Salinicoccus carnicancri sp. nov., a halophilic bacterium isolated from a Korean fermented seafood

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A novel, moderately halophilic bacterium belonging to the genus *Salinicoccus* was isolated from crabs preserved in soy sauce: a traditional Korean fermented seafood. Colonies of strain Crm^T were ivory and the cells were non-motile, Gram-positive cocci. The organism was non-sporulating, catalase-positive and oxidase-negative. The major fatty acids of strain Crm^T were iso-$C_{15:0}$ (22.0 %), anteiso-$C_{15:0}$ (40.6 %) and anteiso-$C_{17:0}$ (12.1 %). The cell wall peptidoglycan contained lysine and glycine, and the major isoprenoid quinone was MK-6. The polar lipids were phosphatidylglycerol, diphosphatidylglycerol and an unidentified glycolipid. The genomic DNA G+C content was 47.8 mol%. Strain Crm^T was closely related to the type strain of *Salinicoccus halodurans*, with which it shared 96.9 % 16S rRNA gene sequence similarity. The DNA–DNA hybridization value between strains Crm^T and *S. halodurans* DSM 19336^T was 7.6 %. Based on phenotypic, genetic and phylogenetic data, strain Crm^T should be classified as a novel species within the genus *Salinicoccus*, for which the name *Salinicoccus carnicancri* sp. nov. is proposed. The type strain is Crm^T (=KCTC 13301^T =JCM 15796^T).

The genus *Salinicoccus* was first proposed by Ventosa *et al.* (1990) as an aerobic, Gram-positive, coccus-shaped and moderately halophilic bacterium isolated from a solar saltern. At the time of writing, there are 12 identified species in the genus *Salinicoccus*: *S. roseus* (Ventosa *et al.*, 1990), *S. hispanicus* (Ventosa *et al.*, 1992), *S. alkaliphilus* (Zhang *et al.*, 2002), *S. salsiraiae* (França *et al.*, 2006), *S. jeotgali* (Aslam *et al.*, 2007), *S. luteus* (Zhang *et al.*, 2007), *S. siamensis* (Pakdeeto *et al.*, 2007), *S. kunmingensis* (Chen *et al.*, 2007), *S. iranensis* (Amoozegar *et al.*, 2008), *S. halodurans* (Wang *et al.*, 2008), '*S. salitudinis*' (Chen *et al.*, 2008) and *S. albus* (Chen *et al.*, 2009). Members of the genus *Salinicoccus* are chemotaxonomically characterized by having menaquinone-6 as the predominant isoprenoid quinone, a cell-wall peptidoglycan type based on L-Lys–Gly_5, and a DNA G+C content of 46–51 mol% (Ventosa *et al.*, 1992). In this study, we describe strain Crm^T as a novel species belonging to the genus *Salinicoccus*, based on phenotypic and chemotaxonomic characterization and phylogenetic analysis.

Strain Crm^T was isolated from the Korean traditional fermented seafood called 'ganjang-gejang' in Korean, which are crabs marinated in soy sauce. The strain was isolated by dilution plating at 30 °C on the DSMZ medium no. 372. The isolate was repeatedly restreaked to obtain a pure culture on marine 2216 agar plates (MA; BBL). Growth at different temperatures (0, 4, 10, 15, 20, 25, 30, 37, 45 and 50 °C) was tested on MA supplemented with 10 % (w/v) NaCl. Growth at different pH (5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0) was examined using marine broth (MB; BBL) supplemented with 10 % (w/v) NaCl. The following buffers were used: pH 5.0, 0.1 M acetic acid/0.1 M sodium acetate; pH 6.0, 7.0 and 8.0, 0.1 M KH_2PO_4/0.1 M NaOH; pH 9.0 and 10.0, 0.1 M $NaHCO_3$/0.1 M Na_2CO_3; pH 11.0, 0.05 M Na_2HPO_4/0.1 M NaOH; pH 12.0, 0.2 M KCl/0.2 M NaOH. The NaCl requirements and tolerance of various NaCl concentrations (0, 3, 5, 6, 7, 8, 9, 10, 11, 12, 15, 20, 25 and 30 %) were determined in MB. Strain Crm^T grew in 0–20 % (w/v) NaCl, at 4–45 °C and at pH 6.0–11.0, with optimal growth occurring in 12 % (w/v) NaCl, at 30–37 °C and at pH 7.0–8.0. Unless stated otherwise, all tests were performed on MA supplemented with 10 % NaCl at 30 °C and pH 7.5±0.2. Cellular morphology of strain Crm^T was determined using a light microscope (ECLIPSE 80*i*; Nikon). Gram-reaction was determined by using a Gram Stain kit (BBL) according to

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Crm^T is FJ182049.

