

Zhihengliuella somnathii sp. nov., a halotolerant actinobacterium from the rhizosphere of a halophyte *Salicornia brachiata*

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Two novel, Gram-stain-positive, rod-shaped, halotolerant bacteria, strains JG 03^T and JG 05 were isolated from the rhizosphere of *Salicornia brachiata*, an extreme halophyte. Comparative analyses of 16S rRNA gene sequences showed that they were closely related to members of the genus *Zhihengliuella*, with sequence similarities of 96.9–99.1 %. The sequence similarity of strains JG 03^T and JG 05 with each other was 99.4 %. DNA–DNA hybridization of JG 03^T and JG 05 with other species of the genus *Zhihengliuella* with validly published names showed reassociation values of 19.8 %–53.4 % and a value of 91.4 % between each other. The peptidoglycan type of both strains was A4 α and MK-9 and MK-10 were the predominant menaquinones. The predominant fatty acid in JG 03^T was anteiso-C₁₅:0 and anteiso-C₁₇:0. However, iso-C₁₅:0, anteiso-C₁₅:0 and anteiso-C₁₇:0 were the major fatty acids in strain JG 05. The DNA G + C content of strains JG 03^T and JG 05 was 70.0 and 70.1 mol%, respectively. In nutrient broth medium both strains grew at NaCl concentrations of up to 15 % (w/v). On the basis of chemotaxonomic characteristics and phylogenetic analyses, strains JG 03^T and JG 05 should be affiliated to the genus *Zhihengliuella*. Strains JG 03^T and JG 05 represent a novel species of the genus *Zhihengliuella* for which the name *Zhihengliuella somnathii* sp. nov. is proposed. The type strain is JG 03^T (=DSM 23187^T=IMCC 253^T).

The genus *Zhihengliuella* was proposed by Zhang *et al.* (2007). At the time of writing, the genus *Zhihengliuella* contains five species with validly published names: *Zhihengliuella halotolerans* (Zhang *et al.*, 2007), *Zhihengliuella alba* (Tang *et al.*, 2009), *Zhihengliuella salsuginis* (Chen *et al.*, 2010), *Zhihengliuella aestuarii* (Baik *et al.*, 2011) and *Zhihengliuella flava* (Hamada *et al.*, 2013).

The roots of *Salicornia brachiata*, an extreme halophytic plant is a potential source of novel bacteria (Schmid *et al.*, 2009; Jha *et al.*, 2012). Strains JG 03^T and JG 05 were isolated from roots of *S. brachiata*, collected from coastal marshy

swamps in Bhavnagar, Gujarat (21° 45' N 72° 14' E), India. The bacteria were isolated using N₂-free semisolid NFb (Döbereiner, 1995) and DYGS (Kirchhof *et al.*, 2001) medium following the method described previously (Gontia *et al.*, 2011). Strains JG 03^T and JG 05 grew in up to 4 % (w/v) NaCl on semisolid N₂-free NFb medium. Tolerance to NaCl was tested using nutrient broth supplemented with NaCl (1–15 %, w/v, at increments of 1 %, w/v). Growth of both strains (JG 03^T and JG 05) was observed in up to 15 % (w/v) NaCl; the optimum concentration of NaCl for growth was 4 % (w/v). The temperature range for growth was determined by keeping the bacterial culture (in nutrient broth) at different temperatures in an incubator shaker. The optimum growth temperature of the novel strains was 30 °C. However, these strains grew over a temperature range of 10–37 °C. The pH range and optimum pH for growth were tested using nutrient broth with the pH was

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Three supplementary figures and one supplementary table are available with the online Supplementary Material.

adjusted to 4–12 (intervals of one pH unit) using the following buffer systems: pH 4.0–5.0, 0.1 M citric acid/0.1 M sodium citrate; pH 6.0–8.0, 0.1 M KH_2PO_4 /0.1 M NaOH; pH 9.0–12.0, 0.1 M NaHCO_3 /0.1 M Na_2CO_3 . The bacteria grew over a pH range of 6–10 with optimum growth at pH 8.

