Identification and classification of an actinomycete strain producing wanlongmycin

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Abstract: One actinomycete strain GAAS2507 (stored in the China Center for Type Culture Collection, CCTCC), producing a new agricultural antibiotic wanlongmycin, was isolated from a soil sample collected from Malaysia. According to the colour of its spore stacks, it was tentatively classified to the cineteus group. Based on its morphological, physiological, biochemical characteristics, and 16S rDNA sequence analysis, it was classified as a new subspecies and name as *Streptomyces griseovariabilis* subsp. *bandungensis* subsp. nov..

Key words: *Streptomyces griseovariabilis*; wanlongmycin producing strain; identification; classification
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新农抗万隆霉素产生菌的鉴定与分类

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摘要: 新农抗万隆霉素产生菌是从来自马来西亚的土壤样品中分离筛选出来的一株放线菌菌株(保存于中国典型培养物保藏中心, 编号为GAAS2507), 根据菌株GAAS2507孢子堆的颜色, 其可以归入到烬灰类群. 又通过对其形态、生理生化特性的比较发现, 其与灰色变异链霉菌 *Streptomyces griseovariabilis* 非常相似, 但是又有一定的差异, 故将其命名为灰色变异链霉菌万隆亚种 *Streptomyces griseovariabilis* subsp. *bandungensis* subsp. nov..

关键词: 灰色变异链霉菌; 万隆霉素产生菌; 鉴定; 分类

In the course of our screening program for new antibiotics in 1996, a new actinomycete strain, GAAS2507, was isolated from a soil sample collected from Malaysia. From the fermentation broth of this strain, a natural cyclic depsipeptide antibiotic, named as wanlongmycin, was found. It is believed to be a new member of quinoxaline-like antibiotics by structural identification. It has a strong activity against a lot of *Oomyces* diseases[1].

Because it is an original wild strain, its systematic status and strain name are unclear. In the present study, we described the morphologic properties, cultural and physiological-biochemical characteristics as well as the 16S rDNA sequences and the taxonomy of the producing strain GAAS2507.

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1 Materials and Methods

1.1 Isolation of producing strain

The air dried soil sample collected from Malaysia was pretreated for 1 h at 120 ℃, then the strain GAAS2507 was isolated from HV-agar cultural medium[2] containing 50 mg/L $KCrO_4$. The isolated strain was incubated at 28 ℃ for 3 to 4 weeks, then purified into single colony, and inoculated on Gause NO. 1 agar cultural medium.

1.2 Observation of morphological properties

Micro-morphological studies of samples cultured for 7,14,21 and 28 d on Gause No. 1 cultural medium at 28 ℃ were carried out under a light microscope (AXCOSROP, CARL ZEISS Inc., Germany) and a environmental scanning electron microscope (X130E, ELECTRONIC, Inc., Japan).

1.3 Determination of cultural and physiological-biochemical characteristics

Cultural and physiological-biochemical characteristics as well as the carbon source utilization were studied by the method of Shirling and Gottlieb[3]. The colour changes were recorded according to ISCC Color Charts[4].

1.4 Whole-cell hydrolysis analysis

The methods used for the analysis of the amino acid components of cell wall and sugars produced by the whole-cell hydrolysis were described by Stanek[5].

1.5 The 16S rDNA sequence analysis

Total DNA preparation was carried out according to SAMBROOK[6]. Cells were harvested by centrifugation (8 000 r/min, 15 min), and lysed in 2 volumes of 10 mmol/L TE buffer (pH8.0) by incubating for 20 min at 65 ℃. Cell debris was removed by centrifugation, and the supernatant was extracted with *V* (phenol): *V*(chloroform): *V*(isoamyl alcohol) = 25: 24: 1. The DNA was precipitated by adding 1/10 volume of 3 mol/L potassium acetate and 2 volumes of ethanol, separated by centrifugation (10 000 r/min), and dried by air.

