Amycolatopsis tucumanensis sp. nov., a copper-resistant actinobacterium isolated from polluted sediments

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A novel actinomycete strain, ABO^T, isolated from copper-polluted sediments showed remarkable copper resistance as well as high bioaccumulation abilities. Classical taxonomic methods, including chemotaxonomy and molecular techniques, were used to characterize the isolate. Strain ABO^T developed a honey-yellow substrate mycelium on all ISP media tested. Abundant, white, aerial mycelium was only formed on ISP 2, 5 and 7 and MM agar. Both types of hyphae fragmented into squarish rod-shaped elements. The aerial mycelium displayed spore-like structures with smooth surfaces in long, straight to flexuous chains. The organism has a type-IV cell wall lacking mycolic acids and type-A whole-cell sugar pattern (*meso*-diaminopimelic acid, arabinose and galactose) in addition to a phospholipid type-II profile. 16S rRNA gene sequence studies indicated that this organism is a member of the family *Pseudonocardiaceae* and that it forms a monophyletic clade with *Amycolatopsis eurytherma* NT202^T. The DNA–DNA relatedness of strain ABO^T to *A. eurytherma* DSM 44348^T was 39.5 %. It is evident from these genotypic and phenotypic data that strain ABO^T represents a novel species in the genus *Amycolatopsis*, for which the name proposed is *Amycolatopsis tucumanensis* sp. nov. The type strain is ABO^T (=DSM 45259^T =LMG 24814^T).

Copper is an essential heavy metal required for numerous enzymic functions in all cells. However, the same chemistry that makes copper essential also makes it a potent cytotoxin when copper homeostatic controls fail (Solioz & Stoyanov, 2003). Copper's many uses in several industrial applications has led to its wide distribution in soil, silt, waste and wastewater and to significant environmental problems that need to be addressed (Albarracín *et al.*, 2008b). Micro-organisms that are able to accumulate and immobilize toxic metals are considered key tools for the bioremediation of polluted environments (Polti *et al.*, 2007; Albarracín *et al.*, 2008a). Because of their great metabolic and morphological diversity, actinobacteria have been proposed as potential tools for remediation biotechnologies (Polti *et al.*, 2007; Schmidt *et al.*, 2005). However, copper resistance in actinobacteria has been little studied (Amoroso *et al.*, 1998; Richards *et al.*, 2002; Schmidt *et al.*, 2005; Albarracín *et al.*, 2005, 2008a, b).

The genus *Amycolatopsis*, proposed by Lechevalier *et al.* (1986), has been classified in the family *Pseudonocardiaceae* by the application of the polyphasic taxonomic approach to actinomycete systematics (Stackebrandt *et al.*, 1997; Kim & Goodfellow, 1999). It currently contains 39 species with validly published names (http://www.bacterio.cict.fr/a/amycolatopsis.html), and their representatives have been thoroughly studied because of their important secondary metabolism and applications in medicine and industry (Wink *et al.*, 2004). Recently, Albarracín *et al.* (2008a) made a report of a copper-resistant *Amycolatopsis* strain,

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain ABO^T is DQ886938.

A table showing the fatty acid composition of strain ABO^T, a micrograph of the aerial mycelium of *A. eurytherma* DSM 44348^T and an extended neighbour-joining tree based on 16S rRNA gene sequences are available as supplementary material with the online version of this paper.

isolated from copper-polluted sediments and designated ABO^T, which has a high bioaccumulation ability and the potential for use in bioremediation biotechnologies.

The actinobacterial strain ABO^T (code of the culture collection at PROIMI, Tucumán, Argentina) was isolated from groundwater sediments polluted with copper (Albarracín *et al.*, 2005). It was stored at 4 °C on starch-casein agar slants, containing (l^{-1}): 10.0 g starch, 1.0 g casein, 0.5 g K_2HPO_4, 15 g agar; pH 7.0. The reference strain *Amycolatopsis eurytherma* DSM 44348^T was included in morphological and physiological studies for comparison.

Morphological and physiological characteristics were observed on various media as described by Shirling & Gottlieb (1966): yeast extract-malt extract agar (ISP 2), inorganic salts-starch agar (ISP 4), glycerol-asparagine agar (ISP 5), peptone-yeast extract-iron agar (ISP 6) and tyrosine agar (ISP 7). Cultures were incubated for 10 days at 30 °C. Honey-yellow-coloured substrate mycelium developed on all ISP media tested (RAL colour code 1024; Deutsches Institut für Gütesicherung und Kennzeichnung, Reichsausschuß für Lieferbedingungen). White, spore-producing aerial mycelium (RAL colour code 9003) was only formed on ISP 2, 5 and 7 and minimal medium (MM) agar, containing (l^{-1}): 0.5 g L-asparagine, 0.5 g K_2HPO_4, 0.2 g $MgSO_4 . 7H_2O$, 0.01 g $FeSO_4 . 7H_2O$, 10.0 g glucose, 15.0 g agar; pH 7.0. Gram and acid–alcohol-fast stains were carried out on a 3-day-old culture as described by Doetsch (1981). The cells stained Gram-positive and were non-acid–alcohol-fast.

