Corrigendum: Saliniramus fredricksonii gen. nov., sp. nov., a heterotrophic halophile isolated from Hot Lake, Washington, a member of a novel lineage (Salinarimonadaceae fam. nov.) within the order Rhizobiales, and reclassification of the genus Salinaronas Liu et al. 2010 into Salinarimonadaceae

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Abstract

There was an error in the proposed genus name in the published article, in that the genus ‘Salinivirga’ was effectively published while this article was in review. Therefore, the genus ‘Salinivirga’ should be replaced with ‘Saliniramus’. For the convenience of future readers, we have included the complete corrected article below, in which all occurrences of the incorrect genus name have been amended:

A halophilic bacterial strain, HL-109T, was isolated from the unicellular bacterial consortium UCC-O, which was obtained from the photosynthetic mat of Hot Lake (Washington, USA). A polyphasic approach using phenotypic, genotypic and chemotaxonomic data was used to classify the strain within the order Rhizobiales. The organism stained Gram-negative and was a moderate thermophile with a growth optimum of 45 °C. It was obligately aerobic, heterotrophic and halophilic, growing in both NaCl and MgSO4 brines. The novel isolate had a polymorphic cellular morphology of short rods with occasional branching, and cells were monotrichous. The major fatty acids detected were C18:1ω7ω, C18:0, C16:0 and C18:ω7ωω. Phylogenetic analysis of the 16S rRNA gene placed the strain in the order Rhizobiales and it shared 94 % identity with the type strain of its nearest relative, Salinaronas ramus. Morphological, chemotaxonomic and phylogenetic results did not affiliate the novel organism with any of the families in the Rhizobiales; therefore, HL-109T is representative of a new lineage, for which the name Saliniramus fredricksonii gen. nov., sp. nov. is proposed, with the type strain HL-109T (=JCM 31876T=DSM 102886). In addition, examination of the phylogenetics of strain HL-109T and its nearest relatives, Salinaronas ramus and Salinaronas rosea, demonstrates that these halophiles form a clade distinct from the described families of the Rhizobiales. We further propose the establishment of a new family, Salinarimonadaceae fam. nov., to accommodate the genera Saliniramus and Salinaronas (the type genus of the family).
The order *Rhizobiales* is a phenotypically-diverse taxonomic group defined primarily by phylogenetic analyses of SSU rRNA gene sequences [1, 2]. Cultured representatives tend to be aerobic, heterotrophic, mesophilic rods isolated from soils and aquatic environments, although the group includes atypical physiologies such as methanotrophs and carbon monoxide oxidizers. In addition, many members are found in association with plants [3, 4] and animals [5, 6], either as commensals or as pathogens, and others have been identified in association with various types of microbial autotrophs [7–9]. A number of species are symbiotic with legumes, exchanging fixed nitrogen for carbon with the host plant [10], and others function as the endosymbionts of lichens [11]. As such, the ecological niches occupied by members of the *Rhizobiales* are diverse, and they may tend towards close associations with other organisms in their environments.

Strain HL-109<sup>T</sup> was isolated from a 28-day-old biofilm of the unicyanobacterial consortium UCC-O [12]. The consortium itself was obtained from the laminated photosynthetic mat of Hot Lake, Washington, USA [13]. Strain HL-109<sup>T</sup> was isolated by repeated subcultivation of single colonies on agar plates of Hot Lake Heterotroph (HLH) medium at 30°C in the dark. Cultivation and experimentation were performed using HLH-700 broth (HLH containing 700 mM MgSO<sub>4</sub> instead of the 400 mM described [12]) or HLH solid media as previously described [12], both amended with Wolfe’s vitamins [14], at 43°C unless otherwise specified.

Electron microscopy was performed using cultures grown for 3 days at 43°C. The cells were concentrated by centrifugation at 5000 × g until the optical density at 600 nm (OD<sub>600</sub>) of the supernatant approached 0.0 (~60 min), assessed using a Smart Spec Plus spectrophotometer (Bio-Rad). The concentrated cellular suspension was used for scanning electron microscopy (SEM) as well as for whole-mount and thin-section transmission electron microscopy (TEM). The SEM and whole-mount samples were prepared as described previously [15] and the whole-mount images were used to measure cell length and diameter. TEM samples were prepared using high-pressure freezing and automatic freeze substitution, followed by plastic embedding as described previously [16].

