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Physiological features of *Halomonas lionensis* sp. nov., a novel bacterium isolated from a Mediterranean Sea sediment

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Abstract:

A novel halophilic bacterium, strain RHS90^T, was isolated from marine sediments from the Gulf of Lions, in the Mediterranean Sea. Its metabolic and physiological characteristics were examined under various cultural conditions, including exposure to stressful ones (oligotrophy, high pressure and high concentrations of metals). Based on phylogenetic analysis of the 16S rRNA gene, the strain was found to belong to the genus *Halomonas* in the class *Gammaproteobacteria*. Its closest relatives are *Halomonas axialensis* and *Halomonas meridiana* (98% similarity). DNA–DNA hybridizations indicated that the novel isolate is genotypically distinct from these species. The DNA G + C content of the strain is 54.4 mol%. The main fatty acids ($C_{18:1}\omega7c$, 2-OH iso-$C_{15:0}$, $C_{16:0}$ and/or $C_{19:0}$ cyclo $\omega8c$), main polar lipids (diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine and an unidentified phosphoglycolipid) and major respiratory quinone (ubiquinone Q9) were determined. The novel isolate is heterotrophic, mesophilic, euryhaline (growth optimum ranging from 2 to 8% w/v NaCl) and is able to grow under stressful conditions. The strain accumulates poly-β-hydroxyalkanoates granules and compatible solutes. Based on genotypic, chemotaxonomic and phenotypic distinctiveness, this isolate is likely to represent a novel species, for which the name *Halomonas lionensis* is proposed. The type strain of *H. lionensis* is RHS90^T (DSM 25632^T = CIP 110370^T = UBOCC 3186^T).

Keywords : Halomonas ; Taxonomy ; Environmental adaptation ; Metal tolerance ; Poly-β-hydroxyalkanoate ; Compatible

1.Introduction

At the time of writing, the genus *Halomonas*, within the class *Gammaproteobacteria*, encompasses more than 76 recognized species (Oren and Ventosa, 2013). It comprises mostly marine halophilic aerobic heterotrophs well known for their metabolic versatility (Arahal and Ventosa, 2006; De la Haba R. R. et al., 2011). Microorganisms belonging to the genus *Halomonas* were initially found in hypersaline environments such as the Dead Sea, hypersaline lakes, hypersaline soils and solar salterns (Vreeland et al., 1980; Franzmann et al., 1987; Mormile et al., 1999; Oueriaghli et al., 2013). Later, culture-based and molecular-based studies revealed that *Halomonas* microorganisms are also present in numerous non-hypersaline environments such as animal tissues (Romanenko et al., 2002), factories (Dobson and Franzmann, 1996), non-marine biofilms (Heyrman et al., 2002), human blood (Kim et al., 2010) and in environments considered as stressful from an anthropocentric point of view, such as highly polluted/alkaline waters (Berendes et al., 1996; Yang et al., 2010) and non-hypersaline ices from Antarctica (Reddy et al., 2003). The use of molecular techniques in microbial ecology has also enlarged the list of environments associated with *Halomonas* species, as they have been found in deep oceans (Takami et al., 1999), hydrothermal vents (Kaye and Baross, 2004b; Simon-Colin et al., 2008; Kaye et al., 2011), subsurface environments (Durbin and Teske., 2011) and crustal fluids and rocks (Santelli et al., 2008). Thus, members of the genus *Halomonas* are widespread in the biosphere and colonize common to extreme environments. This distribution suggests that these bacteria display broad physiological plasticity and metabolic versatility and have developed specific adaptations that allow them to maintain or grow under extreme physical (pressure), chemical (pollutants, high concentrations of metals) and energetic (starvation) conditions, thus allowing them to colonize a variety of habitats.

For instance, different halophilic archaea and bacteria, including several *Halomonas* species, accumulate poly-β-hydroxyalkanoates (PHA) (carbon and energy storage materials) to cope with nutrient-depleted conditions (Simon-Colin et al., 2008; Kulkarni et al., 2011). Also, some halophilic strains develop specific osmoadaptation mechanisms to prevent molecular damage from cellular freezing and dehydration. These mechanisms include (i) transmembrane exchange of salts to balance osmotic pressure through specific membrane transport proteins and (ii) accumulation of protective compatible solutes such as betaine or ectoine. *Halomonas* species are known to accumulate compatible solutes by uptake and/or by synthesis (Zhu et al., 2011). Comparative genomic analyses have shown that gene clusters *pha* (responsible for PHA synthesis) and *ect* (responsible for ectoine synthesis) are subject to horizontal gene transfer (HGT) events within halophilic species and that the genomic organization of *phaC* (coding for PHA synthase) and *pha**P* (coding for phasin) is conserved in *Halomonas elongata* and *Halomonas* sp. TD01(Cai et al., 2011). This conservation suggests that selective pressure is exerted on these genes, which may be partly responsible for the adaptive success and colonization capabilities of *Halomonas* species.

Even though the metabolic diversity of several *Halomonas* species has been described, very few studies have focused on the capacity of these microorganisms to confront various physical, chemical and nutritional conditions. In this study, we report the isolation and physiological characterization of a novel *Halomonas* species, strain RHS90^{T}, isolated from Mediterranean Sea sediments, which exhibits wide physiological flexibility.

2. Materials and methods

2.1.Bacterial isolation

In October 2008, a sediment core was recovered in the Gulf of Lions (42°41'.596 N, 03°50'.493E; water depth: 291 m), in the western Mediterranean Sea and subsampled for microbiological analyses, as described elsewhere (Ciobanu et al., 2012). A sediment sample from 84 cm below the seafloor was spread on an agar plate composed of modified R2A medium (Ciobanu et al., 2012) and then incubated at 25 °C. After 10 days of incubation, a beige colony was picked, purified by repeated streaking on marine agar 2216 (MA; Difco) plates and referenced as strain RHS90^T. Stock cultures were stored at -80°C, in marine broth 2216 (MB, Difco) supplemented with 5% (v/v) DMSO, until characterization.

2.2.Culture conditions

Unless stated otherwise, cultures were carried out aerobically in sterile MB 2216 medium (Difco) aliquoted into 50 mL vials or 10 mL aerobic tubes. Fifty or 25 µL of an overnight preculture were inoculated in 10 mL of MB 2216 medium and then incubated at 30 °C in the dark with shaking at 90 or 100 rpm. All solutions and media used for microbiological experiments were sterile and all reagents used for molecular biology experiments were of molecular biology grade.

2.3.Growth monitoring

Growth of strain RHS90^T was routinely monitored by optical density measurement and ATP assay. The correlation (n=81, r^2=0.92) between cell counting and optical density was determined by measuring the optical density at 600 nm of cultures diluted at different dilution factors (1/10th, 1/100th, 1/1,000th) with a spectrophotometer (Genesys 20, Thermo Scientific). The same diluted cultures were counted in parallel in a modified Thoma chamber (depth 10

µm, Preciss Europe). The ATP content of cultures was determined with a Kikkoman Lumitester C-110 (Isogen Life Science) using the Bac Titer-Glo Microbial Cell Viability assay (Promega) according to the manufacturer's instructions with a few modifications: 75 µL of culture and 75 µL BacTiter-Glo buffer were used; internal calibration was performed with 10 µL of a 100 nM ATP solution and maximal fluorescence emissions values were considered.

2.4.Microscopic observations of PHA inclusions and viability assay

Cells were observed with a phase-constrast light microscope (Olympus BX60) at 40× and 100× magnifications. PHA cytoplasmic inclusions were stained with oxazine dye Nile Blue A following a modified procedure of the Gram-negative viable-colony staining technique of Spiekermann (Spiekermann et al., 1999): 0.5 µg Nile Blue A (Sigma) were added per mL of liquid culture medium. After one day of cultivation, cells were observed under ultraviolet light with an epifluorescence microscope (Olympus BX60). *Escherichia coli* CM237^{T}, which does not produce PHA, was used as a negative control. Cell viability and structural integrity of cultures grown under high hydrostatic pressure were determined using the LIVE/DEAD® BacLight Bacterial Viability kit (Invitrogen). A volume of 200 µL culture exposed to 60 MPa hydrostatic pressure for 9 h was stained in the dark for 15 min with 3 µL propidium iodide/SYTO®9 (Invitrogen) and then observed under UV. Scanning electron microscopy (FEI Quanta 200) observations of cultures were done with standard HMDS-based (HexaMethylDiSilasane) preparation. Transmission electron microscopy (Jeol JEM 100 CX II) observations were made after negative staining with uranyl acetate (2 % v/v).

