Amycolatopsis decaplanina sp. nov., a novel member of the genus with unusual morphology

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Strain DSM 44594^T, which produces the glycopeptide antibiotic decaplanin, is a member of the genus *Amycolatopsis* based on 16S rRNA gene sequence analysis and chemotaxonomic properties. It is the first member of this genus that is reported to form pseudosporangia, which resemble those of members of the genus *Kibdelosporangium*. Phylogenetically, the novel taxon is related to *Amycolatopsis orientalis*, *Amycolatopsis lurida*, *Amycolatopsis azurea*, *Amycolatopsis japonica* and *Amycolatopsis keratiniphila*. Morphological, cultural and physiological properties, the production of a unique glycolipid and DNA–DNA similarity of $<55\%$ with phylogenetically related strains reveal that strain DSM 44594^T represents a novel species of the genus, for which the name *Amycolatopsis decaplanina* sp. nov. (type strain, FH 1845^T = DSM 44594^T = NRRL B-24209^T) is proposed.

In the course of a screening programme in Hoechst, Frankfurt, for new antibiotics that are active against methicillin-resistant strains of *Staphylococcus aureus*, the strain that produces the new antibiotic decaplanin (Eur. Pat., 1990, EP 356894) was isolated from a soil sample from India. Strain FH 1845^T (= DSM 44594^T = NRRL B-24209^T) displays activity against a wide range of Gram-positive bacteria, including enterococci and clinical isolates, that are resistant to commonly applied antibiotics (Sanchez *et al.*, 1992).

Micrographs of the strain described in this study are shown in Fig. 1. Morphological and physiological characteristics of DSM 44594^T were observed on various agar cultures as described by Shirling & Gottlieb (1966): yeast extract/malt extract agar (ISP 2), oatmeal agar (ISP 3), inorganic salt/starch agar (ISP 4), glycerol/asparagine agar (ISP 5), peptone/yeast extract/iron agar (ISP 6) and tyrosine agar (ISP 7), incubated for 10 days at 28 °C. For scanning electron microscopy (Grabley *et al.*, 1992), the strain was grown on ISP 3 agar. A honey-yellow vegetative mycelium developed on all ISP media tested (RAL colour code 1005; Deutsches Institut für Gütesicherung und Kennzeichnung e.V. – Reichsausschuß für Lieferbedingungen). Aerial mycelium was only formed on ISP 3 medium and a soluble red pigment was produced on ISP 7 medium. After 7–10 days on ISP 3 medium, sporangium-like elements were formed. These elements showed a smooth surface and a regular shape under the scanning electron microscope. Spores were not detected either inside or outside the pseudosporangia.

Utilization of carbohydrates was investigated on ISP 9 medium (Shirling & Gottlieb, 1966) by using a 12-well microtitre plate technique. Sodium chloride tolerance was also tested on 6-well microtitre plates by using a technique based on the method of Kutzner *et al.* (1986). A fingerprint of enzymic activities was obtained by using API 20E and API ZYM test strips (Smith *et al.*, 1972; Humble *et al.*, 1977; Kilian, 1978). Reactions are indicated in the species description.

To determine the antimicrobial spectrum (Williams *et al.*, 1989), bacteria were grown on Mueller–Hinton agar and fungi on Czapek Dox agar. Antibacterial activity was seen after cultivation on ISP 2, ISP 3 and starch media, especially against *Staphylococcus aureus*, *Micrococcus luteus*, *Streptomyces murinus* and *Bacillus subtilis*. Antifungal activity was not detected.

For metabolite production, *Amycolatopsis* strains were incubated in four different media: a soymeal medium, a

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Abbreviation: A_2pm, diaminopimelic acid.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains DSM 44594^T, DSM 44213^T and DSM 43134^T are AJ508237, AJ508236 and AJ577997, respectively.