For scanning electron microscopy, cultures were grown on ISP 2 agar. Agar blocks were cut from the growth medium, fixed in 4.25 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 3 h at 4 °C and dehydrated in a graded acetone series. The critical drying point was obtained by exchanging the acetone through liquid CO_2. The samples were coated with gold and visualized in a JEOL JSM 35CF scanning electron microscope with 15 kV voltage acceleration. After 7–10 days on ISP 2, strain ABO^T produced a well-developed branched substrate mycelium as well as a profuse aerial mycelium (Fig. 1). Both types of hyphae fragmented into squarish rod-shaped elements. The aerial mycelium displayed spore-like structures (0.3×0.8–1.5 μm) with smooth surfaces in long, straight to flexuous chains (Fig. 1). All of these properties are consistent with the classification of the strain in the genus *Amycolatopsis*. For a comparison, a micrograph of the reference strain *A. eurytherma* DSM 44348^T is presented in Supplementary Fig. S1 (available in IJSEM Online).

Utilization of carbohydrates was investigated in ISP 9 (Shirling & Gottlieb, 1966) by using a 12-well microtitre plate technique. Lysozyme resistance and sodium chloride tolerance were tested on six-well microtitre plates (Wink *et al.*, 2004). Acid production from sugars was determined according to Gordon *et al.* (1974). A fingerprint of enzymic activities was obtained by using API 20E, API ZYM and API Coryne test strips (bioMérieux). The temperature for

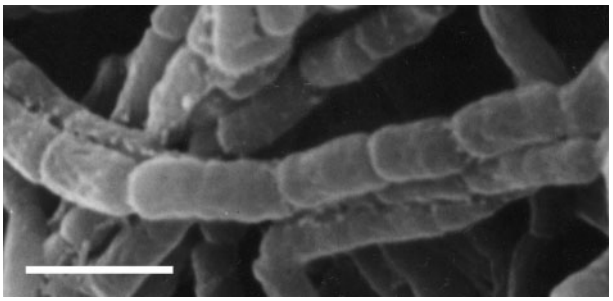

Fig. 1. Micrograph of strain ABO^T grown on ISP2 agar for 7 days at 30 °C, showing the fragmenting aerial mycelium with smooth-surfaced spore-like structures in long, straight to flexuous chains. Bar, 1 μm.

growth was tested at 10, 15, 20, 25, 30, 35, 45 and 55 °C on ISP 2 while the pH for growth was tested in the range pH 2.0–10.0 in ISP 2 broth (30 °C, 180 r.p.m.). Strain ABO^T was able to utilize all of the tested carbon sources except xylose, rhamnose and cellulose (Table 1) and could grow in the presence of 5 % NaCl and up to 100 μg lysozyme ml^{-1}. The strain showed a wide tolerance to temperature and pH, in the ranges 15–55 °C and pH 5.0–10.0.

Copper resistance was measured by incubating cultures at 30 °C for 4 days on MM agar with $CuSO_4$ added to different concentrations (0.5–3 mM), as described previously (Albarracín *et al.*, 2005). For this assay, an additional copper-sensitive control, *Streptomyces coelicolor* DSM 40783^T, was used. Fig. 2 illustrates the growth of the three strains. While strain ABO^T was able to grow up to the maximum concentration tested, *S. coelicolor* DSM 40783^T was notably inhibited at 0.5 mM, with which sparse substrate mycelium was formed. *A. eurytherma* DSM 44348^T presented a moderate copper-resistance profile but it is clearly lower than that depicted by strain ABO^T. Strain ABO^T could be distinguished from its closest phylogenetic relatives on the basis of its copper-resistance profile, broader pH and temperature ranges for growth and differential carbohydrate assimilation and enzymic activity profiles (Table 1).

