

## *Rheinheimera tangshanensis* sp. nov., a rice root-associated bacterium

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A Gram-negative, aerobic, rod-shaped bacterium, designated strain JA3-B52<sup>T</sup>, was isolated from the roots of fresh rice plants (*Oryza sativa*). The cells were motile by means of polar single or lateral flagella. The colonies were non-pigmented. On the basis of 16S rRNA gene sequence comparisons, the strain was phylogenetically related to species of the genus *Rheinheimera*, having the greatest level of sequence similarity with respect to *Rheinheimera texasensis* A62-14B<sup>T</sup> (97.16%). The bacterium grew at temperatures from 10 to 37 °C, with an optimum at 30 °C. The strain exhibited growth with 0–3.0% (w/v) NaCl and at pH 6.0–8.5. The main cellular fatty acids were C<sub>16:1ω7c</sub>, C<sub>17:1ω8c</sub>, C<sub>16:0</sub>, C<sub>18:1ω7c</sub> and C<sub>12:0</sub> 3-OH. The DNA G+C content was 47.0 mol%. The levels of similarity between the 16S rRNA gene sequence of strain JA3-B52<sup>T</sup> and those of the type strains of *Rheinheimera* species ranged from 95.38 to 97.16%. The mean level of DNA–DNA relatedness between strain JA3-B52<sup>T</sup> and *R. texasensis* A62-14B<sup>T</sup>, the strain most closely related to the isolate, was 20.4%. On the basis of physiological and biochemical characteristics and genotypic data obtained in this work, strain JA3-B52<sup>T</sup> represents a novel species of the genus *Rheinheimera*, for which the name *Rheinheimera tangshanensis* sp. nov. is proposed. The type strain is JA3-B52<sup>T</sup> (=CGMCC 1.6362<sup>T</sup> =DSM 19460<sup>T</sup>).

The genus *Rheinheimera* comprises Gram-negative, flagellated, rod-shaped to coccoid, oxidase- and catalase-positive bacterial cells that are commonly isolated from marine or estuarine environments (Brettar *et al.*, 2002). At the time of writing, this genus is represented by six species: *Rheinheimera baltica* (Brettar *et al.*, 2002), *R. pacifica* (Romanenko *et al.*, 2003), *R. perlucida* (Brettar *et al.*, 2006), *R. chironomi* (Halpern *et al.*, 2007), *R. aquimaris* (Yoon *et al.*, 2007) and *R. texasensis* (Merchant *et al.*, 2007). In this study, we report on the taxonomic characterization of a *Rheinheimera*-like bacterial strain, JA3-B52<sup>T</sup>, isolated from the roots of fresh rice plants from the Agricultural Experimental Demonstration Base at the village of Sangyuan, Luannan County, Hebei Province, China, during a study of bacterial diversity in rice (*Oryza sativa*) roots.

Rice plants were sampled at the tillering stage. Clumps loosely adhering to the roots were removed gently as soon

as the samples reached the laboratory. More firmly adhering soil was washed away with sterile double-distilled water three times. The root samples were then ground in a sterile pottery mortar. This produced a suspension that included bacteria from the rhizoplane and from the inner tissues of the roots. Subsequently, the suspension was diluted with double-distilled water using the standard dilution plating technique and then incubated at 28 °C on Luria–Bertani (LB) agar medium for 2 days. Strain JA3-B52<sup>T</sup> was isolated from the plate, purified using the streak plate method and then stored at 4 °C in freeze-drying ampoules.

The morphology of cells of strain JA3-B52<sup>T</sup> grown on LB agar in the exponential phase was observed by means of light microscopy and transmission electron microscopy. Acid production from carbohydrates, hydrolysis of starch and formation of H<sub>2</sub>S from thiosulfate were tested using the methods described by Dong & Cai (2001). Growth at temperatures from 4 to 41 °C was measured on TSA (Difco). The pH range for growth was determined in TSB

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of JA3-B52<sup>T</sup> is DQ874340.

