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Glycomyces anabasis sp. nov., a novel endophytic actinobacterium isolated from roots of *Anabasis aphylla* L.

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Abstract

A novel endophytic actinobacterium, designated strain EGI 6500139^T, was isolated from the surface-sterilized roots of *Anabasis aphylla* L., collected from Xinjiang, northwest PR China, and subjected to polyphasic taxonomic characterization. Strain EGI 6500139^T formed sparse aerial mycelium with rod-like spores. Whole-cell hydrolysates of the isolate contained *meso*-diaminopimelic acid as the cell-wall diamino acid, glucose as major sugar, and mannose, galactose, xylose and ribose as minor sugars. The polar lipids were phosphatidylglycerol, diphosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannoside, one unidentified glycolipid, one unidentified phospholipid and four unidentified polar lipids. The major fatty acids identified were anteiso-$C_{17:0}$, anteiso-$C_{15:0}$, iso-$C_{15:0}$ and iso-$C_{16:0}$. The predominant menaquinones detected were MK-11 and MK-11(H_2). The G+C content of the genomic DNA of strain EGI 6500139^T was 70.4 mol%. Strain EGI 6500139^T showed the highest 16S rRNA gene sequence similarity to *Glycomyces lacisalsi* XHU 5089^T (96.3 %). Phylogenetic analysis showed that strain EGI 6500139^T fell within the clade of the genus *Glycomyces*, and formed a clade with *G. lacisalsi* XHU 5089^T and *G. albus* CCTCC AA 2013004^T. Based on phenotypic, chemotaxonomic and phylogenetic data, strain EGI 6500139^T represents a novel species of the genus *Glycomyces*, for which the name *Glycomyces anabasis* sp. nov. (type strain EGI 6500139^T=JCM 30088^T=KCTC 29495^T) is proposed.

The genus *Glycomyces*, the type genus of the family *Glycomycetaceae*, was first proposed by Labeda *et al.* [1], and latterly emended by Labeda and Kroppenstedt [2]. The genus has features distinct from other actinobacterial genera on the basis of phylogeny and chemotaxonomic characteristics, such as a type II cell wall, whole-cell sugar pattern D, and PI phospholipid pattern [3]. At the time of writing, the genus *Glycomyces* comprises 17 validly published species (http://www.bacterio.net/glycomyces.html). Most members of the genus are from soil or saline environments, while others, such as *Glycomyces artemisiae* [4], *Glycomyces endophyticus* [5], *Glycomyces mayteni* [6], *Glycomyces phytohabitans* [7], *Glycomyces sambucus* [8] and *Glycomyces scopariae* [6] are from plant tissues and have great potential in biotechnology [9]. During a study on the diversity and taxonomy of endophytic actinobacteria associated with eremophytes from Karamay, Xinjiang, northwestern PR China, a novel strain EGI 6500139^T was isolated from the roots of *Anabasis aphylla* L. The isolate was subjected to polyphasic taxonomy, the results of which suggest that strain EGI 6500139^T represents a new species of the genus *Glycomyces*, for which the name *Glycomyces anabasis* sp. nov. is proposed.

Healthy root samples of *Anabasis aphylla* L. were pretreated according to the method of Qin *et al.* [5], and the crumbled pieces were diluted with sterilized water and plated onto tapwater–yeast extract agar [10] supplemented with 3 % (w/v) NaCl. After 3 weeks' incubation, the isolate was purified and maintained on yeast extract–malt extract agar (ISP 2) [11] at 4 °C, and as 20 % (v/v) glycerol suspensions at −80 °C. Biomass for chemical and molecular studies was obtained by cultivation in shake flasks (at about 160 r.p.m.) containing ISP 2 after incubation at 30 °C for 5 days.

Culture characteristics were determined after 4 weeks' incubation at 30 °C according to the methods described by Shirling and Gottlieb [11] except that the media used were

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Two supplementary tables and three supplementary figures are available with the online version of this article.

supplemented with 5 % NaCl (w/v). The colours of aerial and substrate mycelia and of soluble pigments were determined with ISCC-NBS colour charts [12]. Strain EGI 6500139^T grew well on oatmeal agar (ISP 3), inorganic starch agar (ISP 4), Czapek's agar and nutrient agar, moderately on yeast extract–malt extract agar (ISP 2) and potato–dextrose agar, and weakly on glycerol–asparagine agar (ISP 5). No soluble pigments were observed on the media tested. The detailed colours of substrate mycelium and aerial hyphae varied depending on the medium used (Table S1, available with the online version of this article).

