Bacillus gossypii sp. nov., isolated from the stem of *Gossypium hirsutum*

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A Gram-stain-positive, facultatively anaerobic, endospore-forming organism, isolated from the stem of *Gossypium hirsutum*, was studied to determine its taxonomic position. On the basis of 16S rRNA gene sequence similarity comparisons, strain JM-267^T was grouped in the genus *Bacillus*, related most closely to the type strains of *Bacillus simplex* and *Bacillus huizhouensis* (both 97.8 %), *Bacillus muralis* (97.7 %), *Bacillus butanolivorans* and *Bacillus psychrosaccharolyticus* (both 97.3 %). 16S rRNA gene sequence similarity to the sequences of the type strains of other *Bacillus* species was <97.0 %. The fatty acid profile supported the grouping of the strain to the genus *Bacillus*. As major fatty acids, anteiso-$C_{15:0}$, iso-$C_{15:0}$, iso-$C_{14:0}$ and iso-$C_{16:0}$ were detected. The polar lipid profile contained the major components diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine. The major quinone was menaquinone 7 (MK-7). DNA–DNA hybridizations with *B. simplex* DSM 1321^T, *B. huizhouensis* GSS03^T, *B. muralis* LMG 20238^T, *B. butanolivorans* LMG 23974^T and *B. psychrosaccharolyticus* DSM 6^T resulted in values clearly below 70 %. In addition, physiological and biochemical test results allowed the clear phenotypic differentiation of strain JM-267^T from the most closely related species. Hence, strain JM-267^T is considered to represent a novel species of the genus *Bacillus*, for which the name *Bacillus gossypii* sp. nov. is proposed. The type strain is JM-267^T (=DSM 100034^T=LMG 28742^T).

The genus *Bacillus*, delineated by Ash *et al.* (1991) on the basis of analysis of the 16S small-subunit rRNA gene sequence, is a rapidly growing genus, harbouring now more than 200 recognized species (http://www.bacterio.cict.fr/index.html). Species of the genus *Bacillus* have been shown to grow aerobically or facultatively anaerobically. Members of the genus *Bacillus* have been isolated from various habitats, including many terrestrial and aquatic habitats (Ivanova *et al.*, 1999; Siefert *et al.*, 2000), marine sediments (Miranda *et al.*, 2008), high- and low-temperature environments (Caccamo *et al.*, 2000; Logan *et al.*, 2000) and the inner tissue of different plants, such as wheat (Liu *et al.*, 2009) and maize (Rijavec *et al.*, 2007). Here we report the description of a *Bacillus*-like strain isolated from cotton, which has been found recently to harbour another novel species (Kämpfer *et al.*, 2015).

The GenBank/EMBL/DDBJ accession number of the 16S rRNA gene sequence of strain JM-267^T is KT240114.

Two supplementary figures are available with the online Supplementary Material.

Strain JM-267^T was isolated as an endophyte from the internal tissues of healthy cotton stem (*Gossypium hirsutum*, cultivar 'DES-119') 21 days after planting in the field at the Plant Breeding unit facility, E.V. Smith Research Center in Tallassee (Macon county), AL, USA, according to the method described by McInroy & Kloepper (1995).

The strain was initially isolated on nutrient agar (NA; Sigma-Aldrich) at 30 °C and also further maintained and subcultivated on this agar at 30 °C for 48 h. Analyses of the 16S rRNA gene sequence, the fatty acid methyl ester composition of whole-cell hydrolysates, and further biochemical and physiological features were conducted to characterize the strain. In addition, DNA–DNA hybridizations were performed with the type strains of those species most closely related on the basis of 16S rRNA gene sequence similarities, among them *Bacillus simplex* DSM 1321^T, *Bacillus huizhouensis* GSS03^T, *Bacillus muralis* LMG 20238^T, *Bacillus butanolivorans* LMG 23974^T and *Bacillus psychrosaccharolyticus* DSM 6^T. All cultural and morphological characteristics were recorded from cultures grown on

trypticase soy agar (TSA; Oxoid). Gram staining was performed according to Gerhardt *et al.* (1994). Cell size measurements and motility test were done under a light microscope (Axioplan 2; Zeiss) at 1000-fold magnification with cells grown for 3 days in trypticase soy broth (TSB; Oxoid) at 30 °C. Temperature-dependent growth was tested at 4, 10, 15, 20, 30, 36, 40 and 45 °C on TSA. NaCl tolerance was investigated at different concentrations of NaCl [0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0 % (w/v)] in TSB. pH-dependent growth was tested in TSB adjusted with HCl and NaOH to pH values between 4.0 and 12.0. Growth under anaerobic conditions was determined on anaerobic agar (Difco) at 30 °C using a GasPak jar (<1 % O_2; ≥13 % CO_2) (Merck) for 7 days.

