

***Echinicola shivajiensis* sp. nov., a novel bacterium of the family "Cyclobacteriaceae" isolated from brackish water pond**

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Abstract

Strain AK12^T, an orange pigmented Gram-negative, rod shaped, non-motile bacterium, was isolated from a mud sample collected from a brackish water pond at Rampur of West Bengal, India. Strain was positive for oxidase, catalase and phosphatase. The predominant fatty acids were iso-C_{15:0} (42.7%), iso-C_{17:0} 3OH (13.2%), C_{16:1 ω7c}/C_{16:1 ω6c} (summed Feature 3) (8.0%), iso-C_{17:1 I}/anteiso-C_{17:1 B} (summed Feature 4) (6.1%) and iso-C_{17:1 ω9c}/C_{16:0} 10-methyl (summed Feature 9) (9.4%). Strain AK12^T contained MK-7 as the major respiratory quinone and phosphatidylethanolamine, one unidentified aminophospholipid and six unidentified lipids as the polar lipids. The G + C content of DNA of the strain AK12^T was 46.2 mol%. The 16S rRNA gene sequence analysis indicated that strain AK12^T was member of the genus *Echinicola* and closely related to *Echinicola vietnamensis*, *Echinicola pacifica* and *Echinicola jeungdonensis* with pair-wise sequence similarity of 96.8, 96.3 and 96.0% respectively. Phylogenetic analyses indicated that the strain AK12^T clustered with *Echinicola vietnamensis* and together with *Echinicola pacifica* and *Echinicola jeungdonensis* with a phylogenetic distance of 5.1, 6.3 and 6.6% (94.9, 93.7 and 93.4% similarity) respectively. Based on data from the current polyphasic study, strain AK12^T is proposed as a novel species of the genus *Echinicola*, for which the name *Echinicola shivajiensis* sp. nov. is proposed. The type strain of *Echinicola shivajiensis* is AK12^T (= MTCC 11083^T = JCM 17847^T).

Key words: *Echinicola shivajiensis*; 16S rRNA gene based phylogeny; *Bacteroidetes*.

Introduction

The genus *Echinicola* comprises Gram-negative, motile by gliding, rod-shaped, with non-diffusible carotenoid pigments, chemoorganoheterotrophic, strictly aerobic bacteria with MK-7 as major respiratory quinone, iso-C_{15:0}, C_{16:1 ω5c}, iso-C_{17:1 ω9c}, C_{17:1 ω6c}, iso-C_{17:0} 3-OH and summed feature 3, comprising iso-C_{15:0} 2-OH and/or C_{16:1 ω7c} as main cellular fatty acids and the DNA G + C content ranging between 44-46.9 mol%. Species of the genus *Echinicola* were isolated from different marine habitats like from sea urchin *Strongylocentrotus intermedius*, seawater collected in a mussel farm and from a solar saltern (Nedashkovskaya et al. 2006; Nedashkovskaya et al. 2007; Kim et al. 2010). In the present study we focused on the characterization and classification of the strain AK12^T, which was isolated from brackish water pond, by polyphasic approach (Vandamme et al. 1996). The strain is affiliated to the genus *Echinicola* and is proposed as a novel species, *Echinicola shivajiensis* sp. nov.

Materials and Methods

Isolation and growth

Strain was isolated from a mud sample collected from a brackish water pond at Rampur of West Bengal, India on 26th March, 2011. The sample that yielded strain AK12^T had a pH of 7.5. For isolation of bacteria, 1 ml of the mud sample was serially diluted in 1% saline water and 100 µl was then plated on ZoBell marine agar medium (Zobell, 1941) and incubated at room temperature for 15 days. Out of the different morphotypes obtained one pale-orange colony was selected and characterized in the present study. Sub-cultivation of the isolate was carried out on marine agar medium at 30°C. Stock culture of the isolate in marine broth with 10% glycerol was preserved at -80°C.

Type strains used for comparative study

Echinicola vietnamensis LMG 23754^T and *Echinicola pacifica* LMG 23350^T were procured from LMG bacteria collection, Belgium; strain AK12^T was characterized simultaneously with *Echinicola vietnamensis* LMG 23754^T and *Echinicola pacifica* LMG 23350^T.

