Rubrobacter taiwanensis sp. nov., a novel thermophilic, radiation-resistant species isolated from hot springs

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Two novel bacteria, with an optimum growth temperature of approximately 60 °C, were isolated from Lu-shan hot springs in the central region of Taiwan. These isolates were aerobic, thermophilic, halotolerant, pink-pigmented, heterotrophic and resistant to gamma-radiation. Both pleomorphic, short, rod-shaped cells and coccoid cells were observed. Strains LS-286 (=ATCC BAA-452=BCRC 17198) and LS-293^T (=ATCC BAA-406^T=BCRC 17173^T) represented a novel species of the genus *Rubrobacter*, according to a phylogenetic analysis of the 16S rRNA gene, DNA–DNA hybridization, biochemical features and fatty acid composition. The name *Rubrobacter taiwanensis* sp. nov. is proposed for this novel species, with LS-293^T as the type strain.

Alternative pre-treatment methods, such as physical or chemical treatments, are necessary in many cases to isolate samples of minor microbial populations from hot springs. For example, *Rubrobacter radiotolerans* (Suzuki *et al.*, 1988), formerly named *Arthrobacter radiotolerans* (Yoshinaka *et al.*, 1973), was isolated from a hot spring in Japan after water samples were irradiated with gamma-rays. *Rubrobacter xylanophilus*, the second validly named species of the genus *Rubrobacter*, was recovered, without prior gamma-irradiation, from the thermally polluted run-off of a carpet factory in the UK (Carreto *et al.*, 1996). Although strains of *R. xylanophilus* and *R. radiotolerans* were isolated by different methods, both strains exhibited novel characteristics and survived extreme gamma-radiation. Other radiation-resistant bacteria, such as members of the well-known genus *Deinococcus* (*Deinococcus radiodurans*, *Deinococcus geothermalis* and *Deinococcus murrayi*), can survive extreme irradiation (Yoshinaka *et al.*, 1973; Ferreira *et al.*, 1997, 1999): *D. radiodurans* was isolated from irradiated cans whereas *D. geothermalis* and *D. murrayi* were isolated from geothermal areas. In previous studies, the numbers of radiation-resistant bacteria isolated without irradiating the samples reveals that the extreme radiation resistance of the organisms is not a result of selection by irradiation, but is instead an inherent characteristic of these microbes (Sanders & Maxcy, 1979; Ferreira *et al.*, 1999).

In this study, two pink-pigmented, thermophilic isolates from natural hot springs in the Lu-shan area of Taiwan were isolated from non-irradiated samples from hot springs, and were similar to species of the genus *Rubrobacter* in various morphological, physiological, biochemical, cellular and chemotaxonomic characteristics. These isolates, strains LS-286 and LS-293^T, exhibited unusual fatty acid compositions and extreme resistance to gamma-radiation.

Samples of water, thermally heated soil and mud were collected from hot springs in the Lu-shan area, Nantou, Taiwan. Aliquots (100 µl) of untreated water samples were spread directly onto *Thermus* agar plates (Williams & da Costa, 1992), which were subsequently sealed in plastic bags and incubated at 50 °C for 7 days. Pink-pigmented colonies were picked from the plates and subcultured for isolation of pure clones. Strains LS-293^T and LS-286 and other isolates were preserved at −70 °C in *Thermus* medium containing 15 % (v/v) glycerol.

A Zeiss Axioscop light microscope equipped with a Nikon Coolpix 990 digital camera was employed to obtain

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains LS-293^T and LS-286 are AF465803 and AF479791, respectively.

Images produced using scanning electron microscopy and atomic-force microscopy are available, together with pH and salinity data and fatty acid profiles, as supplementary material in IJSEM Online.

photomicrographs. For transmission electron microscopy, bacterial strains were grown for 48 h and then washed using water with centrifugation. For negative-staining, 5 μl liquid culture was dropped onto Formvar/carbon-coated grids (300 mesh) and stained with 1 % phosphotungstic acid (w/v, pH 7·0). Electron micrographs were generated with a Hitachi model H7100 electron microscope as described previously (Chen *et al.*, 2002a, b). Living bacterial cells were fixed to the glass slide with 0·01 % (w/v) poly-L-lysine solution and all experiments were conducted using a Solver Bio atomic-force microscope (NT-MDT). Standard procedures for atomic-force microscopy imaging were used, as described by Hansma & Hoh (1994). Silicon nitrite tips were used, with a constant force of 5·5 kg. Atomic-force microscopy images were generated at line frequencies between 2 and 3 Hz, with 256 lines per image. Images were obtained using semi-contact (tapping)-mode atomic-force microscopy, at a resonant frequency of 147·78 kHz.

