Nitrincola alkalisediminis sp. nov., an alkaliphilic bacterium isolated from an alkaline lake

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Two Gram-stain-negative, aerobic, alkaliphilic bacteria (strains MEB087T and MEB142) were isolated from sediment and water samples, respectively, collected from the alkaline Lonar Lake in Maharashtra, India. Strains MEB087T and MEB142 shared 99.8 % 16S rRNA gene sequence similarity and were 85 % related on the basis of DNA–DNA hybridization. The 16S rRNA gene sequences of both strains showed close relationship with the genus Nitrincola, and their closest neighbour was Nitrincola lacisaponensis 4CAT with 97.7 % sequence similarity. MEB087T and MEB142 exhibited only 45 % and 54 % DNA–DNA relatedness, respectively, with Nitrincola lacisaponensis DSM 16316T. Both strains were asporogenous, short, non-motile rods capable of utilizing a limited range of organic acids as sole carbon and energy sources. They were oxidase- and catalase-positive, able to reduce nitrate and nitrite; but unable to degrade DNA, urea, gelatin, casein or starch. They grew optimally at pH 9.5 (tolerating up to pH 11) and could withstand up to 0.6 M NaCl. The predominant cellular fatty acids were summed feature 8 comprising C18:1ω7c/C18:1ω6c (47–49 %) followed by summed feature 3 comprising C16:1ω7c/C16:1ω6c (28–32 %). The predominant polar lipids were diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylcholine. The DNA G+C content was 49.3–49.7 mol%. On the basis of the phylogenetic analysis and chemotaxonomic characteristics, strains MEB087T and MEB142 represent a novel species in the genus Nitrincola, for which the name Nitrincola alkalisediminis sp. nov. is proposed. The type strain is MEB087T (=KCTC 42948T=JCM 19317T) with MEB142 (=KCTC 42949=JCM 19318) as an additional strain.

The genus Nitrincola (Dimitriu et al., 2005) in the order Oceanospirillales (Garrity et al., 2005) and family Oceanospirillaceae belongs to the class Gammaproteobacteria. It accommodates Gram-stain-negative, non-pigmented, motile, rod-shaped bacteria. At present, the genus Nitrincola comprises one recognized species, Nitrincola lacisaponensis (Dimitriu et al., 2005), isolated from decomposing wood from the shore of a saline, alkaline lake in Grant County, WA, USA. Members of the genus Nitrincola are alkaliphilic, halotolerant, heterotrophic, chemo-organotrophic, and oxidase- and catalase-positive. NO3 and O2 can be used as electron acceptors and these bacteria do not grow on fermentable carbon sources. They require sodium for growth. The genus is differentiated from other related genera, Neptunomonas, Marinobacterium and Oceanospirillum, mainly by morphological and physiological characteristics like growth in alkaline conditions and the ability to grow in the absence of NaCl. In this study, we propose a novel species based on two strains (MEB087T and MEB142) isolated from sediment and water samples collected from Lonar Lake, an alkaline saline lake (pH 9.8) situated in Buldhana district of Maharashtra state, India.

Six sediment and water samples were collected in sterile containers from the alkaline Lonar lake at a depth of 0.46 m (1.5 feet) in October 2010 and brought to the laboratory at 4 °C. The temperature and pH of the lake at the time of sampling were 28 °C and pH 9.8, respectively. While strain MEB087T was isolated from a sediment sample, strain MEB142 was isolated from a water sample. For isolating strain MEB087T, 1 g of sediment sample was inoculated in 99 ml of sterile Lonar lake water.
The suspension was serially diluted and 100 µl aliquots of each dilution were streaked on modified seawater agar (Borsodi et al., 2011) (containing, g l−1: NaCl, 24; KCl, 0.7; MgCl₂, 6H₂O, 5.33; MgSO₄·7H₂O, 7.0; CaCl₂, 0.1; peptic digest of animal tissue, 5.0; yeast extract, 5.0; beef extract, 3.0; agar 1.5). The pH of the medium was adjusted to pH 9.8 by addition of Na₂CO₃/NaHCO₃. After incubation at 28 °C for 72 h, individual colonies were picked up and purified by subculturing on the same medium. Strain MEB142 was isolated by spreading 100 µl water sample on modified seawater agar plates. The isolated strains along with Nitrincola laciapsonensis DSM 16316ᵀ were maintained as 20% glycerol stocks at −80 °C.