(Difco) (pH 5.0–11.0, in increments of 0.5 pH units). Sodium ion tolerance and requirements were examined by cultivating the isolate on LB medium with different NaCl concentrations (0, 1, 2, 3, 4, 5, 6 and 8%, w/v). The following were determined using GN2 MicroPlates (Biolog) and the API 20NE identification system (bioMérieux), as described by the manufacturers: utilization of carbohydrates, hydrolysis of gelatin and aesculin, indole production, *p*-nitrophenyl  $\beta$ -D-galactopyranoside (PNPG) hydrolysis, production of urease and arginine dihydrolase and nitrate reduction. Antibiotic sensitivity was examined by placing 6 mm discs containing 20  $\mu$ l antibiotic suspension centrally on plates of LB agar seeded with 200  $\mu$ l from a 3 day LB medium culture. The antibiotic concentrations used were as follows: 15  $\mu$ g ml<sup>-1</sup> for erythromycin, kanamycin and lincomycin,

10  $\mu$ g ml<sup>-1</sup> for tetracycline, chloramphenicol and ampicillin and 30  $\mu$ g ml<sup>-1</sup> for rifampicin. Inhibition zones were observed after 3–5 days incubation at 30 °C.

The strain was shown to comprise Gram-negative rods (1.3–2.5  $\mu$ m long and 0.4–1.0  $\mu$ m wide) that were motile by means of polar or lateral flagella. Circular, non-pigmented, entire colonies were produced. Strain JA3-B52<sup>T</sup> was oxidase- and catalase-positive. Growth was observed at 10–37 °C and in 0–3% NaCl, but growth was slow in 3% NaCl. The Biolog GN2 MicroPlate system showed that JA3-B52<sup>T</sup> could utilize more than 30 different carbon sources. The morphological, physiological and biochemical characteristics of strain JA3-B52<sup>T</sup> are given in Table 1.

For 16S rRNA gene sequencing, a loop of biomass was scraped off the LB agar plate, suspended in 20  $\mu$ l sterile

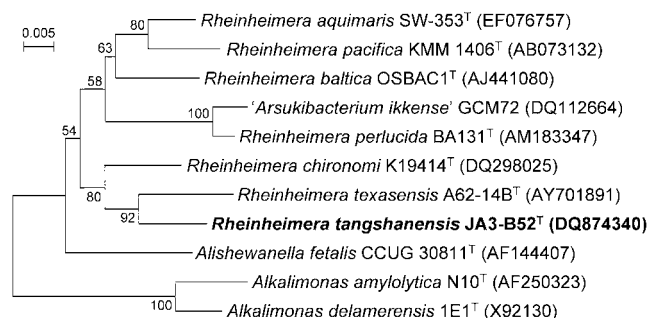
**Table 1.** Phenotypic characteristics of strain JA3-B52<sup>T</sup> and the type strains of related species

Strains: 1, JA3-B52<sup>T</sup> (data from this study); 2, *R. texasensis* A62-14B<sup>T</sup> (Merchant *et al.*, 2007); 3, *R. chironomi* K19414<sup>T</sup> (Halpern *et al.*, 2007); 4, *R. baltica* OSBAC1<sup>T</sup> (Brettar *et al.*, 2002); 5, *R. pacifica* KMM 1406<sup>T</sup> (Romanenko *et al.*, 2003); 6, *R. perlucida* BA131<sup>T</sup> (Brettar *et al.*, 2006); 7, *A. fetalis* CCUG 30811<sup>T</sup> (Fonnesbech Vogel *et al.*, 2000; Romanenko *et al.*, 2003). +, Positive; –, negative; ND, no data available.

