***Streptomyces malaysiensis* sp. nov., a new streptomycete species with rugose, ornamented spores**

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The taxonomic position of a streptomycete strain isolated from Malaysian soil was established using a polyphasic approach. The organism, designated strain ATB-11^T, was found to have chemical and morphological properties consistent with its classification in the genus *Streptomyces*. An almost complete 16S rRNA gene (rDNA) sequence determined for the test strain was compared with those of previously studied streptomycetes by using two treeing algorithms. The 16S rDNA sequence data not only supported classification of the strain in the genus *Streptomyces* but also showed that it formed a distinct phyletic line. At maturity, the aerial hyphae of strain ATB-11^T differentiated into tight, spiral chains of rugose, cylindrical spores. The organism was readily distinguished from representatives of validly described *Streptomyces* species with rugose spores by using a combination of phenotypic features. It is proposed, therefore, that strain ATB-11^T be classified in the genus *Streptomyces* as *Streptomyces malaysiensis* sp. nov.

Keywords: *Streptomyces malaysiensis* sp. nov., polyphasic taxonomy, mesophile, soil

INTRODUCTION

The genus *Streptomyces* was proposed by Waksman & Henrici (1943) for aerobic, spore-forming actinomycetes. Members of this genus are now known to share many phenotypic characteristics and to constitute a distinct phyletic line, and they are currently assigned to over 450 validly described species (Williams *et al.*, 1989; Embley & Stackebrandt, 1994; Goodfellow *et al.*, 1997). It is also clear that minimal standards for the description of *Streptomyces* species need to be based on a judicious selection of genotypic and phenotypic properties (Manfio *et al.*, 1995; Chun *et al.*, 1997b; Labeda *et al.*, 1997; Kim *et al.*, 1996, 1998, 1999).

Streptomycetes continue to be a rich source of novel bioactive compounds, including antibiotics, anti-tumour agents and enzymes (Bérdy, 1995; Chun *et al.*, 1997b; Labeda *et al.*, 1997). During a routine screening programme, an actinomycete, designated strain ATB-11^T, was isolated from a soil sample and shown to have a broad spectrum of activity against pathogenic fungi

(Al-Tai *et al.*, 1995). A cursory examination of the organism showed that it had properties consistent with its assignment to the genus *Streptomyces*. In the present investigation, strain ATB-11^T was the subject of a polyphasic study designed to establish whether or not it represents a new species in the genus *Streptomyces*.

METHODS

Organisms and cultural conditions. Strain ATB-11^T was isolated on a yeast extract/malt extract agar (ISP Medium 2; Difco) plate, supplemented with 0.3% (w/v) NaCl, which had been seeded with a soil suspension and incubated at 28 °C for 14 d. The environmental sample was collected from Tasek Bera, Pahang Province, Malaysia; Tasek Bera is one of two major, freshwater, swamp lakes in Malaysia. The isolate was maintained on modified Bennett's Agar slants (Jones, 1949) at 4 °C and as glycerol suspensions (20%, v/v) at –20 °C. Biomass for the chemical and molecular systematic studies was obtained by growing the test organism in shake flasks (about 150 r.p.m.) of ISP Medium 2 broth, supplemented with 0.3% (w/v) NaCl, at 30 °C for 4 d. The cells for the chemical studies were washed in distilled water and freeze-dried; those required for the molecular systematic investigations were washed in NaCl/EDTA buffer (0.1 M

The GenBank accession number for the 16S rDNA sequence of ATB-11^T is AF117304.

Table 1. Growth and cultural characteristics of strain ATB-11^T

+++ , Abundant growth; ++ , moderate growth; + , poor growth; ± , sparse growth.

