

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

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15 Running title: Halotolerant bacteria from *Salicornia* root

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17 The NCBI GenBank accession number for the 16S rRNA gene sequence of strain JG 06<sup>T</sup> is  
18 EU937750.

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## 1 **Summary**

2 A Gram-positive, aerobic, non-motile, coccoid shaped, halotolerant bacterium (JG 06<sup>T</sup>) was isolated  
3 from roots of *Salicornia brachiata*, an extreme halophyte. Phylogenetic analysis based on 16S rRNA  
4 gene sequence showed sequence similarities of 99.2% to *Brachybacterium paraconglomeratum*,  
5 99.0% to *B. conglomeratum*, and 98.2% to *B. faecium*. DNA-DNA hybridization with the close  
6 relatives *Brachybacterium paraconglomeratum* (DSM 46341<sup>T</sup>), *B. conglomeratum* (DSM 10241<sup>T</sup>),  
7 *B. faecium* (DSM 4810<sup>T</sup>), *B. tyrofermentans* (DSM 10673<sup>T</sup>), *B. alimentarium* (DSM 10672<sup>T</sup>), *B.*  
8 *fresconsis* (DSM 14564<sup>T</sup>), *B. sacelli* (DSM 14566<sup>T</sup>) and *B. muris* (DSM 15460<sup>T</sup>) resulted in re-  
9 association values of 36.2%, 36.5%, 35.8%, 27.6%, 27.9%, 28.2%, 28.7% and 11.2%, respectively.  
10 The peptidoglycan type of strain JG 06<sup>T</sup> was variant A4 $\gamma$ . The menaquinone content was MK7  
11 (100%). The polar lipid profile consisted of diphosphatidylglycerol, phosphatidylglycerol,  
12 monogalactosyl diglyceride and three unidentified phospholipids and three glycolipids. The  
13 predominant fatty acid was anteiso-C<sub>15:0</sub> (52.07%); significant amounts of iso-C<sub>16:0</sub>(12.38%), iso-  
14 C<sub>15:0</sub>(8.59%) and anteiso-C<sub>17:0</sub>(10.03%) were also present. The G+C content of DNA was 73.0 mol%.  
15 The strain formed a growth pellicle in nitrogen-free semisolid NFb medium containing NaCl up to  
16 4% and reduced acetylene to ethylene, characteristic for N<sub>2</sub> fixation. In nutrient broth medium it  
17 grew up to 15% NaCl. It also had the ability to produce IAA, siderophore, utilized ACC as sole  
18 source of nitrogen and possessed ACC deaminase enzyme. On the basis of physiological,  
19 biochemical data and phylogeny, strain JG 06<sup>T</sup> should be placed in the genus *Brachybacterium*. This  
20 bacterium represents a novel species of genus *Brachybacterium* for which the name  
21 *Brachybacterium saurashtrense* sp. nov. is proposed, with type strain JG 06<sup>T</sup> (= DSM 23186<sup>T</sup> =  
22 IMCC 252<sup>T</sup>).

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1 The genus *Brachybacterium* was proposed by Collins *et al.* (1988). Until now, the genus  
2 *Brachybacterium* has been represented by following species, *Brachybacterium faecium* (Collins *et*  
3 *al.*, 1988), *Brachybacterium nesterenkovi* (Gvozdyak *et al.*, 1992), *Brachybacterium*  
4 *conglomeratum*, *Brachybacterium paraconglomeratum*, *Brachybacterium rhamnosum* (Takeuchi *et*  
5 *al.*, 1995), *Brachybacterium alimentarium*, *Brachybacterium tyrofermentans* (Schubert *et al.*, 1996),  
6 *Brachybacterium fresconis*, *Brachybacterium sacelli* (Heyrman *et al.*, 2002), *Brachybacterium*  
7 *muris* (Buczolits *et al.*, 2003), *Brachybacterium zhongshanense* (Zhang *et al.*, 2007) and  
8 *Brachybacterium phenoliresistens* (Chou *et al.*, 2007).