A one-dimensional TLC of the phospholipids and glycolipids from strain Crm^T and *Salinicoccus halodurans* DSM 19336^T is available with the online version of this paper.

the manufacturer's instructions and was confirmed by the non-staining method (Buck, 1982). Spore formation was examined using the spore-staining method (Schaeffer & Fulton, 1933). Flagella were determined using the staining method (Heimbrook *et al.*, 1989). Motility was examined by the method of Tittsler and Sandholzer (1936) using semi-solid agar (Motility Test Medium; BBL). Catalase and oxidase activities were individually determined by using a 3 % (v/v) hydrogen peroxide solution and an oxidase reagent (bioMérieux). H_2S production, citrate utilization, Voges–Proskauer reaction and methyl red test were performed according to methods described by Benson (1994). API ZYM and API 20NE strips (bioMérieux) were used according to the manufacturer's instructions to examine the enzyme activities of strain Crm^T. Substrate utilization from sole carbon sources and acid production from carbon carbohydrate were determined with API 50CH test strips (bioMérieux) and Biolog GP2 plates with GN/GP inoculating fluid with salinity adjusted to 10 % (w/v) NaCl, according to the manufacturer's instructions. Degradation of casein, Tween 80, starch, DNA [using DNA agar (BBL)], tyrosine (Atlas, 1993) and cellulose (Gerhardt *et al.*, 1994) was performed on MA supplemented with 10 % (w/v) NaCl.

Cells of strain Crm^T were Gram-stain-positive cocci, approximately 1.0–2.5 μm, and existed singly, or as pairs, tetrads or clumps. Colonies grown on MA supplemented with 10 % (w/v) NaCl for 3 days were 1.0–2.0 mm in diameter and round with an opaque ivory-coloured pigment. Flagella, spores and motility were not observed. Catalase activity of the strain was positive, but oxidase activity, H_2S production, citrate utilization, Voges–Proskauer reaction and methyl red test were negative. The morphological, cultural, physiological and biochemical characteristics of strain Crm^T and related species are shown in Table 1.

Strain Crm^T and reference strain *S. halodurans* DSM 19336^T were grown on MA supplemented with 10 % (w/v) NaCl for 3 days at 30 °C and used for the analysis of cellular fatty acid composition. The cellular fatty acids were extracted and prepared according to standard protocols provided by the MIDI/Hewlett Packard Microbial Identification System (MIDI, 1999; Sasser, 1990). Total lipids were extracted by the modified method of Xin *et al.* (2000). The amino-acid composition of the cell-wall hydrolysate was determined by using one-dimensional TLC on cellulose sheets (Bousfield *et al.*, 1985). Quinone extraction and identification were performed according to the method of Komagata & Suzuki (1987). The major fatty acids were iso-$C_{15:0}$ (22.0 %), anteiso-$C_{15:0}$ (40.6 %) and anteiso-$C_{17:0}$ (12.1 %). The value for anteiso-$C_{15:0}$ of strain Crm^T is less than that of the reference strain *S. halodurans* DSM 19336^T, whereas the value for iso-$C_{15:0}$ is higher than that of the reference strain. The cellular fatty acid composition of strain Crm^T is shown in Table 2. Major cellular polar lipids were phosphatidylglycerol, diphosphatidylglycerol and an unknown glycolipid, and show the same polar lipids pattern as the reference strain *S. halodurans* DSM 19336^T (Supplementary Fig. S1, available in IJSEM Online). The murein type of the cell-wall contained lysine and glycine, and the major menaquinone was MK-6. These chemotaxonomic properties are similar to those of the species belonging to the genus *Salinicoccus*.

