Tumebacillus ginsengisoli sp. nov., isolated from soil of a ginseng field

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A Gram-reaction-positive, rod-shaped, spore-forming bacterium, designated Gsoil 1105^T, was isolated from soil of a ginseng field in Pocheon Province in South Korea and characterized in order to determine its taxonomic position. Comparative analysis of the 16S rRNA gene sequence showed that the isolate belongs to the order *Bacillales*, showing the highest level of sequence similarity with respect to *Tumebacillus permanentifrigoris* Eur1 9.5^T (94.6%). The phylogenetic distances from other described species with validly published names within the order *Bacillales* were greater than 9.0%. Strain Gsoil 1105^T had a genomic DNA G+C content of 55.6 mol% and menaquinone 7 (MK-7) as the major respiratory quinone. The major fatty acids were iso-C_{15:0} and anteiso-C_{15:0}. On the basis of its phenotypic properties and phylogenetic distinctiveness, strain Gsoil 1105^T represents a novel species of the genus *Tumebacillus*, for which the name *Tumebacillus ginsengisoli* sp. nov. is proposed. The type strain is Gsoil 1105^T (=KCTC 13942^T =DSM 18389^T).

The genus *Tumebacillus* was proposed by Steven *et al.* (2008) to accommodate a Gram-positive, aerobic, spore-forming, rod-shaped, non-motile bacterium. Only one species of the genus, *Tumebacillus permanentifrigoris*, is currently recognized. The genus *Tumebacillus* forms a phylogenetically distinct line of descent that is close to, but separate from, members of the genus *Alicyclobacillus*. During the course of a study on the culturable aerobic and facultatively anaerobic bacterial community present in a soil sample from a ginseng field in Pocheon Province, South Korea, a large number of novel bacterial strains were isolated (Im *et al.*, 2005). In this study, we have characterized one of these isolates, strain Gsoil 1105^T. Phenotypic, chemotaxonomic and phylogenetic analyses establish the affiliation of the isolate to the genus *Tumebacillus*. The data obtained also suggest that this isolate represents a novel species of the genus *Tumebacillus*.

The ginseng field soil sample was thoroughly suspended in 50 mM phosphate buffer (pH 7.0) and the suspension was spread on one-fifth-strength modified R2A agar plates

[containing (l⁻¹): 0.25 g tryptone, 0.25 g peptone, 0.25 g yeast extract, 0.125 g malt extract, 0.125 g beef extract, 0.25 g Casamino acids, 0.25 g soytone, 0.5 g glucose, 0.3 g soluble starch, 0.2 g xylan, 0.3 g sodium pyruvate, 0.3 g K₂HPO₄, 0.05 g MgSO₄, 0.05 g CaCl₂, 15 g agar] after being serially diluted with 50 mM phosphate buffer (pH 7.0). The plates were incubated for a month at room temperature. Single colonies on the plates were incubated once again under aerobic conditions using modified R2A agar or half-strength modified R2A agar. Purified colonies were tentatively identified from partial sequences of the 16S rRNA gene (Im *et al.*, 2005). Strain Gsoil 1105^T was one of the isolates that appeared on modified R2A agar plates grown under aerobic conditions; this strain was routinely cultured on R2A agar (BD) at 30 °C and maintained as a glycerol suspension (20%, w/v) at −70 °C.

The Gram reaction was assessed by the non-staining method as described by Buck (1982). Cell morphology and motility were examined by phase-contrast microscopy, using cells grown for 2 days at 30 °C on R2A agar. Catalase activity was determined from bubble production in 3% (v/v) H₂O₂. Oxidase activity was determined using 1% (w/v) tetramethyl *p*-phenylenediamine. Anaerobic growth was tested in serum bottles containing R2A broth supplemented with thioglycolate (1 g l⁻¹) and in which the headspace

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Gsoil 1105^T is AB245375.