Cell morphology was observed using scanning electron microscopy (LEO 1430VP, Oxford Instruments), according to Yumoto *et al.* (2001). The presence or absence of flagella was visualized using transmission electron microscopy (JEM 2100, JEOL) according to Näther *et al.* (2006). The genomic DNA of strains was isolated by following the method of Sambrook & Russell (2001). The 16S rRNA genes of each strain were amplified according to the method described by Weisburg *et al.* (1991). The purified PCR products were sequenced by Macrogen (Seoul, South Korea). Phylogenetic analysis was performed using MEGA version 6 (Tamura *et al.*, 2013) and neighbour-joining and maximum-likelihood methods were applied to infer the phylogenetic trees (Saitou & Nei, 1987). Bootstrap analysis was carried out (Felsenstein, 1985) and maximum composite likelihood algorithms were used for determination of the evolutionary distances (Tamura *et al.*, 2004). The highest sequence similarity, for both strains JG 03^T and JG 05, was 99.1 % with *Z. flava* DSM 26152^T. Strains JG 03^T and JG 05 had 98.4 % and 98.3 % 16S rRNA gene sequence similarities, respectively, with *Z. salsuginis* DSM 21149^T and 98.2 % and 98.1 % sequence similarities, respectively, with *Z. alba* DSM 21143^T. Both strains showed 98.0 % and 96.9 % sequence similarity to *Z. halotolerans* DSM 17364^T and to *Z. aestuarii* KCTC 19557^T, respectively. Strains JG 03^T and JG 05 had a 16S rRNA gene sequence similarity of 99.4 % with each other. The 16S rRNA gene sequence-based tree showing the position of strains JG 03^T and JG 05 within the genus *Zhihengliuella* is presented in Fig. 1 and Fig. S1 (available in the online Supplementary Material),

reconstructed by the neighbour-joining and maximum-likelihood methods, respectively.

Chemotaxonomic analyses such as of respiratory quinones (menaquinones), peptidoglycan types, cell-wall sugars and polar lipids of strains JG 03^T and JG 05 were performed by DSMZ (Deutsche Sammlung für Mikroorganismen und Zellkulturen, Braunschweig, Germany). Analyses of respiratory quinones and polar lipids were carried out by previously described procedures (Tindall, 1990a, b; Tindall *et al.*, 2007). Determination of the peptidoglycan structure was carried out as described by Schumann (2011). The cell-wall sugar was determined according to Stanek & Roberts (1974). The menaquinone types for JG 03^T were MK-10 (50 %), MK-9 (43 %) and MK-8 (6 %), whereas for JG 05 they were MK-9 (48 %), MK-10 (46 %) and MK-8 (6 %). Menaquinones MK-10 and MK-9 are predominant in all other species of the genus *Zhihengliuella* (Baik *et al.*, 2011; Hamada *et al.*, 2013). The peptidoglycan type of both strains was A4 α (molar ratio of amino acids were strain JG 03^T, 1.9 Ala : 1.9 Glu : 1.0 Lys : 1.0 Mur : 0.1 Asp and for strain JG 05, 1.6 Ala : 1.9 Glu : 1.0 Lys : 1.0 Mur : 0.1 Asp with an interpeptide bridge of L-Lys-L-Ala-L-Glu) consistent with those determined for the other members of the genus *Zhihengliuella* (Hamada *et al.*, 2013). The major component of the cell-wall sugar was galactose with minor amounts of glucose, mannose and rhamnose also detected in both strains (JG 03^T and JG 05). Glucose and tyvelose were the major cell-wall sugars in *Z. halotolerans* and *Z. aestuarii* (Zhang *et al.*, 2007; Baik *et al.*, 2011), whereas mannose and tyvelose were those in *Z. alba* and *Z. salsuginis* (Tang *et al.*, 2009; Chen *et al.*, 2010). In the case of *Z. flava*, however, galactose was also present as a major constituent (Hamada *et al.*, 2013). The polar lipids diphosphatidylglycerol (DPG), phosphatidylglycerol (PG) and phosphatidylinositol (PI) were present

