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

Peter Kämpfer,[1] Hans-Jürgen Busse,[2] John A. McInroy[3] and Stefanie P. Glaeser[1]

Correspondence
Peter Kämpfer
peter.kaempfer@umwelt.uni-giessen.de

[1]Institut für Angewandte Mikrobiologie, Universität Giessen, Giessen, Germany
[2]Institut für Mikrobiologie, Veterinärmedizinische Universität, A-1210 Wien, Austria
[3]Department of Entomology and Plant Pathology, Auburn University, AL 36849, USA

A beige-pigmented bacterial strain (JM-310^T), isolated from the healthy internal root tissue of 4-week-old cotton (*Gossypium hirsutum*, cultivar 'DES-119') in Tallassee (Macon county), Alabama, USA, was studied taxonomically. The isolate produced small rod-shaped cells, which showed a Gram-negative staining behaviour. A comparison of the 16S rRNA gene sequence of the isolate revealed 99.2, 98.8, 98.7, 98.7, 98.1 and 97.6 % similarity to the 16S rRNA gene sequences of the type strains of *Variovorax paradoxus*, *Variovorax boronicumulans*, *Variovorax ginsengisoli*, *Variovorax soli*, *Variovorax defluvii* and *Variovorax dokdonensis*, respectively. In phylogenetic trees based on 16S rRNA gene sequences, strain JM-301^T was placed within the monophyletic cluster of *Variovorax* species. The fatty acid profile of strain JM-310^T consisted mainly of the major fatty acids $C_{16:0}$, $C_{10:0}$ 3-OH and summed feature 4 (iso-$C_{15:0}$ 2-OH/$C_{16:1}\omega7c/t$). The quinone system of strain JM-310^T contained predominantly ubiquinone Q-8 and lesser amounts of Q-7 and Q-9. The major polyamine was putrescine and the diagnostic polyamine 2-hydroxyputrescine was detected as well. The polar lipid profile consisted of the major lipids phosphatidylethanolamine, phosphatidylglycerol, diphospatidylglycerol and several unidentified lipids. DNA–DNA hybridization experiments with *V. paradoxus* LMG 1797^T, *V. boronicumulans* 1.22^T, *V. soli* KACC 11579^T and *V. ginsengisoli* 3165^T gave levels of relatedness of <70 %. These DNA–DNA hybridization results in addition to differential biochemical properties indicate clearly that strain JM-310^T is a member of a novel species, for which the name *Variovorax gossypii* sp. nov. is proposed. The type strain is JM-310^T (=LMG 28869^T=CIP 110912^T=CCM 8614^T).

The genus *Variovorax* was initially proposed along with the reclassification of *Alcaligenes paradoxus* as *Variovorax paradoxus* (Willems *et al.*, 1991). Phylogenetic analyses based on 16S rRNA gene sequences revealed that this genus was grouped within the family *Comamonadaceae* in the class *Betaproteobacteria* (Anzai *et al.*, 2000). The name refers to the organotrophic and facultatively chemolithotrophic metabolism of species of the genus (Willems *et al.*, 1991), and species are characterized by their versatility in using a wide variety of organic acids, including aminoacids. The major fatty acids are palmitoleic acid ($C_{16:1}$), palmitic acid ($C_{16:0}$) and *cis*-vaccenic acid ($C_{18:1}$). The majority of the described species have been isolated from soil, wastewater and plants. The genus harbours, at the time of writing, six recognized species, *Variovorax paradoxus*, *V. dokdonensis* (Yoon *et al.*, 2006), *V. soli* (Kim *et al.*, 2006), *V. boronicumulans* (Miwa *et al.*, 2008), *V. ginsengisoli* (Im *et al.*, 2010) and *V. defluvii* (Jin *et al.*, 2012).

Strain JM-310^T was isolated in 1990 as an endophyte from the healthy internal root tissue of 4-week-old cotton (*Gossypium hirsutum*, cultivar 'DES-119') grown at the Plant Breeding Unit facility at the E.V. Smith Research Center in Tallassee (Macon county), Alabama, USA.

Initial isolation and the testing of cultural and morphological characteristics were determined from fresh cultures grown on trypticase soy agar (TSA; Oxoid) at 28 °C. For Gram staining, a slightly modified method of that of Gerhardt *et al.* (1994) was used and the motility test was done under light microscopy (Axiophot2; Zeiss) at 1000× on cells grown for 3 days in trypticase soy broth (TSB; Oxoid) at 28 °C. Temperature-dependent growth was tested at 4, 10, 15, 20, 30, 36 and 45 °C on nutrient agar (NA; Oxoid). NaCl tolerance was investigated at different concentrations of NaCl [0.5–8.0 % (w/v)] in TSB.

