Lactococcus chungangensis sp. nov., a lactic acid bacterium isolated from activated sludge foam

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The taxonomic position of a Gram-positive coccus, designated strain CAU 28^T, isolated from activated sludge foam was determined by using a polyphasic approach. Based on its cellular morphology and the results of biochemical tests, strain CAU 28^T was identified tentatively as a member of the genus *Lactococcus*. Comparative 16S rRNA gene sequence analysis showed that levels of similarity between strain CAU 28^T and the type strains of recognized *Lactococcus* species ranged from 90.4 to 97.2%. DNA–DNA hybridization studies showed that strain CAU 28^T displayed less than 26.1% relatedness to the type strains of recognized *Lactococcus* species. The rep-PCR fingerprints revealed that strain CAU 28^T was well separated from reference *Lactococcus* species. The combined genotypic and phenotypic data indicate that strain CAU 28^T represents a novel species of the genus *Lactococcus*, for which the name *Lactococcus chungangensis* sp. nov. is proposed. The type strain is CAU 28^T (=KCTC 13185^T =CCUG 55099^T).

Lactococcus Schleifer *et al.* 1986 was first established as a separate genus distinct from the genus *Streptococcus* by Schleifer *et al.* (1985). At the time of writing, *Lactococcus* comprises five recognized species (Euzéby, 1997; last full update 1 December 2007, <http://www.bacterio.net>), *Lactococcus garvieae* (Collins *et al.* 1984) Schleifer *et al.* 1986, *L. plantarum* (Collins *et al.* 1984) Schleifer *et al.* 1986, *L. raffinolactis* (Orla-Jensen and Hansen 1932) Schleifer *et al.* 1988, *L. piscium* Williams *et al.* 1990 and *L. lactis* (Lister 1873) Schleifer *et al.* 1986 (the type species). The last of these species has been divided into three subspecies: *L. lactis* subsp. *cremoris* (Orla-Jensen 1919) Schleifer *et al.* 1986, *L. lactis* subsp. *hordniae* (Latorre-Guzman *et al.* 1977) Schleifer *et al.* 1986 and *L. lactis* subsp. *lactis* (Lister 1873) Schleifer *et al.* 1986. Members of the genus *Lactococcus* have been identified as lactic acid bacteria that contribute significantly to the properties of fermented dairy products while others produce antimicrobial compounds. Members of the genus have been isolated largely from food-related

sources and are generally regarded as safe (GRAS) organisms (Salminen *et al.*, 1998). However, rare cases of invasive disease in humans, sometimes severe, have been reported in association with *L. garvieae* (Vinh *et al.*, 2006; Wang *et al.*, 2007; Yiu *et al.*, 2007) and *L. lactis* (Mannion & Rothburn, 1990) infections. The case study by Wang *et al.* (2007) raised awareness of a potential risk factor for gastrointestinal disease from *L. garvieae* linked to the consumption of raw seafood (squid) in the summer months, although their 16S rRNA gene sequence analysis may not have been the best method of establishing an epidemiological link. The exact source of infection in squid and the farm of its origin could not be identified, and a much larger study is needed to confirm risk factors for potential gastrointestinal infection with *L. garvieae*. Several reports show that *L. garvieae* is an important pathogen of fish, squid and prawns, causing economic losses both in marine and in freshwater aquaculture (Carson *et al.*, 1993; Chen *et al.*, 2001, 2002; Pereira *et al.*, 2004; Vendrell *et al.*, 2006). In addition, *L. garvieae* has been isolated from cases of bovine mastitis (Collins *et al.*, 1983; Teixeira *et al.*, 1996). Until now, however, there have been no reports of

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CAU 28^T is EF694028.

the isolation of lactococci from activated sludge foam. During the course of routine screening of bacteria for industrial purposes, a *Lactococcus*-like strain (designated CAU 28^T) was isolated from activated sludge foam from the wastewater treatment plant in Cheonan, Republic of Korea. The purpose of the present study was to establish the taxonomic position of this novel bacterial strain.

