Echinicola Salinicaeni sp. nov., A Novel Bacterium Isolated from Saltern

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Echinicola salinicaeni sp. nov., a novel bacterium isolated from saltern

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Abstract

A Gram-stain-negative, aerobiotic, pink, motile, rod-shaped bacterium, designated P51^T was isolated from saline silt samples in Yantai, China. The novel isolate was able to grow at 4–42 °C (optimum 33 °C), pH 4.0–9.0 (optimum 7.0) and with 0–11.0% NaCl (optimum 4.0%, w/v). It could grow at 4 °C, which was different from the neighbour strains. The draft genome consisted of 4111 genes with a total length of 5139782 bp and 39.9% G + C content. The 16S rRNA gene sequence analysis indicated that strain P51^T was a member of the genus *Echinicola* and most closely related to '*Echinicola shivajiensis*' with pair-wise sequence similarity of 97.7%. Genome analysis identified genes encoding proteins relating to carbon source utilization such as glycoside hydrolases and glycosyl transferases; carotenoid biosynthesis pathway and *β*-lactam resistance pathway were observed. Strain P51^T shared average nucleotide identity value below 84.7%, average amino acid identity value between of 70.8–89.3%, digital DNA-DNA hybridization identity of between 17.9–28.2% with the closely related type strains within the genus *Echinicola*. The sole menaquinone was MK-7; the major fatty acids were iso-$C_{15:0}$, summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$), summed

feature 4 (anteiso-$C_{17:1}$ B and/or iso-$C_{17:1}$ I) and summed feature 9 (iso-$C_{17:1}\omega 9c$ and/or 10-methyl $C_{16:0}$); the polar lipids included one phosphatidylethanolamine, one unidentified aminophospholipid, one unidentified phospholipid, three unidentified aminolipids and one unknown lipid. Based on phenotypic, chemotaxonomic, and phylogenetic analyses, strain P51^T represents a novel species of the genus *Echinicola*, for which the name *Echinicola salinicaeni* sp. nov. is proposed. The type strain is P51^T (KCTC 82513^T= MCCC 1K04413^T).

Keywords: *Echinicola salinicaeni* sp. nov. · 16S rRNA gene sequence · Novel species · Polyphasic taxonomy

Abbreviations

MA	Marine agar 2216
MB	Marine broth 2216
MEGA	Molecular Evolutionary Genetics Analysis
HPLC	High Performance Liquid Chromatography
HEPES	N-(2Hydroxyethyl)Piperazine-N'-2-Ethanesulfonic Acid
CAPSO	3-Cyclohexylamino-2-Hydroxypropanesulfonic Acid Sodium Salt
MES	2-(N-Morpholino) ethane-sulfonic acid
PIPES	Piperazine-1,4-Bisethanesulfonic Acid
Tricine	N-[Tris(Hhydroxymethyl)Methyl] Glycine
KCTC	Korean Collection for Type Cultures
JCM	Japan Collection of Microorganisms
MCCC	Marine Culture Collection of China
GHs	Glycoside Hydrolases
GTs	Glycosyl Transferases
CBM	Carbohydrate-Binding Modules
ANI	Average Nucleotide Identity
AAI	Average Amino Acid identity
dDDH	digital DNA-DNA Hybridization

Introduction

In 2006, the genus *Echinicola* was first established (Nedashkovskaya et al. 2006), which belonged to the family *Cyclobacteriaceae* in the phylum *Bacteroidetes*. Presently,

the genus *Echinicola* comprises seven species, which were motile by gliding, rod-shaped, heterotrophic, with non-diffusible carotenoid pigments, strictly aerobic bacteria with menaquinone-7 (MK-7) as major respiratory quinone, iso-$C_{15:0}$ and summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$) as major cellular fatty acids and the DNA G + C content ranging between 43.8 and 46.9 mol% (Jung et al. 2017; Kim et al. 2011; Lee et al. 2017; Liang et al. 2016; Nedashkovskaya et al. 2007; Nedashkovskaya et al. 2006; Srinivas et al. 2012; Xing et al. 2020). Species of the genus *Echinicola* were isolated from different marine habitats such as brackish water pond, coastal sediment and the sea urchin *Strongylocentrotus intermedius* (Lee et al. 2017; Nedashkovskaya et al. 2006; Srinivas et al. 2012). For the first time, we isolated a novel strain P51^T from saline silt, which showed high similarity to other strains of genus *Echinicola*. In this study, the novel strain was *Echinicola salinicaeni*, described by a polyphasic approach (Vandamme et al. 1996).

