Salinicoccus jeotgali sp. nov., isolated from jeotgal, a traditional Korean fermented seafood

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A novel, moderately halophilic, Gram-positive coccus, designated strain S2R53-5^T, was isolated from jeotgal, a traditional Korean fermented seafood. The organism was strictly aerobic, non-motile, non-sporulating and catalase- and oxidase-positive. Strain S2R53-5^T grew in the presence of 0.5–15 % (w/v) NaCl and at pH 6.5–11.0, with optimum growth at 5 % (w/v) NaCl and pH 7.0. The temperature range for growth was 20.0–30.0 °C, with an optimum temperature of 30 °C. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain S2R53-5^T belongs to the family *Staphylococcaceae* and was most closely related to *Salinicoccus roseus* DSM 5351^T (96.8 % gene sequence similarity), *Salinicoccus hispanicus* DSM 5352^T (96.1 %), *Salinicoccus alkaliphilus* T8^T (95.2 %) and *Jeotgalicoccus halotolerans* YKJ-101^T (95.1 %). The genomic DNA G + C content was 47.0 mol%, which is in the range of 46–51 mol% that is characteristic for the genus *Salinicoccus*. Levels of DNA–DNA relatedness between strain S2R53-5^T and *S. roseus* DSM 5351^T, *S. hispanicus* DSM 5352^T and *S. alkaliphilus* KCTC 13928^T were 32.2, 15.4 and 4.6 %, respectively. Chemotaxonomic data (major menaquinone, MK-6; major fatty acids, iso-C_{15:0} and anteiso-C_{15:0}; cell-wall murein type, Lys and Gly) and 16S rRNA gene sequence analysis supported the affiliation of strain S2R53-5^T with the genus *Salinicoccus*. The combined evidence from the low DNA–DNA relatedness, physiological, biochemical and other genotypic data indicate that strain S2R53-5^T clearly represents a novel species of the genus *Salinicoccus*, for which the name *Salinicoccus jeotgali* sp. nov. is proposed. The type strain is S2R53-5^T (=KCTC 13030^T =LMG 23640^T).

Jeotgal is an important traditional fermented seafood in Korea. There are more than 100 types of jeotgal, made mostly from fish, crustaceans and molluscs. Jeotgal is important not only as a source of protein, with its peculiar flavour, relative abundance of free amino acids and taste related components, but also because of its high content of lactic acid and non-lactic acid bacteria. Jeotgal has a high salt concentration of almost 8 % (Park *et al.*, 2002). Salt-resistant aerobic and anaerobic bacteria exist in most jeotgals (Lee, 1993). Recent studies have reported the isolation of many novel species and genera from jeotgal: *Bacillus jeotgali* (Yoon *et al.*, 2001a), *Jeotgalibacillus alimentarius* (Yoon *et al.*, 2001b), *Planomicrobium koreense* (Yoon *et al.*, 2001c), *Halomonas alimentaria* (Yoon *et al.*,

2002), *Jeotgalicoccus halotolerans* and *Jeotgalicoccus psychrophilus* (Yoon *et al.*, 2003a), *Psychrobacter jeotgali* (Yoon *et al.*, 2003b), *Bacillus cibi* (Yoon *et al.*, 2005a), *Psychrobacter alimentarius* (Yoon *et al.*, 2005b) and *Psychrobacter cibarius* (Jung *et al.*, 2005).

The genus *Salinicoccus* was first proposed by Ventosa *et al.* (1990) based on a single species, *Salinicoccus roseus* DSM 5351^T. *Marinococcus hispanicus* was later transferred to the genus *Salinicoccus* as *Salinicoccus hispanicus* DSM 5352^T (Ventosa *et al.*, 1992). At the time of writing, there were three recognized species in the genus *Salinicoccus*; *S. roseus* DSM 5351^T, *S. hispanicus* DSM 5352^T and *Salinicoccus alkaliphilus* T8^T (Zhang *et al.*, 2002). The genus *Salinicoccus* is characterized chemotaxonomically by having menaquinone-6 as the predominant isoprenoid quinone, a cell-wall peptidoglycan type based on L-Lys–Gly₅ and a DNA G + C content of 46–51 mol% (Ventosa *et al.*, 1992).

In this study, a polyphasic approach, including phylogenetic analysis based on 16S rRNA gene sequences,

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain S2R53-5^T is DQ471329.

