Taxonomic study of a chromomycin-producing strain and reclassification of *Streptomyces cavourensis* subsp. *washingtonensis* as a later synonym of *Streptomyces griseus*

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A chromomycin-producing actinomycete, strain AP19, was isolated from a sample of faeces collected from Foping national nature reserve in China. Chemotaxonomic and morphological properties indicated that the novel isolate was a member of the genus *Streptomyces*. Phylogenetic analyses based on an almost complete 16S rRNA gene sequence of the strain and on the 120-nt nucleotide variable γ -region of this molecule revealed that it was closely related to *Streptomyces griseus* ISP 5236^T and *Streptomyces cavourensis* subsp. *washingtonensis* ATCC 27732^T. DNA–DNA relatedness values among these strains were above 70%. *Streptomyces cavourensis* subsp. *washingtonensis* could be readily distinguished from *Streptomyces cavourensis* ATCC 14889^T by differing BOX-PCR fingerprinting patterns, relatively low 16S rRNA gene sequence similarity and a low DNA–DNA relatedness value. It is proposed, therefore, that *Streptomyces cavourensis* subsp. *washingtonensis* is a later synonym of *Streptomyces griseus*.

Conventional classification methods for the identification of species within the genus *Streptomyces* have mainly relied on the morphological and phenotypic characteristics of the organisms. During the last decade, molecular biological methods such as 16S rRNA gene sequencing and BOX-PCR fingerprinting have had an increasing impact on streptomycete taxonomy (Kim & Goodfellow, 2002; Kim *et al.*, 2004; Lanoot *et al.*, 2004; Saintpierre *et al.*, 2003; Williams *et al.*, 1983). Molecular systematic data show that the genus is clearly overspeciated (Hatano *et al.*, 2003; Lanoot *et al.*, 2002, 2004), as exemplified by the proposal that *Streptomyces lipmanii* LMG 20047^T, *Streptomyces willmorei* LMG 21046^T, *Streptomyces griseus* subsp. *alpha* LMG 19953^T and *Streptomyces griseus* subsp. *cretosus* LMG 19946^T should be recognized as heterotypic synonyms of *Streptomyces microflavus* LMG 19327^T (Lanoot *et al.*, 2005).

The present study describes the taxonomic position of a chromomycin-producing strain, AP19, isolated from faeces. The sample of faeces was collected from Foping national nature reserve, Shanxi province, China.

Abbreviation: DAP, diaminopimelic acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain AP19 is EF466100.

A figure showing the BOX-PCR patterns of strain AP19 and other closely related strains is available with the online version of this paper.

Phenotypic and phylogenetic data showed that the novel strain was closely related to *Streptomyces cavourensis* subsp. *washingtonensis* NRRL B-8030^T and *Streptomyces griseus* ISP 5236^T. In the phylogenetic tree based on 16S rRNA gene sequences, these strains formed a clade in the *Streptomyces* tree, indicating that they could belong to the same taxon.

Strain AP19 was isolated on Gause's synthetic agar plates supplemented with 2 µg potassium dichromate. The plates were seeded with a faeces sample suspension and incubated at 28 °C for 14 days. The isolate was maintained on Gause's synthetic slopes at 4 °C and as glycerol suspensions (20%, v/v) at –20 °C. All other strains used in this study, namely *Streptomyces albobiridis* NRRL B-3633^T, *Streptomyces cavourensis* ATCC 14889^T, *S. cavourensis* subsp. *washingtonensis* ATCC 27732^T, *Streptomyces erumpens* NRRL B-3163^T, *Streptomyces fulvorobeus* NRRL B-24329^T, *Streptomyces griseus* AS 4.1419^T, *S. griseus* ATCC 13273, *S. griseus* subsp. *solivifaciens* NRRL B-1561^T and *Streptomyces microflavus* AS 4.1428^T, were grown on medium 65 agar (0.4% w/v glucose, 0.4% w/v yeast extract, 1% w/v malt extract, 0.2% w/v CaCO₃; final pH 7.2) and incubated at 30 °C. For large-scale strain preparation, a 250 ml flask with 50 ml medium 65 broth (0.4% w/v glucose, 0.4% w/v yeast extract, 1% w/v malt extract; final pH 7.2) was shaken for 36 h at 30 °C.

The morphological characteristics of strain AP19 grown on inorganic salt/starch agar at 28 °C for 14 days were observed by light microscopy and scanning electron microscopy. The cultural, physiological and biochemical characteristics of the tested strains were determined as described by Williams *et al.* (1983). The diaminopimelic acid (DAP) isomer and whole-organism sugars pattern were analysed according to the method of Hasegawa *et al.* (1983). The G + C content of the DNA of the novel strain was determined using the thermal denaturation method (Marmur & Doty, 1962).