Strain JM-267^T showed a Gram-positive staining behaviour (Fig. S1, available in the online Supplementary Material) and formed visible (diameter about 2 mm) beige-coloured colonies within 48 h at 30 °C. The isolate did not grow below 10 °C or above 36 °C. Strain JM-267^T grew very slowly at 15 °C and was able to grow at NaCl concentrations of 0.5–4 % (w/v).

Colonies showed a beige, slightly glistening appearance. Oxidase activity tested positive using oxidase reagent (bioMérieux) according to the instructions of the manufacturer. Cells were non-motile rods (approx. 1 μm wide and 3.5 μm long). Subterminal spores were observed. The strain grew well on NA, brain heart infusion agar, R2A agar and TSA.

The strain was physiologically/biochemically characterized using the 96-well plate test system (Kämpfer *et al.*, 1991) and by some additional biochemical tests: 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.

The analysis of cellular fatty acid profiles was performed as described by Kämpfer & Kroppenstedt (1996) using an HP 6890 gas chromatograph with the Sherlock MIDI software version 2.11 and the TSBA peak naming table version 4.1. Prior to fatty acid extraction the strains were cultured on TSA at 28 °C for 48 h. The results revealed a profile typical of the genus *Bacillus* for strain JM-267^T with the following most abundant fatty acids: anteiso-$C_{15:0}$, iso-$C_{15:0}$, iso-$C_{14:0}$, iso-$C_{16:0}$ and anteiso-$C_{15:0}$. Only minor differences could be identified in comparison with those of the type strains of the most closely related *Bacillus* species (Table 2).

Quinones and polar lipids were extracted from biomass that was grown on 3.3× PYE (1.0 % peptone from casein, 1 % yeast extract, pH 7.2) at 28 °C and harvested at the stationary growth phase. Analyses of quinones and polar lipids were carried out according to Tindall (1990a, b) and Altenburger *et al.* (1996). The HPLC equipment used was as described by Stolz *et al.* (2007). The quinone system was composed of menaquinones MK-7 (87 %) and MK-6 (12 %). In the polar lipid profile (Fig. 1) diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine were predominant. In addition, minor to moderate amounts of three unidentified phospholipids, one unidentified aminophospholipid and one unidentified lipid that was detectable only after total lipid staining were found. The presence of the three major lipids is in agreement with data for *Bacillus subtilis*, the type species of the genus, but the absence of any glycolipid distinguishes strain

Table 1. Differential phenotypic characteristics between strain JM-267^T and closest related members of the genus *Bacillus*

Strains: 1, JM-267^T; 2, *B. simplex* DSM 1321^T; 3, *B. huizhouensis* GSS03^T; 4, *B. muralis* LMG 20238^T; 5, *B. butanolivorans* LMG 23974^T; 6, *B. psychrosaccharolyticus* DSM 6^T. Data for strains 2–4 and 6 were obtained in this study, and in agreement with those from Li *et al.* (2014). +, Positive; −, negative; w, weakly positive; ND, not determined.

Characteristic	1	2	3	4	5	6
Gram reaction	+	+	+	+	+	+
Nitrate reduction	−	+	−	+	−	+
Growth at 4 °C	−	−	−	−	+	+
Growth at 40 °C	−	−	+	+	+	+
NaCl range (%, w/v)	0–4	0–5	0–2	0–7	0–5	0–2
pH range	5–8	5–9	6.5–8	7–9	6–9	5.2–8
Catalase	+	+	−	+	+	+
Oxidase	+	−	+	+	+	+
Hydrolysis of:						
Casein	−	+	+	+	−	+
Gelatin	−	+	−	−	−	−
Starch	−	+	−	+	−	+
Tween 20	−	+	+	+	−	+
Tween 80	−	+	+	+	−	+
Acid production from:						
Glycerol	−	−	+	w	−	+
Galactose	−	−	+	−	−	+
Fructose	−	−	+	+	−	+
Maltose	−	+	+	+	−	+
Lactose	−	−	+	+	−	+
Trehalose	−	+	+	+	−	+
Raffinose	−	−	+	−	−	−
Sorbitol	−	+	−	−	−	−
Sucrose	−	−	+	−	−	−
L-Arabinose	−	+	+	w	−	+
D-Glucose	−	+	+	+	−	+
D-Mannose	−	+	−	+	−	+
D-Mannitol	−	+	−	−	−	−
D-Xylose	−	+	−	−	−	+

