Reclassification of *Amycolatopsis mediterranei* DSM 46095 as *Amycolatopsis rifamycinica* sp. nov.

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Previous experiments have suggested that the rifamycin-producing strain DSM 46095 might not belong to *Amycolatopsis mediterranei*. Analysis of its 16S rRNA gene sequence and construction of a phylogenetic tree showed most similarity to *Amycolatopsis kentuckyensis* NRRL B-24129^T, *Amycolatopsis lexingtonensis* NRRL B-24129^T and *Amycolatopsis pretoriensis* NRRL B-24133^T, but the strain was probably not a member of any of these species. Results from DNA–DNA hybridization experiments and comparison of DNA profiling patterns using pulsed-field gel electrophoresis also supported the assignment of strain DSM 46095 to a novel species. Analyses of phospholipids, fatty acid methyl esters and physiological characteristics also showed that the differences between different isolates of *A. mediterranei* and *A. mediterranei* DSM 46095 were as large as those between *Amycolatopsis* species. Strain DSM 46095 represents a novel species of the genus *Amycolatopsis* for which the name *Amycolatopsis rifamycinica* sp. nov. is proposed, with the type strain NT 19^T (=DSM 46095^T=ATCC 27643^T).

Amycolatopsis mediterranei has been the focus of much research because it produces an important antibiotic, rifamycin, whose derivative rifampicin is used extensively against *Mycobacterium tuberculosis* and *Mycobacterium leprae*, the causative agents of tuberculosis and leprosy, respectively. *A. mediterranei* was originally classified as '*Streptomyces mediterranei*', later as *Nocardia mediterranei* and, finally, was transferred to the novel genus *Amycolatopsis* by Lechevalier *et al.* (1986). *Amycolatopsis* belongs to the actinomycete family *Pseudonocardiaceae* and was first established by Lechevalier *et al.* (1986) to accommodate four species. The DNA sequences of the rifamycin-biosynthetic gene clusters of strains *A. mediterranei* S669 (August *et al.*, 1998) and *A. mediterranei* LBGA3136 (Schupp *et al.*, 1998) have been determined and are essentially identical. The rifamycin-biosynthetic genes of strain *A. mediterranei* DSM 46095 were cloned, but the restriction pattern was very different from those of the sequenced clusters of strains S669 and LBGA3136 (Kaur *et al.*, 2001). Limited DNA sequencing showed about 10 % sequence divergence between the clusters. This degree of sequence divergence suggested that DSM 46095 might represent a different species, but it was also possible that one of the clusters had been introduced into the strain by horizontal transfer. Because of the commercial importance of rifamycin, details of strain derivation are not always clear. It was therefore decided to examine five further strains (Table 1) and to compare them with *A. mediterranei* DSM 43304^T and DSM 46095.

Published online ahead of print on 9 January 2004 as DOI 10.1099/ijs.0.02901-0.

The GenBank accession numbers for the 16S rRNA gene sequences of strains DSM 46095 and F1/24 are respectively AY083603 and AY083604.

16S rDNA sequence analysis

Genomic DNA was isolated from *A. mediterranei* DSM 46095 and from *A. mediterranei* F1/24, the latter an industrially improved derivative of *A. mediterranei* ATCC 13685^T, using the method described by Kaur *et al.* (2001). PCR amplification of the 16S rRNA genes and sequencing were carried out as described by Reddy *et al.* (2000). The sequences for DSM 46095 and F1/24 show nine mismatches and nine nucleotides in insertions/deletions in 1461/1465 nucleotides, respectively, which supports the suggestion that the strains belong to different species. Similarity searches using BLAST showed closest similarity of the two sequences to *A. mediterranei* NRRL B-3240^T and six further accepted *Amycolatopsis* species whose 16S rDNA sequences were included in the analysis. The sequences were aligned using