Supplementary tables comparing additional characteristics and the fatty acid content of strain S2R53-5^T with members of the genus *Salinicoccus* and other related genera are available in IJSEM Online.

chemotaxonomic and phenotypic properties, was adopted to determine the precise taxonomic position of strain S2R53-5^T. The results obtained in this study indicate that strain S2R53-5^T can be assigned to the genus *Salinicoccus* and the novel strain could be clearly distinguished from the other *Salinicoccus* species with validly published names. Here, we propose that strain S2R53-5^T represents a novel species.

During the study of the bacterial diversity of jeotgal, it has been identified as a source not only of lactic acid bacteria and yeast, but of many other bacteria belonging to the phyla *Actinobacteria*, *Firmicutes* and *Proteobacteria*. To investigate the bacterial diversity of jeotgal, we took fish- (final pH 5.4) and shrimp-jeotgal (final pH 7.5) samples from the Daejeon and Suwon fish markets in Korea. One sample of shrimp-jeotgal (final pH 7.5) packed in a glass jar (net weight 160 g, www.han-sung.co.kr) was also tested. A small portion of each of these samples was blended with a homogenizer (Ace homogenizer; Nihonseiki Kaisha) for 2 min, serially diluted and then applied to R2A (Difco) medium containing 0.5 g yeast extract, 0.5 g proteose peptone, 0.5 g Casamino acids, 0.5 g glucose, 0.5 g soluble starch, 0.3 g sodium pyruvate, 0.3 g dipotassium phosphate, 0.05 g magnesium sulphate and 15.0 g agar dissolved in 1 l of distilled water with different concentrations of NaCl (0, 5, 10 and 20 %, w/v) at pH 7.0 and incubated at 30 °C. After one week of incubation, many pigmented and non-pigmented colonies appeared on the R2A agar with 5 % NaCl (w/v). One orange-pigmented strain, strain S2R53-5^T, was transferred to new saline medium [R2A agar + 5 % NaCl (w/v)]. All procedures were conducted in an aerobic atmosphere. This strain was derived from a sample of the shrimp-jeotgal packed in a glass jar. This novel strain was grown on saline medium at pH 7.0 and at 30 °C and was also preserved as a glycerol suspension (20 %, w/v) at -70 °C.

The Gram reaction was performed by the non-staining method as described by Buck (1982). Cell morphology was observed under a Nikon light microscope at ×1000, with cells grown for 3 days at 30 °C on saline agar. Catalase and oxidase tests were performed according to Cappuccino & Sherman (2002). Substrate utilization as a sole carbon and energy source, the fermentation/oxidation profile and some other physiological characteristics were determined with API 32GN, API 20E and API 50CH (with API 50 CHB/E medium) galleries according to the manufacturer's instructions (bioMérieux). Growth at 4, 10, 15, 20, 25, 30, 37 and 45 °C was tested in saline agar. Salt tolerance was tested in R2A broth medium supplemented with 1–20 % (w/v) NaCl. Growth at different temperatures, pH values and NaCl concentrations was assessed after one week of incubation. Degradation of DNA [using DNA agar (Difco) flooded with 1 M HCl], casein, chitin, Tween 80, starch (Atlas, 1993), xylan and cellulose (Ten *et al.*, 2004) were tested using 5 % NaCl (w/v) and 1.5 % agar (w/v). These tests were evaluated after two weeks of incubation.

Duplicate antibiotic sensitivity tests were performed using filter-paper disks containing the following: ampicillin,

tetracycline, kanamycin (Sigma) and rifampicin, each containing 5, 10, 50 and 100 µg ml⁻¹. Disks were placed on saline agar plates spread with strain S2R53-5^T which were then incubated at 30 °C for 7 days. The physiological and biochemical characteristics of the novel strain and some related type strains are summarized in Table 1.

A nearly complete 16S rRNA gene sequence for strain S2R53-5^T was determined as described below. Genomic DNA was extracted and purified from each of the isolates using DNA extraction kits (Solgent). The 16S rRNA gene was amplified from the chromosomal DNA using the universal bacterial primer set 9F and 1512R and the purified PCR products were sequenced by Solgent (Kim *et al.*, 2005). The nearly complete 16S rRNA gene sequences were compiled using SeqMan software (DNASTAR). The 16S rRNA gene sequences of related taxa were obtained from the GenBank database. Multiple alignments were performed using the CLUSTAL_X program (Thompson *et al.*, 1997). Gaps were edited using the BioEdit program (Hall, 1999). Evolutionary distances were calculated by the method of Jukes & Cantor (1969). The phylogenetic tree was constructed by using a neighbour-joining method (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) using MEGA3 software (Kumar *et al.*, 2004), with bootstrap values based on 1000 replications (Felsenstein, 1985).

