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Lysinibacillus tabacifolii sp. nov., a Novel Endophytic Bacterium Isolated from *Nicotiana tabacum* Leaves[§]

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A Gram-positive, catalase- and oxidase-positive, strictly aerobic, endospore-forming rod bacterium, designated K3514^T, was isolated from the leaves of *Nicotiana tabacum*. The strain was able to grow at temperatures of 8–40°C, pH 5.0–10.0 and NaCl concentrations of 0–7%. The predominant quinones (>30%) of this strain were MK-7(H2) and MK-7. Phylogenetic analysis of 16S rRNA gene sequence showed that strain K3514^T was affiliated to the genus *Lysinibacillus*, with its closest relatives being *Lysinibacillus mangiferihumi* (98.3% sequence similarity), *Lysinibacillus sphaericus* (97.9% sequence similarity), *Lysinibacillus fusiformis* (97.4% sequence similarity), and *Lysinibacillus xylanilyticus* (97.3% sequence similarity). However, low levels of DNA-DNA relatedness values suggested that the isolate was distinct from the other closest *Lysinibacillus* species. Additionally, based on analysis of morphological, physiological, and biochemical characteristics, the isolate could be differentiated from the closest known relatives. Therefore, based on polyphasic taxonomic data, the novel isolate likely represents a novel species, for which the name *Lysinibacillus tabacifolii* sp. nov. and the type strain K3514^T (=KCTC 33042^T =CCTCC AB 2012050^T) are proposed.

Keywords: *Lysinibacillus tabacifolii*, polyphasic taxonomy, *Nicotiana tabacum*

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Introduction

Ahmed *et al.* (2007) proposed the genus *Lysinibacillus* and the reclassification of two *Bacillus* species based on the description of a novel species and a polyphasic taxonomic study. As a Gram-positive, catalase-positive, spore-forming rod (Ahmed *et al.*, 2007; Jung *et al.*, 2012), the genus *Lysinibacillus* was classified in the family *Bacillaceae*; it is distinguished from other members of the family by the presence of lysine and aspartate in the peptidoglycan of its cell wall. There are currently 10 species in this genus, including *Lysinibacillus boronitolerans* (type species), *Lysinibacillus fusiformis*, *Lysinibacillus macroides*, *Lysinibacillus mangiferihumi*, *Lysinibacillus massiliensis*, *Lysinibacillus odysseyi*, *Lysinibacillus parviboronicapiens*, *Lysinibacillus sinduriensis*, *Lysinibacillus sphaericus*, and *Lysinibacillus xylanilyticus* (http://www.bacterio.cict.fr/L/*Lysinibacillus*.html; Miwa *et al.*, 2009; Lee *et al.*, 2010; Yang *et al.*, 2012). During the course of an investigation on the diversity of endophytic microorganisms in leaves of *N. tabacum* in Yunnan province, southwestern China, a *Lysinibacillus*-like strain, designated K3514^T, was isolated. Using polyphasic characterisation, the position of the strain was determined. Based on minimal standards for describing new taxa of aerobic, endospore-forming bacteria (Logan *et al.*, 2009), the novel strain is proposed to represent a novel species of the genus *Lysinibacillus*.

Materials and Methods

Isolation of bacterial strain and culture conditions

Strain K3514^T was isolated from the surface-sterilised leaves of *N. tabacum* collected from Yunnan province, southwestern China. Samples were thoroughly washed in running water and a sonication step (160 W) (Branson 8040) was employed to dislodge any soil and organic matter from the surface. After drying at room temperature, the tissue segments were surface-sterilised by immersion in 0.1% Tween 20 for 1 min, followed by 5% (available Cl^-) NaClO for 4 min (leaves only). The samples were then rinsed in 2.5% (w/v) $Na_2S_2O_3$ for 10 min to remove residual chlorine (Katsivela *et al.*, 1999; Miché and Balandreau, 2001; Qin *et al.*, 2009) and washed with sterile H_2O at least three times. The last disinfection step consisted of immersing the samples in 70% (v/v) ethanol for 4 min (leaves only), followed by a minimum of three washes with sterile water. Surface-sterilised samples were put in sterile plates with filter papers and left to dry in a laminar flow cabinet. To confirm that the surface disinfection process was successful, 0.2 ml of the water used for the final wash of plant samples was spread onto each of

the isolation media and Luria-Bertani (LB) agar medium (Bacto-Tryptone 10 g, Bacto-yeast extract 5 g, NaCl 10 g, ddH_2O 1 L, pH 7.0, total volume 1 L) (Shirling and Gottlieb, 1966) and incubated at 28°C for one to three weeks.

