Methylobacterium jeotgali sp. nov., a non-pigmented, facultatively methylotrophic bacterium isolated from jeotgal, a traditional Korean fermented seafood

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A novel facultatively methylotrophic, Gram-negative, rod-shaped bacterium, designated strain S2R03-9^T, was isolated from jeotgal, a traditional Korean fermented seafood. The organism was strictly aerobic, motile by means of a single polar flagellum, non-sporulating and catalase- and oxidase-positive. Strain S2R03-9^T grew in the presence of 0–1 % (w/v) NaCl and at pH 6.0–10.0, with optimum growth in the absence of NaCl and at pH 7.0. It grew at temperatures in the range 20.0–30.0 °C, with optimum growth at 30 °C. Colonies grown on R2A medium were non-pigmented, opaque and creamy white. Phylogenetic analysis based on 16S rRNA gene sequences indicated that it was most closely related to *Methylobacterium organophilum* JCM 2833^T (96.6 % similarity) and the phylogenetic similarities to all other *Methylobacterium* species with validly published names were less than 95.0 %. The DNA G + C content was 64.9 mol%. The phylogenetic analysis, the phenotypic assessment and the chemotaxonomic data (major ubiquinone, Q-10; major fatty acids, C_{18:1} and C_{18:0}) showed that S2R03-9^T represents a novel species within the genus *Methylobacterium* in the class *Alphaproteobacteria*, for which the name *Methylobacterium jeotgali* sp. nov. is proposed. The type strain is S2R03-9^T (=KCTC 12671^T =LMG 23639^T).

The genus *Methylobacterium* was originally proposed to accommodate facultatively methane-utilizing bacteria, i.e. bacteria that are able to use methane as well as multi-carbon substrates as sole carbon and energy sources. This description was based on a single strain, *Methylobacterium organophilum* XX^T (=JCM 2833^T) (Patt *et al.*, 1976). However, as a result of the demonstration that *Methylobacterium organophilum* had lost the ability to use methane as a sole carbon source (Green & Bousfield, 1983; Romanovskaya *et al.*, 1978; Urakami & Komagata, 1984), Green & Bousfield (1983) proposed the emendation of the description of *Methylobacterium* as comprising facultatively methylotrophic bacteria. The genus belongs to the *Alphaproteobacteria* and has a serine pathway for formaldehyde assimilation. It includes strictly aerobic, Gram-negative, rod-shaped, pink-pigmented, facultatively methylotrophic bacteria that

can grow on single-carbon compounds (such as formate, formaldehyde and methanol) as sole sources of carbon and energy and also on a wide range of multi-carbon growth substrates (Green, 1992). Members of the genus *Methylobacterium* are ubiquitous and have been found in a variety of natural and man-made environments, including soil, dust, lake sediments, freshwater, seawater, leaf surfaces, tree tissues and root nodules, rice grains, air, face-creams, fermented products, water supplies, bathrooms, air-conditioning systems, hospital environments and masonry (Green, 1992; Hiraishi *et al.*, 1995; Trotsenko *et al.*, 2001; Van Aken *et al.*, 2004). In the present study, we propose a novel *Methylobacterium* species for a strain isolated from jeotgal, a traditional fermented Korean seafood.

The aim of the present study was to determine the exact taxonomic position, using polyphasic characterization, of a facultatively methylotrophic strain (S2R03-9^T) isolated from jeotgal.

In the course of studying the bacterial diversity of jeotgal, we found that this traditional food is a source of not only lactic

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain S2R03-9^T is DQ471331.

A phase-contrast micrograph of cells of strain S2R03-9^T is available as a supplementary figure in IJSEM Online.

acid bacteria and yeast but also numerous other bacteria belonging to the *Actinobacteria*, *Firmicutes* and *Proteobacteria*. During the analysis of the bacterial diversity in jeotgal samples, collected from fish markets in Daejeon and Suwon in Korea, we investigated a sample of shrimp-jeotgal, packed in a glass jar (net weight, 160 g; Hansung, <http://www.han-sung.co.kr>). A small portion of this sample was blended in a homogenizer (Ac; Nihonseiki Kaisha) for 2 min. It was then serially diluted and applied to plates of R2A (Difco) medium (pH 7.0, incubated at 30 °C) containing different concentrations of NaCl. Amongst the many pigmented and non-pigmented colonies arising on R2A plates, small, non-pigmented, creamy white colonies were clearly observed after incubation for 1 week. A representative colony was purified and designated as strain S2R03-9^T. This strain was maintained on R2A agar at pH 7.0 and 30 °C and was preserved at -70 °C in 20 % (w/v) glycerol stock.

