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TAXONOMIC DESCRIPTION

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Streptomyces aquilus sp. nov., a novel actinomycete isolated from a Chinese medicinal plant

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Abstract

The taxonomic position of a novel actinomycete isolate, designated strain GGCR-6^T, isolated from the healthy leaves of *Xanthium sibiricum* collected from the botanic garden of Hunan University of Science and Technology in Hunan province, PR China, was determined by a polyphasic approach. GGCR-6^T grew well on ISP series media and formed well-developed, branched substrate hyphae and aerial mycelium that differentiated into straight spore chains consisting of cylindrical spores with smooth surfaces. The diagnostic diamino acid was LL-diaminopimelic acid. The major menaquinones were MK-9(H_8), MK-9(H_2), MK-9 and MK-9(H_6). The polar lipids were diphosphatidylglycerol, phosphatidylethanolamine, phosphotidylinositol and phosphatidylinositol mannosides. The predominant fatty acids were $C_{16:1}\omega9c$, iso-$C_{16:0}$ and $C_{16:0}$. The phenotypic characteristics of GGCR-6^T indicated that it represented a member of the genus *Streptomyces*. Phylogenetic analysis based on the 16S rRNA gene sequence indicated that GGCR-6^T was most closely related to *Streptomyces cyaneus* NRRL B2296^T and *Streptomyces griseoruber* NRRL B1818^T. However, the digital DNA–DNA hybridization, the average nucleotide identity and the multi locus sequence analysis evolutionary distance clearly separate GGCR-6^T from the phylogenetically closely related species. Furthermore, the novel isolate was distinctly differentiated from *S. cyaneus* NRRL B2296^T and *S. griseoruber* NRRL B1818^T by morphological, physiological and biochemical characteristics. Based on these data, strain GGCR-6^T should be designated as a representative of a novel species of the genus *Streptomyces*, for which the name *Streptomyces aquilus* sp. nov. is proposed. The type strain is strain GGCR-6^T (=CICC 11055^T=JCM 33584^T).

Antimicrobials, especially antibiotics, have played a crucial role in modern medicine. But in the past 25 years, their misuse and overuse has made them less effective as bacteria develop resistance [1, 2]. How can the challenges of resistance emergence be met? An important pathway is the development of new bioactive products, particularly those derived from microorganisms. Endophytes are microorganisms that exist inside plant tissues without having any negative effects on the host plant [3]. There is evidence that endophytic actinomycetes are found in almost all medicinal plants studied [4, 5], and possess the potential to produce unique secondary metabolites, which can be exploited in pharmaceutical, agricultural and other industries [6–9]. Thus, there is a growing interest by researchers in bioprospecting of endophytic actinobacteria communities inhabiting plants from various ecosystems. Recently, during our ongoing survey on diversity and biopotential of plant-associated endophytic actinobacteria, strain GGCR-6^T, which exhibited weak antimicrobial activity against *Staphylococcus aureus*, was isolated from *Xanthium sibiricum* Patrin ex Widder. Phylogenetic analysis based on the 16S rRNA gene sequences indicated that GGCR-6^T was most closely related to *Streptomyces cyaneus* NRRL B-2296^T and *Streptomyces griseoruber* NRRL B-1818^T. However, there were distinctly differences in morphological and cultural characteristics between GGCR-6^T and these strains. In the present work, the results of a polyphasic taxonomic study of a novel *Streptomyces* strain GGCR-6^T, are presented.

GGCR-6^T was isolated from the healthy leaves of a medicinal plant *X. sibiricum* collected from the botanic garden of Hunan University of Science and Technology in Hunan province (27°

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Keywords: *Streptomyces aquilus* sp. nov.; *Xanthium sibiricum*; Polyphasic taxonomy.
Abbreviations: JCM, Japan correction of Microorganisms.
1. The GenBank accession numbers for 16S rRNA gene sequences of strain GGCR-6^T is MH718844.
2. The GenBank accession numbers for whole genome sequences of strain GGCR-6^T is CP034463.
†These authors contributed equally to this work
Five supplementary tables and three supplementary figures are available with the online version of this article.

