Exiguobacterium alkaliphilum sp. nov. isolated from alkaline wastewater drained sludge of a beverage factory

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A facultatively anaerobic, alkaliphilic, Gram-stain-positive, rod-shaped bacterium, designated strain 12/1T, isolated from alkaline wastewater drained sludge of a beverage industry facility located near New Delhi, India, was subjected to a polyphasic taxonomic study. Phylogenetic analysis based on 16S rRNA gene sequence comparisons revealed that strain 12/1T belonged to the genus Exiguobacterium and was most closely related to Exiguobacterium aurantiacum DSM 6208T (99.46 %), E. aquaticum IMTB-3094T (99.18 %), E. mexicanum 8N¹ (99.06 %), E. profundum 10C¹ (98.17 %), E. aequatus TF-16¹ (98.1 %) and E. marinum TF-80¹ (98.03 %). The DNA G+C content of strain 12/1T was 55.6 mol%, major respiratory isoprenoid quinone was MK-7, major polar lipids were phosphatidylglycerol, diphosphatidylglycerol and phosphatidylethanolamine and the cell-wall peptidoglycan was of the A3 type. The DNA–DNA hybridization of less than 70 % with the closely related species of the genus Exiguobacterium. Strain 12/1T showed levels of DNA–DNA hybridization of less than 70 % with the closely related species of the genus Exiguobacterium. Overall, the phenotypic, chemotaxonomic and phylogenetic data presented in this study suggest that strain 12/1T represents a novel species of the genus Exiguobacterium, for which the name Exiguobacterium alkaliphilum sp. nov. is proposed. The type strain is 12/1T (=CCM 8459T = DSM 21148T).

The genus Exiguobacterium (type species Exiguobacterium aurantiacum) was described by Collins et al. (1983) representing some slightly alkaliphilic strains isolated from potato wastewater effluent. The genus is characterized by distinct chemotaxonomic properties such as A3 type of peptidoglycan in the cell wall, MK7 as the major respiratory quinone and the presence of diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine as the major polar lipids. At the time of writing, 14 species with validly published names were included in the genus Exiguobacterium: E. aurantiacum (Collins et al., 1983), E. acuticicum (Farrow et al., 1994), E. antarcticum and E. undae (Frühling et al., 2002), E. oxidotolerans (Yumoto et al., 2004), E. aequatus (Kim et al., 2005), E. mexicanum and E. artemiae (López-Cortés et al., 2006), E. indicium (Chaturvedi & Shivaji, 2006), E. sibiricum (Rodrigues et al., 2006), E. profundum (Crampart et al., 2007), E. soli (Chaturvedi et al., 2008) and E. aquaticum (Raichand et al., 2012). In this study, a facultatively alkaliphilic bacterial strain (12/1T) capable of neutralizing highly alkaline wastewater which had been isolated from alkaline wastewater drainage sludge of a soft-drink beverage factory located near New Delhi (Kulshreshtha et al., 2010) is described. The 16S rRNA gene sequence comparisons indicated that the isolate belongs to the genus Exiguobacterium. The aim of the present work was to determine the exact taxonomic position of the isolate by detailed characterization combining phenotypic, chemotaxonomic and phylogenetic analyses.

Strain 12/1T was routinely maintained on alkaline Bacillus medium (ABM) pH 10.5 (Atlas, 2005) or Zobell marine broth 2216 (ZMB; HiMedia). ABM was used for the determination of growth at various temperatures (4, 5, 15, 22, 28, 35, 37, 40 and 42 °C), pH values (5–12 with 0.5-unit intervals) and NaCl concentrations (0–20 % with 0.5 % intervals). The pH of the medium was adjusted as described previously (Kulshreshtha et al., 2010). Cell morphology was examined by light microscopy (E600; Nikon) and transmission electron microscopy. Phenotypic characteristics such as colony morphology and sensitivity...
Characterization of Exiguobacterium alkaliphilum

