Brachybacterium saurashtrense sp. nov., a halotolerant root-associated bacterium with plant growth-promoting potential

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A Gram-positive-staining, aerobic, non-motile, coccoid shaped, halotolerant bacterium (strain JG 06^T) was isolated from the roots of *Salicornia brachiata*, an extreme halophyte. Phylogenetic analysis based on 16S rRNA gene sequence showed that the novel strain had sequence similarities of 99.2 % to *Brachybacterium paraconglomeratum* JCM 11608^T, 99.0 % to *Brachybacterium conglomeratum* DSM 10241 and 98.2 % to *Brachybacterium faecium* DSM 4810^T. DNA–DNA hybridization with *B. paraconglomeratum* DSM 46341^T, *B. conglomeratum* DSM 10241^T, *B. faecium* DSM 4810^T, *Brachybacterium tyrofermentans* DSM 10673^T, *Brachybacterium alimentarium* DSM 10672^T, *Brachybacterium fresconsis* DSM 14564^T, *Brachybacterium sacelli* DSM 14566^T and *Brachybacterium muris* DSM 15460^T resulted in reassociation values of 36.2 %, 36.5 %, 35.8 %, 27.6 %, 27.9 %, 28.2 %, 28.7 % and 11.2 %, respectively. The peptidoglycan type of strain JG 06^T was variant A4γ. The menaquinone content was MK7 (100 %). The polar lipid profile consisted of diphosphatidylglycerol, phosphatidylglycerol, monogalactosyl diglyceride, three unidentified phospholipids and three glycolipids. The predominant fatty acid was anteiso-$C_{15:0}$ (52.07 %); significant amounts of iso-$C_{16:0}$ (12.38 %), iso-$C_{15:0}$ (8.59 %) and anteiso-$C_{17:0}$ (10.03 %) were also present. The G+C content of the DNA was 73.0 mol%. The strain formed a growth pellicle in nitrogen-free semisolid NFb medium containing NaCl at levels of up to 4 % (w/v) and reduced acetylene to ethylene, a result indicative of N_2 fixation. In nutrient broth medium the novel strain grew at NaCl concentrations up to 15 % (w/v). It also had the ability to produce indole-3-acetic acid (IAA) and siderophores, utilized 1-aminocyclopropane-1-carboxylate (ACC) as a sole source of nitrogen and possessed the ACC deaminase enzyme. On the basis of physiological, biochemical data and phylogenetic analyses, strain JG 06^T should be placed in the genus *Brachybacterium*. Strain JG 06^T represents a novel species of the genus *Brachybacterium* for which the name *Brachybacterium saurashtrense* sp. nov. is proposed (type strain JG 06^T= DSM 23186^T=IMCC 252^T).

The genus *Brachybacterium* was proposed by Collins *et al.* (1988). At the time of writing, the genus *Brachybacterium* contained the following species, *Brachybacterium faecium* (Collins *et al.*, 1988), *Brachybacterium nesterenkovii* (Gvozdyak *et al.*, 1992), *Brachybacterium conglomeratum*, *Brachybacterium paraconglomeratum*, *Brachybacterium rhamnosum* (Takeuchi *et al.*, 1995), *Brachybacterium alimentarium*, *Brachybacterium tyrofermentans* (Schubert *et al.*, 1996), *Brachybacterium fresconis*, *Brachybacterium sacelli* (Heyrman *et al.*, 2002), *Brachybacterium muris* (Buczolits *et al.*, 2003), *Brachybacterium zhongshanense* (Zhang *et al.*, 2007) and *Brachybacterium phenoliresistens* (Chou *et al.*, 2007).

Strain JG 06^T was isolated from roots of *Salicornia brachiata* plants collected from coastal marshy swamps, Bhavnagar district, Gujarat (21° 45′ N 72° 14′ E), India.

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylate; DPG, diphosphatidylglycerol (DPG); IAA, indole-3-acetic acid; MGDG, monogalactosyl diglyceride; PG, phosphatidylglycerol.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain JG 06^T is EU937750.

