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Amycolatopsis cihanbeyliensis sp. nov., a halotolerant actinomycete isolated from a salt mine

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A novel halotolerant actinomycete, designated strain BNT52^T, was isolated from soil collected from Cihanbeyli Salt Mine in the central Anatolia region of Turkey, and examined using a polyphasic taxonomic approach. The isolate was found to have chemical and morphological properties typical of the genus *Amycolatopsis* and formed a distinct phyletic line in the 16S rRNA gene tree. Strain BNT52^T was most closely related to *Amycolatopsis nigrescens* CSC17Ta-90^T (96.7 %), *Amycolatopsis magusensis* KT2025^T (96.6 %), *Amycolatopsis sulphurea* DSM 46092^T (96.6 %), *Amycolatopsis dongchuanensis* YIM 75904^T (96.5 %), *Amycolatopsis ultiminotia* RP-AC36^T (96.4 %) and *Amycolatopsis sacchari* DSM 44468^T (96.4 %). Sequence similarities with other strains of species of the genus *Amycolatopsis* were lower than 96.2 %. The isolate grew at 20–37 °C, pH 6–12 and in the presence of 0–10 % (w/v) NaCl. The cell wall of the novel strain contained *meso*-diaminopimelic acid and arabinose and galactose as the diagnostic sugars. Major fatty acids were iso-$C_{16:0}$ 2-OH and iso-$C_{16:0}$. The predominant menaquinone was MK-9(H_4). The polar lipids detected were diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylmethylethanolamine. The genomic DNA G+C content was 68.8 mol%. On the basis of the data from this polyphasic taxonomic study, strain BNT52^T represents a novel species within the genus *Amycolatopsis* for which the name *Amycolatopsis cihanbeyliensis* sp. nov. is proposed (type strain BNT52^T=KCTC 29065^T=NRRL B-24886^T=DSM 45679^T).

Introduction

The genus *Amycolatopsis*, first established by Lechevalier *et al.* (1986), belongs to the family *Pseudonocardiaceae* and is a member of the suborder *Pseudonocardineae* (Embley *et al.*, 1988; Warwick *et al.*, 1994; Stackebrandt *et al.*, 1997). The members of the genus are Gram-reaction-positive, non-acid-fast, non-motile actinomycetes that form branched vegetative hyphae that undergo fragmentation into rod-like and squarish elements. The genus contains halophilic, alkaliphilic, mesophilic, thermophilic and pathogenic species (Saintpierre-Bonaccio *et al.*, 2005; Lee, 2006; Lee *et al.*, 2006; Groth *et al.*, 2007). The species of the genus *Amycolatopsis* have *meso*-diaminopimelic acid, arabinose and galactose in the wall peptidoglycan (wall chemotype IV *sensu* Lechevalier & Lechevalier, 1970), fatty acids rich in iso- and anteiso-branched components and a lack of mycolic acids (Takahashi, 2001). The predominant menaquinone type is MK-9(H4); phosphatidylethanolamine is a diagnostic phospholipid (type II *sensu* Lechevalier *et al.*, 1977) and the G+C content of genomic DNA ranges from 66 to 73 mol%. The aim of the present polyphasic study was to clarify the taxonomic position of the novel strain BNT52^T of the genus *Amycolatopsis*, isolated from a soil sample, collected from Cihanbeyli Salt Mine, in Turkey.

During research on biodiversity of culturable actinomycetes from a soil sample collected from Cihanbeyli Salt Mine (GPS coordinates for the sampling site are 38° 45′ 50″ N 33° 09′ 26″ E), located in the central Anatolia region, actinomycete strain BNT52^T was isolated on modified Bennett's agar (Jones, 1949) supplemented with 5 % NaCl (w/v), which had been inoculated with a soil suspension and incubated at 28 °C for 21 days. The organism was maintained on modified Bennett's agar slopes containing 5 % (w/v) NaCl at room temperature and as glycerol suspensions (20 %, v/v) at −20 °C.

The GenBank accession number for the 16S rRNA gene sequence of *Amycolatopsis cihanbeyliensis* BNT52^T is JN989302.

Six supplementary figures and a supplementary table are available with the online version of this paper.

