***Bacillus jeotgali* sp. nov., isolated from jeotgal, Korean traditional fermented seafood**

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Two Gram-variable, rod-shaped, endospore-forming bacterial strains, which are motile with peritrichous flagella, were isolated from a Korean traditional fermented seafood, jeotgal. The two isolates (strains YKJ-10^T and YKJ-11) were proven to be members of the same species on the basis of the results of phenotypic and phylogenetic analyses and DNA relatedness. Strains YKJ-10^T and YKJ-11 were characterized by having cell wall peptidoglycan based on meso-diaminopimelic acid, MK-7 as the predominant menaquinone, and iso-C_{15:0} as the major fatty acid. The G+C content of the DNA was 41 mol%. Strains YKJ-10^T and YKJ-11 showed only a 1 bp sequence difference in the 16S rDNA sequences. The two strains formed distinct phylogenetic lineages within the radiation of the cluster comprising *Bacillus* species. Levels of 16S rDNA similarity between strains YKJ-10^T and YKJ-11 and *Bacillus* species were less than 96.6%. Levels of DNA–DNA relatedness were found to be low enough to distinguish strains YKJ-10^T and YKJ-11 from some phylogenetically related *Bacillus* species. On the basis of phenotypic properties, phylogeny and genomic distinctiveness, strains YKJ-10^T and YKJ-11 represent a new species of the genus *Bacillus*, for which a new name, *Bacillus jeotgali* sp. nov., is proposed. The type strain of the new species is strain YKJ-10^T (= KCCM 41040^T = JCM 10885^T).

Keywords: *Bacillus jeotgali* sp. nov., polyphasic taxonomy, Korean traditional food, jeotgal

INTRODUCTION

Jeotgal is one of the representative traditional foods in Korea. It is prepared through blending of various kinds of seafoods, including seawater and other ingredients, and becomes palatable through subsequent preservation and fermentation. Jeotgal has been used as an important additive for improving the taste of other foods as well as being food itself. Recently, our preliminary study on the microflora of jeotgal has shown that a variety of bacterial strains exist in it. Such organisms found in jeotgal appear to mainly originate from seafoods and seawater, because most of them have halotolerant or halophilic physiological properties. Gram-positive, endospore-forming bacilli were found to occupy the highest proportion of

micro-organisms existing in jeotgal. In this study, we describe two rod-shaped, endospore-forming bacteria (strains YKJ-10^T and YKJ-11). Traditionally, Gram-positive, rod-shaped, endospore-forming bacteria have been classified in the genus *Bacillus* (Claus & Berkeley, 1986; Slepecky & Hemphill, 1991). However, a number of Gram-positive, endospore-forming rods that were previously assigned to the genus *Bacillus* have recently been reclassified to the following genera: *Alicyclobacillus* (Wisotzkey *et al.*, 1992), *Aneurinibacillus* (Shida *et al.*, 1996), *Brevibacillus* (Shida *et al.*, 1996), *Paenibacillus* (Ash *et al.*, 1993), *Salibacillus* (Wainø *et al.*, 1999) and *Virgibacillus* (Heyndrickx *et al.*, 1998). In addition, some genera such as *Amphibacillus* (Niimura *et al.*, 1990), *Halobacillus* (Spring *et al.*, 1996) and *Gracilibacillus* (Wainø *et al.*, 1999), which are associated with the genus *Bacillus*, have been proposed. Now, it is generally recognized that a polyphasic taxonomic study integrating various characterization methods, especially 16S rDNA

The GenBank accession numbers for the 16S rDNA sequences of strains YKJ-10^T and YKJ-11 are AF221061 and AF221062, respectively.

sequencing, is essential for classification of Gram-positive or variable, rod-shaped, endospore-forming strains (Shida *et al.*, 1997; Wainø *et al.*, 1999; Yoon *et al.*, 1998b; Arahall *et al.*, 1999). This approach is also very important for assigning novel strains to one of the genera associated with the genus *Bacillus* or for determining their positions within a genus. Accordingly, the aim of this study was to determine the exact taxonomic positions of the two rod-shaped, endospore-forming bacterial strains isolated from jeotgal by polyphasic characterization, including morphological and physiological properties, phylogeny based on 16S rDNA sequence, and genomic relatedness. On the basis of data described below, we propose a new species of the genus *Bacillus*, *Bacillus jeotgali*, for strains YKJ-10^T and YKJ-11.