Materials and methods

Isolation of strain P51^T

Strain P51^T was isolated from silt samples taking from Muping saltern in Yantai, Shandong Province, China (121°40'20" E, 37°26'39" N). The silt samples were diluted with sterile water. The dilution was then inoculated on marine agar 2216 (MA, Becton Dickinson). After incubation at 30 °C for 2 days, pink colonies were obtained and designated as strain P51^T. Subcultivation was performed routinely on MA at 30 °C. The type strains, '*Echinicola shivajiensis*' JCM 17847^T, *Echinicola sediminis* KCTC 52495^T, *Echinicola pacifica* KCTC 12368^T, *Echinicola vietnamensis* MCCC 1K04353^T, *Echinicola*

jeungdonensis KCTC 23122^{T} were purchased from their respective collection institutions. All strains were cultured under comparable conditions for physiological and chemotaxonomic characterizations, unless otherwise specified. They were preserved at −80 °C in sterile seawater supplemented with 15.0% (v/v) glycerol.

Phylogenetic analysis of 16S rRNA gene and whole genome sequencing

The 16S rRNA gene was amplified by PCR technique using universal primers, 27F (5’ - AGA GTT TGA TCM TGG CTC AG -3’) and 1492R (5’ - TAC CTT GTT ACG ACT T -3’)(Kim et al. 2014). Comparison of 16S rRNA gene sequences was carried out using NCBI BLAST and the EzBioCloud (Yoon et al. 2017). Phylogenetic trees were inferred using neighbour-joining (NJ), maximum-likelihood (ML) and maximum-parsimony (MP) trees carried out in MEGA version 7.0 (Kumar et al. 2016). The robustness of the topologies for the trees was evaluated by bootstrap analysis based on 1000 resamplings.

The draft genome sequence was sequenced on the Illumina HiSeq-PE150 platform. The sequenced reads were assembled using SOAP, Spades, and Abyss *De novo* software. The annotation of genome sequence was processed using the GeneMarkS, rRNAmmer, and Rfam software. These operations were all implemented by Beijing Novogene Bioinformatics Technology Co, Ltd. (Beijing, China). The genes involved in metabolic pathways were analysed in detail by using information from the Kyoto Encyclopedia of Genes and Genomes (KEGG) databases (Kanehisa et al. 2016). Protein-encoding regions were performed with the Rapid Annotation using Subsystem Technology (RAST) server (http://rast.nmpdr.org/rast.cgi) (Aziz et al. 2008) and the

UniProtKB/Swiss-Prot (Consortium 2019). The presence of gene clusters encoding secondary metabolites was predicted by using the antiSMASH 5.0 database (Blin et al. 2019).

To further clarify the genetic relatedness between strain P51^T and related species of the genus *Echinicola*, the Average Nucleotide Identity (ANI) was calculated according to the online ANI Calculator (www.ezbiocloud.net/tools/ani)(Yoon et al. 2017). The Average Amino Acid identity (AAI) and digital DNA-DNA Hybridization (dDDH) were compared online by using (http://enve-omics.ce.gatech.edu/aai/) (Rodríguez-R and Konstantinidis 2014) and the Genome-to-Genome Distance Calculator (GGDC2.1)(http://ggdc.dsmz.de)(Meier-Kolthoff et al. 2013) respectively.

The DNA G + C content of strain P51^T was determined from the genome sequence. In this study, we have obtained and submitted the draft genome sequence of strain P51^T, the GenBank/EMBL/DDBJ accession number for the whole genome sequence is JACODZ000000000. Genomic data of other type strains within the genus *Echinicola* were obtained from the GenBank/ENA/DDBJ databanks.

Physiology and Chemotaxonomy

Gram staining was performed as described earlier (Moyes et al. 2009). After incubating the bacterium on MA at 37 °C for 2 days, cell morphology and flagella were observed using an electron microscope (Jem-1200; JEOL). To investigate growth under anaerobic conditions, the strain was cultured on MA with or without 0.1% (w/v) $NaNO_3$ under anaerobic conditions at 37 °C for 2 weeks.