Genomic DNA extraction, PCR-mediated amplification of the 16S rRNA gene and purification of the PCR product were carried out following the protocols of Chun & Goodfellow (1995). The almost complete (1460 bp) 16S rRNA gene sequence of strain BNT52^T was determined using a PRISM 3730 XL automatic sequencer (ABI). The identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarity were achieved using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net; Kim *et al.*, 2012). Multiple alignment with sequences from closely related species was performed by using the program CLUSTAL W in MEGA5 (Tamura *et al.*, 2011). Phylogenetic trees were reconstructed with the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Kluge & Farris, 1969) and maximum-likelihood (Felsenstein, 1981) algorithms in MEGA5 (Tamura *et al.*, 2011). Evolutionary distances were calculated using the model of Jukes & Cantor (1969). Topologies of the resultant trees were evaluated by bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings.

The 1455 bp sequence corresponding to the 16S rRNA gene region of strain BNT52^T was compared with sequences deposited in the public databases. The results of these comparative 16S rRNA gene sequence analyses indicated that strain BNT52^T was phylogenetically affiliated to the genus *Amycolatopsis*. The highest levels of sequence similarity were found with sequences of members of the genus *Amycolatopsis*, namely *Amycolatopsis nigrescens* CSC17Ta-90^T (96.7 %), *Amycolatopsis magusensis* KT2025^T (96.6 %), *Amycolatopsis sulphurea* DSM 46092^T (96.6 %), *Amycolatopsis dongchuanensis* YIM 75904^T (96.5 %), *Amycolatopsis ultiminotia* RP-AC36^T (96.4 %) and *Amycolatopsis sacchari* DSM 44468^T (96.4 %). Sequence similarities with other strains of species of the genus *Amycolatopsis* were lower than 96.2 %. The corresponding 16S rRNA gene sequence similarity values with the type strains of the remaining species of the genus *Amycolatopsis* ranged from 93.7 % to 96.2 %. The phylogenetic trees based on the neighbour-joining, maximum-parsimony and maximum-likelihood algorithms indicated that strain BNT52^T formed a distinct branch with *A. dongchuanensis* YIM 75904^T, *A. sacchari* DSM 44468^T, *Amycolatopsis minnesotensis* 32U-2^T and *A. nigrescens* CSC17Ta-90^T within members of the genus *Amycolatopsis* (Figs 1 and S1, S2 and S3 available in IJSEM Online).

Chemotaxonomic analyses were carried out to support the phylogenetic affiliation of strain BNT52^T to the genus *Amycolatopsis*. The strain was grown in ISP 2 broth (Shirling & Gottlieb, 1966; supplemented with 5 %, w/v, NaCl) under aerobic conditions in flasks on a rotary shaker at 160 r.p.m. and 28 °C for 10 days. Biomass was harvested by centrifugation, washed twice in distilled water and recentrifuged and freeze-dried. Isomers of diaminopimelic acid in whole-cell hydrolysates and sugars were prepared according to the methods of Lechevalier & Lechevalier (1970) and analysed by TLC (Staneck & Roberts, 1974). The acid methanolysis procedure was used to detect mycolic acids (Minnikin *et al.*, 1980). Polar lipid and respiratory quinones analyses were carried out by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany. Respiratory quinones were extracted from 100 mg of freeze-dried cells based on the two-stage method described by Tindall (1990a, b). Respiratory quinones were separated into their different classes (menaquinones and ubiquinones) by TLC on silica gel (art. no. 805 023; Macherey–Nagel), using hexane/tert-butylmethylether (9 : 1, v/v) as solvent. UV-absorbing bands corresponding to menaquinones or ubiquinones were removed from the plate and further analysed by HPLC. This step was carried out on a LDC Analytical (Thermo Separation Products) HPLC fitted with a reverse-phase column (2×125 mm, 3 μm, RP18; Macherey–Nagel) using methanol as the eluant. Respiratory lipoquinones were detected at 269 nm.

A starter collection for the fatty acid analyses was prepared in a flask containing 20 ml trypticase soy broth (Difco) (supplemented with 5 % w/v NaCl) which was shaken at 150 r.p.m. at 28 °C for 5 days. A 5 ml volume of the resultant culture was used to inoculate 50 ml TSB, which was incubated under the same conditions; the biomass was harvested by cellulose filtration (pore size 0.45 μm) and the wet cells (200 mg) were placed in an extraction tube. Cellular fatty acids were extracted, methylated and separated by gas chromatography using a 6890N instrument (Agilent Technologies), fitted with an autosampler and a 6783 injector, according to the standard protocol of the Sherlock Microbial Identification (MIDI) system (Sasser 1990; Kämpfer & Kroppenstedt, 1996), the fatty acid methyl ester peaks were quantified using TSBA 5.0 software. The DNA G + C content of the isolate was determined following the procedure of Gonzalez & Saiz-Jimenez (2005).

