***Saccharopolyspora flava* sp. nov. and *Saccharopolyspora thermophila* sp. nov., novel actinomycetes from soil**

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The generic position of two aerobic, Gram-positive, non-acid-alcohol-fast actinomycetes was established following the isolation of their PCR-amplified 16S rRNA genes and alignment of the resultant sequences with the corresponding sequences from representatives of the families Actinosynnemataceae and Pseudonocardiaceae. The assignment of the organisms to the genus *Saccharopolyspora* was strongly supported by chemotaxonomic and morphological data. The strains were distinguished both from one another and from representatives of validly described *Saccharopolyspora* species on the basis of a number of phenotypic properties. It is proposed that the organisms, strains 07^T (= AS4.1520^T = IFO 16345^T = JCM 10665^T) and 216^T (= AS4.1511^T = IFO 16346^T = JCM 10664^T), be classified in the genus *Saccharopolyspora* as *Saccharopolyspora flava* sp. nov. and *Saccharopolyspora thermophila* sp. nov., respectively.

Keywords: *Saccharopolyspora flava* sp. nov., *Saccharopolyspora thermophila* sp. nov., polyphasic taxonomy

INTRODUCTION

The application of the polyphasic taxonomic approach to actinomycete systematics led to the circumscription of the family *Pseudonocardiaceae* Embley *et al.* 1988 for mycolateless actinomycetes characterized by the presence of *meso*-diaminopimelic acid (*meso*-A₂pm), arabinose and galactose in their peptidoglycan (wall chemotype IV *sensu* Lechevalier & Lechevalier, 1970). Members of this family are currently assigned to the genera *Actinobispora*, *Amycolatopsis*, *Kibdelosporangium*, *Prauserella*, *Pseudonocardia* (including *Amycolata*), *Saccharomonospora*, *Saccharopolyspora* and *Thermocrispum* (Kim & Goodfellow, 1999; Xu *et al.*, 1999; Labeda & Kroppenstedt, 2000).

The family *Pseudonocardiaceae* recognized by Stackebrandt *et al.* (1997) also included the genera *Actinosynnema*, *Saccharothrix* and *Streptoalloteichus*;

these taxa were excluded from the family by Warwick *et al.* (1994), who considered that they might form a 'sister' group to the *Pseudonocardiaceae* clade. This now seems to be the case, as Labeda & Kroppenstedt (2000) have proposed that members of the genera *Actinokineospora*, *Actinosynnema*, *Lentzea* and *Saccharothrix* be classified in a new taxon, i.e. the family *Actinosynnemataceae*. Members of both clades can be distinguished from one another using chemical and morphological markers (Kim & Goodfellow, 1999; Labeda & Kroppenstedt, 2000).

The genus *Saccharopolyspora* contains eight validly described species: *Saccharopolyspora erythraea* Labeda 1987; *Saccharopolyspora gregorii* Goodfellow *et al.* 1989; *Saccharopolyspora hordei* Goodfellow *et al.* 1989; *Saccharopolyspora rectivirgula* (Krasil'nikov and Agre 1964) Korn-Wendisch *et al.* 1989; *Saccharopolyspora spinosa* Mertz and Yao 1990; *Saccharopolyspora spinosporotrichia* Zhou *et al.* 1998; *Saccharopolyspora taberi* (Labeda 1987) Korn-Wendisch *et al.* 1989; and *Saccharopolyspora hirsuta* Lacey and Goodfellow 1975, the type species. Representatives of these species form a distinct phyletic line within the evolutionary radiation encompassed by the

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The GenBank accession numbers for the 16S rDNA sequences of strains 07^T (= AS4.1520^T) and 216^T (= AS4.1511^T) are AF154128 and AF127526, respectively.

family *Pseudonocardiaceae* and can be distinguished using a combination of biochemical, morphological and physiological properties (Zhou *et al.*, 1998). Genus-specific primers are available for the PCR identification of *Pseudonocardia* and *Saccharopolyspora* strains (Morón *et al.*, 1999).

Two actinomycetes isolated from soil samples collected in China were provisionally assigned to the genus *Saccharopolyspora*, using chemical and morphological properties. In the present investigation, these organisms, namely strains 07^T and 216^T, were examined for a range of genotypic and phenotypic properties and found to form distinct new centres of taxonomic variation within the genus *Saccharopolyspora*; the names *Saccharopolyspora flava* and *Saccharopolyspora thermophila* are proposed for these strains, respectively.