Two supplementary figures are available with the online version of this paper.

was replaced with nitrogen gas. Tests for the degradation of DNA [DNase agar (Scharlau), tested by flooding plates with 1.0 M HCl], casein, chitin, starch (Atlas, 1993), lipid (Kouker & Jaeger, 1987), xylan and cellulose (Ten *et al.*, 2004) were evaluated after 5 days. Nitrate reduction was determined aerobically in a serum bottle supplemented with 10 mM KNO₃ as an electron donor and was monitored by using an ion chromatograph (Metrohm) equipped with a conductivity detector and anion exchange column. Growth on R2A medium at 4, 10, 15, 20, 30, 37, 42 and 50 °C and at pH 4.0–10.0 (in increments of 0.5 pH units) was assessed after 5 days of incubation. Salt tolerance was tested on R2A medium supplemented with 1–10% (w/v) NaCl after 5 days of incubation. Growth on nutrient agar, trypticase soy agar and MacConkey agar was also evaluated at 30 °C.

For studies on the assimilation of single carbon sources, a defined agar medium was used that contained a basal salt mixture (l⁻¹: 1.8 g K₂HPO₄, 1.08 g KH₂PO₄, 0.5 g NaNO₃, 0.5 g NH₄Cl, 0.1 g KCl, 0.1 g MgSO₄, 0.05 g CaCl₂, 15.0 g agar). A vitamin solution (Widdel & Bak, 1992), a trace element solution (SL-10; Widdel *et al.*, 1983) and selenite/tungstate solution (Tschech & Pfennig, 1984) were added to this medium and the medium was adjusted to pH 6.8 by the addition of 1.0 M HCl. Filter-sterilized carbon sources were added individually to the agar medium [at 0.1% (w/v)] and the plates were incubated at 30 °C for up to 5 days. The negative control agar plate did not contain any carbon source and a positive control culture was grown on R2A agar. Acid production from glucose was examined in BBL phenol red broth base (Difco) prepared with 1% (w/v) glucose added. Duplicate antibiotic sensitivity tests were performed using filter-paper discs containing the following: ampicillin, chloramphenicol, erythromycin, gentamicin, lincomycin, penicillin and streptomycin, each at 5, 10, 30, 50 and 100 µg ml⁻¹. Discs were placed on R2A agar plates spread with strain Gsoil 1105^T and

T. permanentifrigoris DSM 18773^T (reference strain) and the plates were then incubated at 30 °C for 5 days.

Extraction of genomic DNA was performed with a commercial genomic DNA-extraction kit (Core Biosystem) and PCR-mediated amplification of the 16S rRNA gene and sequencing of the purified PCR product were carried out according to Ten *et al.* (2008). The 16S rRNA sequence of strain Gsoil 1105^T was assembled with Seqman II 5.0 (DNASTAR) (Swindell & Plasterer, 1997) and compared with all bacterial 16S rRNA gene sequences available in the GenBank database. Alignment of 23 related sequences was carried out with CLUSTAL_X (Thompson *et al.*, 1997). Gaps at the 5' and 3' ends and ambiguous bases were removed from the alignment using BioEdit (Hall, 1999). Phylogenetic trees based on comparison of 1405 bases were reconstructed by the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) algorithms using MEGA version 3.1 (Kumar *et al.*, 2004). The maximum-likelihood method was also used for phylogenetic analysis, using the program DNAML in the PHYLIP software package (Felsenstein, 1989). Evolutionary distances were calculated using Kimura's two-parameter model (Kimura, 1983) and bootstrap values were based on 1000 replications (Felsenstein, 1985).

To determine the G + C content of the chromosomal DNA, genomic DNA of the novel strain was extracted and purified as described by Moore & Dowhan (1995) and enzymically degraded into nucleosides. The DNA G + C content was determined as described by Mesbah *et al.* (1989) using a reversed-phase HPLC. Isoprenoid quinones were extracted with chloroform/methanol (2:1, v/v), evaporated under a vacuum and re-extracted in n-hexane/water (1:1, v/v). The crude quinone in the n-hexane solution was purified using Sep-Pak (Waters) and subsequently analysed by HPLC as described previously (Hiraishi *et al.*, 1996). Cellular fatty acids of strain Gsoil 1105^T and *T. permanentifrigoris* DSM 18773^T were determined after growth on R2A agar (Difco)

Fig. 1. Neighbour-joining phylogenetic tree, reconstructed from a comparative analysis of 16S rRNA gene sequences, showing the relationships between strain Gsoil 1105^T and related species. Filled circles indicate generic branches that were also recovered by using the maximum-parsimony algorithm. Bootstrap values (expressed as percentages of 1000 replications) greater than 65% are shown at branch points. Bar, 0.02 substitutions per nucleotide position.

for 2 days. Cellular fatty acids methyl esters were prepared and identified following the protocol of the Sherlock Microbial Identification System (MIDI). Determination of peptidoglycan structure were carried out by the methods described by Schleifer & Kandler (1972).

