

Tumebacillus lipolyticus sp. nov., isolated from river water

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An aerobic, endospore-forming, alkali-tolerant, Gram-stain-positive, non-motile, rod-shaped bacterium, designated strain NIO-S10^T, was isolated from a surface water sample collected from the Godavari River, Kapileswarapuram, India. Colonies on nutrient agar were circular, 3–4 mm in diameter, creamish and raised after incubation for 36 h at 37 °C. Growth occurred at 20–40 °C, at pH 6–9 and in the presence of 0–2 % (w/v) NaCl. Strain NIO-S10^T was positive for oxidase, caseinase, DNase, gelatinase, lipase and urease activities, and negative for catalase, aesculinase, amylase and cellulase activities. The fatty acids were dominated by branched and saturated fatty acids, with a high abundance of iso-C_{15:0}, anteiso-C_{15:0} and C_{18:0}. The cell-wall peptidoglycan contained *meso*-diaminopimelic acid as the diagnostic diamino acid, and MK-7 was the major menaquinone. The DNA G + C content of strain NIO-S10^T was 54.4 mol%. A BLAST sequence similarity search based on 16S rRNA gene sequences indicated that *Tumebacillus ginsengisoli* Gsoil 1105^T was the nearest phylogenetic neighbour to strain NIO-S10^T, with a pairwise sequence similarity of 94.9 %. Phylogenetic analysis showed that strain NIO-S10^T was clustered with members of the genus *Tumebacillus* and together with members of the genus *Effusibacillus*. Based on phenotypic characteristics and phylogenetic inference, strain NIO-S10^T represents a novel species of the genus *Tumebacillus*, for which the name *Tumebacillus lipolyticus* sp. nov. is proposed. The type strain is NIO-S10^T (=KCTC 32289^T=MTCC 12483^T).

The genus *Tumebacillus* is a member of the family *Alicyclobacillaceae*, order *Bacillales*, class *Bacilli*, phylum *Firmicutes*, and was established by Steven *et al.* (2008). Members of the family *Alicyclobacillaceae* are Gram-stain-positive, rod-shaped, motile or non-motile, spore-forming, aerobic bacteria, with a respiratory, chemo-organotrophic mode of metabolism. The family members are also positive for catalase and protease activities; contain *meso*-diaminopimelic acid as the diagnostic cell-wall diamino acid, MK-7 as the predominant menaquinone and iso-C_{15:0} and anteiso-C_{15:0} as major fatty acids; and have a DNA G + C content of 53.1–55.6 mol% (*T_m*) (Wang *et al.*, 2013). Members of the genus *Tumebacillus* have been isolated from Canadian high Arctic permafrost, soil of a ginseng field and cassava wastewater (Steven *et al.*, 2008; Baek *et al.*, 2011; Wang *et al.*, 2013). At the time of writing the genus *Tumebacillus* comprises three species with validly published names

(<http://www.bacterio.net/b/tumebacillus.html>). In the present taxonomic study, we focused on the characterization and classification of strain NIO-S10^T by a polyphasic approach (Vandamme *et al.*, 1996), and based on the results, assign the novel strain to the genus *Tumebacillus*.

Strain NIO-S10^T was isolated from a surface water sample collected from the Godavari River near Kapileswarapuram, India. The sample that yielded strain NIO-S10^T had a pH of 7.6. For isolation of bacteria, 1 ml water sample was serially diluted in 1 % saline water and 100 µl each dilution was spread on nutrient agar (NA; HIMEDIA) and incubated at room temperature for 5 days. From the different colony morphotypes that appeared, one cream colony was selected and characterized. Sub-cultivation of the isolate was carried out on NA at 37 °C. A stock culture of the isolate in nutrient broth (NB) with 20 % glycerol was preserved at –80 °C.

Tumebacillus ginsengisoli KCTC 13942^T was obtained from the Korean Collection for Type Cultures (KCTC); strain NIO-S10^T was characterized simultaneously with *T. ginsengisoli* KCTC 13942^T.

