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研究报告

Celeribacter ethanolicus sp. nov., isolated from seawater of the South China Sea

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Abstract: [Objective] A novel *Celeribacter* strain NH195^T was isolated from seawater of the South China Sea and was subjected to polyphasic taxonomic study. **[Methods]** Phenotypic, genotypic and chemotaxonomic characterizations, as well as phylogenetic inference were performed in this study. **[Results]** Strain NH195^T was a Gram-stain-negative, aerobic, rod-shaped, non-motile bacterium that accumulates poly-*β*-hydroxybutyrate (PHB). The isolate grew in media containing 0.5%−10.0% (*W/V*) NaCl (optimally at 1.0%−3.0%) at 20−40 °C and pH 5.0−9.0, and was positive for oxidase, catalase and urease. The respiratory quinone was Q-10. The major fatty acids (>10%) were $C_{18:1}\omega7c$, $C_{18:1}\omega6c$ and 11 methyl $C_{18:1}\omega7c$. The major polar lipids were phosphatidylcholine (PC), phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), one unidentified aminolipid and two unidentified lipids. The genomic DNA G+C content was 61.3 mol%. Phylogenetic analysis based on the 16S rRNA gene sequences indicated that strain NH195^T could be assigned to the genus *Celeribacter*. The 16S rRNA gene sequence similarities between the isolate and the type strains of *Celeribacter* species with validly published names were in the range 94.4%−97.7%. Strain NH195^T exhibited average nucleotide identity (ANI) values of 78.6% and 78.0% to *C. halophilus* ZXM137^T and *C. indicus* P73^T respectively. The genome-to-genome distance analysis revealed that strain NH195^T shared 26.1% DNA relatedness with *C. halophilus* ZXM137^T and 23.0% with *C. indicus* P73^T, respectively. **[Conclusion]** On the basis of phenotypic and genotypic characteristics, strain NH195^T represents a novel species within the genus *Celeribacter*, for which the name *Celeribacter ethanolicus* is proposed. The type strain is NH195^T (=CGMCC 1.15406^T=JCM 31095^T).

Keywords: *Celeribacter ethanolicus*, Polyphasic taxonomy, Seawater, South China Sea

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来源于中国南海的微生物新种乙醇速生杆菌(*Celeribacter ethanolicus*)多相分类研究

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摘 要:【目的】研究来源于南海海水的一株速生杆菌属菌株 NH195^T 的多相分类。【方法】采用表型、基因型和化学分类方法，并综合系统发育关系结果，分析菌株 NH195^T 的分类学地位。【结果】菌株 NH195^T 是一株革兰氏阴性、好氧、杆状、无运动性细菌；能积累 Poly-β-hydroxy butyrate (PHB)；能在 0.5%−10.0% (质量体积比) NaCl 浓度，pH 5.0−9.0 和 20−40 °C 条件下生长，最适 NaCl 生长浓度为 1.0%−3.0%；氧化酶、触酶和脲酶反应结果阳性。菌株 NH195^T 主要呼吸醌为 Q-10，主要脂肪酸为 $C_{18:1}\omega7c$、$C_{18:1}\omega6c$ 和 11 methyl $C_{18:1}\omega7c$，主要极性脂为磷脂酰胆碱、磷脂酰甘油、双磷脂酰甘油、一个未知的氨基脂和两个未知脂。基因组 G+C 含量为 61.3 mol%。基于 16S rRNA 基因的系统发育结果显示，菌株 NH195^T 隶属于速生杆菌属；其与速生杆菌属标准菌株的 16S rRNA 基因相似性范围为 94.4%−97.7%。菌株 NH195^T 与速生杆菌属标准菌株 *C. halophilus* ZXM137^T 和 *C. indicus* P73^T 的平均核苷酸一致性(ANI)分别为 78.6%和 78.0%；基于基因组数据计算所得的 DNA 杂交同源率分别为 26.1%和 23.0%。【结论】基于表型和基因型结果，菌株 NH195^T 代表了速生杆菌属一个新物种，命名为 *Celeribacter ethanolicus*，标准菌株为 NH195^T (CGMCC 1.15406^T=JCM 31095^T)。

