Sphingobacterium zeae sp. nov., an endophyte of maize

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A yellow-pigmented strain (JM-1081^T) isolated from healthy stem tissue of *Zea mays* was taxonomically characterized. Cells of the strain were rod-shaped and Gram-stain-negative. Comparative 16S rRNA gene sequence analysis revealed closest relationship to the type strains of *Sphingobacterium multivorum* (98.1 % similarity), *Sphingobacterium mucilaginosum* (97.9 %) and *Sphingobacterium siyangense* (97.8 %). 16S rRNA gene sequence similarities to the type strains of all other *Sphingobacterium* species were below 97.8 %. Fatty acid analysis of whole-cell hydrolysates of the strain resulted in a pattern typical of the genus *Sphingobacterium* with iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7c$, iso-$C_{15:0}$, iso-$C_{17:0}$ 3-OH and $C_{16:0}$ and as major compounds. The polyamine pattern contained predominantly *sym*-homospermidine. The major quinone was menaquinone MK-7 and the only identified lipids in the polar lipid profile were phosphatidylethanolamine and phosphatidylserine. In addition, 15 unidentified lipids were detected in moderate to major amounts. Sphingolipid was detected. The diagnostic diamino acid of the peptidoglycan was *meso*-diaminopimelic acid. DNA–DNA hybridizations with two of the closely related type strains, those of *S. multivorum* and *S. siyangense*, as well as *Sphingobacterium canadense* resulted in values below 70 %. In addition to the genotypic differences, differential biochemical and chemotaxonomic properties confirmed that the isolate JM-1081^T represents a novel species, for which the name *Sphingobacterium zeae* sp. nov. is proposed. The type strain is JM-1081^T (=LMG 29191^T=CCM 8652^T).

The family *Sphingobacteriaceae* (Steyn *et al.*, 1998) of the phylum *Bacteroidetes* currently contains seven genera including *Sphingobacterium* and *Pedobacter*. The type genus *Sphingobacterium* was proposed by Yabuuchi *et al.* (1983) to accommodate Gram-negative rods that are positive for catalase and oxidase, and having fatty acid profiles with iso-$C_{15:0}$, iso-$C_{15:0}$ 3-OH, $C_{16:1}\omega7c$ and $C_{17:0}$ 3-OH as the most prevalent compounds. Characteristic chemotaxonomic markers are the high content of sphingophospholipids among cellular lipids and menaquinone 7 (MK-7) as the predominant respiratory quinone (Yabuuchi *et al.*, 1983). At the time of writing, the genus comprised 32 species with validly published names, including four species that were isolated from plant material, *Sphingobacterim canadense* isolated from corn root (Mehnaz *et al.*, 2007), *Sphingobacterium nematocida*, a tobacco endophyte (Liu *et al.*, 2012), *Sphingobacterium yanglingense* isolated from the nodule surface of soybean (Peng *et al.*, 2014) and *Sphingobacterium pakistanense*, a plant-growth-promoting rhizobacterium isolated from the rhizosphere of *Vigna mungo* (Ahmed *et al.*, 2014).

Here we describe strain JM-1081^T, which was isolated as an endophyte from the healthy internal stem tissue of mature maize (*Zea mays*, cultivar 'Sweet Belle'). The strain was isolated and grown on tryptic soy agar (TSA; Oxoid) at 30 °C and also further maintained and subcultivated on this agar at 30 °C for 48 h.

The cultural and morphological characteristics were taken from observation with cultures grown on TSA. Gram staining was done according to Gerhardt *et al.* (1994) and a motility test was done under a light microscope with cells grown for 3 days in tryptic soy broth (TSB; Oxoid) at 30 °C. Temperature-dependent growth was tested at 4, 10, 15, 25, 28, 30, 36, 42, 45 and 50 °C on nutrient agar. NaCl tolerance was investigated at different concentrations of NaCl [1.0–8.0 % (w/v); in 1 % increments] in TSB. pH-dependent growth was tested using TSB adjusted to pH values of pH

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain JM-1081^T is KU201960.

