Sphingobacterium changzhouense sp. nov., a bacterium isolated from a rice field

Hongming Liu,[1] Jun Zhang,[2] Dingbin Chen,[1] Li Cao,[1] Peng Lu,[1] ZhiGuo Wu,[1] Fan Yang,[1] Shunpeng Li[1] and Qing Hong[1]

Correspondence
Qing Hong
hongqing@njau.edu.cn

[1]Key Laboratory of Agricultural Environmental Microbiology, Ministry of Agriculture, College of Life Sciences, Nanjing Agricultural University, Nanjing, Jiangsu 210095, PR China

[2]College of Resources and Environmental Sciences, Nanjing Agricultural University, 210095, PR China.

A Gram-stain-negative, yellow, non-spore-forming, rod-shaped bacterium, designated N7^T, was isolated from a soil sample collected from a rice field in Jiangsu, China, and was characterized using a polyphasic taxonomic approach. Strain N7^T grew optimally at 25–30 °C, pH 6.0–8.0, and in the presence of 1 % NaCl (w/v). 16S rRNA gene sequence analysis indicated that strain N7^T was a member of the genus *Sphingobacterium* and was closely related to *Sphingobacterium multivorum* IAM14316^T (97.49 %) and *Sphingobacterium canadense* CR11^T (97.11 %), sharing less than 97 % sequence similarities with other species of the genus *Sphingobacterium*. The predominant respiratory quinone of strain N7^T was MK-7 and major fatty acids were summed features 3 ($C_{16:1}\omega6c$ and/or $C_{16:1}\omega7c$), iso-$C_{15:0}$, $C_{16:0}$ and iso-$C_{17:0}$ 3-OH. The G+C content of the DNA was 40.9±0.5 mol%. The levels of DNA–DNA relatedness between strain N7^T and the most closely related species *S. multivorum* IAM 14316^T and *S. canadense* CR11^T were 21 % and 15 %, respectively. Based on these results, strain N7^T is proposed to represent a separate species within the genus *Sphingobacterium*. The name *Sphingobacterium changzhouense* sp. nov. is suggested and the type strain is N7^T (=CCTCC AB 2012100^T=KACC 16854^T).

Yabuuchi *et al.* (1983) first proposed the genus *Sphingobacterium*, the members of which are Gram-negative rods, positive for catalase and oxidase activities, negative for heparinase and gelatinase activities and for indole production, and contain iso-$C_{15:0}$, iso-$C_{15:0}$ 2-OH, $C_{16:1}\omega7c$ and $C_{17:0}$ 3-OH as the main fatty acids (Takeuchi & Yokota, 1992; Steyn *et al.*, 1998). At the time of writing, the genus *Sphingobacterium* comprises 26 species (http://www.bacterio.net/s/sphingobacterium.html).

Strain N7^T was isolated from the soil of a rice field in Changzhou, Jinagsu, PR China (118° 50′ E 32° 02′ N), using the dilution plating method on LB agar at 30 °C. Routine cultivation of strain N7^T and phenotypic tests were performed on LB agar at 30 °C. The 16S rRNA gene of strain N7^T was amplified with primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTCCTTGTTACGACTT-3′) from its genomic DNA. PCR procedures were similar to those described by Suzuki & Yamasato (1994). The PCR product was obtained using a PCR purification kit (Promega), ligated into the pMD18-T simple vector (TaKaRa Biotechnology) and then transformed into *Escherichia coli* DH5α. An automatic sequencer (model 3730; Applied Biosystems) was used to determine the 16S rRNA gene sequence. The resulting sequence was compared with 16S rRNA gene sequences of related type strains, using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim *et al.*, 2012). Phylogenetic analysis was performed using software package MEGA version 5.0 (Tamura *et al.*, 2011) after multiple alignment of the sequence data with CLUSTAL_X (Thompson *et al.*, 1997). Evolutionary distances were calculated according to Kimura's two-parameter model (Kimura, 1980) and clustering was performed with the neighbour-joining method (Saitou & Nei, 1987). Bootstrap analysis was used to determine the confidence values for the branches of phylogenetic trees based on 1000 resamplings (Felsenstein, 1985).