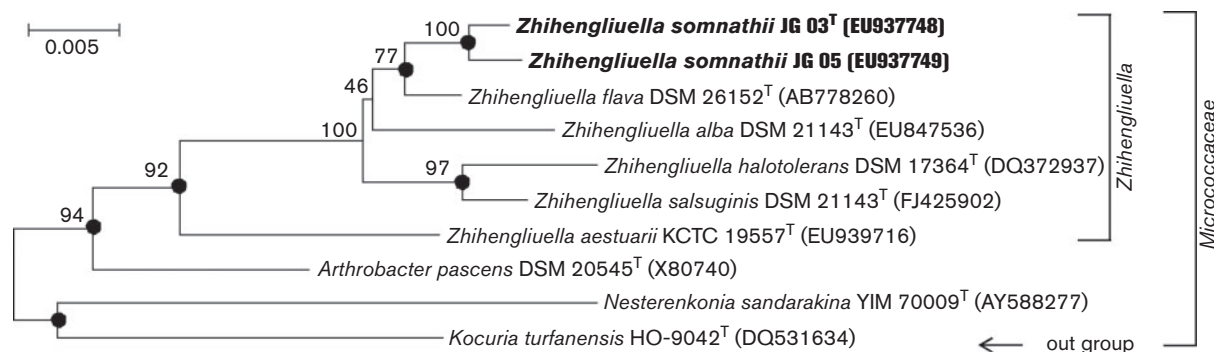


Fig. 1. The phylogenetic tree of strains JG 03^T and JG 05 with taxonomic neighbours. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates). Numbers at nodes are percentage bootstrap values. Circles at the nodes indicate that the corresponding nodes were also recovered in the trees generated by the maximum-likelihood algorithm. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated by the complete deletion option. Phylogenetic analyses were conducted in MEGA version 6. Bar, 0.005 substitutions per nucleotide position.

in both strains. In addition one unidentified polar lipid, two unidentified phospholipids and one unidentified glycolipid were also present (Fig. S2). The presence of the polar lipids, DPG and PG, is consistent with other species of the genus *Zhihengliuella*.

For cellular fatty acid analysis, strains JG 03^T, JG 05 and reference strains (*Z. salsuginis* DSM 21149^T, *Z. halotolerans* DSM 17364^T, *Z. alba* DSM 21143^T, *Z. aestuarii* KCTC 19557^T and *Z. flava* DSM 26152^T) were grown in tryptic soy yeast agar for 24 h at 30 °C. Fatty acid methyl esters were prepared, separated and identified according to the instructions of the Microbial Identification System (MIDI, Microbial ID; Sasser, 1990; Whittaker *et al.*, 2005). Peaks were identified using the RTSBA6 6.10 database. The major fatty acids, anteiso-C_{15:0} (55.1 %) and anteiso-C_{17:0} (14.3 %), were present in JG 03^T; iso-C_{15:0} (35.4 %), anteiso-C_{15:0} (34.8 %), and anteiso-C_{17:0} (10.3 %) were present in strain JG 05 (Table 1). Strain JG 05 had highest iso-C_{15:0} rather than anteiso-C_{15:0}, which is commonly high in other species of the genus *Zhihengliuella* with validly published names, as well as in strain JG 03^T.

