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Halorubrum cibi sp. nov., an Extremely Halophilic Archaeon from Salt-Fermented Seafood

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Strain B31^T is a Gram-staining-negative, motile, and extremely halophilic archaeon that was isolated from salt-fermented seafood. Its morphology, physiology, biochemical features, and 16S rRNA gene sequence were determined. Phylogenetic analysis of its 16S rRNA gene sequence and composition of its major polar lipids placed this archaeon in the genus *Halorubrum* of the family *Halobacteriaceae*. Strain B31^T showed 97.3, 97.2, and 96.9% 16S rRNA similarity to the type strains of *Halorubrum alkaliphilum*, *Hrr. tibetense*, and *Hrr. vacuolatum*, respectively. Its major polar lipids were phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (PGP-Me) and sulfated diglycosyl diether (S-DGD). Genomic DNA from strain B31^T has a 61.7 mol% G+C content. Analysis of 16S rRNA gene sequences, as well as physiological and biochemical tests, identified genotypic and phenotypic differences between strain B31^T and other *Halorubrum* species. The type strain of the novel species is B31^T (=JCM 15757^T =DSM 19504^T).

***Keywords*:** *Halorubrum cibi* sp. nov., archaeon, taxonomy, salt-fermented seafood

Aerobic extremely halophilic archaea can grow optimally in NaCl solutions of 2.6 M or higher (Ventosa *et al.*, 1998) and these are classified within the family *Halobacteriaceae* in the order *Halobacteriales*. The genus *Halorubrum* belongs to the family *Halobacteriaceae*, as first proposed by McGenity and Grant (1995). Members of the genus *Halorubrum* contain the polar lipids such as phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (PGP-Me), phosphatidylglycerol sulfate (PGS), and a sulfated diglycosyl diether (S-DGD) (McGenity and Grant, 2001) and have the G+C content in the range of 60.2~71.2 mol% (McGenity and Grant, 2001; Hu *et al.*, 2008). This genus contains *Halorubrum saccharovorum* (McGenity and Grant, 1995) as a type species as well as other 21 species (http://www.bacterio.net/) (Euzeby, 1997)

We isolated an extremely halophilic archaeon, designated strain B31^T while studying the microbiota of salt-fermented seafood ('jeotgal' in Korean) whose salt concentration of the liquid part is close to or at saturation (approximately 35%, w/v). Shrimp jeotgal is one of the traditional Korean seafood made by combining fresh, tiny shrimps with rock salt and then fermenting the mixture for several months (Suh and Yoon, 1987). It has been reported that a number of archaea and bacteria have been isolated from salt-fermented seafood in Korea (Euzeby, 1997). In this paper, we characterize strain B31^T and describe the features of this new species.

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Materials and Methods

Isolation of the archaeal strain

Strain B31^T was isolated from shrimp jeotgal by repeated streaking onto the complex medium (DSM medium 954) containing (g/L): casamino acids (BBL), 5; yeast extract (BBL), 5; $MgCl_2 \cdot 6H_2O$, 20; KCl, 2; Tris, 12; $CaCl_2 \cdot 2H_2O$, 0.2; NaCl, 200, and antimicrobial compounds (100 μg/ml of penicillin G, erythromycin, and cycloheximide), which inhibit bacteria and eukarya but not archaea (Purdy *et al.*, 2004). The complex medium was adjusted to pH 7.4, followed by incubation at 37°C for 5~7 days.

Morphology and physiological characterization

The characterization of a novel halophilic archaeon was guided by Oren *et al.* (1997) with the proposed minimal standards for description of new taxa in the order *Halobacteriales*. Cell morphology was examined by light microscopy (ECLIPSE 80*i*, Nikon) and electron microscopy (JEM 1010, JEOL). Gram-staining was performed using the standard staining method for haloarchaea (Dussault's technique) (Dussault, 1955). The motility was examined on semi-solid agar plates and cell lysis in distilled water was detected by microscopic examination. The phenotypic tests for nitrate reduction, indole formation, and hydrolysis of casein, starch and urea, were performed as described by Gerhardt *et al.* (1994). Hydrolysis of gelatin and Tween 80 were conducted simultaneously as described by Gutierrez and Gonzalez (1972). Deoxyribonuclease activity was detected as described by Gonzalez *et al.* (1978). Mg^{2+} requirement for growth was determined using the medium 954 containing 0.01% of yeast extract without $MgCl_2 \cdot 6H_2O$ after 2 month incubation. Growth on different complex media: DSM medium 97, 205,

371, 372, and 1018, was also determined. Optimal conditions for growth were determined in the medium 954 at different NaCl concentrations (0, 1, 3, 5, 7.5, 10, 15, 20, 23, 25, 28, and 30%, w/v), at the pH range from pH 5.0 to 11.0 at intervals of 0.5, and at different temperatures (4, 10, 15, 17, 21, 25, 30, 37, 45, 50, and 60°C). Utilization of sole carbon and energy sources from sugars were determined as described by Montalvo-Rodriguez *et al.* (2000) after 2 month incubation. Test of anaerobic growth in the presence of nitrate, sulfate, thiosulfate, and DMSO, was performed as described by Sehgal and Gibbons (1960) for 1 month. Oxidase activity was determined by oxidation of tetramethyl-*p*-phenylenediamine and catalase activity was determined by bubble production in a 3% (v/v) hydrogen peroxide solution.