Genomic DNA was extracted and the 16S rRNA gene sequence was amplified as described by Chun & Goodfellow (1995). The PCR products were ligated into the PCR2.1-TOPO vector and transformed into *Escherichia coli* TOP 10. The 16S rRNA gene sequence of strain AP19, inserted into the plasmid vector, was sequenced on a model 3730 Applied Biosystems DNA sequencer.

The sequence was analysed along with the sequences of closely related reference organisms retrieved from the DDBJ/EMBL/GenBank databases. Sequence data were aligned with CLUSTAL_X software, version 1.8 (Thompson *et al.*, 1997). Phylogenetic trees were constructed by using neighbour-joining (Saitou & Nei, 1987), minimum-evolu-

tion (Edwards & Cavalli-Sforza, 1963) and maximum-parsimony (Kluge & Farris, 1969) algorithms with the MEGA 3 software package (Kumar *et al.*, 2004). Evolutionary distance matrices were generated as described by Kimura (1980). Tree topologies were evaluated by bootstrap analyses based on 1000 resamplings (Fig. 1). The partial sequence covering the variable γ -region (120 nt, positions 158–277 according to the *E. coli* numbering system) of the 16S rRNA gene sequence of strain AP19 was also compared with the corresponding nucleotide sequences of type strains of the genus *Streptomyces* retrieved from GenBank. A phylogenetic tree based on these partial sequences was constructed using the neighbour-joining algorithm (Saitou & Nei, 1987) (Fig. 2).

BOX-PCR fingerprint patterns were analysed as described by Lanoot *et al.* (2004), apart from some modifications in the electrophoresis conditions. A 25 μ l sample mixed with 5 μ l loading dye (6 $\times$) was loaded on a 2.5% agarose gel (Biowest Agar, 10 $\times$ 10 cm). Every fourth well was loaded with 5 μ l molecular ruler (200 bp). Electrophoresis was performed using 90 V, 60 mA for 210 min in 0.5 $\times$ TBE (0.54% Tris, 0.27% boric acid, 2 ml 0.5 M EDTA; pH 8.0).

The chemical and morphological properties of isolate AP19 were consistent with its assignment to the genus

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences from strain AP19 and other related organisms. The tree was constructed using the neighbour-joining algorithm. Asterisks indicate branches that were recovered using the minimum-evolution and maximum-parsimony algorithms; m indicates branches that were also formed when minimum-evolution tree-making algorithm was used. Bootstrap values (>50%) based on 1000 replications are shown at nodes of the tree. Bar, 0.002 substitutions per nucleotide position.

Fig. 2. Neighbour-joining tree based on the variable γ -region (120 nt, positions 158–277) of the 16S rRNA gene of strain AP19 and other related species of the genus *Streptomyces*. Evolutionary distance matrices were generated as described by Kimura (1980). Bootstrap values (>50 %) based on 1000 replications are shown at nodes of the tree. Bar, 0.01 substitutions per nucleotide position.

Streptomyces. It formed extensive branching substrate mycelium and aerial hyphae with smooth-surfaced spores in rectiflexible spore chains. DAP analysis revealed the presence of LL-diaminopimelic acid in the cell-wall peptidoglycan. No characteristic sugar was detected in the whole-cell hydrolysates. The G+C content of the genomic DNA of strain AP19 was 71 mol%.

An almost complete 16S rRNA gene sequence (1478 nt) was determined for the novel strain. Primary sequence analysis with sequences of representatives of the family *Streptomycetaceae* confirmed that the novel isolate was closely related to species of the genus *Streptomyces*. In the phylogenetic tree based on almost complete 16S rRNA gene sequences (Fig. 1), sequence similarity values between strain AP19 and its nearest neighbours, namely *Streptomyces griseorubiginosus* NBRC 13047^T, *S. albobiridis* NBRC 13013^T, *S. globosus* NBRC 15874, *Streptomyces microflavus* NBRC 13062^T and *Streptomyces fulvorubens* NBRC 15897^T, were 99.93 % (1 nt difference at 1476 sites), 99.93 % (1 nt difference at 1475 sites), 99.93 % (1 nt difference at 1473 sites), 99.93 % (1 nt difference at 1478 sites) and 99.86 % (2 nt differences at 1472 sites). Besides the strains mentioned above, two strains that shared many phenotypic characteristics with strain AP19, *S. cavourensis* subsp. *washingtonensis* NRRL B-8030^T and *S. griseus* ISP 5236^T, also showed high 16S rRNA gene sequence similarities of 99.93 % (1 nt difference at 1478 sites) and 99.65 % (5 nt differences at 1448 sites), respectively.

