***Streptomyces thermogriseus*, a new species of the genus *Streptomyces* from soil, lake and hot-spring**

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Many thermophilic actinomycetes were isolated from samples collected from a hot-spring, lake and soil in Yunnan, China. Chemical and molecular classification of four selected strains of thermophilic *Streptomyces* with an upper limited growth temperature of 65–68 °C and autolytic characteristics was carried out. A new species, *Streptomyces thermogriseus* sp. nov. is described. The type strain is Y-14046^T (= CCTCC AA97014^T).

Keywords: *Streptomyces thermogriseus* sp. nov., thermophile

INTRODUCTION

Thermophilic actinomycetes are useful as producing organisms of antibiotics, enzymes and other bioactive metabolites because of their rapid growth rate and rapid autolysis of mycelium (11, 12, 17). They are useful for studying the evolution and adaptation mechanism of organisms to extreme environments. They are also an important resource for development of industrial products (2, 11, 12, 15, 16, 17).

We have isolated many thermophilic streptomycetes from hot-springs, lakes and soils in the whole of Yunnan. We studied the morphological, cultural, physiological and biochemical characteristics, gel electrophoretic patterns of isoenzymes, cell composition and sequences of 16S rRNA genes of four selected isolates of *Streptomyces*. A new species with the name *Streptomyces thermogriseus* sp. nov. was described by comparing relative species of the genus.

METHODS

Actinomycete strains. The soil or sediment samples were collected from forests, hot-springs and lakes in Yunnan. Thermophilic streptomycetes were isolated using ISP 2, ISP 3, ISP 5, colloidal chitin agar (4), AV agar (13) and HV agar (3) media after the pretreatment of air-dried samples at 120 °C for 1 h, and incubated at 55 or 60 °C for 3–7 d. After primary purification, the isolates were incubated on ISP 2 agar medium. Strain Y-4027 was isolated from soil samples in Kunming, Y-5114 from Chenghai Lake, Y-14046^T and Y-14082 from a hot-spring in Eryuan, Yunnan, China. These four strains, which have autolytic characteristics, were selected for the classification studies. Some reference strains for comparison were obtained from ATCC, IFO, NRRL, CCRC and CCCC.

Morphology. The media used for micromorphological studies were yeast extract-malt extract agar (ISP 2) and oatmeal agar (ISP 3) (14), and the incubation time was 5–10 d at 55 °C. Morphological observations of spores were made with a light microscope and a model EPMA-8705 electron microscope.

Cultural, physiological and biochemical tests. The media and procedures used for determining the cultural and physiological characteristics, and carbon and nitrogen source utilization of the strains were those described by Shirling & Gottlieb (14) and Locci (8). Colour determinations were made by comparing the cultures with colour chips from the ISCC-NBS color charts standard samples no. 2106 (6).

Extraction of isoenzymes. Each strain was grown on yeast extract-malt extract broth with shaking at 55 °C to late-exponential growth phase. The cells were harvested by centrifugation at 4000 g for 10 min and were washed with distilled water. The cells were stored at –20 °C until use. The cells were disrupted with 0.1 mm glass beads in a mortar for 20 min in 0.1 M Tris/HCl buffer (pH 7.5). Homogenates were centrifuged at 15000 g for 20 min, and supernatants containing isoenzymes were decanted out. The isoenzyme extracts were stored at –20 °C.

Polyacrylamide gels were prepared by the method of Maurer (9). A 3.0% (w/v) (pH 6.7) large-pore upper gel and a 7.5% (pH 8.9) small-pore separation gel were prepared. The electrode buffer contained 0.005 M Tris, 0.038 M glycine (pH 8.3). The gel electrophoresis was performed at 10–20 mA per gel slab for 5–6 h.

Staining procedures. The six isoenzymes tested were alcohol dehydrogenase (ADH; EC 1.1.1.1), malate dehydrogenase (MDH; EC 1.1.1.37), glutamate dehydrogenase (GDH; EC 1.4.1.4), glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49), esterase (EST; EC 3.1.1.1) and superoxide dismutase (SOD; EC 1.15.1.1). The staining procedures used for these enzymes were described by Weeden & Wendel (18) and Wendel & Weeden (19). After

staining, the gels were dried with a model 583 gel dryer. The relative mobilities (R_m) of the enzyme bands were calculated as the ratio between the mobility distance of the enzymes and the mobility distance of the tracking dye from the origin. A sample was tested at least twice for each enzyme.

Chemical composition of the cell wall. Cell walls were purified and analysed using the method of Lechevalier & Lechevalier (7). The procedures of Becker *et al.* (1) and Lechevalier & Lechevalier (7) were used for analysis of whole-cell chemical composition.