The cell-wall diamino acid in the peptidoglycan layer of strain BNT52^T was *meso*-diaminopimelic acid (Type III; Lechevalier & Lechevalier, 1970) and whole-cell sugars included arabinose, galactose and glucose. The polar lipids included phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylinositol, phosphatidylmethylethanolamine, an unknown phospholipid and an unknown aminolipid (i.e. phospholipid pattern type 2 *sensu* Lechevalier *et al.*, 1977) (Figs S4 and S5). The major menaquinone found was MK-9(H_4) (85 %); followed by MK-7(H_4) (5 %), MK-8(H_4) (3 %), MK-10(H_2) (3 %) and MK-10(H_4) (3 %). These data are in line with the classification of the isolate in the genus *Amycolatopsis* (Lechevalier *et al.*, 1986; Lee, 2009). The major cellular fatty acids are iso $C_{16:0}$ 2-OH (19.3 %) and iso-$C_{16:0}$ (18.6 %) (Table S1). Mycolic acids were not detected. The G + C content of the DNA is 68.8 mol%.

Cultural characteristics of strain BNT52^T were determined (except for temperature tests) after incubation at 28 °C for 14 days on various media as described by Shirling & Gottlieb (1966): yeast extract–malt extract agar [International *Streptomyces* Project (ISP 2)], oatmeal agar (ISP 3), inorganic salt–starch agar (ISP 4), glycerol–asparagine agar (ISP 5), peptone–yeast extract–iron agar (ISP 6), tyrosine agar (ISP 7), modified Bennett's agar (Jones,1949), Czapek's and tryptic soy agar (TSA; Difco). National Bureau of Standards