Agar medium	Growth	Aerial mycelium	Substrate mycelium	Soluble pigment
Asparagine	±	White/grey; sparse	Pale yellow/grey	None
Modified Bennett's	++	White; moderate	Brown	Light brown
Corn meal	++	Grey; moderate	Yellow/grey	None
Inorganic salts/starch	+++	Smoky black; abundant	Grey	Yellow
Nutrient	++	White; sparse	Light yellow	None
Oatmeal	+++	Smoky black; abundant	Yellow/brown	None
Potato dextrose	+++	Grey; abundant	Brown/grey	Brown
Sabouraud	+++	White/brown; moderate	Light brown	Light yellow/brown
Tryptic soy	+	White; sparse	Yellow	None
Tyrosine	+++	Grey; abundant	Brown/grey	Dark brown
Yeast extract/malt extract	+++	Dark grey; abundant	Brown/grey	Brown

Fig. 1. Scanning electron micrograph showing the spore-chain morphology and spore-surface ornamentation of strain ATB-11^T grown on inorganic salts/starch agar for 12 d at 28 °C. Bar, 1 µm.

EDTA, pH 8.0; 0.1 M NaCl) and stored at -20 °C until required.

Morphology and cultural characteristics. The spore-chain morphology of strain ATB-11^T was examined by light- and scanning electron microscopy (SEM) of 10–12 d cultures grown on inorganic salts/starch agar (ISP Medium 4; Difco). Spore-surface ornamentation was determined from SEM preparations, as described previously (O'Donnell *et al.*, 1993). Aerial spore-mass colour, substrate mycelial pigmentation and the production of diffusible pigments were recorded on a number of agar media (Table 1) incubated at 28 °C for 14 d.

Phenotypic tests. Degradation of casein, starch, tyrosine, and xanthine was determined according to Gordon *et al.* (1974), hydrogen sulphide production and coagulation of milk were determined as described by Cowan & Steel (1974), lysozyme resistance was determined according to Gordon & Barnett (1977), growth in the presence of sodium chloride was determined according to Tresner *et al.* (1968), and

growth on 16 sole carbon sources (Table 2) was determined on carbon utilization agar (ISP Medium 9; Difco) as described by Williams *et al.* (1983). Strain ATB-11^T was also examined for its ability to grow in the presence of nine antibiotics (Table 2); 6-mm sensitivity discs (Difco) were placed centrally in plates of ISP Medium 2 seeded with a loopful of 14 d ISP Medium 2 broth culture and inhibition zones observed after 2–5 d incubation at 28 °C were scored positive. Temperature and pH requirements were determined on ISP Medium 2 agar plates incubated for 14 d at 28 °C and the ability of the organism to inhibit the growth of three bacterial, one yeast, and five fungal strains (Table 2) was examined using an agar plug technique; the bacterial, yeast and fungal strains were inoculated on to nutrient (Difco), Sabouraud dextrose (Difco), and potato dextrose agar (Difco) plates, respectively. Agar discs (8–10 mm) of a 7 d culture of strain ATB-11^T, grown on inorganic salts/starch agar at 28 °C, were placed centrally on the seeded cultures and the preparations were then incubated at 37 °C for 24 h (bacteria), at 30 °C for 36 h (yeast), and at 26 °C for 5 d (fungi); zones of inhibition seen around the agar plugs were scored positive.

Chemotaxonomy. The isomeric form of diaminopimelic acid (A₂pm) of the test strain was determined according to Stanek & Roberts (1974), and the analysis of whole-organism sugars carried out using the procedure of Saddler *et al.* (1991). Menaquinones and phospholipids were extracted from freeze-dried biomass (about 100 mg) and examined using the integrated method of Minnikin *et al.* (1984), as described by Chun *et al.* (1997a). Freeze-dried biomass (50 mg) was also degraded by acid methanolysis (Minnikin *et al.*, 1980) and the resultant fatty acid methyl esters purified by preparative TLC then separated and quantified using GC according to Chun *et al.* (1997a).