to antibiotics at 37 °C were ascertained using standard methods (Smibert & Krieg, 1994). Motility was determined using a soft medium (López-Cortés et al., 2006). Anaerobic growth was examined using the Anaerocult system (Merck). Catalase activity was determined by the presence of bubbles in a 3% hydrogen peroxide solution. Oxidase activity was tested by using Bio-Rad oxidase discs. Acid production and the ability of the culture to assimilate various carbon compounds were evaluated according to the method described by Chaturvedi et al. (2008). For fatty acid analysis of strain 12/1T and the reference strains, fatty acid methyl esters were prepared according to the method of Sato & Murata (1988) from the cells grown on tryptic soy medium, pH 7.3 (M322; Himedia) at 37 °C and analysed by the Microbial Type Culture Collection and Gene Bank (Chandigarh, India). Analysis of polar lipids, respiratory quinones, peptidoglycan and cell-wall sugars and determination of G+C content of DNA were carried out by the Identification service of Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). Polar lipids were extracted using a protocol modified after Bligh & Dyer (1959), recovered into the chloroform phase and separated by two-dimensional silica gel TLC. Total lipid material and specific functional groups were detected using the method given in Tindall et al. (2007). Respiratory lipoquinones were extracted using the two-stage method described by Tindall (1990a,b) and were separated by TLC on silica gel and further analysed by HPLC. The peptidoglycan was isolated according to the method of Schleifer (1985), hydrolysed to various degrees using HCl and analysed by TLC and 2D-TLC on cellulose plates by using the solvent systems of Rhuland et al. (1955) and Schleifer & Kandler (1972), respectively. For analysis of whole-cell sugars, the cells were hydrolysed and the sugars were analysed by TLC on cellulose plates according to the protocol of Staneck & Roberts (1974). For analysis of G+C content of the cells, the cells were disrupted by French press and DNA was purified on hydroxyapatite according to the protocol of Cashion et al. (1977). The DNA was hydrolysed and the nucleotides were dephosphorylated (Mesbah et al., 1989). The resulting deoxyribonucleosides were analysed by HPLC (Tamaoka & Komagata, 1984). DNA G+C contents were calculated from the ratio of deoxyguanosine and thymidine according to the method of Mesbah et al. (1989). For hybridization, DNA was isolated manually using the method of Marmur (1961). DNA–DNA hybridization was performed by the membrane filter method (Prakash et al., 2007; Tourova & Antonov, 1988) using labelled DNA from strain 12/1T as probe and DNA from E. aurantiacum DSM 6208T, E. aestuarii DSM 16306T, E. marinus DSM 16307T, E. mexicanum DSM 16483T, E. profundum DSM 17289T and E. aquaticum IMTB-3094T for blotting.

To establish the phylogenetic position of strain 12/1T, DNA was purified and the 16S rRNA gene was amplified and sequenced as described previously (Kulshreshtha et al., 2010). The almost complete sequence (1477 nt) of the 16S rRNA gene of strain 12/1T was aligned against those of the closely related species by CLUSTAL W (Thompson et al., 1994). Evolutionary history was inferred using the maximum-parsimony and neighbour-joining methods (Saitou & Nei, 1987). The evolutionary distances were computed using the Kimura two-parameter method (Kimura, 1980). Phylogenetic trees were reconstructed using the maximum-parsimony and neighbour-joining tree-making algorithms in MEGA4 (Tamura et al., 2007). Confidence in the tree topology was determined by bootstrap analysis using 500 replicates of the sequences (Felsenstein, 1985).

Phenotypic and chemotaxonomic characteristics of strain 12/1T are given in species description, in Table 1 and in Table S1 (available in IJSEM Online). Strain 12/1T was found to comprise cells that were Gram-stain-positive, rod-shaped and non-spor-forming, to possess MK-6, MK-7 and MK-8 (5:85:10) as respiratory quinones, to possess a cell-wall peptidoglycan of the Lys-Gly type and to have diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine as the major polar lipids; and it was therefore similar to species of the genus Exiguobacterium with validly published names (Collins et al., 1983; Farrow et al., 1994; Frühling et al., 2002; Yumoto et al., 2004; Kim et al., 2005; López-Cortés et al., 2006; Raichand et al. 2012). Phylogenetic analysis based on 16S rRNA gene sequence (1477 nt) further confirmed the affiliation of strain 12/1T to the genus Exiguobacterium (Fig. 1). Strain 12/1T formed a robust cluster with E. aurantiacum DSM 6208T (DQ019166), E. aestuarii DSM 16306T (AY594264), E. marinus DSM 16307T (AY594266), E. mexicanum DSM 16483T (AM072764), E. profundum DSM 17289T (AY818050) and E. aquaticum IMTB-3094T (FJ775503). The remaining strains, E. antarcticum DSM 14480T, E. soli MTCC 4816T, E. undae DSM 14481T, E. artemiae DSM 16484T, E. sibiricum DSM 17290T, E. oxidotolerans JCM 12280T, E. acetylicum DSM 20416T and E. indicum LMG 23471T, formed another clade. This observation is in accordance with previous reports (López-Cortés et al., 2006; Kim et al., 2005; Chaturvedi & Shivaji, 2006).