2.5.Determination of optimal growth parameters

Determinations of temperature, pH and NaCl ranges for growth were performed in triplicate in 10 mL aerobic tubes incubated with shaking (90 or 100 rpm) in the dark. Growth rates were calculated using linear regression analysis of 5 to 9 points along the linear portions of the logarithmically transformed growth curves. Determinations of the temperature, NaCl concentration and pH ranges for growth were tested over the range 4-45 °C (4 °C, 10 °C, 16 °C, 22 °C, 30 °C, 37 °C, 40 °C, 43 °C and 45 °C) at pH 7 and with 2 % (w/v) NaCl for temperature determination; over the range 0-30 % (w/v) NaCl (0 %, 0.5 %, 2 %, 4 %, 6 %, 8 %, 15 %, 20 % and 30 %) at 20 °C and pH 7 for NaCl concentration analysis; and over the range pH 3-11 (3, 3.5, 4, 5, 6, 7, 8, 9, 10 and 11) at 20 °C and with 2 % NaCl for pH determination. Exposure to hydrostatic pressure (0.1, 20, 40, 50 and 60 MPa) was done in 0.6 L autoclaves (TopIndustrie, Vaux le Penil, France), in triplicate, at room temperature, with 5 mL syringes containing 3 mL MB medium and 1 mL tetradecafluorohexane (Sigma Aldrich) to facilitate oxygen diffusion.

2.6.Substrate utilization

To investigate the capacity of the strain to catabolize different substrates as sole carbon and energy sources with oxygen as a terminal electron acceptor, the strain was grown in the dark on the mineral basis of MB medium (depleted of all carbon and energy sources) supplemented with one substrate for each test. Carbon utilization tests were performed at concentrations of 1 mM for amino acids, 1 mM for organic acids, 1 % (w/v) for alcohols and 10 mM for sugars except for cellulose, D(+)cellobiose, dextrin, D(+)galactose, poly-D(+)galacturonic acid, D(-)fructose, D(+)lactose, pectin and xylan, which were all tested at 1 $g.L^{-1}$. Tween 80 degradation was investigated on Noble agar (Sigma-Aldrich) plates prepared with the mineral basis of MB medium and covered with the substrate (0.75 mM). The ability of the strain to grow anaerobically and to ferment complex organic matter or carbohydrates

(yeast-extract 1 $g.L^{-1}$, peptone 5 $g.L^{-1}$ and glucose 10 mM) was investigated under an N_2 atmosphere (100 % w/v) on an MB mineral basis degassed and reduced with 0.05 % (w/v) Na_2S $9H_2O$. The ability of the strain to reduce nitrate, nitrite, sulfate or DMSO was investigated on an MB mineral basis prepared with 10 mM nitrate, 10 mM nitrite, 10 mM sulfate or 10 mM DMSO, respectively, and reduced with 10 µL of $Na_2S.9H_2O$ 5 % (v/v). *Aminomonas paucivorans* (DSM 12260^T) and *Shewanella profunda* (DSM 15900^T), which are respectively fermentative and nitrate-reducing microorganisms, were used as positive controls for fermentation and nitrate reduction tests. The utilization of amino acids as sole nitrogen sources was tested in artificial sea water with fumarate and D(-)fructose (2 mM each) as carbon sources.

2.7.Growth under oligotrophic conditions

The capacity of strain $RHS90^T$ to grow under oligotrophic conditions was investigated in duplicate with 20 mL of late-exponential phase cultures centrifuged at 6000 x *g* for 15 min at 4 °C. Cell pellets were then washed and suspended in 200 mL artificial sea water (pH= 6.8) and stored at 4 °C for 30 days. Cellular density and cellular activity were measured every 3 days by cell counting and by ATP content measurements as described above. To discriminate between hypothetical ATP released after cellular lysis and intracellular ATP representative of cellular activity, the extracellular ATP content was also measured: 1 mL of cells suspended in artificial sea water and stored at 4 °C was filtered onto 0.2 µm syringe filters (Millipore) to retain cells and the total ATP content of the filtrate was measured as described above. The viability of stored cells was further evaluated by inoculation of 50 mL vials containing 10 mL MB 2216 medium with 1 mL of the stored suspension diluted at different factors ($1/100^{th}$, $1/1,000^{th}$, $1/10,000^{th}$, $1/100,000^{th}$, $1/1,000,000^{th}$) and then incubated as described above.

2.8.Metal exposure

Tolerance to metal exposure of the novel isolate was investigated in triplicate in MB medium supplemented with different metals [$AgSO_4$, $CdCl_2$, $CrK(SO_4)_2$, $CuSO_4$, $CoSO_4$, $ZnSO_4$, $MnSO_4$, CsCl] at several concentrations (0.0005, 0.001, 0.005, 0.01 and 0.05 mM for $AgSO_4$; 0.05, 0.2, 0.4, 0.6 and 0.8 mM for $CdCl_2$; 0.5, 0.75, 1, 1.5 and 2 mM for $CrK(SO_4)_2$; 0.5, 1, 1.5, 2 and 2.5 mM for $CuSO_4$; 1, 1.5, 2 and 2.5 mM for $CoSO_4$; 0.5, 1, 1.5, 2, 3 and 4 mM for $ZnSO_4$; 10, 20, 30, 40, 50 and 60 mM for $MnSO_4$; 80, 100, 125, 150 and 200 mM for CsCl). Growth was monitored by ATPmetry after 12-15 h incubation at 30 °C with shaking (100 rpm). Minimal inhibitory concentrations (MICs) of metals were defined by the concentration of metals leading to the same ATP content as the inoculum after 12 h of incubation.

The multiresistant strain *Cupriavidus metallidurans* $CH34^T$, used as a control, was grown in DSMZ medium n°1 (http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium1.pdf) supplemented with different concentrations of metals. Its growth and MIC values were determined as described above.

2.9.Chemotaxonomic analyses

Chemotaxonomic analyses were performed on mid- to late-exponential phases of growth cultures grown for 1 day in MB medium at 30 °C with shaking (100 rpm). The determination of whole-cell fatty acid composition was made by the standard protocol of the Sherlock Microbial Identification System (MIDI Inc., Newark, NJ, USA) and separation of polar lipids was performed by two-dimensional silica gel thin layer chromatography followed by development of total lipids and specific functional groups, as described previously. The

analysis of respiratory quinones was carried out by thin-layer chromatography and then HPLC, as described previously (Tindall et al., 1990).

2.10.Susceptibility to antibiotics

Susceptibility to ampicillin, vancomycin, streptomycin, chloramphenicol nitrofuratoin, nalidixic acid, erythromycin, ampicillin (diluted in ethanol), kanamycin, rifampicin (diluted in DMSO), penicillin G and tetracycline was investigated at 10, 30 and 100 ng at 30 °C on MA plates, using the diffusion disc method.

2.11.RMN spectroscopy

Intracellular accumulation of organic compatible solutes was analyzed on cells grown on a rich medium containing 1 $g.L^{-1}$ yeast extract and 5 $g.L^{-1}$ peptone on a mineral basis of MB medium prepared with or without NaCl. It was studied by ^{13}C NMR spectroscopy on 4 L of culture either with and without NaCl 12.5 % (w/v), incubated at 30 °C with shaking. Cells were harvested by centrifugation (6,000 x *g*, 15 min at 4°C) in late-exponential growth phase. Cell pellets were suspended in 20 mL RNase-free water mixed with 80 mL absolute ethanol, and then shaken for 2 h at room temperature. These suspensions were then pelleted (15,000x*g*, 20 min at 4 °C) and supernatants were transferred into 50 mL tubes before being dried in a rotary evaporator. One-dimensional ^{13}C NMR spectra were recorded at 25 °C on a BRUKER DRX 300 spectrometer equipped with a 5 mm QNP probehead 1H/13C/31P/19F. NMR analyses were performed on samples dissolved in 700 µl D_2O at 99.96 %. The spectra were obtained with BRUKER pulse programs, using standard pulse sequences of 2s delay, a 30° pulse and 5000 scans. Chemical shifts were expressed in ppm relative to TMS (tetramethylsilane) as an external reference.