Table 1. Strains used for the present study

Strain	Characteristic	Reference/source
A. mediterranei:		
DSM 43304^T (=ATCC 13685^T=KCTC 1739^T)	Rifamycin SV	Margalith & Beretta (1960)
T-195*	Protorifamycin	Ghisalba *et al.* (1984)
F1/24*	Protorifamycin	Ghisalba *et al.* (1984)
DSM 40773	Rifamycin	Lechevalier *et al.* (1986)
MTCC17 (=ATCC 21271)	Rifamycin SV	Lancini & Hengeller (1971)
DSM 46096 (=ATCC 21411)	Rifamycin derivative	Hengeller *et al.* (1973)
DSM 46095 (=ATCC 27643)	Rifamycin SV	Birner *et al.* (1972)
A. kentuckyensis NRRL B-24129^T	Placentitis in horses	D. P. Labeda, NRRL
A. lexingtonensis NRRL B-24131^T	Placentitis in horses	D. P. Labeda, NRRL
A. pretoriensis NRRL B-24133^T	Placentitis in horses	D. P. Labeda, NRRL

*CIBA GEIGY, presently known as Novartis, Switzerland.

the CLUSTAL W program (Thompson *et al.*, 1994) at the European Bioinformatics Institute web site (http://www.ebi.ac.uk). The alignment was edited to remove terminal nucleotides not present in all nine sequences. All phylogenetic analyses were carried out using the PHYLIP package, version 3.5c (Felsenstein, 1993). The evolutionary distance matrix was calculated using the Kimura two-parameter method (Kimura, 1980) and an evolutionary tree (Fig. 1) was constructed using the neighbour-joining method (Saitou & Nei, 1987). The tree topology was evaluated by carrying out bootstrap analysis based on 1000 resamplings using the SEQBOOT and CONSENSE programs (Fig. 1). Parsimony analysis was performed for the aligned sequence data using DNAPARS, including a bootstrap analysis with 100 resamplings (data not shown). This produced a tree topology very similar to that constructed using the neighbour-joining method. Fig. 1 shows that DSM 46095 grouped with three recently described species of *Amycolatopsis*, *Amycolatopsis kentuckyensis* NRRL B-24129^T, *Amycolatopsis lexingtonensis* NRRL B-24129^T and *Amycolatopsis pretoriensis* NRRL B-24133^T (Labeda *et al.*, 2003). However, DSM 46095 seems to be distinct from these three species, with six mismatches and four insertions/deletions in comparison with the closest species, *A. kentuckyensis*. The 16S rRNA gene sequence of strain F1/24 is very similar to that of the type strain, DSM 43304^T (two mismatches), and, as F1/24 is derived from this strain, it is likely that the differences reflect sequencing errors.

DNA–DNA hybridization

DNA was extracted from the three strains of *A. mediterranei* (DSM 43304^T, F1/24 and DSM 46095), *A. kentuckyensis*, *A. lexingtonensis* and *A. pretoriensis* following the procedure described by Kaur *et al.* (2001). DNA–DNA hybridization was carried out using the membrane filter method (Tourova & Antonov, 1987). The quality of the DNA preparation was checked by spectrophotometry and agarose gel electrophoresis (Sambrook & Russell, 2000). Ten micrograms of denatured DNA was immobilized on each nitrocellulose membrane filter and DNA was labelled with [α-^{32}P]dATP (BRIT, India) using a nick translation kit (Amersham Pharmacia). Hybridization was performed overnight at 65 °C. After hybridization, the filters were washed with SSC and SDS to remove the unbound probe. The amount of bound probe DNA was estimated using a scintillation counter (Beckman Instruments) and levels of hybridization were expressed as percentages of the probe bound relative to the homologous reaction. In initial experiments performed with triplicate filters, DNA from strains DSM 43304^T, F1/24 and 46095 was bound to the filter and hybridized with DNA probe from DSM 43304^T. This gave a level of hybridization of 100 % for DSM 43304^T and F1/24, as expected. By contrast, DSM 46095 gave a level of hybridization of only

