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Paracoccus aurantiacus sp. nov., isolated from shallow-sea hydrothermal systems off Kueishantao Island

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Abstract

An orange-pigmented, short-rod-shaped, aerobic and non-motile bacterial strain, designated TK008^T, was isolated from the shallow-sea hydrothermal systems off Kueishantao Island in Taiwan, China, and it was studied by using a polyphasic taxonomic approach. Cells were Gram-stain-negative, and catalase- and oxidase-positive. Strain TK008^T exhibited highest 16S rRNA gene sequence similarity of 97.1% to *Paracoccus pacificus* F14^T. The phylogenetic trees based on 16S rRNA gene sequences showed that strain TK008^T was a member of the genus *Paracoccus*. Digital DNA–DNA hybridization values between strain TK008^T and two closely related species (*Paracoccus zhejiangensis* and *Paracoccus tegillarcae*) were 20.6 and 20.9%. The average nucleotide identity values of strain TK008^T compared with *P. zhejiangensis* and *P. tegillarcae* were 75.2 and 74.6% respectively. The major isoprenoid quinone was ubiquinone-10. The predominant fatty acids (>10 %) were summed feature 8 ($C_{18:1}\omega6c$ and/or $C_{18:1}\omega7c$). The polar lipids were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, three unidentified phospholipids, two unidentified lipids and an unidentified glycolipid. The DNA G+C content of strain TK008^T from genomic sequence data was 62.54mol%. On the basis of polyphasic analysis, strain TK008^T represents a novel species, for which the name *Paracoccus aurantiacus* sp. nov. is proposed. The type strain is TK008^T (=CGMCC 1.13898^T=JCM 33630^T).

The genus *Paracoccus,* which was first proposed by Davis *et al.* [1], is a clade of *Rhodobacteraceae* within the class *Alphaproteobacteria*. At the time of writing, the genus *Paracoccus* was represented by more than 56 species with validly published names (http://www.bacterio.net/paracoccus.html). Members of the genus *Paracoccus* have been isolated from different environments, such as mangrove [2], air [3], saline–alkaline soil [4], freshwater spring [5], tidal flat [6] and sediment [7]. They are chemotaxonomically characterized as having ubiquinone-10 (Q-10) as the major isoprenoid quinone, and $C_{18:1}\omega7c$ as the predominant fatty acid [5]. During the course of biodiversity investigation of a shallow-sea hydrothermal system, a *Paracoccus*-like bacterium was isolated, designated strain TK008^T. Here we use a polyphasic taxonomic approach to clarify the taxonomic position of strain TK008^T, and show that it represents a novel species of the genus *Paracoccus*.

A seawater sample was obtained by scuba divers in June 2018, collected from 5 m above the yellow vent in the shallow-sea hydrothermal system located near Kueishantao Island (24.834° N 121.962° E), Taiwan, China. The *in situ* temperature, salinity and pH were 25.9 °C, 34.4% and 6.3, respectively. Seawater supplemented with 15% (v/v) glycerol was diluted 10 times and spread on seawater yeast-peptone-glucose agar (5.0 g yeast extract, 10.0 g peptone, 20.0 g NaCl, 10.0 g glucose, 1.0 litre distilled water). After incubation at 28 °C for 6 days, a light orange-pigmented bacterium, strain TK008^T, was observed. Subsequently strain TK008^T was cultivated on marine agar 2216 (Haibo) at 28 °C. The culture was stored at −80 °C in marine broth 2216 (Haibo) with 15 % (v/v) glycerol for long-term preservation.

Genomic DNA was extracted using a GENEray Bacteria DNA kit (Generay) according to the manufacturer's instruction. DNA concentration and purity were assessed with a Nanodrop 2000 spectrophotometer (Thermo Scientific). Whole-genome sequencing of strain TK008^T was carried out on an Illumina HiSeq instrument (Illumina). The reads

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Keywords: *Paracoccus aurantiacus*; *Paracoccus*; Kueishantao Island; polyphasic taxonomic approach.
Abbreviations: ANI, average nucleotide identity; CGMCC, the China General Microbiological Culture Collection; DDH, DNA–DNA hybridization; HPLC, high-performance liquid chromatography; KCTC, the Korean Collection for Type Cultures; NCBI, the National Center for Biotechnology Information; PAGE, polyacrylamide gel electrophoresis; TLC, thin-layer chromatography.
The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence and the whole genome shotgun sequence of strain TK008^T are MN708965 and VOPL00000000, respectively.
Four supplementary figures are available with the online version of this article.