A nearly full-length 16S rRNA gene sequence (1489 bp) of strain N7^T was determined. The phylogenetic tree (Fig. 1) based on the neighbour-joining algorithm showed that strain N7^T belonged to the genus *Sphingobacterium* and shared highest similarity with *Sphingobacterium multivorum* IAM 14316^T (97.49 %) and *Sphingobacterium canadense* CR11^T (97.11 %), and less than 97 % similarity with other species of the genus *Sphingobacterium*.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain N7^T is KC843944

A supplementary figure is available with the online version of this paper.

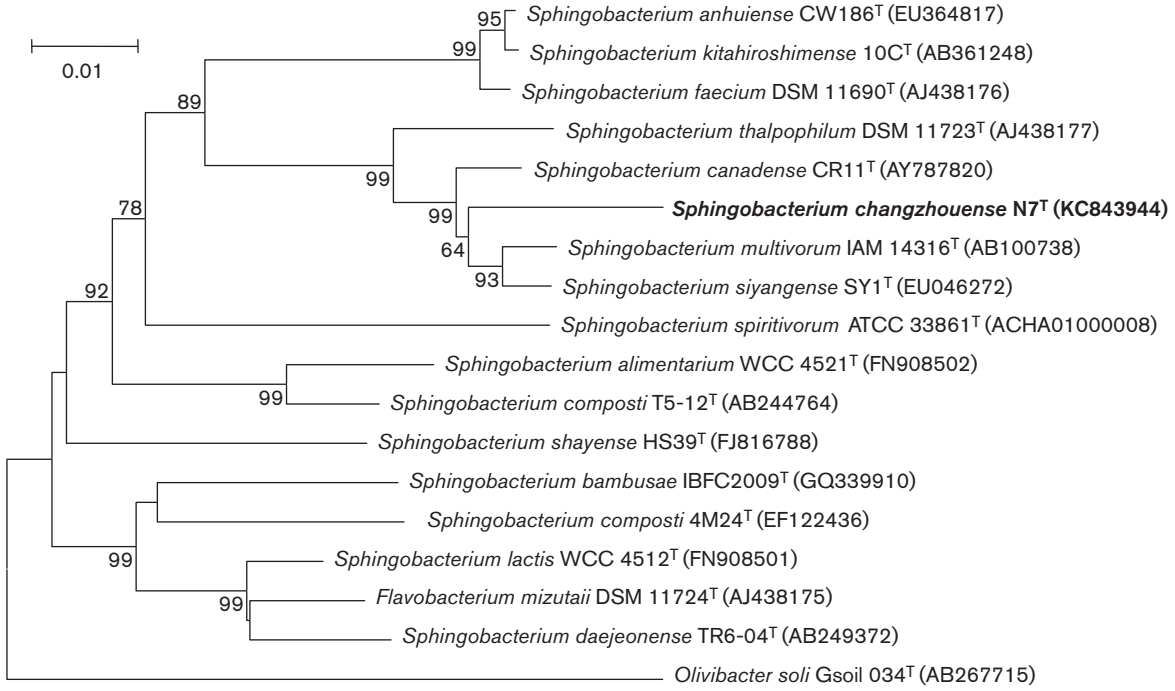

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships between strain N7^T and related species. Bootstrap values (expressed as percentages of 1000 replications) >70 % are shown at branching points. GenBank accession numbers are shown in parentheses. Bar, 0.01 substitutions per nucleotide position. *Olivibacter soli* Gsoil 034^T was used as an outgroup.

Strain N7^T was cultivated aerobically on LB for 2 days at 30 °C. Cell morphology and dimensions were observed by transmission electron microscopy (H-7650; Hitachi) and cell motility was tested on LB swarming agar plates (0.3 % agar, w/v). Gram stain was determined according to the classical Gram procedure (Buck, 1982). Catalase and oxidase activities were investigated as described by Ohta & Hattori (1983). Growth at 5, 10, 20, 25, 30, 37, 40 and 41 °C, at various pH values (5.0–10.0 at intervals of 1 pH unit) and NaCl concentration ranges (0–5 %, w/v) was evaluated on LB medium. Hydrolysis of starch, Tween 20 and DNA was performed according to the method of Cowan & Steel (1965). Sensitivity to antibiotics was determined with the routine disc-diffusion (8 mm diameter; Tianhe, Hangzhou) plate method. The following antibiotics were tested: lincomycin (2 μg), ampicillin (10 μg), neomycin (30 μg), streptomycin (10 μg), tetracycline (30 μg), polymyxin B (300 IU), carbenicillin (100 μg), gentamicin (10 μg), kanamycin (30 μg), chloramphenicol (5 μg), rifampicin (5 μg), vancomycin (30 μg), erythromycin (15 μg), novobiocin (30 μg), cefuroxim (75 μg), piperacillin (100 μg), amoxicillin (10 μg), cefotaxime (30 μg), and roxithromycin (15 μg). Additional physiological characteristics were investigated with the API 20NE, API 20E and API 32GN (bioMérieux) systems according to the manufacturer's instructions.