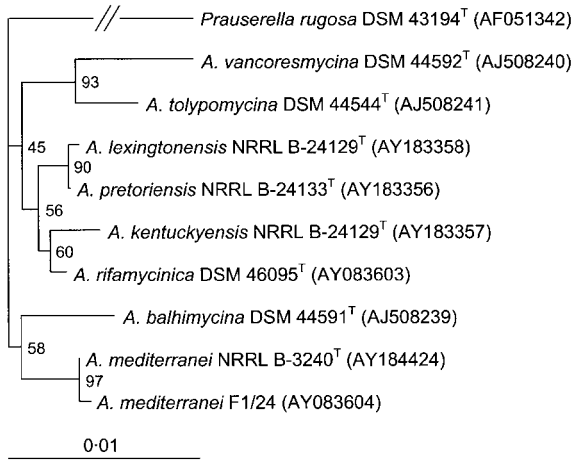

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences showing *Amycolatopsis* species related to *Amycolatopsis rifamycinica* sp. nov. The tree was constructed by the neighbour-joining method and rooted by using *Prauserella rugosa* as an outgroup. Bootstrap values, expressed as percentages based on 1000 replicates, are given at branch points. Bar, 0·01 nucleotide substitutions per site.

40 %, supporting the suggestion that this strain represents a different species, because two members of the same species should usually show at least 70 % hybridization. These results were confirmed from similar experiments in which DSM 46095 DNA was used as probe. Experiments in which DNA from DSM 46095 was bound to filters and hybridized with DNA from *A. kentuckyensis* NRRL B-24129^T, *A. lexingtonensis* NRRL B-24131^T and *A. pretoriensis* NRRL B-24133^T gave hybridization values of 67, 47 and 48 %, respectively. The reciprocal hybridization experiment in which DSM 46095 DNA was used as a probe gave similar results.

Pulsed-field gel electrophoresis of strains classified as *A. mediterranei*

The seven strains that had been classified as *A. mediterranei* were also examined using pulsed-field gel electrophoresis. Strains F1/24 and T-195 were derived from the type strain (DSM 43304^T) in an industrial strain-improvement programme involving successive rounds of mutagenesis (Ghisalba *et al.*, 1984). Strains MTTC 17 and DSM 46096 (Table 1) were also derived by mutagenesis from the type strain. DNA was prepared in agarose blocks using the method of Beyazova *et al.* (1995). Restriction digests were performed as in Pandza *et al.* (1997). Electrophoresis was performed in 0·5 × TBE buffer at 14 °C using a CHEF DR III system (Bio-Rad). Lambda DNA concatemers (New England Biolabs) and *Ase*I digests of total DNA of *Streptomyces coelicolor* M145 (Kieser *et al.*, 1992) were used as molecular size standards. Four different restriction enzymes (*Ase*I, *Dra*I, *Bfr*I and *Xba*I), which cut infrequently in DNA of high G+C content, were tested. *Ase*I gave the most suitable restriction profiles, with 10–20 fragments (Fig. 2). It can be seen that the patterns for five of the strains (lanes 2–6) are similar, whereas the patterns for strains DSM 46096 and DSM 46095 (lanes 7 and 8) resemble each other, but differ from those of the other five strains. The *Ase*I DNA fragments revealed no significant differences in genome size between the seven strains (between 7·9 and 8·4 Mb; data not shown). These data thus indicate that five of the strains are *A. mediterranei*, whereas DSM 46096 appears to represent a second member of the novel species represented by strain DSM 46095. This result is unexpected, because DSM 46096 is considered to be a mutant of the type strain *A. mediterranei* ATCC 13685^T (Hengeller *et al.*, 1973). It has clearly different morphological and physiological properties from DSM 46095 (see below).

