International Journal of Systematic and Evolutionary Microbiology (2010), **60**, 620–626 **DOI** 10.1099/ijs.0.008847-0

Cobetia crustatorum sp. nov., a novel slightly halophilic bacterium isolated from traditional fermented seafood in Korea

Min-Soo Kim, Seong Woon Roh and Jin-Woo Bae

Department of Life and Nanopharmaceutical Sciences and Department of Biology, Kyung Hee University, Seoul 130-701, Republic of Korea

Correspondence
Jin-Woo Bae
baejw@khu.ac.kr

A slightly halophilic, Gram-stain-negative, straight-rod-shaped aerobe, strain JO1^T, was isolated from jeotgal, a traditional Korean fermented seafood. Cells were observed singly or in pairs and had 2–5 peritrichous flagella. Optimal growth occurred at 25 °C, in 6.5 % (w/v) salts and at pH 5.0–6.0. Strain JO1^T was oxidase-negative and catalase-positive. Cells did not reduce fumarate, nitrate or nitrite on respiration. Acid was produced from several carbohydrates and the strain utilized many sugars and amino acids as carbon and nitrogen sources. The main fatty acids were $C_{12:0}$ 3-OH, $C_{16:0}$, $C_{17:0}$ cyclo and summed feature 3 ($C_{16:1}\omega 7c$/iso-$C_{15:0}$ 2-OH). DNA–DNA hybridization experiments with strain JO1^T and *Cobetia marina* DSM 4741^T revealed 24 % relatedness, although high 16S rRNA gene sequence similarity (98.9 %) was observed between these strains. Based on phenotypic, genotypic and phylogenetic analyses, it is proposed that the isolate from jeotgal should be classified as a representative of a novel species, *Cobetia crustatorum* sp. nov., with strain JO1^T (=KCTC 22486^T=JCM 15644^T) as the type strain.

The genus *Cobetia* comprises aerobic, Gram-stain-negative, slightly halophilic, rod-shaped micro-organisms. It is distinct from the genus *Halomonas* (Arahal *et al.*, 2002a) and is one of 11 genera of the family *Halomonadaceae*. Historically, *Cobetia marina* was first described as *Arthrobacter marinus* by Cobet *et al.* (1970), but was later identified as *Pseudomonas marina* (Baumann *et al.*, 1972). Approximately 10 years later, *Cobetia marina* was reclassified once again within the genus *Deleya* (Baumann *et al.*, 1983) and then transferred to the genus *Halomonas* when the genera *Deleya*, *Halomonas* and *Halovibrio*, and *Paracoccus halodenitrificans* were unified by Dobson & Franzmann (1996). After that, the number of the *Halomonas* species increased following studies of micro-organisms from different saline environments. As a result, the genus *Halomonas* contained species that were, in some cases, too different to justify their placement within the genus and the genus became heterogeneous (Arahal *et al.*, 2002a; Romanenko *et al.*, 2002). Delineation of the species in this genus was performed based on 16S and 23S rRNA sequence similarities (Arahal *et al.*, 2002a, b). The sequence of *Halomonas marina* could be distinguished clearly from those of *Halomonas* and *Chromohalobacter* species based on 16S and 23S rRNA analyses and additional phenotypic evidence supported the inclusion of *Halomonas marina* in the novel genus *Cobetia* (Arahal *et al.*, 2002a) as *Cobetia marina*. In a recent study of the microbial diversity of Korean traditional fermented seafood, a *Cobetia*-like strain, JO1^T, was isolated.

Jeotgal, a traditional Korean fermented seafood, tastes salty with a slightly sour flavour. There are about 150 types of jeotgal, which has been consumed by Koreans since 683 AD according to ancient records (Suh & Yoon, 1987). A strain, designated strain JO1^T, was isolated from a traditional fermented seafood in Korea called 'Jogae jeotgal'. It is generally made from the meat of thin-shelled surf clams, large clams and plenty of salt. Strain JO1^T was isolated on marine agar (MA; BBL) containing 7.5 % salts. In this study, it is proposed that, based on molecular biological analyses and biochemical and phenotypic characteristics, strain JO1^T represents a novel species belonging to the genus *Cobetia*.