Sequencing of 16S rDNA. Extraction of genomic DNA and PCR-amplification of the 16S rDNA from the test strain were carried out as described by Pitcher *et al.* (1989), using the modification of Kim *et al.* (1996). The amplified fragments were purified by gel electrophoresis and sequenced directly by using a Taq DyeDeoxy terminator cycle sequencing kit (Applied Biosystems) and previously described oligonucleotide primers (Chun & Goodfellow, 1995). Sequencing gel electrophoresis was carried out and the nucleotide sequences were automatically obtained by using

Table 2. Some phenotypic properties of strain ATB-11^T

Test	Result	Test	Result
Antimicrobial activity against:		Growth on sole carbon sources (1 %, w/v)	
<i>Bacillus subtilis</i> NCIB 3610	+	L-Arabinose	+
<i>Escherichia coli</i> NCIB 9132	—	D-Fructose	+
<i>Staphylococcus aureus</i> ATCC 12600	—	D-Galactose	+
<i>Candida albicans</i> *	+	D-Glucose	+
<i>Cuvularia lunata</i> †	+	meso-Inositol	+
<i>Fusarium solani</i> †	+	D-Maltose	+
<i>Pestalotiopsis guepinii</i> †	+	D-Mannitol	+
<i>Pyricularia oryzae</i> †	+	D-Mannose	+
<i>Trichoderma viridae</i> †	+	D-Raffinose	+
		α-L-Rhamnose	+
Decomposition of:		D-Sorbitol	—
Casein	+	Starch	+
Starch	+	D-Sucrose	—
L-Tyrosine	+	D-Xylose	+
Xanthine	—	CM-cellulose (0.1 %, w/v)	—
Growth at:		Sodium acetate (0.1 %, w/v)	—
10 °C	—	Sodium citrate (0.1 %, w/v)	—
20–40 °C	+	Resistance to antibiotics (μg):	
45 °C	—	Ampicillin (10)	+
pH 4.0	—	Chloramphenicol (30)	—(15)‡
pH 5–10	+	Erythromycin (15)	—(18)
pH 11.0	—	Gentamicin sulphate (10)	—(28)
Growth in the presence of:		Kanamycin sulphate (30)	—(34)
Lysozyme (0.005 %, w/v)	+	Neomycin sulphate (30)	—(30)
Sodium chloride (3 %, w/v)	+	Novobiocin sulphate (30)	—(40)
Sodium chloride (5 %, w/v)	—	Streptomycin sulphate (10)	—(35)
Production of:		Tetracycline hydrochloride (30)	—(20)
Hydrogen sulphide	—		

* Obtained from the Institute of Medical Research, Kuala Lumpur, Malaysia.

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‡ Numbers in parentheses indicate the diameters of the inhibition zones.

an Applied Biosystems DNA sequencer (model 373A) and software provided by the manufacturer.

Phylogenetic analysis. The 16S rDNA sequence of the test strain was aligned manually with available streptomycete nucleotide sequences retrieved from the Ribosomal Database Project (Maidak *et al.*, 1997) and EMBL/GenBank databases by using the AL16s program of Chun (1995). The resultant data set contained information on 1138 positions for 55 streptomycetes. Evolutionary trees were inferred by using two tree-making algorithms, namely, the neighbour-joining (Saitou & Nei, 1987) and least squares (Fitch & Margoliash, 1967) methods. Evolutionary distance matrices were generated as described by Jukes & Cantor (1969), and phylogenetic trees were constructed using the PHYLIP package (Felsenstein, 1993). The resultant unrooted tree topologies were evaluated by carrying out bootstrap analyses of the neighbour-joining method data based on 1000 resamplings using the SEQBOOT and CONSENSE programs in the PHYLIP package (Felsenstein, 1993). The root position of the unrooted tree based on the neighbour-joining method was estimated by using four outgroup organisms,

namely, *Arthrobacter globiformis* DSM 20124^T (accession no. M23411), *Bacillus subtilis* (K00637), *Escherichia coli* (J01695) and *Nocardia asteroides* ATCC 19247^T (Z36934), as described by Swofford & Olsen (1990).

DNA base composition. The base composition of genomic DNA isolated from strain ATB-11^T was determined as described by Kim *et al.* (1998).