16S rRNA gene sequence determination and analysis. Genomic DNAs were extracted using the DNA extraction procedures (5, 20) and were used for PCR amplification of 16S rRNA genes. The 16S rRNA gene was amplified by PCR in a reaction mixture containing 1 × PCR buffer (Sino-American Biotechnology), primer A 5–24r (5'-CCG TCG ACG AGC TCA GAG TTT GAT CCT GGC TCA G-3') and primer B 1525–1506r (5'-CCC GGG TAC CAA GCT TAA GGA GGT GAT CCA GCC GCA-3'). The final volume of the PCR mixture was adjusted to 50 µl by adding distilled H₂O and the reaction mixture was overlaid with 100 µl sterile mineral oil. Thermal cycling was performed with a model 1109 DNA Amplifier made in China. The samples were subjected to an initial denaturing step consisting of 3 min at 95 °C, after which 2 U *Taq* polymerase was added to each sample at 90 °C. The thermal profile used was 35 cycles consisting of 1 min of primer annealing at 56 °C, 2 min extension at 72 °C and 1 min denaturation at 95 °C. A final extension step consisting of 10 min at 72 °C was included. Amplified DNA was detected by agarose gel electrophoresis and visualized by UV fluorescence after ethidium bromide staining.

The PCR product was purified and concentrated using agarose gel electrophoresis. The purified PCR products were directly sequenced. The sequencing reactions were performed with a DyeDeoxy Terminator Cycle Sequencing Kit using AmpliTaq FS (Applied Biosystems). The sequencing primers used were A, B and C 1115–1100r (5'-AGG GTT GCG CTC GTT G-3'). The sequencing reaction mixtures were electrophoresed on a 6% polyacrylamide sequencing gel using a model 377 Prism Automatic DNA sequencer (Applied Biosystems).

The 16S rRNA gene sequences were manually aligned with the sequences of members of the order *Actinomycetales* using the ac 2 editor (10). Genetic distances were calculated by using the PAUA 3.11. Phylogenetic dendrograms were reconstructed using treeing algorithms contained in the PHYLIP package. Bootstrap analyses were used to evaluate the tree topologies of the neighbour-joining data by performing 100 or 500 resamplings.

RESULTS AND DISCUSSION

Morphological observation

Morphological observation of the 3–7 d-old culture of strains Y-4027, Y-5114, Y-14046^T and Y-14082 grown on yeast extract-malt extract agar revealed an abundant aerial mycelium (0.5–1.5 µm in diameter) which could arise from substrate mycelium. Spore chains with many spores were *Rectiflexibiles*, *Retinaculiaperti* or occasionally *Spirales*. The spores were spherical or subspherical, and non-motile. The spores of strain Y-4027 were spherical or subspherical (1 µm in diameter),

Fig. 1. Morphological characteristics of *Streptomyces thermogriseus*.

those of strain Y-5114 were short rods (1 × 1.5–2.0 µm) and those of strains Y-14046^T and Y-14082 were spherical or subspherical (0.7 µm in diameter). The spore surface was smooth (Fig. 1).

Cultural characteristics

Cultural characteristics of the four strains are shown in Table 1. Strain Y-4027 produced sparse aerial mycelium while aerial mycelium of Y-5114, Y-14046^T and Y-14082 was abundant and white, grey or yellow, becoming black after autolysis. The substrate mycelium was yellowish brown. Diffusible pigments and melanin were not produced.

Physiological and biochemical characteristics

The physiological and biochemical characteristics of the four strains are shown in Table 2. The characteristics of the four strains were almost the same except for growth in 7% (w/v) NaCl and production of lecithinase. The upper limits of growth temperature were 65–68 °C.

Electrophoretic pattern of isoenzymes

The electrophoretic patterns of six isoenzymes of strains Y-4027, Y-5114, Y-14046^T, Y-14082 and six typical strains are shown in Fig. 2. The patterns of G6PD, SOD and EST were the same for the four strains. The electrophoretic pattern of the MDH of Y-5114 had two extra bands at R_m 0.2 and 0.6. The ADH pattern of Y-4027 and Y-14082 showed one extra band at R_m 0.2 and that of GDH of Y-5114 and Y-14046^T

Table 1. Cultural characteristics of four strains

From Inter-Society Color Council-National Bureau of Standards color-name charts.

Medium	Mycelium*	Strain:			
		Y-4027	Y-5114	Y-14046 ^T	Y-14082
Czapek agar	AM	—	White	Grey	Light grey-brown
	SM	Yellowish grey	Light brown-grey	Grey	Light grey
	SP	—	—	—	—
Glycerol asparagine agar	AM	—	Grey	—	White
	SM	Pale yellow	Yellowish grey	Yellowish grey	Grey-yellow
	SP	—	—	—	—
Inorganic salts-starch agar	AM	—	—	—	—
	SM	Yellowish grey	Yellowish grey	Yellowish grey	Yellowish grey
	SP	—	—	—	—
Oatmeal agar	AM	—	Yellowish white	—	Grey yellow
	SM	Yellowish white	Yellowish white	Yellowish grey	Yellowish grey
	SP	—	—	—	—
Yeast extract-malt extract agar	AM	—	Olive-black	Yellowish grey	Light grey
	SM	Light olive-brown	Olive-brown	Light olive-brown	Light olive-brown
	SP	—	—	—	—
Potato extract agar	AM	—	Deep grey	Grey-yellow	—
	SM	Grey-yellow	Grey-yellow	Grey-yellow	Pale yellow
	SP	—	—	—	—

* AM, Aerial mycelium; SM, substrate mycelium; SP, soluble pigments.

