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A report of 7 unrecorded haloarchaea species from seawater samples near Dokdo island, Korea

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In July 2023, the seawater samples were collected near Dokdo island to obtain unrecorded haloarchaea in Korea. These samples were suspended in a 20% NaCl (w/v) solution and enriched for one month at 37°C in fresh DB Characterization media No. 2 broth with simply modification. Further, the enriched culture was spread on agar medium to obtain single colonies of halophilic archaea. Phylogenetic analysis based on the 16S rRNA gene showed that the isolates from this study exhibited minimum 98.7% sequence similarity with previously reported species. Finally, 7 haloarchaeal species, which had not been reported in Korea but were validly published under the International Code of Nomenclature of Prokaryotes (ICNP), were obtained. These isolates were classified into the orders *Halobacteriales*. The 7 *Halobacteriales* species were further categorized into the family *Haloferacaceae*, comprising 2 genus, *Haloferax* and *Haloplanus*. Collectively, these unrecorded haloarchaeal species spanned 1 order, 1 family, and 2 genera. This research highlights the potential for discovering previously unknown species in domestic seawater environment. Comprehensive analyses, including Gram staining, cell morphology, physiological and basic biochemical parameters, and phylogenetic analysis, were conducted and are detailed for each species.

Keywords: Dokdo island, Haloarchaea, seawater, unrecorded species

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Introduction

Extremophiles are microorganisms capable of growing, adapting, and thriving in extreme environments, such as those with high salinity, extreme temperatures, or acidic and alkaline conditions (Rampelotto, 2013). Among these, haloarchaea are the members of the class *Halobacteria*, which belongs to the *Methanobacteriota* phylum of the archaea (Goker and Orea, 2023). They can thrive in hypersaline habitats, including natural brine, the Dead Sea, alkaline salt lakes, marine solar salterns, and rock salt deposits (Kato *et al*., 1995; Stan-Lotter and Fendrihan, 2015). Beyond high salinity, haloarchaea are exposed to additional extreme conditions such as elevated temperatures, UV radiation, severe ionic stresses, and alkaline pH. To survive and grow under such harsh conditions, these archaea produce compounds such as carotenoids, halorhodopsin, and polyhydroxyalkanoates (PHA). Carotenoids are abundant in haloarchaea, which have a role of antioxidants, protect cell to external stress (Oren, 2014; Squillaci *et al*., 2017). Bacterioruberin, a representative C_{50} carotenoid produced by haloarchaea, is capable to membrane stabilizers, contributing to their adaptability, and survival (Grivard *et al*., 2022). PHA is a biopolymer that accumulates as carbon and energy storage inclusions, serving as a survival strategy to overcome physical, geochemical, and environmental stresses in archaea (Kumar and Kim, 2018; Kumar *et al*., 2020). Halorhodopsin is the known anion pump detected in haloarchaea that is energized by light (Oesterhelt, 1998). Haloarchaea thrive in saturated brines with isoosmolar salt concentrations in their cytoplasm. During cell growth, halorhodopsin plays a crucial role by

using light energy to help maintain osmotic balance (Kolbe *et al*., 2000). The unique metabolites and proteins of these halophilic archaea make them valuable for various industrial and research applications (Singh and Singh, 2017). Therefore, discovering haloarchaea from halophilic environment is considered highly valuable.

Research on the microbial biodiversity of Dokdo is limited, particularly regarding the isolation of halophilic archaea from seawater around Dokdo. To contribute to the understanding of domestic biodiversity, this study focuses on unrecorded haloarchaeal species that have not yet been reported in Korea. In this study, we identified 7 species of haloarchaea from the orders *Halobacteriales*, belonging to 2 genera (*Haloferax* and *Haloplanus*) and one family *Haloferacaceae*, based on 16S rRNA gene sequence. The phenotypic characteristics of these species were confirmed their status as previously unrecorded haloarchaea in Korea.