2.12.DNA extraction and amplification

Briefly, DNA was extracted after centrifugation (20 min, 10,000 x *g* at 4°C) of 10 and 20 mL of mid-log phase culture. The pellet was suspended in 1 mL buffer (Tris 100 mM-pH8, EDTA 50 mM-pH8, NaCl 100 mM) and cellular lysis was achieved with 50 µL sarkosyl 20 %, 100 µL SDS 10 % and 20 µL proteinase K at 20 mg/mL (1 h, 55 °C). One mL phenol/chloroform/isomaylic acid (25/24/1; Sigma) was added and gently mixed with the lysis buffer. After centrifugation (10,000 x *g*, 15 min at 4 °C), the aqueous phase was gently mixed with 1 mL chloroform (Carlo Erba) and centrifuged (10,000 x *g*, 15 min at 4 °C). The aqueous phase was then transferred, mixed with 400 µL of sodium acetate (3M, pH=5.2) and a 0.8 volume of isopropanol. DNA pellet was precipitated 30 min at -20 °C, centrifuged (15,000 x *g*, 10 min at 4 °C), dried and finally resuspended in 50 µL DEPC water. Amplification by polymerase chain reaction (PCR) was performed with Go*Taq*® Flexi DNA polymerase (Promega), following the manufacturer's instructions. The 16S rRNA gene was amplified with the Bac8F and Bac1492R primers (DeLong, 1992) using the following protocol: 3 min at 95 °C; 30 cycles of 1 min at 95 °C, 1 min 30 s at 52 °C and 2 min at 72 °C; 6 min at 72 °C. The amplification of genes encoding ectoine synthase (*ectC*), PHA synthase (*phaC*) and phasin (*phaP*) was performed using degenerated oligonucleotide primers (Eurogentec) designed with *Halomonas elongata* sequences as references: ectc_R_141 (TAC-CGA-GAC-SCA-YAT-CCA-YT), ectc_F_7 (GTT-CGC-AAB-MTB-GAA-GAA-GC), phaC_F_767 (CGC-CCT-GGA-TCA-ACA-AGT-AT), phaC_R_998 (CCG-ACA-CAG-TAG-CTC-AGC-AG), phaC_F_727 (AGC-ACC-GAG-AAG-GTC-TTC-AA), phaC_R_1037 (CTG-GTC-AGG-TAG-GCC-ACT-GT), phaP_F_69 (CAA-TGC-CTT-GAT-GCT-GGA-C), phaP_R_251 (AGC-ATR-TGS-TTG-GAC-AGC-TC). The program used for PCR amplification was the same as that described above except

that the hybridization temperatures were 60 °C, 64 °C and 62 °C for gene *ectC*, *phaP* and *phaC*, respectively.

2.13.Genotypic and phylogenetic analyses

DNA-DNA hybridization experiments were performed by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany), with *H. axialensis* (DSM-15723) and *H. meridiana* (DSM-5425), using a Cary 100 Bio UV/VIS-spectrophotometer.

Blast-based research of most similar 16S rRNA sequences was done against the GenBank database and against the web-based EzTaxon-e server (Kim et al., 2012). Phylogenetic analyses were done with SeaView4 (Gouy et al., 2010) using the Muscle Multiple Alignment option to align sequences. Sequences of the nearest neighbors used to perform the alignment were imported from the Ribosomal Database Project (RDP) website (http://rdp.cme.msu.edu/). Phylogenetic trees were constructed using SeaView4 software, on the basis of Neighbor Joining and PhyML (GTR model) algorithms. The robustness of the inferred topologies was assessed by bootstrap analyses based on 1,000 replications. The 19 nucleotidic signatures of the family *Halomonadaceae* (Dobson and Franzmann, 1996) were manually invastigated with SeaView4 using the *E. coli* 16S rRNA gene as reference numbering (Accession number NR_102804). The 16S rRNA gene sequence of *Halomonas lionensis* RHS90^T was deposited in the GenBank/EMBL/DDBJ databases under the accession number HE661586.

The genomic DNA G+C content of the isolate was determined by the Identification Service of the DSMZ, by HPLC analysis.

3. Results and discussion

3.1.Genotypic and phylogenetic analyses

Based on a BLASTN search against GenBank and the EzTaxon-e Server, the 16S rRNA gene of strain RHS90^T shared highest sequence similarity with *Halomonas axialensis* (97.96 %), *Halomonas meridiana* (98.03 %) and *Halomonas aquamarina* (97.89 %). The 19 nucletotidic signatures of the family *Halomonadaceae* defined by Dobson and Franzmann (1996) were also all found in RHS90^T 16S rRNA gene. Phylogenetic analyses performed with this gene confirmed these results, positioning the novel isolate RHS90^T close to *H. axialensis* and *H. meridiana*, within the genus *Halomonas*, in the family *Halomonadaceae*, class *Gammaproteobacteria* (Fig. 1).

To further determine whether or not strain RHS90^T represents a novel species, DNA-DNA hybridizations were performed with the two closest relatives. Levels of DNA-DNA relatedness with *H. axialensis* and *H. meridiana* were 57.1 % and 62.4 %, respectively, and were therefore below the threshold value of 70 % for species delineation (Wayne et al., 1987), indicating that the novel isolate was likely a novel *Halomonas* species.

3.2.Morphology

Cells were rod-shaped, with a size of 4.4-2.2 × 0.8-0.6 µm (n=30). They were motile. This motility feature is characteristic of the genus *Halomonas* as the vast majority of *Halomonas* species are flagellated.

3.3.Physiological characteristics

Strain RHS90^T is mesophilic and moderately alkaliphilic, since its optimal temperature is 30°C (upper limit 45°C and positive growth at 4°C, the minimal tested temperature) and has a pH range from 6 to 10 (optimum 7-9). It has a euryhaline phenotype, growing at NaCl

concentrations from 0 % to 20 % NaCl (w/v) with a wide optimum of 2 % to 8 %. The strain was shown to be a heterotrophic and obligate aerobic bacterium. It was able to use the following substrates as sole energy and carbon sources, with O_2 as a terminal acceptor: the carbohydrates D(-)fructose, D(-)ribose, sucrose, D(+)galacturonate, pectin, D(-)trehalose, N-acetylglucosamine, and xylan; the alcohols glycerol and mannitol; the organic acids propionate, fumarate and succinate; the amino acids L-alanine, L-arginine, L-asparagine, L-glutamine, L-glutamate, L-methionine, L-proline, L-serine, L-valine, L-cysteine, L-glycine, L-leucine and L-aspartate; and creatine. The strain did not use nitrate and nitrite as terminal electron acceptors with lactate or acetate as the carbon source, which is in agreement with the fact that no amplification of the *nirK* and the *nirS* genes could be obtained. It respired neither sulfate nor DMSO. Growth was not observed under fermentative conditions. No amino acid could be used as sole nitrogen source. Its metabolic versatility regarding carbon and energy sources may allow the strain to use refractory organic matter and detrital macromolecules such as proteins, polypeptides and polysaccharides from dead marine organisms, that may become available to them. Strain RHS90^T presents a distinctive carbon source utilization profile compared with its closest relatives (Table 1): it cannot, for example, use glucose or ethanol as its sole carbon source, whereas *H. axialensis*, *H. meridiana* and *H. aquamarina* are able to use these compounds. On the contrary, strain RHS90^T is able to grow on a minimal medium with D(-) ribose,while its closest relatives cannot.

Similarly to numerous other *Halomonas* species, this euryhaline strain, isolated from a marine sediment with an interstitial water salinity of 4 % (w/v) (Ciobanu et al., 2012), was able to grow under strict halophilic conditions. Indeed, it was shown to be able to grow at concentrations from 0 to 20 % NaCl and its upper and optimal salinities (2-8 %) for growth were higher than the values generally accepted to discriminate halophilic from halotolerant microorganisms (optimum NaCl concentration ≥5 %; upper NaCl concentration ≥10 %)

(Oren, 2008). Strain RHS90^T was also able to grow under high hydrostatic pressure. Its growth rate was optimal at atmospheric pressure, but was slightly affected by an increase in hydrostatic pressure up to 40 MPa (Fig. 2). Above 40 MPa, its growth rate decreased sharply. When grown under 50 or 60 MPa, the growth rate of the novel isolate was about one fifth of its growth rate under atmospheric pressure, but microscopic observations confirmed that cells were still dividing. However, these cells were non-motile and exhibited atypical elongated cellular shapes. LIVE/DEAD® staining of cells exposed to high pressure demonstrated that cells remained intact and that membranes were not permeabilized (Fig. S1). Since its growth rate is higher under atmospheric pressure, this strain can be considered as piezotolerant. Even though *H. meridiana* has already been reported to be capable of growing under 55 MPa (Kaye and Baross, 2004a), this is the first time that growth of a *Halomonas* species has been described under 60 MPa. Piezotolerant strains have already been described among *Gammaproteobacteria* and *Halomonas* species. For example, enrichment cultures under high pressure have already been performed and efficient growth of *Halomona*s-related organisms has been described under 30 MPa (Takami et al., 1999). As the pressure of 60 MPa is much higher than the pressure measured in situ, it can be hypothesized that strain RHS90^T would be capable of growing in deeper environments, at 6,000 m depth where hydrostatic pressure reaches this level of high pressure. The effects of hydrostatic pressure have already been studied in *H. axialensis*, *H. meridiana* and *H. hydrothermalis*, showing a change in membrane lipid composition and in the protein expression level (Kaye and Baross, 2004a). These properties may explain the fact that several *Halomonas* species have also been isolated from deep marine environments (Kaye et al., 2004b; Simon-Colin et al., 2008).

