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ORIGINAL ARTICLE

Glycomyces phytohabitans sp. nov., a novel endophytic actinomycete isolated from the coastal halophyte in Jiangsu, East China

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A novel endophytic actinomycete, designated strain KLBMP 1483^T, was isolated from the stem of the coastal plant *Dendranthema indicum* (Linn.) Des Moul collected from Nantong, in East China. Phylogenetic analysis showed that strain KLBMP 1483^T was affiliated with the genus *Glycomyces* within the family *Glycomycetaceae* and shared the highest 16S rRNA gene sequence similarities with the type strains of *Glycomyces arizonensis* NRRL B-16153^T (96.7%) and *Glycomyces tenuis* IFO 15904^T (96.2%), and lower similarities (94.1–95.1%) to the other members of the genus *Glycomyces*, which distinguished KLBMP 1483^T from representatives of the genus *Glycomyces*. The whole-cell hydrolysates contained *meso*-diaminopimelic acid, glucose, xylose and galactose. The polar lipids were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannosides, two unknown aminophospholipids, two phosphoglycolipids, two unknown phospholipids and one unknown lipid. MK-10(H_4) was the predominant menaquinone. The major fatty acids were iso-$C_{15:0}$, anteiso-$C_{15:0}$, iso-$C_{16:0}$, iso-$C_{16:1}$ G and anteiso-$C_{17:0}$. On the basis of the phenotypic and genotypic characteristics presented in this study, strain KLBMP 1483^T represents a novel species, for which the name *Glycomyces phytohabitans* sp. nov. is proposed. The type strain is KLBMP 1483^T (NBRC 109116^T = DSM 45766^T).
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INTRODUCTION

Actinomycetes are widely distributed in different habitats and are well-known producers of an enormous variety of secondary metabolites, including antibiotics, antitumor agents, enzyme and immunosuppressive agents.[1] More recently, endophytic actinomycetes have attracted significant interest for their enormous biodiversity and capacity to produce a vast array of secondary metabolites.[2–5] During a study on the culturable diversity and bioactivities of endophytic actinomycetes from coastal halophytes, an aerobic actinomycete strain, KLBMP 1483^T, was isolated from the stem of *Dendranthema indicum* (Linn.) Des Moul collected from Nantong, Jiangsu Province, East China. In this study, identification and classification of the isolate are reported by a polyphasic taxonomic study, with the proposal of the name *Glycomyces phytohabitans* sp. nov.

The genus *Glycomyces* representing Gram-positive actinobacteria belongs to class *Actinobacteria*, suborder *Glycomycineae* and family *Glycomycetaceae*.[6] It was first reported by Labeda *et al.*[7] in 1985 when the type strains of *Glycomyces harbinensis* NRRL 15337^T and *Glycomyces rutgersensis* NRRLB-16106^T were, respectively, isolated from soil samples from Harbin, People's Republic of China and Rutgers University, NJ, USA during the course of isolation of actinomycete strains for antibiotic screening. Its description was later emended by Labeda and Kroppenstedt.[8] Members of the genus are characterized chemotaxonomically by type II cell walls (*meso*-diaminopimelic acid and glycine are present) and type PI phospholipid pattern with significant amounts of phosphatidylinositol mannosides. The menaquinones predominantly contain 10, 11 and/or 12 isoprene units, but the degree of saturation varies within each species. In addition, the predominant fatty acids present in the members of this genus mostly consist of iso $C_{15:0}$, anteiso $C_{15:0}$, iso $C_{16:0}$ and anteiso $C_{17:0}$.[8] At present, the genus *Glycomyces* consists of 11 species with validly published names: *Glycomyces algeriensis*, *Glycomyces arizonensis*, *Glycomyces harbinensis*, *Glycomyces lechevalierae*, *Glycomyces rutgersensis*, *Glycomyces tenuis*, *Glycomyces sambucus*, *Glycomyces mayteni*, *Glycomyces endophyticus*, *Glycomyces scopariae* and *Glycomyces halotolerans* (http://www.bacterio.net/g/glycomyces.html).[7–13]