Materials and Methods

Samples were collected from seawater near Dokdo island in July 2023. The obtained seawater samples (5 mL) were mixed in 10 mL of 20% NaCl (v/v) and vortexed enough. The sample solutions (15 mL) were inoculated directly to 100 mL of modified DB characterization medium No. 2 (DBCM2) broth in 500 mL shaking flask as following components (l^{-1}): 833 mL MDS salt water (7 g of KCl, 35 g of $MgSO_4 \cdot 7H_2O$, 30 g of $MgCl_2 \cdot 6H_2O$, 240 g of NaCl, and 5 mL of 1 M $CaCl_2$ solution), 1 mL of $FeCl_2$ solution, 0.05 g of peptone (Oxoid, Hampshire, UK), 0.25 g of yeast extract (BD, Franklin Lakes, NJ, USA), 5 mL of 1 M NH_4Cl, 2 mL of potassium phosphate buffer, 1 mL of trace element solution (2.0 mg of $FeSO_4 \cdot 7H_2O$, 141.5 mg of $ZnSO_4 \cdot 7H_2O$, 57.6 mg of $MnSO_4 \cdot H_2O$, 6.0 mg of H_3BO_3, 190.0 mg of $CoCl_2 \cdot 6H_2O$, 2.0 mg of $CuCl_2 \cdot 2H_2O$, 24.0 mg of $NiCl_2 \cdot 6H_2O$, and 36.0 mg of $Na_2MoO_4 \cdot 2H_2O$ per liter), 3 mL of vitamin solution (3.0 mg of biotin, 4.0 mg of folic acid, 50.0 mg of pyridoxine · HCl, 33.0 mg of thiamine · HCl, 10.0 mg of riboflavin, 33.0 mg of nicotinic acid, 17.0 mg of DL-calcium pantothenate, 17.0 mg of vitamin B_{12}, 13.0 mg of *p*-aminobenzoic acid, and 10 mg of lipoic acid per liter), 10 mL of 1 M sodium pyruvate solution, supplemented 0.25 g of fish peptone (HiMedia, Maharashtra, India), 1 g of D-glucose (Samchun chemical, Pyeongtaek, Korea), and 1 g of sucrose (SAMCHUN CHEMICALS, Pyeongtaek, Korea), and finally adjusted to pH 7.0–7.3 using 1 M Tris-base buffer solution. The DBCM2 agar plate was added with 2% (w/v) agarose (KisanBio, Seoul, Korea). Each enrichment culture was incubated at 37°C for one month. Subsequently, the enriched culture broths were serially diluted with DBCM2 broth. Different colonies were selected and repeatedly subcultured into fresh culture media at intervals of 3–7 days. After more than three subculturing steps, pure colonies were obtained and stored at −80°C in a 20% (w/v) glycerol with 15% (w/v) NaCl stock solution for preservation.

Phenotypic experiments were conducted on cultures grown at 37°C on DBCM2 agar plates. The cell morphology of isolated species was examined using light microscopy (model CX 23; Olympus, Tokyo, Japan) and transmission electron microscopy (LIBRA 120; Carl Zeiss, Oberkochen, Germany). Gram staining of the isolated species was performed using a Gram stain kit (BioWORLD, Dublin, OH) according to the manufacturer's instructions. Each species was cultured in DBCM2 broth with NaCl concentrations ranging from 5% to 30% (w/v), in increments of 5%, for 3–7 days at 37°C and 200 rpm to evaluate cell growth at varying salinities. The pH range for cell growth was assessed by growing the cells for 3–7 days at 30°C and 200 rpm, using buffer solutions with pH increments of 1.0 unit from pH 4.0 to 12.0: 100 mM of CH_3COOH/CH_3COONa buffer (pH 4.0–6.0), 100 mM of NaH_2PO_4/Na_2HPO_4 buffer (pH 7.0–8.0), 100 mM $NaHCO_3/Na_2CO_3$ buffer (pH 9.0–10.0), and 100 mM of Na_2CO_3/NaOH buffer (pH 11.0–12.0). To determine the temperature range for growth, each species was cultivated on DBCM2 agar and incubated at 4, 10, 15, 20, 25, 30, 35, 37, 40, 45, and 55°C for 3–7 days. Catalase and oxidase tests were performed using 3% (v/v) H_2O_2 and 1% tetramethyl-p-phenylenediamine, respectively. Energy sources used in assimilation tests included D-glucose, D-mannose, D-galactose, D-maltose, D-sucrose, D-lactose, D-fructose, D-ribose, D-arabinose, L-rhamnose, D-xylose, trehalose, raffinose, glycerol, L-sorbose, pyruvate, D-mannitol, succinate, DL-lactate, L-malate, fumarate, citrate, acetate, L-glycine, L-histidine, L-alanine, L-glutamate, L-glutamic acid, L-arginine, L-lysine, and L-ornithine, each at 0.1% (w/v) based on DBCM2 broth without any carbon and nitrogen sources.