Morphological characteristics

Colony morphology was examined following growth of the strain on MA at 30 °C for 48 h. Cell morphology and motility were observed by using phase contrast microscopy. Motility was also assessed on Motility-Indole-Lysine HiVegTM medium (cat. no. MV847; HIMEDIA) with agar 2 g l⁻¹.

Physiological and biochemical characteristics

Growth was tested on MA (HiMedia), Tryptone Soya Agar (TSA; HiMedia) and Nutrient Agar (NA; HiMedia). Growth at 4, 10, 18, 30, 37 and 40°C were ascertained using ZoBell marine agar medium and salt tolerance [0 (without NaCl), 1, 2, 3, 4, 5, 6, 8 and 10% (w/v) NaCl] was ascertained using nutrient agar (NA) medium containing (l⁻¹) peptone (5 g), beef extract (3 g) and agar (20 g). Growth of strain AK12^T at pH 5, 6, 7, 7.5, 8, 8.5, 9, 9.5, 10, 11 and 12 was assessed on TSA buffered with citric acid/NaOH (for pH 5 and 6), NaHPO₄/Na₂HPO₄ (for pH 7 and 8), glycine/NaOH (for pH 9 and 10) or Tris-HCl or NaOH (for pH 11 and 12). Biochemical characteristics such as activity of oxidase, catalase, lysine decarboxylase, ornithine decarboxylase, nitrate reduction, hydrolysis of aesculin, gelatin, ONPG,

starch, Tween 40, carbon source assimilation, H₂S production and the sensitivity to 24 different antibiotics using the disc diffusion method with commercially available discs (HiMedia) were determined by previously described methods (Lányi, 1987; Smibert and Krieg, 1994). Anaerobic growth was assessed by streaking the cultures on anaerobic agar (HiMedia) slants and flushing with N₂ to expel all the air. Biochemical and enzymatic characteristics were also assessed using Vitek 2 GN (bioMérieux) with incubation at 30 °C, according to the manufacturer's protocol.

Chemotaxonomic analysis

Fatty acid methyl esters were prepared (classical method) and analyzed using the Sherlock Microbial Identification System (MIDI-6890 with database TSBA6) according to the protocol described by Sherlock Microbial Identification System. For this purpose, strain AK12^T, *Echinicola vietnamensis* LMG 23754^T and *Echinicola pacifica* LMG 23350^T were grown on MA at 30°C for two days. Polar lipids were extracted and analyzed according to the method described by Komagata and Suzuki (1987). Menaquinones and polar lipids were determined in freeze-dried cells. Menaquinones were extracted as described by Collins et al. (1977) and were analyzed by HPLC (Groth et al. 1997).

Determination of DNA G + C content

The DNA of strain AK12^T was isolated according to the procedure of Marmur (1961) and the G+C content was determined from melting point (*T_m*) curves (Sly et al. 1986) obtained by using a Lambda 2 UV-Vis spectrophotometer (Perkin Elmer) equipped with the Templab 2.0 software package (Perkin Elmer). *Escherichia coli* strain DH5- α DNA was used as a standard in determining the DNA G + C content.

16S rRNA gene sequence determination and phylogenetic analysis

For 16S rRNA gene sequencing, DNA was prepared using a microbial DNA isolation kit (Mo Bio Laboratories Inc.) and sequenced as described previously (Lane, 1991). The resultant sequence of the 16S rRNA gene (1409 nt) was subjected to BLAST sequence similarity searches (Altschul et al. 1990) and the EzTaxon server (Chun et al. 2007) were used to identify the nearest taxa. All the 16S rRNA gene sequences of members of the family “*Cyclobacteriaceae*” were downloaded from the NCBI database (<http://www.ncbi.nlm.nih.gov>) and aligned using the CLUSTAL_X program (Thompson et al. 1997) and the alignment was then corrected manually. Phylogenetic tree was constructed using the

maximum-likelihood method using the PhyML program (Guindon et al. 2005). The topologies of the maximum-likelihood tree were evaluated based on 100 resamplings.