MATERIALS AND METHODS

Strains and cultural conditions

Plant samples were collected from the coastal region of Nantong, Jiangsu Province, East China and used as the source for the isolation of endophytes. The samples were first surface sterilized using previously described five-step

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procedures.[14] After that, the samples were aseptically crumbled into smaller fragments using a commercial blender and spread onto cellulose agar medium (per liter: cellulose 2.5 g, KNO_3 1.0 g, $MgSO_4$ 0.2 g, K_2HPO_4 0.2 g, $CaCl_2$ 0.5 g, $FeSO_4$ 0.01 g, NaCl 30 g, 0.5 g amino-acid mixture and agar 15.0 g, pH 7.2). Strain KLBMP 1483^T was maintained on ISP 4 agar[15] containing 3% (w/v) NaCl at 4 °C for short-term preservation and as a glycerol suspension (20%, w/v in distilled water) at −80 °C for long-term preservation.

Morphological, physiological and biochemical characterization

Growth on various culture media was investigated by using yeast extract-malt extract agar (ISP 2), oatmeal agar (ISP 3), inorganic salts-starch agar (ISP 4), glycerol-asparagine agar (ISP 5),[15] as well as potato-dextrose (PDA), Czapek's and nutrient agars,[16] for 21 days at 28 °C. The colony and mycelia color was determined with the ISCC–NBS color charts.[17] Cell morphology was observed with light microscopy (SA3300-PL) and scanning electron microscopy (Hitachi, Tokyo, Japan; S-3400N) and cells grown for 28 days at 28 °C. Growth at different temperatures (4, 10, 15, 20, 28, 37, 45, 50 and 55 °C) was tested on ISP 2 agar containing 3% (w/v) NaCl. The ability of strain KLBMP 1483^T to grow at different salt concentrations (1–15% NaCl, w/v, at intervals of 1%) was examined by growing the novel strain on ISP 2 basal medium. The ability to grow at different pH values (4.0 to 10.0, adjusted with 1.0 M HCl or 1.0 M NaOH, at intervals of 1 pH unit) was examined in ISP 2 broth at 28 °C. Carbon and nitrogen source utilization tests were assessed according to Kurup and Schmitt[18] and Williams *et al.*[19] Other phenotypic characteristics were carried out according to Gordon *et al.*[20] All the media used were added with 3% (w/v) NaCl.

16S rRNA gene sequencing and phylogenetic analyses

Isolation of chromosomal DNA, PCR amplification and direct sequencing of the PCR products of isolate KLBMP 1483^T were carried out as described by Qin *et al.*[21] The identification of phylogenetic neighbors was initially carried out by using the BLAST program. Calculation of pairwise 16S rRNA gene sequence identities was achieved using the EzTaxon-e database.[22] The phylogenetic relationship between the isolate and closely related strains was investigated using the neighbor-joining,[23] maximum-parsimony[24] and maximum-likelihood[25] algorithms. Phylogenetic analysis was performed using MEGA version 5.[26] Tree stability was assessed by comparison with other trees constructed with the maximum-parsimony and maximum-likelihood methods. The topology of the phylogenetic tree was evaluated by using the bootstrap resampling method of Felsenstein[27] with 1000 replicates.

Chemotaxonomy

Chemotaxonomic characteristics of strain KLBMP 1483^T were determined by using biomass obtained from cultures grown in ISP 4 broth for 7 days at 28 °C with shaking. Whole-cell sugars and isomers of diaminopimelic acid in whole-cell hydrolysates were prepared and analyzed by thin-layer chromatography.[28] Purified cell wall was obtained by using the method of Kawamoto *et al.*,[29] and the amino-acid composition of hydrolyzed cell walls was determined by thin-layer chromatography. The acyl type of the muramic acid in the cell wall was determined by the method of Uchida and Aida.[30]