The procedure for isolation of strain CAU 28^T followed that of Gordon & Mihm (1962) by using glucose-yeast extract agar (GYEA) supplemented with cycloheximide (50 g ml⁻¹) and nalidixic acid (20 g ml⁻¹). An activated sludge foam sample was diluted with sterilized distilled water, and appropriate dilutions were spread onto GYEA medium and incubated for 3 days at 30 °C. An opaque, white bacterial colony with a diameter of less than 1 mm was isolated and designated strain CAU 28^T. The pure culture of CAU 28^T was preserved in 25 % (v/v) glycerol at -70 °C. *Lactococcus* reference strains were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ; Braunschweig, Germany), Korean Collection for Type Cultures (KCTC; Daejeon, Korea) or Korean Culture Center of Microorganisms (KCCM; Seoul, Korea). The cell morphology of colonies of strain CAU 28^T on GYEA was observed at different incubation times, every 24 h for 5 days, with a microscope following Gram staining. The general biochemical and physiological properties of the strain were tested. In addition, API 20 Strep and API 50 CHL (bioMérieux) test kits were used according to the manufacturer's instructions to assist in the rapid characterization of this strain. For cellular fatty acid analysis, cell mass of strain CAU 28^T and of the type strains of recognized *Lactococcus* species, except *L. piscium* DSM 6634^T, was harvested from trypticase soy agar plates after incubation for 3 days at 30 °C. *L. piscium* DSM 6634^T was cultured under anaerobic conditions in Acetomicrobium faecalis medium (DSMZ medium 412) for 3 days at 37 °C. Cellular fatty acid methyl esters were extracted by acid methanolysis (Minnikin *et al.*, 1980) and analysed by using a Hewlett Packard series II gas chromatograph model 5890A (Microbial Identification System instrument) equipped with a 5 % phenyl methyl silicon-fused silica capillary column (HP 19091B-102). Strain CAU 28^T was cultured on GYEA medium at 30 °C for 3 days for extraction of chromosomal DNA. PCR amplification and sequencing of the 16S rRNA gene were carried out following established procedures (Nam *et al.*, 2004). The resultant 16S rRNA gene sequence was compared with corresponding sequences of the type strains of all recognized *Lactococcus* species, representatives of the genus *Streptococcus*, *Enterococcus faecalis* and *Vagococcus fluvialis* as described by Nam *et al.* (2004). The level of DNA-DNA relatedness between strain CAU 28^T and the type strains of the seven recognized *Lactococcus* species/subspecies was estimated by using the fluorometric microplate method (Ezaki *et al.*, 1989), as modified by Goris *et al.* (1998). These strains also underwent discriminatory rep-PCR fingerprinting by using five different

primers: BOX (BOXA1R), ERIC (ERIC 1R and ERIC 2), (GTG)₅, REP (REP 1R-I and REP 2-I) and SERE (Versalovic *et al.*, 1991, 1994; Rajashekara *et al.*, 1998). DNA fingerprints were analysed by using BioNumerics software (version 4.5; Applied Maths).

Colonies of strain CAU 28^T on GYEA plates growing aerobically or under aerobic conditions at 30 °C for 3 days were white, opaque and very small (diameter ~0.3 mm). After several days growth, colony enlargement on GYEA plates was difficult to distinguish by eye. Cells examined microscopically were Gram-positive, regular coccoid or ovoid-shaped, occurring singly, in pairs, as short chains and as irregular clusters. No mycelium was observed. There were no spores and cells were catalase- and oxidase-negative. API 20 Strep and API 50 CHL test strips provided adequate phenotypic data to describe the new isolate and a detailed description of the key phenotypic characteristics of the isolate is given in Table 1 and in the species description. No particular set of phenotypic characteristics could be identified to differentiate strain CAU 28^T from recognized *Lactococcus* species. Conventional tests were not performed alongside the API tests as no distinguishing API test could be identified. The fatty acid profile of strain CAU 28^T comprised straight-chain saturated and unsaturated com-

Table 1. Differential phenotypic properties between strain CAU 28^T and the type strains of recognized *Lactococcus* species

Strains: 1, CAU 28^T; 2, *L. garvieae* KCTC 3772^T; 3, *L. piscium* DSM 6634^T; 4, *L. plantarum* DSM 20686^T; 5, *L. raffinolactis* DSM 20443^T; 6, *L. lactis* subsp. *cremoris* KCCM 40699^T; 7, *L. lactis* subsp. *hordniae* KCTC 3768^T; 8, *L. lactis* subsp. *lactis* KCTC 3769^T. +, Positive; -, negative; w, weakly positive. Data are from the present study.