Colony morphology was examined following growth of the strain on NA at 37 °C for 2 days. Cell morphology

All authors contributed equally to this manuscript.

Abbreviations: FAMES, fatty acid methyl esters.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain NIO-S10^T is KC462738.

was investigated by light microscopy (Nikon) at $\times 1000$ magnification. The Gram reaction was determined by using the HIMEDIA Gram Staining kit according to manufacturer's protocol. Endospore formation was determined by malachite-green staining of the isolate grown on MA for 1 week. Motility was assessed on Motility-Indole-Lysine HiVeg medium (HIMEDIA) with agar (2 g l^{-1}), and also assessed under light microscopy using the hanging-drop method.

Growth was tested on ZoBell marine agar (MA; HIMEDIA) and trypticase soy agar (TSA; HIMEDIA). Growth at 4, 10, 20, 30, 37, 40 and 45 °C was ascertained using trypticase Soy broth (TSB; HIMEDIA) and salt tolerance [0, 0.5, 1, 2, 3 and 4 % (w/v) NaCl] was ascertained using nutrient

broth containing peptone (5 g l^{-1}) and beef extract (3 g l^{-1}). Growth of strain NIO-S10^T at pH 4, 5, 6, 6.5, 7, 7.5, 8, 8.5, 9 and 10 was assessed on TSB buffered either with acetate or citrate buffer (for pH 4–6), 100 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer (pH 7–8), or 100 mM $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$ buffer (pH 9–10). Different biochemical tests, the results of which are listed in the species description and Table 1, were carried out using cultures grown at 37 °C on NA medium as described by Lányi (1987) (catalase and oxidase activities, nitrate reduction, indole production and aesculin hydrolysis) and Smibert & Krieg (1994) (H_2S production, gelatin and urea hydrolysis). Extracellular enzymic activities like amylase, lipase and protease, were studied as described by Srinivas

Table 1. Features that distinguish strain NIO-S10^T from type strains of species of the genus *Tumebacillus*

Strains: 1, NIO-S10^T (data from this study); 2, *Tumebacillus ginsengisoli* KCTC 13942^T (this study); 3, *Tumebacillus permanentifrigoris* Eur1 9.5^T (Steven *et al.*, 2008); 4, *Tumebacillus flagellatus* GST4^T (Wang *et al.*, 2013). All strains were positive for hydrolysis of casein and assimilation of D-glucose, but negative for catalase activity. All strains were sensitive to ampicillin, tetracycline, kanamycin and streptomycin. +, Positive; w, weakly positive; –, negative; R, resistant; S, sensitive.

Characteristic	1	2	3	4
Cell size (μm)	3.5–4.0 \times 0.5–0.6	3.4–3.6 \times 0.5	3–3.5 \times 0.5	3.1–4.2 \times 0.5
Colony colour	Cream	White	Yellowish	Light yellow
Motility	–	–	–	+
Flagella	–	–	–	+
Peritrichous flagella	–	–	–	+
Salinity growth range (%)	0–2	0–1.0	0–0.5	0–0.5
Temperature growth range (°C)	20–40	20–45	10–37	20–45
pH growth range	6.0–9.0	5.0–9.0	5.5–9.0	4.5–8.5
Oxidase activity	+	+	–	+
Hydrolysis of:				
Gelatin	+	–	–	+
Starch	–	+	+	+
Utilization of:				
Lactose	–	+	+	+
D-Xylose	–	+	–	+
Maltose	–	–	+	+
D-Fructose	–	–	+	+
D-Galactose	w	+	+	+
Trehalose	–	+	+	+
D-Sorbitol	–	w	–	+
Melibiose	–	+	w	–
Sucrose	–	+	w	–
L-Arabinose	–	+	w	–
D-Mannose	–	+	w	–
D-Mannitol	–	+	+	+
Cellobiose	–	+	+	+
Citrate	+	–	w	+
Antibiotic susceptibility				
Chloramphenicol	R	S	S	S
Erythromycin	R	R	S	S
Gentamicin	S	R	S	R
Penicillin G	S	S	R	S
DNA G + C content (mol%)	54.4	55.6*	53.1	53.7

*Data from Baek *et al.* (2011).

et al. (2009). Biochemical and enzymic characterizations, carbon substrate utilization and acid production of the strains were performed using previously described methods (Anil Kumar *et al.*, 2012). Antibiotic sensitivity and anaerobic growth were tested as described by Baek *et al.* (2011). Biochemical and enzyme characterization of strain NIO-S10^T was also performed using the Vitek 2 GP system (bioMérieux) according to the manufacturer's protocol.

