Carnobacterium jeotgali sp. nov., isolated from a Korean traditional fermented food

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A Gram-positive, facultatively anaerobic bacterium, designated strain MS3^T, was isolated from a traditional Korean fermented food made with freshwater shrimp. Strain MS3^T was able to grow at 4–37 °C, at pH 5.5–9.0 and in the presence of 0–5 % (w/v) NaCl. Optimal growth occurred at 30 °C, at pH 8.5 and in the presence of 2 % NaCl. The strain was catalase- and oxidase-negative. It was able to metabolize various carbohydrates as energy sources. 16S rRNA gene sequence analysis showed that strain MS3^T was most closely related to *Carnobacterium pleistocenium* FTR1^T (98.95 % similarity), but the level of DNA–DNA relatedness between the two taxa was less than 16.0 %. The genomic G+C content of strain MS3^T was 43.9 mol% and the major fatty acid components were $C_{16:0}$, $C_{16:1}\omega9c$ and $C_{18:1}\omega9c$. On the basis of its genotypic, physiological and biochemical characteristics, strain MS3^T is considered to represent a novel species of the genus *Carnobacterium*, for which the name *Carnobacterium jeotgali* sp. nov. is proposed. The type strain is MS3^T (=KCTC 13251^T=JCM 15539^T).

Species of the genus *Carnobacterium* are heterofermentative, predominantly (+)-L-lactic acid-producing, Gram-positive, facultative anaerobes (Collins *et al.*, 1987). They may act as the primary agents responsible for the formation of conditions that favour the development of strict anaerobes (Franzmann *et al.*, 1991). They are found in vacuum-packed meat and are capable of growing in products stored at low temperatures, including refrigerated food (Collins *et al.*, 1987). At the time of writing, the genus *Carnobacterium* comprised ten recognized species. *Carnobacterium funditum* and *Carnobacterium alterfunditum* were isolated from the waters of Ace Lake, Antarctica (Franzmann *et al.*, 1991). *Carnobacterium piscicola* was reported to be a pathogen of salmonids (Collins *et al.*, 1987). *Carnobacterium inhibens* was isolated from the intestinal tract of salmon and has the ability to inhibit the growth of fish pathogens (Joborn *et al.*, 1999). Most recently, *Carnobacterium pleistocenium* was isolated from permafrost in Fox, Alaska (Pikuta *et al.*, 2005).

Here we describe a novel strain, designated MS3^T, isolated from a traditional Korean fermented food, jeotgal. Jeotgal is salty with a slightly sour flavour. Strain MS3^T was isolated from 'toha jeotgal', which is generally made with freshwater shrimp meat and salt. Strain MS3^T was isolated on trypticase soy agar medium (TSA; BBL). Based on the data presented, we propose that strain MS3^T represents a novel species of the genus *Carnobacterium*.

To verify the phylogenetic status of the new isolate, its 16S rRNA gene sequence was determined. The isolate was transferred several times on TSA medium to obtain a pure culture. Next, colony PCR was performed with PCR Pre-Mix (SolGent) and two bacteria-specific primers (8F, 1492R; Baker *et al.*, 2003). After purification, the PCR product was sequenced by using a BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) according to the manufacturer's instructions. The reaction mixtures were analysed with an automated DNA analyser system (PRISM 3730XL DNA Analyzer; Applied Biosystems). The 16S rRNA gene sequence obtained was compared with all sequences in the GenBank database (NCBI database). After comparison, four primers (8F, 968F, 518R and 1492R) were used to obtain the complete 16S rRNA gene sequence of strain MS3^T. The 16S rRNA gene sequences were assembled by using SeqMan software (DNASTAR) and were aligned by using the multiple sequence alignment program CLUSTAL_X (v. 1.83) (Thompson *et al.*, 1997). The sequences of the new isolate and relatives in the GenBank database were used to construct a phylogenetic consensus tree via the BioEdit program (Hall, 1999). A phylogenetic tree constructed by MEGA4 (Tamura *et al.*, 2007) revealed the taxonomic status of the new isolate in relation to its relatives. The neighbour-joining and maximum-parsimony algorithms were used to construct

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain MS3^T is EU817500.

a consensus phylogenetic tree (Kluge & Farris, 1969; Saitou & Nei, 1987). Bootstrap analysis was used to evaluate the tree topology by means of 1000 randomly chosen replications. Strain MS3^T formed a separate phylogenetic clade within the cluster comprising *C. pleistocenium*, *C. inhibens* and *Carnobacterium viridans* (Fig. 1). Strain MS3^T shared highest 16S rRNA gene sequence similarity with *C. pleistocenium* FTR1^T (98.95 %), *C. viridans* MPL-11^T (98.88 %) and *C. inhibens* K1^T (98.74 %). These three species were closest to the new isolate based on both phylogenetic and genetic analyses. Given that strain MS3^T shared greater than 97 % 16S rRNA gene sequence similarity with its closest relatives, DNA–DNA hybridization experiments were also performed.