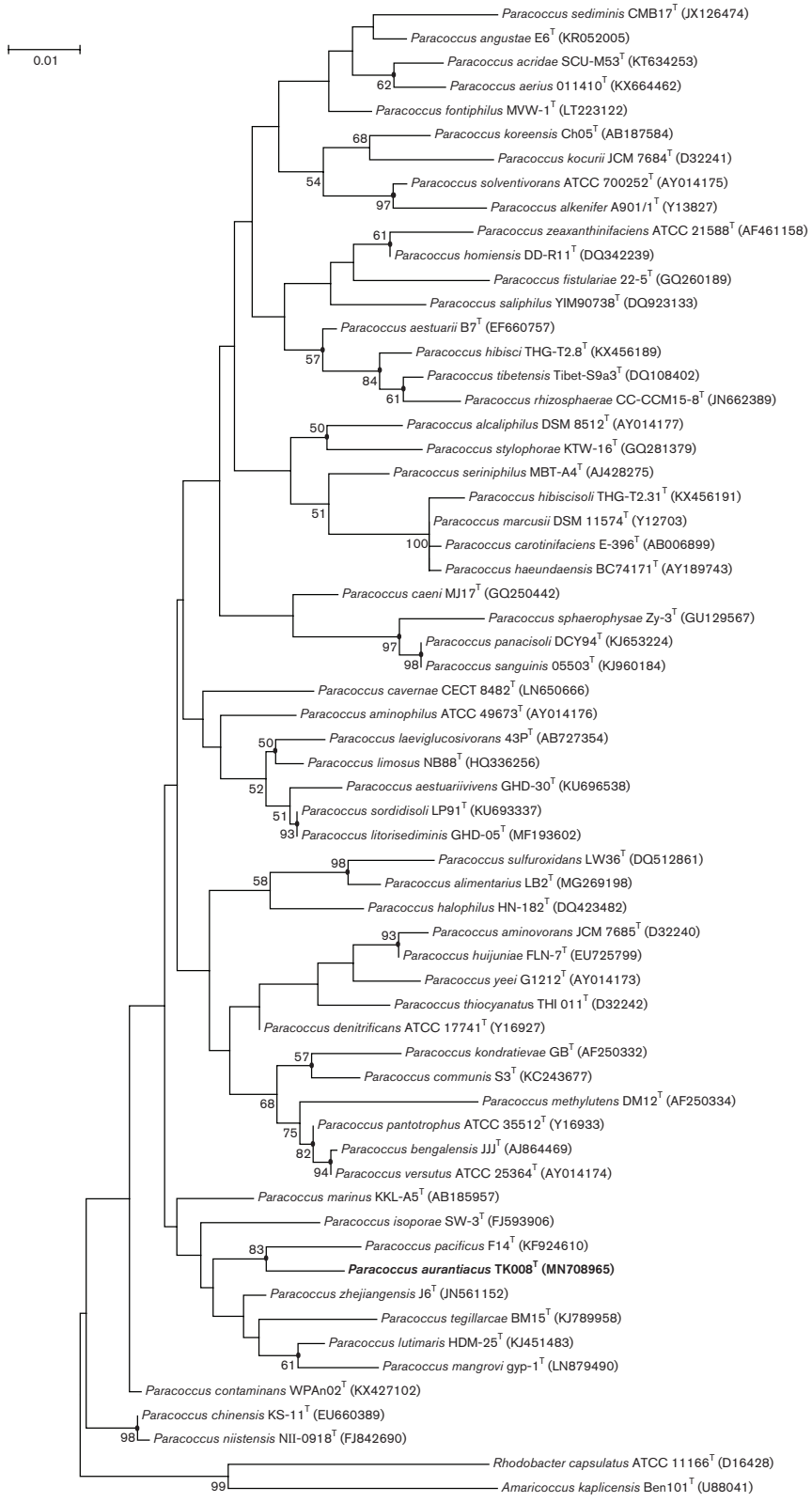

Fig. 1. Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences showing the relationships between strain TK008^T and members of the genus *Paracoccus*. *Rhodobacter capsulatus* ATCC 11166^T and *Amaricoccus kaplicensis* Ben101^T were used to root the tree. Bootstrap values (≥50 %) from the maximum-likehood approach are shown at branch points (expressed as percentages of 1000 replications). Filled circles indicate that the corresponding nodes were also recovered in the trees generated with the neighbour-joining and maximum-parsimony algorithms. Bar, 0.01 substitutions per nucleotide position.

Table 1. Cellular fatty acid composition (%) of strain TK008^T and of closely related type strains

Strains: 1, TK008^T 2, *P. pacificus* F14^T (=CGMCC 1.12755^T); 3, *P. caeni* MJ17^T (=KCTC 22480^T); 4, *P. lutimaris* HDM-25^T (=KCTC 42007^T). All the data were obtained in this study. Fatty acids with ≥1.0% abundance are shown (–, not detected).

Fatty acid	1	2	3	4
Straight-chain				
$C_{16:0}$	4.9	7.1	5.7	11.6
$C_{18:0}$	10.0	6.5	8.9	3.1
Unsaturated				
$C_{19:0}$ cyclo ω8*c*	1.0	13.1	8.8	–