For chemotaxonomic analyses, strain ABO^T was grown in trypticase soy broth in flasks on a rotary shaker at 90 r.p.m. and 28 °C and the biomass was harvested, washed in distilled water and freeze-dried. Cell walls were prepared according to the method of Schleifer (1985). The peptidoglycan structure was studied in whole- and partial-cell-wall hydrolysates using TLC on cellulose (Schleifer & Kandler, 1972). Analysis of sugars in the purified cell walls was carried out as described by Staneck & Roberts (1974). Menaquinones were extracted and purified by the method of Minnikin *et al.* (1984) and analysed by HPLC (Hewlett Packard 1100). Methyl esters of cellular fatty acids from strain ABO^T were prepared from cells of cultures grown for 24 h on trypticase soy agar at 28 °C and

Table 1. Characteristics of strain ABO^T and its closest phylogenetic relatives in the genus *Amycolatopsis*

Strains: 1, *Amycolatopsis tucumanensis* sp. nov. ABO^T; 2, *A. eurytherma* DSM 44348^T; 3, *A. methanolica* IFO 15065^T; 4, *A. thermoflava* IFO 14333^T. Data in columns 1 and 2 were obtained in this study; data in columns 3 and 4 were taken from Kim *et al.* (2002), Chun *et al.* (1999) and De Boer *et al.* (1990). +, Positive; W, weakly positive; −, negative; ND, no data available. All strains form a white aerial mycelium.

Characteristic	1	2	3	4
Production of soluble pigment	−	−	−	+
Acid production from:				
(+)-D-Fructose	W	+	+	+
myo-Inositol	−	+	−	−
(+)-Raffinose	−	−	−	+
α-(+)-L-Rhamnose	W	+	+	−
(−)-Sucrose	−	−	+	−
(+)-Trehalose	W	+	+	+
(+)-D-Xylose	W	+	+	+
Utilization of:				
(+)-Cellobiose	−	−	+	+
(+)-Raffinose	+	−	ND	+
α-(+)-L-Rhamnose	−	+	+	ND
(+)-D-Xylose	−	+	+	+
Growth at:				
15 °C	W	W	−	+
20 °C	+	W	−	+
25 °C	+	+	−	+
55 °C	+	+	−	+
Degradation of:				
Aesculin	+	−	W	+
Casein	+	+	−	+
Gelatin	+	+	+	−
Production of:				
β-Galactosidase	+	−	ND	ND
N-Acetyl-β-glucosaminidase	+	−	ND	ND
Nitrate reductase	−	+	+	−
Urease	−	+	−	+

were analysed by GLC (Schröder *et al.*, 1997). Polar lipids were extracted and identified by two-dimensional TLC (Minnikin *et al.*, 1984). Chemotaxonomic data also supported the assignment of strain ABO^T to the genus *Amycolatopsis*. The micro-organism has a type-IV cell wall (*meso*-diaminopimelic acid) and a type-A whole-cell sugar pattern, with arabinose, galactose and ribose as the major constituents. The detected phospholipids included phosphatidylinositol, phosphatidylethanolamine, hydroxyl phosphatidylethanolamine and diphosphatidylglycerol, which corresponds to a phospholipid type-II profile. The major menaquinone present was MK-9(H_4), and MK-9(H_2), MK-9(H_6) and MK-10(H_2) were found in minor amounts. Strain ABO^T contained major amounts of 14-methyl pentadecanoic acid (23 %), hexadecanoic acid (12 %) and 14-methyl hexadecanoic acid (11.4 %). In accordance with its non-acid–alcohol-fastness, mycolic acids were absent in this strain. The complete fatty acid profile is shown in Supplementary Table S1.

DNA extraction and PCR cloning and sequencing of the 16S rRNA gene were carried out as described previously (Albarracín *et al.*, 2005). Multiple alignments of the 16S rRNA gene sequences from strain ABO^T (1488 nt) and reference sequences from the NCBI databases were performed by using the CLUSTAL W program (Thompson *et al.*, 1994). A phylogenetic tree was constructed according to the neighbour-joining method (Saitou & Nei, 1987) using the maximum-composite-likelihood method (Tamura *et al.*, 2004) and compared with a tree constructed according to the maximum-parsimony method (Fitch, 1971). In both cases, 1000 resamplings were used for bootstrap analyses (Felsenstein, 1985). All analyses were carried out with the MEGA4 program (Kumar *et al.*, 2001). The phylogenetic analysis with corresponding nucleotide sequences from representatives of the family *Pseudonocardiaceae* showed that the organism belongs to the genus *Amycolatopsis* (Fig. 3 and Supplementary Fig. S2). It is clear from the phylogenetic trees that strain ABO^T forms a monophyletic clade with *A. eurytherma* NT202^T. This relationship is supported by the 100 % bootstrap value for the node in the neighbour-joining tree. The 16S rRNA gene sequence similarity between strain ABO^T and *A. eurytherma* NT202^T is 99.8 %; this value corresponds to two differences in the 1466 nucleotide positions that were

