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***Marinilabilia nitratireducens* sp. nov., a lypolytic
bacterium of the family *Marinilabiliaceae* isolated
from marine solar saltern**

Authors **Shalley, S¹., Pradip Kumar, S¹., Srinivas, T. N. R²., Suresh, K¹., Anil
Kumar P*¹**

Institution ¹CSIR - Institute of Microbial Technology, MTCC-Microbial Type Culture
Collection & Gene Bank, Chandigarh-160036, India
²CSIR - National Institute of Oceanography, Regional Centre,
Visakhapatnam-530017, India

Address for correspondence* Dr. P. Anil Kumar
Microbial Type Culture Collection and Gene bank
Institute of Microbial Technology, Sector 39A,
Chandigarh – 160 036, INDIA
Email: apinnaka@imtech.res.in
Phone: +91-172-6665170

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A Gram-negative, rod shaped, motile bacterium, was isolated from a marine solar saltern sample collected from Kakinada, India. Strain AK2^T was positive for nitrate reduction, catalase, Ala-Phe-Pro-arylamidase, β -galactosidase, β -N-acetylglucosaminidase, β -glucosidase, β -xylosidase, α -glucosidase, α -galactosidase and phosphatase activities, hydrolysis of aesculin, Tween 20/40/60/80 and urea. It was negative for oxidase, lysine decarboxylase and ornithine decarboxylase activities and could not hydrolyze agar, casein, gelatin and starch. The predominant fatty acids were iso-C_{15:0} (28.2%), anteiso-C_{15:0} (23.2%), iso-C_{13:0} (19.9%) and iso-C_{15:0} 3-OH (13.9%). Strain AK2^T contained menaquinone with seven isoprene units (MK-7) as the sole respiratory quinone and phosphatidylethanolamine, one unidentified phospholipid and three unidentified lipids as polar lipids. The 16S rRNA gene sequence analysis indicated the strain AK2^T as a member of the genus *Marinilabilia* and closely related to *Marinilabilia salmonicolor* with pair-wise sequence similarity of 98.2%. Phylogenetic analysis of 16S rRNA gene revealed that the strain AK2^T clustered with *Marinilabilia salmonicolor*. However, DNA-DNA hybridization with *Marinilabilia salmonicolor* JCM 21150^T showed a relatedness of 48% with respect to strain AK2^T. The DNA G + C content of the strain was 40.2 mol%. Based on the phenotypic characteristics and phylogenetic inference, it is confirmed that the strain AK2^T represents a novel species of the genus *Marinilabilia*, for which the name *Marinilabilia nitratireducens* sp. nov. is proposed. The type strain of *Marinilabilia nitratireducens* sp. nov. is AK2^T (= MTCC 11402^T = JCM 17679^T).

Key words: *Marinilabilia nitratireducens* sp. nov., 16S rRNA gene based phylogeny, chemotaxonomy.

The recent reclassification of the phylum *Bacteroidetes* results creation of one more new class *Cytophagia* (earlier three classes *Bacteroidia*, *Flavobacteriia* and *Sphingobacteriia*) based on more robust phylogenetic analyses (Nakagawa, 2011). The family *Marinilabiliaceae* (Ludwig et al. 2012) created within the order *Bacteroidales*, class *Bacteroidia* comprises six genera *Alkaliflexus*, *Anaerophaga*, *Geofilum*, *Mangroviflexus*, *Marinilabilia* and *Natronoflexus* with type genus *Marinilabilia* (<http://www.bacterio.cict.fr/classifphyla.html#Bacteroidetes>). The genus *Marinilabilia* was established with the reclassification of species *Cytophaga agarovorans* (Veldkamp, 1961; Reichenbach, 1989) and *Cytophaga salmonicolor* (Veldkamp, 1961) to *Marinilabilia agarovorans* and *Marinilabilia salmonicolor* by Nakagawa and Yamasato, (1996) and later emended by Suzuki *et al.*

(1999). Members of the genus *Marinilabilia* are Gram-negative, slender, flexible, cylindrical rods with rounded or slightly tapering ends, without resting stages, motile by gliding, salmon to pink-pigmented, chemoorganotrophic growth exhibiting both respiratory and fermentative metabolisms, catalase positive. Usually they, require elevated salt concentration for growth, with optimum temperature between 28 to 37°C and pH 7. They are known to decompose several kinds of bio-macromolecules and complex nutrients. Spermidine and MK-7 are major polyamine and respiratory quinone and the DNA G+C content was found between 41.2-41.5 mol% (Veldkamp 1961; Reichenbach 1989; Nakagawa and Yamasato 1996; Suzuki et al. 1999). Both species *Marinilabilia agarovorans* and *Marinilabilia salmonicolor* can be differentiated based on their ability to degrade agar. However, genetic analysis (*gyrB* sequences, DNA-DNA hybridization and 16S rDNA sequences) led to the conclusion that *Marinilabilia agarovorans* is a later heterotypic synonym of *Marinilabilia salmonicolor* (Suzuki et al. 1999). In fact, based on the prominent agar-degrading ability of the type strain *Marinilabilia agarovorans* (ATCC 19043 = IFO (now NBRC) 14957), it was renamed as *Marinilabilia salmonicolor* biovar *Agarovorans* (Suzuki et al. 1999). In the present study made an attempt to characterize a *Marinilabilia* strain AK2^T isolated from a solar saltern using polyphasic approach and determined the taxonomic position as a new species of the genus *Marinilabilia* for which the name *Marinilabilia nitratireducens* sp. nov. is proposed.