Utilization of different substrates by strain JG 03^T and JG 05 and by the reference strains was tested using Biolog GEN III MicroPlates of the Microlog system (Biolog). The results

are summarized in Table S1. Additionally, activity of some important enzymes, such as amylase, catalase, oxidase, pectinase, gelatinase, protease, lipase and cellulase were tested for strains JG 03^T and JG 05 (Gontia *et al.*, 2011). Both strains tested positive for the production of amylase, catalase, pectinase, gelatinase, protease and lipase enzyme, but negative for oxidase and cellulase. All species of the genus *Zhihengliuella* are catalase-positive and oxidase-negative. Antibiotic resistance was determined with the disc diffusion method using commercial antibiotic-impregnated discs. The strains were tested for sensitivity (quantity per disc) to ampicillin (10 µg), azireonam (10 µg), bacitracin (10 U), chloramphenicol (30 µg), ciprofloxacin (5 µg), clindamycin (2 µg), co-trimoxazole (25 µg), erythromycin (10 µg), gatifloxacin (10 µg), gentamicin (10 µg), levofloxacin (5 µg), neomycin (30 µg), nitrofurantoin (300 µg), norfloxacin (10 µg), ofloxacin (5 µg), penicillin G (1 U), polymyxin (300 U), sulphamethoxazole (23.75 µg) and tetracycline (25 µg) using standard disc diffusion protocols, whereas aztreonam, fusidic acid, guanidine HCl, lincomycin, lithium chloride, minocycline, nalidixic acid, niaproof 4, potassium tellurite, rifamycin SV, 1 % sodium lactate, sodium butyrate, sodium bromated, tetrazolium violet, tetrazolium blue, troleandomycin and vancomycin were tested with a chemical sensitivity assay using Biolog GEN III MicroPlates. Strain JG 03^T was sensitive to erythromycin, fusidic acid, troleandomycin, rifamycin SV, minocycline, lincomycin, niaproof 4 and vancomycin, whereas JG 05 was sensitive to fusidic acid, troleandomycin, minocycline, lincomycin, niaproof 4 and vancomycin.

Table 1. Whole-cell fatty acid profiles of JG 03^T and JG 05 and type strains of species of the genus *Zhihengliuella*

Taxa: 1, strain JG 03^T; 2, strain JG 05; 3, *Z. aestuarii* KCTC 19557^T; 4, *Z. alba* DSM 21143^T; 5 *Z. salsuginis* DSM 21149^T; 6, *Z. halotolerans* DSM 17364^T; *Z. flava* DSM 26152^T. –, Not detected.

Fatty acid	1	2	3	4	5	6	7
C _{10:0}	–	–	–	–	–	0.2	–
C _{12:0}	0.1	0.2	0.2	0.4	0.1	0.2	0.2
C _{14:0}	1.6	0.5	1.6	2.5	1.0	1.0	1.2
C _{16:0}	6.0	2.4	4.3	6.5	4.9	4.0	3.8
C _{17:0}	0.2	–	–	0.5	0.2	0.5	0.2
C _{18:0}	0.4	–	–	0.5	0.4	5.7	0.3
C _{19:0}	–	–	–	–	–	0.3	–
C _{20:0}	–	–	–	–	–	2.7	–
iso-C _{13:0}	–	0.3	–	–	0.1	0.4	0.2
iso-C _{14:0}	2.0	1.4	1.2	2.6	0.5	2.8	1.4
iso-C _{15:0}	8.2	35.4	9.0	4.2	7.2	15.0	14.3
iso-C _{16:0}	9.8	6.9	7.7	8.0	3.0	2.1	6.0
iso-C _{17:0}	1.1	7.0	0.7	0.4	1.0	3.3	1.6
iso-C _{18:0}	0.1	–	–	–	–	0.5	–
iso-C _{19:0}	–	–	–	–	–	0.6	–
anteiso-C _{13:0}	0.2	0.2	–	0.4	–	0.2	0.2
anteiso-C _{15:0}	55.1	34.8	64.9	63.5	58.4	52.1	58.1
anteiso-C _{17:0}	14.3	10.3	9.3	9.2	16.6	7.6	12.3
anteiso-C _{19:0}	–	–	–	–	–	0.7	–
C _{18:1} ω9c	0.3	–	0.4	0.4	0.3	0.2	0.2
C _{15:0} 2-OH	–	–	0.2	0.3	–	–	–
C _{17:0} 2-OH	0.3	0.1	0.2	0.2	–	–	0.1

Scanning electron micrographs showed that strains JG 03^T and JG 05 were rod-shaped (Fig. S3a, c), similarly to *Z. halotolerans*, *Z. alba*, *Z. aestuarii* and *Z. flava*. However, *Z. salsuginis* has coccoid morphology. Transmission electron microscopy of strains JG 03^T and JG 05 revealed the absence of flagella (Fig. S3b, d), which is consistent with all other members of the genus *Zhihengliuella*. Strains JG 03^T and JG 05 differed from the other species of the genus *Zhihengliuella* with respect to the major biochemical and physiological characteristics as summarized in Table 2.