In order to confirm whether strain S2R53-5^T represented a novel species, DNA–DNA hybridizations were carried out with *S. roseus* DSM 5351^T, *S. hispanicus* DSM 5352^T and *S. alkaliphilus* KCTC 13928^T using photobiotin-labelled probes in microplate wells, as described by Ezaki *et al.* (1989). A microplate fluorescence reader (FLX 800; Bio-Tek) was employed for fluorescence measurements. For the measurement of the G+C content of the chromosomal DNA, genomic DNA of the strains was extracted and purified as described by Ausubel *et al.* (1995). The DNA was then enzymically degraded into nucleosides and the G+C content was determined as described by Mesbah *et al.* (1989) using reversed-phase HPLC. Isoprenoid quinones were extracted with chloroform/methanol (2:1, v/v), evaporated under vacuum conditions and reextracted in n-hexane-water (1:1, v/v). The crude n-hexane–quinone solution was then purified using Sep-Pak Vac silica cartridges (Waters) and analysed by HPLC as described previously (Hiraishi *et al.*, 1996). Cellular fatty acids were analysed from micro-organisms grown on saline agar for 2 days. Cellular fatty acids were saponified, methylated and extracted according to the protocol of the Sherlock Microbial Identification System (MIDI). Fatty acids analysed by GC (6890; Hewlett Packard) were identified by the microbial identification software package (Sasser, 1990). The peptidoglycan structure of the cell wall was determined by one-dimensional TLC (Schleifer & Kandler, 1972) followed by derivatization (MacKenzie, 1987).

Cells of strain S2R53-5^T were strictly aerobic, Gram-positive cocci, 1.0–2.0 µm in diameter (Fig. 1). Colonies grown on saline agar plates (Difco) for 3 days were smooth, circular,

Table 1. Comparison of selected characteristics of *Salinicoccus jeotgali* S2R53-5^T with related taxa

Taxa: 1, *Salinicoccus jeotgali* S2R53-5^T (data from this study); 2, *Salinicoccus roseus* DSM 5351^T (Ventosa *et al.*, 1990); 3, *Salinicoccus hispanicus* DSM 5352^T (Marquez *et al.*, 1990; Ventosa *et al.*, 1992); 4, *Salinicoccus alkaliphilus* T8^T (Zhang *et al.*, 2002); 5, *Jeotgalicoccus halotolerans* YKJ-101^T; 6, *Jeotgalicoccus psychrophilus* YKJ-115^T (data for taxa 5–6 from Yoon *et al.*, 2003a). +, Positive; –, negative; w, weakly positive reaction; ND, not determined; D, differs among strains. All species are positive for Gram-staining and catalase- and oxidase-activities. All species are negative for spore formation and motility.

Characteristic	1	2	3	4	5	6
Diameter of cells (µm)	1.0–2.0	1.0–2.5	1.0–2.5	0.5–0.8	0.6–1.1	0.6–1.1
Colony pigmentation	Orange	Pink–red	Reddish orange	Pinkish	Light yellow	Light yellow
Anaerobic growth	–	–	–	–	+	+
pH for growth:						
Range	6.5–11.0	5.0–9.0	6.0–9.0	6.5–11.5	5.5–8.0	5.5–8.0
Optimum	7.0	7.5	8.0	9.5	7.0–8.0	7.0–8.0
Salt range for growth (%; w/v)	0.5–15.0	0.5–25.0	0.9–25.0	0–25.0	0–20.0	1.0–14.0
Temperature for growth (°C):						
Range	20–30	15–37	15–40	10–49	4–42	4–34
Optimum	30	37	37	32	30–35	20–25
Acid production from:						
Arabinose	–	–	–	+	+	–
Fructose	+	–	+	–	–	–
Galactose	–	–	+	–	–	–
D-Glucose	+	–	+	+	–	–
Glycerol	+	–	+	–	ND	ND
Maltose	+	–	+	–	–	–
D-Mannitol	–	–	+	+	+	–
Sucrose	–	–	D	–	–	W
Hydrolysis of:						
Aesculin	+	–	+	+	–	–
Casein	–	+	–	–	–	–
Gelatin	–	+	+	–	ND	ND
Starch	–	+	–	–	–	–
Tween 80	–	+	–	–	–	–
Tyrosine	ND	+	–	–	+	+
Nitrate reduction	+	–	+	+	–	–
Methyl red test	+	–	+	–	ND	ND
Urease	–	–	+	+	–	–
Peptidoglycan type	L-Lys–Gly ₅	L-Lys–Gly ₅	L-Lys–Gly ₅	L-Lys–Gly ₅	L-Lys–Gly _{3–4} –L-Ala (Gly)	L-Lys–Gly _{3–4} –L-Ala (Gly)
Predominant menaquinone	MK-6	MK-6	MK-6	MK-6	MK-7	MK-7
DNA G + C content (mol%)	47.0	51.2	45.6–49.3	49.6	42.0	42.0