Strain AK2^T was isolated from a marine solar saltern sample collected from Kakinada, India. Approximately 100 mg of the sediment sample was suspended in 900 ml of sterile normal saline and subjected to vigorous shaking for 2 h. The supernatant was serially diluted and 100 µl of each dilution was plated on ZoBell marine agar (ZMA) plates and incubated at 30 °C. A shiny, smooth, translucent, reddish-orange-pigmented colony was observed upon five days incubation and it was sub-cultured, tested for purity and preserved as -70 °C glycerol stock for further characterization.

Cell morphology studies of the strain AK2^T were done by phase contrast microscopy (Olympus) and also using electron microscopy after staining with 1% phosphotungstic acid and drying on carbon coated copper grids were observed on transmission electron microscope (TEM, JEM 2100). Flagellar motility was assessed on Motility-Indole-Lysine HiVegTM medium (HIMEDIA) with 2 g l⁻¹ of agar, by phase contrast microscopy and gliding motility was determined by previously described method (Bernardet et al. 2002). Growth of strain AK2^T was assessed (i) on MA, nutrient agar (NA; HIMEDIA) and Tryptone Soya agar (TSA; HIMEDIA); (ii) at 5, 10, 18, 25, 30, 35, 37, 42, 45, 50 and 55 °C in MB;

(iii) in the presence of 0, 1, 2, 3, 4, 5, 6, 8, 10, 15 and 20% (w/v) NaCl in nutrient broth initially devoid of NaCl; and (iv) at pH 4, 5, 6, 7, 7.5, 8, 8.5, 9, 10, 11 and 12 in MB buffered with acetate buffer (pH 4-6), 100 mM NaH₂PO₄/Na₂HPO₄ buffer (pH 7-8), 100 mM NaHCO₃/Na₂CO₃ buffer (pH 9-10) or 100 mM Na₂CO₃/NaOH buffer (pH 11-12) (Anil Kumar et al. 2010; Srinivas et al. 2011). Different biochemical tests listed in description of species and as well as table 1 were carried out as described by Lányí (1987) and Smibert & Krieg (1994) using the culture that was grown at 30 °C on ZMA medium. The sensitivity of strain to antibiotics was tested on ZMA medium using various antibiotic discs (HIMEDIA). Strain AK6^T was also tested in the Vitek 2 GN system (bioMérieux), according to the manufacturer's protocol, except that a 2.0 % (w/v) NaCl sterile solution was used to prepare the inoculum. Standardization of the physiological age of strains AK2^T and *Marinilabilia salmonicolor* JCM 21150^T was done based on the protocol (http://www.microbialid.com/PDF/TechNote_101.pdf) given by Sherlock Microbial Identification System (MIDI, USA). For cellular fatty acids analysis, strains AK2^T and *Marinilabilia salmonicolor* JCM 21150^T were grown on MA plates at 30 °C for four and two days respectively. Cellular fatty acid methyl esters (FAMES) were obtained from cells by saponification, methylation and extraction following the standard protocol of the Sherlock Microbial Identification System. Cellular FAMES were separated by Gas Chromatography (Agilent 6890 series GC system), identified and quantified with the Sherlock Microbial Identification System Software (version.6.0, using the aerobe TSBA6 method and the TSBA6 database). Polar lipids and respiratory quinones were analysed on freeze-dried cells. The polar lipids of strains AK2^T and *Marinilabilia salmonicolor* JCM 21150^T were extracted from cells grown on MA at 30 °C for 3 days under aerobic condition (Bligh & Dyer, 1959) and analyzed by two dimensional thin layer chromatography followed by spraying with appropriate detection reagents (Komagata & Suzuki, 1987). Isoprenoid quinones were extracted as described by Collins et al. (1977) and analyzed by HPLC (Groth et al. 1997). The solvent was acetonitrile:isopropanol (65:35) and the flow rate was 1 ml min⁻¹. Genomic DNA was isolated by using the procedure of Marmur (1961) and the mol% G + C content was determined from melting point (T_m) curves (Sly et al. 1986) using Lambda 35; Perkin Elmer spectrophotometer equipped with Templab 2.0 software package.

DNA isolation, 16S rRNA gene amplification and sequencing were done as described previously (Srinivas et al. 2011; Surendra et al. 2011). The 16S rRNA gene was sequenced using primers 530f and 907r (Johnson, 1994) in addition to the PCR primers and the dideoxy chain-termination method (Pandey et al. 2002). The 16S rRNA gene sequence of strain AK2^T was subjected to BLAST sequence similarity

