Novosphingobium gossypii sp. nov., isolated from *Gossypium hirsutum*

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A Gram-stain-negative, rod-shaped, non-spore-forming bacterium (strain JM-1396^T) producing a yellow pigment, was isolated from the healthy internal stem tissue of post-harvest cotton (*Gossypium hirsutum*, cultivar 'DES-119') grown at the Plant Breeding Unit at the E. V. Smith Research Center in Tallassee (Macon county), AL, USA. 16S rRNA gene sequence analysis of strain JM-1396^T showed high sequence similarity values to the type strains of *Novosphingobium mathurense, Novosphingobium panipatense* (both 98.6 %) and *Novosphingobium barchaimii* (98.5 %); sequence similarities to all other type strains of species of the genus *Novosphingobium* were below 98.3 %. DNA–DNA pairing experiments of the DNA of strain JM-1396^T and *N. mathurense* SM117^T, *N. panipatense* SM16^T and *N. barchaimii* DSM 25411^T showed low relatedness values of 8 % (reciprocal 7 %), 24 % (reciprocal 26 %) and 19 % (reciprocal 25 %), respectively. Ubiquinone Q-10 was detected as the dominant quinone; the fatty acids $C_{18:1}\omega7c$ (71.0 %) and the typical 2-hydroxy fatty acid, $C_{14:0}$ 2-OH (11.7 %), were detected as typical components. The polar lipid profile contained the diagnostic lipids diphosphatidylglycerol, phosphatidylethanolamine, sphingoglycolipid and phosphatidylcholine. The polyamine pattern contained the major compound spermidine and only minor amounts of other polyamines. All these data revealed that strain JM-1396^T represents a novel species of the genus *Novosphingobium*. For this reason we propose the name *Novosphingobium gossypii* sp. nov. with the type strain JM-1396^T (=LMG 28605^T=CCM 8569^T=CIP 110884^T).

Representatives of the genus *Novosphingobium* have been isolated from a large variety of environmental sources, including coastal and freshwater sediments and soil (Balkwill *et al.*, 1997; Sohn *et al.*, 2004; Liu *et al.*, 2005), surface water layers of lakes (Glaeser *et al.*, 2009; 2013a, b), activated sludge and wastewater treatment plants (Neef *et al.*, 1999; Fujii *et al.*, 2003) and contaminated groundwater bioremediation reactors (Tiirola *et al.*, 2002; 2005), and have also been recently found associated with plants (Lin *et al.*, 2014; Kämpfer *et al.*, 2015). At the time of writing, 29 species of the genus *Novosphingobium* have been described with validly published names: *Novosphingobium acidiphilum* (Glaeser *et al.*, 2009), *N. aquaticum* (Glaeser *et al.*, 2013a), *N. arabidopsis* (Lin *et al.*, 2014), *N. aromaticivorans* (Balkwill *et al.*, 1997), *N. aquiterrae* (Lee *et al.*, 2014b), *N. barchaimii* (Niharika *et al.*, 2013), *N. capsulatum* (Yabuuchi *et al.*, 1990), *N. chloroacetimidivorans* (Chen *et al.*, 2014), *N. fuchskuhlense* (Glaeser *et al.*, 2013b), *N. hassiacum* (Kämpfer *et al.*, 2002), *N. indicum* (Yuan *et al.*, 2009), *N. kunmingense* (Xie *et al.*, 2014), *N. lentum* (Tiirola *et al.*, 2005), *N. lindaniclasticum* (Saxena *et al.*, 2013), *N. malaysiense* (Lee *et al.*, 2014a), *N. mathurense* and *N. panipatense* (Gupta *et al.*, 2009), *N. naphthalenivorans* (Suzuki & Hiraishi, 2007), *N. nitrogenifigens* (Addison *et al.*, 2007), *N. pentaromativorans* (Sohn *et al.*, 2004), *N. rosa* (Takeuchi *et al.*, 1995), *N. resinovorum* (Lim *et al.*, 2007), *N. sediminicola* (Baek *et al.*, 2011), *N. soli* (Kämpfer *et al.*, 2011), *N. stygium* (Balkwill *et al.*, 1997), *N. subarcticum* (which is a later subjective synonym of *N. resinivorum*; Lim *et al.*, 2007), *N. subterraneum* (Balkwill *et al.*, 1997),

Abbreviations: LTP, All Species Living Tree Project database; NA, nitroanilide; NP, nitrophenyl.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain JM-1396^T is KP657488.