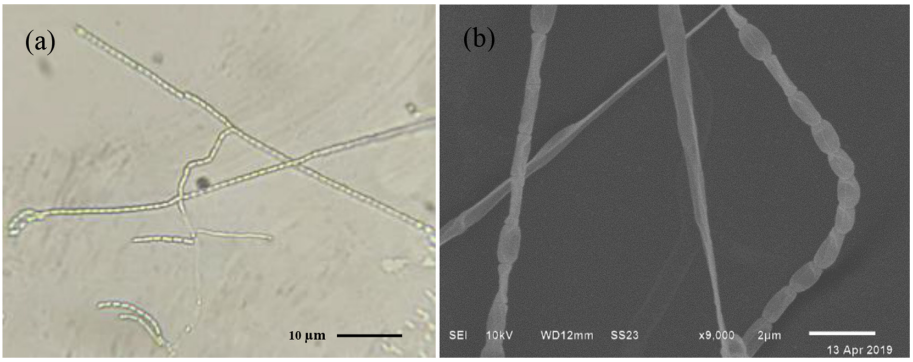

Fig. 1. Optical micrograph (a) and scanning electron micrograph (b) of GGCR-6^T grown on Gause's synthetic medium at 28 °C after incubation for 14 days.

54′ N, 112° 54′ E), PR China. The leaf segments were firstly washed in ultrapure water to remove adhered epiphytes and soil debris. Then, the tissue surfaces were sterilized according to a procedure described previously [10]. GGCR-6^T was isolated and purified by the methods described by Mo *et al.* [11]. The purified isolate was maintained on Gauze's synthetic agar [12] at 4 °C and stored in a 30% (w/v) glycerol suspension at −80 °C. *S. cyaneus* CGMCC 4.1671^T(=NRRL B-2296^T) was purchased for reference from the China General Microbiological Culture Collection Centre (CGMCC). The reference strain was cultured under the same conditions for comparative testing. For chemical and molecular analyses, biomass was prepared by culturing in Gauze's synthetic medium for 4–7 days at 28 °C in a rotary shaker (180 r.p.m.) and was then collected at the exponential phase of growth by centrifugation.

The morphology of the spore chain and the spore surface ornamentation of GGCR-6^T were observed by light microscope (BX41, Olympus) and scanning electron microscopy (JSM-6610LV, JEOL) of 14-day-old cultures on Gauze's synthetic agar, respectively. The cultural properties of GGCR-6^T were evaluated according to the guidelines of the International *Streptomyces* Project (ISP) as described by Shirling and Gottlieb [13]. The colour of colonies and soluble pigments were determined according to the colour standard [14]. The optimum pH range, temperature and NaCl tolerance for cell growth were determined according to the methods of Verma *et al.* [7]. The utilization of carbon and nitrogen sources was determined by the methods of Shirling and Gottlieb [13]. Susceptibility to antimicrobial agents was examined by the disc diffusion method [15] using a set of antibiotic-impregnated discs (Product code: S1100, Hangzhou Microbial Reagent). The other physiological and biochemical tests, such as aesculin hydrolysis, gelatin liquefaction, hydrogen sulfide production, nitrate reduction, starch hydrolysis and degradation tests for tweens (20, 40, 60 and 80), were carried out according to the methods described by Xu *et al.* [16].

Cellular fatty acids analysis was carried out by the China Centre of Industrial Culture Collection (CICC; Beijing, PR China) according to the protocol of the Sherlock Microbial ID System (http://www.midi-inc.com/). Menaquinones were extracted according to the method of Collins *et al.* [17] and analyzed by HPLC [18]. The polar lipids analysis was performed as described by Komagata and Suzuki [19]. The isomer of diaminopimelic acid analysis and sugar analysis of whole-cell hydrolysates were performed according to the procedures described by Hasegawa *et al.* [20] and Lechevailer and Lechevailer [21].