search (Altschul et al. 1990) to identify the nearest taxa and the 16S rRNA gene sequences of nearest taxa belonging to families *Marinilabiliaceae* and *Porphyromonadaceae* were downloaded from the NCBI database (<http://www.ncbi.nlm.nih.gov>). The sequences were aligned using the CLUSTAL_W program in MEGA5 (Tamura et al. 2011) and the evolutionary history was inferred by using the maximum-likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated. There were a total of 1214 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al. 2011). Phylogenetic trees were also constructed using other tree making algorithms including neighbor-joining (NJ) method (Saitou & Nei, 1987) and maximum-parsimony method (Nei & Kumar, 2000) using the MEGA5 package (Tamura et al. 2011). The DNA-DNA hybridization was performed by the membrane filter method (Tourova & Antonov, 1987). Purified genomic DNA (10 µg) was taken in 6 X SSC buffer and boiled for ten minutes, and chilled immediately on ice and then immobilized onto a nylon membrane (Hybond- N from Amersham) using a dot-blot apparatus. The wells were then washed with 100 µl of 0.5 N NaOH after which the filter was removed and fixed in UV cross linker volt -1200, 2-5 min. The DNA probe of each strain was labelled with [α -³²P] ATP using nick translation kit (Genei, Bangalore). The UV cross linked nylon membrane was then soaked in pre-hybridization buffer for four hours and then pre-hybridization buffer was discarded and probe dissolved in pre-hybridization buffer was added and hybridization was performed for 16 hrs at 65 °C and the nylon membrane was washed with 0.5 X SSC and 0.1% w/v SDS for 10 min at 25 °C and then with 0.1 X SSC to remove any unbound probe and the membrane was exposed to BAS-MS 20/25 cm imaging plate (fuzi photo film, CO, Tokyo, Japan) for 1 hr and analyzed using Fuji film FLA 9000, autoradiogram analyzer.

Cells of the strain AK2^T were Gram-negative, slender long rods, motile by gliding, 0.2-0.3 x 20-25 µm (Fig. 1.). Colonies were circular, 1-2 mm in diameter, shiny, smooth, translucent, reddish-orange and raised with entire margin on ZMA plates at 37 °C. The strain grew between 25-42 °C temperature and optimum being 30-37 °C and from pH 7-12, with the optimum growth at pH 8.0. Optimum growth occurred between salinity 2-4% (NaCl, w/v) and tolerates up to 10% (NaCl, w/v). Other phenotypic characteristics of the strain AK2^T are listed in the species description and in Table 1.

The fatty acid profile was dominated by branched and hydroxy fatty acids, like iso-C_{15:0} (28.2%), anteiso-C_{15:0} (23.2%), iso-C_{13:0} (19.9%) and iso-C_{15:0} 3-OH (13.9%) (Table 2). Although the major fatty acid composition of strain AK2^T was found to be similar to the reference strain, it could be clearly distinguished from the reference strain by absence or low amount of iso-C_{12:0}, C_{15:0} 2OH and iso-C_{17:0} 3OH (Table 2). The MK-7 is the sole menaquinone present in AK2^T, which was in accordance with the type strain. The polar lipids of strain AK2^T consisted of phosphatidylethanolamine, one unidentified phospholipid and three unidentified lipids (Supplementary Fig. S3a). The polar lipids of *Marinilabilia salmonicolor* JCM 21150^T were phosphatidylethanolamine, one unidentified phospholipid and two unidentified lipids as in strain AK2^T and five unidentified phospholipids and one unidentified lipid (differed with AK2^T) (Supplementary Fig. S3b). DNA G + C content of the strain AK2^T was 40.2 mol% (T_m) (Table 1).

Phylogenetic analysis based on 16S rRNA gene sequences of strain AK2^T and other close relatives revealed that the strain AK2^T was close to the *Marinilabilia salmonicolor* with pair-wise sequence similarity of 98.2%. Phylogenetic trees constructed with maximum-likelihood, neighbor-joining and maximum-parsimony algorithms yielded a clade of the strain AK2^T with *Marinilabilia salmonicolor* and together clustered with the other members of the family *Marinilabiales* (Fig. 2, Supplementary Figures S1 and S2). However, DNA-DNA hybridization of strain AK2^T with *Marinilabilia salmonicolor* JCM 21150^T showed a relatedness of only 48%, which is lower than the proposed threshold value of 70% (Wayne et al. 1987), confirming that the strain AK2^T represents a novel species of the genus *Marinilabilia*.

The main features of the strain AK2^T are in line with the original and emended descriptions of the genus *Marinilabilia* but it could be distinguished from closely related species in phenotypic and genotypic characteristics (Tables 1 and 2). Thus, the cumulative differences that strain AK2^T exhibits with the closely related type strain unambiguously supports the creation of a new species of the genus *Marinilabilia* for which the name *Marinilabilia nitratireducens* sp. nov. is proposed.

Description of *Marinilabilia nitratireducens* sp. nov.

Marinilabilia nitratireducens (ni.tri.ti.re.du'cens. N.L. n. nitras -atis, nitrate; L. part. adj. reducens, leading back, bringing back and in chemistry converting to a different oxidation state; N.L. part. adj. *nitratireducens*, reducing nitrate).