Menaquinones were extracted and purified as described by Collins *et al.*[31] and analyzed by HPLC.[32] Analysis of polar lipids by thin-layer chromatography was performed as described by Minnikin *et al.*[33] Cellular fatty acids were extracted from cells cultivated in ISP 2 broth at 28 °C for 7 days. Fatty acids were extracted, methylated and analyzed by using the Sherlock Microbial Identification System (MIDI) according to the manufacturer's instructions. Fatty acid methyl esters were analyzed by gas chromatography using the Microbial Identification software package (MIDI, Sherlock Version 6.1; database, TSBA6; gas chromatograph, model 6890 N, Agilent Technologies, Santa Clara, CA, USA). The G + C content of the DNA was determined by using the method of Mesbah *et al.*[34]

Nucleotide sequence accession number

The 16S rRNA gene sequence of strain KLBMP 1483^T determined in this study has been deposited in GenBank under the accession number JQ819256.

RESULTS AND DISCUSSION

An almost complete 16S rRNA gene sequence (1436 nt) was obtained for the strain KLBMP 1483^T. Comparative 16S rRNA gene sequence analysis showed that the strain was phylogenetically affiliated with species of *Glycomyces*, the highest similarities being found with the sequences of *G. arizonensis* NRRL B-16153^T (96.7% 16S rRNA gene sequence similarity) and *G. tenuis* IFO 15904^T (96.2% 16S rRNA gene sequence similarity). Levels of 16S rRNA gene sequence similarities of strain KLBMP 1483^T with the other recognized species of the genus *Glycomyces* ranged from 94.1 to 95.1%. Similarities between the 16S rRNA gene sequences of KLBMP 1483^T and all the *Glycomyces* species were observed to be less than 97%. It is generally accepted that organisms displaying 16S rRNA sequence similarity values of 97% or less belong to different species.[35] Thus, the strain KLBMP 1483^T represents a distinct species of the genus *Glycomyces*. It is evident from the phylogenetic tree (Figure 1) based on the neighbor-joining method that the strain KLBMP 1483^T clusters with the nearest neighbors *G. arizonensis* NRRL B-16153^T and *G. tenuis* IFO 15904^T in a separate branch with high bootstrap support. This topology was also confirmed in the maximum-likelihood and maximum-parsimony trees.

Morphological observation of the 4-week-old culture of strain KLBMP 1483^T revealed that white aerial mycelia were produced on some of the tested media (ISP 3, ISP 4, NA and Czapek's agar). The substrate mycelium varies from white to gray/deep gray. Black diffusible pigments were produced on ISP 3 and ISP 4 media agar. Strain KLBMP 1483^T showed good growth on all the media tested except moderate growth on ISP 5 agar and poor growth on ISP 3 media. Chains of square-ended conidia were produced on aerial mycelia (Figure 2). Growth of the strain KLBMP 1483^T occurred in the pH range 6.0–9.0 and 0–7% NaCl (w/v), with optimum growth at pH 7.0 and 3% NaCl (w/v). The temperature range for growth was 10–37 °C, with the optimum temperature being 28 °C. The detailed physiological features are indicated in Table 1 and in the species description. Several test results were obtained that enable the differentiation of strain KLBMP 1483^T from its most closely related two *Glycomyces* species (Table 1).