关键词: 乙醇速生杆菌，多相分类，海水，中国南海

1 Introduction

The genus *Celeribacter*, belonging to the family Rhodobacteraceae, was proposed by Ivanova *et al*. (2010)[1]. At the time of writing, the genus *Celeribacter* contains seven species: *C. neptunius* (type species), *C. baekdonensis*, *C. halophilus*, *C. indicus*, *C. manganoxidans*, *C. marinus* and *C. naphthalenivorans*[1-7]. The habitats which *Celeribacter* species have been isolated are mainly marine environments, including seawaters[1,3,6-7] and sediments[2,4-5]. Members of the genus *Celeribacter* are characterized as being Gram-staining-negative, non-spore-forming, chemo-organotrophic with rod-shaped cells. The genus contains Q-10 as the major respiratory quinone and phosphatidylglycerol and aminolipids as the major lipids. Members of this genus show diverse physiological features. For example, *C. indicus* and *C. naphthalenivorans* participate in polycyclic aromatic hydrocarbon degradation[2,5]. *C. manganoxidans* is a manganese-oxidizing bacterium[4]. A novel *Celeribacter* strain NH195^T was isolated from a surface seawater sample collected from the South China Sea and was subjected to polyphasic taxonomic study.

2 Materials and Methods

2.1 Organisms and maintenance conditions

A surface seawater sample was collected from the South China Sea (119°E, 19°N) by a CTD multiparameter sonde (SBE911 plus, Sea-Bird Electronics, Inc. USA) from the vessel *Xiang yang Hong Shi Hao* during the winter cruise in December, 2014. Aboard the ship, the seawater samples were subsampled aseptically. Approximate 100 μL subsample was spread on modified marine agar (prepared according to the Difco formula for marine 2216 agar, but the amount of peptone and yeast extract was reduced to 0.5 g and 0.1 g, final pH 7.2) by using the standard ten-fold dilution plating technique. After ten days of aerobic incubation at 28 °C, one white

colony, designed NH195^T, was picked. The isolate was purified by repeated restreaking. Purity was confirmed by the uniformity of cell morphology. Unless otherwise stated, strain NH195^T was routinely cultured on marine broth 2216 (MB, BD, Difco^{TM}) at 30 °C.

Strain NH195^T can be maintained for short-term storage on Marine Agar (MA, BD, Difco^{TM}) slants at 4 °C for 6 weeks. For long-term preservation, cultures can be preserved in liquid nitrogen, by lyophilization or frozen at −80 °C. Strain NH195^T has been deposited in the CGMCC (China General Microbiological Culture Collection) and the JCM (Japan Collection of Microorganisms).

2.2 Reference strains

Three reference strains were used for biochemical and physiological comparisons, *C. halophilus* ZXM137^T, *C. indicus* P73^T and *C. manganoxidans* D2-5^T.

2.3 Growth and nutritional characteristics

The temperature range for growth was determined by incubation at 4, 15, 20, 25, 28, 30, 37, 40, 45, 50 and 55 °C. The pH range for growth was determined in MB that was adjusted to pH 5.0–10.5 (0.5 pH unit intervals) using appropriate buffers (MES for pH 5.0−6.0, PIPES for 6.5−7.0, Tricine for 7.5−8.5 and CAPSO for 9.0−10.5) at a concentration of 50 mmol/L. Evaluation of the pH values after autoclaving revealed only minor changes. The optimal conditions for growth were tested by using NaCl-free MB (prepared according to the MB formula, but without NaCl) with different NaCl concentrations (0, 0.5%, 1.0%, 3.0%, 5.0%, 7.5%, 10.0%, 12.0%, 15.0% and 20.0% as final concentration, *W/V*). The requirement of strain NH195^T for artificial sea salts was evaluated on YP medium (yeast extract 5 g/L and peptone 1 g/L) with the addition of 0, 1%, 2%, 3%, 4.5% and 5% (*W/V*) sea salts (Sigma). Growth was monitored by measuring OD_{590} in a UV/Visible Spectrophotometer (Ultrospec 6300 pro, Amersham Biosciences).

The utilization of carbohydrates as sole carbon and energy sources was determined in BM medium[8]. The corresponding filter-sterilized sugars (0.2%, *W/V*), alcohols (0.2%, *W/V*), organic acids (0.1%, *W/V*) or amino acids (0.1%, *W/V*) were added into liquid BM medium.

2.4 Light and electron microscope examination

The Gram reaction was tested by using the Gram staining method as described by Dong and Cai (2001)[9]. Cell morphology, size and motility were examined using optical microscopy (BX40; Olympus), confocal laser scanning microscopy (TCS SP5; Leica) and transmission electron microscopy (JEM-1230, JEOL). Cells were harvested from marine agar 2216 (MA, BD) at 30 °C and prepared for microscope examination. For whole cell microscopy, cells were stained with 1% (*W/V*) uranyl acetate solution. For section electron microscopy, cells were fixed with 2.5% (*V/V*) glutaraldehyde and 1% (*W/V*) osmium tetroxide, dehydrated in gradient concentrations of ethanol (50%, 70%, 80%, 90% and 95%, *V/V*), transferred to pure acetone, sectioned in a microtome and stained with 2% (*W/V*) lead citrate and uranyl acetate.

