Nocardioides alpinus sp. nov., a psychrophilic actinomycete isolated from alpine glacier cryoconite

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A Gram-positive, non-motile, rod-shaped, psychrophilic actinomycete, designated strain Cr7-14T, was isolated from alpine glacier cryoconite. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain Cr7-14T was related to members of the genus Nocardioides and shared highest 16S rRNA gene sequence similarities with the type strains of Nocardioides furvisabuli (98.6 %), Nocardioides ganghwensis (98.2 %), Nocardioides oleivorans (98.1 %) and Nocardioides exalbidus (97.6 %). The predominant cellular fatty acids of strain Cr7-14T were C17:0 3OHc (39.5 %) and iso-C16:0 (32.4 %). The major menaquinone was MK-8(H4). The diagnostic diamino acid in the cell-wall peptidoglycan was LL-2,6-diaminopimelic acid. The predominant cell-wall sugars were galactose and rhamnose. The polar lipid pattern contained diphosphatidylglycerol, phosphatidylglycerol, phosphatidylcholine, phosphatidylinositol, four unknown glycolipids and two unknown polar lipids. The genomic DNA G+C content was 71.9 mol%. On the basis of phenotypic characteristics, phylogenetic analysis and DNA–DNA relatedness data, a novel species, Nocardioides alpinus sp. nov., is proposed, with Cr7-14T (=DSM 23325T =LMG 26053T =CGMCC 1.10697T) as the type strain.

The genus Nocardioides was originally created by Prauser (1976) to accommodate Gram-positive, aerobic, mesophilic nocardioform actinomycetes that contained LL-2,6-diaminopimelic acid (LL-Dpm) in the cell-wall peptidoglycan and MK-8(H4) as the major menaquinone (Prauser, 1976; O’Donnell et al., 1982; Urzi et al., 2000; Zhang et al., 2009). At the time of writing, the genus Nocardioides comprised at least 50 species with validly published names. Members of this genus have rarely been found in cold environments, although Nocardioides aquaticus was isolated from an Antarctic lake (Lawson et al., 2000). Several Nocardioides species are able to grow at 4 °C (e.g. Lawson et al., 2000; Lee, 2007, Tóth et al., 2008), however, the maximum growth temperature of these strains is never lower than 28–30 °C. The present study deals with the characterization of a novel psychrophilic representative of the genus Nocardioides isolated from alpine glacier cryoconite.

Strain Cr7-14T was isolated from alpine glacier cryoconite collected from the Pitztaler Jöchl glacier in the Ötztaler Alps in Tyrol, Austria, at an altitude of 2875 m above sea-level as described previously (Zhang et al., 2011). The sample was collected under sterile conditions in the spring of 2006. A portion of the sample (1 g dry mass) was shaken with 9 ml sterile 1 % sodium pyrophosphate for 20 min at 150 r.p.m. Appropriate dilutions, prepared with sterile saline solution [0.9 % (w/v) NaCl], were plated (0.1 ml) on R2A agar (0.05 % yeast extract, 0.05 % peptone, 0.05 % Casamino acids, 0.05 % glucose, 0.05 % starch, 0.03 % sodium pyruvate, 0.03 % K2HPO4, 0.005 % MgSO4, 1.5 % agar, pH 7; Reasoner & Geldreich, 1985) and incubated at 10 °C. One of the pure cultures was light-yellow-pigmented

Abbreviation: Dpm, 2,6-diaminopimelic acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of Nocardioides alpinus Cr7-14T is GU784866.

Two supplementary figures and a supplementary table are available with the online version of this paper.
and was designated strain Cr7-14<sup>T</sup>. Nocardioides furvisabuli DSM 18445<sup>T</sup>, Nocardioides ganghwensis KACC 20321<sup>T</sup>, Nocardioides oleivorans DSM 16090<sup>T</sup> and Nocardioides exalbidus DSM 22017<sup>T</sup> were used as reference strains; they were routinely grown on R<sub>2</sub>A agar at 25 °C.