Cells are Gram-negative, single, slender long rods (0.2-0.3 wide x 20-25 long μm) and exhibited gliding motility. Chemoorganoheterotrophic. Colonies on ZMA plates are circular, 1-2 mm in diameter, shiny, smooth, translucent, reddish-orange and raised with entire margin upon growing at 37 °C for four days. Grows in the temperature range 25-42 °C (optimum, 30-37 °C), and from pH 7-12 (optimum, 8). Optimum growth occurred at salinities from 2 to 4% (NaCl, w/v) and tolerates up to 10% (NaCl, w/v). NaCl is required for growth. Positive for catalase and negative for oxidase activities. In the VITEK GN ID card positive for, Ala-Phe-Pro-arylamidase, β -galactosidase, β -*N*-acetylglucosaminidase, β -glucosidase, β -xylosidase, α -glucosidase, α -galactosidase and phosphatase activities, and negative for lysine decarboxylase, ornithine decarboxylase, L-pyrrolydonyl-arylamidase, lipase, glutamyl arylamidase, γ -glutamyl-transferase, β -alanine arylamidase, L-proline arylamidase, tyrosine arylamidase, β -*N*-acetylgalactosaminidase, glycine arylamidase, β -glucuronidase and glu-gly-arg-arylamidase activities. Positive for aesculin, Tween 20/40/60/80 and urea hydrolysis and negative for agar, casein, gelatin, starch hydrolysis. It could not grow under anaerobic conditions. Nitrate reduction observed. No growth on MacConkey's agar, negative for H₂S and indole production, methyl red and Voges Proskauer's reactions, phenylalanine deamination. Acid production observed from melibiose and sorbitol but not from inositol, inulin, raffinose, cellubiose, mannose, rhamnose, galactose, salicin, trehalose and xylose, ELLMAN reaction is positive. Negative for fermentation glucose, L-lactate and succinate alkalisation, courmarate, 0/129 resistance; 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-histidine, L-malate and L-lactate utilization in VITEK GN ID card. Susceptible to antibiotics ($\mu\text{g}/\text{disc}$) amoxycillin (25), ampicillin (30), gentamicin (10), ceftazidime (30), spectinomycin (100), chloremphenicol (30), cephadroxil (30), novamycin (30), penicillin-G (2 units), bacitracin (8), ciproflaxin (10), vancomycin (30), tetracyclin (30), lincomycin (2) and chlorotetracyclin (30); resistant to polymixin B (300), kanamycin (30) and neomycin (30). The cellular fatty acids composition is as in Table 2. MK-7 is the sole respiratory quinone. The polar lipids are phosphatidylethanolamine, one unidentified phospholipid and three unidentified lipids. The G + C content of the genomic DNA is 40.2 mol%.

The type strain, AK2^T (= MTCC 11402^T = JCM 17679^T) was isolated from a marine solar saltern sample collected from Kakinada, India.

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Legends to Figures

Fig. 1. Electron micrograph of a negatively stained cell of strain AK2^T. Bar, 1 μ m.

Fig. 2. Maximum-likelihood phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships between strain AK2^T and closely related members of the families *Marinilabiliaceae* and *Porphyromonadaceae*. Numbers at the nodes are bootstrap values >50%. *Lishizhenia tianjinensis* H6^T (EU183317) was used as an outgroup. Bar, 0.02 substitutions per nucleotide position.

Supplementary Fig. S1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships between strain AK2^T and closely related members of the families *Marinilabiliaceae* and *Porphyromonadaceae*. Numbers at the nodes are bootstrap values >50%. *Lishizhenia tianjinensis* H6^T (EU183317) was used as an outgroup. Bar, 0.02 substitutions per nucleotide position.

Supplementary Fig. S2. Maximum-parsimony phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships between strain AK2^T and closely related members of the families *Marinilabiliaceae* and *Porphyromonadaceae*. Numbers at the nodes are bootstrap values >50%. *Lishizhenia tianjinensis* H6^T (EU183317) was used as an outgroup.

Supplementary Fig. S3. Two-dimensional thin-layer chromatogram of the total polar lipids of strain AK2^T (a) and *Marinilabilia salmonicolor* JCM 21150^T (b) revealed by 5% ethanolic molybdatophosphoric acid. PE, phosphatidylethanolamine; L1-4, unidentified lipids; PL1-6, unidentified phospholipid.

Table 1. Features that distinguish strain AK2^T from the closely related species *Marinilabilia salmonicolor*.

Data were from present study. Both strains were rod-shaped, positive for catalase, Ala-Phe-Pro-arylamidase, β -galactosidase, β -*N*-acetylglucosaminidase, β -glucosidase, β -xylosidase, α -glucosidase, α -galactosidase and phosphatase activities; aesculin, Tween 20/40/60/80 hydrolysis; acid production from sorbitol; and ELLMAN reaction. Both strains were negative for growth on MacConkey's agar, H₂S and indole production, methyl red and Voges Proskauer's reactions, phenylalanine deamination, lysine decarboxylase, ornithine decarboxylase, L-pyrrolydonyl-arylamidase, glutamyl arylamidase, γ -glutamyl-transferase, β -alanine arylamidase, L-proline arylamidase, tyrosine arylamidase, β -*N*-acetylgalactosaminidase, glycine arylamidase, β -glucuronidase and glu-gly-arg-arylamidase activities; agar, casein, gelatin, starch hydrolysis; acid production from inositol, inulin, raffinose, cellubiose, rhamnose, galactose, trehalose and xylose; fermentation glucose, L-lactate and succinate alkalinisation, courmarate, 0/129 resistance; 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-histidine, L-malate and L-lactate utilization. Both strains were susceptible to antibiotics (μ g/disc) amoxycillin (25), ampicillin (30), gentamicin (10), ceftazidime (30), spectinomycin (100), chloremphenicol (30), cephadroxil (30), novamycin (30), penicillin-G (2 units), bacitracin (8), ciproflaxin (10), vancomycin (30), tetracyclin (30), lincomycin (2) and chlorotetracyclin (30); resistant to kanamycin (30) and neomycin (30). +, positive; (+), weak positive; -, negative; R, resistant; S, sensitive.