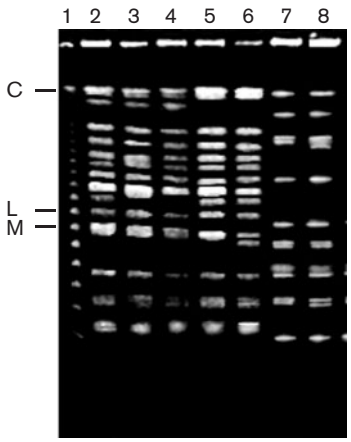

Fig. 2. Restriction profiles of *Ase*I-digested DNA of *A. mediterranei* strains (pulse time: 5–90 s, run time: 36 h, voltage: 200 V). Lanes: 1, lambda ladder PFGE marker; 2, DSM 43304^T; 3, T-195; 4, F1/24; 5, DSM 40773; 6, MTCC17; 7, DSM 46096; and 8, DSM 46095.

Phenotypic properties

The colour, shape, size and contour of colonies of the seven strains producing rifamycin or related compounds were observed on YM medium at 28 °C for 7 days. The culture conditions were as described by Lal *et al.* (1998). All strains showed the expected branched mycelial growth, but there was considerable variation between strains in colony morphology and pigmentation. DSM 46095 sporulated poorly and produced two types of colonies (orange with a pitted mucoid-like surface and pale orange with a smooth mucoid-like surface); poor sporulation and the production of two colony types (on glucose/asparagine medium) were reported previously by Birner *et al.* (1972). DSM 46096 produced yellowish colonies and showed poor to moderate sporulation.

Polar lipids were extracted from DSM 46095 as in Kates (1972) and identified by TLC. The predominant phospholipids were cardiolipin, phosphatidylethanolamine, phosphatidylglycerol and a small amount of phosphatidylinositol. Some other unidentified spots were also seen on the TLC plate. Fatty acid methyl esters were extracted from wet cells of DSM 46095 as described by Sato & Murata (1988) and were separated on a gas chromatograph (HP5890 series) using a DB-23 capillary column (30 m × 0·25 mm; J and W Scientific). The fatty acid methyl ester profile was: 25 % 18 : 1, 24 % 16 : 0 iso, 11 % 17 : 0 anteiso, 9 % 17 : 0 iso, 8 % 16 : 0, 4 % 17 : 1, 4 % 18 : 4, 3 % 15 : 0 iso and other minor components.

The seven strains were subjected to various physiological tests. Growth at different temperatures was analysed and catalase tests were carried out as described by McCarthy & Cross (1984) using YM medium. Hydrolysis of Tweens 20 and 80 and the ability of the strains 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 examined as described by Gordon *et al.* (1974). Urease activity was detected as in Christensen (1946). The other physiological tests and methods were as described by Collins *et al.* (1989). All seven strains were urease-positive and none produced amylase. In addition, all the strains could utilize dextrin, D-fructose, D-glucose, sucrose, trehalose, Tweens 20 and 80,

hypoxanthine and aesculin and could not utilize xanthine or allantoin. They grew in the temperature range 10–37 °C but not at 45 °C. Results were compared with published results for the other strains in the monophyletic clade shown in Fig. 1 (Table 2). The differential properties may not be species specific because five of the strains (DSM 43304^T, F1/24, T-195, MTCC 17 and DSM 40733) and the other two strains (DSM 46095^T and DSM 46096) of *A. mediterranei* showed differences within the species as large as those between species. Although the loss of many properties by F1/24 and T-195 compared to the parent DSM 43304^T (Table 2) could be explained by mutagenesis during strain improvement, it seems less likely that the ability of DSM 46096 to utilize lactose, maltose and mannitol has arisen by mutagenesis from a non-utilizing ancestor similar to DSM 46096. However, evaluation of the gross morphological characteristics and differential physiological properties of strain DSM 46095 is generally consistent with the molecular systematic observations and clearly demonstrated that strain DSM 46095 represents a novel species of the genus *Amycolatopsis* for which the name *Amycolatopsis rifamycinica* sp. nov. is proposed, with the type strain NT 19^T.

Description of *Amycolatopsis rifamycinica* sp. nov.

Amycolatopsis rifamycinica (N.L. n. *rifamycinum -i* rifamycin; L. suff. *icus -a -um* related to; N.L. fem. adj. *rifamycinica* referring to the ability to produce rifamycin).