DNA was extracted and purified as described by Sambrook et al. (1989). The 16S rRNA gene was amplified by PCR with a pair of universal primers, 27F (5′-AGAGTTTGATCCTG-GCTCAG-3′) and 1541R (5′-AAGGAGGTGATCCAGCC-GCA-3′). PCR products were cloned in pGEM-T vectors (Promega) according to the manufacturer’s instructions. FASTA similarity searches were carried out using the GenBank and EMBL databases. Strain Cr7-14<sup>T</sup> exhibited 16S rRNA gene sequence similarity values of 98.6, 98.2, 98.1 and 97.6% with <i>N. furvisabuli</i> DSM 18445<sup>T</sup>, <i>N. ganghwensis</i> KACC 20321<sup>T</sup>, <i>N. oleivorans</i> DSM 16090<sup>T</sup> and <i>N. exalbidus</i> DSM 22017<sup>T</sup>, respectively. <i>Nocardioides albus</i>, the type species of the genus, shared low 16S rRNA gene similarity (95.0%) with strain Cr7-14<sup>T</sup>.

Phylogenetic analysis was performed using the software MEGA4.0 (Tamura et al., 2007) as well as the PHYLIP 3.69 package (Felsenstein, 2009) after multiple alignment of data using CLUSTAL X 1.8 (Thompson et al., 1997). For neighbour-joining analysis (Saitou & Nei, 1987), genetic distances were calculated using Kimura’s two-parameter model (Kimura, 1980). Phylogenetic analysis based on the neighbour-joining method showed that strain Cr7-14<sup>T</sup> grouped with members of the genus <i>Nocardioides</i> and formed a distinct cluster with <i>N. furvisabuli</i> DSM 18445<sup>T</sup> (Fig. 1; Supplementary Fig. S1 available in IJSEM Online). The relative position of strain Cr7-14<sup>T</sup> was also confirmed in the maximum-likelihood tree (Felsenstein, 2009).

**Fig. 1.** Neighbour-joining tree based on 16S rRNA gene sequence data (1302 nt) showing the phylogenetic position of strain Cr7-14<sup>T</sup> and recognized members of the genus <i>Nocardioides</i>. Terrabacter tumescens KCTC 9133<sup>T</sup> (AF005023) was used as an outgroup. Asterisks indicate nodes that were also found in the maximum-likelihood tree. Bootstrap values (expressed as percentages of 1000 replicates) greater than 50% are shown at branch points. GenBank accession numbers are given in parentheses. Bar, 1% sequence divergence.
Strain Cr7-14\textsuperscript{T} was routinely cultured on R2A medium at 20 °C and maintained as a suspension in skimmed milk (10 %, w/v) at −80 °C. Cell morphology was examined by phase-contrast microscopy (×1000) of cells grown on R2A agar plates at 20 °C. Colony morphology was also observed on R2A agar plates. All tests described in the following paragraph were carried out with strain Cr7-14\textsuperscript{T} and four reference strains (\textit{N. furvisabuli} DSM 18445\textsuperscript{T}, \textit{N. ganghwensis} KACC 20321\textsuperscript{T}, \textit{N. oleivorans} DSM 16090\textsuperscript{T} and \textit{N. exalbidus} DSM 22017\textsuperscript{T}).

The API M system (bioMérieux) and microscopic examination (×1000) were used to evaluate cell motility. The Gram-reaction was tested by Gram-staining. Catalase activity was determined by bubble production in 3 % (v/v) H\textsubscript{2}O\textsubscript{2}; cytochrome oxidase activity was determined using API strips (API 20 E, API 20 NE, API Coryne, API ZYM; bioMérieux) incubated at 25 °C to determine physiological and biochemical characteristics and enzyme activities. Activities of β-galactosidase, amylase, protease and lipase esterase were also tested by using R2A agar plates supplemented with the appropriate substrates (Margesin \textit{et al.}, 2003). Growth under anaerobic conditions was assessed on nutrient agar (NA: 0.5 % peptone, 0.3 % meat extract, 1.5 % agar; pH 7) supplemented with 10 mM KNO\textsubscript{3} after 5 days of incubation at 25 °C in an anaerobic jar [containing Anaerocult A (Merck) to produce anaerobic conditions]. Growth at different temperatures (1–42 °C) was monitored daily for up to 20 days on R2A agar plates and in R2A liquid medium. Growth at various pH values (pH 5, 6, 7, 8, 9) and salt concentrations [0, 1, 2, 3, 5, 7 and 10 % (w/v) NaCl] was determined on R2A agar plates after 3–7 days at 25 °C. Growth on various media [R2A, NA, trypticase soy agar (TSA; 1.5 % casein peptone, 0.5 % soy peptone, 0.5 % NaCl, 1.5 % agar; pH 7)] was assessed on agar plates. Morphological, physiological and biochemical characteristics of strain Cr7-14\textsuperscript{T} are given in the species description (see below) and are shown in Table 1. Features that serve to differentiate strain Cr7-14\textsuperscript{T} from the reference strains (\textit{N. furvisabuli} DSM 18445\textsuperscript{T}, \textit{N. ganghwensis} KACC 20321\textsuperscript{T}, \textit{N. oleivorans} DSM 16090\textsuperscript{T} and \textit{N. exalbidus} DSM 22017\textsuperscript{T}) are given in Table 1.

