Larsenia salina gen. nov., sp. nov., a new member of the family *Halomonadaceae* based on multilocus sequence analysis

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Note: Nucleotide sequence data for the 16S rRNA, *atpA, rpoD* and *secA* genes are available in the GenBank/EMBL/DDBL databases under the accession numbers: HF678441, KJ182932, KJ182934 and KJ182936 (strain M1-18^T) and HG917900, KJ182931, KJ182933 and KJ182935 (strain L1-16), respectively.

Abstract

Two Gram-staining-negative, moderately halophilic bacteria, strains M1-18^T and L1-16, were isolated from a saltern located in Huelva (Spain). They were motile, strictly aerobic rods, growing in the presence of 3-25% (w/v) NaCl (optimal growth at 7.5-10% [w/v] NaCl), between pH 4.0 and 9.0 (optimal at pH 6.0-7.0) and at temperatures between 15 and 40 ºC (optimal at 37 ºC). Phylogenetic analysis based on 16S rRNA gene sequence comparison showed that both strains showed the higher similarity values with *Chromohalobacter israelensis* ATCC 43985^T (95.2-94.8%) and *Chromohalobacter salexigens* DSM 3043^T (95.0-94.9%), and similarity values lower than 94.6% with other species of the genera *Chromohalobacter, Kushneria, Cobetia* or *Halomonas*. Multilocus sequence analysis (MLSA) based on the partial sequences of *atpA, rpoD* and *secA* housekeeping genes indicated that the new isolates formed an independent and monophyletic branch that was related to the peripheral genera of the family *Halomonadaceae*, *Halotalea, Carnimonas* and *Zymobacter*, supporting their placement as a new genus of the *Halomonadaceae*. The DNA-DNA hybridization between both strains was 82 %, whereas the values between strain M1-18^T and the most closely related species of *Chromohalobacter* and *Kushneria* were equal or lower to 48 %. The major cellular fatty acids were $C_{18:1}\omega7c/C_{18:1}\omega6c$, $C_{16:0}$, and $C_{16:1}\omega7c/C_{16:1}\omega6c$, a profile that differentiate this new taxon from species of the related genera. We propose the placement of both strains as a novel genus and species, within the family *Halomonadaceae*, with the name *Larsenia salina* gen. nov., sp. nov. The type strain is M1-18^T (=CCM 8464 =CECT 8192^T =IBRC-M 10767^T =LMG 27461^T).

49 *Keywords:* Moderately halophilic bacteria, *Larsenia*, *Larsenia salina*,
50 *Gammaproteobacteria*, hypersaline environments, salterns.

51 Scope: Systematics

52

53 **Introduction**

54 Moderately halophilic bacteria are characterized by their optimal growth in
55 media containing 3 to 15 % (w/v) NaCl [37]. They contribute to the microbiota of saline
56 habitats, such as saline and alkaline lakes, salterns, saline soils and salted foods as well
57 as other salted materials [35]. The family *Halomonadaceae* includes a large number of
58 species, most of them are moderately halophilic and have been isolated from
59 hypersaline habitats. This family comprises currently 10 genera, being *Halomonas* and
60 *Chromohalobacter* the genera that include a larger number of species [10]. The family
61 *Halomonadaceae* is phylogenetically coherent, according to studies carried out using
62 16S rRNA and 23S rRNA gene sequences [5,8] and more recently by a Multilocus
63 Sequence Analysis (MLSA) based on five housekeeping genes [9]. However, the 16S
64 rRNA gene is not an adequate phylogenetic marker for the delineation of taxa within the
65 *Halomonadaceae* due to the low evolution rate and the close relationship of the species,
66 especially in the genus *Halomonas* and for those reasons recently it has been suggested
67 that MLSA should be used in order to define adequately new taxa within this family [9];
68 this view has been endorsed by the ICSP-Subcommittee on the taxonomy of the family
69 *Halomonadaceae*, and a recommendation for including such studies on the delineation
70 of new taxa has been adopted [24].

During the course of studies on the microbial diversity of salterns in south Spain we isolated two new halophilic microorganisms, designated as strains M1-18^T and L1-16, which according to preliminary identification could represent a new genus and species of the *Halomonadaceae*. The aim of this study was to determine the taxonomic position of these two strains using a polyphasic approach and a complete MLSA analysis in order to define their taxonomic status. We propose the placement of these two strains in a new genus of the family *Halomonadaceae,* for which we propose the new designation *Larsenia salina* gen. nov., sp. nov.

