Reclassification of *Amycolatopsis orientalis* DSM 43387 as *Amycolatopsis benzoatilytica* sp. nov.

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Amycolatopsis orientalis DSM 43387, a clinical isolate from submandibular mycetoma tissue, is one of three plasmid-bearing strains of the genus *Amycolatopsis*. It degrades aromatic compounds such as *m*-hydroxybenzoate, but does not produce any antibiotics, in contrast to *Amycolatopsis orientalis* NBRC 12806^T. Phylogenetic analysis based on a complete 16S rRNA gene sequence placed the strain in the clade of *Amycolatopsis albidoflavus* IMSNU 22139^T, distant from the clade of *A. orientalis* NBRC 12806^T. The strain showed low DNA–DNA hybridization values of 24.1 and 45.7% with *A. orientalis* NBRC 12806^T and *A. albidoflavus* DSM 44639^T (=IMSNU 22139^T), respectively. It could also be readily distinguished from *A. orientalis* NBRC 12806^T and all species with validly published names classified in the genus *Amycolatopsis*, by using a combination of chemical and physiological markers such as utilization of lactose, degradation of xanthine, hypoxanthine, gelatin and casein and hydrolysis of Tween 80, indicating that it represents a novel species. Strain DSM 43387 could also be differentiated from *A. orientalis* NBRC 12806^T and its nearest neighbour *A. albidoflavus* IMSNU 22139^T on the basis of fatty acid and phospholipid profiles. Based on genotypic and phenotypic differences, the name *Amycolatopsis benzoatilytica* sp. nov. is proposed for strain DSM 43387 that was previously classified as *Amycolatopsis orientalis*. The type strain is AK 16/65^T (=DSM 43387^T=ATCC 55165^T=IMRU 1389^T).

The genus *Amycolatopsis*, proposed by Lechevalier *et al.* (1986), comprises a large group of medically and commercially important amycolate, nocardioform actinomycetes. It belongs in the family *Pseudonocardiaceae* (Embley *et al.*, 1988; Warwick *et al.*, 1994), which is characterized by the presence of *meso*-diaminopimelic acid, arabinose and galactose in the wall peptidoglycan (wall chemotype IV *sensu* Lechevalier & Lechevalier, 1970), *N*-acetylated muramic acid residues and fatty acids rich in iso- and anteiso-branched components, but its members lack mycolic acids (Takahashi, 2001). Currently, 26 species with validly published names constitute the genus *Amycolatopsis*.

Most members of the genus *Amycolatopsis* are commercially important because of their ability to produce bioactive compounds, such as antibiotics, and to degrade a wide range of aromatic compounds. *Amycolatopsis orientalis* DSM 43387 does not appear to produce antibiotics. Interestingly, it

metabolizes *m*-hydroxybenzoate through a central intermediate protocatechuate, unlike other species of *Amycolatopsis* and *Streptomyces* that are not able to utilize this compound as a substrate for growth (Grund *et al.*, 1990). In addition, it also contains a 29.6 kb conjugative plasmid, pA387 (Lal *et al.*, 1991), and is thus one of three plasmid-bearing strains of *Amycolatopsis* so far reported (Dhingra *et al.*, 2003). Strain DSM 43387 was isolated in Czechoslovakia from a patient suffering from submandibular mycetoma (Scharfen, 1971). The strain was initially identified as *Nocardia brasiliensis* (Scharfen, 1971) and deposited in IMRU under the accession number 1389. *N. brasiliensis* IMRU 1389 was reclassified as a member of *Nocardia orientalis* by Gordon *et al.* (1978), based on morphological and biochemical properties (acid production from erythritol and arabinose and decomposition of casein, hypoxanthine, tyrosine, urea, aesculin and starch) typical of *N. orientalis* (Mishra *et al.*, 1980). However, the authors emphasized that the availability of more strains from different sources and a detailed study of their properties would be required to establish the status of the test strain as representing a distinct species (Gordon *et al.*, 1978). At the time when the mycolic acid-lacking nocardioform actinomycetes were separated from the genus *Nocardia*, *N. orientalis* was renamed *Amycolatopsis orientalis* (Lechevalier *et al.*, 1986).

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain DSM 43387^T is AY957506.

Tables detailing DNA–DNA relatedness values and the differential distribution of phospholipids of *Amycolatopsis benzoatilytica* sp. nov. DSM 43387^T and the type strains of *A. orientalis* and *A. albidoflavus* are available as supplementary data in IJSEM Online.