The 16S rRNA gene sequence of the isolate was subjected to colony PCR using a PCR master mix solution (iNtRON Biotechnology) with universal primer set as described by Baker *et al.* (2003). The PCR product was purified with a QIAquick PCR Purification kit (Qiagen) and sequenced using the 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 analyser; Applied Biosystems). The partial 16S rRNA gene sequences were assembled using SeqMan software (DNASTAR) and pair-wise 16S rRNA gene sequence similarity was determined using the NCBI website to locate phylogenetic neighbours. The 16S rRNA gene sequence of the isolate was aligned with 13 reference sequences (Fig. 1) using the multiple sequence alignment program CLUSTAL_X (1.83) (Thompson *et al.*, 1997). The phylogenetic relationships of representatives of the genus *Salinicoccus* were determined using the MEGA version 4 software program (Tamura *et al.*, 2007). Phylogenetic trees were determined by neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Kluge & Farris, 1969) and a phylogenetic consensus tree was reconstructed by randomly selecting 1000 bootstrap replicates (Felsenstein, 1985). Chromosomal DNA was extracted using a G-spin DNA extraction kit (iNtRON Biotechnology), and the G+C content was determined by using a fluorometric method employing SYBR Green I and a real-time PCR thermocycler (Gonzalez & Saiz-Jimenez, 2002). Genomic DNA of *Escherichia coli* K-12 was used as the calibration reference (Gonzalez & Saiz-Jimenez, 2002). DNA–DNA hybridization was performed using the fluorometric method of Ezaki *et al.* (1989) with modifications (Hirayama *et al.*, 1996). The genomic DNA G+C content of the recognized species belonging to the genus *Salinicoccus* is in the range 46–51 mol% (Ventosa *et al.*, 1992). The G+C content of genomic DNA of strain Crm^T is 47.8 mol%, which falls within the range for the genus *Salinicoccus*. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain Crm^T is associated with the genus *Salinicoccus*. Strain Crm^T was most closely related to *S. halodurans* DSM 19336^T (96.9 %), *S. hispanicus* DSM 5352^T (95.6 %), *S. roseus* DSM 5351^T (95.3 %), *S. salsiraiae* LMG 22840^T (95.2 %) and *S. jeotgali* KCTC 13030^T (95.2 %). Other species of the genus *Salinicoccus* had 16S rRNA gene sequence similarities of less than 95.0 % with strain Crm^T. The genomic DNA hybridization value between strain Crm^T and *S. halodurans* DSM 19336^T was 7.6 %. It has been shown that two strains with 16S rRNA gene sequence similarity values of less than 97.0 % and DNA–DNA hybridization values of less than 70 % represent different species (Stackebrandt & Goebel, 1994; Wayne *et al.*, 1987).

Table 1. Taxonomic characteristics of strain Crm^T and closely related type strains of the genus *Salinicoccus*