For genome sequencing, high-molecular-weight gDNA of strain HL-109<sup>T</sup> was extracted using a modified hexadecltritri-methylammonium bromide (CTAB)-based extraction protocol [17]. All enzymes used were from New England BioLabs. Cell pellets were resuspended in 140 µl of TE (10 mM Tris, 1 mM EDTA, pH 8.0; Sigma-Aldrich), to which lysozyme (final concentration 100 mg ml<sup>−1</sup>) was added and incubated at 37°C for 30 min. Further cell lysis was achieved by addition of 30% (w/v) SDS and Proteinase K (final concentration 10 mg ml<sup>−1</sup>) and incubation at 56°C for 1 h, followed by treatment with CTAB solution (342 mM CTAB/873 mM NaCl) and incubation at 65°C for 10 min. Next, three sequential organic extractions were performed: chloroform/isoamyl alcohol (24:1), phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1). RNase A (final concentration 50 µg ml<sup>−1</sup>) was added and the extracts were incubated at 37°C for 30 min. The DNA was pelleted using ethanol precipitation, resuspended in 1 × TE, and quantified using the Qubit dsDNA HS assay kit (Life Technologies). The draft genome of HL–109<sup>T</sup> was generated using the Pacific Biosciences (PacBio) sequencing technology [18] at the U.S. Department of Energy Joint Genome Institute [19]. The whole genome sequence revealed three complete, identical copies of the 16S rRNA gene in strain HL-109<sup>T</sup> and another partial copy adjacent to a contig edge.

Routine DNA extractions were performed using a modified protocol designed for use with cultures containing high levels of magnesium sulfate, which otherwise frustrated extraction efforts. Cell pellets were washed with 1 ml of a sterile solution (pH 8.0) of 550 mM NaCl (Fisher)/500 mM EDTA (Sigma-Aldrich), mixed using a vortex mixer for 10 min at high speed, and centrifuged at 16 000 × g at 4°C for 5 min. The supernatant was aspirated and the washing procedure was repeated for a total of three washes. Each pellet was resuspended in 700 µl of a solution (pH 8.0) of 50 mM Tris-HCl (Sigma-Aldrich)/25 mM EDTA and transferred to sterile Lysing Matrix E tubes (MP Biomedicals). The cells were lysed by disruption in a Mini-BeadBeeater-24 device (BioSpec) for 2 min, followed by centrifugation at 16 000 × g for 90 s. The supernatants were transferred to 2-ml microcentrifuge tubes, incubated at 85°C for 5 min, and cooled to room temperature. Then, 70 µl of 10% SDS (Sigma-Aldrich) was added and tubes were mixed. Proteinase K (New England BioLabs) was added to a final concentration of 0.2 mg ml<sup>−1</sup> and tubes were incubated at 56°C with shaking at 1000 r.p.m. for 60 min. One hundred microlitres of a solution of NaCl (5 M) was added to each sample, followed by 100 µl of a 65°C solution of CTAB (0.274 M; Sigma-Aldrich)/NaCl (0.702 M). The extractions were mixed and then incubated at 65°C for 10 min.

Each sample was extracted with one volume of chloroform/isooamyl alcohol (24:1; Sigma-Aldrich), mixed at high speed for 1 min, and centrifuged at 16 000 × g for 10 min. The aqueous phases were transferred to new microcentrifuge tubes and the extraction was repeated with a phenol/chloroform/isooamyl alcohol mixture (25:24:1; Sigma-Aldrich), followed by an extraction using chloroform/isooamyl alcohol (24:1). Ten micrograms of RNase A (Thermo Scientific) was added to each tube and incubated at 37°C for 30 min. One-tenth volume of 3 M sodium acetate (pH 5.5) and 2.5 volumes of ice-cold 200-proof ethanol (Decon Laboratories) were added and tubes were inverted by hand for 1 min. The samples were incubated at −80°C for 30 min, centrifuged at 16 000 × g at 4°C for 10 min, and the supernatants were aspirated. The DNA pellets were washed twice with 0.5 ml of ice-cold 70% ethanol and centrifuged at 16 000 × g at 4°C for 10 min. The pellets were desiccated in a SC100 SpeedVac Concentrator (Savant) on medium heat until dry and resuspended in 100 µl of Tris-EDTA buffer solution (pH 7.4; Fluka). DNA was quantified using a Qubit 2.0 and HS dsDNA Assay (Thermo Fisher) as per the manufacturer’s instructions.
The sequence of the 16S rRNA gene was obtained by performing PCR with primers 9bF (5'-GRGTTTGAATCTGG CTCAG-3') [20] and 1512uR (5'-ACGGHTACCTGTG TACGACTT-3') [21]. Amplification was performed using Phusion High-Fidelity polymerase (New England Biolabs) as per the manufacturer’s instructions, using an annealing temperature of 55°C and 30 cycles. The PCR product was purified using a QIAquick PCR Purification Kit (Qiagen) and shipped to Functional Biosciences (Madison, WI, USA) for Sanger dideoxy sequencing. Sequencing was performed using BioEdit v. 7.2.0 [23] and checked for chimeric properties using Bellerophon software [24]. The sequence comprised 1404 nt and is available in NCBI GenBank under accession number KR560061.1.