Extraction of genomic DNA was carried out by using the microwave-based method [22]. The 16S rRNA gene was amplified with the universal primers 27 f and 1492 r [23]. The PCR product was sequenced by Sangon Biotech (Shanghai, PR China). The obtained sequence was compared with available 16S rRNA gene sequences of species with validly published names from the EzBioCloud public databases (http://www.ezbiocloud.net/eztaxon) in order to determine an approximate phylogenetic affiliation. The *atp*D, *gyr*B, *rec*A, *rpo*B and *trp*B genes sequences were directly downloaded from GenBank or drawn from draft genome sequences, and concatenated head to tail in-frame. Phylogenetic trees based on the 16S rRNA gene sequences and on the concatenated protein-coding sequences were reconstructed by using the neighbor-joining (NJ) [24], maximum likelihood (ML) [25] and maximum parsimony (MP) [26] methods with 1000 bootstrap replications in MEGA 7.0 [27]. For the multilocus sequence analysis (MLSA) [28], the Kimura two-parameter model [29] was chosen to calculate the genetic distances. The 16S rRNA sequence has been deposited in the GenBank and assigned the accession number MH718844.

Complete genome sequencing of GGCR-6^T was finished by Beijing Novogene Bioinformatics (Beijing, PR China). The average nucleotide identity (ANI) and digital DNA–DNA hybridization (dDDH) values between the genomes of GGCR-6^T and other relatives were calculated using the JSpeciesWS online service [30] and the genome-to-genome distance calculator [31], respectively. The G+C content of the genomic DNA of GGCR-6^{T}was calculated using ChunLab's online Average Nucleotide Identity (ANI) calculator [32]. The DNA–DNA hybridization experiment was performed using a method described by De Ley *et al.* [33]. The genome sequence