N. taihuense (Liu *et al.*, 2005) and *N. tardaugens* (Fujii *et al.*, 2003). A further species proposal was recently published, but the species name has not yet been validly published at the time of writing, '*N. ginsenosidimutans*' (Kim *et al.*, 2013).

Strain JM-1396^T was isolated from the healthy internal stem tissue of post-harvest cotton (*Gossypium hirsutum*, cultivar 'DES-119') grown at the Plant Breeding Unit at the E. V. Smith Research Center in Tallassee (Macon county), AL, USA. This isolate formed small yellow colonies (<0.5 mm) showing a smooth surface after 48 h at 25 °C on nutrient agar. Cell morphological features investigated with cells grown on nutrient agar at 25 °C by phase-contrast microscopy showed growth as single cells with a rod-shaped morphology with cells 2.0 ± 0.4 μm long and 1.0 ± 0.2 μm wide. The cells stained Gram-negative and were positive for cytochrome oxidase, as determined using an oxidase test (Merck). Endospores could not be detected by light microscopy. The strain was able to produce catalase, after 24 h of growth on nutrient agar with detected by bubble formation after addition of H_2O_2 on the agar plates.

The 16S rRNA gene of strain JM-1396^T was PCR-amplified and sequenced by the dideoxy method using bacterial 16S rRNA gene targeting primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-ACGGCTACCTTGTTACGACTT-3′; Lane, 1991). Sequence processing was performed in MEGA5 (Tamura *et al.*, 2011). Unclear 5′and 3′ ends were removed and the electropherograms were controlled manually. Phylogenetic analyses were performed using the software package ARB release 5.2 (Ludwig *et al.*, 2004) and the 'All Species Living Tree Project' (LTP) database of the current release LTPs119, November 2014 (Yarza *et al.*, 2008). The 16S rRNA gene sequence of strain JM-1396^T was aligned with the SILVA Incremental Aligner (SINA; v1.2.11) (Pruesse *et al.*, 2012), against the LTP database. The sequence alignment was checked manually before further analysis. Pairwise sequence similarities among type strains of species of the genus *Novosphingobium* were calculated using the ARB neighbour-joining tool, without the application of an evolutionary model. Phylogenetic trees were reconstructed by the maximum-likelihood method using RAxML version 7.04 (Stamatakis, 2006) with GTR-GAMMA and rapid bootstrap analysis and by the maximum-parsimony method using DNAPARS version 3.6 (Felsenstein, 2005). Both phylogenetic trees considered 100 resamplings (bootstrap analysis; Felsenstein, 1985) and were based on 16S rRNA gene sequences between gene termini 68 and 1450 (according to the *Escherichia coli* numbering, Brosius *et al.*, 1981).

The sequenced 16S rRNA gene fragment of strain JM-1396^T was 1404 nt long, spanning 16S rRNA gene positions 16 to 1474 (according to *E. coli* numbering). The signature gap at 16S rRNA gene sequence positions 1257 to 1278 (*E. coli* numbering) obtained for *N. acidiphilum* (Glaeser *et al.*, 2009) and *N. nitrogenifigens* (Addison *et al.*, 2007) was not present in the 16S rRNA gene sequence of strain JM-1396^T, as obtained for all other species of the genus *Novosphingobium*. Pairwise 16S rRNA gene sequence similarities of strain JM-1396^T to type strains of species of the genus *Novosphingobium* ranged from 94.1 % to 98.6 % with the highest 16S rRNA gene sequence similarity to type strains of *N. mathurense*, *N. panipatense* (both 98.6 %) and *N. barchaimii* (98.5 %). Similarities to all other *Novosphingobium* type strains were below 98.2 %. Both reconstructed phylogenetic trees showed clustering of JM-1396^T with *N. mathurense*, *N. panipatense* and *N. pentaromativorans*; however, this was not supported by high bootstrap values (Fig. 1). A direct clustering with *N. barchaimii* was not obtained.