The three strains, MEB087ᵀ, MEB142 and Nitrincola laciapsonensis DSM 16316ᵀ were grown on nutrient agar (pH 9.5; Hi-Media) and incubated at 30 °C for 48 h. Genomic DNA for 16S rRNA gene sequencing was prepared using the DNeasy kit (Qiagen) in accordance with the manufacturer’s instructions. The 16S rRNA gene was PCR-amplified using the universal primers 27F (GTGCTGCAGAGATTTGATCCTGGCTCAG) (Brosius et al., 1978) and 1488R (CGGTTACCTTTGATACGACTTCACC) (positions 11–27 and 1488–1511 for 27F and 1488R, respectively, according to the Escherichia coli 16S rRNA numbering system; Lane et al., 1985). The PCR and DNA sequencing was done as described by Ahire et al. (2012).

Strains MEB087ᵀ and MEB142 shared 99.8% sequence similarity between their nearly complete 16S rRNA genes (1477 and 1401 bp, respectively). Their closest phylogenetic neighbour on the basis of EzTaxon-e search analysis (Kim et al., 2012) was Nitrincola laciapsonensis DSM 16316ᵀ, which shared 97.7% and 97.8% 16S rRNA gene sequence similarity, respectively, with the two strains, followed by <93% similarity with the other sequences in the database. Sequences representing the closest neighbours of MEB087ᵀ and MEB142 in the EzTaxon-e database were aligned using CLUSTAL X, version 2.0.9 (Larkin et al., 2007). Phylogenetic analysis was carried out using maximum-likelihood (ML), maximum-parsimony (MP) and neighbour-joining (NJ) methods using MEGA 5.04 (Tamura et al., 2011). NJ and ML trees were reconstructed using Kimura two-parameter with gamma distribution as a model of nucleotide substitution and 1000 bootstrap replicates. The MP tree was obtained using the close-neighbour-interchange algorithm with search level 1 in which the initial trees were obtained with the random addition of sequences (10 replicates). The total number of bootstrap replicates used was 1000. Strains MEB087ᵀ and MEB142 clustered with Nitrincola laciapsonensis 4CAᵀ with 97.7% sequence similarity (Fig. 1) followed by Pseudospirillum japonicum ATCC 19191ᵀ, Marinomonas communis LMG 2864ᵀ, Neptuniibacter caesariensis MED92ᵀ and Corallomonas stylophorae KTSW-65ᵀ with <91.7% similarity. Similar tree topologies were obtained using ML and MP methods (Fig. 1).

The taxonomic relationship between strains MEB087ᵀ, MEB142 and Nitrincola laciapsonensis DSM 16316ᵀ was examined using DNA–DNA hybridization carried out by the modified method of De Ley et al. (1970) as described by Huss et al. (1983). The analysis was done by fluorometry in a 96-well plate using a Step One Plus Real-Time PCR system (Applied Biosystems) fitted with a 96-well thermal cycling block (Loveland-Curtze et al., 2011). DNA suspended in 2× SSC (saline sodium citrate) was used for the analysis and values reported are the mean of triplicates. The reassociation of DNA was carried out at an optimum renaturation temperature of 72.0 °C (Tm = 0.51 × (% G + C) + 47.0) according to De Ley et al. (1970). The two strains, MEB087ᵀ and MEB142, were closely related to each other with 85% DNA–DNA relatedness, while both strains were more distantly related to Nitrincola laciapsonensis DSM 16316ᵀ with only 45% and 54% relatedness, respectively. The hybridization values were below the recommended threshold to delineate bacterial species (Stackebrandt & Goebel, 1994).