Table 2. Cellular fatty acid content of strain JM-267^T and related members of the genus *Bacillus*

Strains: 1, JM-267^T; 2, *B. simplex*, DSM 1321^T; 3, *B. huizhouensis* GSS03^T; 4, *B. muralis* LMG 20238^T; 5, *B. butanolivorans* LMG 23974^T; 6, *B. psychrosaccharolyticus* DSM 6^T. Data are expressed as percentages of the total fatty acids. All data are from this study. Fatty acid analysis was performed with the standard MIDI procedures. All data presented were obtained on the basis of the same method (strains were cultivated under the same conditions prior to extraction of the fatty acid). Fatty acids representing <0.5 % are not included; −, not found.

Fatty acid	1	2	3	4	5	6
Saturated						
$C_{14:0}$	1.2	1.6	4.0	1.9	1.1	3.5
$C_{15:0}$	−	−	1.0	−	1.1	0.9
$C_{16:0}$	1.1	3.1	3.9	2.8	6.6	9.7
iso-$C_{13:0}$	0.7	−	0.9	−	−	−
iso-$C_{14:0}$	5.8	27.9	8.7	3.1	5.5	7.0
iso-$C_{15:0}$	26.2	17.0	25.7	23.2	15.7	5.7
iso-$C_{16:0}$	3.6	11.6	1.5	1.8	8.2	8.6
iso-$C_{17:0}$	−	1.2	−	1.9	3.7	1.6
anteiso-$C_{15:0}$	57.8	25.5	44.6	56.7	39.9	59.1
anteiso-$C_{17:0}$	3.5	1.2	1.7	2.4	5.8	3.0
Others						
$C_{16:1}\omega7c$ alcohol	−	8.8	1.8	1.9	4.7	−
$C_{16:1}\omega11c$	−	2.1	5.8	3.0	5.4	0.7
iso-$C_{17:0}\omega10c$	−	−	−	−	−	−
iso-$C_{17:1}\omega10c$	−	−	−	1.4	1.8	−

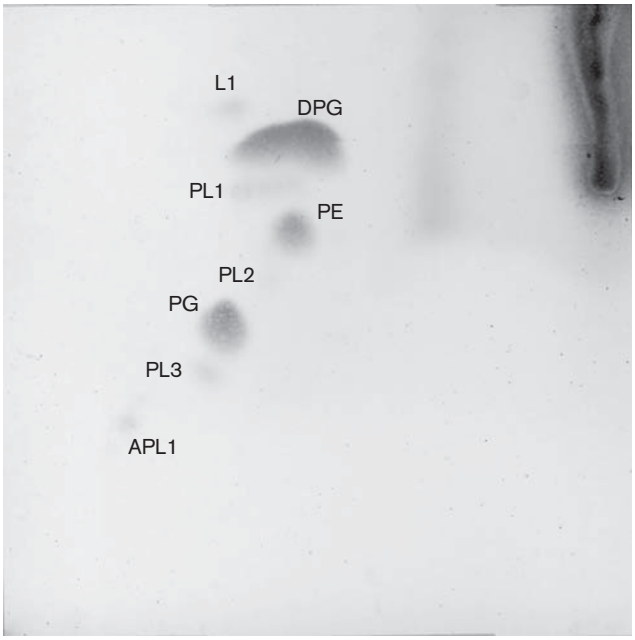

Fig. 1. Polar lipid profile of strain JM-267^T. Total lipids were visualized after two-dimensional TLC applying molybdatophosphoric acid. DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PL1–3, unidentified phospholipids; APL1, unidentified aminophospholipid; L1, unidentified polar lipid only visible after total lipid staining.

