Terrimonas rhizosphaerae sp. nov., isolated from ginseng rhizosphere soil

Song-Ih Han,¹ Ye-Rim Lee,¹ Ju-Ok Kim¹ and Kyung-Sook Whang¹,2,*

Abstract

The novel isolate belonging to the genus Terrimonas, designated CR94¹, was isolated from rhizosphere soil of a ginseng field in Geumsan, Korea. Cells of strain CR94¹ were strictly aerobic, Gram-stain-negative, non-motile, non-filamentous single rods. Growth was observed at 10–37°C (optimum 28°C) and at pH 4.0–10.0 (optimum pH 6.0). Phylogenetic analyses based on 16S rRNA gene sequences indicated that strain CR94¹ belonged to the genus Terrimonas, showing highest sequence similarity to Terrimonas lutea ÐY¹ (97.3 %), Terrimonas pekingsensis QH¹ (97.1 %), Terrimonas aquatica RIB¹–6¹ (95.6 %), Terrimonas rubra M-8¹ (94.7 %) and Terrimonas ferruginea ATCC 13524¹ (93.8 %). DNA–DNA relatedness values between strain CR94¹ and T. lutea KACC 13047¹, T. pekingsensis KACC 18795¹, T. ferruginea KACC 11130¹ and T. aquatica LMG 24825¹ were 30.5, 28.9, 17.8 and 13.5 %, respectively. The DNA G+C content was 46.5 mol% and the major respiratory quinone was menaquinone-7 (MK-7). The major cellular fatty acids of strain CR94¹ were iso-C₁₅:₀ G and iso-C₁₆:₀. On the basis of the polyphasic analysis, strain CR94¹ represents a novel species of the genus Terrimonas, for which the name Terrimonas rhizosphaerae sp. nov. is proposed. The type strain is CR94¹ (=KACC 17564¹=NCAIM B 025317¹).

The genus Terrimonas, a member of the family Chitinophagaceae, phylum Bacteroidetes, was proposed by Xie and Yokota [1] and currently comprises five recognized species, Terrimonas ferruginea (the type species of the genus), Terrimonas lutea [1], Terrimonas aquatica [2], Terrimonas rubra [3] and Terrimonas pekingsensis [4]. During the screening of cellulose-producing bacteria from ginseng rhizosphere soil, a novel bacterial strain, CR94¹, was isolated and preliminary 16S rRNA gene sequence analysis showed that it was most closely related to the genus Terrimonas. In this study, strain CR94¹ was characterized using a polyphasic taxonomic approach.

Strain CR94¹ was isolated from rhizosphere soil of a ginseng field (36°11’ 11” N 127°54’ 32” E) located at Geumsan in Korea. For isolation, a ginseng rhizosphere soil sample (10 g) was dispersed using a sonic oscillator (Vibracell VCX750; Sonics) for 40 s at 30 W in 90 ml of sterilized water. It was serially diluted with sterile distilled water and aliquots (100 μl) of the dilutions were transferred onto plates of a 10-fold dilution of nutrient broth (NB) medium (Difco) containing 1.2 % (w/v) agar (10⁻¹ NA) medium, followed by aerobic incubation at 28°C for 3 days. The pH of the medium was adjusted to pH 7.0 with 1 M NaOH. The strain was subsequently purified three times by plating on 10⁻¹ NA medium and maintained on the same medium. The strain was stored at –80°C in NB supplemented with 20 % (v/v) glycerol. To characterize strain CR94¹ phenotypically, the isolate was routinely grown aerobically on 10⁻¹ NA for 3 days at 28°C and at pH 7.0, except where indicated otherwise. The type strains of species of the genus Terrimonas, T. pekingsensis KACC 18795¹, T. lutea KACC 13047¹, T. ferruginea KACC 11130¹ and T. aquatica LMG 24825¹, were obtained from the respective culture collection for comparative taxonomic analysis.

The morphology of the isolate was observed by Gram staining and scanning electron microscopy (Hitachi High-Technologies Canada) using cells from exponentially growing cultures on 10⁻¹ NA for 3 days at 28°C. Gram staining was performed by the Hucker method [5]. Catalase activity was determined by assessing bubble production by cells in 3 % (v/v) H₂O₂, and oxidase activity was determined using 1 % (w/v) N,N,N,N-tetramethyl-1,4-phenylenediamine reagent (bioMérieux). Hydrolysis of aesculin, casein, DNA, gelatin, hypoxanthine, l-tyrosine, CM-cellulose, colloidal chitin, starch, Tween 80, xanthine and urea, the Voges–Proskauer reaction and production of indole were tested as recommended by Smibert and Krieg [6]. A DNase test was conducted using DNase test agar (Difco) [7]. Determination of acid production from

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CR94¹ is FJ772030.

