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ORIGINAL PAPER

Salinarchaeum laminariae gen. nov., sp. nov.: a new member of the family *Halobacteriaceae* isolated from salted brown alga *Laminaria*

Heng-Lin Cui · Xin Yang · Yun-Zhuang Mou

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Abstract Halophilic archaeal strains R26^T and R22 were isolated from the brown alga *Laminaria* produced at Dalian, Liaoning Province, China. Cells from the two strains were pleomorphic rods and Gram negative, and colonies were red pigmented. Strains R26^T and R22 were able to grow at 20–50°C (optimum 37°C) in 1.4–5.1 M NaCl (optimum 3.1–4.3 M) at pH 5.5–9.5 (optimum pH 8.0–8.5) and neither strain required Mg^{2+} for growth. Cells lyse in distilled water and the minimum NaCl concentration required to prevent cell lysis was 8% (w/v) for strain R26^T and 12% (w/v) for strain R22. The major polar lipids of the two strains were phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester and minor phosphatidylglycerol sulfate; glycolipids were not detected. Phylogenetic analyses based on 16S rRNA genes and *rpoB′* genes revealed that strains R26^T and R22 formed a distinct clade with the closest relative, *Natronoarchaeum mannanilyticum*. The DNA G+C content of strains R26^T and R22 was 65.8 and 66.4 mol%, respectively. The DNA–DNA hybridization value between strains R26^T and R22 was 89%. The phenotypic, chemotaxonomic and phylogenetic properties suggest that the strains R26^T and R22 represent a novel species in a new genus within the family *Halobacteriaceae*, for which the name *Salinarchaeum laminariae* gen. nov., sp. nov. is proposed. The type strain is R26^T (type strain R26^T = CGMCC 1.10590^T = JCM 17267^T, reference strain R22 = CGMCC 1.10589).

Keywords *Salinarchaeum laminariae* gen. nov., sp. nov. · Halophilic archaea · Salted kelp

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H.-L. Cui (✉) · X. Yang · Y.-Z. Mou
School of Food and Biological Engineering,
Jiangsu University, 301 Xuefu Road, Jingkou District,
Zhenjiang 212013, People's Republic of China
e-mail: cuihenglin@sohu.com

Introduction

Extremely halophilic archaea members belonging to the family *Halobacteriaceae*, order *Halobacteriales*, class *Halobacteria* and phylum *Euryarchaeota* are widely distributed in diverse natural and artificial hypersaline environments in which salt concentrations exceed 150–200 g/l (Oren 2006). Based on the polyphasic taxonomy criterion (Oren et al. 1997), the extremely halophilic archaea are currently classified within 35 validly published genera (Oren et al. 2009; Burns et al. 2010; Cui et al. 2010a, b, c, 2011; Shimane et al. 2010; Minegishi et al. 2010a; Inoue et al. 2011).

In addition to natural hypersaline environments, salt lakes, soda lakes and salt mines, the artificial heavily salted fish and hides are always the ideal artificial habitats for halophilic archaea of the order *Halobacteriales*. The diverse strains of *Halobacterium salinarum*, *Halococcus morrhuae*, *Natrinema pallidum* and *Natrinema pellirubrum* were originally isolated from these salted hides and salted codfish (Lochhead 1934, 1943; Kocur and Hodgkiss 1973; McGenity et al. 1998). The traditional Korean salt-fermented seafood also contained *Haladaptatus cibarius*, *Halalkalicoccus jeotgali*, *Halorubrum cibi*, *Haloterrigena jeotgali* and *Natronococcus jeotgali* (Roh et al. 2007a, b, 2009, 2010; Roh and Bae 2009). Thailand fish sauce has been the source of *Haloarcula salaria*, *Haloarcula*

