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ORIGINAL PAPER

Variovorax guangxiensis sp. nov., an aerobic, 1-aminocyclopropane-1-carboxylate deaminase producing bacterium isolated from banana rhizosphere

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Abstract A 1-aminocyclopropane-1-carboxylate deaminase producing bacterium, designated GXGD002^T, was isolated from the rhizosphere of banana plants cultivated in Guangxi province, China. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain GXGD002^T is a member of the genus *Variovorax*. High levels of 16S rRNA gene sequence similarity are found between strain GXGD002^T and *Variovorax paradoxus* DSM 30034^T (99.4 %), *Variovorax ginsengisoli* KCTC 12583^T (99.1 %), *Variovorax boronicumulans* KCTC 22010^T (99.0 %), *Variovorax soli* DSM18216^T (98.7 %), *Variovorax defluvii* DSM 27259^T (98.1 %) and *Variovorax dokdonensis* KCTC 12544^T (97.4 %) respectively. However, the DNA–DNA hybridization values between strain GXGD002^T and its closely related species *V. paradoxus* DSM 30034^T, *V. ginsengisoli* KCTC 12583^T and *V. boronicumulans* KCTC 22010^T were found to be 40.7, 30.9 and 23.7 %, respectively. The DNA G + C content of strain GXGD002^T was found to be 67.8 mol%. The major fatty acids of strain GXGD002^T are $C_{16:0}$ (20.3 %), $C_{10:0}$ 3OH (18.4 %), $C_{17:0}$ cyclo (18.9 %), $C_{18:1}w_7c$ (12.3 %) and summed feature 3 (13.9 %). The predominant respiratory quinone was identified as ubiquinone-8 (Q-8) and the major polar lipids as phosphatidylethanolamine and phosphatidylglycerol. The results of polyphasic taxonomic study including physiological and biochemical tests, whole-cell SDS-PAGE profiles and chemotaxonomic analysis allowed a clear differentiation of strain GXGD002^T from the other species in the genus *Variovorax*. Based on these results, a new species, *Variovorax guangxiensis,* is proposed. The type strain is GXGD002^T (=DSM 27352^T = ACCC 05911^T).

Keywords *Variovorax guangxiensis* sp. nov. · Taxonomy · 1-Aminocyclopropane-1-carboxylate deaminase · Banana rhizosphere

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Introduction

Ethylene is an important signaling molecule in plants which under pathogen attack or abiotic stress results in plant growth inhibition (Abeles et al. 1992). 1-amino-cyclopropane-1-carboxylate (ACC) deaminase, commonly found in plant-growth-promoting rhizobacteria (Shah et al. 1998), cleaves ACC, the immediate precursor of the plant hormone ethylene, to produce a-ketobutyrate and ammonia (Todorovic and Glick 2008). It has been reported that some ACC deaminase-producing bacteria promote plant growth under a variety of stressful conditions, such as flooding, saline conditions, and drought by lowering plant ethylene levels (Onofre-Lemus et al. 2009). Importantly, using ACC deaminase-producing bacteria in association with plants subjected to a wide range of different kinds of biotic and abiotic stresses could enhance plant tolerance to the stresses (Glick 2014).

The genus *Variovorax* was erected with the reclassification of *Alcaligenes paradoxus* as *Variovorax paradoxus* (Willems et al. 1991). Phylogenetic analysis based on 16S rRNA gene sequence similarities showed that the genus *Variovorax* belongs to the family *Comamonadaceae* and class *Betaproteobacteria* (Anzai et al. 2000). At the time of writing, the genus comprises six validly named species, *V. paradoxus* (Willems et al. 1991), *Variovorax dokdonensis* (Yoon et al. 2006), *Variovorax soli* (Kim et al. 2006), *Variovorax boronicumulans* (Miwa et al. 2008), *Variovorax ginsengisoli* (Im et al. 2010) and *Variovorax defluvii* (Jin et al. 2012). Most of the members of the genus have been isolated from soils, except *V. defluvii* which has been isolated from sewage. Members of the genus *Variovorax* are typical Gram-negative, rod-shaped motile bacteria that form yellowish colonies. During an investigation of the diversity of ACC deaminase-producing bacteria in plant rhizosphere, a novel strain, designated GXGD002^T, was isolated. Polyphasic taxonomy data indicated that this strain represents a novel species of the genus *Variovorax*.