$C_{17:1}$ω9*c*	1.0	0.6	1.0	0.6
$C_{18:1}$ω9*c*	1.2	–	–	–
Hydroxy				
$C_{10:0}$ 3-OH	4.5	7.4	4.2	6.1
Branched-chain				
iso-$C_{14:0}$	–	–	–	2.9
iso-$C_{15:0}$	–	–	–	2.4
iso-$C_{16:0}$	–	–	–	5.2
anteiso-$C_{15:0}$	–	–	–	1.6
iso-$C_{16:1}$ G	–	–	–	1.7
Summed features*				
2	–	–	2.4	1.4
8	74.9	64.0	67.9	61.5

*Summed features are groups of two or three fatty acids that cannot be separated by GLC with the MIDI System. Summed feature 2, $C_{14:0}$ 3-OH and/or iso-$C_{16:1}$ I; summed feature 8, $C_{18:1}\omega 6c$ and/or $C_{18:1}\omega 7c$.

were quality-checked and then assembled using velvet, gap-filled with SSPACE and GapFiller [8–12]. The draft genome sequence of strain TK008^T resulted in 42 contigs. Contigs varied in length from 281 to 1134541 bp (N50=435274). The accession number for the whole genome sequence of strain TK008^T is VOPL00000000. The DNA G+C content of strain TK008^T was 62.54 mol%. The genome size was 3788400 bp. The genome contains a total of 3643 protein coding genes, three complete rRNA genes, two partial rRNA genes, 45 tRNA genes and four other non-coding RNA genes.