Characteristic	1	2	3	4	5	6	7	8
Growth at:								
4 °C	+	-	+	-	-	-	-	-
40 °C	-	+	-	-	-	-	-	+
Growth in 4 % NaCl	-	+	-	+	-	-	-	+
Ammonia from arginine	-	+	-	-	-	-	+	+
Acid from:								
Amygdalin	+	+	+	+	-	-	-	+
Galactose	-	+	+	+	w	+	-	+
Lactose	-	-	+	-	-	+	-	+
Maltose	+	+	+	+	+	+	-	+
Melibiose	-	-	+	-	+	+	-	-
Melezitose	-	-	+	+	-	-	-	-
Methyl α-D-glucoside	-	-	+	+	-	-	-	w
Methyl α-D-mannoside	-	-	+	-	-	-	-	-
Raffinose	-	-	+	-	+	+	-	-
Sucrose	+	-	+	+	+	+	+	-
Trehalose	w	+	+	+	+	+	+	+
Turanose	w	-	+	+	-	-	-	-
D-Xylose	-	-	+	-	+	+	-	+
Hydrolysis of aesculin	+	+	+	+	-	-	-	-

ponents, branched-chain components plus summed features (groups of two or three fatty acids that could not be separated by GLC with the MIDI system) and an unknown fatty acid. Saturated fatty acids $C_{16:0}$ and $C_{14:0}$ and unsaturated fatty acid $C_{18:1\omega7c}$ were the major components present in strain CAU 28^T. The fatty acid profile of strain CAU 28^T was similar to those of reference *Lactococcus* type strains except *L. piscium* DSM 6634^T (Table 2). Interestingly, strain CAU 28^T contained an unknown fatty acid, and it is suggested that this should be characterized as it may provide taxonomically valuable data for the differentiation of species within the genus.

The nearly complete 16S rRNA gene sequence of strain CAU 28^T was determined and compared with the corresponding sequences of other bacterial strains in the GenBank database. There were at least seven sequences showing over 99% 16S rRNA gene sequence similarity to strain CAU 28^T, which originated from six uncultured bacteria (GenBank accession nos AB286356, AB286530, AB286522, EU015102, AB286488 and AM697410) and isolate *Lactococcus* sp. F116 (EF204365). It appears that strain CAU 28^T might be a relative of one or more of these seven isolates or clones. A phylogenetic tree, based on 16S rRNA gene sequence data from strain CAU 28^T and

Table 2. Cellular fatty acid compositions of strain CAU 28^T and type strains of recognized *Lactococcus* species

Strains: 1, CAU 28^T; 2, *L. garvieae* KCTC 3772^T; 3, *L. piscium* DSM 6634^T (cultured under anaerobic conditions in Acetomicrobium faecalis medium for 3 days at 37 °C); 4, *L. plantarum* DSM 20686^T; 5, *L. raffinolactis* DSM 20443^T; 6, *L. lactis* subsp. *cremoris* KCCM 40699^T; 7, *L. lactis* subsp. *hordniae* KCTC 3768^T; 8, *L. lactis* subsp. *lactis* KCTC 3769^T. Values are percentages of total fatty acids. tr, Trace (<1.0%); —, not detected. Data are from the present study. ALDE, Aldehyde; DMA, dimethylacetal; FAME, fatty acid methyl ester; ECL, equivalent chain-length.