4.5–10.5 (in 1.0 pH units intervals). The pH values were adjusted using 1 M HCl and 1 M KOH and stabilized by the addition of 5 mM phosphate buffer adjusted to the same pH values.

Strain JM-1081^T showed a Gram-negative staining behaviour and produced visible (diameter about 2 mm) yellow colonies within 48 h at 30 °C. The isolate did not grow below 4 °C or above 45 °C. The strain grew very slowly at 45 °C and at NaCl concentrations of 1–4 % (w/v), but not at 5 % (w/v) and above. In addition, the strain grew at pH values of 5.5–9.0 under the conditions described above, but not at pH values below 5.5 or above 9.0.

Colonies revealed a translucent glistening appearance with entire edges. The yellow pigment was shown to be of the flexirubin type (KOH method according to Reichenbach, 1989). Oxidase activity was positive with oxidase reagent (bioMérieux). Cells of the strain were non-motile rods (approx. 1 μm wide and 2 μm long). Spores could not be detected. The strain grew well on complex agar media, such as nutrient agar, brain heart infusion agar, R2A agar and TSA, but not on MacConkey agar (Oxoid)

For phylogenetic identification, the nearly full-length 16S rRNA gene of strain JM-1081^T was PCR-amplified with primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-ACGGCTACCTTGTTACGACTT-3′, Lane, 1991) and sequenced by the didesoxy sequencing method with primers 8F and E786F (5′-GATTAGATACCCTGG-TAG-3′). The manually corrected 16S rRNA gene sequence represented a continuous stretch of 1436 nt spanning gene positions 20–1477 (according to the *Escherichia coli* numbering published by Brosius *et al.,* 1978). A BLAST analysis in the EzTaxon type strain 16S rRNA gene sequence database (Kim *et al.*, 2012) showed that strain JM-1081^T shared highest 16S rRNA gene sequence similarity with the type strains of *Sphingobacterium multivorum* (98.1 %), *Sphingobacterium mucilaginosum* (97.9 %) and *Sphingobacterium siyangense* (97.8 %). All other *Sphingobacterium* type strains showed a gene sequence similarity to strain JM-1081^T that was below 97.8 %. The next closest related plant-derived species, *S. canadense*, showed a 16S rRNA gene sequence similarity to strain JM-1081^T of 97.7 %. Sequence similarities to the type strains of all described species was below the proposed 16S rRNA gene sequence cut-off value of 98.65 % for species delineation (Kim *et al.*, 2014). The phylogenetic relationship between strain JM-1081^T and all *Sphingobacterium* type strains was investigated in ARB release 5.2 (Ludwig *et al.*, 2004) using the 'All-Species Living Tree' Project (LTP; Yarza *et al.*, 2008) release LTPs123 (September 2015). Sequences not included in the database were aligned in version v1.2.11 of the SILVA Incremental Aligner (SINA; Pruesse *et al.*, 2012) and implemented into the LTP database. Phylogenetic trees were reconstructed including 16S rRNA gene sequences of all *Sphingobacterium* type strains after the sequence alignment was rechecked manually. A maximum-parsimony tree (Fig. 1) was reconstructed using DNAPARS version 3.6 (Felsenstein, 2005). The stability of the phylogenetic relationship was underlined by the reconstruction of a maximum-likelihood tree using RAxML v7.04 (Stamatakis, 2006) with GTR-GAMMA and rapid bootstrap analysis (100 resamplings) and a neighbour-joining tree using the ARB-Neighbor-Joining tool and the Jukes–Cantor model (Jukes & Cantor, 1969). All trees were based on 100 resamplings (bootstrap analysis, Felsenstein, 1985) and gene sequences between gene termini 50 and 1440 (*E. coli* numbering, Brosius *et al.*, 1978). The type strains of *Mucilaginibacter gracilis* and *Mucilaginibacter paludis* were used as the outgroup. The phylogenetic trees clearly showed the placement of strain JM-1081^T within the genus *Sphingobacterium*, clustering with the type strains of *S. multivorum*, *S. mucilaginosum* and *Sphingobacterium changzhouense* (Fig. 1). The last named, however, shared only 96.6 % 16S rRNA gene sequence similarity with strain JM-1081^T. The clustering of the four strains was furthermore not supported by high bootstrap values and varied between the phylogenetic trees reconstructed with the different treeing algorithms.