Genomic DNA was extracted using the LaboPass™ Tissue Genomic DNA Isolation kit (Cosmogenetech, Seoul, Korea). The partial 16S rRNA gene was amplified by polymerase chain reaction (PCR) using archaeal-specific primers (Cui *et al*., 2009). The obtained PCR products were purified using the LaboPass™ PCR purification kit (Cosmogenetech, Seoul, Korea) and sent to Macrogen Co., Ltd. (Seoul, Korea) for sequence analysis. Sequencing was performed using archaeal universal primer including 18F (5′-ATTCCGGTTGATCCTGCC-3′), 344F (5′-CGGG YGCASCAGGCGCGAA-3′), 520R (5′-GWATTACCGC GGCKGCTG-3′), 787F (5′-ATTAGATACCCSBGTAG TCC-3′), and 1518R (5′-AGGAGGTGATCCAGCCGC-3′) (Abdallah *et al*., 2018). The sequences were assembled using the SeqMan™ II expert sequence analysis software (Thombre *et al*., 2016). Assembled sequences were identified based on the EzBioCloud 16S-based ID (https://

www.ezbiocloud.net/identify). Closely related taxa were obtained from the EzTaxon-e server (http://www.ezbiocloud.net/eztaxon) (Yoon *et al*., 2017). Multiple sequence alignments were conducted using the Clustal W multiple sequence alignment program in BioEdit 7.2.6.1 software (Thompson *et al*., 1994; Hall, 1999). Phylogenetic trees were constructed using the MEGA X software (Kumar *et al*., 2018), based on the 16S rRNA gene sequence. Sequence relatedness was calculated using the Maximum likelihood (ML) algorithm (Felsenstein, 1981). Phylogenetic tree robustness was assessed using the bootstrap method with 1,000 replications. The Kimura two-parameter model was applied to calculate evolutionary distances (Kimura, 1980).

Results and Discussion

The 16S rRNA gene sequence analyses revealed that a total of 7 species belonged to previously unreported species in Korea. The strain information, identification, taxonomic assignment from species to classes, isolation source, and sequence accession numbers, including the National Institute of Biological Resources (NIBR) in Korea and GenBank, are listed in Table 1. The 7 species were distributed across the order *Halobacteriales* and belonged to the family *Haloferacaceae*. The family *Haloferacaceae* included the genera *Haloferax* (4 species) and *Haloplanus* (3 species). The isolated unrecorded haloarchaeal species exhibited various morphologies, including pleomorphic coccus and coccus shaped (Fig. 1). All these species were Gram-staining negative and lysed in distilled water. Some of the species required Mg^{2+} for growth, and most of them were found to grow optimally in to be mesophilic (20–40°C) and neutrophilic (pH 7.0) conditions. Most species were found to grow more than 5% NaCl (w/v). All species tested positive for catalase and negative for oxidase. Additionally, growth was observed in all species when using D-glucose, pyruvate, and fumarate as sole energy source. The identification of the 7 unrecorded species based on the 16S rRNA gene sequence similarities, was supported by the phylogenetic tree using the ML algorithm (Fig. 2). Detailed morphological, physiological, and basic biochemical characteristics of the 7 unrecorded haloarchaeal species are described in the following strain descriptions.

