

***Marinobacterium alkalitolerans* sp. nov., with nitrate reductase and urease activity isolated from green algal mat collected from a solar saltern**

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain AK62^T is LN558833. The GenBank/EMBL/DDBJ accession number for the whole genome of strain AK62^T is JACVEW000000000.

Abstract

A novel Gram-staining-negative, rod-shaped, 0.6-0.8 µm wide and 2.0–3.0 µm in length, motile bacterium designated strain AK62^T, was isolated from the green algal mat collected from saltpan, Kakinada, Andhra Pradesh, India. Colonies on ZMA were circular, off-white, shiny, moist, translucent, 1–2 mm in diameter, flat, with an entire margin. The major fatty acids include C_{16:0}, C_{18:1 ω7c}, and summed feature 3 (C_{16:1 ω7c} and/or C_{16:1 ω6c} and/or iso-C_{14:0 3-OH}). Polar lipids include diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, one unidentified aminophospholipid, three unidentified phospholipids, and one unidentified lipid. Polyamine includes Spermidine. The DNA G+C content of the strain AK62^T was 58.8 mol%. Phylogenetic analysis based on 16S rRNA gene sequence revealed that strain AK62^T was closely related to the type strains *Marinobacterium sediminicola*, *Marinobacterium coralli* and *Marinobacterium stanieri* with a pairwise sequence similarity of 96.9, 96.6 and 96.6%, respectively, forming a distinct branch within the genus *Marinobacterium* and clustered with *M. stanieri*, *M. sediminicola*, *M. coralli* and *M. maritimum* cluster. Strain AK62^T shares average nucleotide identity (ANI_b, based on BLAST) of 78.44, 76.69, and 76.95% with *Marinobacterium sediminicola* CGMCC 1.7287^T, *Marinobacterium stanieri* DSM 7027^T, and *Marinobacterium halophilum* Mano11^T respectively. Based on the observed phenotypic, chemotaxonomic characteristics, and phylogenetic analysis, strain AK62^T is described in this study as a novel species in the genus *Marinobacterium*, for which the name *Marinobacterium alkalitolerans* sp. nov. is proposed. The type strain of *Marinobacterium alkalitolerans* is AK62^T (= MTCC 12102^T = JCM 31159^T = KCTC 52667^T).

Keywords: *Marinobacterium alkalitolerans*; 16S rRNA gene-based phylogeny; chemotaxonomy, genome.

Introduction

The genus *Marinobacterium* is a member of the family *Alteromonadaceae*, order *Alteromonadales*, class *Gammaproteobacteria*, phylum *Proteobacteria* and was established by González et al. (1997). The family *Alteromonadaceae* comprises of Gram-staining-negative, aerobic, rod-shaped motile bacteria (Ivanova and Mikhaïlov 2001), does not form endospores or microcysts, chemo-organotrophic, uses oxygen as the electron acceptor, grows aerobic or facultative anaerobically, is negative for denitrification, arginine dihydrolase activity, requires Na⁺ ion for growth and currently comprises 26 genera (Euzéby, <https://lpsn.dsmz.de/family/alteromonadaceae>, October 2020). The genus *Marinobacterium* is Gram-staining-negative, rod-shaped, with numerous vesicles on their surface, growth is strictly aerobic, oxidase and catalase-positive, and utilizes sugars fatty acids, aromatic compounds, and amino acids, which requires a sea salt-based medium for growth (González et al. 1997). The type species is *Marinobacterium georgiense* (González et al. 1997). The genus *Marinobacterium* presently comprises 19 species (Euzéby, <https://lpsn.dsmz.de/genus/marinobacterium>, October 2020). In the present study, we focused on the characterization and classification of the strain AK62^T, which was isolated from an algal mat sample by a polyphasic taxonomic approach (Vandamme et al. 1996).

Materials and methods

Isolation of the novel strain and cultivation

Strain AK62^T was isolated from the green algal mat and water collected from saltpan, Kakinada (GPS Positioning: 16° 54' 23.30" N, 82° 14' 07.97" E), Andhra Pradesh, India. For the isolation of strain AK62^T, 1 ml of water sample was serially diluted in 2% saline water, and 100 µl of which was plated on Zobell's marine agar (ZMA, HIMEDIA) and incubated for five days at 30 °C. Among the different colony, morphotypes appeared, an off-white coloured colony was selected and further characterized. The strain AK62^T was preserved with 10% glycerol in marine broth at -80 °C for long-term storage.

Type strains used in the analysis

The type strains of *M. stanieri* DSM 7027^T, *M. georgiense* DSM 11526^T, *M. halophilum* DSM 17586^T, *M. mangrovicola* DSM 27697^T and *M. lutimaris* DSM 22012^T were also cultivated under the same conditions as strain AK62^T for comparative studies.

Phylogenetic analysis

Isolation of genomic DNA, polymerase chain reaction (PCR) amplification (Rotor-Gene Q, Qiagen), and sequencing of the 16S rRNA gene were performed as described in Surendra et al. (2012). The 16S rRNA gene sequence obtained was 1488 bp long and subjected to EZBioCloud (<http://ezbiocloud.net/>) search (Yoon et al. 2017) to find the nearest taxa. The 16S rRNA gene sequences of the species of the genus *Marinobacterium* and type species of the genera belonging to the family *Alteromonadaceae* were downloaded from NCBI (<http://www.ncbi.nlm.nih.gov>) and aligned using the CLUSTAL_W tool of MEGA version 6 (Tamura et al. 2013). The neighbour-joining method was used to construct the phylogenetic tree using MEGA 6. Bootstrap analysis of 10000 replicates was performed to infer the robustness of the phylogenetic tree and the evolutionary distances were calculated using Kimura-2-parameter (Kimura 1980). The evolutionary history was inferred by using the Maximum Composite Likelihood approach based on the Tamura-Nei model (Tamura and Nei 1993) and the Maximum Parsimony method based on the Subtree-Pruning-Regrafting algorithm (Nei and Kumar 2000). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 30 sequences. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). The G+C content of strain AK62^T was determined from the melting point (T_m) curves obtained (Sly et al. 1986) using a Lambda 2 UV-Vis spectrophotometer with the Templab 2.0 software package (Perkin Elmer). For the determination of G+C content, the genome of *Escherichia coli* DH5 α DNA was used as a standard.

Genome information and analysis of genome encoded features

The genomic DNA of strain AK62^T was sequenced using the platform Illumina HiSeq with a 2*150bp paired-end library. The reads were checked for quality using FastQC v0.11.9 (Andrews 2010), and further genome was assembled using SPAdes 3.14.0 (Bankevich et al. 2012). The genomic contamination of strain A62^T was analyzed using the ContEst16S algorithm (Lee et al. 2016). The G+C content of the genome was obtained by annotating the genome on DFAST (DDBJ) (Tanizawa et al. 2018). OrthoANI values of the strain AK62^T and other closely related species were calculated and plotted as a heatmap using OAT: OrthoANI Tool version 0.93.1 (Lee et al. 2016). The DNA-DNA hybridization (DDH) value and percent difference of G+C content for strain AK62^T and other closely related species were calculated on the online platform GGDC (Lee et al. 2017). The phylogenomic tree of strain AK62^T was obtained from TYGS (Meier-Kolthoff and Göker 2019).

Pangenome analysis

Pangenome studies provide a platform to estimate the genomic diversity, effect of environmental factors on the gene pool of species and to further study the species evolution (Chaudhari et al. 2016). For this, we performed the pan-genome analysis of AK62^T and its close relatives (from available genomes) using the Bacterial Pan Genome Analysis (BPGA) pipeline. The pangenome includes the core genome consisting of common genes present in all species, the accessory or dispensable genome that contains the genes present in few individuals, and the unique genes that consist of the genes exclusively present in specific individuals and are absent in others (Tettelin et al. 2005). The unique genes of strain AK62^T were further annotated using the WebMGA webserver (Wu et al. 2011).

Morphological analysis

The cell morphology was observed by light microscopy (Nikon, Eclipse, 80i, at 1000X), the structure of the cell was studied after staining with 1% phosphotungstic acid viewed through a transmission electron microscope (H-7500; Hitachi). The cell motility was performed as described in Bernardet et al. (2002), and Gram staining was made using a Gram staining kit (HIMEDIA, India) according to the manufacturer's instructions. The colony morphology of strain AK62^T was examined, the following growth at ZMA for 48 hours at 30 °C.