Results and Discussion

Cells of strain AK12^T were Gram-negative, non-motile, rod shaped 0.5-1.0 µm wide and 1.5-2.5 µm long (Supplementary Fig. S1) and multiply by binary fission. Colonies were circular, 1-3 mm in diameter, smooth, shiny, orange, translucent and raised with entire margin on marine agar. The strain was positive for oxidase, catalase, phosphatase and arginine dihydrolase activities and negative for lysine decarboxylase, ornithine decarboxylase and phenylalanine deaminase activities. Strain AK12^T reduced nitrate. Strain AK12^T hydrolysed aesculin but not gelatin, Tween 40, cellulose, casein, starch and urea. It was negative for indole production. Strain AK12^T was negative for methyl red and Voges Proskauer's reactions and H₂S was not produced. Growth was observed at 5-40 °C with optimum growth at 30-37 °C, at 0.5-12% (w/v) NaCl, with optimum growth at 1-2%, and at pH 6-12, with optimum growth at pH 7.0-8.5. Other characteristics are presented in Table 1 and also listed in the species description.

The cellular fatty acid composition of strain AK12^T showed a spectrum of 26 fatty acids with a pronounced dominance of (>5%) of iso-C_{15:0}, iso-C_{17:0} 3OH, C_{16:1 ω7c}/C_{16:1 ω6c} (summed Feature 3), iso-C_{17:1 I}/anteiso-C_{17:1 B} (summed Feature 4) and iso-C_{17:1 ω9c}/C_{16:0} 10-methyl (summed Feature 9) (Table 2). Saturated fatty acids constituted 67.2% of the total fatty acids. Strain *Echinicola vietnamensis* LMG 23754^T also comprises 26 fatty acids but the composition differed considerably with strain AK12^T. Compared with *Echinicola pacifica* LMG 23350^T the number of fatty acids detected in strain AK12^T were less and the composition differed considerably (Table 2). The menaquinone present was MK-7 and the major polar lipids consisted of phosphatidylethanolamine, one unidentified aminophospholipid and six unidentified lipids (Supplementary Fig. S2). The G + C content of DNA of the strain AK12^T was 46.2 ± 0.8 mol%.

The 16S rRNA gene analysis placed strain AK12^T within the genus *Echinicola*. The results indicated that at the 16S rRNA gene sequence level strain AK12^T was close to the three phylogenetic neighbors, *Echinicola vietnamensis*, *Echinicola pacifica* and *Echinicola jeungdonensis* with a pair-wise sequence similarity of 96.8, 96.3 and 96.0% respectively. Phylogenetic tree obtained using the maximum-likelihood (Fig. 1) method revealed clustering of the strain AK12^T with *Echinicola*

vietnamensis and together with *Echinicola pacifica* and *Echinicola jeungdonensis* with a phylogenetic distance of 5.1, 6.3 and 6.6% (94.9, 93.7 and 93.4% similarity) respectively.

Based on the phylogenetic analysis a comparison was made between the characteristics of strain AK12^T with the type strains of the genus *Echinicola*, (*Echinicola pacifica* and *Echinicola vietnamensis*) of the family “*Cyclobacteriaceae*” (Tables 1 and 2) and a number of differences were observed. For instance, strain AK12^T differed from both strains with respect to colony color, cell size, salt tolerance, biochemical activities, hydrolysis of complex substrates, different carbon substrate utilization, G + C content, fatty acid and polar lipid composition (Tables 1 and 2). Thus, the cumulative differences that strain AK12^T exhibits with the above two closely related type strains unambiguously supports the creation of a new species of the genus *Echinicola* for which the name *Echinicola shivajiensis* sp. nov. is proposed.

Description of *Echinicola shivajiensis* sp. nov.

Echinicola shivajiensis (Shi.va.ji.en'sis. N. L. masc. adj. *shivajiensis* named after Dr. S. Shivaji, an eminent Indian microbiologist who has made a significant contribution to our knowledge of heterotrophic bacteria from diverse habitats).

