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TAXONOMIC DESCRIPTION
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Salinivirga fredricksonii gen. nov., sp. nov., a heterotrophic halophile isolated from a photosynthetic mat, a member of a novel lineage (*Salinarimonadaceae* fam. nov.) within the order *Rhizobiales*, and reclassification of the genus *Salinarimonas* Liu *et al.* 2010 into *Salinarimonadaceae*

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Abstract

A halophilic bacterial strain, HL-109^T, was isolated from the unicyanobacterial 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 $MgSO_4$ 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 $C_{18:1}$, $C_{18:0}$, $C_{16:0}$ and $C_{18:cyc}$. 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, *Salinarimonas ramus*. Morphological, chemotaxonomic and phylogenetic results did not affiliate the novel organism with any of the families in the *Rhizobiales*; therefore, HL-109^T is representative of a new lineage, for which the name *Salinivirga fredricksonii* gen. nov., sp. nov. is proposed, with the type strain HL-109^T (=JCM 31876^T=DSM 102886^T). In addition, examination of the phylogenetics of strain HL-109^T and its nearest relatives, *Salinarimonas ramus* and *Salinarimonas 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 *Salinivirga* and *Salinarimonas* (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

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Keywords: meromictic; hypersaline; epsomitic lake; halophile; epsophile.
Abbreviations: CL, cardiolipin; CTAB, hexadecyltrimethylammonium bromide; DG, diacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; SEM, scanning electron microscopy; TEM, transmission electron microscopy; TG, triacylglycerol.
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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain HL-109^T is KR560061.1. The Joint Genome Institute Sequencing Project ID for the improved draft genome of strain HL-109^T is 1064087.

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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^T 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^T 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 $MgSO_4$rather than 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_{600}) 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^T was extracted using a modified hexadecyltrimethylammonium 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^{-1}) was added and incubated at 37 °C for 30 min. Further cell lysis was achieved by addition of 10 % (w/v) SDS and Proteinase K (final concentration 10 mg ml^{-1}) 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^{-1}) 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^T 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^T 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-BeadBeater-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^{-1} 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/isoamyl 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/isoamyl alcohol mixture (25 : 24 : 1; Sigma-Aldrich), followed by an extraction using chloroform/isoamyl 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′-GRGTTTGATCCTGG CTCAG-3′) [20] and 1512uR (5′-ACGGHTACCTTGT-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

Table 1. Differential characteristics between strain HL-109^T and other related members of the order *Rhizobiales*

Strains: 1, HL-109^T; 2, *Salinarimonas rosea* YD3^T; 3, *Salinarimonas ramus* SL014B-41A4^T; 4, *Chelatococcus asaccarovorans* TE2^T; 5, *Chelatococcus daeguensis* K106^T. All strains are Gram-stain negative and capable of aerobic respiration. +, Positive; –, negative; W, weak; ND, no data.

Characteristic	1	2	3	4	5
Isolation environment	Hypersaline lake	Salt mine	Saline soil	Textile wastewater	Textile wastewater
Cell morphology	Rods, branches	Rods	Rods, branches	Diplococci	Rods
Motility	+	+	+	–	+
Colony pigmentation	Pink	Pink	Red	White	Yellow
Temperature (range, optimum) (°C)	20–45, 45	15–37, 28–30	4–50, 28	ND, 36	ND, 30–37
pH (range, optimum)	5.0–9.0, 7.0–9.0	6.0–9.0, 7.0–8.0	6.0–9.0, 7.0	5.5–9.5, 7.0–8.0	ND, 7.0–7.5
NaCl (range, optimum) (mM)	100–1400	0–85, 51	0–171, 68	0–427.59, ND	0–855, 0–171
$MgSO_4$ (range, optimum) (mM)	100–1400	ND	ND	ND	ND
Facultative anaerobic growth	–	+	+	–	+
Sulfate respiration	–	ND	ND	ND	ND
Nitrate respiration	–	–	ND	–	+
Storage polymer production					
Polyhydroxyalkanoates	+	–	+	ND	ND
Cyanophycin	+	ND	ND	ND	ND
Enzyme activity					
Amylase	+	–	W	–	–
Catalase	+	+	+	+	+
Lipase	+	+	–	–	–
Nitrate reductase	–	+	+	+	+
Oxidase	+	+	–	+	+
Urease	–	+	–	–	–
Chemotaxonomic data					
Major fatty acids	$C_{18:1}$, $C_{18:0}$, $C_{16:0}$	$C_{18:1}\omega7c$, $C_{18:1}\omega9c$, $C_{16:0}$, $C_{19:0}$ cyclo $\omega8c$	$C_{18:1}\omega7c$, $C_{16:0}$, 10-methyl $C_{19:0}$	$C_{18:1}\omega7c$, $C_{19:0}$ cyclo $\omega8c$, $C_{16:0}$	$C_{18:1}\omega7c$, $C_{19:0}$ cyclo $\omega8c$
Major polar and neutral lipids	CL, DG, PC, PE, PG, TG	CL, PME, PG, PC, PL	DPG, PE, PG, PC, PI, PIM	PC, PG, PE	PC, PG, PE
DNA G+C content (mol %)	64.57	71.80	67.68	63.3–63.5	68.3
References	Present study	[32]	[31]	[40, 41]	[41, 42]