Standardization of the physiological age of strains NIO-S10^T and *T. ginsengisoli* KCTC 13942^T was done based on the protocol (Sasser, 1990) of the Sherlock Microbial Identification System (MIDI). For cellular fatty acids analysis, strains NIO-S10^T and *T. ginsengisoli* KCTC 13942^T were grown on TSA at 37 °C for 2 days and were of the same physiological age (at exponential phase of growth). Cellular fatty acid methyl esters (FAMES) were obtained from cells by saponification, methylation and extraction following the protocol of MIDI. Cellular FAMES were separated by GC (6890; Agilent) and analysed using the Sherlock Microbial Identification System software (MIDI; database TSBA6) according to the protocol described by the Sherlock Microbial Identification System. For the quinone analysis, freeze-dried cells were obtained from cultures grown on NA at 37 °C for 2 days under aerobic conditions. Quinones were extracted as described by Collins *et al.* (1977) and analysed by HPLC (Groth *et al.*, 1997). Peptidoglycan was prepared and analysed according to the method described by Komagata & Suzuki (1987).

The DNA of strain NIO-S10^T was isolated according to the procedure of Marmur (1961) and the G+C content was determined from melting point (*T_m*) curves (Sly *et al.*, 1986) obtained by using a Lambda 2 UV-Vis spectrophotometer (Perkin Elmer) equipped with the Templab 2.0 software package (Perkin Elmer). *Escherichia coli* DH5- α DNA was used as a standard in determining the DNA G+C content.

For 16S rRNA gene sequencing, DNA was prepared using a microbial DNA isolation kit (Mo Bio Laboratories) and sequenced as described previously (Lane, 1991). The resultant sequence of the 16S rRNA gene was subjected to BLAST sequence similarity searches (Altschul *et al.*, 1990), and the EzTaxon-e server (Kim *et al.*, 2012) was used to identify the nearest taxa. The 16S rRNA gene sequences of members of the family *Alicyclobacillaceae* were downloaded from the NCBI database (<http://www.ncbi.nlm.nih.gov>) and aligned using the CLUSTAL W program in the MEGA5 software package. A phylogenetic tree was created using the neighbour-joining method (Saitou and Nei, 1987; Tamura *et al.*, 2004). All positions containing gaps and missing data were eliminated from the dataset (complete deletion option); there were 1331 positions in the final dataset. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10 000 replicates) are shown next to the branches (Felsenstein, 1985). Phylogenetic trees were also reconstructed using the maximum-likelihood and maximum-parsimony methods. All phylogenetic analyses were conducted in MEGA5 software (Tamura *et al.*, 2011).

Cells of strain NIO-S10^T were Gram-stain-positive, non-motile rods, 0.5–0.6 μ m wide and 3.5–4.0 μ m long. Colonies were circular, 3–4 mm in diameter, creamish and raised after incubation for 36 h on NA at 37 °C. Forms terminal endospores. Growth was observed at 20–40 °C (optimum 37 °C), in the presence of 0–2 % (w/v) NaCl (optimum 0–1 %) and at pH 6–9 (optimum pH 7). Other characteristics are presented in the species description and in Table 1.