Phylogenetic analysis based on 16S rRNA gene sequences

The chromosomal DNA was extracted and purified as described by Sambrook *et al*. (1989), and the determination of 16S rRNA gene sequences was performed as described previously (Roh *et al.*, 2008). The identification of phylogenetic neighbors and the calculation of pairwise sequence similarity against the database of type strains of validly named species using global alignment algorithm, were performed by using the EzTaxon tool (server 2.0) (Chun *et al.*, 2007). The MEGA 4 software program (Tamura *et al.*, 2007) and PHYLIP software package (Felsenstein, 2005) were used to determine the phylogenetic relationships between our isolate and the type strains of species of the genus *Halorubrum*. Distance matrices were determined using the method by Kimura (1980) and used to generate dendrograms by the neighbor-joining (Saitou and Nei, 1987), maximum-parsimony (Kluge and Farris, 1969) and maximum-likelihood (Felsenstein, 1981) methods. Bootstrap analysis was used to evaluate the stability of phylogenetic trees. It was performed using a consensus tree that was based on 1,000 randomly-generated trees, except for maximum-likelihood method, which was done based on 300 replications.

Nucleotide sequence accession number

The GenBank/EMBL/DDBJ accession number for the strain B31^T 16S rRNA gene sequence is EF077639.

Determination of G+C content, DNA-DNA hybridization and polar lipid profile

The genomic DNA G+C content was determined by a fluorimetric method using SYBR Green and a real-time PCR thermocycler as described by Gonzalez and Saiz-Jimenez (2002) with *Escherichia coli* K12 as a calibration reference. DNA-DNA hybridization experiment was performed by the fluorometric method of Ezaki *et al*. (1989). The composition of polar lipids of the new isolate was determined with the extraction method described by Xin *et al.* (2000) and the detection method with specific reagents sprayed on a Merck silica gel 60 F254 aluminium-backed plate, as described by Tindall (1990). The designations of all spots on silica gel plate were as referred to by Cui *et al.* (2006).

Results and Discussion

Morphology and physiological characteristics

The strain B31^T is motile, Gram-negative, and rod-shaped. The colonies are red-colored and circular. The strain B31^T does not reduce nitrate to nitrite, is catalase-positive and oxidase-negative, and is negative for indole formation. The characteristics of B31^T and various reference strains are compared in Table 1 and a detailed species description is presented below. As shown in Table 1, the new isolate could be readily differentiated from the reference species on the basis of several characterastics.

Phylogenetic analysis based on 16S rRNA gene sequence and DNA-DNA hybridization

Comparison of 16S rRNA gene sequences from the strain

Table 1. Differential characteristics of *Halorubrum cibi* with respect to other closely related *Halorubrum* species

Characteristic	1	2	3	4	5	6
Motility	+	+	-	-	+	+
Growth at 17°C	+	-	-	-	-	-
Growth at 50°C	-	-	-	+	+	+
Growth at pH 10.5	-	+	+	+	-	-
Mg^{2+} requirement	+	-	-	-	-	+
Indole production	-	+	-	NR	-	-
Nitrate reduction	-	+	+	+	+	+
Oxidase	-	+	+	+	+	+
Utilization of:						
Sucrose	-	-	+	+	NR	+
Glucose	-	+	+	+	+	+
Lactose	+	-	+	NR	-	+
Acetate	-	-	+	+	-	NR
Presence of PGS	-	-	-	-	-	+
Presence of S-DGD	+	-	-	-	+	+
G+C content (mol%)	61.7	62.1	63.3	62.7	64.2	71.2

Taxa: 1, *Halorubrum cibi* B31^T sp. nov. (data from this study); 2, *Hrr. alkaliphilum* DZ-1^T (Feng *et al*., 2005); 3, *Hrr. tibetense* 8W8^T (Fan *et al*., 2004); 4, *Hrr. vacuolatum* M24^T (Mwatha and Grant, 1993); 5, *Hrr. orientale* EJ-52^T (Castillo *et al.*, 2006); 6, *Hrr. saccharovorum* M6^T (McGenity and Grant, 2001). +, Positive; -, negative; NR not reported; PGS, phosphatidyl glycerol sulfate; S-DGD, sulfated mannosyl-glucosyl-glycerol diether.