Cells are Gram-negative, strictly aerobic, rod shaped, non-motile, 0.5-1.0 µm wide and 1.5-2.5 µm long. Colonies on marine agar are circular, 1-3 mm in diameter, smooth, shiny, orange, translucent and raised with entire margins. Grows at 5 to 40 °C with an optimum temperature of 30-37 °C and tolerates up to 12.0% (w/v) NaCl. Grows at pH 6 to 12 with an optimum pH of 7-8.5. Oxidase, catalase, arginine dihydrolase, Ala-Phe-Pro-arylamidase, β-galactosidase, β-N-acetylglucosaminidase, glutamyl arylamidase pNA, β-glucosidase, tyrosine arylamidase, L-pyrrolydonyl-arylamidase, L-proline arylamidase, β-N-acetylgalactosaminidase, α-glucosidase, α-galactosidase and phosphatase activities are present but lysine decarboxylase, ornithine decarboxylase, β-galactosidase, phenylalanine deaminase, γ-glutamyl-transferase, lipase, β-xylosidase, β-alanine arylamidase pNA, glycine arylamidase, β-glucuronidase and glu-gly-arg-arylamidase activities are absent. The methyl red and Voges-Proskauer reactions are negative. Nitrate is reduced. H₂S and indole are not produced. Negative for glucose fermentation, L-lactate and succinate alkalisation. Aesculin is hydrolysed but agar, casein, cellulose, DNA, gelatin, starch, Tween 20, 40, 60, 80 and urea are not hydrolysed. Acid is produced from fructose, cellobiose, glucose, melibiose, sucrose, galactose, sorbitol, trehalose and raffinose, but not from

arabinose, rhamnose, mannose, xylose, lactose, maltose, adonitol, dulcitol, inositol and mannitol. Assimilates fructose, cellobiose, glucose, melibiose, sucrose, galactose, sorbitol, trehalose and raffinose but not citrate, adonitol, L-arabitol, D-cellobiose, D-glucose, D-maltose, D-mannitol, D-mannose, palatinose, D-sorbitol, sucrose, D-tagatose, D-trehalose, citrate, malonate, 5-keto-D-gluconate, L-malate, L-lactate, L-histidine and courmarate. MK-7 is the predominant respiratory quinone and the major polar lipids consisted of phosphatidylethanolamine (PE), one unidentified aminophospholipid (AP) and six unidentified lipids (L). The G + C content of DNA of the strain AK12^T was 46.2 mol%.

The type strain AK12^T (= MTCC 11083^T = JCM 17847^T), was isolated from a mud sample collected from brackish water pond at Rampur of West Bengal, India.

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Legends to Figures

Fig. 1. Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences, showing the relationships between strain AK12^T and representatives of the genus *Echinicola*. Numbers at the nodes are bootstrap values >50%. *Parapedobacter soli* DCY14^T was used as an out group. Bar, 0.02 substitutions per nucleotide position.

Supplementary Fig. S1. Phase contrast photograph of cells of strain AK12^T. Bar, 5.0 μm.

Supplementary Fig. S2. Two-dimensional thin-layer chromatogram of polar lipids of the strains AK12^T (a), LMG 23350^T (b) and LMG 23754^T (c). Detection of total lipids by molybdotophosphoric acid. Abbreviations: PE, phosphatidylethanolamine; AL, unidentified aminolipid; AP, unidentified aminophospholipid; L, unidentified lipid.

Table 1. Phenotypic features that distinguish strain AK12^T from the closely related species of the genus *Echinicola*.

All the strains were rod-shaped and contain MK-7 as the respiratory quinone. All strains were positive for the following tests: oxidase, catalase, Ala-Phe-Pro-arylamidase, β -galactosidase, β -N-acetylglucosaminidase, glutamyl arylamidase pNA, β -glucosidase, tyrosine arylamidase, α -glucosidase, α -galactosidase and phosphatase activities. All strains were negative for the following tests: Voges Proskauer reaction, glucose fermentation, L-lactate and succinate alkalisation, H₂S and indole production; lipase, lysine decarboxylase, ornithine decarboxylase, γ -glutamyl-transferase, β -xylosidase, β -alanine arylamidase pNA, glycine arylamidase, β -glucuronidase, glu-gly-arg-arylamidase activities; hydrolysis of casein, cellulose, DNA, urea; acid production from adonitol, dulcitol, inositol and mannitol; and utilization of L-arabitol, D-cellobiose, D-glucose, D-maltose, D-mannitol, D-mannose, palatinose, D-sorbitol, sucrose, D-tagatose, D-trehalose, citrate, malonate, 5-keto-D-gluconate, L-malate, L-lactate, L-histidine and courmarate.