tradensis, *Halobacterium piscisalsi*, *Halococcus thailandensis* and *Natrinema gari* (Namwong et al. 2007, 2011; Tapingkae et al. 2008; Yachai et al. 2008). Other halophilic archaea, *Halarchaeum acidiphilum* (Minegishi et al. 2010a), *Halostagnicola kamekurae* (Nagaoka et al. 2010), *Halostagnicola alkaliphila* (Nagaoka et al. 2011), *Natrialba taiwanensis* (Hezayen et al. 2001), *Natronoarchaeum mannanilyticum* (Shimane et al. 2010) and *Salarchaeum japonicum* (Shimane et al. 2011), can be recovered from commercial salt, which indicates that the halophilic archaea in the salted fish, salted hides and salty fermented food may be transferred from the salt used in processing.

The brown alga *Laminaria* is the main seaweed species under commercial cultivation in China (Xu et al. 2009). The annual production of fresh *Laminaria* is about 4 million tons (Tseng 2001). Large amounts of fresh *Laminaria* are preserved with crude salt which is supersaturated and endow the fresh *Laminaria* with a shelf life of about 24 months while being stored at −10°C temperature. However, the salted fresh *Laminaria* will fade, redden and rot at room temperature, such as during transport or on the supermarket shelves during the hot season. During our surveys on the microbiological nature of the peculiar reddening of salted *Laminaria*, we isolated two halophilic archaeal strains phylogenetically related to the closest relative, *Natronoarchaeum mannanilyticum* (with 90.2–90.6% 16S rRNA gene similarities) (Shimane et al. 2010). In this study, we characterize these two strains, R26^T and R22, as a novel species in a new genus of the family *Halobacteriaceae*.

Materials and methods

Isolation and cultivation of halophilic archaeal strains

Strains R26^T and R22 were isolated from the red brine of salted *Laminaria* produced from Dalian, Liaoning Province, China. The brine had a pH of 7.5 and a total salinity of 293 g/l. A neutral oligotrophic haloarchaeal medium (NOM) solidified with 2.0% agar was used for the isolation procedure and the medium comprised the following ingredients (g/l): yeast extract, 0.05; fish peptone, 0.25; sodium pyruvate, 1.0; KCl, 5.4; K_2HPO_4, 0.3; $CaCl_2$, 0.25; NH_4Cl, 0.25; $MgSO_4{\cdot}7H_2O$, 26.8; $MgCl_2{\cdot}6H_2O$, 23.0; NaCl, 184.0 (pH adjusted to 7.0–7.2 with 1 M NaOH solution). The red brine was subjected to tenfold serial dilution and 0.2 ml of each dilution was plated onto the NOM agar plates. The plates were incubated in the dark at 37°C for at least 10 weeks in sealed plastic bags. A plate with 50–100 colonies was selected for picking single colonies. To insure purity, a single colony of each strain was re-streaked twice onto NOM plates. The strains were routinely grown aerobically at 37°C in NOM-3 medium (NOM series medium) with the following modifications (g/l): yeast extract, 1.0; fish peptone, 0.25; sodium formate, 0.25; sodium acetate, 0.25; sodium lactate, 0.25; sodium pyruvate, 0.25.

Phenotypic determination

Phenotypic tests were performed according to the proposed minimal standards for description of new taxa in the order *Halobacteriales* (Oren et al. 1997). The type strains *Natronoarchaeum mannanilyticum* YSM-123^T, *Halovivax asiaticus* CGMCC 1.4248^T, *Natrinema pellirubrum* JCM 10476^T, *Haloterrigena turkmenica* CGMCC 1.2364^T, *Haladaptatus paucihalophilus* JCM 13897^T, *Halobacterium jilantaiense* NG4^T and *Haloferax volcanii* CGMCC 1.2150^T were selected as reference strains in positive and negative testing. Cell morphology and motility in exponentially growing liquid cultures were examined using a Nikon microscope equipped with phase-contrast optics (model: E400). Minimal salt concentration to prevent cell lysis was tested by suspending washed cells in serial sterile saline solutions containing NaCl ranging from 0 to 15% (w/v), and the stability of the cells was detected by light microscopic examination.