Morphological characteristics of strain EGI 6500139^T were observed by light microscopy (BH-2 microscope; Olympus) and scanning electron microscopy (Quanta 200; FEI) after incubation on ISP 3 agar modified with addition of 5 % (w/v) NaCl at 30 °C for 14–28 days. The strain formed branched and non-fragmented substrate mycelium (Fig. 1a), which was in strong olive green or deep olive green in colour. The isolate formed sparse aerial hyphae, and some rod-like spores on aerial mycelium (Fig. 1b).

Growth at different temperatures (5–60 °C, at intervals of 5 °C) was tested on ISP 2 agar modified with addition of 5 % (w/v) NaCl. Tolerance to various NaCl concentrations (0–15 %, w/v, at intervals of 1 %) was examined by growing the strain on ISP 2 agar. The pH growth range was tested between 4.0 and 12.0, at intervals of 1.0 pH unit, on ISP 2 broth medium supplemented with 5 % (w/v) NaCl, using the buffer system described by Xu *et al.* [13]. Carbon-source utilization tests were performed according to the methods of Shirling and Gottlieb [11] except that the basic medium was modified with addition of 5 % (w/v) NaCl. Nitrogen-source utilization tests were carried out as described by Williams *et al.* [14] except that the basic medium was modified with addition of 5 % (w/v) NaCl. Catalase activity was determined using 3 % (v/v) H_2O_2, and gas production was identified as a positive reaction. Physiological and biochemical characteristics were examined as described previously [14, 15]. Strain EGI 6500139^T could grow at 15–40 °C, pH 6.0–10.0 and 0–10 % (w/v) NaCl. The optimal growth was determined to occur at 30 °C, pH 7.0–8.0 and 4–6 % (w/v) NaCl. Other physiological characteristics of strain EGI 6500139^T are given in Table 1 and the species description below.

Amino acids in whole-cell hydrolysates were analysed by TLC as described by Staneck and Roberts [16]. Cell-wall sugars were detected according to the method used by Tang *et al.* [17]. Polar lipids were extracted and identified by two-dimensional TLC following the method of Minnikin *et al.* [18]. Menaquinones were extracted and prepared as described previously [19], and analysed by HPLC [20]. For fatty acid analysis, all the strains studied were cultured on tryptic soy broth (TSB) medium modified with addition of 5 % (w/v) NaCl, at 160 r.p.m. and 30 °C for 5 days. Cellular fatty acid analysis was performed as described by Sasser [21] according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System. The prepared fatty acid methyl esters were separated using the Sherlock Microbial Identification System (MIS; MIDI) and analysed with the Sherlock Microbial Identification software package (version 6.1). During the procedure, the Agilent Technologies 7890A GC system (gas chromatography column, 0.2 mm ×25 m, Ultra, 25 % phenyl methyl silox) and MIDI database (TSBA6) were used. For determination of G+C content, the genomic DNA of strain EGI 6500139^T was prepared according to Marmur [22]. The G+C content of the DNA was determined by the HPLC method [23].