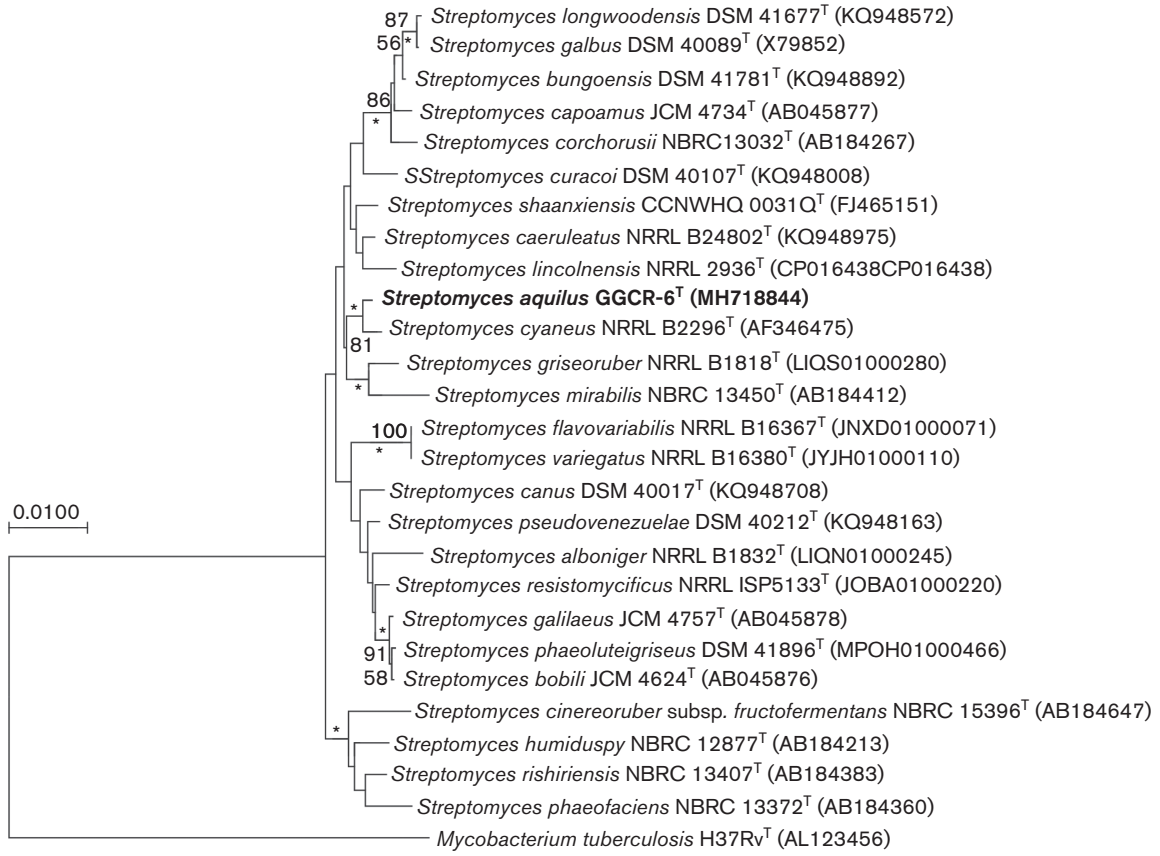

Fig. 2. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship between selected species of the genus *Streptomyces*. *Mycobacterium tuberculosis* H37Rv^T was used as an outgroup. Bootstrap percentages over 50% derived from 1000 replications are shown at the nodes. Asterisks indicate branches also recovered in the maximum-likelihood and maximum-parsimony trees. Bar, 0.01 nucleotide substitutions per site.

obtained in this work was deposited in Genbank and assigned the accession number CP034463.

GGCR-6^T exhibited typical characteristics of members of the genus *Streptomyces* and formed well-developed, branched substrate mycelium and aerial hyphae. The colour of aerial hyphae and substrate mycelium was white and light yellow to brown on Gause's agar, respectively. Aerial mycelia produced straight chains of cylindrical and smooth-surfaced spores (Fig. 1). GGCR-6^T grew well on ISP (27) media. No distinct soluble pigment was produced on all tested media. The detailed cultural characteristics are presented in Table S1 (available in the online version of this article). The growth temperature range of GGCR-6^T was 10–45 °C, with an optimal temperature at 30 °C. NaCl tolerance was up to 7%. GGCR-6^T could grow at between pH 5.0 and 12.0, with an optimum pH of 7.0. The detailed physiological and biochemical characteristics are presented in the species description.

The cell wall of GGCR-6^T contained alanine, asparagine, glutamate, glycine and LL-diaminopimelic acid. Whole-cell hydrolysates contained glucose, mannose and trace amounts of xylose. Cellular fatty acid compositions of strain GGCR-6^T and reference strains are shown in Table S2. The predominant cellular fatty acids (>10%) of strain GGCR-6^T were $C_{16:1}\omega9c$ (27.1%), iso-$C_{16:0}$ (18.2%) and $C_{16:0}$ (14.1%); The fatty acids present in smaller amounts (>1%) were anteiso-$C_{15:0}$ (8.5%), iso-$C_{14:0}$ (7.1%), $C_{14:0}$ (5.1%), iso-$C_{15:0}$ (3.7%), $C_{15:0}$ (3.4%), iso-$C_{16:1}$H (2.7%), anteiso-$C_{17:0}$ (1.6%), $C_{15:1}$B (1.3%) and summed feature 6 (1.2%). The polar lipids were diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphotidylinositol (PI) and phosphatidylinositol mannosides (PIM) (Fig. S1). The predominant menaquinones were MK-9(H_8) (36.3%), MK-9(H_2) (25.7%), MK-9 (17.3%) and MK-9(H_6) (15.7%). The G+C content of the DNA was 70.9 mol% from the whole genome sequence data. It is within the range (69–78%) observed for the members of the genus *Streptomyces* [34]. All the data indicated that GGCR-6^T represented a member of the genus *Streptomyces*.