The GenBank/EMBL/DDBJ accession number of the 16S rRNA gene sequence of strain JM-310^T is KR349468.

Table 1. Differential physiological characteristics between strain JM-310^T and the type strains of recognized *Variovorax* species

Strains: 1, JM-310^T; 2, *V. ginsengisoli* Gsoli 3165^T; 3, *V. paradoxus* DSM 66^T; 4, *V. soli* GH9-3^T; 5, *V. boronicumulans* BAM-48^T; 6, *V. dokdonensis* DS-43^T. All data (except for *V. dokdonensis*) are from this study. All strains were Gram-stain-negative, motile, rod-shaped and positive for catalase and oxidase. +, Positive; −, negative; w, weakly positive.

Characteristic	1	2	3	4	5	6
Cell shape	Rods	Rods	Rods	Rods	Rods	Oval or rods
Cell width (μm)	0.3–0.5	0.3–0.5	0.5–0.6	0.5–0.7	0.5–0.7	0.3–0.5
Cell length (μm)	1.5–3.0	1.5–3.0	1.2–3.0	1.0–1.5	1.0–2.0	0.7–2.8
α-Glucosidase	−	+	+	−	−	w
Utilization of substrates:						
D-Glucose	+	−*	+*	+*	+†	+
L-Arabinose	+	+*	+*	+*	w*	−
D-Mannose	−	+*	+*	−*	−	−
D-Mannitol	−	+*	+*	+*	w*	−
Gluconate	+	−*	+*	+*	−*	+
Adipate	+	−*	+*	+*	−*	+
Citrate	−	−*	−*	−*	−†	−
Phenylacetate	−	+*	+*	+†	−*	−
D-Sorbitol	+	−*	+*	+*	−*	−

*Results in congruence with those reported by Im *et al.* (2010) obtained with the API systems.
†Results not in congruence with those reported by Im *et al.* (2010) obtained with the API systems.

The strain stained Gram-negative and produced visible (diameter about 2 mm) beige colonies within 48 h at 28 °C. No growth was observed at 4 or 45 °C. The strain grew at 10–36 °C and at NaCl concentrations of 1–3 % (w/v).

Colonies of the isolate showed a beige colour with entire edges. Oxidase activity was positive when tested with Oxidase reagent (bioMérieux). Cells were rod-shaped (0.5–0.8 μm in width and 1.5–2.0 μm in length). No spores were detected. Good growth of strain JM-310^T was observed on NA, brain heart infusion agar, R2A agar and TSA and also on MacConkey agar (Oxoid).

Physiological/biochemical characterization (96-well plate test system) and additional biochemical tests were performed as described previously (Kämpfer *et al.*, 1991). The biochemical/physiological data are given in Table 1 and in the species description.

Isolate JM-310^T was maintained and subcultivated on NA at 30 °C for 48 h and subsequently analysed for its 16S rRNA gene sequence, fatty acid methyl ester composition of the whole-cell hydrolysate, quinone, polyamine and polar lipid patterns, further phenotypic characteristics, and DNA–DNA relatedness to those species most closely related on the basis of 16S rRNA gene sequence similarities.

Phylogenetic analysis was based on nearly full-length 16S rRNA gene sequences. The sequenced 16S rRNA gene fragment of strain JM-310^T was a continuous stretch of 1437 nt spanning gene positions 19–1466 according to the *Escherichia coli rrnB* numbering (Brosius *et al.*, 1981). A first phylogenetic placement and pairwise sequence similarities to the closest related type strains were obtained by BLAST analysis against the EzTaxon type strain database (Kim *et al.*, 2012). Phylogenetic trees were calculated in the software package ARB release 5.2 (Ludwig *et al.*, 2004) and the 'All-Species Living Tree' Project (LTP; Yarza *et al.*, 2008) database release LTPs119 (November 2014). The sequence of strain JM-310^T and all other sequences not included in the database were aligned using the SINA online tool (v.1.2.11; Pruesse *et al.*, 2012) and implemented into the LTP database. The alignment of sequences used for tree reconstruction was checked manually. Pairwise 16S rRNA gene sequence similarities were calculated using the ARB neighbour-joining tool without an evolutionary substitution model. Phylogenetic trees were reconstructed with different treeing methods, including the maximum-likelihood method using RAxML v.7.04 (Stamatakis, 2006) with GTR-GAMMA and rapid bootstrap analysis and the maximum-parsimony method using DNAPARS v.3.6 (Felsenstein, 2005). Both trees were calculated with 100 resamplings (bootstrap analysis; Felsenstein, 1985) and based on 16S rRNA gene sequence positions between gene termini 75 and 1407 (according to the *E. coli rrnB* numbering; Brosius *et al.*, 1981).