Materials and methods

Strains and culture conditions

Strain GXGD002^T was isolated from the rhizosphere of banana(*Musa paradisiaca)* planted in Guangxi province, People's Republic of China. For enrichment and isolation, one g the soil samples were enriched in 50 ml sterile TSB medium (tryptone soy broth, CM129, Oxoid) in a 250-ml flask. The flask and its contents are incubated in a shaking water bath (200 r.p.m.) at a temperature 28 °C. After 24 h, one ml aliquot was removed from the growing culture, transferred to 50 ml of sterile DF medium (Penrose and Glick 2003) in a 250-ml flask and incubated at the same condition for 24 h, then one ml aliquot of the growing culture was transferred to 50 ml of sterile ADF medium (Penrose and Glick 2003) in a 250-ml flask and incubated at the same condition for 48 h. Following these three incubations, the final growing culture was diluted using the standard dilution plating technique and spread on the plates of ADF medium. Inoculated plates were incubated at 28 °C for 2–3 days. Colonies that appeared on the plates were then picked and re-streaked on fresh media, until pure cultures were obtained. Strain GXGD002^T was selected and pure culture was maintained at −80 °C in 30 % (v/v) glycerol. The type strains of *V. paradoxus* DSM 30034^T, *V. ginsengisoli* KCTC 12583^T, *V. boronicumulans* KCTC 22010^T, *V. soli* DSM18216^T, *V. defluvii* DSM 27259 ^{T} and *V. dokdonensis* KCTC 12544^T were used as reference strains under the same conditions.

Phenotypic characterization

Colony macromorphology of the isolate was observed on LB medium agar plate(Miller 1972). Cell morphology was examined using light microscopy and scanning electron microscopy (SEM). Gram staining was determined using the bioMérieux Gram Stain kit according to the manufacturer's instructions. Motility was observed under a phase-contrast microscope (Leica DM5500; 1,000 × magnification) with cells grown on R2A agar plates (BD) (Reasoner and Geldreich 1985) for 3 days. Catalase activity was determined based on bubble production in 3 % (v/v) H_2O_2 and oxidase activity was determined by using 1 % (w/v) tetramethyl p-phenylenediamine. Growth was tested at 5, 10, 25, 30, 35, 37, and 40 °C on LB medium agar plates and pH tolerance was tested using the same medium adjusted to various pHs (4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0). Carbon source utilization tests, acid production tests and additional physiological and biochemical characteristics were performed

using Biolog GEN III microtest system (Biolog, USA) and API 20NE, API 50CH and API ZYM systems (bioMe′rieux, France) according to the manufacturer's instructions. Activity of ACC deaminase of strain GXGD002^T was determined according to Penrose and Glick (2003).

Electrophoresis of whole-cell proteins

Strain GXGD002^T and its closely related species that share more than 99.0 % 16S rRNA gene sequence similarity with strain GXGD002^T, *V. paradoxus* DSM 30034^T, *V. ginsengisoli* KCTC 12583^T, *V. boronicumulans* KCTC 22010^T were grown in LB broth, and were subjected to SDS-PAGE analysis of whole-cell proteins as described elsewhere(Tan et al. 1997). The normalized densitometric traces of the electrophoretic protein patterns were grouped using the Gelcompar II software package (Applied Maths). The similarity between each pair of samples (strains) was expressed by DICE and an UPGMA dendrogram was constructed (Vauterin and Vauterin 1992).

Chemotaxonomy

For determination of cellular fatty acid composition, strain GXGD002^T and the type strains of related *Variovorax* species were grown for 2 days at 28 °C on R2A medium agar plates (BD) (Reasoner and Geldreich 1985). The cellular fatty acids were derivatized to methyl esters (Sasser 1990) and analysed using gas chromatograph (Hewlett Packard 6890) according to the Microbial Identification System (MIDI; Sherlock Version 6.0). Polar lipids were extracted according to the method of Minnikin et al. (1984) and identified as described by Collins and Jones (1980). Respiratory quinones were extracted according to the method of Collins et al. (1977), purified using thin layer chromatography (TLC) and analyzed using HPLC- spell out (HPLC) as described by Collins and Jones (1980).

