the 16S rDNA gene (Fig. 3).

The DNA sequences from the isolated bacteria were submitted to GenBank under the accession numbers AY509953–AY509973.

Discussion

Previous microbiological studies carried out in SNF storage installations have shown the presence of microbial activity [6, 40, 41]. However, little is known about the ability of microorganisms to survive and grow in such extreme conditions (oligotrophic and radioactive water). The identification of such microorganisms is very interesting due to the deterioration that they could cause to the systems in the nuclear power facilities, as well as to their potential use in the bioremediation of radionuclide-contaminated sites.

From the water filling the SNF pools of the Cofrentes NPP, 21 different bacteria were isolated. Among these, the most abundant were *Ralstonia* species. Bacteria related to *Pseudomonas*, *Burkholderia*, *Stenotrophomonas*, *Sphingomonas*, *Methylobacterium*, *Afipia*, *Bradyrhizobium*, *Rhizobium*, *Streptococcus*, *Staphylococcus*, *Microbacterium*, *Nocardia*, and *Chryseobacterium* were also found.

The oligotrophic character of the water filling the SNF pools should be enough to avoid microbial growth. However, some studies have demonstrated the presence

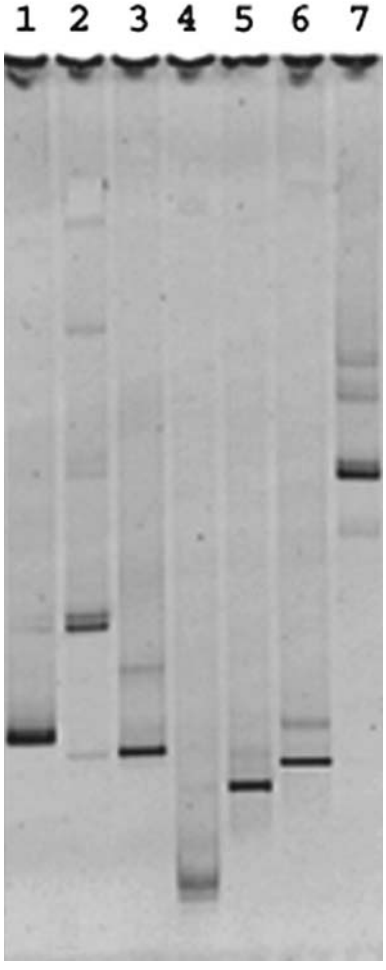


Fig. 2 The denaturing gradient gel electrophoresis (DGGE) profiles of amplified 16S rDNA fragments from different water samples used as pre-screening to select microorganisms to be sequenced. Lanes: *1 Ralstonia* sp. isolate 3F-1, *2 Ralstonia* sp. isolate 3F-2, *3 Ralstonia* sp. isolate 2F-1, *4 Nocardia* sp. isolate 3F-4, *5 Afipia* sp. isolate 3F-5, *6 Sphingomonas* sp isolate 3F-6, *7 Staphylococcus haemolyticus* isolate 3F-7

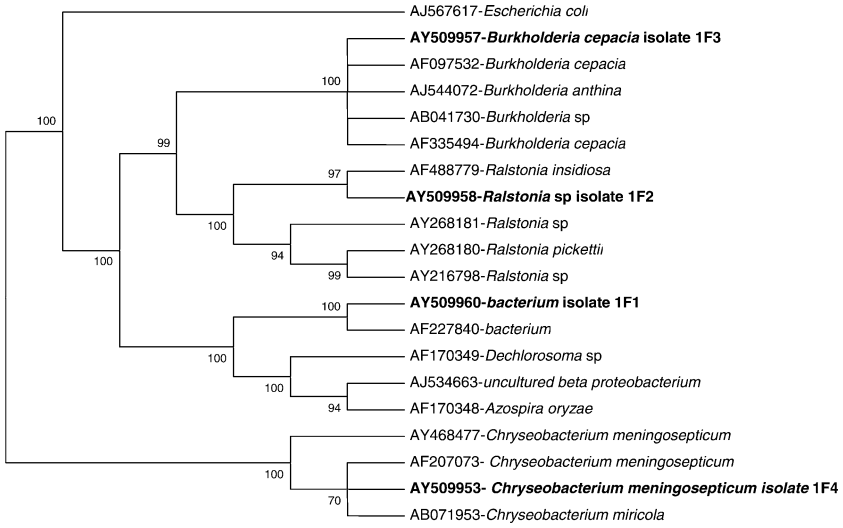


Fig. 3a,b Phylogenetic analysis of 16S rDNA gene sequences from the strains isolated from the nuclear pools (*in bold*). Trees were constructed by the neighbour-joining approach using the molecular evolutionary genetics analysis (MEGA) program on the basis of the complete 16S rDNA sequence (**a**) or a 500 bp fragment (**b**). Bootstrap values (percentages) are given at the nodes

of microbial life in tap water [24], ultrapure water systems [25] and distilled water [11]. Moreover, some of the microorganisms isolated in this research, such as *Ralstonia, Pseudomonas saccharophila* and *Stenotrophomonas maltophilia*, have also been found in oligotrophic environments [25].