Biochemical and tolerance tests were performed on isolates LS-286 and LS-293^T and on type strains *R. xylanophilus* DSM 9441^T and *R. radiotolerans* DSM 5868^T, as described previously (Santos *et al.*, 1989; Manaia & da Costa, 1991; Tenreiro *et al.*, 1995) in *Thermus* medium or on *Thermus* agar incubated at appropriate temperatures for 3 days. Media with different pH values and NaCl concentrations were prepared using appropriate biological buffers (Chung *et al.*, 1997). Filter-sterilized carbon sources (2·0 g l^{-1}), ammonium sulfate (0·5 g l^{-1}) and yeast extract (0·2 g l^{-1}) were added to *Thermus* basal salts to perform single-carbon-source assimilation tests. The growth rate was determined by measuring the turbidity (660 nm) of liquid cultures. Positive and negative control cultures were grown in *Thermus* and minimal media, respectively. All growth experiments were performed in triplicate.

The protocols used to evaluate radiation resistance were as described in previous studies (Carreto *et al.*, 1996; Ferreira *et al.*, 1997). Bacteria were grown in *Thermus* medium until they entered the exponential growth phase; they were then washed once in 0·067 M potassium phosphate buffer (pH 7·0) with centrifugation at 4 °C and resuspended to a concentration of 1×10^7 to 1×10^8 c.f.u. ml^{-1} in 0·067 M potassium phosphate buffer at pH 7·0. The suspensions were divided into 2 ml aliquots and exposed to a ^{60}Co source at a dose rate of 0·45 kGy min^{-1} at room temperature (1 kGy = 10^5 rads). The gamma-radiation doses were from zero to 18·0 kGy, in steps of 2·0 kGy. Treated samples were diluted with the same buffer in suspensions, and 100 μl each suspension was plated, in triplicate, on *Thermus* agar at the optimum temperatures for each strain. Colony-forming units were counted daily for up to 15 days. The viability of irradiated cells was evaluated using unirradiated suspensions of each strain under the same conditions.

The cultures used for fatty acid analyses were grown in *Thermus* medium at 37 °C (*R. radiotolerans*), 45 °C (all strains tested) or 60 °C (all strains except *R. radiotolerans*) until they reached the middle of the exponential phase of growth. Fatty acid methyl esters were obtained from freeze-dried biomass by saponification, methylation and extraction, as described previously (Kuykendall *et al.*, 1988). Picolinyl esters were prepared according to the method described by Harvey (1982), as modified by Wait & Hudson (1985). Fatty acid methyl esters and fatty acid picolinyl esters were separated and analysed by GC/MS, using an HP 6890 gas chromatograph fitted with a 5 % (v/v) phenylmethyl siloxane capillary column (30 m × 0·25 mm; Hewlett Packard 5MS) and equipped with an HP 5973 mass-selective detector. The fatty acid methyl esters and picolinyl esters were identified and quantified and numerically analysed by using standard MIS Library Generation software (Microbial ID) as described previously (Chen *et al.*, 2002b).

The G+C content of the DNA was obtained by HPLC, as described by Mesbah & Whitman (1989). Bacterial DNA was isolated using a Qiagen DNAeasy tissue kit; λ phage DNA was used as a control. DNA–DNA hybridization was performed by using a modification of the microplate method described in previous studies (Ezaki *et al.*, 1989; Willems *et al.*, 2001). PCR-mediated amplification of bacterial 16S rRNA genes and sequencing of the purified PCR products were performed according to Rainey *et al.* (1996). The 16S rRNA gene sequences of the two novel strains were compared with those in the EMBL database (Maidak *et al.*, 1994), using FASTA (Pearson & Lipman, 1988). The 16S rRNA gene sequences of the species most closely related to the two novel strains were retrieved from the database and all of the sequences were aligned using the CLUSTAL W program (Thompson *et al.*, 1994) that is included in the BioEdit software package (version 5.0.6; Hall, 1999). Evolutionary distances were calculated according to the algorithm of Jukes & Cantor (1969). The phylogenetic dendrogram was generated from evolutionary distances by using the neighbour-joining method (Saitou & Nei, 1987), with the MEGA software package (version 2.1; Kumar *et al.*, 2001).