| Characteristic | <i>Echinicola shivajiensis</i> AK12 ^T | <i>Echinicola pacifica</i> LMG 23350 ^T | <i>Echinicola vietnamensis</i> LMG 23754 ^T |
|--|---|--|--|
| Colony colour | Orange | Pink | Light pink |
| Cell size (μ m) | 0.5-1.0 x 1.5-2.5 | 0.3-0.5 x 1.0-2.0 | 0.4-0.5 x 1.0-2.0 |
| Salinity growth range (%) | 0.5-12 | 0-12 | 0-15 |
| Temperature growth range ($^{\circ}$ C) | 5-40 | 6-41 | 6-44 |
| Temperature optimum ($^{\circ}$ C) | 30-37 | 25-28 | 30-32 |
| Biochemical: | | | |
| Citrate utilization | - | + | - |
| Nitrate reduction | + | - | - |
| H ₂ S production | - | + | - |
| Arginine dihydrolase | + | - | - |
| L-Pyrrolydonyl-arylamidase | + | - | - |
| L-Proline arylamidase | + | - | + |
| β -N-acetylgalactosaminidase | + | - | - |
| ELLMAN | + | + | - |
| Hydrolysis of: | | | |
| Aesculin | + | + | - |
| Agar | - | + | - |
| Gelatin | - | + | - |
| Starch | - | + | + |
| Tween 20/40/60/80 | - | -/+/-/- | -/+/-/- |
| Acid production from: | | | |
| Fructose | + | + | - |
| Arabinose | - | + | - |
| Cellobiose | + | + | - |
| Glucose | + | + | - |
| Rhamnose | - | + | - |
| Mannose | - | + | - |
| Melibiose | + | - | - |
| Sucrose | + | + | - |
| Galactose | + | + | - |
| Xylose | - | + | - |

| Characteristic | <i>Echinicola shivajiensis</i> AK12 ^T | <i>Echinicola pacifica</i> LMG 23350 ^T | <i>Echinicola vietnamensis</i> LMG 23754 ^T |
|--------------------------|---|--|---|
| Sorbitol | + | - | - |
| Lactose | - | + | - |
| Trehalose | + | + | - |
| Raffinose | + | - | - |
| Maltose | - | + | - |
| Major fatty acids (> 5%) | iso-C _{15:0} ; iso-C _{17:0} 3OH; Summed Feature 3 [#] ; Summed Feature 4 [#] and Summed Feature 9 [#] | iso-C _{15:0} ; C _{16:1} ω _{5c} ; iso-C _{17:0} 3OH; C _{17:1} ω _{6c} ; Summed Feature 3 [#] and Summed Feature 9 [#] | iso-C _{15:0} ; C _{16:1} ω _{5c} ; iso- C _{17:0} 3OH; Summed Feature 3 [#] ; Summed Feature 4 [#] and Summed Feature 9 [#] |
| Polar lipids | PE, AP, L1 to L6 | PE, AL1, L7 to L10, L12 to L14 | PE, AL1, L10, L11, L13 to L17 |
| DNA G + C content (mol%) | 46.2 | 44-45* | 45.9* |

[#]Summed features represent groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed Feature 3 contains C_{16:1} ω_{7c}/C_{16:1} ω_{6c}/C_{16:1} ω_{6c}/C_{16:1} ω_{7c}; Summed Feature 4 contains iso-C_{17:1} I/anteiso-C_{17:1} B; anteiso-C_{17:1} B/ iso-C_{17:1} I; Summed Feature 9 contains iso-C_{17:1} ω_{9c}/C_{16:0} 10-methyl. PE, phosphatidylethanolamine; AL, unidentified aminolipid; AP, unidentified aminophospholipid; L, unidentified lipid. * Data for DNA G + C content of strain *Echinicola pacifica* was from Nedashkovskaya et al. 2006 and of strain *Echinicola vietnamensis* was from Nedashkovskaya et al. 2007.

Table 2. Fatty acid profiles of strain AK12^T with the closely related species of the genus *Echinicola*

Data from the present study. All three strains were grown on MA plates at 30 °C for two days. Results are presented as a percentage of the total fatty acids. Fatty acids amounting to 5% or more of the total fatty acids are in bold. ND, not detected. Data in parentheses is from Nedashkovskaya et al. 2006; Nedashkovskaya et al. 2007.