The Gram stain was performed by following the method outlined by Dussault (1955). Most miscellaneous biochemical tests and nutritional tests were performed as described and proposed by Oren et al. (1997). Briefly, growth and gas formation with nitrate as electron acceptor were tested in 9-ml stoppered tubes, completely filled with liquid NOM medium to which $NaNO_3$ (5 g/l) had been added, containing an inverted Durham tube. The formation of gas from nitrate was detected by the presence of gas bubbles in Durham tubes, and the formation of nitrite was monitored colorimetrically. Anaerobic growth in the presence of L-arginine and DMSO (5 g/l) was tested in completely filled 9-ml stoppered tubes. Starch hydrolysis was determined on NOM agar plates supplemented with 2 g/l soluble starch and detected by flooding the plates with Lugol's iodine solution. Gelatin hydrolysis was performed by growing colonies on NOM agar plates amended with 1% (w/v) gelatin and flooding the plates with Frazier's reagent (McDade and Weaver 1959) after growth was established. Esterase activity was detected as outlined by Gutiérrez and González (1972). Tests for catalase and oxidase activities were performed as described by Gonzalez et al. (1978). Production of H_2S was tested by growing the isolates and reference strains in a tube with the NOM liquid medium supplemented with 0.5% (w/v) sodium thiosulfate; a filter-paper strip impregnated with lead acetate was used for H_2S detection (Cui et al. 2007). To test for growth on single carbon sources, fish peptone and

sodium pyruvate were omitted from the NOM medium and the compound to be tested was added at a concentration of 5 g/l. Antimicrobial susceptibilities were determined by Gutiérrez et al. (2008) methods on NOM agar plates with antimicrobial compound discs.

Chemotaxonomic characterization

Polar lipids were extracted using a chloroform/methanol system and analyzed using one- and two-dimensional TLC, as described previously (Kates 1986). Merck silica gel 60 F_{254} aluminum-backed thin-layer plates were used in TLC analysis. In two-dimensional TLC, the first solvent is chloroform–methanol–water (65:25:4, by vol.) and the second solvent is chloroform–methanol–acetic acid–water (80:12:15:4, by vol.), which is also used in one-dimensional TLC. Two specific detection spray reagents, phosphate stain reagent for phospholipids and α-naphthol stain for glycolipids, were used. The general detection reagent, sulfuric acid–ethanol (1:2, by vol.), was also used to detect total polar lipids.

Phylogenetic analysis

Genomic DNAs from halophilic archaeal strains were prepared by the following procedure. Liquid cultures at the late logarithmic stage were centrifuged to pellet cells and the cells were re-suspended in 20% NaCl solution; 1 ml of the thick cell suspension was added into 9 ml of DNA extract solution (8 ml TE and 1 ml 10% g/ml SDS). The lysate was extracted with an equal volume of TE-saturated phenol for 5 min, centrifuged and the upper phase was collected and extracted with an equal volume of chloroform/isoamyl alcohol (24:1, v/v) for 5 min. RNase A was added to a final concentration of 50 μg/ml and incubated for 2 h at 37°C. Then the lysate was subjected to TE-saturated phenol and chloroform/isoamyl alcohol extractions as mentioned above. To the supernatant, equal volume of DNA binding buffer (4 M guanidine hydrochloride, 1 M potassium acetate, pH 5.5) was added, mixed gently and applied to a DNA purification column (UNIQ-500, Sangon Biotech Co., Ltd., Shanghai) and centrifuged at 4000×g for 5 min. Subsequently, the purification column was washed twice by centrifugation using 2 ml of 75% ethanol. The DNA was eluted with 1 ml of 0.1 SSC.

