Exiguobacterium enclense sp. nov., isolated from sediment

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A Gram-stain-positive bacterium, designated strain NIO-1109T, was isolated from a marine sediment sample from Chorao Island, Goa, India. Phenotypic and chemotaxonomic characteristics and data from phylogenetic analysis based on 16S rRNA gene sequences indicated that strain NIO-1109T was related to the genus Exiguobacterium. Strain NIO-1109T exhibited >98.0 % 16S rRNA gene sequence similarity with respect to Exiguobacterium indicum HHS 31T (99.5 %) and Exiguobacterium acetylicum NCIMB 9889T (99.1 %); the type strains of other species showed <98 % similarity. Levels of DNA–DNA relatedness between strain NIO-1109T and E. acetylicum DSM 20416T and E. indicum LMG 23471T were less than 70 % (33.0 ± 2.0 and 37 ± 3.2 %, respectively). Strain NIO-1109T also differed from these two closely related species in a number of phenotypic traits. Based on phenotypic, chemotaxonomic and phylogenetic data, strain NIO-1109T is considered to represent a novel species of the genus Exiguobacterium, for which the name Exiguobacterium enclense sp. nov. is proposed. The type strain is NIO-1109T (=NCIM 5457T=DSM 25128T=CCTCC AB 2011124T).

Bacterial species of the genus Exiguobacterium are globally diverse organisms, and have been found in a variety of environments, including extreme environments such as microbialites (White et al., 2013), arsenic-rich lakes (Ordoñez et al., 2013), tidal flats (Kim et al., 2005), freshwater lakes (Raichand et al., 2012), Himalayan ice (Chaturvedi & Shivaji, 2006), Himalayan soil (Singh et al., 2013), hydrothermal vents (Crapart et al., 2007), brine shrimp (López-Cortés et al., 2006) and microbial biofilms (Carneiro et al., 2012). Exiguobacterium was proposed by Collins et al. (1983) with the description of Exiguobacterium aurantiacum as the type species. Farrow et al. (1994) transferred Brevibacterium acetylicum incertae sedis to the genus Exiguobacterium, as Exiguobacterium acetylicum. At the time of writing, the genus Exiguobacterium comprised 15 recognized species, including the recently proposed Exiguobacterium aquaticum (Raichand et al., 2012) and Exiguobacterium alkaliphilum (Mohan Kulshreshtha et al., 2013). The genus Exiguobacterium comprises psychrophilic, mesophilic and moderately thermophilic species and strains (Vishnivetskaya et al., 2009), with pronounced morphological diversity (ovoid, rods, double rods and chains) depending on species, strain and environmental conditions (Vishnivetskaya et al., 2007). During an investigation of the bacterial diversity in the marine environment of Chorao Island (15’ 32’ 34” N 73’ 55’ 15” E), Goa State, India, a yellow-pigmented bacterial strain, designated NIO-1109T, was isolated and subjected to a polyphasic taxonomic investigation.

Strain NIO-1109T was isolated on marine agar (MA; Hi-media) by the standard serial dilution method. MA plates were incubated at 28 ± 2 °C for 3–5 days. Nutrient agar (NA) was used as a growth medium. Isolated colonies were purified by repeated streaking on fresh MA plates, and maintained on NA slants at 4 °C and as glycerol suspensions (20 %, v/v) at −80 °C. Biomass for chemotaxonomic and molecular systematic studies was obtained following growth in trypticase soy broth (Hi-media) under shaking conditions of 150 r.p.m. at 28 ± 2 °C for 72 h. Gram-staining was carried out by using the standard Gram

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of Exiguobacterium enclense NIO-1109T is JF893462.

