Salibacterium halotolerans gen. nov., sp. nov., a bacterium isolated from a salt pan, reclassification of Bacillus qingdaonensis as Salibacterium qingdaonense comb. nov. and Bacillus halochares as Salibacterium halochares comb. nov.

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Two novel Gram-stain-positive, rod-shaped, non-motile, non-endospore-forming bacterial strains, S7T and IB5, were isolated from Khavda, India. Based on 16S rRNA gene sequence analysis they were identified as belonging to the class Bacilli, order Bacillales, family Bacillaceae, and were most closely related to Bacillus qingdaonensis CGMCC 1.6134T (97.3 %, sequence similarity), Bacillus halochares LMG 24571T (96.9 %), Bacillus salarius KCTC 3912T (95.6 %) and Bacillus aidingensis DSM 18341T (95.3 %). However, these strains shared only 88.2 % 16S rRNA gene sequence similarity with Bacillus subtilis subsp. subtilis DSM 10T, indicating that strains S7T and IB5 might not be members of the genus Bacillus. The DNA–DNA relatedness of these strains with B. qingdaonensis CGMCC 1.6134T was 42.9 ± 0.8. The cell-wall peptidoglycan of strains S7T and IB5 contained meso-diaminopimelic acid, while the polar lipids included diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, a phospholipid and three unknown lipids. The predominant isoprenoid quinone was MK-7. anteiso-C15 : 0 was the predominant fatty acid. The results of the phylogenetic, chemotaxonomic and biochemical tests allowed a clear differentiation of strains S7T and IB5, suggesting that they represent a novel member of the family Bacillaceae, for which the name Salibacterium halotolerans gen. nov., sp. nov. is proposed. The type strain of Salibacterium halotolerans is S7T (=KCTC 33658T=CGMCC 1.15324T). Based on the results of the present study, it is also suggested that B. qingdaonensis and B. halochares should be transferred to this novel genus, as Salibacterium qingdaonense comb. nov. and Salibacterium halochares comb. nov., respectively.

Halophiles, the salt-loving organisms that inhabit hypersaline environments, include mainly prokaryotic and eukaryotic micro-organisms with the capacity to balance the osmotic pressure of the environment and resist the denaturing effects of salts (Oren, 2002; Ventosa, 2006).

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequences of strains S7T and IB5 are LN812017 and LN850139, respectively.

One supplementary figure and one supplementary table are available with the online Supplementary Material.

Halophilic bacteria that grow optimally in media containing 3–15 % (w/v) NaCl are widely distributed throughout various types of saline environments, such as salt lakes, salterns and salty foods (Ventosa et al., 1998). During a study on the microbial population in the salt pans of Kutch, India, two aerobic, Gram-stain-positive bacteria, designated S7T and IB5, were isolated. In this study, we describe the results of the polyphasic study aimed at the characterization of these strains.

Strains S7T and IB5 were isolated from the Khavda, located near Buj, Gujarat, India (23° 98′ 33.20″ N 69° 74′ 51.99″ E).
A 1g sample of air-dried marine sediment was serially diluted up to \(10^{-6}\) and 100 µl was spread on a growth medium containing (g l\(^{-1}\)): NaCl (200), KH\(_2\)PO\(_4\) (1), MgSO\(_4\).7H\(_2\)O (0.2), casein enzyme hydrolysate-type - L (5), SL7 trace elements solution 1 ml [(mg l\(^{-1}\)) : HCl (25 %, v/v) (1 ml); ZnCl\(_2\) (70); MnCl\(_2\).4H\(_2\)O (100); H\(_2\)BO\(_3\) (60); CoCl\(_2\).6H\(_2\)O (200) NiCl\(_2\).6H\(_2\)O (20); Na\(_2\)MoO\(_4\).2H\(_2\)O (40); CuCl\(_2\).2H\(_2\)O (20)] and agar (30) in 1 litre NaHCO\(_3\)/Na\(_2\)CO\(_3\) buffer (100 mM in demionized water; pH 10). Pure cultures were obtained by repeated streaking of the isolate on the same medium. Pure cultures were then preserved at 4 °C for further use.

Genomic DNA was extracted and purified according to the method of Marmur (1961). The 16S rRNA gene sequences of strains S\(_7\)\(^T\) and IB5 were obtained by PCR as described earlier (Reddy et al., 2013). Identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarities were achieved using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim et al., 2012).

