**Nocardiaceae halotolerans** sp. nov., a halotolerant actinomycete isolated from saline soil

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A novel halotolerant actinomycete, strain Chem15 T, was isolated from soil around Inche-Broun hypersaline wetland; its taxonomic position was determined based on a polyphasic approach. Strain Chem15 T was strictly aerobic and tolerated NaCl up to 12.5 %. The optimum temperature and pH for growth were 28–30 °C and pH 7.0–7.5, respectively. The cell wall of strain Chem15 T contained meso-diaminopimelic acid as diamino acid and galactose, arabinose and ribose as whole-cell sugars. The major phospholipids were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol mannosides. The cellular fatty acids profile consisted of C16 : 0, iso-C18 : 0, C18 : 0 10-methyl and C18 : 1 ω9 c, and the major respiratory quinone was MK-8(H4cyc). The G+C content of the genomic DNA was 68.0 mol%. The novel strain constituted a distinct phyletic line within the genus *Nocardia*, based on 16S rRNA gene sequence analysis, and was closely associated with *Nocardia sungurluensis* DSM 45714 T and *Nocardia alba* DSM 44684 T (98.2 and 98.1 % 16S rRNA gene sequence similarity, respectively). However DNA–DNA relatedness and phenotypic data demonstrated that strain Chem15 T was clearly different from closely related species of the genus *Nocardia*. It is concluded that the organism should be classified as a representative of a novel species of the genus *Nocardia*, for which the name *Nocardia halotolerans* sp. nov. is proposed. The type strain is Chem15 T (= IBRC-M 10490 T = LMG 28544 T).

The family *Nocardiaceae* includes the genera *Nocardia*, *Rhodococcus*, *Gordonia*, *Millisia*, *Skermania*, *Williamsia* and *Smaragdicoccus*, based on nucleotide signatures in the 16S rRNA genes and some chemotaxonomic features, significantly the presence of mycolic acids (Adachi *et al.*, 2007; Goodfellow *et al.*, 1999; Goodfellow & Maldonado, 2006). The genus *Nocardia*, as type genus of the family, encompasses aerobic and acid–alcohol-fast actinomycetes, in which the substrate hyphae can be fragmented to coccoid to rod-shaped and non-motile elements.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Chem15 T is KM577163.

Two supplementary figures are available with the online Supplementary Material.

Over the long-term programme of the Iranian Biological Resource Center (IBRC) for isolation, authentication and preservation of native microbial resources, ecologically diverse regions of Iran have been explored. The Inche-Broun wetland is a unique natural hypersaline ecosystem located below the Caspian Sea near the border with Turkmenistan (37° 13′–14′ 57″ N 54° 50′–51′ 57″ E) and our previous investigations clearly illustrated its microbial richness, especially actinomycetes reservoirs (Nikou *et al.*, 2014). The aim of this study was the characterization of a novel actinomycete belonging to the genus *Nocardia* based on a polyphasic approach.

The strain isolation was performed by diluting a sample obtained from soil around the Inche-Broun hypersaline wetland in sterile 3 % (w/v) salt solution, plating on glycerol-
casein agar (Küster & Williams, 1964) and incubating aerobically at 28 °C for 3 weeks. The purified strain was maintained on yeast extract-malt extract agar medium (ISP 2; Shirling & Gottlieb, 1966) at 4 °C and as glycerol suspensions (20 %, v/v) at −20 °C and −80 °C. The strains Chem15T, Nocardia sungurluensis DSM 45714T and Nocardia alba DSM 44684T were cultivated and simultaneously subjected to polyphasic taxonomy studies.

The macroscopic morphology and growth were examined on media recommended by Shirling & Gottlieb (1966) and Waksman (1961). The colour of aerial mycelium (spore mass) and substrate mycelium was determined on yeast extract-malt extract agar (ISP 2), and oatmeal agar (ISP 3 at 28 °C for 3 weeks, using colour chips from the ISCC-NBS charts (Kelly, 1964). Spore motility was investigated under light microscopy using sterile distilled water. Ability to produce diffusible pigment was assayed on ISP 2, ISP 3, inorganic salt-starch agar (ISP 4) and glycerol-asparagine agar (ISP 5) media. Melanin production was evaluated on peptone-yeast extract iron agar (ISP 6) and tyrosine agar (ISP 7) at 28 °C after 7 days. The strain was Gram-stained according to Gerhardt et al. (1994). The acid-fastness degree of the strain was evaluated using the modified Ziehl–Neelsen method detailed by Gordon (1967). Substrate mycelium and spore chain microscopic morphology of a five-day-old culture grown on ISP 2 agar were observed using a Nikon phase-contrast Eclipse 80i model microscope.