It therefore appears that *A. orientalis* DSM 43387 (= IMRU 1389) has been misclassified in this species. The present investigation was designed to establish the taxonomic position of strain DSM 43387 on the basis of the 16S rRNA gene sequence, DNA–DNA hybridization, chemotaxonomy and morphological and biochemical properties. The genotypic and phenotypic data show that strain DSM 43387 should be reclassified as the type strain of a novel species of *Amycolatopsis*, for which the name *Amycolatopsis benzoatilytica* sp. nov. is proposed.

Genomic DNA was isolated from strain DSM 43387 by using the salting-out method described by Kieser *et al.* (2000). PCR amplification and sequencing of the 16S rRNA gene were carried out as described by Reddy *et al.* (2000). An almost complete 16S rRNA gene sequence was determined for strain DSM 43387 (1417 nucleotides). The resultant sequence was first manually aligned with corresponding 16S rRNA gene sequences of 24 species of the genus *Amycolatopsis* with validly published names retrieved from GenBank (<http://www.ncbi.nlm.nih.gov/>), using the CLUSTAL_X program (version 1.8b; Thompson *et al.*, 1997). Phylogenetic comparison of the 16S rRNA gene sequence of strain DSM 43387 with the corresponding sequences of representative members of the family *Pseudonocardiaceae* showed that the organism belongs to the genus *Amycolatopsis*. The levels of 16S rRNA gene sequence similarity between strain DSM 43387 and other members of species of the genus *Amycolatopsis* with validly published names ranged from 91.0 to 95.4%. Strain DSM 43387 had a sequence similarity of 93.9% with *A. orientalis* NBRC 12806^T and it was also revealed that its closest relative was *Amycolatopsis albidoflavus* IMSNU 22139^T, with a sequence similarity of 95.4%. These values corresponded to 63 and 83 nucleotide differences out of 1372 nucleotides positions compared. For the construction of the phylogenetic tree, the sequence alignment was optimized manually and the terminal nucleotides were removed. The tree was constructed using the neighbour-joining method (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and parsimony analysis based on 1372 nucleotides present in all the strains, using the PHYLIP package (version 3.5c; Felsenstein, 1993). *Prauserella rugosa* DSM 43194^T was used as an outgroup. The evolutionary distance matrix was calculated using the distance model of Jukes & Cantor (1969). The resultant tree topologies were evaluated by bootstrap analysis based on 1000 resamplings using the programs SEQBOOT and CONSENSE. It is clear from the phylogenetic tree that strain DSM 43387 forms a monophyletic clade with *A. albidoflavus* IMSNU 22139^T (Fig. 1). The type strain, *A. orientalis* NBRC 12806^T, forms a distinct phyletic clade distant from strain DSM 43387. Similar tree topologies were obtained using maximum-likelihood and parsimony analysis (data not shown). This clearly indicates that strain DSM 43387 forms a new centre of taxonomic variation in the genus *Amycolatopsis* and is quite distant from *A. orientalis* NBRC 12806^T in the 16S rRNA gene-based phylogenetic tree. Thus

phylogenetic clustering indicates that strain DSM 43387 represents a novel species of the genus *Amycolatopsis*.

The 16S rRNA gene does not have enough discriminatory power for use for the delineation of closely related species. Therefore the gold-standard method for species delineation to date is DNA–DNA hybridization (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994). To further clarify the taxonomic status of strain DSM 43387, *A. orientalis* NBRC 12806^T and its phylogenetic neighbour *A. albidoflavus* DSM 44639^T (= IMSNU 22139^T) were obtained from NBRC and DSMZ, respectively, and DNA–DNA hybridization was performed using the membrane filter method (Tourova & Antonov, 1987). First, genomic DNA was isolated from strain DSM 43387, *A. orientalis* NBRC 12806^T and *A. albidoflavus* DSM 44639^T by using the salting-out method described by Kieser *et al.* (2000). Experiments to determine DNA–DNA relatedness between the strains were performed as described by Bala *et al.* (2004). DNA (10 µg) of each strain was transferred onto a nylon membrane (Hybond-N; Amersham) using a dot-blot apparatus (Bio-Rad). The membrane was air-dried and UV cross-linked. The DNA probe of each strain was labelled with [α -³²P] (BRIT) using a nick translation kit (Bangalore Genei). Hybridization was done overnight at 65 °C. After hybridization, the nylon membrane was washed twice, first with 0.5 × SSC and 0.1% SDS for 10 min at room temperature and then with 0.1 × SSC and 0.5% SDS for 20 min at 65 °C, to remove the unbound probe. The amount of bound probe DNA was estimated by using a scintillation counter (Beckman Instruments) and the hybridization values obtained were expressed as the percentage of the probe bound relative to the homologous reaction. In the experiments, DNA of each of the three strains was bound to the filters and hybridized with DNA probes prepared from each strain. The DNA–DNA hybridization data showed that strain DSM 43387 had DNA–DNA relatedness values of 24.1 and 45.7%, respectively, with *A. orientalis* NBRC 12806^T and *A. albidoflavus* DSM 44639^T (= IMSNU 22139^T) at the genome level. The hybridization value of below 70%, which has been suggested as the threshold value for delineating a bacterial species (Wayne *et al.*, 1987), clearly indicated that strain DSM 43387 represents a novel species of *Amycolatopsis*. The percentage relatedness calculated on the basis of the data obtained (mean of four replicates) by DNA–DNA hybridization is summarized in Supplementary Table S1 in IJSEM Online.