The cellular fatty acid composition of strain NIO-S10^T showed a spectrum of 14 fatty acids with a pronounced dominance (>5 %) of iso-C_{15:0}, anteiso-C_{15:0} and C_{18:0} (Table 2). The fatty acids were dominated by saturated and branched fatty acids. The presence of high concentrations of branched and saturated fatty acids is common among members of the genus *Tumebacillus* (Wang *et al.*, 2013). MK-7 was the major respiratory quinone present in strain NIO-S10^T and *T. ginsengisoli* KCTC 13942^T. Strain NIO-S10^T contained *meso*-diaminopimelic acid as the diagnostic diamino acid, similar to the other members of the genus *Tumebacillus* (Wang *et al.*, 2013). The DNA G+C content of strain NIO-S10^T was 54.4 mol%. The DNA G+C content of strain NIO-S10^T is in agreement with other species of the genus *Tumebacillus*, which range from 53 to 56 mol% (Wang *et al.*, 2013).

Table 2. Comparison of the fatty acid composition of strain NIO-S10^T with the type strains of species of the genus *Tumebacillus*

Strains: 1, NIO-S10^T (data from this study); 2, *Tumebacillus ginsengisoli* KCTC 13942^T (this study); 3, *Tumebacillus permanentifrigoris* Eur1 9.5^T (Steven *et al.*, 2008); 4, *Tumebacillus flagellatus* GST4^T (Wang *et al.*, 2013). Results are presented as a percentage of the total fatty acids. Fatty acids amounting to \geq 5 % of the total fatty acids are in bold. Values of <1 % for all strains are not shown. ND, Not detected; TR, traces (<1 %).

Fatty acid	1	2	3	4
C _{12:0}	1.3	ND	ND	ND
iso-C _{13:0}	TR	TR	1.1	TR
C _{14:0}	1.4	ND	ND	ND
iso-C _{14:0}	1.1	8.5	2.8	TR
iso-C _{15:0}	49.2	49.5	56.2	74.6
anteiso-C _{15:0}	21.4	31.6	13.7	12.9
C _{16:0}	3.8	4.5	2.4	1.6
iso-C _{16:0}	1.1	3.4	TR	1.8
C _{16:1ω11c}	1.9	ND	ND	ND
iso-C _{17:0}	1.4	TR	ND	2.0
iso-C _{17:0ω10c}	3.2	ND	ND	ND
anteiso-C _{17:0}	TR	TR	ND	1.3
C _{18:0}	7.2	ND	ND	ND
Summed feature 4*	4.6	ND	ND	ND

*Summed features are groups of two or three fatty acids that could not be separated by GC with the MIDI system. Summed feature 4 comprised anteiso-C_{17:1} B and/or iso-C_{17:1} I.

A 16S rRNA gene sequence of 1475 nt was determined for strain NIO-S10^T. The phylogenetic relationship of strain NIO-S10^T was ascertained based on the 16S rRNA gene sequence similarity with other strains using BLAST sequence similarity search. The 16S rRNA gene sequence analysis placed strain NIO-S10^T within the family *Alicyclobacillaceae*. The results indicated that at the 16S rRNA gene sequence level, *Tumebacillus ginsengisoli* Gsoil 1105^T was the nearest relative, with a pairwise sequence similarity of 94.9 %. Phylogenetic analysis based on the neighbour-joining algorithm further revealed clear affiliations of the novel isolate with the family *Alicyclobacillaceae* and showed that strain NIO-S10^T clustered with *Tumebacillus ginsengisoli* Gsoil 1105^T that together clustered with other members of the genus *Tumebacillus*, which as a whole clustered with members of the genus *Effusibacillus* (Fig. 1).

As strain NIO-S10^T was sufficiently different from members of the genus *Tumebacillus* at the 16S rRNA gene

sequence similarity level but clearly clustered with the members of the genus *Tumebacillus*, we considered *T. ginsengisoli* KCTC 13942^T, which was phylogenetically close to the novel strain, for comparative characterization. The characteristics that differentiate strain NIO-S10^T from the type species of the genus *Tumebacillus*, *T. ginsengisoli* are given in Tables 1 and 2. Strain NIO-S10^T differed from *T. ginsengisoli* KCTC 13942^T with respect to various phenotypic characteristics such as colony colour, tolerance to salt, temperature growth range, pH growth range, biochemical characteristics, hydrolysis of complex substrates, utilization of various carbon sources, fatty acid composition, antibiotic sensitivity and DNA G+C content (Tables 1 and 2). Thus, the cumulative differences that strain NIO-S10^T exhibits from *Tumebacillus ginsengisoli* unambiguously supports the creation of a novel species to accommodate strain NIO-S10^T, for which the name *Tumebacillus lipolyticus* sp. nov. is proposed.