The genomic distinction between strain JM-1081^T and its closest related species was investigated in more detail using DNA–DNA hybridization experiments performed with strain JM-1081^T and the type strains of two of the most closely related *Sphingobacterium* species, *S. siyangense* SY1^T and *S. multivorum* NCTC 11343^T, and the closest related plant-derived species, *S. canadense* LMG 23727^T. Hybridization was done according to the method of Ziemke *et al.* (1998) (except that for nick translation 2 μg DNA was labelled during 3 h of incubation at 15 °C) using DNA extracted according to the method of Pitcher & Saunders (1989). Strain JM-1081^T showed low levels of DNA–DNA relatedness to all three reference strains, *S. multivorum* NCTC 11343^T (29 %, reciprocal 29 %), *S. siyangense* SY1^T (47 %, reciprocal 20 %) and *S. canadense* LMG 23727^T (56 %, reciprocal 37 %).

Strain JM-1081^T was physiologically/biochemically studied by using the methods described by Kämpfer *et al.* (1991) and by additional biochemical tests, among them the production of hydrogen sulphide using the lead acetate paper and triple-sugar-iron methods, indole reaction with Ehrlich's and Kovacs' reagents, activity of arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, DNase (Oxoid CM321; supplemented with 0.01 % toluidine blue), β-galactosidase (ONPG) and urease on Christensen's urea agar (Kämpfer, 1990); and hydrolysis of casein, gelatin (plate method), starch and tyrosine (Smibert & Krieg, 1994). The biochemical/physiological data are given in Table 1 and in the species description.

Analysis of the cellular fatty acid profiles from whole-cell hydrolysates was performed as described previously (Kämpfer & Kroppenstedt, 1996) by using an HP 6890 gas chromatograph with Sherlock MIDI software version 2.11 and a TSBA peak naming table version 4.1. Prior to fatty acid extraction the strain was cultured on TSA at 28 °C for 48 h. The results are shown in Table 2 in comparison with

the most closely related type strains (including *S. multivorum* JCM 21156^T, *S. siyangense* SC-1^T and *S. canadense* CR11^T) and revealed a profile typical for the genus *Sphingobacterium*, with the following most abundant fatty acids: iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7$, detected as summed feature 3, but as shown in several previous studies could be clearly identified as iso-$C_{15:0}$ 2-OH (Montero-Calasanz *et al.*, 2013), iso-$C_{15:0}$, iso-$C_{17:0}$ 3-OH and $C_{16:0}$. Only minor differences were found in comparison with the profiles of the type strains of the most closely related *Sphingobacterium* species.

Polyamines, quinones, sphingolipids, polar lipids and *meso*-diaminopimelic acid were extracted from biomass grown in PYE broth [0.3 % (w/v) peptone from casein, 0.3 % (w/v) yeast extract, pH 7.2]. Polyamines were extracted according to Busse & Auling (1988) and analysed according to Busse *et al.* (1997). Quinones and polar lipids were extracted and analysed as described by Tindall (1990a, b) and Altenburger *et al.* (1996). The HPLC apparatus applied was as reported by Stolz *et al.* (2007). The presence of *meso*-diaminopimelic acid was shown after extraction and analysis following the protocol of Schumann (2011). The analysis was carried out applying one-dimensional TLC on pre-coated silica TLC aluminium sheets (0.20 mm thick, ALUGRAM SIL G/UV$_{254}$; Macherey-Nagel) in chloroform/methanol/glacial acetic acid/water (65 : 12 : 15 : 4) and detection using molybdatophosphoric acid, ninhydrin and molybdenum blue, respectively. Sphingolipids were purified from polar lipid extracts after mild alkaline hydrolysis in methanolic 0.5 M KOH for 20 h followed by neutralization with 1 M HCl as described by Kato *et al.* (1995). The presence of a sphingolipid was unambiguously demonstrated by a single spot which stained positive for an amino group and a phosphate group. The polyamine pattern consisted of *sym*-homospermidine [36.7 µmol (g dry weight)$^{-1}$], spermidine [4.1 µmol (g dry weight)$^{-1}$], spermine [1.6 µmol (g dry weight)$^{-1}$],