2.5 Physiological and biochemical characteristics

Unless otherwise noted, physiological and biochemical tests were carried out in MB (or MA) medium at 30 °C.

Anaerobic growth was tested using AnaeroPack (Mitsubishi) with nitrate (20 mmol/L) or nitrite (10 mmol/L) as a potential electron acceptor. Catalase activity was detected by bubble production in 3% hydrogen peroxide solution (*V/V*). Oxidase activity was determined by oxidation of 1% (*W/V*) *p*-aminodimethylaniline oxalate. Tests for hydrolysis of DNA (0.2%, *W/V*), gelatin (1%, *W/V*), starch (0.2%, *W/V*) and Tweens (1%, *W/V*) were performed on plates to examine the existence of a transparent circle or aureole according to Dong & Cai (2001)[9]. DNA plates were flooded with 5 mol/L HCl, gelatin plates were flooded with Frazier reagent (150 g $HgCl_2$ and 200 mL hydrochloric acid per liter distilled water), and starch plates were flooded with Lugol's iodine solution (3.3 g iodine and 6.6 g potassium iodide per liter distilled water). Acid production was tested using the marine oxidation-fermentation (MOF) medium supplemented with 0.5% sugars[10].

Polycyclic aromatic hydrocarbon (PAH) degradation was tested using the clear zone method[11] on NH medium[5] supplemented by different PAHs as sole carbon source. The NH medium was sprayed with tested PAH over the surface of the agar plate. The PAHs included naphthalene, 2-methylnaphthalene, 2,6-dimethylnaphthalene, phenanthrene, anthracene,

fluorene, acenaphthene, pyrene, fluoranthene, dibenzothiophene, 4-methyldibenzothiophene and dibenzofuran. Heavy metal tolerance was studied in MA supplemented with different concentrations of $MnCl_2$ (0, 1, 5, 10 and 50 mmol/L), $ZnCl_2$ (0, 0.5, 1 and 5 mmol/L), $CuSO_4$ (0, 0.25, 0.5, 0.75, 1 and 2 mmol/L), $HgCl_2$ (0, 0.01, 0.05, 0.1 and 0.5 mmol/L) and $CdCl_2$ (0, 0.05, 0.1, 0.5, 1 and 5 mmol/L). Extracellular accumulation of manganic oxide were examined on K agar plates[4] supplemented with 10 mmol/L $MnCl_2$. After incubation, the presence of brownish manganic oxide deposits in the colonies could be confirmed by leucoberbelin blue (LBB) reagent.

Additional enzyme activities and biochemical characteristics were determined using API 20 NE, API 20E and API ZYM kits (bioMérieux) at 30 °C. Strips were inoculated with a heavy bacterial suspension (*MacFarland 5 Standard*) in AUX medium supplemented with 2% (*W/V*) sea salts (Sigma) according to Park *et al.* (2005)[12].

Sensitivity to antimicrobial agents was determined on MA at 30 °C and observed after 1, 3 and 5 days by plating antibiotics disks (Hangzhou Microbial Reagent Co. LTD, Hangwei™) on the medium and observing the appearance of inhibition zone. The concentrations of antibiotics was enumerated below (μg unless otherwise stated): ampicillin (10), amoxicillin (20), bacitracin (0.04 IU), chloramphenicol (30), clindamycin (2), erythromycin (10), gentamicin (10), kanamycin (30), nalidixic acid (30), nitrofurantoin (300), novobiocin (30), polymyxin B (300 IU), rifampicin (5), streptomycin (10), tetracycline (30), tobramycin (10) and vancomycin (30). A diameter of inhibition zones > 8 mm (including the 6 mm diameter of the filter paper) was considered sensitive to antimicrobial agents.

2.6 Fatty acid, quinone and polar lipid analysis

Cellular fatty acids methyl esters were obtained from cells grown in MB for one day at 30 °C and analyzed by using GC/MS[13] according to the instructions of the Microbial Identification System (MIDI Inc.).

Isoprenoid quinone was extracted from lyophilized cells (200 mg) with chloroform/methanol (2:1, *V/V*). The filtered extracts were evaporated to dry at 35 °C, and suspended with chloroform/methanol (2:1, *V/V*). Isoprenoid quinone was separated through chromatography on GF254 silica gel plates (Branch of Qingdao Haiyang Chemical Co Ltd) with n-hexane/ether (17:3, *V/V*). The separated fractions were analyzed by HPLC-MS[14].