Cells of strain N7^T were Gram-stain-negative, short rods, non-motile, non-spore-forming and formed circular, convex, yellow colonies after 2 days of incubation. Cells were about 0.6–0.8 μm × 1.3–2.2 μm and had pili (Fig. S1, available in IJSEM Online). Other phenotypic properties of strain N7^T are given in the species description and Table 1.

The G+C content of the genomic DNA was determined by thermal denaturation (Mandel & Marmur, 1968) with *E. coli* K-12 used as the standard. The G+C content of strain N7^T was 40.9±0.5 mol%, which was in accordance with the values of 36.6–44.2 mol% observed for other members of the genus *Sphingobacterium* (Yoo *et al.*, 2007; Choi *et al.*, 2012). Menaquinones of strain N7^T were extracted from dried cells using the method of Collins *et al.* (1977) and separated by HPLC (Tamaoka *et al.*, 1983). For whole-cell fatty acids, strain N7^T and the two reference strains were cultivated on TSB at 30 °C for 48 h. Cells were collected and analysis of fatty acid methyl esters was carried out according to the manufacturer's instructions (Sherlock Microbial Identification System; MIDI) (Sasser, 1990).

The fatty acid patterns of strain N7^T and two other closely related species of the genus *Sphingobacterium* are given in Table 2. Strain N7^T contained iso-$C_{15:0}$ (25.5 %), $C_{16:0}$ (7.9 %), iso-$C_{17:0}$ 3-OH (7.0 %) and summed feature 3 (comprising $C_{16:1}\omega6c$ and/ or $C_{16:1}\omega7c$; 42.1 %) as the major fatty acids (>5 %). This fatty acid profile of strain N7^T was very similar to the two reference strains grown under the same conditions except for small qualitative and

Table 1. Differential characteristics of strain N7^T and related species of the genus *Sphingobacterium*

Strains: 1, N7^T; 2, *S. canadense* CR11^T; 3, *S. multivorum* IAM 14316^T. +, Positive; −, negative; W, weakly positive.

Characteristic	1	2	3
Nitrate reduction	+	+	−
Arginine dihydrolase	−	−	+
Casein hydrolysis	−	+	−
Gelatinase	−	+	−
Assimilation of:			
Citrate	−	−	+
L-Rhamnose	−	−	+
L-Arabinose	W	+	+
Glycogen	−	+	+
D-Mannose	+	W	W
N-Acetylglucosamine	+	−	−
Maltose	+	−	−
Acid production from:			
L-Rhamnose	−	W	W
Melibiose	W	+	+
Amygdalin	−	W	W

Table 2. Cellular fatty acid compositions (%) of strain N7^T and closely related members of the genus *Sphingobacterium*.

Strains: 1, N7^T; 2, *S. canadense* CR11^T; 3, *S. multivorum* IAM 14316^T. Fatty acids that represented <0.5 % for all strains are omitted; ND, Not detected.

Fatty acid	1	2	3
$C_{14:0}$	2.8	2.5	5.6
$C_{16:0}$	7.9	8.2	9.7
$C_{18:0}$	0.6	1.2	0.3
$C_{13:1}$ at 12-13	0.2	0.1	2.0
anteiso-$C_{15:0}$	0.6	1.2	3.6
iso-$C_{15:0}$	25.5	21.0	16.1
iso-$C_{15:0}$ 3-OH	2.4	2.6	1.0
$C_{16:0}$ 2-OH	0.8	0.1	0.1
$C_{16:0}$ 3-OH	2.1	3.0	1.9
iso-$C_{16:0}$ 3-OH	0.8	ND	0.8
$C_{16:1}\omega5c$	0.5	0.5	0.9
iso-$C_{17:0}$ 3-OH	7.0	7.0	1.6
anteiso-$C_{17:1}\omega9c$	0.2	0.2	0.6
$C_{18:1}\omega9c$	0.9	1.2	1.0
Summed feature 1	0.5	1.0	1.2
Summed feature 3	42.1	43.0	46.1
Summed feature 4	0.2	0.3	0.7
Summed feature 8	1.3	1.7	0.7
Summed feature 9	0.8	1.0	1.6

Summed feature 1 contains $C_{13:0}$ 3-OH/iso-$C_{15:1}$ H; summed feature 3 contains $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$; summed feature 4 contains anteiso-$C_{17:1}$ B and/or iso-$C_{17:1}$ I; summed feature 8 contains $C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$; summed feature 9 contains iso-$C_{17:1}\omega9c$ and/or 10-methyl $C_{16:0}$.

quantitative differences in some fatty acids (Table 2).The major respiratory quinone of strain N7^T was MK-7.

