

Amycolatopsis saalfeldensis sp. nov., a novel actinomycete isolated from a medieval alum slate mine

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Three actinomycetes isolated from the surfaces of rocks in a medieval slate mine were examined in a polyphasic taxonomic study. Chemotaxonomic and morphological characteristics of the isolates were typical of strains of the genus *Amycolatopsis*. The isolates had identical 16S rRNA gene sequences and formed a distinct phyletic line towards the periphery of the *Amycolatopsis mediterranei* clade, being most closely related to *Amycolatopsis rifamycinica*. The organisms shared a wide range of genotypic and phenotypic markers that distinguished them from their closest phylogenetic neighbours. On the basis of these results, a novel species, *Amycolatopsis saalfeldensis* sp. nov., is proposed. The type strain is HKI 0457^T (= DSM 44993^T = NRRL B-24474^T).

The genus *Amycolatopsis* is classified in the family *Pseudonocardiaceae*, suborder *Pseudonocardineae* (Stackebrandt *et al.*, 1997). The genus currently contains 34 recognized species, most of which have been described in the past five years using polyphasic taxonomic approaches (Goodfellow *et al.*, 2001; Labeda *et al.*, 2003; Groth *et al.*, 2007). Members of most of these species have been isolated from geographically diverse soils (Kim *et al.*, 2002; Saintpierre-Bonaccio *et al.*, 2005; Lee *et al.*, 2006; Tan *et al.*, 2006a), and others from clinical material (Labeda *et al.*, 2003; Huang *et al.*, 2004), vegetable matter (Goodfellow *et al.*, 2001) and from the wall of a hypogean Roman catacomb (Groth *et al.*, 2007).

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains HKI 0457^T, HKI 0473 and HKI 0474 are DQ792500, DQ792501 and DQ792502, respectively.

A dendrogram showing relationships between the isolates and between them and the type strains of closely related *Amycolatopsis* species based on MALDI-TOF MS data is available with the online version of this paper.

Amycolatopsis strains can be distinguished from members of other genera classified in the family *Pseudonocardiaceae* by using a combination of chemotaxonomic and morphological markers (Kim & Goodfellow, 1999) and genus-specific oligonucleotide primers based on 16S rRNA gene sequences (Tan *et al.*, 2006b). A range of phenotypic markers can be weighted to distinguish between species with validly published names (Saintpierre-Bonaccio *et al.*, 2005; Lee *et al.*, 2006; Groth *et al.*, 2007). The present polyphasic study was designed to determine the taxonomic position of three strains which had been isolated from a medieval alum slate mine. The strains were assigned to the genus *Amycolatopsis* on the basis of their morphological properties and ability to produce the diagnostic amplification products when probed with the genus-specific 16S rRNA oligonucleotide primers AMY2 (5'-GGTGTGGGCGACATCCACGTTGT-3') and ATOP (5'-GTATCGCAGCCCTCTGTACCAGC-3') as described by Tan *et al.* (2006b). The resultant data showed that the isolates represent a novel *Amycolatopsis* species for which the name *Amycolatopsis saalfeldensis* sp. nov. is proposed.

The three strains were isolated from the surfaces of acidic and heavy-metal-containing rocks of two galleries in the medieval alum slate mine Feengrotten in Saalfeld, Thuringia,

Germany. Strain HKI 0457^T was isolated from rock in the central grotto (second level of the mine) by touching it with a sterile cotton swab and dispersing adhering bacteria in 1 ml sterile distilled water. Aliquots of the resultant suspension were spread over casein mineral agar plates (Altenburger *et al.*, 1996), supplemented with cycloheximide (50 µg ml⁻¹), and incubated at 28 °C for 4 weeks. Strains HKI 0473 and HKI 0474 were isolated from rock surfaces towards the end of the Hess von Wichdorff Grotto (first level of the mine) by using the same procedure, but plating out onto humic acid agar (Hayakawa & Nonomura, 1987).