Antibiotics have many roles in natural environments, shaping microbial physiology such as motility or biofilm formation at low concentrations (Raaijmakers and Mazzola, 2012). Considering these multiple effects, we considered it would be interesting to find out whether

the novel isolate was resistant to antibiotics. The strain presented variable sensitivities towards different antibiotics. On solid medium, it was sensitive to nalidixic acid, chloramphenicol, ampicillin, rifampicin and penicillin G at 10 ng, to streptomycin, kanamycin and tetracycline at 30 ng and to vancomycin at 100 ng. The strain was resistant to nitrofurantin, erythromycin and ampicillin at 100 ng. This variability in antibiotic sensitivities of strain RHS90^T may reflect complex cellular communication mediated by diffusive secondary metabolites within natural communities.

3.4.Fatty acids, polar lipids and quinone composition

The main fatty acid component of strain RHS90^T was $C_{18:1}$ *ω7c*(48.6%). The fatty acids $C_{16:1}$ *ω7c*/$C_{15:0}$ iso-2-OH (13 %), $C_{16:00}$ (11.9 %),$C_{19:0}$ cyclo *ω8c*(9.3 %), $C_{12:0}$ 3-OH (6.3 %) and $C_{17:0}$ cyclo (4.3 %) were also present in significant proportions (Table S1). The polar lipid pattern indicated the presence of phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, one phosphoglycolipid, two glycolipids and two phospholipids (Fig. S2). The major respiratory quinone was ubiquinone 9 (90 %), which is the typical dominant quinone in *Halomonas* species. In a previous study, Franzman and Tindall (1990) showed that there was no clear distinction between the genera *Halomonas* and *Deleya*, 2 genera of the family *Halomonadaceae*, on the sole basis of respiratory quinones, polar lipids and fatty acid composition. All species of these genera were described as containing $C_{16:1}$ cis 9, $C_{16:0}$, $C_{17:0}$ cyclo, $C_{18:1}$ and $C_{19:0}$ $cyclo_{11–12}$ as major fatty acid components. Interestingly, $C_{16:1}$ cis 9 and $C_{19:0}$ $cyclo_{11–12}$ were not detected in strain RHS90^T, although a $C_{19:0}$ cyclo *ω8c* fatty acid represented a significant proportion.

3.5. Tolerance to metals

Metals can have beneficial or deleterious effects on cells, mainly depending on which metal is considered and at what concentration. Metals become toxic for a cell when they disturb molecular and cellular functions and structures. In environments such as polluted sites or hydrothermal vents, metals can be present at high concentrations and can diffuse more rapidly into cells. Over their evolution, cells have developed strategies to overcome these problems (Nies, 2003) and the tree of life contains microorganisms with a range of metal sensitivities that are more or less adapted to metal-rich environments. To investigate the capacity of strain RHS90^T to grow in the presence of metals, the MICs of strain RHS90^{T}were determined for 9 metals and compared with the MICs of *Cupriavidus metallidurans* strain CH34^T (determined in this study), a highly metal-resistant bacterium (Mergeay et al., 1985), and to MICs of the model bacterium *E. coli* strain CM237^T determined by Mergeay et al. (2003) (Table 2). The novel isolate was highly sensitive to Ag (MIC: 0.01 mM) and Cd (MIC: 0.75 mM), which inhibited its growth at very low concentrations, but grew very well at high concentrations of Cs (MIC: 200 mM). Strain RHS90^T was also particularly resistant to Mn (MIC: 60 mM). Metal MIC values of strain RHS90^T differed substantially from those of *C. metallidurans* CH34^T and from those of *E. coli* CM237^T. They were higher overall than those of *E. coli* CM237^T, but lower than those of *C. metallidurans*. This trend was observed after exposure to Cd, Cu, Co, Ni and Cs. However, strain RHS90^T had higher MIC for Mn than *C. metallidurans* CH34^T and *E. coli* CM237^T, higher and lower MIC values for Ag than *C. metallidurans* and *E. coli* CM237^T respectively, and was as resistant to Cr as *C. metallidurans*.

Strain RHS90^T might possess specific mechanisms to detoxify cells of an excess of metals. In *C. metallidurans* str. CH34^T, it has been shown that metal tolerance is conferred by different plasmid encoded-systems such as the czc (cobalt-zinc-cadmium) or cnr (cobalt-nickel) tolerance systems (Nies, 2000; Monsieurs et al., 2011). These highly regulated

systems involve the sensing of metals and gene expression activation in order to release metals into the extracellular medium through efflux pumps. Many *Halomonas* species have been reported to harbor plasmids of ~600 Mbp and ~70 Mbp, as well as other extrachromosomal elements (Argandoña et al., 2003). These plasmids could be responsible for some of the adaptive advantages in the genus *Halomonas*, including tolerance to metals. Interestingly, plasmid extraction could be performed on cells of strain RHS90^T, revealing one or several plasmids > 10 kbp (data not shown) that might possibly be involved in metal tolerance. CMI values of strain RHS90^T were higher (Ag, Cu, Cd) or comparable (Co and Cr) to those previously determined for *H. elongata* and *H. subglaciescola* (Nieto et al., 1989), two organisms harboring ~600 kbp and ~70 kbp plasmids (Argandoña et al., 2003).

3.6.Growth under oligotrophic conditions

The isolation of *Halomonas* species and detection of *Halomonas*-related sequences from oligotrophic environments have been extensively described. For example, sediments from the Arctic and Antarctic seas, Mediterranean sea, deep-sea waters or deep-sea bed (Durbin and Teske, 2011; Kaye et al., 2011) have been shown to harbor representatives of the genus *Halomonas*. This widespread representation of *Halomonas* species in nutrient-depleted habitats raises questions about their adaptation to oligotrophic conditions and may reflect a strong capability to thrive in such conditions. In order to ascertain whether strain RHS90^T can survive in extremely nutrient-depleted environments, the strain was stored for 4 weeks in artificial sea water without any carbon source at 4 °C (Fig. 3). During this storage period, cellular density remained constant (~8.10^6 cells.mL^{-1}). The viability of counted cells was demonstrated by the positive growth of starved cultures when these were transferred to nutrient-rich media (MB2216) inoculated with the stored cell suspension diluted from the 1/100th to the 1/100,000th. Total ATP content (with 85-97 % representing intracellular ATP)

determination showed that cellular activity remained relatively constant after 15 days storage and dropped off sharply after 25 day storage. This can be explained by (i) a decrease in metabolic activity and/or (ii) a decrease in cell size, as was microscopically observed (data not shown). These results show that strain RHS90^T remained viable and maintained its population size under extremely oligotrophic conditions and at low temperatures over a period of one month.

3.7.Amplification of PHA synthesis genes

Many prokaryotes respond to starvation or to imbalanced ratios between carbon and nitrogen through the accumulation of carbon substrates in the form of polyhydroxyalkanoate (PHA) granules. PHA metabolism relies mostly on PHA synthase (*phaC*), PHA depolymerase (*phaZ*) and phasin, a protein associated with PHA granule inclusions (Matsumoto et al., 2002). PHA granules are synthesized by *phaC* when carbon sources are abundant and used under starvation. The phasin gene is generally located upstream of *phaC* and this genomic organization is conserved in many *Proteobacteria* (Cai et al., 2011). It is likely that PHA accumulation confers a strong adaptive advantage in natural environments where carbon source concentrations fluctuate. To determine whether strain RHS90^T had the genetic potential to synthesize PHA granules, PCR amplifications of the *phaC* gene were performed on DNA extracts. A single stretch of 234 nucleotides was obtained with the primer pairs phaCF998- phaCR767. Sequence comparison showed that this sequence was highly similar to poly(R)-hydroxyalkanoic acid synthase of some other *Halomonas* species. The highest similarity (96 % identity) was shared with *Halomonas* sp. HAL1, *Halomonas* sp. GFAJ and *Halomonas* sp. TD01, isolated from a gold mine and from two salt lakes (California, USA and Xinjiang, China), respectively (Lin et al., 2011; Tan et al., 2011; Kim and Rensing, 2012). This suggests that the genome of strain RHS90^T encodes a PHA synthase gene. Four classes

of PHA synthases have been described, differing in subunit numbers and product chain-lengths (Cai et al., 2011). Phylogenetic reconstruction demonstrated that this sequence belongs to class I of *phaC* genes (Fig. S3). Class I *phaC* comprises enzymes with one subunit that synthesizes short chains (3-5 carbon atoms) and medium chains (6 - 14 carbon atoms). Interestingly, other *phaC* genes belonging to class I have been sequenced in *Halomonas* sp. TD01 and *Halomonas elongata* (Cai et al., 2011).