9 Strain JG 06<sup>T</sup> was isolated from roots of *Salicornia brachiata* plants collected from coastal marshy  
10 swamps, Bhavnagar district, Gujarat (N 21<sup>o</sup> 45'; E 72<sup>o</sup> 14'), India. Roots were washed thoroughly in  
11 0.5 x PBS solution. After washing, the roots (0.5 g fresh weight) were homogenized with a sterile  
12 mortar in 9.5 ml 0.5 x PBS solution. Aliquots of 50 µl of serial dilutions (up to 10<sup>-7</sup>) were inoculated  
13 into vials containing 5 ml of the nitrogen-free semisolid NFb medium (Döbereiner, 1995). After  
14 incubation for six to seven days at 30 °C a diffuse subsurface growth pellicle appeared up to 10<sup>-5</sup>  
15 dilution vials. Bacteria from the highest dilution vial showing the pellicle formation were transferred  
16 to new sterile semisolid medium for second and third incubation. After new pellicle formation, cells  
17 were plated on NFb solid medium supplemented with trace amount of yeast extract. Single, separated  
18 colonies growing on these plates were re-inoculated into new semisolid medium. Bacteria from  
19 growth pellicles in these vials were finally transferred to ½ DYGS agar plates (Kirchhof *et al.*, 2001).  
20 It could grow up to 4% NaCl on nitrogen-free NFb semisolid medium. On nutrient broth medium  
21 growth was observed up to 15% NaCl. On this medium it grew at 10-45 °C, with optimum growth at  
22 30 °C. This strain grew at pH range from 6-11, with optimum of pH 8.

23 Cell morphology was observed using scanning electron microscopy according to Yumoto *et al.*  
24 (2001). The presence of bacterial flagella was investigated using transmission electron microscopy  
25 according to Nather *et al.* (2006). The 16S rRNA gene was amplified as described previously by

1 Weisburg *et al.* (1991). The 16S rRNA gene sequence was determined by direct sequencing of PCR-  
2 product done by Macrogen (Korea). Phylogenetic analysis of the 16S-rRNA gene sequences were  
3 performed with the software MEGA version 4 (Tamura *et al.*, 2007). The phylogenetic trees were  
4 inferred by using the Neighbor-Joining method (Saitou & Nei, 1987) and bootstrap analysis were  
5 done (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite  
6 Likelihood method (Tamura *et al.*, 2004). The 16S rRNA gene sequence was 1485 bp (GenBank  
7 accession no. EU937750). It showed maximum sequence similarity 99.2% to *Brachybacterium*  
8 *paraconglomeratum* (EU660352), 99.0% to *B. conglomeratum* (AB537169) and 98.2% to *B. faecium*  
9 (X83810). The 16S rRNA gene- based tree assigning the position of strain JG 06<sup>T</sup> is shown in Fig.1.

10 The chemotaxonomic analyses of the respiratory quinones (menaquinones) and the peptidoglycan  
11 type of the strain JG 06<sup>T</sup> were performed by German Collection of Microorganisms and Cell  
12 Cultures (DSMZ), Braunschweig, Germany. The determined menaquinone type (100% MK7) and  
13 the peptidoglycan type A4 $\gamma$  were consistent with other members of the genus *Brachybacterium*  
14 (Heyrman *et al.*, 2002; Buczolits *et al.*, 2003). Polar lipids were extracted from the strain JG 06<sup>T</sup>  
15 along with 4 reference strains (*Brachybacterium paraconglomeratum*, *B. conglomeratum*, *B. faecium*  
16 and *B. muris*) and analysed by TLC according to Christie (2003). The lipids were identified by using  
17 authentic standards (Phospholipids PH9 – KT, monogalactosyl diglyceride and digalactosyl  
18 diglyceride, Sigma, USA). Strain JG 06<sup>T</sup> contained diphosphatidylglycerol (DPG),  
19 phosphatidylglycerol (PG) and monogalactosyl diglyceride (MGDG). In addition, three unknown  
20 phospholipids and three unknown glycolipids were also present. The occurrence of DPG and PG are  
21 similar to those for other species of *Brachybacterium* (Chou *et al.*, 2007). However, the presence of  
22 MGDG was unique for strain JG 06<sup>T</sup> (see Supplementary Fig. S1A and S1B). Unlike other  
23 *Brachybacterium* species, two additional unidentified phospholipids were also present in JG 06<sup>T</sup>.

