Pseudoclavibacter chungangensis sp. nov., isolated from activated sludge

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The taxonomic position of a Gram-positive, non-spore-forming strain, designated CAU 59^T, from activated sludge was investigated. Colony morphology, biochemical tests and chemotaxonomic investigations revealed that strain CAU 59^T possessed the characteristics of the genus *Pseudoclavibacter*. Comparative 16S rRNA gene sequence analysis showed sequence divergence values between strain CAU 59^T and other described *Pseudoclavibacter* species of more than 3.6 %, and the strain formed a hitherto-unknown subline within the genus *Pseudoclavibacter*. DNA–DNA hybridization studies showed that strain CAU 59^T displayed 20.9 % relatedness to its closest phylogenetic neighbour, *Pseudoclavibacter helvolus* DSM 20419^T. The DNA G + C content was 66.2 mol%. The phenotypic, chemotaxonomic and genotypic data indicated that strain CAU 59^T represents a novel species of the genus *Pseudoclavibacter*, for which the name *Pseudoclavibacter chungangensis* sp. nov. is proposed. The type strain is CAU 59^T (=KCTC 22691^T =CCUG 58142^T).

The family *Microbacteriaceae* embraces a large group of actinomycetes with cell-wall peptidoglycan of the B type (Schleifer & Kandler, 1972) and unsaturated major menaquinones (Collins & Jones, 1981). Initially, the family *Microbacteriaceae* was proposed to accommodate the genera *Agromyces*, *Aureobacterium*, *Clavibacter*, *Curtobacterium* and *Microbacterium* (Park *et al.*, 1993). At the time of writing, *Microbacteriaceae* comprises 31 recognized genera, which are distinguishable at the phenotypic level by a number of chemotaxonomic characteristics, including peptidoglycan diamino acids and respiratory menaquinone composition (Collins & Bradbury, 1992; Evtushenko & Takeuchi, 2003; Sheridan *et al.*, 2003). The genus *Pseudoclavibacter* was created to accommodate '*Brevibacterium helvolum*' (Zimmermann, 1890; Lochhead, 1955; Manaia *et al.*, 2004), an organism isolated from butter; the name is an earlier homotypic synonym of '*Zimmermannella helvola*' (Lin *et al.*, 2004). The genus name '*Zimmermannella*' is illegitimate based on nomenclature rules. Other related organisms, namely '*Zimmermannella alba*', '*Z. bifida*', '*Z. faecalis*' and *Pseudoclavibacter soli*, have been isolated from

human and animal specimens and soil (Lin *et al.*, 2004; Kim & Jung, 2009).

During the course of routine screening of bacteria for industrial purposes, a *Pseudoclavibacter*-like strain (designated CAU 59^T) was isolated from activated sludge at the wastewater treatment plant in Cheonan, Republic of Korea. The procedure for isolation of strain CAU 59^T followed that of Gordon & Mihm (1962) using glucose-yeast extract agar (GYEA; 10 g yeast extract 1⁻¹, 10 g glucose 1⁻¹, 15 g agar 1⁻¹) supplemented with 50 mg cycloheximide 1⁻¹ and 20 mg nalidixic acid 1⁻¹. An activated sludge sample was diluted with sterilized distilled water and appropriate dilutions were spread on GYEA medium and incubated aerobically for 3 days at 30 °C.

The Gram-staining reaction and production of spores were determined by microscopic examination, following described procedures (Doetsch, 1981; Smibert & Krieg, 1981). Catalase activity was determined by bubble production in a 3 % (v/v) hydrogen peroxide solution. Oxidase activity was tested by means of the oxidation of 1 % tetramethyl *p*-phenylenediamine (Merck). Hydrolysis of casein, starch and urea was determined on GYEA according to described methods (Cowan & Steel, 1965; Lányi, 1987; Smibert & Krieg, 1994). Other enzymic activities were tested using the API ZYM and API 20E

Abbreviations: GYEA, glucose-yeast extract agar; GYEB, glucose-yeast extract broth.

The GenBank/EMBL/DBJ accession number for the 16S rRNA gene sequence of strain CAU 59^T is FJ514934.

systems (bioMérieux), following the manufacturer's instructions. The fermentation of carbohydrates was characterized using the API 50CH system and defined medium B (Manaia *et al.*, 2003). Growth with 0–15 % NaCl was investigated on glucose-yeast extract broth (GYEB) at 30 °C. Growth at 4–50 °C was determined on GYEA. Growth at pH 3.0, 4.5 and 6.0 was determined in GYEB buffered with 12 mM MES (Sigma) and at pH 8.0, 9.0, 10.0 and 11.0 in GYEB buffered with 15 mM CAPS (Sigma).