Determination of temperature, salinity, and pH optimum

Growth at 4, 10, 15, 20, 25, 30, 37, 42 and 55 °C temperatures was ascertained using Zobell marine broth medium and salt tolerance (0, 0.5, 1, 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 % (w/v) NaCl) was determined using nutrient broth medium containing (L⁻¹) peptone (5 g), beef extract (3 g) and agar (20 g). Growth of strain AK62^T at pH 5, 6, 7, 7.5, 8, 8.5, 9, 9.5, 10, 11 and 12 was assessed in marine broth 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) (Srinivas et al. 2014).

Determination of biochemical tests

Different biochemical tests listed in the description of species and as well as in Table 1 were carried out for both strains under identical conditions using cultures grown at 37 °C on ZMA medium as described by Lányi (1987). Substrate hydrolysis (Aesculin, casein, gelatin, starch and Tweens), biochemical characteristics (Catalase and oxidase activity, production of H₂S and reduction of nitrate, Voges-Proskauer and methyl red test, oxidation-fermentation tests, oxidation of tetramethyl-p-phenylenediamine dihydrochloride) were determined as previously described (Lányi 1987; Cowan and Steel 1965). The utilization of different carbohydrate sources was determined by analyzing the

acid production by the isolate using bromocresol purple as an acid-base indicator (Gordon et al. 1974). ONPG discs were used to determine the β -galactosidase activity (HIMEDIA). Biochemical and characterization of different enzyme activity of strain AK62^T was also performed using the Vitek 2 GN system (bioMérieux) according to the manufacturer's protocol. The inoculum for Vitek analysis was prepared using sterile 2.0% (w/v) NaCl and incubated at 30 °C. Antibiotic sensitivity was determined using commercially available antibiotic discs (HIMEDIA, India) as described by Baek et al. (2011).

Determination of chemotaxonomic characteristics

Standardization of the physiological age of strains AK62^T, *M. stanieri* DSM 7027^T, *M. georgiense* DSM 11526^T, *M. halophilum* DSM 17586^T, *M. mangrovicola* DSM 27697^T and *M. lutimaris* DSM 22012^T were done based on the protocol (Sasser 1990) given by Sherlock Microbial Identification System (MIDI, USA). For cellular fatty acids analysis, all strains were grown on ZMA plates at 30 °C for two days and were in the comparable growth phase (i.e., exponential phase). Cellular fatty acid methyl esters (FAMES) were obtained from cells by saponification, methylation and extraction following the protocol of MIDI. Cellular FAMES were separated by GC (6890, Agilent) and analyzed using the Sherlock Microbial Identification System (MIDI-6890, Agilent with database TSBA6) according to the protocol described by MIDI (Sasser 1990). Extraction of polar lipids was performed as described in Bligh and Dyer (1959) and analyzed using two-dimensional TLC followed by spraying with suitable detection reagents (Komagata and Suzuki 1987). The spots identified as phospho-, amino- or glyco- lipids by spraying molybdenum blue, ninhydrin and α -naphthol reagents, respectively.

Results and discussion

Phylogenetic analysis

For phylogenetic assessment of strain AK62^T, 16S rRNA gene sequence was compared with the database of type strains with validly published prokaryotic names (Available online at <http://ezbiocloud.net/>), which revealed its affiliation to the genus *Marinobacterium* of the family *Alteromonadaceae*. Based on pair-wise sequence similarity, strain AK62^T was closest to *M. sediminicola*, *M. coralli* and *M. stanieri* with 96.9, 96.6 and 96.6% pair-wise sequence similarity respectively and with other members of the genus, *Marinobacterium* is between 92.3 to 96.2% pair-wise sequence similarity. These values are below the proposed species threshold of 98.7%, but above the threshold for a separate genus of 94.5% (Yarza et al. 2014), indicating that strain AK62^T represents a distinct species in the genus *Marinobacterium*. There were a total of 1272 positions in

the final dataset. Phylogenetic analysis based on a neighbour-joining tree further revealed clear affiliations of the novel isolate with the genus *Marinobacterium* and clustered with *M. stanieri*, *M. sediminicola* and *M. coralli* cluster and *M. maritimum* and together clustered with *M. georgiense*, *M. iners* and *M. halophilum* cluster (Fig. 1). Similar topology was observed in maximum parsimony tree but slight variation in clustering in maximum likelihood tree observed, i.e., *M. stanieri* clustered with AK62^T, *M. sediminicola* and *M. coralli* cluster and *M. maritimum*.

Genomic analyses

OrthoANI values of strain AK62^T and other closely related species are shown in Fig. 2, results here showed OrthoANI values are less than defined demarcation cut off of 95-96% for the delineation of novel species (Kim et al. 2014; Konstantinidis and Tiedje 2005). The digital DNA-DNA hybridization (dDDH) values of the strain AK62^T with other closely related species were 20.40%, 21.10%, 22.20% with *Marinobacterium halophilum* DSM 17586^T, *Marinobacterium sediminicola* CGMCC 1.7287^T and *Marinobacterium stanieri* DSM 7027^T, respectively. The DDH values of the strain AK62^T with type strains are well below the standard cut-off (<70%) for species delineation (Wayne et al. 1987). The phylogenomic tree is shown in Fig. 3 and Supplementary Fig. S1 confirmed the evolutionary novelty of the strain AK62^T among the members of the genus *Marinobacterium* and clustering is similar to Fig. 1.

Comparison of genotypic features of strain AK62^T with closely related type strains was given in Table 1. The draft genome of AK62^T yielded a genome of 3,335,703 bp in length after assembly with N50 value 125046, and the assembly produced 49 contigs. All contigs were larger than 1661 bp and the largest was 261,172 bp. The calculated G + C % of the bacterium was 58.2%, which was closest to the experimentally deduced value of 58.8 mol%. Annotation of the genome consisted of a total of 3192 genes, out of which CDSs were 3127 genes and total coding genes are 3114. A total of 65 RNA genes are present. Out of which 55 tRNA genes, 5 rRNA genes (three genes of 23S and one each of 5S and 16S) and 5 non-coding RNA genes are present. The annotation also consisted of 47 genes for different stress responses, including 9 genes for cell division-ribosomal stress proteins cluster, 3 genes for detoxification, 7 genes for the heat shock response, 1 gene for the nitrosative stress, 9 genes for the oxidative stress response, 5 genes for periplasmic stress response, 5 genes for SigmaB stress response regulation, and 8 genes for universal stress protein. Besides, there were 35 genes for resistance against antibiotics and toxic compounds, including 2 genes for resistance against arsenic, 1 gene for resistance against chromium compounds, 13 genes for cobalt–zinc–cadmium resistance, 2 genes for resistance against fluoroquinolones, 1 gene for glyoxalase/bleomycin resistance, 1 gene for mercury resistance, 11 genes for multidrug resistance efflux pumps, 2 genes

for small multidrug resistance, 1 gene for Polymyxin resistance and 1 gene for Tetracycline resistance. The cells AK62^T are gram-negative which is supported by the presence of 26 genes related to cell wall and capsule. It contains four genes related to Gram-Negative cell wall components and no genes related to the Gram-Positive cell wall components. The cells of AK62^T are motile and in the genome 75 genes related to motility and chemotaxis were present. It does not have any genes related to photosynthesis supports its heterotrophic nature of growth/metabolism. AK62^T genome comprises several genes related to carbohydrate metabolism especially genes related to Glycolysis/Gluconeogenesis, citrate cycle, pentose phosphate pathway, pentose and glucuronate interconversions, fructose, mannose, galactose, ascorbate, aldarate, starch, sucrose, amino sugars, nucleotide sugars, pyruvate, glyoxylate, dicarboxylate, propionate, butanoate, C5-branched dibasic acid and inositol phosphate metabolism. The energy generation through oxidative phosphorylation. Pairwise dDDH values and percent G+C difference of strain AK62^T with selected type strain genomes were given in Supplementary Table S1. Dataset of selected type strains to compare strain AK62^T were given in Supplementary Table S2.

