Planococcus donghaensis sp. nov., a starch-degrading bacterium isolated from the East Sea, South Korea

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A novel Gram-positive, aerobic bacterium, strain JH1^T, was isolated from deep-sea sediment of the East Sea, South Korea, and identified by methods of polyphasic taxonomy. The strain was oxidase-positive, motile and coccus-shaped. The genomic DNA G + C content of strain JH1^T was 47 mol%. The major fatty acid of strain JH1^T was anteiso-C_{15:0} and the predominant menaquinones were MK-7 and MK-8. Similarity of the 16S rRNA gene sequence (1452 nt) of strain JH1^T to those of species of the genera *Planococcus* and *Planomicrobium* was 96.0–98.2%. The signature nucleotides in the 16S rRNA gene sequence were compared with those of previously studied type strains of species in the genera *Planococcus* and *Planomicrobium*, and suggested that strain JH1^T belongs to the genus *Planococcus*. In addition, phylogenetic analysis showed that strain JH1^T was located within the cluster comprising *Planococcus antarcticus* and *Planococcus kocurii*. DNA–DNA hybridization showed that it had 9.3% genomic relatedness with *Planococcus antarcticus* DSM 14505^T and 22.9% with *Planococcus kocurii* DSM 20747^T. On the basis of the phenotypic, phylogenetic and genomic data, a novel species of the genus *Planococcus*, *Planococcus donghaensis* sp. nov., is proposed, with type strain JH1^T (=KCTC 13050^T=LMG 23779^T).

The genus *Planococcus* was first established by Migula (1894) to accommodate Gram-positive, motile cocci of the family *Micrococcaceae*. However, the genus name *Planococcus* was validly published by Kocur *et al.* (1970) for the first species, *Planococcus citreus*. Yoon *et al.* (2001) created the novel genus *Planomicrobium* on the basis of menaquinone profiles and phylogenetic data, as well as cellular morphology. Consequently, *Planococcus okeano-koides* (Nakagawa *et al.*, 1996), *Planococcus mcmeekinii* (Junge *et al.*, 1998), *Planococcus alkanoclasticus* (Engelhardt *et al.*, 2001) and *Planococcus psychrophilus* (Reddy *et al.*, 2002) were transferred to the genus *Planomicrobium* (Yoon *et al.*, 2001; Dai *et al.*, 2005). At present, the genus *Planococcus* comprises eight recognized species (Euzéby, 1997). In this study, the taxonomic status of a novel

bacterial strain, JH1^T, was investigated by using a combination of phenotypic properties, phylogeny based on 16S rRNA gene sequence and genomic relatedness. The polyphasic evidence presented in this paper indicates that strain JH1^T represents a novel species of the genus *Planococcus*.

During screening of starch-degrading bacteria, strain JH1^T was isolated from marine sediment of the East Sea, South Korea, and selected to determine its exact taxonomic position. A bottom sediment sample was retrieved at a depth of 200–500 m, placed in a sterile tube and diluted serially with filtered deep-sea water. An aliquot of each dilution was spread on 100-fold-diluted Marine agar (MA; Difco) containing 1% starch and incubated at 17 °C for 3–10 days. Colonies showing a halo on the medium were selected as starch-degrading bacteria. Single colonies with the ability to degrade starch were purified by streaking onto new plates and subjected to additional incubation for

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain JH1^T is EF079063.

Table 1. Comparison of the phenotypic characteristics of strain JH1^T with those of close relatives in the genera *Planococcus* and *Planomicrobium*