For the isolation step, 1 g of each sample was sectioned using a commercial blender and then ground with a mortar and pestle, after which 9 ml sterile water was added. One milliliter of the resulting suspension was serially diluted to 10^{-3} and spread on isolation medium plates. The isolation medium used was sodium propionate-asparagine-salt agar [3% (w/v) NaCl added to the sodium propionate-asparagine agar described previously by Qin *et al.* (2009), pH 7.2]. Nalidixic acid (25 mg/L) and nystatin (50 mg/L) were added to this medium after incubation at 28°C for two weeks. Pure cultures were obtained by repeated streaking on half-strength LB agar medium. The purified strain was routinely cultivated on KMB medium at 28°C and stored as a glycerol suspension (20%, v/v) at -80°C.

The strain was deposited in the Korean Collection for Type Cultures (KCTC) and the Collection Center of Typical Cultures, China (CCTCC). The strain deposition numbers were as follows: K3514^T =KCTC 33042^T =CCTCC AB 2012050^T. The closely related *Lysinibacillus* species *L. sphaericus*, *L. fusiformis*, *L. xylanilyticus*, and *L. mangiferihumi* were obtained from the DSMZ and YIM (Yunnan Institute of Microbiology, Yunnan University, China) as reference strains.

Phenotypic characteristics

The phenotypic characteristics of strain K3514^T and the reference strains were examined on Kings Medium B agar (KMB; Difco) at 32°C and pH 7.0, unless noted otherwise. Gram staining was performed using the standard Gram reaction and the KOH lysis method, according to the previously established protocol (Gregersen, 1978). The oxidase activity of the strains were detected using the API oxidase reagent according to the manufacturer's instructions. Catalase activity was assessed by bubble production in 3% (v/v) H_2O_2. The cell suspension was treated with ultrasonication for 25 sec to separate the thallus into single cells that were then fixed with glutaraldehyde (2%) for 2 h and dehydrated with a gradient series of alcohol (30, 50, 70, 90, and 100%, respectively). The cell specimens were sputter-coated with gold powder for 200 s and examined. The morphology of cells and endospores in each individual sample was examined by scanning electron (Philips XL30; ESEM-TMP) and phase-contrast microscopy (BH-2; Olympus), using cells grown on KMB medium at 32°C for three days. Motility was observed by scanning electron microscopy (ESEM-TMP) using the same cell cultures. The temperature range (4, 10, 15, 20, 28, 37, 45, 50, and 55°C) and sodium chloride conditions (0–12% w/v, in 1% increments) for the strains' growth were determined using KMB agar and up to three days of incubation. The pH range for growth was determined in KMB liquid incubated at 32°C by monitoring OD_{600} using a mini-photometer (model 518R; TAITEC); 1% (v/v) phosphatic buffer was added to maintain the pH (pH range from 4.0 to 11.0, in increments of 1.0). Anaerobic growth was examined in serum bottles with sodium thioglycolate (1 g/L) added to KMB media and the upper air layer was replaced with nitrogen. Production of nitrate reductase, indole, H_2S, urease and gelatinase was examined using the API 20E kit (bioMérieux, France). Carbon source utilisation, acid-production profiles and other enzyme activities were assessed using the Biolog GEN III system (MicroPlate, USA), API 50 NH test strips (bioMérieux), API 50 CH test strips (bioMérieux) and the API ZYM system (bioMérieux). Hydrolysis of Tween (20, 40, 60, and 80), starch and cellulose, gelatin liquefaction, and catalase, oxidase and urease activity were assessed using the media described by Williams *et al.* (1989) and MacFaddin (1980).