The CLUSTAL W algorithm of MEGA 5 (Tamura et al., 2011) was used for sequence alignments and phylogenetic analysis of the 16S rRNA gene was performed using MEGA 5. Distances were calculated by using the Kimura correction in a pairwise deletion manner (Kimura, 1980). Neighbour-joining, minimum-evolution, maximum-likelihood and maximum-parsimony methods in the MEGA 5 software (Tamura et al., 2011) were used to reconstruct phylogenetic trees. Gaps were treated as complete deletions. A nearest-neighbour-interchange heuristic search in the maximum-likelihood method and a subtree-pruning-regrafting search in the maximum-parsimony method were used for tree reconstruction. Percentage support values were obtained using a bootstrap procedure for 1000 pseudoreplcates. The taxonomic relationship between strains S\(_7\)\(^T\) and IB5 was determined using MEGA 5 and Bacillus qingdaonensis CGMCC 1.6134\(^T\) (97.3 % 16S rRNA gene sequence similarity), Bacillus halochares LMG 24571\(^T\) (96.9 %), Bacillus salarius KCTC 3912\(^T\) (95.6 %) and Bacillus aidingensis DSM 18341\(^T\) (95.3 %). However, these strains shared only 88.2 % 16S rRNA gene sequence similarity with Bacillus subtilis subsp. subtilis DSM 10\(^T\), a value lower than those obtained with respective species of the genera Alteribacillus (93.9 %), Salinibacillus (91.4 %), Virgibacillus (91.2 %), Scopulibacillus (91.0 %), Sediminibacillus (91.0 %), Salibahadus (90.6 %), Marinococcus (90.5 %), Lentibacillus (90.3 %), Salsuginibacillus (89.7 %), Bhargavaea (89.7 %), Amphibacillus (89.6 %), Sinibacillus (88.9 %) and Fictibacillus (88.9 %). The level of DNA–DNA relatedness between strains S\(_7\)\(^T\) and IB5 was 89.6 ± 0.9, indicating that the two strains represent a single species. DNA–DNA relatedness of these strains with B. qingdaonensis CGMCC 1.6134\(^T\) was 42.9 ± 0.8. Based on the hybridization results, strains S\(_7\)\(^T\) and IB5 are distinctly related to B. qingdaonensis CGMCC 1.6134\(^T\) and the hybridization values are within the recommended standards to delineate a bacterial species (Stackebrandt & Goebel, 1994; Stackebrandt & Ebers, 1990; Meier-Kolthoff et al., 2013).

The mean DNA G+C content of strains S\(_7\)\(^T\) and IB5 was 48.4 ± 0.4 mol%, which was similar to that of their nearest phylogenetic neighbours (Table 1).