Phylogenetic calculations placed strain JM-310^T in the genus *Variovorax* within the family *Comamonadaceae*, clustering most closely with the type strains of *V. paradoxus*, *V. boronicumulans* and *V. ginsengisoli* within the monophyletic cluster of the genus *Variovorax* (Fig. 1). Pairwise calculations revealed highest 16S rRNA gene sequence similarities to the type strains of *V. paradoxus* (99.2 %), *V. boronicumulans* (98.8 %), and *V. ginsengisoli* and *V. soli* (both 98.7 %). Sequence similarities to all other type strains

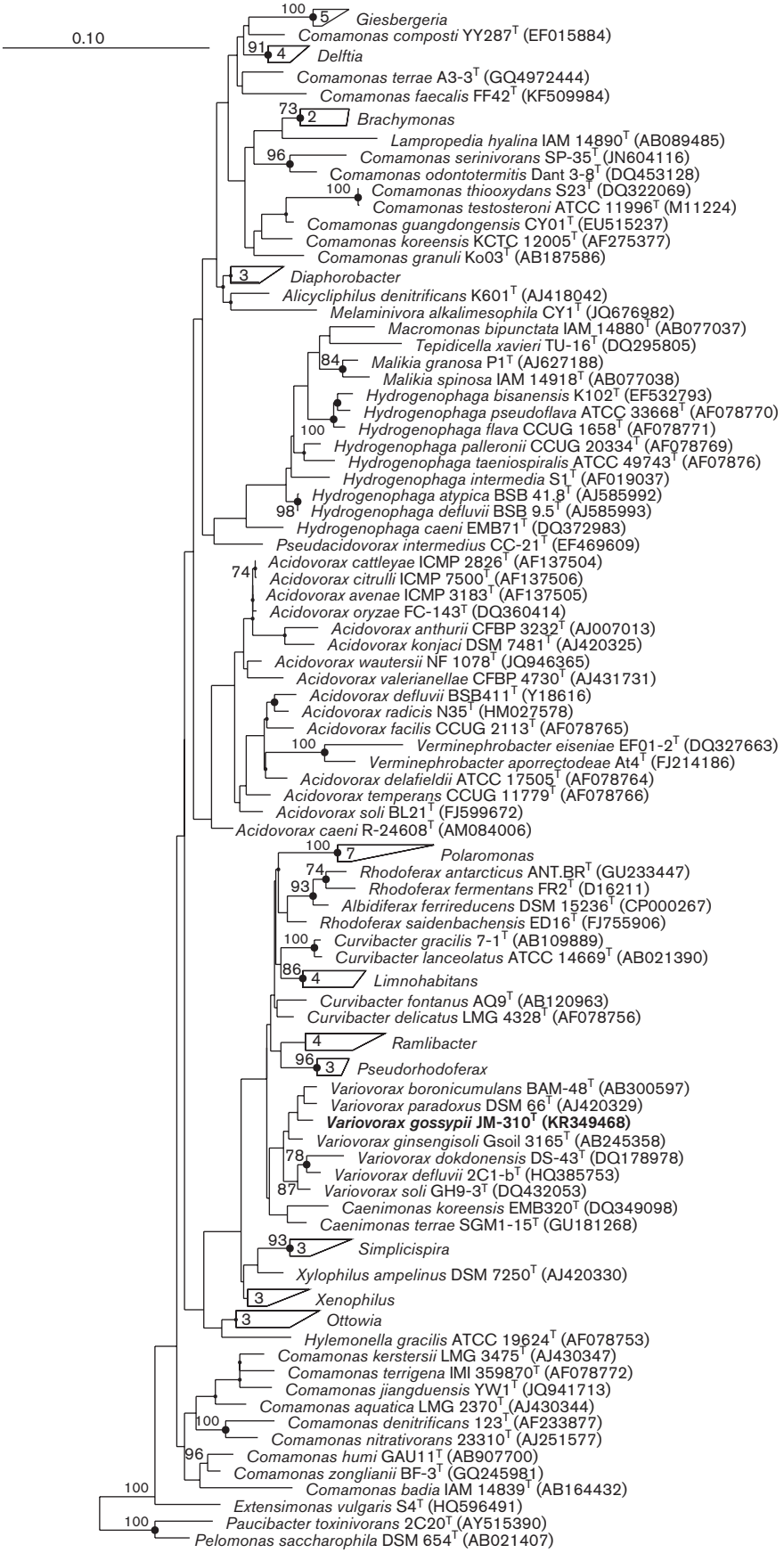

Fig. 1. Maximum-parsimony tree based on nearly full-length 16S rRNA gene sequences showing the phylogenetic placement of strain JM-310^T within the genus *Variovorax* of the family *Comamonadaceae*. The tree was generated in ARB using DNAPARS v.3.6 and based on 16S rRNA gene positions 75–1407 (Brosius *et al.*, 1981). Numbers in the 'boxes' represent numbers of strains (sequences) considered in that 'box'. Numbers at nodes represent bootstrap values >70 % (100 replications). Nodes marked with filled circles were also present in the maximum-likelihood tree calculated in parallel. Larger circles mark those nodes that were also supported by high bootstrap values (>70 %) in the maximum-likelihood tree. The type strains of *Paucibacter toxinivorans* and *Pelomonas saccharophila* were used to root the tree (outgroup). Bar, 0.10 nt substitutions per nucleotide position.

were below 98.5 %, the recommended threshold for the differentiation of two species (Kim *et al.*, 2014). Sequence similarities to the type strains of the other two recognized *Variovorax* species, *V. defluvii* and *V. dokdonensis*, were 98.1 and 97.6 %, respectively.

Cellular fatty acid analysis was performed for strain JM-310^T and with the same method for *V. ginsengisoli* Gsoli 3165^T, *V. paradoxus* LMG 1797^T, *V. soli* KACC 11579^T and *V. boronicumulans* 1.22^T after extraction of whole-cell hydrolysates according to Kämpfer & Kroppenstedt (1996) using the Sherlock Microbial Identification System (Sherlock MIDI software v.2.11 and a TSBA peak naming table v.4.1).