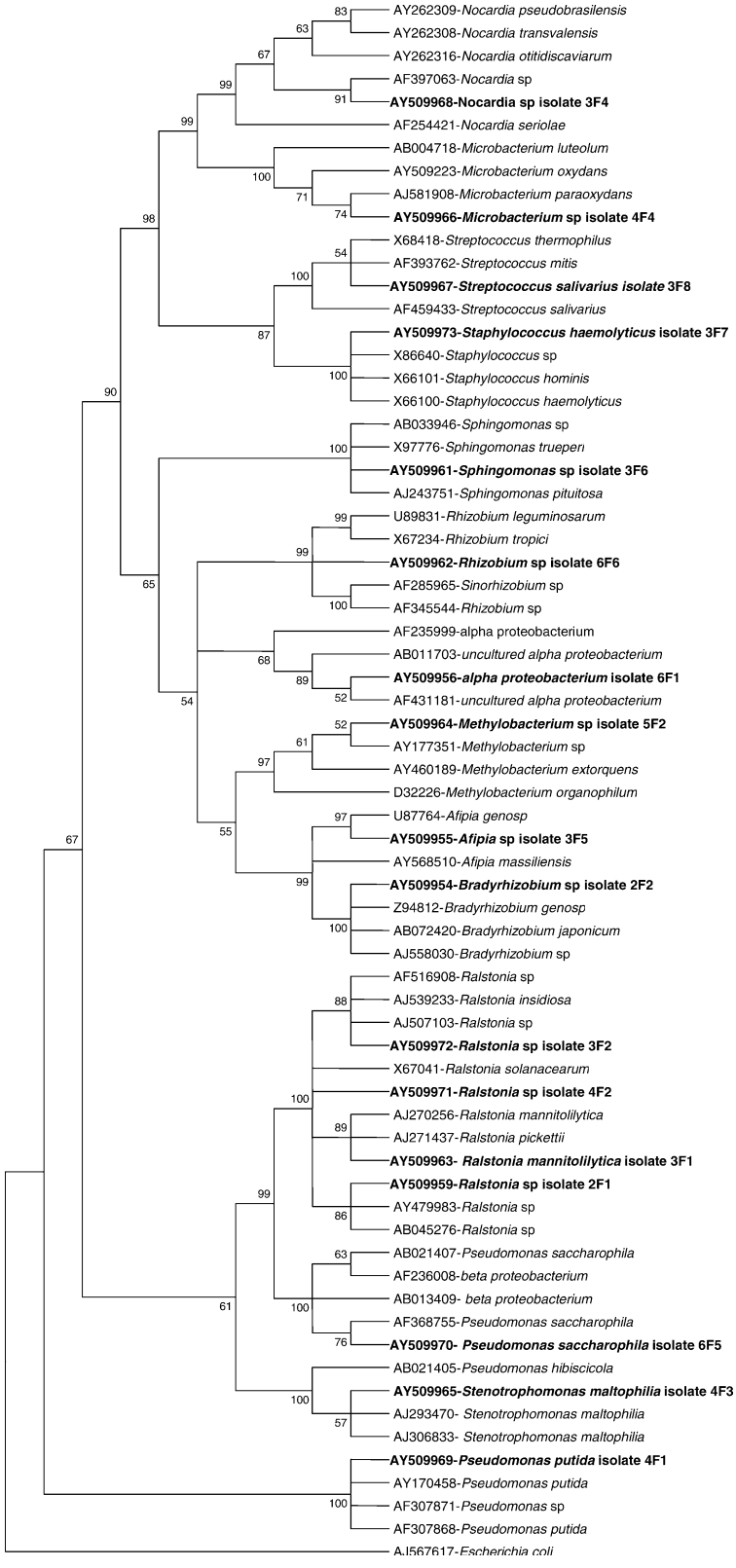
The resistance of microbes to gamma radiation varies. Gram-negative bacteria are more sensitive than Gram-positive bacteria. Bacterial spores are relatively radiation-resistant, 3–4 kGy irradiation is required to cause 90% mortality, while most vegetative cells require only one-tenth of this dosage to suffer the same percentage mortality [14]. However, non-spore forming bacteria have been isolated from SNF pool water, and most of them are Gram-negative.