Chemotaxonomy

All strains, including reference strains, were cultured to extract and analyse cellular fatty acids under the same conditions, with trypticase soy agar (TSA; Difco) incubated at 32°C for 48 h. Using a gas chromatograph (Agilent Technologies 7890A GC System) with the Microbial identification software package (Sherlock Version 6.1; MIDI database: TSBA6) according to the manufacturer's instructions, the fatty acids were derivatised to methylesters and analysed. To obtain the biomass for other chemotaxonomic studies, these strains were cultured at 32°C for three days in shake flasks containing trypticase soy liquid medium. Respiratory quinines were extracted and purified from 300 mg dried cells of strain K3514^T using previously described methods (Collins *et al.*, 1977) and examined by HPLC (Groth *et al.*, 1997). Polar lipids were extracted and purified from 100 mg dried cells using the protocol described by Minnikin *et al.* (1979) and identified by two-dimensional TLC (Collins and Jones, 1980; Tang *et al.*, 2009). Spray reagents, molybdatophosphoric acid, ninhydrin, molybdenum, and α-naphthol were applied to separate plates for detection. Using pre-column derivatisation with *o*-phthalaldehyde (OPA), the amino acid contents of the cell-wall peptidoglycan were assessed by HPLC using a routine protocol (Schleifer and Kandler, 1972; Groth *et al.*, 1996).

Molecular analysis

Extraction of genomic DNA and amplification of the 16S rRNA were undertaken as previously described (Li *et al.*, 2007). The 16S rRNA sequence was aligned and compared to reference sequences retrieved from the GenBank database using BLAST searches (http://www.ncbi.nlm.nih.gov/blast/) and Clustal_X (Thompson *et al.*, 1997). Phylogenetic trees were built using neighbour-joining (Saitou and Nei, 1987), maximum-likelihood (Felsenstein *et al.*, 1981) and maximum parsimony (Fitch *et al.*, 1971) tree-making algorithms in the software packages MEGA version 4.0 (Tamura *et al.*, 2007) and PhyML 3.0 (Guindon and Gascuel, 2003). The topologies of the phylogenetic trees were evaluated by the bootstrap resampling method described by Felsenstein *et al.* (1985) with 1,000 replicates. The G+C content of the genomic DNA was determined using previously described protocols (Mesbah *et al.*, 1989). DNA-DNA hybridisation was performed in five replicates using the fluorometric micro-well method (Ezaki *et al.* 1989; Christensen *et al.*, 2000) at 42°C with photobiotin-belled DNA and microplates. For fluorescence measurements, an HTS7000 Bio Assay Reader (Applied Biosystems, USA) was used.

Nucleotide sequence accession number

The GenBank/EMBL/DDBJ accession number for the 16S rRNA sequence of strain K3514^T is JQ754706.

Results and Discussion

Strain K3514^T was Gram-positive, catalase- and oxidase-ositive, non-motile, endospore-forming, aerobic and rod-haped. Cells of the strain were 0.4–1.5×5.0–10.0 μm and generated ellipsoidal endospores in terminally swollen sporangia (Supplementary data Fig. S3). After three days of incubation on KMB agar at 32°C, colonies of the strain were circular, entirely surrounded by a margin, white and approximately 0.6–1.0 mm in diameter. Using a variety of tests, the growth conditions for the novel isolate were determined: temperature range 8–40°C, pH range 5.0–10.0 and NaCl concentration range 0–7%. The optimal pH, temperature and NaCl concentration for growth were 6.0–8.0, 32°C and 0%, respectively. Detailed physiological and biochemical characteristics of strain K3514^T and the closely related *Lysinibacillus* species are given in Table 1. Although strain K3514^T exhibited many phenotypic features in common with the genus *Lysinibacillus*, there are several characteristics that distinguish the strain from the closest *Lysinibacillus* species (such as pH, salt concentration, temperature, hydrolysis of urea and patterns of carbohydrate oxidisation). Based on these phenotypic analyses, the strain was classified into the genus *Lysinibacillus*, but slightly distinct from closely related *Lysinibacillus* species *L. sphaericus*, *L. fusiformis*, *L. xylanilyticus*, and *L. mangiferihumi* (Table 1). Moreover, tests for milk peptonisation, melanin formation and hydrolysis of cellulose, casein, Tween 20, 40, 60, 80, and gelatin were negative. Nitrate was reduced to nitrite and H_2S was not produced. Tests for milk coagulation and starch hydrolysis were positive.