In order to define the optimal culture conditions for strain MS3^T, growth was tested in trypticase soy broth medium (TSB; BBL) at various NaCl concentrations (0, 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12 and 14 %, w/v) for 1 week at 30 °C. Growth of the isolate was tested in TSB at 0, 4, 10, 15, 25, 30, 37 and 40 °C and at pH 4–13 (at increments of 0.5 pH units at 30 °C) for 1 week. Growth was observed at 4–37 °C, at pH 5.5–9.0 and in the presence of 0–5 % NaCl. Optimal growth occurred at 30 °C, at pH 8.5 and in the presence of 2 % (w/v) NaCl (Table 1). All subsequent tests were performed at these optimal conditions, unless stated otherwise. Although the strain was isolated under aerobic conditions, anaerobic cultures were grown by using the medium for *C. pleistocenium* FTR1^T with modifications (Pikuta *et al.*, 2005). The medium consisted of (l^{-1} distilled water): 0.2 g yeast extract, 3.0 g peptone, 20.0 g NaCl, 0.5 g $MgCl_2.6H_2O$, 0.2 g KH_2PO_4, 0.3 g NH_4Cl, 0.3 g KCl, 0.015 g $CaCl_2.2H_2O$, 1 ml trace element solution A (Löffler *et al.*, 1996), 0.001 g resazurin, 0.525 g L-cysteine, 4.0 g Na_2CO_3 and 2 ml vitamin solution (Wolin *et al.*, 1963). The medium was boiled and cooled rapidly while being flushed with oxygen-free N_2/CO_2 (80 : 20). Sodium carbonate was added after cooling and then reducing compound was added over 5 min. The final pH was adjusted to 7.1–7.5 by varying the CO_2 flow. The medium (25 ml) was transferred to 100 ml serum bottles, which had been flushed with oxygen-free N_2/CO_2 (80 : 20). The serum bottles were closed with butyl rubber stoppers and capped with tear-off aluminium seals. The vitamin solution was filter-sterilized and added to the autoclave-sterilized medium. The serum bottles were inoculated with a 1.0 % (v/v) aliquot of an exponentially growing culture and incubated at 30 °C for 48 h. Cell growth was determined based on the opacity of the medium in the serum bottles. The standard anaerobic technique used throughout was a serum bottle modification of the Hungate technique (Miller & Wolin, 1974) and the methods of Löffler *et al.* (1996). Cells of strain MS3^T were able to grow under anaerobic as well as aerobic conditions, but the growth rate was higher under anaerobic conditions. Gram-staining was accomplished by the method of Gram (1884). Light microscopy (Eclipse 80*i*; Nikon) and transmission electron microscopy (JEM 1010; JEOL) were used to determine the cell shape and size, the presence of flagella and Gram-staining.

Catalase and oxidase activities were tested with solutions of 3 % (v/v) hydrogen peroxide and 1 % (w/v) *p*-tetramethyl phenylenediamine (bioMérieux), respectively. The carbohydrate metabolism of the isolate (Table 1) was examined by using API 50CH strips (bioMérieux) according to the manufacturer's instructions, with minimal salt medium consisting of (l^{-1} distilled water): 1.0 g NaCl, 0.5 g $MgCl_2.6H_2O$, 0.2 g KH_2PO_4, 0.3 g NH_4Cl, 0.3 g KCl, 0.015 g $CaCl_2.2H_2O$ and trace element solutions A and B (Löffler *et al.*, 1996). The API ZYM and API 20NE kits (bioMérieux) were used according to the manufacturer's protocols to determine the enzyme activities of strain MS3^T.