CL, cardiolipin; DG, diacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; TG, triacylglycerol; PME, phosphatidylmethylethanolamine; PL, unknown phospholipids; DPG, diphosphatidylglycerol; PI, phosphatidylinositol; PIM, phosphatidylinositol mannosides.

purified using a QIAquick PCR Purification Kit (Qiagen) and shipped to Functional Biosciences (Madison, WI, USA) for Sanger dideoxy sequencing. Sequencing was performed from primers 9bF, 1512uR, and a pair of primers internal to the *rrnA* gene sequence of strain 109^T (109_133R: 5′-TCGGCGATAAATCTTTCTGCTC-3′; 109_1248F: 5′-CAT GCCACGGTGAATACGTT-3′) designed using the Primer3 software v. 4.0.0 [22]. The 16S rRNA gene was assembled 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*, *Methlyobacteriaceae* and *Beijerinckiaceae*, 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 % cut-off) 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.

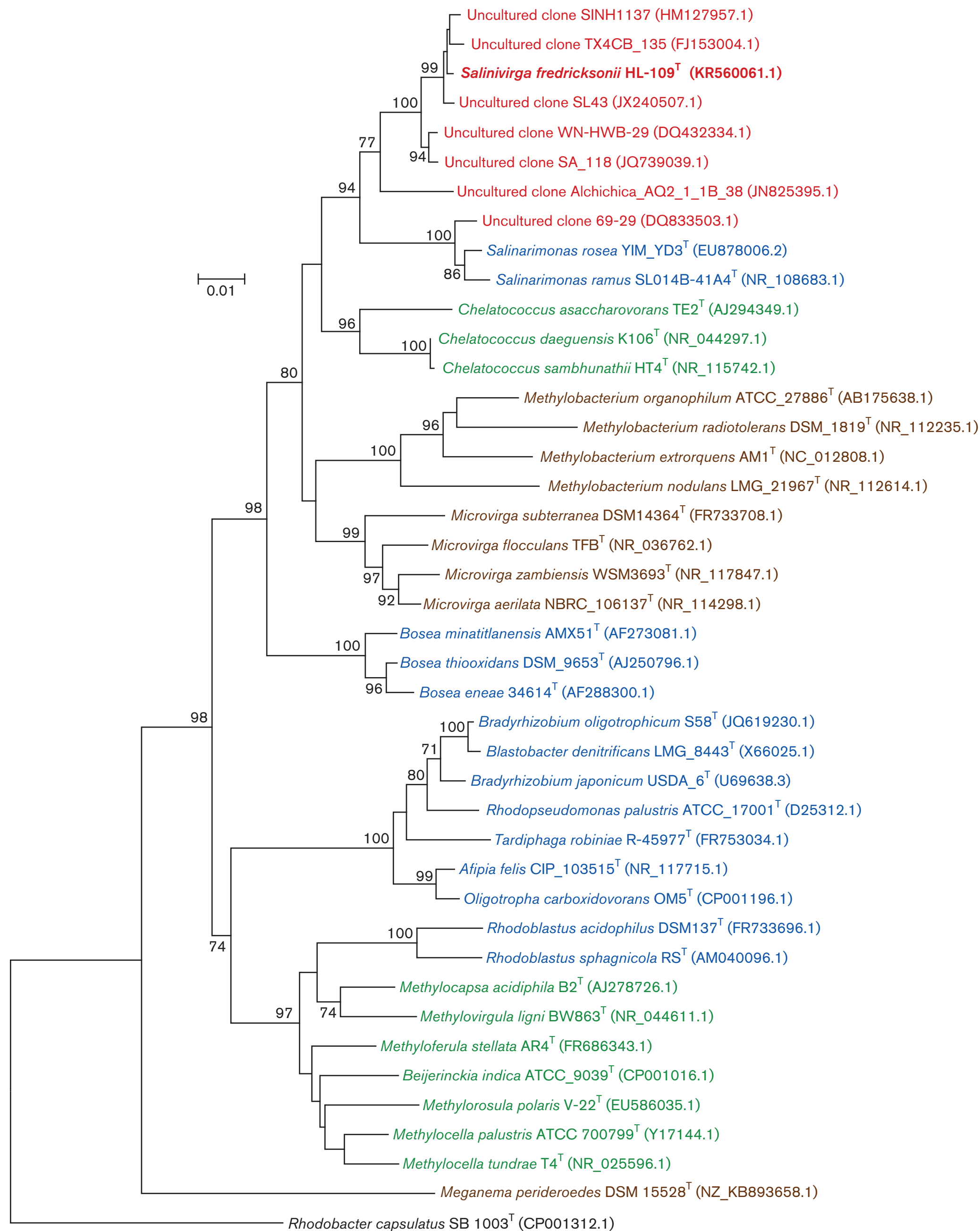

Fig. 1. Phylogenetic tree illustrating the relatedness of strain HL-109^T (red, bold) to type strains of the families *Bradyrhizobiaceae* (blue), *Methylobacteriaceae* (brown), and *Beijerinckiaceae* (green). Published clone sequences (red) related to strain HL-109^T are also included. *Rhodobacter capsulatus* 1003^T was used as the outgroup. The tree was generated using the neighbour-joining method over 1367 positions and with 10 000 bootstrap replicates. Bootstrap values of at least 70 % are included at nodes.

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 $MgSO_4$ 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 spectrophotometer (Shimadzu). Cultures grown in the dark or