Four supplementary figures and one supplementary table are available with the online Supplementary Material.
carbohydrates, as well as utilization of carbohydrates as sole carbon and energy sources, activities of constitutive enzymes and other additional biochemical characteristics were performed by using API 50CH (with API 50CHB medium), API 20NE and API ZYM galleries according to the instructions of the manufacturer (bioMerieux) supplemented with 0.5 % (w/v) NaCl. Growth on NA, trypticase soy agar (TSA), R2A agar, Luria–Bertani (LB) agar and potato dextrose agar (all from Difco) was evaluated at 28 °C. Growth at 4, 10, 15, 20, 25, 28, 30, 37, 40, 45 and 50 °C and at pH 3.0–10.0 (in increments of 0.5 pH units) was assessed in 10−1 NB after 3 days. The pH values of <6, 6–9 and >9 were obtained by using sodium acetate/acetate acid, Tris/HCl and glycine/sodium hydroxide buffers, respectively. Tolerance to NaCl was tested in 10−1 NB supplemented with 0–10 % (w/v) NaCl (in increments of 1 %) after 5 days. Production of flexirubin-type pigments was checked using the KOH test according to Bernardet et al. [8]. Growth was monitored based on the turbidity at OD600 by using a spectroscopic method (model UV-1650PC; Shimadzu). Anaerobic growth was determined by incubation in the BBL GasPak Anaerobic System (Difco) for 5 days at 28 °C on 10−1 NA.

Genomic DNA from strain CR94T was prepared using a commercial genomic DNA extraction kit (Solgent). The 16S rRNA gene was amplified by PCR with the forward primer 27F and the reverse primer 1492R [9]. The identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarity were achieved by using the EzTaxon-e server (www.eztaxon-e.ezcloud.net/) [10]. Sequence alignment was performed using CLUSTAL W 2.0 software [11]. Gaps at the 5′ and 3′ ends of the alignment were omitted in further analyses. Phylogenetic trees were reconstructed by using three different methods: the neighbour-joining [12], maximum-likelihood [13] and maximum-parsimony [14] algorithms within the MEGA6 software package [15]. Evolutionary distance matrices for the neighbour-joining method were calculated using the algorithm of Kimura’s two-parameter model [16]. To evaluate the stability of the phylogenetic tree, a bootstrap analysis (1000 replications) was performed [17]. The 16S rRNA gene sequences used for phylogenetic comparisons were obtained from the GenBank database and their strain designations and GenBank accession numbers are shown in Fig. 1.

To determine genomic relatedness, DNA–DNA hybridization was performed using modifi method of Ezaki et al. [18]. Probe labelling for DNA–DNA hybridization was conducted by using the non-radioactive DIG-High prime system (Roche); hybridized DNA was visualized using the DIG luminescent detection kit (Roche) and the level of DNA–DNA relatedness was quantified by using a densitometer (Bio-Rad). For determination of the G+C content, the genomic DNA of strain CR94T was extracted, purified and enzymatically degraded into nucleosides by P1 nuclease. The G+C content was determined according to the methods of Mesbah et al. [19].

For analysis of fatty acids, strain CR94T was cultured on TSA at 28 °C for 3 days and cells were obtained at the late-exponential growth phase, and similarly for the reference strains. Cellular fatty acids were extracted and analysed by GC (6890N; Agilent Technologies) according to the standard protocol of the Sherlock Microbial Identification System (version 4.5; MIDI database TSBA40 4.10) [20]. Respiratory quinones of strain CR94T were extracted from 100 mg of freeze-dried cells with chloroform/methanol (2:1, v/v) and analysed by HPLC (Shimadzu SPD-10AV), as described by Collins and Jones [21]. For polar lipid analysis, strain CR94T was grown in R2A broth at 30 °C for 3 days. The cellular lipids were extracted twice, washed and hydrolysed as described by Minnikin et al. [22]. The total lipids were separated on silica-gel plates by two-dimensional TLC with a solvent system composed of chloroform/methanol/water (65:25:4, by vol.) in the first direction and chloroform/methanol/acetate acid/water (80:15:12:4, by vol.) in the second direction [23]. To detect spots and their colour reaction, 30 % sulfuric acid solution, Dittmer’s reagent, ninhydrin solution and α-naphthol reagent were used for total lipids, phospholipids, amino lipids and glycolipids, respectively.

