

***Aliiglaciecola coringensis* sp. nov., isolated from a water sample collected from mangrove forest in Coringa, Andhra Pradesh, India**

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A Gram-negative, rod shaped, motile, aerobic bacterium, designated as strain AK49^T was isolated from a water sample from a mangrove forest in Coringa village, Andhra Pradesh, India. Strain AK49^T was observed to form yellow coloured, smooth, circular, convex colonies on marine agar, with entire margins. Cells of strain AK49^T are 0.5–1.0 µm wide and 1.5–3.5 µm long. Growth was observed at 25–37 °C (optimum 30 °C), 2–6% NaCl (optimum 2%) and pH 6–8 (optimum 7). Phylogenetic analysis based on 16S rRNA gene sequences showed that the strain AK49^T is closely related to two species recently reclassified as members of the the genus *Aliiglaciecola*: *Aliiglaciecola lipolytica* JCM 15139^T (sequence similarity 95.43%) and *Aliiglaciecola litoralis* JCM 15896^T (sequence similarity 96.91%). The major cellular fatty acids of strain AK49^T were found to include C_{16:0}, C_{18:1ω7c} and summed feature 3 (C_{16:1ω7c} / C_{15:0} iso-2-OH). The polar lipid content of cell membrane was found to include phosphatidylethanolamine, phosphatidylglycerol, an unidentified aminolipid, an unidentified lipid and an unidentified glycolipid. The genomic DNA G+C content of strain AK49^T was determined to be 41.9 mol%. Based on the taxonomic methods, including chemotaxonomic, phenotypic and phylogenetic approaches, strain AK49^T is described here as a novel species belonging to the genus *Aliiglaciecola*, for which the name *Aliiglaciecola coringensis* sp. nov. is proposed. The type strain of *A. coringensis* sp. nov. is AK49^T (= MTCC 12003^T = JCM 19197^T).

The family *Alteromonadaceae* is a family of Gram-negative, aerobic, motile, rod shaped bacteria (Ivanova *et al.*, 2004), currently comprising of 17 genera including the genus *Aliiglaciecola* (<http://www.bacterio.net/aliiglaciecola.html>). The genus *Aliiglaciecola* has been recently described as a novel genus incorporating two species previously classified in the genera *Glaciecola* and *Aestuariibacter* (Jean *et al.*, 2013). *Aliiglaciecola lipolytica* was previously included in the genus *Glaciecola* (Chen *et al.*, 2009) and *Aliiglaciecola litoralis* was previously included in the genus *Aestuariibacter* (Tanaka *et al.*, 2010). The members of the genus *Aliiglaciecola* are aerobic, Gram-negative, rods, belonging to the family *Alteromonadaceae* in the class Gammaproteobacteria in the (Jean *et al.*, 2013). In this paper, based on chemotaxonomic, phenotypic and phylogenetic approaches, strain AK49^T is described as a novel species belonging to the genus *Aliiglaciecola*, for which the name *Aliiglaciecola coringensis* sp. nov. is proposed.

Material and methods

Isolation and growth

Strain AK49^T was isolated from water a sample from a mangrove forest in Coringa village (16° 48' N 82° 14' E), Andhra Pradesh, India. One ml of water sample was serially diluted 10 fold in 2% (w/v) NaCl and spread on marine agar (MA; HIMEDIA) and incubated at 30 °C. A yellow coloured colony was isolated and preserved at -80 °C in marine broth with 10% glycerol for long term storage.

Type strains used for comparative study

The type strains of *A. lipolytica* (JCM 15139^T) and *A. litoralis* (JCM 15896^T) were obtained from the JCM culture collection, Japan; strain AK49^T was characterized simultaneously with *A. lipolytica* JCM 15139^T and *A. litoralis* JCM 15896^T.

Morphological characteristics

Colony morphology was examined after 24h growth on MA. Cell morphology was investigated by light microscopy (Nikon, at 1000X) and Gram reaction was observed by staining using a Gram staining kit (HIMEDIA) according to the manufacturer's protocol. Motility was assessed under phase contrast microscopy.

Physiological and biochemical characteristics

Optimum temperature and salt concentration for growth were assessed by growing the strains in marine broth over a temperature range of 5, 10, 15, 20, 25, 30, 34, 37, 40 and 45 °C and 0–12 % (w/v) NaCl concentrations with 1% increment respectively. The optimum pH for growth was determined by growing the strains in marine broth with suitable buffers to obtain a pH range of 5–12 (Srinivas et al., 2013). Biochemical characteristics, H₂S production, carbon source assimilation and Voges–Proskauer and methyl red test were determined as already described (Lányi, 1987) and confirmed using HiCarbohydrate kit (HIMEDIA), Hi *Enterobacteriaceae* kit (HIMEDIA) and Vitek analysis using Vitek 2 GN system (bioMérieux) according to the manufacturer's protocol. Sensitivity to different antibiotics was determined using commercially available antibiotic discs (HIMEDIA).