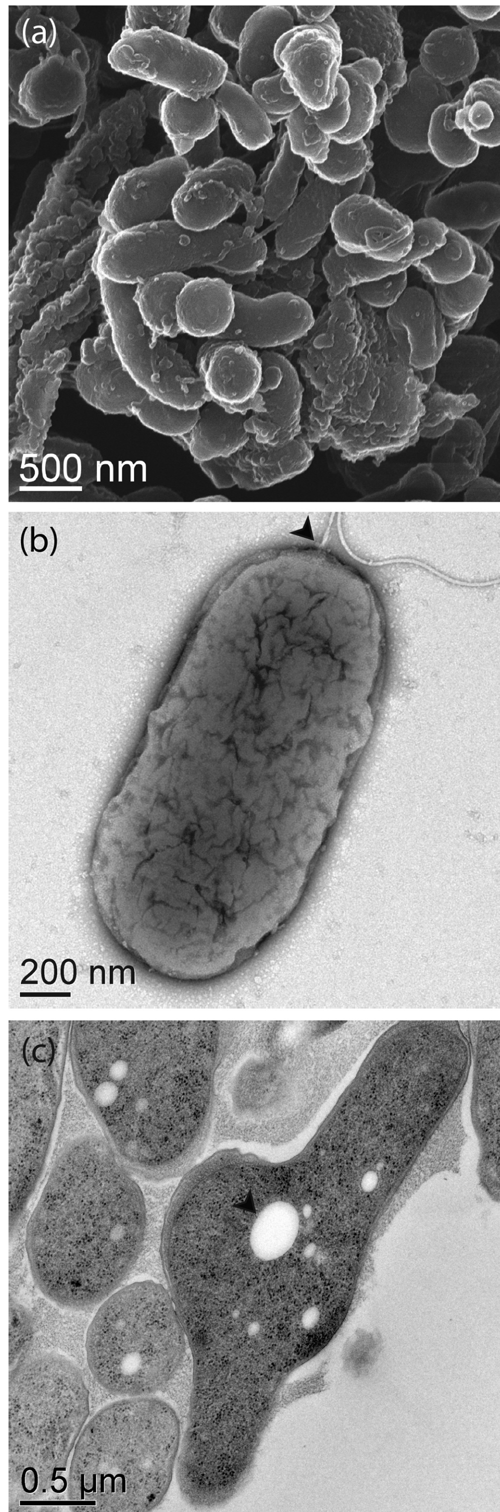

Fig. 2. Electron micrographs of strain HL-109^T. (a) SEM image of a cluster of cells. (b) TEM image of a whole mount of a single cell with arrowhead indicating a single, intact polar flagellum. (c) TEM image of a thin section with arrowhead indicating one of many inclusion bodies within a branched cell.

under diel illumination by an incandescent bulb, both intact and sonicated, were analysed. The presence of bacteriochlorophylls 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 differential or hydrolysis media, HLH-700 medium was modified 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 OD_{600} 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) H_2O_2 (Grainger) to biomass grown on a Petri dish and the test for oxidase was performed using Fluka Oxidase Strips (Sigma-Aldrich) as per the manufacturer'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 supernatant approached an OD_{600} 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 performed 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-109^T grown in Petri dishes of HLH were pink, circular, convex and punctiform with a glossy sheen and entire perimeter. The cells stained Gram-negative. 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 pleomorophic 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), phosphatidycholine (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 $C_{18:cyc}$ (9.7 %), $C_{18:0}$ (1.4 %), $C_{16:1(2)}$ (1.3 %), $C_{19:0}$ (0.9 %), $C_{21:1}$ (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 3-hydroxypentanoate. Cyanophycin was also detected, in addition to aspartate and arginine. Glucose was detected after acid hydrolysis, the source of which is attributed to trehalose, but the possibility that glycogen could have also produced the glucose cannot be ruled out. The G+C of the gDNA was 64.57 mol%.