Supplementary figures are available with the online version of this paper.

Roots were washed thoroughly in 0.5 × PBS solution. After washing, the roots (0.5 g fresh weight) were homogenized in 9.5 ml 0.5 × PBS solution with a sterile mortar. Aliquots of 50 µl of serial dilutions (up to 10^{-7}) were inoculated into vials containing 5 ml nitrogen-free semisolid NFb medium (Döbereiner, 1995). After incubation for six to seven days at 30 °C, a diffuse subsurface growth pellicle appeared in the vials containing dilutions of up to 10^{-5}. Bacteria from the highest dilution vial showing pellicle formation were transferred to new sterile semisolid medium for second and third incubations. After new pellicle formation, cells were plated on NFb solid medium supplemented with a trace amount of yeast extract. Single, separated colonies growing on these plates were reinoculated into new semisolid medium. Bacteria from growth pellicles in these vials were finally transferred to half-strength DYGS agar plates (Kirchhof *et al.*, 2001). The organism could grow at NaCl concentrations of up to 4 % (w/v) on nitrogen-free NFb semisolid medium. On nutrient broth medium, growth was observed at NaCl concentrations of up to 15 % (w/v). On this medium, the novel strain grew at temperatures of 10–45 °C, with optimum growth at 30 °C. The strain grew over a pH range of 6–11, with optimum growth at pH 8.

Cell morphology was observed using scanning electron microscopy according to Yumoto *et al.* (2001). The presence of bacterial flagella was investigated using transmission electron microscopy according to Näther *et al.* (2006). The 16S rRNA gene was amplified as described previously by Weisburg *et al.* (1991). The 16S rRNA gene sequence was determined by direct sequencing of the PCR product and was performed by Macrogen (Korea). Phylogenetic analysis of the 16S rRNA gene sequences was performed with MEGA version 4 software (Tamura *et al.*, 2007). The phylogenetic trees were inferred by using the neighbour-joining method (Saitou & Nei, 1987) and bootstrap analysis was performed (Felsenstein, 1985). The evolutionary distances were computed using the maximum composite likelihood method (Tamura *et al.*, 2004). The 16S rRNA gene sequence comprised 1485 bp (GenBank accession no. EU937750). It showed maximum sequence similarity 99.2 % to *B. paraconglomeratum* CT24 (AJ415377), 99.0 % to *B. conglomeratum* JCM 11608^T (AB537169) and 98.2 % to *B. faecium* DSM 4810^T (X83810). The 16S rRNA gene sequence-based tree showing the position of strain JG 06^T within the genus *Brachybacterium* is presented in Fig. 1.

Chemotaxonomic analyses of the respiratory quinones (menaquinones) and determination of the peptidoglycan type of strain JG 06^T were performed by the DSMZ, Germany. The menaquinone type (100 % MK7) and the peptidoglycan type (A4γ) were consistent with those determined for the other members of the genus *Brachybacterium* (Heyrman *et al.*, 2002; Buczolits *et al.*, 2003). Polar lipids were extracted from strain JG 06^T and four reference strains (*B. paraconglomeratum* DSM 46341^T, *B. conglomeratum* DSM 10241^T, *B. faecium* DSM 4810^T and *B. muris* DSM 15460^T) and analysed by TLC according to Christie (2003). The lipids were identified by using authentic standards (phospholipids PH9–KT, monogalactosyl diglyceride and digalactosyl diglyceride; Sigma). Strain JG 06^T contained diphosphatidylglycerol (DPG), phosphatidylglycerol (PG) and monogalactosyl diglyceride (MGDG). In addition, three unknown phospholipids and three unknown glycolipids were also present. The presence of DPG and PG was consistent with analyses of other species of the genus *Brachybacterium* (Chou *et al.*, 2007). However, the presence of MGDG was unique for strain JG 06^T (see Supplementary Fig. S1a and b in IJSEM Online). Unlike other species of the genus *Brachybacterium*, two additional unidentified phospholipids were also present in strain JG 06^T.