Intracytoplasmic granules of PHA were observed by microscopy after Nile Blue A staining, suggesting that the amplified *phaC* gene is functional and allows the synthesis of PHA.

3.8.Production of ectoine

Compatible solutes such as ectoine, hydroxyectoine, betaine or glutamate are commonly produced by halophilic microorganisms to adapt to osmotic pressure caused by high extracellular salt concentrations. These compatible solutes prevent molecular and cellular structures from dehydration or freezing (Zhu et al., 2011). In order to discover whether such compatible solutes are produced by strain RHS90^T, the metabolites of cells grown in MB without or with 12.5 % NaCl were analyzed with nuclear magnetic resonance (NMR) spectroscopy (Fig. S4).

When grown in rich medium without NaCl, cells did not accumulate ectoine (Fig. S4A). On the contrary, when cells were grown in rich medium supplied with 12.5 % NaCl, peaks attributed to ectoine, glycine betaine and glutamate were detected and represented the vast majority of metabolites accumulated (Fig. S4B). These results demonstrate that ectoine is accumulated by biosynthesis under our hypersaline growth conditions and suggest that the genome of strain RHS90^T carries the ectoine biosynthetic pathway genes. Similar results were previously obtained with *H. pantelleriense*. Ectoin was indeed the most abundant compatible

480 solute detected in *H. pantelleriense* when grown in rich medium, and hydroxyectoine, betaine,
481 glycine and glutamate were also detected (Romano et al., 2001). The proportion of ectoine
482 increased with increasing NaCl concentration. This phenomenon was observed in rich (yeast-
483 extract) medium but appeared less pronounced in minimal (glucose) medium (Romano et al.,
484 2001). In another study, Zhu et al. (2011) showed that the presence of ectoine or
485 hydroxyectoine increased the cellular growth of the halophile *Halomonas ventosae* DL7^T after
486 both thermal and osmotic stresses.

487 In order to confirm that an ectoine synthase (*ectC*) encoding gene is indeed borne by the
488 genome of strain RHS90^T, PCR amplifications were performed. Unfortunately, no positive
489 amplification could be obtained. This lack of amplification may be attributed to the use of a
490 non-specific primer, since primer sequences were determined on the basis of the *H. elongata*
491 *ectC* gene sequence (NCBI Accession number: YP_003897659). The corresponding regions
492 might not be conserved in strain RHS90^T, thus leading to mismatches.

493

494

In conclusion, this study demonstrates the physiological plasticity of strain RHS90^T. From the results of polyphasic taxonomic analysis and based on genetic, physiological and chemotaxonomic distinctness, it is proposed that strain RHS90^T be considered a novel species within the genus *Halomonas,* for which the name *Halomonas lionensis* is proposed. This novel species presents interesting growth features, especially in terms of salinity, metal concentration and hydrostatic pressure tolerance. It has developed adaptive mechanisms based notably on PHA and ectoine accumulation, to overcome extreme environmental conditions. This flexibility might allow strain RHS90^T to colonize environments associated with a variety of environmental conditions and may be related to the ecological success and the ubiquitous presence of *Halomonas* species in natural settings. More studies focusing on the adaptive mechanisms are needed to fully understand the interaction of *Halomonas* species with their natural biogeochemical environments. It would be, for instance, relevant to undertake comparative genomics studies within the genus *Halomonas*, notably to investigate the role of plasmids in the ecological success of this genus.

Description of Halomonas lionensis sp. nov.

(li.on.en'sis. N.L. fem. adj. lionensis, of or belonging to *Golfe du Lion* [Gulf of Lions], in reference to the origin of the type strain).

Cells are Gram-negative, rod-shaped, motile, 0.7-2.5 µm in length x 0.4-1 µm in width. Colonies on MA are white, regularly circular, convex, translucent, smooth with an entire edge, creamy and do not produce exopolysaccharides. Grows aerobically at ≤ 4– 45 °C with an optimum at 30 °C, pH 6-10 with an optimum at 7-9 and with NaCl concentrations ranging

fom 0-20 % (w/v) with an optimum at 2-8 %. Negative for nitrate and nitrite reduction, fermentation of peptone or yeast extract, Voges-Proskauer test and Methyl red test, indole formation, β-galactosidase (ONPG), arginine dihydrolase, gelatinase, *β*-glucosidase, lysine decarboxylase, ornithine decarboxylase, tryptophane deaminase, potassium gluconate assimilation, capric acid assimilation, adipic acid assimilation. Positive for urease, oxidase and catalase. The following substrates can be used as sole carbon source: citrate, fumarate, propionate, succinate, glycerol, D-mannitol, pectin, xylan, D(-)fructose, poly-D-(+)galacturonic acid, N-acetylglucosamine, D(+)mannose, D(+)rhamnose, D(-)ribose, sucrose, D(-)trehalose, L-alanine L-arginine, L-asparagine, L-glutamate L-glutamine, L-glycine, L-leucine L-proline, L-serine, L-valine, creatine. The following substrates cannot be used as sole carbon source: collagen, elastine, keratine, tween 80, acetate, ascorbate, benzoate, betain, caprylate, citrate, formate, gluconate, hippurate, lactate, malate, malonate, tartrate, *myo*-inositol, ethanol, isopropanol, sorbitol, D-melezitose, threalose, L(+)arabinose, cellulose, dextrine, D(+)cellobiose, D(+)glucose, D(+)galactose, D(+)lactose, D(+)maltose, D(+)xylose, L-aspartate, L-cysteine, L-glycine, L-histidine, L-isoleucine, L-lysine, L-methionine, L-ornithine, L-phenylalanine, L-threonine, L-tryptophane, L-tyrosine and L-valine. None of the 20 proteic amino acids can be used as sole nitrogen source.

The main fatty acids are C_{16} (11.85 %), $C_{17:00}$ cyclo (4.32 %), $C_{19:0}$ CYCLOω8*c* (9.32 %), $C_{18:1}\omega 7c$ (48.6 %), 3-OH $C_{12:0}$ (6.25 %) and $C_{16:1}\ \omega 7c$ and/or 2-OH iso-$C_{15:0}$ (13 %). The main polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine and an unidentified phosphoglycolipid. Ubiquinone 9 (Q-9) is the major quinone (90 %). The DNA G + C content is 54.4 mol %.

The type strain RHS90^T (DSM 25632^T, CIP 110370^T, UBOCC3186) was isolated from surficial sediments (84 cm below the seafloor) of the Gulf of Lions, in the western Mediterranean Sea.

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Figure captions

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences showing the relationships between *Halomonas lionensis* RHS90^T and its related phylogenetic neighbours. The topology shown was calculated with the neighbour-joining algorithm. Accession numbers are indicated in brackets. Bootstrap values (%) are indicated at the branch nodes and were calculated from 1000 resampled datasets. *Chromohalobacter canadensis* and *Chromohalobacter israelensis* were used as outgroups.

Fig. 2. Effects of hydrostatic pressure on the growth rate of strain RHS90^T. Bars indicate standard deviation (n=3).

Fig. 3. Cellular activity, as determined by total ATP content and cellular density of cells of strain RHS90^T stored in carbon source-depleted artificial sea water. The total and extracellular ATP contents of artificial sea water are represented by black and grey bars, respectively. Cellular densities determined by cell counts are shown by white squares.

718

719 **Tables**

720 **Table 1**

721 Phenotypic characteristics that differentiate strain RHS90^T from related species of the genus

722 *Halomonas*.