The phylogenetic tree was generated from 42 16S rRNA gene sequences, including those derived from type strains from the families Bradyrhizobiaceae, Methylobacteriaceae and Beijerinckiacaeae, in addition to published clones from NCBI GenBank found by BLASTn searches. Sequences were aligned against the SILVA alignment using mothur v. 1.36.1 [25] using default parameters. The alignment was inspected using Unipro UGENE v. 1.20.0 [26] and, after filtering, was 1367 nt in length. Phylogenetic trees were reconstructed using the neighbour-joining (1000 bootstraps, pairwise deletion of missing data) and maximum-likelihood algorithms (100 bootstraps, partial deletion of missing data at 95%
cutoff) using the Molecular Evolutionary Genetics Analysis tool v. 6.06 [27]. The neighbour-joining tree is presented in Fig. 1 and the maximum-likelihood tree displayed a similar topology.

Growth curves to identify the ranges of growth for temperature, salinity, pH and oxygen were determined in triplicate. Absorbance readings were taken at 4-h intervals for 12 h a day for 2 days. Growth was tested at temperatures of 4, 10, 20, 30, 33, 37, 40, 45, 50 and 60 °C. Growth in NaCl or MgSO₄ was tested at concentrations between 0 and 1400 mM in 100 mM increments. The pH range for growth was tested from pH 3.0 to 9.0 in increments of 1.0 pH unit. A pH greater than 9.0 could not be tested as these pH values induced precipitation, probably of magnesium hydroxide or...
magnesium carbonate. Growth was tested at 0.0, 0.2, 1.0,
2.6, 5.0, 6.6, 8.5, 10.0, 15.0 and 20.9 % oxygen.

Due to the pink coloration of the isolate, the organism was
analysed for the production of pigments. The presence of
proteorhodopsin was assessed by scanning the spectra of
cultures from 200 to 900 nm using a BioSpec-1610 spectro-
photometer (Shimadzu). Cultures grown in the dark or
under diel illumination by an incandescent bulb, both intact
and sonicated, were analysed. The presence of bacterio-
chlorophylls was tested by extracting biomass in a solution
of acetone/methanol (7:2, v/v) at 4 °C in the dark for
30 min and then scanning the spectrum of the supernatant.

As this halophilic isolate would not grow in traditional dif-
ferential or hydrolysis media, HLH-700 medium was modi-
fied for use in metabolic tests based on the recipes detailed
by Leboffe and Pierce [28] Secretion of amylase and lipase
were tested by cultivation on HLH plates with starch
(1 %, w/v) or tributyrin (10 ml l⁻¹); starch plates were
flooded with Gram’s iodine and examined for zones of
clearing around the colonies. Nitrate reduction was tested
by modifying HLH-700 broth to mimic traditional nitrate
broth by excluding ammonium chloride and including
18.7 mM sodium nitrate, and inverted Durham vials were
included to test for denitrification. Urease activity was tested
in HLH broth prepared at pH 7.0 with 1.25 g l⁻¹ of yeast
extract and 333 mM urea and the OD₆₀₀ and pH were
recorded after cultivation. The isolate would not grow in
HLH media modified to mimic gelatin iron agar, motility
agar, milk plates, triple-sugar iron agar or thioglycollate
broth. The test for catalase was performed by applying one
drop of 3 % (v/v) H₂O₂ (Grainger) to biomass grown on a
Petri dish and the test for oxidase was performed using
Fluka Oxidase Strips (Sigma-Aldrich) as per the manufac-
turer’s instructions. Gram staining was performed using a
stabilized Gram-stain set (Fisher Healthcare Protocol)
according to the manufacturer’s instructions.

Samples for metabolomics were prepared by centrifugation
of 3-day-old broth cultures at 5000 × g until the superna-
tant approached an OD₆₀₀ of 0.00 (~60 min). The pellets
were washed twice with sterile HLH-700 (yeast extract
excluded) to remove residues and the cell pellets were frozen
at −80 °C until analysis. Polar metabolites were extracted
and analysed as reported previously [29]. Analysis of fatty
acid methyl esters was performed using methanolic HCl
solution (1.25 M; Sigma-Aldrich) [30]. Analysis was per-
fomed on two biological replicates, the relative abundances
of the fatty acids were averaged between the two replicates,
and the average values for the major fatty acids detected are
reported in Table 1.

