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.

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-293T, 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-286 and LS-293T, among other isolates collected from non-irradiated samples from hot springs, were recovered, without prior gamma-radiation, from the thermally polluted run-off of a carpet factory in the UK (Carreto et al., 1996). These isolates were aerobic, thermophilic, halotolerant, pink-pigmented, heterotrophic and resistant to gamma-radiation.

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 nitride 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-293T and on type strains R. xylanophilus DSM 9441T and R. radiotolerans DSM 8568T, 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 1×10⁷ to 1×10⁸ 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 ⁶⁰Co source at a dose rate of 0.45 kGy min⁻¹ at room temperature (1 kGy=10²⁰ 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; φ 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-293T, were isolated and selected for further studies. Cells of strains LS-286 and LS-293T 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-293T 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...
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-293T. Both strains grew on all of the media tested, but grew particularly well on Thermus medium. Strains LS-286 and LS-293T 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 9441T but different from that of the mesophilic species *R. radiotolerans* DSM 5868T (Fig. 1). Strains LS-286 and LS-293T 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-293T was in the range 0–5% (w/v); strains of *R. xylanophilus* DSM 9441T and *R. radiotolerans* DSM 5868T 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-293T, 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-293T 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-293T, LS-286 and other *Rubrobacter* species.

The fatty acid profiles of strains LS-293T 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-293T 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 acyl fatty acids in strain LS-293T 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 9441T and *R. radiotolerans* DSM 5868T. 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 roseus*, 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-293T (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-293T and LS-286 are sigmoid. The shoulder doses of radiation (the dose required before the number of c.f.u. declines) of strains LS-293T 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 9441T (4.0 kGy) and *R. radiotolerans* DSM 5868T (5.8 kGy), which were comparable to the shoulder dose of *D. radiodurans* DSM 12573T. These results presented here clearly indicate that the novel thermophilic bacterial isolates LS-293T 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
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-293T 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. geothermali* 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

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

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>LS-293T</th>
<th>LS-286</th>
<th>R. <em>xylanophilus</em> DSM 9441T</th>
<th>R. <em>radiotolerans</em> DSM 5868T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pigmentation</td>
<td>Pink</td>
<td>Pink</td>
<td>Light pink</td>
<td>Bright pink</td>
</tr>
<tr>
<td>Optimum growth (°C)</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>45</td>
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<tr>
<td>Hydrolisis of:</td>
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<tr>
<td>Aesculin</td>
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<td>–</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Xylan</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
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<td>DNA</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<td>Utilization of:</td>
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<tr>
<td>D-Mannitol</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<tr>
<td>D-Xylene</td>
<td>–</td>
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<tr>
<td>Glycerol</td>
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<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>L-Rhamnose</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
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<tr>
<td>D-Galactose</td>
<td>+</td>
<td>+</td>
<td>–</td>
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<tr>
<td>D-Glucose</td>
<td>+</td>
<td>+</td>
<td>–</td>
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<tr>
<td>D-Melibioso</td>
<td>+</td>
<td>+</td>
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<td>Malate</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>myo-Inositol</td>
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<td>+</td>
<td>–</td>
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<tr>
<td>Ribitol</td>
<td>–</td>
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<tr>
<td>Succinate</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>L-Asparagine</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L-Glutamine</td>
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<td>+</td>
<td>W</td>
<td>–</td>
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<tr>
<td>L-Serine</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Menaquinone</td>
<td>MK-8</td>
<td>MK-8</td>
<td>MK-8</td>
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<tr>
<td>DNA G+C content (mol%)</td>
<td>68.5</td>
<td>67.9</td>
<td>67.6</td>
<td>64.9</td>
</tr>
</tbody>
</table>

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

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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-293T was 68.9 mol% and that of LS-286 was 67.9 mol%, as determined by the HPLC method. This result reveals that LS-293T and LS-286 are high-G + C, Gram-positive bacteria. Strains LS-293T 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-293T showed 59.6% relatedness to *R. xylanophilus* DSM 9441T and 42.4% relatedness to *R. radiotolerans* DSM 5868T. Strain LS-286T showed 52.8% relatedness to *R. xylanophilus* DSM 9441T and 46.2% relatedness to *R. radiotolerans* DSM 5868T. The relatedness between strains LS-293T and LS-286 was 85.7%.

Following PCR amplification and sequencing, 16S rRNA gene sequences of 1476 nt (strain LS-293T) and 1475 nt (strain LS-286) were determined. A comparison of the 16S rRNA genes, DNA–DNA relatedness, biochemical features and fatty acid composition presented in this study, the 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-293T represent a novel species of the genus *Rubrobacter*. The name *Rubrobacter taiwanensis* sp. nov. is proposed.

**Description of Rubrobacter taiwanensis sp. nov.**

*Rubrobacter taiwanensis* (ta.i.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-xylene, 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-293T and LS-286 are highly resistant to gamma-radiation.

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

The authors would like to thank the National Science Council of the Republic of China for financially supporting this research under contract no. NSC 92-2317-B-002-027. The authors also thank Dr C. Hu (Institute of Nuclear Energy Research, Atomic Energy Council) for assistance provided during gamma-irradiation. The authors are grateful to Professor L.-L. Huang, Dr S.-J. Chen, Miss C.-Y. Lin and Mr C.-Y. Tang (all of National Taiwan University) for electron microscopy. We are also indebted to Mr Y.-C. Wang and C.-C. Lin (Nanotake Technology Co. Ltd) for conducting the atomic-force microscopy.

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