For fatty acid analysis, strain JG 06^T and five closely related reference strains 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). The RTSBA6 6.10 database was used for identification of peaks. The predominant fatty acids in strain JG 06^T were anteiso-$C_{15:0}$ (52.07 %), anteiso-$C_{17:0}$ (10.03 %), iso-$C_{15:0}$ (8.59 %) and iso-$C_{16:0}$ (12.38 %). This profile was in agreement with the major characteristics of members of the genus *Brachybacterium* (Collins *et al.*, 1988; Gvozdyak *et al.*, 1992; Takeuchi *et al.*, 1995; Schubert *et al.*, 1996; Heyrman *et al.*, 2002; Buczolits *et al.*, 2003; Chou *et al.*, 2007) except for the lower percentage of iso-$C_{15:0}$ (8.59 %) and iso-$C_{14:0}$ (2.06 %) in strain JG 06^T (Table 1).

The results of the physiological characterization are given in the species description and in Table 2. Biochemical tests for citrate utilization, activities of lysine decarboxylase, ornithine decarboxylase, urease, and phenylalanine deaminase, nitrate reduction and H_2S production were performed using a Biochemical Easy kit (Himedia, India), following the manufacturer's protocol. The activity of some important enzymes, such as oxidase, catalase, amylase (using a standard protocol), gelatinase (Smibert & Krieg, 1994), cellulase and pectinase (Mateos *et al.*, 1992), protease (Sánchez-Porro *et al.*, 2003) and lipase (Sierra, 1957) were tested. Tests for carbohydrate assimilation for maltose, mannose, fructose, ribose, xylose, arabinose, galactose, sucrose, malic acid, glucose, adonitol, lactose and sorbitol were conducted according to standard protocols (Collee *et al.*, 1996). The novel strain was tested for antibiotic sensitivity to penicillin G (1 U), ampicillin (10 µg), erythromycin (10 µg), clindamycin (2 µg), gentamicin (10 µg), fusidic acid (10 µg), tetracycline (25 µg), co-trimoxazole (25 µg), ciprofloxacin (5 µg), oflaxacin (5 µg), norfloxacin (10 µg), levofloxacin (5 µg), azireonam (10 µg), gatifloxacin (10 µg), nitrofurantoin (300 µg), sulphamethoxazole (23.75 µg), bactracin (10 U), chloramphenicol (30 µg), polymyxin (300 U) and neomycin (30 µg) using standard protocols.

The microscopic characteristics of strain JG 06^T (coccoid morphology and non-motile) (see Supplementary Fig. S2) were similar to those of *B. paraconglomeratum*, *B. faecium*