The 16S rRNA genes were amplified, cloned and sequenced as described previously (Cui et al. 2009). PCR-mediated amplification and sequencing of the *rpoB′* genes were carried out according to Minegishi et al. (2010b). Multiple sequence alignments were performed using the ClustalW program integrated in MEGA 5 software (http://www.megasoftware.net/) (Kumar et al. 2008). Phylogenetic trees were reconstructed using the neighbor-joining (NJ) (Saitou and Nei 1987), maximum-parsimony (MP) (Fitch 1971) and maximum-likelihood (ML) (Felsenstein 1981) algorithms in MEGA 5 software. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The gene sequence similarity was calculated using pairwise-distance computing function of MEGA 5 comparison with those of related halophilic archaea. The DNA G+C content was determined from the midpoint value (T_m) of the thermal denaturation method (Marmur and Doty 1962) at 260 nm with a Beckman-Coulter DU800^{TM} spectrophotometer equipped with a high-performance temperature controller. DNA–DNA hybridization analyses were performed according to the thermal denaturation and renaturation method of De Ley et al. (1970) as modified by Huß et al. (1983), using the Beckman-Coulter DU800^{TM} spectrophotometer equipped with a high-performance temperature controller. The DNA–DNA hybridizations were carried out in 2× SSC at 80°C and the experiment was carried out in triplicate.

Results and discussion

Cells of strain R26^T and strain R22 were motile, pleomorphic rods when grown in NOM-3 liquid medium (Supplementary Fig. S1). The cells stained Gram negative and their colonies were red pigmented. Strains R26^T and R22 were able to grow at 20–50°C (optimum 37°C) in 1.4–5.1 M NaCl (optimum 3.4–3.9 M) at pH 5.5–9.5 (optimum pH 7.0–7.5) and neither strain required Mg^{2+} for growth. Cells lysed in distilled water and the minimal NaCl concentrations to prevent cell lysis were 8% (w/v) for strain R26^T and 12% (w/v) for strain R22. Strains R26^T and R22 hydrolyzed gelatin but did not hydrolyze starch, Tween 80 and casein. Both strains produced H_2S from sodium thiosulfate, but did not produce indole from tryptophan. The main phenotypic characteristics differentiating strains R26^T and R22 from *Natronoarchaeum*, *Halovivax*, *Natronococcus* and *Natrinema* are shown in Table 1 and more detailed results of phenotypic tests and nutritional features of strains R26^T and R22 are given in the species descriptions.

The major polar lipids of strains R26^T and R22 were phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (PGP-Me) and minor phosphatidylglycerol sulfate (PGS); glycolipids were not detected (Supplementary Fig. S2). The polar lipid profile sets strains R26^T and R22 apart from *Natronoarchaeum* which contains S_2-DGD (disulfated mannosyl glucosyl diether) and an unidentified glycolipid (Shimane et al. 2010), *Haloterrigena* and *Natrinema* which contain S_2-DGD (disulfated mannosyl glucosyl diether) and unidentified glycolipids

Table 1 Differential characteristics between strain R26^T, strain R22 and closely related genera within the order *Halobacteriales*

Characteristic	1	2	3	4	5	6
Cell morphology	Rods	Rods	Pleomorphic	Pleomorphic	Cocci	Pleomorphic
Gram stain	−	−	−	−	+/−	−
Motility	+	+	−	+	−	+
Cells lyse in distilled water	+	+	+	+	−	+
Optimum NaCl (M)	4.3	3.1	4.0–4.5	3.4	2.6–3.4/3.9–4.3	2.6–3.4/3.4–4.3
Mg^{2+} required	−	−	+	+/−	+	+
Optimum pH	8.5	8.0	7.5–8.0	7.0–7.5	7.5/9.0–9.5	6.5–7.0/7.0–7.5
Reduction of nitrate to nitrite	−	−	−	−	+	+/−
Utilization of:						
D-Glucose	+	+	+	−	+	+
Lactose	−	−	+	+	+/−	+/−
Maltose	−	−	+	−	+/−	+/−
Indole formation	−	−	+	−	+/−	+/−
Starch hydrolysis	−	−	+	−	+/−	+/−
Gelatin hydrolysis	+	+	−	+	+/−	+/−
Presence of PGS	+	+	−	−	−	+
Types of glycolipids	/	/	S_2-DGD, UG	S-DGD-3, S-DGD-1, 2UG	/	3UG
G+C content (mol%)	65.8	66.4	63	60.3–65.0	61.2–63.5	62.9–65.6