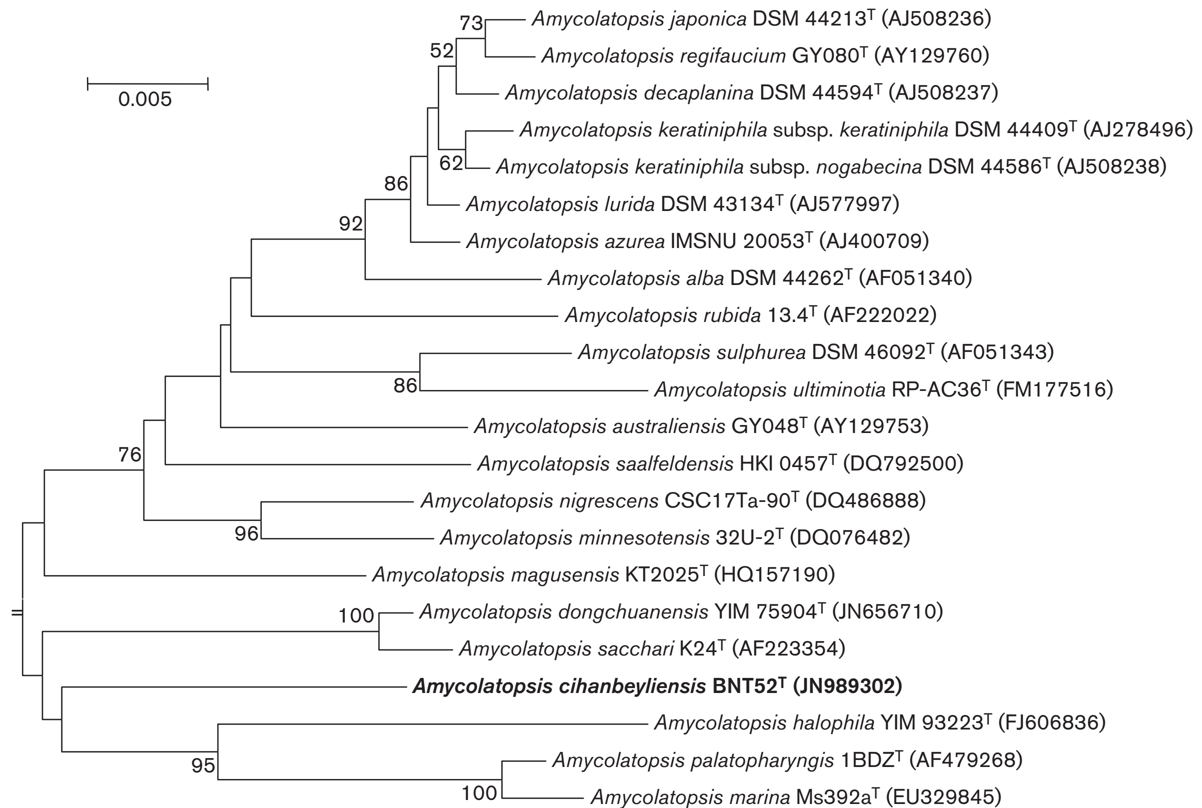

Fig. 1. Neighbour-joining tree (Saitou & Nei, 1987) based on almost complete 16 rRNA gene sequences showing the position of strain BNT52^T amongst its phylogenetic neighbours. Numbers at the nodes indicate the levels of bootstrap support (%); only values $\geqslant$50 % are shown. GenBank accession numbers are given in parentheses. Bar, 0.005 substitutions per site.

(NBS) Colour Name Charts (Kelly, 1964) were used for determining colour designation and names. Growth was tested at different temperatures (4, 10, 20, 28, 37, 45, 50 and 55 °C) and at pH 4.0–11.0 (at intervals of 1.0 pH unit), and for NaCl tolerance (0–15 %, w/v) using ISP 2. Established methods were used to determine whether the strains degraded chitin (Hsu & Lockwood, 1975), RNA (Goodfellow *et al.*, 1979) and Tweens 20, 40 and 80 (Nash & Krent, 1991); the remaining degradation tests were examined employing methods described by Williams *et al.* (1983) but using marine agar as a basal medium. Carbon source utilization was tested using carbon source utilization (ISP 9) medium (Shirling & Gottlieb, 1966) supplemented with a final concentration of 1 % of the tested carbon sources and 0.1 % succinic acid. Nitrogen source utilization was examined using the basal medium recommended by Williams *et al.* (1983) supplemented with a final concentration of 0.1 % of the tested nitrogen sources. The type strains *A. magusensis* KT2025^T, *Amycolatopsis halophila* DSM 45216^T, *A. nigrescens* DSM 44992^T, *A. minnesotensis* DSM 44988^T and *A. sacchari* DSM 44468^T were included for comparison in all tests. Colony morphology and micromorphological properties of BNT52^T were determined by examination of gold-coated dehydrated specimens of 14-day-old cultures from ISP 2 medium using a JSM 6060 instrument (JEOL).

Cells of strain BNT52^T were Gram-reaction-positive, aerobic, non-motile, non-acid–alcohol-fast, catalase-positive actinomycetes, which formed branched substrate hyphae that fragmented into rod-like elements (Fig. S6) when grown on modified Bennett's (Jones, 1949) agars. Strain BNT52^T grew well on modified Bennett's, GYM, ISP 2, ISP 3, ISP 4, ISP 5, ISP 6 and ISP 7 agar. The colour of aerial mycelium was bluish-white on ISP 2 and ISP 4, light cream on ISP 3 and ISP 5, white on ISP 6 and Czapek's and light grey on ISP 7 and the vegetative mycelium was greenish-yellow on ISP 2, ISP 3, ISP 4, ISP 5, ISP 6 and Bennett's, light olive-brown on ISP 7 and pale orange–yellow on Czapek's agar media. Diffusible pigments were not produced. Melanoid pigments were not produced on ISP 6 or ISP 7. The physiological properties that distinguished strain BNT52^T from closely related species of the genus *Amycolatopsis* are listed in Table 1. Furthermore, a comparison with the physiologically most closely related species *A. nigrescens* CSC17Ta-90^T and *A. magusensis* KT2025^T, showed differences in the degradation of xanthine and utilization of maltose, L-cysteine, L-phenylalanine, L-threonine and L-valine.

On the basis of the genotypic and phenotypic data presented, it is concluded that isolate BNT52^T represents a novel species within the genus *Amycolatopsis*. It is, therefore, proposed

Table 1. Phenotypic properties of strain BNT52^T and closely related type species

Strains: 1, BNT52^T; 2, *A. nigrescens* DSM 44992^T; 3, *A. magusensis* KT2025^T; 4, *A. sacchari* DSM 44468^T; 5, *A. dongchuanensis* DSM 45675^T; 6, *A. minnesotensis* DSM 44988^T; 7, *A. halophila* DSM 45216^T. All strains were positive for degradation of Tween 40 (1.0 %), ability to grow using D-galactose as a sole carbon source (1.0 %), glycine, L-alanine, L-histidine, L-proline and L-serine as sole nitrogen sources (0.1 %), but negative for degradation of adenine (0.5 %). All data were obtained in this study except for those for *A. dongchuanensis* (data taken from Nie *et al.*, 2012). +, Positive; −, negative.