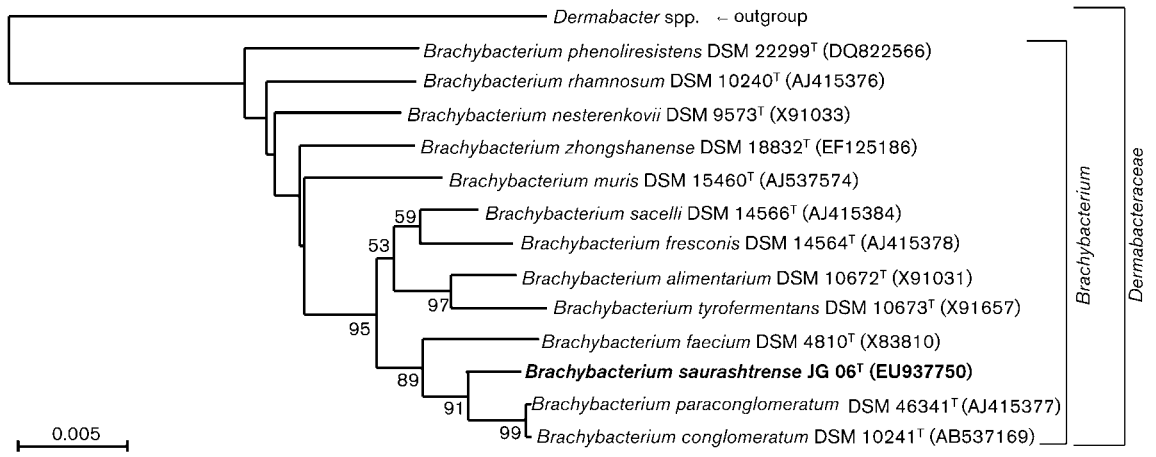

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences and inferred by using the neighbour-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (only bootstrap values >50 % are shown). Evolutionary distances were computed using the maximum composite likelihood method. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). Phylogenetic analyses were performed by MEGA version 4. Bar, 0.005 substitutions per site.

and *B. conglomeratum*. No flagella could be observed using transmission electron microscopy (Supplementary Fig. S2b). Strain JG 06^T differed from the other three most closely related species of the genus *Brachybacterium* as regards the following major biochemical and physiological characteristics. Strain JG 06^T had a pale yellow colony colour whereas other species showed pale brown coloured colonies. Strain JG 06^T showed growth over the temperature range 10–45 °C, while the growth temperatures for *B. faecium* and *B. conglomeratum* ranged between 4 and 42 °C and 15 and 42 °C, respectively. The novel strain showed growth over the pH range 6–11, while *B. paraconglomeratum*, *B. faecium* and *B. conglomeratum* grew well between pH 6 and pH 9. Strain JG 06^T was positive for the methyl red test and in tests for the hydrolysis of gelatin, casein, tributyrin and Tween 80, while *B. paraconglomeratum* and *B. faecium* gave negative results for these tests (Takeuchi *et al.*, 1995). The novel strain hydrolysed starch, but was not able to hydrolyse cellulose, pectin, lysine, ornithine or phenylalanine.

Table 1. Fatty acid content (%) of strain JG 06^T and five reference species of the genus *Brachybacterium*

Taxa: 1, strain JG 06^T; 2, *B. paraconglomeratum* DSM 46341^T; 3, *B. conglomeratum* DSM 10241^T; 4, *B. faecium* DSM 4810^T; 5, *B. alimentarium* DSM 10672^T; 6, *B. muris* DSM 15460^T. –, <0.5 %.

Fatty acid	1	2	3	4	5	6
$C_{14:0}$ iso	2.06	4.57	1.61	1.71	2.97	2.17
$C_{14:0}$	1.58	2.20	1.91	1.15	0.92	0.89
$C_{15:0}$ iso	8.59	16.24	9.20	11.77	10.44	5.88
$C_{15:0}$ anteiso	52.07	51.73	63.48	50.83	30.75	59.45
$C_{16:0}$	2.62	2.00	1.78	2.51	3.78	1.88
$C_{16:0}$ iso	12.38	11.36	6.60	10.26	1.45	6.41
$C_{17:0}$ iso	1.76	1.12	1.04	2.16	4.99	1.02
$C_{17:0}$ anteiso	10.03	4.15	8.39	9.36	7.98	11.22
$C_{17:1}\omega 9c$	1.04	1.28	0.67	1.01	–	–
$C_{18:0}$	–	–	–	1.47	11.56	–
$C_{18:0}$ iso	0.52	–	–	–	0.82	–
$C_{18:1}\omega 7c$	3.83	2.66	3.09	–	–	3.62
$C_{19:0}$ iso	–	–	–	–	2.32	–
$C_{19:0}$ anteiso	0.50	–	–	–	2.32	–
$C_{19:1}\omega 7c$	0.72	0.91	0.87	1.02	–	1.11
$C_{20:0}$	–	–	–	–	11.34	–
$C_{20:1}\omega 7c$	0.59	–	–	0.87	–	2.83