Strains: *Salinicoccus carnicancri* sp. nov. Crm^T (data from this study); 2, *S. halodurans* DSM 19336^T (Wang *et al.*, 2008); 3, *S. hispanicus* DSM 5352^T (Ventosa *et al.*, 1992); 4, *S. jeotgali* KCTC 13030^T (Aslam *et al.*, 2007); 5, *S. alkaliphilus* JCM 11311^T (Zhang *et al.*, 2002); 6, *S. salsiraiae* LMG 22840^T (França *et al.*, 2006); 7, *S. roseus* DSM 5351^T (Ventosa *et al.*, 1990); 8, *S. siamensis* JCM 12822^T (Pakdeeto *et al.*, 2007); 9, *S. kunmingensis* DSM 17847^T (Chen *et al.*, 2007); 10, '*S. salitudinis*' DSM 17846^T (Chen *et al.*, 2008); 11, *S. albus* DSM 19776^T (Chen *et al.*, 2009); 12, *S. luteus* KCTC 3941^T (Zhang *et al.*, 2007); 13, *S. iranensis* DSM 18903^T (Amoozegar *et al.*, 2008). +, Positive reaction; −, negative reaction; ND, data not available.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13
Cell diameter (μm)	1.0–2.5	0.6–0.9	1.0–2.0	1.0–2.0	0.5–0.8	1.0–2.5	1.0–2.5	0.6–0.8	0.8–1.2	0.9–1.5	0.6–1.1	0.9	0.8–1.0
Pigmentation	Ivory	White	Reddish orange	Orange	Pinkish	Pink–red	Pink–red	Orange	Yellow	Pale yellow	White	Orange	Orangish pink
pH for growth													
Range	6.0–11.0	5.5–9.0	5.0–9.0	6.5–11.0	6.5–11.5	6.5–9.5	6.0–9.0	6.0–9.0	6.0–10.0	6.0–11.0	6.0–10.0	7.0–11.0	6.5–10
Optimum	7.0–8.0	7.5	7.5	7.0	9.5	8.0	8.0	8.5	8.0	8.0	8.5	8.0–9.0	7.5
Temperature for growth (°C)													
Range	4–45	8–43	15–37	20–30	10–49	20–45	15–40	15–45	4–45	4–37	5–40	4–45	5–45
Optimum	30–37	28	37	30	32	37	37	37	37	25	25	30	35
Salinity for growth (%, w/v)													
Range	0–20	0–24	0.5–25	0–22	0–25	0–22	0.9–25	1.5–25	0.5–25	1–25	1–30	1–25	1–25
Optimum	12	8	10	4	10	4	10	10	8–10	10–12	10	10	7.5–10
Acid production from:													
Sucrose	+	+	+	−	−	−	−	−	+	+	−	+	−
Maltose	+	+	+	+	−	+	−	−	−	−	−	+	−
D-Mannitol	+	+	+	−	+	−	−	−	+	−	−	ND	+
Trehalose	+	+	−	+	+	+	−	+	−	−	−	ND	ND
D-Galactose	−	−	+	−	−	−	−	−	−	−	−	−	−
Hydrolysis of:													
Aesculin	+	+	+	+	+	−	−	−	+	+	−	+	−
Casein	+	−	−	−	−	+	+	−	−	−	−	−	−
Gelatin	−	+	+	−	−	+	+	−	−	−	−	−	−
Nitrate reduction	−	+	+	+	+	+	+	−	+	+	+	+	+
Urease	+	+	+	−	+	−	−	−	−	−	−	−	−
DNA G+C content (mol%)	47.8	45.8	45.7	47.0	49.6	46.2	51.2	46.0	46.2	46.5	46.1	49.7	54.5

Table 2. Fatty acid composition (%) of strain Crm^T and *Salinicoccus halodurans* DSM 19336^T

Strains: 1, *S. carnicancri* sp. nov. Crm^T; 2, *S. halodurans* DSM 19336^T. All data shown are from the present study. Values are percentages of total fatty acids. tr, Trace (less than 1.0 %); −, not detected.

Fatty acid	1	2
iso-$C_{14:0}$	1.18	1.37
iso-$C_{15:1}$ AT 5	−	tr
iso-$C_{15:0}$	22.00	14.33
anteiso-$C_{15:0}$	40.61	43.01
$C_{16:1}\omega7c$ alcohol	2.01	2.03
iso-$C_{16:0}$	3.66	4.02
$C_{16:1}\omega11c$	tr	−
Unknown (ECL 15.669)	−	1.05
$C_{16:0}$	tr	tr
$C_{17:1}\omega10c$	5.29	5.65
iso-$C_{17:0}$	6.17	5.73
anteiso-$C_{17:0}$	12.12	15.57
iso-$C_{19:0}$	1.51	1.08
anteiso-$C_{19:0}$	1.46	2.19

Characteristics such as major fatty-acid profile, predominant isoprenoid quinone and cell-wall peptidoglycan type, as well as genomic DNA G+C content, indicate that strain Crm^T belongs to the genus *Salinicoccus*. However, the morphological, cultural, physiological and biochemical characteristics of strain Crm^T can be used to distinguish this novel strain from the described species of the genus *Salinicoccus*. In addition, the low 16S rRNA gene sequence similarities with species of the genus *Salinicoccus* and the low level of DNA–DNA relatedness clearly support the recognition of strain Crm^T as a novel species of the genus *Salinicoccus*.

Thus, on the basis of phenotypic, genotypic and phylogenetic comparisons to previously described taxa, strain Crm^T is a novel species of the genus *Salinicoccus*, for which the name *Salinicoccus carnicancri* sp. nov. is proposed.