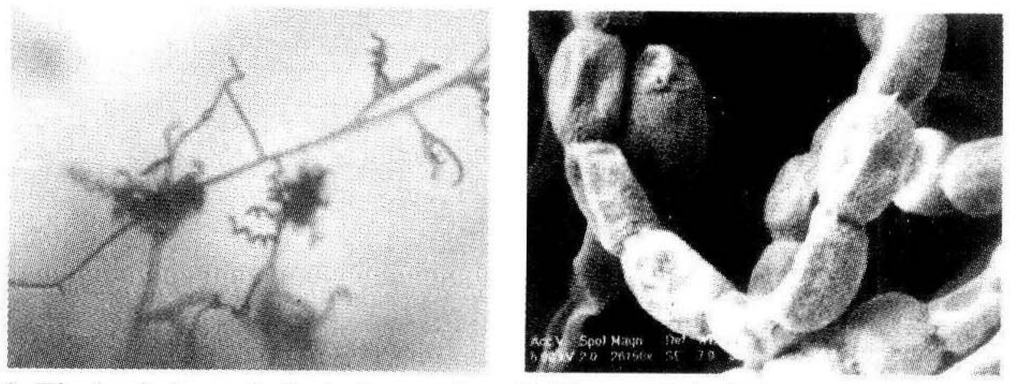
PCR amplification of the 16S rRNA gene of strain GAAS2507 was performed using 2 primers: primer A: 5′-AGGAGTTTGATCCTGGCTCAG-3′, and primer B: 5′-AAGGAGGTGATCCAGCCG CA-3′. Amplification was performed in automatic thermocycler (Perkin-Elmer) and the recommended buffer system according to the following amplification profile: 95 ℃ (1 min) followed by 30 cycles of denaturation at 95 ℃ (30 s), annealing at 55 ℃ (40 s) and extension at 72 ℃ (2 min). Predenaturation 95 ℃ (5 min) before the reaction, and extension after the cycles at 72 ℃ (7 min). The PCR reaction mix was analyzed by agarose gel (0.8 g/mL) electrophoresis and the DNA was purified, then its sequence was determined with ABI PRISMTM-377 DNA automatic sequencing system. Reactions were performed with a Big Dye™ terminator cycle sequencing ready reaction sequencing kit (Amersham) and the following 3 specific primers are primer A: 5′-AGAGTTTGATCCTGGCTCAG-3′, primer B: 5′-TTAAGGTGATCCAGCCGC-3′ and primer C: 5′-AGGGTTGCGCTCGTT-3′.

Homology studies were performed by BLAST software (Search algorithm). According to the sequencing results, the Megaline (DNAStar 5.01, DNAStar Inc.) was used to calculate the homology rate between the strain GAAS2507 and 11 typical *Streptomyces* strains, and the calculation method is Clustal W (Slow/Accurate IUB). Multisequence comparison of the strain GAAS2507 and other 11 typical *Streptomyces* strains selected from the GenBank and EMBL databases was calculated by the adopt DNAMAN 5.2.9[7]. The phylogenetic tree was constructed by the Neighbour-joining method[8].

2 Results and analyses

2.1 Morphological and cultural characterastics

The strain GAAS2507 is characterised by incompact spiral spore chains, with a smooth spore surface (Fig. 1). It formed grey aerial mycelium and colourless to yellowish white substrate mycelium on Gause NO. 1 medium. Pigments were not observed (Tab. 1). The cultural characteristics of strain GAAS2507 are summarized in Tab. 1.



A: The hyphal morphological screening under photo-microscopy(×130)　B: The morphological screening of spores. under electrical scan microscopy(×2600)

Fig. 1　The morphological characteristics of strain GAAS2507

2.2 Physiological-biochemical characteristics

Physiological-biochemical characteristics of strain GAAS2507 are shown in Tab. 2, such as unsure gelatin liquefaction , strong starch hydrolysis, milk coagulattion and fierce peptonization . The strain could not grow on the cellulose agar medium and produce hydrogen sulfide.

Tab. 1 Culture characteristics of strain GAAS2507[1)]

culture medium	aerial mycelium		substrate mycelium		soluble pigment
	growth	colour	growth	colour	
Gause No. 1 agar	+ +	smoky grey	+ +	colourless to yellow	none
czapek's sucrose	+ +	greyish white	+ +	colourless to grey	none
carrot NO. 1	+ + +	smoky grey	+ + +	colourless to yellow	none
glucose asparagine	+ +	white	−		none
glycerol asparagine	−	−	+ +	light brown	parrot-crest yellow
potato piece	+ + +	smoky grey	+ + +	smoky grey	blackish brown
oatmeal	+ + +	dark grey	+ +	colourless to greyish white	none
tyrosine	−	−	+	light grey	brown
inorganic salt-starch	+	light grey	−		none
glucose yeast	+ +	smoky grey	+ +	white to yellowish white	none
calcium malate	+ + +	greyish white	+ + +	greyish white	jasmine yellow