Chemotaxonomic analysis

For cellular fatty acid analysis, strains AK49^T, *A. lipolytica* JCM 15139^T and *A. litoralis* JCM 15896^T were grown on MA plates at 30 °C for 1 day. Cellular fatty acid methyl esters (FAMES) were obtained from cells by saponification, methylation and extraction following the protocols of Sherlock Microbial Identification System (MIDI, USA). Cellular FAMES were separated by gas chromatography (6890), identified and quantified with Sherlock Microbial Identification System Software (version.6.0, using the aerobe TSBA6 method and TSBA6 database). Polar lipids were estimated as previously described (Komagata & Suzuki, 1987) and detected by two-dimensional thin layer chromatography using molybdenum blue, ninhydrin and α -naphthol reagents.

Determination of DNA G+C content

Genomic DNA was isolated using a ZR Bacterial/Fungal DNA isolation kit (D6005, Zymo, USA) according to the manufacturer's protocol. *Escherichia coli* DNA was used as a control for the G+C content estimation of the genomic DNA. The G+C content was determined using the melting point (T_m) curves (Sly *et al.*, 1986) acquired by a Lambda 2 UV-Vis spectrophotometer and Templab 2.0 software package (Perkin Elmer).

16S rRNA gene sequence determination and phylogenetic analysis

For 16S rRNA gene sequencing, DNA was prepared using a ZR Bacterial/Fungal DNA isolation kit (D6005, Zymo, USA). Polymerase chain reaction (PCR) amplification of the 16S rRNA gene was performed with bacterial universal primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-TACGGYTACCTTGTTACGACTT-3'). PCR amplification of the 16S rRNA gene, sequencing and assembly was performed as described in Surendra *et al.* (2012). The resulting 16S rRNA gene sequence of strain AK49^T was subjected to an EzBioCloud (<http://eztaxon-e.ezbiocloud.net/>) search (Kim *et al.*, 2012) to identify the closely related taxa. For phylogenetic analysis, sequences for the different type species in the family *Alteromonadaceae* and strains in the genus *Aliiglaciecola* were downloaded from NCBI (<http://www.ncbi.nlm.nih.gov>). The sequence for *Aeromonas hydrophila* subsp. *hydrophila* ATCC 7966^T was used as an out group. All the sequences were aligned and phylogenetic trees were constructed using neighbour joining, maximum likelihood and Maximum Parsimony methods using MEGA version 5.2 (Tamura *et al.*, 2011). The evolutionary distances were calculated using Kimura-2-parameter (Kimura, 1980) and bootstrap analysis was performed to determine the robustness of trees.

Results and Discussion

The cells of strain AK49^T were observed to be Gram-negative, rod shaped, motile and showed aerobic growth characteristics. Strain AK49^T was observed to form yellow coloured, smooth, circular, convex colonies of 1 mm diameter with entire margins on MA. Cells were found to be 0.5–1.0 µm wide and 1.5–3.5 µm long. The strain was found to grow at pH 6-8, temperature 25-37 °C and 2-6% salt concentration (NaCl, w/v). Optimal growth was found to occur at pH 7, 30 °C temperature and 2% (w/v) NaCl concentration. A detailed description of the morphological and biochemical features for strain AK49^T is provided in Table 1, and in species description. Strain AK49^T was found to be resistant to (µg per disc) bacitracin (8), cefmetazole (30) and methicillin (5) and sensitive to amoxicillin (30), ampicillin (25), chloramphenicol (30), cefalexin (30), ceftazidime (30), cefprozil (30), cinoxacin (100), cephadroxil (30), chlortetracycline (30), clarithromycin (15), enoxacin (10), enrofloxacin (5), lincomycin (2), neomycin (30), novobiocin (30), penicillin (2), streptomycin (25), spectinomycin (100), tetracycline (30) and vancomycin (30).