Cells of strain CR94T were Gram-stain-negative, non-motile, non-filamentous single rods (Fig. S1, available in the online Supplementary Material). Colonies were circular, convex, entire, white and 1.0–2.0 mm in diameter on 10−1 NA after 3 days of incubation at 28 °C. Strain CR94T was able to grow at 10−37 °C, at pH 4.0–10.0 and in the presence of 0–1.0 % (w/v) NaCl. Optimal growth was observed at 28 °C, at pH 6.0 and in the absence of NaCl. Growth was also observed on NA, R2A agar and LB agar. Strain CR94T was catalase-negative and oxidase-positive. Flexirubin-type pigments were not produced. Starch, CM-cellulose and DNA were hydrolysed but casein, chitin, gelatin and Tween 80 were not. Other phenotypic features are included in the species description, and characteristics that differentiate strain CR94T from the type strains of recognized species of the genus Terrimonas are summarized in Table 1.

The almost-complete 16S rRNA gene sequence (1408 bp) of strain CR94T was obtained and used for initial BLAST searches in GenBank (www.ncbi.nlm.nih.gov/) and phylogenetic analysis. The results showed that strain CR94T was phylogenetically related to T. lutea DY1T (97.3 % 16S rRNA gene sequence similarity), T. pekingensis QH1T (97.1 %), T. aquatica RIB1-6T (95.6 %), T. rubra M-8T (94.7 %) and T. ferruginnea ATCC 13524T (93.8 %). 16S rRNA gene sequence similarity with members of other genera in the family Chitinophagaceae was <93.1 %. In the neighbour-joining tree (Fig. 1), strain CR94T clustered with T. lutea DY1T and T. pekingensis QH1T, while the two other members of the genus Terrimonas formed another cluster with the genus Niabella; however, 16S rRNA gene sequence similarity between T. ferruginnea ATCC 13524T and T. aquatica RIB1-6T and members of the genus Niabella was <92.5 %. The topology of the minimum-evolution tree was essentially the same. Strain
CR94<sup>T</sup> joined the clade comprising <i>T. ferruginea</i>, <i>T. lutea</i>, <i>T. aquatica</i>, <i>T. rubra</i> and <i>T. pekingensis</i> with a bootstrap resampling value of 100%; this tree topology was also found in trees generated with the maximum-likelihood and maximum-parsimony algorithms (Figs S2 and S3). DNA–DNA hybridization values between strain CR94<sup>T</sup> and the type strains of other species of the genus Terrimonas, <i>T. lutea</i> KACC 13047<sup>T</sup>, <i>T. pekingensis</i> KACC 18795<sup>T</sup>, <i>T. ferruginea</i> KACC 11310<sup>T</sup> and <i>T. aquatica</i> DSM 24825<sup>T</sup>, were 30.5, 28.9, 17.8 and 13.5%, respectively, which were significantly lower than 70%, the threshold value recommended for the assignment of genomic species [24]. The DNA G+C content of strain CR94<sup>T</sup> was 46.5 mol%.

The major respiratory quinone of strain CR94<sup>T</sup> was menaquinone-7 (MK-7), which is in agreement with the genus Terrimonas and the family Chitinophagaceae. Total polar lipids of strain CR94<sup>T</sup> were phosphatidylethanolamine, an unidentified aminophospholipid, unidentified glycolipids and unidentified polar lipids (Fig. S4). The major fatty acids in strain CR94<sup>T</sup> were iso-C<sub>15:0</sub> (27.9%) and iso-C<sub>15:1</sub> (37.8%) (Table S1).

Therefore, on the basis of evidence from this polyphasic taxonomic study, we propose that strain CR94<sup>T</sup> represents a novel species of the genus Terrimonas, for which the name Terrimonas rhizosphaerae sp. nov. is proposed.

**DESCRIPTION OF TERRIMONAS RHIZOSPHAERAE SP. NOV.**

Terrimonas rhizosphaerae (rhi.zo.sphae‘rae. Gr. fem. n. rhiza a root; L. fem. n. sphaera ball, sphere; N.L. gen. fem. n. rhizosphaerae of the rhizosphere, referring to the site from which the type strain was isolated).