Growth at 4, 15, 20, 25, 28, 33, 37, 40, 42, 45 and 50 °C were determined using MA.

The effect of NaCl on growth was tested in NaCl-free artificial seawater medium supplemented with 5.0 g peptone, 1.0 g yeast extract, and various concentrations of NaCl (final concentration 0–15.0%, in increments of 0.5%)(Yang and Cho 2008). The pH range for growth was determined in MB adjusted to pH 5.5–9.5 with a concentration of 20 mM using the following buffer systems: MES (pH 5.5 and 6.0), PIPES (pH 6.5 and 7.0), HEPES (pH 7.5 and 8.0), Tricine (pH 8.5), and CAPSO (pH 9.0 and 9.5). According to the Bowman's method (Bowman 2000), gliding motility was investigated using oil-immersion phase-contrast microscopy (AX70; Olympus). The activities of catalase and oxidase, hydrolysis of cellulose and agar, were tested according to the method described before (Tindall et al. 2007). Antibiotic sensitivity was assessed on MA plates with discs (Tianhe) containing various antibiotics for 3 days at 37 °C. According to the manufacturers' instructions, the oxidation and fermentation of carbohydrates were determined after growth on MA at 33 °C for 2 days using the Biolog GEN III Micro Plates and API 50CHB Fermentation Kit (bioMérieux). Other physiological tests were carried out using API 20E, API 20NE, and API ZYM strips (bioMérieux).

Chemotaxonomic characterisation

The bacteria of strain P51^T at the late stage of the exponential growth phase were collected and lyophilized to obtain freeze-dried cells. The five related type strains were both treated the same way. For fatty acid analysis, strain P51^T and related strains were incubated in MB at 33 °C until the mid-exponential phase. Under identical conditions, fatty acid methyl esters were extracted and analysed according to the

142 standard MIDI (Microbial Identification) system (Sasser 1990) and identified by the
143 TSBA6.0 database. For the analysis of respiratory quinone and polar lipids, the strain
144 was harvested after incubation in MB at 33 °C for 3 days. Respiratory quinones in
145 strain P51^T were determined with HPLC (Hiraishi et al. 1996). Polar lipid analysis
146 was performed by the Marine Culture Collection of China (MCCC), Xiamen, Fujian
147 Province, P. R. China.

148 Results and discussion

149 Phylogenetic analysis and whole genome sequencing

150 The nearly complete 16S rRNA gene sequence (1444 bp) was measured. 16S rRNA
151 gene sequence comparisons showed that strain P51^T was most closely related to
152 members of the genus *Echinicola*, with 16S rRNA gene sequence similarities of
153 97.7% with '*E. shivajiensis*' AK12^T and 97.0% with *E. sediminis* 001-Na2^T. The
154 results of the phylogenetic analysis based on 16S rRNA gene sequences showed that
155 strain P51^T fell within the cluster comprising the genus *Echinicola* (Fig. 1). Similar
156 tree topologies were also obtained with the ML algorithms (Fig. S1).
157 The genome of strain P51^T was comprised of 4111 genes and 25 contigs with a total
158 length of 5139782 bp. The N50 value was 513723 bp. There were 5S rRNAs of 6, 16S
159 rRNA of 2, 23S rRNAs of 2 and tRNAs of 43. According to the genome sequence, the
160 DNA G + C content was 39.9 mol%, which was outside the range of existing species
161 of genus *Echinicola* (Kim et al. 2011; Liang et al. 2016)(43.8-46.9 mol%). However, it
162 was similar to that of unpublished species submitted in the NCBI, such as 39.8 mol%
163 at *Echinicola* sp. CAU 1574 (JACYTQ000000000) and 39.9 mol% at *Echinicola* sp.

