Gulosibacter chungangensis sp. nov., an actinomycete isolated from a marine sediment, and emended description of the genus *Gulosibacter*

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A Gram-positive, strictly aerobic, non-spore-forming, irregular short rod, strain CAU 9625^T, was isolated from a sediment of the Yellow Sea in the Republic of Korea. Strain CAU 9625^T grew optimally at 37 °C, at pH 8.0 and in the presence of 1 % (w/v) NaCl. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain CAU 9625^T belonged to the genus *Gulosibacter*, which has one known member, *Gulosibacter molinativorax*. Strain CAU 9625^T and *G. molinativorax* ON4^T shared 97.8 % 16S rRNA gene sequence similarity and formed a distinct cluster (99 % bootstrap support) within the family *Microbacteriaceae*. DNA–DNA relatedness between strain CAU 9625^T and *G. molinativorax* DSM 13485^T was 35.4 ± 0.9 %. The predominant menaquinone was MK-9. The major whole-cell sugars were ribose and glucose. The major polar lipids were diphosphatidylglycerol, phosphatidylglycerol, an unidentified phospholipid and an unidentified lipid. The fatty acid composition was similar to that of *G. molinativorax* DSM 13485^T, with anteiso-C_{15:0} as the predominant fatty acid. The DNA G + C content of strain CAU 9625^T was 66.2 mol%. The phylogenetic and genetic distinctiveness and several differentiating phenotypic and chemotaxonomic properties revealed that strain CAU 9625^T was distinguishable from *G. molinativorax* and other phylogenetic neighbours. On the basis of these data, strain CAU 9625^T represents a novel species of the genus *Gulosibacter*, for which the name *Gulosibacter chungangensis* sp. nov. is proposed. The type strain is CAU 9625^T (=KCTC 13959^T =CCUG 60841^T).

The family *Microbacteriaceae*, of the suborder *Micrococcineae*, embraces a large group of actinomycetes with cell-wall peptidoglycan of the B-type (Schleifer & Kandler, 1972) and unsaturated major menaquinones (Collins & Jones, 1981). The suborder *Micrococcineae* was established on the basis of a characteristic set of 16S rRNA gene signature nucleotides (Stackebrandt *et al.*, 1997; Stackebrandt & Schumann, 2006; Zhi *et al.*, 2009). At the time of writing, the suborder comprises 16 recognized families and 97 genera (Euzéby, 1997), including the genus *Gulosibacter*, which was created by Manaia *et al.* (2004). Phylogenetically, it constitutes a distinct evolutionary lineage, which is most closely related to the genus *Pseudoclavibacter*. At present, the genus *Gulosibacter* contains a single species with a validly published name, *Gulosibacter molinativorax*, which was described on the basis of a single strain isolated from a

herbicide-contaminated mixture of soil and water. The members of this genus are Gram-positive rods and contain D-ornithine as the diagnostic diamino acid in the peptidoglycan (Manaia *et al.*, 2004).

In the course of screening bacteria from marine samples for their biotechnological potential, a bacterium, designated strain CAU 9625^T, was isolated from a marine sediment near Oh Island (33° 29' 31.15" N 126° 57' 08.01" E) in the Republic of Korea. The procedure for the isolation of strain CAU 9625^T followed that of Gordon & Mihm (1962) using glucose-yeast extract agar (GYE; containing 1⁻¹: 10 g yeast extract, 10 g glucose, 15 g agar; pH 7.0) supplemented with 50 mg cycloheximide 1⁻¹ and 20 mg nalidixic acid 1⁻¹. A sediment sample was diluted with sterilized distilled water and appropriate dilutions were spread on GYE agar and incubated under aerobic conditions at 30 °C for 3 days. The isolate was preserved at -70 °C in GYE broth supplemented with 25 % (v/v) glycerol. The study followed the proposed minimal standards for describing new genera and species of the suborder *Micrococcineae* (Schumann *et al.*, 2009).

To determine the morphological, physiological and biochemical characteristics, strain CAU 9625^T was cultivated

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Abbreviation: DAB, diaminobutyric acid.