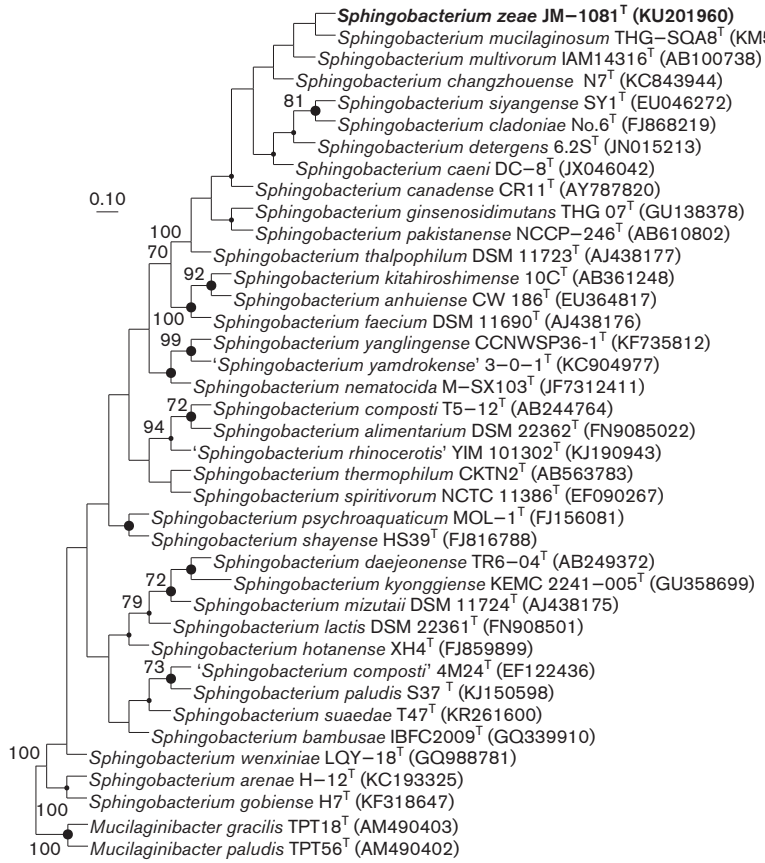

Fig. 1. Maximum-parsimony tree based on nearly full-length 16S rRNA gene sequences showing the phylogenetic affiliation of strain JM-1081^T among the type strains of all *Sphingobacterium* species. The tree was calculated in ARB and based on 16S rRNA gene sequences between gene termini 59 and 1440 (according to the *E. coli* numbering; Brosius *et al.*, 1978). Numbers at nodes represent bootstrap values $\geq$70 % (100 replications). Nodes marked with filled circles were also present in the respective maximum-likelihood and neighbour-joining trees. Larger circles mark nodes with >70 % bootstrap support in the other treeing methods, small circles <70 %. *Mucilaginibacter gracilis* TPT18^T and *Mucilaginibacter paludis* TPT56^T were used as the outgroup. Bar, 0.1 substitutions per nucleotide position.