Because strain S2R03-9^T showed low levels of 16S rRNA gene sequence similarity with respect to all *Methylobacterium* species with validly published names, the strain was investigated further to determine its taxonomic position.

The Gram reaction was performed by using the non-staining method, as described by Buck (1982). Cell morphology was observed under a Nikon light microscope at $\times 1000$ and a transmission electron microscope (CM-20; Philips), with cells grown for 3 days at 30 °C on R2A agar (Yoon *et al.*, 2002). Catalase and oxidase tests were performed by using the procedures outlined by Cappuccino & Sherman (2002). Carbon-source utilization, the carbohydrate fermentation/oxidation profile and some physiological characteristics were determined with the API 32GN, API 20E and API 50 CH (with CHB/E medium) galleries according to the instructions of the manufacturer (bioMérieux). Aliquots (30 ml) of MMS broth (Green, 1992) were used, in 50 ml serum bottles, in tests for the utilization of betaine, methane, formaldehyde and methylamine as sole carbon sources. MMS agar medium was used in tests for the utilization of the following sole carbon sources: ethanol, isopropanol and methanol. All of the carbon sources mentioned were used at a concentration of 0.1 % (w/v or v/v). Methane (as a sole carbon and energy source) was provided by filtering (through a syringe-driven 0.22 μ m filter unit; Millex) in an atmosphere of methane/air (1:4) into 50 ml serum bottles containing 25 ml MMS broth. Vitamin solution was also added in the aforementioned tests (Patt *et al.*, 1974). Growth at 4, 10, 15, 20, 25, 30, 37 and 45 °C was tested in R2A agar. Salt tolerance was tested in R2A medium supplemented with 1–10 % (w/v) NaCl. Growth at different temperatures, pHs and NaCl concentrations was assessed after incubation for 1 week. Degradation of DNA (using DNA agar from Difco, flooded with 1 M HCl), casein, chitin, Tween 80, starch (Atlas, 1993), xylan and cellulose (Ten *et al.*, 2004) was tested and evaluated after incubation for 2 weeks.

For nitrate- and nitrite-reduction tests, strain S2R03-9^T was inoculated into serum bottles (25 ml) containing 13 ml R2A

medium, while nitrate and nitrite were added in the form of KNO₃ and NaNO₂, respectively, at a concentration of 10 mM. The reduction of nitrate and nitrite was monitored via an ion chromatograph (model 790 personal IC; Metrohm) equipped with a conductivity detector and an anion-exchange column (Metrosep Anion Supp 4; Metrohm). Duplicate antibiotic-sensitivity tests were conducted using filter-paper discs containing the following: ampicillin, tetracycline, kanamycin (Sigma) and rifampicin, each at concentrations of 5, 10, 20, 50 and 100 μ g ml⁻¹. Discs were placed on R2A agar plates spread with strain S2R03-9^T and then incubated at 30 °C for 7 days. Chlorine-resistance was assessed according to the method of Hiraishi *et al.* (1995).

Extraction of genomic DNA was accomplished using a commercial genomic DNA-extraction kit (Core Biosystems); PCR-mediated amplification of the 16S rRNA gene and sequencing of the purified PCR product were carried out according to the procedure of Kim *et al.* (2005). 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 in the BioEdit program (Hall, 1999). Evolutionary distances were calculated by the method of Jukes & Cantor (1969). The phylogenetic tree was constructed using a neighbour-joining method (Saitou & Nei, 1987) and maximum parsimony (Fitch, 1971) in the MEGA3 program (Kumar *et al.*, 2004) with bootstrap values based on 1000 replications (Felsenstein, 1985).