The 16S rRNA gene sequence for strain AK49^T was determined (1469 nucleotides; GenBank/EMBL/DDBJ accession number HG529994). Based on an EzBioCloud sequence similarity

search with closely related type strains, strain AK49^T shows similarity to *A. litoralis* (96.92%), *Aestuariibacter aggregatus* (96.34%), *Alteromonas litorea* (95.67%), *Aestuariibacter halophilus* (95.60%), *Alteromonas marina* (95.53%) and *A. lipolytica* (95.43%). Phylogenetic trees constructed using the neighbour joining, maximum likelihood and maximum parsimony methods indicated that strain AK49^T is closely related to *A. litoralis* (KMM 3894^T = JCM 15896^T) and *A. lipolytica* (E3^T = JCM 15139^T), with a strong bootstrap support value (Fig.1). The overall topology of the phylogenetic trees generated by the neighbour joining, maximum likelihood and maximum parsimony methods was similar (Fig. 1, Supplementary Figs. S1 and S2).

The major cellular fatty acids of the strain AK49^T were identified as C_{16:0}, C_{18:1}ω7c and summed feature 3 (C_{16:1}ω7c and/or C_{15:0} iso-2-OH, as defined by MIDI; Table 2). The fatty acid composition of strain AK49^T was found to be comparable to that of *A. litoralis* (JCM 15896^T) and *A. lipolytica* (JCM 15139^T) in terms of the major fatty acids mentioned above but different with respect to the presence (C_{17:0}; C_{17:0} 10 CH₃), absence (C_{17:0} cyclo; C_{18:3} ω6,9,12c) or abundance (C_{17:1} ω8c) or low abundance (C_{16:1} N OH) of fatty acids (Table 2). The polar lipid content of the cell membrane was found to include phosphatidylethanolamine, phosphatidylglycerol, an unidentified aminolipid, an unidentified glycolipid and an unidentified lipid (Fig. 2a). Both reference type strains also comprised phosphatidylethanolamine and phosphatidylglycerol, like strain AK49^T, but were considerably different with respect to other polar lipids such as aminophospholipid, unidentified glycolipid GL2 and several unidentified lipids (Fig. 2 and Table 1). The genomic DNA G+C content of strain AK49^T was determined to be 41.9 mol%.

The main features of the strain AK49^T are in line with the original description of the genus *Aliiglaciecola* (Jean *et al.*, 2013). Strain AK49^T could be distinguished from *A. litoralis* with respect to cell size, temperature and salinity growth range, G+C content (mol%), nitrate reduction to nitrate, H₂S production, β-N-acetyl-glucosaminidase and urease activities, hydrolysis of gelatin and starch, utilization of galactose, resistance to lincomycin, polar lipids and fatty acid profile; and from *A. lipolytica* regarding cell size, temperature and salinity growth range, G+C content (mol%), nitrate reduction to nitrate, arginine dihydrolase activity, hydrolysis of gelatin and starch, inability to utilise a number of organic substrates, resistance to lincomycin, polar lipids and fatty acid profiles. Thus, the cumulative differences of strain AK49^T from the closely related type strains indicate that the strain should be classified as a new species of the genus *Aliiglaciecola*, for which the name *Aliiglaciecola coringensis* sp. nov. is proposed.

Description of *Aliiglaciecola coringensis* sp. nov.

Aliiglaciecola coringensis sp. nov. (co.ring.en'sis N.L. fem. adj. *coringensis* pertaining to Coringa village, Andhra Pradesh, India, from where the water sample was collected from which the organism was isolated).