24 For fatty acid analysis JG 06<sup>T</sup> along with five closely related reference strains were grown in Tryptic  
25 soy yeast agar for 24hrs at 30<sup>0</sup>C. Fatty acid methyl esters were prepared, separated and identified

1 according to the instructions of the Microbial Identification System (MIDI; Microbial ID)  
2 (sasser,1990). For peak identification RTSBA6 6.10 database was used. The predominant fatty acids  
3 in strain JG 06<sup>T</sup> were anteiso-C<sub>15:0</sub> (52.07%), anteiso-C<sub>17:0</sub>(10.03%), iso-C<sub>15:0</sub>(8.59%) and iso-  
4 C<sub>16:0</sub>(12.38%). This profile is in agreement with the major characteristics of members of the genus  
5 *Brachybacterium* (Collins et al., 1988; Gvozdyak et al., 1992; Takeuchi et al., 1995; Schubert et al.,  
6 1996; Heyrman et al., 2002; Buczolits et al., 2003; Chou et al., 2007) except the presence of iso-C<sub>15:0</sub>  
7 (8.59%) and a lower per centage of iso-C<sub>14:0</sub>(2.06%) in JG 06<sup>T</sup> (Table 1).

8 The results of physiological characterization are given in the species description and in Table 2.  
9 Biochemical tests for citrate utilization, lysine decarboxylase, ornithine decarboxylase, urease,  
10 phenylalanine deaminase, nitrate reduction and H<sub>2</sub>S production were performed using biochemical  
11 easy kit (Himedia, India), following the manufacture's protocol. Activity of some of the important  
12 enzymes, such as oxidase, catalase, amylase (using standard protocol) gelatinase (Smibert *et al.*,  
13 1994), cellulase and pectinase (Mateos *et al.*, 1992), protease (Sánchez-Porro *et al.*, 2003), and lipase  
14 (Sierra, 1957) were tested as described. Carbohydrate assimilation for maltose, mannose, fructose,  
15 ribose, xylose, arabinose, galactose, sucrose, malic acid, glucose, adonitol, lactose and sorbitol were  
16 carried according to the standard protocols (Collee *et al.*, 1996). Antibiotic sensitivity for penicillin  
17 G (1 unit), ampicillin (10 µg), erythromycin (10 µg), clindamycin (2 µg), gentamicin (10 µg), fusidic  
18 acid (10 µg), tetracycline (25 µg), co-trimazole (25 µg), ciprofloxacin (5 µg), ofloxacin (5 µg),  
19 norfloxacin (10 µg), levofloxacin (5 µg), azireonam (10 µg), gatifloxacin (10 µg), nitrofurantoin  
20 (300 µg), sulphametroxazon (23.75 µg), bactracin (10 unit), chloroamphenicol (30 µg), polymyxin  
21 (300 unit) and neomycin (30 µg), were tested for the bacterial strain according to the standard  
22 protocols.

23 Microscopic characteristics of strain JG 06<sup>T</sup> (cocci morphology and non-motile) (Supplementary  
24 Fig. S2) showed similarity with *B. paraconglomeratum*, *B. faecium* and *B. conglomeratum*. No  
25 flagella could be observed using transmission electron microscopy (Fig. S2b). Strain JG 06<sup>T</sup> differed

1 from the other three species of *Brachybacterium* in the following major biochemical and  
2 physiological characteristics. JG 06<sup>T</sup> has a pale yellow colony colour whereas other species have  
3 pale brown colour. It showed growth within the temperature range 10-45 °C while growth  
4 temperature for *B. faecium* and *B. conglomeratum* ranged between 4-42 °C and 15-42 °C,  
5 respectively. It showed growth within the pH range 6-11, while *B. paraconglomeratum*, *B. faecium*  
6 and *B. conglomeratum* grew well within pH range 6-9. Strain JG 06<sup>T</sup> was positive for methyl red  
7 test, hydrolysis of gelatin, casein, tributyrin and tween 80 while the other two species (*B.*  
8 *paraconglomeratum*, *B. faecium*) were negative for them (Takeuchi *et al.*, 1995). It hydrolysed  
9 starch, but was negative for hydrolysis of cellulose, pectin, lysine, ornithine and phenylalanine.