DNA–DNA hybridization was carried out to prove further the taxonomic status of strain N7^T with respect to its closest phylogenetic neighbours, *S. multivorum* IAM 14316^T and *S. canadense* CR11^T. Total genomic DNA of the three strains was extracted and purified, and hybridization was performed according to the method described by Ezaki *et al.* (1989). The values for DNA–DNA relatedness between strain *Sphingobacterium changzhouense* N7^T and the reference strains *S. multivorum* IAM 14316^T and *S. canadense* CR11^T were 21 % and 15 %, respectively, which were significantly lower than the value of 70 % that is commonly accepted to define a novel bacterial species (Wayne *et al.*, 1987).

Therefore, on the basis of the phenotypic, phylogenetic and chemotaxonomic results, we deduced that strain N7^T is a novel species belonging to the genus *Sphingobacterium*, and the name *Sphingobacterium changzhouense* sp. nov. is proposed.

Description of *Sphingobacterium changzhouense* sp. nov.

Sphingobacterium changzhouense (chang.zhou.en′se. N.L. neut. adj. *changzhouense* pertaining to Changzhou in Jiangsu Province, China, the city where the strain was isolated).

Cells are Gram-stain-negative, non-motile, non-spore-forming, short rods, 0.6–0.8 μm × 1.3–2.2 μm in size and have pili (Fig. S1). Colonies on LB are yellow, circular, and convex, grow at 10–40 °C (optimum, 25–30 °C), pH 5.0–10.0 (optimum, pH 6.0–8.0) and with 0–5 % NaCl (w/v) (optimum, 1 %). Cells are positive for catalase and oxidase activities and can hydrolyse starch, DNA and Tween 20. Using API 20E and 20NE kits, strain N7^T is positive for β-galactosidase, β-glucosidase, the Voges–Proskauer reaction, nitrate reduction, fermentation of glucose and urease activity, but negative for arginine dihydrolase, gelatinase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase, and H_2S and indole production. Acid production occurs on D-glucose and L-arabinose. D-Glucose, *N*-acetylglucosamine, salicoside, sucrose, D-mannose, melibiose and maltose are used as growth substrates, while D-mannitol, valeric acid, capric acid, malate, sodium acetate, L-serine, potassium 5-ketogluconate, glycogen, citrate, adipic acid, L-fucose, D-sorbitol, propionic acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, 3-hydroxybutyric acid, gluconate, L-histidine, amygdalin, potassium 2-ketogluconate, phenylacetic acid, L-proline, lactic acid, itaconic acid, suberic acid, sodium malonate, L-rhamnose, D-ribose, inositol and L-alanine are not used as sole carbon sources. The predominant respiratory quinone is MK-7. The major fatty acids are iso-$C_{15:0}$, $C_{16:0}$, iso-$C_{17:0}$ 3-OH, and summed feature 3 (comprising $C_{16:1}\omega6c$ and/or $C_{16:1}\omega7c$).

The type strain N7^T (=CCTCC AB 2012100^T=KACC 16854^T) was isolated from the soil of a rice field in Changzhou, Jinagsu, PR China. The DNA G+C content of

the type strain is 40.9±0.5 mol%. The type strain N7^T is resistant to ampicillin (10 μg), kanamycin (30 μg), streptomycin (10 μg), gentamicin (10 μg), amoxicillin (10 μg), carbenicillin (100 μg), erythromycin (15 μg), neomycin (30 μg) and polymyxin B (300 IU), but sensitive to lincomycin (2 μg), neomycin (30 μg), tetracycline (30 μg), chloramphenicol (5 μg), rifampicin (5 μg), vancomycin (30 μg), novobiocin (30 μg), cefuroxim (75 μg), piperacillin (100 μg), cefotaxime (30 μg), neomycin (30 μg) and roxithromycin (15 μg).