Cellular fatty acid methyl esters were extracted after incubation for 3 days on tryptic soy agar (Difco) by acid methanolysis (Minnikin *et al.*, 1980) and analysed using a gas chromatograph (model 56890N; Agilent) equipped with a cross-linked 5 % phenyl methyl silicon-fused silica capillary column (HP 19091B-102; Hewlett Packard). Menaquinones were analysed as described by Komagata & Suzuki (1987) using reversed-phase HPLC. For strain CAU 59^T, preparation of cell walls and analysis of peptidoglycan structures were carried out using the methods described by Schleifer (1985), except that TLC was performed on cellulose sheets instead of paper. An analysis of the cell-wall diamino acid was performed as described elsewhere (Groth *et al.*, 1996; MacKenzie, 1987; Schleifer & Kandler, 1972).

Genomic DNA of strain CAU 59^T was isolated and purified by the method described by Marmur (1961). The G+C content of the genomic DNA was determined using SYBR Green I and a real-time thermocycler (model 7300; Applied Biosystems) and the fluorimetric method (Gonzalez & Saiz-Jimenez, 2002). The extent of genomic DNA–DNA relatedness between the isolate and *P. helvolus* DSM 20419^T was estimated using the fluorometric microplate method (Ezaki *et al.*, 1989), as modified by Goris *et al.* (1998). PCR amplification of the 16S rRNA gene sequence of strain CAU 59^T was carried out following established procedures (Nam *et al.*, 2004). The PCR products were sequenced directly using a BigDye Terminator Cycle Sequencing kit and ABI 3730 automatic DNA sequencer (Applied Biosystems). The sequence obtained from strain CAU 59^T was compared with corresponding sequences of other bacterial strains in the GenBank database. Multiple alignments with 16S rRNA gene sequences from a broad selection of related species were carried out using CLUSTAL X (Thompson *et al.*, 1997). Sequence similarity levels were calculated using PHYDIT and the EzTaxon server (Chun *et al.*, 2007). A phylogenetic tree was constructed using neighbour-joining (Saitou & Nei, 1987), least-squares (Fitch & Margoliash, 1967) and maximum-likelihood (Felsenstein, 1981) algorithms from the PHYLIP suite of programs (Felsenstein, 1989). Evolutionary distances for the neighbour-joining analysis were based on the correction of Jukes & Cantor (1969). The topology of the final tree was evaluated by the bootstrap method (Felsenstein, 1985) with 1000 replicates of the neighbour-joining dataset with the SEQBOOT and CONSENSE programs from the PHYLIP package.

Colonies of strain CAU 59^T were strictly aerobic, opaque, yellow and circular, with a diameter of 1–3 mm after 3 days of cultivation at 30 °C on GYEA medium. Cells were short rods, approximately 0.1–0.2 µm in width and 1.3–1.7 µm in length. Growth was observed at 4–50 °C (optimum 30 °C) and at pH 3.0–11.0 (optimum pH 7.0) on GYEA. Strain CAU 59^T grew with 0–15 % (w/v) NaCl (optimum 5 % NaCl). Cells of strain CAU 59^T were catalase-positive and oxidase-negative.

Table 1. Differential phenotypic properties between strain CAU 59^T and the type strains of closely related taxa of family Microbacteriaceae

Strains: 1, *Pseudoclavibacter chungangensis* sp. nov. CAU 59^T; 2, *P. helvolus* DSM 20419^T; 3, *P. soli* KP02^T; 4, '*Zimmermannella alba*' NBRC 15616; 5, '*Z. bifida*' IAM 14848; 6, '*Z. faecalis*' NBRC 15706; 7, *Gulosibacter molinivorax* ON4^T; 8, *Curtobacterium pusillum* DSM 20527^T. Data were obtained in this study. +, Positive; –, negative.