The major fatty acid components (>3% of total fatty acids) of strain K3514^T were iso-$C_{14:0}$, iso-$C_{15:0}$, anteiso-$C_{15:0}$, iso-$C_{16:0}$, $C_{16:0}$, iso-$C_{17:0}$, $C_{16:1}$ $\omega7c$ alcohol and $C_{17:0}$. The complete fatty acid compositions are described in detail in Table 2. The fatty acid profile of strain K3514^T differed from that of related species: there was a higher proportion of iso-$C_{14:0}$ (7.0%), a lower proportion of iso-$C_{15:0}$ (32.6%), anteiso-$C_{17:0}$ (0.7%) and iso-$C_{17:1}$ $\omega10c$ (0.9%) and an absence of $C_{16:1}$ $\omega11c$. The levels of anteiso-$C_{15:0}$ (19.0%), iso-$C_{16:0}$ (8.9%), iso-$C_{17:0}$ (5.2%), and $C_{16:1}$ $\omega7c$ alcohol (3.7%) distinguished strain K3514^T from related species. The predominant isoprenoid quinones of strain K3514^T were MK-7(H_2) (40%) and MK-7 (36%), and the major polar lipids were diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and two unknown phospholipids (PL_1, PL_2) (Supplementary data Fig. S4); of the level of phosphatidylinositol (PI) distinguished this strain from closely related species. Moreover, the novel isolate contained alanine, glutamic acid, lysine and aspartic acid as the diagnostic amino acids (alanine, glutamic acid, lysine, and aspartic acid in a molar ratio of 2.03 : 1.70 : 1.00 : 1.20), corresponding to

Table 1. Differential characteristics of strain K3514^T and its closely related *Lysinibacillus* species

Strains: 1, strain K3514^T; 2, *L. mangiferihumi* M-GX18^T; 3, *L. sphaericus* DSM 28^T; 4, *L. fusiformis* DSM 2898^T; 5, *L. xylanilyticus* DSM 23493^T. Data are from this work, unless otherwise indicated. DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PL, unknown polar lipid; NPG, ninhydrin-positive phosphoglycolipid; PI, phosphatidylinositol. +, positive; -, negative. All members of the genus showed positive results for hydrolysis of gelatin, naphthol-AS-BI-phosphohydrolase, catalase and oxidase but negative results for production of H_2S, reduction of nitrate and oxidation of sucrose and *N*-acetyl-D-glucosamine. Data marked with * were taken from Ahmed *et al.* (2007), Lee *et al.* (2010), and Yang *et al.* (2012). Other data were obtained in this study.

Characteristic	1	2*	3*	4*	5*
pH growth range	5.0–10.0	6.0–9.0	6.0–9.0	6.0–9.0	5.0–9.0
Growth temperature	8–40	10–45	10–37	15–37	10–37
Growth in 7% NaCl	+	-	-	+	-
Hydrolysis of urea	+	-	+	-	-
Voges–Proskauer test	-	+	-	-	-
Arginine dihydrolase	+	-	-	-	-
Lysine decarboxylase	+	-	-	-	-
Ornithine decarboxylases	+	-	-	-	-
Esterase lipase C8	+	+	+	+	-
Trypsin	-	-	+	+	-
α-Chymotrypsin	+	+	+	+	-
Acid phosphatase	+	+	-	-	+
Oxidation of:					
D-maltose	-	+	-	-	+
D-cellobiose	-	-	+	+	+
D-raffinose	-	+	-	-	-
D-melibiose	+	+	-	-	-
α-D-glucose	+	-	+	+	+
D-mannose	+	-	-	+	-
D-fructose	-	-	+	+	+
D-sorbitol	+	-	-	-	-
Polar lipids	DPG, PG, PE, PL, PI	DPG, PG, PE, NPG, PL	DPG, PG, NPG, PL	DPG, PG, PE, NPG	DPG, PG, PE

Table 2. Comparison of cellular fatty acids in strain K3514^T and closely related *Lysinibacillus* species

Strains: 1, strain K3514^T; 2, *L. mangiferihumi* M-GX18^T; 3, *L. sphaericus* DSM 28^T; 4, *L. fusiformis* DSM 2898^T; 5, *L. xylanilyticus* DSM 23493^T. Values are percentages of total fatty acids; -, not detected or <0.5%. All data are from the present study.