Colonies of strain HL-109ᵀ grown in Petri dishes of HLH
were pink, circular, convex and punctiform with a glossy
sheen and entire perimeter. The cells stained Gram-nega-
tive. Phase contrast microscopy revealed cells to be capable
of motility and electron microscopy showed the isolate to
be monotrichous (arrowhead, Fig. 2b). Cells had a

![Fig. 2. Electron micrographs of strain HL-109ᵀ. (a) SEM image of a
cluster of cells. (b) TEM image of a whole mount of a single cell with
arrowhead indicating a single, intact polar flagellum. (c) TEM image of
a thin section with arrowhead indicating one of many inclusion bodies
within a branched cell.](image-url)
pleomorphic morphology composed of short rods with occasional branching (Fig. 2a) and contained inclusions of low electron density (arrowhead, Fig. 2c). Cell lengths averaged 1.9 µm (range 1.0–3.2 µm) and diameters averaged 0.9 µm (range 0.8–1.0 µm).

The isolate grew between 20 and 45 °C with an optimal temperature of 45 °C. The isolate grew between 100 and 1400 mM MgSO$_4$ and NaCl. Final cell yields decreased linearly in relation to oxygen concentration. The isolate was positive for amylase, catalase and lipase but negative for nitrate reductase and urease. The groups of intact polar lipids detected were cardiolipin (CL), diphosphatidylglycerol (DG), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and triacylglycerols (TGs). The major fatty acids (>10 %) were C$_{18:1}$ (46.8%), C$_{18:0}$ (22.5%) and C$_{16:0}$ (15.1%); the minor fatty acids were C$_{18:1}$cyc (9.7%), C$_{18:0}$ (1.4%), C$_{16:1(3)}$ (1.3%), C$_{19:0}$ (0.9%), C$_{21:0}$ (0.9%), C$_{16:1(9)}$ (0.7%), C$_{14:0}$ (0.3%) and C$_{22:1}$ (0.3%), and 3-hydroxy fatty acids (C$_{14:0}$, C$_{16:0}$, C$_{18:0}$) were observed at less than 0.2 %.

Major metabolites were betaine, trehalose, sucrose, glutamate and lysine. Polyhydroxyalkanoates were detected and the most abundant components were 3-hydroxybutyrate and malate and lysine. Polyhydroxyalkanoates were detected and the most abundant components were 3-hydroxybutyrate and 3-hydroxyfatty acids (C$_{14}$, C$_{16}$, C$_{18}$). The major polar lipids are CL, DG, PC, PE and PG; the major neutral lipid is TG. The genus is a member of the family Salinarimonadaceae. The type species is Salinarimus fredricksonii.

**DESCRIPTION OF SALINIRAMUS FREDRICKSONII SP. NOV.**

Salinarimus fredricksonii (fred.rick.so’ni.i. N.L. gen. n. fredricksonii of Fredrickson, the American microbial ecologist who founded the study of the Hot Lake microbial mat and has contributed to the fields of subsurface microbiology and biogeochemistry).

In addition to the characteristics reported for the genus, cells average 1.9 µm in length and 0.9 µm in diameter and are monotrichous. Colonies are circular, convex and punctiform with a glossy sheen and entire perimeter. Halophilic growing between 100 and 1400 mM MgSO$_4$ and NaCl in HLH medium. Grows between 20 and 45 °C with an optimum temperature of 45 °C. Positive for amylase, catalase and lipase, but negative for nitrate reductase and urease.

The type strain is HL-109$^T$ (=JCM 31876$^T$=DSM 102886$^T$), isolated from a hypersaline unicyanobacterial consortium. The DNA G+C content of the type strain is 64.57 mol%.

**DESCRIPTION OF SALINARIMONADACEAE FAM. NOV.**

Salinarimonadaceae (Sa.li.na.ri.mo.na.da.ce’ae. N.L. fem. n. Salinarimonas type genus of the family; L. pl. suff. -aceae ending to denote a family; N.L. fem. pl. n. Salinarimonadaceae the family of the genus Salinarimonas).

Aerobic or facultatively anaerobic, rod-shaped cells that may form branches. Gram-negative, motile and oxidase-positive. Produce pink- or red-pigmented colonies. The predominant fatty acids include C$_{18:0}$, C$_{18:1}$ and C$_{16:0}$. The predominant respiratory quinone is Q-10. The major polar lipids include PG and PC. The family is a member of the order Rhizobiales. The type genus is Salinarimus.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical statement

No portion of this experimental work included research involving human samples or animals.

References