Two novel pink-pigmented isolates, LS-286 and LS-293^T, were isolated and selected for further studies. Cells of strains LS-286 and LS-293^T were Gram-positive. Colonies were pink in colour (when grown at 45 °C) or light pink in colour (at 60 °C) on the surface of the *Thermus* agar plates. In liquid culture, cells often grew in chains that were wrapped around each other, forming large aggregates. Transmission electron microscopy with negative staining revealed that the morphology of strains LS-286 and LS-293^T was pleomorphic, with short rod-shaped or coccoid cells approximately 0·9–1·0 μm in diameter and 1·0–3·0 μm in length, although much longer cells were occasionally observed in fresh cultures. Motility and endospores were not observed under phase-contrast microscopy. Flagella were not observed under transmission electron microscopy. In liquid culture, under transmission electron microscopy and atomic-force microscopy, cells presumably at a stage preceding cellular

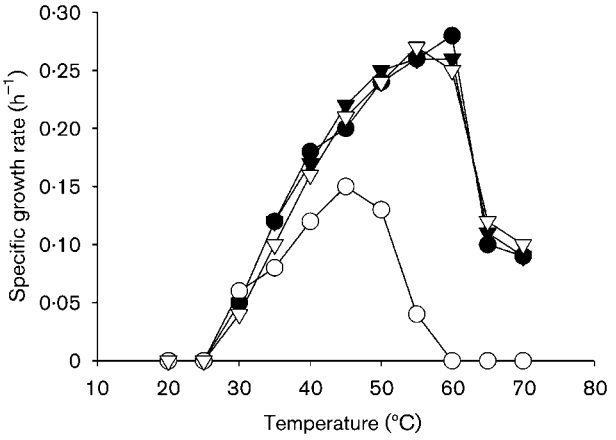

Fig. 1. Effect of temperature on the growth rates of isolates LS-286 (▽) and LS-293^T (▼), *R. radiotolerans* DSM 5868^T (○) and *R. xylanophilus* DSM 9441^T (●).

division were frequently observed and cells often grew in chains that were wrapped around each other, forming large aggregates. Images from scanning electron microscopy and atomic-force microscopy are available as Supplementary Fig. A in IJSEM Online.

Various media, including nutrient medium, trypticase–soy medium, Luria–Bertani medium, medium 162 (Degryse *et al.*, 1978) and *Thermus* medium, were tested initially to determine whether they supported the growth of strains LS-286 and LS-293^T. Both strains grew on all of the media tested, but grew particularly well on *Thermus* medium. Strains LS-286 and LS-293^T grew between 30 and 70 °C; the optimum growth temperature was 60 °C in *Thermus* medium, which is similar to that for the thermophilic species *R. xylanophilus* DSM 9441^T but different from that of the mesophilic species *R. radiotolerans* DSM 5868^T (Fig. 1). Strains LS-286 and LS-293^T were assessed in *Thermus* medium over a broad range of pH values (pH 6–11): the optimum pH was 8·0 at the optimum growth temperature. The NaCl concentration for growth of strains CB-286 and CB-293^T was in the range 0–5 % (w/v); strains of *R. xylanophilus* DSM 9441^T and *R. radiotolerans* DSM 5868^T exhibited the same ranges (0–4 %, w/v) for growth (data for pH and salinity are available as Supplementary Fig. B in IJSEM Online). Strains LS-293^T, LS-286 and other strains of the genus *Rubrobacter* shared various biochemical characteristics, including the hydrolysis of carbohydrate polymers and the utilization of single carbon sources. The main differences in biochemical characteristics were observed during the assimilation of single carbon sources. Two carbon sources, L-glutamine and L-serine, were utilized by strains LS-293^T and LS-286 but not by other strains of the genus *Rubrobacter*. The two isolated strains could also be distinguished by their utilization of L-rhamnose, L-asparagine and D-glucose. Table 1 presents the biochemical characteristics of strains LS-293^T, LS-286 and other *Rubrobacter* species.