These data support the 16S rRNA gene sequence results and lead to the conclusion that strain DSM 43387 represents a novel species of *Amycolatopsis*.

The polar lipids of strain DSM 43387 have already been reported (Yassin *et al.*, 1993). Analysis of the polar lipids revealed that the phospholipid pattern of strain DSM 43387 is type PII (Lechevalier *et al.*, 1977). The polar lipid profile contained the acidic lipids diphosphatidylglycerol, phosphatidylglycerol and phosphatidylinositol, and the neutral lipids phosphatidylethanolamine and phosphatidylmethylethanolamine, as predominant phospholipids, together with

Fig. 1. Phylogenetic tree based on nearly complete 16S rRNA gene sequences of species of *Amycolatopsis* with validly published names and *Amycolatopsis benzoatilytica* sp. nov. The tree was constructed by using the neighbour-joining method (Saitou & Nei, 1987) and rooted using *Prauserella rugosa* DSM 43194^T as an outgroup. Numbers at nodes are bootstrap values based on 1000 resamplings. Bar, 0.1 nucleotide substitution per nucleotide position.

several unidentified ninhydrin-positive phospholipids that were not present in *A. orientalis* NBRC 12806^T. In contrast, several phospholipids possessing vicinal OH groups (designated P-OH) and reacting with periodate–Schiff's reagent were present in *A. orientalis* NBRC 12806^T, in addition to the major components of the PII type phospholipid pattern, but phosphatidylinositol was absent. As shown in Supplementary Table S2 in IJSEM Online, phosphatidylglycerol, which is present as a major component in strain DSM 43387, was not present in *A. albidoflavus* IMSNU 22139^T. Whereas the polar lipid composition underlies the integrity of the genus *Amycolatopsis*, it clearly differentiates strain DSM 43387 from *A. orientalis* ATCC 19795^T (=NBRC 12806^T) (Yassin *et al.*, 1993) and *A. albidoflavus* IMSNU 22139^T (Lee & Hah, 2001).

Because of the uneven distribution of fatty acids among different species of actinomycetes, they are a useful criterion

for the delineation of species. Cellular fatty acid analysis of strain DSM 43387, *A. orientalis* NBRC 12806^T and *A. albidoflavus* DSM 44639^T was carried out by Microbial ID using the following procedure. Cultures of all three strains were grown on tryptic soy agar (TSBA; Himedia Laboratories Pvt. Ltd) under similar growth conditions and fatty acid methyl esters from 40 mg cells scraped from Petri plates were analysed by saponification, methylation and extraction, using the method of Sasser (1990). The fatty acid methyl ester mixtures were separated using the Sherlock Microbial Identification System (Microbial ID), which consisted of an Agilent model 5980 gas chromatograph fitted with a phenylmethyl silicon column (25 m × 0.2 mm) and FID detector. Identification and comparison was carried out using the Aerobe (TSBA50, version 5) database of the Sherlock Microbial Identification System. The predominant fatty acids detected in the cell hydrolysate of strain DSM 43387 included 12-methyltridecanoic acid (iso-C_{14:0};

11.25%), pentadecanoic acid ($C_{15:0}$; 14.60%), pentadecenoic acid ($C_{15:1\omega6c}$; 12.81%), 14-methylpentadecanoic acid (iso- $C_{16:0}$; 12.80%), heptadecanoic acid ($C_{17:0}$; 11.31%) and heptadecenoic acid ($C_{17:1\omega8c}$; 11.31%). The predominant fatty acids of *A. orientalis* NBRC 12806^T and *A. albidoflavus* DSM 44639^T were heptadecenoic acid ($C_{17:1\omega8c}$; 17.08%) and 14-methylpentadecanoic acid (iso- $C_{16:0}$; 16.74%), respectively. A comparison of the fatty acid compositions of strain DSM 43387, *A. orientalis* NBRC 12806^T and *A. albidoflavus* DSM 44639^T is given in Table 1. However, the fatty acid profiles of *A. orientalis* NBRC 12806^T (=IMSNU 20058^T) and *A. albidoflavus* DSM 44639^T (=IMSNU 22139^T) determined using TSBA varied from those obtained previously for these strains on YMG broth (Lee & Hah, 2001).