The 16S rRNA gene sequence similarity values of strain 12/1T with the species of the genus Exiguobacterium with validly published names as determined by using EzTaxon server 2.0 (Chun et al., 2007) were as follows: 99.46% with E. aurantiacum DSM 6208T (DQ019166), 99.18% with E. aquaticum IMTB-3094T (FJ775503), 99.06% with E. mexicanum 8NT (AM072764), 98.17% with E. profundum 10CT (AY818050), 98.10% with E. aestuarii TF-16T (AY594264), 98.03% with E. marinus TF-80T (AY594266). Thus, to merit the status of a novel species, strain 12/1T must exhibit 97% similarity at the 16S rRNA gene level. At the whole-genome level, as determined by DNA–DNA hybridization, the levels of DNA–DNA relatedness between strain 12/1T and E. aurantiacum DSM 6208T, E. aquaticum IMTB-3094T, E. aestuarii DSM 16306T, E. marinus DSM

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Table 1. Differential phenotypic characteristics between strain 12/1T and closely related type strains of species of the genus *Exiguobacterium*

Strains: 1, 12/1T (data from the present study); 2. *E. aurantiacum* DSM 6208T (Collins et al. 1983); 3. *E. aestuarii* DSM 16306T (Kim et al., 2005); 4. *E. marinum* DSM 16307T (Kim et al., 2005); 5. *E. mexicanum* DSM 16483T (López-Cortés et al., 2006); 6. *E. profundum* DSM 17289T (Crapart et al., 2007); 7. *E. aquaticum* IMTB-3094T (Raichand et al., 2012). All strains were Gram-stain-positive, rod-shaped, motile, non-endospore forming, catalase-positive, positive for acid production from D-mannitol and contained peptidoglycan of type A3 x L-Lys–Gly. +, Positive; w, weakly positive; –, negative; ND, no data available.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
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<th>4</th>
<th>5</th>
<th>6</th>
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<td>Potato processing effluent</td>
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<td>Tidal flat from yellow sea, Korea</td>
<td>Commercial can of <em>Artemia franciscana</em> cysts</td>
<td>Deep-sea hydrothermal vent</td>
<td>Tikkar Tal Lake, Haryana</td>
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<td>Colony size (mm)*</td>
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<td>2–3</td>
<td>3–4</td>
<td>3–4</td>
<td>2–3</td>
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<td>Rods to almost coccoid</td>
<td>Short rods to cocci</td>
<td>Short rods to cocci</td>
<td>Rods</td>
<td>Rods</td>
<td>Short rods</td>
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<td>Colony colour*</td>
<td>Orange</td>
<td>Orange–yellow</td>
<td>Pale orange</td>
<td>Deep orange</td>
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<td>Creamy</td>
<td>Orange–yellow</td>
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<td>12–30</td>
<td>10–47</td>
<td>10–43</td>
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<td>12–49</td>
<td>15–42</td>
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<td>+</td>
<td>w</td>
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<td>Ribose</td>
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<td>–</td>
<td>+</td>
<td>–</td>
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<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
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<td>+</td>
<td>–</td>
<td>+</td>
<td>w</td>
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<td>ND</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>w</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>w</td>
<td>+</td>
<td>ND</td>
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<tr>
<td>Cellobiose</td>
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<td>+</td>
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<td>Raffinose</td>
<td>–</td>
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<td>–</td>
<td>w</td>
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<td>Carbon source utilization</td>
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<td>Mannan</td>
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<td>–</td>
<td>+</td>
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<td>N-acetylgalactosamine</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>w</td>
<td>+</td>
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<td>N-acetylmannnosamine</td>
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<td>–</td>
<td>w</td>
<td>+</td>
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<td>+</td>
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<td>Cellobiose</td>
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<td>–</td>
<td>w</td>
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<tr>
<td>D-Galactose</td>
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<tr>
<td>D-Mannitol</td>
<td>w</td>
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<td>+</td>
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<td>Raffinose</td>
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<td>–</td>
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<td>D-Sorbitol</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td>ND</td>
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<td>Acetic acid</td>
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<td>+</td>
<td>+</td>
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<td>Methylpyruvate</td>
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<td>+</td>
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<td>Methyl</td>
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<td>–</td>
<td>–</td>
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</table>
Strain 12/1\(^T\) differed from the above six phylogenetically closely related species in terms of a number of phenotypic characteristics (Table 1) and the pattern of fatty acids present (Table S1). The major fatty acids were iso-C\(_{17:0}\) (24.49%), anteiso-C\(_{13:0}\) (17.53%), iso-C\(_{15:0}\) (13.78%) and iso-C\(_{13:0}\) (12.57%). According to the criteria generally used for discriminating species (Stackebrandt & Goebel, 1994), strain 12/1\(^T\), which exhibits 70% similarity at the DNA–DNA level with respect to \(E.\) aurantiacum DSM 6208\(^T\), \(E.\) aquaticum IMTB-3094\(^T\), \(E.\) aestuarii DSM 16306\(^T\), \(E.\) marinum DSM 16307\(^T\), \(E.\) mexicanum DSM 16483\(^T\) and \(E.\) profundum DSM 17289\(^T\) (the most closely related species) and which also differs phenotypically from these six species (Tables 1 and S1), represents a novel species of the genus \(Exiguobacterium\). The name \(Exiguobacterium\) alkaliphilum sp. nov. is proposed for this novel species.