METHODS

Organisms and cultural conditions. Strain 07^T was isolated on an oatmeal agar plate (Shirling & Gottlieb, 1966) seeded with a soil suspension; the latter was prepared from a garden soil collected from Xishan Mountain, Beijing, and incubated at 28 °C for 5 d. Similarly, strain 216^T was isolated on selective medium 2 (Lu *et al.*, 2000) inoculated with a soil suspension prepared from a grassland soil sample taken from Nanning, Guangxi Province, Southern China, and incubated at 45 °C for 7 d; the selective medium consisted of Stevenson's basal medium (Stevenson, 1967) supplemented with melezitose (1%, w/v) and neomycin (4 µg ml⁻¹). The strains were maintained on glucose yeast-extract agar (GYEA) (Gordon & Mihm, 1962) at room temperature and as glycerol suspensions (20%, v/v) at -20 °C. Biomass for the chemotaxonomic and molecular systematic studies was prepared by growing the strains in shake flasks of modified Sauton's broth (Mordarska *et al.*, 1972) at either 28 °C (strain 07^T) or 50 °C (strain 216^T) for 5 d. At maximum growth, the broth cultures were checked for purity, killed with formaldehyde (1%, v/v), washed three times with distilled water and freeze-dried.

Cultural and morphological properties. The undisturbed arrangement of hyphae, notably aerial hyphae and spore-chain morphology, was observed on modified Sauton's agar (Mordarska *et al.*, 1972) and ISP medium 2 (Shirling & Gottlieb, 1966) after 7 d, using the coverslip technique of Kawato & Shinobu (1959). Growth on the coverslips was fixed and examined according to the procedure described by Zhou *et al.* (1998). Additional morphological properties were observed by examining gold-coated, dehydrated specimens of the organisms, using a Hitachi S-570 scanning electron microscope.

Biochemical and physiological properties. The strains were examined for a range of phenotypic properties following incubation for 14 d at either 28 °C (strain 07^T) or 50 °C (strain 216^T), in most cases according to procedures described in previous studies on saccharopolysporae (Lacey & Goodfellow, 1975; Zhou *et al.*, 1998). To determine lysozyme sensitivity, a 0.05% (w/v) solution of lysozyme was sterilized by membrane filtration and added to sterilized Sauton's broth (McCarthy & Cross, 1984) to give a final concentration of 0.0025% (w/v). Test tubes of the basal medium with and without lysozyme were inoculated with

fresh cultures and examined for growth after 10 d at either 28 °C or 50 °C. The antibiotic-sensitivity tests were carried out by placing impregnated filter-paper discs (Goodfellow & Orchard, 1974) over Sauton's agar and incubating the samples for 7–10 d at the incubation temperatures cited above. The two organisms were also examined for their ability to grow at either 20, 25, 37 and 45 °C (strain 07^T) or at 40, 45, 50, 55 and 60 °C (strain 216^T).

Chemotaxonomy. Established procedures were used to determine the diagnostic isomers of A₂pm, the predominant whole-organism sugars and the major polar lipids (Lechevalier & Lechevalier, 1980; Hasegawa *et al.*, 1983). Isoprenoid quinones were extracted and purified according to Collins (1985) and were analysed by HPLC (Wu *et al.*, 1989), with *Streptomyces griseus* as the control. The acid methanolysis procedure was used to screen for mycolic acids (Minnikin *et al.*, 1980). The DNA base ratios of the two strains were determined using the thermal denaturation method of Marmur & Doty (1962), with *Escherichia coli* AS1.365 as the control.

16S rDNA sequencing. Genomic DNA was isolated from the strains by following the procedure described by Kim *et al.* (1995). PCR amplification of the 16S rDNA preparations was carried out as described previously (Kim *et al.*, 1996). The resultant PCR products were purified by using the Wizard PCR purification system (Promega) according to the procedure provided by the manufacturer. The purified products were directly sequenced using a *Taq* DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems) and the universal primers 27f (5'-GAGAGTTTGATCCTGGC-TCAG-3'), 704f (5'-GTACGGTGAARTGCGCAGA-3') 765r (5'-CTGTTTCGCTCCCCACGCTTTC-3') and 1495r (5'-CTACGGCTACCTTGTTACGA-3'). Sequence gel electrophoresis was carried out and nucleotide sequences automatically obtained by using an Applied Biosystems DNA sequencer (model 373A) and software provided by the manufacturer.

