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研究报告

Halolamina litorea sp. nov., a haloarchaeon isolated from a marine solar saltern

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Abstract: [Objective] A halophilic archaeal strain YJ-41^T was isolated from Yangjiang marine solar saltern in the south region of China. In the present work, strain YJ-41^T was characterized in detail to elucidate its taxonomic position. **[Methods]** The taxonomic status of strain YJ-41^T was studied by using a polyphasic taxonomic method including determining phenotype, chemotype and genotype. **[Results]** Cells of strain YJ-41^T were rod-shaped, Gram-staining negative and formed red-pigmented colonies on agar plates. Strain YJ-41^T was able to grow between 20 and 50 °C (optimum 37 °C), 2.1 to 4.8 mol/L NaCl (optimum 3.1 mol/L), 0 to 1.0 mol/L $MgCl_2$ (optimum 0.05 mol/L) and pH 5.0 to 9.0 (optimum 7.0). Cells lysed in distilled water, and the minimal NaCl concentration required to prevent cell-lysis was 10% (*W/V*). The major polar lipids of the strain were phosphatidic acid, phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, phosphatidylglycerol sulfate and eight glycolipids; three of these glycolipids chromatographically identical to sulfated mannosyl glucosyl diether, galactosyl mannosy glucosyl diether and mannosyl glucosyl diether. The 16S rRNA and *rpoB'* genes of strain YJ-41^T were phylogenetically related to the corresponding genes of *Halolamina* members (97.5% to 98.4% and 93.1% to 94.4% similarities, respectively). The DNA G+C content of strain YJ-41^T was 61.4 mol%. **[Conclusion]** The phenotypic, chemotaxonomic and phylogenetic properties suggested that strain YJ-41^T (=CGMCC 1.12859^T=JCM 30237^T) represents a new species of *Halolamina*, for which the name *Halolamina litorea* sp. nov. is proposed.

Keywords: *Halolamina litorea* sp. nov., Halophilic archaeon, Marine solar saltern

海滨盐薄片菌——分离自海洋盐田的一个嗜盐古菌新种

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摘 要:【目的】研究分离自我国华南地区阳江盐田的一株嗜盐古菌菌株 YJ-41^T，探究其分类学地位。【方法】运用多相分类学方法即通过表型和遗传型特征鉴定，研究菌株 YJ-41^T 的分类学地位。【结果】菌株 YJ-41^T 的细胞为杆状、革兰氏染色阴性、菌落呈红色。菌株 YJ-41^T 的生长温度范围 20–50 °C (最适为 37 °C)、NaCl 浓度范围 2.1–4.8 mol/L (最适为 3.1 mol/L)、$MgCl_2$

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浓度范围 0–1.0 mol/L (最适为 0.05 mol/L)、pH 范围 5.0–9.0 (最适为 pH 7.0)。细胞在蒸馏水中会裂解，维持细胞形态的最低 NaCl 浓度为 10% (质量体积比)。菌株 YJ-41^T 的极性脂为磷脂酸(Phosphatidic acid，PA)、磷脂酰甘油(Phosphatidyl glycerol，PG)、磷脂酰甘油磷酸甲基酯(Phosphatidyl glycerol phosphate methyl ester，PGP-Me)、磷脂酰甘油硫酸酯(Phosphatidyl glycerol sulphate，PGS)和 8 种糖脂；其中 3 种糖脂为硫酸甘露糖苷葡萄糖二醚(Sulfated mannosyl glucosyl diether，S-DGD-1)、半乳糖苷甘露糖苷葡萄糖二醚(Galactosyl mannosy glucosyl diether，TGD-1)和甘露糖苷葡萄糖二醚(Mannosyl glucosyl diether，DGD-1)，其余为未知糖脂。菌株 YJ-41^T 的 16S rRNA 基因和 *rpoB′*基因与盐薄片菌属(*Halolamina*)的成员相关基因相似性分别为 97.5%–98.4%和 93.1%–94.4%。菌株 YJ-41^T 的 G+C mol%为 61.4 mol%。【结论】表型、化学分类和系统发育的特性表明，菌株 YJ-41^T (=CGMCC 1.12859^T=JCM 30237^T)代表 *Halolamina* 属的一个新种，建议命名为海滨盐薄片菌(*Halolamina litorea*)。