Fatty acids	1	2	3	4	5
Branched fatty acids:					
iso-$C_{14:0}$	7.0	1.8	2.5	2.4	1.7
iso-$C_{15:0}$	32.6	56.0	45.8	42.1	33.7
anteiso-$C_{15:0}$	19.0	16.8	3.5	11.8	28.1
iso-$C_{16:0}$	8.9	7.8	9.4	11.5	5.9
iso-$C_{17:0}$	5.2	4.3	7.4	4.7	7.8
anteiso-$C_{17:0}$	0.7	3.5	1.2	4.8	7.6
$C_{16:1}$ $\omega 7c$ alcohol	3.7	2.9	6.7	10.2	0.7
Saturated fatty acids:					
$C_{14:0}$	0.9	1.1	-	0.8	1.2
$C_{16:0}$	3.0	2.8	0.8	2.4	6.1
Unsaturated fatty acids:					
$C_{16:1}$ $\omega 11c$	-	-	15.2	3.4	1.2
iso-$C_{17:1}$ $\omega 10c$	0.9	-	3.1	2.0	1.1
Summed features 4[a]:	0.8	-	1.7	2.0	-

[a] Fatty acids that could not be separated by GC using Microbial Identification System (Microbial ID) software were considered as summed features. The summed features 4 group includes iso-$C_{17:1}$ I and/or anteiso-$C_{17:1}$ B.

peptidoglycan type A4α (Lys-Asp) (Schleifer and Kandler, 1972). All of the chemotaxonomic properties of strain K3514^T were consistent with its classification in the genus *Lysinibacillus* but slightly different from the most closely related *Lysinibacillus* species, *L. sphaericus*, *L. fusiformis*, *L. xylanilyticus*, and *L. mangiferihumi*.

The DNA G+C content of strain K3514^T was 36.5%. Based on the phylogenetic analysis of 16S rRNA sequences, strain

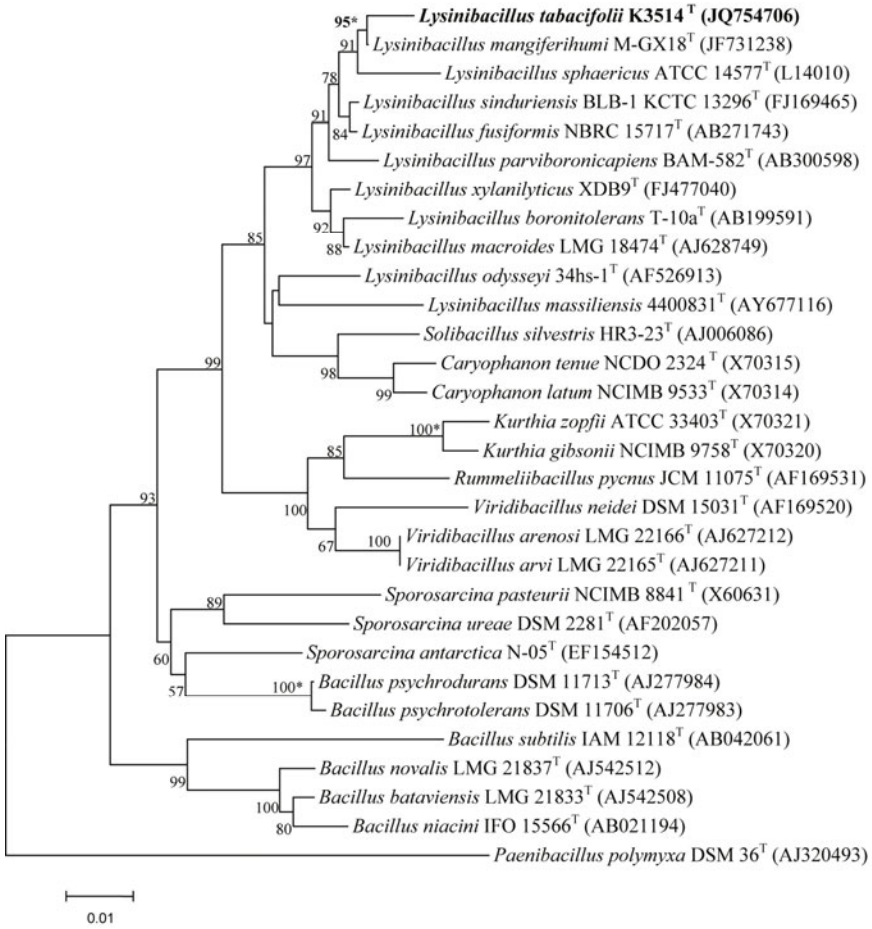

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA sequences, showing the position of K3514^T within the family *Bacillaceae*. Bootstrap values are shown as percentage of 1,000 replicates, and only bootstrap values above 50% are shown. GenBank accession nos. are shown in parentheses. The sequence of *Paenibacillus polymyxa* NCDO1774^T was used as the outgroup. The asterisk shows that the result was also supported by maximum-parsimony and maximum-likelihood tree-making algorithms with high bootstrap values. Bar, 0.01 nucleotide substitutions per 100 nt.