Taxa: 1, strain R26^T; 2, strain R22; 3, *Natronoarchaeum*; 4, *Halovivax*; 5, *Natronococcus*; 6, *Natrinema*. Symbols: +, positive; −, negative; UG, unidentified glycolipid

(Cui et al. 2006), *Halovivax* which contains S-DGD-3 (a kind of sulfated mannosyl glucosyl diether), S-DGD-1 (sulfated mannosyl glucosyl diether) and unidentified glycolipids (Castillo et al. 2006).

Eight complete 16S rRNA gene sequences of strain R26^T and nine complete 16S rRNA gene sequences of strain R22 were obtained. Sequence comparisons indicated that strains R26^T and R22 have one kind of 16S rRNA gene sequence and are 99.9% similar to each other. Both strains showed low levels of 16S rRNA gene sequence similarity to other members of the family *Halobacteriaceae* and the closest related recognized species were *Natronoarchaeum mannanilyticum* (90.2–90.6% similarity) and *Halovivax asiaticus* (90.9–91.0% similarity). Phylogenetic analysis using the NJ algorithm revealed that strains R26^T and R22 formed a distinct clade with *Natronoarchaeum mannanilyticum* (Fig. 1). The phylogenetic position was also confirmed in other trees generated using the MP and ML algorithms (data not shown). The result based on phylogenetic analysis of 16S rRNA gene reveals that both strains represent a novel phylogenetic taxon.

The *rpoB′* gene lengths of strains R26^T and R22 are both 1830 bp, and the nucleotide sequences are 99.5% similar to each other and also closely similar to the corresponding gene of *Natronoarchaeum mannanilyticum* (85 and 85.1%, respectively). In phylogenetic tree reconstructions, strains R26^T and R22 distantly clustered with *Natronoarchaeum mannanilyticum* (Fig. 1b) and formed a monophyletic group separated from the related genera *Halovivax* and *Haladaptatus*. The phylogenetic position was also confirmed in the trees generated using the MP and ML algorithms (data not shown). The *rpoB′* gene-based phylogenetic analysis results supported the placement of strains R26^T and R22 in a novel genus.

The DNA G+C content of strains R26^T and R22 are 65.8 and 66.4 mol%, respectively. These values are higher than those of *Natronoarchaeum* (63 mol%) (Shimane et al. 2010), *Halovivax* (60.3–65.0 mol%) (Castillo et al. 2007), *Natronococcus* (61.2–63.5 mol%) (Roh et al. 2007a) and *Natrinema* (62.9–65.6 mol%) (Tapingkae et al. 2008). The DNA–DNA hybridization value between strains R26^T and R22 was 89%, lower than the accepted threshold value (70%) to separate two species (Stackebrandt and Goebel 1994).

This polyphasic taxonomic study provides evidence that strains R26 and R22 represent a novel species of a new genus within the family *Halobacteriaceae*, for which the name *Salinarchaeum laminariae* gen. nov., sp. nov. is proposed. The type strain is R26^T (=CGMCC 1.10590^T = JCM 17267^T) and the reference strain is R22 (=CGMCC 1.10589). Characteristics that distinguish strains R26 and R22 from other genera within *Halobacteriaceae* are shown in Table 1.

