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Rheinheimera aquatica sp. nov., Antimicrobial Activity-Producing Bacterium Isolated from Freshwater Culture Pond

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A bacterial strain designated GR5^T, previously isolated from a freshwater culture pond in Taiwan while screening for bacteria for antimicrobial compounds, was characterized using a polyphasic taxonomic approach. Strain GR5^T was found to be Gram-negative, aerobic, greenish-yellow colored, rod-shaped, and motile by means of a single polar flagellum. Growth occurred at 10–40°C (optimum, 35°C), pH 7.0–8.0 (optimum pH 8.0), and with 0–2.0% NaCl (optimum, 0.5–1.0%). The major fatty acids were $C_{16:1}\omega7c$ (36.3%), $C_{16:0}$ (16.6%), $C_{12:0}$ 3-OH (12.5%), and $C_{18:1}\omega7c$ (9.1%). The major respiratory quinone was Q-8, and the DNA G+C content of the genomic DNA was 51.9 mol%. Phylogenetic analyses based on 16S rRNA gene sequences showed that strain GR5^T belongs to the genus *Rheinheimera*, where its most closely related neighbors are *Rheinheimera texasensis* A62-14B^T and *Rheinheimera tangshanensis* JA3-B52^T with sequence similarities of 98.1% and 97.5%, respectively, and the sequence similarities to any other recognized species within *Gammaproteobacteria* are less than 96.5%. The mean level of DNA–DNA relatedness between strain GR5 ^{T} and *R. texasensis* A62-14B^T, the strain most closely related to the isolate, was 26.5±7.6%. Therefore, based on the phylogenetic and phenotypic data, strain GR5^T should be classified as a novel species, for which the name *Rheinheimera aquatica* sp. nov. is proposed. The type strain is GR5^T (=BCRC 80081^T= LMG 25379^T).

Keywords: *Rheinheimera aquatica* sp. nov., antimicrobial activity, polyphasic taxonomy

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The genus *Rheinheimera*, first described by Brettar *et al.* [1], is characterized as Gram-negative, flagellated, rod-shaped to coccoid, oxidase- and catalase-positive, aerobic, and chemoheterotrophic, where chemotaxonomically it has Q-8 as the predominant ubiquinone, $C_{16:1}\omega7c$, $C_{16:0}$, and $C_{18:1}\omega7c$ as the major fatty acids, and DNA G+C contents of 47.0–50.5 mol% [1, 30, 31]. At the time of writing, the genus *Rheinheimera* comprises eight recognized species, namely *Rheinheimera baltica* [1], *R. pacifica* [22], *R. perlucida* [2], *R. aquimaris* [30], *R. chironomi* [13], *R. texasensis* [18], *R. soli* [23], and *R. tangshanensis* [31], which were isolated from seawater, freshwater, chironomid egg mass, soil, and rice roots, indicating that members of this genus are widely distributed in various environments.

In order to screen bacteria producing antimicrobial compounds, a water sample was collected from a freshwater pond (GPS location: 22° 29' 16.8" N, 120° 27' 28.9" E) used to culture soft-shell turtles located in the Pingtung countryside in southern Taiwan. The temperature of the pond water was about 25°C, the pH value was approximately 7.2, and the NaCl concentration was about 0.1% (w/v). In a previous study, several bacterial strains were already isolated from this water sample and subjected to an antibiogram assay for antimicrobial activity based on inhibition zone formation against *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas putida*, and *Escherichia coli* [4]. As a result, a greenish yellow pigmented strain that exhibited significant antimicrobial activity was chosen and designated as GR5. Strain GR5 possesses a broad spectrum of antimicrobial activities, owing to the generation of hydrogen peroxide by the enzymatic activity of L-lysine oxidase [4]. Accordingly, this study describes the morphological, biochemical, and phylogenetic distinctiveness of strain

GR5, which indicate that it should be classified as a novel species, for which the name *Rheinheimera aquatica* sp. nov. is proposed.

Materials and Methods

Bacterial Strains

Strain GR5^T was isolated on R2A agar (BD Difco) plates after incubation at 25°C for 3 days, and then subcultured on the R2A agar at 25°C for 48–72 h. The strain was preserved at −80°C in an R2A broth with 20% (v/v) glycerol or by lyophilization. Two closely related strains, *R. texasensis* A62-14B^T and *R. tangshanensis* JA3-B52^T, were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) and used as the reference strains for the phenotypic and genotypic tests.