The isolate was transferred 2 or 3 times onto MA containing 7.5 % salts to obtain a pure culture. The 16S rRNA gene sequence of the isolate was amplified by Colony PCR with PCR Pre-Mix (SolGent) and two bacteria-specific primers (8F, 1492R) (Baker *et al.*, 2003). After purification (QIAquick PCR Purification kit), the PCR product was sequenced using a BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) according to the manufacturer's instructions. The reaction mixtures were analysed by using an automated DNA analyser system (PRISM 3730XL DNA analyzer; Applied Biosystems). The 16S rRNA gene sequence of the isolate was then compared with other sequences in the GenBank (NCBI) database. As a result, the isolate was identified as a member of the genus *Cobetia* in the

Abbreviation: PHB, poly-β-hydroxybutyric acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain JO1^T is EU909460.

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Gammaproteobacteria; its 16S rRNA gene sequence shared 98.9 % similarity with that of *C. marina* DSM 4741^{T}. Moreover, 16S rRNA gene sequence similarities between the isolate and members of the genus *Halomonas* were less than 95.0 %. Three primers (8F, 1088R and 1492R) were introduced to obtain a nearly complete 16S rRNA gene sequence of the isolate. The 16S rRNA gene sequences were subsequently assembled by SEQMAN software (DNASTAR) and aligned using the multiple sequence alignment program CLUSTAL_X (1.83) (Thompson *et al.*, 1997). The trimmed alignment was converted to MEGA and PHYLIP formats for phylogenetic analyses. The relationships between strain JO1^{T} and strains of other closely related species were shown using phylogenetic consensus trees based on neighbour-joining and maximum-parsimony methods constructed using MEGA 4 (Tamura *et al.*, 2007) and a phylogenetic consensus tree based on the maximum-likelihood method constructed using PHYLIP (Felsenstein, 2004); relationships were assessed by randomly selecting 1000, 1000 and 300 bootstrap replicates for the neighbour-joining, maximum-parsimony and maximum-likelihood algorithms, respectively (Felsenstein, 1981; Kluge & Farris, 1969; Saitou & Nei, 1987). Phylogenetic consensus trees were visualized with MEGA 4 (Tamura *et al.*, 2007). Phylogenetic analysis indicated that strain JO1^{T} was closely related to *Cobetia marina* in the family *Halomonadaceae* (Fig. 1).

To determine optimal culture conditions of strain JO1^{T}, growth of the isolate was tested under various conditions. Growth was determined in marine broth (MB; BBL) containing various concentrations of NaCl (0, 0.5, 1, 2, 3, 5, 7.5, 10, 15, 20, 25 and 30 %, w/v) for 48 h. Growth was assessed at various temperatures (0, 4, 10, 15, 25, 30, 37, 40 and 43 °C) for 96 h and at different pH (4, 5, 6, 7, 8, 9 and 10) for 48 h in MB supplemented with 6.5 % salts. Growth of the isolate occurred under a range of conditions:

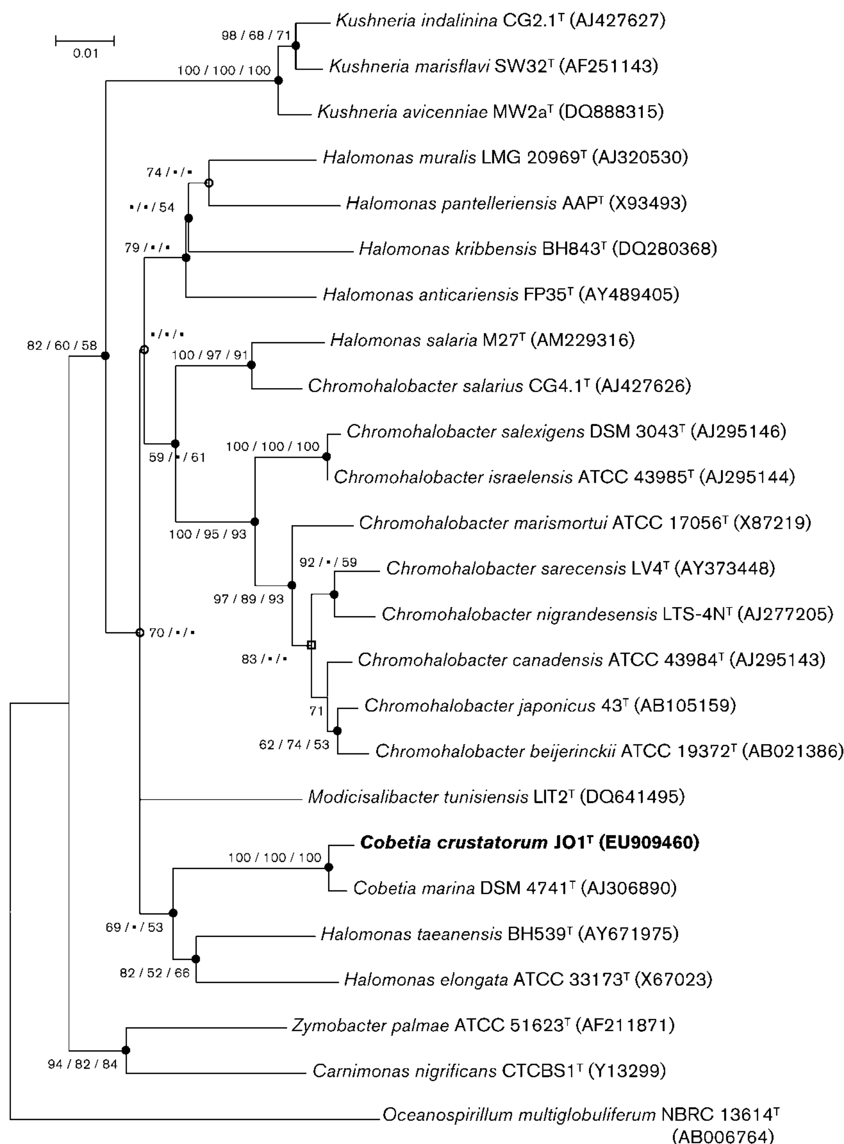

Fig. 1. Phylogenetic consensus tree based on 16S rRNA gene sequences. Filled circles, generic branches that are present in phylogenetic consensus trees generated by using the neighbour-joining, maximum-parsimony and maximum-likelihood algorithms; open circles, generic branches that are present in trees generated by using the neighbour-joining and maximum-parsimony algorithms; open squares, generic branches that are present in trees generated by using the neighbour-joining and maximum-likelihood algorithms. Numbers at nodes indicate bootstrap values as percentages of 1000, 1000 and 300 replicates, respectively (neighbour-joining probability/maximum-parsimony probability/maximum-likelihood probability). Values lower than 50 % are not indicated at the branch points. Bar, 1 substitution per 100 nt positions.

3.5–11.5 % salts, 4–30 °C and pH 5–10. Growth was best on 6.5 % (w/v) salts at 25 °C and pH 5.0–6.0. All tests used to characterize the isolate were performed at 25 °C and pH 7.5 ± 0.2 on MA containing 6.5 % salts unless stated otherwise. Gram-staining was accomplished using the method of Gram (1884). Poly-*β*-hydroxybutyric acid (PHB) was examined by using the Sudan Black B method (Smibert & Krieg, 1994). Production of exopolysaccharide was examined after cultivation on MH medium (Quesada *et al.*, 1983) for 5 days. Both light microscopy (ECLIPSE 80*i*; Nikon) and transmission electron microscopy (JEM 1010; JEOL) were used to determine cell shape, size, colour, flagella, PHB staining and Gram-staining. Respiration on fumarate, nitrate and nitrite was studied according to Callies & Mannheim (1978) with MH medium at 30 °C for 7 days in an anaerobic chamber maintained in an atmosphere of $N_2:CO_2:H_2$ (8 : 1 : 1). Catalase and oxidase activities were investigated with a 3 % (v/v) hydrogen peroxide solution and 1 % (w/v) *p*-tetramethyl phenylene-diamine (bioMérieux), respectively. DNA degradation was assessed on DNase agar (BBL). Hydrolysis of L-tyrosine was tested by incubating the isolate on optimal medium supplemented with L-tyrosine, after each component was autoclaved separately (Gordon *et al.*, 1974). For the Tweens (80 and 20) decomposition test, the optimal growth medium was supplemented with 0.01 % (w/v) $CaCl_2$ and 1 % (v/v) Tween (80 or 20) (Holding & Collee, 1971). Hydrolysis of starch and casein were tested by incubating the isolate on optimal medium supplemented with 0.5 % (w/v) soluble starch (BBL) or skim milk agar, respectively. Skim milk agar was prepared by mixing 5 % (w/v) skim milk (BBL) with 2 % agar and 5 % NaCl in distilled water, after each component had been individually autoclaved and cooled to 45 °C. Citrate utilization was tested on Simmons citrate agar (BBL). Phenylalanine agar (BBL) was used to test phenylalanine deaminase activity. Hydrogen sulfide production from L-cysteine was tested with Kligler iron agar (BBL). Methyl red and Voges–Proskauer reactions were performed using MR-VP medium (BBL). *β*-Galactosidase (ONPG test), indole production, urease activity, lysine decarboxylase, ornithine decarboxylase, reduction of nitrate to nitrite under aerobic conditions and oxidation/fermentation of D-glucose were assessed by using API 20E and 20NE strips (bioMérieux). API ZYM (bioMérieux) was used to assess other enzyme activities. Oxidative metabolism of carbohydrates by strain JO1^T on the basis of acid reaction was tested using oxidation/ fermentation (OF) basal medium (BBL). Carbohydrates in API 50 CH (bioMérieux) tubes were provided for use in OF basal medium. Utilization of substrates as sole carbon or nitrogen sources was also demonstrated as recommended by Arahal *et al.* (2007) and Mata *et al.* (2002) by introducing API 50 CH strips and Biolog GN2 MicroPlates according to the manufacturers' instructions. All suspension media were added to 5 % NaCl and all strips were incubated at 25 °C. Susceptibility of the isolate to antibiotics was defined by the disc diffusion method. The following discs were used: ampicillin (10 μg), kanamycin (30 μg), chloramphenicol (30 μg), erythromycin (15 μg), streptomycin (10 μg) and polymyxin B (300 IU) (Mata *et al.*, 2002). The results of phenotypic characterization are given in Table 1 and the species description.