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A supplementary figure and table are available with the online version of this paper.

routinely on brain heart infusion agar (BHI; Difco) at 30 °C except for spore formation and fatty acid analysis. The Gram-staining and KOH lysis tests were carried out according to Gram (1884) and Cerny (1978). Growth at 4, 10–45 (at intervals of 5 °C) and 37 °C was determined on BHI agar in an aerobic incubator (model MIR-253; Sanyo) or an anaerobic chamber (model Bactron; Sheldon) for 3 days. Growth at pH 5.0–11.0 (at intervals of 0.5 pH unit) was investigated at 30 °C for 3 days in BHI broth with the pH adjusted with sodium acetate/acetic acid and Na₂CO₃ buffers. Growth with 0–15 % (w/v) NaCl (at intervals of 1 %) was investigated in BHI broth at 30 °C for 3 days. A nutrient sporulation medium was used to test for spore production (Nicholson & Setlow, 1990). After 3 days at 30 °C, the presence of endospores was determined using malachite green staining (Smibert & Krieg, 1994). Catalase activity was determined by bubble production in 3 % (v/v) hydrogen peroxide. Oxidase activity was tested by means of the oxidation of 1 % tetramethyl-*p*-phenylenediamine (Merck) as described by Cowan & Steel (1965). Enzymic activities and acid production from carbohydrates were tested using the API 50 CHB, API ZYM and API Coryne systems (bioMérieux) according to the manufacturer's instructions, as recommended by Schumann *et al.* (2009).

For the determination of fatty acid composition, cell mass was harvested from tryptic soy agar (Difco) after cultivation for 3 days at 30 °C. The physiological age of the harvested biomass was standardized by observing growth development during incubation and choosing the time of harvest according to a standard protocol (Sherlock Microbial Identification System; MIDI). Cellular fatty acid methyl esters were obtained as described by Minnikin *et al.* (1980) and separated by an automated GC system (model 6890N and 7683 autosampler; Agilent). Peaks were identified using the Microbial Identification software (version 6.1, database TSBA6). Menaquinones were analysed as described by Komagata & Suzuki (1987) by reversed-phase HPLC. Polar lipids were extracted and analysed by two-dimensional TLC according to Minnikin *et al.* (1984). Whole-cell sugars were analysed by TLC according to the method of Komagata & Suzuki (1987). Peptidoglycan analysis was performed by the identification service at the DSMZ as described by Schleifer (1985), with the modification that TLC and two-dimensional TLC were substituted for paper chromatography. The molar amino-acid ratio in the peptidoglycan was determined by GC and GC/MS according to MacKenzie (1987).

Genomic DNA of strain CAU 9625^T was isolated and purified by the method of Marmur (1961). PCR amplification and sequencing of the 16S rRNA gene was carried out following established procedures (Cho *et al.*, 2010). The amplified 16S rRNA gene was sequenced directly using a BigDye Terminator Cycle Sequencing kit and an automatic DNA sequencer (model 3730; Applied Biosystems). Multiple alignment of the 16S rRNA gene sequence of strain CAU 9625^T with 16S rRNA gene sequences from a broad selection of closely related sequences in the GenBank

database and calculation of sequence similarity were performed using CLUSTAL X (Thompson *et al.*, 1997) and the EzTaxon server (Chun *et al.*, 2007). A phylogenetic tree was reconstructed using the neighbour-joining (Saitou & Nei, 1987), least-squares (Fitch & Margoliash, 1967) and maximum-likelihood (Felsenstein, 1981) algorithms included in the PHYLIP suite of programs (Felsenstein, 1989). Evolutionary distances for the neighbour-joining analysis were based on the correction of Jukes & Cantor (1969). Tree topologies were evaluated by the bootstrap resampling method with 1000 replicates (Felsenstein, 1985) of the neighbour-joining dataset using the SEQBOOT and CONSENSE programs in the PHYLIP package. DNA–DNA relatedness between the isolate and its nearest phylogenetic neighbour was estimated using the fluorometric microplate method (Ezaki *et al.*, 1989), as modified by Goris *et al.* (1998). The G + C content of the genomic DNA was determined by HPLC with a YMC-Pack ODS-A 4.6 × 250 mm column and the eluent 0.55 M (pH 4.0) NH₄H₂PO₄/acetonitrile (20:0.5, v/v), according to the method of Tamaoka & Komagata (1984).