Working cultures of the isolates were maintained on organic medium 79 agar (Prauser & Falta, 1968). The cultures were preserved as mixtures of hyphae and fragmented spores in organic medium 79 broth and in glycerol medium (Groth *et al.*, 2007) at -80 °C. Stock cultures were also kept in liquid organic medium 79 supplemented with 5 % DMSO in the vapour phase of liquid nitrogen. Biomass for the chemotaxonomic and molecular systematic studies was prepared by growing the isolates and *Amycolatopsis kentuckyensis* DSM 44652^T, *Amycolatopsis lexingtonensis* DSM 44653^T, *A. mediterranei* DSM 43304^T, *Amycolatopsis pretoriensis* DSM 44654^T, *A. rifamycinica* DSM 46095^T and *Amycolatopsis tolypomycina* DSM 44544^T in liquid organic medium 79 and bacto-tryptic soy broth (Sigma-Aldrich) for 24 to 48 h at 28 °C. For MALDI-TOF MS analysis the strains were cultivated as described by Groth *et al.* (2007).

Chromosomal DNA was extracted from the three isolates using slight modifications of the method of Pospiech & Neumann (1995). PCR amplification of 16S rRNA genes was achieved using the conserved primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1522R (5'-AAGGAGGTGATCCAGCCGCA-3') (Edwards *et al.*, 1989) and the following conditions: initial denaturation at 95 °C for 5 min, 35 cycles of 94 °C for 15 s, 55 °C for 15 s, 72 °C for 1 min; and a final extension for 10 min at 72 °C. After electrophoretic separation the 16S rRNA genes were extracted from the agarose gel using the MinElute Gel Extraction Kit (Qiagen), according to the manufacturer's instructions. Forward and reverse strands of amplified DNA fragments were directly sequenced using a Big Dye Terminator v. 3.1 Cycle Sequencing Kit and an ABI Prism 3100 sequencer (both Applied Biosystems).

The resultant 16S rRNA gene sequences were aligned manually using the PHYDIT program (<http://plaza.snu.ac.kr/~jchun/phydit/>), against corresponding sequences of representatives of the family *Pseudonocardiaceae* retrieved from the GenBank/EMBL/DDBJ databases. Unrooted phylogenetic trees were inferred using the least-squares (Fitch & Margoliash, 1967), maximum-parsimony (Fitch, 1971) and neighbour-joining (Saitou & Nei, 1987) tree-making algorithms. Evolutionary distance matrices were generated for the least-squares and neighbour-joining algorithms, using the method of Jukes & Cantor (1969). All of the phylogenetic analyses were carried out using the

PHYLP suite of programs (Felsenstein, 1993). The robustness of the resultant trees was evaluated by bootstrap analysis (Felsenstein, 1985) of neighbour-joining data based on 1000 resamplings using the TREECON program (Van de Peer & De Wachter, 1994). The root position of the tree was estimated using *Prauserella rugosa* DSM 43194^T (accession no. AF051342) as the outgroup organism.

Almost complete 16S rRNA gene sequences were generated for the three novel isolates (>1447 nt), all of which had identical sequences. Comparison of the 16S rRNA gene sequences with corresponding sequences of representatives of the family *Pseudonocardiaceae* showed that the isolates belong to the genus *Amycolatopsis* (data not shown). The high 16S rRNA gene sequence similarities found between the isolates and representatives of the genus *Amycolatopsis* (94.3–98.4 %) support the addition of these strains to the genus.

It is apparent from Fig. 1 that the isolates are most closely associated with the *A. mediterranei* 16S rRNA subclade, though this relationship is not supported by a high bootstrap value in the neighbour-joining analysis. Strain HKI 0457^T was most closely related to *A. rifamycinica* DSM 46095^T. The two organisms shared a 16S rRNA gene sequence similarity of 98.4 %, a value that corresponded to 23 differences at 1432 locations. The isolates also shared relatively high 16S rRNA gene sequence similarities with the type strains of *A. kentuckyensis* (98.1 %), *A. lexingtonensis* (98.1 %), *A. mediterranei* (98.2 %), *A. pretoriensis* (98.2 %) and *A. tolypomycina* (98.1 %). DNA–DNA relatedness studies were not carried out between isolate HKI 0457^T and its closest phylogenetic neighbours, as it is known that the type strains of *Amycolatopsis* species classified in the *A. mediterranei* 16S rRNA subclade share much higher 16S rRNA gene similarities than those cited above but have lower DNA–DNA relatedness values (Labeda *et al.*, 2003; Wink *et al.*, 2003; Bala *et al.*, 2004), that is, values well below the 70 % cut-off point recommended for the delineation of genomic species (Wayne *et al.*, 1987).