20G (PRJDB10725). The KEGG pathway annotation annotated genes and most genes related to global and overview maps. A large number of genes were found to be involved in carbohydrate metabolism(194) and amino acid metabolism(171). Result of further genome sequences analysis showed that strain P51^T harboured pathway for carotenoid biosynthesis (Fig. S4), which may associated with the colour pigmented in the bacteria colony (Paniagua-Michel et al. 2012). Based on the genome sequence annotation, the genome contained several genes coding for Glycoside Hydrolases (GHs), Glycosyl Transferases (GTs) and Carbohydrate-Binding Modules (CBMs), such as fucosidase (14 GH29), mannosidase (13 GH2), cellulose synthase (25 GT2) and sucrose synthase (27 GT4), which indicated that the strain played an important role in the carbon cycle. The secondary metabolite of strain P51^T were predicted to terpene and bacteriocin, which were considered as important raw materials for drug synthesis. Genome mining analysis using antiSMASH-4.0.2 led to the identification of 2 putative gene clusters in the genome of strain P51^T, including 23 genes that showed similarity to the known gene clusters of terpene and 10 genes that showed similarity to bacteriocin. Hence, it had great potential to biosynthesize secondary metabolite and produce novel bioactive compounds. One cluster showed 75% similarity to the reported biosynthetic gene cluster of terpene in *Anditalea andensis* ANESC-S^T (Shi et al. 2012).

A complete 16S rRNA gene sequence (1444 bp) obtained from the genome sequence was 100.0% similar with the clone sequence (2665 bp and 1700 bp) deposited in GenBank under accession number MW110902.According to the data obtained, the ANI between strain P51^T and the other species tested were no more than 84.7%, which were

below the standard ANI criteria identity (95.0-96.0%)(Kim et al. 2014). The dDDH comparison with the draft genome of strain P51^T yielded low percentages (<28.2%) with all the species tested, which were far below the boundary (70.0%) for new species identification (Meier-Kolthoff et al. 2013). The AAI values were between 70.8–74.0% with the closely related type species. All the data were showed in Table. S1. Based on the genome sequence analysis, strain P51^T should be located within the genus *Echinicola* as a novel species.

Morphology, physiology, and biochemical analysis

Cells were Gram-stain-positive, aerobiotic, motile, gliding, lacking flagella, and rod-shaped (0.3–0.4 μm in width, 1.5–2.8 μm in length) (Fig. S2). A round pink colony with a diameter of 1.5 mm was obtained after incubating on MA for 2–3 days at 33 °C. Catalase-positive and oxidase-negative. Growth was observed at 4–42 °C (optimum 33 °C), pH 4.0–9.0 (optimum 7.0). It could grow at 4 °C, which was different from related strains. The NaCl concentrations for growth were 0–11.0% (optimum 4.0%). Susceptible to penicillin, cefazolin, erythromycin, norfloxacin, ciprofloxacin, trimethoprim-sulfamethoxazole, chloramphenicol. Resistant to ampicillin, amikacin, gentamicin. The resistance to ampicillin was consistent with genome analysis about *β*-lactam resistance in KEGG pathway annotation (Fernández and Hancock 2012) (Fig. S5). Other phenotypic characteristics of the strain P51^T and related strains are shown in Table 1.

Chemotaxonomic characterization

The main fatty acids (>5.0%) of strain P51^T were iso-$C_{15:0}$ (34.5%), summed feature 3

208 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$) (12.7%), summed feature 4 (anteiso-$C_{17:1}$ B and/or iso-$C_{17:1}$
209 I) (5.9%) and summed feature 9 (iso-$C_{17:1}\omega9c$ and/or 10-methyl $C_{16:0}$) (8.5%). Fatty
210 acid profiles of the strain P51^T and related strains were shown in Table 2. The sole
211 menaquinone of strain P51^T was MK-7, which was consistent with other members of
212 the genus *Echinicola* (Kim et al. 2011; Lee et al. 2017; Nedashkovskaya et al. 2007;
213 Nedashkovskaya et al. 2006; Srinivas et al. 2012). The polar lipids included one
214 phosphatidylethanolamine, one unidentified aminophospholipid, one unidentified
215 phospholipid, three unidentified aminolipids and one unknown lipid (Fig. S3). Species
216 in the genus *Echinicola* also had the same polar lipids as phosphatidylethanolamine and
217 one unidentified aminophospholipid. Besides they were different in the number of
218 unidentified lipids from '*E. shivajiensis*' AK12^T (six)(Srinivas et al. 2012) and *E.*
219 *sediminis* 001-Na2^T (three)(Lee et al. 2017), which was the distinction between strain
220 P51^T and the related strains.
221 In conclusion, the phenotypic properties and phylogenetic analyses suggest that the
222 strain P51^T can be differentiated from other species of the genus *Echinicola,* for which
223 the name *Echinicola salinicaeni* is proposed.
224 **DESCRIPTION OF *ECHINICOLA SALINICAENI* SP. NOV.**
225 *Echinicola salinicaeni* (sa.li.ni.cae'ni. N.L. masc. adj. *salinus* saline; L. neut. n. *caenu*
226 *m* mud, silt; N.L. gen. n. *salinicaeni* of saline silt).
227 Cells are Gram-stain-positive, aerobiotic, motile, gliding, and rod-shaped. Colonies on
228 MA are convex, circular, smooth and pink with entire margins. Cells are 0.3–0.4 μm
229 in width and 1.5–2.8 μm in length after incubating on MA at 37 °C for 2 days. Growth