Table 1. Differential characteristics between strain JM-1081^T and related *Sphingobacterium* species

Strains: 1, JM-1081^T; 2, *S. multivorum* JCM 21156^T; 3, *S. siyangense* SY1^T; 4, *S. canadense* CR11^T; 5, *S. mucilaginosum* THG-SQA8^T; 6, *S. thalpophilum* ATCC 43320^T; 7, *S. faecium* DSM 11690^T; 8, *S. mizutaii* ATCC 33299^T; 9, *S. daejeonense* LMG 23402^T; 10, *S. spiritivorum* ATCC 33861^T; 11, *S. composti* DSM 1885^T. Data for taxa 1–4 are from this study. For taxa 2–4, the data were congruent with those reported by Liu *et al.* (2008). All other data were from Du *et al.* (2015), Steyn *et al.* (1998), Kim *et al.* (2006), Yoo *et al.* (2007), Liu *et al.* (2008) and Mehnaz *et al.* (2007). +, Positive; –, negative; V, results vary between reference source; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Growth at:											
4 °C (5 °C)	(+)	–	+	–	–	–	+	–	–	–	–
42 °C (41 °C)	+	–	+	–	–	+	–	–	+	–	+
Hydrolysis of:											
Starch	+	+	+	+	–	+	+	+	–	+	–
Aesculin	+	+	–	+	+	+	+	+	–	+	+
Urea	–	+	+	+	+	+	+	+	–	+	–
Utilization of:											
L-Rhamnose	+	–	+	–	+	+	+	–	–	+	–
L-Arabinose	+	+	+	+	–	+	+	V	–	–	+
Gluconate	–	–	+	–	ND	–	–	–	–	–	–
Acid production from:											
D-Glucose	+	+	–	+	ND	+	+	+	+	+	–

and traces of putrescine and cadaverine [each 0.1 μmol (g dry weight)$^{-1}$]. The quinone system consisted of menaquinone MK-7 (99 %) and MK-6 (1 %). Both polyamine pattern (Hamana & Matsuzaki, 1991; Albert *et al.,* 2013) and quinone system were in accordance with the characteristics of other species of the genus *Sphingobacterium*. In the polar lipid profile (Fig. 2) phosphatidylethanolamine, phosphatidylserine, one phospholipid and four polar lipids

Table 2. Fatty acid contents (%) of strain JM-1081^T and related *Sphingobacterium* species

Strains: 1, JM-1081^T; 2, *S. multivorum* JCM 21156^T; 3, *S. siyangense* SC-1^T; 4, *S. canadense* CR11^T; 5, *S. mucilaginosum* THG-SQA8^T; 6, *S. thalpophilum* ATCC 43320^T; 7, *S. faecium* DSM 11690^T; 8, *S. mizutaii* ATCC 33299^T; 9, *S. daejeonense* LMG 23402^T; 10, *S. spiritivorum* ATCC 33861^T; 11, *S. composti* DSM 18850^T. Data for taxa 1 and 3–5 are from this study. All other data, and those in parentheses for taxon 4, are from Du *et al.* (2015), Steyn *et al.* (1998), Kim *et al.* (2006), Yoo *et al.* (2007), Liu *et al.* (2008) and Mehnaz *et al.* (2007). Fatty acids amounting to less than 1.0 % in all strains tested are not listed. TR, Trace (<1.0 %); –, not detected; ECL, equivalent chain-length.