Molecular characterization

Preparation of genomic DNA was carried out according to the method of Marmur (1961). The DNA G + C content was determined using the thermal denaturation method (Marmur and Doty 1962) and *Escherichia coli* K-12 was used as a control. DNA–DNA relatedness was determined by the initial renaturation rate method in 2 × SSC (De Ley 1970). A loop of biomass was scraped off the LB medium agar plate, suspended in 100 µl ddH_2O and lysed by boiling for 10 min and freezing for 5 min. Following centrifugation, the supernatant was used as the template for 16S rRNA gene amplification. The 16S rRNA gene was amplified using the universal primers 27F and 1492R (Lane 1991). Purified PCR products approximately 1.5 kb in length were sequenced by the machine using an ABI 3730 DNA Analyzer (Applied Biosystems Company). The almost complete 16S rRNA gene sequence (1,395 bp) was determined by direct sequencing and compared with available 16S rRNA gene sequences in the EzTaxon database (http://eztaxon-e.ezbiocloud.net/; Kim et al. 2012). Phylogenetic analysis was performed using the software package MEGA 4.1 after multiple alignments of the sequences data using Clustal X (Thompson et al. 1997). Phylogenetic trees were constructed using on the neighbour-joining (Saitou and Nei 1987), the maximum parsimony (Fitch 1971) and maximum-likelihood (Felsenstein 1981) algorithms and the bootstrapanalysis were based on 1,000 replicates.

Results and discussion

Phenotypic characteristics

Morphological, cultural, physiological and biochemical characteristics of strain GXGD002^T are given in the species description. The distinctive phenotypic features of the strain GXGD002^T and the other species in the genus *Variovorax* are shown in Table 1. Strain GXGD002^T exhibited ACC deaminase activity; it was able to cleave 59 nmol a-ketobutyrate (mg protein)$^{-1}$ min^{-1}. It has been reported that ≥20 nmol a-ketobutyrate (mg protein)$^{-1}$ min^{-1} is sufficient to initiate plant-growth-promoting effects (Penrose and Glick 2003).

SDS-PAGE of whole-cell proteins

Analysis of whole-cell protein profiles distinct clustering of strain GXGD002^T and four other species, *V. paradoxus* DSM 30034^T, *V. ginsengisoli* KCTC 12583^T, *V. boronicumulans* KCTC 22010^T and *V. dokdonensis* KCTC 12544^T, included in this study at a similarity level of approximalety 80 %. The dendrogram based on protein patterns is presented in Fig. 1.

Table 1 Phenotypic characteristics that distinguish strain GXGD002^T from other *Variovorax* species, Species: 1, Strain GXGD002^T; 2, *Variovorax paradoxus* DSM 30034^T; 3, *V. boronicumulans* KCTC 22010^T; 4, *V. dokdonensis* KCTC 12544^T; 5, *V. ginsengisoli* KCTC 12583^T; 6, *V. soli* DSM18216^T; 7, *V.defluvii* DSM 27259^T

Characteristic	1	2	3	4	5	6	7
Nitrate reduction	−	+	−	−	+	+	−
Indole production	+	−	+	+	−	+	−
Esculine hydrolysis	+	+	−	−	−	−	+
Gelatin hydrolysis	−	−	(+)	−	−	−	(+)
Assimilation of							
lucose	+	−	+	+	−	−	−
Arabinose	+	−	+	−	−	+	−
Mannose	−	−	+	−	−	−	−
Mannitol	+	+	+	−	−	+	−
N-acetyl-glucosamine	+	−	−	−	−	−	−
Potasium gluconate	+	+	+	+	−	−	−
Capric acid	+	−	+	−	−	−	−
Adipic acid	−	−	−	+	−	−	−
Malic acid	+	−	+	−	−	−	+
Trisodium citrate	−	−	+	−	−	−	−
Phenylacetic acid	−	−	+	−	−	−	−
D-Ribose	+	−	−	−	−	−	−
D-Adonitol	+	−	+	−	−	−	−
D-Galactose	+	−	−	−	−	−	−
D-Fucose	+	−	+	−	−	−	−
D-Sorbitol	+	−	+	−	−	−	−
D-Lactose	+	−	(+)	−	−	−	−
Xylitol	+	−	+	−	−	−	−
D-Arabitol	+	−	+	−	−	−	−
L-Arabitol	+	−	+	−	−	−	−
Enzyme activities							
Valine arylamidase	−	+	−	−	(+)	+	(+)
Cystine arylamidase	−	−	−	(+)	−	(+)	−
ß-galactosidase	−	−	(+)	−	−	−	−
α-glucosidase	(+)	+1	+	+	−	−	−
Urease	−	−	−	+	−	−	−
Utilization of (Biolog GN3)							
Dextrin	(+)	−	+	−	−	−	−
D-Fucose	+	−	+	−	−	−	−
L-Fucose	+	−	−	−	−	−	−
1 % Sodium lactate	−	−	+	+	−	+	+
D-Aspartic acid	−	+	+	−	−	−	−
Troleandomycin	+	+	+	−	−	−	−
L-Aspartic acid	−	+	+	−	−	(+)	+
L-Glutamic acid	−	+	+	−	−	(+)	−
L-Pyroglutamic acid	−	+	+	−	−	−	−
D-Gluconic acid	+	+	−	−	−	−	−
D-Glucuronic acid	+	+	−	−	−	−	−