Two supplementary figures are available with the online Supplementary Material.
reaction (Smibert & Krieg, 1981) and cell motility was confirmed by the development of turbidity throughout a tube containing semisolid medium. For motility testing, isolated colonies were picked from a sterile needle and stabbed in 0.4% semisolid NA medium, then incubated at 28±2°C for 18 h. Motility was indicated by a turbid area extending away from the line of inoculation (Leifson, 1960). The morphological characteristics of strain NIO-1109T were assessed by light and scanning electron microscopy (Philips XL30; ESEM-TMP) of 72 h cultures grown on NA. Growth at 4, 10, 20, 30, 37, 45, 50 and 55°C was tested in nutrient broth by incubating the cultures for 72 h and measuring the optical density at 660 nm; distilled water was used as a control. The pH range for growth (pH 4, 5, 6, 7, 8, 9, 10 and 12, using the buffer system described by Xu et al., 2005) and NaCl tolerance (0, 1, 3, 5, 7, 9, 10, 12 and 15%, w/v) were tested at 28±2°C for 72 h by culturing the strains in nutrient broth. Catalase activity was determined based on production of bubbles after the addition of a drop of 3% H2O2. Oxidase activity was determined using API oxidase reagent. Hydrolysis of urea was determined on peptone-glucose agar [per litre: peptone, 1 g; glucose, 1 g; NaCl, 5 g; and KH2PO4, 2 g, containing 2% (w/v) urea and 0.001% (w/v) phenol red as an indicator]. Gelatin liquefaction was determined by incubation on peptone-gelatin medium (per litre: peptone, 5 g; and gelatin, 120 g) and milk coagulation and peptonization was determined using 20% (w/v)

Table 1. Phenotypic characteristics that differentiate strain NIO-1109T from its phylogenetic neighbours in the genus *Exiguobacterium*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
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</thead>
<tbody>
<tr>
<td>Colony morphology</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Size (mm)</td>
<td>2–4</td>
<td>2–5</td>
<td>2–4</td>
</tr>
<tr>
<td>Shape</td>
<td>Round</td>
<td>Irregular</td>
<td>Round</td>
</tr>
<tr>
<td>Growth temperature (°C)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>37</td>
<td>+</td>
<td>w</td>
<td>–</td>
</tr>
<tr>
<td>Optimum growth temperature (°C)</td>
<td>28±2.0</td>
<td>37</td>
<td>30</td>
</tr>
<tr>
<td>Gelatinase</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ornithine decarboxylase</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Voges-Proskauer reaction</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Aesculine hydrolysis</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Starch hydrolysis</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Lysine decarboxylase</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Acid production from:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-Glucose, sucrose, d-fructose, trehalose, maltose</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Inulin</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
<td>D-Mannose</td>
<td>–</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Cellobiose</td>
<td>+</td>
<td>+</td>
<td>–</td>
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<tr>
<td>D-Adonitol</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Carbon-source utilization</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>D-Galactose</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>L-Rhamnose, melibiose, D-xylose</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>L-Xylose</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>D-Mannitol</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Raffinose</td>
<td>+</td>
<td>–</td>
<td>+</td>
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<tr>
<td>L-Sorbitol, dulcitol</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
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<td>DNA G+C content (mol%)</td>
<td>46.9</td>
<td>47.0*</td>
<td>48.0*</td>
</tr>
<tr>
<td>Quinone(s)</td>
<td>MK-7, MK-8</td>
<td>MK-7*</td>
<td>MK-7, MK-8*</td>
</tr>
<tr>
<td>Polar lipids†</td>
<td>PG, DPG, PE</td>
<td>DPG, PS*</td>
<td>PG, DPG, PE*</td>
</tr>
<tr>
<td>Source of isolation</td>
<td>Marine sediment</td>
<td>Creamery waste</td>
<td>Glacial water</td>
</tr>
</tbody>
</table>

*Data from Chaturvedi & Shivaji (2006).
†DPG, Diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PS, phosphatidylserine.
skimmed milk as medium with incubation for 48–72 h at 28 ± 2 °C. Hydrolysis of starch was determined on peptone-beef extract agar containing 0.2 % (w/v) soluble starch by flooding of the plates with iodine solution. Nitrate reduction and urease activity were assessed as described by Smibert & Krieg (1994). The strain was characterized biochemically using the API CH50 and API ZYM systems (bioMérieux). NA plates were used to examine hydrolysis of starch and Tweens 20, 40, 60 and 80 [final concentration of 1 % (v/v)].