The fatty acid compositions of strain MS3^T and its two closest phylogenetic relatives (*C. pleistocenium* FTR1^T and

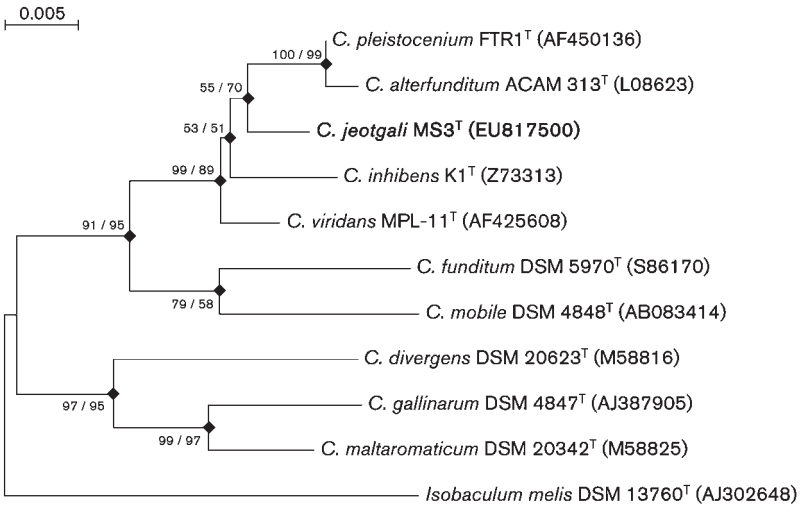

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences. Filled diamonds indicate generic branches that are present in phylogenetic trees generated with the neighbour-joining and maximum-parsimony algorithms. Numbers at nodes indicate bootstrap values as percentages of 1000 replicates (neighbour-joining probability/maximum-parsimony probability). Values <50 % are not shown. Bar, 5 substitutions per 1000 nt positions.

Table 1. Differential phenotypic characteristics between strain MS3^T and the type strains of *Carnobacterium pleistocenium* and *Carnobacterium inhibens*

Strains: 1, MS3^T; 2, *C. pleistocenium* FTR1^T (data from Pikuta *et al.*, 2005); 3, *C. inhibens* K1^T (Joborn *et al.*, 1999). +, Positive; −, negative; (+), weakly positive; ND, not determined.

Characteristic	1	2	3
Temperature range (optimum) (°C)	4–37 (30)	0–28 (24)	0–30
pH range (optimum)	5.5–9.0 (8.5)	6.5–9.5 (7.3–7.5)	5.5–9.0
NaCl range (optimum) (%)	0–5 (2)	0.1–5.0 (0.5)	0–6
Growth on:			
Aesculin	+	ND	+
D-Arabinose	−	+	ND
L-Arabinose	−	ND	−
Erythritol	+	ND	−
D-Glucose	−	+	+
Inositol	+	ND	−
Lactose	−	+	(+)
Maltose	−	+	+
D-Mannose	−	+	+
Starch	−	+	−
Sucrose	−	+	+
Trehalose	−	+	+
DNA G+C content (mol%)	43.9	42±1.5	ND
Isolation source	Jeotgal	Permafrost of thermokarst	Atlantic salmon

C. inhibens K1^T) were determined by GC (6890; Hewlett Packard) and components were identified with the Microbial Identification software package (Sasser, 1990) from cells extracted as described according to the Sherlock Microbial Identification System (MIDI, 1999). The three strains were grown under the same conditions (TSB medium with 0.5 % NaCl at 25 °C and pH 7.3). The predominant fatty acids in all three strains were $C_{14:0}$, $C_{16:0}$, $C_{16:1}\omega9c$ and $C_{18:1}\omega9c$, athough their proportions were different in each strain. The major fatty acid profiles of of strain MS3^T and the two reference strains are presented in Table 2.

The level of genetic relatedness between strain MS3^T and its closest phylogenetic relatives (*C. pleistocenium* FTR1^T, *C. inhibens* K1^T and *C. viridans* MPL-11^T) was further assessed based on DNA–DNA hybridization following extraction with the G-spin Genomic DNA extraction kit (iNtRON Biotechnology). DNA–DNA hybridization experiments were performed according to Ezaki *et al.* (1989) with modifications (Hirayama *et al.*, 1996). It has been reported that bacterial strains showing greater than 97 % 16S rRNA gene sequence similarity do not represent the same species unless the level of DNA–DNA relatedness between them is greater than 70 % (Stackebrandt & Goebel, 1994). The levels of DNA–DNA relatedness between strain MS3^T and *C. pleistocenium* FTR1^T, *C. inhibens* K1^T and *C. viridans* MPL-11^T were 16.0, 5.7 and 9.7 %, respectively. 16S rRNA gene sequence analysis was not sufficient to classify strain MS3^T as representing a novel species. However, DNA–DNA hybridization experiments indicated that strain MS3^T differs from the reference species at the genomic level. To determine the DNA G+C content of strain MS3^T, a fluorimetric method employing SYBR Green I and real-time PCR was utilized