A sequence inculding the 16S rRNA gene of strain TK008^T was amplified by PCR using two primers, 5′-CTCTACCAACT-GAGCTAAGG-3′ and 5′-TTGACGGCGCCGAATATGTA-3′. PCR amplification consisted of a hot start at 94 °C for 5 min, and 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 90 s, followed by an elongation at 72 °C for 10 min. The PCR product was purified by PAGE and Sanger-sequenced. The PCR-amplified 16S rRNA gene sequence (1459 bp) showed an identical nucleotide sequence with the 16S rRNA gene from the genome of strain TK008^T. The 16S rRNA gene sequence of strain TK008^T was compared with sequences available in the EzBioCloud database [13] and NCBI database to determine its closest matches. Strain TK008^T exhibited highest 16S rRNA gene sequence similarity of 97.1% to *Paracoccus pacificus* F14^T and less than 97% similarity to all other type strains of the genus *Paracoccus*. Phylogenetic trees were reconstructed by the neighbour-joining [14], maximum-parsimony [15] and maximum-likelihood [16] algorithms in the MEGA 7 software [17] after sequence alignment was performed via CLUSTALW. All positions containing missing data were eliminated. Phylogenetic distance matrices were estimated by the Kimura two-parameter model [18]. In each case, bootstrap values were calculated based on 1000 replications to evaluate the phylogenetic tree topology. In the phylogenetic trees, strain TK008^T clustered with *P. pacificus* F14^T (Figs 1, S1 and S2, available in the online version of this paper). *P. pacificus* F14^T (97.1% similarity), *Paracoccus caeni* MJ17^T (96.8%) and *Paracoccus lutimaris* HDM-25^T (96.6%) were chosen as reference strains. *P. pacificus* F14^T (=CGMCC 1.12755^T) was obtained from the China General Microbiological Culture Collection Center (CGMCC). *P. caeni* MJ17^T(=KCTC 22480^T) and *P. lutimaris* HDM-25^T (=KCTC 42007^T) were both obtained from the Korean Collection for Type Cultures (KCTC).

The genomic relatedness between strain TK008^T and the closely related strains was estimated by calculating average nucleotide identity (ANI) and digital DNA–DNA hybridization (DDH). Complete genomes of *Paracoccus zhejiangensis* (GenBank accession number, CP025430.1) and *Paracoccus tegillarcae* (GenBank accession number, CP025408) were available in the public database. ANI values of strain TK008^T compared with *P. zhejiangensis* and *P. tegillarcae* were 75.2 and 74.6%, respectively, by using the ANI calculator (https://www.ezbiocloud.net/tools/ani) [19], clearly below the suggested ANI value (95–96%) for demarcating genomic species [20]. The estimated digital DDH values between strain TK008^T and its closest related species (*P. zhejiangensis* and *P. tegillarcae*) were 20.6 and 20.9% by using the genome-to-genome distance calculator (GGDC2.1) (http://ggdc.dsmz.de/ggdc.php) [21] with the alignment method of BLAST+ and formula 2, as recommended. This was below the standard cut-off value (70%) [22], which confirmed that strain TK008^T represents a novel species of the genus *Paracoccus*.

Cell growth was studied on a rich organic (RO) medium [23], Luria–Bertani and marine agars under aerobic conditions at 28 °C. Cells formed orange, convex circular colonies after incubation for 2 days. Cells used for tests were all at exponential and stationary phases of growth. NaCl requirement and tolerance were tested in RO medium at 28 °C with NaCl concentrations ranging from 0 to –10% (0, 0.5 and 1.0–10.0%, intervals of 1%). The range of temperature (4, 10, 15, 23, 28, 30, 35, 37, 40, 45 °C) for growth was tested in marine broth 2216. Growth at different pH was evaluated in RO medium (intervals of 1 pH unit, pH 3–12, Na_2HPO_4/citric acid for pH 3–8, glycine/NaOH for pH 9–10, Na_2HPO_4/NaOH for pH 11–12) at 28 °C. To achieve sterility, the medium for pH

Table 2. Characteristics that distinguish strain TK008^T from the type strains of species of the genus *Paracoccus*

Strains: 1, TK008^T; 2, *P. pacificus* F14^T (=CGMCC 1.12755^T); 3, *P. caeni* MJ17^T (=KCTC 22480^T); 4, *P. lutimaris* HDM-25^T (=KCTC 42007^T). +, Positive; –, negative; ND, no data available. Data for the reference strains were obtained from this study unless otherwise indicated. Values in parentheses indicate optimum conditions.