关键词：海滨盐薄片菌，嗜盐古菌，海洋盐田

Marine solar salterns harbour diverse halophilic archaea of the class *Halobacteria*[1-3]. There are about 100 marine solar salterns located in tropical and subtropical offshore areas along 18 000 km coastline of marginal sea at Eastern China[4-5]. During our survey on halophilic archaeal diversity of the Yangjiang marine solar saltern in the southern region of China[6-11], a halophilic archaeal strain YJ-41^T was isolated which is most closely related to members of the genus *Halolamina*.

The genus *Halolamina*, belonging to the family Haloferacaceae[12], was proposed to accommodate the species *Halolamina pelagica* based on two strains isolated from an artificial marine solar saltern in Eastern China[13]. In 2013, another two species of the genus *Halolamina*, *Halolamina salifodinae* and *Halolamina salina*, isolated from a salt mine in Wensu county, Xinjiang province, China were described[14]. Recently, *Halolamina rubra* and *Halolamina sediminis* were isolated from solar saltern located in Republic of Korea[15-16]. Currently, the genus *Halolamina* contains five species and the members of genus *Halolamina* are differentiated from other members of the family Halobacteriaceae. Cells of species of the genus *Halolamina* are pleomorphic with shapes ranging from rods to pleomorphic shapes under optimal growth conditions, and stain Gram-negative. Colonies are red or pink due to the presence of bacterioruberin carotenoids. Cells lyse in distilled water. The major polar lipids are PA (phosphatidic acid), PG (phosphatidylglycerol), PGP-Me (phosphatidylglycerol phosphate methyl ester), PGS (phosphatidylglycerol sulfate) and several diverse glycolipids. In this study, strain YJ-41^T was characterized as a new member of the genus *Halolamina*, for which the name *Halolamina litorea* sp. nov. is proposed.

1 Materials and Methods

1.1 Isolation and cultivation of halophilic archaeal strain

Strain YJ-41^T was isolated from a sediment sample from Yangjiang marine solar saltern in the south region of China (21°31′48″N, 111°28′5″E; elevation, sea level) in 2012. The neutral haloarchaeal medium (NHM) was used for the isolation procedure and contained the following ingredients (g/L): yeast extract (Oxoid) 0.05, fish peptone (Sinopharm Chemical Reagent Co., Ltd.) 0.25, sodium pyruvate 1.0, KCl 5.4, K_2HPO_4 0.3, $CaCl_2$ 0.29, NH_4Cl 0.27, $MgSO_4·7H_2O$ 26.8, $MgCl_2·6H_2O$ 23.0, NaCl 184.0 (pH adjusted to 7.0–7.2 with 1 mol/L NaOH solution). The medium was solidified with 15 g/L agar. The sediment sample was serially diluted in liquid NHM medium and spread onto NHM agar plates. Incubated for 3 months at 37 °C. Colonies were successively re-streaked on NHM agar plates at least three times to obtain pure colonies. The strain was routinely grown aerobically at 37 °C for 7 days in NHM and preserved at −20 °C as a suspension in NHM broth supplemented with glycerol (150 g/L).

1.2 Phenotypic determination

Phenotypic tests were performed by the following method descripted by Oren[17]. Strains *Halolamina pelagica* TBN21^T, *Halolamina rubra* JCM 19436^T, *Halolamina salifodinae* CGMCC

1.12371^T, *Halolamina salina* CGMCC 1.12285^T, *Halolamina sediminis* JCM 30187^T were selected as reference strains in the present work. All strains were routinely grown aerobically on NHM medium.