Analysis of cell-wall amino acids was performed using the methods of Collins et al. (1983). Polar lipids were extracted, examined by two-dimensional TLC and identified using standard procedures (Minnikin et al., 1984). Polar lipids were separated by two-dimensional TLC (silica-gel plate 60; Merck). The first direction was developed in chloroform/methanol/water (65 : 25 : 4, v/v) and the second was developed in chloroform/methanol/acetic acid/water (80 : 12 : 15 : 4, v/v). Total lipid material and specific functional groups were detected by using Dittmer and Lester reagent (phosphate), ninhydrin (free amino groups), Dragendorff reagent (quaternary nitrogen) and anisaldehyde-sulfuric acid (glycolipids). Menaquinones were isolated according to Minnikin et al. (1984) and were separated by HPLC (Kroppenstedt, 1982). For analysis of fatty acids, strain NIO-1109T was cultured on trypticase soy agar (TSA; Hi-Media, Mumbai) at 28 ± 2 °C for 72 h. Preparation and analysis of fatty acid methyl esters were performed as described by Sasser (1990) by using the Microbial Identification System (MIDI) and the Microbial Identification software package (Sherlock version 6.1; MIDI database: TSBA6).

Extraction of genomic DNA, PCR amplification and sequencing of the 16S rRNA gene from strain NIO-1109T were performed as described by Li et al. (2007). The generated 16S rRNA gene sequence was used to query the EzTaxon-e server to identify its closest relatives, and a subsequent alignment and phylogenetic analysis was then performed (Kim et al., 2012). Multiple sequence alignments with closely related species of the genus *Exiguobacterium* and levels of similarity were determined using CLUSTAL X (Thompson et al., 1997). Phylogenetic analyses were performed using the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) tree-making algorithms. A phylogenetic tree was reconstructed based on Kimura's two-parameter distances (Kimura, 1980) using the neighbour-joining method as implemented in MEGA version 6.0 (Tamura et al., 2013). The topology of the phylogenetic tree was evaluated by the bootstrap resampling method of Felsenstein (1985) based on 1000 replicates.

The genomic DNA of the isolate for determination of the G+C content was prepared according to the method of Marmur & Doty (1962). The G+C content of the DNA was determined by reversed-phase HPLC analysis and calculated from the ratio of deoxyguanosine (dG) and thymidine (dT) according to the method of Mesbah et al. (1989). DNA–DNA

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**Fig. 1.** Neighbour-joining phylogenetic tree based on nearly complete (1415 nt) 16S rRNA gene sequences showing the position of NIO-1109T among members of the genus *Exiguobacterium*. Asterisks indicate that the corresponding nodes (groupings) are also recovered in Fitch–Margoliash, maximum-parsimony and maximum-likelihood trees. Bootstrap values (>70 %; 1000 resamplings) are given at branch points. *Bacillus idriensis* SMC 4352-2T was used as an outgroup. Bar, 0.01 nt substitutions per position.
hybridization was carried out by applying the optical renaturation method (De Ley et al., 1970; Huss et al., 1983; Jahnke, 1992) under optimal hybridization conditions.

Cells of strain NIO-1109T were aerobic, Gram-stain-positive, motile, short rods 0.5–0.6 × 0.8–1.3 μm in size (Fig. S1, available in the online Supplementary Material). Strain NIO-1109T grew well on MA and NA and moderate growth was recorded on International Streptomyces project 5 (ISP-5) and ISP-7 media. On NA, strain NIO-1109T formed opaque, yellowish orange, circular colonies with entire margins after 48 h of incubation. Strain NIO-1109T was oxidase-positive. It grew well between 25 and 42 °C (optimum, 28 ± 2 °C with an OD<sub>660</sub> of 1.83), at pH 6–12 (optimum, pH 7.0 with an OD<sub>660</sub> of 1.79) and in the presence of 0–15% (w/v) NaCl. Strain NIO-1109T was negative for hydrolysis of starch and urea but positive for hydrolysis of Tweens 20, 40, 60 and 80. A detailed species description is presented below. A phenotypic comparison of strain NIO-1109T and related species of the genus Exiguobacterium is presented in Table 1, and in the species description. Strain NIO-1109T showed marginal differences in phenotypic properties from Exiguobacterium indicum DSM 23471<sup>T</sup> and E. acetylicum DSM 20416<sup>T</sup> (Table 1).