Strains/species: 1, JH1^T (data from this study); 2, *Planococcus antarcticus* DSM 14505^T (Reddy *et al.*, 2002); 3, *Planococcus kocurii* DSM 20747^T (Hao & Komagata, 1985; Reddy *et al.*, 2002); 4, *Planococcus maritimus* JCM 11543^T (Yoon *et al.*, 2003); 5, *Planococcus maitriensis* DSM 15305^T (Alam *et al.*, 2003); 6, *Planococcus rifietoensis* DSM 15069^T (Romano *et al.*, 2003); 7, *Planococcus citreus* DSM 20549^T (Hao & Komagata, 1985; Reddy *et al.*, 2002); 8, *Planococcus stackebrandtii* MTCC 6226^T (Mayilraj *et al.*, 2005); 9, *Planomicrobium koreense* KCTC 3684^T (Yoon *et al.*, 2001); 10, *Planomicrobium okeanokoites* IFO 12536^T (Nakagawa *et al.*, 1996); 11, *Planomicrobium chinense* AS 1.3454^T (Dai *et al.*, 2005). +, Positive; −, negative; +/−, weakly positive; v, variable reaction; ND, not determined.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Cell morphology	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci/rods	Rods	Cocci/rods
Growth temperature (°C)	4–37	0–30	4–37	4–41	0–30	5–42	4–37	15–30	4–38	20–37	12–43
Oxidase	+	−	−	−	−	+	−	−	−	+/−	−
Hydrolysis of:											
Aesculin	+	+/−	−	−	+	ND	−	ND	+	−	−
Tween 80	−	+/−	−	−	ND	ND	−	ND	−	−	−
Starch	+	−	−	−	−	ND	−	−	−	−	−
Gelatin	−	+	v	+	+	+	+	+/−	+	+	+
Casein	+	ND	ND	+	+	−	ND	−	+	+	−
Acid production from:											
Glucose	−	+	v	+	−	ND	+	−	+/−	−	+
Mannitol	+	ND	−	+	−	ND	−	−	−	−	−
Melibiose	−	ND	−	−	ND	ND	−	ND	+	−	−
NaCl tolerance (%)	12	12	3.3	17	12.5	15	10	7	7	7	10
Predominant menaquinones	MK-7, MK-8	MK-7, MK-8	MK-7, MK-8	MK-8, MK-7, MK-6	MK-8, MK-7	MK-8	MK-8, MK-7	MK-7, MK-8	MK-8, MK-7, MK-6	MK-8, MK-7	MK-8, MK-7
DNA G + C content (mol%)	47	42	40–43	48	39	48	48–51	40	47	46	35

3 days at 25 °C. Cultures were stored at −80 °C in Marine broth (MB; Difco) supplemented with 25 % (v/v) glycerol. Strain JH1^T was grown routinely on MA or trypticase soy agar (TSA) or broth (TSB; Difco). *Planococcus kocurii* DSM 20747^T, *Planococcus antarcticus* DSM 14505^T, *Planomicrobium koreense* KCTC 3684^T and *Planomicrobium okeanokoites* KCTC 3672^T were used as reference strains for some analyses. For polar lipid analysis, menaquinone and cell-wall analyses and DNA extraction, strain JH1^T was cultured in MB at 25 °C. For fatty acid analysis, cells were grown on MA plates at 25 °C.

Morphology and cell size were determined by phase-contrast microscopy (80i; Nikon). Gram staining was performed with a BD Gram stain kit, according to the instructions of the manufacturer, and with a non-staining method, as described by Buck (1982). Catalase and oxidase activities were determined by using a bioMérieux Orientation test kit according to the manufacturer's instructions after cultivation for 2 days. Acid production from carbohydrates, single carbon-source assimilation and additional physiological characteristics were determined by using the API 20E, API 20NE and API ID32 galleries according to the instructions of the manufacturer

(bioMérieux). Tolerance to NaCl was measured on nutrient agar (Difco) containing 0–14 % (w/v) NaCl. Plates were incubated at 25 °C for 10 days. The temperature range for growth was determined on TSA incubated for 6 days at 0, 4, 10, 15, 20, 25, 30, 35, 37, 40, 45 and 50 °C.

Cellular fatty acid profiles were determined for strains grown on MA 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 then analysed by gas chromatography (6890; Hewlett Packard) using the Microbial Identification software package (Sasser, 1990). Isoprenoid quinones were extracted with chloroform/methanol (2:1), evaporated under vacuum conditions and re-extracted in n-hexane/water (1:1). Then, the crude menaquinone in n-hexane was purified by using Sep-Pak Vac Cartridges Silica (Waters) and subsequently analysed by HPLC as described by Hiraishi *et al.* (1996). Peptidoglycan structure was determined according to Schleifer & Kandler (1972). Polar lipids were extracted by a biphasic mixture of petroleum ether and methanolic saline and examined by two-dimensional TLC (Minnikin *et al.*, 1984).

Table 2. Comparison of fatty acid compositions of *Planococcus donghaensis* JH1^T and phylogenetically related species of the genera *Planococcus* and *Planomicrobium*

Strains/species: 1, JH1^T (data from this study); 2, *Planococcus antarcticus* DSM 14505^T (Reddy *et al.*, 2002); 3, *Planococcus kocurii* DSM 20747^T (Yoon *et al.*, 2003); 4, *Planococcus maritimus* JCM 11543^T (Yoon *et al.*, 2003); 5, *Planomicrobium koreense* KCTC 3684^T (Yoon *et al.*, 2001); 6, *Planomicrobium okeanokoites* IFO 12536^T (Yoon *et al.*, 2001). Percentage of total fatty acids is shown.