Characteristic	1	2	3	4
Source of isolation	Seawater	Sediment*	Sludge†	Sediment‡
Colony pigmentation	Orange	Pale yellow	Pale yellow	Pale yellow
Growth temperature (°C)	15–40 (28)	4–40 (28-30)*	25–30 (30)†	10–37 (30)‡
NaCl requirement (%)	0–9 (4–5)	0–7 (1–2)*	0–6 (3–4)†	0–7 (2–3)‡
pH requirement	4–11 (6–7)	6–12 (7–8)*	6–9 (6.5–7)†	5–ND (7–8)‡
Enzyme activity (API ZYM)				
α-Chymotrypsin	–	+	+	–
β-Glucosidase	+	–	–	–
N-Acetyl-*β*-glucosaminidase				
Hydrolysis of (API 20NE):				
Aesculin	+	–	–	+
p-Nitrophenyl-*β*-D- galactopyranoside	+	–	–	–
Utilization of (GEN III):				
D-Aspartic acid	–	–	+	–
Dextrin	+	–	+	+
Formic acid	–	–	+	–
myo-Inositol	+	–	+	+
N-Acetyl-D-glucosamine	+	–	+	–
Pectin	–	–	–	+
L-Serine	–	–	+	+
DNA G+C content (mol%)	62.54	61.4*	58.7†	65.9‡

*Data taken from Zhang *et al.* [31].
†Data taken from Lee *et al.* [32].
‡Data taken from Jung *et al.* [7].

range testing of strain TK008^T was filtered through 0.22 µm pore-size polycarbonate filters (Millipore).

Gram staining was performed using a Gram stain kit (Haibo). Cell morphology and size were studied by transmission electron microscopy (Tecnai Spirit G2; FEI). A transmission electron micrograph of cells of strain TK008^T is shown in Fig. S3. Motility and growth under anaerobic conditions were determined by the semi-solid puncture method [24].

Activities of catalase and oxidase were determined according to the methods of Smibert and Krieg [25]. The oxidation of organic substrates was assessed by using Biolog GEN III microplates with cells suspended in IF-A (Biolog). Sodium thioglycollate (20 µl) at 7.66% concentration was added to the IF-A in advance. Other biochemical and enzymatic characterizations of strain TK008^T were investigated using the API ZYM and API 20NE (bioMérieux) systems according to the manufacturer's instructions.

For cellular fatty acid analysis, cell masses of strain TK008^T, *P. caeni* and *P. lutimaris* were harvested from marine agar plates after cultivation for 42 h at 28 °C. The fatty acid profiles of *P. pacificus* was determined using cells grown on marine agar at 28 °C for 2 days. The analysis was carried out by the method described by Komagata and Suzuki [26]. The Microbial Identification System (MIDI) was Sherlock version 6.0 and the library was TSBA6 6.00. The fatty acids (≥1.0% of the total) of strain TK008^T were summed feature 8 ($C_{18:1}$ω6*c* and/or $C_{18:1}$ω7*c*, 74.9%), $C_{18:0}$ (10.0%), $C_{16:0}$ (4.9%), $C_{10:0}$ 3-OH (4.5%), $C_{18:1}$ω9*c* (1.2%), $C_{19:0}$ cyclo *ω*8*c* (1.0%) and $C_{17:1}$ω9*c* (1.0%). Isoprenoid quinones were extracted from 400 mg of freeze dried cell material, then separated into their different

classes by TLC on silica gel [27, 28], and analysed by HPLC. The isoprenoid quinones detected in strain TK008^T were Q-10 (94.6%), Q-9 (4.2%) and Q-8 (1.2%). Polar lipids were extracted as described by Kates [29] using a chloroform/methanol system and analysed using two-dimensional TLC. Merck silica gel 60 F_{254} aluminium-backed thin-layer plates were used in TLC analysis. The plate dotted with sample was subjected to two-dimensional development, with the first solvent of chloroform/methanol/water (65:25:4, by vol.) followed by second solvent of chloroform/methanol/acetic acid/water (85:12:15:4, by vol.). Polar lipids were identified by spraying with molybdatophosphoric acid (for total lipids), phosphomolybdic acid (for phospholipids), ninhydrin (for aminolipids) and naphthol–sulfuric acid reagent (for glycolipids) [30]. The polar lipids of strain TK008^T consisted of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, three unidentified phospholipids, two unidentified lipids and an unidentified glycolipid (Fig. S4).