The cell wall of strain KLBMP 1483^T contained *meso*-diaminopimelic acid and glycine. Whole-cell hydrolysates contained glucose, xylose and galactose. The strain KLBMP 1483^T was found to contain *N*-glycolylmuramic acid. The polar lipids detected in strain KLBMP 1483^T were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannosides, two unknown aminophospholipids, two phosphoglycolipids, two unknown phospholipids and one unknown lipid (Supplementary Figure S1). The menaquinones determined in the strain KLBMP 1483^T were MK-10 (4%), MK-10(H_4) (71%), MK-11 (8%), MK-11(H_2) (11%) and MK-11(H_4) (6%). The predominant menaquinone components of strain KLBMP 1483^T were similar to *G. arizonensis* NRRL B-16153^T but different from the strain *G. tenuis* IFO 15904^T (Table 1). Major fatty acids (>10%) detected in the strain KLBMP 1483^T were iso-$C_{15:0}$ (21.8%), anteiso-$C_{15:0}$ (14.1%), iso-$C_{16:1}$ G (11.4%), iso-$C_{16:0}$ (16.3%) and anteiso-$C_{17:0}$ (10.2%). Table 2 summarizes the cellular fatty acid profiles of strain KLBMP 1483^T and its closest phylogenetic relative *G. arizonensis* NRRL B-16153^T. The fatty acid profiles of strain KLBMP 1483^T were similar to those members of the genus *Glycomyces* but differed from *G. arizonensis* NRRL B-16153^T based on the presence or proportions of $C_{18:1}$ ω9*c*, iso-$C_{15:0}$, anteiso-$C_{15:0}$, iso-$C_{16:1}$ G and iso-$C_{16:0}$. The DNA G + C content was 71.6 mol%, which is in accordance with its placement in the proposed genus too.

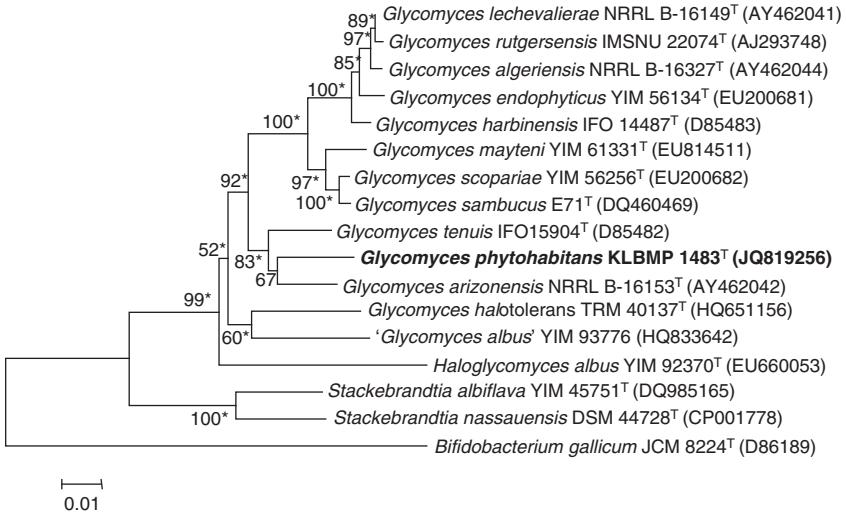

Figure 1 Phylogenetic tree based on 16S rRNA gene sequence analysis, constructed using the neighbor-joining method showing the inter-relationship of strain KLBMP 1483^T and type strains of related *Glycomyces* species. Numbers at branching points refer to bootstrap values of 1000 resamplings. The sequence of *Bifidobacterium gallicum* JCM 8224^T (D86189) was used as outgroup. Asterisks indicate the clades that were conserved when maximum-parsimony and maximum-likelihood methods were used to construct phylogenetic trees. Bar, 0.01 substitutions per nucleotide position.