Fatty acid	1	2	3	4	5	6	7	8
Saturated								
$C_{12:0}$	1.6	4.0	—	1.8	—	—	—	—
$C_{14:0}$	17.3	19.4	—	10.8	8.2	10.0	3.4	8.9
$C_{14:0}$ FAME	—	—	12.7	—	—	—	—	—
$C_{15:0}$	—	0.8	—	—	0.4	0.5	0.3	—
$C_{16:0}$	37.6	34.6	—	51.1	26.7	40.3	32.6	45.7
$C_{16:0}$ ALDE	—	—	2.7	—	—	—	—	—
$C_{16:0}$ DMA	—	—	12.1	—	—	—	—	—
$C_{16:0}$ FAME	—	—	35.1	—	—	—	—	—
$C_{18:0}$	1.2	0.9	—	2.1	0.7	0.7	1.3	2.7
Unsaturated								
$C_{16:1\omega5c}$	0.9	2.5	—	—	—	—	—	—
$C_{16:1}$ (<i>cis</i> 7) FAME	—	—	1.7	—	—	—	—	—
$C_{16:1\omega9c}$	0.8	2.1	—	—	—	—	—	—
$C_{18:1}$ (<i>cis</i> 11) DMA	—	—	2.9	—	—	—	—	—
$C_{18:1\omega7c}$	19.0	31.3	—	27.0	12.6	9.9	60.5	22.9
$C_{18:1\omega9c}$	3.2	—	—	2.0	—	—	—	—
$C_{20:2\omega6,9c}$	—	—	—	—	1.3	1.5	tr	—
$C_{20:4\omega6,9,12,15c}$	1.6	—	—	—	—	—	—	—
Branched-chain								
anteiso- $C_{15:0}$	1.3	—	—	—	—	—	—	—
anteiso- $C_{17:0}$	4.1	—	—	—	—	—	—	—
cyclo- $C_{17:0}$	—	—	—	—	0.5	0.7	—	—
cyclo- $C_{19:0\omega8c}$	—	—	—	—	43.8	31.5	—	12.5
Cyclopropane								
$C_{19:0}$ cyc 11,12 DMA	—	—	5.5	—	—	—	—	—
C_{19} cyc 11,12/1 FAME	—	—	19.9	—	—	—	—	—
11-Methyl $C_{18:1\omega7c}$	—	—	—	—	1.9	1.9	—	0.5
Summed features*								
3	4.7	4.5	—	5.3	4.0	3.1	1.9	6.9
4	1.8	—	—	—	—	—	—	—
10	—	—	7.4	—	—	—	—	—
Unknown fatty acid (ECL 17.834)	4.9	—	—	—	—	—	—	—

*Summed feature 3: $C_{16:1\omega7c}$ /iso- $C_{15:0}$ 2-OH. Summed feature 4: iso- $C_{17:1}$ I/anteiso- $C_{17:1}$ B. Summed feature 10: $C_{18:1c11/t9/t6}$ FAME.

corresponding sequences from the type strains of recognized *Lactococcus* and *Streptococcus* species, as well as *Enterococcus faecalis* ATCC 19433^T and *V. fluvialis* NCDO 2497^T, was constructed according to the neighbour-joining algorithm (Saitou & Nei, 1987) (Fig. 1). Trees were also constructed based on the least-squares (Fitch & Margoliash, 1967) and maximum-likelihood (Felsenstein, 1981) methods. The tree topology, supported by high bootstrap values, clearly separated strain CAU 28^T within the genus *Lactococcus* lineage from the genus *Streptococcus*. *Enterococcus faecalis* ATCC 19433^T and *V. fluvialis* NCDO 2497^T formed a separate group distant from the genera *Lactococcus* and *Streptococcus*, with *Escherichia coli* K-12 (GenBank accession no. E05133) forming an outlying single membered cluster. Members of the genus *Lactococcus* occurred as two groups. Strain CAU 28^T formed a group with *L. raffinolactis* DSM 20443^T, *L. piscium* CCUG 32732^T and *L. plantarum* DSM 20686^T, and shared 16S rRNA gene sequence similarities of 97.2, 95.8 and 95.5 %, respectively, with these three type strains. Lower levels of 16S rRNA gene sequence similarity were observed between strain CAU 28^T and members of the second group, namely *L. lactis* subsp. *lactis* NCDO 604^T (90.4 %), *L. lactis* subsp. *hordniae* KCTC 3768^T (91.0 %), *L. lactis* subsp. *cremoris* NCDO 607^T (90.7 %) and *L. garvieae* ATCC 49156^T (90.8 %). It is evident from these results that strain CAU 28^T represents a distinct species belonging to the genus *Lactococcus* (Stackebrandt & Goebel, 1994). Levels of DNA–DNA relatedness between strain CAU 28^T and the seven *Lactococcus* reference strains ranged between 18.5 ± 0.1 and 26.1 ± 0.3 % (means ± SD of triplicate determinations). These values are below the 70 % cut-off point recommended by Wayne *et al.* (1987) for the

