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Characterization and radiation resistance of new isolates of *Rubrobacter radiotolerans* and *Rubrobacter xylanophilus*

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Abstract In this study we characterized new strains of the slightly thermophilic species *Rubrobacter radiotolerans* and the thermophilic species *Rubrobacter xylanophilus*, both of which were previously represented only by the type strains isolated, respectively, from Japan and the United Kingdom. The new isolates were recovered from two hot springs in central Portugal after gamma irradiation of water and biofilm samples. We assessed biochemical characteristics, performed DNA–DNA hybridization, and carried out 16S rDNA sequence analysis to demonstrate that the new *Rubrobacter* isolates belong to the species *R. radiotolerans* and *R. xylanophilus*. We also show for the first time that the strains of *R. xylanophilus* and other strains of *R. radiotolerans* are extremely gamma radiation resistant.

Key words *Rubrobacter radiotolerans* · *Rubrobacter xylanophilus* · Radiation resistance · Hot springs · Distribution

Introduction

Some thermophilic species are frequently isolated from geothermal sites or man-made thermal environments, while other thermophiles have been rarely recovered from environments where their isolation would be expected. These observations lead to the view that some thermophiles have a limited geographical distribution because of specific physicochemical parameters of the hot springs, or constitute minor populations that are difficult to isolate by conventional enrichment methods. The genus *Rubrobacter* contains two validly named species; the species *Rubrobacter radiotolerans* (Suzuki et al. 1988), initially named *Arthrobacter radiotolerans* by Yoshinaka et al. (1973), was described from one strain isolated from a hot spring in Japan after gamma irradiation of the water samples. The description of *Rubrobacter xylanophilus*, was also based on one strain recovered from a thermally polluted runoff of a carpet factory in the United Kingdom, without gamma irradiation of the sample (Carreto et al. 1996).

The genus *Rubrobacter* belongs to the recently created class *Actinobacteria* and represents one of the most deeply branching lineages of this class (Stackebrandt et al. 1997). *Rubrobacter radiotolerans* can be distinguished from *Rubrobacter xylanophilus* by the lower temperature range of the former species and differences in phenotypic characteristics. *Rubrobacter radiotolerans* is also known to be highly radiation resistant (Yoshinaka et al. 1973). Selection methods and selective media could offer the advantage of isolating new species or strains of known species that would otherwise not be frequently isolated, but have rarely been used to isolate thermophilic organisms. Herein, we report the isolation of *Rubrobacter* strains, after gamma irradiation of the water samples, from hot spring sites in central Portugal with temperatures ranging from 48°C to 57°C, where strains of the genus *Rubrobacter* had never been isolated using conventional methods (Ferreira et al. 1997; Santos et al. 1989; Tenreiro et al. 1995). Biochemical, chemotaxonomic, and morphological characteristics, DNA–DNA hybridization results, and phylogenetic analysis showed that the new isolates belong to *Rubrobacter radiotolerans* and *Rubrobacter xylanophilus*. Moreover, we found that the type strain of *Rubrobacter xylanophilus* and the new isolates of both species were also gamma radiation resistant.

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Methods and materials

Several strains designated RSPS were isolated from the hot spring at São Pedro do Sul in central Portugal, while strains RALC-4 and RALC-5 were isolated from the hot spring at Alcafache about 20 km away. The strains were isolated after irradiation of the samples with a cobalt-60 source, 24 h after sampling, at a dose of 8.06 kGy and a rate of 1.5 kGy h^{-1}. Water samples were transported without temperature control and were filtered through membrane filters (Gelman, Ann Arbor, USA, type GN-6; pore size, 0.45 μm; diameter, 47 mm). The filters were placed on the surface of agar plates containing solidified *Thermus* medium (Brock 1978); the plates were wrapped in plastic bags and incubated at 45°C and 60°C for up to 7 days. Cultures were purified by subculturing and were preserved at −80°C in *Thermus* medium containing 15% glycerol. The type strain of *Rubrobacter radiotolerans* (DSM 46359) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; the type strain of *Rubrobacter xylanophilus* PRD-1 (=DSM 9941) was our strain. All morphological, biochemical, and tolerance tests were performed in liquid or solidified *Thermus* medium. The type strain of *R. radiotolerans* and strains RALC-4 and RSPS-4 were grown at 45°C, and the type strain of *R. xylanophilus* and strains RSPS-10 and RSPS-21 were grown at 60°C, as described previously (Carreto et al. 1996; Manaia and da Costa 1991; Santos et al. 1989).