METHODS

Bacterial strains and culture conditions. Strains YKJ-10^T and YKJ-11 were isolated from a jeotgal by the dilution plating technique on trypticase soy agar (BBL) supplemented (1^{-1}) with 24 g NaCl, 7 g MgSO₄·7H₂O, 5.3 g MgCl₂·6H₂O, 0.7 g KCl and 0.1 g CaCl₂ (ASW) (pH 7.5). For the investigation of morphological and physiological characteristics, strains YKJ-10^T and YKJ-11 were in most cases cultivated at 30 °C on trypticase soy agar (TSA+ASW) and trypticase soy broth (TSB+ASW) supplemented with ASW. Cell mass for the analyses of cell wall and menaquinone and for DNA extraction was obtained from TSB+ASW cultures. Some reference strains were cultivated for DNA extraction as follows: *Bacillus circulans* KCTC 3347^T was cultivated at 30 °C on TSB, *Bacillus lentus* DSM 9^T was cultivated at 26 °C on TSB, *Bacillus firmus* KCTC 3626^T was cultivated at 30 °C on TSB+ASW and *Bacillus benzoovorans* KCTC 3392^T was cultivated at 30 °C on TSB (Difco) supplemented with 0.4% (w/v) yeast extract and 0.2% (w/v) benzoate. All strains were cultivated on a horizontal shaker at 150 r.p.m. and the broth cultures were checked for purity microscopically before being harvested by centrifugation. Strains YKJ-10^T and YKJ-11 were also cultivated at 30 °C for 2 d on TSA+ASW for fatty acid methyl ester analysis.

Morphological and physiological characterization. The morphology of cells was examined by phase-contrast microscopy and transmission electron microscopy (TEM). The flagellum type was examined by TEM using cells from the exponentially growing culture. The cells were negatively stained with 1% (w/v) phosphotungstic acid, and after air drying the grids were examined by using a model CM-20 transmission electron microscope (Philips). Motility was determined with an optical microscope using the hanging drop technique (Skerman, 1967). Catalase activity was determined by bubble production in a 3% hydrogen peroxide solution. Oxidase activity was determined by oxidation of 1% *p*-aminodimethylaniline oxalate. Hydrolyses of casein, starch, hypoxanthine, tyrosine, Tween 80 and xanthine and production of urease were determined as described by Cowan & Steel (1965). Hydrolysis of aesculin and nitrate reduction were tested as described previously (Lanyi, 1987). Tolerance of sodium chloride and growth at various temperatures were tested on TSB+ASW. Acid production from carbohydrates was determined by using the API 50CHB system (bioMérieux). Other physiological tests were performed with the API 20NE system (bioMérieux).

Isolation of DNA. Chromosomal DNA was isolated and purified according to the method described previously (Yoon *et al.*, 1996), with the exception that ribonuclease T1 was used together with ribonuclease A.

Chemotaxonomic characterization. The isomer type of diaminopimelic acid in the peptidoglycan was determined as described by Komagata & Suzuki (1987). Menaquinones were analysed as described previously (Komagata & Suzuki, 1987) using reversed-phase HPLC. For quantitative analysis of cellular fatty acid compositions, a loop of cell mass grown was harvested and fatty acid methyl esters were prepared and identified following the instructions of the Microbial Identification System (MIDI).

Determination of G+C content. The G+C content was determined by the method of Tamaoka & Komagata (1984). DNA was hydrolysed and the resultant nucleotides were analysed by reversed-phase HPLC.

DNA–DNA hybridization. DNA–DNA hybridization was performed fluorometrically by the method of Ezaki *et al.* (1989) using photobiotin-labelled DNA probes and microdilution wells.