Analysis of sequence data. The 16S rDNA sequences of the strains were aligned manually using the CLUSTAL X program (version 1.64b, Thompson *et al.*, 1997) against corresponding nucleotide sequences of representatives of the families *Actinosynnemataceae* (Labeda & Kroppenstedt, 2000) and *Pseudonocardiaceae* (Kim & Goodfellow, 1999) retrieved from the DDBJ (Thompson *et al.*, 1997) and GenBank (Benson *et al.*, 1997) databases. Evolutionary trees were inferred by using the neighbour-joining (Saitou & Nei, 1987) and least-squares (Fitch & Margoliash, 1967) treeing algorithms from the PHYLIP package (Felsenstein, 1993). Evolutionary distance matrices were generated as described by Kimura (1980). Tree topologies were evaluated by bootstrap analysis based on 1000 resamplings of the neighbour-joining dataset using the SEQBOOT and CONSENSE options from the PHYLIP suite of programs (Felsenstein, 1993).

RESULTS AND DISCUSSION

Almost complete 16S rDNA sequences were generated for strains 07^T (1443 nucleotides) and 216^T (1441 nucleotides). Comparison of these nucleotide sequences with those of representatives of the families *Actinosynnemataceae*, *Pseudonocardiaceae* and related taxa (Labeda & Kroppenstedt, 2000) clearly showed that the organisms belong to the genus *Saccharo-*

Fig. 1. Neighbour-joining tree (Saitou & Nei, 1987) based on almost complete 16S rDNA sequences, showing relationships between *Saccharopolyspora* strains 07^T and 216^T and representatives of the families Actinosynnemataceae and Pseudonocardiaceae and related taxa. Asterisks indicate branches that were also recovered using the Fitch-Margoliash treeing algorithm. The numbers at the nodes indicate the levels of bootstrap support, based on a neighbour-joining analysis of 1000 resampled datasets; only values greater than 50 % are given. Bar, 0.02 substitutions per nucleotide position.

polyspora (Lacey & Goodfellow, 1975) (Fig. 1). The chemical properties of strain 07^T are also consistent with its assignment to this taxon. The organism contains *meso*-A₂pm as the wall diamino acid, arabinose and galactose as the major wall sugars, a phospholipid pattern containing phosphatidylcholine and tetrahydrogenated menaquinones with nine isoprene units as the predominant isoprenoid quinone, but lacks mycolic acids. This chemical profile distinguishes strain 07^T from members of wall-chemotype-IV taxa, apart from those classified in the genus *Saccharopolyspora* (Zhou *et al.*, 1998; Kim & Goodfellow, 1999). Strain 216^T also showed a range of chemical markers typical of saccharopolysporae, though it was unusual in having hexa- and decahydrogenated menaquinones with nine isoprene units as the predominant menaquinones. This apparently anomalous result may reflect the time at which the culture was sampled, as there is evidence that the menaquinone composition of *Amycolatopsis* and *Streptomyces* strains can be age-dependent (Saddler *et al.*, 1986; Yassin *et al.*, 1991). In addition, strains 07^T

and 216^T were found to have G+C-rich DNA (73.1 and 67.0 mol %, respectively).

The phenotypic properties of the strains are also typical of saccharopolysporae. The organisms are aerobic, non-motile, Gram-positive, non-acid-alcohol-fast actinomycetes which form extensively branched substrate mycelia. The latter fragment into rod-shaped elements which carry aerial hyphae that differentiate into short chains of smooth-surfaced spores (Fig. 2). Strain 07^T bears aerial hyphae which differentiate into chains of three to five spores (Fig. 2, right); the aerial hyphae of strain 216^T differentiate into chains of four to six spores separated by 'empty' hyphae (Fig. 2, left).

The close relationship found between *Saccharopolyspora* strain 07^T and the type strain of *S. spinosa* is supported both by treeing algorithms and by a high bootstrap value (Fig. 1). The two strains share a 16S rDNA nucleotide similarity value of 96.2 %, which corresponds to 53 nucleotide differences. In contrast, *Saccharopolyspora* strain 216^T is not particularly

Fig. 2. Scanning electron micrographs showing (left) vesicular chains of smooth-surfaced spores of a 7-d-old culture of strain 216^T grown on ISP medium 2 at 45 °C and (right) short chains of smooth-surfaced spores of a 7-d-old culture of strain 07^T grown on oatmeal agar at 28 °C. Bars, 3 µm.