The NaCl range for growth was determined by incubating the strain at 0.9, 1.4, 1.7, 2.1, 2.6, 3.1, 3.4, 3.9, 4.3, 4.8 and 5.1 mol/L. The $MgCl_2$ range for growth was determined by incubating the strain at 0, 0.005, 0.01, 0.03, 0.05, 0.1, 0.3, 0.5, 0.7 and 1.0 mol/L. The pH range for growth was determined at pH 5.0–10.0 (with intervals of 0.5) using following buffers: MES (pH 5.5–6.7), PIPES (pH 6.1–7.5), MOPS (pH 6.5–7.9), HEPES (pH 6.8–8.2), Tricine (pH 7.4–8.8) and CHES (pH 8.6–10.0) at a concentration of 25 mmol/L. The temperature range for growth was determined by incubating the strain at 10, 15, 20, 25, 30, 37, 40, 42, 45, 50, 55 and 60 °C.

The Gram stain was performed by following method outlined by Dussault[18]. Cell morphology and motility in exponentially growing liquid cultures were examined using a light microscope equipped with phase-contrast optics. The minimum salt concentration preventing cell lysis was determined by suspending cells in serial dilutions of sterile saline with NaCl concentrations ranging from 0 to 150 g/L and the stability of the cells was detected by light microscopic examination. Growth and gas formation with nitrate as an electron acceptor were tested in 9 mL stoppered tubes (with Durham tubes) completely filled with liquid NHM medium and to which $NaNO_3$ (5 g/L) had been added. The formation of gas from nitrate was detected by the presence of gas bubbles in the Durham tubes and the formation of nitrite was detected and analyzed by the Griess reaction. Anaerobic growth in the presence of L-arginine or DMSO (5 g/L) was tested in completely filled 9 mL stoppered tubes. Starch hydrolysis was determined on NHM 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 NHM agar plates amended with 5 g/L gelatin and detected by flooding the plates with Frazier's reagent[19]. Tests for catalase, oxidase and esterase activities were performed as described previously[20-21]. Production of H_2S was tested by growing the isolates and reference strains in a tube containing NHM liquid medium supplemented with 5 g/L sodium thiosulfate and detected using a filter-paper strip impregnated with lead acetate[22]. To test for growth on sole carbon sources, fish peptone and sodium pyruvate were omitted from the NHM medium and the compound to be tested was added at a concentration of 5 g/L. Production of acid from the carbohydrates and sugar alcohols is tested in unbuffered growth medium supplemented with 0.5 g of a substrate per liter. Antimicrobial susceptibilities were determined on NHM agar plates with antimicrobial compound discs.

1.3 Chemotaxonomic characterization

Strain YJ-41^T and *Halolamina pelagica* TBN21^T were routinely grown aerobically at 37 °C in NHM medium. Halophilic archaeal polar lipids were extracted using a chloroform-methanol system and analyzed using one- and two-dimensional TLC, as described previously[5]. Two specific detection spray reagents, phosphate stain reagent for phospholipids and α-naphthol stain reagent for glycolipids, were used. The general detection reagent, sulfuric acid:ethanol (1:2, *V/V*), was also used to detect total polar lipids. The presence of phospholipids and glycolipids on the two-dimensional TLC was confirmed by comparison with one-dimensional TLC on which the polar lipid profile of reference strains was developed.

1.4 Phylogenetic and genotypic analysis

Genomic DNA from halophilic archaeal strain was extracted and purified using a genomic DNA extraction kit (CW0552, Beijing ComWin Biotech Co., Ltd.) and the 16S rRNA gene was amplified with the forward primer 0018F (5′-ATTCCGGTTG ATCCTGCC-3′) and reverse primer 1518R (5′-AGG AGGTGATCCAGCCGC-3′), then cloned and sequenced according to a previous protocol[23]. The *rpoB′* gene was amplified using the primer pair HrpoB2 1420F (5′-TGTGGGCTNGTGAAGAAC TT-3′) and HrpoA 153R (5′-GGGTCCATCAGCCC CATGTC-3′)[24], and the PCR product was sequenced using the following primers: HrpoB2 1420F, HrpoA 153R and B1-628F (5′-CCNGCNGS VCAGAACTTC-3′). These sequences were aligned using the ClustalW program integrated in the MEGA 5 software[25] and the phylogenetic trees