The potential for aerobic anoxygenic photosynthesis was determined at the physiological level by determining bacteriochlorophyll *a* content *in vitro* (Allgaier *et al.*, 2003). Nitrogen-fixation ability was assessed by using the forward primer IGK and the reverse primer AQE (5'-TACGGYAARGGBGGYATCGG-3' and 5'-GACGATGAT-YTCCTG-3', respectively) to amplify the *nifH* gene from the extracted genomic DNA (Xie & Yokota, 2004). The *msaF* gene, encoding the α -subunit of the methanol dehydrogenase, was sequenced for strains S2R03-9^T and *Methylobacterium zatmanii* DSM 5688^T and a phylogenetic analysis was performed according to Sy *et al.* (2001).

To measure the G+C content of the extracted chromosomal DNA, it was enzymically degraded into nucleosides and investigated as described by Mesbah *et al.* (1989), using reversed-phase HPLC. Isoprenoid quinones were extracted with chloroform/methanol (2:1, v/v), evaporated under a vacuum and re-extracted in n-hexane/water (1:1, v/v). The crude n-hexane quinone solution was then purified using silica Sep-Pak Vac cartridges (Waters) and subsequently analysed by HPLC, as described previously (Hiraishi *et al.*, 1996). Cellular fatty acids were analysed using bacteria grown on R2A agar for 2 days. The cellular fatty acids were saponified, methylated and extracted according to the protocol of the Sherlock Microbial Identification System (MIDI). The fatty acids were analysed by GC (Hewlett

Packard 6890) and identified using the Microbial Identification software package (Sasser, 1990).

Strain S2R03-9^T was determined as comprising Gram-negative, rod-shaped (0.7–1.0 × 1.0–1.6 µm) bacteria bearing single polar flagella (Fig. 1). The cells occurred singly, in pairs and in rosettes (see Supplementary Fig. S1 available in IJSEM Online). Colonies grown on R2A agar plates (Difco) for 7 days were smooth, circular, non-pigmented, opaque, creamy white and 1–2 mm in diameter. Growth was strictly aerobic. No budding morphology was observed. On R2A agar, strain S2R03-9^T was able to grow at 20–30 °C but not at 4 or 37 °C. On R2A agar, strain S2R03-9^T was able to grow at pH 6.0–10.0, the optimum being pH 7.0 at 30 °C. NaCl at concentrations up to 1.0 % (w/v) was tolerated. Catalase and oxidase activities were present.

The physiological and biochemical characteristics of strain S2R03-9^T are summarized in the species description, and selective characteristics are compared with those of related type strains in Table 1.

The 16S rRNA gene sequence of the strain S2R03-9^T was a continuous stretch of 1380 bp. The phylogenetic tree based on the neighbour-joining algorithm showed that strain S2R03-9^T falls within the radiation of the cluster comprising

Methylobacterium species, forming a cluster with related strains at a high bootstrap resampling value (91 %; Fig. 2). Sequence-similarity calculations indicated that the closest relative of strain S2R03-9^T was *Mtb. organophilum* JCM 2833^T (96.6 %); the similarities with respect to other members of this genus were low (< 95.0 %). It has been suggested that in bacterial strains with less than 97 % 16S rRNA gene sequence identity, the DNA–DNA hybridization level is less than 70 % (Stackebrandt & Goebel, 1994) (i.e. the threshold for defining a genomic species; Wayne *et al.*, 1987). In previous studies we have found corresponding trends (Aslam *et al.*, 2005a, b). Thus, on the basis of the 16S rRNA gene sequence analyses, a novel taxon could be proposed.

The G + C content of the genomic DNA of strain S2R03-9^T was 64.9 mol% and the major ubiquinone was Q-10. The fatty acids of strain S2R03-9^T were C_{18:1}ω7c (84.8 %), C_{18:0} (10.7 %) and C_{16:0} (4.5 %). These data are compatible with the assignment of strain S2R03-9^T to the genus *Methylobacterium*.

The following characteristics of strain S2R03-9^T are consistent with its assignment to the genus *Methylobacterium* (Patt *et al.*, 1976; Green & Bousfield, 1983; Green, 1992): the 16S rRNA gene sequence, phylogenetic data (Fig. 2), the profile for single-carbon-source assimilation, the presence of the *mxoF* gene and the resulting phylogenetic analysis (Fig. 3), morphology (cell and colony size; polar flagellum) and chemotaxonomic results (major ubiquinone, Q-10; major fatty acids, C_{18:1} and C_{18:0}).