orange-coloured with transparent edges and 1–2 mm in diameter. On saline agar, strain S2R53-5^T was able to grow at 20–30 °C, but not at 15 or 37 °C. The physiological characteristics of strain S2R53-5^T are summarized in the species description and a comparison of selected characteristics with those of related type strains is presented in Table 1. Further characteristics that differentiate strain S2R53-5^T from other similar Gram-positive, coccus strains are given in Supplementary Table S1 (available in IJSEM Online).

The 16S rRNA gene sequence of strain S2R53-5^T was a continuous stretch of 1480 bp. Sequence similarity calculations indicated that the closest relatives of strain S2R53-5^T were *S. roseus* DSM 5351^T (96.8%), *S. hispanicus* DSM

5352^T (96.1%) and *S. alkaliphilus* T8^T (95.2%). The levels of DNA–DNA relatedness between strain S2R53-5^T and *S. roseus* DSM 5351^T, *S. hispanicus* DSM 5352^T and *S. alkaliphilus* KCTC 13928^T were 32.2, 15.4 and 4.6%, respectively. It has been suggested that bacterial strains with less than 97% 16S rRNA gene sequence identity and a DNA–DNA relatedness level of less than 70% are not members of the same species (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994). In previous studies, we have found corresponding trends (Aslam *et al.*, 2005a, b). Thus, based on the 16S rRNA gene sequence analysis and DNA–DNA hybridization data, a novel taxon was detected.

Chemotaxonomic data obtained from strain S2R53-5^T are consistent with those expected for members of the genus

Fig. 1. Phase-contrast photomicrograph of cells of *Salinicoccus jeotgali* S2R53-5^T grown on saline medium. Bar, 4 µm.

Salinicoccus (Ventosa *et al.*, 1990, 1992). The G + C content of the genomic DNA of strain S2R53-5^T was 47.0 mol% and the novel strain had MK-6 as its major menaquinone and MK-7 present in trace amounts. The major fatty acids of strain S2R53-5^T were iso-C_{15:0} (22.8 %) and anteiso-C_{15:0} (32.4 %). The fatty acid profiles of strain S2R53-5^T and related type strains are given in Supplementary Table S2, available in IJSEM Online. The cell-wall murein type of the novel strain was L-Lys and Gly₅. These data are also

compatible with the assignment of strain S2R53-5^T to the genus *Salinicoccus*.

In a previous study (Ventosa *et al.*, 1992), *Marinococcus hispanicus* was transferred to the genus *Salinicoccus* as *S. hispanicus* on the basis that it contains MK-6 as a major menaquinone, contains straight and branched chain fatty acids and has L-Lys and Gly₅ as the cell-wall murein type along with some other phenotypic characteristics. It is clear that strain S2R53-5^T belongs to the genus *Salinicoccus* on the basis of chemotaxonomic data (major menaquinone, MK-6; major fatty acids, iso-C_{15:0} and anteiso-C_{15:0}; cell-wall murein type, L-Lys and Gly₅), phylogenetic analysis and some other morphological and biochemical characteristics. However, strain S2R53-5^T is also different to the other recognized species of the genus *Salinicoccus*. Strain S2R53-5^T has a low 16S rRNA gene sequence similarity with *S. roseus* DSM 5351^T (96.8 %), *S. hispanicus* DSM 5352^T (96.1 %) and *S. alkaliphilus* T8^T (95.2 %), low levels of DNA–DNA relatedness and a separate position in the phylogenetic analysis (Fig. 2). In addition, the G + C content of genomic DNA (47.0 %), fatty acid profile and growth factors, such as pH and temperature range and NaCl tolerance, and some biochemical characteristics, such as acid production from maltose, glycerol, glucose and fructose (Table 1), clearly support the recognition of strain S2R53-5^T as a novel species within the genus *Salinicoccus*, for which the name *Salinicoccus jeotgali* sp. nov. is proposed.