Characteristic	1	2	3	4	5	6	7
O <sub>2</sub> requirement	Aerobic	Facultative	Aerobic	Aerobic	Aerobic	Facultative	Facultative
Pigmentation	None	None	None	Blue	None	None	None
Cell size ( $\mu$ m)							
Length	1.3–2.5	1.25–2.5	1.0–2.4	1.8–2.0	0.9–4.5	0.9–2.4	2.0
Width	0.4–1.0	0.7–0.8	0.3–0.7	0.5–1.5	0.6–0.8	0.6–1.2	0.5–1.0
Motility	+	+	+	+	+	+	–
Flagella	Single; polar or lateral	Single or multiple; polar and lateral	Single; polar	Single; polar	Multiple; polar and lateral	Single; polar	None
Temperature for growth (°C)							
Range	10–37	25–37	4–40	4–30	4–37	4–37	25–41
Optimum	30	30–37	30–37	20–25	ND	20–30	ND
pH for growth							
Range	6.0–8.5	6.5–9.6	ND	5.7–10	ND	5.7–10	ND
Optimum	7.0	7.5–8.0	ND	7.0	ND	7.0	ND
NaCl concentration for growth (% w/v)							
Range	0–3	0–1	0–2	0–6	0–8	0–8	3–8
Optimum	1	0	0.5–1	1–3	ND	1–3	ND
Starch hydrolysis	+	+	ND	+	+	+	–
Reduction of nitrate to nitrite	–	+	+	–	–	+	+
Utilization of:							
Arabinose	+	+	–	–	+	–	ND
Glycerol	–	–	–	–	+	–	ND
<i>N</i> -Acetylglucosamine	+	+	+	+	+	+	–
Cellobiose	+	+	–	–	–	–	ND
D-Glucose	+	+	+	+	–	+	–
D-Lactose	+	+	–	ND	ND	ND	ND
Trehalose	+	+	–	–	+	–	ND
$\beta$ -Cyclodextrin	–	+	–	ND	ND	ND	ND
Citrate	–	–	–	–	+	–	–
DNA G + C content (mol%)	47.0	48.2	49.9	48.9	49.6	48.9	50.6
Isolation source	Rice roots	Lake water	Chironomid egg mass	Surface sea-water (Baltic)	Deep-sea water (Pacific)	Surface sea-water (Baltic)	Human fetus

double-distilled water and lysed by boiling for 10 min followed by freezing ( $-20^{\circ}\text{C}$ ) for 5 min. Following centrifugation at 13 000 g, the supernatant was used as the template for a PCR with universal primers 27F and 1492R (Lane, 1991). Automated sequencing was performed using the ABI Big Dye Primer cycle sequencing ready reaction kit (Perkin-Elmer Applied Biosystems) and a DNA sequencer (3730; Applied Biosystems). A partial 16S rRNA gene sequence (1390 bp) was obtained: it was compared with sequences deposited in GenBank (National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov/>) by using the BLAST program. Multiple alignments of the sequences were performed using CLUSTAL\_X (version 1.83) (Thompson *et al.*, 1997). Aligned sequences were analysed using MEGA 3.1 software (Kumar *et al.*, 2004). The evolutionary distances (using distance options according to Kimura's two-parameter model; Kimura, 1980) and clustering with the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Lake, 1987) methods were determined by using a bootstrap analysis based on 1000 replications (Felsenstein, 1985). Similarity values were calculated using the same software. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain JA3-B52<sup>T</sup> belongs to the genus *Rheinheimera*, with bootstrap confidence values of 92% (neighbour-joining method; Fig. 1) and 73% (maximum-parsimony method; data not shown). Analysis of 16S rRNA gene sequences indicated that the sequence of strain JA3-B52<sup>T</sup> showed the highest similarity (97.16%) with respect to *R. texasensis* A62-14B<sup>T</sup>, followed by *R. chironomi* K19414<sup>T</sup> (96.77%), *R. baltica* DSM 14885<sup>T</sup> (96.03%), *R. pacifica* KMM 1406<sup>T</sup> (95.46%), *R. perlucida* BA131<sup>T</sup> (95.38%) and *Alishewanella fetalis* DSM 16032<sup>T</sup> (95.29%).