The fatty acid profiles of strain TK008^T, *P. pacificus* F14^T, *P. caeni* MJ17^T and *P. lutimaris* HDM-25^T were similar in that summed feature 8 ($C_{18:1}\omega6c$ and/or $C_{18:1}\omega7c$) were major fatty acids (Table 1). The major isoprenoid quinone of strain TK008^T and its closely related strains (*P. pacificus* F14^T [31] and *P. lutimaris* HDM-25^T [7]) was Q-10. In terms of the polar lipid composition, diphosphatidylglycerol, phosphatidylglycerol and an unidentified lipid were detected in strain TK008^T, *P. pacificus* F14^T [31] and *P. lutimaris* HDM-25^T [7]. An unidentified phospholipid was only found in strain TK008^T.

The morphological, physiological and biochemical characteristics of strain TK008^T are given in the species description. The results of the phylogenetic analysis and chemotaxonomic studies (predominant fatty acids and major isoprenoid quinone component) presented above support the view that strain TK008^T should be assigned to the genus *Paracoccus*. However, the low 16S rRNA gene sequence similarity and differences in physiological and biochemical properties between TK008^T and its closest related strains (Table 2) indicate that strain TK008^T represents a novel species of the genus *Paracoccus*, for which the name *Paracoccus aurantiacus* sp. nov. is proposed.

DESCRIPTION OF *PARACOCCUS AURANTIACUS* SP. NOV.

Paracoccus aurantiacus (au.ran.ti'a.cus. N.L. masc. adj. *aurantiacus* orange-coloured).

Cells are aerobic, non-motile, non-flagellated, short-rod-shaped, about 0.4–0.6 µm wide and 0.8–1.0 µm long. Cells are Gram-stain-negative, and catalase- and oxidase-positive. Cells form orange, convex circular colonies after incubation for 2 days on marine agar 2216 at 28 °C. Growth occurs at 15–40 °C (optimum, 28 °C), at pH 4–11 (optimum, 6–7) and in the presence of 0–9.0% (w/v) NaCl (optimum, 4.0–5.0%). In assays with API ZYM strips, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, *α*-glucosidase, *β*-glucosidase and *N*-acetyl-*β*-glucosaminidase are present, but lipase (C14), trypsin, *α*-chymotrypsin, *α*-galactosidase, *β*-galactosidase, *β*-glucuronidase, *α*-mannosidase and *α*-fucosidase are absent. Cells are positive for hydrolysis of aesculin and *p*-nitrophenyl-*β*-D-galactopyranoside, but negative for nitrate reduction, indole production, D-glucose fermentation, arginine dihydrolase, urease, and hydrolysis of gelatin in API 20NE test strips. The following substrates are oxidized as carbon sources in Biolog GEN III tests: acetic acid, acetoacetic acid, L-alanine, D-arabitol, cellobiose, dextrin, D-fructose, L-fucose, D-galactose, D-glucose 6-phosphate, L-aspartic acid, D-gluconic acid, L-glutamic acid, glycerol, *β*-hydroxy-DL-butyric acid, inosine, *α*-ketobutyric acid, L-lactic acid, D-malic acid, L-malic acid, maltose, D-mannose, *myo*-inositol, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, propionic acid, L-pyroglutamic acid, quinic acid, L-rhamnose, D-saccharic acid, D-sorbitol, sucrose, trehalose and turanose. The major isoprenoid quinone is Q-10 and the major fatty acids (>10%) are summed feature 8 ($C_{18:1}\omega6c$ and/or $C_{18:1}\omega7c$). The polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, three unidentified phospholipids, two unidentified lipids and an unidentified glycolipid.

The type strain, TK008^T (=CGMCC 1.13898^T=JCM 33630^T), was isolated from seawater in shallow-sea hydrothermal systems off Kueishantao Island, Taiwan, China. The DNA G+C content of the type strain is 62.54 mol% (from genome sequence data). The GenBank accession numbers for the 16S rRNA gene sequence and the whole genome shotgun sequence of strain TK008^T are MN708965 and VOPL00000000, respectively.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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