Pangenome analysis

The BPGA pipeline was used to analyze the orthologous cluster for strain AK62^T and its other closely related species using standard protein sequences available on NCBI as input file (Fasta format). The functions of the core genes are mostly related to translation, ribosomal structure and biogenesis. The part of core genes along with most accessory genes are dedicated to transcription, amino acid transport and metabolism, general functions and proteins coding for the unknown functions. Most of the unique genes are involved in the signal transduction mechanisms, general functions, cell wall and membrane biogenesis, and a few unknown functions (Supplementary Fig. S1). The BPGA pipeline was used to analyze the KEGG distribution plot of the gene pool of strain AK62^T and its related species. The pool's core genes and accessory genes are involved in amino acid metabolism, carbohydrate metabolism, energy and metabolism of cofactors and vitamins, and translation. The few of the cell motility, metabolism of energy, lipid, membrane transport, in signal transduction, xenobiotic metabolism and biodegradation. The unique genes are involved in carbohydrate metabolism, cell motility, drug resistance, infectious diseases, metabolism of lipids, membrane transport and signal transduction (Supplementary Fig. S1b).

The comparative genomics of genera investigated here revealed the open pangenome (Supplementary Fig. S2) as the number of accessory and unique genes increases with the addition of genomes. For the genus *Marinobacterium*, the pangenome and core genome contains 19,280 and 932 genes, respectively. The pangenome analysis showed that strain AK62^T has 268 unique genes in its

genome. We found that most of the unique genes codes for the proteins involved in chemotactic movement will help the bacterium move towards attractants (nutrients) and help the bacterium re-orient itself in a new direction on detection of repellent (decline nutrients, inhibitors, toxins etc.). The pangenome is larger than the core genome, and the size of the pangenome increases with the addition of a new genome.

Polyamine analysis

We annotated the genome sequence of AK62^T and compared it with other closely related species, and we have found the genes associated with the spermidine synthesis pathway in all species. The spermidine synthase gene (*speE*) catalyzes the final synthesis of spermidine from putrescine. The *speE* gene of strain AK62^T is 90.53%, 85.21% and 86.57%, similar to the *speE* gene of *Marinobacterium staineri*, *Marinobacterium georgiense* and *Marinobacterium halophilum* respectively.

Morphological and physiological analyses

The cells of strain AK62^T were Gram-staining-negative, rod-shaped, 0.6-0.8 µm wide and 2.0–3.0 µm in length (Supplementary Fig. S3), aerobic, and motile with a single polar flagellum. Colonies on ZMA were circular, off-white, shiny, moist, translucent, 1–2 mm in diameter, flat, with an entire margin. The temperature growth range for AK62^T was 20–42 °C, with optimum growth at 30-37 °C. Growth was observed at 0.5–18% NaCl (w/v) with optimum growth at 2-4% NaCl; and pH range 6–11, with optimum growth at pH 7-8. Strain AK62^T was sensitive to antibiotics (µg/disc) chloramphenicol (30), ciprofloxacin (10), cefprozil (30), cephalexin (30), cefazolin (30), clarithromycin (15), cefmetazole (30), gentamicin (10), polymyxin-B (300), spectinomycin (100); resistance streptomycin (25), bacitracin (8), kanamycin (30), ceftazidime (30), ampicillin (25), penicillin-g (2 units), amoxicillin (30), novobiocin (30) and vancomycin (30); and intermediate to neomycin (30) and tetracycline (30). The genomic DNA G+C content of strain AK62^T was 58.8 % was within the range of values (54.9–62.1 %) reported for established *Marinobacterium* species (Supplementary Table S2). The list of distinguishing features of strain AK62^T with the reference strains is provided in Table 1.

Chemotaxonomic analyses

The cellular fatty acid composition of strain AK62^T showed a spectrum of 6 fatty acids with a pronounced dominance of (>10 %) of C_{16:0}, C_{18:1 ω7c}, and summed feature 3 (C_{16:1 ω7c} and/or C_{16:1 ω6c} and/or iso-C_{14:0 3-OH}) (Table 2). The fatty acids were dominated by saturated and unsaturated fatty acids. Strain AK62^T showed similar fatty acid compositions with all the type strains compared

in terms of major fatty acids such as C_{16:0}, C_{18:1} ω7c, and summed feature 3. Strain AK62^T differed with *M. lutimaris* DSM 22012^T in terms of major fatty acid C_{10:0} 3-OH. Strain AK62^T differed with all type strains compared in the present study concerning the absence of several fatty acids (Table 2). Polar lipids of strain AK62^T included diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), one unidentified aminophospholipid (APL1), three unidentified phospholipids (PL1-3) and one unidentified lipid (L1) (Fig. 2a). However, the reference strains *M. stanieri* DSM 7027^T, *M. georgiense* DSM 11526^T, *M. halophilum* DSM 17586^T, *M. mangrovicola* DSM 27697^T and *M. lutimaris* DSM 22012^T comprises nearly similar composition (PE, PG and DPG) but differed with strain AK62^T either in the absence of APL1, PL1-3, L1 or the presence of APL2, PL4, PL5, L2 to L5 (Fig. 4).

Based on the phylogenetic analysis a comparison was made between the characteristics of strain AK62^T with the type strains *M. stanieri* DSM 7027^T, *M. georgiense* DSM 11526^T, *M. halophilum* DSM 17586^T, *M. mangrovicola* DSM 27697^T and *M. lutimaris* DSM 22012^T of the family *Alteromonadaceae* (Table 1) and some differences were observed. Strain AK62^T and type strains exhibited similar characteristics concerning Gram-staining-negative, cell shape, motility, H₂S and indole production, oxidase, catalase and several enzymatic activities, carbon substrate utilization and sensitivity to different antibiotics. However, strain AK62^T differed from the type strains in several characteristics such as cell size, temperature growth range and optimum, salinity growth range and optimum, pH growth range and optimum, hydrolysis of starch, Tween 20/40/60/80 and urea, utilization of several carbon substrates, lysine decarboxylase, ornithine decarboxylase and tyrosine arylamidase activities, sensitivity to different antibiotics, G+C content, fatty acid and polar lipid composition (Table 1). Based on the observed phenotypic, chemotaxonomic characteristics and phylogenetic analysis, strain AK62^T is sufficiently different from the existing species in the genus *Marinobacterium*, for which the name *Marinobacterium alkalitolerans* sp. nov. is proposed.

Description of *Marinobacterium alkalitolerans* sp. nov.

Marinobacterium alkalitolerans (al.ka.li.to'le.rans. N.L. n. *alkali*, alkali; L. pres. Part. *tolerans*, tolerating; N.L. part. Adj. *alkalitolerans*, alkali-tolerating).

The major characteristics of *Marinobacterium alkalitolerans* strain AK62^T are similar to the description provided for the genus. Cells are Gram-staining-negative, aerobic, motile rods, 0.6-0.8 μm wide and 2.0–3.0 μm in length. Colonies on ZMA were circular, off-white, shiny, moist, translucent, 1–2 mm in diameter, flat, with an entire margin. Growth occurs at 20-42 °C (optimum 30-37 °C) at 0.5–18% NaCl (w/v) (optimum 2-4%) and at pH 6-11 (optimum 7-8). Positive for

nitrate reduction, methyl red test; oxidase, catalase, L-proline arylamidase, tyrosine arylamidase, and urease activities; fructose, rhamnose, sorbose, and xylose utilization; weak growth with lactose; L-lactate alkalisation. Negative for phenylalanine deamination, H₂S and indole production, Voges Proskauer's test; aesculin, gelatin, ONPG, starch, Tween 40, 60 and 80 hydrolysis; lysine and ornithine decarboxylase, Ala-Phe-Pro-arylamidase, L-pyrrolidiny-arylamidase, β -galactosidase, β -N-acetyl glucosaminidase, glutamyl arylamidase, γ -glutamyl-transferase, β -glucosidase, β -xylosidase, β -alanine arylamidase, lipase, α -glucosidase, β -N-acetylgalactosaminidase, α -galactosidase, phosphatase, glycine arylamidase, β -glucuronidase and glu-gly-arg-arylamidase activities; arabinose, adonitol, rhamnose, cellobiose, citrate, coumarate, malonate, melibiose, raffinose, glucose, maltose, mannose, inulin, dextrose, galactose, glycerol, sodium gluconate, salicin, inositol, sorbitol, mannitol, cellobiose, dulcitol, adonitol, arabitol, erythritol, α -methyl-D-glucoside, melezitose, α -methyl-D-mannoside, xylitol, L-arabitol, D-arabinose, D-cellobiose, D-glucose, D-maltose, D-mannitol, D-mannose, palatinose, D-sorbitol, sucrose, D-tagatose, D-trehalose, citrate (sodium), malonate, 5-keto-D-gluconate, L-arabinose, L-histidine, L-lactate, L-malate, trehalose and N-acetyl- β -D-mannosamine utilization; fermentation glucose, succinate alkalisation, 0/129 resistance and ELLMAN reaction. The major fatty acids included C_{16:0}, C_{18:1} ω 7c, and summed feature 3 (C_{16:1} ω 7c and/or C_{16:1} ω 6c and/or iso-C_{14:0} 3-OH). Polar lipids included diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, one unidentified aminophospholipid, three unidentified phospholipids and one unidentified lipid. Polyamine includes Spermidine (Genome).