Fatty acids of strain JO1^T and *C. marina* DSM 4741^T were extracted as described by the Sherlock Microbial Identification System (MIDI, 1999) from cells grown together for 72 h on MA with 2 % NaCl at 30 °C and pH 7.6. Fatty acids were analysed by GC (Hewlett Packard 6890) and identified with the Microbial Identification software package (Sasser, 1990). The cellular fatty acid compositions of these strains are presented in Table 2. The genomic DNA of strain JO1^T and *C. marina* DSM 4741^T were assessed for DNA–DNA hybridization and G + C content following extraction by using a G-spin Genomic DNA extraction kit (iNtRON Biotechnology). Preliminary comparison of the 16S rRNA gene sequence of strain JO1^T with those of references in GenBank showed that the isolate had 98.9 % similarity with *C. marina* DSM 4741^T in the family *Halomonadaceae*. It is recommended that a DNA–DNA reassociation value of about 70 % plays a dominant role in classification at the species level (Stackebrandt & Goebel, 1994; Wayne *et al.*, 1987). To verify the extent of genetic relatedness, DNA–DNA hybridization experiments (Ezaki *et al.*, 1989) were performed according to a modified method of Hirayama *et al.* (1996); specifically, probe DNA was labelled by boiling at 100 °C for 30 min with photobiotin instead of illuminating the probe under a 400 W mercury vapour lamp. Reportedly, bacterial strains with 16S rRNA gene sequence similarities greater than 97 % do not belong to the same species unless the extent of DNA–DNA hybridization is greater than 70 % (Stackebrandt & Goebel, 1994; Wayne *et al.*, 1987). DNA–DNA hybridization between strains JO1^T and *C. marina* DSM 4741^T was 24 %. To predict the DNA G + C content of strain JO1^T, a fluorimetric method employing SYBR Green I and real-time PCR was carried out (Gonzalez & Saiz-Jimenez, 2002). Genomic DNAs from *Escherichia coli* K-12 and *C. marina* DSM 4741^T were used as the calibration references (Gonzalez & Saiz-Jimenez, 2002). The DNA G + C content of the isolate was estimated as 61.4 mol%. Not only is this value within the 52–68 mol% range of the genus *Halomonas*, but is comparable with the DNA G + C content of *C. marina*.

Based on phenotypic, genotypic and phylogenetic analyses, it is proposed that strain JO1^T represents a novel species belonging to the genus *Cobetia*.

Description of *Cobetia crustatorum* sp. nov.

Cobetia crustatorum (cru.sta.to′rum. N.L. part. n. *crustatus* crustated; N.L. gen. pl. n. *crustatorum* of shellfish).