10 GC content determination and DNA-DNA hybridization experiments were performed by German  
11 Collection of Microorganisms and Cell Cultures (DSMZ) Braunschweig, Germany. The G+C  
12 content of DNA for strain JG 06<sup>T</sup> was 73.0 mol% which is similar to the values (68-73 mol%)  
13 reported for the other species belonging to *Brachybacterium* (Collins *et al.*, 1988; Takeuchi *et al.*,  
14 1995; Heyrman *et al.*, 2002). DNA-DNA hybridization experiments between strain JG 06<sup>T</sup> and  
15 *Brachybacterium paraconglomeratum* (DSM 46341<sup>T</sup>), *B. conglomeratum* (DSM 10241<sup>T</sup>), *B.*  
16 *faecium* (DSM 4810<sup>T</sup>), *B. tyrofermentans* (DSM 10673<sup>T</sup>), *B. alimentarium* (DSM 10672<sup>T</sup>), *B.*  
17 *fresconsis* (DSM 14564<sup>T</sup>), *B. sacelli* (DSM 14566<sup>T</sup>) and *B. muris* (DSM 15460<sup>T</sup>) reference species  
18 showed re-association values of 36.2%, 36.5%, 35.8%, 27.6%, 27.9%, 28.2%, 28.7% and 11.2%  
19 respectively. DNA relatedness has been used as a genotypic parameter to delineate species  
20 (Caballero-Mellado *et al.*, 1995). DNA-DNA hybridization percentage values below 70% are  
21 considered to show that the organisms belong to different species (Stackebrandt & Goebel, 1994).

22 Strain JG 06<sup>T</sup> was grown in LB medium at 30°C till the midlog phase was reached. Equal amounts of  
23 cells were inoculated in 5 ml of nitrogen-free semisolid Nfb medium in a 10 ml culture bottle  
24 incubated at 30 °C for the formation of pellicle. After 4 days of incubation, bottles were made airtight  
25 with suba-seal caps, 1 ml of acetylene gas was injected into the bottles and incubated at 30 °C for 24

1 h. Strain JG 06<sup>T</sup> along with a positive control, *Herbaspirillum frisingense* GSF 30, were tested for  
2 acetylene-reducing activity by measuring the amount of ethylene produced from acetylene using HP  
3 6890 series gas chromatograph equipped with a flame ionization detector and a GS Alumina column.  
4 Strain JG 06<sup>T</sup> converted acetylene to ethylene, which was denoted by a peak at its retention time.  
5 Acetylene reduction activity is a measure of N<sub>2</sub> fixing ability of the bacteria. In addition, the *nifH*  
6 gene could be PCR-amplified successfully from strain JG 06<sup>T</sup> (Jha et al, unpublished). IAA  
7 production was determined using colorimetric method described by Gordon & Weber (1951). With  
8 addition of 0.05% tryptophan, strain JG 06<sup>T</sup> produced IAA which was 100.0 µg ml<sup>-1</sup> of culture  
9 supernatant. Test for phosphate solubilization was performed as per the method of Goldstein (1986).  
10 The strain could not solubilize phosphate from the complex tri-calcium phosphate containing  
11 medium. To study the utilization of 1- aminocyclopropane-1-carboxylic acid (ACC) as sole nitrogen  
12 source, the strain was grown in NFb medium supplemented with 3 mM ACC at 30 °C for 72 h at 175  
13 rpm. The bacterial growth was measured by taking absorbance at 600 nm. Strain JG 06<sup>T</sup> showed  
14 growth in this medium suggesting that the strain might possess ACC deaminase enzyme. ACC  
15 deaminase enzyme activity was carried out according to Penrose & Glick (2003). JG 06<sup>T</sup> showed a  
16 high ACC deaminase activity (0.220 µmol α-ketobutyrate µg<sup>-1</sup> h<sup>-1</sup>). Siderophores production was  
17 detected by the formation of orange halos surrounding bacterial colony on CAS agar plates after 48 h  
18 incubation at 30 °C (Schwyn & Neilands, 1987). To the best of our knowledge this is the first report  
19 of genus *Brachybacterium* to be isolated from the rhizosphere of any plant and this is the first  
20 demonstration of the genus *Brachybacterium* for N<sub>2</sub> fixing ability. The enrichment of bacteria in  
21 nitrogen-free NFb semisolid medium has obviously resulted in the isolation of this new diazotrophic  
22 *Brachybacterium* species. Additionally, strain JG 06<sup>T</sup> also has the ability to produce the plant  
23 hormone IAA, siderophores, utilizes ACC as sole source of nitrogen and ACC deaminase activity,  
24 which may contribute to plant growth promotion. It has been reported that greater or equal to 20  
25 nmol α-ketobutyrate mg<sup>-1</sup> h<sup>-1</sup> is sufficient to show plant-growth-promoting effects (Penrose & Glick,  
26 2003).