The determination of the G+C content of the DNA and DNA–DNA hybridization experiments were performed by the DSMZ, Braunschweig, Germany. The G+C content was determined by DNA isolation (Cashion *et al.*, 1977), followed by DNA degradation (Mesbah *et al.*, 1989), HPLC (Tamaoka & Komagata, 1984) and finally calculation of G+C content (Mesbah *et al.*, 1989). For DNA–DNA hybridization, cells were disrupted by using a Constant Systems TS 0.75 kW (IUL Instruments). DNA in the crude lysate was purified by chromatography on hydroxyapatite, as described by Cashion *et al.* (1977). DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970) under consideration of the modifications described by Huss *et al.* (1983). The G+C content of strains JG 03^T and JG 05 was 70.0 mol% and 70.1 mol%, respectively, which are similar to the values (59.1–70.3 %) reported for other species of the genus *Zhihengliuella* (Zhang *et al.*, 2007;

Table 2. Differential phenotypic characteristics of strains JG 03^T, JG 05 and type strains of species of the genus *Zhihengliuella*

Taxa: 1, strain JG 03^T; 2, strain JG 05^T; 3, *Z. aestuarii* KCTC 19557^T; 4, *Z. alba* DSM 21143^T; 5, *Z. salsuginis* DSM 21149^T; 6, *Z. halotolerans* DSM 17364^T; 7, *Z. flava* DSM 26152^T. +, Positive; –, negative; ±, weakly positive; Gal, galactose; Glc, glucose; Man, mannose; Tyv, tyvelose.

Characteristic	1	2	3	4	5	6	7
Colony colour	Pale yellow	White	Yellow	White	Light yellow	Pale yellow	Pale yellow
Morphology	Short rod	Short rod	Ovoid to short rod*	Short rod†	Cocci‡	Short rod§	Rod-shaped
NaCl range (% w/v)	0.5–15	0.5–15	0–7*	0–15†	0.5–20‡	0–25§	0–10
pH range	6–10	6–10	6–10*	5–9†	6.5–11.5‡	6–10§	6–11
Temperature range (°C)	10–37	10–37	4–37*	4–45†	10–40‡	4–45§	10–37
Carbon source utilization							
Acetic acid	+	±	+	+	±	+	+
D-Arabitol	+	–	+	+	–	+	±
Citric acid	+	+	±	±	–	–	±
Formic acid	±	–	+	+	±	±	±
Gentiobiose	±	+	±	±	±	+	±
L-Histidine	+	–	+	+	–	+	±
α-Hydroxy-butyric acid	+	±	+	±	–	+	±
D-Malic acid	±	–	–	±	–	+	±
D-Mannose	±	+	+	±	±	±	±
D-Mannitol	±	+	+	+	–	±	±
Mucic acid	±	–	–	±	–	+	±
Propionic acid	+	±	±	+	±	±	+
Quinic acid	±	+	±	+	–	+	±
Tween 40	±	±	+	±	±	–	±
Cell-wall sugar	Gal	Gal	Tyv, Glc*	Tyv, Man†	Tyv, Man‡	Tyv, Glc§	Gall
DNA G + C content (mol%)	70.0	70.1	59.1*	70.3†	67.8‡	66.5§	70.3

*Data from Baik *et al.* (2011).

†Data from Tang *et al.* (2009).

‡Data from Chen *et al.* (2010).