Characteristic	**1**	**2**	**3**	**4**
Isolation source	Mediterranean Sea sediments	Temperate ocean	Cold hypersaline lake	Low temperature hydrothermal fluid
Motility	Y	Y	Y	Y
Size (µm)	4.4-2.2 × 0.8-0.6	4-6 × 0.4-0.6	1.9-4.5 × 0.6-1.0	ND
Temperature range (opt)	≤ 4 – 45 (30)	5 – 40 (20-25)	-5 – 45 (28–40)	-1 – 35 (30)
pH range (opt)	6 – 10 (7-9)	5 – 10	5 – 10	5 – 12
NaCl range %w:v (opt)	0 – 20 (2-8)	0 – 20 (7.5-10)	0.01 – 2 5 (1-3)	0.5 – 24 (4)
Hydrolysis of:				
Tween 80	–	+	+	–
Growth with:				
L(+)Arabinose	–	+	-	+
D(-)Fructose	+	-	-	+
D(+)Galactose	–	-	+	–
D(+)Glucose	–	+	+	+
D(+)Lactose	–	+	–	–
D(+)Maltose	–	–	+	+
D(-)Ribose	+	–	–	–
Citrate	–	+	–	–
Lactate	–	+	+	–
Malonate	–	+	–	–
Propionate	+	+	–	–
Succinate	+	+	+	–
Ethanol	–	+	+	+
Glycerol	+	+	+	–
Mannitol	+	+	–	–
L-Alanine	+	+	+	–
L-Arginine	+	–	ND	ND
L-Asparagine	+	–	ND	–

L-Glutamine	+	–	ND	ND
L-Glutamate	+	–	–	+
Lysine	–	+	+	–
Proline	+	–	+	–
Serine	+	–	+	–
Valine	+	–	–	–
DNA G+C content (mol %)	54.4	57-58	58.2-59.9	54.4

723

724 Taxa: 1, strain RHS90^T (this study); 2, *H. aquamarina* (Kaye et al. 2004; Arahal

725 and Ventosa 2006); 3, *H. meridiana* (Kaye et al. 2004; Arahal and Ventosa 2006) ; 4, *H.*

726 *axialensis* (Kaye et al. 2004) ; +, Positive; –, Negative; ND, no data available; Y, Yes.

727

728 **Table 2.**

729 Comparison of Minimal Inhibitory Concentrations (MIC) of different metals for *H. lionensis*

730 strain RHS90^T, *C. metallidurans* CH34^T and *E. coli* CM237^T.

	$AgSO_4$	$CdCl_2$	$CrK(SO4)_2$	$CuSO_4$	$CoSO_4$	$NiCl_2$	$ZnSO_4$	$MnSO_4$	CsCl
Halomonas lionensis									
RHS90^T	0.01	0.75	1.75	2	3	8	3	60	200
Cupriavidus metallidurans									
CH34^T	0.0005	8	1.75	3	35	13	12	30	250
***E.coli strain*CM237^T** [a]	0.02	0.5	0.2	1	1	1	1	20	50

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732 Values are expressed in mM.

733 [a] The MIC values of *E. coli* strain CM237^T correspond to those previously determined by

734 Monsieur et al. (2011).

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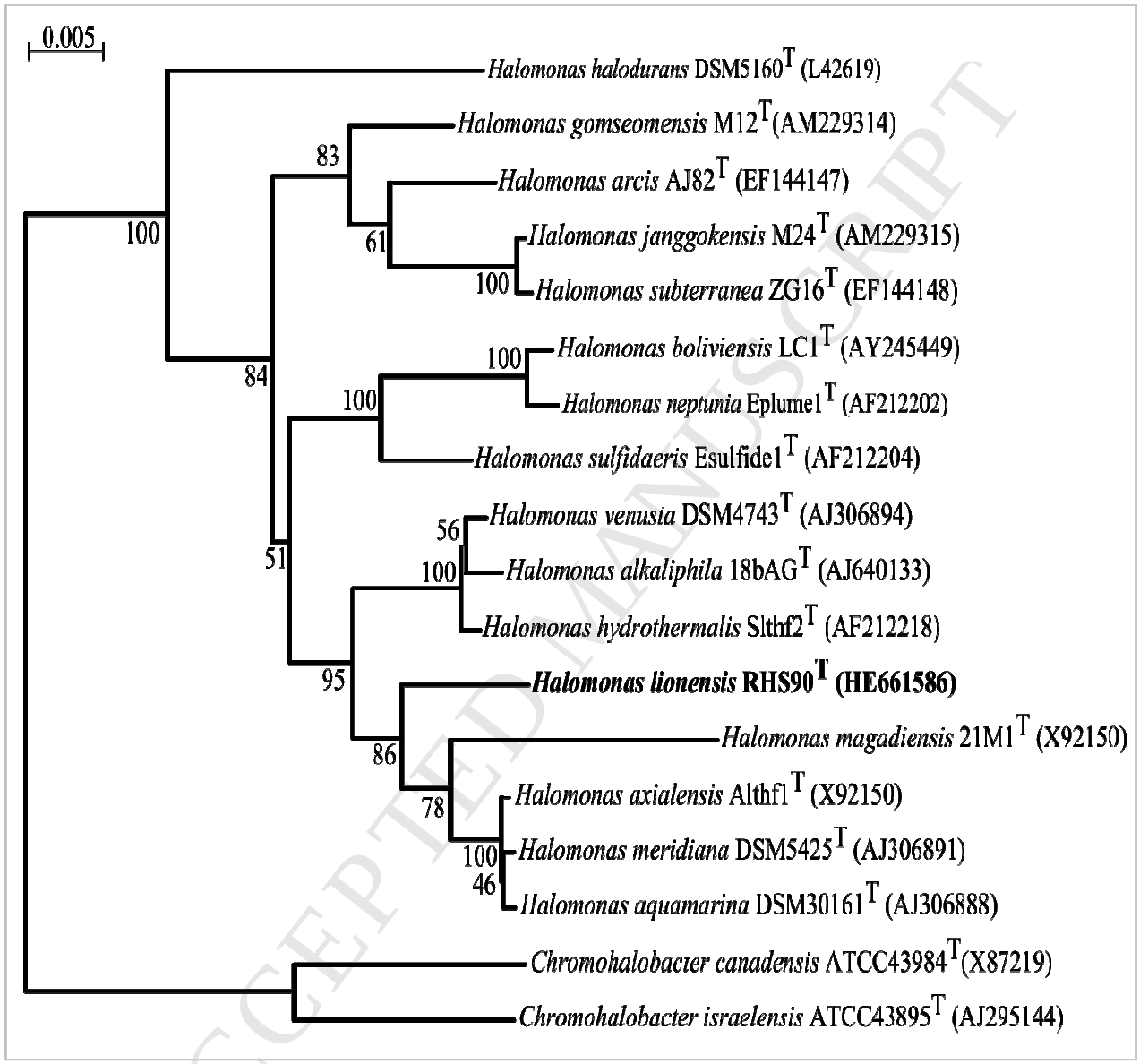
737 **Figures**

738 **Fig. 1.**



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747 **Fig. 2.**

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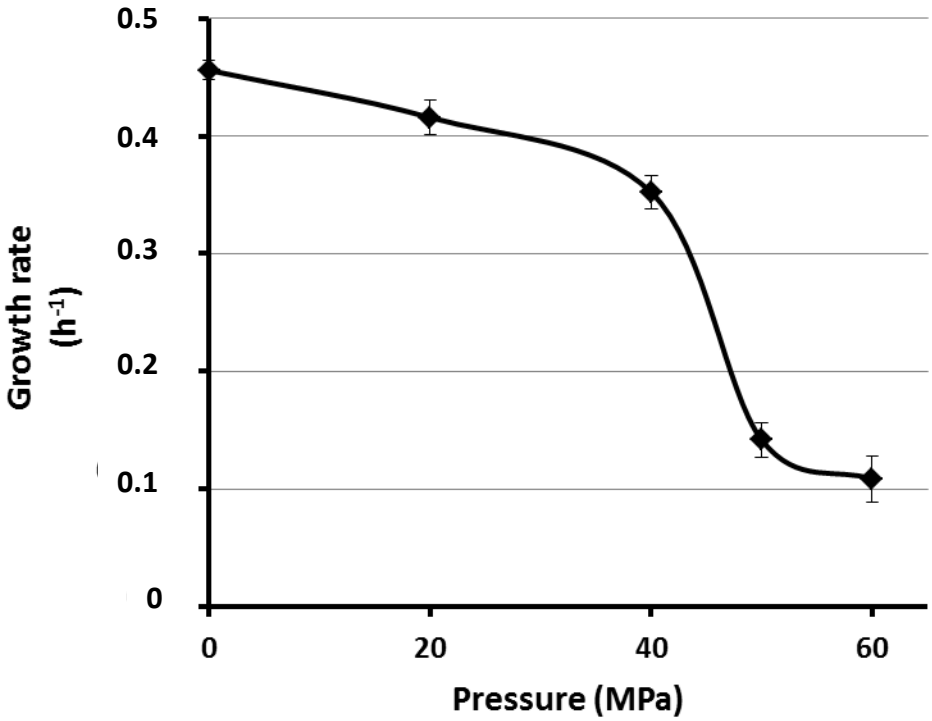