16S rDNA sequencing and phylogenetic analysis. The 16S rDNA was amplified by PCR using two universal primers as described previously (Yoon *et al.*, 1998a). The PCR product was purified by using the QIAquick PCR purification kit (Qiagen). The purified 16S rDNA was sequenced using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) as recommended by the manufacturer. The purified sequencing reaction mixtures were automatically electrophoresed using an Applied Biosystems model 310 automatic DNA sequencer. The 16S rDNA sequences of strains YKJ-10^T and YKJ-11 were aligned with 16S rRNA gene sequences of *Bacillus* species and some other related taxa by using CLUSTAL W software (Thompson *et al.*, 1994). The 16S rDNA similarity values were calculated from the alignment. Gaps at the 5' and 3' ends of the alignment were omitted from further analyses. Evolutionary distance matrices were calculated by using the algorithm of Jukes & Cantor (1969) with the DNADIST program within the PHYLIP package (Felsenstein, 1993). A phylogenetic tree was constructed by using the neighbour-joining method (Saitou & Nei, 1987) as implemented within the NEIGHBOR program of the same package. The stability of relationships was assessed by a bootstrap analysis of 1000 data sets by using the programs SEQBOOT, DNADIST, NEIGHBOR and CONSENSE of the PHYLIP package.

Nucleotide sequence accession numbers. GenBank and EMBL accession numbers for reference 16S rDNA sequences used in this analysis are in Fig. 1.

RESULTS

Morphological and physiological characteristics

Strains YKJ-10^T and YKJ-11 were facultatively anaerobic and Gram-variable bacteria. The two strains were motile by means of peritrichous flagella. Cells were rods measuring 0.8–1.1 µm wide and 4.0–6.0 µm long in 48 h culture on TSA+ASW at 30 °C. Ellipsoidal spores were formed in swollen sporangia. Colonies of the two strains were smooth, irregular and flat after 2–3 d on TSA+ASW. Colonies of strain

Table 1. Phenotypic characteristics of strains YKJ-10^T and YKJ-11

+, Positive reaction; –, negative reaction; w, weakly positive reaction. The two strains were positive for catalase, nitrate reduction, decomposition of aesculin, gelatin, starch and urea, and acid production from glucose, fructose, aesculin, maltose, trehalose, starch and glycogen. The two strains were negative for oxidase, production of indole, decomposition of casein, hypoxanthine, tyrosine and xanthine, and acid production from glycerol, erythritol, D-arabinose, L-arabinose, ribose, D-xylose, L-xylose, adonitol, β -methyl D-xyloside, galactose, mannose, sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, α -methyl D-mannoside, α -methyl D-glucoside, *N*-acetylglucosamine, amygdalin, arbutin, salicin, lactose, melibiose, inulin, melezitose, raffinose, xylitol, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol and 2-ketogluconate.

Characteristic	Strain YKJ-10 ^T	Strain YKJ-11
Colour of colonies	Cream-yellow	Light orange-yellow
Decomposition		
Tween 80	–	+
Acid production from:		
Cellobiose	+	–
Sucrose	+	–
Gentiobiose	+	–
Gluconate	–	w
5-Ketogluconate	+	–
Growth at 50 °C on TSA + ASW	w	–

Table 2. Cellular fatty acid profiles of strains YKJ-10^T and YKJ-11

Strain	Fatty acid composition (%)												
	i-C _{14:0}	C _{14:0}	i-C _{15:0}	ai-C _{15:0}	C _{15:0}	i-C _{16:0}	C _{16:0}	C _{16:1} ω 7c alcohol	C _{16:1} ω 11c	i-C _{17:0}	ai-C _{17:0}	i-C _{17:1} ω 10c	Summed feature 4*
YKJ-10 ^T	1.9	1.3	49.3	8.8	–	2.3	3.2	4.5	5.0	4.1	3.7	7.5	6.6
YKJ-11	4.3	1.6	46.0	6.4	0.9	5.4	3.9	8.5	5.3	4.3	3.3	5.7	4.1

* Summed feature represents groups of two or three fatty acids which could not be separated by GLC with the MIDI system. Summed feature 4 contained one or more of the following fatty acids: iso-C_{17:1} I and/or anteiso-C_{17:1} B.