Aerobic, Gram-stain-negative and slightly halophilic micro-organism. Cells are rod-shaped, 0.6–1.5 μm in length, 0.6 μm in width, and generally observed as single cells or pairs. Cells have 2–5 peritrichous flagella. Colonies are less than 3 mm in diameter, round, glistening, raised

Table 1. Differential features of strain JO1^T (*C. crustatorum* sp. nov.) and *C. marina* DSM 4741^T

Data are from this study, except where indicated. Both strains were positive for catalase activity and hydrolysis of tyrosine and DNA. Both strains were negative for Gram staining, oxidase activity, nitrate reduction to nitrite, indole production, urease, lysine and ornithine decarboxylases, hydrolysis of casein, starch, gelatin, aesculin and Tweens (80 and 20), phenylalanine deaminase, methyl red and Voges–Proskauer tests, H_2S production, and respiration on fumarate, nitrate and nitrite. They both metabolized glycerol, D-galactose, D-glucose, D-fructose, D-mannose and trehalose in API 50 CH tests and D-fructose, D-galactose, α-D-glucose, α-ketoglutaric acid, bromosuccinic acid, L-asparagine and L-glutamic acid in the Biolog system.

Characteristic	Strain JO1^T	*C. marina* DSM 4741^T
Temperature range (°C)*	4–30	10–42
Optimal temperature (°C)*	25	37
Salts range (%)*	3.5–11.5	0.5–20
Motility*	+	−
β-Galactosidase (ONPG)	+	−
D-Glucose fermentation	−	+
Simmons citrate test	−	+
Acid production from:		
Glycerol	+	−
D-Xylose	+	−
D-Fructose	+	−
D-Lactose	−	+
5-Ketogluconate	−	+
Growth on:		
Inositol	−	+
Methyl α-D-glucoside	−	+
Amygdalin	−	+
Salicin	−	+
Cellobiose	−	+
Maltose	+	−
Sucrose	+	−
Melezitose	−	+
Starch	−	+
Turanose	−	+
D-Arabitol	−	+
Gluconate	−	+
Biolog system		
Glycogen	−	+
Tween 80	+	−
L-Arabinose	+	−
Lactulose	+	−
Maltose	+	−
D-Mannitol	+	−
D-Psicose	+	−
Sucrose	+	−
Turanose	+	−
Pyruvic acid methyl ester	+	−
cis-Aconitic acid	+	−
Citric acid	+	−
D-Gluconic acid	+	−
β-Hydroxybutyric acid	−	+
γ-Hydroxybutyric acid	+	−
p-Hydroxybutyric acid	+	−
DL-Lactic acid	+	−
Succinamic acid	−	+
L-Alanine	+	−
L-Alanyl glycine	+	−
L-Aspartic acid	−	+
Glycyl L-aspartic acid	−	+

Table 1. cont.

Characteristic	Strain JO1^T	*C. marina* DSM 4741^T
Glycyl L-glutamic acid	+	−
L-Proline	+	−
L-Pyroglutamic acid	+	−
L-Serine	+	−
γ-Aminobutyric acid	+	−
Inosine	−	+
Glycerol	+	−
DL-α-Glycerol phosphate	+	−
DNA G+C content (mol%)	61.4	62.4
Isolation source	Salt-fermented food	Coastal sea sample

*Data are from Arahal *et al.* (2002a), Mata *et al.* (2002) and the present study.