Cells are aerobic, rod shaped, Gram-negative, motile, forming yellow coloured, smooth, circular, convex colonies of 1 mm diameter with entire margins on marine agar. The cells are 0.5–1.0 μm wide and 1.5–3.5 μm long. Grows at 25–37 °C (optimum 30 °C), pH 6–8 (optimum 7) and 2–6% (w/v) NaCl (optimum 2%). Aesculin and Tween 20, Tween 40, Tween 60 and Tween 80 are hydrolysed but casein, gelatin and starch are not hydrolysed. Methyl red and Voges Proskauer's reactions are negative. Positive for oxidase, catalase and H₂S production but negative for arginine hydrolase, nitrate reduction, phenylalanine deamination, glucose fermentation, β -galactosidase activity and indole production. Does not utilise lactose, xylose, maltose, fructose, dextrose, galactose, raffinose, trehalose, melibiose, sucrose, L-arabinose, mannose, inulin, sodium gluconate, glycerol, salicin, dulcitol, inositol, sorbitol, mannitol, adonitol, arabitol, erythritol, α -methyl-D-glucoside, α -methyl-D-mannoside, rhamnose, cellobiose, melezitose, xylitol, D-arabinose, citrate, malonate and sorbose. Vitek GN analysis shows positive activity for Ala-Phe-Pro-arylamidase, β -galactosidase, β -N-acetyl-glucosaminidase, glutamyl arylamidase, β -glucosidase, L-proline arylamidase, lipase, tyrosine arylamidase, urease, α -glucosidase, α -galactosidase, phosphatase and Ellman's reaction; and negative activity for L-pyrrolydonyl-arylamidase, γ -glutamyl-transferase, β -xylosidase, β -alanine arylamidase, glycine arylamidase, ornithine decarboxylase, lysine decarboxylase, β -glucuronidase, glu-gly-arg-arylamidase, L-malate assimilation, L-lactate assimilation, L-histidine assimilation, L-lactate alkalisation, succinate alkalisation, H₂S production, citrate utilization, malonate utilization and 0/129 resistance. Vitek GN analysis also confirms the negative assimilation of the sugars adonitol, L-arabitol, D-cellobiose, D-glucose, glucose fermentation, D-maltose, D-mannitol, D-mannose, palatinose, D-sorbitol, saccharose/sucrose, D-tagatose, D-trehalose, 5-keto-D-gluconate and coumarate. The major cellular fatty acids include C_{16:0}, C_{18:1 ω 7c} and summed feature 3 (C_{16:1 ω 7c} and/or C_{15:0} iso-2-OH). The polar lipids include phosphatidylethanolamine, phosphatidylglycerol, an unidentified aminolipid, an unidentified lipid and an unidentified glycolipid. The genomic DNA G+C content of the type strain is 41.9 mol%.

The type strain of *Aliiglaciecola coringensis* sp. nov. is AK49^T (= MTCC 12003^T = JCM 19197^T) and was isolated from water sample of mangrove forest of Coringa village in Andhra Pradesh, India. The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain AK49^T is HG529994.

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Legends

Fig. 1. Neighbour joining tree showing phylogenetic relationships of strain AK49^T with representative type species and type strains of the family *Alteromonadaceae* based on 16S rRNA sequence dataset. Bootstrap support values more than 50% are shown at the nodes. *Aeromonas hydrophila* subsp. *anaerogenes* CECT 4221^T was used as an out group. Scale bar represents 0.01 substitutions per nucleotide position.

Fig. 2. Two-dimensional thin-layer chromatogram of the total polar lipids of the strain AK49^T (a), *Aliiglaciecola litoralis* JCM 15896^T (b) and *Aliiglaciecola lipolytica* JCM 15139^T (c) after spraying the plates with molybdato-phosphoric acid. Abbreviations: PE, phosphatidylethanolamine; PG, phosphatidylglycerol; AL, unidentified aminolipid; APL, unidentified aminophospholipid; GL, unidentified glycolipid; L, unidentified lipid; PL, unidentified phospholipid. The spots were identified as phospho-, amino- or glyco-lipids by spraying molybdenum blue, ninhydrin and α -naphthol reagents, respectively.

Table 1. List of distinguishing features of strain AK49^T compared with the closely related type strains *Aliiglaciecola lipolytica* JCM 15139^T and *Aliiglaciecola litoralis* JCM 15896^T

Data are from present study. All strains were rod-shaped and motile, require 2% NaCl (w/v) for optimum growth, pH growth range is 6-8 and optimal growth at pH 7; positive for oxidase activity and Aesculin, Tween 20/40/60/80 hydrolysis. All strains were negative for casein hydrolysis, methyl red and Voges Proskauer's reactions, glucose fermentation, fructose, dextrose, glycerol, dulcitol, inositol, sorbitol, adonitol, arabitol, erythritol, rhamnose, melezitose, xylitol, citrate, malonate, sorbose and ONPG. All strains are sensitive to antibiotics ($\mu\text{g}/\text{disc}$) amoxycillin (30), ampicillin (25), chloramphenicol (30), cefalexin (30), ceftazidime (30), cefprozil (30), cinoxacin (100), cephadroxil (30), chlortetracycline (30), clarithromycin (15), enoxacin (10), enrofloxacin (5), neomycin (30), novobiocin (30), penicillin (2), streptomycin (25), spectinomycin (100), tetracycline (30) and vancomycin (30) resistant to bacitracin (8), cefmetazole (30) and methicillin (5). +, positive; -, negative; (+), partially positive; S, sensitive; R, resistance; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; AL, unidentified aminolipid; APL, unidentified aminophospholipid; GL, unidentified glycolipid; L, unidentified lipid; PL, unidentified phospholipid.