A sequence homology search based on the full-length 16S rRNA gene sequence (1530 bp) of GGCR-6^T indicated that it represented a member of the genus *Streptomyces* and exhibited 99.5, 99.1, 99.0, 99.0, 98.8, 98.8, 98.8, 98.8, 98.7 and 98.7 % similarities to *S. cyaneus* NRRL B-2296^T, *S. shaanxiensis* CCNWHQ 0031^T, *S. pseudovenezuelae* DSM 40212^T, *S. caeruleatus* NRRL B-24802^T, *S. curacoi* DSM 40107^T, *S. canus*

Fig. 3. Neighbor-joining tree based on five-gene concatenated sequences (*atp*D, *gyr*B, *rec*A, *rpo*B and *trp*B, 2458 nt) showing the relationships between the related members of the genus *Streptomyces*. *Mycobacterium tuberculosis* H37Rv^T was used as an outgroup. Bootstrap percentages over 50% derived from 1000 replications are shown at the nodes. Asterisks indicate branches that were also found using the maximum-likelihood method and the maximum-parsimony method. Bar, 0.05 substitutions per site.

DSM 40017^T, *S. griseoruber* NRRL B-1818^T, *S. rishiriensis* NBRC 13407^T, *S. resistomycificus* NRRL-ISP 5133^T and *S. phaeoluteigriseus* DSM 41896^T, respectively, and ≤98.2% similarities to the other species in the genus *Streptomyces*. A NJ tree based on the 16S rRNA gene sequences indicated that the phylogenetic neighbours of GGCR-6^T were *S. cyaneus* NRRL B-2296^T and *S. griseoruber* NRRL B-1818^T (Fig. 2). The close association of GGCR-6^T, *S. cyaneus* NRRL B-2296^T and *S. griseoruber* NRRL B-1818^T was further supported by the ML and MP trees (Figs S2 and S3) based on the 16S rRNA gene sequences and the MLSA tree (Fig. 3) based on the five house-keeping gene (*atp*D, *gyr*B, *rec*A, *rpo*B and *trp*B) sequences (Table S3). However, the MLSA distances between this strain and the closely related species of the genus *Streptomyces* mentioned above were 0.041 and 0.039, respectively (Table S4), which was well above the species level threshold of 0.007 recommended by Rong and Huang [28], indicating that GGCR-6^T represented a distinct species of the genus *Streptomyces*. Results of previous studies have indicated that the DNA–DNA reassociation experiments should be mandatory for testing the genomic uniqueness of a novel isolate which has more than or equal to 98.7% 16S rRNA gene sequence similarities to the related species [35]. Thus, considering the higher 16S rRNA gene sequence similarities (≥98.7%), DNA–DNA relatedness studies were carried out between GGCR-6^T and the ten related type strains listed above. In the present work, the ANI and dDDH values were used for calculating the relatedness between their genome sequences. However, considering that the genome sequence data of *S. shaanxiensis* CCNWHQ 0031^T is not available, DNA–DNA relatedness between it and strain GGCR-6^T was determined using the DDH method [33]. Results indicated that the ANI and dDDH or DDH values between the whole-genome sequences of GGCR-6^T and the other strains were 86.5–87.2 and 24.5–31.3% (or 33.8±0.9%), which were well below the 95–96 and 70% cut-off points recommended for delineating species (Table S5) [36, 37]. Furthermore, distinct differences in phenotypic traits between GGCR-6^T and its phylogenetic neighbours, namely *S. cyaneus* NRRL B2296^T and *S. griseoruber* NRRL B1818^T, also further indicated that GGCR-6^T did not represent any known species of the genus *Streptomyces* phenotypically (Tables 1, S1 and S5). Therefore, based on a combination of the genotypic and phenotypic data,

Table 1. Phenotypic features distinguishing GGCR-6^T from *S. cyaneus* CGMCC 4.1671^T and *S. griseoruber* NRRL B-1818^T

Strains: 1, GGCR-6^T; 2, *S. cyaneus* CGMCC 4.1671^T; 3, *S. griseoruber* NRRL B-1818^T (data from Landwehr *et al*. [38]). **+**, Positive; **–**, negative; w, weakly positive.