Materials and methods

Isolation and bacterial strains

Strains M1-18^T and L1-16 were isolated from water samples of two ponds of Isla Bacuta saltern, located in Huelva (37º12'31"N 7º19'49"W), South West Spain. Samples were collected in sterile containers and transported within 3-4 h of its collection to the laboratory, plated immediately on SW medium supplemented with 0.1 % calcium acetate or 0.09 % glycerol and incubated at 37 ºC for one month. The isolation medium for strain M1-18^T contained (g l^{-1}): NaCl, 117; $CaCl_2$, 0.5; KCl, 3; $NaHCO_3$, 0.1; NaBr, 0.35 [37], supplemented with (g l^{-1}) yeast extract, 1.0 and calcium acetate, 1.36, solidified with 1.8 % agar. The same medium was used for the isolation of strain L1-16 but supplemented with (g l^{-1}) yeast extract, 0.5 and glycerol, 0.9. The pH was adjusted to 7.5 with 1 M KOH. These strains were routinely grown in SW 7.5 % medium at 37 ºC; its composition is the following: (g l^{-1}): NaCl, 58.5; $MgCl_2.6H_2O$, 9.75; $MgSO_4.7H_2O$, 15.25; $CaCl_2$, 0.25; KCl, 1.5; $NaHCO_3$, 0.05; NaBr, 0.175 and

94 yeast extract, 5.0. The pH was adjusted to 7.5. These cultures were maintained at -80 ºC
95 in SW 7.5 % medium containing 50 % (v/v) glycerol.

96 The type strains *Chromohalobacter israelensis* CECT 5287^T, *Chromohalobacter*
97 *canadensis* ATCC 43984^T, *Chromohalobacter marismortui* ATCC 17056^T,
98 *Chromohalobacter beijerinckii* DSM 7218^T, *Chromohalobacter salexigens* DSM 3043^T,
99 *Kushneria aurantia* A10^T and *Kushneria avicenniae* MW2a^T were used as reference
100 strains for comparative purposes. They were cultivated under the same conditions than
101 strains M1-18^T and L1-16.

102 *Phenotypic characterization*

103 The proposed minimal standards for describing new taxa of the family
104 *Halomonadaceae* as recommended by Arahal et al. [6] were followed. For the
105 determination of cellular morphology and motility, a culture from liquid 7.5 % SW
106 medium was examined by light microscopy under a phase-contrast microscope. The
107 morphology of colonies, their size and pigmentation were observed on the 7.5 % SW
108 solid medium after 48 h of incubation at 37 ºC. Optimal conditions for growth were
109 determined by growing the strains in SW medium at 0.5, 1, 2, 3, 5, 7.5, 10, 15, 20, 25
110 and 30 % (w/v) NaCl, and at temperatures of 4, 15, 20, 28, 30, 37, 40 and 45 °C,
111 respectively. The pH range for the isolates was tested in SW7.5 medium adjusted to the
112 following pH values: 3.0, 3.5, 4.0, 5.0, 6.0, 7.0, 7.5, 8.0, 9.0 and 10.0 with the addition
113 of the appropriate buffering capacity to each medium [28]. All biochemical tests were
114 carried out at 7.5 % NaCl and 37 °C, unless it is stated otherwise. Growth under
115 anaerobic conditions (with H_2/CO_2) was determined by incubating strains in an
116 anaerobic chamber in SW7.5 medium. Tests for anaerobic growth on nitrate or arginine
117 was determined as previously described [7]. Catalase activity was determined by adding

a 1 % (w/v) H_2O_2 solution to colonies on SW7.5 agar medium. Oxidase test was performed using the Dry Slide Assay (Difco). Hydrolysis of aesculin, casein, DNA, gelatin, starch, Tween 80, Voges-Proskauer and methyl red tests, production of indole, phenylalanine deaminase, phosphatase, urease and nitrate reduction were determined as described by Cowan & Steel [7] with the addition of a 7.5% total salts to the medium [26,37]. Citrate utilization was determined on Simmon's Citrate medium supplemented with SW7.5. Acid production from carbohydrates was determined using phenol red base supplemented with 1 % of the carbohydrate and SW7.5 medium [37]. For determining the range of substrates used as carbon and energy sources or as carbon, nitrogen and energy sources, the classical medium of Koser [18] as modified by Ventosa et al. [37] was used: 75 g NaCl l^{-1}, 2 g KCl l^{-1}, 0.2 g $MgSO_4 \cdot 7H_2O$ l^{-1}, 1 g KNO_3 l^{-1}, 1 g $(NH_4)_2HPO_4$ l^{-1}, 0.5 g KH_2PO_4 l^{-1} and 0.05 g yeast extract (Difco) l^{-1}. Substrates were added as filter-sterilized solutions to give a final concentration of 1 g l^{-1}, except for carbohydrates, which were used at 2 g l^{-1}. When the substrate was an amino acid, it was tested as carbon, nitrogen and energy source, and the basal medium was therefore prepared without KNO_3 and $(NH_4)_2HPO_4$.