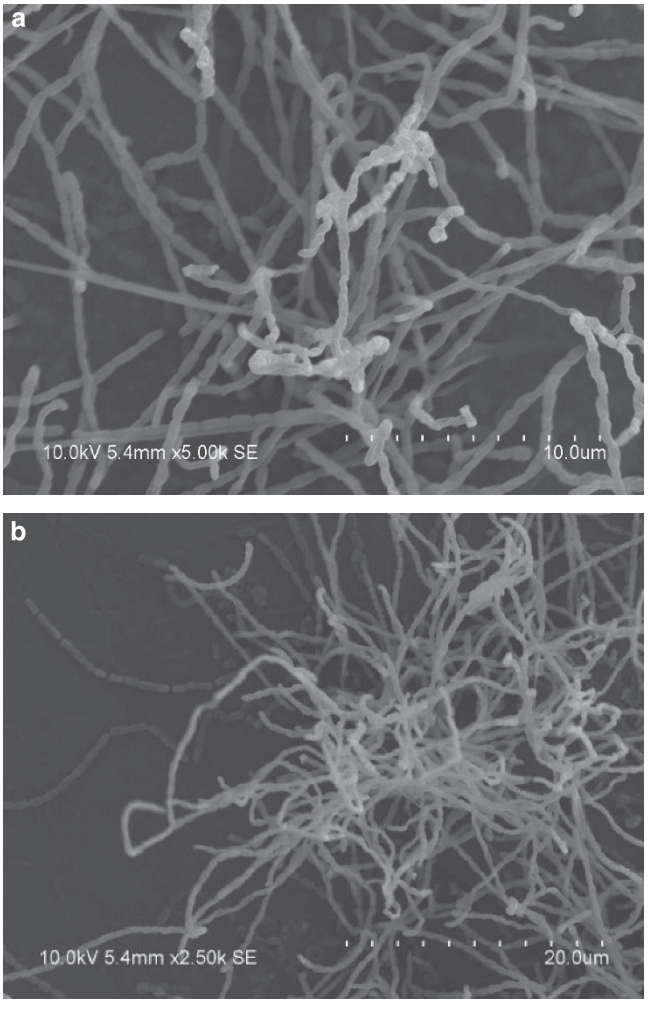

Figure 2 Scanning electron micrographs of strain KLBMP 1483^T grown on ISP 4 medium for 28 days at 28 °C. Bar indicates 10 (**a**) or 20 (**b**) μm.

The morphological and chemotaxonomic properties of strain KLBMP 1483^T were consistent with those members of the genus *Glycomyces*. The 16S rRNA gene sequence similarities between strain KLBMP 1483^T and related *Glycomyces* species were low (<97%). Furthermore, the strain KLBMP 1483^T can be distinguished from related phylogenetic closest species based on cultural and physiological and chemotaxonomic characteristics, as shown in Tables 1 and 2. Thus, on the basis of the phenotypic and genotypic characteristics, the strain KLBMP 1483^T represents a novel species within the genus *Glycomyces*, for which the name *Glycomyces phytohabitans* sp. nov. is proposed.

Description of *Glycomyces phytohabitans* sp. nov

G. phytohabitans (Phy.to.ha'bi.tans. Gr. n. phyton, plant; L. part. adj. habitans, inhabiting; N. L. part. adj. used as a masc. n. *phytohabitans*, plant inhabiting, isolated from a plant).

Cells are aerobic and Gram positive. Aerial mycelia are present and vegetative mycelia have branched hyphae. Chains of square-ended conidia were produced on aerial mycelia. Black soluble pigment produced on some media. Temperature and pH ranges for growth are 10–37 °C and pH 6.0–9.0, with optimal at 28 °C and pH 7.0. The NaCl concentration range for growth is 0–7%, with optimal growth occurring at 3%. It is positive for nitrate reduction and milk peptonization and coagulation. Urease is positive. Casein, chitin, starch, Tween 20, Tween 40 and Tween 80 are hydrolyzed, but negative for gelatin liquefaction, cellulose hydrolysis and H_2S production. D-Arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, lactose, maltose, D-mannose, rhamnose, D-ribose, trehalose and xylose are utilized as sole carbon sources, but not D-raffinose. Uses L-arginine, L-cysteine, L-proline and L-histidine as sole nitrogen sources, but negative for assimilation of L-alanine, L-asparagine, L-glycine, L-serine, L-tyrosine and L-lysine. Whole-cell hydrolysates contain *meso*-diaminopimelic acids, glycine, glucose, xylose and galactose. Menaquinones present include MK-10, MK-10(H_4), MK-11, MK-11(H_2) and MK-11(H_4). The polar lipid profile consists of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannosides, two unknown aminophospholipids, two phosphoglycolipids, two unknown phospholipids and one unknown lipid.

