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, cocccoid shaped, halotolerant bacterium (strain JG 06T) 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 11608T, 99.0 % to Brachybacterium conglomeratum DSM 10241 and 98.2 % to Brachybacterium faecium DSM 4810T. DNA–DNA hybridization with B. paraconglomeratum DSM 46341T, B. conglomeratum DSM 10241T, B. faecium DSM 4810T, Brachybacterium tyrofermentans DSM 10673T, Brachybacterium alimentarium DSM 10672T, Brachybacterium fresconis DSM 14564T, Brachybacterium sacelli DSM 14566T and Brachybacterium muris DSM 15460T 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 06T was variant A4c. 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-C15 : 0 (52.07 %); significant amounts of iso-C15 : 0 (12.38 %), iso-C16 : 0 (8.59 %) and anteiso-C17 : 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 N2 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 06T should be placed in the genus Brachybacterium. Strain JG 06T represents a novel species of the genus Brachybacterium for which the name Brachybacterium saurashtrense sp. nov. is proposed (type strain JG 06T = DSM 23186T = IMCC 252T).

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 conglom eratum, 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 06T 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, diphostatidylglycerol (DPG); IAA, indole-3-acetic acid; MGDG, monogalactosyldiglyceride; PG, phosphatidylglycerol.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain JG 06T 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 10⁻⁵) 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 using the neighbour-joining method (Saitou & Nei, 1985) and bootstrap analysis was performed (Felsenstein, 1987) and Tamura (1985). The evolutionary distances were computed using the maximum composite likelihood method (Tamura 1985). The RTSBA6 6.10 database was used for identification of peaks. The predominant fatty acids in strain JG 06T were anteiso-C₁₅ : ₀ (52.07 %), anteiso-C₁₇ : ₀ (10.03 %), iso-C₁₅ : ₀ (8.59 %) and iso-C₁₇ : 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-C₁₅ : ₀ (8.59 %) and iso-C₁₇ : ₀ (2.06 %) in strain JG 06T (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 H₂S 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), azirenam (10 μg), gatifloxacin (10 μg), nitrofurantoin (300 μg), sulphamethoxazole (23.75 μg), bacitracin (10 U), chloramphenicol (30 μg), polymyxin (300 U) and neomycin (30 μg) using standard protocols.

The microscopic characteristics of strain JG 06T (coccolid morphology and non-motile) (see Supplementary Fig. S2) were similar to those of B. paraconglomeratum, B. faecium
and *B. conglomeratum*. No flagella could be observed using transmission electron microscopy (Supplementary Fig. S2b). Strain JG 06T differed from the other three most closely related species of the genus *Brachybacterium* as regards the following major biochemical and physiological characteristics. Strain JG 06T had a pale yellow colony colour whereas other species showed pale brown coloured colonies. Strain JG 06T 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 06T 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.

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 06T 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 06T and *B. paraconglomeratum* DSM 46341T, *B. conglomeratum* DSM 10241T, *B. faecium* DSM 4810T, *B. tyrofermentans* DSM 10673T, *B. alimentarium* DSM 10672T, *B. fresconsis* DSM 14564T, *B. sacelli* DSM 14566T and *B. muris* DSM 15460T 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 06T was grown in LB medium at 30 °C until the midexponential phase was reached. Equal amounts of cells
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 06T and a positive control, *Herbaspirillum frisingense* GS30T, 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 06T converted acetylene to ethylene, which was denoted by a peak at its retention time. Acetylene reduction activity is a measure of the N₂-fixing ability of bacteria. In addition, the nifH gene could be PCR-amplified successfully from strain JG 06T (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 06T 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-amino-cyclopropane-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 06T 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 06T 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 & Neillands, 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 N₂-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 06T 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 ≥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 N₂-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 *Thermo-monosporaceae* (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 N₂-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 06T represents a novel species of the genus *Brachybacterium*. The name *Brachybacterium sau-rashtrense* sp. nov. is proposed for this novel species.

### Description of *Brachybacterium sau-rashtrense* sp. nov.

*Brachybacterium sau-rashtrense* (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 H₂S. Sensitive to ampicillin, erythromycin, clindamycin, fusidic acid, nitrofurantoin and sulphonamethoxazole. 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-C₁₅:₀ with significant amounts of iso-C₁₅:₀, iso-C₁₆:₀ and anteiso-C₁₇:₀.

The type strain, JG 06₇ (= DSM 23186T = IMCC 252T) 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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References