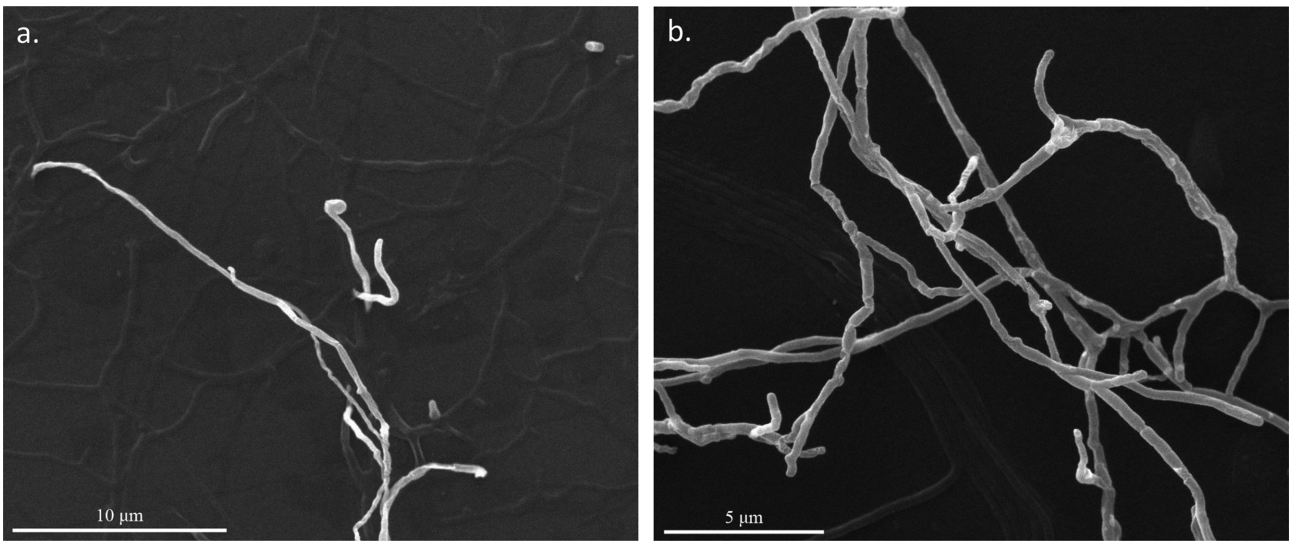

Fig. 1. Scanning electron micrographs of spore chains of strain EGI 6500139^T grown on ISP 3 agar modified with addition of 5 % (w/v) NaCl at 30 °C for 4 weeks. (a) Branched substrate mycelium; (b) rod-like spores on aerial mycelium. Bars: 10 µm (a); 5 µm (b).

Table 1. Characteristics that differentiate strain EGI 6500139^T from phylogenetically closely related neighbours

Taxa: 1, strain EGI 6500139^T; 2, *Glycomyces lacisalsi* XHU 5089^T; 3, *Glycomyces albus* CCTCC AA 2013004^T. All strains form branched substrate mycelia, and contain *meso*-diaminopimelic acid as the cell-wall diamino acid, show positive reactions for hydrolysis of cellulose and Tweens 20, 40, 60 and 80, and are positive for oxidase activities, but are negative for H_2S production, and for coagulation and peptonization of skimmed milk. All strains grow optimally at 30 °C, and utilize D-galactose, but not raffinose, trehalose or D-xylitol. +, Positive; –, negative; DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIM, phosphatidylinositol mannoside; PL, unidentified phospholipid; GL, unidentified glycolipid; PGL, phosphoglycolipid; UL, unidentified polar lipid; Ara, arabinose; Gal, galactose; Glu, glucose; Man, mannose; Rib, ribose; Xyl, xylose.

Characteristic	1	2	3
Aerial mycelium	Sparse	Sparse	Abundant
Growth:			
pH range	6.0–10.0	6.0–12.0	6.0–12.0
Optimal pH	7.0–8.0	7.0–8.0	8.0–9.0
NaCl range (%)	0–10	0–10	0–12
Optimal NaCl (%)	4–6	1–3	5–7
Temperature range (°C)	15–40	20–50 °C	15–40
Hydrolysis of:			
Casein	–	–	+
Starch	+	–	+
Methyl Red test	–	+	–
Nitrate reduction	–	–	+
Oxidase	+	–	+
Carbon source utlization:			
L-Arabinose	–	–	+
Cellobiose	–	+	–
D-Fructose	+	–	+
D-Glucose	–	+	+
Glycerol	–	+	–
Inositol	–	+	+
D-Mannose	–	+	–
Melibiose	–	+	–
L-Rhamnose	–	–	+
D-Ribose	–	–	+
D-Sorbitol	–	–	+
D-Xylose	–	–	+
Whole-cell sugars	Glu, Man, Rib, Gal, Xyl	Glu, Gal	Xyl, Rib, Ara
Major menaquinones	MK-11, MK-11(H_2)	MK-10(H_4), MK-9(H_4), MK-10(H_2), MK-11	MK-9(H_4), MK-9(H_6)
Major fatty acids (>10 %)	anteiso-$C_{17:0}$, anteiso-$C_{15:0}$, iso-$C_{15:0}$, iso-$C_{16:0}$	anteiso-$C_{17:0}$, anteiso-$C_{15:0}$, iso-$C_{15:0}$	anteiso-$C_{17:0}$, anteiso-$C_{15:0}$, iso-$C_{15:0}$, iso-$C_{16:0}$
Major phospholipids	PG, DPG, PI, PIM, GL, PL, ULs	DPG, PG, PI, GL, PL	PG, DPG, PI, PIM, GL, PGL
DNA G+C (mol%)	70.4	68.6*	71*

*DNA G+C contents data for the reference strains are from previously published articles [31, 33].