JM-267^T (Kämpfer *et al.*, 2006). Polyamines were extracted from cells grown on 3.3× PYE, which were harvested at the late exponential growth phase as recommended by Busse & Auling (1988). Extraction and analysis of polyamines were carried out according to Altenburger *et al.* (1997) applying the HPLC apparatus described by Stolz *et al.* (2007). The major polyamine was spermidine [13.1 μmol (g dry weight)$^{-1}$]. In addition, moderate amounts of spermine [2.8 μmol (g dry weight)$^{-1}$] and trace amounts of putrescine [<0.1 μmol (g dry weight)$^{-1}$] were detected.

The full-length 16S rRNA gene of strain JM-267^T was sequenced for detailed phylogenetic analysis using the primer system 8F and 1492R (Lane, 1991). After manual sequence correction the sequence used for analysis had a length of 1435 nt spanning gene termini 47–1475 (numbered according to the *Escherichia coli rrnB* gene sequence; Brosius *et al.*, 1981). Pairwise sequence similarities to closest related type strains were obtained using the EzTaxon type strain database (Kim *et al.*, 2012). Phylogenetic trees were calculated in 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). Sequences not included in the database were added after alignment with the SILVA Incremental Aligner (SINA, v. 1.2.11; Pruesse *et al.*, 2012). Phylogenetic trees were calculated with the maximum-likelihood method using RAxML v7.04 (Stamatakis, 2006) with GTR-GAMMA as the evolutionary model and rapid bootstrap analysis, the maximum-parsimony method using DNAPARS version 3.6 (Felsenstein, 2005) and the neighbour-joining method using the ARB neighbour-joining tool with the Jukes–Cantor correction as the evolutionary model (Jukes & Cantor, 1969). The analysis was based on 16S rRNA gene sequences between gene termini 144 and 1375 (Brosius *et al.*, 1981) and a bootstrap analysis (Felsenstein, 1985) with 100 resamplings. To reduce the size of the final tree, strain JM-267^T was added to the LTP database tree including all *Bacillus* species and further trees were calculated including the 74 closest clustering *Bacillus* species. Strain JM-267^T formed a distinct cluster with the type strains of six species of the genus *Bacillus* (supported by a bootstrap value >70 %; Fig. 2; Fig. S2). Within this cluster, strain JM-267^T showed no further distinct clustering with the other type strains. The relationship of strain JM-267^T varied between the treeing methods used and was not supported by high bootstrap values. Pairwise 16S rRNA gene sequence analysis also showed that the species in this cluster showed highest similarity to strain JM-267^T, namely the type strains of *B. simplex* and *B. huizhouensis* (both 97.8 %), *B. muralis* (97.7 %), *B. butanolivorans* and *B. psychrosaccharolyticus* (both

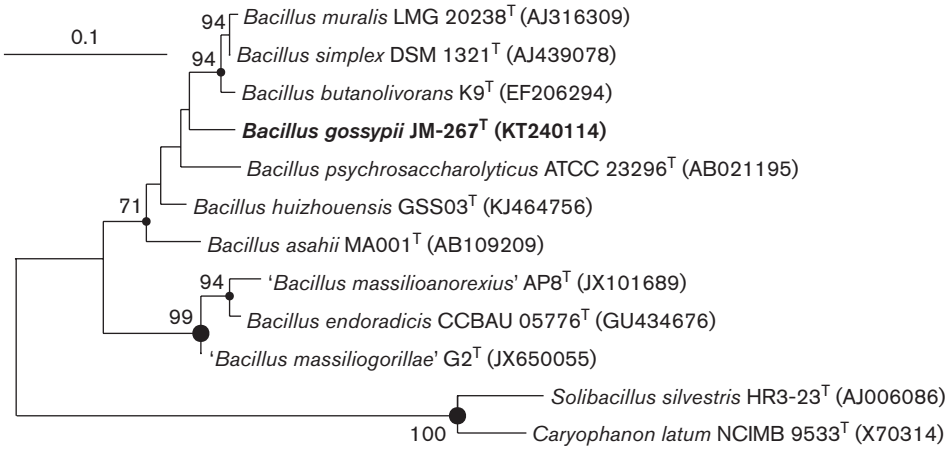

Fig. 2. Maximum-likelihood tree based on nearly full-length 16S rRNA gene sequences showing the position of strain JM-267^T among closest related type strains of the genus *Bacillus*. The tree was calculated in ARB and based on 16S rRNA gene sequence positions 144–1375 (*E. coli* numbering; Brosius *et al.*, 1981) and 100 resamplings (bootstrap analysis; only values >70 % are shown). Nodes marked with filled circles were also present in the maximum-parsimony tree calculated in parallel; larger circles represent nodes supported by bootstrap values >70 % in the maximum-parsimony tree. The type strains of *Solibacillus silvestris* and *Caryophanon latum* were used as outgroups. Bar, 0.1 substitutions per nucleotide position.