Fig. 2. Phylogenetic tree constructed from a comparative analysis of 16S rRNA gene sequences showing the relationships of *Salinicoccus jeotgali* sp. nov. S2R53-5^T with related taxa. The phylogenetic tree was constructed by using the neighbour-joining method and Jukes & Cantor evolutionary distance matrix data obtained from unambiguous aligned nucleotides. Solid circles indicate that the corresponding clades were also recovered in maximum-parsimony trees. Bootstrap values (expressed as percentage of 1000 replications) greater than 50 % are shown at the branch points. Bar, 1 substitution per 100 nucleotide positions.

Description of *Salinicoccus jeotgali* sp. nov.

Salinicoccus jeotgali (je.ot.ga'li. N.L. gen. n. *jeotgali* of jeotgal, a traditional Korean fermented seafood, from which the type strain was isolated).

Cells are strictly aerobic, Gram-positive, coccus-shaped, non-motile and 1.0–2.0 µm in diameter. Colonies on R2A supplemented with 5% NaCl for 2–4 days are smooth, circular, orange-coloured with transparent edges and 1–2 mm in diameter. Growth occurs at pH 6.5–11.0 (optimum, pH 7.0), at temperatures of 20–30 °C (optimum, 30 °C) and in R2A media supplemented with 0.5–15.0% (w/v) NaCl [optimum, 5.0% (w/v) NaCl]. Tests for catalase and oxidase activity are positive. H₂S and indole are produced. Nitrate is reduced to nitrite, but not to nitrogen gas. Aesculin is hydrolysed. Urease, β-glucosidase, cellulase, xylanase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, amylase, lipase, chitinase, caseinase, DNase and gelatinase are not produced. Acetoin is produced. Tryptophan deaminase activity is positive. Acid is produced from D-glucose, D-fructose, D-maltose, D-trehalose, N-acetyl-D-glucosamine and glycerol, but not from D-mannitol, sucrose, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, D-galactose, D-mannose, L-sorbose, L-rhamnose, D-sorbitol, D-cellobiose, D-lactose, D-melibiose, D-melezitose, D-raffinose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, erythritol, methyl β-D-xylopyranoside, dulcitol, inositol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, amygdalin, arbutin, salicin, inulin, starch, glycogen, xylitol, gentiobiose, potassium gluconate, potassium 2-ketogluconate or potassium 5-ketogluconate. The following are utilized as sole carbon and energy sources: salicin, L-arabinose, D-glucose, citrate, histidine, L-rhamnose, N-acetyl-D-glucosamine, D-maltose, D-lactate, L-alanine and 5-ketogluconate. The following are not utilized as sole carbon and energy sources: mannitol, D-melibiose, L-fucose, D-sorbitol, propionate, caprate, valerate, 2-ketogluconate, 3-hydroxybutyrate, L-proline, D-ribose, inositol, D-sucrose, itaconate, suberate, malonate, acetate, glycogen, 3-hydroxybenzoate, 4-hydroxybenzoate and L-serine. The type strain is resistant to 100 µg ampicillin ml⁻¹, 100 µg tetracycline ml⁻¹, 100 µg rifampicin ml⁻¹ and 100 µg kanamycin ml⁻¹. The major cellular fatty acids are iso-C_{15:0} (22.8%) and anteiso-C_{15:0} (32.4%). The G + C content of the genomic DNA of the type strain is 47.0 mol% (as determined by HPLC). The predominant lipoquinone is MK-6 and MK-7 is present in trace amounts. The assumed cell-wall murein type is L-Lys–Gly₅.

The type strain S2R53-5^T (= KCTC 13030^T = LMG 23640^T), was isolated from jeotgal, a traditional Korean fermented seafood.

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