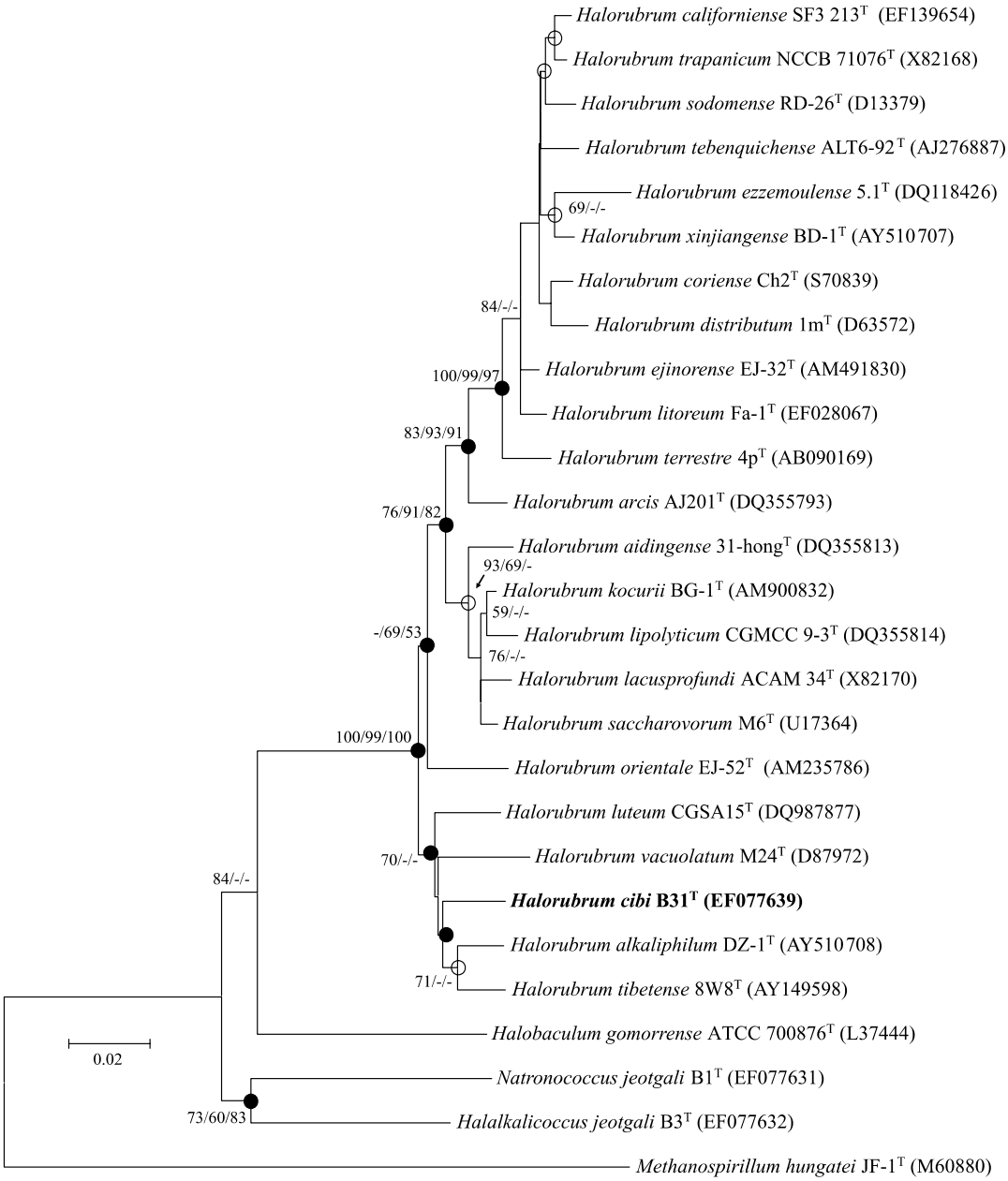

Fig. 1. Phylogenetic tree generated with 16S rRNA gene sequences using the neighbor-joining method, indicates the position of strain B31^T with respect to other species of the genus *Halorubrum* and other haloarchaea. The 16S rRNA sequence accession numbers used are included in brackets and the 16S rRNA gene of *Methanospirillum hungatei* JF-1^T was used as an outgroup. Filled circles indicate the nodes that were also recovered by using maximum-parsimony (MP) and maximum-likelihood (ML) algorithms; and open circles indicate the nodes that were also recovered by using one of MP and ML algorithms of MP and ML. Numbers at nodes indicate bootstrap values as calculated by neighbor-joining/ maximum-parsimony/ maximum-likelihood probabilities in percent. Bootstrap values greater than 50% are shown at the branch points. Bar, 0.01 substitutions per nucleotide position.