| Fatty acid composition | <i>Echinicola shivajiensis</i> AK12 ^T | <i>Echinicola pacifica</i> LMG 23350 ^T | <i>Echinicola vietnamensis</i> LMG 23754 ^T |
|-----------------------------------|---|--|--|
| C _{15:0} | ND | ND (0.8-1.0) | ND (1.5) |
| C _{15:0} 3OH | ND | ND (2.5-2.6) | ND (ND) |
| iso-C _{15:0} | 42.7 | 20.0 (17.3-18.0) | 36.3 (20.0) |
| anteiso-C _{15:0} | 1.2 | 2.4 (2.1-2.8) | 3.1 (1.4) |
| iso-C _{15:0} 3OH | 2.9 | 2.8 (3.4-5.0) | 3.1 (3.7) |
| iso-C _{15:1} G | 1.1 | ND (ND) | ND (ND) |
| C _{15:1} ω _{6c} | 1.7 | 2.7 (1.1-1.2) | 0.9 (1.2) |
| C _{16:0} | 0.3 | 1.4 (0.8-0.9) | 0.6 (ND) |
| C _{16:0} 3OH | 1.3 | 1.5 (0.9-1.4) | 1.8 (2.3) |
| iso-C _{16:0} | 1.6 | 1.1 (1.1-1.2) | 1.5 (ND) |
| C _{16:1} ω _{5c} | 1.6 | 6.9 (6.7-7.8) | 7.07 (4.9) |
| C _{17:0} | ND | ND (ND) | ND (1.0) |
| iso-C _{17:0} | 1.2 | 0.6 (ND) | 0.8 (ND) |
| iso-C _{17:0} 3OH | 13.2 | 7.8 (9.4-10.0) | 9.5 (10.0) |
| C _{17:1} ω _{6c} | 3.3 | 7.4 (4.3-4.8) | 3.1 (4.5) |
| C _{17:1} ω _{8c} | 0.7 | 1.3 (ND) | 0.5 (ND) |
| Summed Feature 3 [#] | 8.0 | 24.9 (30.7-30.8) | 14.3 (34.5) |
| Summed Feature 4 [#] | 6.1 | 4.6 (ND) | 5.5 (5.0) |
| Summed Feature 9 [#] | 9.4 | 8.7 (6.3-6.9) | 8.3 (4.4) |

[#]Summed features represent groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed Feature 3 contains C_{16:1} ω_{7c}/C_{16:1} ω_{6c}/C_{16:1} ω_{6c}/C_{16:1} ω_{7c}; Summed Feature 4 contains iso-C_{17:1} I/anteiso-C_{17:1} B; anteiso-C_{17:1} B/ iso-C_{17:1} I; Summed Feature 9 contains iso-C_{17:1} ω_{9c}/C_{16:0} 10-methyl.