Phylogenetic analysis based on the 16S rRNA gene sequence comparison

Genomic DNA from strains M1-18^T and L1-16 was prepared using the method described by Marmur [21]. Their 16S rRNA gene was amplified by PCR with the forward primer 16F27 and the reverse primer 16R1488 [23]. Direct sequence determination of the PCR-amplified DNA was carried out using an automated DNA sequencer (model ABI 3130XL; Applied Biosystems). The 16S rRNA gene sequence analysis was performed with the ARB software package [20]. The 16S rRNA gene sequences were aligned with the published sequences of the closely related bacteria and

142 the alignment was confirmed and checked against both primary and secondary
143 structures of the 16S rRNA molecule using the alignment tool of the ARB software
144 package. Phylogenetic trees were constructed using three different methods: maximum
145 likelihood [12], maximum parsimony [14] and neighbour-joining [27], algorithms
146 integrated in the ARB software for phylogenetic inference. Bootstrap test [13] was
147 performed by calculating 1000 replicate trees in order to assess the robustness of the
148 topology. The 16S rRNA gene sequences used for phylogenetic comparisons were
149 obtained from the GenBank database and their strain designations and accession
150 numbers are shown in Figure 1.

151 *Multilocus sequence analysis (MLSA)*

152 In addition to the 16S rRNA gene, three protein-encoding genes were sequenced
153 and analyzed, according to the recommendations by de la Haba et al. [9]: *atpA* (F1-ATP
154 synthase, α subunit), *rpoD* (RNA polymerase, β subunit) and *secA* (protein translocase,
155 SecA subunit). PCR amplification and sequencing was carried out using primers
156 atpA493F and atpA1120R, rpoD88F and rpoD1321R, and secA555F and secA1131R
157 [9]. The housekeeping gene sequences obtained were aligned with the respective
158 sequences from members of the family *Halomonadaceae* retrieved from
159 GenBank/EMBL/DDBJ databases using CLUSTAL_X 2.0 [19] and BioEdit [16],
160 taking into account the corresponding amino acid alignments. Sequence data for
161 *Pseudomonas aeruginosa* strain PAO1, extracted from the GenBank/EMBL/DDBJ
162 databases, were used to polarize the phylogenies. Phylogenetic trees were constructed
163 using the maximum likelihood algorithm [12] as implemented in PhyML software [15],
164 as indicated elsewhere [9]. Bootstrap analyses [12] were performed using 1000
165 replications.

166 *Chemotaxonomic analysis*

167 Fatty acids analysis was performed using the MIDI system (Microbial
168 Identification System). Cells were cultured on TSA medium (Difco) supplemented with
169 10 % NaCl at 37 ºC for 24 h. The extraction and analysis of fatty acids were performed
170 according to the recommendations of the MIDI system [29]. This analysis was carried
171 out by the Identification Service of the CECT Culture Collection (Valencia, Spain). The
172 peptidoglycan structure of strains M1-18^T was determined as described by Schleifer
173 [30] and Schleifer and Kandler [31] by chromatography on a cellulose thin-layer plate
174 (Merck). Polar lipid analysis and isoprenoid quinone determination were carried out by
175 the Identification Service of the DSMZ (Braunschweig, Germany). Lipids were
176 extracted from freeze-dried cells and analysed by two-dimensional thin layer
177 chromatography (TLC) according to Tindall et al. [34]. The quinones were separated by
178 TLC on silica-gel plates and then further analysed by HLPC.

179 *DNA fingerprinting*

180 For genotypic differentiation, strains M1-18^T and L1-16 were compared using two DNA
181 fingerprint methods, two repetitive element primed (rep)-PCRs, BOX- and $(GTG)_5$-
182 PCR. All PCRs were carried out in a total volume of 50 µl including 50 ng/µl genomic
183 DNA, 10X reaction buffer, 25 mM $MgCl_2$, 1.25 mM each dNTP, 12 µm each primer
184 and 5 U/µl *Taq* DNA polymerase. BOX-PCR was performed with primer BOXA1R (5'-
185 CTACGGCAAGGCGACGCTGACG-3') and $(GTG)_5$-PCR with primer $(GTG)_5$ (5'-
186 GTGGTGGTGGTGGTG-3'[39]. PCR conditions were as follows: 95 ºC for 3 min
187 followed by 30 cycles of 94 ºC for 30 s, 53 ºC for 1 min and 70 ºC for 8 min (BOX-
188 PCR) or 3 min ($[GTG]_5$-PCR) and finally 70 ºC for 16 min. PCR products were

189 separated by electrophoresis on 1.5 % agarose gels in 1 X TAE buffer for 2.5 h at 50 V,
190 stained with ethidium bromide.