The fatty acid profiles of strains LS-293^T and LS-286 were very similar under the same growth conditions. An internally branched fatty acid with a methyl group at position 14, 14-methyl-18 : 0, was the major fatty acid (33·27 % in strain LS-293^T and 31·44 % in strain LS-286 at 60 °C). Other branched fatty acids, with a methyl group at position 12, including 12-methyl-16 : 0 (12·32 %) and 12-methyl-17 : 0 (13·25 %), were also present in acryl fatty acids in strain LS-293^T at 60 °C. The main distinctive characteristic of the isolates was the large proportion of saturated fatty acid 19 : 0, which was not obtained in previously described profiles of *R. xylanophilus* DSM 9441^T and *R. radiotolerans* DSM 5868^T. Fatty acid methyl esters are the common derivatives subjected to GC-MS analysis of fatty acids. In this study, several strong chromatographic peaks were not consistent with the equivalent lengths of the fatty acid methyl esters in the MIDI (Microbial ID) database. Alternative methods of identification, including GC-MS of fatty acid picolinyl esters, were employed to confirm the cellular fatty acid constituents (Harvey, 1982; Wait & Hudson, 1985; Carreto *et al.*, 1996). In the thermophilic bacterium *Thermococcus roseum*, 12-methyl-18 : 0 is the predominant fatty acid and other internally branched fatty acids are also present, but this bacterium belongs to the phylum *Thermomicrobia* and is not related to *R. radiotolerans*, *R. xylanophilus* or strains LS-286 and LS-293^T (Pond *et al.*, 1986; Garrity & Holt, 2001). Fatty acid profiles are available in Supplementary Table A in IJSEM Online.

Previous studies have identified strong resistance to gamma-radiation as a special characteristic of the genus *Rubrobacter* (Suzuki *et al.*, 1988; Yoshinaka *et al.*, 1973; Ferreira *et al.*, 1999). The survival curves for resistance to gamma-radiation of the type strains of *R. radiotolerans* and *R. xylanophilus* and isolates LS-293^T and LS-286 are sigmoid. The shoulder doses of radiation (the dose required before the number of c.f.u. declines) of strains LS-293^T and LS-286 were 4·8 and 5·0 kGy, respectively. The doses required to reduce the number of viable units after the shoulder to 37 % (the mean dose required to inactivate a single c.f.u. of the irradiated population) were approximately 9·8 and 10·0 kGy, respectively. The shoulder doses for the isolates were between those of *R. xylanophilus* DSM 9441^T (4·0 kGy) and *R. radiotolerans* DSM 5868^T (5·8 kGy), which were comparable to the shoulder dose of *D. radiodurans* DSM 12573^T. These results presented here clearly indicate that the novel thermophilic bacterial isolates LS-293^T and LS-286 were highly resistant to radiation (Fig. 2). Radiation-resistant bacterial strains including *D. radiodurans*, *D. geothermalis*, *D. murrayi*, *R. radiotolerans* and *R. xylanophilus* have exhibited variable radiation resistance in previous studies (Moseley, 1967; Moseley & Mattingly, 1971; Moseley & Evans, 1983; Ferreira *et al.*, 1997; Suzuki *et al.*, 1988; Yoshinaka *et al.*, 1973; Carreto *et al.*, 1996). Of these radiation-resistant bacteria, the

Table 1. Biochemical features that distinguish strains of the genus *Rubrobacter*

+, Positive result or growth; −, negative result or no growth; W, weak result. All tested strains grew in medium containing 5 % (w/v) NaCl, hydrolysed gelatin and were cytochrome oxidase-, oxidase-, β-galactosidase- and β-glucosidase-positive. D-Cellobiose, D-raffinose, D-trehalose, D-arabinose, D-fructose, D-mannose, lactose, L-glutamate and pyruvate were utilized by all of the strains. All of the organisms were α-galactosidase-negative and α-glucosidase-negative. None of the strains hydrolysed starch, casein, cellulose or tributyrin and none utilized D-sorbitol.

Characteristic	LS-293^T	LS-286	*R. xylanophilus* DSM 9441^T	*R. radiotolerans* DSM 5868^T
Pigmentation	Pink	Pink	Light pink	Bright pink
Optimum growth (°C)	60	60	60	45
Hydrolysis of:				
Aesculin	−	−	+	+
Xylan	−	−	+	−
DNA	+	+	−	−
Utilization of:				
D-Mannitol	−	−	−	+
D-Xylose	+	+	+	−
Glycerol	−	−	−	+
L-Rhamnose	−	+	+	+
D-Galactose	+	+	+	−
D-Glucose	+	−	+	+
D-Melibiose	+	+	+	−
Malate	−	−	+	+
myo-Inositol	+	+	+	−
Ribitol	−	−	−	+
Succinate	−	−	+	−
L-Asparagine	+	−	+	+
L-Glutamine	+	+	W	−
L-Serine	+	+	−	−
Menaquinone	MK-8	MK-8	MK-8	MK-8
DNA G+C content (mol%)	68·5	67·9	67·6	64·9