DNA–DNA pairing experiments with strain JM-1396^T and the type strains of the three closest related species were done as described previously (Kämpfer *et al.*, 2002) with DNA extracted using the method of Pitcher *et al.* (1989). Hybridization analyses between JM-1396^T and *N. mathurense* SM117^T, *N. panipatense* SM16^T and *N. barchaimii* DSM 25411^T showed low relatedness values of 8 % (reciprocal 7 %), 24 % (reciprocal 26 %) and 19 % (reciprocal 25 %), respectively. DNA–DNA hybridization experiments were not performed with type strains of species showing 16S rRNA gene sequences <98.5 %. This level of agreement is below the value for which the probability is less than 1 % (Meier-Kolthoff *et al.* 2013). Kim *et al.* (2014) recently recommended a threshold of 98.65 % 16S rRNA gene sequence similarity. If the similarity is higher, DNA-DNA hybridizations are necessary.

Strain JM-1396^T was further characterized using a substrate assimilation panel and enzyme tests with chromogenic substrates [*p*-nitrophenyl (NP)- and *p*-nitroanilide (NA)-linked substrates] (Kämpfer *et al.*, 1991) and additional tests, including the indole reaction with Ehrlich's and Kovacs' reagent, the activity of arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, reduction of nitrate to nitrite and urease (Kämpfer, 1990).

The results of the comparative characterizations showing differences from the most closely related species are given in Table 1. Strain JM-1396^T can be clearly differentiated from the most closely related species of the genus *Novosphingobium*.

Fatty acids were analysed from biomass grown on tryptone soy agar (TSA; Difco) at 28 °C, as described previously (Kämpfer & Kroppenstedt, 1996) and harvested in their exponential growth phase after 24 h of incubation. The fatty acids were analysed in methyl ester format and identified using the Sherlock Microbial Identification System (Sherlock MIDI software version 2.11 and TSBA peak naming table version 4.1).

The major fatty acids of JM-1396^T were $C_{18:1}\omega7c$ (56 %) and the characteristic 2-hydroxy fatty acid $C_{14:0}$ 2-OH (7.5 %). In addition, $C_{16:0}$ 2-OH (3.0 %) could be detected, but not $C_{15:0}$ 2-OH, which is found in other species of the genus *Novosphingobium* (Kämpfer *et al.*, 2002; Glaeser *et al.*, 2009). The detailed fatty acid profile of strain JM-1396^T is shown in Table 2.

Biomass for analyses of the quinone system and the polar lipids was harvested after growth of strain JM-1396^T in