1 The genus *Brachybacterium* has been placed among the Actinobacteria group. There is a report of  
2 N<sub>2</sub>-fixation by two non-*Frankia* actinobacterial strains, isolated from the roots of *Casuarina*  
3 *equisetifolia*. One of these isolates showed closest similarity with *Micromonospora aurantiaca* and  
4 the other showed similarity with the members of the family *Thermomonosporaceae* (Valdés *et al.*,  
5 2005). The ability of N<sub>2</sub>-fixation by *Brachybacterium* - a member of the actinobacteria family  
6 besides *Frankia* - can be added to the list of N<sub>2</sub>-fixing bacteria.

7 From the results of 16S rRNA gene sequencing, differences in biochemical characteristics, polar  
8 lipid profile, fatty acid composition and low DNA-DNA hybridization re-association values with the  
9 closest relatives, it is evident that strain JG 06<sup>T</sup> is different from previously described species of  
10 *Brachybacterium*. Hence, the strain JG 06<sup>T</sup> was proposed as *Brachybacterium saurashtrense* sp. nov.  
11 a new species of the genus *Brachybacterium*.

#### 12 **Description of *Brachybacterium saurashtrense* sp. nov.**

13 *Brachybacterium saurashtrense* (sau.rasht.ren'se, N.L. neutr. adj. saurashtrense, of or belonging to  
14 Saurashtra the name of the Western coast in Gujarat State, India, where *Salicornia* plants are  
15 growing, from which this bacteria was isolated).

16 Cells are Gram-positive coccoid to ovoid having diameter 0.3-0.75µm. Aerobic and non-motile.  
17 Colonies were pale yellow, circular, entire margin and opaque within 24 h with a diameter of  
18 approximately 2 mm. It was mesophilic, exhibiting optimum growth temperature of 30 °C, but was  
19 able to grow between 10-45 °C and at pH 6-11 (optimum pH 8) and it tolerates NaCl up to 15% with  
20 an optimal growth at 8% NaCl. Assimilation of maltose, mannose, fructose, galactose, xylose,  
21 sucrose, malic acid, glucose and lactose is positive; ribose, arabinose, adonitol, sorbitol and citrate  
22 were not assimilated. It was positive for catalase and methyl red but negative for oxidase, urease and  
23 Voges-Proskauer test. Carbon source utilization and hydrolysis of substrates (including  
24 differentiating characters for some *Brachybacterium* species) are indicated in Table 2. It was capable  
25 of reducing nitrate but did not produce H<sub>2</sub>S. Strain JG 06<sup>T</sup> was sensitive to ampicillin, erythromycin,  
26 clindamycin, fusidic acid, nitrofurantoin and sulphametroxazon. It also possessed several plant

1 growth promoting traits like, IAA production, siderophore production, ACC utilization and ACC  
2 deaminase activity and conversion of acetylene to ethylene. The peptidoglycan type is variant A4 $\gamma$   
3 and the menaquinone is MK7 (100%). The polar lipid profile consists of diphosphatidylglycerol,  
4 phosphatidylglycerol, monogalactosyl diglyceride, three unknown phospholipids and three unknown  
5 glycolipids. The predominant fatty acid is anteiso-C<sub>15:0</sub> with significant amounts of iso-C<sub>15:0</sub>, iso-  
6 C<sub>16:0</sub> and anteiso-C<sub>17:0</sub>. The DNA G+C content is 73.0 mol%. The DNA-DNA hybridization of the  
7 strain JG 06<sup>T</sup> with close relatives, *Brachybacterium paraconglomeratum*, *B. conglomeratum*, *B.*  
8 *faecium*, *B. tyrofermentans*, *B. alimentarium*, *B. fresconsis*, *B. sacelli* and *B. muris* showed re-  
9 association value of 36.2%, 36.5%, 35.8%, 27.6%, 27.9%, 28.2%, 28.7% and 11.2%, respectively.  
10 The type strain, JG 06<sup>T</sup> (=DSM 23186<sup>T</sup> = IMCC 252<sup>T</sup>) was isolated from roots of *Salicornia*  
11 *brachiata* from coastal marshy swamps, Bhavnagar district, Gujarat, India.