The fatty acid profile of isolate JM-310^T was characterized by large amounts of $C_{16:0}$ and summed feature 4 (iso-$C_{15:0}$ 2-OH/$C_{16:1}\omega7c/t$), which is in agreement with the results obtained by Im *et al.* (2010). The detailed fatty acid pattern is given in Table 2.

For analysis of the polyamines, respiratory quinones and polar lipids, strain JM-310^T was grown on PYE medium [0.3 % (w/v) peptone from casein and 0.3 % (w/v) yeast extract, pH 7.2]. Biomass subjected to polyamine analysis was harvested at the late exponential growth phase and extracted as described by Busse & Auling (1988). HPLC analysis was carried out as described by Busse *et al.* (1997) using the equipment reported by Stolz *et al.* (2007). Biomass for analyses of quinones and polar lipids was harvested at the stationary growth phase.

Quinones and polar lipids were extracted and analysed applying an integrated procedure (Tindall, 1990a, b; Altenburger *et al.*, 1996; Stolz *et al.*, 2007). The quinone system was composed of ubiquinones Q-8 (69 %), Q-7 (29 %) and Q-9 (2 %). Similar quinone systems have also been reported for other species of the genus *Variovorax*, including the type species of the genus, *V. paradoxus* (Oyaizu-Masuchi & Komagata, 1988). The polar lipid profile (Fig. 2), here analysed for the first time for a strain of the genus *Variovorax*, consisted of the major lipids phosphatidylethanolamine, phosphatidylglycerol, diphospatidylglycerol and minor amounts of four unidentified glycolipids (GL1–4), one unidentified phospholipid (PL1), one unidentified aminolipid (AL1) and one unidentified lipid (L1) not containing an amino residue, a phosphate residue or a sugar moiety. Major polyamines were putrescine [66.1 μmol (g dry weight)$^{-1}$] and the diagnostic polyamine 2-hydroxyputrescine [11.1 μmol (g dry weight)$^{-1}$]. Minor amounts of

Table 2. Cellular fatty acid profiles of strain JM-310^T and the type strains of recognized *Variovorax* species

Strains: 1, JM-310^T; 2, *V. ginsengisoli* Gsoli 3165^T; 3, *V. paradoxus* DSM 66^T; 4, *V. soli* GH9-3^T (Kim *et al.*, 2006); 5, *V. boronicumulans* BAM-48^T; 6, *V. dokdonensis* DS-43^T. All data except those in parentheses are from this study; all strains were grown on TSA for 2 days. Data in parentheses are from Im *et al.* (2010), with all strains grown on R2A agar for 2 days.