### Description of \(Exiguobacterium\) alkaliphilum sp. nov.

\(Exiguobacterium\) alkaliphilum sp. nov. al.ka.li'phi.lum. N.L. n. alkali (from Arabic alqali ashes of salt wort); Gr. adj. philos loving; N.L. neut. adj. philum loving, friendly to; N.L. neut adj. alkaliphilum loving alkaline conditions.

Cells are facultatively anaerobic, Gram-stain-positive, motile and rod to almost coccoid in shape (2.0–3.0 \(\mu\)m in length and 0.7–0.9 \(\mu\)m in width). Colonies (2–3 mm) on ABM are round, shiny, regular, elevated and yellowish orange-coloured after 24 h at 37°C. No spores are observed. Growth occurs between 5°C and 40°C and at pH 6.5–12. The optimum temperature and pH for growth are 35°C and 9.5 respectively on ABM. NaCl is tolerated at 9.5% and growth also occurs in the absence of salt. Positive in tests for catalase and starch hydrolysis. Negative for oxidase, aminopeptidase and nitrate reduction. The following substances are utilized: glycogen, N-acetylglucosamine, N-acetylmannosamine and D-sorbitol. The following are not utilized: mannan, cellobiose, D-galactose, raffinose, acetic acid, methylpuruvate, methylsuccinate, propionic acid, L-alanine, L-alanyl glycine, L-serine, 2,3-butanediol, adenosine 5'-monophosphate, thymidine 5'-monophosphate, uridine 5'-monophosphate, fructose-6-phosphate and glucose-1-phosphate. Acid is produced from glycerol, ribose, mannitol, methyl-D-glucoside, amygdalin, arbutin and cellobiose but not from D-galactose, D-mannose, melibiose and raffinose. Resistant to the following antibiotics: kanamycin (30 \(\mu\)g), nalidixic acid (30 \(\mu\)g) and polymixin B (100 IU). Sensitive to the following antibiotics: ampicillin (25 mg), carbenicillin (100 mg), chloramphenicol (50 mg), gentamicin (50 \(\mu\)g), penicillin (10 IU), rifampicin (15 mg), streptomycin (10 mg), tetracycline (10 \(\mu\)g) and vancomycin (10 mg). Among the respiratory quinones, MK-7 is predominant component and MK-6 and MK-8 are minor components.