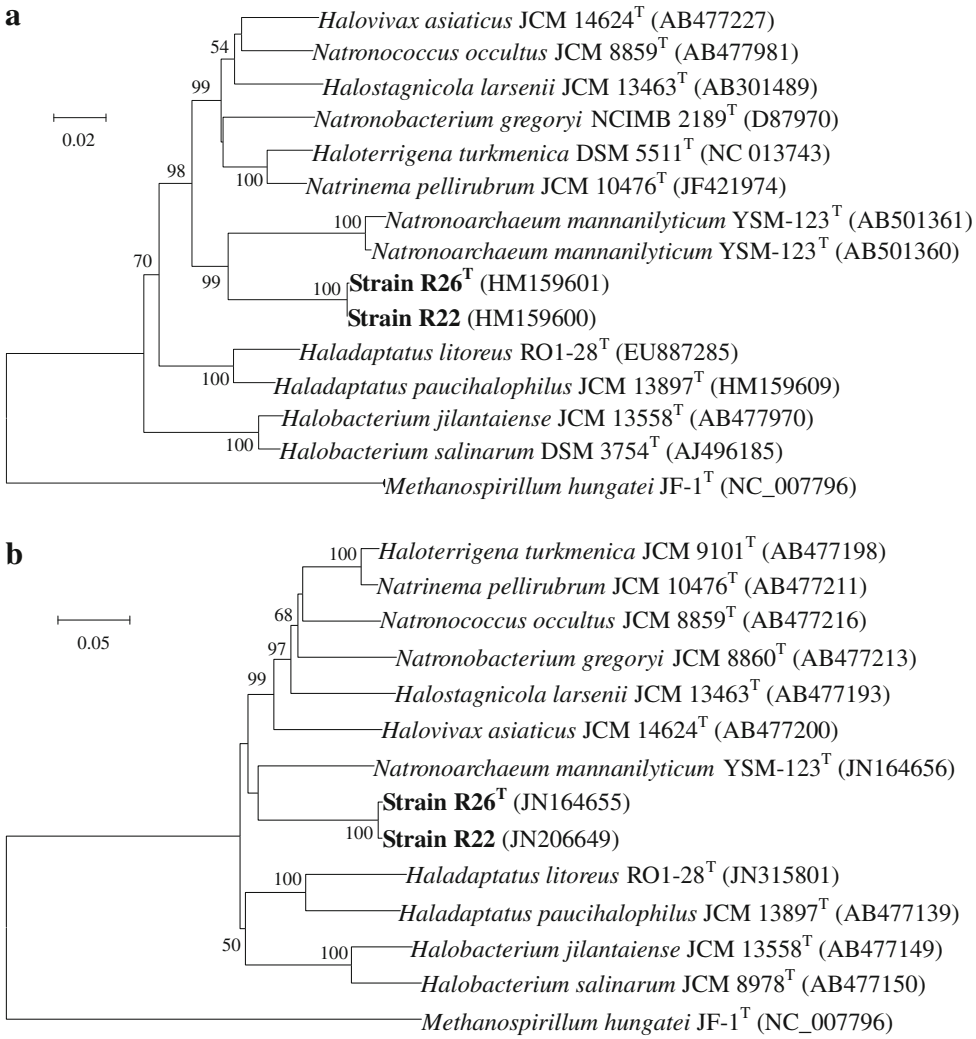

Fig. 1 Neighbor-joining phylogenetic trees based on 16S rRNA gene (**a**) and *rpoB′* gene (**b**) sequences showing the relationships between strains R26^T and R22 and related members within the family *Halobacteriaceae*. Bootstrap values (%) are based on 1000 replicates and are shown for branches with more 50% bootstrap support. *Bar* represents expected changes per site

Description of *Salinarchaeum* gen. nov.

Salinarchaeum (Sa.li.nar.chae'um. L. fem. pl. n. *salinae* salterns, salt works; N.L. neut. n. *archaeum* archaeon from Gr. adj. *archaios-ê-on* ancient; N.L. neut. n. *Salinarchaeum* the archaeon from salt works).