Description of *Haloferax sulfurifontis* MBLA0249

Cells are Gram-stain-negative and pleomorphic coccus shaped with 1.5–1.7 μm in diameter. Colonies are red, circular, and flat after 3 days of incubation on DBCM2 at 37°C. Catalase is positive, but oxidase is negative. Growth occurs in the 20–50°C (optimum, 37°C) at 5–25% NaCl (optimum, 15%) at pH 5.0–9.0 (optimum, pH 7.0) and

Table 1. Summary of all strains isolated in this study and their taxonomic affiliations.

Order	Family	Order	Strain ID	Accession/ NIBRARC number	Closest species	Similarity (%)	Media	Isolation source
Halobacteriales	*Haloferacaceae*	*Haloferax*	MBLA0249	OR646564 NIBRARC000510481	*Haloferax sulfurifontis*	99.93	DBCM2 at 37°C, 3 days	Seawater
			MBLA0250	OR646565 NIBRARC000510482	*Haloferax elongans*	99.78		
			MBLA0251	OR646566 NIBRARC000510483	*Haloferax larsenii*	99.85		
			MBLA0252	OR646567 NIBRARC000510484	*Haloferax namakaokahaiae*	99.68		
		Haloplanus	MBLA0253	OR646568 NIBRARC000510485	*Haloplanus litoreus*	99.78	DBCM2 at 37°C, 7 days	
			MBLA0254	OR646569 NIBRARC000510486	*Haloplanus ruber*	99.56		
			MBLA0255	OR646570 NIBRARC000510487	*Haloplanus aerogenes*	99.85		

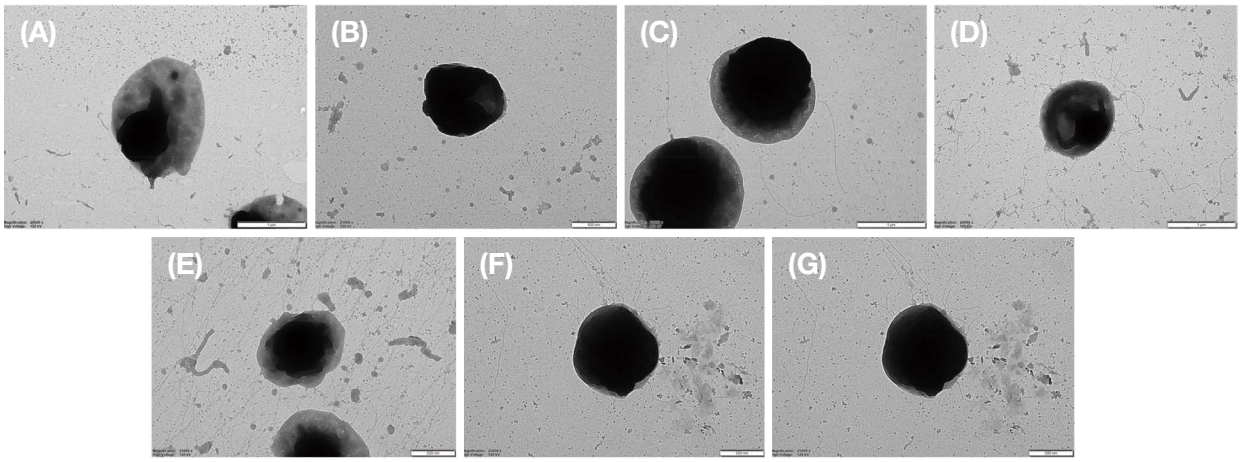

Fig. 1. Transmission electron micrographs of the 7 unrecorded species isolated in this study. Strains: A, MBLA0259; B, MBLA0250; C, MBLA0251; D, MBLA0252; E, MBLA0253; F, MBLA0254; G, MBLA0255.