Table 1 continued

Characteristic	1	2	3	4	5	6	7
Glucuronamide	+	(+)	−	−	−	−	−
Quinic acid	+	+	+	−	−	+	+
Vancomycin	+	+	+	−	−	+	+
α-Ketoglutaric acid	−	+	+	−	−	−	−
L-Malic acid	+	−	−	−	−	−	−
ß-Hydroxy-D, L-butyric acid	−	+	+	−	−	−	−
Acetoacetic acid	(+)	−	−	+	−	−	−
Acetic acid	+	+	+	−	−	−	−
DNA G + C content (mol%)	67.8	67.0[a]*	71.2[b]	66.0[c]	66.0[d]	67.1[e]	65.5[f]

All data are from this study unless indicated. All strains were grown on R2A for 2 days.+ positive; (+) weak positive; − negative

[a] Willems et al. (1991)

[b] Miwa et al. (2008)

[c] Yoon et al. (2006)

[d] Im et al. (2010)

[e] Kim et al. (2006)

[f] Jin et al. (2012)

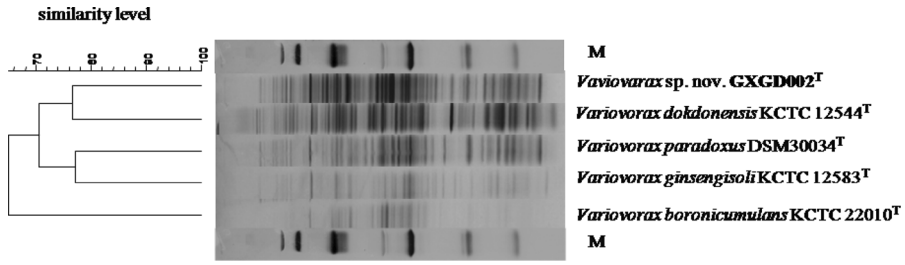

Fig. 1 Dendrogram based on whole-cell protein electrophoretic profiles of strain GXGD002^T and type strains of *Variovorax paradoxus, Variovorax dokdonensis, Variovorax ginsengisoli and Variovorax boronicumulans. M* markers

Chemotaxonomic characteristics

The major fatty acids of strain GXGD002^T are $C_{16:0}$ (20.3 %), $C_{10:0}$ 3OH (18.4 %),$C_{17:0}$ cyclo (18.9 %), $C_{18:1}w_7c$ (12.3 %) and summed feature 3(13.9 %). This fatty acid profile is typical for the genus *Variovorax*, although there are some differences in the proportions of the fatty acids (Table 2). The dominant phospholipids of strain GXGD002^T were found to be phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) (Supplementary Fig. S1). The predominant respiratory quinone was ubiquinone-8 (Q-8).

Molecular characterization

The DNA G + C content of GXGD002^T was found to be 67.8 mol%. Genomic DNA relatedness between strain GXGD002^T and its closely related species *V. paradoxus* DSM 30034^T, *V. ginsengisoli* KCTC 12583^T, *V. boronicumulans* KCTC 22010^T was 40.7, 30.9 and 23.7 %, respectively. These values are below 70 %, which is the recommended value for species definition (Wayne et al. 1987).