YKJ-10^T were cream-yellow coloured, whereas colonies of strain YKJ-11 were light orange-yellow coloured. Growth on TSA and nutrient agar was poor. Strains YKJ-10^T and YKJ-11 grew in the presence of 13 % (w/v) NaCl but did not grow in the presence of more than 14 % (w/v) NaCl. The two strains grew at 10 and 45 °C but not at 55 °C. The optimal growth temperature of the two strains was 30–35 °C. The two strains grew well at a pH range of 7.0–8.0 but growth was inhibited at pH values below 5.0. Strains YKJ-10^T and YKJ-11 had catalase and urease activities but no oxidase activity. Nitrite was not produced from nitrate and indole was not formed. Aesculin, gelatin and starch were hydrolysed but no hydrolysis of casein, hypoxanthine, tyrosine and xanthine was observed in the two strains. Hydrolysis of Tween 80 was observed only in strain YKJ-11. Phenotypic properties of the two strains are summarized in Table 1.

Chemotaxonomic characteristics and DNA base composition

Strains YKJ-10^T and YKJ-11 contained *meso*-diaminopimelic acid as diamino acid in the cell wall peptidoglycan. The predominant isoprenoid quinone was unsaturated menaquinone with seven isoprene units (MK-7). The cellular fatty acid profiles of strains YKJ-10^T and YKJ-11 were similar and characterized by having iso-C_{15:0} (13-methyltetradecanoic acid) as the major fatty acid (Table 2). The G + C contents of strains YKJ-10^T and YKJ-11 were the same, 41 mol %.

Phylogenetic analysis based on 16S rDNA sequence

The almost complete 16S rDNA sequences of strains YKJ-10^T and YKJ-11 were directly determined following PCR amplification. The 16S rDNA sequences of strains YKJ-10^T and YKJ-11 determined in this

Fig. 1. Phylogenetic tree based on 16S rDNA sequences showing the positions of strains YKJ-10^T and YKJ-11, *Bacillus* species and some other taxa. The scale bar represents 0.01 substitutions per nucleotide position. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are shown at the branch points.

Table 3. Levels of DNA–DNA relatedness

Strain	Percentage reassociation with:	
	Strain YKJ-10 ^T	Strain YKJ-11
YKJ-10 ^T	100.0	93.5
YKJ-11	92.2	100.0
<i>Bacillus benzoovorans</i> KCTC 3392 ^T	7.5	9.8
<i>Bacillus circulans</i> KCTC 3347 ^T	10.3	10.9
<i>Bacillus firmus</i> KCTC 3626 ^T	14.5	13.4
<i>Bacillus lentus</i> DSM 9 ^T	12.7	10.7

study comprised 1505 nucleotides, representing approximately 96% of the *Escherichia coli* 16S rRNA sequence. Strains YKJ-10^T and YKJ-11 showed only a 1 bp sequence difference in their 16S rDNA sequences. A phylogenetic tree showed that both strains YKJ-10^T and YKJ-11 fall within the radiation of the cluster comprising *Bacillus* species (Fig. 1). Strains YKJ-10^T and YKJ-11 exhibited less than 96.5% and 96.6% 16S rDNA similarity, respectively, with *Bacillus* species whose 16S rDNA sequences are known. Levels of 16S rDNA similarity between strain YKJ-10^T and the type

strains of some phylogenetically related *Bacillus* species are as follows: *Bacillus benzoovorans*, 95.8%; *Bacillus circulans*, 96.2%; *Bacillus firmus*, 96.5%; *Bacillus lentus*, 95.5%; and *Bacillus methanolicus*, 96.1%.

DNA–DNA relatedness

A DNA–DNA relatedness test was performed between strains YKJ-10^T and YKJ-11 and between the two strains and the type strains of some *Bacillus* species

that are phylogenetically related to strains YKJ-10^T and YKJ-11. Strains YKJ-10^T and YKJ-11 exhibited two independent levels of DNA–DNA relatedness of 92.2% and 93.5%. Levels of DNA–DNA relatedness between strains YKJ-10^T and YKJ-11 and the type strains of some *Bacillus* species are shown in Table 3.

DISCUSSION

Strains YKJ-10^T and YKJ-11 show similar phenotypic characteristics except for some properties such as colour of colonies and acid production from carbohydrates (Table 1). The two strains are also phylogenetically and genetically similar. The 16S rDNA sequences have only a 1 bp sequence difference (Fig. 1). The genomic DNA relatedness between the two strains is more than 70%, the level of DNA relatedness for defining a species in current bacterial systematics (Wayne *et al.*, 1987). Accordingly, on the basis of the phenotypic, phylogenetic and genomic data, strains YKJ-10^T and YKJ-11 should be considered as members of the same species.