closely related to any of the type strains of *Saccharopolyspora* species, or to *Saccharopolyspora* strain 07^T. Its closest relationship is with the type strain of *S. spinosporotrichia*. The 16S rDNA similarity between these two strains is 93.0%, a value that is equivalent to 94 nucleotide differences. The two most closely related type strains of *Saccharopolyspora* species, namely, those of *S. erythraea* and *S. spinosporotrichia*, share a 16S rDNA similarity of 97.5%, a value that corresponds to 31 nucleotide differences. It is evident, therefore, that *Saccharopolyspora* strains 07^T and 216^T represent two new centres of taxonomic variation within the genus *Saccharopolyspora*. The two organisms can also be distinguished from one another, and from the type strains of the validly described species of *Saccharopolyspora*, by using a combination of phenotypic properties (Table 1).

The chemotaxonomic, molecular systematic and phenotypic data show that strains 07^T and 216^T should each be given species status within the genus *Saccharopolyspora*. It is proposed, therefore, that strain 07^T be classified as *Saccharopolyspora flava* sp. nov. and strain 216^T as *Saccharopolyspora thermophila* sp. nov.

It is also evident from the 16S rDNA tree that the genera *Actinobispora* Jiang *et al.* 1991 and *Pseudonocardia* Henssen 1957 emend. Reichert *et al.* 1998 are closely related. Comparative taxonomic studies on representative strains are needed to determine the detailed relationships that exist between members of these genera.

Description of *Saccharopolyspora flava* Lu *et al.*

Saccharopolyspora flava (fla'va. L. adj. *flaveus* yellow, referring to the colour of the substrate mycelium).

Aerobic, Gram-positive, non-acid-alcohol-fast, non-motile actinomycete which forms an extensively branched, yellow substrate mycelium which fragments into rod-shaped elements after 3–4 d at 28 °C. Aerial hyphae are produced upon prolonged cultivation on oatmeal agar. The organism also grows well on glucose-asparagine, ISP medium 2 and modified

Sauton's agars. The aerial mycelium carries abundant chains of 3–5 smooth-surfaced spores (0.4 × 0.5–0.6 µm). Diffusible pigments are not produced. Adenine, cellulose, hypoxanthine, starch, xanthine and xylan are degraded, but casein, chitin, elastin, guanine and tyrosine are not degraded. Aesculin and urea are hydrolysed and nitrate is reduced. Uses adonitol, cellobiose, erythritol, fructose, galactose, glucose, glycerol, inositol, lactose, maltose, mannitol, methyl α-D-glucoside, raffinose, salicin, sorbitol, sucrose, trehalose, xylose, acetate, adipate, benzoate, butyrate, citrate, fumarate, H-malate, propionate, pyruvate and succinate as sole carbon sources for energy and growth but does not use L-arabinose, melezitose, sebacic acid or tartrate. Weak growth occurs in the presence of 7% (w/v) NaCl. Growth is inhibited by lysozyme, gentamicin sulphate (discs soaked in 100 µg antibiotic ml⁻¹), streptomycin sulphate (100 µg ml⁻¹) and rifampicin (50 µg ml⁻¹). The temperature growth range is between 28 and 37 °C. The G+C content of the DNA is 67 mol%. Isolated from garden soil collected from Xishan Mountain, Beijing, China. The type strain is strain 07^T [= AS4.1520^T (Chinese Culture Collection of Microorganisms) = IFO 16345^T = JCM 10665^T].

Description of *Saccharopolyspora thermophila* Lu *et al.*

Saccharopolyspora thermophila (ther.mo'phi.la. Gr. n. *therme* heat; Gr. adj. *philus* loving; M.L. adj. *thermophila* heat-loving organism).

Aerobic, Gram-positive, non-acid-alcohol-fast, non-motile actinomycete which forms an extensively branched colourless to buff substrate mycelium which fragments into rod-shaped elements after 4–5 d at 45 °C. Good growth occurs on ISP medium 2 and modified Sauton's and oatmeal agars. An abundant aerial mycelium carries long hooked to flexuous chains of 4–6 smooth-surfaced, vesicular spores (0.7–1.1 × 0.85–1.5 µm). Diffusible pigments are not produced. Adenine, guanine, starch, tyrosine and xylan are degraded, but casein, cellulose, chitin, elastin, hypoxanthine and xanthine are not degraded. Aesculin and urea are hydrolysed but nitrate is not reduced.