Isolates and clones within the clade of strain HL-109^T originated from sediments or soils of alkaline and/or saline environments [31–39]. Strain HL-109^T formed a monophyletic group with its related clones and the two species of the genus *Salinarimonas*, *S. rosea* and *S. ramus* (Fig. 1). The clade was strongly supported at a bootstrap recovery value of 94 % and is distinct from clades of the families *Methylocystaceae*, *Bradyrhizobiaceae* and *Beijerinckiaceae*.

Phylogenetic, morphological and chemotaxonomic results did not affiliate the novel organism with any of the families in the *Rhizobiales*. We thus propose that strain HL-109^T is representative of a new lineage, *Salinivirga fredricksonii* gen. nov., sp. nov., with the type strain HL-109^T (=JCM 31876^T =DSM 102886^T). In addition, examination of strain HL-109^T and its nearest relatives, *S. ramus* and *S. rosea*, demonstrate that these halophiles form a clade distinct from the families of the *Rhizobiales*. We therefore propose the establishment of a new family, the *Salinarimonadaceae* fam. nov., to accommodate the genera *Salinivirga* and *Salinarimonas*, with *Salinarimonas* as the type genus of the family.

DESCRIPTION OF *SALINIVIRGA* GEN. NOV.

Salinivirga (Sa.li.ni.vir′ga. N.L. adj. *salinus* saline; L. fem. n. *virga* twig; N.L. fem. n. *Salinivirga* a twig-shaped salt-associated organism).

Cells are rods that may branch. Colonies are pink. Do not form endospores or other resting structures. Stain Gram-negative and obligately aerobic. Cells are motile and produce oxidase and catalase. The major fatty acids are $C_{18:1}$, $C_{18:0}$ and $C_{16:0}$. 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 *Salinivirga fredricksonii*.

DESCRIPTION OF *SALINIVIRGA FREDRICKSONII* SP. NOV.

Salinivirga fredricksonii (fred.rick.so′ni.i. N.L. gen. n. *fredricksonii* of Fredrickson, named in honour of James K. 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 1 400 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 *Salinarimonas*.

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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.

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