The organism was a Gram-staining-positive, strictly aerobic and acid-fast actinomycete. The strain formed powdery-surfaced colonies and produced short whitish aerial mycelium. Extensive branches of substrate mycelium fragmented into rod- or cocci-shaped elements (Fig. S1, available in the online Supplementary Material). The colour of the substrate mycelium of strain Chem15T was light orange. Diffusible pigment was not produced on ISP 2, ISP 3, ISP 4 or ISP 5 media. The motility of spores was negative. The cultural characteristics and significant colour variations of aerial and substrate mycelium on different media are listed in Table 1, which also shows some morphological differences between this strain, N. sungurluensis DSM 45714T and N. alba DSM 44684T.

Physiological and biochemical tests were carried out using standard methods (Gordon et al., 1974; Williams et al., 1983) at 28 °C; tests included catalase and oxidase determination, decomposition of aesculin, arbutin, adenine, hypoxanthine, tyrosine, urea, guanine, xanthine, xylan and Tweens 20, 40, 60 and 80, and hydrolysis of starch, casein, gelatin, urea and DNA. Carbohydrate utilization was determined according to the methods described by Shirling & Gottlieb (1966) using a basal medium supplemented with a final concentration of 1.0 % (w/v) of the tested carbon sources. The growth temperature range was examined on ISP 4 agar at 0, 4, 10, 15, 20, 25, 28–37 (at intervals of 1.0 °C), 40, 45 and 50 °C. The growth pH range was assessed on ISP 2 broth at pH 4.0–10.0 using 50 mM buffers: MES (pH 4–6.5), HEPES (pH 6.5–8) and CHES (pH 8.5–10) (at 0.5 pH unit intervals). Tolerance to NaCl was assessed on ISP 4 agar medium containing 0, 1, 3, 5, 7.5, 10, 12.5, 15, 17.5, 20, 23 and 25 % (w/v) NaCl. Reduction of nitrate and production of melanoid pigment were determined by the method of the International Streptomyces Project (ISP; Shirling & Gottlieb, 1966).

### Table 1. Macroscopic cultural characteristics of strain Chem15T and its closest phylogenetic neighbours grown on different media

<table>
<thead>
<tr>
<th>Strains: 1, Chem 15T; 2, N. sungurluensis DSM 45714T; 3, N. alba DSM 44684T.</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Characteristic</strong></td>
<td><strong>Yeast extract-malt extract agar (ISP 2)</strong></td>
<td><strong>Oatmeal agar (ISP 3)</strong></td>
<td><strong>Inorganic salts-starch agar (ISP 4)</strong></td>
</tr>
<tr>
<td>Growth</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Aerial mycelium</td>
<td>White</td>
<td>Pale yellow to white</td>
<td>Light grey</td>
</tr>
<tr>
<td>Soluble pigment</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Reverse colour</td>
<td>Light orange</td>
<td>Pale orange</td>
<td>Moderate orange</td>
</tr>
<tr>
<td><strong>Yeast extract-malt extract agar (ISP 2)</strong></td>
<td><strong>Oatmeal agar (ISP 3)</strong></td>
<td><strong>Inorganic salts-starch agar (ISP 4)</strong></td>
<td></td>
</tr>
<tr>
<td>Growth</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Aerial mycelium</td>
<td>White</td>
<td>Pale yellow to white</td>
<td>White</td>
</tr>
<tr>
<td>Soluble pigment</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Reverse colour</td>
<td>Light yellow</td>
<td>Pale orange</td>
<td>Light yellow</td>
</tr>
<tr>
<td><strong>Yeast extract-malt extract agar (ISP 2)</strong></td>
<td><strong>Oatmeal agar (ISP 3)</strong></td>
<td><strong>Inorganic salts-starch agar (ISP 4)</strong></td>
<td></td>
</tr>
<tr>
<td>Growth</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Aerial mycelium</td>
<td>White</td>
<td>Pale yellow to white</td>
<td>Pink white</td>
</tr>
<tr>
<td>Soluble pigment</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Reverse colour</td>
<td>Strong orange</td>
<td>Pale orange</td>
<td>Pink white</td>
</tr>
<tr>
<td><strong>Glycerol-asparagine agar (ISP 5)</strong></td>
<td><strong>Yeast extract-malt extract agar (ISP 2)</strong></td>
<td><strong>Oatmeal agar (ISP 3)</strong></td>
<td></td>
</tr>
<tr>
<td>Growth</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Aerial mycelium</td>
<td>White</td>
<td>Pale yellow to white</td>
<td>Yellowish white</td>
</tr>
<tr>
<td>Soluble pigment</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Reverse colour</td>
<td>Not determined</td>
<td>Pale orange</td>
<td>Yellowish white</td>
</tr>
</tbody>
</table>
Production of H₂S was tested by growing strain Chem15ᵀ on ISP 6 agar slant medium at 28 °C. All tests were recorded after 7–21 days. The miniaturized identification system API ZYM kit (bioMérieux) was used to examine enzymic activity. The antibiotic susceptibility of the strain was assayed using the method of Bauer et al. (1966).