were reconstructed using Maximum-Likelihood algorithm in the MEGA 5 software. Sequence similarity was analysed by comparing the 16S rRNA gene sequence of strain YJ-41^T with known sequences from the EzTaxon-e database (http://www.ezbiocloud.net/eztaxon)[26].

The DNA G+C content was determined from the mid-point value (T_m) of the thermal denaturation method[27] at 260 nm with a Beckman-Coulter DU800^{TM} spectrophotometer equipped with a high-performance temperature controller.

2 Results and Discussion

Cells of strain YJ-41^T were motile, rod-shaped ((0.8–1.0) μm×(2.0–4.0) μm) when grown in NHM liquid medium (Figure 1). They stained Gram-negative and the colonies were red-pigmented. Strain YJ-41^T was able to grow at 20–50 °C (optimum 37 °C), at 2.1–4.8 mol/L NaCl (optimum 3.1 mol/L NaCl), at 0–1.0 mol/L $MgCl_2$ (optimum 0.05 mol/L $MgCl_2$) and at pH 5.0–9.0 (optimum pH 7.0). The cells lysed in distilled water and the minimal NaCl concentration to prevent cell-lysis was found to be 10% (*W/V*). The strain was able to grow under anaerobic conditions using nitrate, L-arginine and DMSO. It was found to be positive for H_2S formation and indole formation. Strain YJ-41^T did not hydrolyze starch, gelatin, casein or Tween 80. Strain YJ-41^T was sensitive to the following antimicrobial compounds (μg per disc, unless otherwise indicated): novobiocin (30), bacitracin (0.04 IU per disc), nitrofurantoin (300), vancomycin (30). It was resistant to the following antimicrobial compounds: rifampin (5), mycostatin (100), trimethoprim (5), erythromycin (15), penicillin G (10 IU per disc), ampicillin (10), chloramphenicol (30), neomycin (30), norfloxacin (10), ciprofloxacin (5), streptomycin (10), kanamycin (30), tetracycline (30), gentamicin (10) and nalidixic acid (30). The main phenotypic characteristics differentiating strain YJ-41^T from the members of the genus *Halolamina* are colonial morphology, motility, Mg^{2+} requirement, utilization of specific carbon sources, indole formation and H_2S formation (Table 1). More detailed results of phenotypic features of strain YJ-41^T are given in the species description.

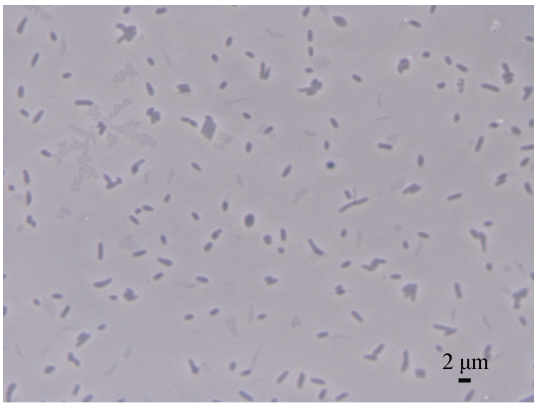

Figure 1 Phase-contrast micrograph of strain YJ-41^T
图 1 菌株 YJ-41^T 的相差显微镜照片

The major polar lipids of strain YJ-41^T were identified as PA (phosphatidic acid), PG (phosphatidylglycerol), PGP-Me (phosphatidylglycerol phosphate methyl ester), PGS (phosphatidylglycerol sulfate) and eight glycolipids, three of these glycolipids chromatographically identical to S-DGD-1 (sulfated mannosyl glucosyl diether), TGD-1 (galactosyl mannosy glucosyl diether) and DGD-1 (mannosyl glucosyl diether) respectively, other five unidentified lipids may be present (Figure 2). Since the polar lipid profile of strain YJ-41^T was identical to those of the most members of the genus *Halolamina*[13-15], the major polar lipid composition supports the classification of strain YJ-41^T in the genus *Halolamina*.