Colonies of strain CAU 9625^T were strictly aerobic, opaque, white and circular, with a diameter of 0.3–0.5 mm after 1 day of cultivation at 30 °C on BHI agar. Cells were irregular short rods, approximately 0.5–0.7 µm in width and 1.0–1.3 µm in length. Growth was observed at 15–40 °C (optimum 37 °C), at pH 6.0–10.0 (optimum pH 8.0) and with 0–5 % (w/v) NaCl (optimum 1 % NaCl). Cells were catalase- and oxidase-positive. API tests provided adequate phenotypic data to differentiate the isolate from *G. molinativorax* DSM 13485^T (Table 1). The isolate reduced nitrate, utilized pyrazine carboxamide as a sole nitrogen source, produced esterase (C4) and produced acid from fructose, rhamnose, sorbitol and 5-ketogluconate. The isolate did not produce gelatinase, urease, 2-naphthyl-phosphatase, 2-naphthyl-β-D-galactopyranosidase, alkaline phosphatase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-glucuronidase or α-glucosidase. The key phenotypic characteristics of the isolate are given in the species description.

The peptidoglycan of strain CAU 9625^T contained alanine, glycine, 2,4-diaminobutyric acid, ornithine and glutamic acid in the approximate molar ratio of 0.4:1.1:0.4:0.3:1.0. Peptidoglycans of this type have been reported for the genera *Agreia* (Evtushenko *et al.*, 2001) and *Humibacter* (Vaz-Moreira *et al.*, 2008). On the basis of these data, the isolate has peptidoglycan of the B-type with a Gly-D-Glc motif like strain *G. molinativorax* DSM 13485^T (Manaia *et al.*, 2004). However, *G. molinativorax* contains only D-ornithine. The peptidoglycan type of strain CAU 9625^T is different from those of the closely related strains *G. molinativorax* DSM 13485^T, *Zimmermannella bifida* IAM 14848^T, *Zimmermannella faecalis* NBRC 15706^T, *Pseudoclavibacter chungangensis* KCTC 22691^T, *Pseudoclavibacter helvolus* DSM 20419^T and *Pseudoclavibacter soli* KP02^T, which contain D-ornithine, L-diaminobutyric acid (L-DAB), Hsr and L-DAB, DAB, DAB, and DAB,

Table 1. Differential phenotypic properties of strain CAU 9625^T and the type strains of closely related taxa of the family Microbacteriaceae

Strains: 1, *Gulosibacter chungangensis* sp. nov. CAU 9625^T; 2, *G. molinativorax* DSM 13485^T; 3, *Zimmermannella bifida* IAM 14848^T; 4, *Z. faecalis* NBRC 15706^T; 5, *Pseudoclavibacter chungangensis* KCTC 22691^T; 6, *P. helvolus* DSM 20419^T; 7, *P. soli* KP02^T. Data were taken from this study. +, Positive; w, weakly positive; –, negative.