BOX-PCR fingerprinting has been reported as a powerful tool to distinguish finer taxonomic relationships in the genus *Streptomyces* (Lanoot *et al.*, 2004). The BOX-PCR fingerprinting patterns of strain AP19 and the closely related strain, *S. cavourensis* subsp. *washingtonensis* ATCC 27732^T were very similar (see Supplementary Fig. S1 in IJSEM Online). These two strains formed a phylogenetic cluster, joining the phylogenetic clade comprising *S. griseus* AS 4.1419^T, *S. griseus* ATCC 13273 and *S. griseus* subsp. *solivifaciens* NRRL B-1561^T. It was also evident that strain AP19 and *S. cavourensis* subsp. *washingtonensis* ATCC 27732^T were separated from the nearest phylogenetic neighbours, *S. albobiridis* NRRL B-3633^T, *S. microflavus* AS 4.1428^T, *S. erumpens* NRRL B-3163^T and *S. fulvorubens* NRRL B-24329^T. Furthermore, distinct BOX-PCR fingerprinting patterns were found between *S. cavourensis* ATCC 14889^T and *S. cavourensis* subsp. *washingtonensis* ATCC 27732^T. This was consistent with the relatively low levels of 16S rRNA gene sequence similarity (98.9 %) between these strains and the fact that they belonged to different clusters in the phylogenetic tree based on 16S rRNA gene sequences. These data indicate that strains AP19 and *S. cavourensis* subsp. *washingtonensis* ATCC 27732^T can be classified as *S. griseus*.

A DNA–DNA hybridization study provided further evidence for the taxonomic classification of strain AP19. High DNA–DNA relatedness values, in the range from 75.2 to 94.2 %, were found between strain AP19, *S. cavourensis*

Table 1. DNA–DNA reassociation values between strain AP19 and some related species of the genus *Streptomyces*

Strain	Hybridization (%) with labelled DNA from:			
	1	2	3	4
1. AP19	100			
2. <i>S. griseus</i> ATCC 13273	94.2	100		
3. <i>S. cavourensis</i> subsp. <i>washingtonensis</i> ATCC 27732 ^T	75.2	80.1	100	
4. <i>S. cavourensis</i> ATCC 14889 ^T	54.8	64.3	51.3	100

Table 2. Phenotypic characteristics of strain AP19 and some related strains

Strains: 1, AP19; 2, *S. cavourensis* subsp. *washingtonensis* ATCC 27732^T; 3, *S. griseus* ATCC 13273; 4, *S. cavourensis* ATCC 14889^T. All strains produced white aerial spore mass on oatmeal agar and had a rectiflexible spore chain arrangement and a smooth spore surface. For *S. cavourensis* ATCC 14889^T, melanin was produced during tests for the degradation of casein, guanine, L-tyrosine and xanthine. +, Positive; –, negative.

Characteristic	1	2	3	4
Melanin production	–	–	–	+
Production of diffusible pigments	Greenish yellow	Greenish yellow	Yellow	Pale orange–yellow
Degradation of:				
Cellulose	+	+	+	–
Guanine	+	+	+	–
Xanthine	+	+	+	–
Growth on sole carbon source (1.0 %, w/v):				
Adonitol	+	+	+	–
Production of antibiotic	Chromomycin	Chromomycin	Chromomycin	Flavensomycin

subsp. *washingtonensis* ATCC 27732^T and *S. griseus* ATCC 13273 (Table 1). The DNA–DNA relatedness value between *S. cavourensis* subsp. *washingtonensis* ATCC 27732^T and *S. cavourensis* ATCC 14889^T was 51.3 %, which is below the 70 % cut-off point for recognition of genomic species (Wayne *et al.*, 1987). Furthermore, *S. cavourensis* subsp. *washingtonensis* ATCC 27732^T could be distinguished from *S. cavourensis* ATCC 14889^T by distinct BOX-PCR fingerprinting patterns, different cultural characteristics on standard media and a set of physiological features (Table 2). Phylogenetic trees based on 16S rRNA gene sequences and the 120 nt γ -region of the 16S rRNA gene sequence in which *S. cavourensis* subsp. *washingtonensis* ATCC 27732^T and *S. griseus* ISP 5236^T formed a clade, distinct from *S. cavourensis* ATCC 14889^T, also supported this assignment (Figs 1, 2).

Based on the genotypic and phenotypic evidence, *Streptomyces cavourensis* subsp. *washingtonensis* ATCC 27732^T should be considered as a later heterotypic synonym of *Streptomyces griseus* ISP 5236^T and strain AP19 should also be assigned to this taxon.

Acknowledgements

This contribution was supported by grants from the National Nature Science Foundation of China. The authors are grateful to Dr Ying Huang (Chinese Academy of Sciences, China) for her help in the identification of strain AP19 and to Mrs Zhiyun Wang for her

assistance in strain isolation. The authors are indebted to Dr Labeda (Microbial Genomics and Bioprocessing Research Unit, America) for providing some of the type strains.

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