The cultures for polar lipid and fatty acid analyses were grown in *Thermus* medium at 45°C and 60°C. Growth of cultures, harvesting, extraction, two dimensional thin-layer chromatography, and fatty acid analysis were performed as described previously (Ferreira et al. 1997). Bacteria for radiation resistance tests were grown in the appropriate liquid medium until the exponential phase of growth, washed once by centrifugation at 4°C with 0.067 M potassium phosphate buffer at pH 7.0, and resuspended, in the same buffer, at a concentration of 1×10^8 colony-forming units per milliliter. The suspensions were divided into 1.0-ml aliquots and exposed to a cobalt-60 source with a dose rate of 2.57 kGy h^{-1} (1 kGy = 1×10^5 rads) at room temperature. At appropriate intervals, 0.1 ml of each suspension was removed and diluted in the same buffer, plated in triplicate on *Thermus* agar, and incubated at 45°C and 60°C. The colony-forming units were counted daily for up to 15 days, and viability was assessed by using unirradiated suspensions of each strain maintained under the same conditions. DNA isolation, DNA–DNA reassociation, and 16S rRNA sequence analysis was performed as described previously (Carreto et al. 1996). The 16S rDNA sequence data are available from EMBL under accession number AJ243870 for *R. radiotolerans* strain RALC-4, and accession number AJ243871 for *R. xylanophilus* strain RSPS-15.

Results

The hot spring at Alcafache had a temperature of 50°C and a pH of 8.6, while the sites at S. Pedro do Sul had temperatures ranging between 50°C and 57°C and a pH of 8.9. Twenty-three pink isolates were recovered from samples of water and biofilms from these sites after gamma irradiation. All the sites at S. Pedro do Sul, irrespective of the water temperature, yielded strains with optimum growth temperatures of 45°C and 60°C. The only other organisms recovered were spore-forming gram-positive rods. The site at Alcafache yielded strains of *Rubrobacter* only on plate enrichments incubated at 45°C, namely strain RALC-4. Isolates RALC-4 and RSPS-4 had optimum growth temperatures similar to that of *R. radiotolerans* (about 45°C). Strains with optimum growth temperature similar to those of *R. xylanophilus* (about 60°C), namely RSPS-21 and RSPS-10, were only isolated at S. Pedro do Sul. The pH range of the type strains and the new isolates were very similar (Carreto et al. 1996). Strains RALC-4 and RSPS-4 had biochemical and tolerance characteristics identical to those reported for *R. radiotolerans*, while strains RSPS-21 and RSPS-10 were nearly identical to the type strain of *R. xylanophilus*. All organisms grew in medium containing 6% NaCl (w/v), degraded hide powder azure, gelatin, hippurate, arbutin, and esculin, were cytochrome oxidase, catalase, and β-galactosidase positive and produced nitrite. Strains RSPS-21 and RSPS-10 showed only minor differences in the assimilation of some carbon sources to the original description of *R. xylanophilus*, namely D-raffinose, lactose, D-sorbitol and L-serine.

The polar lipids patterns of strains RALC-4, RSPS-4, RSPS-21, and RSPS-10 were in agreement with previous results for *R. radiotolerans* and for *R. xylanophilus* (Carreto et al. 1996; Suzuki et al. 1988). The fatty acid composition of the type strain of *R. radiotolerans* and strains RALC-4 and RSPS-4 was dominated by 12-methyl-16:0, which accounted for about 70% of the total, respectively, while 14-methyl-18:0 was present in lower relative amounts of 15.7%–17.8% of the total fatty acids. On the other hand, in the type strain of *R. xylanophilus* and strains RSPS-21 and RSPS-10, 14-methyl-18:0 constituted about 75% of the total fatty acid while 12-methyl-16:0 only reached about 8% of the total (results not shown).