Morphological, Physiological, and Biochemical Characteristics

The cell morphology was observed using phase-contrast microscopy (DM 2000; Leica) during the lag, exponential, and stationary phases of growth. The motility was tested using the hanging drop and semi-solid agar methods. A Spot Test Flagella Stain (BD Difco) was used for flagellum staining. A Gram Stain Set S kit (BD Difco) and the Ryu non-staining KOH method [21] were adopted to test the Gram reaction. The colony morphology was observed on an R2A agar using a stereoscopic microscope (SMZ 800; Nikon).

The pH range for growth was determined by measuring the optical densities (wavelength 600 nm) of R2A broth cultures. Prior to sterilization, the pH was adjusted to 3.0–10.0 (at intervals of 1.0 pH unit) using appropriate biological buffers [6]. Verification of the pH values after autoclaving revealed only minor changes. Additionally, the temperature range for growth was determined on R2A agar at 4, 10, 15, 20, 25, 30, 35, 40, 45, and 50°C. To investigate the tolerance to NaCl, an R2A broth was prepared based on a combination of the BD Difco medium and NaCl concentrations of 0%, 0.5%, 1.0%, 1.5%, and 2.0%–6.0% at intervals of 1.0% (w/v). Growth under anaerobic conditions was determined after incubating strain GR5^T in an Oxoid AnaeroGen system.

Standard approaches were used to examine strain GR5^T and the two reference strains for catalase, oxidase, DNase, urease, and lipase (corn oil) activities, along with the hydrolysis of starch, casein, gelatin, agar, chitin, lecithin, and Tweens 20, 40, 60, and 80 [8, 26]. The antimicrobial activity was previously examined and reported by Chen *et al*. [4]. Additional biochemical tests were performed using API ZYM and API 20NE kits (bioMérieux), and the carbon source utilization was evaluated using a GN2 microplate (Biolog). All the commercial phenotypic tests were performed according to the manufacturers' recommendations, and the results of the API 20NE kit and GN2 microplate were read after 72 h at 30°C.

The sensitivities to antibiotics of strain GR5^T and the two reference strains were tested using the disc diffusion method after spreading cell suspensions (0.5 McFarland) on R2A agar (BD Difco) plates. The discs (Oxoid) contained the following antibiotics: ampicillin (10 μg), chloramphenicol (30 μg), gentamicin (10 μg), kanamycin (30 μg), nalidixic acid (30 μg), novobiocin (30 μg), penicillin G (10 μg), rifampicin (5 μg), streptomycin (10 μg), erythromycin (15 μg), sulfamethoxazole (23.75 μg), trimethoprim (1.25 μg), and tetracycline (30 μg). The diameter of the antibiotic disc was 8 mm. The effects of the antibiotics on cell growth were assessed after 3 days of incubation at 30°C. A strain was considered susceptible when the diameter of the inhibition zone was >13 mm, intermediate at 10–12 mm, and resistant at <10 mm, as described by Nokhal and Schlegel [20].

Determination of Cellular Fatty Acids, Isoprenoid Quinones, and DNA G+C Content

After cultivating the strains on R2A agar at 30°C for 2 days, the fatty acid methyl esters were prepared, separated, and identified according to the instructions of a Microbial Identification System (Microbial ID; [25]). In addition, the isoprenoid quinones were extracted and purified according to the method of Collins [7], and then analyzed by HPLC. The DNA G+C content of strain GR5^T was determined by HPLC according to Mesbah *et al*. [19].

16S rRNA Gene Sequencing and Phylogenetic Analysis

The 16S rRNA gene sequence was analyzed as previously described by Chen *et al.* [3] using the software packages BioEdit [12] and MEGA version 3.1 [16], after multiple alignments of the data using CLUSTAL_X [27]. An almost-complete 16S rRNA gene sequence was then compared against 16S rRNA gene sequences available from the EzTaxon server [5], Ribosomal Database Project [17], and GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/). The distances (corrected according to Kimura's two-parameter model; [14]) were calculated and clustering was performed using the neighbor-joining method [24]. The maximum-likelihood [10] and maximum-parsimony [15] trees were generated using the treeing algorithms contained in the PHYLIP software package [11]. In each case, the bootstrap values were calculated based on 1,000 replications.