These results suggest that the oligotrophic and radioactive environment of the SNF pools and the deionisation treatments commonly applied to prevent electrochemical corrosion in these facilities do not prevent microbial colonisation and survival. The SNF pools are clad in austenitic stainless steel. Resistance to corrosion of this material is due to the formation of a thin passive film [34]. However, passivity can break down in different types of environments and, indeed, biofouling and microbial corrosion of stainless steel has already been documented [16]. Biofilm formation can accelerate rates of partial reactions in corrosion and seems to be a universal requirement for biocorrosion, although it is not necessarily sufficient [29]. The microorganisms isolated in the SNF pools of the Cofrentes NPP have been shown to form biofilms under different conditions. For example, *Ralstonia* and *Pseudomonas* have been isolated from the biofilm covering the Space Shuttle water system [23]; *Pseudomonas*, *Sphingomonas*, and *Afipia* species have been detected in biofilms found in dental unit water systems [43]; species phylogenetically related to *Afipia*and *Stenotrophomonas* were present in biofilms of the polymer granules from a fully operating reactor for denitrification of drinking water [33]. *Staphylococcus* sp. and its ability to form biofilms are well known [15], as are *Streptococcus* sp. [12]. Therefore, microorganisms that grow in the water of SNF pools could develop and form biofilms on the pool walls or around the zircaloy-clad fuel rods and the boron-containing stainless steel racks immersed in the pool water, causing several problems such as biofouling or biocorrosion. Indeed, in another study conducted in the upper nuclear pool of the same NPP, microorganisms have been detected attached to the pool walls [6], although no signs of biocorrosion were observed in the pools. Moreover, most of the microorganisms isolated in the present research, e.g. *Methylobacterium* sp., *S. maltophilia*, *P. saccharophila*, *Staphylococcus haemolyticus*, *Ralstonia* sp., *Burkholderia* sp., *Microbacterium* sp., and *Nocardia*, have also been found in biofilms developed on stainless steel specimens immersed in the same nuclear pools [41].

Due to the difficulties imposed by radiation on investigation of the biodeterioration of materials in nuclear fuel storage facilities, there is a lack of information in this field [20]. Thus, it is important to identify opportunities to pursue research in this area. Although the qualitative conditions under which biocorrosion can occur are well known, there are at present no models that allow quantitative predictions to be made [37]. Microbes are present in all repositories and waste storage areas, and the minimization of their impact on materials needs to be studied.

From another angle, we wanted to emphasise that the bacteria isolated in this research could be useful in bioremediation of radionuclides. Their ability to grow on different materials and to form biofilms in this particular radioactive environment makes them good candidates for this purpose. They belong to groups frequently used in the bioremediation of heavy metals and organic compounds [9]; therefore, their use in the bioremediation of radioactive contaminated sites needs to be considered. In this context, information exists on *Pseudomonas* and its ability to interact with certain radionuclides like uranium [31], cobalt [3] or chromium [36]. This bacterium is resistant to high concentrations of certain heavy

Fig. 3a,b (Contd.)



metals, and exhibits a remarkable ability to degrade a variety of toxic compounds [4]. *Ralstonia* sp. has genes encoding tolerance to heavy metals such as zinc and cobalt [27], and such genes have been cloned in the radiation-resistant bacterium *Deinococcus radiodurans* for the bioremediation of groundwater contaminated by radioactive waste [9]. More interesting is its ability to form hydroxide and carbonate complexes with

radionuclides that take part in biomineralization or chemisorption mechanisms [35]. *Microbacterium* sp. takes up uranium and plutonium in a process mediated by siderophores [19], and *Sphingomonas* sp. can survive in the presence of cadmium at high concentration as has been documented in a study of biosorption of this element. The biosorption of this element by living cells has been documented [45]. *Nocardia* sp. binds nickel, copper, and cadmium [22] and *Streptococcus* sp. resists high concentrations of heavy metals such as cobalt, zinc, and manganese [42]. Therefore, such microorganisms, growing in a radioactive site such as the water of nuclear pools, could be useful for the development of processes to decontaminate radioactive waters following the principles governing heavy metal bioremediation [13]. We have already reported that some of these microorganisms form biofilms on the surfaces of stainless steel specimens immersed in the SNF pools, and that these biofilms can accumulate radionuclides reaching radioactivity values of about 5,500 Bq cm^{-2} [41]. Although some bacterial species have been described to be able to transform, immobilise, or detoxify different metallic and/or organic pollutants, they could not be used in the bioremediation of radioactive environments due to their sensitivity to gamma radiation [9]. Therefore, the isolation of microorganisms such as those isolated in the oligotrophic and radioactive environment studied here is of great interest.

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