The phenotypic features of strain S\(_7\)\(^T\) were determined following the minimum standards for describing new taxa, as recommended by Logan et al. (2009). Morphological properties, such as cell shape, cell size and motility, were observed by phase-contrast light microscopy (MLX; Magnus). The effect of pH (range 6.0–12.0, with intervals of 0.5) on growth was tested (with K\(_2\)HPO\(_4\)/KH\(_2\)PO\(_4\) buffer for pH 6.0–8.0; NaHCO\(_3\)/NaOH buffer for pH 8.5–11.0 and Na\(_2\)CO\(_3\)/NaOH buffer for pH 11.5–12.0). The temperature (0, 4, 10, 16, 20, 28, 35, 37, 40, 45, 50, 55, 65 and 70 °C) and salt concentration [0–30 % (w/v) with intervals of 0.5 % (w/v)] ranges for growth were examined in marine broth consisting of (g l\(^{-1}\)) : NaCl (24); MgCl\(_2\).6H\(_2\)O (11); Na\(_2\)SO\(_4\) (4); CaCl\(_2\).6H\(_2\)O (2); KCl (0.7); KBr (0.1); H\(_2\)BO\(_3\) (0.03); Na\(_2\)SiO\(_3\).9H\(_2\)O (0.005); SrCl\(_2\).6H\(_2\)O (0.004); NaF (0.003); NH\(_4\)NO\(_3\) (0.002); Fe\(_2\)PO\(_4\).4H\(_2\)O (0.001); bacteriological peptone (5); and yeast extract (1); pH 7.5 ± 2. The results were recorded after 5 days of incubation. Growth was measured turbidimetrically at 540 nm in a colorimeter (Systronics). The tests for temperature and salt concentration tolerance were performed in the above medium emended with 10 % NaHCO\(_3\) to maintain a pH of 9.0. Growth under anaerobic conditions was determined on modified nutrient agar [(g l\(^{-1}\)) : peptone (5), NaCl (120), beef extract (1.5), yeast extract (1.5), agar (15) with a final pH of 7.5 supplemented with 0.5 % (w/v) glucose and with or without 0.1 % (w/v) nitrate] using the anaerobic systems of HiMedia. Various biochemical tests such as for the hydrolysis of starch, casein, tyrosine, xanthine, http://ijs.microbiologyresearch.org
hypoxanthine and gelatin, as well as urease and nitrate reduction, the Voges–Proskauer test, the methyl red test, H2S production, indole production, and oxidase and catalase activities, were carried out as described by Smibert and Krieg (1981, 1994), in Zobell marine broth 2216 (HiMedia) or in specified medium. Utilization of various substrates as sole carbon and energy sources or carbon, nitrogen and energy sources was determined using a basal medium with the following composition (g l−1): yeast extract (0.01), KH2PO4 (0.5), MgSO4.7H2O (0.2), (NH4)2HPO4 (1.0) and NaCl (100). To this liquid medium, 0.1% (w/v) filter-sterilized substrate was added. Carbohydrates were used at a final concentration of 0.2% (w/v) and the tests for their utilization were performed as described by Ventosa et al. (1982). Antibiotic sensitivity tests were performed using the standard disc assay method (Ventosa et al., 1982).

Colonies of strain S7T grown on DSMZ medium 514d were cream-coloured, circular (0.9–1.7 mm in diameter), convex, opaque with entire margins. Cells were Gram-stain-positive and non-motile rods, 0.1–0.3 μm in diameter and 1.5–2.8 μm in length. The strain did not form endospores, similarly to B. qingdaonensis CGMCC 1.6134T. Growth occurred over a pH range of 6.5–10.5 with an optimum at pH 9. NaCl was essential for growth (minimum 2%, w/v) and could be tolerated up to 25% (w/v) with optimum growth seen at 12% (w/v). Optimum growth occurred at 37°C and over a range of 25–45°C. Casein, aesculin, tyrosine, DNA, starch, cellulose, hippurate, xanthine, hypoxanthine and Tween 20 were not hydrolysed by strain S7T. Gelatin was not liquefied. Oxidase and catalase activities were positive, whereas lipase and urease activities were negative. Indole production from tryptophan was positive. Strain S7T neither produced H2S nor reduced nitrate, but showed positive results for citrate utilization and the Voges–Proskauer test. The strain showed a negative result for the methyl red test. Strain S7T was a facultative anaerobe and the substrates which supported growth are indicated in the species description. The strain was sensitive to penicillin.
Table 1. Characteristics distinguishing strain S7\textsuperscript{T} from the type strains of phylogenetically related species of the genus Bacillus

<table>
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<th>Characteristic</th>
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<td>Endospore formation</td>
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<td>Spore shape</td>
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<td>NA</td>
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<td>S</td>
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<td>Range of NaCl concentration for growth</td>
<td>2–25 (12)</td>
<td>2.5–20 (12)</td>
<td>6–23 (15)</td>
<td>8–33 (12)</td>
<td>3–20 (10–12)</td>
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<td>pH range for growth (optimum)</td>
<td>6.5–10.5 (9.0)</td>
<td>6.5–10.5 (9.0)</td>
<td>6.0–9.0 (8.0)</td>
<td>6.0–9.5 (7.2)</td>
<td>6.8–9.5 (8.0)</td>
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<td>Temperature range (°C) (optimum)</td>
<td>25–45 (37)</td>
<td>25–45 (37)</td>
<td>26–45 (37)</td>
<td>22–44 (37)</td>
<td>15–40 (30)</td>
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<td>Urease activity</td>
<td>–</td>
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<td>Nitrate reduction</td>
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<td>Hydrolisis of gelatin</td>
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<td>Acid production from:</td>
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<td>D-Xylose</td>
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<td>+</td>
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<tr>
<td>DNA G+C content (mol%)</td>
<td>48.4 ± 0.4</td>
<td>48</td>
<td>47.2</td>
<td>48.1</td>
<td>43</td>
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</table>

(10 μg), tetracycline (30 μg), ciprofloxacin (5 μg), kanamycin (30 μg), gentamicin (120 μg), vancomycin (30 μg), streptomycin (10 μg) and chloramphenicol (30 μg) but resistant to ampicillin (10 μg), amikacin (30 μg), erythromycin (15 μg) and nalidixic acid (30 μg). The characteristics differentiating strain S7\textsuperscript{T} from related species of the genus Bacillus are summarized in Table 1.