Preparation of genomic DNA was carried out using the method described by Marmur (1961). The G+C content was determined according to the method of Marmur & Doty (1962). The level of DNA–DNA relatedness between the novel isolate and the reference strain *R. texasensis* A62-14B<sup>T</sup> was determined by using the reassociation-rate method (Dong *et al.*, 2000). The cellular fatty acid composition was determined by using the Microbial Identification System (MIDI) as described by Kämpfer & Kroppenstedt (1996) and Sasser (1990). Strain JA3-B52<sup>T</sup> was grown on TSA at 30 °C for 2 days. Because some of the recognized species of the genus *Rheinheimera* require different media for optimum growth, direct comparisons of their fatty acid profiles are difficult. Strain JA3-B52<sup>T</sup> contained C<sub>16:1</sub>ω7c (33.72%) as the major cellular fatty acid and also had significant amounts of C<sub>17:1</sub>ω8c (15.15%), C<sub>18:1</sub>ω7c (13.41%), C<sub>16:0</sub> (7.75%) and C<sub>12:0</sub> 3-OH (7.21%). A comparison of the detailed fatty acid composition of strain JA3-B52<sup>T</sup> with those of related species is shown in Table 2. C<sub>16:1</sub>ω7c was also a major cellular fatty acid in all of the recognized species of the genus *Rheinheimera*. The genomic DNA G+C content of strain JA3-B52<sup>T</sup> was 47.0 mol%, being very close to the values for species of the genus *Rheinheimera* (47.8–



**Fig. 1.** Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, for strain JA3-B52<sup>T</sup> and species of related genera. Bootstrap percentages (based on 1000 replicates) are shown at nodes. GenBank accession numbers are given in parentheses. Bar, 0.005 nucleotide substitutions per site.

49.6 mol%) (Merchant *et al.*, 2007; Halpern *et al.*, 2007; Brettar *et al.*, 2002, 2006; Romanenko *et al.*, 2003). The DNA–DNA relatedness for strain JA3-B52<sup>T</sup> and *R. texasensis* A62-14B<sup>T</sup> was 20.4%. Since the recommended DNA–DNA relatedness threshold for the definition of a species is 70% (Wayne *et al.*, 1987), this result indicates that strain JA3-B52<sup>T</sup> does not belong to any known species

**Table 2.** Cellular fatty acid compositions of strain JA3-B52<sup>T</sup> and related type strains

Strains: 1, JA3-B52<sup>T</sup> (grown on TSA; data from this study); 2, *R. texasensis* A62-14B<sup>T</sup> (R2A; data from Merchant *et al.*, 2007); 3, *R. chironomi* K19414<sup>T</sup> (TSA; Halpern *et al.*, 2007); 4, *R. baltica* OSBAC1<sup>T</sup> (blood agar; Brettar *et al.*, 2002); 5, *R. pacifica* KMM 1406<sup>T</sup> (marine agar; Romanenko *et al.*, 2003); 6, *R. perlucida* BA131<sup>T</sup> (blood agar; Brettar *et al.*, 2006); 7, *A. fetalis* CCUG 30811<sup>T</sup> (blood agar; Fonnesbech Vogel *et al.*, 2000; Romanenko *et al.*, 2003; Brettar *et al.*, 2006). Values are percentages of total fatty acids; –, not detected or <2%.