Cells are Gram-stain-negative, non-motile, non-filamentous single rods. Colonies are circular, convex, entire, white and 1.0–2.0 mm in diameter on 10<sup>−1</sup> NA after 3 days of incubation at 28 °C. Growth was observed at 0–1% (w/v) NaCl (optimum in the absence of NaCl) and at pH 4.0–10.0 (optimum at pH 6.0). Catalase-negative and oxidase-positive. Flexirubin-type pigments are not produced. Starch, CM-cellulose and DNA are hydrolysed but casein, chitin, gelatin and Tween
80 are not. In API 20NE tests, positive for nitrate reduction, β-glucosidase activity, and assimilation of glucose, arabinose, N-acetylglucosamine, mannos, mannotol and maltose, but negative for indole production, glucose acidification, arginine dihydrolase, urease and β-galactosidase activities, and assimilation of gluconate, caprate, adipate, malate, citrate and phe- nylacetate. In API ZYM tests, positive for alkaline phosphatase, esterase (C4), leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase. All strains are negative for lipase (C14), arginine dihydrolase, urease, production of indole and assimilation of malate, caprate, adipate and citrate. +, Positive; –, Negative.

### Table 1. Differential characteristics between strain CR94^T^ and members of the genus Terrimonas

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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</thead>
<tbody>
<tr>
<td>Colony colour</td>
<td>White</td>
<td>White</td>
<td>Yellow</td>
<td>Yellow</td>
<td>Yellowish orange</td>
</tr>
<tr>
<td>pH range for growth</td>
<td>4.0–10.0</td>
<td>5.0–8.0</td>
<td>6.0–8.0</td>
<td>5.0–8.0</td>
<td>7.0–8.0*</td>
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<td>NaCl range for growth (% w/v)</td>
<td>0–1</td>
<td>0–1</td>
<td>0–1</td>
<td>0–2</td>
<td>0–1*</td>
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<tr>
<td>Nitrate reduction</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>Enzyme activity (API ZYM):</td>
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<tr>
<td>Esterase</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Esterase lipase</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Valine arylamidase</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<td>Cystine arylamidase</td>
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<td>+</td>
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<tr>
<td>α-Chymotrypsin</td>
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<td>α-Galactosidase</td>
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<tr>
<td>α-Glucosidase</td>
<td>–</td>
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<td>β-Glucosidase</td>
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<tr>
<td>N-Acetyl-β-glucosaminidase</td>
<td>–</td>
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<td>α-Mannosidase</td>
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<td>Assimilation of (API 20 NE):</td>
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<tr>
<td>Glucose</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Arabinose</td>
<td>+</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Mannose</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Mannotol</td>
<td>+</td>
<td>–</td>
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<tr>
<td>N-Acetylglucosamine</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Maltose</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<tr>
<td>DNA G+C content (mol%)</td>
<td>46.5</td>
<td>41.0†</td>
<td>47.2‡</td>
<td>48.9‡</td>
<td>47.3*</td>
</tr>
</tbody>
</table>

*Data obtained from Sheu et al. [2].
†Data obtained from Jin et al. [4].
‡Data obtained from Xie and Yokota [1].

80 are not. In API 20NE tests, positive for nitrate reduction, β-glucosidase activity, and assimilation of glucose, arabinose, N-acetylglucosamine, mannos, mannotol and maltose, but negative for indole production, glucose acidification, arginine dihydrolase, urease and β-galactosidase activities, and assimilation of gluconate, caprate, adipate, malate, citrate and phenylacetate. In API ZYM tests, positive for alkaline phosphatase, leucine arylamidase, trypsin, α-chymotrypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase, but negative for esterase (C4), esterase lipase (C8), lipase (C14), valine arylamidase, cystine arylamidase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. Acid is produced from erythritol, D-arabinose, L-arabinose, D-xylitol, L-xylitol, D-adenitol, D-galactose, D-glucose, D-fructose, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, N-acetyl-glucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, D-lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose, amidon, glycogen, xylitol, gentiobiose, turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, L-arabitol and L-arabitol, but not produced from glycerol, methyl β-D-xylpyranoside, D-mannitol or D-sorbitol. The major fatty acids (>10%) are iso-C_{15:0} and iso-C_{15:1}. The major respiratory quinone is MK-7.

The type strain, CR94^T^ (=KACC 17564^T^=NCAIM B 025317^T^), was isolated from ginseng rhizosphere soil in Geumson, Korea. The DNA G+C content of the type strain is 46.5 mol%.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

References


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