For analysis of peptidoglycan structure and cell-wall sugars, cells of strain Cr7-14\textsuperscript{T} were cultured in shaken flasks of medium 92 (http://www.dsmz.de/) at 20 °C for 3 days. The peptidoglycan structure was determined by using hydrolysates of purified cell walls according to Schleifer (1985). Amino acids and peptides were separated by two-dimensional ascending TLC on cellulose plates with the solvent systems of Schleifer & Kandler (1972). Molar ratios of amino acids were determined by GC as described by MacKenzie (1987). The peptidoglycan of strain Cr7-14\textsuperscript{T} contained the amino acids L-Dpm, glutamic acid, glycine and alanine in an approximate molar ratio of 1.1 : 1.0 : 0.4 : 1.2. In addition, a partial hydrolysate (4 M HCl, 100 °C, 0.75 h) of the peptidoglycan contained the peptides L-Ala–D-Glu and L-Dpm–D-Ala. From these data, it was concluded that the peptidoglycan of strain Cr7-14\textsuperscript{T} is of type A3\textsubscript{2}L\textsubscript{2}Dpm–Gly (A41.1 according to http://www.peptidoglycan-types.info).

For sugar analysis, cell walls were hydrolysed in 0.5 M H\textsubscript{2}SO\textsubscript{4} at 100 °C for 2 h. H\textsubscript{2}SO\textsubscript{4} was removed by shaking with 20 % N,N-dioctylmethyamine in chloroform according to Whiton \textit{et al.} (1985) and sugars in the hydrolysate were analysed by TLC on cellulose plates according to Staneck & Roberts (1974). The predominant cell-wall sugars of strain Cr7-14\textsuperscript{T} were galactose and rhamnose. Xylose and mannose were detected in trace amounts.

For fatty acid methyl ester analysis, cell mass of strain Cr7-14\textsuperscript{T} and four reference strains (\textit{N. furvisabuli} DSM 18445\textsuperscript{T}, \textit{N. exalbidus} DSM 22017\textsuperscript{T}, \textit{N. ganghwensis} KACC 20321\textsuperscript{T} and \textit{N. oleivorans} DSM 16090\textsuperscript{T}) was harvested from R2A agar plates after incubation at 25 °C for 48 h. All type strains included in the fatty acid analyses corresponded in terms of their growth behaviour and sufficient cells of comparable physiological age could be harvested from the third streak quadrant of the agar plates after cultivation under the conditions described. Fatty acid methyl esters were extracted and prepared according to the standard protocol of the Sherlock Microbial Identification System (MIDI, version 6.0) (Sasser, 1990) and were identified using the database TSBA (version 6.0). Strain Cr7-14\textsuperscript{T} contained the following cellular fatty acids (>2 %): C\textsubscript{17}:0 (39.5 %), iso-C\textsubscript{16}:0 (32.4 %), iso-C\textsubscript{16}:1\textsubscript{H} (3.5 %), C\textsubscript{17}:0 (3.5 %), C\textsubscript{18}:1\textsubscript{ω9c} (3.3 %), summed feature 3 (2.5 %); comprising C\textsubscript{16}:1\textsubscript{ω7c} and/or C\textsubscript{16}:1\textsubscript{ω6c} and C\textsubscript{17}:1\textsubscript{ω6c} (2.3 %). 10-Methyl-C\textsubscript{17}:0 (1.7 %), which is typically found in the genus \textit{Nocardioïdes} (Yoon \textit{et al.}, 2004; Choi \textit{et al.}, 2007), was also detected in strain Cr7-14\textsuperscript{T} (Supplementary Table S1, available in IJSEM Online).

Respiratory quinones were extracted and purified according to Collins (1985) and analysed by HPLC (Wu \textit{et al.}, 1989). Strain Cr7-14\textsuperscript{T} contained MK-8(H\textsubscript{4}) (86 %) and MK-8(H\textsubscript{2}) (14 %).