Characteristics	<i>Aliiglaciecola coringensis</i> AK49 ^T	<i>Aliiglaciecola litoralis</i> JCM 15896 ^T	<i>Aliiglaciecola lipolytica</i> JCM 15139 ^T
Cell size (μm)	1.5-3.5x0.5-1.0	1.0-2.5x0.4-0.5	1.0-2.5x0.4-0.5
Temperature growth range ($^{\circ}\text{C}$)	25-37	4-37	4-37
Optimum temperature ($^{\circ}\text{C}$)	30	30	30
Salinity growth range (%)	2-6%	2-8%	2-8%
G+C content (mol%)	41.9	43.0	40.8
Biochemical characteristics			
Catalase	(+)	+	+
Arginine dihydrolase	-	-	+
Nitrate reduction to nitrite	-	+	+
H ₂ S production	+	-	+
Indole	-	-	-
β -N-acetyl-glucosaminidase	+	-	+
Urease	+	-	+
Hydrolysis of			
Gelatin	-	(+)	+
Starch	-	+	+
Carbon source utilisation			
Lactose	-	-	+
Xylose	-	-	+
Maltose	-	-	+
Galactose	+	-	+
Raffinose	-	-	+
Trehalose	-	-	+
Melibiose	-	-	+
Sucrose	-	-	+
L-arabinose	-	-	+
Mannose	-	-	+

Characteristics	<i>Aliiglaciecola coringensis</i> AK49 ^T	<i>Aliiglaciecola litoralis</i> JCM 15896 ^T	<i>Aliiglaciecola lipolytica</i> JCM 15139 ^T
Inulin	-	-	+
Salicin	-	-	+
Mannitol	-	-	+
Cellobiose	-	-	+
D-arabinose	-	-	+
Antibiotic sensitivity			
Lincomycin (2 µg/disc)	S	R	R
Polar lipids	PE, PG, AL, GL, L6	PE, PG, APL, GL, PL, L5-8	PE, PG, AL, APL, GL1, GL2, L1-6, L9, L10

Table 2. Comparisons of fatty acid composition of strain AK49^T with the closely related type strains *Aliiglaciecola lipolytica* JCM 15139^T and *Aliiglaciecola litoralis* JCM 15896^T

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. Data is from present study. All the strains were grown on MA plates at 30°C for two days. ND, not detected. Values of less than 1% for all strains are not shown. tr, traces (<1%).

Fatty acid	<i>Aliiglaciecola</i> <i>coringensis</i> AK49 ^T	<i>Aliiglaciecola</i> <i>litoralis</i> JCM 15896 ^T	<i>Aliiglaciecola</i> <i>lipolytica</i> JCM 15139 ^T
C _{12:0}	2.9	2.5	2.5
C _{12:0} 3OH	4.3	1.3	2.9
C _{12:1} 3OH	1.2	1.3	1.4
C _{14:0}	4.7	1.3	2.0
C _{16:0}	28.7	30.1	30.8
C _{16:1} N OH	4.5	1.9	8.7
C _{16:1} ω7c OH	2.2	2.2	4.1
C _{17:0} cyclo	ND	9.9	3.5
C _{17:1} ω8c	1.2	tr	tr
C _{17:0}	1.5	ND	ND
C _{17:0} 10 CH ₃	3.0	ND	ND
C _{18:0}	2.6	1.1	1.8
C _{18:1} ω7c	11.8	11.6	11.2
C _{18:3} ω6,9,12c	ND	tr	2.9
Summed in feature 3	27.2	28.4	23.2

Summed features are groups of two or three fatty acids that could not be separated by GC with the MIDI system. Summed feature 3 comprised C_{16:1} ω7c and/or iso-C_{15:0} 2-OH.