RESULTS

Morphological and cultural characteristics

Strain ATB-11^T grew on a range of agar media (Table 1), showing morphology typical of streptomycetes. The colour of the substrate mycelium tended to be either brown/grey or yellow/grey and that of the aerial spore mass grey/smoky black. The organism also produced brownish, diffusible pigments on several agar media and formed melanin pigments on tyrosine agar plates. Aerial hyphae differentiated into long,

Fig. 2. Neighbour-joining tree (Saitou & Nei, 1987) of streptomycetes, based on partial 16S rDNA sequences (1138 nucleotide positions). The asterisks denote the branches that were also recovered using the least-squares treeing algorithm (Fitch & Margoliash, 1967). The numbers at the nodes indicate the level of bootstrap support (%) based on a neighbour-joining analysis of 1000 resampled data sets. The arrow indicates the estimated root position of the tree. Scale bar, 0.01 substitutions per nucleotide position.

spiral chains of cylindrical spores with rugose ornamentation (Fig. 1).

Phenotypic properties

The phenotypic profile of strain ATB-11^T is shown in Table 2.

Chemotaxonomy

The absence of characteristic sugars and the presence of LL-A₂pm in whole-organism hydrolysates showed that strain ATB-11^T had a wall chemotype I *sensu* Lechevalier & Lechevalier (1970). The organism also contained hexahydrogenated menaquinone with nine

isoprene units as the predominant isoprenologue, was rich in saturated straight-chain and iso- and anteiso-fatty acids (fatty acid type 2c *sensu* Kroppenstedt, 1985), but lacked mycolic acids. Diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol mannosides were present in the polar lipid fraction (phospholipid type II *sensu* Lechevalier *et al.*, 1977). The G + C ratio of the DNA of the strain was 72.2 mol %.

16S rDNA sequences

An almost complete 16S rDNA sequence was determined for strain ATB-11^T (1467 nucleotides). This sequence served to distinguish it from other streptomycetes for which full or partial nucleotide sequences are available (Fig. 2, Table 3), notably from its nearest neighbours, namely, '*Streptomyces vellosus*' NRRL 8037 (97.21% nucleotide similarity), *Streptomyces griseocarneus* DSM 40004^T (97.14%), *Streptomyces mashiensis* ISP 5221^T (97.14%), *Streptomyces thermoviolaceus* subsp. *apingens* DSM 41392^T (97.11%), *Streptomyces thermocarboxydus* DSM 44293^T (96.94%) and *Streptomyces abikoensis* DSM 40831^T (96.94%).

DISCUSSION

The chemical and morphological properties of the test strain are consistent with its assignment to the genus *Streptomyces* (Williams *et al.*, 1989; Manfio *et al.*, 1995). It is also clear that the organism forms a distinct phyletic line in the 16S rDNA tree and belongs to the group of streptomycetes which characteristically produce spores with rugose ornamentation. Streptomycetes with rugose spores were assigned to a single taxon, cluster 32, in the numerical phenetic survey of Williams *et al.* (1983). However, this cluster was subsequently shown to be heterogeneous on the basis of both DNA relatedness (Labeda & Lyons, 1991) and extensive numerical taxonomic (Kämpfer *et al.*, 1991) data, although the type strains of *Streptomyces hygroscopicus* and *Streptomyces violaceusniger* share an identical ribosomal AT-L30 protein sequence (Ochi, 1995).

Streptomycetes that form rugose spores are currently assigned to four validly described species, namely, *S. hygroscopicus*, *Streptomyces melanosporofaciens*, *Streptomyces sparsogenes* and *S. violaceusniger*, though there is evidence that they are underspecified (Kämpfer *et al.*, 1991; Labeda & Lyons, 1991). Representatives of the four validly described taxa form distinct genomic species (Labeda & Lyons, 1991) and can also be distinguished using a combination of phenotypic features (Williams *et al.*, 1983; Kämpfer *et al.*, 1991; Labeda & Lyons, 1991). It is now apparent that members of these taxa were assigned to cluster 32 *sensu* Williams *et al.* (1983), mainly because of a test sampling error; the strains in this taxon shared 85 out of 139 unit characters (Manfio, 1995).