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 of the DNA for strain JG 06^T was 73.0 mol%, which was similar to the values (68–73 mol%) reported for other species belonging to the genus *Brachybacterium* (Collins *et al.*, 1988; Takeuchi *et al.*, 1995; Heyrman *et al.*, 2002). DNA–DNA hybridization experiments between strain JG 06^T and *B. paraconglomeratum* DSM 46341^T, *B. conglomeratum* DSM 10241^T, *B. faecium* DSM 4810^T, *B. tyrofermentans* DSM 10673^T, *B. alimentarium* DSM 10672^T, *B. fresconsis* DSM 14564^T, *B. sacelli* DSM 14566^T and *B. muris* DSM 15460^T showed reassociation values of 36.2 %, 36.5 %, 35.8 %, 27.6 %, 27.9 %, 28.2 %, 28.7 % and 11.2 %, respectively. DNA relatedness has been used as a genotypic parameter to delineate species (Caballero-Mellado *et al.*, 1995). DNA–DNA hybridization percentage values <70 % are considered to show that organisms belong to different species (Stackebrandt & Goebel, 1994).

Strain JG 06^T was grown in LB medium at 30 °C until the midexponential phase was reached. Equal amounts of cells

Table 2. Physiological characteristics of strain JG 06^T and closest related species of the genus *Brachybacterium*

Taxa: 1, strain JG 06^T; 2, *B. paraconglomeratum* DSM 46341^T; 3, *B. conglomeratum* DSM 10241^T; 4, *B. faecium* DSM 4810^T. +, Positive; −, negative; (+), weakly positive. All strains are coccoid, non-motile, and give positive results in tests for nitrate reduction, catalase and amylase activities and for the assimilation of maltose and glucose. All strains give a negative result for the Voges–Proskauer reaction, for oxidase activity and for sorbitol assimilation.

Characteristic	1	2	3	4
Indole	+	+	(+)	(+)
Urease	−	+	+	−
H_2S	−	+	−	−
Hydrolysis of:				
Gelatin	+	−	(+)	−
Tween 80	+	−	(+)	−
Assimilation of:				
Adonitol	−	+	+	−
Arabinose	−	−	−	(+)
Fructose	+	+	+	−
Galactose	+	+	+	−
Lactose	+	(+)	(+)	−
Mannose	+	+	+	−
Ribose	−	−	−	(+)
Sucrose	+	+	+	−
Xylose	+	−	+	−

were inoculated in 5 ml nitrogen-free semisolid NFb medium in a 10 ml culture bottle incubated at 30 °C for the formation of the pellicle. After 4 days of incubation, bottles were made airtight with suba-seal caps, 1 ml acetylene gas was injected into the bottles and the bottles incubated at 30 °C for 24 h. Strain JG 06^T and a positive control, *Herbaspirillum frisingense* GSF30^T, were tested for acetylene-reducing activity by measuring the amount of ethylene produced from acetylene using a GC (HP6890; Hewlett Packard) equipped with a flame-ionization detector and a GS-Alumina column. Strain JG 06^T converted acetylene to ethylene, which was denoted by a peak at its retention time. Acetylene reduction activity is a measure of the N_2-fixing ability of bacteria. In addition, the *nifH* gene could be PCR-amplified successfully from strain JG 06^T (B. Jha and others, unpublished data). IAA production was determined using a colorimetric method as described by Gordon & Weber (1951). After the addition of 0.05 % tryptophan, strain JG 06^T produced indole-3-acetic acid (IAA) at a concentration of 100.0 μg ml^{-1} in the culture supernatant. The test for phosphate solubilization was performed as according to the method of Goldstein (1986). The novel strain could not solubilize phosphate from the complex tri-calcium phosphate-containing medium. To study the utilization of 1-aminocyclopropane-1-carboxylic acid (ACC) as a sole nitrogen source, the novel strain was grown in NFb medium supplemented with 3 mM ACC at 30 °C for 72 h at 175 r.p.m. Bacterial growth was measured by monitoring the absorbance at 600 nm. Strain JG 06^T showed growth in this medium suggesting that the strain might possess the ACC deaminase enzyme. A test for ACC deaminase enzyme activity was carried out according to Penrose & Glick (2003). Strain JG 06^T showed high ACC deaminase activity (0.220 μmol α-ketobutyrate μg^{-1} h^{-1}). Siderophore production was detected by the formation of orange halos surrounding bacterial colonies on CAS agar plates after 48 h incubation at 30 °C (Schwyn & Neilands, 1987). To the authors' knowledge, this is the first report of a member of the genus *Brachybacterium* to be isolated from the rhizosphere of any plant and also the first demonstration of N_2-fixing ability from a member of this genus. The enrichment of the bacteria in nitrogen-free NFb semisolid medium may have been a factor in the isolation of this novel diazotrophic species of the genus *Brachybacterium*. Additionally, strain JG 06^T also showed the ability to produce the plant hormone IAA and siderophores and was able to utilize ACC as a sole source of nitrogen and showed ACC deaminase activity. These factors may contribute to plant growth promotion. It has been reported previously that concentrations of α-ketobutyrate of $\geqslant$20 nmol mg^{-1} h^{-1} are sufficient to show plant-growth-promoting effects (Penrose & Glick, 2003).