The survival curves for all the strains had sigmoid shapes. The shoulder dose (the dose required before reduction of the colony-forming units is observed) was 4.7, 5.7, 3.7, and 4.3 kGy, respectively, for *R. radiotolerans*, strain RSPS-4, *R. xylanophilus*, and strain RSPS-21 (Fig. 1). The dose of radiation required to reduce the number of viable units after the shoulder to 37%, that is, the dose required, on average, to inactivate a single colony-forming unit of the irradiated population, was 7.6 and 9.0 kGy for *R. radiotolerans* and strain RSPS-4, and 4.6 and 5.2 kGy for *R. xylanophilus* and strain RSPS-21.

PCR amplification and sequencing allowed the determination and comparison of 1504 and 1509 nucleotide positions, respectively, of the 16S rDNA of strains RALC-4 and RSPS-15. The 16S rRNA gene sequence of RALC-4 was observed to be most similar to that of the type strain of *R. radiotolerans*, with only two nucleotide differences detected (99.8%) (Table 1). The 16S rRNA gene sequence of RSPS-15 was observed to be most similar to that of the type strain of *R. xylanophilus*, with only one nucleotide difference de-

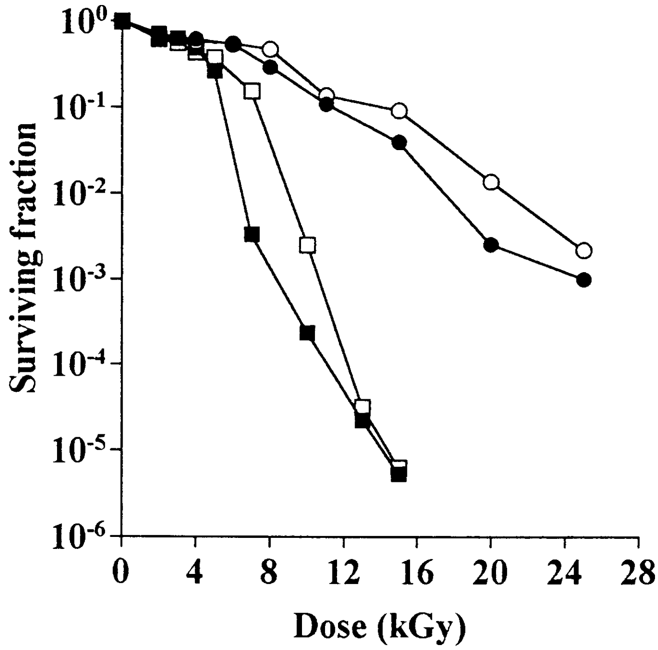

Fig. 1. Gamma radiation survival curves of the type strain of *Rubrobacter radiotolerans* (*closed circles*), strain RSPS-4 (*open circles*), type strain *R. xylanophilus* (*closed squares*), and strain RSPS-21 (*open squares*)

Table 1. Sequence similarities between the 16S rDNA genes of the validly described species of the genus *Rubrobacter* and the hot spring isolates

Strain	Sequence similarities (%) with:	
	R. radiotolerans[a]	*R. xylanophilus*[b]
RALC-4	99.8	89.7
RSPS-4	99.8[c]	89.4[c]
RSPS-15	89.9	99.9
RSPS-10	89.3[c]	99.9[c]
RSPS-21	89.3[c]	99.9[c]
RSPS-31	89.3[c]	99.9[c]

[a] *R. radiotolerans* (DSM 46359^T), 16S rRNA gene sequence EMBL accession number X87134