The phylogenetic analysis based on the 16S rRNA gene sequence (1,395 bp) of GXGD002^T clearly indicated that strain GXGD002^T belongs to the genus *Variovorax* showing 99.4, 99.1, 99.0, 98.7, 98.1 and 97.4 % 16S rRNA gene sequence similarities with the type strains of *V. paradoxus*, *V.ginsengisoli*, *V. boronicumulans*, *V. soli*, *V. defluvii* and *V. dokdonensis* respectively. Strain GXGD002^T formed a robust cluster with *V. paradoxus* DSM 30034^T, *V. ginsengisoli* KCTC 12583^T, *V. boronicumulans* KCTC 22010^T

Table 2 Cellular fatty acid compositions (%) of strain GXGD002^T and the type strains of other *Variovorax* species

Fatty acid	1	2	3	4	5	6	7
$C_{8:0}$ 3-OH	0.17	0.34	0.22	–	–	–	1.09
$C_{9:0}$ 3-OH	–	–	–	–	–	0.16	0.14
$C_{10:0}$	–	–	–	0.28	–	–	0.11
$C_{10:0}$ 3-OH	18.42	4.01	10.43	2.31	2.94	4.25	4.33
$C_{12:0}$	4.85	4.82	3.90	3.95	3.77	3.98	7.21
$C_{12:0}$ 3-OH	0.43	–	–	1.73	–	–	–
$C_{13:0}$	–	–	–	–	–	0.37	0.22
$C_{14:0}$	0.68	0.75	0.52	2.13	4.93	0.79	1.46
$C_{14:0}$ 2-OH	3.14	2.95	2.55	–	–	–	4.87
$C_{15:0}$	0.32	1.61	–	1.12	0.81	2.46	1.91
$C_{15:1}$ w6c	–	0.45	–	0.55	–	1.70	0.99
$C_{16:0}$	20.33	23.27	29.61	27.21	35.68	29.14	24.57
$C_{16:0}$ 2-OH	0.86	–	1.95	–	–	–	–
$C_{16:1}$ 2-OH	2.34	0.35	1.63	–	–	–	–
$C_{17:0}$	–	0.43	–	0.44	–	1.40	0.48
$C_{17:0}$ cyclo	18.92	23.09	31.84	29.35	17.21	23.63	26.30
$C_{18:0}$	0.54	–	0.75	0.31	–	0.29	–
$C_{18:1}$ 2-OH	1.23	–	0.52	–	–	–	–
$C_{18:1}$ w7c	12.26	6.46	5.03	9.77	10.81	6.74	6.79
$C_{19:0}$ cyclo w8c	0.53	0.47	6.14	–	–	–	–
$C_{20:0}$	0.33	–	–	–	–	–	–
Summed feature 3	13.93	9.61	4.25	20.26	21.94	23.75	17.81
Summed feature 4	0.16	0.24	–	–	–	0.11	0.19

All data are from this study. Cells of all strains were harvested after growth on R2A medium at 30 °C for 2 days. –, Not detected

Summed feature 3 contains16:1 w7c/15 iso 2OH; Summed feature 4 contains Contains 17:1 ISO I/ANTEI B

Strains: *1* GXGD002^T; *2 Variovorax paradoxus* DSM 30034^T; *3 V. boronicumulans* KCTC 22010T; *4 V. dokdonensis* KCTC 12544T; *5 V. ginsengisoli* KCTC 12583T; *6 V. soli* D18216; *7V. defluvii* DSM 27259

and *V. dokdonensis* KCTC 12544^T in the neighbour-joining phylogenetic tree (Fig. 2) constructed using 16S rRNA gene sequences of all species of the genus *Variovorax* and some other related species within the family *Comamonadaceae*. The same topologies were retrieved in the maximum-parsimony and maximum-likelihood phylogenetic trees (Supplementary Fig. S2 and Fig. S3).

Taxonomic conclusion

Strain GXGD002^T could be easily distinguished from the other species in the genus *Variovorax* by distinct whole-cell SDS-PAGE profile, differences in the fatty acid composition and phenotypic characteristics. The results of the phylogenetic, morphological and chemotaxonomic analyses together with low DNA–DNA hybridization values support the proposal that strain GXGD002^T should be assigned to the genus *Variovorax* as a new species, for which the name *Variovorax guangxiensis* sp. nov. is proposed.

Description of *Variovorax guangxiensis* sp. nov.

Variovorax guangxiensis (gu.ang.xi.en'sis. N.L. fem. adj. guangxiensis referring to Guangxi Province, China, pertaining to the geographic location where the type strain was isolated).