The isolates were examined for a range of key chemical markers to establish whether they had a chemotaxonomic profile typical of members of the genus *Amycolatopsis*. To this end, standard HPLC and TLC procedures were used to determine the isomers of diamino pimelic acid (A₂pm) in whole-organism hydrolysates (Hasegawa *et al.*, 1983), menaquinone profiles (Collins *et al.*, 1977; Groth *et al.*, 1996), muramic acid type (Uchida & Aida, 1984), the presence of mycolic acids (Minnikin *et al.*, 1975), predominant whole-organism sugars (Becker *et al.*, 1965; Schön & Groth, 2006) and polar lipid patterns (Minnikin *et al.*, 1979; Collins & Jones, 1980), using appropriate standards. All three isolates contained *meso*-A₂pm, arabinose and galactose in whole-organism hydrolysates (wall chemotype IV *sensu*; Lechevalier & Lechevalier, 1970) together with glucose, mannose and rhamnose, *N*-acetylated muramic acid, diphosphatidylglycerol, phosphatidylethanolamine, hydroxyphosphatidylethanolamine, phosphatidylglycerol,

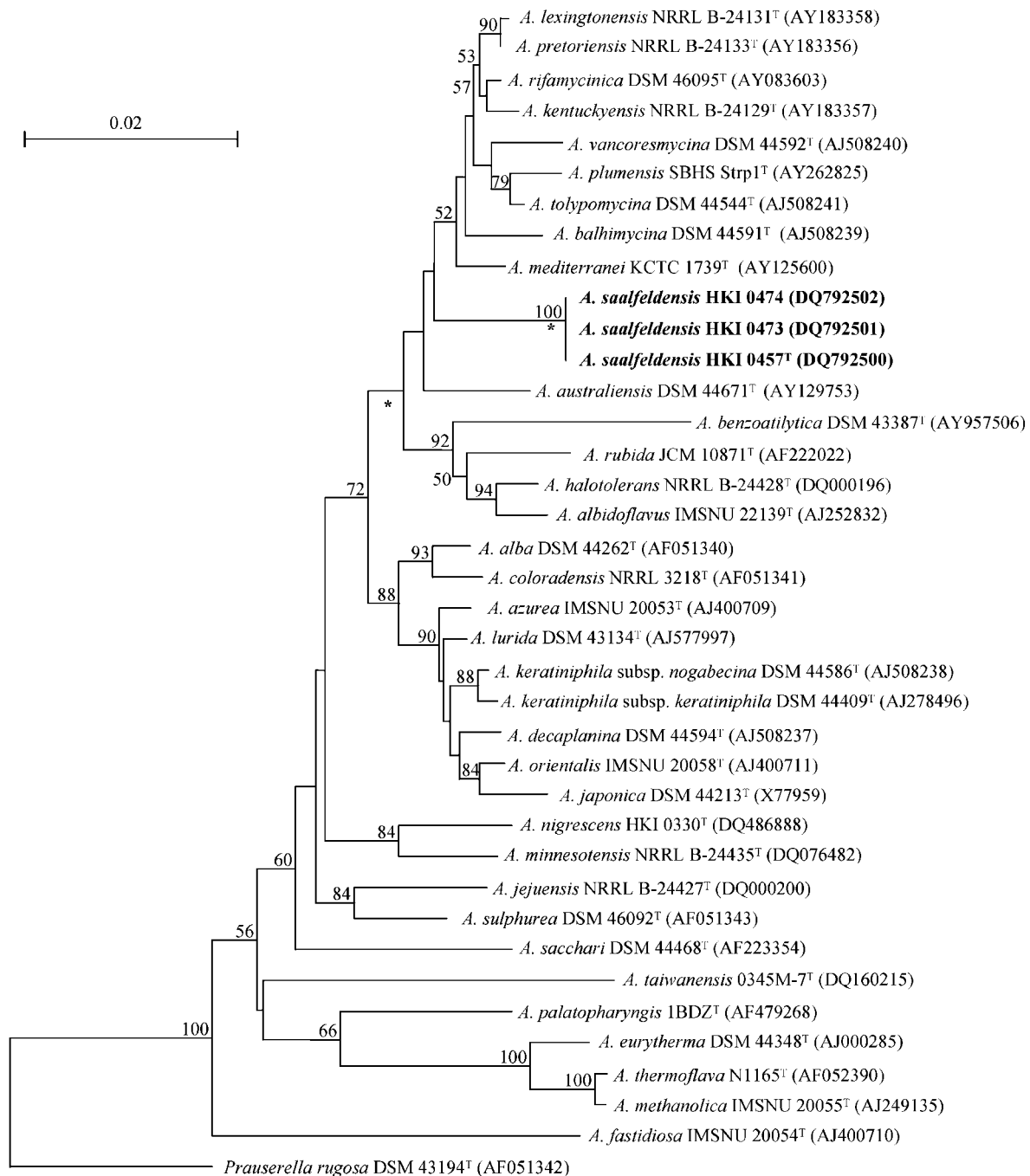


Fig. 1. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences showing relationships between isolates HKI 0457^T, HKI 0473 and HKI 0474 and between them and representatives of the genus *Amycolatopsis*. Asterisks indicate branches of the tree that were also found using the least-squares and maximum-parsimony tree-making algorithms. The numbers at the nodes indicate the level of bootstrap support (%) based on a neighbour-joining analysis of 1000 resampled datasets; only values above 50% are shown. Bar, 2 substitutions per 100 nt.

phosphatidylinositol, phosphatidylserine and two uncharacterized glycolipids (phospholipid pattern 2 *sensu* Lechevalier *et al.*, 1977), and tetrahydrogenated menaquinones with nine isoprene units [MK-9(H₄)] as the major

isoprenologue (86–88% of total) with minor proportions of MK-8(H₄) (4–5%), MK-9(H₆) (1–3%) and MK-10(H₄) (2%), but they lacked mycolic acids. These data serve to distinguish the isolates from all other wall chemotype IV