Strain Chem15ᵀ grew at a temperature range of 15–45 °C (optimum temperature 28–30 °C), pH 6–8 (optimum pH 7.0–7.5) and NaCl concentrations of 0–12.5 % (optimum growth at 0–3 % (w/v) NaCl). The strain reduced nitrate to nitrite; however, nitrite reduction was not observed. Urease activity was positive, while proteolytic and amylolytic activity were not. Gelatin was not liquefied. H₂S was not produced. The strain was susceptible to chloramphenicol (30 μg), amoxiclav, amoxicillin (30 μg), tetracycline (30 μg), nitrofurantoin (300 μg), amikacin (30 μg), rifampicin (5 μg) and carbenicillin (100 μg), but resistant to penicillin G (10 U), nalidixic acid (30 μg), cefoxitin (30 μg), polymixin B (100 U), cefalotin (30 μg) and ceftazidime (30 μg). Other phenotypic properties of strain Chem15ᵀ compared with N. sungurluensis DSM 45714ᵀ and N. alba DSM 44684ᵀ are listed in Table 2.

For comparative chemotaxonomic investigation, strain Chem15ᵀ, N. sungurluensis DSM 45714ᵀ and N. alba DSM 44684ᵀ were cultivated in shake flasks containing ISP 2 broth medium for 3–4 days. The biomasses were harvested

Table 2. Some significant characteristics that distinguish strain Chem15ᵀ from its closest phylogenetic neighbours.

<table>
<thead>
<tr>
<th>Feature</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature range</td>
<td>28–30</td>
<td>28</td>
<td>28–30</td>
</tr>
<tr>
<td>pH optimum</td>
<td>7.0–7.5</td>
<td>7.0</td>
<td>6.5–7.5</td>
</tr>
<tr>
<td>Hydrolysis of:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Starch</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Tween 20</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Tween 60</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Arbutin</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Utilization of carbon and energy source:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>D-Xylose</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lactose</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Maltose</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>myo-Inositol</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>d-Mannitol</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>L-Rhamnose</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>d-Mannose</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>d-Ribose</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Enzyme activity:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Galactosidase</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>β-Glucuronidase</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>β-Glucosidase</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>DNA G + C content (mol%)</td>
<td>68.0</td>
<td>69.8ᵃ</td>
<td>74ᵇ</td>
</tr>
<tr>
<td>Polar lipids</td>
<td>Diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannosides, unknown glycolipid</td>
<td>Diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannosides, unknown glycolipidᵃ</td>
<td>Diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol mannosides, unknown glycolipidᵇ</td>
</tr>
<tr>
<td>Menquinones</td>
<td>MK-8(H₄cyc), MK-8(H₂), MK-8(H₄)</td>
<td>MK-8(H₄cyc), MK-8 (H₄)ᵃ</td>
<td>MK-8(H₄cyc)b</td>
</tr>
<tr>
<td>Whole sugar content</td>
<td>Galactose, arabinose, ribose</td>
<td>Arabinose, galactose, glucose and xylose</td>
<td>Galactose, arabinose</td>
</tr>
</tbody>
</table>