Cells of strain Gsoil 1105^T were found to be Gram-reaction-positive, aerobic, non-motile rods, 0.5–0.8 µm wide and 3.0–6.0 µm long. Phase-contrast microscopy revealed that cells of strain Gsoil 1105^T formed ellipsoidal spores in large terminal sporangia (Supplementary Fig. S1, available in IJSEM Online). The novel strain was able to grow aerobically using nitrate as a terminal electron acceptor. Colonies grown on R2A agar plates (Difco) for 2 days were circular, concentric, serrate, white, raised and 1–2 mm in diameter. Growth was not observed on nutrient

agar, MacConkey agar or trypticase soy agar. Strain Gsoil 1105^T was able to grow on R2A agar at 20–42 °C; no growth was observed at 15 or 50 °C.

The 16S rRNA gene sequence of strain Gsoil 1105^T determined in this study was 1498 bp long. Sequence similarity calculations after a simple alignment analysis indicated that the closest relative of strain Gsoil 1105^T was *T. permanentifrigoris* Eur1 9.5^T (94.6%). Lower levels of sequence similarity (<91.0%) were found to type strains of two other species in the order *Bacillales*, *Alicyclobacillus pohliae* MP4^T (91.0%) and *A. contaminans* 3-A191^T (89.4%). This relationship between strain Gsoil 1105^T and other members of the order *Bacillales* was confirmed in the neighbour-joining phylogenetic tree (Fig. 1; using 1405 bases) and the maximum-likelihood phylogenetic tree (Supplementary Fig. S2; using 1405 bases). The phylogenetic trees indicate that strain Gsoil 1105^T is closely related to the genus *Tumebacillus*. Gsoil 1105^T formed a distinct cluster with *T. permanentifrigoris* Eur1 9.5^T. The same results were obtained when the maximum-parsimony method was applied. Detailed characteristics that differentiate strain Gsoil 1105^T from the type strain of *T. permanentifrigoris* are shown in Table 1.

The DNA G+C content of strain Gsoil 1105^T was 55.6 mol%. Strain Gsoil 1105^T contained menaquinone 7 (MK-7) as the predominant isoprenoid quinone. The fatty

Table 1. Phenotypic and physiological characteristics of strain Gsoil 1105^T and *T. permanentifrigoris* DSM 18773^T

Strains: 1, Gsoil 1105^T; 2, *T. permanentifrigoris* DSM 18773^T. Data for both strains were from this study. Both strains tested positive for hydrolysis of casein and starch, sensitivity to ampicillin, chloramphenicol, penicillin and streptomycin and assimilation of cellobiose, D-galactose, D-glucose, lactose, D-mannitol, trehalose and lactate. Both strains were negative for catalase activity, hydrolysis of xylan, chitin, aesculin, cellulose and DNA, acid production from D-glucose and assimilation of D-arabinose, L-sorbose and L-xylose. +, Positive; w, weakly positive; –, negative; R, resistant; S, sensitive.

Characteristic	1	2
Colony colour	White	Yellow
Cell size (µm)	3–6 × 0.5–0.8	3–3.5 × 0.5
Ranges for growth		
Temperature (°C)	20–42	10–37
pH	5.0–8.5	5.5–9.0
Oxidase	+	–
Response to antibiotics (per disc)		
Erythromycin (10 µg)	R	S
Gentamicin (10 µg)	R	S
Lincomycin (30 µg)	R	S
Penicillin (5 µg)	S	R
Assimilation of:		
Sucrose	+	w
L-Arabinose	+	w
Melibiose	+	w
Alanine	+	w
D-Mannose	+	w
Salicin	+	w
Pyruvic acid	+	–
D-Xylose	+	–
D-Sorbitol	w	–
D-Fructose	–	+
Maltose	–	+
Fumaric acid	–	w
Citrate	–	w
N-Acetylglucosamine	–	w
Gluconate	–	w