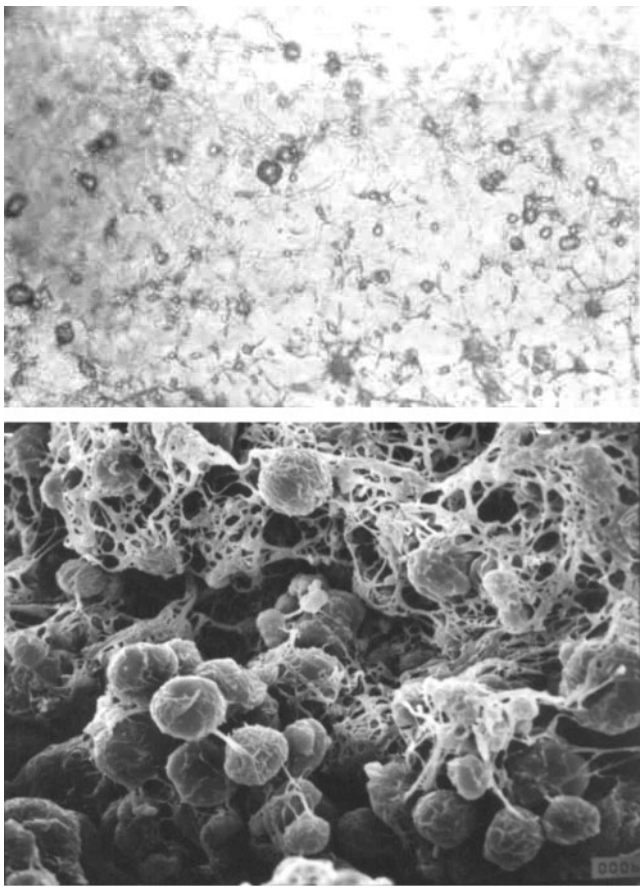

Fig. 1. Pseudosporangia formation in strain DSM 44594^T grown on ISP 3 medium for 14 days at 28 °C. Top, light microscopy (× 200); bottom, scanning electron microscopy (× 1000).

starch medium and ISP 2 and 3 media for 7 days in a shaking flask culture at 28 °C. After cultivation, the whole culture was extracted with methanol, evaporated and dissolved in water.

Analysis of whole-cell diaminopimelic acid (A_2pm) isomers and sugars was done by the method of Hasegawa *et al.* (1983). Phospholipids and menaquinones were analysed by the method of Kutzner *et al.* (1986). To determine the whole-cell fatty acid profile, the fast method with trimethylsulfonium hydroxide (TMSH) was used (Müller *et al.*, 1990). Major fatty acids were i-$C_{15:0}$ (22·5 %), $C_{17:0}$ (11·4 %) and i-$C_{16:0}$ (10·3 %), whilst i-$C_{14:0}$ (6·4 %), ai-$C_{15:0}$ (9·4 %), i-$C_{15:0}$ 2-OH (8·7 %), $C_{15:0}$ (7·6 %), $C_{17:1}$ (5·8 %), $C_{16:0}$ (3·3 %), $C_{15:1}$ (2·1 %), i-$C_{17:0}$ (1·7 %), ai-$C_{17:0}$ (3·4 %), i-$C_{16:0}$ 2-OH (2·8 %), ai-$C_{15:0}$ 2-OH (1·9 %), $C_{17:0}$ 2-OH (1·5 %) and 10-methyl i-$C_{17:0}$ (1·2 %) occurred in smaller amounts. Phospholipids were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, hydroxyphosphatidylethanolamine phosphatidylinositol and phosphatidylinositol mannoside. Menaquinones were MK-8(H_4) and MK-9(H_4). The peptidoglycan diamino acid was *meso*-A_2pm; cell-wall sugars were arabinose and galactose.

Genomic DNA extraction, PCR-mediated amplification of the 16S rRNA gene and purification of PCR products were carried out as described previously (Rainey *et al.*, 1996). The almost-complete 16S rRNA gene sequence of strain DSM 44594^T (1464 nt) was aligned manually against 16S rRNA gene sequences of representatives of the main actinobacterial lineages and then against members of the genus *Amycolatopsis*. Pairwise evolutionary distances were computed by using the correction of Jukes & Cantor (1969). A phylogenetic dendrogram (Fig. 2) was reconstructed from the distance matrix by using the treeing algorithm of DeSoete (1983). Strain DSM 44594^T was related closely to *Amycolatopsis azurea* DSM 43854^T (99·2 % sequence similarity), *Amycolatopsis orientalis* IMSNU 20058^T (99·0 %), *Amycolatopsis japonica* DSM 44213^T (99·1 %) and *Amycolatopsis keratiniphila* DSM 44409^T (99·1 %) (Al-Musallam *et al.*, 2003). Sequence similarity values with less closely related members of the genus ranged between 95·0 and 98·8 %.

Automated ribotyping of the isolates was accomplished by using the RiboPrinter (Qualicon) system (Bruce, 1996) and *Pvu*II as the standard restriction enzyme for cutting genomic DNA. Whilst the patterns of the type strains of *A. japonica* and *A. orientalis* bear some similarity, those of other strains, including DSM 44594^T, are different (Fig. 3).