The type strain AK62^T (= MTCC 12102^T = JCM 31159^T = KCTC 52667^T) was isolated from the green algal mat and water collected from saltpan, Kakinada, Andhra Pradesh, India. The DNA G+C content of the strain AK62^T was found to be 58.8 mol%. The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and genome sequence of type strain AK62^T are LN558833 and JACVEW000000000, respectively.

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Code availability: .here is a software application or custom code developed)

Authors' contributions: Anil Kumar P., Srinivas T. N. R.: Conceptualization, designed study, analyzed data, methodology, writing - original draft, Performed research: Vasundhara Gupta, Gunjan Vasudeva, Sudha Rani P, Nishant Kashyap, Swarnaprava Behera, Sampath Kumar G.

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Table 1. List of distinguishing features of strain AK62^T with the closely related type strains

1. Strain AK62^T; 2. *M. stanieri* DSM 7027^T; 3. *M. georgiense* DSM 11526^T; 4. *M. halophilum* DSM 17586^T; 5. *M. mangrovicola* DSM 27697^T; 6. *M. lutimaris* DSM 22012^T.

Data of taxa from the present study. All strains were Gram-staining-negative, rod-shaped, motile, positive for catalase, oxidase and L-proline arylamidase activities. All strains were negative for phenylalanine deamination, H₂S and indole production, Voges proskauer's test, aesculin, gelatin, ONPG hydrolysis, Ala-Phe-Pro-arylamidase, L-pyrrolydonyl-arylamidase, β-galactosidase, β-N-acetyl glucosaminidase, glutamyl arylamidase, γ-glutamyl-transferase, β-glucosidase, β-xylosidase, β-alanine arylamidase, lipase, α-glucosidase, β-N-acetylgalactosaminidase, α-galactosidase, phosphatase, glycine arylamidase, β-glucuronidase and glu-gly-arg-arylamidase activities; arabinose, adonitol, rhamnose, cellobiose, melibiose, raffinose, glucose, maltose, dextrose, galactose, glycerol, salicin, dulcitol, adonitol, arabitol, erythritol, α-methyl-D-glucoside, melezitose, α-methyl-D-mannoside, xylitol, L-arabitol, D-cellobiose, D-glucose, D-maltose, D-mannitol, D-mannose, palatinose, D-sorbitol, D-tagatose, D-trehalose, citrate (sodium), malonate, 5-keto-D-gluconate, L-histidine, L-lactate, L-malate and N-acetyl-β-D-mannosamine utilization; fermentation glucose, succinate alkalisation, 0/129 resistance and ELLMAN reaction. All strains are sensitive to antibiotics (μg/disc) chloramphenicol (30), ciprofloxacin (10), gentamicin (10) and polymyxin-B (300), and spectinomycin (100) and resistance novobiocin (30) and vancomycin (30). +, positive; -, negative; w, weakly positive; I, intermediate; S, sensitive; R, resistance; NA, no data available.

Characteristics	1	2	3	4	5	6
Cell size (μm)	0.6-0.8 x 2.0-3.0	0.6-0.8 x 1.3-2.2	0.5-0.7 x 1.6-2.3	0.5-0.7 x 2.1-3.0	0.5-0.8 x 1.2-1.6	0.5-0.8 x 1.2-1.6
Temperature growth range (°C)	20-42	10-37	4-42	4-42	10-37	25-42
Optimum temperature (°C)	30-37	25-30	30-37	25-30	25-30	30-37
Salinity growth range (%)	0.5-18	0.5-8	0-12	2-12	0.5-12	2-10
Optimum NaCl concentration (% w/v)	2-4	2	0.5-2	2	2-4	2
pH growth range (Optimum)	6-11 (7-8)	5-12 (7)	5-10 (7-8)	5-10 (7)	5-11 (7-8)	5-10 (7)
Biochemical characteristics						
Lysine decarboxylase	-	+	-	+	-	-
Ornithine decarboxylase	-	+	-	+	-	-
Nitrate reduction	+	-	-	+	-	+
Methyl red	+	-	-	+	-	+
Hydrolysis of						
Starch	-	-	-	-	+	-
Tween 20/40/60/80	+/-/-/-	-/-/-/-	-/-/-/-	-/-/-/-	-/+/>+/+	+/-/-/-
Urease	+	-	-	-	+	+
Carbon source utilization						
Xylose	+	+	-	-	+	-
Trehalose	-	-	+	-	-	-
Lactose	W	-	+	+	-	-
Fructose	+	-	+	+	-	-
Sucrose	-	-	+	-	-	-
L-Arabinose	-	-	+	-	-	-
Mannose	-	-	+	-	-	-
Inulin	-	-	-	+	-	-
Sodium gluconate	-	-	-	+	-	-
Inositol	-	-	-	+	-	-
Characteristics	1	2	3	4	5	6
Sorbitol	-	-	+	+	-	-
Mannitol	-	-	+	-	-	-

Rhamnose	+	–	–	+	–	–
Cellobiose	–	–	–	+	–	–
D–Arabinose	–	–	–	+	–	–
Citrate	–	–	+	+	–	–
Malonate	–	–	–	+	–	–
Sorbose	+	–	–	+	–	–
Vitek GN information:						
Tyrosine arylamidase	+	–	–	+	–	+
Urease	+	+	–	+	+	+
L–Lactate alkalisation	+	+	+	+	+	–
Courmarate	–	–	+	–	–	–
Antimicrobial susceptibility ($\mu\text{g}/\text{disc}$)						
Tetracycline (30)	I	R	R	S	S	S
Streptomycin (25)	R	S	S	S	S	S
Bacitracin (8)	R	R	R	R	I	S
Cefprozil (30)	S	I	R	R	S	S
Cephalexin (30)	S	S	R	R	S	S
Cefazolin (30)	S	S	R	S	S	S
Clarithromycin (15)	S	S	R	R	S	S
Kanamycin (30)	R	I	S	S	S	S
Ceftazidime (30)	R	S	R	R	R	S
Ampicillin (25)	R	R	R	S	R	S
Cefmetazole (30)	S	R	R	I	R	S
Neomycin (30)	I	I	S	S	I	S
Penicillin–G (2 Units)	R	R	R	R	R	S
Amoxicillin (30)	R	R	R	S	R	S
G+C mol%*	58.8	56.3	54.9	56.3	57.0	58.0
Genomic characteristics						
Genome size (bp)	3335705	4680330	3925261	3653655	4979947	5568333
G + C (%)	58.2	55.6	54.9	56.0	57.1	57.4
Scaffold N50	125046	388460	167945	130350	922765	869882
Number of contigs	49	24	51	47	15	21
Completeness (%)	100	100	100	100	100	100
Total genes	3192	4409	3742	3443	4518	5129
All protein-coding genes	3114	4328	3625	3326	4414	5002
Protein-coding genes/Mb	3.1	4.3	3.6	3.3	4.4	5.0
Hypothetical proteins	579	1371	1125	913	1204	1496
Total RNAs	65	81	69	78	75	82
tRNAs	55	75	56	71	68	69
16S rRNA genes	1	1	1	1	-	1

*Data were taken from Baumann et al (1983); González et al (1997); Chang et al (2007); Alfaro–Espinoza and Ullrich (2014); Kim et al (2010).

Table 2. Comparisons of the fatty acid composition of strain AK62^T with the related type strains

1. Strain AK62^T; 2. *M. stanieri* DSM 7027^T; 3. *M. georgiense* DSM 11526^T; 4. *M. halophilum* DSM 17586^T; 5. *M. mangrovicola* DSM 27697^T; 6. *M. lutimaris* DSM 22012^T.

Data of taxa are from the present study. Results are presented as a percentage of the total fatty acids. Fatty acids amounting to 10% or more of the total fatty acids are in bold. ND, not detected. Values of less than 0.5% for all strains are not shown. tr, traces (<0.5%).