Characteristic	1	2	3	4	5	6	7	8
Enzyme activity								
Alkaline phosphatase	+	–	–	+	+	–	–	–
Cystine arylamidase	–	–	–	–	+	+	–	–
Esterase (C4)	+	–	–	–	–	–	–	–
Esterase lipase (C8)	+	–	–	–	–	+	–	–
Valine arylamidase	+	–	+	–	–	–	–	+
α-Glucosidase	+	–	–	–	–	–	–	+
β-Glucosidase	+	–	–	–	–	–	–	+
α-Mannosidase	–	–	–	–	–	–	–	+
Assimilation of:								
Glycerol	+	+	+	+	+	–	–	+
D-Arabinose	–	+	–	+	+	–	–	+
L-Arabinose	–	+	+	–	+	–	–	+
D-Arabitol	–	+	–	–	+	–	–	+
L-Arabitol	–	+	–	+	+	–	–	–
Arbutin	–	+	–	–	+	–	–	+
L-Arginine	–	–	–	+	–	–	–	+
Aesculin	–	+	+	–	–	–	–	+
D-Fructose	+	+	+	–	–	+	–	+
D-Fucose	–	+	–	–	+	–	–	–
L-Fucose	–	–	–	–	+	–	–	+
D-Glucose	–	+	+	+	–	+	–	+
Inositol	–	+	–	–	–	+	–	+
Inulin	–	–	–	–	+	–	–	–
D-Lyxose	–	+	–	–	–	–	–	–
Melibiose	–	+	–	–	–	–	+	+
D-Mannitol	+	+	+	+	+	+	–	+
Raffinose	+	+	–	+	–	–	–	+
D-Ribose	–	+	–	+	+	+	–	+
Salicin	–	+	+	+	–	–	–	+
Sucrose	+	+	–	–	–	–	+	–
Starch	–	–	–	+	+	–	–	–
D-Tagatose	–	+	+	+	–	–	–	+
Trehalose	–	+	–	+	+	–	–	+
Turanose	–	+	–	–	+	–	–	+
Xylitol	–	+	–	–	–	–	–	+
L-Xylose	–	+	–	+	–	–	–	–

Table 2. Chemotaxonomic characteristics of strain CAU 59^T and other closely related species

Strains: 1, *Pseudoclavibacter chungangensis* sp. nov. CAU 59^T; 2, *P. helvolus* DSM 20419^T; 3, *P. soli* KP02^T; 4, '*Zimmermannella alba*' NBRC 15616; 5, '*Z. bifida*' IAM 14848; 6, '*Z. faecalis*' NBRC 15706; 7, *Gulosibacter molinivorax* ON4^T; 8, *Curtobacterium pusillum* DSM 20527^T. Data were obtained in this study unless otherwise indicated. +, Positive; tr, trace (<1.0 %); –, not detected. DAB, 2,4-diaminobutyric acid; ECL, equivalent chain-length; Hsr, homoserine.

Fatty acid (%)	1	2	3	4	5	6	7	8
Saturated								
C _{14:0}	1.2	tr	tr	–	tr	tr	–	tr
C _{16:0}	3.6	4.7	3.6	1.4	tr	5.4	1.9	1
Unsaturated								
C _{18:1ω7c}	–	–	–	–	–	–	–	32.7
C _{20:1ω9c}	–	–	5.7	–	–	–	7.9	–
Branched-chain								
anteiso-C _{13:0}	–	1.6	tr	tr	1.6	tr	–	–
anteiso-C _{15:0}	35.0	59.4	48	36.2	68.4	51.2	53.9	29.3
anteiso-C _{17:0}	17.5	25.2	21.7	55.6	16.2	13.8	17.4	23.4
cyclo-C _{19:0ω8c}	1.3	–	–	–	–	–	–	–
iso-C _{14:0}	2.1	tr	tr	–	1.4	2	tr	tr
iso-C _{15:0}	–	–	3.9	tr	3.5	2.7	1.6	4.4
iso-C _{16:0}	27.3	6.8	13.4	tr	5.7	–	16.7	6.3
iso-C _{17:0}	–	–	1.6	1.6	tr	tr	–	1.9
Summed feature 1*	1.1	–	–	tr	–	–	–	–
Unknown fatty acid ECL	5.5	–	–	–	–	–	–	–
Cell-wall amino acid(s)	DL-DAB	DL-DAB†	DL-DAB‡	L-DAB§	L-DAB§	Hsr, L-DAB§	D-Orn†	D-Orn
Cell-wall sugar(s)	Fuc	Xyl, Gal	Xyl	Rha	Rha	Rha, Fuc	Fuc	Rha, Glu, Gal
Predominant menaquinone	MK-9	MK-9†	MK-9, MK-8‡	MK-10§	MK-8, MK-9§	MK-9§	MK-9†	MK-9

*Summed features represent two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature 1 consisted of iso-C_{15:0} H and/or C_{13:0} 3-OH.

†Data from Manaia *et al.* (2004).

‡Data from Kim & Jung (2009).

§Data from Lin *et al.* (2004).

||Data from Behrendt *et al.* (2002) and Manaia *et al.* (2004).

The API systems provided adequate phenotypic data to describe the new isolate. A detailed description of the phenotypic characteristics is given in Table 1 and the species description. Using API 50CHB, acids were produced from D-fructose, glycerol, 5-ketogluconate, D-mannitol, raffinose and D-xylose. API ZYM detected acid phosphatase, alkaline phosphatase, esterase (C4), esterase lipase (C8), α- and β-glucosidase, leucine arylamidase, valine arylamidase and naphthol-AS-BI-phosphohydrolase. API 20E demonstrated the utilization of L-tryptophan and sodium pyruvate.