Cells are Gram-stain negative, motile, aerobic rods with approximately 0.4–0.5 μm wide and 1.2–4.2 μm long (Supplementary Fig. S4). Colonies are circular with entire margins, convex, yellowish, glossy and 1–2 mm in diameter on LB agar plates after 3–4 days incubation at 28 °C. The temperature range for growth is 20–35 °C, with optimal growth at 30 °C. The pH range for growth is 5.0–9.0, with optimum pH 7.0. Enzyme activities for catalase and oxidase are positive. In the API systems, positive for assimilation of some substrates, including glucose, arabinose, mannitol, *N*-acetyl-glucosamine, potassium gluconate, capric acid, malic acid, D-ribose, D-adonitol, D-galactose, D-fucose, D-sorbitol, D-lactose, xylitol, D-arabitol and L-arabitol; but negative for assimilation of

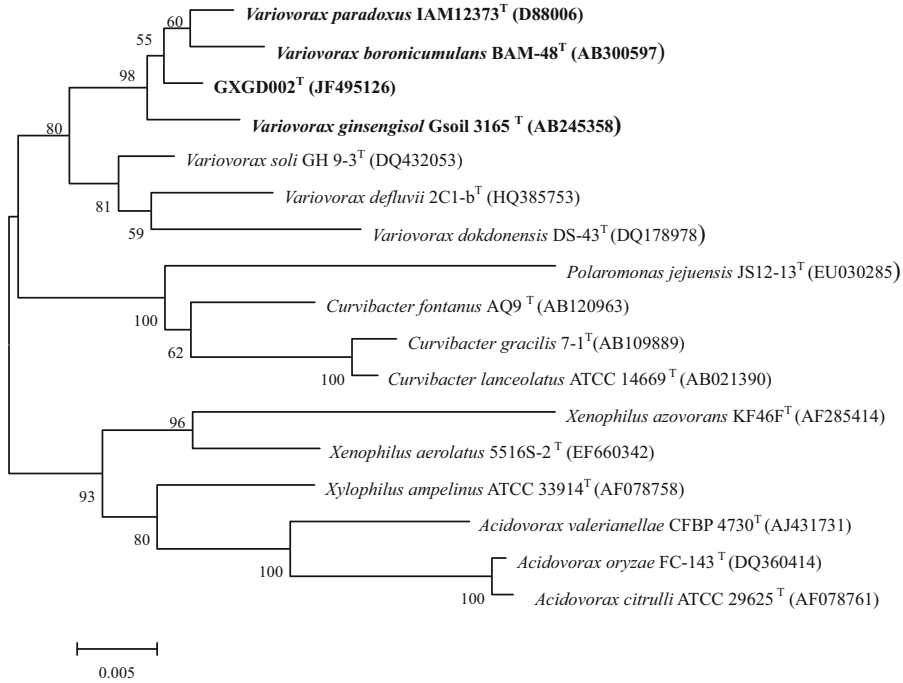

Fig. 2 Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strain GXGD002^T among species of the genus *Variovorax* and related genera with validly published names. Bootstrap analyses were determined based on 1,000 resamplings; values >50 % were shown. *Bar* 0.005 substitutions per nucleotide position

other substrates. Nitrate reduction, production of indole and hydrolysis of esculine are positive. In the tests of API ZYM, only one enzyme activity is detected for α-glucosidase; but no enzyme activities are detected for valine arylamidase, cystine arylamidase, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine, arylamidase, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-galactosidase, β-glucuronidase, β-glucosidase, lipase (C14), trypsin, α-galactosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. In the Biolog GEN III microtest system, dextrin, D-fucose, L-fucose, D-gluconic acid, D-glucuronic acid, glucuronamide, quinic acid, L-malic acid, acetoacetic acid, acetic acid, troleandomycin, vancomycin are utilised. The predominant respiratory quinone is ubiquinone-8 (Q-8). The major fatty acids consist of $C_{16:0}$, $C_{10:0}$ 3OH, $C_{17:0}$ cyclo, $C_{18:1}\omega_7c$ and summed feature 3. The dominant phospholipids are PE and PG. The G + C content of the DNA of the type strain is 67.8 mol%. The type strain, GXGD002^T(= DSM 27352^T = ACCC 05911^T), was isolated from the rhizosphere soil of banana sampled from Guangxi province, People's Republic of China.

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