§Data from Zhang *et al.* (2007).

||Data from Hamada *et al.* (2013).

Tang *et al.*, 2009; Chen *et al.*, 2010; Baik *et al.*, 2011; Hamada *et al.*, 2013). DNA–DNA hybridization of JG 03^T with *Z. alba* DSM 21143^T, *Z. halotolerans* DSM 17364^T, *Z. salsuginis* DSM 21149^T, *Z. flava* DSM 26152^T and *Z. aestuarii* KCTC 19557^T showed reassociation values of 53.4 %, 49.6 %, 37.2 %, 25.9 % and 19.8 %, respectively. For strain JG 05 the reassociation values with *Z. salsuginis* DSM 21149^T, *Z. halotolerans* DSM 17364^T, *Z. flava* DSM 26152^T, *Z. alba* DSM 21143^T and *Z. aestuarii* KCTC 19557^T were 35.6 %, 35 %, 26.4 %, 25 % and 24.6 %, respectively. DNA–DNA hybridization between JG 03^T and JG 05 resulted in a reassociation value of 91.4 %. DNA–DNA relatedness values have been used as a genotypic parameter to delineate species (Wayne *et al.*, 1987). DNA–DNA hybridization values <70 % are considered to show that organisms belong to different species (Stackebrandt & Goebel, 1994). Thus, according to accepted criteria for novel species, these two strains belong to the same species, but represent a novel species of the genus *Zhihengliuella*.

From the results of the phylogenetic analysis based on 16S rRNA gene sequences, differences in biochemical

characteristics, the polar lipid profile, the fatty acid composition and the low reassociation values of DNA–DNA hybridization with its closest relatives, it is evident that strains JG 03^T and JG 05 represent a novel species of the genus *Zhihengliuella*. The name *Zhihengliuella somnathii* sp. nov. is proposed for this novel species.

Description of *Zhihengliuella somnathii* sp. nov.

Zhihengliuella somnathii (som.nath'i.i. N.L. gen. n *somnathii* of somnath, the presiding deity, dating back to pre-historic era of the area of Saurashtra, Gujarat, India, from where the type strain was isolated).

Cells are Gram-stain-positive, rod-shaped, 1.1–1.9 × 0.3–0.5 µm, aerobic and non-motile. Colonies are pale yellow and white, circular, have an entire margin and are opaque within 24 h with a diameter of approximately 2 mm on nutrient agar. Mesophilic, with an optimum growth temperature of 30 °C, but are able to grow between 10 and 37 °C and at pH 6–10 (optimum pH 8.0). Able to tolerate concentrations of NaCl up to 15 % (w/v) with optimal

growth at 4 % (w/v). Positive for amylase, catalase, pectinase, gelatinase, protease and lipase, but negative for oxidase and cellulase. Utilizes N-acetyl-D-glucosamine, L-alanine, L-aspartic acid, citric acid, D-cellobiose, D-fructose, D-Galactose, α -D-glucose, D-gluconic acid, L-glutamic acid, glycerol, inosine, L-malic acid, D-maltose, L-rhamnose, sucrose, L-serine and D-trehalose as sole carbon sources. The peptidoglycan type is A4 α and major menaquinones are MK-10 and MK-9. The predominant cell-wall sugar is galactose and minor amounts of glucose, mannose and rhamnose are also present. The polar lipid profile consists of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, one unidentified polar lipid, two unidentified phospholipids and one unidentified glycolipid. The predominant fatty acids are anteiso-C_{15:0} and anteiso-C_{17:0} or iso-C_{15:0}, anteiso-C_{15:0} and anteiso-C_{17:0}.

The type strain, JG 03^T (=DSM 23187^T=IMCC 253^T) and strain JG 05 (=DSM 23191=IMCC 254), were isolated from roots of an extreme halophyte *Salicornia brachiata* from the coastal region of Bhavnagar district, Gujarat, India. The DNA G+C content of the type strain and strain JG 05 are 70.0 mol% and 70.1 mol%, respectively.

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