Complete 16S rRNA gene sequence comparisons indicated that strain YJ-41^T has one kind of 16S rRNA gene sequence (1 473 bp in length). The 16S rRNA gene of the strain was phylogenetically related to *Halolamina rubra* CBA1107^T (98.4% similarity), *Halolamina salifodinae* WSY15-H1^T (98.3% similarity), *Halolamina pelagica* TBN21^T (98.2% similarity), *Halolamina sediminis* halo-7^T (97.6% similarity) and *Halolamina salina* WSY15-H3^T (97.5% similarity). These 16S rRNA gene similarities are well lower than the recently recommended thresholds (98.65%) to separate two prokaryotic species[28]. Phylogenetic tree reconstructions using the maximum-likelihood (ML) algorithm revealed that strain YJ-41^T tightly clustered with the current five members of *Halolamina* (Figure 3). The *rpoB'* gene of strain YJ-41^T was closely similar to the corresponding gene of *Halolamina salifodinae*

Table 1 Differential characteristics between strain YJ-41^T and members of *Halolamina*
表 1 菌株 YJ-41^T 与 *Halolamina* 物种的差异特征

Characteristic	1	2	3	4	5	6
Colonial morphology	Red	Red	Red	Pink	Red	Red
Motility	+	–	+	–	–	+
Mg^{2+} required	–	–	+	–	–	+
Utilization of:						
D-mannose	+	+	+	–	–	+
D-galactose	–	+	+	–	+	–
D-fructose	+	–	–	–	–	–
Maltose	+	–	–	–	–	+
Sucrose	+	–	–	+	+	+
Lactose	+	–	–	–	–	–
Glycerol	+	–	–	–	–	+
D-sorbitol	–	–	–	–	–	+
Acetate	+	+	–	+	+	+
DL-lactate	–	+	–	+	–	+
L-malate	–	–	–	+	–	+
Fumarate	+	–	–	–	–	+
Citrate	–	–	–	–	–	+
Glycine	–	–	+	–	–	+
L-alanine	–	+	–	+	–	–
L-aspartate	–	–	–	–	–	+
L-lysine	–	–	+	–	–	+
Indole formation	+	–	–	–	–	–
H_2S formation	+	+	–	–	–	–
G+C content (mol%)	61.4	64.8	65.1	65.4	66.2	68.0

Note: 1: YJ-41^T; 2: *Halolamina pelagica* TBN21^T; 3: *Halolamina rubra* JCM 19436^T; 4: *Halolamina salifodinae* CGMCC 1.12371^T; 5: *Halolamina salina* CGMCC 1.12285^T; 6: *Halolamina sediminis* JCM 30187^T. +: Positive; –: Negative.

WSY15-H1^T (94.4% similarity), *Halolamina pelagica* TBN21^T (93.7% similarity), *Halolamina rubra* CBA1107^T (93.6% similarity), *Halolamina salina* WSY15-H3^T (93.5% similarity) and *Halolamina sediminis* halo-7^T (93.1% similarity). In phylogenetic tree reconstructions using the *rpoB′* (Figure 4), strain YJ-41^T tightly clustered with the members of *Halolamina*. The 16S rRNA gene and *rpoB′* gene-based phylogenetic analysis results supported the placement of strain YJ-41^T in the genus *Halolamina*.