Total lipids were extracted by the modified method of Kamekura & Kates (1988)[15] and identified by two-dimensional TLC. Merck silica gel 60 F254 aluminium-backed thin-layer plates were used. For two-dimensional TLC, the first dimension was developed using chloroform/methanol/water (65:25:4, *V/V/V*) and the second dimension used chloroform/methanol/acetic acid/water (80:12:15:4, *V/V/V/V*). Molybdophosphoric acid and sulfuric acid were used for the detection of all lipids, ninhydrin reagent for lipids containing free amino lipids, ammonium molybdate reagent for phosphorus containing lipids and α-naphthol reagent for glycolipid[16].

2.7 16S rRNA gene sequence analysis

Genomic DNA was obtained using the method described by Marmur (1961)[17]. The 16S rRNA genes were amplified with the following primers (position number given according to *Escherichia coli* 16S rRNA gene): primer 8F: 5′-AGAGTTTGATCCTGGCTCAG-3′, positions 8–27; primer 1492R: 5′-GGYTACCTTGTTACGACTT-3′, positions 1 510–1 492. PCR products were cloned into vector pMD 19-T (TaKaRa) and then sequenced to determine the almost-complete sequence of 16S rRNA gene.

The 16S rRNA gene sequence of strain NH195^T was compared with its closely related sequences from the EzTaxon-e service[18]. Phylogenetic analysis was performed in ARB release 6.0.2[19] in the All-Species Living Tree Project database (LTP_s119, November 2014)[20]. The 16S rRNA gene sequences of NH195^T were aligned with SINA (version 1.2.11) according to the SILVA seed alignment (http://www.arb-silva.de)[21] and implemented into the LTPs database. The alignment was checked manually based on secondary structure information. Based on the obtained phylogenetic resolution and EzTaxon-e results, further trees were reconstructed including 18 type strains of the closest related species and performed using the software tool MEGA 5 program package. Sequence data were aligned with

ClustalW[22]. Phylogenetic trees were constructed by the neighbour-joining[23], maximum-parsimony[24] and maximum-likelihood methods[25] with the MEGA 5 program package[26]. Evolutionary distances were calculated according to the algorithm of the Kimura two-parameter model[27] for the neighbour-joining method.

2.8 Genomic analysis

The genomes of strain NH195^T and *C. halophilus* ZXM137^T were sequenced in this study. Genomic DNA was isolated with the AxyPrep™ Bacterial Genomic DNA Miniprep Kit (Corning Life Sciences, USA). High-throughput sequencing was carried out on an Illumina HiSeq2000 platform (Novogene Bioinformatics Technology Co. Ltd, Beijing). One paired-end library was constructed with the average insert size of ~500 bp. The sequencing generated about 1 G clean data with ~400-fold genome coverage on average. Reads were assembled *de novo* into contigs and subsequently joined into scaffolds using SOAPdenovo[28] version 2.0.1 and Abyss[29] version 1.5.2. The assembly k-mer was tested from k=36 to 64 for seeking the optimal value. MUMmer[30] were used to estimate the assembly quality for choosing a best one for further analysis. The genome of *C. indicus* P73^T (CP004393-CP004398) was retrieved from the GenBank database. The rRNA genes were found via RNAmmer 1.2 Server[31]. The open reading frames (ORFs) and the functional annotation of translated ORFs were predicted and achieved by using the RAST server online[32]. Classification of some predicted genes and pathways were analyzed using COG databases[33] and KEGG databases[34-35].

The average nucleotide identity (ANI) between two genomes was calculated using the algorithm of EZGenome web service[18]. The genome-to-genome distance were calculated by GGDC[36].

3 Results and Discussion

3.1 Genotypic characterizations

The almost-complete 16S rRNA gene sequence of strain NH195^T (1 455 nt) was obtained (GenBank accession number KT852989). The 16S rRNA gene sequence similarities between strain NH195^T and the type strains of *Celeribacter* species with validly published names were in the range 94.4%−97.7%. Strain NH195^T was most closely related to *C. halophilus* (97.8%), *C. indicus* (97.5%), *C. naphthalenivorans* (97.5%) and *C. neptunius* (97.4%) in the genus *Celeribacter*. Sequence comparisons to representative bacteria with validly published names indicated that strain NH195^T belongs to the genus *Celeribacter*.

The All-Species Living Tree indicated that strain NH195^T fell within the cluster comprising species of the genus *Celeribacter* and formed a clade with *C. halophilus* and *C. indicus*. The neighbour-joining, maximum likelihood and maximum-parsimony phylogenetic tree topologies constructed by MEGA 5 supported the notion that strain NH195^T formed a clade with *C. halophilus* and *C. naphthalenivorans* (Figure 1).