For ΔTm analysis, genomic DNA was extracted and purified according to the method of Marmur (1961). DNA suspended in 0.1x SSC buffer was used for thermal denaturation in a 96-well plate using a Step One Plus Real-Time PCR system (Applied Biosystems) fitted with a 96-well thermal cycling block. Each well was loaded with 10 µl of sample containing 1 µg of DNA, in triplicates. The fluorescent dye SYBR Green I (1 : 100000 dilution; Invitrogen) was used to analyse the melting profiles of homo- and heteroduplex DNA. DNA duplexes were prepared by denaturation followed by optimum reassociation using the equation Tm = 0.51 × (% G + C) + 47.0 (De Ley et al., 1970; Gillis et al., 1970). ΔTm of the homoduplex DNA (MEB087ᵀ) and heteroduplex DNA (MEB087ᵀ + DSM 16316ᵀ) was assessed as described by Gonzalez & Saiz-Jimenez (2005). A value of ΔTm >5 °C also supported a novel species delineation for MEB087ᵀ as recommended by Wayne et al. (1987). The ΔTm of homoduplex DNA (MEB087ᵀ) and heteroduplex DNA (MEB087ᵀ + DSM 16316ᵀ) was 6.9±0.3 °C. DNA G+C content for strains MEB087ᵀ and MEB142 was determined from mid-point value (Tm) as 49.3 and 49.7 mol%, respectively, estimated according to the method of Gonzalez & Saiz-Jimenez (2002).

To support the genotypic data, comparative chemotaxonomic and phenotypic analyses were performed on strains MEB087ᵀ, MEB142 and Nitrincola laciapsonensis DSM 16316ᵀ. For analysis of the cellular fatty acids, cells were grown on trypticase soy broth agar plates (pH 9.5; Hi-Media) at 28 °C and harvested at exponential phase. Cellular fatty acids were extracted as their methyl esters and analysed by GC (7890A, Agilent Technologies) according to the rapid Microbial Identification System software (MIS, MIDI; version 6.0), and peaks were identified based on the RITSBA6 database (Sasser 1990; http://www.midi-inc.com). Strains MEB087ᵀ, MEB142 and Nitrincola laciapsonensis DSM 16316ᵀ had summed feature 8 comprising C₁₈ : 1ω7c/C₁₈ : 1ω6c as the major fatty acid (Table S1 available in the online Supplementary Material). Significant amounts of summed feature 3 comprising C₁₆ : 1ω7c/C₁₆ : 1ω6c, C₁₆ : 0 and minor amounts (less
than 5 %) of C_{10:0} 3-OH, C_{12:0} and C_{10:0} were present in all three strains, with some percent variation.

For extraction of polar lipids, the cultures were grown in nutrient broth (pH 9.5; Hi-Media) at 30 °C. The cultures were harvested at exponential phase and the pellet was freeze-dried and was used for polar lipid extraction with methanol/chloroform/saline (2 : 1 : 0.8, by vol.) as described by Bligh & Dyer (1959) considering the modifications of Card (1973). Lipids were separated using silica gel TLC (Kieselgel 60 F254, Merck) by two-dimensional chromatography using chloroform/methanol/water (65 : 25 : 4, by vol.) in the first dimension and chloroform/methanol/acetate acid/water (86 : 16 : 15 : 4, by vol.) in the second dimension (Tindall, 1990). The dried plates were subjected to spraying with 5 % ethanolic molybdophosphoric acid for 5 min in the first dimension and chloroform/methanol/acetic acid/water (86 : 16 : 15 : 4, by vol.) in the second dimension (Tindall, 1990). The dried plates were subjected to spraying with ninhydrin (specific for amino groups), Dragendorff’s reagent (specific for phosphates), molybdenum blue (specific for amino groups), and silver nitrate (specific for sugars).

To determine whether the isolate required Na⁺ or Cl⁻, medium was prepared separately with KCl, Na_{2}SO_{4}, NaNO_{3} or Na_{2}CO_{3} to substitute for these ions. Growth at different pH (7–11) and NaCl concentration up to 3.5 M was determined by measuring OD_{600} after 48 h of incubation at 28 °C. Tolerance to temperature was determined by growth at different temperatures (4, 22, 30 and 37 °C) in SBM with acetate for 48 h.