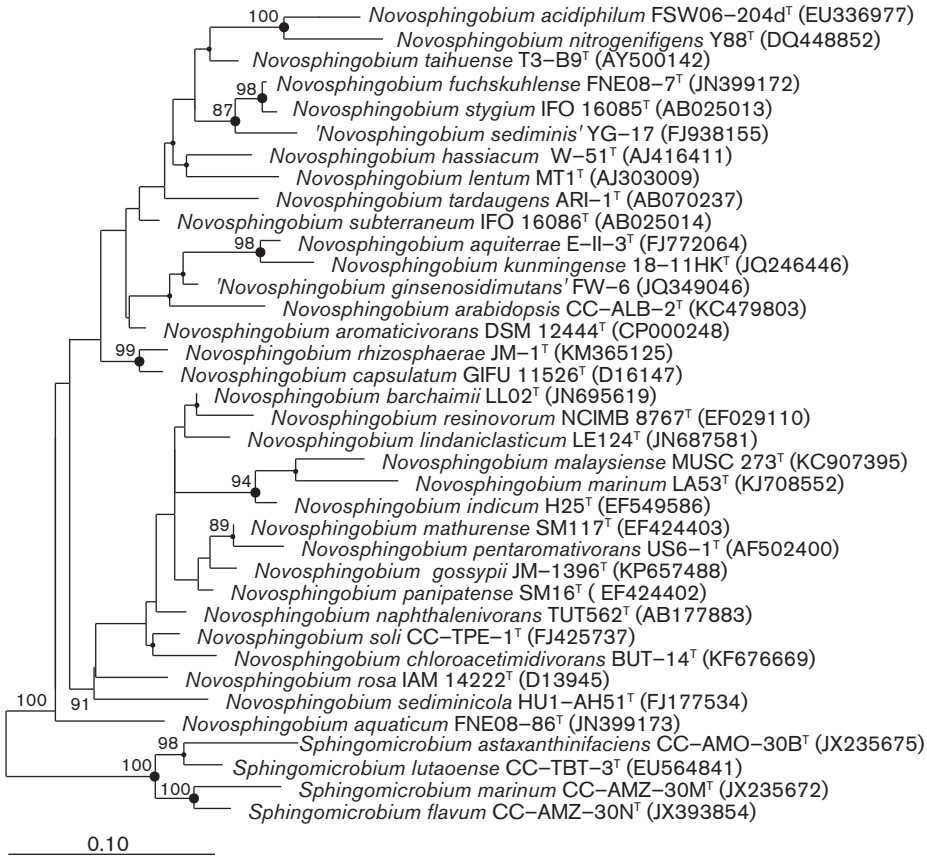

Fig. 1. Maximum-likelihood tree showing the phylogenetic position of JM-1396^T among all type strains of currently proposed species of the genus *Novosphingobium*. The phylogenetic tree is based on nearly full-length 16S rRNA gene sequences and was calculated in ARB using the RAxML treeing method (GTR-GAMMA, rapid bootstrap analysis). Type strains of species of the genus *Sphingomicrobium* were used as an outgroup. Numbers at nodes represent bootstrap values based on 100 resamplings; only values >70 % are depicted. Nodes marked with filled circles were also conserved in the maximum-parsimony tree, whereas nodes marked with larger circles were supported by a bootstrap value >70 %. Bar, 0.1 substitutions per nucleotide position.

tryptic soy broth medium (TSB, Difco) for 24 h at 28 °C. The harvested biomass was lyophilized and used for the analyses. Polyamines were extracted from biomass of the strain grown on 3.3× peptone-yeast-extract-agar [1.0 % (w/v) peptone from casein, 1.0 % (w/v) yeast extract, pH 7.2]. For analysis of polyamines, biomass harvested at the late exponential growth phase was used, as recommended by Busse & Auling (1988). Polyamines were extracted as reported by Busse & Auling (1988) and analysed according to the conditions reported by Busse *et al.* (1997). HPLC analyses were carried out using the equipment reported by Stolz *et al.* (2007).

Respiratory quinones of the strain were extracted and separated, as described previously by Collins *et al.* (1977), and analysed by HPLC. A JASCO X-LC HPLC system, containing two pumps, autosampler, intelligent column thermostat and fluorescence detector, was used. For instrument control, data acquisition and analysis, JASCO Chrompass software was employed. Menaquinones were eluted from a RP 18 column (250 mm by 4 mm inside diameter) using a solution containing acetonitrile and 2-2-propanol (65/35, v/v) at a flow rate of 1.3 ml min^{-1} and a temperature of 20 °C. The detection wavelength was 269 nm. Strain JM-1396^T exhibited a quinone system consisting of Q-10 (90 %) and Q-9 (10 %). This is in agreement with the described species of the family *Sphingomonadaceae*.