ᵃData taken from: a, Camas et al. (2014); b, Li et al. (2004).
by centrifugation at 3000 g. for 20 min, washed twice in distilled water and lyophilized. Isomers of dianaminopimelic acid, and whole-cell sugars were determined by established TLC methods (Staneck & Roberts, 1974; Lechevalier & Lechevalier, 1970). Polar lipids were extracted and identified by the method of Collins et al. (1977) and were analysed by HPLC (Wu et al., 1989). The cellular mycolic and fatty acid composition were determined according to standard protocols, as described for the Microbial Identification System (MIDI). Genomic DNA G+C content of strain Chem15T was determined by reversed-phase HPLC of nucleosides according to Mesbah et al. (1989).

The cell wall peptidoglycan of strain Chem15T contained meso-diaminopimelic acid, galactose and ribose. Diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannosides along with unknown phospholipid, glycolipid and aminolipid were detected in the membrane of strain Chem15T (Fig. S2). The predominant menaquinones were MK-8(H₄)cycl, MK-8(H₂) and MK-8(H₄) (90.0, 8.0 and 2.0 %, respectively). The presence of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannosides and MK-8(H₄)cycl are significant chemotaxonomical markers, providing evidence that strain Chem15T is a member of the genus Nocardia.

The major fatty acid methyl esters of strain Chem15T were C₁₆:0 (19.6 % of the total), C₁₇:0 (4.8 %), iso-C₁₈:0 (10.9 %) and C₁₈:1 (6.5 %). The mycolic acid chain length ranged from C₃₈ to C₅₈ for strain Chem15T. The DNA G+C content was 68.0 mol%, which was similar to that of other related species in the genus Nocardia. Some significant chemical differences and similarities between Chem15T and other reference strains are highlighted in Table 2.

Genomic DNA of strain Chem15T was isolated according to a modified salting out procedure (Pospiech & Neumann, 1995). Approximately 50–100 mg of the strain biomass was obtained from ISP 2 broth medium and transferred to a 150 μl microfuge tube. The biomass was resuspended in 567 μl SET buffer [NaCl 75 mM, EDTA 25 mM (pH 8.0), Tris/HCl 20 mM (pH 7.5)], mixed with lysis solution buffer, which contained 100 μl lysosome (30 mg ml⁻¹ in 10 mM Tris, pH 8.0), and incubated at 37°C for one hour. This procedure was accomplished by adding 9.0 μl proteinase K (600 U ml⁻¹), 100 μl SDS 10 % (w/v) and 3.0 μl RNase (10 mg ml⁻¹) followed by brief mixing and incubation at 55 °C for 2 h. The lysate was centrifuged at 1000 g. for 15 min and the resulting preparation was extracted according to the reference protocol. Finally, DNA was precipitated with 2-propanol and rinsed with ethanol 70 % (v/v). The pellet was dried at room temperature and the resulting DNA was dissolved in 50 μl Tris/EDTA solution. Amplification of 16S rRNA gene was performed using 27F (5'-AGAGTTTGTATCMTGCGCTCAG-3') and 1492R (5'-GTTACCTTGTAGGACGT-3') universal primers. The PCR conditions involved initial denaturation at 94 °C for 5 min followed by 25 cycles including denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min and extension at 72 °C for 90 s. This was followed by a final extension at 72 °C for 10 min. The purified PCR product was sequenced using an ABI 3730/3730XL DNA sequencer at Bioneer (Daejeon, South Korea). Identification of phylogenetic neighbours was initially carried out by BLAST (Altschul et al., 1997) and MEGA BLAST (Zhang et al., 2000). The aligned 16S rRNA gene sequences of Chem15T with most closely related type strains were analysed using MEGAversion 6 (Tamura et al., 2011). The tree reconstructions were based on near full-length 16S rRNA gene sequences of type strains of the most closely related taxa to Chem15T and conducted by neighbour-joining (Saitou & Nei, 1987), minimum-evolution (Rzhetsky & Nei, 1992) and maximum-likelihood (Felsenstein, 1981) methods to assess the support for each node of the phylogenetic tree.