Fatty acid	1	2	3	4	5	6
Straight-chain fatty acids						
C _{15:0}	1.7	14.2	7.2	3.1	2.4	—
C _{16:0}	6.3	4.2	1.4	0.8	—	1.1
C _{17:0}	1.3	1.0	—	2.5	—	—
Branched fatty acids						
iso-C _{14:0}	3.4	1.0	13.1	13.1	21.0	40.4
iso-C _{15:0}	4.7	1.3	5.1	9.5	6.4	—
anteiso-C _{15:0}	43.8	43.2	40.9	30.6	26.1	4.0
iso-C _{16:0}	9.4	4.0	7.2	18.5	11.1	24.5
iso-C _{17:0}	6.4	0.3	1.7	3.1	1.6	—
anteiso-C _{17:0}	15.5	9.5	2.6	4.4	1.6	—
iso-C _{17:1} ω10c	0.7	Unknown	2.5	0.8	1.9	—
iso-C _{18:0}	0.4	—	—	2.1	—	0.9
Unsaturated fatty acids						
C _{16:1} ω7c alcohol	1.6	Unknown	11.4	8.9	23.6	26.1
C _{16:1} ω11c	1.3	Unknown	2.5	0.7	1.7	2.1
Summed feature 5*	1.9	4.2	4.5	1.3	—	—

*Summed feature 5 represents iso-C_{17:1} I and/or anteiso-C_{17:1} B, which could not be separated by GLC with the MIDI system.

For phylogenetic analysis of strain JH1^T, DNA was extracted by using a commercial genomic DNA extraction kit (Solgent). The 16S rRNA gene was amplified from the chromosomal DNA by using the universal bacterial primer set 27F and 1492R, and the purified PCR products were sequenced by Solgent, South Korea (Park *et al.*, 2006). The full sequences of the 16S rRNA gene were compiled by using SeqMan software (DNASTAR). The 16S rRNA gene sequences of related taxa were obtained from GenBank. Multiple alignments were performed by using the CLUSTAL_X program (Thompson *et al.*, 1997). Gaps were edited in the BioEdit program (Hall, 1999). Evolutionary distances were calculated by using the Kimura two-parameter model (Kimura, 1980). A phylogenetic tree was constructed by using the neighbour-joining method (Saitou & Nei, 1987) in the MEGA3 program (Kumar *et al.*, 2004), with bootstrap values based on 1000 replications (Felsenstein, 1985).

For measurement of the G + C content of the chromosomal DNA, the extracted genomic DNA was degraded enzymically into nucleosides and the DNA G + C content was determined as described by Mesbah *et al.* (1989) by using reverse-phase HPLC. DNA–DNA hybridization experiments were performed between strain JH1^T and

Planococcus antarcticus DSM 14505^T, *Planococcus kocurii* DSM 20747^T, *Planomicrobium koreense* KCTC 3684^T and *Planomicrobium okeanokoites* KCTC 3672^T, using photo-biotin-labelled DNA probes and microdilution wells as described by Ezaki *et al.* (1989). Hybridization was performed with five replications for each sample. The highest and lowest values obtained for each sample were excluded and the means of the remaining three values were quoted as the DNA relatedness value.

Strain JH1^T appeared as Gram-positive, aerobic and motile cocci in all growth phases, as shown by all members of the genus *Planococcus*. Morphological, physiological, biochemical and chemotaxonomic characteristics are given in Table 1. Phenotypically, strain JH1^T could hydrolyse starch and could not hydrolyse gelatin, characteristics that are unique in the genera *Planococcus* and *Planomicrobium*. In addition, strain JH1^T differed from related species by having positive reactions for oxidase and hydrolysis of aesculin. The amino acids in the cell wall were lysine, glutamic acid and alanine. Accordingly, the peptidoglycan type of strain JH1^T was A4α, based on L-Lys–D-Glu, as described by Schleifer & Kandler (1972). This result shows that strain JH1^T had an identical cell-wall peptidoglycan type (L-Lys–D-Glu) to *Planococcus* species, whereas *Planomicrobium* species, except for *Planomicrobium koreense*, have cell-wall peptidoglycan based on L-Lys–D-Asp (Yoon *et al.*, 2001). The major menaquinones in strain JH1^T were found to be similar amounts of MK-7 and MK-8, as in most other members of the genus *Planococcus* (Hao & Komagata, 1985).

Strain JH1^T contained anteiso-C_{15:0} (43.8 %) as the major fatty acid and significant amounts of anteiso-C_{17:0} (15.5 %) and C_{16:0} (6.3 %) (Table 2). The cellular phospholipids found in strain JH1^T were phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol. The genomic DNA G + C content of strain JH1^T was 47 mol%.