Table 3. 16S rDNA similarity values between strain ATB-11^T and representatives of the genus *Streptomyces*

Strains were type cultures unless otherwise stated. ATCC, American Type Culture Collection, 10801 University Boulevard, Manassas, VA, USA; DPDU, Instituto di difesa delle Plante, Universita degli Studi di Udine, Udine, Italy; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; ISP, International *Streptomyces* Project; NRRL, Northern Regional Research Laboratory, Agricultural Research Service, US Department of Agriculture, Peoria, IL, USA.

<i>Streptomyces</i> species	Similarity to strain ATB-11 ^T	No. nucleotide differences/ Total no. nucleotides compared
<i>S. abikoensis</i>	96.94	34/1110
<i>S. acidiscabies</i>	96.87	46/1469
<i>S. albidoflavus</i>	96.83	46/1451
<i>S. albus</i> subsp. <i>albus</i>	96.37	43/1186
<i>S. ambofaciens</i>	96.05	58/1469
<i>S. bikiniensis</i>	96.05	58/1469
<i>S. bluensis</i>	96.60	50/1472
<i>S. bottropensis</i>	95.85	61/1470
<i>S. brasiliensis</i>	95.70	51/1186
<i>S. caelestis</i>	96.32	54/1469
' <i>S. cinnamoneus</i> subsp. <i>azacoluta</i> ' DPDU 0074	94.93	57/1124
<i>S. cinnamoneus</i> subsp. <i>cinnamoneus</i>	96.56	40/1163
<i>S. diastaticus</i> subsp. <i>diastaticus</i>	95.60	50/1136
<i>S. diastatochromogenes</i>	95.92	60/1470
' <i>S. espinosus</i> ' NRRL 5729	95.99	59/1470
<i>S. eurythermus</i>	96.26	55/1471
<i>S. galbus</i>	96.12	57/1469
<i>S. glaucescens</i> DSM 40716	96.87	46/1469
<i>S. gougerotii</i>	96.34	53/1450
<i>S. griseocarneus</i>	97.14	42/1469
<i>S. griseus</i> subsp. <i>griseus</i>	96.46	52/1468
' <i>S. hygroscopicus</i> subsp. <i>limoneus</i> ' ATCC 21431	95.92	60/1469
<i>S. intermedius</i>	96.34	53/1450
<i>S. lavendulae</i> subsp. <i>lavendulae</i>	96.08	57/1454
<i>S. lincolnensis</i>	95.98	59/1469
' <i>S. luteoreticuli</i> ' ISP 5509	95.11	55/1124
<i>S. macrosporus</i>	95.80	61/1452
<i>S. mashuensis</i>	97.14	42/1471
<i>S. megasporus</i>	94.91	74/1454
<i>S. mobaraensis</i> ISP 5587	96.37	41/1129
<i>S. neyagawaensis</i>	96.39	53/1470
<i>S. olivoreticuli</i> subsp. <i>cellulophilus</i>	96.03	46/1159
<i>S. pseudogriseolus</i> NRRL 3985	96.39	53/1467
<i>S. purpureus</i> DSM 43360	95.41	52/1134
<i>S. rimosus</i> R6-554	96.73	48/1468
<i>S. roseovorticillatus</i> DPDU 0819	95.61	48/1093
<i>S. rutgersensis</i> subsp. <i>rutgersensis</i>	96.41	52/1450
<i>S. salmonis</i>	96.02	43/1080
<i>S. scabiei</i>	96.19	56/1469
<i>S. seoulensis</i>	95.38	67/1451
<i>S. setonii</i>	96.35	53/1454
<i>S. subrutilus</i>	95.78	62/1468
<i>S. tendae</i>	96.53	51/1469
<i>S. thermocarboxydovorans</i>	96.53	51/1469
<i>S. thermocarboxydus</i>	96.94	45/1472
<i>S. thermodiastaticus</i>	96.49	51/1455
<i>S. thermolineatus</i>	95.94	59/1452
<i>S. thermoviolaceus</i> subsp. <i>apingens</i>	97.11	42/1455
<i>S. thermoviolaceus</i> subsp. <i>thermoviolaceus</i>	96.84	46/1455
<i>S. thermovulgaris</i>	96.56	50/1453
<i>S. thermovulgaris</i> ISP 5579	96.49	51/1454
' <i>S. vellosus</i> ' NRRL 8037	97.21	41/1472
<i>S. violaceoruber</i> A(3)2	95.92	60/1469
<i>S. virginiae</i>	96.18	56/1465