Characteristic	<i>Marinilabilia nitratireducens</i> AK2 ^T	<i>Marinilabilia salmonicolor</i> JCM 21150 ^T
Salinity growth range (% w/v) NaCl (optimum)	2-10 (2-4)	2-12 (2-6)
Growth temperature range (°C) (optimum)	25-42 (30-37)	25-42 (28-37)
pH growth range (optimum)	7-12 (8)	6-12 (7)
Pigmentation	Reddish-orange	Pale yellow-salmon
Anaerobic growth	-	+
Nitrate reduction	+	(+)
Oxidase	-	-
Lipase	+	-
Urease	+	-
<i>Acid Production from:</i>		
Salicin	-	+
Melibiose	(+)	+
Mannose	-	+
Susceptibility to Polymixin B (300 μ g/disc)	-	+
Polar lipids	PE, L1-L3, PL1	PE, L1, L2, L4, PL1-PL6
Habitat	Saltpan	Marine

Table 2. Fatty acid composition of the strains AK2^T, *Marinilabilia salmonicolor* JCM 21150^T

Results are presented as a percentage of the total fatty acids. Fatty acids are designated as follows: total number of carbon atoms : number of double bonds, followed by the position of the double bond from the aliphatic end of the molecule. Prefixes i, a and OH represent iso-branched, anteiso-branched and hydroxy fatty acids, respectively. The suffix c represents a cis isomer. ECL, equivalent chain-length. Fatty acids amounting to 5% or more of the total fatty acids are in bold. Strains AK2^T and *Marinilabilia salmonicolor* JCM 21150^T were grown on MA plates at 30°C for four and two days respectively. ND, not detected. Values of less than 1% for all strains are not shown. Data were from present study. Data in the parenthesis is taken from Miyazaki et al. 2011.

Fatty acid	<i>Marinilabilia nitratireducens</i> AK2 ^T	<i>Marinilabilia salmonicolor</i> JCM 21150 ^T
i-C _{12:0}	2.0	ND (ND)
i-C _{13:0}	19.9	2.1 (19.3)
a-C _{13:0}	4.4	ND (3.8)
C _{13:1} at 12-13	ND	1.5 (ND)
i-C _{14:0}	ND	ND (6.1)
C _{15:0} 2OH	1.7	ND (ND)
i-C _{15:0}	28.2	47.6 (39.0)
a-C _{15:0}	23.2	23.3 (17.6)
i-C _{15:0} 3OH	13.9	5.4 (6.8)
i-C _{16:0} 3OH	1.2	1.9 (ND)
i-C _{17:0} 3OH	1.8	ND (0.8)
C _{18:1}	ND	ND (5.4)
Unknown fatty acid, ECL 11.543	ND	2.8 (ND)
Unknown fatty acid, ECL 13.565	ND	15.5 (ND)