K3514^T clustered consistently within the genus *Lysinibacillus* and formed a stable clade with *L. mangiferihumi* but did not cluster with *L. sphaericus* or *L. fusiformis* in the neighbour-joining phylogenetic tree (Fig. 1). The result was supported by maximum-parsimony and maximum-likelihood tree-making algorithms with high bootstrap values (Supplementary data Figs. S1 and S2). Furthermore, strain K3514^T exhibited high 16S rRNA sequence similarity with *L. sphaericus* (97.4%), *L. xylanilyticus* (97.9%), *L. fusiformis* (97.3%), and *L. mangiferihumi* (98.3%). The novel isolate showed greater than 97% 16S rRNA gene sequence similarity with these four *Lysinibacillus* species. However, the isolate was evidently identified as a separate species based on low DNA-DNA relatedness values (*L. xylanilyticus*, 44.5%; *L. sphaericus*, 47.4%; *L. fusiformis*, 58.4%; *L mangiferihumi*, 52.7%). These results indicate that strain K3514^T represents a novel species of the genus *Lysinibacillus*.

The fatty acid and polar lipid profiles and peptidoglycan type, as well as the 16S rRNA sequence analysis, showed that strain K3514^T is associated with the genus *Lysinibacillus*. On the basis of the observed phenotypic distinctions (Tables l and 2), the differences in the 16S rRNA sequences and DNA-DNA relatedness, we conclude that strain K3514^T represents a novel species of genus *Lysinibacillus* and propose the name *Lysinibacillus tabacifolii* sp. nov.

Description of *Lysinibacillus tabacifolii* sp. nov.

Lysinibacillus tabacifolii (N.L. n. *tabaci*, scientific name of *Nicotiana tabacum*, the tobacco plant; L. n. *folii*, gen. *folium* meaning from a leaf of the tobacco plant.).

Lysinibacillus tabacifolii cells are Gram-positive, non-motile, aerobic and rod-shaped, 0.4–1.5×5.0–10.0 μm in size. Terminal ellipsoidal endospores are observed in swollen sporangia. Colonies are white, circular, completely surrounded by a margin, and 0.6–1.0 mm in diameter after three days of incubation at 32°C on KMB agar. The temperature range for growth is 8–40°C (optimal temperature for growth 28–32°C). The pH range for growth is 5.0–10.0 (optimum pH 6.0–8.0). The NaCl concentration range for growth is 0–7% (optimum NaCl concentration 0%). When being assayed with API 20E kits, catalase and oxidase activity, hydrolysis of gelatin and urease, arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase are positive; while tests for the Voges-Proskauer reaction, nitrate reduction, β-galactosidase, tryptophan deaminase, H_2S and indole production are negative. Acid phosphatase, C_8-esterase lipase and α-chymotrypsin, naphthol-AS-BI-phosphohydrolase activity are detected as positive by using the API ZYM system, but there is no activity of C_{14}-lipase, C_4-esterase, valine, arylamidase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, α-mannosidase or fucosidase. With the Biolog GEN III assay, positive activity of D-melibiose, α-D-glucose, D-mannose, trypsin, D-sorbitol, dextrin, pectin, L-galactonic acid lactone, D-lactic acid methyl ester, γ-aminobutryric acid, and α-hydroxybutyric acid is observed. The activity of other compounds is not examined. Acid is produced only from α-D-glucose (API 50 CH system). The cell wall peptidoglycan type is A4α (Lys-Asp). The predominant quinones (>30%) are MK-7(H_2) and MK-7. The major fatty acid profiles (>3%) are iso-$C_{14:0}$, iso-$C_{15:0}$, anteiso-$C_{15:0}$, iso-$C_{16:0}$, $C_{16:0}$, iso-$C_{17:0}$, $C_{16:1}$ *ω7c* alcohol, and $C_{17:0}$. The polar lipids are diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and two unknown phospholipids. The G+C content of genomic DNA is 36.5 mol%.

The type strain K3514^T (=KCTC 33042^T =CCTCC AB 2012050^T) was isolated from surface-sterilised leaves of *N. tabacum* collected in Yunnan province, southwestern China.

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