To determine the phylogenetic position of strain JH1^T, the 16S rRNA gene sequence (1452 nt) was compared with those of type strains of species of the genera *Planococcus* and *Planomicrobium* retrieved from GenBank. Sequence similarity of strain JH1^T to species in the genera *Planococcus* and *Planomicrobium* ranged from 96.0 to 98.2 %. Phylogenetic analyses based on the neighbour-joining method (Fig. 1) and maximum-parsimony method (data not shown) showed that strain JH1^T falls within the evolutionary radiation of the genus *Planococcus*, with moderate bootstrap support (Fig. 1). In addition, at positions 183 and 190 (*Escherichia coli* numbering), JH1^T had the signature nucleotides of the genus *Planococcus* (T and A), rather than the C and G nucleotides characteristic of the genus *Planomicrobium* (Dai *et al.*, 2005). Strain JH1^T falls within the cluster comprising *Planococcus antarcticus* and *Planococcus kocurii*. JH1^T exhibited highest levels of sequence similarity to the type strains of these species (98.2 and 97.2 %, respectively).

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences (1452 nt), showing the phylogenetic relationship of *Planococcus donghaensis* JH1^T with type strains of related species of the genera *Planococcus* and *Planomicrobium*. Bootstrap values (expressed as a percentage of 1000 replications) >50% are given at nodes. The *Bacillus subtilis* and *Exiguobacterium aurantiacum* sequences were used as an outgroup. Bar, 1% sequence variation.

DNA–DNA relatedness values of strain JH1^T are 9% with *Planococcus antarcticus* DSM 14505^T, 22% with *Planococcus kocurii* DSM 20747^T, 6% with *Planomicrobium okeanokoites* KCTC 3672^T and 24% with *Planomicrobium koreense* KCTC 3684^T, suggesting clearly that it is a novel species. When 16S rRNA gene sequence similarity is $\geq 97\%$, members of other species of the genus can be excluded from DNA–DNA hybridization studies (Stackebrandt & Goebel, 1994). The DNA–DNA relatedness between the two strains is <70%, indicating a level of DNA relatedness appropriate for defining a species in current bacterial systematics (Wayne *et al.*, 1987). Levels of DNA–DNA relatedness provide decisive evidence that strain JH1^T is genetically different from the phylogenetically related species *Planococcus antarcticus*, *Planococcus kocurii*, *Planomicrobium koreense* and *Planomicrobium okeanokoites* (Wayne *et al.*, 1987). Therefore, on the basis of morphology, growth characteristics, biochemical and chemotaxonomic characteristics, molecular systematic studies including 16S rRNA gene sequence analysis, and DNA–DNA hybridization analysis, strain JH1^T should be placed in the genus *Planococcus* as the type strain of a novel species, for which the name *Planococcus donghaensis* sp. nov. is proposed.

Description of *Planococcus donghaensis* sp. nov.

Planococcus donghaensis (dong.ha.en'sis. N.L. masc. adj. *donghaensis* of Donghae, the Korean name for the East Sea in Korea, from which the strain was isolated).

Cells are Gram-positive, aerobic, non-spore-forming, motile cocci, 0.8–1.2 μm in diameter, and occur singly, in pairs, in groups of three or in tetrads. Colonies are opaque, smooth, glistening, low convex in the centre, circular, orange in colour and 2.0–3.0 mm in diameter after 3 days cultivation at 25 °C on TSA. Growth occurs at 4 and 37 °C, but not above 40 °C. Optimal growth temperature is 25–30 °C. Optimal pH for growth is 7.5–8.0. Growth occurs at pH 7.0, but not at pH 6.0. Growth occurs in the presence of 12% (w/v) NaCl. Optimal growth occurs in the presence of 2% (w/v) NaCl. Aesculin, starch and casein are hydrolysed. Tween 80 and gelatin are not hydrolysed. Positive for β -galactosidase, citrate utilization and Voges–Proskauer test, but negative for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase, urease, hydrogen sulfide production and indole production. Glucose, mannitol, *N*-acetylglucosamine, maltose, gluconate, salicin, propionate, valerate, 3-hydroxybutyrate, proline, ribose, sucrose, acetate, alanine and serine are utilized, but mannose, caprate, adipate, malate, phenylacetate, melibiose, *L*-fucose, sorbitol, *L*-arabinose, histidine, 2-ketogluconate, 4-hydroxybenzoate, *L*-rhamnose, inositol, itaconate, suberate, malonate, *DL*-lactate, 5-ketogluconate, glycogen and 3-hydroxybenzoate are not. Acid is produced from *D*-mannitol, *D*-sucrose and amygdalin, but not from *D*-glucose, inositol, *D*-sorbitol, *L*-rhamnose, *D*-melibiose or *L*-arabinose. The type of cell-wall peptidoglycan is *L*-Lys–*D*-Glu and the predominant polar lipids are phosphatidylethanolamine, phosphatidylglycerol

and diphosphatidylglycerol. The DNA G+C content of the type strain is 47 mol%. Other characteristics are given in Table 1.

The type strain, strain JH1^T (=KCTC 13050^T=LMG 23779^T), was isolated from deep-sea sediment of the East Sea in South Korea.

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