Table 1 Different characteristics of strain KLBMP 1483^T and the closest phylogenetic relatives

Characteristic	*Strain 1*	*Strain 2*	*Strain 3*
Growth on ISP 4 and Czapek's media	Good	Poor	ND
Aerial mycelia and colour	White	None	None
Diffusible pigment	+	−	−
Growth at 10 °C	w	−	+
Growth on 7% NaCl	+	−	−
Hydrolysis of			
Aesculin	+	−	+
Casein	+	−	+
Starch	+	−	+
Xylan	−	+	ND
Nitrate reduction	+	−	ND
Assimilation of sole carbon sources			
Dextrine	+	−	ND
Erythrityl	+	−	ND
Inositol	+	−	ND
D-Lactose	+	−	+
D-Raffinose	−	+	+
D-Rhamnose	+	−	+
D-Sorbitol	+	−	ND
Trehalose	+	−	ND
Acid produced from			
D-Arabinose	+	−	−
D-Fructose	+	−	+
Maltose	−	−	+
D-Sorbitol	+	−	−
Assimilation of sole nitrogen sources			
L-Arginine	+	−	ND
L-Cysteine	+	−	ND
L-Histidine	+	+	−
L-Tyrosine	−	+	ND
Menaquinone:	MK-10 (4%), MK-10(H_4) (71%), MK-11 (8%), MK-11(H_2) (11%), MK-11(H_4) (6%)	MK-10(H_2) (18%), MK-10(H_4) (52%), MK-11(H_2) (8%), MK-11(H_4) (22%)	Predominant: MK-9(H_6), MK-10(H_6), MK-11(H_6)
G+C content (mol%)	71.6	ND	72

Abbreviations: +, Positive or present; −, negative or absent; ND, not determined; w, weakly positive. Strain 1, KLBMP 1483^T (data from present study); Strain 2, *Glycomyces arizonensis* NRRL B-16153^T (data from present study except the menaquinone component); Strain 3, *Glycomyces tenuis* IFO 15904^T (data from Evtushenko *et al.*[9]).

The major fatty acids were iso-$C_{15:0}$, anteiso-$C_{15:0}$, iso-$C_{16:0}$, iso-$C_{16:1}$ G and anteiso-$C_{17:0}$. The genomic DNA G+C content was 71.6 mol%. The type strain, KLBMP 1483^T (=NBRC 109116^T=DSM 45766^T) was isolated from surface-sterilized stems of the coastal halophyte *Dendranthema indicum* (Linn.) Des Moul collected from the coastal region of Nantong, Jiangsu Province, East China.

Table 2 Fatty acid profiles (%) of strain KLBMP 1483^T and the closest phylogenetic relative

Fatty acid	*Strain 1*	*Strain 2*
$C_{14:0}$	−	1.6
$C_{16:0}$	4.9	3.2
$C_{18:0}$	1.2	−
anteiso-$C_{17:1}$ A	6.4	5.2
iso-$C_{14:0}$	1.7	7.7
iso-$C_{15:0}$	21.8	10.1
anteiso-$C_{15:0}$	14.1	24.2
iso-$C_{16:1}$ G	11.4	9.4
iso-$C_{16:0}$	16.3	23.0
iso-$C_{17:0}$	1.5	−
anteiso-$C_{17:0}$	10.2	12.6
$C_{18:1}$ ω9*c*	5.6	−
Sum In Feature 3[a]	−	0.6
Sum In Feature 5[a]	2.8	−

Strain 1, KLBMP 1483^T (data from present study); Strain 2, *Glycomyces arizonensis* NRRL B-16153^T (data from present study). Fatty acids amounting to less than 0.5% in all species are not shown; −, not detected.
[a]Summed features represent groups of two or three fatty acids that cannot be separated by GC with the MIDI system. Summed features 3, 5 comprised 16:1ω7*c*/16:1ω6*c*, 18:2ω6, 9*c* /18:0 ante, respectively.

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