The genus *Brachybacterium* has been placed in the class *Actinobacteria*. There is a report of N_2-fixation by two non-*Frankia* actinobacterial strains, isolated from the roots of *Casuarina equisetifolia*. One of these isolates showed closest similarity with *Micromonospora aurantiaca* and the other showed similarity with members of the family *Thermomonosporaceae* (Valdés *et al.*, 2005). Members of a second genus of the family *Actinobacteridae*, besides the genus *Frankia*, can thus be added to the list of N_2-fixing bacteria.

From the results of 16S rRNA gene sequencing, differences in biochemical characteristics, the polar lipid profile, the fatty acid composition and the low DNA–DNA hybridization reassociation values with its closest relatives, it is evident that strain JG 06^T represents a novel species of the genus *Brachybacterium*. The name *Brachybacterium saurashtrense* sp. nov. is proposed for this novel species.

Description of *Brachybacterium saurashtrense* sp. nov.

Brachybacterium saurashtrense (sau.rash.tren′se. N.L. neutr. adj. *saurashtrense* of or belonging to Saurashtra, the name of the Western coast in Gujarat State, India, where *Salicornia* plants grow and from where this strain was isolated).

Cells are Gram-positive-staining, are coccoid to ovoid and have a diameter 0.3–0.75 μm. Aerobic and non-motile. Colonies are pale yellow, circular, have an entire margin and are opaque within 24 h with a diameter of approximately 2 mm. Mesophilic, with an optimum growth temperature of 30 °C, but able to grow between 10 and 45 °C and at pH 6–11 (optimum pH 8). Able to tolerate

concentrations of NaCl up to 15 % (w/v) with optimal growth at 8 % (w/v) NaCl. Tests for assimilation of maltose, mannose, fructose, galactose, xylose, sucrose, malic acid, glucose and lactose give positive results. Ribose, arabinose, adonitol, sorbitol and citrate are not assimilated. Positive in tests for catalase and methyl red, but negative for oxidase and urease activity and the Voges–Proskauer reaction. Carbon source utilization and hydrolysis of substrates (including characteristics that differentiate the species from other members of the genus *Brachybacterium*) are indicated in Table 2. Able to reduce nitrate, but does not produce H_2S. Sensitive to ampicillin, erythromycin, clindamycin, fusidic acid, nitrofurantoin and sulphamethoxazole. Possesses several plant growth-promoting traits such as IAA production, siderophore production, ACC utilization, ACC deaminase activity and conversion of acetylene to ethylene. The peptidoglycan type is variant A4γ and the menaquinone is MK7 (100 %). The polar lipid profile consists of DPG, PG, MGDG, three unknown phospholipids and three unknown glycolipids. The predominant fatty acid is anteiso-$C_{15:0}$ with significant amounts of iso-$C_{15:0}$, iso-$C_{16:0}$ and anteiso-$C_{17:0}$.

The type strain, JG 06^T (=DSM 23186^T=IMCC 252^T) was isolated from roots of *Salicornia brachiata* from coastal marshy swamps, Bhavnagar district, Gujarat, India. The DNA G+C content of the type strain is 73.0 mol%.

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