97.3 %) and *Bacillus asahii* (97.0 %). 16S rRNA gene sequence similarities between strain JM-267^T and the type strains of all other *Bacillus* species were below 97.0 %.

For further genotypic analysis, high molecular mass genomic DNA was extracted as described by Pitcher *et al.* (1989) and DNA–DNA hybridization experiments were performed with strain JM-267^T and the type strains of the five most closely related *Bacillus* species 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). Strain JM-267^T showed moderate to low levels of DNA–DNA relatedness to *B. simplex* DSM 1321^T (43 %, reciprocal 44 %), *B. huizhouensis* GSS03^T (64 %, reciprocal 35 %), *B. muralis* LMG 20238^T (20 %, reciprocal 25 %), *B. butanolivorans* LMG 23974^T (22 %, reciprocal 10 %) and *B. psychrosaccharolyticus* DSM 6^T (15 %, reciprocal 17 %).

On the basis of the results of this polyphasic study, it is clear that strain JM-267^T represents a novel species, for which the name *Bacillus gossypii* sp. nov. is proposed.

Description of *Bacillus gossypii* sp. nov.

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

Cells show a Gram-positive staining behaviour. They are non-motile, endospore-forming rods, approx. 1 µm in width and 3 µm in length. Facultatively anaerobic; oxidase- and catalase-positive. Good growth occurs after 48 h on NA, brain heart infusion agar, TSA and R2A agar (all Oxoid) at 20–30 °C.

Unable to grow below 10 °C or above 37 °C. Optimum temperature for growth is 28–30 °C. Cells grow in the presence of 0.5–4.0 % (w/v) NaCl as an additional ingredient of NA [optimum: 1–2 % (w/v) NaCl]. Colonies on NA produce a beige colour and appear circular and slightly translucent.

Acid is produced from D-glucose (weakly). No acid is produced from maltose, trehalose, sucrose, L-arabinose, adonitol, D-arabitol, dulcitol, erythritol, *i*-inositol, lactose, D-mannitol, melibiose, methyl α-D-glucoside, raffinose, L-rhamnose, salicin, D-sorbitol or D-xylose. Negative for urease activity, hydrolysis of casein, gelatin, starch, DNA and tyrosine, indole production, hydrogen sulphide production, and activity of arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and β-galactosidase. The following compounds are weakly utilized as a sole source of carbon: D-glucose and maltose.

The following compounds are not utilized as a sole source of carbon: acetate, propionate, *N*-acetylgalactosamine, *N*-acetylglucosamine, L-arabinose, cellobiose, D-galactose, gluconate, D-mannose, D-fructose, glycerol, D-mannitol, maltitol, α-melibiose, L-rhamnose, D-ribose, salicin, sucrose, 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 and *p*-nitrophenyl-β-D-glucopyranoside (weak) are hydrolysed, but not 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, L-proline-*p*-nitroanilide, *p*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-β-D-xylopyranoside or *p*-nitrophenyl-β-D-glucuronide. The major cellular fatty acids are anteiso-$C_{15:0}$, iso-$C_{15:0}$, iso-$C_{14:0}$ and iso-$C_{16:0}$. In the polyamine pattern spermidine is the major component. The quinone system contains predominantly menaquinone MK-7. Diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine are major components in the polar lipid profile. In addition, moderate to minor amounts of unidentified lipids are present (three phospholipids, one aminophospholipid, and one lipid not containing a sugar moiety, an amino residue or a phosphate residue).

The type strain is JM-267^T (=DSM 100034^T=LMG 28742^T) isolated from the internal tissues of healthy cotton stem (*Gossypium hirsutum*), AL, USA.

Acknowledgements

We thank Gundula Will, Katja Grebing and Maria Sowinsky for excellent technical assistance, and Professor Dr Shungui Zhou, Guangdong Institute of Eco-environmental and Soil Sciences, Guangdong, China, for kindly sending the type strain of *B. huizhouensis*.

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