The motility of strains MEB087^T and MEB142 was observed by the hanging-drop method (Suzuki et al., 2001), which showed both strains as non-motile. When grown for 2 days at 28 °C on solid nutrient agar medium (pH 9.5), colonies of strains MEB087^T and MEB142 were 2–5 mm diameter, cream coloured, mucoid, glistening, circular and convex, with entire margins. Liquid nutrient broth also supported growth of strains MEB087^T and MEB142. Both strains produced uniform turbidity until the cultures aged while Nitrincola lacisaponensis DSM 16316^T had a slight tendency to clump as the culture aged. pH tolerance was determined using salt basal medium (SBM) containing acetate (Dimitriu et al., 2005) by adjusting the pH from 7 to 11 (at intervals of 0.5 pH units) by using KH_{2}PO_{4}/K_{2}HPO_{4} or Na_{2}CO_{3}/NaHCO_{3} buffer systems. pH of the medium was checked before and after autoclaving. Growth with various concentrations of NaCl up to 3.5 M (at intervals of 0.1 M) was measured. To determine whether the isolate required Na⁺ or Cl⁻, medium was prepared separately with KCl, Na_{2}SO_{4}, NaNO_{3} or Na_{2}CO_{3} to substitute for these ions. Growth at different pH (7–11) and NaCl concentration up to 3.5 M was determined by measuring OD_{600} after 48 h of incubation at 28 °C. Tolerance to temperature was determined by growth at different temperatures (4, 22, 30 and 37 °C) in SBM with acetate for 48 h.

When tested on finely buffered SBM, both strains MEB087^T and MEB142 had the ability to grow between pH 7.5 and 11.0, with optimal growth observed at pH 9.5; hence, the strains were considered to be alkaliphilic.
They could grow at NaCl concentrations of 0–0.6 M, with optimum growth occurring at 0.4 M NaCl. Growth at pH 9.5 and low NaCl concentrations differentiated strains MEB087ᵀ and MEB142 from *Nitrincola laciapenosins* DSM 16316ᵀ (Table 1). The novel strains could also grow at Na₂SO₄ concentrations of 0–0.5 M, NaNO₃ concentrations of 0–0.8 M and Na₂CO₃ concentrations of 0.1–1.0 M. When NaCl and other sources of Na⁺ were removed from the medium and replaced with KCl, isolate MEB087ᵀ did not grow, indicating that it has a requirement for Na⁺. It grew best at 30–37 °C and some growth was detected at 22 °C, but it failed to grow at 4 °C.

Gram staining, catalase and oxidase activities and endospore production were determined as described by Smibert & Krieg (1994), and all three strains stained Gram-stain-negative. Tests for hydrolysis of casein, starch, gelatin and DNA were performed while the ability to reduce nitrate and nitrite under aerobic conditions was assessed in nitrate and nitrite broth, respectively (Smibert & Krieg, 1994). King’s media A and B were used to determine the production of pigments (King et al., 1954). Indole production, methyl red and Voges-Proskauer tests and the utilization of citrate using Simmons’ medium and replaced with KCl, isolate MEB087ᵀ did not grow, indicating that it has a requirement for Na⁺.

Under aerobic conditions, MEB087ᵀ was able to utilize malate, succinate, D-glucose, pyruvate, sodium citrate and sucrose as sole carbon and energy sources. The results of differential tests with *Nitrincola laciapenosins* DSM 16316ᵀ are shown in Table 1. All physiological tests were performed in triplicate.

Antibiotic susceptibilities were determined by using anti-biotic discs on SBM at pH 9.5 (Dimitriu et al., 2005). Strains MEB087ᵀ and MEB142 were resistant to carbenicillin (100 µg) and ampicillin (25 µg) and sensitive to neomycin (30 µg), erythromycin (15 µg), bacitracin (10 units), polymyxin B (35 µg), gentamicin (5 µg), streptomycin (10 µg), rifampicin (2 µg), tetracycline (30 µg), nalidixic acid (30 µg), neomycin (30 µg), chloramphenicol (30 µg) and penicillin (10 µg).