1) − : no growth ; + : poor growth ; + + : moderate growth ; + + + : good growth

Tab. 2 Comparison of colony and physiological-biochemical characteristics of strain GAAS2507 with *Streptomyces griseovariabilis*[1)]

item	features	GAAS2507	*S. griseovariabilis*
features of colony	aerial mycelium[2)]	smoky grey	light grey to grey
	substrate mycelium[2)]	colourless or yellowish white	colouress
	pigment[2)]	none	none
	aerial mycelium[3)]	light grey	light grey
	substrate mycelium[3)]	none	colourless
	pigment[3)]	none	none
	shape of spore	smooth	smooth
	shape of spores chain	incompact spiral	incompact spiral
physiological and biochemical features	coagulation of milk	+	+
	peptonization of milk	+	+
	starch hydrolysis	+	+
	celluloytic activity	−	−
	sulfureted hydrogen	−	−
	gelatin liquefaction	N. D.	+
carbon sources used for growth	D-glucose	+	+
	maltose	+	+
	sorbinose	−	−
	mannitol	+	+
	inositol	+	+
	D-xylose	−	−
	rhamnose	+	−
	D-fructose	−	+
	arabinose	−	+
	raffinose	+	+
	sucrose	−	+
	galactose	+	−
	inulin	−	−
	mannose	+	+
	lactose	+	+
	sorbitol	−	−

1) − : not utilize; + : utilize ; N. D. : not described;2) cultured on Gause No. 1; 3) cultured on inorganic salt − starch medium

The strain could utilize glucose, lactose, raffinose, maltose, mannitol, inositol, rhamnose and galactoses, but not *D*-fructose, sorbinose, arabinose, xylose, sucrose, inulin, dulcitol and sorbitol. The metabolite from the fermentation broths of strain GAAS2507 showed a potent antibiotic activity against *Bacillus subtilis*, *Phytophthora melonis*, *Fusarium oxysporum* (SCHL.) f. sp. *cucumerinum*, *Xanthomonas oryzae* pv. *oryzae* and *Xanthomonas oryzae* pv. *oryzicola*[9].

2.3 The chemical type of cell wall

The amino acids of the whole cell contained ,*L*-diaminopimelic acid (*L*-DAP)but not diagnostic sugars, suggesting the cell wall of this strain belonged to the type I.

2.4 Analysis of 16S rDNA sequence

The complete length of the 16S rDNA sequences were 1 427 bp, n (G + C): n (G + C + A + T) = 58.4%. The information of this sequence has been submitted to GenBank under the accession number AY 654298. The similarity in 16S rDNA sequences between strain GAAS2507 and eleven related *Streptomyces* typical strains chosen from GenBank was calculated and shown in Tab. 3. Almost all of these similarity values are greater than 96%.

Tab. 3 The typical strains of *Streptomyces* and their homologous ratio with strain GAAS2507

strain names	accession number	similarity/%
S. melanosporofaciens	AJ391837	96.2
S. yogyakartensis	AJ391827	96.4
S. somaliensis	AJ007399	96.9
S. platensis	AB045882	97.1
S. albulus	AB024443	96.3
S. albofaciens	AB045880	96.2
S. nogalater	AB045886	97.0
S. neyagawaensis	D63869	96.8
S. virginiae	D85123	97.1
S. megasporus	Z68100	94.0
S. longisporus griseus	AJ399475.1	97.3

The neighbour-joining tree based on nearly complete 16S rDNA sequences of 12 streptomycetes (Fig. 2). The numbers at the nodes indicate the levels of bootstrap support based on a neighbour-joining analysis of 1 000 resampled data sets[10]. The strain *S. virginiae*, GAAS2507, *S. longisporus griseus* and *S. neyagawaensis* belong to the same branches, and the corresponded bootstrap support value is 68%. This indicates that they are closely affiliated.