lysed in distilled water. Mg^{2+} is required for growth. D-Glucose, D-galactose, D-fructose, D-sucrose, D-mannose, D-xylose, L-rhamnose, starch, DL-lactate, fumarate, pyruvate, citrate, acetate, glycine, L-asparagine, and L-glutamine are utilized as sole energy sources for growth, but D-maltose, D-lactose, D-trehalose, D-raffinose, D-mannitol, D-sorbitol, glycerol, DL-malate, succinate, L-alanine, L-arginine, L-histidine, L-ornithine, and L-glutamic acid are not utilized. The NCBI accession number for the 16S rRNA gene sequence is OR646564. Strain MBLA0249 (=NIBRARC000510481) was isolated from seawater near Dokdo island, Korea (37°14′29.4″N 131°52′00.9″E).

Description of *Haloferax elongans* MBLA0250

Cells are Gram-stain-negative and pleomorphic-coccus shaped with 1.7−1.9 μm in diameter. Colonies are red, circular, and flat after 3 days of incubation on DBCM2 at 37°C. Catalase is positive, but oxidase is negative. Growth occurs in the 25−50°C (optimum, 37°C) at 10−30% NaCl (optimum, 15%) at pH 6.0−8.0 (optimum, pH 7.0) and lysed in distilled water. Mg^{2+} is required for growth. D-Glucose, D-fructose, D-sucrose, L-rhamnose, D-sorbitol, fumarate, pyruvate, glycine, L-histidine D-xylose, glycerol, DL-lactate, succinate, pyruvate, DL-malate, fumarate, citrate, acetate, and L-alanine are utilized as sole energy sources for growth, but D-galactose, D-maltose, lactose, D-mannose, D-xylose, D-trehalose, D-raffinose, D-mannitol, glycerol, starch, citrate DL-lactate, acetate, succinate, L-alanine, L-arginine, L-ornithine, L-asparagine, L-glutamine, and L-glutamic acid are not utilized. The NCBI accession number for the 16S rRNA gene sequence is OR646565. Strain MBLA0250 (=NIBRARC000510482) was isolated from seawater near Dokdo island, Korea (37°14′29.4″N 131°52′00.9″E).

Description of *Haloferax larsenii* MBLA0251

Cells are Gram-stain-negative and coccus shaped with 1.5−1.7 μm in diameter. Colonies are red, circular, and flat after 3 days of incubation on DBCM2 at 37°C. Catalase is positive, but oxidase is negative. Growth occurs in the 30−55°C (optimum, 37°C) at 5−25% NaCl (optimum, 15%) at pH 6.0−8.0 (optimum, pH 7.0) and lysed in distilled water. Mg^{2+} is required for growth. D-Glucose, D-fructose, D-sucrose, L-rhamnose, starch, DL-lactate, fumarate, pyruvate, citrate, acetate, glycine, L-alanine, L-histidine, L-ornithine, L-asparagine, L-glutamine, and L-glutamic acid are utilized as sole energy sources for growth, but D-galactose, D-maltose, lactose, D-mannose, D-xylose, D-trehalose, D-raffinose, D-mannitol, D-sorbitol, glycerol, DL-malate, and succinate are not utilized. The NCBI accession number for the 16S rRNA gene sequence is OR64 6566. Strain MBLA0251 (=NIBRARC000510483) was isolated from seawater near Dokdo island, Korea (37°14′ 29.4″N 131°52′00.9″E).