Cells are pleomorphic rods under optimal growth conditions and stain Gram negative. Aerobic heterotrophs. Cells lyse in distilled water. Oxidase and catalase tests are positive. Extremely halophilic, with growth occurring in media containing 1.4–5.1 M NaCl; most strains grow best at 3.1–4.3 M NaCl. The optimum magnesium concentration varies between 0.05 and 0.5 M. Temperatures between 20 and 50°C and pH between 5.5 and 9.5 may support growth. Sugars are metabolized, in some cases with the formation of acids. The polar lipids were PG, PGP-Me and minor PGS; glycolipids were not detected. Phylogenetically related to *Natronoarchaeum* in the *Halobacteriaceae*. The genomic DNA G+C content is between 65.8 and 66.4 mol%. Isolated from the salted brown alga *Laminaria*. The type species is *Salinarchaeum laminariae*. Recommended three-letter abbreviation: *Saa*.

Description of *Salinarchaeum laminariae* sp. nov.

Salinarchaeum laminariae (la.mi.na'ri.ae, N.L. gen. n. *laminariae*, of *Laminaria*, the brown alga *Laminaria*, from which the halophilic archaea were isolated).

Cells are motile, pleomorphic rods under optimal growth conditions and stain Gram negative. Colonies on agar plates containing 3.1–4.3 M NaCl are red, elevated and round. Chemoorganotrophic and aerobic. Growth occurs at 20–50°C (optimum 37°C), 1.4–5.1 M NaCl (optimum 3.1–4.3 M), 0–1.0 M $MgCl_2$ (optimum 0.05–0.5 M) and at pH 5.5–9.5 (optimum pH 8.0–8.5). Cells lyse in distilled water and the minimal NaCl concentration to prevent cell lysis is 8–12% (w/v). Catalase- and oxidase positive. Do not grow under anaerobic conditions with nitrate, arginine or DMSO. Nitrate reduction to nitrite is not observed in some strains. H_2S is not produced from

sodium thiosulfate. Indole formation is negative. Hydrolyze gelatin but do not hydrolyze starch, casein and Tween 80. The following substrates are utilized as single carbon and energy sources for growth: D-glucose, D-mannose, D-galactose, sucrose, glycerol, pyruvate and DL-lactate. L-Glutamate is utilized as single carbon, nitrogen or energy sources for growth. The following substrates are not utilized as single carbon and energy sources for growth: D-fructose, L-sorbose, D-ribose, D-xylose, maltose, lactose, starch, D-mannitol, D-sorbitol, acetate, succinate, L-malate, fumarate and citrate. The following substrates are not utilized as single carbon, nitrogen or energy sources for growth: glycine, L-alanine, L-arginine, L-aspartate, L-lysine and L-ornithine. Acid is produced from D-glucose, D-mannose and D-galactose. Susceptible to the following antimicrobial compounds (micrograms per disc, unless otherwise indicated): bacitracin (0.04 IU per disc), mycostatin (100) and nitrofurantoin (300). Resistant to the following antimicrobial compounds: novobiocin (30), rifampin (5), trimethoprim (5), erythromycin (15), neomycin (30), norfloxacin (10), chloramphenicol (30), ampicillin (10), penicillin G (10 IU per disc), ciprofloxacin (5), streptomycin (10), kanamycin (30), tetracycline (30), vancomycin (30), gentamicin (10) and nalidixic acid (30). The polar lipids are PG, PGP-Me and minor PGS, glycolipids were not detected. The DNA G+C content of $R26^T$ is 65.8 mol% (T_m). The type strain $R26^T$ (type strain $R26^T$ = CGMCC 1.10590^T = JCM 17267^T) and the reference strain R22 (=CGMCC 1.10589) were isolated from the salted brown alga *Laminaria* produced at Dalian, Liaoning Province, China.

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