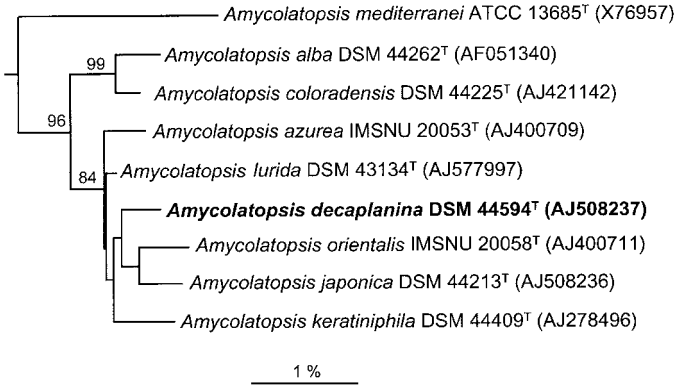

Fig. 2. 16S rDNA dendrogram (DeSoete, 1983), displaying the phylogenetic position of strain DSM 44594^T and phylogenetically related members of the genus *Amycolatopsis*. Numbers indicate percentages of 1000 bootstrap resamplings. More distantly related members of the genus served as a root. Bar, 1 % sequence divergence.

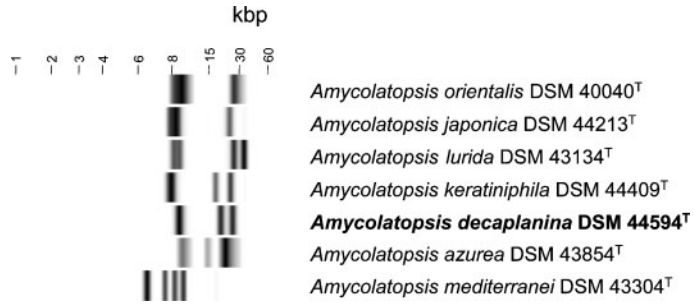

Fig. 3. Diversity of normalized *Pvu*II ribotype patterns found within strain DSM 44594^T and phylogenetically related *Amycolatopsis* strains.

For DNA–DNA reassociation experiments, DNA was isolated by using a French pressure cell (Thermo Spectronic) and was purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977). DNA–DNA hybridizations were carried out in duplicate, according to methods described by De Ley *et al.* (1970) and Huß *et al.* (1983), at 69 °C in 2× SSC that contained 10 % DMSO. Renaturation rates were calculated by using the computer program TRANSFER.BAS (Jahnke, 1992). DNA similarities were determined for strain DSM 44594^T and its closest phylogenetic relatives. Strain DSM 44594^T and *A. azurea* DSM 43854^T, which share high 16S rRNA gene sequence similarity, showed 55·7 % DNA–DNA hybridization (mean of two determinations of 55·2 and 56·2 %). Similarly low values were found for strain DSM 44594^T and the type strains of *A. orientalis* DSM 40040^T (50·5 %, mean of 47 and 54 %) and *A. lurida* DSM 43134^T (31·5 %, mean of 32 and 31 %). The type strains of the latter two species share only 44·5 % DNA similarity. The remote DNA relatedness of organisms that share >99·0 % 16S rRNA gene sequence similarity has recently been reported by Wink *et al.* (2003).

On the basis of 16S rRNA gene sequence similarity and chemotaxonomic properties, strain DSM 44594^T is classified as a member of the genus *Amycolatopsis*. Species of this genus have not previously been described to form sporangium-like structures that are surrounded by a well-defined wall and in which spores are not observed. Strain DSM 44594^T produces the glycopeptide antibiotic decaplanin. The formation of glycopeptide antibiotics has also been reported for some phylogenetically related species, such as vancomycin by *A. orientalis* (Pittenger & Brigham, 1956), ristocetin by *A. lurida* (Grundy *et al.*, 1956–1957) and azureamycin by *A. azurea* (Omura *et al.*, 1979). *Amycolatopsis mediterranei*, on the other hand, being less closely related to these organisms, produces the antibiotic rifamycin.

Strain DSM 44594^T differs from related *Amycolatopsis* species in cultural properties. Whilst the substrate mycelium is honey-yellow on all ISP media, *A. azurea* forms steel-blue to purple–violet colonies, *A. orientalis* and *A. lurida* form yellow to beige colonies, *A. japonica* forms white colonies on some ISP media and *Amycolatopsis keratiniphila* has sand-yellow colonies. Based on carbohydrate utilization patterns and the API ZYM and API 20E panels (Table 1), strain DSM 44594^T has highest similarity to *A. orientalis* and *A. lurida*, but has more pronounced differences from *A. azurea*, *A. japonica* and other species (data not shown; Chun *et al.*, 1999; Kim *et al.*, 2002).