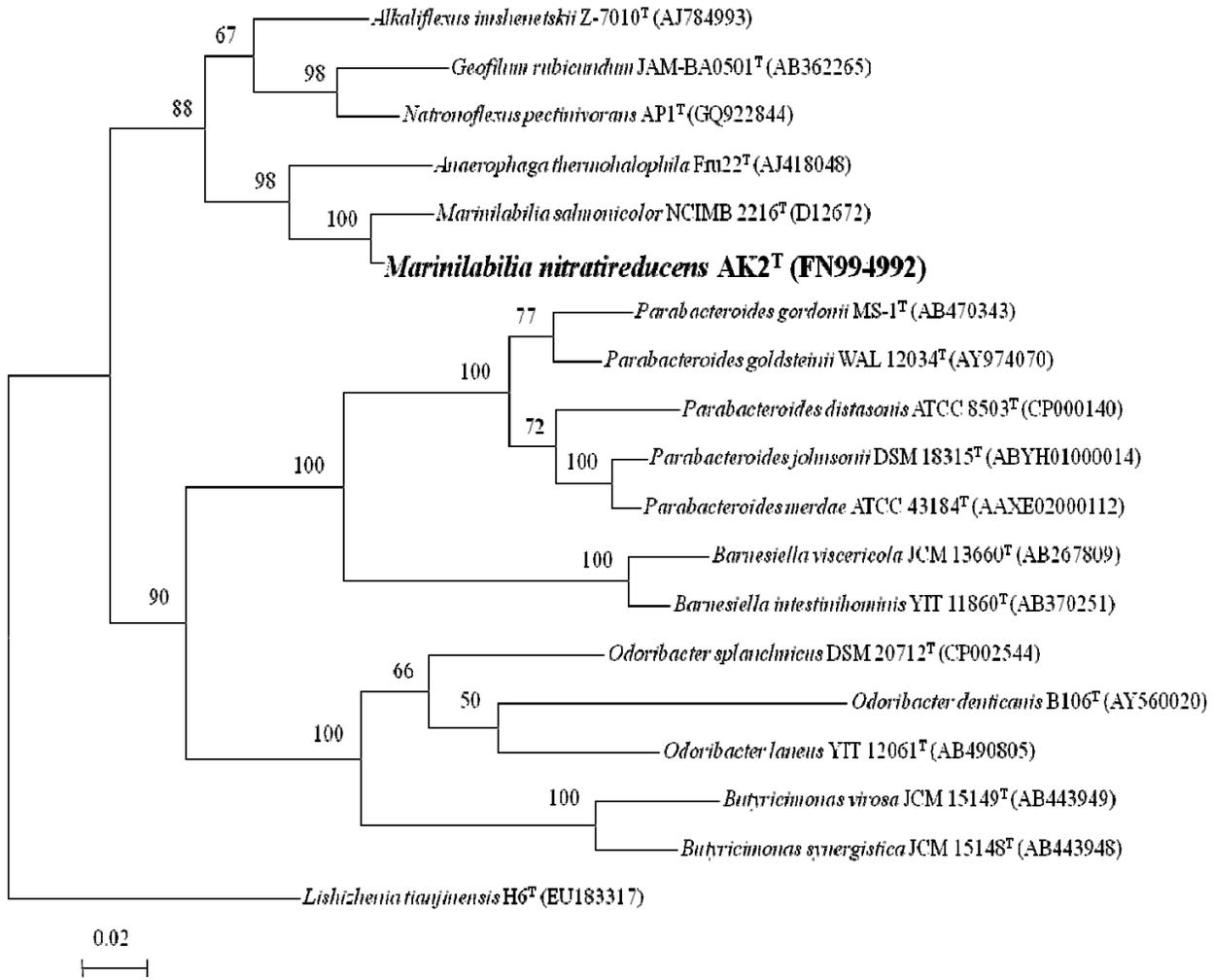


Fig. 1

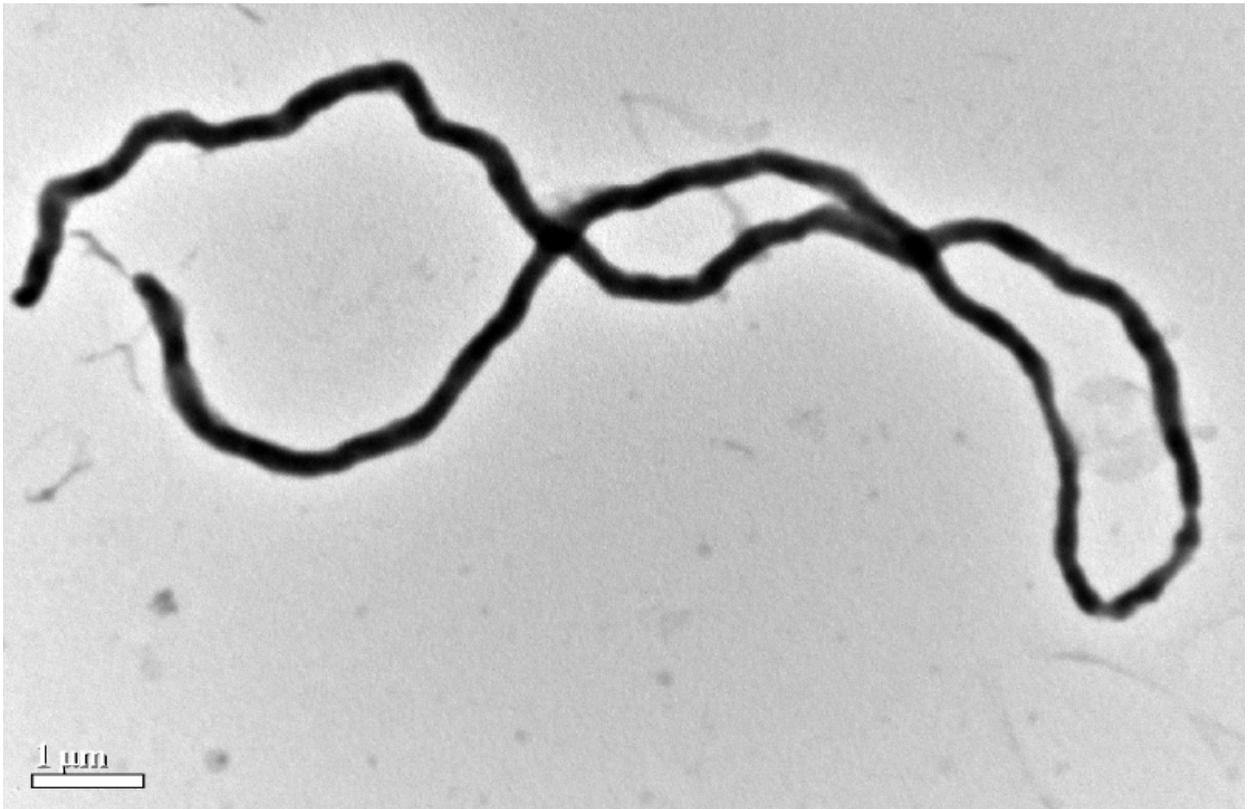