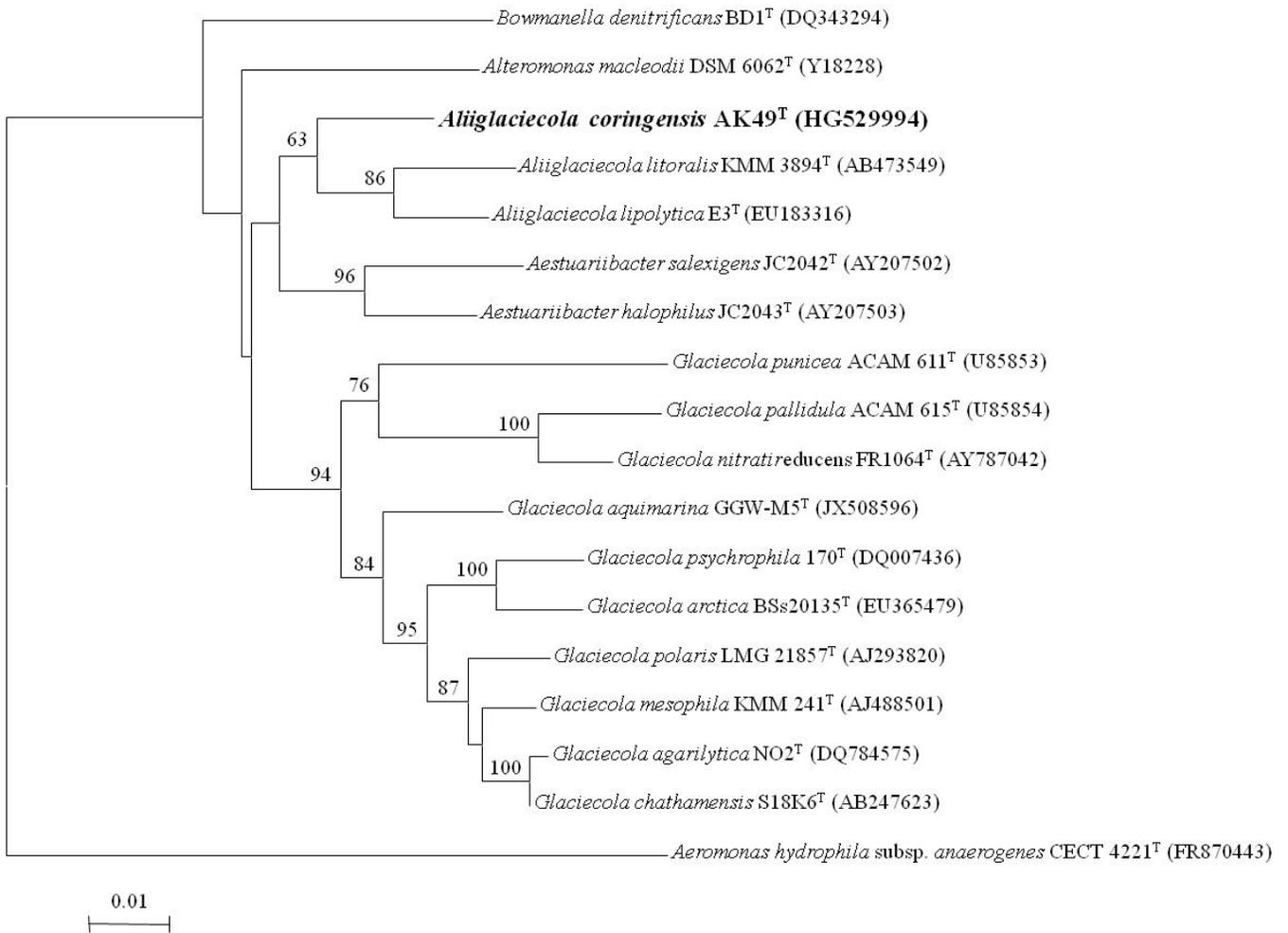


Fig. 1

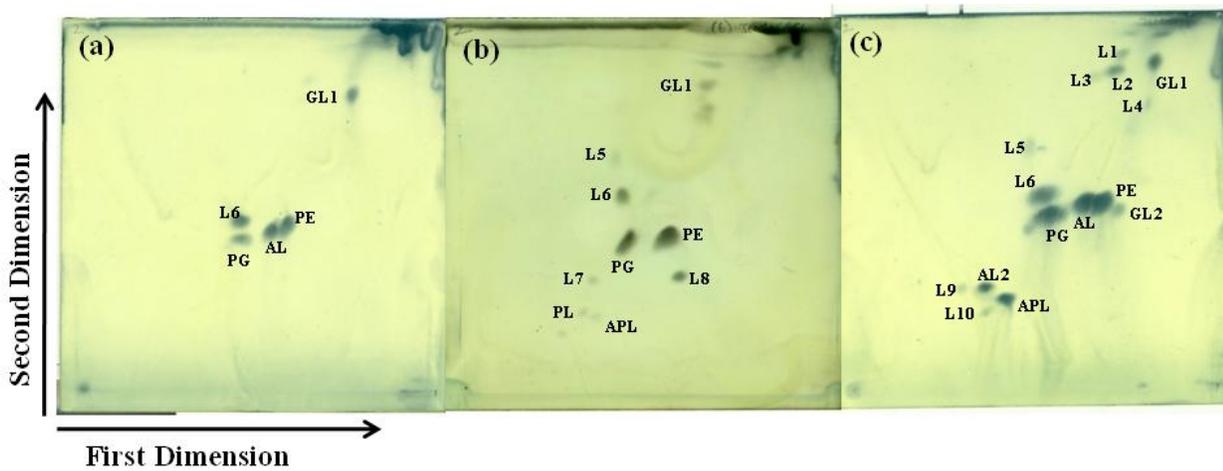
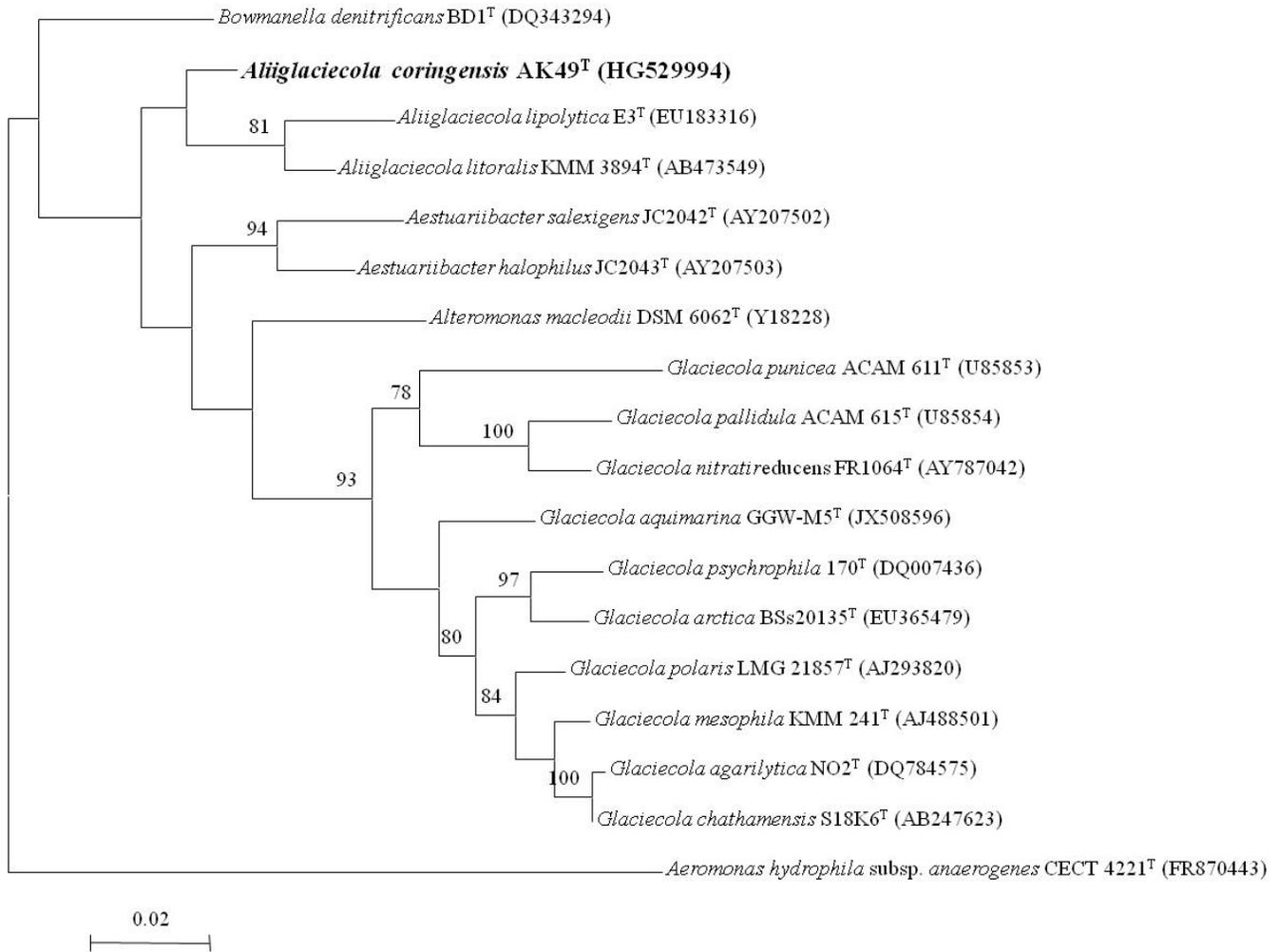
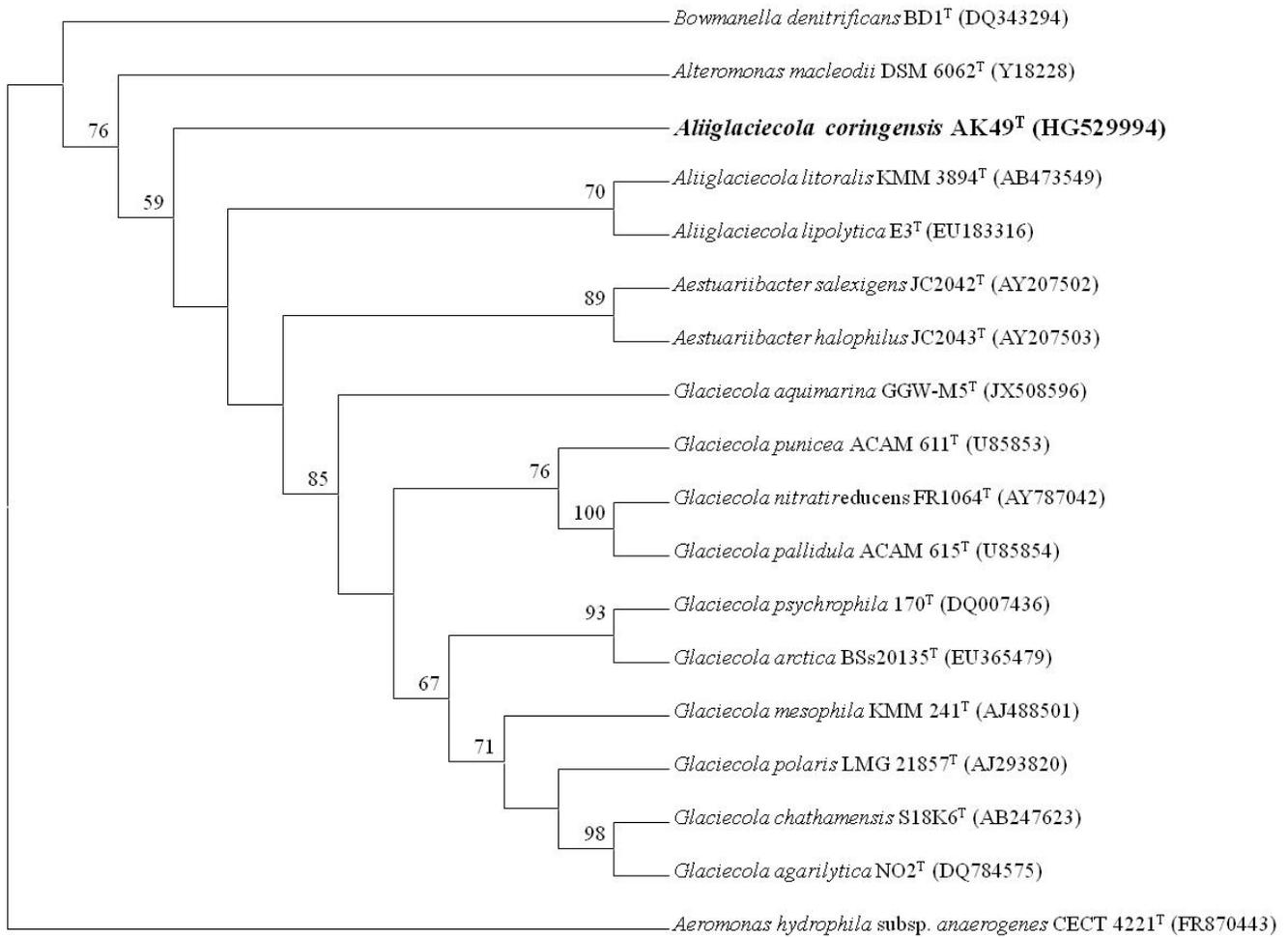


Fig. 2



Supplementary Fig. S1. Maximum likelihood tree showing phylogenetic relationships of strain AK49^T with representative type species and type strains of the family *Alteromonadaceae* based on 16S rRNA sequence dataset. Bootstrap support values more than 50% are shown at the nodes. *Aeromonas hydrophila* subsp. *anaerogenes* CECT 4221^T was used as an out group taxa. Scale bar represents 0.02 substitutions per nucleotide position.



Supplementary Fig. S2. Maximum parsimony tree showing phylogenetic relationships of strain AK49^T with representative type species and type strains of the family *Alteromonadaceae* based on 16S rRNA sequence dataset. Bootstrap support values more than 50% are shown at the nodes. *Aeromonas hydrophila* subsp. *anaerogenes* CECT 4221^T was used as an out group taxa.