The genomes of strains NH195^T and *C. halophilus* ZXM137^T were sequenced and annotated (GenBank accession numbers LRUC00000000 and LRUD00000000). The DNA G+C content of strain NH195^T is 61.3 mol%. Several important encoding genes related to phenotypic and chemotaxonomic characterizations were found in genome and analyzed, such as the RodA protein which determines cells shape and mediates antibiotics resistance via beta-lactamase, and the UbiB protein which participates in ubiquinone biosynthesis. Besides, alcohol dehydrogenase (EC 1.1.1.1) and aldehyde dehydrogenase (EC 1.2.1.3) involved in ethanol metabolism were detected in the genome.

Strain NH195^T exhibited ANI values of 78.6% and 78.0% to *C. halophilus* ZXM137^T and *C. indicus* P73^T, respectively. The ANI values were far below the threshold of 94%−96% that corresponds to the proposed species boundary[37], indicating a low taxonomic relatedness between strain NH195^T and its closely related species. The genome-to-genome distance analysis revealed that strain NH195^T shared 26.1% DNA relatedness with *C. halophilus* ZXM137^T and 23.0% with *C. indicus* P73^T, respectively. The calculated DDH values were well below the 70% cut-off point recommended for the assignment of strains to the same genospecies[38]. Therefore, strain NH195^T represents a separate genospecies in the genus *Celeribacter*.

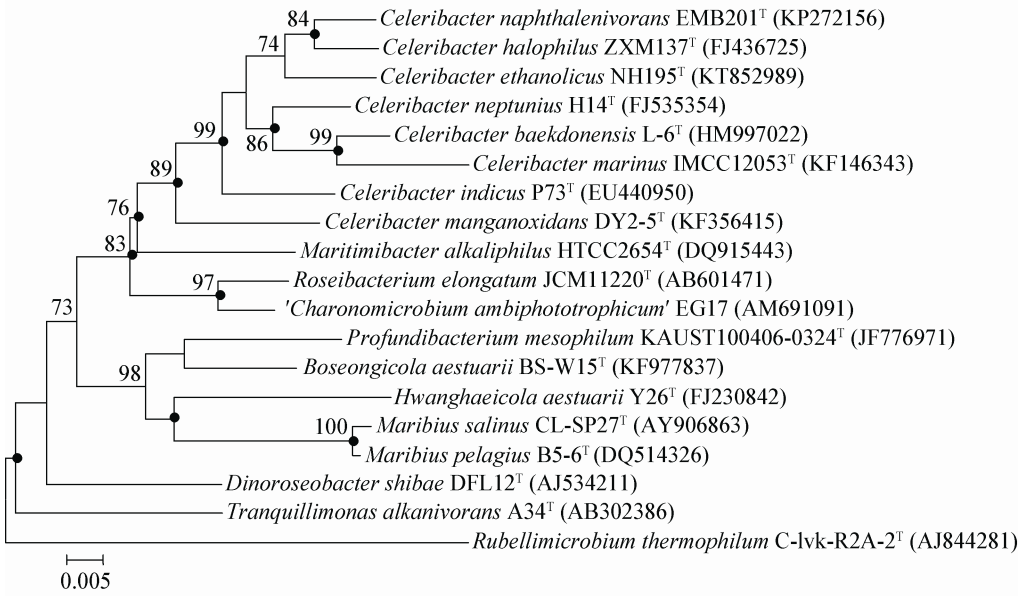

Figure 1 Neighbour-Joining phylogenetic tree based on 16S rRNA genes showing the phylogenetic relationship of strain NH195^T and its related species

图 1 基于 16S rRNA 基因采用邻接法构建的菌株 NH195^T 和相关菌株系统发育树

Note: Only bootstrap values above 70% are shown. Filled circles indicate nodes recovered in maximum-likelihood and maximum-parsimony trees.

注：只显示置信度大于 70%分支节点；圆点表示采用最大似然法法和最大简约法构建的系统发育树具有一致的分支结构.

3.2 Phenotypic characterization

Strain NH195^T is Gram-stain-negative, aerobic and non-motile rod (0.5−1.0) μm×(1.0−2.0) μm. No flagellum was observed (Figure 2). Cells divided by binary fission. Strain NH195^T accumulates poly-*β*-hydroxybutyrate as intracellular reserve product. Colonies are circular, slightly convex and 1−2 mm in diameter after one day incubation at 30 °C on marine agar. Strain NH195^T was positive for oxidase, catalase and urease activities as well as Voges-Proskauer reaction, and was susceptible to (μg per disc unless otherwise stated) ampicillin (10), amoxicillin (20), chloramphenicol (30), erythromycin (10), gentamicin (10), kanamycin (30), nitrofurantoin (300), novobiocin (30), polymyxin B (300 IU), rifampicin (5), streptomycin (10), tetracycline (30) and tobramycin (10), and resistant tobacitracin (0.04 IU), clindamycin (2), nalidixic acid (30) and vancomycin (30). Detailed results of morphological characteristics, nutritional tests, physiological tests and biochemical tests are given in the species description.