Fatty acid	1	2	3	4	5	6	7	8	9	10	11
$C_{14:0}$	1.8	2.7	3.9	1.0 (1.4)	2.8	3.2	TR	TR	–	1.0	–
anteiso-$C_{15:0}$	–	–	2.7	1.8 (0.3)	–	–	TR	TR	2.6	TR	–
iso-$C_{15:0}$	26.9	22.2	32.9	30.9 (22.0)	23.0	17.7	24.6	30.0	45.6	30.1	29.5
iso-$C_{15:0}$ 3-OH	3.2	3.2	3.0	2.3 (2.8)	4.7	4.3	3.7	3.0	1.5	2.2	2.3
$C_{16:0}$	8.3	7.8	10.9	3.5 (8.7)	10.8	6.0	4.5	TR	3.4	3.5	2.2
$C_{16:0}$ 2-OH	–	TR	TR	TR (0.4)	–	3.2	–	–	–	–	–
$C_{16:0}$ 3-OH	2.9	5.3	6.4	2.2 (3.2)	3.1	6.3	2.1	TR	–	2.7	1.2
$C_{16:0}$ 10-methyl	–	–	–	–	–	–	1.4	–	–	–	–
$C_{16:1}\omega5c$	–	–	TR	–	–	–	1.5	TR	TR	TR	–
iso-$C_{17:0}$ 3-OH	9.8	7.1	5.9	8.3 (9.8)	5.9	10.0	10.0	22.1	16.6	12.5	19.7
$C_{17:1}\omega9c$	–	TR	1.1	–	1.2	–	–	3.7	2.9	1.7	2.9
$C_{18:0}$	–	–	–	–	1.6	–	–	–	–	–	–
$C_{18:1}\omega7c$	–	–	–	TR (1.0)	–	–	–	–	–	–	–
Summed feature 3*	45.1	49.0	24.1	42.6 (45.9)	37.8	47.8	48.1	35.1	23.8	42.7	37.5
ECL 13.566	1.9	–	TR	1.8 (–)	–	1.4	TR	1.3	1.0	TR	–

*Summed feature 3 contains iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7$.

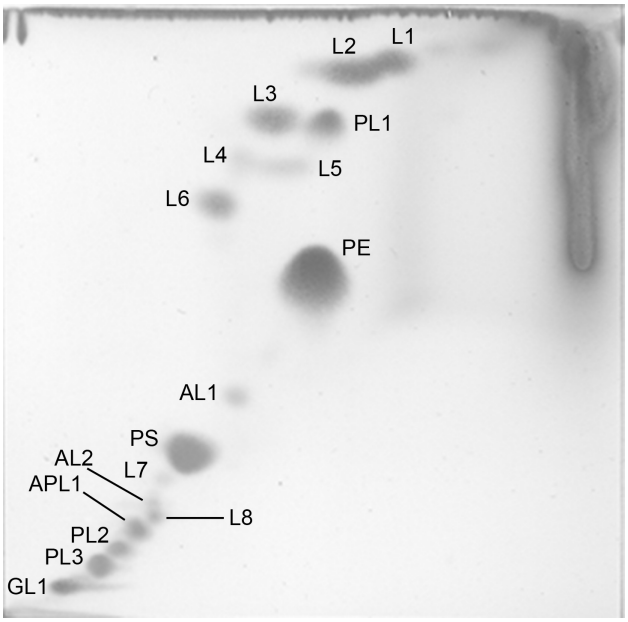

Fig. 2. Polar lipid profile of strain JM-1081^T after separation by two-dimensional TLC and detection using 5 % ethanolic molybdatophosphoric acid. PE, phosphatidylethanolamine; PS, phosphatidylserine; PL1–PL3, unidentified phospholipids; AL1, AL2, unidentified aminolipids; APL1, unidentified aminophospholipipid; GL1, unidentified glycolipid; L1–L8, unidentified polar lipids not detectable with any of the spray reagents specific for phosphate, an amino group or a sugar moiety.

detectable only after total lipid staining were detected. In addition, moderate to minor amounts of two unidentified aminolipids, two unidentified phospholipids, one unidentified aminophospholipid, one unidentified glycolipid and four unidentified polar lipids were detected.

On the basis of the results of this polyphasic study, it is clear that strain JM-1081^T represents a novel species, for which the name *Sphingobacterium zeae* sp. nov. is proposed.

Description of *Sphingobacterium zeae* sp. nov.

Sphingobacterium zeae (ze′ae. L. gen. n. *zeae* of spelt, of *Zea mays*).