Table 2. Physiological and biochemical properties of four strains

+, Positive, utilized; —, negative, not utilized.

Test	Strain				Test	Strain			
	4027	5114	14046 ^T	14082		4027	5114	14046 ^T	14082
Milk coagulation	+	+	+	+	Lecithinase	—	+	+	+
Milk peptonization	+	+	+	+	Pectolase	—	—	—	—
Starch hydrolysis	+	+	+	+	Use of carbon sources:				
Nitrate reduction	+	+	+	+	Glucose	++	+	+	—
H ₂ S production	—	—	—	—	Fructose	++	++	+++	—
Degradation of urea	—	—	—	—	Xylose	++	++	++	—
Growth in cellulose	—	—	—	—	Raffinose	+	—	—	+
Growth in phenol (0.1 %, w/v)	+	+	+	+	Rhamnose	++	++	+	+
Growth in sodium chloride (7 %, w/v)	—	—	+	+	Inositol	—	—	—	—
					Mannitol	++	++	++	++
					Ribitol	+	+	—	+
					Cellobiose	+	+	—	—
					Resistance to antibiotics (50 µg ml ⁻¹)				
					Neomycin	—	—	—	—
					Rifampicin	—	—	—	—
					Antimicrobial activity:				
					<i>Bacillus subtilis</i>	—	—	—	—
					<i>Aspergillus niger</i>	—	—	—	—

Fig. 2. Diagrammatic representation of electrophoretic patterns of six isoenzymes of 10 strains of actinomycetes.

had one extra band at R_m 0.3. The number of patterns of the six isoenzymes of the four strains was greater than those of *Streptomyces griseus* and different from those of other genera.

Fig. 3. Phylogenetic tree based on 16S rRNA gene sequences showing relationships among four strains of *Streptomyces thermogriseus*, related species of the same genus and other taxa.

Chemotype of cell wall

The cell wall of all the four strains contained L-diaminopimelic acid and glycine. The whole-cell hydrolysates contained galactose.

16S rRNA gene sequences

The almost complete 16S rRNA gene sequences of strains Y-4027, Y-5114, Y-14046^T and Y-14082 which we determined contained 1467, 1476, 1423 and 1383 nucleotides, respectively. A result of the phylogenetic analysis of the four strains and some typical strains of the genus *Streptomyces* and other genera is shown in Fig. 3.

The strict consensus parsimony trees (not shown) and the neighbour-joining phylogram (Fig. 3) constructed using the Kimura-2 model based on the entire sequence data of 16S rRNA genes were extremely similar. The four strains of autolytic thermophilic *Streptomyces* were related with 99.5% similarity of 16S rRNA gene sequences.

Based on the morphological characteristics, cell wall chemotype and sequences of 16S rRNA genes, strains Y-4027, Y-14082, Y-5114 and Y-14046^T should be included in the genus *Streptomyces*. The four strains should be identified as a single species because of the

high similarity value (99.5%) of their 16S rRNA gene sequences, despite their morphological, cultural, physiological and biochemical characteristics, and the different electrophoretic patterns of some of the six isoenzymes tested. However, similarity values of 16S rRNA gene sequences among the four strains and *Streptomyces hygroscopicus* (8), *S. griseus* and other members of the genus *Streptomyces* with thermophilic and autolytic characteristics were less than 95%. Thus, we propose that the four strains should be a new species, *Streptomyces thermogriseus* sp. nov.

Description of *Streptomyces thermogriseus* sp. nov.

Streptomyces thermogriseus (ther.mo.gri.seus. M.L. adj. *therme* heat; adj. *griseus* grey; M.L. *thermogriseus* heat, grey).

Brown or yellow vegetative hyphae. Grey or white aerial hyphae, becoming black after autolysis. The spores are spherical, subspherical or short rods. Spore surface is smooth. Neither diffusible pigment nor melanin is produced. Milk is coagulated and peptonized. Lecithinase may or may not be produced. Hydrolyses starch. Nitrate reduction. No H₂S production. No growth on cellulose. Growth in 0.1% phenol. Growth or no growth in 7% NaCl. No degradation of urea. Utilizes rhamnose and mannitol. Utilizes or does not utilize glucose, fructose, xylose, raffinose, ribitol and cellobiose. Does not utilize inositol. No resistance to neomycin and rifampicin. No antimicrobial activities are detected. Growth at 65–68 °C. The cell wall contains L-diaminopimelic acid and glycine. The whole-cell hydrolysate contains galactose. Strain Y-4027 (= CCTCC AA97012) was isolated from soil samples of Kunming, Y-5114 (= CCTCC AA97013) was from Chenghai Lake, and Y-14046^T (= CCTCC AA97014^T) and Y-14082 (= CCTCC AA97015) were from a hot-spring in Eryuan, Yunnan, China. The type strain is Y-14046^T.

ACKNOWLEDGEMENTS

The authors gratefully thank Dr Zheng-Fu Lu for his help in the preparation of the phylogenetic trees. The work was supported by the National Natural Science Foundation of China and International Cooperation Foundation of Yunnan.

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