Fatty acid	1	2	3	4	5	6
C _{10:0}	2.2	1.0	3.0	3.3	0.7	3.1
C _{10:0} 3OH	3.1	5.9	2.2	2.9	3.5	29.0
C _{12:0}	2.2	4.7	2.4	3.1	3.9	9.8
C _{12:0} 2OH	ND	ND	ND	ND	1.1	6.6
C _{14:0}	ND	0.5	0.6	1.0	tr	ND
C _{14:1} ω5 <i>c</i>	ND	ND	tr	0.7	ND	ND
iso-C _{15:0}	ND	ND	1.1	ND	tr	ND
anteiso-C _{15:0}	ND	tr	1.1	0.5	tr	ND
C _{16:0}	25.0	18.2	27.2	27.2	21.4	16.1
iso-C _{16:0}	ND	tr	0.6	ND	tr	ND
anteiso-C _{17:0}	ND	tr	0.6	ND	tr	ND
iso-C _{17:1} ω5 <i>c</i>	ND	tr	ND	ND	0.7	ND
C _{18:0}	ND	tr	0.9	0.8	tr	ND
C _{18:1} 2OH	ND	ND	ND	1.0	ND	ND
C _{18:1} ω7 <i>c</i>	41.5	35.6	38.8	20.0	39.9	21.2
C _{18:1} ω7 <i>c</i> 11-methyl	ND	0.6	ND	ND	ND	ND
C _{18:3} ω6,9,12 <i>c</i>	ND	tr	tr	1.1	1.3	ND
Sum in feature 3	26.1	29.1	20.2	38.6	21.9	14.2

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} ω7*c* and/or C_{16:1} ω6*c* and/or iso-C_{14:0} 3-OH.

Supplementary Table S1. Pairwise dDDH values and percent G+C difference of strain AK62^T with selected type strain genomes. The dDDH values are provided with the formula d4; the sum of all identities found in HSPs divided by overall HSP length.

Reference genome	d4 DDH	G+C difference
<i>Marinobacterium stanieri</i> DSM 7027 ^T	22.2	2.54
<i>Marinobacterium halophilum</i> DSM 17586 ^T	20.4	2.16
<i>Marinobacterium lutimaris</i> DSM 22012 ^T	20.4	0.7
<i>Marinobacterium nitratireducens</i> CGMCC 1.7286 ^T	20.2	3.93
<i>Marinobacterium georgiense</i> DSM 11526 ^T	20.2	3.23
<i>Marinobacterium litorale</i> DSM 23545 ^T	20.2	1.74
<i>Marinobacterium mangrovicola</i> DSM 27697 ^T	20.0	1.07
<i>Marinobacterium zhoushanense</i> CGMCC 1.15341 ^T	19.9	0.27
<i>Amphritea balenae</i> JCM 14781 ^T	19.5	10.44
<i>Marinobacterium profundum</i> PAMC 27536 ^T	19.5	0.97
<i>Marinobacterium jannaschii</i> DSM 6295 ^T	19.1	2.94
<i>Nitrincola alkalilacustris</i> ZV-19 ^T	19.0	4.67
<i>Pseudomonas fluvialis</i> CCM 8778 ^T	18.0	4.5
<i>Halomonas alimentaria</i> DSM 15356 ^T	18.0	8.15
<i>Pseudomonas alcaligenes</i> NBRC 14159 ^T	17.6	6.62

Supplementary Table S2. Strains in the dataset to compare with AK62^T.

Species cluster	Subspecies cluster	Reference genome	Genome size in base pairs	Percent G+C	No. of proteins	Assembly accession No.
1	0	<i>Marinobacterium litorale</i> DSM 23545 ^T	4380 194	56.4	4223	GCA_000428985
2	1	<i>Marinobacterium stanieri</i> DSM 7027 ^T	4679 482	55.6	4332	GCA_900155945
3	2	<i>Nitrincola alkalilacustris</i> ZV-19 ^T	4058 106	53.5	3652	GCA_008973755
4	3	<i>Marinobacterium jannaschii</i> DSM 6295 ^T	5176 235	55.2	4692	GCA_000620085
5	4	<i>Marinobacterium halophilum</i> DSM 17586 ^T	3653 180	56.0	3387	GCA_003014615
6	5	<i>Marinobacterium mangrovicola</i> DSM 27697 ^T	4979 440	57.1	4466	GCA_004339595
7	6	<i>Marinobacterium georgiense</i> DSM 11526 ^T	3922 811	54.9	3713	GCA_900107855
8	7	<i>Pseudomonas alcaligenes</i> NBRC 14159 ^T	4823 390	64.8	4531	GCA_000467105
9	8	<i>Marinobacterium zhoushanense</i> CGMCC 1.15341 ^T	4722688	58.4	4342	GCA_014641945
10	9	<i>Marinobacterium nitratireducens</i> CGMCC 1.7286 ^T	5538044	62.1	3092	GCA_014645375
11	10	<i>Pseudomonas fluvialis</i> CCM 8778 ^T	3304463	62.65	3092	GCA_014635625
12	11	<i>Amphritea balenae</i> JCM 14781 ^T	4663163	47.7	4086	GCA_014646975
13	12	<i>Marinobacterium profundum</i> PAMC 27536 ^T	5637 742	57.2	4981	GCA_001528745
14	13	<i>Marinobacterium lutimaris</i> DSM 22012 ^T	5568 156	57.4	5064	GCA_001528745
15	14	<i>Halomonas alimentaria</i> DSM 15356 ^T	3281 998	66.3	2997	GCA_009902005
16	15	<i>Marinobacterium alkalitolerans</i> AK62 ^T	3335703	58.2	3149	

Legends

Fig. 1. Neighbour-joining tree showing phylogenetic relationships of strain AK62^T with all members of the genus *Marinobacterium* and type species of the other genera of the family *Alteromonadaceae* based on 16S rRNA sequence dataset. Filled circles indicate branching topologies that are common in all three trees constructed. Open circles indicate branching topologies that are common in any two trees. Bootstrap support values of more than 70% are shown at the nodes. *Spongiispira norvegica* Gp_4_7.1^T (AM117931) was used as an outgroup taxon. Scale bar represents 0.01 substitution per nucleotide position.

Fig. 2. Heatmap generated with orthoANI values calculated from OAT software. The dataset contained the genome of 9 type strains of closely related species to strain AK62^T with its assembly accession number is shown in Supplementary (Table S2).

Fig. 3. Phylogenomic tree of strain AK62^T inferred from GBDP distances calculated from genome sequences.

Fig. 4. Two-dimensional thin-layer chromatogram of the total polar lipids of the strain AK62^T (a), *M. stanieri* DSM 7027^T (b), *M. georgiense* DSM 11526^T (c), *M. halophilum* DSM 17586^T (d), *M. mangrovicola* DSM 27697^T (e) and *M. lutimaris* DSM 22012^T (f) after spraying the plates with molybdato-phosphoric acid. Abbreviations: PE, phosphatidylethanolamine; PG, phosphatidylglycerol; DPG, diphosphatidyl glycerol, APL, unidentified aminophospholipid, PL, unidentified phospholipid, UL, unidentified lipid.

Supplementary Fig. S1. (A) The cluster of orthologous groups (COG) analysis of thirteen genomes along with the genome of AK62^T with the comparison of COG distribution between the core, accessory and unique genes. The X-axis indicates the COG categories and Y-axis denotes the percent of genes present in each category and (B) The KEGG distribution plot of AK62^T and its closely related species between core, accessory and unique genes. The X-axis indicates the KEGG categories and Y-axis indicates the per cent of genes in each category.

Supplementary Fig. S2 (A) The Core and pangenome plots of genera *Marinobacterium*. The orange and purple coloured lines indicate the change in the number of pangenome gene and core genome gene with the sequential addition of the new genome, and (B) The ven diagram with a distribution of core, accessory and unique genes among the individual genomes under comparative study. Strain AK62^T; *Amphritea atlantica* DSM 18887^T; *Marinobacterium aestuarii* ST58-10^T; *Marinobacterium halophilum* DSM 17586^T; *Marinobacterium lutimaris* DSM 22012^T; *Marinobacterium mangrovicola* DSM 27697^T; *Marinobacterium profundum* PAMC 27536^T; *Marinobacterium rhizophilum* DSM 18822^T; *Marinobacterium sedimanicola* CGMCC 1.7287^T; *Marinobacterium stanieri* DSM 7027^T; *Neptuniibacter caesariensis* MED92^T; *Neptunomonas antarctica* DSM 22306^T; *Neptunomonas japonica* DSM 18939^T; *Nitriicola alkalilacustris* ZV-19^T.