Table 4. Phenotypic properties separating representative streptomycetes that form spiral chains of rugose spores and a grey, aerial spore mass

Phenotypic property	Strain ATB-11 ^T	<i>S. hygroscopicus</i> ISP 5578 ^{T*}	<i>S. melanosporofaciens</i> ISP 5318 ^{T*}	<i>S. sparsogenes</i> ISP 5356 ^{T*}	<i>S. violaceusniger</i> ISP 5563 ^{T*}
Melanin production on tyrosine agar	+	—	—	—	—
Production of diffusible pigments	+	—	—	—	—
Production of hydrogen sulphide	—	+	+	+	+
Antimicrobial activity against					
<i>Bacillus subtilis</i>	+	+	+	—	+
<i>Candida albicans</i>	+	—	+	—	—
Growth at:					
37 °C	+	+	—	+	+
45 °C	—	+	—	+	—
Growth on sole carbon sources					
D-Fructose	+	+	+	+	—
D-Galactose	+	+	+	—	+
meso-Inositol	+	+	+	—	+
D-Raffinose	+	—	+	+	+
D-Sucrose	—	—	—	+	+
D-Xylose	+	+	+	—	—
Sodium acetate	—	+	+	—	+
Sodium citrate	—	+	+	—	—

* Data for *S. hygroscopicus*, *S. melanosporofaciens*, *S. sparsogenes* and *S. violaceusniger* are from Williams *et al.* (1983).

Strain ATB-11^T can be separated from representative strains of *S. hygroscopicus*, *S. melanosporofaciens*, *S. sparsogenes* and *S. violaceusniger* on the basis of 16S rDNA sequence data (D. Saintpierre & M. Goodfellow, unpublished results) and by using a combination of phenotypic properties (Table 4). The phenotypic properties have high diagnostic scores (Manfio, 1995) based on the use of the DIACHAR program (Sneath, 1980), as implemented in the x program (Chun, 1995). Manfio (1995) also found that representatives of the four validly described species can be distinguished from members of all of the other streptomycete taxa circumscribed in the numerical phenetic survey of Williams *et al.* (1983) by means of a combination of phenotypic properties. These observations are in line with those from recent studies, which show that differential phenotypic properties, notably morphological and pigmentation features, highlighted in numerical phenetic studies can be reliably weighted for the differentiation of closely related streptomycete species (Kim *et al.*, 1998, 1999).

The genotypic and phenotypic data show that strain ATB-11^T forms a new centre of taxonomic variation within the genus *Streptomyces*. Therefore it is proposed that this organism should be recognized as a new species of the genus *Streptomyces*, i.e. *Streptomyces malaysiensis* sp. nov.

Description of *Streptomyces malaysiensis* sp. nov.

Streptomyces malaysiensis (mal.ay.si.en'sis. M.L. adj. *malaysiensis* belonging/pertaining to Malaysia, the

source of the soil from which the organism was isolated).

The organism forms extensively branched substrate hyphae (0.3–0.5 µm in diameter) and aerial hyphae which differentiate into tight, spiral spore chains. The appearance of the spores ranges from cylindrical to barrel-shaped (1.3–1.0 × 1.5 µm) and the spore surface is rugose. On inorganic salts/starch agar the aerial spore-mass colour is grey, becoming smoky black and slimy at maturity; the reverse is grey, and a yellow, diffusible pigment is formed. Growth does not occur at 10 °C or at 45 °C. Xanthine is not degraded. Additional phenotypic properties are listed in Table 2. The G + C ratio of the genomic DNA is 72.2 mol%. The organism was isolated from a soil sample collected at Tasek Bera, Malaysia's first Ramsar Site. The type strain is ATB-11^T (= DSM 41697^T). The species description is based on a single strain and hence serves as the type strain description.

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