Polar lipids, extracted by the method of Minnikin *et al.* (1979), were identified by two-dimensional TLC, as described by Collins & Jones (1980). Strain JM-1396^T exhibited a complex polar lipid profile similar to other species of the genus *Novosphingobium*, with the diagnostic polar lipids phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylglycerol and sphingoglycolipid (Fig. 2). Traces of phosphatidyldimethylethanolamine, avisible shoulder beside phosphatidylethanolamine, two unknown phospholipids, four unknown lipids, two of

Table 1. Physiological test results of strain JM-1396^T in comparison with closely related species of the genus *Novosphingobium*

Strains: 1, JM-1396^T; 2, *N. mathurense* SM117^T; 3, *N. panipatense* SM16^T; 4, *N. barchaimii* DSM 25411^T. All strains were negative for acid production from lactose, sucrose, D-mannitol, dulcitol, salicin, adonitol, inositol, sorbitol, methyl-D-glucoside, erythritol, raffinose, trehalose, cellobiose, D-arabitol, D-mannose; hydrolysis of *o*-NP-*β*-D-galactopyranoside, *p*-NP-phosphorylcholine, L-glutamate-*γ*-3-carboxy-*p*-NA, L-proline-*p*-NA; assimilation of *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, D-ribose, salicin, adonitol, i-inositol, maltitol, D-mannitol, D-sorbitol, putrescine, *cis*-aconitate, adipate, 4-aminobutyrate, azelate, itaconate, mesaconate, suberate, *β*-alanine, L-phenylalanine, L-tryptophan, 3-hydroxybenzoate, 4-hydroxybenzoate, phenylacetate. All strains were positive for acid production from glucose and D-xylose; hydrolysis of *p*-NP-α-D-glucopyranoside, *p*-NP-*β*-D-glucopyranoside, *p*-NP-*β*-D-xylopyranoside, bis-*p*-NP-phosphate, 2-deoxythymidine-5′-thymidine-*p*-NP-phosphate, L-alanine-*p*-NA; assimilation of D-glucose, D-xylose. +, Positive; −, negative; W, weakly positive. All data from this study.

Test	1	2	3	4
Acid produced from:				
L-Arabinose	+	−	W	W
Raffinose	−	−	−	−
Rhamnose	W	−	−	−
Maltose	W	−	W	W
D-Xylose	+	−	W	W
Assimilation of:				
L-Arabinose	+	W	−	W
p-Arbutin	+	−	−	−
Cellobiose	W	W	−	−
D-Fructose	W	W	−	W
D-Galactose	W	W	−	W
Gluconate	W	−	−	−
D-Mannose	W	W	−	W
Maltose	W	−	−	−
α-Melibiose	W	−	−	−
L-Rhamnose	+	W	−	−
Sucrose	W	W	−	−
Salicin	W	−	−	−
Trehalose	W	W	−	−
Acetate	+	W	−	−
Propionate	−	+	−	−
Citrate	+	−	−	W
Fumarate	+	−	−	W
DL-3-Hydroxybutyrate	−	+	W	W
DL-Lactate	+	−	−	−
L-Malate	+	−	−	W
Pyruvate	−	+	W	W
L-Alanine	W	−	−	−
L-Histidine	W	W	−	−
L-Leucine	W	−	W	−
L-Ornithine	W	−	−	−
L-Proline	W	W	−	−
L-Serine	−	W	−	−
Hydrolysis of:				
Aesculin	+	+	−	+
p-NP-*β*-D-glucuronide	−	+	−	−
p-NP-*β*-D-glucopyranoside	+	+	−	−
p-NP-*β*-D-xylopyranoside	+	+	−	−
p-NP-phenylphosphonate	+	−	−	W
p-NP-phosphorylcholine	−	+	−	−

Table 2. Whole-cell fatty acid profiles of strain JM-1396^T, and type strains of related species of the genus *Novosphingobium*

Strains: 1, JM-1396^T; 2, *N. mathurense* SM117^T; 3, *N. panipatense* SM16^T; 4, *N. barchaimii* DSM 25411^T. Values represent the percentage of total fatty acids. All data from this study.