DNA–DNA Hybridization

The DNA–DNA hybridization experiments between strain GR5^T and *R. texasensis* A62-14B^T and *R. tangshanensis* JA3-B52^T were carried out using the method of Ezaki *et al.* [9]. The signal produced by self-hybridization was taken as 100%, and the percentage homology values were calculated from duplicate samples.

Results and Discussion

Morphological, Physiological, and Biochemical Characteristics

Strain GR5^T was Gram-negative, aerobic, rod-shaped, motile by means of a polar single flagellum (Fig. 1), and exhibited antimicrobial activity. The colonies on the R2A agar were greenish-yellow, round, and convex with entire margins. Growth occurred at 10–40°C (optimum, 35°C), pH 7.0–8.0 (optimum pH 8.0), and with 0–2.0% NaCl (optimum, 0.5–1.0%). The phenotypic characterization is listed in the species description in Tables 1 and 2.

The chemotaxonomy analysis indicated that strain GR5^T contained Q-8 as the major respiratory quinone. According to the fatty acid profiles determined for strain GR5^T, *R. texasensis* A62-14B^T, and *R. tangshanensis* JA3-B52^T, the fatty acid profile of strain GR5^T was similar to those of the other two *Rheinheimera* species, although differences

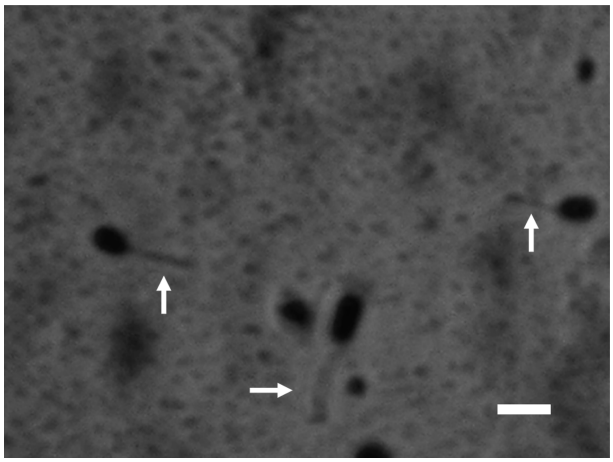

Fig. 1. Phase-contrast photomicrograph of *R. aquatica* GR5^T. Arrows represent the flagellar position. Bar, 2.0 μm.

were found in the proportions of certain components (Table 3). The major fatty acids of strain GR5^T were $C_{16:1}\omega7c$ (36.3%), $C_{16:0}$ (16.6%), $C_{12:0}$ 3-OH (12.5%), and $C_{18:1}\omega7c$ (9.1%).

Phylogenetic Analysis Based on 16S rRNA Gene Sequences

An almost-complete 16S rRNA gene sequence (1,406 bp) of strain GR5^T was obtained. A 16S rRNA gene sequence analysis then indicated that strain GR5^T belonged to the genus *Rheinheimera* in the class *Gammaproteobacteria* and formed a distinct subline within the genus *Rheinheimera* in a neighbor-joining tree (Fig. 2). The phylogenetic trees obtained using the maximum-likelihood and maximum-parsimony methods were similar in their overall topologies, and sequence similarity calculations (over 1,400 bp) indicated that strain GR5^T was closely related to *Rheinheimera*

Table 1. Differential characteristics of *Rheinheimera aquatica* strain GR5^T and related *Rheinheimera* species.

Characteristic	*R. aquatica* GR5^T	*R. texasensis* A62-14B^T	*R. tangshanensis* JA3-B52^T
Colony pigmentation	Greenish yellow	None	None
Growth on			
Nutrient agar	+	-	+
Tryptic soy agar	+	-	+
Luria–Bertani agar	+	-	+
Temperature for growth (°C):			
Range	10–40	20–40	10–40
Optimum	35	30–40	30
NaCl concentration for growth (%, w/v):			
Range	0–2.0	0–0.5	0–3.0
Optimum	0.5–1.0	0	1.5
Antimicrobial activity	+	-	-
Nitrate reduction	+	+	-
Hydrolysis of			
Tween 20	+	-	+
Tween 60	+	-	+
Lecithin	+	-	-
Assimilation of (API 20NE)			
Arabinose	-	+	+
Maltose	+	-	+
Malate	-	-	+
Enzymatic activities (API ZYM):			
C4 esterase	-	+	+
Cystine arylamidase	-	+	-
α-Chymotrypsin	+	+	-
Naphthol-AS-BI-phosphohydrolase	-	+	+
DNA G+C content (mol%)	51.9	48.2	47.0

All data are from this study, except G+C content of *R. texasensis* [18] and *R. tangshanensis* [31]. +, Positive reaction; -, negative reaction.