and cream-coloured. Grows under a range of conditions: 4–30 °C; 3.5–11.5 % salts; and pH 5.0–10.0. Optimal growth occurs at 25 °C, at pH 5.0–6.0 and in 6.5 % salts. Cells produce exopolysaccharide and accumulate PHB. Oxidase-negative and catalase-positive. Hydrolyses L-tyrosine, DNA and ONPG, but not casein, starch, gelatin, urea or Tweens (80 and 20). Oxidative metabolism occurs from D-glucose; indole is not produced. Lysine and ornithine decarboxylases are not produced. The Simmons citrate, methyl red and Voges–Proskauer tests, phenylalanine deaminase, and hydrogen sulfide production from L-cysteine are negative. Respiration on fumarate, nitrate and nitrite is negative. Reduction of nitrate to nitrite under aerobic conditions is negative. Acid is produced from glycerol, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, cellobiose, maltose and D-fucose, but not from erythritol, D-arabinose, L-arabinose, D-ribose, L-xylose, D-adonitol, methyl β-D-xyloside, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl α-D-mannoside, methyl α-D-glucoside, *N*-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, D-lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose, starch, glycogen, xylitol, gentiobiose, turanose, D-lyxose, D-tagatose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate or 5-ketogluconate. Utilizes glycerol, D-galactose, D-glucose, D-fructose, D-mannose, maltose, sucrose and trehalose in API 50 CH strips, but not erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, methyl β-D-xyloside, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl α-D-mannoside, methyl α-D-glucoside, *N*-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, D-lactose, melibiose, inulin, melezitose, raffinose, starch, glycogen, xylitol, gentiobiose, turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate or 5-ketogluconate. In Biolog GN2 MicroPlates, metabolizes L-arabinose, D-fructose, D-galactose, α-D-glucose, maltose, D-mannitol, sucrose, turanose, glycerol, D-gluconic acid, lactulose, D-psicose, Tween 80, pyruvic acid methyl ester, *cis*-aconitic acid, citric acid, γ-hydroxybutyric acid, *p*-hydroxyphenylacetic acid, α-ketoglutaric acid, DL-lactic acid, bromosuccinic acid, L-alanine, L-alanyl glycine, L-asparagine, L-glutamic acid, glycyl L-glutamic acid, L-proline, L-pyroglutamic acid, L-serine, γ-aminobutyric acid and α-D-glucose 1-phosphate, but not glycogen, *N*-acetyl-D-glucosamine, adonitol, D-arabitol, cellobiose, *i*-erythritol, L-fucose, gentiobiose, *myo*-inositol, D-lactose, D-mannose, melibiose, raffinose, L-rhamnose, D-sorbitol, trehalose, xylitol, D-arabinose, dulcitol, methyl α-D-mannoside, methyl α-D-glucoside, amygdalin, arbutin, aesculin, salicin, inulin, melezitose, starch, D-tagatose, L-arabitol, 2-ketogluconate, 5-ketogluconate, methyl β-D-glucoside, α-cyclodextrin, dextrin, Tween 40, *N*-acetyl-D-galactosamine, succinic

Table 2. Cellular fatty acid contents (%) of strain JO1^T (*C. crustatorum* sp. nov.) and *C. marina* DSM 4741^T

All data shown are from the present study and were determined under the same conditions. Values shown are percentages of total fatty acids. tr, Trace (less than 1.0 %); −, not detected.

Fatty acid	Strain JO1^T	*C. marina* DSM 4741^T
$C_{10:0}$	4.5	1.7
$C_{12:0}$	8.0	4.0
$C_{12:0}$ 3-OH	25.3	9.5
$C_{14:0}$	1.2	tr
$C_{16:0}$	24.6	26.0
$C_{16:0}$ 3-OH	−	tr
$C_{17:0}$	−	tr
$C_{17:0}$ cyclo	9.4	13.8
$C_{18:0}$	1.0	tr
$C_{18:1}\omega7c$	3.8	20.7
$C_{19:0}$ cyclo $\omega8c$	−	4.9
10-methyl $C_{19:0}$	−	tr
Summed feature 3*	22.2	16.7

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

acid monomethyl ester, acetic acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-glucosaminic acid, D-glucuronic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, itaconic acid, α-ketobutyric acid, α-ketovaleric acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, succinic acid, succinamic acid, glucuronamide, L-alaninamide, D-alanine, L-aspartic acid, glycyl L-aspartic acid, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, L-phenylalanine, D-serine, L-threonine, DL-carnitine, urocanic acid, inosine, uridine, thymidine, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, DL-α-glycerol phosphate or D-glucose 6-phosphate. Positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, cystine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase and β-galactosidase, but negative for lipase (C14), valine arylamidase, α-chymotrypsin, α-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. Susceptible to ampicillin (10 μg), kanamycin (30 μg), chloramphenicol (30 μg), erythromycin (15 μg), streptomycin (10 μg) and polymyxin B (300 IU). Major fatty acids are $C_{12:0}$ 3-OH, $C_{16:0}$, $C_{17:0}$ cyclo and summed feature 3 ($C_{16:1}\omega7c$/iso-$C_{15:0}$ 2-OH).

The type strain is JO1^T (=KCTC 22486^T=JCM 15644^T), isolated from 'Jogae jeotgal', a type of traditional Korean fermented seafood. The DNA G+C content of the type strain is 61.4 mol%.

Acknowledgements

We thank Professor J. P. Euzéby (Ecole Nationale Vétérinaire, France) for etymological advice. This work was supported by the Environmental Biotechnology National Core Research Center (KOSEF: R15-2003-012-02002-0) and TDPAF (Technology Development Program for Agriculture and Forestry) of the Ministry for Agriculture, Forestry and Fisheries.

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