Fatty acids, quinones and polar lipids of strains S7\textsuperscript{T} and B. qingdaonensis CGMCC 1.6134\textsuperscript{T} were analysed from cells grown in DSMZ medium 514d at 37 °C at pH 9.0 and with 12 % (w/v) NaCl. Cells were harvested by centrifugation (10000 g, 15 min, 4 °C) after reaching a cell density of 70 % of the maximum optical density (100 % = OD\textsubscript{max} of 0.8) and the lyophilized pellet was used for analysis. Cellular fatty acids of strains S7\textsuperscript{T} and B. qingdaonensis CGMCC 1.6134\textsuperscript{T} were methylated, separated and identified according to the instructions for the Microbial Identification System [Microbial ID; MIDI 6.0 version; peak identification was carried out based on the RTSSA6 database; Sasser (1990); www.midi-inc.com]. Fatty acid methyl ester analysis was outsourced to Royal Research Laboratories, Secunderabad, India. Polar lipids were extracted from 1 g freeze-dried cells with methanol/chloroform/saline (2 : 1 : 0.8, by vol.) as described by Kates (1986) and were separated using silica gel TLC (Kieselgel 60 F254; Merck) by two-dimensional chromatography using a chloroform/methanol/water (75 : 32 : 4, by vol.) mixture in the first dimension and a chloroform/methanol/acetic acid/water (86 : 16 : 15 : 4, by vol.) mixture in the second dimension (modified after Tindall 1990a, b; Oren et al., 1996). Total polar lipids profiles were detected by spraying with 5 % ethanol molybdophosphoric acid and further characterized by spraying with ninhydrin (specific for amino groups), molybdenum blue (specific for phosphates), Dragendorff’s reagent (quaternary nitrogen) or z-naphthol (specific for sugars) (Kates, 1972; Oren et al., 1996). Quinones of strain S7\textsuperscript{T} and B. qingdaonensis CGMCC 1.6134\textsuperscript{T} were determined by extraction with a chloroform/methanol (2 : 1, v/v) mixture, purified by TLC and analysed by HPLC (Tamaoka et al., 1983). The peptidoglycan of strain S7\textsuperscript{T} and B. qingdaonensis CGMCC 1.6134\textsuperscript{T} was isolated after disruption of the cells by shaking with glass beads and subsequent trypsin digestion, according to the method of Schleifer (1985). The cell wall was hydrolysed for amino acid analysis and analysed as described by Schleifer & Kandler (1972) and Hasegawa et al. (1983).

Whole-cell fatty acid analysis of strain S7\textsuperscript{T} and B. qingdaonensis CGMCC 1.6134\textsuperscript{T} revealed that anteiso-C\textsubscript{15}:0 (38.4 and 36.4 %, respectively) and anteiso-C\textsubscript{17}:0 (16.9 and 21.9 %, respectively) were the predominant fatty acids present. The other significant (>5 %) fatty acids detected in strain S7\textsuperscript{T} included: C\textsubscript{16}:0 (12.9 %), iso-C\textsubscript{16}:0 (9.7 %) and iso-C\textsubscript{17}:0 (5.7 %). Similarly, notable proportions (>5 %) of iso-C\textsubscript{16}:0 (12.2 %), C\textsubscript{16}:0 (7.8 %) and iso-C\textsubscript{15}:0 (5.9 %) were detected in B. qingdaonensis CGMCC 1.6134\textsuperscript{T} (Table S1, available in the online Supplementary Material). The polar lipids of strain S7\textsuperscript{T} included diphasphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phospholipid and three unknown lipids (Fig. S1). The strain differed from B. qingdaonensis CGMCC 1.6134\textsuperscript{T} based on the presence of a phospholipid and an unknown lipid; it also differed based on the absence of another unknown lipid. The major quinone of strain S7\textsuperscript{T} was MK-7 (98.7 %) with traces of MK-6 (1.3 %),...
The cell-wall peptidoglycan of strain S7T contained meso-diaminopimelic acid as the diagnostic diamino acid.