The DNA G+C content of strain YJ-41^T were determined to be 61.4 mol%, lower than those of *Halolamina pelagica* TBN21^T (64.8 mol%)[13], *Halolamina rubra* CBA1107^T (65.1 mol%)[15], *Halolamina salifodinae* WSY15-H1^T (65.4 mol%)[14], *Halolamina salina* WSY15-H3^T (66.2 mol%)[14] and *Halolamina sediminis* halo-7^T (68.0 mol%)[16].

Based on these phenotypic, chemotaxonomic and phylogenetic properties, a novel species of the genus *Halolamina* is proposed to accommodate the strain, *Halolamina litorea* sp. nov. Characteristics that distinguish strain YJ-41^T from the related members of the genus *Halolamina* are shown in Table 1.

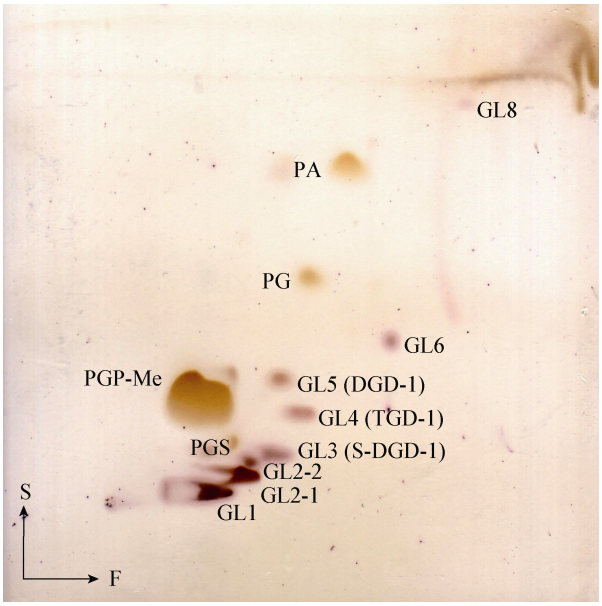

Figure 2 Analysis of the polar lipid composition of strain YJ-41^T using thin layer chromatography
图 2 菌株 YJ-41^T 的极性脂薄层层析图谱
Note: PA: Phosphatidic acid; PG: Phosphatidylglycerol; PGP-Me: Phosphatidylglycerol phosphate methyl ester; PGS: Phosphatidylglycerol sulfate; GL: Glycolipid; DGD-1: Mannosyl glucosyl diether; S-DGD-1: Sulfated mannosyl glucosyl diether; TGD-1: Galactosyl mannosyl glucosyl diether. F: First dimension of TLC; S: Second dimension of TLC.