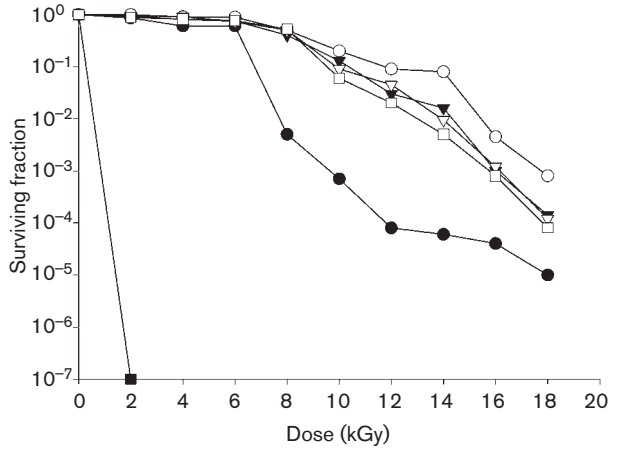

Fig. 2. Gamma-radiation survival curves of the new isolates and other radiation-resistant type strains. Strains: ▽, LS-286; ▼, LS-293^T; ○, *R. radiotolerans* DSM 5868^T; ●, *R. xylanophilus* DSM 9441^T; □, *D. radiodurans* DSM 20539^T; ■, *E. coli* K-12.

thermophilic species *D. murrayi* exhibited stronger resistance to gamma-radiation (shoulder doses, 7·3 kGy) than did the well-known species *D. radiodurans* (shoulder doses, 5·0 kGy) and would be the most radiation-resistant bacterium currently known. Thus, isolates LS-293^T and LS-286 in this study also showed resistance to extreme gamma-radiation. In previous studies, several mesophilic bacteria such as *Acinetobacter radioresistens* (Nishimura *et al.*, 1988) and *Methylobacterium radiotolerans* (Green & Bousfield, 1983) were identified as radiation-resistant, but very little information is known about the degrees of resistance or the mechanisms by which these organisms are resistant. Only those species in the genera *Deinococcus* and *Rubrobacter* with inherent radiation resistance have been studied in detail (Ferreira *et al.*, 1997, 1999; Mattimore & Battista, 1996; Sanders & Maxcy, 1979; Suzuki *et al.*, 1988; Yoshinaka *et al.*, 1973). Of these extremely radiation-resistant bacteria, almost all are thermotolerant (*D. radiodurans* and *R. radiotolerans*) or thermophilic (*D. murrayi*, *D. geothermalis* and *R. xylanophilus*). It is not clear how these radiation-resistant bacteria acquire the ability to resist radiation damage, but further evidence has suggested that

this capacity could be acquired by an evolutionary process resulting from environmental stress, especially drought and heat stress (Mattimore & Battista, 1996). Drought and heat stress, in bacterial cells, would cause damage similar to that caused by gamma-irradiation. DNA oxidation or strand breakdown leads to the death of bacterial cells (Mattimore & Battista, 1996; White *et al.*, 1999). Geothermal areas, such as hot springs, are associated with environmental stressors such as drought and heat, and micro-organisms able to survive in these extreme environments could develop different mechanisms to combat the stress.

The DNA G+C content of isolate LS-293^T was 68·9 mol% and that of LS-286 was 67·9 mol%, as determined by the HPLC method. This result reveals that LS-293^T and LS-286 are high-G+C, Gram-positive bacteria. Strains LS-293^T and LS-286 and all type strains of species of the genus *Rubrobacter* were tested using DNA–DNA hybridization to elucidate their interrelatedness. Strain LS-293^T showed 59·6 % relatedness to *R. xylanophilus* DSM 9441^T and 42·4 % relatedness to *R. radiotolerans* DSM 5868^T. Strain LS-286^T showed 52·8 % relatedness to *R. xylanophilus* DSM 9441^T and 46·2 % relatedness to *R. radiotolerans* DSM 5868^T. The relatedness between strains LS-293^T and LS-286 was 85·7 %.