No clear zone was observed of strain NH195^T after one week of growing on plates of NH medium containing PAHs. The reference strain, *C. indicus* P73^T, degraded phenanthrene, anthracene, fluorene, pyrene, fluoranthene, dibenzothiophene, 4-methyldibenzothiophene and dibenzofuran. Strain NH195^T was able to grow on MA containing low concentrations of heavy metals, including $MnCl_2$ (10 mmol/L), $ZnCl_2$ (1 mmol/L), $CuSO_4$ (1 mmol/L),

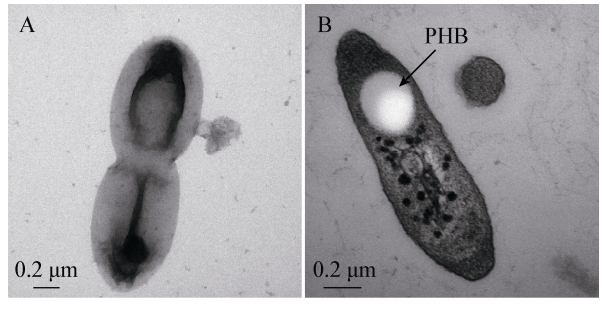

Figure 2 Transmission electron micrographs of cells of strain NH195^T with hanging drop method (A) and section method (B)

图 2 菌株 NH195^T 的悬滴透射电镜图(A)和切片透射电镜图(B)

$HgCl_2$ (0.05 mmol/L) and $CdCl_2$ (0.5 mmol/L). The colony did not turn blue with the addition of LBB reagent after one week growing on K agar plates. It is indicated that strain NH195^T did not accumulate extracellular manganic oxide.

3.3 Fatty acid characterization

Fatty acids analysis revealed that three components, $C_{18:1}\omega7c$, $C_{18:1}\omega6c$ and 11 methyl $C_{18:1}\omega7c$ were the principal fatty acids (>10%) (Table 1). Low contents of branched fatty acids were reported in *Celeribacter*. Comparing to the reference strains, the content of branched fatty of strain NH195^T (14.7%) was similar with *C. halophilus* ZXM137^T (20%), but higher than that of *C. indicus* P73^T (3.1%). In addition, $C_{17:1}\omega8c$ and $C_{18:1}\omega6c$ was detected in strain NH195^T (4.1% and 13.8%, respectively), while it was not detected in both *C. halophilus* ZXM137^T and *C. indicus* P73^T. $C_{19:0}\omega8c$ cyclo was detected in strain *C. indicus* P73^T (16.2%), while it was not detected in both strain NH195^T and *C. halophilus* ZXM137^T. 11 methyl $C_{18:1}\omega7c$ was detected in strain NH195^T and *C. halophilus* ZXM137^T (13.1% and 14.3%, respectively), while it was not detected in *C. indicus* P73^T (Table 1).

Table 1 Fatty acid composition (%) of strain NH195^T and its closely related *Celeribacter* species
表 1 菌株 NH195^T 和相关速生杆菌属物种的脂肪酸含量组成(%)

Fatty acids	Strain NH195^T	*C. halophilus* ZXM137^T	*C. indicus* P73^T
Saturated			
$C_{16:0}$	3.8	3.3	8.5
$C_{18:0}$	2.6	1.6	2.0
10 methyl $C_{19:0}$	1.6	5.7	3.1
Unsaturated			
$C_{15:1}\omega8c$	–	–	1.6
$C_{17:1}\omega8c$	4.1	–	–
$C_{18:1}\omega6c$	13.8	–	–
$C_{18:1}\omega7c$	30.3	51.3	51.3
11 methyl $C_{18:1}\omega7c$	13.1	14.3	–
$C_{19:0}\omega8c$cyclo	–	–	16.2
$C_{20:4}\omega6,9,12,15c$	5.6	2.4	0.9
Hydroxy			
$C_{10:0}$ 3OH	9.0	8.9	4.5
$C_{16:0}$ 3OH	1.8	0.9	1.5
$C_{18:0}$ 3OH	1.8	0.8	1.4
Unknown			
ECL 11.799	7.6	8.1	5.8
ECL 14.959	2.1	0.5	0.6

Note: –: Not detected; ECL: Equivalent chain-length. All strains were grown under identical conditions on MA for one day at 30 ºC. Fatty acids representing less than 1% in all strains were omitted.
注：–：未检测到；ECL：当量链长. 所有菌株生长条件一致，30 °C 生长 1 d. 在所有菌株中含量均小于 1%已舍弃.