Whole-cell hydrolysates of strain EGI 6500139^T contained *meso*-diaminopimelic acid (DAP) as the cell-wall diamino acid. The major sugar was glucose, with mannose, ribose, galactose and xylose as minor sugars. The predominant menaquinones detected were MK-11 (78.4 %) and MK-11 (H_2) (13.7 %), while the minor components were MK-9(H_4) (5.4 %) and MK-9(H_6) (2.5 %). The polar lipids were phosphatidylglycerol, diphosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannosides, one unidentified glycolipid, one unidentified phospholipid and four unidentified polar lipids (Fig. S1). The major fatty acids identified were anteiso-$C_{17:0}$ (40.4 %), anteiso-$C_{15:0}$ (20.2 %), iso-$C_{16:0}$ (18.4 %) and iso-$C_{15:0}$ (10.0 %); the detailed fatty acid profiles are given in Table S2. The genomic DNA G+C content of strain EGI 6500139^T was 70.4 mol%.

Extraction of genomic DNA and PCR amplification of the 16S rRNA gene were performed using procedures described by Li *et al.* [24]. Multiple alignments with sequences of the type strains of the family *Glycomycetaceae*, and calculations of levels of sequence similarity were carried out using the EzTaxon-e server (http://www.ezbiocloud.net/) [25] on the basis of 16S rRNA gene sequence data. Phylogenetic analysis was performed using three tree-making algorithms, the

neighbour-joining [26], maximum-likelihood [27] and maximum-parsimony [28] methods, by means of the software MEGA6 [29]. The topologies of the resultant trees were evaluated by using the bootstrap resampling method of Felsenstein [30] with 1000 replicates. An almost complete 16S rRNA gene sequence (1541 bp) was determined for strain EGI 6500139^T. Blast searches indicated that the isolate showed high 16S rRNA gene sequence similarities to *Glycomyces lacisalsi* XHU 5089^T (96.3 %), *G. albus* CCTCC AA 2013004^T (95.8 %), *G. halotolerans* TRM 40137^T (94.6 %), *G. artemisiae* IXS^T (94.6 %) and *G. arizonensis* DSM 4472^T (94.5 %), and lower 16S rRNA gene sequence similarities to other members of the genus *Glycomyces* (below 94.0 %). Phylogenetic analysis based on the 16S rRNA gene sequences showed that the strain fell within the clade of the genus *Glycomyces*. In the phylogenetic tree based on the neighbour-joining algorithm, strain EGI 6500139^T formed a distinct clade with *G. lacisalsi* XHU 5089^T and *G. albus* CCTCC AA 2013004^T [31] with a high bootstrap value of 99 % (Fig. 2), and the distinction was supported by the other two tree-making methods used in the study (Figs S2 and S3). According to Stackebrandt and Goebel [32], there is no need to carry out DNA–DNA hybridizations between strain EGI 6500139^T and its phylogenetic closest neighbours because their 16S rRNA gene sequence similarities are lower than 97 %.

As described above, strain EGI 6500139^T exhibited typical characteristics of the genus *Glycomyces* (Table 1) – branched substrate mycelium and sparse aerial mycelium, *meso*-diaminopimelic acid as diagnostic diamino acid, whole-cell sugars (mannose and xylose), predominant polar lipids (phosphatidylglycerol, diphosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannosides) – as well as the closest phylogenetic relationship with this genus, which suggests that the novel strain belongs to the genus *Glycomyces*. Although strain EGI 6500139^T forms a clade with *G. lacisalsi* XHU 5089^T and *G. albus* CCTCC AA 2013004^T, the isolate clearly differed from its closest phylogenetic neighbours by the presence of more complicated cell sugars (glucose as major sugar), and MK-11 and MK-11(H_2) as predominant menaquinones, fewer sole carbon sources being utilized, and other characteristics (Tables 1 and S1). These distinct features suggest that strain EGI 6500139^T does not belong to the species *G. lacisalsi* or *G. albus*. Based on the characteristics described above, strain EGI 6500139^T represents a novel member of the genus *Glycomyces*, for which the name *Glycomyces anabasis* sp. nov. is proposed.