Orange-coloured vegetative mycelium is produced in yeast extract agar and glucose/asparagine agar and white to very pale pink aerial mycelium on oatmeal agar and yeast extract/molasses agar. A light pale to brown yellow pigment is produced on tyrosine agar, yeast extract/glucose agar and oatmeal agar. Aerobic and catalase-positive. Casein, aesculin, gelatin, hypoxanthine and urea are hydrolysed. Starch, allantoin and xanthine are not hydrolysed or decomposed. Acid is produced from adonitol, glucose, fructose, arabinose, dextrin, sucrose, trehalose, xylose and cellobiose. No acid is produced from lactose, maltose, mannitol, rhamnose and raffinose. Grows weakly in the presence of 5 % NaCl (w/v). Temperature range for growth is 10–37 °C.

The type strain, NT 19^T (=DSM 46095^T=ATCC 27643^T), was isolated from a soil sample in an arid region near Alice Springs, Northern Territory, Australia, by Birner *et al.* (1972) and produces rifamycin SV.

Acknowledgements

This work was funded by grants from the Department of Biotechnology. We would like to thank the Department of Science and Technology (Government of India) and the Deutsche Akademische Austauschdienst for a project-based exchange programme grant. We are also grateful to D. P. Labeda for providing three strains (*A. kentuckyensis*, *A. lexingtonensis* and *A. pretoriensis*). S. B. and M. D. gratefully acknowledge CSIR (Government of India) for

Table 2. Differences in the physiological characteristics of *Amycolatopsis* strains

Strains: 1–5, *A. mediterranei* strains DSM 43304^T (1), F1/24 (2), T-195 (3), MTCC 17 (4) and DSM 40773 (5); 6, *A. rifamycinica* DSM 46095^T; 7, *A. rifamycinica* DSM 46096; 8, *A. kentuckyensis* NRRL B-14129^T (data from Labeda *et al.*, 2003); 9, *A. lexingtonensis* NRRL B-24131^T (Labeda *et al.*, 2003); 10, *A. pretoriensis* NRRL B-24133^T (Labeda *et al.*, 2003); 11, *A. tolypomycina* DSM 44544^T (Wink *et al.*, 2003); 12, *A. vancoresmycina* DSM 44592^T (Wink *et al.*, 2003); 13, *A. balhimycina* DSM 44591^T (Wink *et al.*, 2003). +, Positive; −, negative; W, weakly positive; ND, not done.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13
Acid production from:													
Adonitol	−	−	−	−	−	+	−	+	+	−	ND	ND	ND
Arabinose	+	−	−	−	+	+	+	+	+	+	+	+	+
Cellobiose	+	−	−	−	+	+	+	+	+	+	ND	ND	ND
D-Galactose	+	−	−	−	+	+	+	+	+	+	ND	ND	ND
meso-Inositol	−	−	−	+	+	+	+	+	+	+	+	+	+
α-Lactose	+	−	−	−	+	−	+	+	+	+	ND	ND	ND
Maltose	+	−	−	+	+	−	+	+	+	+	ND	ND	ND
D-(−)-Mannitol	+	−	−	−	+	−	+	−	W	−	+	+	+
Raffinose	+	−	−	−	−	−	−	+	+	+	−	+	+
L-(+)-Rhamnose	+	−	−	+	−	−	−	+	+	+	+	+	+
D-(−)-Sorbitol	W	−	−	−	−	−	−	+	−	W	ND	ND	ND
D-(+)-Xylose	+	−	−	+	+	+	+	+	+	+	−	−	−
Decomposition of:													
Casein	+	−	+	+	+	+	+	+	+	+	ND	ND	ND
Gelatin	+	−	−	W	+	+	+	+	+	+	ND	ND	ND
Growth in the presence of 5 % NaCl	+	−	−	+	+	W	W	+	+	+	ND	ND	ND
Production of catalase	+	W	W	+	+	+	+	ND	ND	ND	ND	ND	ND

providing senior research fellowships. We would like to thank K. H. Gartemann for his valuable advice and J. P. Euzéby for etymological advice.

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