Table 1 Comparison of properties of strains 07^T and 216^T with those of the type strains of validly described *Saccharopolyspora* species

Data for organisms other than the tested strains are taken from Korn-Wendisch *et al.* (1989) and Zhou *et al.* (1998). Abbreviations: Bf, buff; Br, brown; C, colourless; G, grey; O, orange; P, pink; R, red; W, white; Y, yellow; +, positive; –, negative; N/A, not applicable (aerial mycelium was not observed); ND, not determined. Results were positive for degradation of aesculin and utilization of D-fructose, glycerol, D-mannitol and D-mannose.

Characteristic	07 ^T	216 ^T	<i>S. erythraea</i> DSM 40517 ^T	<i>S. gregorii</i> DSM 44324 ^T	<i>S. hirsuta</i> DSM 43463 ^T	<i>S. horderi</i> DSM 44065	<i>S. rectivirgula</i> DSM 43747 ^T	<i>S. spinosa</i> DSM 44228 ^T	<i>S. spinospirotrichia</i> DSM 44350 ^T	<i>S. taberi</i> DSM 43856 ^T
Spore arrangement	Straight	Hooks or flexuous hyphae	Open spirals	Hooks or flexuous hyphae	Straight to loose spirals	Hooks and spirals	Straight	Hooks and open loops	Spirals	N/A
Spore ornamentation	Smooth	Smooth	Spiny	Smooth	Hairy	Smooth	Smooth or irregularly rough	Spiny	Spiny	N/A
Substrate mycelium	Fragments	Fragments	Branched	Fragments	Fragments	Fragments	Branched	Fragments in lipid media	Fragments	Fragments
Colour of aerial mycelium	W	W	P-BrG-W	W-Y	W	W-Y	W-light P	W-P	W-G	N/A
Colour of substrate mycelium	Y	C-Bf	OY-RBr	C-Bf	C-Bf	C-Bf	Y-O	G-OY-Br	Br-R	C-Y
Colour of soluble pigment	None	None	Y-POBr	None	R-O-Y	None	None	C-Br	Br	O-R
Spores on substrate mycelium	–	–	–	–	–	–	+	–	–	–
Degradation of:										
Adenine	+	+	+	–	+	+	–	–	–	+
Casein	–	–	–	+	+	+	–	–	+	+
Chitin	–	–	+	–	–	+	–	–	–	+
Elastin	–	–	+	+	+	+	–	–	+	+
Starch	+	+	+	+	+	+	+	–	+	+
Tyrosine	–	+	+	+	+	+	+	+	–	+
Xanthine	+	–	+	+	+	+	+	+	+	+
Reduction of nitrate	+	–	+	–	–	–	+	+	–	+
NaCl tolerance (w/v)	7	7	< 5	13	< 7	< 13	< 10	< 11	2–3	7
Temperature range (°C)	28–37	45–55	20–42	10–35	25–50	20–60	37–63	15–37	28–37	20–45
Utilization of carbohydrates as sole carbon source										
L-Arabinose	–	–	+	+	–	+	–	+	–	–
D-Galactose	+	+	+	+	+	+	+	–	+	+
D-Lactose	+	+	–	–	+	+	+	–	–	+
D-Maltose	+	+	+	+	+	+	+	–	+	+
D-Raffinose	+	+	+	+	+	+	+	–	+	+
L-Rhamnose	+	+	+	+	+	+	+	–	+	+
Sucrose	+	+	+	+	+	+	+	–	+	+
D-Xylose	+	–	+	+	+	+	+	–	+	+

Uses adonitol, cellobiose, erythritol, fructose, galactose, glucose, glycerol, inositol, lactose, maltose, mannitol, melezitose, methyl α -D-glucoside, raffinose, rhamnose, salicin, sorbitol, sucrose, trehalose, acetate, adipate, benzoate, butyrate, citrate, fumarate, H-malate, propionate, pyruvate, sebacic acid and succinate as sole carbon sources for energy and growth but does not use L-arabinose or tartrate. Growth occurs in the presence of 7% (w/v) NaCl but is inhibited by lysozyme, gentamicin sulphate, streptomycin sulphate and rifampicin. The temperature growth range is 45–55 °C. The predominant menaquinones are MK-9(H₆) and MK-9(H₈). The G+C content of the DNA is 73.1 mol%. Isolated from

grassland soil collected from Nanning, Guangxi Province, China. The type strain is strain 216^T [= AS4.1511^T (China Culture Collection of Microorganisms) = IFO 16346^T = JCM 10664^T].

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