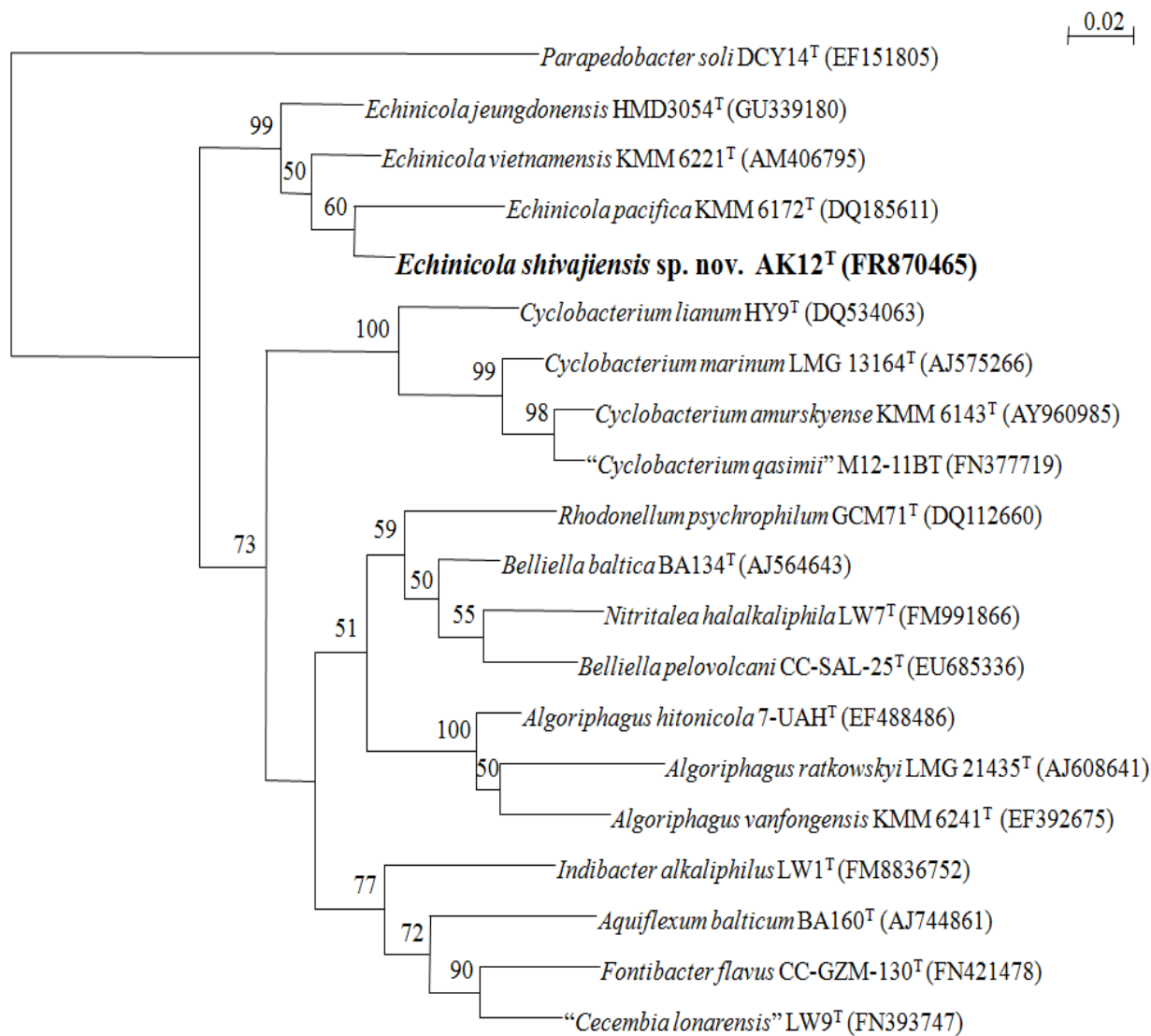
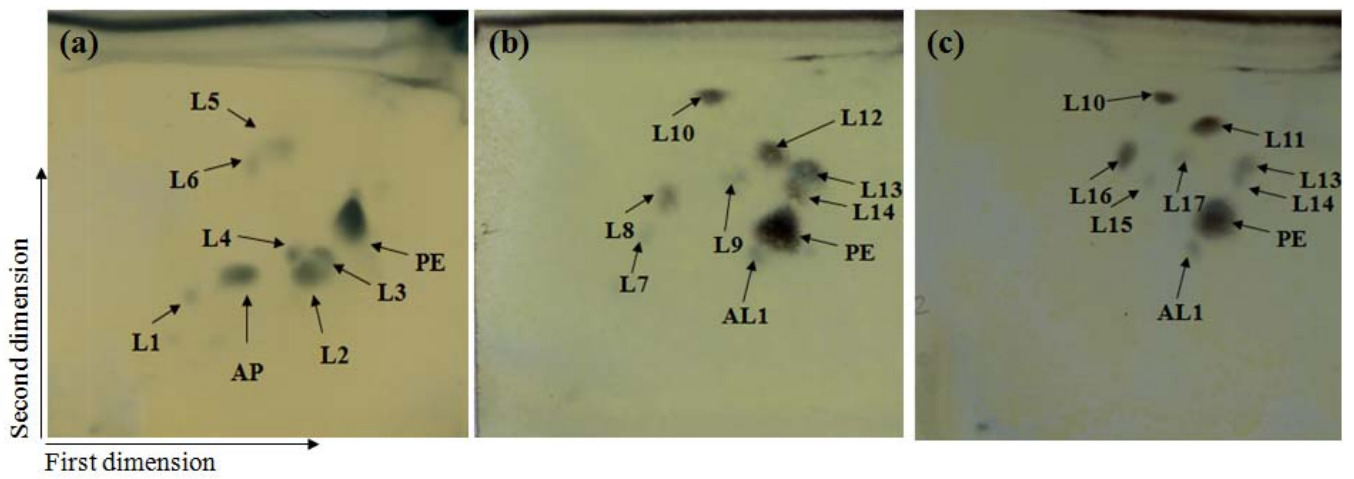


Fig.1



Supplementary Fig. S1



Supplementary Fig. S2