Strains YKJ-10^T and YKJ-11 revealed phylogenetic affiliation to the genus *Bacillus* from 16S rDNA sequence analysis (Fig. 1). In the phylogenetic tree based on 16S rDNA sequences, the two isolates occupy a distinct phylogenetic lineage within the radiation of the cluster comprising *Bacillus* species (Fig. 1). Chemotaxonomic characteristics support the result of the monothetic classification by 16S rDNA sequence analysis, although chemotaxonomic differentiation is not necessarily possible among aerobic or facultatively anaerobic, rod-shaped, endospore-forming genera. Cellular fatty acid profiles, particularly major fatty acid(s), distinguish strains YKJ-10^T and YKJ-11 from some genera associated with the genus *Bacillus* (Table 2). The genus *Paenibacillus* contains anteiso-C_{15:0} as the major fatty acid, whereas the genera *Amphibacillus*, *Brevibacillus*, *Gracilibacillus*, *Salibacillus* and *Virgibacillus* are known to simultaneously contain significant amounts of other fatty acids as well as anteiso-C_{15:0} (Shida *et al.*, 1997; Wainø *et al.*, 1999). The genera *Aneurinibacillus* and *Alicyclobacillus* contain iso-C_{15:0}, C_{16:0} and iso-C_{16:0} and ω -alicyclic acids, respectively, as the major fatty acids (Shida *et al.*, 1997). The genus *Bacillus* comprises variable species having iso-C_{15:0} or anteiso-C_{15:0} as the major fatty acid (Shida *et al.*, 1997). Accordingly, strains YKJ-10^T and YKJ-11 can be allocated to the group of *Bacillus* species that contain iso-C_{15:0} as the major fatty acid.

The taxonomic positions of strains YKJ-10^T and YKJ-11 within the genus *Bacillus* are clear from the results of 16S rDNA sequence comparison with *Bacillus* species (Fig. 1). Levels of 16S rDNA similarity between strains YKJ-10^T and YKJ-11 and *Bacillus* species whose 16S rRNA gene sequences are known are low enough to exclude the possibility of assigning the two strains to one of the *Bacillus* species (Stackebrandt & Goebel, 1994). Accordingly, strains YKJ-10^T and YKJ-11 can be considered as a new

species of the genus *Bacillus*, even if DNA–DNA hybridization with *Bacillus* species is not performed. Levels of DNA relatedness between strains YKJ-10^T and YKJ-11 and some *Bacillus* species which are phylogenetically related to the two strains are shown to be much lower than the value of approximately 70% which has been recognized for delineating a species (Table 3). Therefore, on the basis of the phenotypic and chemotaxonomic characteristics, the phylogeny and the genomic distinctiveness, strains YKJ-10^T and YKJ-11 should be placed within the genus *Bacillus* as a new species, for which a new species name, *Bacillus jeotgali* sp. nov., is proposed. The properties of the new species are summarized below.

Description of *Bacillus jeotgali* sp. nov.

Bacillus jeotgali (je.ot.ga'li. N.L. gen. n. *jeotgali* of jeotgal, Korean traditional food).

Cells are facultatively anaerobic rods that are 0.8–1.1 μ m wide and 4.0–6.0 μ m long in 24 h culture on TSA + ASW. Ellipsoidal spores are observed in swollen sporangia. Gram-variable. Motile by means of peritrichous flagella. Colonies are cream-yellow or light orange-yellow coloured, smooth, irregular and flat on TSA + ASW. See Table 1 for the physiological characteristics. Grows in the presence of 13% (w/v) NaCl. No growth occurs in the presence of more than 14% (w/v) NaCl. Growth occurs at 10 and 45 °C but not at 55 °C. Optimal growth temperature is 30–35 °C. Optimal pH for growth is 7.0–8.0. The growth is inhibited below pH 5.0. The cell wall peptidoglycan contains *meso*-diaminopimelic acid. The predominant menaquinone is MK-7. The major fatty acid is iso-C_{15:0}. The G + C content is 41 mol% (determined by HPLC). Isolated from Korean traditional fermented seafood, jeotgal. The type strain is strain YKJ-10^T, which has been deposited in the Korean Culture Center of Microorganisms as KCCM 41040^T and the Japan Collection of Microorganisms as JCM 10885^T.

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