delineation of a genomic species, supporting the proposal that CAU 28^T represents a separate species. rep-PCR fingerprints of five different primers [BOX, ERIC, (GTG)₅, REP and SERE] and the corresponding consensus dendrogram show that strain CAU 28^T is clearly separate from the seven *Lactococcus* reference strains and forms a single-membered cluster (Fig. 2). Two bands with high intensity at 2.1 and 1.3 kb occur in the ERIC and SERE fingerprints, respectively, for strain CAU 28^T only. A low level of similarity was found between strain CAU 28^T and its phylogenetically closest relatives, namely *L. raffinolactis* DSM 20443^T, *L. piscium* DSM 6634^T and *L. plantarum* DSM 20686^T (37.4, 47.9 and 50.4 %, respectively). The consensus dendrogram showed that, at a similarity cut-off point of approximately 76.0 %, *L. lactis* subsp. *hordniae* KCTC 3768^T forms a cluster with *L. lactis* subsp. *cremoris* KCCM 40699^T, while *L. lactis* subsp. *lactis* KCTC 3769^T occurs as a single-membered cluster.

This study reveals good congruence between three different types of genotypic data, namely levels of DNA–DNA relatedness, phylogenetic position and rep-PCR fingerprints. These data, together with the phenotypic data for strain CAU 28^T, provide sufficient evidence to support the proposal that it represents a novel species, as recommended by Stackebrandt *et al.* (2002). Although a species description is given based on a single isolate, this taxonomic status is warranted because this strain was isolated during the course of routine screening of bacteria for industrial purposes and is associated with a particular industrial application. The name *Lactococcus chungangensis* sp. nov. is proposed for this novel taxon.

Fig. 1. Neighbour-joining phylogenetic tree based on nearly complete 16S rRNA gene sequences showing the relationships between strain CAU 28^T and members of the genera *Lactococcus* and *Streptococcus*, *Enterococcus faecalis*, *Vagococcus fluvialis* and *Escherichia coli* K-12. Branches that were also found using the least-squares and/or maximum-likelihood treeing algorithms are indicated by asterisks (both algorithms), F (least-squares only) or M (maximum-likelihood only). Numbers at nodes indicate levels of bootstrap support (%) based on a neighbour-joining analysis of 1000 resampled datasets; only values above 50 % are given. Bar, 0.02 substitutions per nucleotide position.

Fig. 2. Consensus dendrogram based on combined analysis of REP/ERIC/BOX/(GTG)₅/SERE-PCR fingerprints, showing the position of strain CAU 28^T among closely related species of the genus *Lactococcus*. Data were sorted by the UPGMA clustering method and levels of similarity between patterns were calculated by using Pearson's correlation coefficient.

Description of *Lactococcus chungangensis* sp. nov.

Lactococcus chungangensis (chun.gan.gen'sis. N.L. masc. adj. *chungangensis* named after Chungang University, where taxonomic studies on this species were performed).

Cells are Gram-positive, non-spore-forming, non-mycelium-forming, coccoid or ovoid-shaped, occurring singly, in pairs, as short chains or in irregular clusters. Pinpoint colonies on GYE plates are white and opaque, with a diameter of less than 0.3 mm; colony enlargement is difficult to distinguish by eye. Cells grow under aerobic or facultatively anaerobic conditions, are non-motile, and catalase- and oxidase-negative. Optimal growth occurs at 30 °C. Growth does not occur at 40 °C and occurs only very weakly at 37 °C, but growth occurs at 4 and 10 °C. Commercial API 20 Strep and API 50 CHL testing reveals acid production from D-glucose, D-fructose, D-mannose, mannitol, N-acetylglucosamine, amygdalin, arbutin, salicin, maltose, sucrose and gentiobiose. Acid is produced weakly from cellobiose, trehalose, starch and turanose. The Voges-Proskauer test (acetoin production) is positive. Aesculin and hippuric acid are hydrolysed. Leucine aminopeptidase is produced, but pyrrolidonyl arylamidase, α-galactosidase, β-glucuronidase, β-galactosidase, alkaline phosphatase and arginine dihydrolase are not. The major cellular fatty acids are straight-chain saturated (C_{16:0} and C_{14:0}) and unsaturated (C_{18:1ω7c}) acids.

The type strain, CAU 28^T (=KCTC 13185^T =CCUG 55099^T), was isolated from a foam sample taken from an activated sludge wastewater treatment plant in Cheonan, Republic of Korea.

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