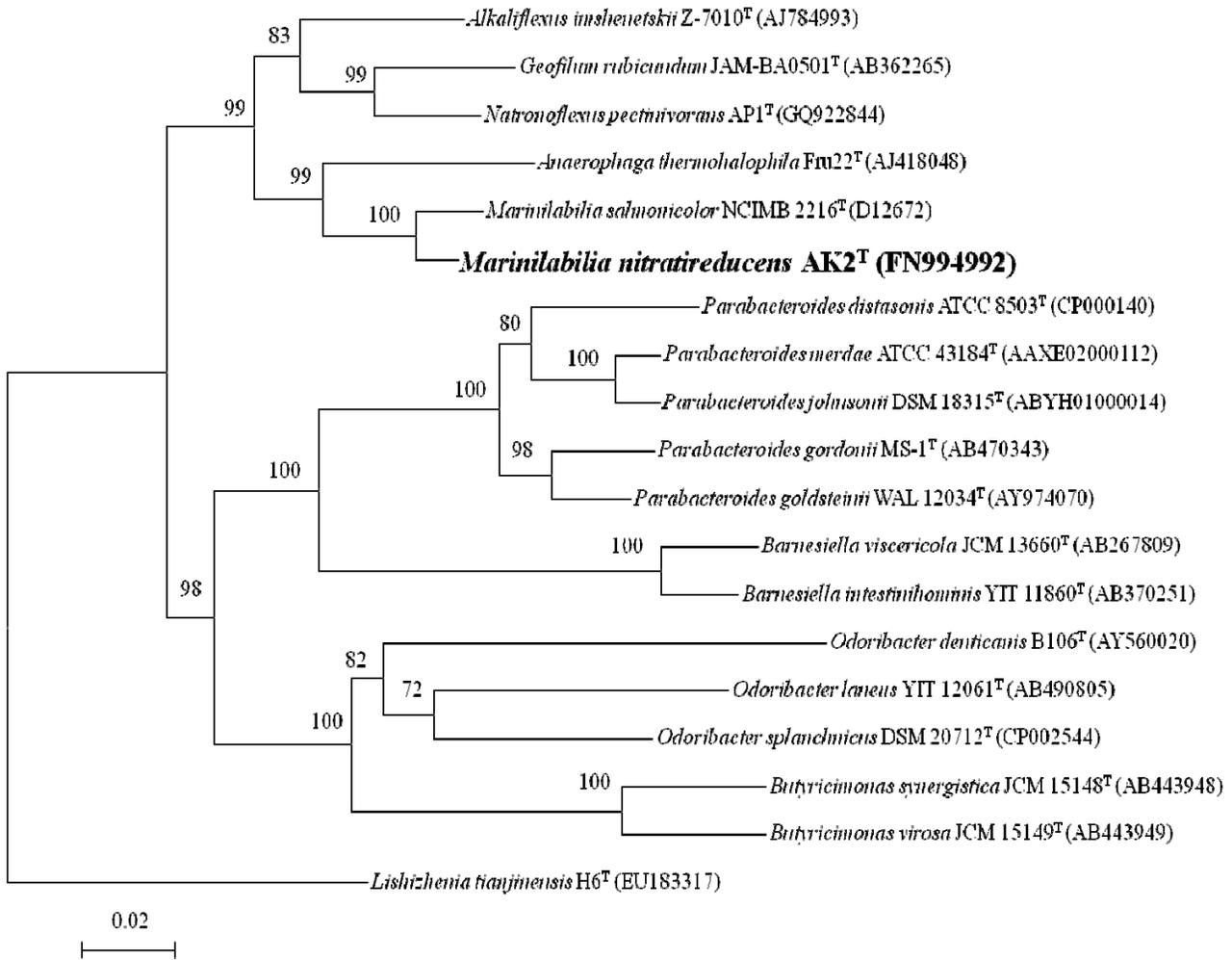
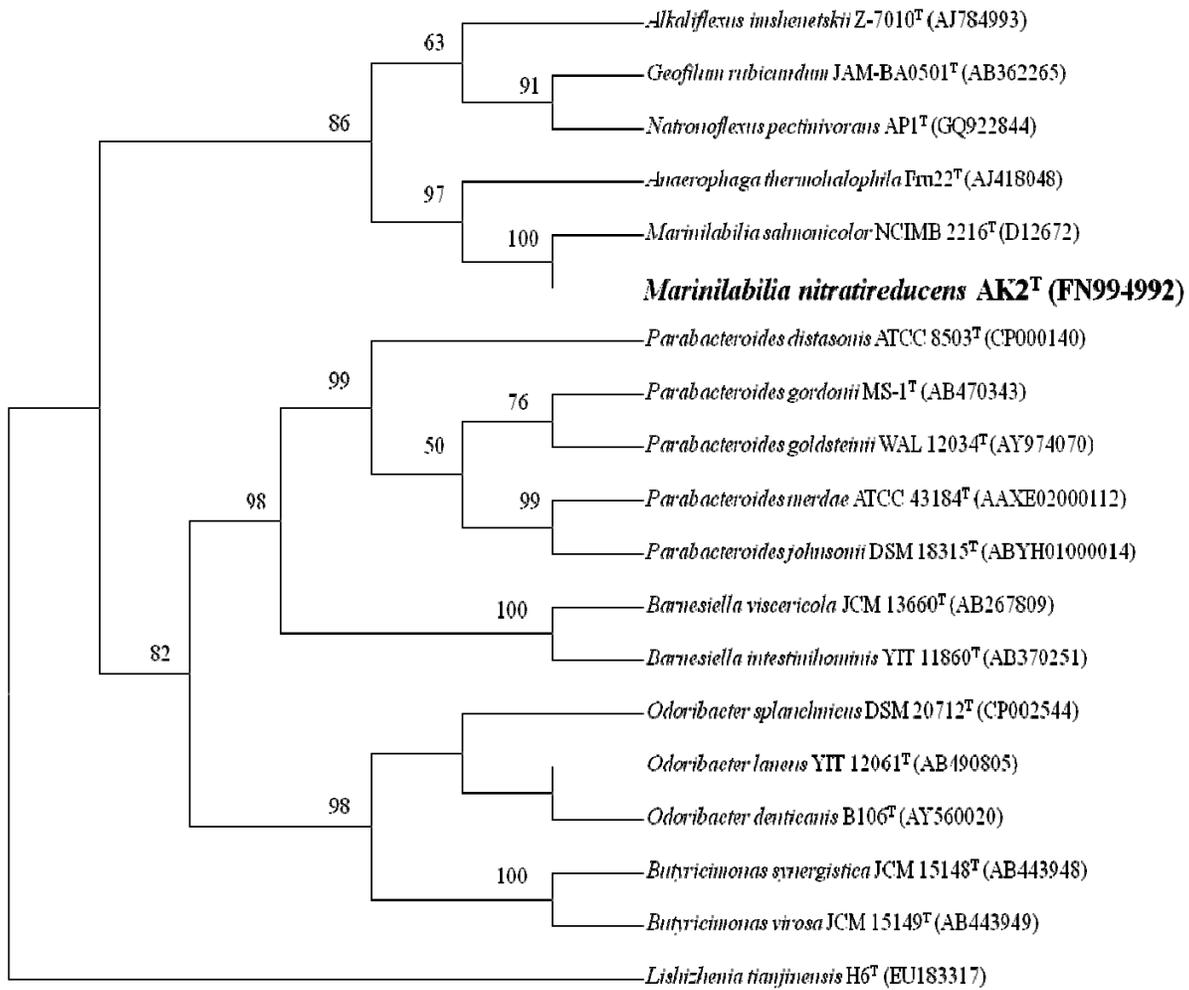