is observed at 4–42 °C (optimum 33 °C) at pH 4.0–9.0 (optimum 7.0), with 0–11.0% NaCl (optimum 4.0%). Catalase and oxidase are positive. Cells cannot hydrolyse alginate, cellulose and agar. Positive for alkaline phosphatase, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, *α*-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, *α*- and *β*-galactosidases, *α*- and *β*-glucosidases and N-acetyl-*β*-glucosaminidase, *α*-mannosidase and *α*-fucosidase are present, but esterase (C4), esterase lipase (C8), lipase (C14) and *β*-glucuronidase are absent. Carbon source oxidation tests are negative for 3-methyl-D-glucoside, D-fucose, inosine, fusidic acid, D-sorbitol, D-mannitol, D-arabitol, D-aspartic-acid, D-serine, troleandomycin, minocycline, L-arginine, L-histidine, L-pyroglutamic acid, lincomycin, niaproof 4, D-gluconic acid, mucic acid, quinic acid, D-saccharic acid, tetrazolium violet, *p*-hydroxyphenylacetic acid, D-lactic acid methyl ester, citric acid, *α*-ketoglutaric acid, D-malic acid, bromosuccinic acid, nalidixic acid, potassium tellurite, *γ*-amino-butryric acid, *α*-hydroxy-butyric acid, *β*-hydroxy-D,L-butyric acid, *α*-keto-butyric acid, acetoacetic acid, acetic acid, sodium butyrate and sodium bromate while positive for all other tested carbon sources. Acid is produced from L-arabinose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, methyl-*α*-D-mannopyranoside, methyl-*α*-D-glucopyranoside, N-acetylglucosamine, arbutin, esculin, D-cellobiose, D-maltose, D-lactose, sucrose, trehalose, inulin, raffinose, turanose and L-fucose, but not from glycerol, erythritol, L-xylose, D-adonitol, dulcitol, inositol, D-sorbitol, glycogen, xylitol, D-lyxose, D-tagatose, D-fucose, D/L-arabitol, potassium gluconate, potassium 2-ketogluconate and potassium 5-ketogluconate. In

the API 20NE tests, strain P51^T is positive for D-glucose fermentation, hydrolysis of esculin and 4-nitrophenyl-*β*-D-galactopyranoside, but negative for nitrate reduction which corresponding to the genomic analysis, indole production, arginine dihydrolase, hydrolysis of gelatin and urea. Additionally, strain P51^T is negative for assimilation of L-arabinose, D-mannose, D-mannitol, N-acetylglucosamine, D-maltose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate and phenylacetic acid. The cells are positive for *β*-galactosidase and glucose acidification, but negative for gelatin, arginine dehydratase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, H_2S production, urease, indole production, tryptophan deaminase.

The type strain, P51^T = (KCTC 82513^T = MCCC 1K04413^T) was isolated from silt samples in Muping saltern in Yantai, Shandong Province, People's Republic of China. The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain P51^T is MW110902 and the number for the whole genome sequence is JACODZ000000000.

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Declarations

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Conflicts of interest

Authors declare that there is no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Data availability statement

All data generated or analysed during this study are included in this published article, its supplementary information files and GenBank/EMBL/DDBJ. The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain P51^{T} is MW110902 and the number for the whole genome sequence is JACODZ000000000. Supplementary figures and Supplementary tables are available with the online version of this paper.

Authors' contributions

HZ drafted the manuscript. CYS, HZ, YJY, and YXZ performed isolation, deposition and identification. RY, HZ and STY performed genome analysis. YXL revised the manuscript. YXZ designed all the experiments and supervised the manuscript.