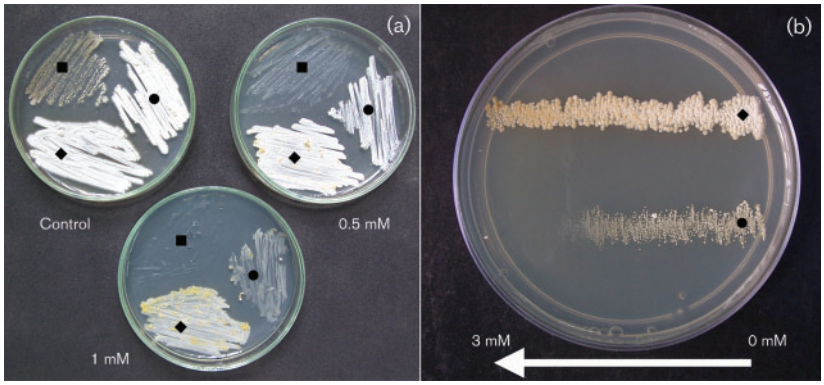

Fig. 2. Copper-resistance assays for *S. coelicolor* DSM 40783^T (■), *A. eurytherma* DSM 44348^T (●) and strain ABO^T (◆) on MM agar plates supplemented with 0.5 or 1 mM copper sulphate (a) and *A. eurytherma* DSM 44348^T (●) and strain ABO^T (◆) inoculated on MM agar plates with a copper sulphate gradient (0–3 mM; arrow indicates the direction of increasing copper concentration) (b).

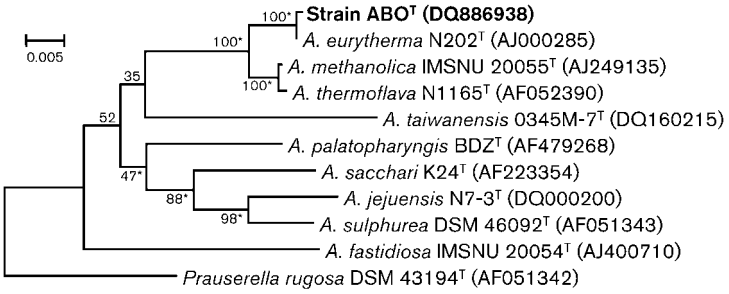

Fig. 3. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences (at least 1402 nt) showing the relationships between strain ABO^T and its closest phylogenetic relatives in the genus *Amycolatopsis*. Percentages at nodes are levels of bootstrap support based on 1000 resamplings. Asterisks indicate that the corresponding nodes were also recovered in trees generated with the maximum-parsimony method. *Prauserella rugosa* DSM 43194^T was used as the outgroup. Bar, 0.005 substitutions per nucleotide position.

compared. Similarity values with other *Amycolatopsis* strains ranged from 93 to 99.8 %.

DNA for DNA–DNA hybridization was isolated using a French pressure cell and purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977). DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970) with some modifications (Huß *et al.*, 1983) using a model Cary 100 Bio UV/Vis spectrophotometer. The DNA–DNA relatedness of strain ABO^T to *A. eurytherma* DSM 44348^T was 39.5 %, indicating that they were distinct genomospecies based on the criteria set by Stackebrandt *et al.* (2002).

In summary, strain ABO^T could be distinguished from its closest phylogenetic neighbours, including *A. eurytherma*, by combined results from phenotypic and molecular tests (Figs 1, 2 and 3 and Table 1). It is evident from these genotypic and phenotypic data that strain ABO^T represents a novel species in the genus *Amycolatopsis*. It is proposed that the organism be assigned to the novel species *Amycolatopsis tucumanensis* sp. nov.

Description of *Amycolatopsis tucumanensis* sp. nov.

Amycolatopsis tucumanensis (tu.cu.ma.nen′sis. N.L. fem. adj. *tucumanensis* pertaining to Tucumán, Argentina, the origin of the soil sample from which the type strain was isolated).

Aerobic, Gram-positive, non-acid–alcohol-fast, catalase-positive, non-motile actinomycete that forms an extensively branched, honey-yellow substrate mycelium which fragments into squarish elements on all ISP media tested (RAL colour code 1024). White, spore-producing aerial mycelium (RAL colour code 9003) is only formed on ISP 2, 5 and 7 and MM agar and also fragments. The spore-like structures (0.3×0.8–1.5 μm) present smooth surfaces and are displayed in long, straight to flexuous chains. No diffusible pigments are produced with any tested media. Produces phosphatase, but not nitrate reductase nor urease. Grows at 15–55 °C and pH 5–10 and with 5 % NaCl. Resistant to lysozyme (100 μg ml^{-1}) and to high concentrations of copper (up to 3 mM). Other phenotypic properties are given in Table 1.

The type strain is ABO^T (=DSM 45259^T =BCCM/LMG 24814^T), which was isolated from a sediment sample polluted with copper and collected in Tucumán, Argentina. The species description is based on a single strain and hence serves as the type strain description.

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