Fatty acid	1	2	3	4	5	6
$C_{10:0}$	–	– (0.5)	– (–)	0.5 (0.6)	– (–)	(0.6)
$C_{12:0}$	3.7	3.4 (3.6)	3.5 (3.9)	2.8 (3.3)	3.7 (3.5)	(6.3)
$C_{14:0}$	–	4.2 (4.9)	– (1.4)	(0.8)	– (1.1)	(1.2)
$C_{15:0}$	–	– (0.5)	2.9 (7.1)	3.4 (1.2)	– (–)	(–)
$C_{16:0}$	27.7	34.4 (39.1)	26.3 (31.6)	30.4 (38.1)	32.7 (36.1)	(34.9)
$C_{17:0}$	–	– (–)	2.3 (2.7)	(1.2)	– (–)	(–)
$C_{8:0}$ 3-OH	–	– (–)	1.0 (–)	(–)	1.2 (–)	(–)
$C_{10:0}$ 3-OH	12.8	4.1 (3.5)	3.6 (2.9)	3.9 (4.8)	4.3 (2.9)	(3.3)
$C_{14:0}$ 2-OH	2.5	– (–)	2.6 (–)	(–)	2.9 (–)	(–)
$C_{17:0}$ cyclo	3.9	13.6 (18.8)	2.3 (23.8)	20.6 (22.5)	7.1 (19.0)	(23.1)
$C_{15:1}\omega6c$	–	– (–)	1.2 (–)	1.6 (–)	– (–)	(–)
Summed feature 4*	29.8	24.4 (19.7)	23.1 (17.0)	21.6 (21.5)	33.8 (21.4)	(23.4)
Summed feature 7*	19.6	16.0 (9.3)	17.9 (11.9)	12.0 (5.1)	14.3 (11.3)	(7.1)

*Summed features are groups of two or three fatty acids which cannot be separated by GLC with the MIDI system. Summed feature 4 comprised $C_{16:1}\omega7c$/iso-$C_{15:0}$ 2-OH; summed feature 7 comprised $C_{18:1}\omega7c/\omega9t/\omega12t$.

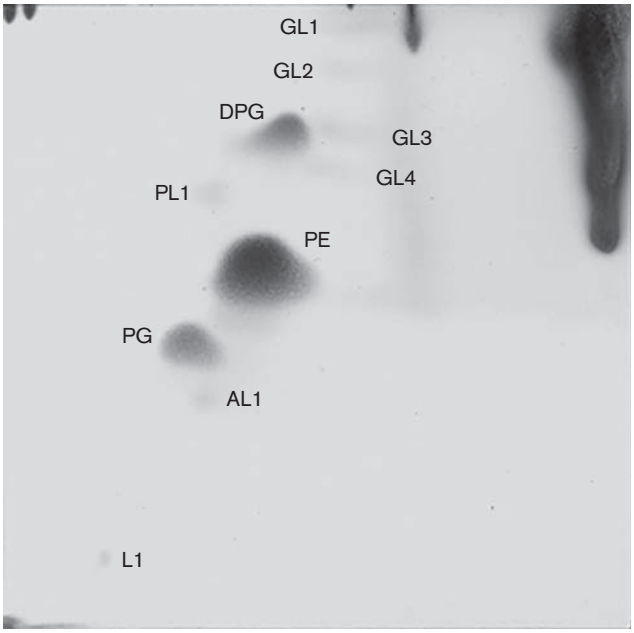

Fig. 2. Total polar lipid profile of strain JM-310^T after staining with molybdatophosphoric acid. PE, phosphatidylethanolamine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; GL1–4, unidentified glycolipids; L1, unidentified polar lipid; PL1, unidentified phospholipid; AL1, unidentified aminolipid.

cadaverine [0.2 μmol (g dry weight)$^{-1}$] and traces of spermidine and spermine [<0.1 μmol (g dry weight)$^{-1}$] were also found. This polyamine profile is in good agreement with those of the majority of betaproteobacteria, including *V. paradoxus* (Busse & Auling, 1988; Hamana *et al.*, 2000, 2006).