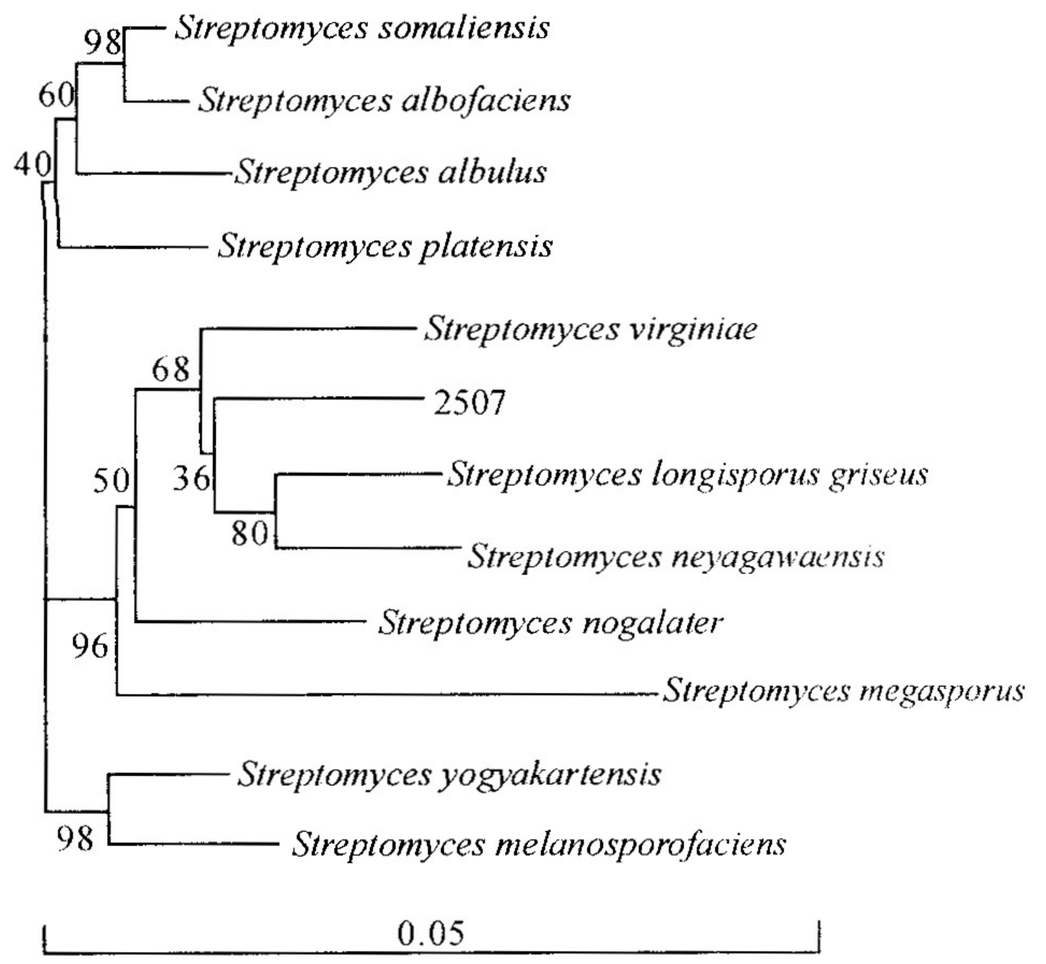


Fig. 2 The phylogenetic tree of strain GAAS2507 and related strains

3 Discussion

The above-mentioned characteristics of strain GAAS2507 revealed that it belonged to the genus *Streptomyces*. The strain GAAS2507 formed grey aerial mycelium, colourless to yellowish white substrate mycelium and smoky gray spore masses, which could be sorted to *Cinerogriseus*[11]. It grew well on Gause NO. 1 medium, its aerial mycelium formed incompact spiral spore chains with 1 to 5 turns per chain, and the spores are oval. The cell wall was classified to the type I based on the amino acid analysis.

The genus *Streptomyces* has species more than *500* at present. According to the previous description of *Streptomyces* species, the taxonomic features of strain GAAS2507 resembled those of "*Streptomyces griseovariabilis*"[12], except for hydrolyzation of starch and several carbon utilization (Tab. 2). Therefore, this strain was designated as *Streptomyces griseovariabilis* subsp. *bandungensis* subsp. nov..

rRNA is a good material for the taxonomy study, especially the 16S rRNA. The information represented by 16S rRNA can not only reveal the evolution in the biosphere, but also be easily operated and used in all levels of classifications[11]. The 16S rDNA sequence a-

nalysis is applied to the classification and identification of strain GAAS2507 in this paper and the results hold out the above opinions as well. Since the 16S rDNA sequence of *S. griseovariabilis* has not yet included in many databases such as GenBank, EMBL, DDBJ or PDB, it is unable to do the cluster analysis. In this paper, it is the first time to analyze the strain GAAS2507 16S rDNA sequence and its sequence have been reserved in GenBank.

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