Polar lipid profiles were analysed according to Tindall (1990a, b). Diphosphatidylglycerol, phosphatidylglycerol, phosphatidylcholine, phosphatidylglycerol, four unknown glycolipids and two unknown polar lipids were detected in the polar lipid profile (two-dimensional TLCs in Supplementary Fig. S2, available in IJSEM Online). Though polar lipids of many recognized members of the genus \textit{Nocardioïdes} have been analysed, only the polar lipid profile of \textit{N. panaciolis} is available as an image accessible for visual comparison (Cho \textit{et al.}, 2010). Strain Cr7-14\textsuperscript{T} can be easily differentiated from close phylogenetic neighbours based on polar lipid profiles; it differs from \textit{N. furvisabuli} (Lee, 2007) by containing diphosphatidylglycerol, phosphatidylglycerol, phosphatidylcholine, phosphatidylglycerol, four unknown glycolipids and two unknown polar lipids, and from \textit{Nocardioïdes hwaseonensis} (Lee \textit{et al.}, 2008) by containing phosphatidylcholine and four unknown glycolipids.
The DNA G+C content was determined by the thermal denaturation method (Marmur & Doty, 1962) with Escherichia coli K-12 as reference; DNA–DNA hybridization was done at 82.5 °C by the liquid renaturation method (De Ley et al., 1970) as modified by Huß et al. (1983). Both experiments were carried out using a model Lambda 35 UV/VIS spectrometer equipped with a temperature programme controller (Perkin–Elmer). The DNA G+C content of strain Cr7-14T was 71.9 mol%. Levels of DNA–DNA relatedness between strain Cr7-14T and the four reference strains N. furvisabuli DSM 18445T, N. exalbidus DSM 22017T, N. ganghwensis KACC 20321T and N. oleivorans DSM 16090T were 48.8 ± 1.1, 45.5 ± 1.5, 38.4 ± 2.0 and 35.5 ± 1.1 %, respectively.

The diagnostic diamino acid in the cell-wall peptidoglycan (LL-Dpm), MK-8(H4) as the major menaquinone, iso-C16:0 as a predominant fatty acid and a DNA G+C content of 71.9 mol% support assignment of strain Cr7-14T to the genus Nocardioides (Urzì et al., 2000; Yi & Chun, 2004; Li et al., 2007; Lee, 2007). The strain is a psychrophilic representative of this genus and is able to grow well over a temperature range of 1–25 °C. We have used the term psychrophile as a general term to describe a microorganism that grows in a cold environment (Margesin et al., 2008), since the use of growth rates to define the optimum growth temperature as described by Morita (1975) has been shown to be ambiguous and inappropriate (Cavicchioli, 2006; Margesin, 2009).

Table 1. Phenotypic characteristics that differentiate strain Cr7-14T from the type strains of phylogenetically closely related Nocardioides species

<table>
<thead>
<tr>
<th>Characteristic</th>
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<th>3</th>
<th>4</th>
<th>5</th>
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<tbody>
<tr>
<td>Isolation source</td>
<td>Alpine glacier</td>
<td>Black sand*</td>
<td>Lichen†</td>
<td>Tidal flat sediment†</td>
<td>Crude oil§</td>
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<td>DNA G+C content (mol%)</td>
<td>71.9</td>
<td>69.1*</td>
<td>74†</td>
<td>72‡</td>
<td>ND</td>
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<tr>
<td>Major fatty acids</td>
<td>iso-C16:0, C17:1ω8c</td>
<td>iso-C16:0, C17:1ω8c, C18:1ω9c</td>
<td>iso-C16:0, C18:1ω9c, iso-C17:0</td>
<td>iso-C16:0, C17:1ω9c</td>
<td>iso-C16:0, C18:1ω9c</td>
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<td>Growth on R2A agar at/in:</td>
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<td>+</td>
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<td>5 °C</td>
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<td>30 °C</td>
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<td>37 °C</td>
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<td>pH 6</td>
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<td>5 % (w/v) NaCl</td>
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<td>Growth on TSA</td>
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<td>Enzyme activities:</td>
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<tr>
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<tr>
<td>β-Galactosidase</td>
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<tr>
<td>Pyrazinamidase</td>
<td>–</td>
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<td>–</td>
<td>+</td>
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<td>Assimilation of (API 20NE):</td>
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<tr>
<td>Glucose</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>l-Arabinose</td>
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<tr>
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<td>w</td>
<td>–</td>
<td>+</td>
<td>+</td>
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</table>