Characteristic	1	2	3	4	5	6	7
Biochemical tests							
Nitrate reduction	−	+	−	+	+	+	−
Urea hydrolysis	+	+	+	+	+	+	−
pH Tolerance							
5.0	−	+	−	+	−	−	−
10.0	+	−	+	+	−	+	−
11.0	+	+	+	+	−	+	−
12.0	+	+	+	+	−	+	−
Temperature (°C)							
20	+	−	−	+	+	−	−
45	−	−	−	+	+	−	+
50	−	−	−	+	−	−	+
55	−	−	−	−	−	−	+
NaCl (%)							
0	+	+	+	+	+	+	−
8.0	+	−	+	+	−	−	+
9.0	+	−	−	−	−	−	+
10.0	+	−	−	−	−	−	+
15.0	−	−	−	−	−	−	+
Degradation							
Tween 80 (1 %)	+	+	+	+	+	+	−
Xanthine (0.4 %)	+	−	−	+	+	−	+
Starch (1.0 %)	−	−	−	−	+	−	−
L-Tyrosine (0.5 %)	+	+	+	+	+	−	+
Use of sole C sources (1.0 %)							
Cellobiose	+	+	+	+	−	+	−
D-Fructose	+	+	+	+	+	+	−
D-Sorbitol	+	+	−	−	−	+	−
D-Mannose	+	+	+	+	+	+	+
D-Mannitol	+	+	+	+	−	+	−
Lactose	+	+	−	+	+	+	−
Maltose	+	−	−	+	+	−	−
Succinic acid (0.1 %)	−	+	−	−	+	−	−
Sucrose	−	−	−	+	+	−	+
Xylitol	+	+	−	+	−	+	+
Xylose	+	+	+	+	+	−	−
Use of sole N sources (0.1 %)							
L-Cysteine	+	−	−	+	+	−	+
L-Phenylalanine	+	−	−	+	+	−	−
L-Threonine	+	−	−	+	+	+	+
L-Valine	+	−	−	+	+	+	+

that the organism be classified in the genus as a representative of *Amycolatopsis cihanbeyliensis* sp. nov.

Description of *Amycolatopsis cihanbeyliensis* sp. nov.

Amycolatopsis cihanbeyliensis (ci.han.bey.li.en′sis. N.L. fem. adj. *cihanbeyliensis* of or belonging to Cihanbeyli, Central Anatolia, the source of the organism).

Aerobic, Gram-reaction-positive, catalase-positive, non-motile, non-acid–alcohol-fast actinomycete which forms an extensively branched vegetative mycelium that fragments into squarish rod-like elements. Growth occurs at pH 6.0–12 and at 20–37 °C, but not at pH 5.0 and at 10 and 45 °C. Optimal growth is at 28 °C and pH 7.2. Grows well in the presence of 0–10.0 % (w/v) NaCl, but does not grow in the presence of more than 10.0 % (w/v) NaCl. Aesculin, allantoin and urea are hydrolysed but arbutin is not hydrolysed. Nitrate reduction is negative. Hypoxanthine, L-tyrosine, Tweens 40 and 80 and xanthine are degraded but adenine, casein, chitin, starch and elastin are not. Utilizes adonitol, D-arabinose, cellobiose, D-fructose, D-sorbitol, D-galactose, D-mannose, D-mannitol, dextrin, inulin, lactose, maltose, *myo*-inositol, xylitol and xylose as sole carbon sources, but not dextran, L-sorbose, L-arabinose, L-glutamate, starch, succinic acid or sucrose. Utilizes α-isoleucine, glycine, L-alanine, L-arginine, L-cysteine, L-histidine, L-hydroxyproline, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine and L-valine as sole nitrogen sources. Contains meso-diaminopimelic acid and arabinose, galactose and glucose. The predominant menaquinone of the type strain is MK-9(H_4) (85 %) with minor amounts of MK-7(H_4), MK-8(H_4), MK-10(H_2) and MK-10(H_4). The polar lipid profile contains diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylmethylethanolamine, an unknown phospholipid and an unknown aminolipid. The major cellular fatty acids are iso-$C_{16:0}$ 2-OH and iso-$C_{16:0}$.

The type strain, BNT52^T (=KCTC 29065^T=NRRL B-24886^T=DSM 45679^T) was isolated from a soil sample collected from Cihanbeyli Salt Mine, located in the central Anatolia region of Turkey. The G+C content of the genomic DNA of the type strain is 68.8 mol%.

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