Fatty acid	1	2*	3	4
Saturated fatty acids				
$C_{14:0}$	–	–	0.5	2.2
$C_{16:0}$	18.2	5.4	6.5	4.7
Unsaturated fatty acids				
$C_{16:1}\omega5c$	2.0	2.8	3.4	–
$C_{17:1}\omega6c$	–	5.2	2.5	3.3
$C_{18:1}\omega7c$	56.0	61.3	57.9	59.6
$C_{18:1}\omega5c$	–	1.4	–	2.6
11-Methyl $C_{18:1}\omega7c$	2.5	1.1	0.6	–
Hydroxy fatty acids				
$C_{14:0}$ 2-OH	7.5	6.2	7.7	11.7
$C_{16:0}$ 2-OH	3.0	4.1	1.4	3.3
Summed feature 3†	10.9	9.8	19.9	15.9

*In addition *N. mathurense* SM117^T produced $C_{15:0}$ and $C_{15:0}$ 2-OH in amounts <1 %.
†Summed feature 3 contained $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH.

them more hydrophilic and two of them hydrophobic, as well as traces of an unknown aminolipid were detected, as well as the hydrophobic yellow pigment, also observed in *N. acidiphilum*, *N. fuchskuhlense*, *N. rhizosphaerae*, *N. sediminicola* and *N. stygium*. Phosphatidylcholine was not detected. This profile showed a high similarity to those of other species of the genus *Novosphingobium*, but a characteristic feature of strain JM-1396^T was the lack of phosphatidylcholine – similar to *N. acidiphilum* (Glaeser *et al.*, 2009). The polyamine pattern consisted of the major compound spermidine [31.2 mmol (g dry weight)$^{-1}$] and traces of spermine [1.7 mmol (g dry weight)$^{-1}$], putrescine [0.1 mmol (g dry weight)$^{-1}$] and 1,3-diaminopropane [0.02 mmol (g dry weight)$^{-1}$], which is in agreement with the polyamine profiles found in members of the genus *Novosphingobium* (Glaeser *et al.*, 2013a,b).

A combination of the observed phylogenetic, chemotaxonomic and physiological differences, production of the characteristic hydroxylated fatty acid and several physiological features warrant the proposal of a separate species of the genus *Novosphingobium*.

Description of *Novosphingobium gossypii* sp. nov.

Novosphingobium gossypii (gos.sy′pi.i. N. L. gen. n. *gossypii* of *Gossypium hirsutum*).

Cells are rod-shaped, 2.0 ± 0.4 μm long and 1.0 ± 0.2 μm wide, and motile in the early exponential phase. Chemoorganotrophic with a respiratory type of metabolism and

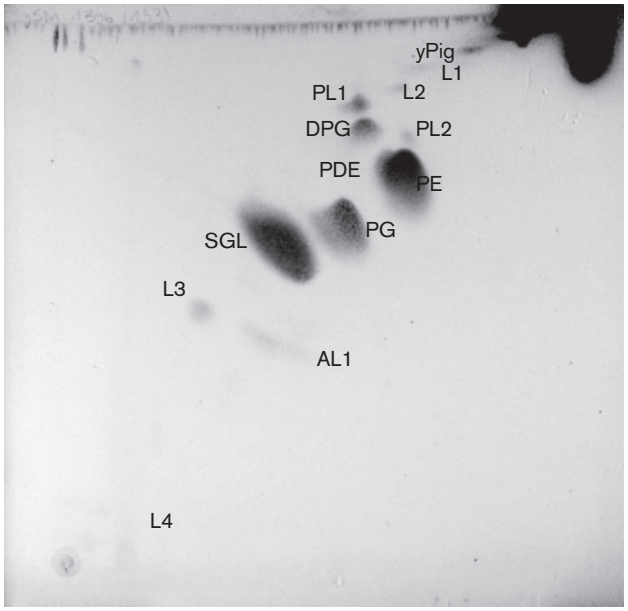

Fig. 2. Total polar lipid profile of strain JM-1396^T after two-dimensional TLC stained with molybdatophosphoric acid. AL1, unknown aminolipid; DPG, diphosphatidylglycerol; L1–L4, unknown lipids; PDE, phosphatidyldimethylethanolamine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PL1, PL2, unknown phospholipids; SGL, sphingoglycolipid; yPig, yellow pigment.