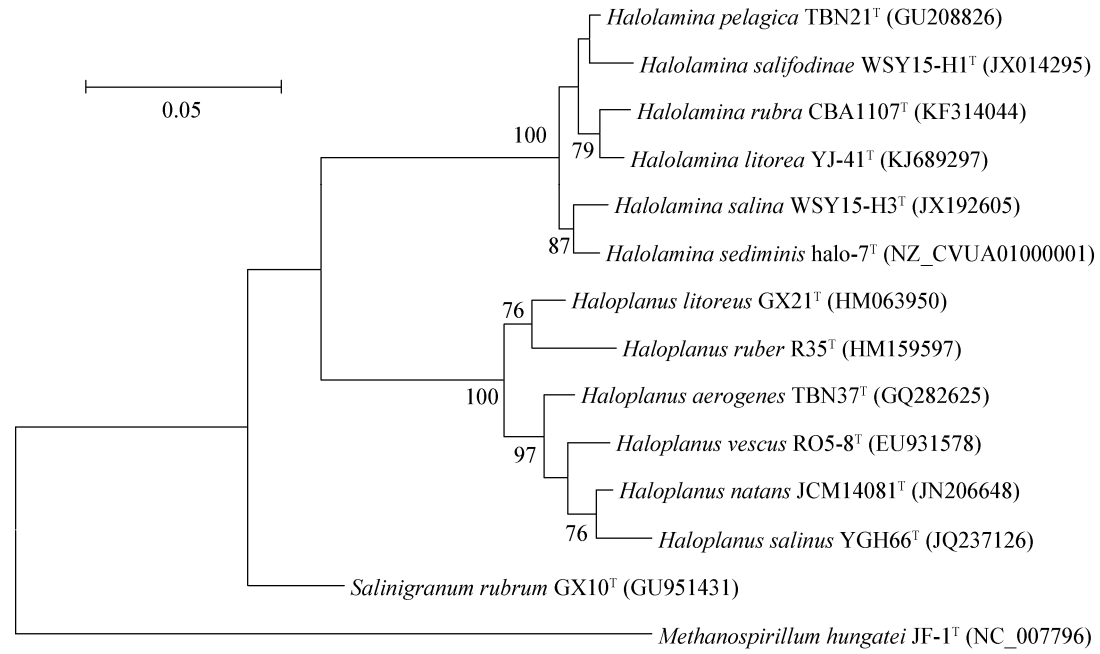

Figure 3 Maximum-Likelihood phylogenetic tree based on 16S rRNA gene sequences, showing the relationships between strain YJ-41^T and members of *Halolamina*

图 3 菌株 YJ-41^T 与 *Halolamina* 物种基于 16S rRNA 基因的系统发育树

Note: Bootstrap values (%) are based on 1 000 replicates and are shown for branches with more 70% bootstrap support. Bar represents expected substitutions per nucleotide position.

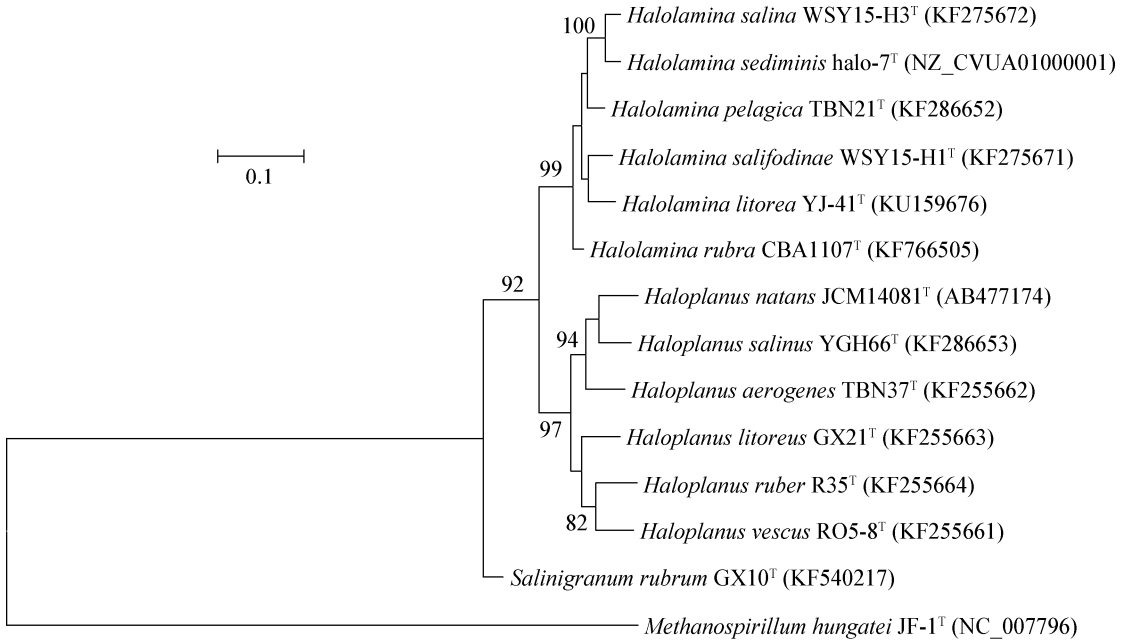

Figure 4 Maximum-Likelihood phylogenetic tree based on *rpoB′* gene sequences, showing the relationships between strain YJ-41^T and members of *Halolamina*

图 4 菌株 YJ-41^T 与 *Halolamina* 物种基于 *rpoB′*基因的系统发育树

Note: Bootstrap values (%) are based on 1 000 replicates and are shown for branches with more 70% bootstrap support. Bar represents expected substitutions per nucleotide position.