All strains are Gram-negative, aerobic, rod-shaped, motile, positive for hydrolysis of starch, casein, DNA, aesculin, gelatin, and Tweens 40 and 80, plus oxidase, catalase, β-galactosidase, alkaline phosphatase, C8 esterase lipase, leucine arylamidase, trypsin, acid phosphatase, and *N*-acetyl-β-glucosaminidase activities, and assimilation of glucose and *N*-acetyl-D-glucosamine.

All strains are negative for hydrolysis of lipid and chitin, indole production, D-glucose fermentation, plus arginine dihydrolase, urease, C14 lipase, valine arylamidase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, α-mannosidase, and α-fucosidase activities, and assimilation of mannose, mannitol, gluconate, caprate, adipate, citrate, and phenyl acetate. All strains are sensitive to ampicillin, chloramphenicol, gentamicin, kanamycin, nalidixic acid, novobiocin, penicillin G, rifampicin, streptomycin, sulfamethoxazole/trimethoprim, and tetracycline.

Table 2. GN2 microplate oxidation data that can be used to distinguish strain GR5^T from *Rheinheimera texasensis* A62-14B^T and *Rheinheimera tangshanensis* JA3-B52^T.

Substrate	*R. aquatica* GR5^T	*R. texasensis* A62-14B^T	*R. tangshanensis* JA3-B52^T
α-Cyclodextrin	-	+	+
Dextrin	+	-	+
L-Arabinose	-	+	+
D-Cellobiose	-	-	+
D-Fructose	-	-	+
L-Fucose	-	-	+
D-Galactose	-	+	+
Gentiobiose	+	-	+
α-D-Lactose	-	+	+
Lactulose	-	-	+
Maltose	+	-	+
D-Melibiose	-	-	+
β-Methyl-D-glucoside	-	-	+
D-Raffinose	-	+	+
D-Sorbitol	-	+	+
D-Trehalose	-	+	+
Turanose	-	+	+
Pyruvic acid methyl ester	-	-	+
Acetic acid	+	-	+
β-Hydroxybutyric acid	-	-	+
α-Ketobutyric acid	-	-	+
α-Ketoglutaric acid	-	-	+
D,L-Lactic acid	-	+	-
Propionic acid	-	-	+
Succinamic acid	-	-	+
L-Alaninamide	-	-	+
D-Alanine	-	+	-
L-Alanine	+	-	-
L-Glutamic acid	-	+	-
Hydroxy-L-proline	-	+	-
L-Leucine	-	-	+
L-Ornithine	-	+	-
L-Phenylalanine	-	+	-
L-Proline	-	+	+
D-Serine	-	+	-

+, Positive reaction; -, negative reaction. The following compounds in the GN2 microplate were utilized as sole carbon sources by all strains: glycogen, Tweens 40 and 80, *N*-acetyl-D-glucosamine, α-D-glucose, sucrose, and D-galacturonic acid.

texasensis A62-14B^T [18] (98.1% 16S rRNA gene sequence similarity) and *Rheinheimera tangshanensis* JA3-B52^T [31] (97.5% 16S rRNA gene sequence similarity). Lower sequence similarities (<96.5%) were also found with representative members of the other genera listed in Fig. 2.

DNA–DNA Hybridization

Based on the results of the 16S rRNA gene sequence analysis and subsequent phylogenetic analysis, DNA–DNA hybridization experiments were performed between strain GR5^T and *R. texasensis* A62-14B^T, and strain GR5^T and *R. tangshanensis* JA3-B52^T, in order to determine the possibility of a novel species. The level of DNA–DNA relatedness of strain GR5^T to *R. texasensis* A62-14B^T and to *R. tangshanensis* JA3-B52^T was 26.5±7.6% and 12.5±5.8%, respectively. Thus, since the recommended DNA–DNA relatedness threshold for the definition of a species is 70% [27, 29], these results indicate that strain GR5^T does not belong to any known species of the genus *Rheinheimera.*

Taxonomic Conclusions

The phenotypic examination revealed many common traits between the novel strain and its closest relatives, *R. texasensis* A62-14B^T and *R. tangshanensis* JA3-B52^T. However, strain GR5^T could still be clearly differentiated from *R. texasensis* A62-14B^T by its colony pigmentation,

Table 3. Cellular fatty acid composition of *Rheinheimera aquatica* strain GR5^T and related *Rheinheimera* species.