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11 Legend for figure

12 Fig. 1. The phylogeny was inferred by using the Neighbor-Joining method. The percentage of  
13 replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is  
14 shown next to the branches (numbers at nodes are percentage bootstrap values). The evolutionary  
15 distances were computed using the Maximum Composite Likelihood method and are in the units of  
16 the number of base substitutions per site. All positions containing gaps and missing data were  
17 eliminated from the dataset (Complete deletion option). Phylogenetic analyses were performed by  
18 MEGA version 4.

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1 Table 1. Fatty acid compositions (%) of JG 06<sup>T</sup> and five reference species of *Brachybacterium*.  
 2 Fatty acid values less than 0.5% for the strains are not given and displayed with symbol '-'.  
 3

Fatty acid	JG 06 <sup>T</sup>	<i>B. paraconglomeratum</i> (DSM 46341 <sup>T</sup> )	<i>B. conglomeratum</i> (DSM 10241 <sup>T</sup> )	<i>B. faecium</i> (DSM 4810 <sup>T</sup> )	<i>B. alimentarium</i> (DSM 10672 <sup>T</sup> )	<i>B. muris</i> (DSM 15460 <sup>T</sup> )
14:0 iso	2.06	4.57	1.61	1.71	2.97	2.17
14:0	1.58	2.20	1.91	1.15	0.92	0.89
15:0 iso	8.59	16.24	9.20	11.77	10.44	5.88
15:0 anteiso	52.07	51.73	63.48	50.83	30.75	59.45
16:0	2.62	2.00	1.78	2.51	3.78	1.88
16:0 iso	12.38	11.36	6.60	10.26	1.45	6.41
17:0 iso	1.76	1.12	1.04	2.16	4.99	1.02
17:0 anteiso	10.03	4.15	8.39	9.36	7.98	11.22
17:1 ω9c	1.04	1.28	0.67	1.01	-	-
18:0	-	-	-	1.47	11.56	-
18:0 iso	0.52	-	-	-	0.82	-
18:1 ω7c	3.83	2.66	3.09	-	-	3.62
19:0 iso	-	-	-	-	2.32	-
19:0 anteiso	0.50	-	-	-	2.32	-
19:1 ω7c	0.72	0.91	0.87	1.02	-	1.11
20:0	-	-	-	-	11.34	-
20:1 ω7c	0.59	-	-	0.87	-	2.83

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1 Table 2. Physiological characteristics of JG 06<sup>T</sup> and the closest relatives

2

Physiological characteristics	JG 06 <sup>T</sup>	<i>B. paraconglomeratum</i> (DSM 46341 <sup>T</sup> )	<i>B. conglomeratum</i> (DSM 10241 <sup>T</sup> )	<i>B. faecium</i> (DSM 4810 <sup>T</sup> )
Indole	+	+	(+)	(+)
Urease	-	+	+	-
H <sub>2</sub> S	-	+	-	-
Hydrolysis of gelatin	+	-	(+)	-
Hydrolysis of tween 80	+	-	(+)	-
Assimilation of carbohydrates				
Mannose	+	+	+	-
Ribose	-	-	-	(+)
Xylose	+	-	+	-
Arabinose	-	-	-	(+)
Adonitol	-	+	+	-
Lactose	+	(+)	(+)	-
Fructose	+	+	+	-
Galactose	+	+	+	-
Sucrose	+	+	+	-

3

4 +, positive; -, negative; (+), weakly positive.

5 All the strains are coccoid, non motile, positive for nitrate reduction, catalase, amylase and assimilation of  
6 maltose, glucose. They are negative for Voges- Proskauer, oxidase and sorbitol assimilation.

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**Fig. 1**