There is a clear consensus that the genus *Bacillus* should be restricted to species that share high 16S rRNA gene sequence similarities with the type strain of the type species, *Bacillus subtilis* (Albert et al., 2007; Kämpfer et al., 2006), and hence strains S7T and IB5 do not fit into the genus *Bacillus* (only 88.2 % sequence similarity with *B. subtilis* subsp. *subtilis* DSM 101T). Furthermore, strains S7T and IB5 were non-sporing forms, contrasting with members of the genus *Bacillus* (Logan & De Vos, 2009). Therefore, on the basis of the morphological, phenotypic and genotypic distinctiveness of strains S7T and IB5 there is support for the proposal that these isolates represent a novel member of the family *Bacillaceae* for which the name *Salibacterium halotolerans* gen. nov., sp. nov., is proposed. Neither do the two strains *B. qingdaonensis* CGMCC 1.6134T and *B. halochares* LMG 24571T fit into the genus *Bacillus*, as they share only 90.3 and 87.7 % 16S rRNA gene sequence similarity with *B. subtilis* subsp. *subtilis* DSM 101T, respectively, and were found to be more closely related to strains S7T and IB5. These strains are also both non-sporing forms, in contrast to members of the genus *Bacillus* (Logan & De Vos, 2009). Hence, a reclassification of *B. qingdaonensis* CGMCC 1.6134T and *B. halochares* LMG 24571T into the new genus proposed here is also suggested.

**Description of Salibacterium gen. nov.**

*Salibacterium* (L. sal salis salt; L. neut. n. bacterium a rod; N.L. neut. n. *Salibacterium* a rod from salt).

Cells are non-motile, rod-shaped, Gram-stain-positive and non-endospore formers. Facultatively anaerobic. Positive for catalase activity and with variable oxidase activity. Indole production from tryptophan is positive, but H2S is not proposed. Positive result for citrate utilization and the Voges–Proskauer test, but negative for the methyl red test. Acids and gas are produced from raffinose, sucrose, d-glucose, maltose, d-xylene, d-arabinose, cellobiose, trehalose, mannose and d-mannitol. Growth is not supported by inulin, salicin, d-galactose, mellibiose, rhamnose, inositol, lactose, d-sorbitol and fructose as the sole carbon sources. Glutamate is the most suitable nitrogen source, but growth is not observed with urea, nitrate, nitrite, ammonium chloride or aspartate.

The type strain and an additional strain, IB5, were isolated from a sediment sample of a salt pan near Khavda, Bhuj, Gujarat, India. The type strain is S7T (KCTC 33658T=CGMCC 1.15324T). The mean DNA G+C content of strains S7T and IB5 is 48.4±0.4 mol%.

**Description of Salibacterium qingdaonense comb. nov.**

*Salibacterium qingdaonense* (qing.da.o.nen’se. N.L. neut. adj. qingdaonense pertaining to Qingdao, the name of the place from which the type strain was isolated).

Basonym: *Bacillus qingdaonensis* Wang et al. 2007.

The description is identical to that given for *B. qingdaonensis* by Wang et al. (2007). The type strain of *Salibacterium qingdaonense* is CM1T (=CGMCC 1.6134T=JCM 14087T).

**Description of Salibacterium halochares comb. nov.**

*Salibacterium halochares* [ha.lo.cha’res. Gr. masc. n. hals salt; N.L. part. adj. chares from Gr. v. chairo to rejoice at, to delight in; N.L. part. adj. halochares finding pleasure in salt (salty environments)].

Basonym: *Bacillus halochares* Pappa et al. 2010.

The description is identical to that given for *B. halochares* by Pappa et al. (2010). The type strain of *Salibacterium halochares* is MSS4T (=DSM 21373T=LMG 24571T).

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**References**

reclassification of *Bacillus arvi*, *Bacillus arenosi* and *Bacillus neidei* as *Viridibacillus arvi* gen. nov., comb. nov., *Viridibacillus arenosi* comb. nov. and *Viridibacillus neidei* comb. nov. *Int J Syst Evol Microbiol* 57, 2729–2737.