Fatty acid	*R. aquatica* GR5^T	*R. texasensis* A62-14B^T	*R. tangshanensis* JA3-B52^T
iso-$C_{10:0}$	1.4	-	-
$C_{10:0}$	1.5	3.5	2.5
$C_{11:0}$ 3-OH	2.1	-	2.5
$C_{12:0}$	2.9	3.0	-
$C_{12:0}$ 3-OH	12.5	9.5	9.6
$C_{14:0}$	1.9	3.2	-
$C_{14:0}$ 2-OH	1.1	-	-
$C_{15:1}$ $\omega 8c$	1.7	-	3.1
iso-$C_{16:0}$	1.2	-	1.2
$C_{16:0}$	16.6	23.9	15.8
$C_{16:1}$ $\omega 7c$	36.3	46.3	32.5
anteiso-$C_{17:0}$	-	-	2.3
$C_{17:0}$	1.9	-	2.6
$C_{17:1}$ $\omega 8c$	4.8	-	8.5
$C_{18:0}$	1.7	4.2	-
$C_{18:1}$ $\omega 7c$	9.1	6.4	10.8

All the data are from this study. All the strains were grown on R2A agar at 30°C for 2 days. Values are percentages of the total fatty acids; -, not detected. Fatty acids amounting to <1% are not shown.
For unsaturated fatty acids, the position of the double bond is located by counting from the methyl (ω) end of the carbon chain. The *cis* isomer is indicated by the suffix *c*.

ability to grow well in rich media, higher NaCl range for growth, antimicrobial activity, ability to hydrolyze Tweens 20 and 60, and lecithin, ability to assimilate maltose and inability to assimilate arabinose, and the absence of C4 esterase, cystine arylamidase, and naphthol-AS-BI-phosphohydrolase activities. The phenotypic properties that distinguished strain GR5^T from *R. tangshanensis* JA3-B52^T included its colony pigmentation, antimicrobial activity, its ability to reduce nitrate to nitrite and hydrolyze lecithin, its inability to assimilate arabinose and malate, the presence of α-chymotrypsin activity, and the absence of C4 esterase and naphthol-AS-BI-phosphohydrolase activities.

Strain GR5^T was also found to be Gram-negative, rod-shaped, motile by means of a single polar flagellum, and catalase- and oxidase-positive. Its growth was aerobic, chemoheterotrophic, and occurred with 0–2.0% NaCl (optimum, 0.5–1.0%), plus the predominant fatty acids were $C_{16:1}\omega 7c$, $C_{16:0}$, and $C_{18:1}\omega 7c$. As such, the characteristics of strain GR5^T were all consistent with the description of the genus *Rheinheimera* [1]. Notwithstanding, on the basis of the data obtained from the 16S rRNA gene sequence comparisons, strain GR5^T occupies a distinct position within the genus *Rheinheimera*, which is also supported by its unique combination of chemotaxonomic and biochemical characteristics. Thus, since the phylogenetic and phenotypic data clearly indicate that strain GR5^T constitutes a novel member of the genus *Rheinheimera*, the name *Rheinheimera aquatica* sp. nov. is proposed for this taxon.

Amended Description of Genus *Rheinheimera*

Rheinheimera (Rhein.hei'me.ra. N.L. n. *Rheinheimera* named after the German marine microbiologist Gerhard Rheinheimer, in recognition of his work on marine and estuarine bacteria).