Cells are Gram-stain-negative. They are non-motile, and appear as non-spore-forming rods, approx. 1 μm in width and 2 μm in length. Aerobic, oxidase-positive and catalase-positive. Good growth is observed after 48 h growth on nutrient agar, brain heart infusion agar, TSA and R2A agar (all Oxoid) at 10–30 °C. No growth occurs on MacConkey agar (Oxoid) at 28 °C. Unable to grow below 4 °C and above 45 °C. Cells grow in the presence of 1.0–4.0 % (w/v) NaCl as an additional ingredient of nutrient agar. Grows at pH values of 5.5–9.0 under the conditions described above, but not at pH values below 5.5 or above 9.0. Colonies on nutrient agar produce a yellow colour and appear circular, translucent and glistening with entire edges. The yellow pigment of the flexirubin type is non-diffusible and non-fluorescent. Acid is produced from D-glucose, lactose, sucrose, salicin, L-arabinose, raffinose, L-rhamnose, maltose, trehalose, cellobiose and methyl α-D-glucoside. No acid is produced from adonitol, D-arabitol, dulcitol, erythritol, *i*-inositol, D-mannitol, melibiose, D-sorbitol or D-xylose. Positive for aesculin, β-galactosidase, and hydrolysis of casein, gelatin and starch, but negative for urease activity, indole production, hydrogen sulphide production, and activity of arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase. The following compounds are weakly utilized as a sole source of carbon: D-glucose, maltose, D-mannose, L-arabinose, acetate, *N*-acetylglucosamine, cellobiose, D-galactose, gluconate, salicin, D-fructose, maltitol, α-melibiose, L-rhamnose, D-ribose and sucrose. The following compounds are not utilized as a sole source of carbon: *N*-acetylgalactosamine, D-mannitol, D-xylose, adonitol, *i*-inositol, D-sorbitol, putrescine, *cis*-aconitate, *trans*-aconitate, 4-aminobutyrate, adipate, azelate, fumarate, glutarate, DL-3-hydroxybutyrate, itaconate, DL-lactate, 2-oxoglutarate, pyruvate, suberate, citrate, mesaconate, L-alanine, β-alanine, L-ornithine, L-phenylalanine, L-serine, L-aspartate, L-histidine, L-leucine, L-proline, L-tryptophan, 3-hydroxybenzoate, 4-hydroxybenzoate and phenylacetate. The chromogenic substrates *p*-nitrophenyl-α-D-glucopyranoside, *p*-nitrophenyl-β-D-glucopyranoside (weak), *p*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-β-D-xylopyranoside, bis-*p*-nitrophenyl-phosphate, bis-*p*-nitrophenyl-phenyl-phosphonate, bis-*p*-nitrophenyl-phosphoryl-choline, 2-deoxythymidine-2′-*p*-nitrophenyl-phosphate, L-alanine-*p*-nitroanilide, γ-L-glutamate-*p*-nitroanilide and L-proline-*p*-nitroanilide are hydrolysed. *p*-Nitrophenyl-β-D-glucuronide is not hydrolysed. The major cellular fatty acids are iso-$C_{15:0}$, $C_{16:0}$, iso-$C_{15:0}$2-OH and iso-$C_{17:0}$3-OH. The diamino acid of the peptidoglycan is *meso*-diaminopimelic acid. The polyamine pattern is characterized by the major compound *sym*-homospermidine and a moderate amount of spermidine. The quinone system contains predominantly menaquinone MK-7. Phosphatidylethanolamine, phosphatidylserine, one phospholipid and four polar lipids are the major lipids. Moderate to minor amounts of two unidentified aminolipids, two unidentified phospholipids, one unidentified aminophospholipid, one unidentified glycolipid and four unidentified polar lipids are detectable. A sphingoglycolipid is also present.

The type strain is JM-1081^T (=LMG 29191^T=CCM 8652^T), which was isolated in 1990 as an endophyte from the healthy internal stem tissue of maize (*Zea mays*, cultivar 'Sweet Belle') at the time of harvest. The field plot was located at the E.V. Smith Research Center in Tallassee (Elmore county), AL, USA.

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