On the basis of their cellular fatty acid composition, strain DSM 43387 differed from *A. orientalis* NBRC 12806^T and its phylogenetic neighbour *A. albidoflavus* DSM 44639^T,

Table 1. Cellular fatty acid compositions (%) of *A. benzoatilytica* sp. nov. DSM 43387^T, *A. orientalis* NBRC 12806^T and *A. albidoflavus* DSM 44639^T

Strains: 1, *A. benzoatilytica* DSM 43387^T; 2, *A. orientalis* NBRC 12806^T; 3, *A. albidoflavus* DSM 44639^T. All data from this study. —, Not detected.

Fatty acid	1	2	3
iso- $C_{13:0}$	0.15	0.18	—
$C_{13:0}$	0.31	0.34	0.36
iso- $C_{14:0}$	11.25	5.97	11.02
$C_{14:1\omega5c}$	—	—	0.27
$C_{14:0}$	0.50	0.51	1.31
iso- $C_{15:0}$	6.75	14.25	9.17
anteiso- $C_{15:0}$	3.04	1.88	2.79
$C_{15:1\omega6c}$	12.81	5.12	12.43
$C_{15:0}$	14.60	7.19	9.00
iso- $C_{14:0}$ 3-OH	—	—	0.31
iso- $C_{16:1}$ H	0.21	0.27	—
iso- $C_{16:0}$	12.80	14.89	16.74
Summed feature 3*	6.79	14.25	15.31
$C_{16:0}$	5.15	4.68	8.95
$C_{15:0}$ 2-OH	1.01	0.79	0.75
Summed feature 4*	—	0.27	—
anteiso- $C_{17:1\omega9c}$	0.35	—	—
iso- $C_{17:0}$	0.37	1.72	0.62
anteiso- $C_{17:0}$	0.82	1.37	1.74
$C_{17:1\omega8c}$	11.31	17.08	4.69
$C_{17:0}$	11.31	7.44	3.85
$C_{18:1\omega7c}$	0.09	—	—
$C_{18:1\omega9c}$	—	0.95	—
$C_{18:0}$	0.37	0.84	0.70

*Summed feature 3, $C_{16:1\omega6c}/C_{16:1\omega7c}$; summed feature 4, 10-methyl $C_{16:0}/iso-C_{17:1\omega9c}$.

indicating that strain DSM 43387 probably represents a novel species of *Amycolatopsis*.

The colour, shape, size and contour of colonies of strain DSM 43387 were observed on YM medium at 28 °C for 7 days. Cream-coloured, irregularly shaped, rough, dull and moderate-sized flat colonies with undulating margins were observed. The vegetative mycelia showed a tendency to fragment when the organism was grown in liquid culture. Similar to *A. orientalis* NBRC 12806^T, strain DSM 43387 did not produce spores in any of the media tested. The strain was subjected to a battery of physiological tests. Growth at different temperatures and catalase tests were carried out as described by McCarthy & Cross (1984) using YM medium. Hydrolysis of Tween 80 and the ability of the strain to grow in the presence of NaCl were tested as described by Arden-Jones *et al.* (1979). Acid production from carbohydrates and degradation of xanthine and hypoxanthine were determined as described by Gordon *et al.* (1974). Urease activity was detected as described by Christensen (1946). Other physiological tests and methods used were as described by Collins *et al.* (1989). Strain DSM 43387 grew at a temperature range of 10–37 °C. The pH range for growth was 5–10. Similar to *A. orientalis* NBRC 12806^T and *A. albidoflavus* IMSNU 22139^T, strain DSM 43387 utilized adonitol, (+)-L-arabinose, (+)-D-cellobiose, meso-erythritol, (+)-D-fructose, (+)-D-galactose, myo-inositol, (+)-D-lactose, (+)-D-mannose, (+)-D-trehalose and (+)-D-xylose and degraded aesculin. However, differences in results from those for *A. albidoflavus* IMSNU 22139^T and *A. orientalis* NBRC 12806^T were observed in acid production from dextrin, (+)-D-glucose, (+)-D-maltose, (+)-L-rhamnose and sucrose and in decomposition of casein, gelatin, xanthine and hypoxanthine and hydrolysis of Tween 80. Differential properties of strain DSM 43387, *A. orientalis* NBRC 12806^T and *A. albidoflavus* IMSNU 22139^T are shown in Table 2. The differences in chemical and physiological data between strain DSM 43387 and the reference strains support the results of the 16S rRNA gene analysis, DNA–DNA hybridization and polar lipid and fatty acid analyses.