Characteristics	1	2	3
Spore chain morphology	Long, straight	Spiral	Spiral
Spore shape	Smooth	Spiny	Smooth
Colour of substrate mycelia on ISP3	Citrine	Purple Violet	Red
Colour of substrate mycelia on ISP4	Light Buff	Dark Blue	Red
Gelatin liquefaction	+	–	w
H_2S production	+	–	+
Melanin production	–	+	+
Growth with 7% NaCl (w/v)	+	–	–
Assimilation of sole carbon sources (1.0%, w/v)			
Sucrose	+	+	–
Mannitol	+	–	–
Raffinose	+	+	–
Xylose	+	w	–
Assimilation of sole nitrogen sources (1.0%, w/v)			
Arginine	+	–	–
Major cellular fatty acids (>10% of total)			
			anteiso-$C_{15:0,}$
	$C_{16:0,}$	anteiso-$C_{15:0,}$	iso-$C_{15:0,}$
	iso-$C_{16:0,}$	$C_{16:0,}$	iso-$C_{16:0,}$
	$C_{16:1}\omega9c$	iso-$C_{16:0}$	anteiso-$C_{17:0}$

GGCR-6^T represents a novel species of genus *Streptomyces*, for which the name *Streptomyces aquilus* sp. nov. is proposed.

DESCRIPTION OF *STREPTOMYCES AQUILUS* SP. NOV.

Streptomyces aquilus sp. nov. (a'qui.lus. L. masc. adj. *aquilus* brown; referring to the ability of the organism to produce brown nutrient hyphae).

Aerobic, Gram-positive actinobacterium; forms well-developed branched aerial mycelium. Produces cylindrical and smooth-surfaced spores arranged in straight chains. The colour of the aerial mycelium is white and the colour of the substrate mycelium is light yellow to brown on ISP (2–7) media. No distinct soluble pigment is produced on tested media. Grows well on ISP (2–7) media. Growth occurs at pH 5.0–12.0, 10–45 °C and with 0–7% NaCl (w/v). Fructose, D-galactose, D-glucose, mannitol, raffinose, ribose and sucrose can be used as sole carbon sources for growth. As a nitrogen source, it utilizes arginine, asparagine, L-cysteine, L-histidine, L-threonine, methionine and tyrosine. Starch hydrolysis, H_2S production, nitrate reduction, milk coagulation and milk peptonization, gelatin liquefaction and tweens (20, 40, 60 and 80) degradation are positive. Resistant to the following antibiotics: ampicillin, carbenicillin, cefoperazone, ceftazidime, clindamycin, norfloxacin, oxacillin, penicillin and vancomycin. The cell wall contains alanine, asparagine, glutamate, glycine and LL-DAP. Whole-cell sugars are glucose, mannose and xylose. The predominant cellular fatty acids are $C_{16:1}\omega9c$, iso-$C_{16:0}$ and $C_{16:0}$. The predominant menaquinones are MK-9(H_8), MK-9(H_2), MK-9 and MK-9(H_6). The polar lipids contain phosphatidylethanolamine, diphosphatidylglycerol, phosphotidylinositol and phosphatidylinositol mannosides.

The type strain is GGCR-6^T (=CICC 11055^T=JCM 33584^T), which was isolated from the healthy leaves of a medicinal plant, *Xanthium sibiricum*, collected from the botanical garden of Hunan University of Science and Technology in Hunan province, PR China. The G+C content of the genomic DNA of the type strain is 70.9 mol%. The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of GGCR-6^T is MH718844. The whole-genome shotgun project has been deposited at DDBJ/ENA/GenBank under the accession number CP034463. The version described in this paper is version CP034463.1.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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