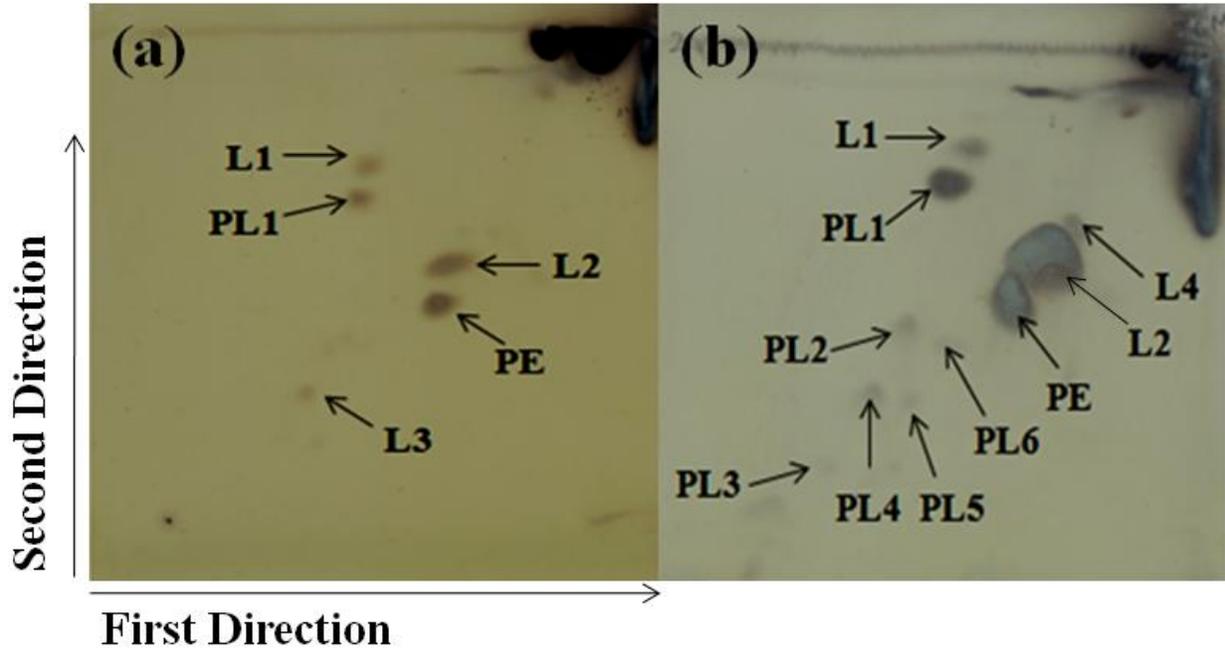
Fig. 2



Supplementary Fig. S1



Supplementary Fig. S2



Supplementary Fig. S3