The diamino acid in the peptidoglycan of strain CAU 59^T was 2,4-diaminobutyric acid and alanine, glycine and glutamic acid were also found: the molar ratios were 2.0:0.8:1.2:1.0. The cell-wall sugars contained fucose. The predominant isoprenoid quinone was the unsaturated menaquinone MK-9. The cellular fatty acid profile contained C_{14:0} (1.2 %), C_{16:0} (3.6 %), anteiso-C_{15:0} (35.0 %), anteiso-C_{17:0} (17.5 %), cyclo-C_{19:0ω8c} (1.3 %), iso-C_{14:0} (2.1 %), iso-C_{16:0} (27.3 %), summed feature 1 (1.1 %) and an unknown fatty acid (5.5 %; Table 2).

The G + C content of the genomic DNA of strain CAU 59^T was approximately 66.2 mol%. A phylogenetic tree based on 16S rRNA gene sequences (1424 bp) from strain CAU 59^T and closely related type strains in the family *Microbacteriaceae* was constructed using combinations of different algorithms (Fig. 1). It is evident that strain CAU 59^T falls within the cluster comprising *Pseudoclavibacter* species, forming a clade with *P. helvolus* DSM 20419^T, and is clearly separated from the members of the genera *Clavibacter*, *Curtobacterium*, *Mycetocola* and *Frigoribacterium*. Pairwise similarity values between strain CAU 59^T and closely related strains were 96.7 % ('*Z. alba*' IFO 15616), 96.4 % (*P. helvolus* DSM 20419^T), 95.5 % ('*Z. faecalis*' IFO 15706), 95.2 % ('*Z. bifida*' IAM 14848), 95.2 % (*Gulosibacter molinivorax* ON4^T) and 94.5 % (*P. soli* KP02^T). The high sequence divergence values with recognized members of the genus *Pseudoclavibacter* (>3.6 %) clearly indicate that strain CAU 59^T represents a novel species within the genus (Stackebrandt & Goebel, 1994). The mean DNA–DNA relatedness value between strain CAU 59^T and *P. helvolus* DSM 20419^T was 20.9 %. This value is well below the 70 % cut-off point recommended by Wayne *et al.* (1987) for the delineation of a

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships between strain CAU 59^T and type strains of closely related taxa of family *Microbacteriaceae*. Bootstrap values (>50%) based on 1000 replications are shown at branch nodes. Asterisks indicate that the corresponding nodes were recovered in trees generated with the concurrent use of the least-squares and maximum-likelihood algorithms. F or M indicates that the corresponding nodes were recovered in trees generated with the least-squares algorithm or maximum-likelihood algorithm, respectively. Bar, 0.01 substitutions per nucleotide position.

genomic species, supporting the proposal that CAU 59^T represents a novel species.

These phenotypic and chemotaxonomic data, together with the 16S rRNA gene sequence data and the DNA–DNA relatedness study, provide sufficient evidence to support the proposal that strain CAU 59^T represents a hitherto-unrecognized species within the genus *Pseudoclavibacter*. The name *Pseudoclavibacter chungangensis* sp. nov. is proposed.

Description of *Pseudoclavibacter chungangensis* sp. nov.

Pseudoclavibacter chungangensis (chung.an.gen'sis. N.L. masc. adj. *chungangensis* named after Chung-Ang University, where the taxonomic studies on the type strain were performed).

Cells are strictly aerobic, Gram-positive, non-spore-forming rods, 0.1–0.2 µm in width and 1.3–1.7 µm in length. Colonies are yellow, opaque and circular on GYEA

medium. Growth occurs at 4–50 °C (optimum 30 °C), at pH 3.0–11.0 (optimum pH 7.0) and with 0–15 % (w/v) NaCl (optimum 5 %). Catalase-positive and oxidase-negative. Acid is produced from D-fructose, glycerol, 5-ketogluconate, D-mannitol, raffinose and D-xylose. Casein, starch and urea are not hydrolysed. Enzyme activities were demonstrated for acid phosphatase, alkaline phosphatase, esterase (C4), esterase lipase (C8), α - and β -glucosidase, leucine arylamidase, valine arylamidase and naphthol-AS-BI-phosphohydrolase. L-tryptophan and sodium pyruvate are utilized. The diamino acid in the peptidoglycan is 2,4-diaminobutyric acid. The major isoprenoid quinone is MK-9. Whole-cell hydrolysates contain mainly fucose. The predominant cellular fatty acids (>10 %) are anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{16:0}. The DNA G + C content of the type strain is 66.2 mol%.

The type strain (CAU 59^T=KCTC 22691^T=CCUG 58142^T) was isolated from activated sludge taken from a wastewater treatment plant in Cheonan, Republic of Korea.

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