Characteristic	1	2	3	4	5	6	7
Optimum temperature (°C)	37	37	30	30	30	30	30
Growth with 7 % (w/v) NaCl	–	+	–	–	+	+	+
API Coryne							
Nitrate reduction	+	+	–	–	–	–	–
Pyrazine carboxamide	+	+	+	+	+	–	–
2-Naphthyl-phosphate	–	–	+	–	–	–	–
2-Naphthyl-β-D-galactopyranoside	–	–	–	–	–	+	+
Acid production from:							
Glycerol	–	–	–	–	w	–	w
Ribose	–	–	w	–	–	–	–
D-Xylose	–	–	–	–	w	–	–
Glucose	–	–	–	+	w	–	–
Fructose	+	–	–	+	+	w	w
Mannose	–	–	–	+	–	–	–
Rhamnose	+	–	+	+	–	–	–
Inositol	–	–	–	+	–	–	–
Mannitol	–	–	–	+	w	–	–
Sorbitol	+	–	–	–	w	–	–
N-Acetylglucosamine	–	–	–	–	+	–	–
Aesculin	–	w	–	–	w	w	–
Inulin	–	–	–	–	w	–	–
Raffinose	–	–	–	–	w	–	–
5-Ketogluconate	+	w	–	–	+	–	–
Enzyme activity							
Alkaline phosphatase	–	w	+	w	–	–	+
Esterase (C4)	+	+	–	–	+	–	+
Valine arylamidase	–	–	–	w	–	–	+
Cystine arylamidase	–	–	–	w	–	–	–
Acid phosphatase	–	–	w	–	w	+	+
Naphthol-AS-BI-phosphohydrolase	–	–	w	–	w	+	+
β-Glucuronidase	–	–	–	–	–	+	–
α-Glucosidase	–	–	–	–	–	+	–
DNA G + C content (mol%)	66.2	64.5	62.0	67.0	66.2	67.0	66.2

respectively (Table 2). Whole-cell hydrolysates of strain CAU 9625^T contained mainly glucose and ribose, similarly to *G. molinativorax*. The predominant isoprenoid quinone was an unsaturated menaquinone, MK-9. The major polar lipids were diphosphatidylglycerol, phosphatidylglycerol, an unidentified phospholipid and an unidentified lipid (Fig. S1, available in IJSEM Online). Strain CAU 9625^T did not contain the unidentified glycolipid reported for *G. molinativorax* (Manaia *et al.*, 2004). The cellular fatty acids were anteiso-C_{15:0} (48.6 %), iso-C_{16:0} (25.2 %), anteiso-C_{17:0} (12.5 %), iso-C_{15:0} (7.5 %), C_{16:0} (3.1 %), iso-C_{14:0} (1.4 %) and iso-C_{17:0} (1.3 %) (Table S1).

A nearly complete 16S rRNA gene sequence of strain CAU 9625^T (1451 bp) was determined. Phylogenetic analysis indicated that the isolate fell into a cluster comprising species of the genera *Gulosibacter*, *Zimmermannella* and

Pseudoclavibacter, but was clearly separated from the genera *Pseudoclavibacter* and *Zimmermannella*. The phylogenetic tree is shown in Fig. 1. Pairwise 16S rRNA gene sequence similarities between strain CAU 9625^T and closely related strains were 97.8 % (*G. molinativorax* ON4^T), 96.7 % (*Z. bifida* IAM 14848^T), 95.8 % (*Z. faecalis* IFO 15706^T), 94.5 % (*P. chungangensis* CAU 59^T), 94.6 % (*P. helvolus* DSM 20419^T), 94.4 % (*Z. alba* IFO 15616^T) and 92.3 % (*P. soli* KP02^T). Mean DNA–DNA relatedness between strain CAU 9625^T and *G. molinativorax* DSM 13485^T was 35.4 ± 0.9 %. This value is well below the 70 % cut-off point recommended by Wayne *et al.* (1987) for the delineation of genomic species and supports the proposal that strain CAU 9625^T represents a separate species. The G + C content of the genomic DNA of strain CAU 9625^T was 66.2 mol%.

Table 2. Differential chemotaxonomic properties of strain CAU 9625^T and the type strains of closely related taxa of the family *Microbacteriaceae*

Strains: 1, *Gulosibacter chungangensis* sp. nov. CAU 9625^T; 2, *G. molinativorax* DSM 13485^T (Manaia *et al.*, 2004); 3, *Zimmermannella bifida* IAM 14848^T (Lin *et al.*, 2004); 4, *Z. faecalis* NBRC 15706^T (Lin *et al.*, 2004); 5, *Pseudoclavibacter chungangensis* KCTC 22691^T (Cho *et al.*, 2010); 6, *P. helvolus* DSM 20419^T (Lin *et al.*, 2004); 7, *P. soli* KP02^T (Kim *et al.*, 2009). ND, no data available.