actinomycetes, apart from those classified in the genus *Amycolatopsis* (Lechevalier *et al.*, 1986; Kim & Goodfellow, 1999; Takahashi, 2001).

The fatty acid profiles of the isolates grown in bacto-tryptic soy broth for 48 h were determined using the MIDI system (www.midi-inc.com/). All of the strains had a very similar fatty acid composition in which 14-methylpentadecanoic acid (iso-C_{16:0}) was the major component (41–42 % of total); the fatty acids found in smaller proportions were iso-C_{17:0} (8–10 %), iso-C_{15:0} (8–9 %), iso-C_{14:0} (7–9 %), iso-2OH C_{16:0} (4–6 %), C_{15:0} (6 %) and C_{17:0} (5–7 %). These profiles are similar to those recorded for members of established *Amycolatopsis* species (Yassin *et al.*, 1993; Wink *et al.*, 2004; Groth *et al.*, 2007).

Morphological properties of the isolates were examined following growth on ISP media 2 and 3 agar plates (Difco; Shirling & Gottlieb, 1966) at 28 °C for up to 21 days. The pH growth range was established using shake flasks of liquid organic medium 79, adjusted to pH values between 4.5 and 10.0 with either 1 M HCl or 20 %, (w/v) Na₂CO₃ solution, and incubated for 6 days at 28 °C. The isolates were also tested for their ability to grow on solidified minimal medium (Amoroso *et al.*, 2000) supplemented with CuSO₄ (2 mM) and NiCl₂ (5 mM), respectively, following growth at 28 °C for 21 days. The remaining physiological tests, including the determination of antibiotic sensitivity and enzymic

activities, were carried out as described by Groth *et al.* (2003). The isolates formed an extensively branched substrate mycelium on the ISP media tested and moderate amounts of aerial hyphae only on ISP medium 3. Substrate and aerial hyphae fragmented into rod-like elements typical of *Amycolatopsis* strains. The strains also grew in the presence of copper and nickel salts, and shared a broad range of phenotypic properties. Some of the latter can be used to distinguish the isolates from the type strains of phylogenetically close *Amycolatopsis* species classified in the *Amycolatopsis* 16S rRNA gene clade (Table 1).