*Data from Lee (2007).
†Data from Schippers et al. (2005).
‡Data from Yi & Chun (2004).
§Data from Li (2007).
Strain Cr7-14<sup>T</sup> can be easily differentiated from its four closest phylogenetic neighbours, i.e. <i>N. furvisabuli</i> DSM 18445<sup>T</sup>, <i>N. exalbidus</i> DSM 22017<sup>T</sup>, <i>N. ganghwensis</i> KACC 20321<sup>T</sup> and <i>N. oleivorans</i> DSM 16090<sup>T</sup>, by its polar lipid profile (see above) and by its inability to grow at 30 °C, grow in the presence of 5% (w/v) NaCl, produce gelatinase and β-galactosidase, and assimilate N-acetylglucosamine. In addition, strain Cr7-14<sup>T</sup> contains a higher amount of the cellular fatty acid C<sub>17:1</sub>ω8c and a lower amount of C<sub>18:1</sub>ω9c. Based on phenotypic, phylogenetic and genomic evidence, strain Cr7-14<sup>T</sup> was identified as a representative of a novel species of <i>Nocardioides</i> for which the name <i>Nocardioides alpinus</i> sp. nov. is proposed.

### Description of <i>Nocardioides alpinus</i> sp. nov.

<i>Nocardioides alpinus</i> (al-pi’nu’s. L. masc. adj. alpinus of or pertaining to the Alps, Alpine).

Cells are Gram-positive, non-motile, short rods, 0.4–0.5 x 1.5–3.0 μm (occasionally 1.2–5.0 μm in length) after 7 days at 20 °C on R<sub>2A</sub> agar. Colonies on R<sub>2A</sub> agar are round, convex, smooth and glossy with entire margins; colony diameter is 0.4–0.6 mm after 7 days at 20 °C. Young colonies are cream–white and become light yellow after 2–3 weeks. Unable to grow under anaerobic conditions. Growth occurs in liquid R<sub>2A</sub> medium and on agar plates at 1–25 °C (with fastest growth rates at 25 °C) but not at 30 °C. On R<sub>2A</sub> agar plates, growth occurs at pH 7–8 and in the presence of 0–3% (w/v) NaCl. Growth at pH 9 is very weak. Grows on R<sub>2A</sub> agar, weak growth is observed on NA; unable to grow on TSA. Cytochrome oxidase-negative and catalase-positive. Gelatin, aesculin and starch are not hydrolysed, positive for hydrolysis of Tween 80 and skimmed milk. Cytochrome oxidase-negative and catalase-positive. Gelatin, aesculin and starch are not hydrolysed, positive for hydrolysis of Tween 80 and skimmed milk.

For positive nitrate reduction, alkaline phosphatase, acid phosphatase, esterase lipase (C8), lipase (C14), valine arylamidase, leucine arylamidase, cystine arylamidase and α-glucosidase. Activities of esterase (C4) and naphthol-AS-BI-phosphohydrolase are weak. Negative for indole production (from tryptophan), H<sub>2</sub>S production, urease, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, arginine dihydrolase, lysine dihydrolase, ornithine dihydrolase, tryptophan deaminase, pyrazinamidase and pyrrolidonyl arylamidase. Assimilates d-glucose, L-arabinose, D-mannitol, maltose and gluconate. Assimilation of D-mannose and malic acid is weak. N-Acetylglucosamine, capric acid, adipic acid, phenylacetic acid and trisodium citrate are not assimilated. Negative for fermentation of glucose, mannitol, sucrose, inositol, sorbitol, rhamnose, melibiose, amygdalin, L-arabinose, ribose, xylose, maltose, lactose and glycogen. The predominant cellular fatty acids are C<sub>17:1</sub>ω9c and iso-C<sub>16:0</sub>. Major menaquinone is MK-8(H<sub>6</sub>); MK-8(H<sub>2</sub>) is a minor menaquinone component. Contains LL-Dpm in its cell wall and type A3γ peptidoglycan (LL-Dpm-Gly). The predominant cell-wall sugars are galactose and rhamnose; traces of xylose and mannose are detected. The polar lipid pattern contains diphasatidylglycerol, phosphatidylglycerol, phosphatidylcholine, phosphatidylinositol, four unknown glycolipids and two unknown polar lipids.

The type strain is Cr7-14<sup>T</sup> (= DSM 23325<sup>T</sup>=LMG 26053<sup>T</sup>=CGMCC 1.10697<sup>T</sup>), isolated from alpine glacier cryoconite in the Ötztaler Alps in Austria. The DNA G+C content of the type strain is 71.9 mol%.

### Acknowledgements

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### References