3 Description of *Halolamina litorea* sp. nov.

Halolamina litorea (li.to're.a. L. fem. adj. *litorea*, of or belonging to the sea-shore).

Cells are motile, rod-shaped ((0.8–1.0) μm× (2.0–4.0) μm) under optimal growth conditions and stain Gram-negative. Colonies on agar plates containing 3.1 mol/L NaCl are red, elevated and round. The type strain is chemoorganotrophic and aerobic. Growth occurs at 20–50 °C (optimum 37 °C), at 2.1–4.8 mol/L NaCl (optimum 3.1 mol/L NaCl), at 0–1.0 mol/L $MgCl_2$ (optimum 0.05 mol/L $MgCl_2$) and at pH 5.0–9.0 (optimum pH 7.0). Cells lyse in distilled water and the minimal NaCl concentration to prevent cell lysis is 10% (*W/V*). Catalase and oxidase positive. Anaerobic growth occurs in the presence of nitrate, arginine and DMSO. Nitrate reduction to nitrite is observed but gas formation from nitrate does not occur. H_2S formation and indole formation are positive. The type strain does not hydrolyze casein, starch, gelatin or Tween 80. The following substrates are utilized for growth: D-glucose, D-mannose, D-fructose, maltose, sucrose, lactose, glycerol, acetate, pyruvate, fumarate, L-arginine, L-glutamate and L-ornithine. No growth occurs on D-galactose, L-sorbose, D-ribose, D-xylose, D-mannitol, D-sorbitol, DL-lactate, succinate, L-malate, citrate, glycine, L-alanine, L-aspartate or L-lysine. Acid is produced from D-glucose, D-mannose, lactose and sucrose. The polar lipids are PA (phosphatidic acid), PG (phosphatidylglycerol), PGP-Me (phosphatidylglycerol phosphate methyl ester), PGS (phosphatidylglycerol sulfate) and eight glycolipids, three of these glycolipids chromatographically identical to S-DGD-1 (sulfated mannosyl glucosyl diether), TGD-1 (galactosyl mannosy glucosyl diether) and DGD-1 (mannosyl glucosyl diether) respectively, other four unidentified lipids may be present. The DNA G+C content of the type strain was 61.4 mol% (T_m).

The type strain is YJ-41^T (=CGMCC 1.12859^T=JCM 30237^T) and was isolated from Yangjiang marine solar saltern, China. The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and *rpoB′* gene sequences of strain YJ-41^T are KJ689297 and KU159676, respectively.

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征 稿 简 则

1 刊物简介与栏目设置

《微生物学通报》是由中国科学院微生物研究所和中国微生物学会主办的，以微生物学应用基础研究及技术创新与应用为主的综合性学术期刊。刊登内容包括：工业微生物学、海洋微生物学、环境微生物学、基础微生物学、农业微生物学、食品微生物学、兽医微生物学、药物微生物学、医学微生物学、病毒学、酶工程、发酵工程、代谢工程等领域的最新研究成果，产业化新技术和新进展，以及微生物学教学研究和改革等。设置的栏目有：研究报告、专论与综述、生物实验室、高校教改纵横、显微世界、专栏、书讯、会讯等。

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3 写作要求

来稿要求论点明确，数据可靠，简明通顺，重点突出。

3.1 参考文献

参考文献按文内引用的先后顺序排序编码，未公开发表的资料请勿引用。我刊参考文献需要注明著者(文献作者不超过 3 人时全部列出，多于 3 人时列出前 3 人，后加“等”或“et al.”，作者姓前、名后，名字之间用逗号隔开)、文献名、刊名、年卷期及页码。国外期刊名必须写完整，不用缩写，不用斜体。参考文献数量不限。

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