UP-PCR reactions were performed on the three isolates and their phylogenetically closest neighbours using the primers AS4/AS15 of Bulat *et al.* (2000). The reactions were prepared in a final volume of 10 µl containing 0.5 U GoTaq (Promega), the GoTaq flexi buffer, 0.2 mM of each of the four dNTPs, 3 mM MgCl₂, 1 µM primer AS4, 2 µM primer AS15 and about 20 ng extracted DNA. For initial calculation of *T_m* and primer concentration, the biomath *T_m*-calculator provided by Promega was used (www.promega.com/biomath/calc11.htm). The different primer concentrations were empirically optimized to give the best possible size range and distribution of the resulting bands. Amplifications were performed three times using Thermo-Fast low profile PCR plates (ABgene) in an MJ research PTC 225 thermal cycler according to the following profile: an initial step at 94 °C for 2 min, 57 °C for 40 s and 72 °C for 30 s to

Table 1. Physiological properties that separate the novel isolates from the type strains of phylogenetically close *Amycolatopsis* species

Strains: 1, *A. saalfeldensis* sp. nov. HKI 0457^T, HKI 0473 and HKI 0474; 2, *A. mediterranei* DSM 43304^T; 3, *A. pretoriensis* DSM 44654^T; 4, *A. rifamycinica* DSM 46095^T. Data for the hypoxanthine and xanthine tests for the reference strains were taken from Wink *et al.* (2003), Lechevalier *et al.* (1986) and Labeda *et al.* (2003). +, Positive; –, negative; w, weakly positive; *, delayed reaction.

Characteristic	1	2	3	4
Colour of aerial mycelium	White	Absent	White to orange	White to orange
Production of soluble pigment	–	–	+(faint)	Light pale brown-yellow
Decomposition of:				
Hypoxanthine	–	+	+	+
Xanthine	+	–	–	–
Production of indole	–	+	–	–
Growth on sole carbon sources:				
Malate (0.2 %, w/v)	+	+	–	+
D-Raffinose (1 %, w/v)	–	+	+	+
API ZYM tests:				
Cystine arylamidase	–	+	+	+
α -Fucosidase	–	+	–	–
β -Galactosidase	–	+	+	+
β -Glucosidase	w	+	–	+
Valine arylamidase	+	+	–	+
Growth at:				
10 °C	w*	+	–	+
42 °C	–	+	+	+

provide templates, followed by 30 cycles at 92 °C for 20 s, 57 °C for 40 s, 72 °C for 30 s and a final extension at 72 °C for 2 min. Amplification products were separated by gel electrophoresis in native polyacrylamide gels (8%) containing 0.5% TBE buffer. Gels were stained with SYBR Safe (Invitrogen). Fingerprints were analysed using GelComparII software (version 4.5, Applied Maths). The banding patterns were automatically matched (optimization value 1%, position tolerance value 0.4%). This computed matching was refined manually afterwards. A phylogenetic clustering of the banding patterns was achieved using the built-in maximum-parsimony tree-making algorithm (Fitch, 1971). The topology of the resulting tree (Fig. 2) was evaluated by bootstrap analysis (Felsenstein, 1985) based on 1000 resamplings. It is clear from Fig. 2 that the isolates gave almost identical UP-PCR fingerprint patterns which serve to distinguish them from corresponding patterns of their closest phylogenetic neighbours.

For MALDI-TOF mass spectrometry, biomass of the isolates taken from cellulose acetate filters was examined as described previously (Kroppenstedt *et al.*, 2005; Groth *et al.*, 2007) using a Voyager mass spectrometer. The resultant spectra were added to an existing *Amycolatopsis* dataset, and the data were analysed using Data Explorer software (Applied Biosystems) and the peak lists compared using SARAMIS software (Anagnos Tec). It is evident from our results (supplementary Fig. S1 available with the online version of this paper) that the isolates have similar profiles that distinguish them from those of *Amycolatopsis* type strains, including those representing phylogenetically close taxa.

It is evident from the genotypic and phenotypic data that isolates HKI 0457^T, HKI 0473 and HKI 0474 form a homogeneous taxon that can be distinguished readily from representatives of phylogenetically close *Amycolatopsis* species classified in the *A. mediterranei* 16S rRNA gene clade. It is, therefore, proposed that the isolates be classified in the genus *Amycolatopsis* as *Amycolatopsis saalfeldensis* sp. nov.

Description of *Amycolatopsis saalfeldensis* sp. nov.

Amycolatopsis saalfeldensis (saal.feld.en'is. N.L. fem. adj. *saalfeldensis* from Saalfeld, named after the place of origin, a town in Thuringia, Germany).