Urakami *et al.* (1993) found some colourless colonies in the species *Methylobacterium aminovorans*, and Jourand *et al.* (2004) also described a non-pigmented species, *Methylobacterium nodulans*, in the genus *Methylobacterium*. Similarly, we propose a novel non-pigmented, creamy white species belonging to the genus *Methylobacterium*. The low levels of 16S rRNA gene sequence similarity, the growth pattern on single carbon sources and the physiological and biochemical characteristics (see Table 1 and the species description) of S2R03-9^T differentiate this strain from all of the *Methylobacterium* species with validly published names. These results clearly support the recognition of S2R03-9^T as a novel species within the genus *Methylobacterium*, for which the name *Methylobacterium jeotgali* sp. nov. is proposed.

Description of *Methylobacterium jeotgali* sp. nov.

Methylobacterium 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-negative, rod-shaped, 0.7–1.0 × 1.0–1.6 µm in size and each possess a single polar flagellum. Cells occur singly, in pairs and in rosettes. Colonies grown on R2A agar plates for 7 days are smooth, circular, non-pigmented, opaque, creamy-white,

Fig. 1. Transmission electron micrograph showing the general cell morphology of strain S2R03-9^T and the presence of a single polar flagellum after growth on R2A agar for 3 days. Bar, 1 µm.

Table 1. Comparison of the characteristics of strain S2R03-9^T with those of members of related taxa

Strains: 1, strain S2R03-9^T (data from this study); 2, *Mtb. organophilum* JCM 2833^T (see footnote for details of data sources); 3, *Methylobacterium hispanicum* GP34^T (data from Gallego *et al.*, 2005); 4, *Methylobacterium aquaticum* DSM 16371^T (Gallego *et al.*, 2005); 5, *Methylobacterium mesophilicum* JCM 2829^T (Green, 1992; Doronina *et al.*, 2002; Austin & Goodfellow, 1979); 6, *Methylobacterium fujisawaense* DSM 5686^T (Green, 1992; Doronina *et al.*, 2002); 7, *Mtb. nodulans* ORS 2060^T (Jourand *et al.*, 2004); 8, *Mtb. aminovorans* CCM 4612^T (Urakami *et al.*, 1993). All strains are motile, strictly aerobic and non-spore-forming. +, Positive; –, negative; V, variable; W, weakly positive; ND, not determined/no data available.

Characteristic	1	2	3	4	5	6	7	8
Colony pigmentation	None	Pink ^{a*}	Light pink	Pink to red	Pink	Pink	None	Pink to red
Source of isolation	Jeotgal	Lake sediment ^a	Drinking Water	Drinking Water	Leaf surfaces	ND	<i>Crotalaria podocarpa</i>	Soil
Cells occur:								
Singly	+	+	+	+	ND	ND	+	+
In pairs	+	ND	+	+	ND	ND	+	+
In aggregates/rosettes	+	+	–	+	ND	ND	–	–
Cell length (µm)	1.0–1.6	1.5–2.0 ^a	2–2.5	4.5–8	3.0–4.0	ND	1.0–1.5	1.5–4.0
Cell width (µm)	0.7–1.0	0.8–1.0 ^a	1–1.5	1.5–1.7	1.0	ND	0.8–1.0	0.8–1.0
Diameter of colonies (mm)	1–2	1–3 ^b	1–2	1–2	1.0	1–3	0.5–1	1–3
Temperature (°C) [range], (optimum)	[25–30], (30)	[5–37] ^b , (28) ^a	[15–30], (28)	[20–30], (28)	[10–30], (25)	[10–37], (ND)	[30–37], (30)	[ND], (30)
pH [range], (optimum)	[6.0–10.0], (7.0–8.0)	[ND], (6.8) ^a	[5.0–8.0], (6.5)	[5.0–7.0], (6.0)	ND	ND	[ND], (6.8–7.5)	[ND], (6.0–8.0)
NaCl tolerance (%) [range], (optimum)	[0–1], (0)	ND	[0], (0)	[0], (0)	[0–1], (0)	ND	ND	[0–2], (0)
Oxidase	+	+	W	–	W	W	W	+
Growth on:								
Citrate	–	– ^b	+	+	+	+	+	–
L-Glutamate	ND	– ^{a,b} , + ^c	+	+	+	+	+	ND
D-Glucose	–	+ ^{a,b} , – ^c	–	+	+	+	–	–
D-Xylose	+	– ^b	–	–	+	+	+	–
Fructose	–	+ ^b	+	+	–	V	–	ND
Methylamine	+	– ^a , + ^{b,c}	ND	ND	–	–	–	+
Acetate	+	+ ^b	ND	ND	–	+	+	ND
Betaine	+	– ^b	ND	ND	–	–	+	+
DNA G+C content (mol%)	64.9	66.0 ^a	67.5–68.0	67.3–67.9	65.8	70.8–71.8	ND	68.0
Ubiquinone	Q-10	Q-10 ^b	ND	ND	ND	ND	ND	Q-10