The differences at genetic, phenotypic and chemotaxonomic levels indicate that strain DSM 43387 is different from *A. orientalis* NBRC 12806^T (=IMSNU 20058^T = ATCC 19795^T) and *A. albidoflavus* DSM 44639^T (=IMSNU 22139^T). Moreover, the tree topographies strongly suggest that strain DSM 43387 represents a novel species of *Amycolatopsis*. These observations clearly demonstrate that DSM 43387 should be reclassified as representing a novel species of the genus *Amycolatopsis*, for which the name *Amycolatopsis benzoatilytica* sp. nov. is proposed.

Description of *Amycolatopsis benzoatilytica* sp. nov.

Amycolatopsis benzoatilytica (ben.zo.a.ti.ly'ti.ca. N.L. n. *benzoas* -atis benzoate; Gr. adj. *lutikos* able to loose, able to dissolve; N.L. adj. *lyticus* -a -um dissolving; N.L. fem. adj. *benzoatilytica* benzoate-degrading).

Table 2. Differential phenotypic characteristics of *A. benzoatilytica* sp. nov. DSM 43387^T

Strains: 1, *A. benzoatilytica* DSM 43387^T; 2, *A. orientalis* NBRC 12806^T; 3, *A. albidoflavus* IMSNU 22139^T (data from Lee & Hah, 2001). +, Positive reaction; –, negative reaction; ND, not determined. All strains are negative for production of soluble pigment. All strains produce acid from adonitol, (+)-D-arabinose, (+)-D-cellobiose, *meso*-erythritol, (+)-D-fructose, (+)-D-galactose, (+)-D-lactose, (+)-D-mannose, *myo*-inositol, (+)-D-trehalose and (+)-D-xylose, but do not assimilate (+)-D-raffinose or (–)-D-sorbitol. All strains decompose aesculin and are catalase-positive.

Characteristic	1	2	3
Acid production from:			
Dextrin	+	+	–
(+)-D-Glucose	+	+	–
(+)-D-Maltose	–	+	–
(+)-L-Rhamnose	+	+	–
Sucrose	–	+	+
Decomposition of:			
Allantoin	+	+	ND
Gelatin	–	+	+
Xanthine	–	+	+
Hypoxanthine	–	+	+
Casein	–	+	+
Hydrolysis of:			
Tween 80	–	+	ND
Starch	–	–	+

Cells are aerobic, non-motile, non acid-fast, Gram-positive, filamentous and differentiated into substrate and aerial mycelia. Vegetative mycelia have the tendency to fragment when the organism is grown in liquid culture. Cream-coloured aerial mycelium is produced on YM, nutrient agar, TSBA and Bennett's agar. Catalase- and urease-positive but amylase-negative. Soluble pigment is not produced. Acid is produced from adonitol, (+)-L-arabinose, (+)-D-cellobiose, dextrin, *meso*-erythritol, (+)-D-fructose, (+)-D-glucose, (+)-D-galactose, *myo*-inositol, (+)-D-lactose, (–)-D-mannitol, (+)-D-mannose, (+)-L-rhamnose, (–)-D-ribose, (+)-D-trehalose and (+)-D-xylose. Utilizes (+)-D-raffinose but not (–)-D-sorbitol or sucrose. Hydrolyses aesculin and decomposes allantoin. Tween 80, starch, casein, gelatin, xanthine and hypoxanthine are not hydrolysed or decomposed. Saturated, unsaturated and branched fatty acids are present. The predominant fatty acids are 12-methyltridecanoic acid (iso-C_{14:0}), pentadecanoic acid (C_{15:0}), pentadecenoic acid (C_{15:1}ω6c), 14-methylpentadecanoic acid (iso-C_{16:0}), heptadecanoic acid (C_{17:0}) and heptadecenoic acid (C_{17:1}ω8c). The phospholipid profile is type PII and contains diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, phosphatidylethanolamine and phosphatidylmethylethanolamine. Growth occurs at pH 5.0–10.0. Grows in the presence of 5% NaCl. The temperature range for growth is 10–37 °C. Degrades *m*-hydroxybenzoate. Contains the 29.6 kb conjugative plasmid pA387.

The type strain, AK 16/65^T (=DSM 43387^T=ATCC 55165^T=IMRU 1389^T), was isolated in Czechoslovakia from a patient with submandibular mycetoma by Scharfen (1971) (Institute of Epidemiology and Microbiology, Prague).

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