Supplementary Fig. S3. Electron micrograph of a negatively stained cell of strain AK62^T. Bar, 0.5 μ m.

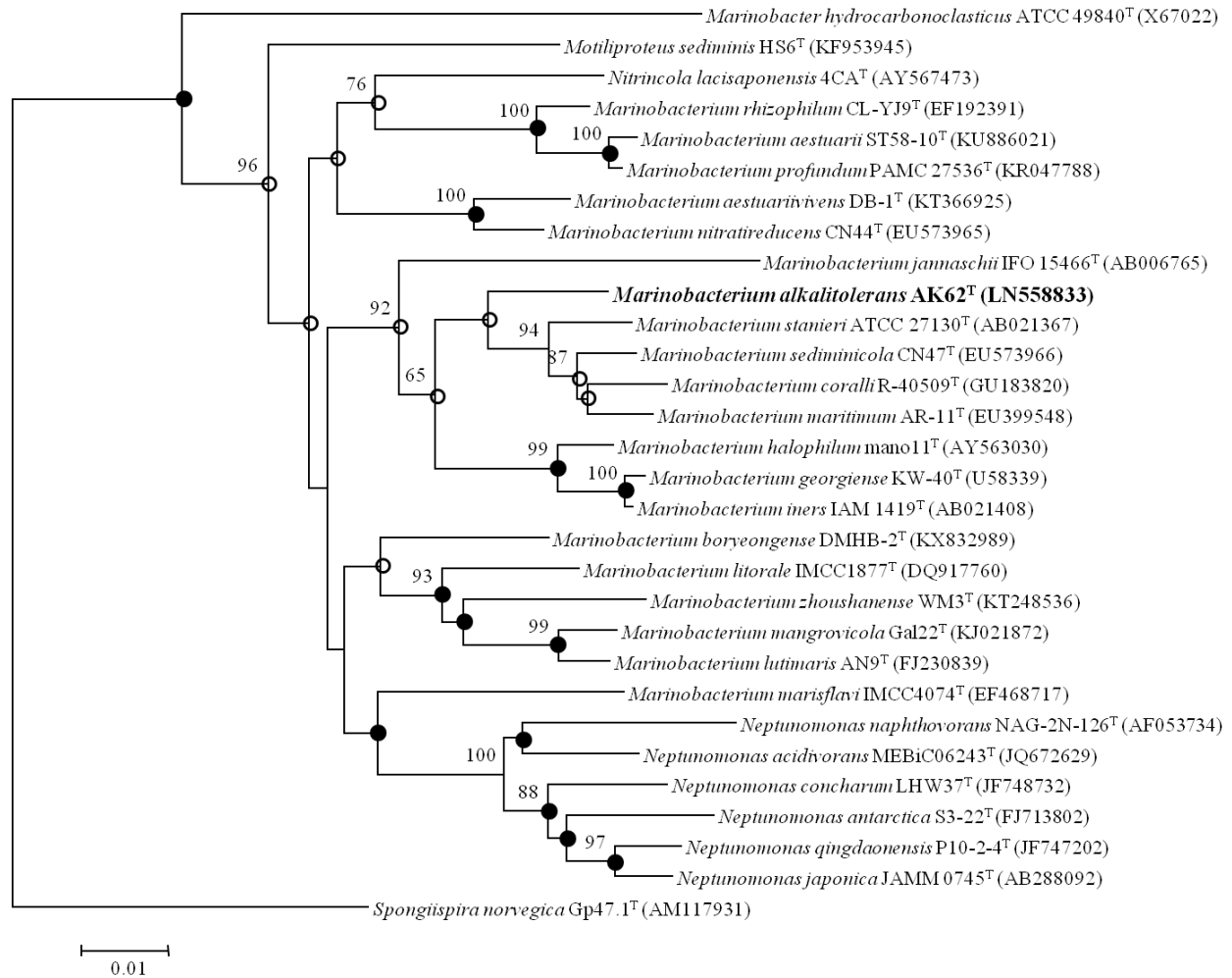


Fig. 1

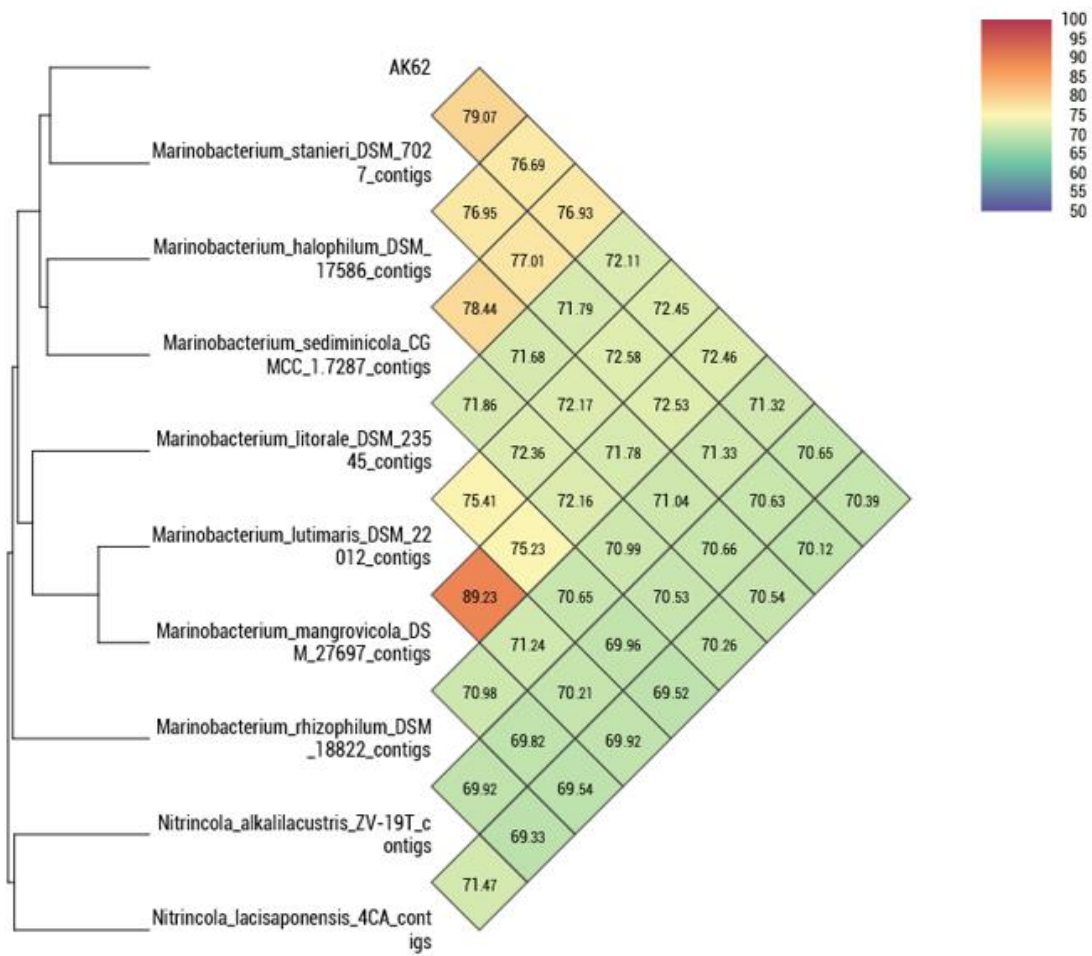


Fig. 2

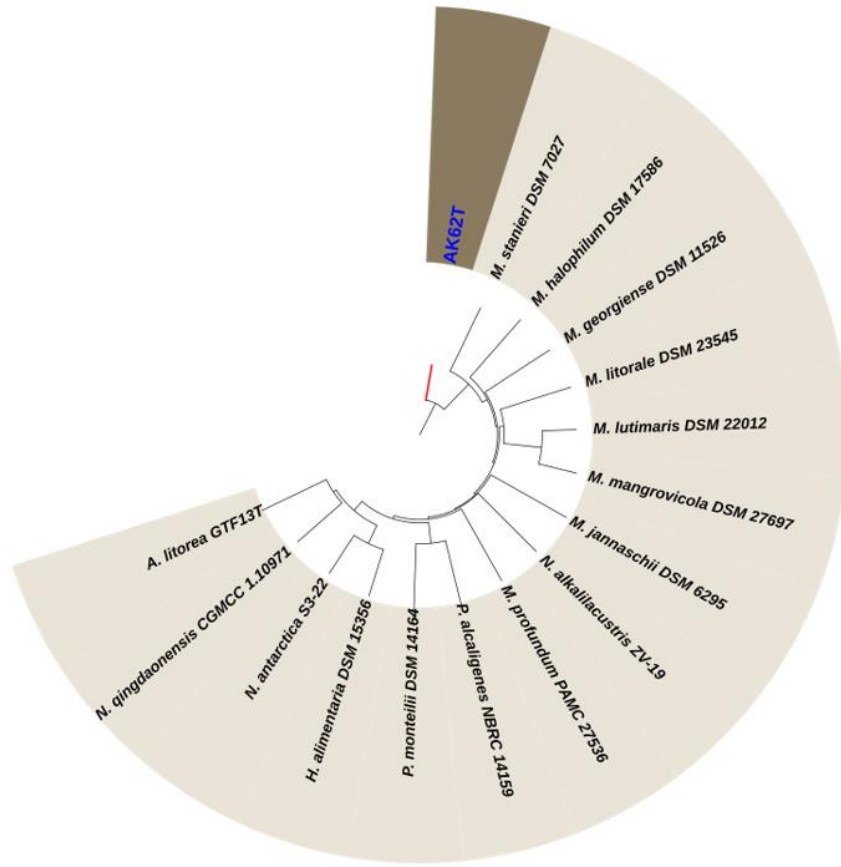