*Data obtained from the following studies: *a*, Patt *et al.* (1976); *b*, Green (1992); *c*, Doronina *et al.* (2002).

slow-growing and 1–2 mm in diameter. No budding morphology is observed. Growth occurs at 20–30 °C but not at 4 or 37 °C. Growth occurs at pH 6.0–10.0, with an optimum pH of 7.0 at 30 °C. NaCl concentrations up to 1.0 % (w/v) are tolerated and no growth occurs with 3.0 % NaCl. Catalase- and oxidase-positive. H₂S and indole are not produced. Nitrate is not reduced to nitrite or to nitrogen gas. Acetoin is produced. Urease, caseinase and arginine dihydrolase are produced but β-glucosidase, cellulase, xylanase, lysine decarboxylase, ornithine decarboxylase, amylase, lipase, chitinase and DNase are not produced. Gelatin and aesculin hydrolysis is weak. Acid is produced from D-arabinose, glycerol, L-xylose and D-lyxose but not from D-glucose, D-fructose, D-mannitol, D-maltose, D-trehalose, N-acetyl-D-glucosamine, D-mannitol, sucrose, L-arabinose, D-ribose, D-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-tagatose, D- or L-fucose, D- or 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 used as sole carbon and energy sources: betaine, formaldehyde, methylamine, isopropanol, ethanol, methanol, rhamnose, malonate, acetate, DL-lactate, L-serine, L-proline, 3-hydroxybenzoate, 3-hydroxybutyrate and 4-hydroxybenzoate. The following are not utilized as sole carbon and energy sources: methane, mannitol, D-glucose, D-melibiose, salicin, L-fucose, D-sorbitol, L-arabinose, propionate, caprate, valerate, citrate, histidine, 2-ketogluconate,

Fig. 2. Phylogenetic tree, constructed from a comparative analysis of 16S rRNA gene sequences, showing the relationships between strain S2R03-9^T and members of related taxa. The 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 percentages of 1000 replications) greater than 70% are shown at the branch points. Bar, 1 substitution per 100 nucleotide positions.

Fig. 3. Phylogenetic tree, constructed from a comparative analysis of *mxoF* gene sequences, showing the relationships between strain S2R03-9^T and members of related taxa. The tree was constructed by using the neighbour-joining method and Jukes–Cantor evolutionary distance matrix data obtained from unambiguous aligned nucleotides. Bootstrap values (expressed as percentages of 1000 replications) greater than 70% are shown at the branch points. Bar, 5 substitutions per 100 nucleotide positions.

N-acetyl-D-glucosamine, ribose, inositol, sucrose, itaconate, glycogen, D-maltose, L-alanine, 5-ketogluconate and suberate. Bacteriochlorophyll *a* is not produced. The *nifH* gene is not detected. The *mxoF* gene is detected. Resistant to (ml⁻¹) 100 µg ampicillin, 100 µg tetracycline and 100 µg rifampicin but sensitive to 50 µg kanamycin. Resistant to chlorine (0.1–0.5 mg l⁻¹). The major ubiquinone is Q-10 and the major fatty acids are C_{18:1}ω7c (84.8%), C_{18:0} (10.7%) and C_{16:0} (4.5%). The G + C content of the genomic DNA of the type strain is 64.9 mol% (as determined by HPLC).

The type strain, S2R03-9^T (=KCTC 12671^T=LMG 23639^T), was isolated from jeotgal, a traditional Korean fermented seafood.

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