not able to reduce nitrate to nitrite. Catalase-positive. Good growth is observed on peptone-yeast-extract-agar, nutrient, tryptone soy and R2A agar at 25 °C. On nutrient agar, almost circular, yellow-pigmented colonies are produced after an incubation of 2–3 days at 25 °C. Cells stain Gram-negative. Cytochrome oxidase-positive. Endospores are not observed. On nutrient agar, growth occurs between 10 and 36 °C, but not at 4 or 42 °C, and between pH values of 4.5 and 10.5, but not at pH 3.5 or 11.5. Strain JM-1396^T is negative for acid production from L-rhamnose, raffinose, lactose, sucrose, D-mannitol, melibiose, trehalose, cellobiose, dulcitol, salicin, adonitol, inositol, methyl-D-glucoside and erythritol, and positive for acid production from D-glucose, L-arabinose, D-xylose, maltose (weakly positive) and D-mannose (weakly positive). No hydrolysis of *p*-NP-*β*-D-glucuronide, *p*-NP-phosphorylcholine, L-glutamate-*γ*-3-carboxy-*p*-NA and L-proline-*p*-NA can be observed, but hydrolysis of aesculin, *o*-NP-*β*-D-galactopyranoside (weakly positive), *p*-NP-*α*-D-glucopyranoside, *p*-NP-*β*-D-glucopyranoside, *p*-NP-*β*-D-xylopyranoside, bis-*p*-NP-phosphate, *p*-NP-phenylphosphonate, 2-deoxythymidine-5′-thymidine-*p*-NP-phosphate and L-alanine-*p*-NA is positive. Assimilation of *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, D-ribose, salicin, adonitol, i-inositol, maltitol, D-mannitol, D-sorbitol, putrescine, propionate, *cis*-aconitate, adipate, 4-aminobutyrate, azelate, DL-3-hydroxybutyrate, itaconate, mesaconate, oxoglutarate, pyruvate, suberate, *β*-alanine, L-leucine, L-phenylalanine, L-serine, L-tryptophan, 3-hydroxybenzoate, 4-hydroxybenzoate, and phenylacetate is negative. L-Arabinose, cellobiose, D-glucose, D-fructose (weakly), D-galactose (weakly), gluconate (weakly), maltose, D-mannose, trehalose, L-rhamnose, sucrose, acetate, citrate, DL-lactate, L-malate, fumarate, L-alanine (weakly), L-aspartate (weakly), L-histidine (weakly), L-ornithine (weakly) and L-proline (weakly) are utilized as sole sources of carbon. Indole formation from tryptophan and the activities of arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, reduction of nitrate to nitrite and urease are negative. The fatty acid profile consists of major amounts of $C_{18:1}\omega7c$, and the 2-hydroxy fatty acids $C_{14:0}$ 2-OH and $C_{16:0}$ 2-OH. Polar lipid profile consists of the predominant lipids diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, the characteristic sphingoglycolipid, traces of phosphatidyldimethylethanolamine and unknown phospholipids (2), lipids (4) and aminolipid (1). The quinone system is ubiquinone Q-10. The major polyamine is spermidine.

The type strain, JM-1396^T (=LMG 28605^T=CCM 8569^T=CIP 110884^T), was isolated from the healthy internal stem tissue of post-harvest cotton (*Gossypium hirsutum*, cultivar 'DES-119') grown at the Plant Breeding Unit at the E. V. Smith Research Center in Tallassee (Macon county), AL, USA.

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