Table 2. Comparative profiles of the cellular fatty acids of strain MS3^T and the type strains of *Carnobacterium pleistocenium* and *Carnobacterium inhibens*

Strains: 1, MS3^T; 2, *C. pleistocenium* FTR1^T; 3, *C. inhibens* K1^T. All data are from the present study. Values shown are percentages of the total fatty acids. tr, Trace (<1.0 %); −, not detected.

Fatty acid	1	2	3
Saturated			
$C_{10:0}$	tr	tr	1.3
$C_{12:0}$	tr	tr	1.2
$C_{14:0}$	4.3	3.6	3.8
$C_{15:0}$	tr	tr	tr
$C_{16:0}$	20.0	19.9	17.7
$C_{18:0}$	tr	1.6	tr
Unsaturated			
$C_{16:1}\omega9c$	40.0	37.3	42.5
$C_{18:1}\omega9c$	30.2	31.1	28.3
$C_{18:1}\omega7c$	tr	1.1	tr
$C_{20:1}\omega9c$	tr	tr	−
Branched			
anteiso-$C_{16:0}$	tr	−	tr
iso-$C_{17:1}\omega9c$	tr	−	tr
$C_{16:0}$ 10 methyl	−	tr	−
Summed feature 3*	2.9	2.6	2.7

*Summed feature 3 comprises $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH.

(Gonzalez & Saiz-Jimenez, 2002). The G+C content of the genomic DNA of strain MS3^T was 43.9 mol% (this compares with 42±1.5 mol% for *C. pleistocenium*; Pikuta *et al.*, 2005).

Based on the phenotypic and genotypic data presented, we suggest that strain MS3^T represents a novel species of the genus *Carnobacterium*, for which the name *Carnobacterium jeotgali* sp. nov. is proposed.

Description of *Carnobacterium jeotgali* sp. nov.

Carnobacterium jeotgali (je.ot.ga′li. N.L. gen. n. *jeotgali* of jeotgal, a traditional Korean fermented seafood).

Gram-positive, non-motile, facultatively anaerobic bacteria. Cells are 3.5±0.7 μm long and 0.7–0.8 μm wide, and occur more frequently in chains than as single cells. Cells lack flagella. Colonies are about 1 mm in diameter, irregular in shape and pale yellow. Colony surface is rough. Grows at 4–37 °C (optimum 30 °C), at pH 5.5–9.0 (optimum pH 8.5) and in the presence of 0–5 % (w/v) NaCl (optimum 2 % NaCl). Oxidase- and catalase-negative. Assimilates erythritol, D-fructose, inositol, D-mannitol and aesculin, but not glycerol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, methyl β-D-xyloside, D-galactose, D-glucose, D-mannose, L-sorbose, L-rhamnose, dulcitol, D-sorbitol, methyl α-D-mannoside, *N*-acetylglucosamine, amygdalin, arbutin, cellobiose, maltose, D-lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose, starch, glycogen, xylitol, gentiobiose, turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate or 2,5-ketogluconate. In API 20NE and API ZYM tests for enzyme activities, negative for nitrate reduction, indole production, D-glucose fermentation, L-arginine dihydrolase and for hydrolysis of β-galactosidase, gelatin and urea, but positive for hydrolysis of aesculin. Positive for acid phosphatase, naphthol-AS-BI-phosphohydrolase and β-glucuronidase, but negative for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, β-glucosidase, lipase (C14), valine arylamidase, α-galactosidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. The predominant fatty acids are $C_{16:0}$, $C_{16:1}\omega9c$ and $C_{18:1}\omega9c$.

The type strain, MS3^T (=KCTC 13251^T=JCM 15539^T), was isolated from 'toha jeotgal', a traditional Korean fermented food. The G+C content of the genomic DNA of the type strain is 43.9 mol%.

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