B31^T and reference species in the family *Halobacteriaceae*, indicated that the isolate, B31^T falls within the *Halorubrum* cluster, supported by the high bootstrap values (100, 99, and 100 by the neighbor-joining, maximum-parsimony and maximum-likelihood method, respectively), and is specifically clustered with *Hrr. alkaliphilum* and *Hrr. tibetense*, as having relatively low bootstrap values (<50) regardless of different tree-making algorithms in the phylogenetic tree (Fig. 1). Strain B31^T exhibited 97.3% 16S rRNA gene similarity to *Hrr. alkaliphilum* DZ-1^T, 97.2% similarity to *Hrr. tibetense* 8W8^T and 96.9% similarity to *Hrr. vacuolatum* M24^T, in the order of the highest 16S rRNA gene sequence similarity. The similarity between the 16S rRNA gene sequence of strain B31^T and other species from the genus *Halorubrum* ranged between 97.3 and 94.4%. The DNA-DNA hybridization study showed an average level of DNA-DNA related-

ness between B31^T and the type strain of its closest relatives that have the 16S rRNA gene sequence similarity of >97.0% with the isolate: *Halorubrum alkaliphilum* (21.5%), *Hrr. tibetense* (16.6%). Therefore, strain B31^T can be considered a distinct genospecies, based on the characteristics of 16S rRNA gene sequence similarity and DNA-DNA reassociation value of less than 70% (Wayne *et al.*, 1987).

Determination of G+C content and polar lipid profile

The G+C content of the strain B31^T is 61.7 mol%. The genomic DNA G+C content in the valid species names of the genus *Halorubrum* is in the range of 60.2~71.2 mol% (McGenity and Grant, 2001; Hu *et al*., 2008). The G+C content of the isolate is in accord with G+C content of other members of the genus *Halorubrum* and confirms the affiliation of strain B31^T within this genus.

The major polar lipids of the strain B31^T are PG, PGP-Me, and S-DGD. The members belonging to the genus *Halorubrum* contain PG, PGP-Me, PGS, and/or a S-DGD (McGenity and Grant, 2001). The polar lipids composition of the novel strain correlates with those of species of *Halorubrum* and the presence of glycolipid, S-DGD in the novel strain could clarify the distinction from valid species having the highest 16S rRNA gene sequence similarity with novel strain: *Hrr. alkaliphilum*, *Hrr. Tibetense*, and *Hrr. vacuolatum* that do not have S-DGD (Mwatha and Grant, 1993; Fan *et al.*, 2004; Feng *et al.*, 2005).

The polyphasic phylogenetic analysis with the 16S rRNA gene sequence, DNA-DNA relatedness, genomic DNA G+C content, polar lipid profile, and results from physiological and biochemical tests, indicates clear genotypic and phenotypic differences between strain B31^T and other *Halorubrum* species. For these reasons, it is concluded that strain B31^T represents a novel species of genus *Halorubrum*, for which the name *Halorubrum cibi* sp. nov. is proposed.

Description of *Halorubrum cibi* sp. nov.

Halorubrum cibi (ci'bi. L. n. *cibus -i* food; L. gen. n. *cibi* of food).

The cells are Gram-staining-negative, motile and rod-shaped (0.4~0.5 μm wide and 0.8~1.1 μm long). The colonies are red-colored, circular with entire margin, convex and shiny with a diameter of 0.5~1.0 mm after incubating 5 days on complex agar medium (DSM medium 954) at 37°C. Growth also occurs on complex media like DSM medium 97, 371, and 372, but not on medium 205 or 1018. Growth occurs at temperatures ranging from 17 to 45°C (optimum, 30~37°C), in the presence of 15~30% (w/v) NaCl (optimum, 23~25%), and in the pH range 7.0 to 8.5 (optimum, pH 7.5). Mg^{2+} is required for growth. Anaerobic growth with nitrate, sulfate, thiosulfate or DMSO does not occur. Cell lysis occurs on hypotonic condition. The isolate does not reduce nitrate to nitrite under aerobic condition, is catalase-positive and oxidase-negative, and is negative for indole formation. Casein, starch, urea, DNA, gelatin, and Tween 80 are not hydrolysed. Lactose is utilized as carbon and energy source, but fructose, acetate, sucrose, glucose, citrate, and formate are not. Resistant to the following antimicrobial compounds (μg/ml): penicillin (50), ampicillin (25), chloramphenicol (50), and erythromycin (50); Sensitive to: novobiocin (25), bacitracin (25), ampicillin (50), anisomycin (25), and aphidicolin (25). The polar lipids are PG, PGP-Me and S-DGD. The G+C content of B31^T genomic DNA is 61.7 mol%.

The type strain is B31^T (=JCM 15757^T =DSM 19504^T) and it was isolated from salt-fermented seafood from Korea.

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