Description of *Haloferax namakaokahaiae* MBLA0252

Cells are Gram-stain-negative and coccus-shaped with 1.1−1.2 μm in diameter. Colonies are red, circular, and flat after 3 days of incubation on DBCM2 at 37°C. Catalase is positive, but oxidase is negative. Growth occurs in the 20−40°C (optimum, 37°C) at 5−25% NaCl (optimum, 15%) at pH 6.0−8.0 (optimum, pH 7.0) and lysed in distilled water. Mg^{2+} is required for growth. D-Glucose, D-galactose, D-fructose, D-sucrose, lactose, D-xylose, L-rhamnose, D-raffinose, D-sorbitol, starch, fumarate, pyruvate, DL-malate, acetate, glycine, L-ornithine, L-asparagine, L-glutamine, and L-glutamic acid are utilized as sole energy sources for growth, but D-maltose, D-mannose,

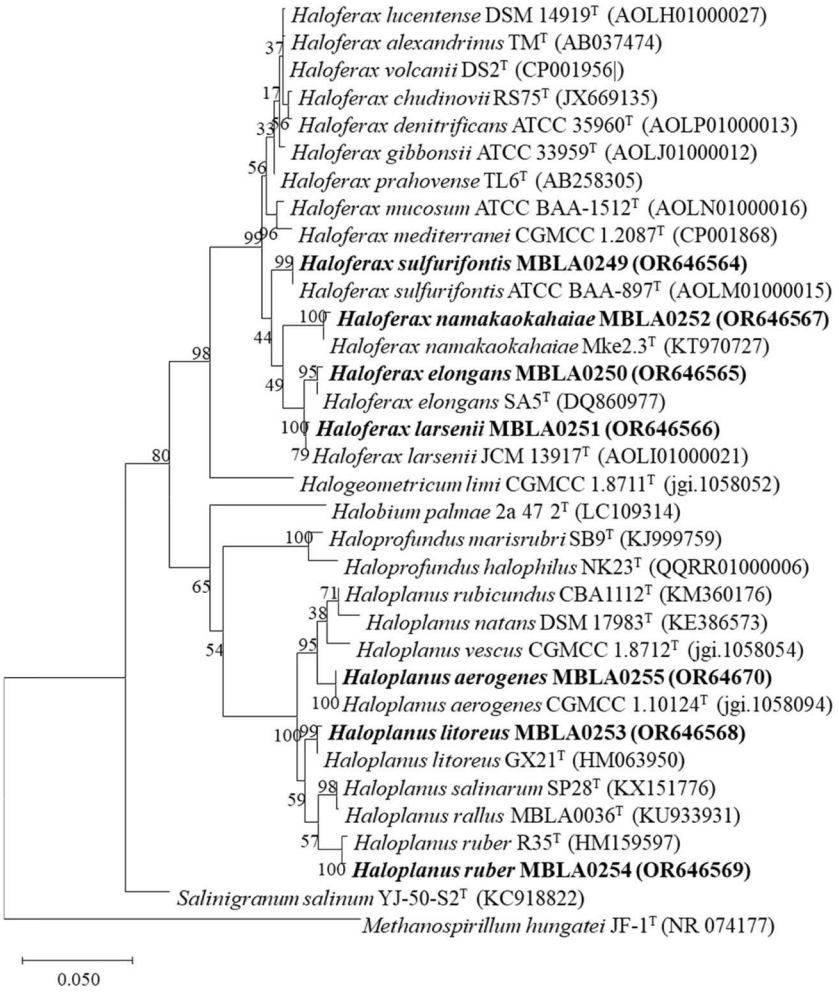

Fig. 2. Maximum likelihood (ML) phylogenetic tree based on the 16S rRNA gene sequences between the species isolated in this study. A phylogenetic tree was constructed with their relatives of the genera *Haloferax*, *Haloplanus*, *Halogeometricum*, *Halobium*, *Haloprofundus*, and *Salinigranum*. The numbers on the nodes indicate the bootstrap values (>70%). Bar, 0.05 accumulated changes per nucleotide, respectively.

D-trehalose, D-mannitol, glycerol, DL-lactate, citrate, succinate, L-alanine, and L-histidine are not utilized. The NCBI accession number for the 16S rRNA gene sequence is OR646567. Strain MBLA0252 (=NIBRARC000510484) was isolated from seawater near Dokdo island, Korea (37°14′29.4″N 131°52′00.9″E).