Fig. 3

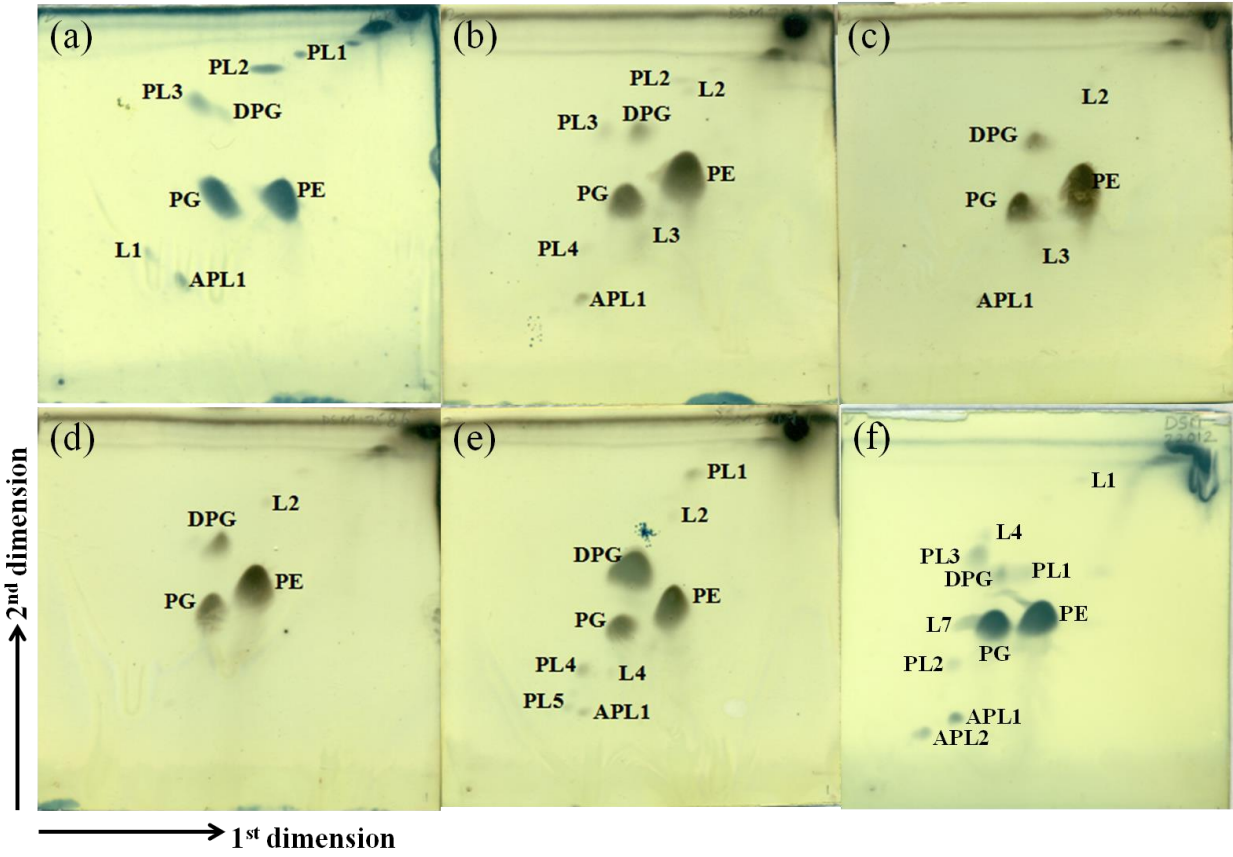
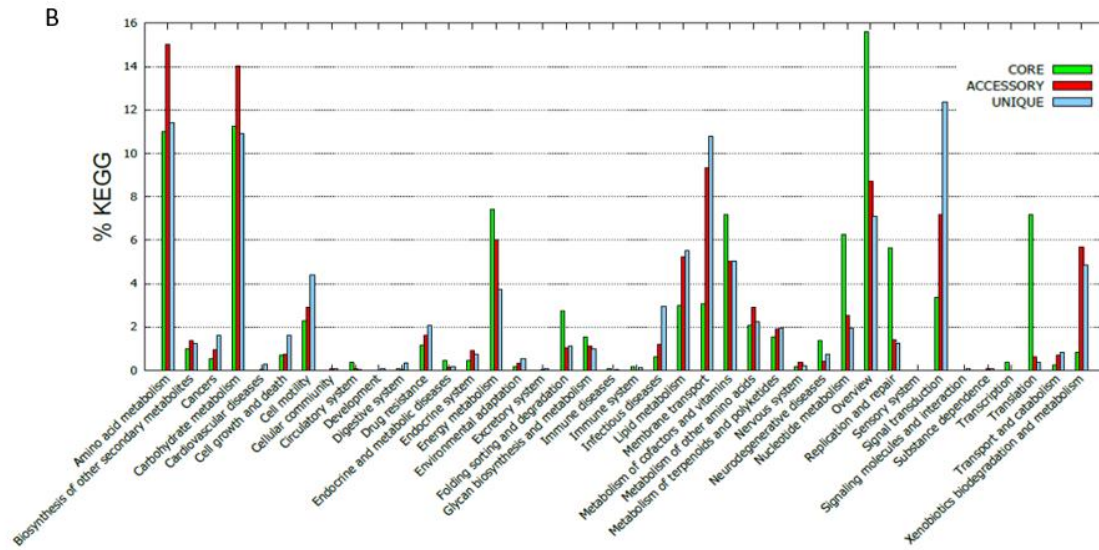
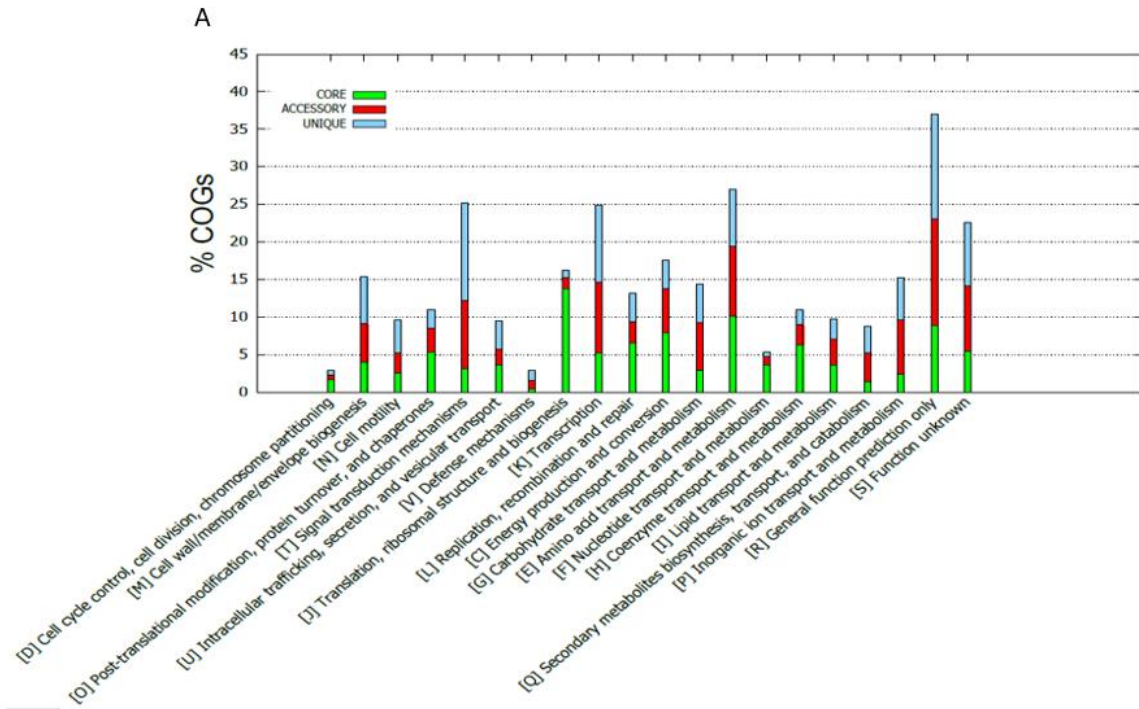
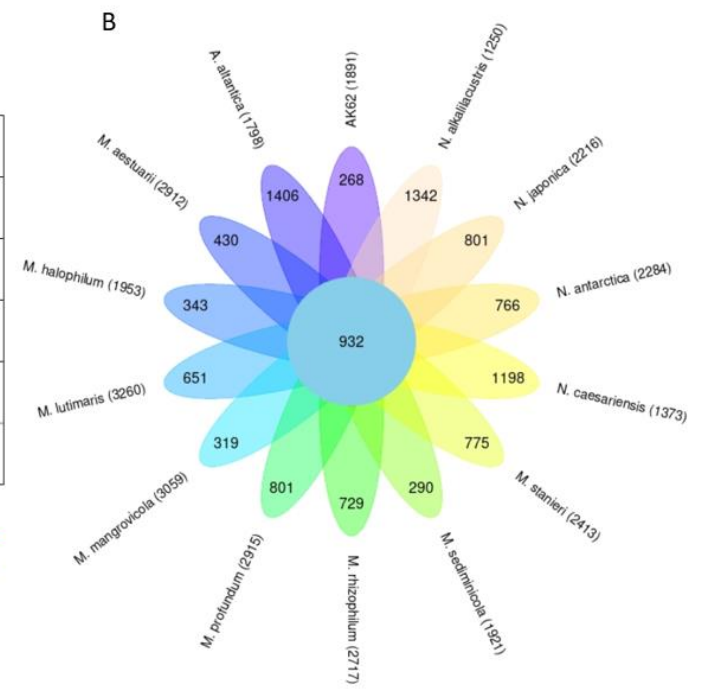
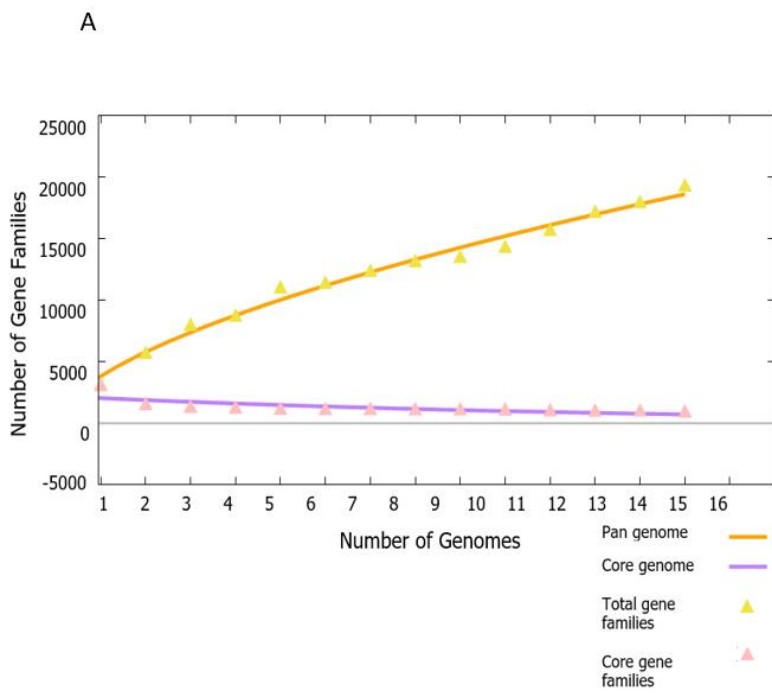