Aerobic, Gram-positive, non-acid/alcohol-fast, non-motile, catalase-positive actinomycete which forms an extensively branched vegetative mycelium (hyphal diameter 0.5–0.6 µm) that fragments into squarish rod-like elements. The substrate mycelium carries moderate amounts of white aerial hyphae which fragment into squarish rod-like elements. Diffusible pigments are not produced. Good growth occurs between 20 and 35 °C, but growth is not evident below 10 or at 42 °C. Grows well between pH 4.5 and 8.0 and in the presence of 2% (w/v) NaCl, but does not grow at pH 9.0 or in the presence of 4% (w/v) NaCl. Grows on minimal medium supplemented with NiCl₂ (5 mM) and CuSO₄ (2 mM), respectively. Oxidase is produced, aesculin and urea hydrolysed, and H₂S produced. Nitrate is not reduced to nitrite. Degrades casein, gelatin, hippurate, Tween 80 and tyrosine, but not adenine or potato starch. L-Arabinose, D-fructose, D-glucose, *meso*-inositol, D-mannitol, L-rhamnose (type strain weakly), sucrose and D-xylose are used as sole carbon sources for energy and growth, but not cellulose (all at 1%, w/v). Similarly, acetate, aconitate, benzoate (weakly), citrate and succinate are used as sole carbon and energy sources for growth, but not DL-tartrate (all at 0.2%, w/v). Produces α-chymotrypsin (weakly), leucine arylamidase, esterase (C4), esterase lipase (C8), N-acetyl-β-glucosamidase, α-glucosidase, naphthol-AS-BI-phosphohydrolase, acid phosphatase and alkaline phosphatase, but not α-galactosidase, β-glucuronidase, lipase (C14), α-mannosidase or trypsin (API ZYM tests). Susceptible to chloramphenicol (30 µg per disc), ciprofloxacin (5 µg per disc, weakly), imipenem (10 µg per disc), kanamycin sulphate (30 µg per disc), lincomycin hydrochloride (2 µg per disc, weakly), ofloxacin (10 µg per disc, weakly), oxytetracycline hydrochloride (30 µg per disc), rifampicin (30 µg per disc), streptomycin sulfate (10 µg per disc) and

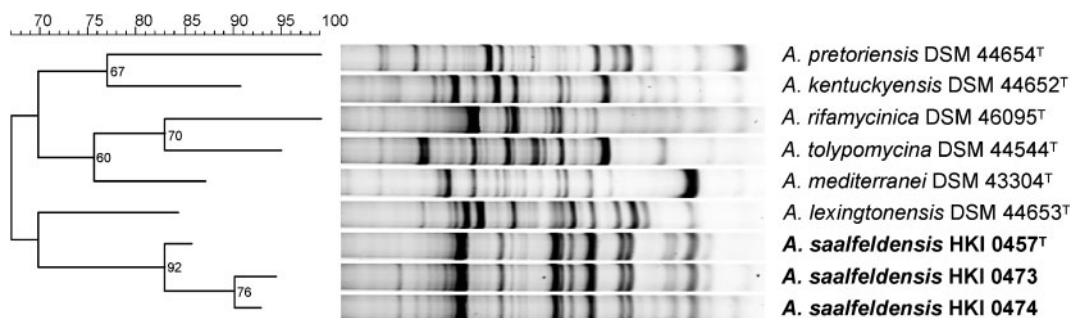


Fig. 2. Maximum-parsimony tree based on UP-PCR generated fingerprint data showing relationships between the isolates and between them and type strains of closely related *Amycolatopsis* species. Bootstrap rates (%) at the nodes are based on 1000 resamplings; only values above 50% are shown.

vancomycin hydrochloride (30 µg per disc), but is resistant to ampicillin (10 µg per disc), methicillin (5 µg per disc), norfloxacin (10 µg per disc), novobiocin (5 µg per disc), penicillin G (10 IU per disc) and polymyxin B (300 IU per disc). Additional phenotypic properties are shown in Table 1. Chemotaxonomic characters are typical for *Amycolatopsis* species.

The type strain, HKI 0457^T (=DSM 44493^T=NRRL B-24474^T), was isolated from the surface of rocks in a medieval alum slate mine.

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