**Table 1. cont.**

<table>
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<tr>
<th>Characteristic</th>
<th>1</th>
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<td>Propionic acid</td>
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<td>+</td>
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<td>+</td>
<td>++</td>
<td>+</td>
<td>++</td>
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<td>L-Alanyl glycine</td>
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<td>L-Serine</td>
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<td>++</td>
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<td>+</td>
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<td>2,3-Butanediol</td>
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<td>++</td>
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<td>++</td>
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<td>DNA G+C content (mol%)</td>
<td>56.6</td>
<td>55.8</td>
<td>55.8</td>
<td>48.6</td>
<td>48.6</td>
<td>55.8</td>
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<td>MK-7</td>
<td>MK-7</td>
<td>PG, DPG, PE</td>
<td>MK-7</td>
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<td>PG, DPG, PE, unidentified (2)</td>
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<td>PG, DPG, PE, unidentified (2)</td>
</tr>
</tbody>
</table>

*Determined on Zobell marine broth (ZMB) for all the strains.

**DG**DPhosphatidylglycerol; **DG**Diphasatidylglycerol; **PG**Phosphatidylglycerol; **PE**Phosphatidylethanolamine; **PS**Phosphatidylserine.
The cell-wall peptidoglycan is of the A3γ l-Lys–Gly type. Phosphatidylglycerol, diphosphatidylglycerol, phosphatidyl-
lethanolamine and two unidentified lipids are present. The 
major fatty acids present are iso-C13:0 (12.57%), anteiso-
C13:0 (17.53%), iso-C15:0 (13.78%), iso-C16:0 (5.44%), iso-
C17:0 (24.49%) and anteiso-C17:0 (7.41%). Galactose 
and rhamnose are present as cell-wall sugars.

The type strain, 12/1\textsuperscript{T} (=CCM 8459\textsuperscript{T}=DSM 21148\textsuperscript{T}), was isolated from alkaline wastewater drainage sludge of a 
beverage industry facility located near New Delhi, India. 
The DNA G+C content of the type strain is 56.6 mol%.

Acknowledgements

The authors would like to acknowledge the Department 
of Biotechnology, New Delhi for financial assistance. N. M. K. is grateful 
to the University Grants Commission for a research fellowship. This 
work was carried out at the Institute of Genomics and Integrative 
Biology, Mall Road, Delhi 110007, India.

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of base substitutions through comparative studies of nucleotide 

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationship between strain 
12/1\textsuperscript{T} and species of the genus Exiguobacterium. Bacillus subtilis DSM 10\textsuperscript{T} was used as the outgroup. Bootstrap values 
greater than 50 % are given at nodes. Bar, 1 substitution per 100 nt.

Exiguobacterium antarcticum DSM 14480\textsuperscript{T} (DQ019164)
Exiguobacterium soli MTCC 4816\textsuperscript{T} (AY864633)
Exiguobacterium undae DSM 14481\textsuperscript{T} (DQ019165)
Exiguobacterium artemiae DSM 16484\textsuperscript{T} (AM072763)
Exiguobacterium sibiricum DSM 17290\textsuperscript{T} (AY444899)
Exiguobacterium oxidotolerans JCM 12280\textsuperscript{T} (AB105164)
Exiguobacterium acetylicum DSM 20416\textsuperscript{T} (DQ019167)
Exiguobacterium indicum LMG 23471\textsuperscript{T} (AJ846291)
Exiguobacterium aestuarii DSM 16306\textsuperscript{T} (AY594264)
Exiguobacterium profundum DSM 17289\textsuperscript{T} (AY818050)
Exiguobacterium marinus DSM 16307\textsuperscript{T} (AY594266)
Exiguobacterium alkalophilum 12/1\textsuperscript{T} (EU379016)
Exiguobacterium auranticum DSM 6208\textsuperscript{T} (DQ019166)
Exiguobacterium mexicanum DSM 16483\textsuperscript{T} (AM072764)
Exiguobacterium aquaticum LMG 1769\textsuperscript{T} (X60646)
Bacillus subtilis NDCO-3094\textsuperscript{T} (JF775503)

0.01
Characterization of Exiguobacterium alkaliphilum