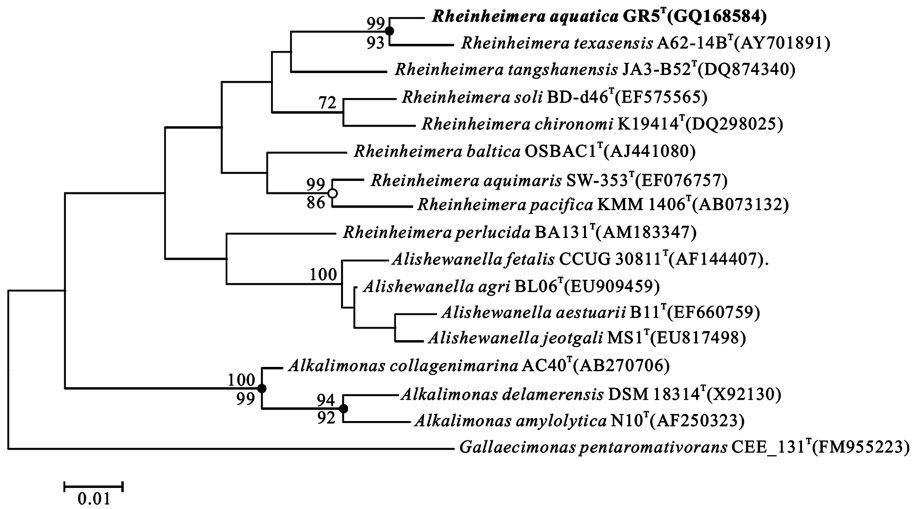

Fig. 2. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the positions of *R. aquatica* GR5^T and related taxa in class *Gammaproteobacteria*.
Numbers at nodes are bootstrap percentages (>70%) based on neighbor-joining (above nodes) and maximum-parsimony (below nodes) tree-making algorithms. Filled circles indicate tree branches that were also recovered when using maximum-likelihood and maximum-parsimony tree-making algorithms. Open circles indicate nodes that were also recovered in tree generated using maximum-parsimony algorithm. *Gallaecimonas pentaromativorans* CEE_131^T was used as the outgroup. Bar, 0.01 substitutions per nucleotide position.

The descriptions of the genus *Rheinheimera* are as given by Brettar *et al.* [1], Yoon *et al.* [30], and Zhang *et al.* [31], but with the following change: the G+C content of the DNA is 47.0–51.9 mol%.

Description of *Rheinheimera aquatica* sp. nov.

Rheinheimera aquatica (a.qua'ti.ca. L. fem. adj. *aquatica* living, growing, or found in water, aquatic).

The cells are Gram-negative, aerobic, rod-shaped, motile by means of a single polar flagellum, and exhibit antimicrobial activity. After 48 h of incubation on R2A agar at 30°C, the mean cell size is 0.5–1.0 μm in diameter and 1.5–2.0 μm in length. The colonies on R2A agar are greenish-yellow, round, and convex with entire margins. The colony size is approximately 2–3 mm in diameter on R2A agar after 48 h of incubation at 25°C. Growth occurs at 10–40°C (optimum, 35°C), pH 7.0–8.0 (optimum pH 8.0), and with 0–2.0% NaCl (optimum, 0.5–1.0%). Positive for oxidase and catalase activities, and the hydrolysis of starch, DNA, casein, and Tweens 20, 40, 60, and 80. Negative for lipase and urease activities, and hydrolysis of chitin and agar. In API 20NE tests, positive reactions for nitrate reduction, aesculin hydrolysis, gelatin hydrolysis, β-galactosidase activity, and the assimilation of D-glucose, *N*-acetyl-glucosamine, and maltose, but negative reactions for indole production, D-glucose fermentation, arginine dihydrolase and urease activities, and the assimilation of arabinose, mannose, mannitol, gluconate, caprate, adipate, malate, citrate, and phenyl acetate. In tests with an API ZYM kit, alkaline phosphatase, C8 esterase lipase, leucine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, and *N*-acetyl-β-glucosaminidase activities are present, whereas C4 esterase, C14 lipase, valine arylamidase, cystine arylamidase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, α-mannosidase, and α-fucosidase activities are absent. The following compounds are utilized as sole carbon sources in a GN2 microplate: dextrin, glycogen, Tweens 40 and 80, *N*-acetyl-D-glucosamine, gentiobiose, α-D-glucose, maltose, sucrose, acetic acid, D-galacturonic acid, and L-alanine. No other substrates in the GN2 microplate are utilized. Sensitive to ampicillin, chloramphenicol, gentamicin, kanamycin, nalidixic acid, novobiocin, penicillin G, rifampicin, streptomycin, erythromycin, sulfamethoxazole/trimethoprim, and tetracycline. The DNA G+C content is 51.9 mol%. The major fatty acids are $C_{16:1}\omega7c$ (36.3%), $C_{16:0}$ (16.6%), $C_{12:0}$ 3-OH (12.5%), and $C_{18:1}\omega7c$ (9.1%). The major respiratory quinone is Q-8.

The type strain is GR5^T (=BCRC 80081^T=LMG 25379^T) isolated from a water sample from a freshwater culture pond for soft-shell turtles located in the Pingtung countryside, southern Taiwan. The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Rheinheimera aquatica* strain GR5^T is GQ168584.

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