Based on the 16S rRNA gene sequence similarity, DNA–DNA hybridization, whole-cell fatty acid profile, polar lipid profiles, pH optima and range, and organic substrate utilization, we suggest that strains MEB087ᵀ and MEB142 represent a single novel species of the genus *Nitrincola*, for which the name *Nitrincola alkalisediminis* sp. nov. is proposed.

**Description of *Nitrincola alkalisediminis* sp. nov.**

*Nitrincola alkalisediminis* (alka.li.se.di’mi.nis. N.L. n. alkali alkali; L. gen. n. sediminis of sediment; N.L. gen. n. alkalisediminis of alkaline sediment).

### Table 1. Differential characteristics of strains MEB087ᵀ and MEB142 and *Nitrincola laciapenosins* DSM 16316ᵀ

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
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</thead>
<tbody>
<tr>
<td><strong>Cell size (width × length, µm)</strong></td>
<td>0.7–1.17 × 1.4–2.2</td>
<td>0.6–1.17 × 1.4–2.2</td>
<td>0.3–0.4 × 1.32–2.3</td>
</tr>
<tr>
<td><strong>Colony consistency</strong></td>
<td>Mucoid</td>
<td>Mucoid</td>
<td>Dry</td>
</tr>
<tr>
<td><strong>Motility</strong></td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td><strong>pH range for growth (optimal)</strong></td>
<td>7.5–11 (9.5)</td>
<td>7.5–11 (9.5)</td>
<td>6–12 (9.0)</td>
</tr>
<tr>
<td><strong>NaCl range (optimum) (M)</strong></td>
<td>0–0.6 (0.4)</td>
<td>0–0.7 (0.4)</td>
<td>0–1.1 (0.8)</td>
</tr>
<tr>
<td><strong>Utilization of single carbon sources</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-Glucose</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Acetate</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>L-Serine</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Sarcosine</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Saccharate</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>L-Asparagine</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>L-Aspartic acid</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Fumarate</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td><strong>DNA G+C content (mol%)</strong></td>
<td>49.3</td>
<td>49.7</td>
<td>49.1</td>
</tr>
</tbody>
</table>

Strains: 1, MEB087ᵀ; 2, MEB142; 3, *Nitrincola laciapenosins* DSM 16316ᵀ. All data are from this study, except where otherwise indicated. All strains are able to utilize pyruvate and malate as sole carbon and energy source but not mannitol, maltose, D-mannose, adonitol, D-arabinose, D-galactose, sorbitol, cellobiose, L-rhamnose, D-xyllose, glycerol, cumaric acid, D-ornithine, succinate, L-arginine, lactose, D-lactate, D-fructose, leucine, L-threonine, L-glutamic acid, hippurate, L-isoleucine, L-phenylalanine, L-methionine, dulcitol, L-alanine, L-glutamine, potassium gluconate, capric acid, adipic acid, malic acid, phenylacetic acid, N-acetylglicosamine, ethanol or propanol. +, Positive; –, Negative.
Cells are short, non-motile rods, occurring singly or in pairs (1.4–2.2 μm in length and 0.7–1.17 μm in width). When grown on nutrient agar at pH 9.5, colonies are 2–5 mm in diameter, cream coloured, entire, glistening, mucoid and convex. Alkaliphilic. In minimal medium (SBM) containing acetate, the conditions for growth are pH 7.5–11.0 and up to 0.6 M NaCl. Optimum growth occurs at pH 9.5 and at 0.4 M NaCl at 37 °C. Positive for oxidase, catalase, nitrite and nitrite reduction and methyl red. Lysine decarboxylase, arginine dihydrolase and ornithine decarboxylase are not produced; H2S, acetoin and indole are not produced. Not able to hydrolyse casein, urea, gelatin, DNA or starch and does not produce visible or fluorescent pigments on solid King’s media B or C. Positive for pyocyanin and fluorescin.

The type strain is MEB087T (=KCTC 42948T=JCM 19317T), isolated from a sediment sample collected from Lonar Lake, India. MEB142 is a second strain of the species. The DNA G+C content of the type strain MEB087T is 49.3–49.7 mol%.

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References