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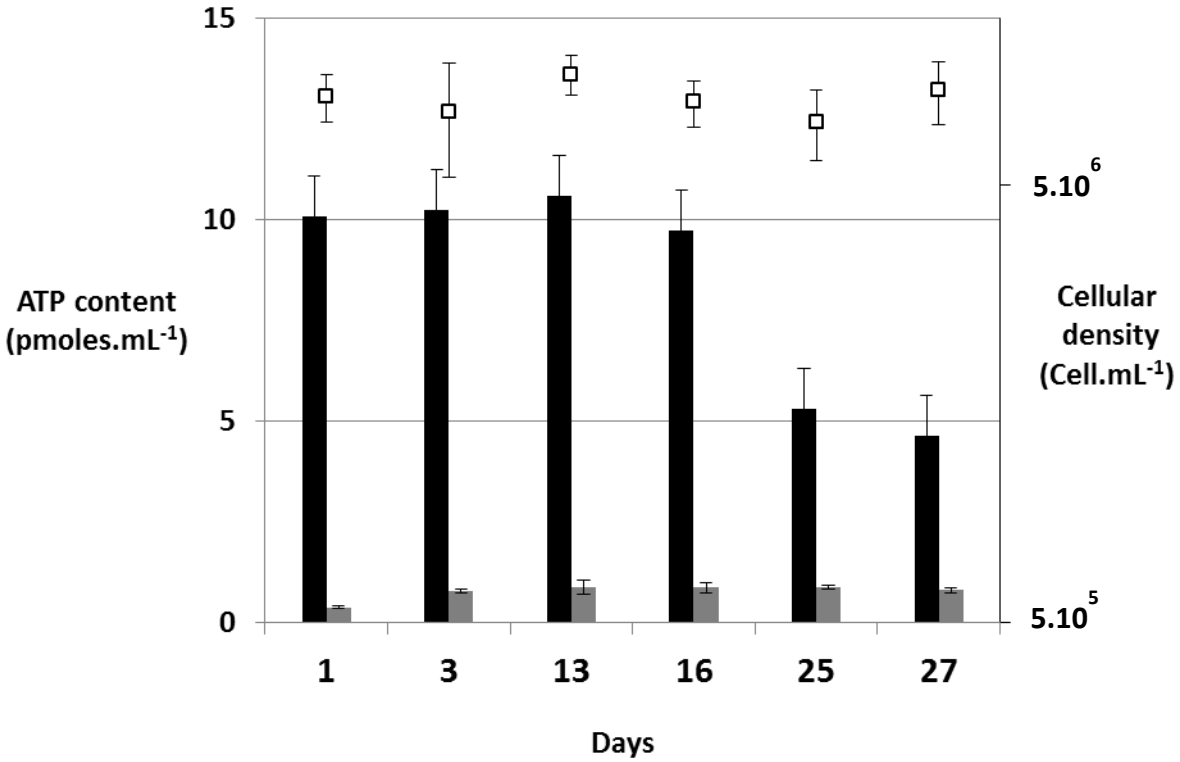
760 **Fig. 3.**



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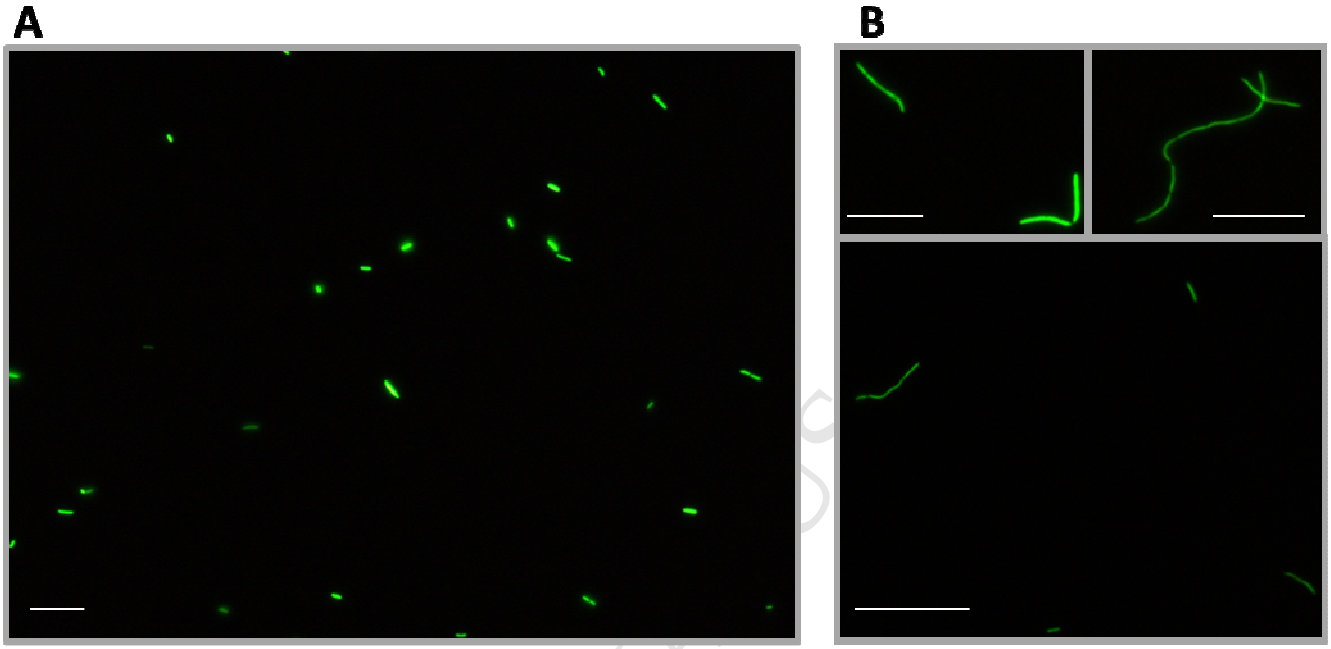
762 **Supplementary materials for on line submission**



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764 **Fig. S1.** UV-exposed micrographs of cells of strain RHS90^T incubated for 9 hours under atmospheric
765 pressure (A) or under 60 MPa (B) and stained with the *LIVE/DEAD® BacLight™* Bacterial Viability
766 mixture. Bars, 5µm.

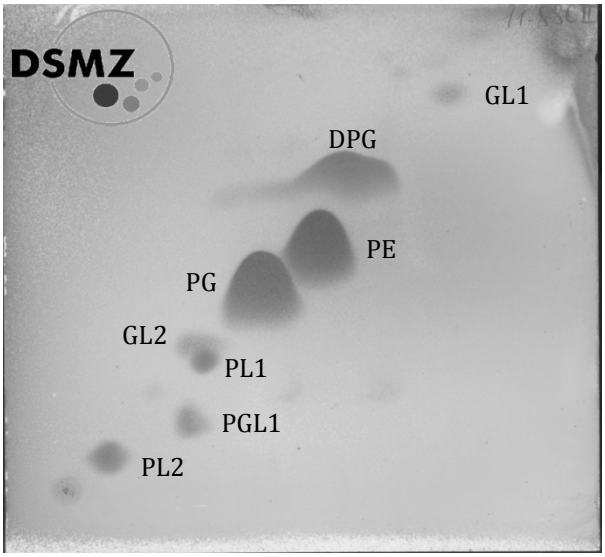

Fig. S2. Polar lipids of strain RHS90^T following separation by two-dimensional TLC. PE, Phosphatidylethanolamine; PG, Phosphatidylglycerol; PC, Phosphatidylcholine; GL1-GL2, Glycolipids; PL1-PL2, Phospholipids; DPG, Diphosphatidylglycerol; PGL1, Phosphoglycolipids.