Fatty acid	1	2	3	4	5	6	7
C <sub>10:0</sub>	2.2	–	–	–	–	–	–
C <sub>11:0</sub> 3-OH	3.3	–	5.5	–	–	–	2.5
C <sub>12:0</sub>	–	2.0	–	5.4	–	–	–
C <sub>12:0</sub> 3-OH	7.2	12.8	8.5	–	–	3.8	2.0
C <sub>13:0</sub> 3-OH	–	–	–	–	–	–	2.7
C <sub>14:0</sub>	–	–	–	4.7	–	–	–
C <sub>15:0</sub>	2.1	–	6.9	–	2.4	–	6.7
C <sub>15:1</sub> ω8c	3.0	–	2.8	5.8	3.3	3.0	4.9
iso-C <sub>16:0</sub>	–	–	–	–	3.7	–	–
C <sub>16:0</sub>	7.8	19.0	14.8	25.2	19.1	18.2	8.9
C <sub>16:1</sub> ω9c	–	–	–	–	25.5	–	–
C <sub>16:1</sub> ω7c	33.7	38.6	25.8	33.1	–	23.6	19.0
C <sub>17:0</sub>	–	–	5.8	2.5	8.1	7.9	10.3
C <sub>17:1</sub> ω8c	15.2	3.8	–	3.1	11.7	18.3	19.5
C <sub>18:0</sub>	–	2.0	–	–	–	–	–
C <sub>18:1</sub> ω7c	13.4	7.7	6.7	3.8	15.7	9.4	7.2

of the genus *Rheinheimera*. In addition, there are a range of phenotypic characteristics that serve to distinguish strain JA3-B52<sup>T</sup> from phylogenetically related species of the genus *Rheinheimera* (Table 1). Therefore, on the basis of the data presented, strain JA3-B52<sup>T</sup> represents a novel species of the genus *Rheinheimera*, for which the name *Rheinheimera tangshanensis* sp. nov. is proposed.

### Description of *Rheinheimera tangshanensis* sp. nov.

*Rheinheimera tangshanensis* (tang.shan.en'sis. N.L. fem. adj. *tangshanensis* pertaining to Tangshan, a city in Hebei Province, PR China, where the type strain was collected).

On LB agar, cells are rods, 1.3–2.5 µm long and 0.4–1.0 µm wide. Motile by means of polar or lateral flagella. Strictly aerobic, Gram-negative and oxidase- and catalase-positive. Colonies are circular, non-pigmented, convex, entire with dark centres and transparent margins on LB agar. Growth occurs at 10–37 °C, but not at or above 41 °C; optimum growth at 30 °C. NaCl concentrations up to 3% (w/v) are tolerated, but the optimal concentration for growth is 1% (w/v). Acids are produced from L-arabinose, maltose and sucrose, but not from D-glucose. H<sub>2</sub>S is not produced. Starch is hydrolysed. With the API 20NE system, positive results are obtained for hydrolysis of gelatin and aesculin, indole production, PNPG hydrolysis and assimilation of glucose, arabinose, N-acetylglucosamine and malate. Negative results are obtained for assimilation of mannose, mannitol, maltose, gluconate, caprate, adipate, citrate and phenyl acetate. Nitrate is not reduced to nitrite. Urease and arginine dihydrolase are not detected. Of the 95 different carbon sources available in the GN2 MicroPlate analysis, the following 30 compounds are positive for utilization: α-cyclodextrin, dextrin, glycogen, N-acetyl-D-glucosamine, L-arabinose, cellobiose, D-galactose, gentiobiose, α-D-glucose, α-D-lactose, lactulose, maltose, melibiose, methyl β-D-glucoside, raffinose, sucrose, trehalose, turanose, pyruvic acid methyl ester, acetic acid, D-galacturonic acid, β-hydroxybutyric acid, succinic acid, L-glutamic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, L-leucine, L-proline, L-serine and L-threonine. Cells are susceptible to (µg ml<sup>-1</sup>) erythromycin (15), tetracycline (10), rifampicin (30) and chloramphenicol (10), but resistant to kanamycin (15), lincomycin (15) and ampicillin (10). The DNA G+C content of the type strain is 47.0 mol%. The major fatty acids (>5%) are C<sub>16:1</sub>ω7c, C<sub>17:1</sub>ω8c, C<sub>18:1</sub>ω7c, C<sub>16:0</sub> and C<sub>12:0</sub> 3-OH.

The type strain, JA3-B52<sup>T</sup> (=CGMCC 1.6362<sup>T</sup> =DSM 19460<sup>T</sup>), was isolated from the roots of fresh rice plants (*Oryza sativa*).

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