3.4 Quinone and lipid characterzation

Ubiquinone Q-10 was determined to be the sole quinone, which is in accord with the description of the genus *Celeribacter*. The major polar lipids were phosphatidylcholine (PC), phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), one unidentified aminolipid (AL1) and two unidentified lipids (L2 and L7) (Figure 3). In addition, moderate to minor amounts of two unidentified glycolipid (GL1 and GL2), one unidentified aminolipid (AL2) and five other lipids (L1, L3–L6) were also present in strain NH195^T. Comparing the available data, the polar lipid profiles of the *Celeribacter* species are different, while all species possess phosphatidylglycerol (PG) as one of the major polar lipids. Comparing to the reference strains, one lipid (L8) was detected in strain *C. halophilus* ZXM137^T, while it was not detected in both strain NH195^T and *C. indicus* P73^T.

3.5 Taxonomic conclusion

Strain NH195^T can be distinguished from the type strains of the closely related *Celeribacter* species by a number of phenotypic characteristics, such as assimilation of carbon sources and acid production (Table 2). Moreover, the DNA-DNA relatedness, lower than the threshold, was another evidence to distinguish strain NH195^T from it closely related *Celeribacter* species. On the basis of the phenotypic, genotypic and chemotaxonomic characterizations, as well as the phylogenetic inference obtained in this study, strain NH195^T represents a novel species within the genus *Celeribacter*, for which the name *Celeribacter ethanolicus* sp. nov. is proposed.

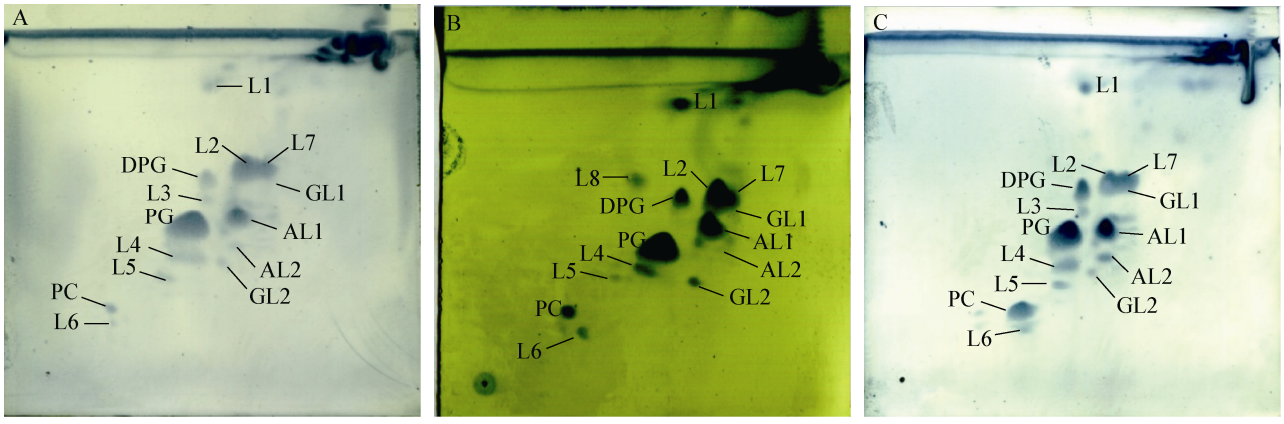

Figure 3 Thin-layer chromatograms after staining with molybdatophosphoric acid showing the total polar lipid profiles of strain NH195^T (A), *C. halophilus* ZXM137^T (B) and *C. indicus* P73^T (C)

图 3 菌株 NH195^T (A)，*C. halophilus* ZXM137^T (B)和 *C. indicus* P73^T (C)磷钼酸染色的总脂层析图

Note: PC: Phosphatidylcholine; PG: Phosphatidylglycerol; DPG: Diphosphatidylglycerol; AL: Aminolipid; GL: Glycolipid; L: Lipid.

注：PC：磷脂酰胆碱；PG：磷脂酰甘油；DPG：双磷脂酰甘油；AL：氨基脂；GL：糖脂；L：脂.