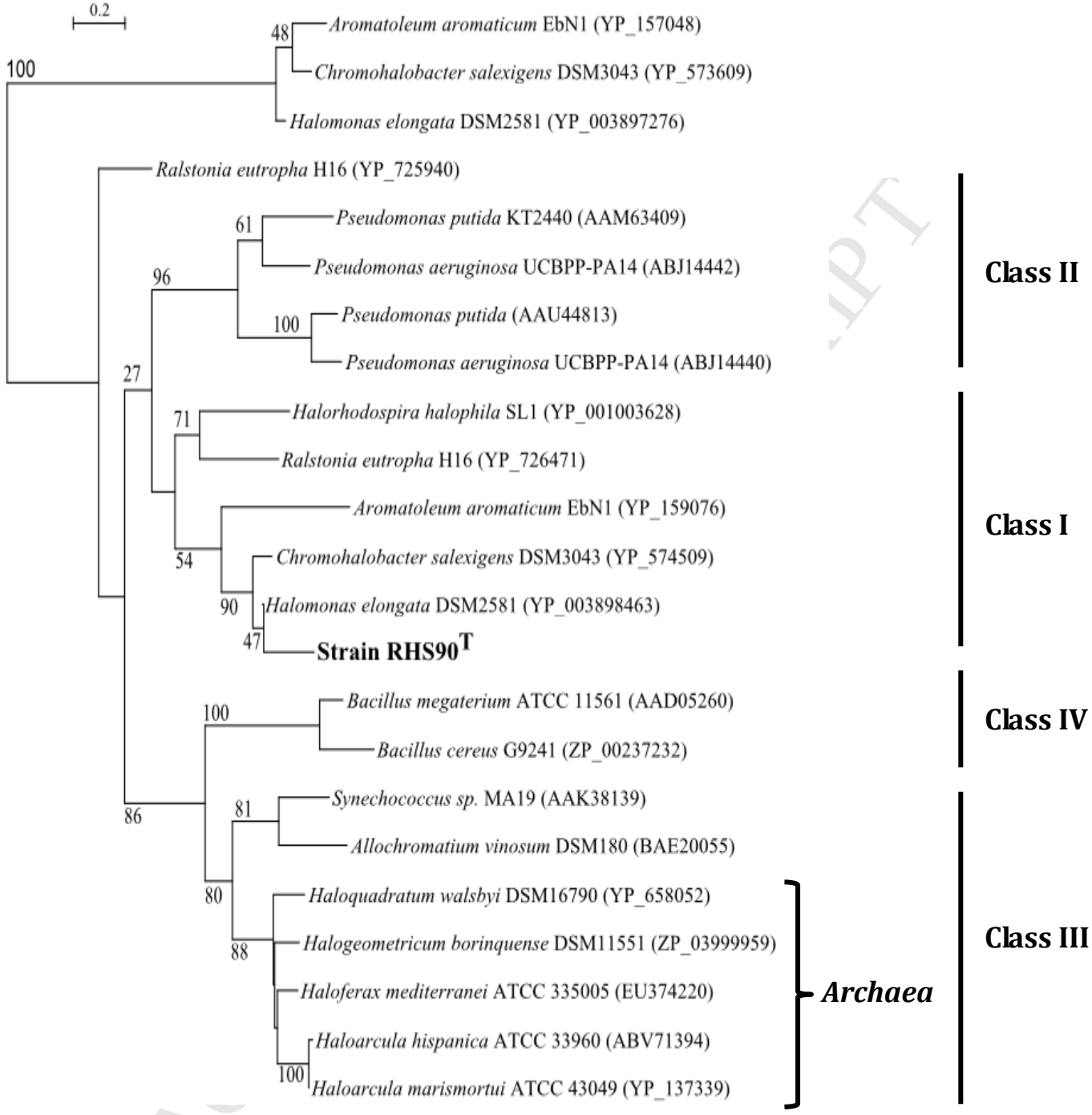

Fig. S3. Phylogenetic position of the putative *phaC* gene sequence of strain RHS90^T.

A multiple alignment was made with ClustalW and the tree was constructed using the neighbour-joining algorithm of Seaview4. The GenBank accession numbers are given in brackets.

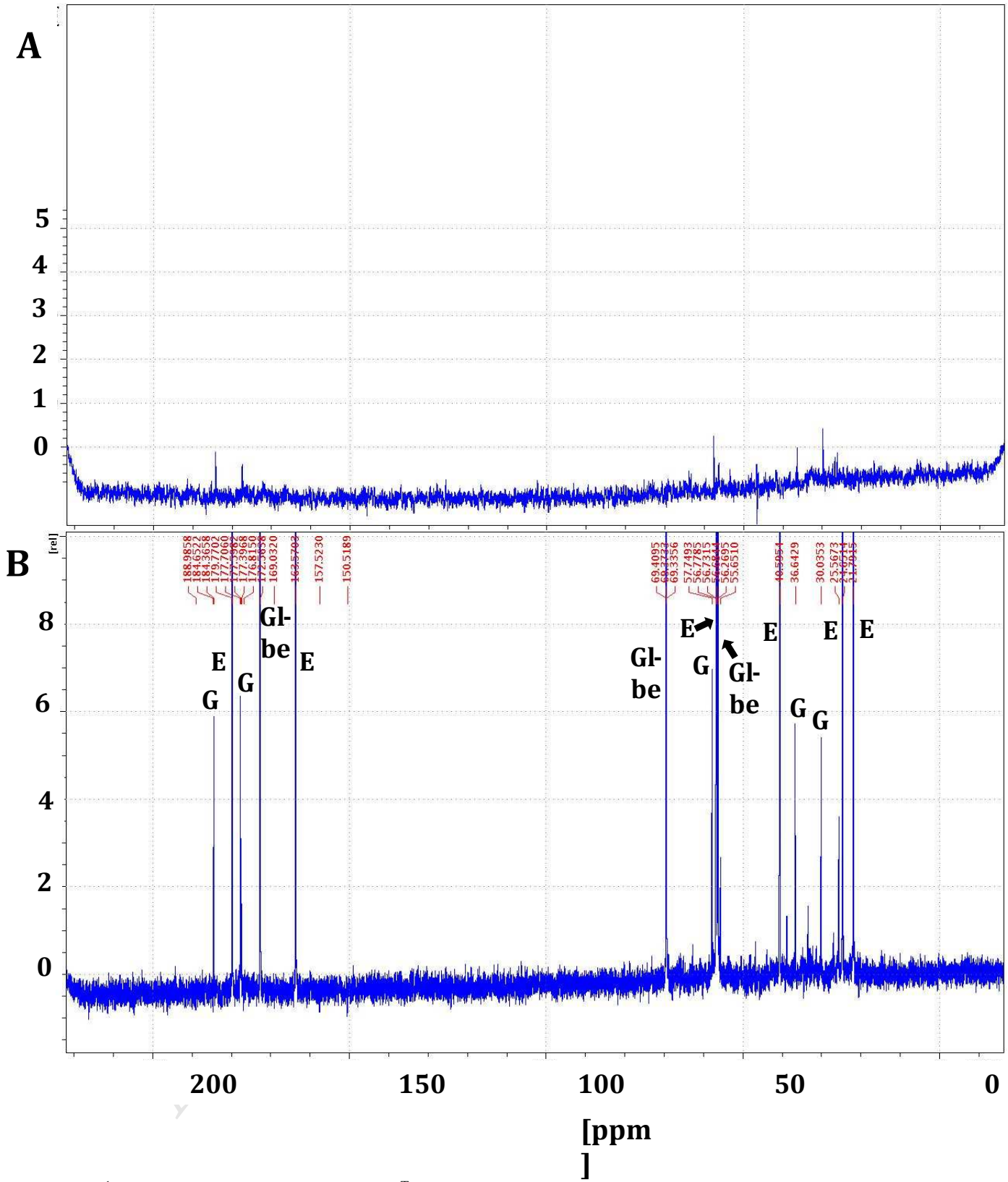

Fig. S4. ^{1}H-NMR spectra of strain RHS90^{T} grown without (A) or with (B) 12.5% (w/v) NaCl. The major solutes were ectoine (E), glycine-betaine (Gl-bt) and glutamate (G).

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829 **Table S1** Whole-cell fatty acid profile of strain RHS90^T cells at mid-exponential growth
830 phase, cultivated on MB2216; 99.59% of the fatty acid peaks could be assigned by the
831 Sherlock Microbial Identification System (MIDI Inc, Newark, USA). Major fatty acids are
832 indicated in bold.

Fatty acid	**Proportion (%)**
Saturated	
$C_{10:0}$	0.17
$C_{12:0}$	0.66
$C_{14:0}$	2.38
$C_{16:0}$	**11.85**
$C_{17:0}$	0.27
$C_{17:0}$ ISO	0.17
$C_{17:0}$ CYCLO	**4.32**
$C_{18:0}$	0.46
$C_{19:0}$ CYCLO $\omega 8c$	**9.32**
Monounsaturated	
$C_{18:1}\omega 7c$	**48.60**
Hydroxy	
3-OH $C_{10:0}$	0.27
3-OH $C_{12:0}$	**6.25**
2-OH $C_{18:1}$	0.12
Methyl-substituted	
11-methyl $C_{18:1}\omega 7c$	1.75
Summed featured	
Summed feature 3[a]	**12.99**
Summed feature 7[a]	0.54

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834 Legend: ECL, equivalent chain-length. [a] Summed feature 3 contains $C_{16:1}$ $\omega 7c$ and/or 2-OH iso-$C_{15:0}$

835 and summed feature 7 contains an unidentifed component with 18.846 ECL and/or ante-$C_{19:1}$ $\omega 6c$.

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