Characteristic	1	2	3	4	5	6	7
Quinones	MK-9	MK-9	MK-8, -9	MK-9	MK-9	MK-9	MK-9, -8
Polar lipids*	DPG, PG, PL, L	DPG, PG, GL	ND	ND	ND	DPG, PG, GL	ND
Cell wall sugars†	Rib, Glc	Rib, Glc	Rha, Glc	Rha	Fuc	Xyl, Gal	Xyl
Peptidoglycan components	D-Orn, DAB	D-Orn	L-DAB	Hsr, L-DAB	DAB	DAB	DAB

*DPG, Diphosphatidylglycerol; GL, unidentified glycolipid; PG, phosphatidylglycerol; PL, unidentified phospholipid; L, unidentified lipid.

†Data from this study. Fuc, fucose; Gal, galactose; Glc, glucose; Rha, rhamnose; Rib, ribose; Xyl, xylose.

The phenotypic and chemotaxonomic data, together with the results of the 16S rRNA gene phylogenetic analysis and DNA–DNA relatedness study, support the proposal that strain CAU 9625^T represents a novel species within the genus *Gulosibacter*, for which the name *Gulosibacter chungangensis* sp. nov. is proposed.

Emended description of the genus *Gulosibacter*

Gulosibacter (Gu.lo'si.bac.ter. L. adj. *gulosus* fond of titbits, dainty feeder; N.L. masc. n. *bacter* rod; N.L. masc. n. *Gulosibacter* rod fond of titbits).

The description of the genus *Gulosibacter* is as given by Manaia *et al.* (2004) with the following amendments. The peptidoglycan is based on ornithine or ornithine and 2,4-diaminobutyric acid. The characteristic polar lipids are diphosphatidylglycerol and phosphatidylglycerol. Whole-cell hydrolysates contain mainly glucose and ribose. The G + C content is 64.5–66.2 mol%.

Description of *Gulosibacter chungangensis* sp. nov.

Gulosibacter chungangensis (chung.ang.en'sis. N.L. masc. adj. *chungangensis* named after Chung-Ang University, where the taxonomic studies on this species were performed).

Colonies are strictly aerobic, opaque, white and circular, with a diameter of 0.3–0.5 mm after 1 day of cultivation at 30 °C on BHI agar. Cells are irregular short rods, approximately 0.5–0.7 µm in width and 1.0–1.3 µm in length. Growth occurs at 15–40 °C (optimum 37 °C), at pH 6.0–10.0 (optimum pH 8.0) and with 0–5 % (w/v) NaCl (optimum 1 % NaCl). Cells are catalase- and oxidase-positive. Nitrate is reduced and pyrazine carboxamide is utilized as a sole nitrogen source. Positive for esterase (C4). The peptidoglycan contains ornithine in combination with 2,4-diaminobutyric acid. Acid is produced from fructose, rhamnose, sorbitol and 5-ketogluconate. The major isoprenoid quinone is MK-9. Whole-cell hydrolysates

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences (1451 bp) showing the relationships between strain CAU 9625^T and the type strains of closely related taxa of the family *Microbacteriaceae*. Bootstrap values (>50%) based on 1000 resampled datasets are shown at branch nodes. Filled circles indicate that the corresponding nodes were also recovered in trees generated with the maximum-likelihood and least-squares algorithms. *Arthrobacter globiformis* DSM 20124^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

contain mainly ribose and glucose. The major polar lipids are diphosphatidylglycerol, phosphatidylglycerol, an unidentified phospholipid and an unidentified lipid. The predominant cellular fatty acids are anteiso-C_{15:0}, iso-C_{16:0} and anteiso-C_{17:0}. The DNA G+C content of the type strain is 66.2 mol%.

The type strain is CAU 9625^T (=KCTC 13959^T =CCUG 60841^T), which was isolated from a marine sediment near Oh Island in the Yellow Sea, Republic of Korea.

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