Following PCR amplification and sequencing, 16S rRNA gene sequences of 1476 nt (strain LS-293^T) and 1475 nt (strain LS-286) were determined. A comparison of the 16S rRNA gene sequences of strains LS-293^T and LS-286 with reference sequence data in the EMBL database suggested that these two strains belonged to the genus *Rubrobacter*. A more detailed analysis, based on a dataset of least 1400 unambiguous nucleotides between positions 28 and 1526 (*Escherichia coli* numbering; Brosius *et al.*, 1978), confirmed this result. Strains LS-293^T and LS-286, which have identical 16S rRNA gene sequences at all positions, exhibited the highest mutual similarity (99·2 %) and constituted a sister cluster next to *R. xylanophilus* DSM 9441^T. The similarities between the 16S rRNA gene sequence of the LS-293^T/LS-286 cluster and those of *R. xylanophilus* and *R. radiotolerans* were 95·1 and 92·4 %, respectively. Fig. 3 presents a 16S rRNA gene sequence-based phylogenetic dendrogram generated by the neighbour-joining method (Saitou & Nei, 1987).

On the basis of the results of phylogenetic analysis of the 16S rRNA genes, DNA–DNA relatedness, biochemical features and fatty acid composition presented in this study, strains LS-286 and LS-293^T represent a novel species of the genus *Rubrobacter*. The name *Rubrobacter taiwanensis* sp. nov. is proposed.

Description of *Rubrobacter taiwanensis* sp. nov.

Rubrobacter taiwanensis (tai.wan.en′sis. N.L. masc. adj. *taiwanensis* of Taiwan, where the micro-organism was first isolated).

Colonies on *Thermus* medium incubated at 60 °C for 7 days are 1·6–2·2 mm in diameter, circular, convex, smooth, opaque and light pink. Cells are aerobic, Gram-positive, pleomorphic, short, rod-shaped or coccoid, 0·9–1·0 μm wide and 1·0–3·0 μm long. They do not exhibit flagella, motility or endospores. Thermophilic, with growth over the temperature range 30–70 °C, and the optimum temperature is 60 °C. The pH range for growth is 6–11, with the optimum pH at about pH 8·0. The G+C content of the DNA is 68·5 mol%. Cytochrome oxidase, catalase and β-galactosidase are present. Growth occurs in *Thermus* medium containing 5·0 % (w/v) NaCl. Gelatin and DNA are hydrolysed. The strain can utilize numerous carbon sources, including D-cellobiose, D-fructose, D-galactose, D-glucose, D-mannose, maltose, D-melibiose, *myo*-inositol, D-raffinose, D-trehalose, D-xylose, lactose, L-arabinose, L-glutamine, L-glutamate, L-serine, L-proline and L-arginine. The predominant fatty acids are 14-methyl-18 : 0, 12-methyl-17 : 0, 12-methyl-16 : 0 and 19 : 0. Strains LS-293^T and LS-286 are highly resistant to gamma-radiation.

The type strain LS-293^T (=ATCC BAA-406^T=BCRC 17173^T) and the reference strain LS-286 (=ATCC BAA-452=BCRC 17198) were isolated from Lu-shan hot springs in Taiwan.

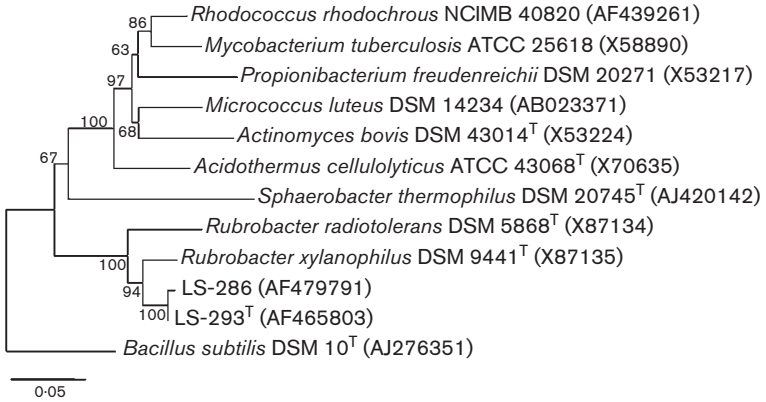

Fig. 3. Phylogenetic relationship dendrogram (based on pairwise 16S rRNA gene sequence comparisons) of the isolated strains LS-286 and LS-293^T, type strains *R. radiotolerans* DSM 5868^T and *R. xylanophilus* DSM 9441^T and reference strains of Gram-positive phyla. Bootstrap values based on 1000 replications are listed as percentages at branching points. Bar, 5 % evolutionary distance.

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