Figures

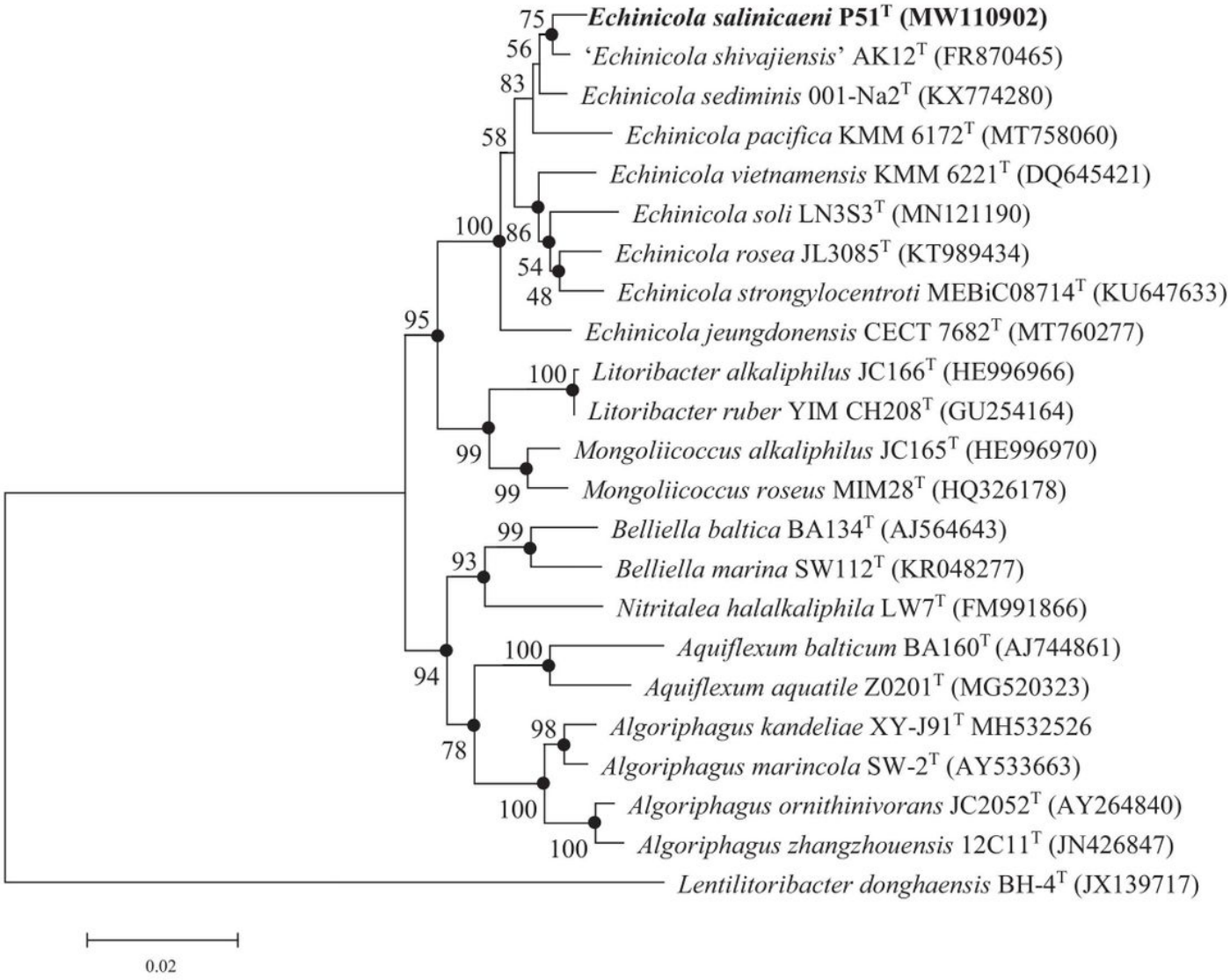

Figure 1

Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences of strain P51T and high similarity of genus. Bootstrap values (>50%) based on 1000 resamplings are shown at branch nodes. Filled circles indicate that the corresponding nodes were recovered in trees generated by the neighbour-joining, maximum-likelihood methods. Lentilitoribacter donghaensis BH-4T was used as an outgroup. Bar, 0.02 substitutions per nucleotide position.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- 4P51SupplementaryMaterials.pdf