Table 2. Cellular fatty acid profiles of strain Gsoil 1105^T and *T. permanentifrigoris* DSM 18773^T

Strains: 1, Gsoil 1105^T; 2, *T. permanentifrigoris* DSM 18773^T. Data for both strains are from this study using biomass grown under identical conditions and analysed with the Microbial Identification System. Values are percentages of total fatty acids; –, not detected. Major components (>10%) are highlighted in bold.

Fatty acid	1	2
C _{14:0}	0.86	0.83
C _{16:0}	2.90	2.37
iso-C _{13:0}	0.49	1.15
iso-C _{14:0}	9.30	2.85
iso-C _{15:0}	43.03	56.21
iso-C _{16:0}	3.45	0.88
iso-C _{17:0}	0.94	–
iso-C _{16:1} G	0.32	–
anteiso-C _{15:0}	35.59	13.66
anteiso-C _{17:0}	0.35	–
Summed features*		
2	1.08	17.70
4	0.27	1.00
5	1.41	3.35

*Summed feature 2 consists of one or more of iso-C_{15:1} I, iso-C_{15:1} H and C_{13:0} 3-OH. Summed feature 4 consists of C_{16:1} ω7c and/or iso-C_{15:0} 2-OH. Summed feature 5 consists of iso-C_{17:1} I and/or anteiso-C_{17:1} B.

acid profile of strain Gsoil 1105^T (Table 2) was composed mainly of iso-C_{15:0} (43.03 %) and anteiso-C_{15:0} (35.59 %). The diagnostic diamino acid of the peptidoglycan was *meso*-diaminopimelic acid, suggesting the presence of peptidoglycan type A1 γ . These chemotaxonomic data support the affiliation of strain Gsoil 1105^T to the order *Bacillales*.

All characteristics determined for strain Gsoil 1105^T were in accordance with those of the genus *Tumebacillus*. However, the phylogenetic distance from the sole recognized *Tumebacillus* species and the combination of unique phenotypic characteristics indicated that strain Gsoil 1105^T is not affiliated to *T. permanentifrigoris*. Therefore, it is concluded that strain Gsoil 1105^T represents a novel species of the genus *Tumebacillus*, for which the name *Tumebacillus ginsengisoli* sp. nov. is proposed.

Description of *Tumebacillus ginsengisoli* sp. nov.

Tumebacillus ginsengisoli (gin.sen.gi.so'li. N.L. n. *ginsengum* ginseng; L. n. *solum* soil; N.L. gen. n. *ginsengisoli* of the soil of a ginseng field, the source of the type strain).

Cells are Gram-reaction-positive, aerobic, non-motile rods, 0.5–0.8 μ m wide and 3.0–6.0 μ m long, after 2 days of culture on R2A agar. Forms white colonies on R2A plates, with no growth in liquid media. Colonies grown on R2A agar for 2 days are circular, concentric, serrate and raised. Terminal endospores are formed in swollen sporangia. Grows well at 20–42 °C and pH 5.0–8.5, but not at 15 or 50 °C. Growth occurs in the absence of NaCl, but not in the presence of 1 % (w/v) NaCl. It can reduce nitrate to nitrite. MK-7 is the predominant menaquinone. The major cellular fatty acids are iso-C_{15:0} and anteiso-C_{15:0}. The peptidoglycan cell wall is of the A1 γ type. In addition to the results given in Table 1, tests positive for utilization of the following substrates: D-fucose, L-rhamnose, acetate, phenylacetate, malate, glycerol, glycogen, dextran, glycine and serine. Utilization of D-ribose, D-raffinose, xylitol, cysteine and glutamic acid is weakly positive. Ethanol, formic acid, propionate, 3-hydroxybutyrate, valerate, caprate, maleic acid, benzoic acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, malonate, succinic acid, glutaric acid, tartaric acid, itaconate, adipate, suberate, oxalic acid, D-adonitol, dulcitol, inositol, amygdalin, inulin, asparagine, aspartic acid, histidine, isoleucine, leucine, phenylalanine, proline, threonine, tyrosine and valine do not serve as carbon sources for growth. The genomic DNA G+C content of the type strain is 55.6 mol% (HPLC).