Stackebrandt & Goebel (1994) recommended a binary threshold value of about 97 % 16S rRNA gene sequence similarity between strains, above which it was advised to perform DNA–DNA reassociation experiments in order to determine whether or not organisms should be affiliated to the same species. Some members of the genus *Amycolatopsis*, however, are significantly more closely related than this by 16S rRNA gene sequence similarity (around 99·0 %), but DNA–DNA reassociation values are significantly below 70 %, the recommended threshold value for species delineation (Wayne *et al.*, 1987). For example, *Amycolatopsis methanolica* NCIB 11946^T and *Amycolatopsis thermoflava* NBRC 14333^T share 99·8 % sequence similarity and the corresponding DNA–DNA binding value was 21 %; similarly, 16S rRNA gene sequence similarity between *Amycolatopsis eurytherma* NCIMB 13795^T and strains NCIB 11946^T and NBRC 14333^T was also 99·2 % and the corresponding DNA–DNA binding values were 60 and 2 %, respectively (Chun *et al.*, 1999; Kim *et al.*, 2002). The phylogenetically closest neighbour of the decaplanin-producing strain DSM 44594^T is *A. azurea* DSM 43854^T, which shares 99·2 % 16S rRNA gene sequence similarity. Similar values were found for the type strains of *A. orientalis* and *A. lurida*. As the corresponding DNA–DNA reassociation value was determined to be <56 %, we refrained from testing the binding rate for strain DSM 44594^T and other *Amycolatopsis* species. We consider that strain DSM 44594^T represents a distinct species with respect to phylogenetic position and genomic, morphological and metabolic uniqueness, for which we propose the name *Amycolatopsis decaplanina* sp. nov.

Description of *Amycolatopsis decaplanina* sp. nov.

Amycolatopsis decaplanina (de.ca.pla.ni′na. N.L. fem. adj. *decaplanina* formed from the name of the antibiotic decaplanin, which is produced by the organism).

Aerobic, non-motile, Gram-positive, catalase-positive actinomycete that forms extensively branched substrate mycelium. After 7–10 days on ISP medium 3, regular-shaped to globose and smooth-surfaced sporangium-like elements (pseudosporangia) are formed. Spores are not

Table 1. Utilization of carbohydrates and enzymic activities of strain DSM 44594^T and type strains of related *Amycolatopsis* species

Taxa: 1, Strain DSM 44594^T; 2, *A. azurea* DSM 43854^T; 3, *A. japonica* DSM 44213^T; 4, *A. keratiniphila* DSM 44409^T; 5, *A. orientalis* DSM 40040^T; 6, *A. lurida* DSM 43134^T. All strains utilized glucose and arabinose and were positive for *N*-acetyl-β-glucosamidase, chymotrypsin and acid phosphatase activities, acetoin production and gelatinase. No strain utilized rhamnose and all were negative for β-glucuronidase, tryptophan deaminase and production of H_2S and indole.

Substrate/activity	1	2	3	4	5	6
Utilization of carbohydrates:						
Sucrose	−	+*	−†	+	−	−
Xylose	−	−	−	+	−	−
Inositol	−	+	+	+	+	−
Mannitol	+	−	+	+	+	+
Fructose	+	−	+	+	+	+
Raffinose	−	+	+	+	−	−
Citrate	−	+	+	+	+	+
Enzymic activities (API ZYM):						
Esterase (C4)	−	−	+	+	+	−
Esterase lipase (C8)	−	+	+	+	+	+
Lipase (C14)	−	−	+	+	−	+
Alkaline phosphatase	+	−	+	+	+	+
Leucine arylamidase	+	+	+	+	−	+
Valine arylamidase	+	−	+	+	−	−
Cystine arylamidase	−	−	+	+	−	−
Trypsin	+	+	+	+	−	+
α-Galactosidase	−	−	+	−	−	−
β-Galactosidase	+	+	+	+	−	+
α-Glucosidase	+	+	+	+	−	+
β-Glucosidase	+	+	+	+	−	+
α-Mannosidase	+	−	+	−	−	−
α-Fucosidase	−	−	+	−	−	−
Enzymic activities (API 20E):						
Arginine dihydrolase	−	+	+	+	+	−
Lysine decarboxylase	−	+	+	+	−	−
Ornithine decarboxylase	−	+	+	+	−	−
Urease	+	+	+	+	−	+

*Similar to the growth that occurs in basal medium with glucose.
†Growth is not better than that that occurs in basal medium with water.

detected either inside or outside the pseudosporangia. Colonies are honey-yellow on ISP media 1–7. Aerial mycelium is formed only on ISP medium 3. A soluble red pigment is only produced on ISP medium 7. Melanoid pigment is not produced. Carbohydrate utilization and enzymic relation towards the API ZYM and API 20E substrate panels are indicated in Table 1. Major fatty acids (>10 %) are i-$C_{15:0}$, i-$C_{16:0}$ and $C_{17:0}$. Menaquinones are MK-8(H_4) and MK-9(H_4). Produces the glycopeptide antibiotic decaplanin.

Type strain is FH 1845^T (=DSM 44594^T=NRRL B-24209^T). Isolated from soil in India.

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