DNA–DNA hybridization experiments were performed between strain JM-310^T and the type strains of the four closest related *Variovorax* species, namely *V. ginsengisoli* Gsoli 3165^T, *V. paradoxus* LMG 1797^T, *V. soli* KACC 11579^T and *V. boronicumulans* 1.22^T, according to the method of Ziemke *et al.* (1998) (except that for nick translation 2 μg of DNA was labelled during 3 h of incubation at 15 °C). These experiments with *V. paradoxus* LMG 1797^T, *V. boronicumulans* 1.22^T, *V. soli* KACC 11579^T and *V. ginsengisoli* 3165^T resulted in levels of DNA–DNA relatedness of 39 % (reciprocal 64 %), 40 % (reciprocal 52 %), 24 % (reciprocal 47 %) and 17 % (reciprocal 10 %), respectively.

Although the chemotaxonomic data demonstrate close similarity between strain JM-310^T with the type strains of species of the genus *Variovorax*, the hybridization experiments, and physiological and chemotaxonomic data allow its clear separation. On the basis of the reported results, we suggest that strain JM-310^T represents a novel species of the genus *Variovorax*, for which the name *Variovorax gossypii* sp. nov. is proposed.

Description of *Variovorax gossypii* sp. nov.

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

Cells are Gram-stain-negative, non-spore forming rods, approx. 0.3–0.5 μm in width and 1.5–3.0 μm in length. Aerobic, oxidase- and catalase-positive. Good growth occurs after 48 h on NA, R2A agar and TSA (all Oxoid) at 11–36 °C. Unable to grow below 4 °C or above 45 °C. Colonies on NA are smooth, beige, circular and translucent. Acid is very weakly produced from D-glucose, L-arabinose, D-mannitol and adonitol. Acid is not produced from maltose, L-rhamnose, sucrose, trehalose, cellobiose, lactose, D-arabitol, dulcitol, erythritol, *i*-inositol, melibiose, methyl α-D-glucoside, raffinose, salicin, D-sorbitol or D-xylose. Aesculin hydrolysis is positive and β-galactosidase activity (ONPG) is negative. The following compounds are utilized as sole source of carbon: *N*-acetyl-D-glucosamine, L-arabinose, D-fructose, D-glucose, gluconate, D-mannose, α-melibiose, D-ribose, D-xylose, D-mannitol, D-sorbitol, acetate, propionate, 4-aminobutyrate, adipate, azelate, fumarate, glutarate, DL-3-hydroxybutyrate, 2-oxoglutarate, pyruvate, L-alanine, β-alanine, L-ornithine, L-aspartate, L-histidine, L-leucine, L-serine, L-proline and L-tryptophan. The following compounds are not utilized as sole source of carbon: *N*-acetyl-D-galactosamine, L-arbutin, cellobiose, maltose, D-galactose, glycerol, maltitol, L-rhamnose, sucrose, salicin, trehalose, adonitol, *i*-inositol, putrescine, *cis*-aconitate, *trans*-aconitate, itaconate, suberate, citrate, mesaconate, L-phenylalanine, 3-hydroxybenzoate, 4-hydroxybenzoate and phenylacetate. The chromogenic substrates *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-α-D-glucopyranoside, bis-*p*-nitrophenyl-phosphate, bis-*p*-nitrophenyl-phenyl-phosphonate, bis-*p*-nitrophenyl-phosphorylcholine, 2-deoxythymidine-2′-*p*-nitrophenyl-phosphate, L-alanine-*p*-nitroanilide, γ-L-glutamate-*p*-nitroanilide and L-proline-*p*-nitroanilide are hydrolysed but *p*-nitrophenyl-β-D-xylopyranoside and *p*-nitrophenyl-β-D-glucuronide are not hydrolysed. The major cellular fatty acids are $C_{16:0}$, $C_{10:0}$ 3-OH and summed feature 4 (iso-$C_{15:0}$ 2-OH/$C_{16:1}\omega7c/t$). The major menaquinone is ubiquinone Q-8 with lesser amounts of Q-7 and Q-9. The major polar lipids are phosphatidylethanolamine, phosphatidylglycerol and diphospatidylglycerol; minor amounts of four unknown glycolipids are also present. The polyamine pattern contains major amounts of putrescine and 2-hydroxyputrescine.

The type strain is JM-310^T (=LMG 28869^T=CIP 110912^T=CCM 8614^T), isolated as an endophyte from the healthy internal root tissue of 4-week-old cotton (*Gossypium hirsutum*, cultivar 'DES-119') grown at the Plant Breeding Unit facility at the E.V. Smith Research Center in Tallassee (Macon county), Alabama, USA.

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