Description of *Haloplanus litoreus* MBLA0253

Cells are Gram-stain-negative and pleomorphic coccus shaped with 1.0−1.1 μm in diameter. Colonies are red, irregular, and flat after 7 days of incubation on DBCM2 at 37°C. Catalase is positive, but oxidase is negative. Growth occurs in the 20−50°C (optimum, 37°C) at 15−25% NaCl (optimum, 15%) at pH 6.0−9.0 (optimum, pH 7.0) and lysed in distilled water. Mg^{2+} is required for growth. D-Glucose, starch, DL-lactate, fumarate, pyruvate, citrate, DL-malate, acetate, L-asparagine, L-glutamine, and L-glutamic acid are utilized as sole energy sources for growth, but D-galactose, D-fructose, D-sucrose, D-maltose, lactose, D-mannose, D-xylose, L-rhamnose, D-trehalose, D-raffinose, D-mannitol, D-sorbitol, glycerol, succinate, glycine, L-alanine, L-arginine, L-histidine, and L-ornithine are not utilized. The NCBI accession number for the 16S rRNA gene sequence is OR646568. Strain MBLA0253 (=NIBRARC000510485) was isolated from seawater near Dokdo island, Korea (37°14′29.4″N 131°52′00.9″E).

Description of *Haloplanus ruber* MBLA0254

Cells are Gram-stain-negative and coccus shaped with 1.0−1.1 μm in diameter. Colonies are red, irregular, and flat after 7 days of incubation on DBCM2 at 37°C. Catalase is positive, but oxidase is negative. Growth occurs in the

20−45°C (optimum, 37°C) at 15−25% NaCl (optimum, 15%) at pH 6.0−9.0 (optimum, pH 7.0), lysed in distilled water, and growth occur without Mg^{2+}. D-Glucose, D-galactose, D-fructose, D-mannose, D-xylose, starch, fumarate, pyruvate, citrate, DL-malate, acetate, glycine, L-alanine, L-asparagine, and L-glutamic acid are utilized as sole energy sources for growth, but D-sucrose, D-maltose, lactose, L-rhamnose, D-trehalose, D-raffinose, D-mannitol, D-sorbitol, glycerol, starch, succinate, L-alanine, L-arginine, L-histidine, and L-glutamine are not utilized. The NCBI accession number for the 16S rRNA gene sequence is OR646569. Strain MBLA0254 (=NIBRARC 000510486) was isolated from seawater near Dokdo island, Korea (37°14′29.4″N 131°52′00.9″E).

Description of *Haloplanus aerogenes* MBLA0255

Cells are Gram-stain-negative and coccus shpaed with 0.8−0.9 μm in diameter. Colonies are red, irregular, and flat after 7 days of incubation on DBCM2 at 37°C. Catalase is positive, but oxidase is negative. Growth occurs in the 25−50°C (optimum, 37°C) at 10−25% NaCl (optimum, 15%) at pH 6.0−9.0 (optimum, pH 7.0) and lysed in distilled water, and growth occur without Mg^{2+}. D-Glucose, D-galactose, D-sucrose, lactose, D-mannose, D-trehalose, D-raffinose, D-mannitol, glycerol, starch fumarate, pyruvate, citrate, DL-malate, acetate, succinate, L-alanine, L-arginine, L-histidine, L-ornithine, L-asparagine, and L-glutamic acid are utilized as sole energy sources for growth, but D-fructose, D-maltose, D-xylose, L-rhamnose, D-sorbitol, DL-lactate, and glycine are not utilized. The NCBI accession number for the 16S rRNA gene sequence is OR646570. Strain MBLA0255 (=NIBRARC000510 487) was isolated from seawater near Dokdo island, Korea (37°14′29.4″N 131°52′00.9″E).

Conflicts of Interest

The author of this paper has no affiliation with any interests and is solely responsible for the paper.

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