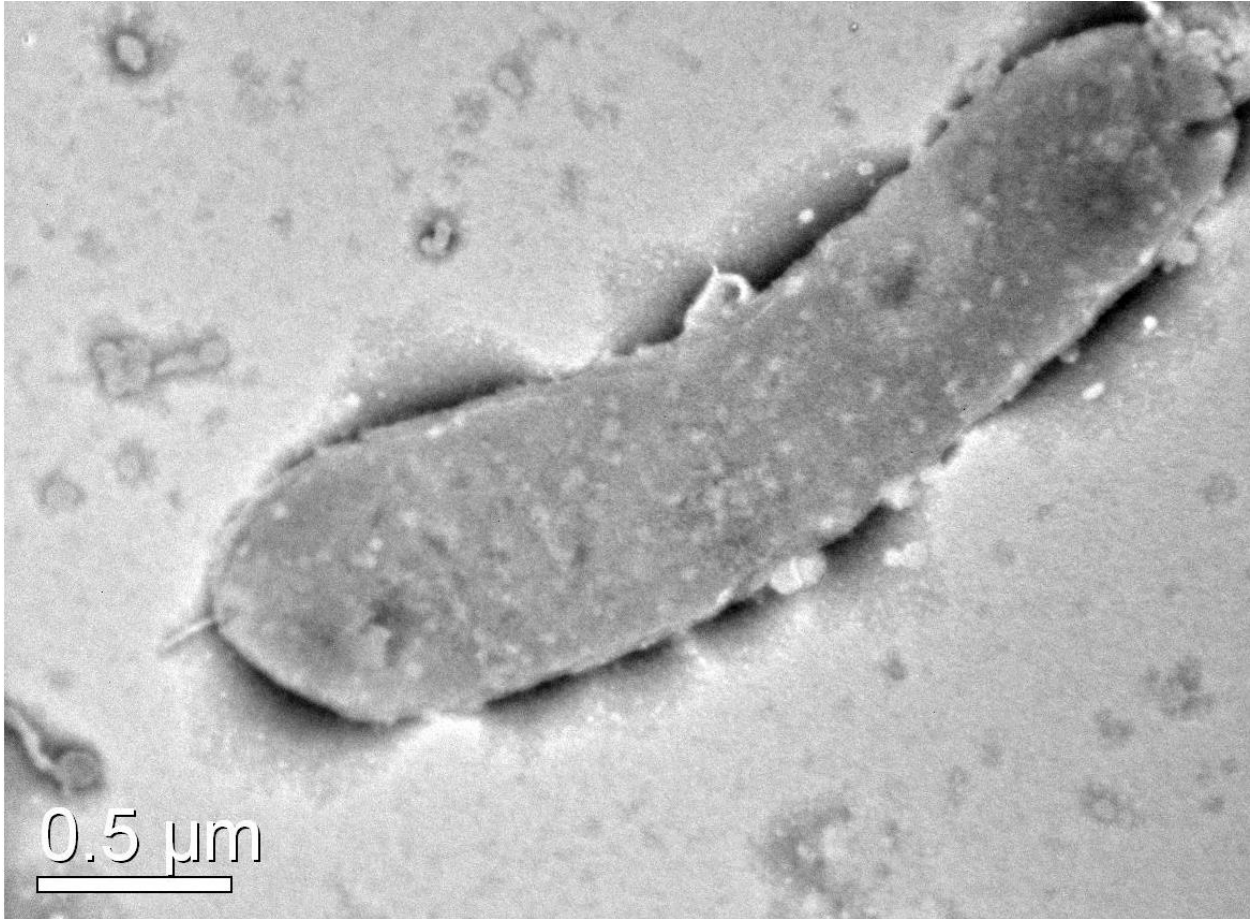
Fig. 4



Supplementary Fig. S1



Supplementary Fig. S2



Supplementary Fig. S3