Acknowledgements

This work was supported by the National High Technology Research and Development Program of China (2012AA101403), the Project for Science and Technology of Jiangsu Province (BE201270608) and the Chinese National Natural Science Foundation (31070099, J1210056).

References

Buck, J. D. (1982). Nonstaining (KOH) method for determination of gram reactions of marine bacteria. *Appl Environ Microbiol* **44**, 992–993.

Choi, H.-A. & Lee, S.-S. (2012). *Sphingobacterium kyonggiense sp. nov.*, isolated from chloroethene-contaminated soil, and emended descriptions of *Sphingobacterium daejeonense* and *Sphingobacterium mizutaii*. *Int J Syst Evol Microbiol* **62**, 2559–2564.

Collins, M. D., Pirouz, T., Goodfellow, M. & Minnikin, D. E. (1977). Distribution of menaquinones in actinomycetes and corynebacteria. *J Gen Microbiol* **100**, 221–230.

Cowan, S. & Steel, K. (1965). *Manual for the Identification of Medical Bacteria*. London: Cambridge University Press.

Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in micro-dilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.

Kim, O.-S., Cho, Y.-J., Lee, K., Yoon, S.-H., Kim, M., Na, H., Park, S.-C., Jeon, Y. S., Lee, J.-H. & other authors (2012). Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. *Int J Syst Evol Microbiol* **62**, 716–721.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.

Mandel, M. & Marmur, J. (1968). Use of ultraviolet absorbance-temperature profile for determining the guanine plus cytosine content of DNA. *Methods Enzymol* **12B**, 195–206.

Ohta, H. & Hattori, T. (1983). *Agromonas oligotrophica* gen. nov., sp. nov., a nitrogen-fixing oligotrophic bacterium. *Antonie van Leeuwenhoek* **49**, 429–446.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Sasser, M. (1990). Identification of bacteria by gas chromatography of cellular fatty acids. *USFCC News Lett* **20**, 16.

Steyn, P. L., Segers, P., Vancanneyt, M., Sandra, P., Kersters, K. & Joubert, J. J. (1998). Classification of *heparinolytic* bacteria into a new genus, *Pedobacter*, comprising four species: *Pedobacter heparinus* comb. nov., *Pedobacter piscium* comb. nov., *Pedobacter africanus* sp. nov. and *Pedobacter saltans* sp. nov. proposal of the family *Sphingobacteriaceae* fam. nov. *Int J Syst Bacteriol* **48**, 165–177.

Suzuki, T. & Yamasato, K. (1994). Phylogeny of spore-forming lactic acid bacteria based on 16S rRNA gene sequences. *FEMS Microbiol Lett* **115**, 13–17.

Takeuchi, M. & Yokota, A. (1992). Proposals of *Sphingobacterium faecium* sp. nov., *Sphingobacterium piscium* sp. nov., *Sphingobacterium heparinum* comb. nov., *Sphingobacterium thalpophilum* comb. nov. and two genospecies of the genus *Sphingobacterium*, and synonymy of *Flavobacterium yabuuchiae* and *Sphingobacterium spiritivorum*. *J Gen Appl Microbiol* **38**, 465–482.

Tamaoka, J., Katayama-Fujimura, Y. & Kuraishi, H. (1983). Analysis of bacterial menaquinone mixtures by high performance liquid chromatography. *J Appl Bacteriol* **54**, 31–36.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* **28**, 2731–2739.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.

Wayne, L., Brenner, D., Colwell, R., Grimont, P., Kandler, O., Krichevsky, M., Moore, L., Moore, W., Murray, R. & other authors (1987). Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.

Yabuuchi, E., Kaneko, T., Yano, I., Moss, C. W. & Miyoshi, N. (1983). *Sphingobacterium* gen. nov., *Sphingobacterium spiritivorum* comb. nov., *Sphingobacterium multivorum* comb. nov., *Sphingobacterium mizutae* sp. nov., and *Flavobacterium indologenes* sp. nov.: Glucose-Nonfermenting Gram-Negative Rods in CDC Groups IIK-2 and IIb. *Int J Syst Bacteriol* **33**, 580–598.

Yoo, S.-H., Weon, H.-Y., Jang, H.-B., Kim, B.-Y., Kwon, S.-W., Go, S.-J. & Stackebrandt, E. (2007). *Sphingobacterium composti* sp. nov., isolated from cotton-waste composts. *Int J Syst Evol Microbiol* **57**, 1590–1593.