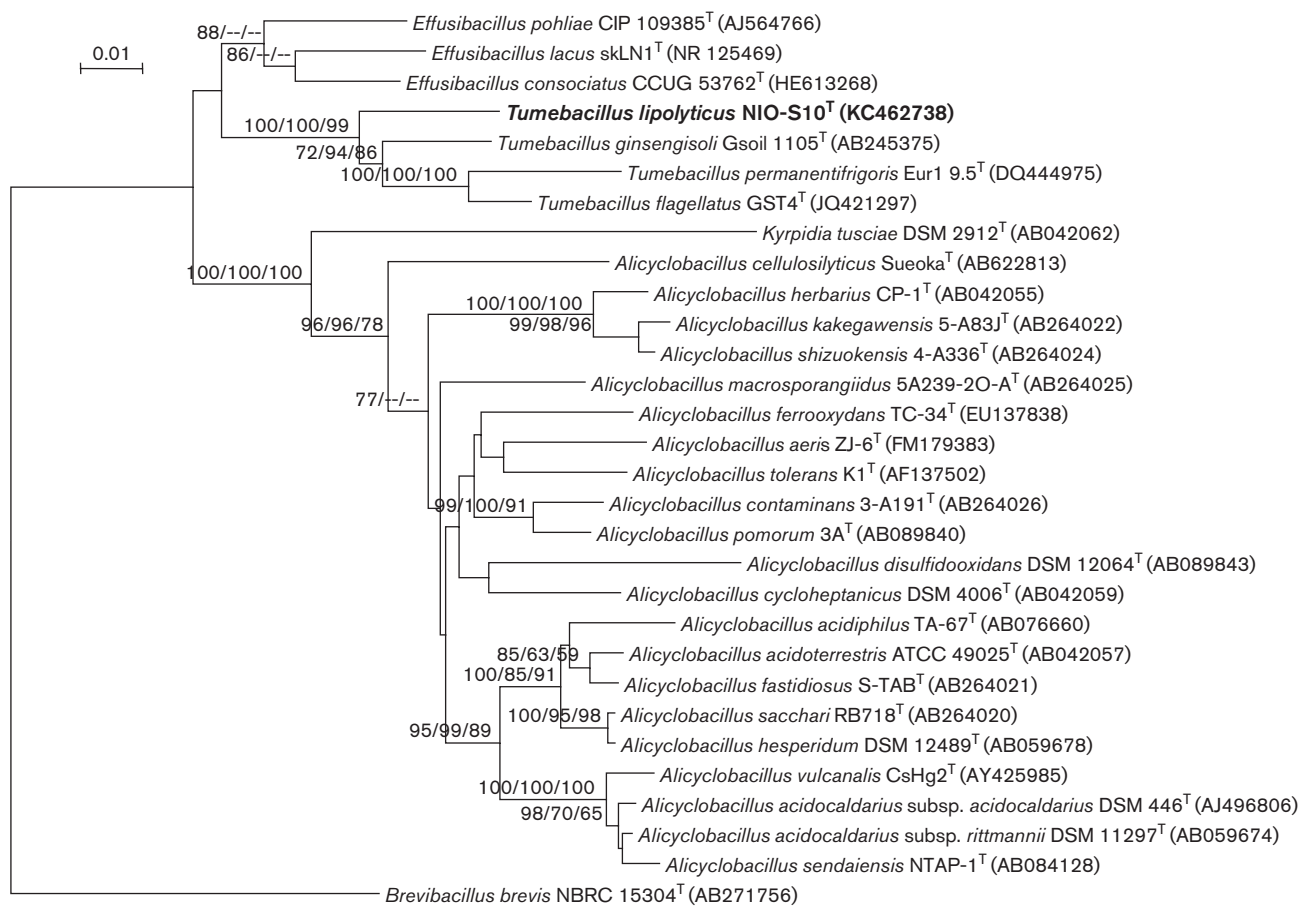


Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the distant relationships between strain NIO-S10^T and representatives of the family *Alicyclobacillaceae*. Numbers at nodes are bootstrap values (NJ/ML/MP; – represents no bootstrap value in ML/MP trees where nodes differ in NJ/ML/MP dendrograms) based on 10 000 replications; only values >70 % are shown. *Brevibacillus brevis* NBRC 15304^T (GenBank accession no. AB271756) was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

Description of *Tumebacillus lipolyticus* sp. nov.

Tumebacillus lipolyticus (li.po.ly'ti.cus. Gr. n. *lipos* fat; Gr. adj. *lytikos* -ē -on able to loosen, able to dissolve; N.L. masc. adj. *lipolyticus* fat-dissolving, referring to the property of being able to hydrolyse lipid).

Cells are rod-shaped, spore-forming and Gram-stain-positive. Growth is aerobic. Cells are 0.5–0.6 μm wide and 3.5–4.0 μm long. Colonies on NA are circular, 3–4 mm in diameter, creamish and raised after incubation at 37 °C for 36 h. Terminal endospores are formed. Grows at 20–40 °C (optimum 37 °C) and tolerates up to 2 % (w/v) NaCl (optimum 0–1 %). NaCl is not a requirement for growth. Grows at pH 6–9, with optimum growth at pH 7. Cells metabolize heterotrophically. Lysine decarboxylase, ornithine decarboxylase, β-galactosidase and phenylalanine deaminase activities are absent. Nitrate is reduced. Indole is produced but not H₂S gas. The methyl red and Voges–Proskauer reactions are negative. Casein, DNA, gelatin, tributyrin, Tweens 20, 40, 60 and 80, and urea are hydrolysed but aesculin, agar, cellulose and starch are not. Positive results in tests for arginine dihydrolase 1, arginine