Table 2 Differential phenotypic characteristics of strain NH195^T and its closely related species

表 2 菌株 NH195^T 和相近物种表型特征差异

Characteristics	Strain NH195^T	*C. halophilus* ZXM137^T	*C. indicus* P73^T
Cell size (μm)	(0.5−1.0)×(1.0−2.0)	0.3×0.8*	(0.6−0.7)×(1.2−1.3)†
Temperature (optimum) (°C)	20−40(37)	4−45(28)*	10−41(28)†
NaCl range (%, *W/V*)	0.5−10.0	0.5−11.0*	0.5−12.0†
Utilization of:			
Citrate	−	+	−
Ethanol	+	−	+
D-Galactose	−	+	−
α-D-Lactose	−	+	−
L-Rhamnose	−	+	+
Sucrose	+	+	−
Acid production from:			
Ethanol	+	−	+
D-Galactose	−	+	−
myo-Inositol	−	+	−
α-D-Lactose	−	+	+
L-Rhamnose	−	+	+
Sorbitol	−	−	+
DNA G+C content (mol%) (by genome)	61.3	58.1	65.7

Note: +: Positive; −: Negative. Data were obtained from this study. *: Data from Wang *et al.*, 2012[7]; †: Data from Lai *et al.*, 2014[5].

注：+：阳性；−：阴性. 所有数据均来自此次研究. *：数据引自 Wang 等，2012[7]；†：数据引自 Lai 等，2014[5].

4 Description of *Celeribacter ethanolicus* sp. nov.

Celeribacter ethanolicus (e.tha.no′li.cus. N.L. n. *ethanol* ethanol; L. suf. *-icus -a -um* suffix used with various meanings; N.L. masc. adj. *ethanolicus* belonging to ethanol, referring to the substrate ethanol, which can be utilized).

Cells are Gram-stain-negative, aerobic, non-motile and rod-shaped, 0.5−1.0 μm in width and 1.0−2.0 μm in length. Colonies are circular, slightly convex and 1−2 mm in diameter after one day incubation at 30 °C on marine agar. Growth occurs at NaCl concentration of 0.5%−10.0% (*W/V*) (optimum at 1.0%−3.0%). Require natural seawater or artificial sea-salts for growth. The pH and temperature ranges for growth are pH 5.0−9.0 and 20−40 °C (optimum at pH 7.5 and 37 °C). No growth is detected at 4 °C or above 45 ºC. Resistant to low concentrations of heavy metals, including $MnCl_2$ (10 mmol/L), $ZnCl_2$ (1 mmol/L), $CuSO_4$ (1 mmol/L), $HgCl_2$ (0.05 mmol/L) and $CdCl_2$ (0.5 mmol/L). No anaerobic growth occurs on marine agar supplemented with nitrate (20 mmol/L) or nitrite (10 mmol/L). Positive for oxidase, catalase, urease and Voges-Proskauer reaction. Accumulates poly-*β*-hydroxybutyrate as intracellular reserve product. Negative for arginine dihydrolase, citrate utilization, glucose fermentation, lysine and ornithine decarboxylases, tryptophan deaminase, indole formation, H_2S production and nitrate reduction.

Gelatin, DNA, starch and Tween 20, 40, 60 and 80 are not hydrolysed. Acid and alkaline phosphatases, esterase (C4), esterase lipase (C8), *α*- and *β*-glucosidases, leucine arylamidase, naphthol-AS-BI-phosphohydrolase and valine arylamidase activities are present. *N*-Acetyl-*β*-glucosaminidase, *α*-chymotrypsin, *β*-fucosidase, *α*- and *β*- galactosidases, *β*-glucuronidase, lipase (C14), *α*-mannosidase and trypsin activities are absent. Cysteine arylamidase activity is weak. The following compounds are utilized as sole carbon and energy sources: alanine, ethanol, D-fructose, glucose, mannitol, D-mannose, sucrose and D-xylose. The following compounds are not utilized as sole carbon and energy sources: adipic acid, *N*-acetyl-glucosamine, L-arabinose, citrate, D-galactose, glycine, α-D-lactose, potassium gluconate, L-rhamnose, raffinose and sorbitol. Acid is produced from ethanol, D-fructose, glucose, maltose, D-mannose, mannitol, sucrose, trehalose and D-xylose, but not fromadonitol, D-galactose, *myo*-inositol, *α*-D-lactose, D-melezitose, L-rhamnose, raffinose, sorbose and sorbitol. The respiratory quinone is Q-10. The major fatty acids (>10%) are $C_{18:1}\omega7c$, $C_{18:1}\omega6c$ and 11 methyl $C_{18:1}\omega7c$. The major polar lipids were phosphatidylcholine (PC), phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), one unidentified aminolipid and two unidentified lipids. In addition, moderate to minor amounts of two unidentified glycolipid, one unidentified aminolipid and five unidentified lipidswere also present. The DNA G+C content is 61.3 mol%.

The type strain is NH195^T (=CGMCC 1.15406^T=JCM 31095^T), isolated from seawater of the South China Sea.

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