The nearly complete 16S rRNA gene sequence of strain NIO-1109<sup>T</sup> (1415 bp) was determined and compared with corresponding sequences from the EzTaxon database. A phylogenetic tree, based on 16S rRNA gene sequence data from strain NIO-1109<sup>T</sup> and the type strains of species of the genus Exiguobacterium, was reconstructed using the neighbour-joining algorithm (Fig. 1). Comparative phylogenetic analysis showed that strain NIO-1109<sup>T</sup> lies in a subclade with E. indicum HHS 31<sup>T</sup> and E. acetylicum NCIMB 9889<sup>T</sup> (supported by high bootstrap values of 98 and 99%, Fig. 1), and with which it shared highest sequence similarity of 99.5 and 99.1%, respectively. The affiliation of strain NIO-1109<sup>T</sup> with its closest neighbours was also supported in the maximum-parsimony and maximum-likelihood trees with high bootstrap values.

The major fatty acids (>5%) of strain NIO-1109<sup>T</sup> were iso-C<sub>17 : 0</sub> (21.8%), iso-C<sub>15 : 0</sub> (13.0%), iso-C<sub>13 : 0</sub> (12.8%), C<sub>16 : 0</sub> (11.6%), C<sub>18 : 0</sub> (8.3%) and anteiso-C<sub>13 : 0</sub> (7.3%). The complete cellular fatty acid profile is given in Table 2. The peptidoglycan of strain NIO-1109<sup>T</sup> contained L-lysine as the diagnostic diamino acid. The major menaquinone was MK-7 (91.3%), and MK-8 (8.4%) was found as a minor component. The polar lipids detected in strain NIO-1109<sup>T</sup> were phosphatidylglycerol (PG), diphosphatidylglycerol (DPG) and phosphatidylethanolamine (PE) (Fig. S2).

The genomic DNA G + C content of strain NIO-1109<sup>T</sup> was 46.9 mol%. Levels of DNA–DNA relatedness between strain NIO-1109<sup>T</sup> and E. indicum DSM 23471<sup>T</sup> and E. acetylicum DSM 20416<sup>T</sup> were 37.0 (±3.2%) and 33.0 (±2.0%), respectively (mean ± SD from triplicate experiments; Table 3). These values were well below the 70% cut-off point for recognition of novel genomic species (Wayne et al., 1987), suggesting that strain NIO-1109<sup>T</sup> should be considered as representing a novel species of the genus Exiguobacterium.

Based on phenotypic, chemotaxonomic and phylogenetic analyses, strain NIO-1109<sup>T</sup> can be distinguished from its closest phylogenetic relatives (Table 1). Therefore, strain NIO-1109<sup>T</sup> is considered to represent a novel species of the genus Exiguobacterium, for which the name Exiguobacterium enclense sp. nov. is proposed.

**Description of Exiguobacterium enclense sp. nov.**

Exiguobacterium enclense (en.clens'e, L.N. neut. adj. enclense arbitrary name formed from NCL, the acronym for the National Chemical Laboratory, India, where taxonomic studies on this species were performed).

Cells are Gram-stain-positive, motile, non-spore-forming rods to coccolbacilli. Colonies grown on TSA are 1.2–1.8 mm in size.
The type strain, NIO-1109T (\textit{\textsuperscript{T}}), was isolated from a marine sediment sample that represent uncultured species.

### References

Genome Announc

strain S17, isolated from hyperarsenic lakes in the Argentinian Puna.

Syst Appl Microbiol 425.

63 alkaline wastewater drained sludge of a beverage factory.

J Liq Chromatogr HPLC using reverse phase (RP-18) and a silver-loaded ion exchanger.

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