The 16S rRNA gene sequence analysis of strain Chem15T (1477 nt) was obtained and used for initial BLAST searches in GenBank and phylogenetic analysis. The identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarity were achieved using the EzTaxon-e database (Kim et al., 2012). The 16S rRNA gene sequence analysis showed that strain Chem15T is a member of the family Nocardiaceae, and is closely associated with N. sungurluensis DSM 45714T and N. alba DSM 44684T, with similarity values of 98.2 % and 98.1 %, respectively. Phylogenetic analysis based on the neighbour-joining algorithm revealed that strain Chem15T comprised a separate branch from N. sungurluensis DSM 45714T and N. alba DSM 44684T within the family Nocardiaceae (Fig. 1). The tree topology in this region is supported by the minimum-evolution and maximum-likelihood methods, which confirmed the phylogenetic cluster formed by strain Chem15T and other neighbours in this family.

DNA–DNA hybridization was conducted by the method described by De Ley et al. (1970) incorporating the modifications described by Huss et al. (1983), using a Cary 100 Bio UV/VIS spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with an in situ temperature probe (Varian). DNA–DNA relatedness values between strain Chem15T and its closest phylogenetic neighbours were 20 % with N. sungurluensis DSM 45714T and 19 % with N. alba DSM 44684T, which are below the 70 % cut-off recommended for species delineation (Wayne et al., 1987).

In conclusion, the strain Chem15T can be distinguished from representatives of all described species of the genus Nocardia with validly published names, including N. sungurluensis DSM 45714T and N. alba DSM 44684T, using a set of phenotypic and genotypic properties. Hence, we propose that strain Chem15T is a representative of a novel species of the genus Nocardia, for which the name Nocardia halotolerans sp. nov. is proposed.
**Description of *Nocardia halotolerans* sp. nov.**

*Nocardia halotolerans* (ha.lo.to’le.rans Gr. n. hals halos salt; L. part. adj. tolerans tolerating; N.L. part. adj. halotolerans salt-tolerating).

Gram-staining-positive, strictly aerobic and acid-fast actinomycete. Extensive substrate and short aerial mycelia produced. The reverse sides of colonies are light orange. White aerial mass is produced on ISP 2, ISP 3, ISP 4 and ISP 5. Grows at 15–45 °C, 0–12.5 % (w/v) NaCl and pH 6–8. Optimal growth occurs at 28–30 °C and pH 7.0–7.5. Catalase and oxidase are positive. Tweens 20, 40, 60 and urea are hydrolysed, but not arbutin, casein, aesculin, gelatin, starch, tyrosine, hypoxanthine, adenine, xylan, guanine,
xanthine or Tween 80. Nitrate is reduced, but nitrite is not. 
H2S is not produced. Utilizes D-glucose and sodium citrate 
although maltose, sorbitol, D-mannose, sucrose, mannositol, 
trehalose, D-galactose, lactose, sodium acetate, D-ribose, D- 
xyllose, melibiose, rhamnose, raffinose, cellobiose, myo-inositol 
and D-arabinose are not utilized as sole carbon source. 
Acid is produced from D-glucose. The activity of alkaline 
phosphatase, esterase (C4), esterase lipase (C8), lipase C14, 
leucine arylamidase, trypsin, 3-chemotrypsin, acid phosphatase, 
naphthol-AS-BI-phosphohydrolase, B-glucosidase, 
B-glucuronidase, N-acetyl-B-glucosaminidase, and z and 
B-glucosidase is positive. Contains meso-diaminopimelic acid in 
cell-wall peptidoglycan and whole-cell sugar pattern 
consists of galactose, arabinose and ribose. Polar lipid pattern 
includes diphosphatidylglycerol, phosphatidylglycerol, 
phosphatidylethanolamine, phosphatidylglycitol, phospho-

tidylinositol mannosides and unknown phospholipid, 
glycolipid and aminolipid. The predominant menaquinone is 
MK-8(H4cyc). The cellular fatty acids consist of C16:0, 
iso-C18:0, C18:0 10-methyl and C18:1ω9c.

The type strain is Chem15T (=IBRC-M 10490T = LMG 
28544T), isolated from Inche-Broun hypersaline wetland 
in Iran. The DNA G+C content of the type strain is 
68.0 mol% (HPLC).

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