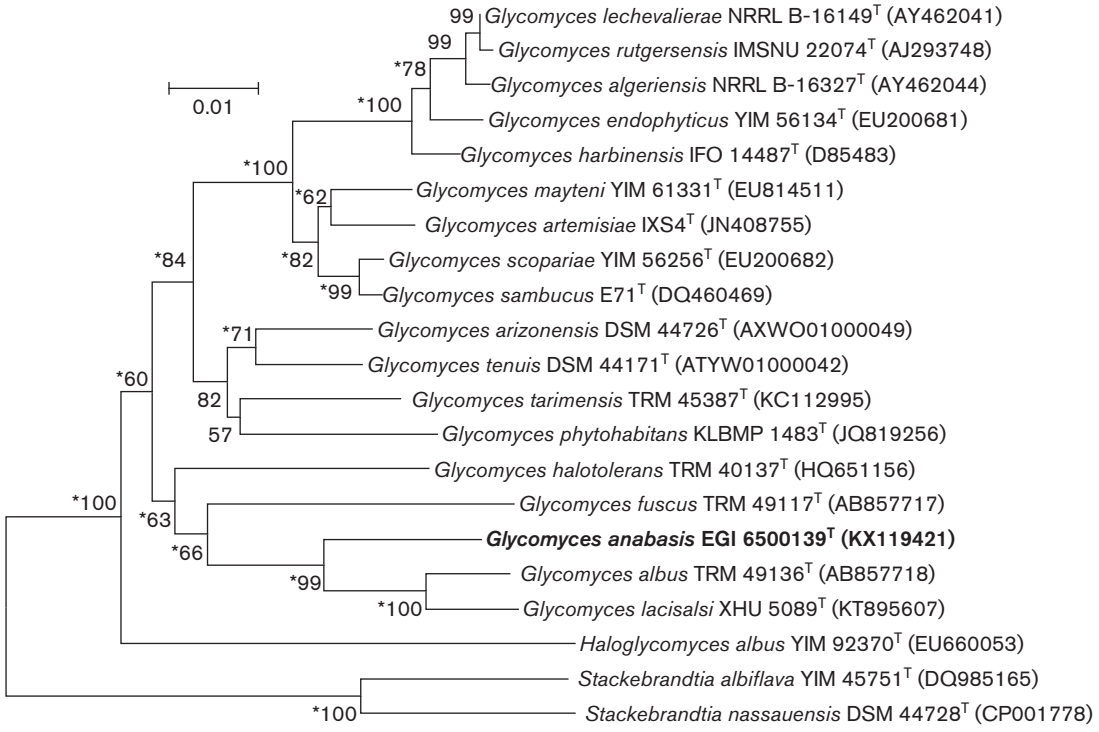

Fig. 2. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strain EGI 6500139^T. Bootstrap values (expressed as percentages of 1000 replications) above 50 % are shown at the branch nodes. Asterisks indicate that the clades were conserved when maximum-parsimony and maximum-likelihood methods were used to reconstruct the phylogenetic trees. Bar, 0.01 nucleotide sequence divergence.

DESCRIPTION OF *GLYCOMYCES ANABASIS* SP. NOV.

Glycomyces anabasis (a.na.ba′sis. N.L. gen. n. *anabasis*, of the plant genus *Anabasis*).

Gram-stain-positive and aerobic. Forms sparse aerial mycelium with rod-like spores and strong-olive-green-coloured substrate mycelia. No pigment is observed on the media tested. Growth occurs at 15–40 °C, pH 6.0–10.0 and 0–10 % (w/v) NaCl, optimally at 30 °C, pH 7.0–8.0 and 4–6 % (w/v) NaCl. Grows well on ISP 3, ISP 4, Czapek's agar and nutrient agar, moderately on ISP 2 and potato–dextrose agar, while weakly on ISP 5. No soluble pigments are observed on the media tested. D-Fructose, D-galactose, lactic acid and L-rhamnose can be used as sole carbon source, but not L-arabinose, cellobiose, D-glucose, glycerol, *myo*-inositol, D-mannose, melibiose, raffinose, D-ribose, D-sorbitol, trehalose, D-xylitol or D-xylose. L-Arginine, L-aspartic acid, L-cysteine, L-glutamic acid, L-histidine, L-isoleucine, L-lysine, L-methionine, L-threonine, L-tryptophan and L-valine can be utilized as sole nitrogen resource, but not glycine, L-phenylalanine or L-serine. Positive reactions for hydrolysis of cellulose, starch and Tweens 20, 40, 60 and 80, positive for oxidase and catalase activities, while negative for hydrolysis of casein, coagulation and peptonization of skimmed milk, H_2S production and nitrate reduction. The whole-cell hydrolysates contain *meso*-diaminopimelic acid as diagnostic diamino acid, glucose as major sugar, and mannose, galactose, xylose and ribose as minor sugars. The predominant menaquinones are MK-11 and MK-11(H_2). The polar lipids are phosphatidylglycerol, diphosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannoside, one unidentified glycolipid, one unidentified phospholipid and four unidentified polar lipids. The major fatty acids identified are anteiso-$C_{17:0}$, anteiso-$C_{15:0}$, iso-$C_{15:0}$ and iso-$C_{16:0}$. The DNA G+C content of the type strain is 70.4 mol %.

The type strain is EGI 6500139^T (=JCM 30088^T=KCTC 29495^T), isolated from surface-sterilized roots of *Anabasis aphylla* L., collected from Xinjiang, northwest PR China.

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

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