The type strain, Gsoil 1105^T (=KCTC 13942^T =DSM 18389^T), was isolated from soil of a ginseng field in Pocheon Province, South Korea.

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References

- Atlas, R. M. (1993). *Handbook of Microbiological Media*. Edited by L. C. Parks. Boca Raton, FL: CRC Press.
- Buck, J. D. (1982). Nonstaining (KOH) method for determination of gram reactions of marine bacteria. *Appl Environ Microbiol* **44**, 992–993.
- Felsenstein, J. (1985). Confidence limit on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.
- Felsenstein, J. (1989). PHYLIP – phylogeny inference package (version 3.2). *Cladistics* **5**, 164–166.
- Fitch, W. M. (1971). Toward defining the course of evolution: minimum change for a specific tree topology. *Syst Zool* **20**, 406–416.
- Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* **41**, 95–98.
- Hiraishi, A., Ueda, Y., Ishihara, J. & Mori, T. (1996). Comparative lipoquinone analysis of influent sewage and activated sludge by high-performance liquid chromatography and photodiode array detection. *J Gen Appl Microbiol* **42**, 457–469.
- Im, W.-T., Jung, H.-M., Cui, Y.-S., Liu, Q.-M., Zhang, S.-L. & Lee, S.-T. (2005). Cultivation of the three hundreds of bacterial species from soil of a ginseng field and mining the novel lineage bacteria. In *Proceedings of the International Meeting of the Federation of Korean Microbiological Societies*, abstract A035, p. 169. Seoul: Federation of Korean Microbiological Societies.
- Kimura, M. (1983). *The Neutral Theory of Molecular Evolution*. Cambridge: Cambridge University Press.
- Kouker, G. & Jaeger, K.-E. (1987). Specific and sensitive plate assay for bacterial lipases. *Appl Environ Microbiol* **53**, 211–213.
- Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* **5**, 150–163.
- Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.
- Moore, D. D. & Dowhan, D. (1995). Preparation and analysis of DNA. In *Current Protocols in Molecular Biology*, pp. 2–11. Edited by F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith & K. Struhl. New York: Wiley.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Schleifer, K. H. & Kandler, O. (1972). Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol Rev* **36**, 407–477.
- Steven, B., Chen, M. Q., Greer, C. W., Whyte, L. G. & Niederberger, T. D. (2008). *Tumebacillus permanentifrigoris* gen. nov., sp. nov., an aerobic, spore-forming bacterium isolated from Canadian high Arctic permafrost. *Int J Syst Evol Microbiol* **58**, 1497–1501.
- Swindell, S. R. & Plasterer, T. N. (1997). SEQMAN. Contig assembly. *Methods Mol Biol* **70**, 75–89.
- Ten, L. N., Im, W.-T., Kim, M.-K., Kang, M.-S. & Lee, S.-T. (2004). Development of a plate technique for screening of polysaccharide-degrading microorganisms by using a mixture of insoluble chromogenic substrates. *J Microbiol Methods* **56**, 375–382.
- Ten, L. N., Jung, H.-M., Im, W. T., Yoo, S. A. & Lee, S.-T. (2008). *Lysobacter daecheongensis* sp. nov., isolated from sediment of stream near the Daechung dam in South Korea. *J Microbiol* **46**, 519–524.
- 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.

Tschech, A. & Pfennig, N. (1984). Growth yield increase linked to caffeate reduction in *Acetobacterium woodii*. *Arch Microbiol* **137**, 163–167.

Widdel, F. & Bak, F. (1992). Gram-negative mesophilic sulfatereducing bacteria. In *The Prokaryotes*, 2nd edn, pp. 3352–3378. Edited by

A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K. H. Schleifer. New York: Springer.

Widdel, F., Kohring, G. & Mayer, F. (1983). Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids. III. Characterization of the filamentous gliding *Desulfonema limicola* gen. nov., sp. nov. and *Desulfonema magnum* sp. nov. *Arch Microbiol* **134**, 286–294.