dihydrolase 2 and urease activities, and L-lactate alkalization. Negative results in tests for α-glucosidase, Ala-Phe-Pro arylamidase, leucine arylamidase, L-proline arylamidase, alanine arylamidase, tyrosine arylamidase, phosphatidylinositol phospholipase C, β-galactosidase, L-aspartate arylamidase, β-galactopyranosidase, α-mannosidase, phosphatase, β-glucuronidase, α-galactosidase, L-pyrrolidonyl arylamidase and β-glucuronidase activities; utilization of cyclodextrin, D-amygdalin, D-sorbitol, D-xylose, D-galactose, D-ribose, lactose, N-acetyl-D-glucosamine, maltose, D-mannitol, D-mannose, methyl β-D-glucopyranoside, pullan, raffinose, salicin, sucrose and trehalose; resistance to polymixin B, bacitracin, O/129, novobiocin and optochin; and growth in 6.5 % NaCl. Utilizes glucose and citrate; weak reactions observed for utilization of galactose and raffinose; and does not utilize L-arabinose, arabinol, cellobiose, erythritol, inositol, inulin, mannose, melibiose, salicin, trehalose, xylitol, fructose, glycerol, malonate, maltose, mannitol, methyl α-D-glucoside, rhamnose, sucrose, xylose, D-arabinose, adonitol, dulcitol, lactose, melezitose, methyl α-D-mannoside, sodium gluconate, sorbitol or sorbose. The major cellular fatty acids are iso-C_{15:0}, anteiso-C_{15:0} and C_{18:0}; the detailed cellular fatty acid composition is given in Table 2. MK-7 is the major respiratory quinone and the cell-wall peptidoglycan is of type A1γ.

The type strain, NIO-S10^T (=KCTC 32289^T=MTCC 12483^T) was isolated from a surface water sample collected from the Godavari River, Kapileswarapuram, India. The DNA G+C content of the type strain is 54.4 mol%.

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