Description of *Salinicoccus carnicancri* sp. nov.

Salinicoccus carnicancri (car.ni.can′cri. L. n. *caro carnis* flesh; L. n. *cancer -cri* a crab; N.L. gen. n. *carnicancri* of the flesh of a crab).

Cells are non-motile, non-sporulating, Gram-stain-positive cocci with a diameter of 1.0–2.5 μm, and exist singly or as pairs, tetrads or clumps. Colonies are ivory-coloured, circular and measure 1.0–2.0 mm in diameter after 3 days culture on MA supplemented with 10 % NaCl at 30 °C. Growth occurs in 0–20 % (w/v) NaCl, at temperatures ranging from 4–45 °C, and in the pH range 6.0–11.0. Optimal growth at 30–37 °C, pH 7.0–8.0 and with a NaCl concentration of 12 %. Catalase-positive and oxidase-negative. Casein and tyrosine hydrolysis occurs, but DNA, starch, Tween 80 and cellulose hydrolysis does not. Possesses alkaline phosphatase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase and β-glucosidase activities (API ZYM). Esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase activities are not observed. Acid is produced from glycerol, D-ribose, D-glucose, D-fructose, D-mannose, D-mannitol, *N*-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, maltose, sucrose, trehalose and 5-ketogluconate. The following substrates of Biolog GP2 plates can be utilized as sole carbon and energy sources: *N*-acetyl-D-glucosamine, *N*-acetyl-β-D-mannosamine, arbutin, D-fructose, α-D-glucose, maltose, D-mannitol, D-mannose, β-methyl-D-glucoside, D-psicose, D-ribose, salicin, trehalose, α-hydroxybutyric acid, β-hydroxybutyric acid, L-lactic acid, pyruvic acid, L-alaninamide, L-alanine, L-alanyl glycine, L-glutamic acid, L-serine and glycerol. The major fatty acids

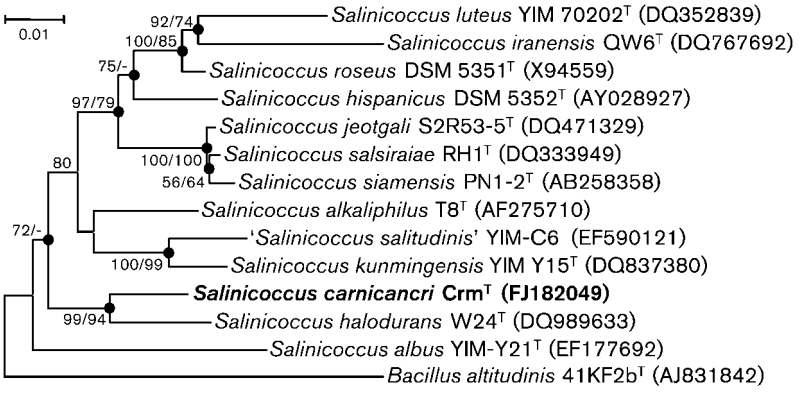

Fig. 1. Phylogenetic consensus tree based on 16S rRNA gene sequence showing the relationship between strain Crm^T and type strains of the most closely related species of the genus *Salinicoccus*. The GenBank accession number for each strain is enclosed in parentheses. Filled circles indicate generic branches that were present in phylogenetic trees generated by both the neighbour-joining algorithm and the maximum-parsimony algorithm. Numbers at nodes indicate percentage bootstrap values, as calculated by neighbour-joining/maximum-parsimony probabilities. Bootstrap analyses were performed with 1000 repetitions and only values higher than 50 % are shown. Bar, 0.01 accumulated changes per nucleotide.

are iso-$C_{15:0}$, anteiso-$C_{15:0}$ and anteiso-$C_{17:0}$. The major cellular polar lipids are phosphatidylglycerol, diphosphatidylglycerol and an unknown glycolipid. The major amino acid constituents of the cell-wall hydrolysate are glycine and lysine, and the major menaquinone is MK-6. The DNA G+C content of the type strain is 47.8 mol%.

The type strain is Crm^T (=KCTC 13301^T =JCM 15796^T), which was isolated from traditional Korean fermented seafood.

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