[b] *R. xylanophilus* (DSM 9941^T), 16S rRNA gene sequence EMBL accession number X87135

[c] Sequence similarities determined from comparisons of approximately 800 nucleotide positions

tected (99.9%). Additional isolates (RSPS-4, RSPS-10, RSPS-21, and RSPS-31) were screened by sequencing a portion of their PCR-amplified 16S rDNAs, corresponding to the region between positions 260 and 1080 (*E. coli* 16S rRNA gene sequence numbering). Within this region, the 16S rRNA gene sequence of RSPS-4 was observed to possess only one nucleotide difference from those of RALC-4 and *R. radiotolerans*. The 16S rDNA sequences of the isolates RSPS-10, RSPS-21, and RSPS-31 were observed to be identical to that of isolate RSPS-15, for the same nucleotide region. Strain RSPS-4 had DNA–DNA reassociation values of 93.9% with *R. radiotolerans*, while strain RSPS-21 had reassociation values of 75.8% with *R. xylanophilus*.

Discussion

The results presented here clearly show that the new isolates from hot springs in central Portugal belong to the species *R. radiotolerans* and *R. xylanophilus*. The strains of the two species of the genus *Rubrobacter* examined are extremely gamma radiation resistant, but both strains of *R. radiotolerans* were more resistant than the strains of *R. xylanophilus*. The degree of radiation resistance of a strain depends on the growth phase of organisms, oxygen tension, or temperature of the irradiated suspension, among other factors, resulting in variations in resistance levels reported for species of the genus *Deinococcus* (Keller and Maxcy 1984; Moseley 1983; Smith et al. 1992). It appears, nevertheless, that the strains of some species of *Deinococcus* are more radiation resistant than strains of other species (Smith et al. 1992). Because we used cells in the same phase of growth, and used the same method to assess radiation resistance of both *Rubrobacter* species, it can be concluded that the strains of *R. radiotolerans* are more resistant to gamma radiation than the strains of *R. xylanophilus*. Our results also show that radiation resistance in these organisms is not selected for by irradiation of samples, because the type strain of *R. xylanophilus*, isolated without irradiation of the sample, was as radiation resistant as strain RSPS-21.

Inherent radiation resistance is also the hallmark of the species of *Deinococcus* (Ferreira et al. 1997; Mattimore and Battista 1996; Sanders and Maxcy 1979). The other radiation-resistant species known are *Acinetobacter radioresistens* (Nishimura et al. 1988) and *Methylobacterium radiotolerans* (Green and Bousfield 1983), but very little is known about the levels of radiation resistance or mechanisms of resistance in these organisms. According to Mattimore and Battista (1996), radiation resistance of the deinococci probably does not represent an adaptation to ionizing radiation because natural environments with high levels of gamma radiation are not known. Instead, radiation resistance is probably related to desiccation resistance, because both stresses produce the same type of DNA damage, and radiation-sensitive mutants become sensitive to desiccation as well. Resistance to desiccation certainly confers a selective advantage to temporary survival of organisms in dry environments and as a means of aerial dispersal. Moreover, desiccation resistance coupled to UV resistance, for example, could be an important advantage for the dispersal of these organisms between suitable habitats for growth.

We can hypothesize that resistance to radiation in *Rubrobacter* spp. probably has the same selective advantage as in the deinococci, although this has not been shown in the former. The distant phylogenetic relationships between *Deinococcus*, *Rubrobacter*, *Acinetobacter*, and *Methylobacterium* reinforces the view that resistance to gamma radiation appeared several times during the evolution of bacteria and confers some selective advantage to these phylogenetically unrelated organisms. This is the first report of isolation of strains of *Rubrobacter xylanophilus* from hot springs. It appears that these organisms are widespread in hot springs, but appear to represent minor populations that may be difficult to isolate using nonselective methods. The isolation of the type strain of *R. xylanophilus* from a thermally polluted stream in the United Kingdom was probably accidental, unless environmental conditions had been created by local physicochemical properties conducive to the extensive colonization by these organisms.

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