191 *Determination of the DNA G+C content and DNA-DNA hybridization*

192 The G+C content of the genomic DNA was determined from the midpoint value
193 of the thermal denaturation profile [22] using the equation of Owen & Hill [25]. DNA-
194 DNA hybridization studies were performed by the competition procedure of the
195 membrane filter method [17]. The hybridization temperature was 52.84ºC, which is
196 within the limit of validity for the filter method [11] and the percentage of hybridization
197 was calculated according to Johnson [17]. The experiments were performed in triplicate.

198

199 **Results and discussion**

200 Strains M1-18^T and L1-16 were Gram-staining-negative, motile and strictly
201 aerobic straight rods. They were able to grow in media containing 3-25 % (w/v) NaCl
202 and optimally in media containing 7.5-10 % and 7.5 % (w/v) NaCl, respectively. Both
203 strains were unable to grow in the absence of NaCl. On the basis of the NaCl
204 requirements these bacteria can be considered as moderately halophilic microorganisms
205 [36]. Their optimal temperature and pH were 37 ºC and pH 7.0. Both strains showed
206 very similar phenotypic features and their characteristics are detailed in the new species
207 description and Table 1. They showed some differences with respect to the Simmons'
208 citrate test and the utilization of some compounds, as reported in the new species
209 description.

210 The 16S rRNA gene sequence analysis showed that the two novel isolates were
211 most closely related to the genus *Chromohalobacter*, sharing 95.2-94.9 % sequence

similarity with *C. israelensis* ATCC 43985^T, 95.0-94.8 % with *C. salexigens* DSM 3043^T, 94.6-94.5 % with *C. beijerinckii* ATCC 19372^T and 94.4-94.2 % with *C. canadensis* ATCC 43948^T. Nevertheless, the 16S rRNA-based phylogenetic trees clustered strains M1-18^T and L1-16 into a monophyletic branch (their 16S rRNAgene sequence similarity was 99.9 %) that was most closely related to the species of the genus *Kushneria* (Fig. 1), which showed lower 16S rRNA sequence similarity (94.0-93.4 %) with respect to the two new isolates than the species of *Chromohalobacter*. Those low similarity values might indicate that the new strains could constitute a novel genus within the family *Halomonadaceae*. In order to elucidate the correct taxonomic position of strains M1-18^T and L1-16, a MLSA study was performed according to the recommendations of de la Haba et al. [9] for members of the *Halomonadaceae*. Partial sequences of *atpA* (643 bp), *rpoD* (1019 bp) and *secA* (563 bp) genes were obtained and analyzed. On the basis of *atpA* gene the novel isolates formed an independent cluster closely related to the genera *Zymobacter*, *Carnimonas*, *Cobetia* and *Halotalea*, showing a very stable topology with high bootstrap values (Supplementary Fig. S1). According to the *rpoD*-based tree the grouping was quite similar, with the exception of the genus *Zymobacter*, which *rpoD* gene was not possible to amplify in the study of de la Haba et al. [9] and, therefore, has not been included within this analysis (Fig. S1). In the case of the *secA*-based tree the cluster formed by strains M1-18^T and L1-16 was slightly different, being the genera *Zymobacter* and *Kushneria* the closest neighbours (Fig. S1). So, concerning the phylogenetic placement of the new strains all the individual gene based trees were in agreement. Concatenation of the 16S rRNA, *atpA*, *rpoD* and *secA* genes showed that the novel strains formed an independent and monophyletic branch related to the peripheral genera of the family (*Zymobacter*, *Halotalea*, *Carnimonas* and *Cobetia*) (Fig. 2). Therefore, the individual and concatenated gene trees (Fig. S1 and

237 Fig. 2) undoubtedly demonstrated that the novel isolates are not members of the genera
238 *Chromohalobacter* or *Kushneria*, as could be expected after 16S rRNA gene analysis
239 (Fig. 1). Actually, they are more related to the genera *Zymobacter*, *Halotalea*,
240 *Carnimonas* and *Cobetia* and, in fact, our MLSA data support that they constitute a new
241 genus within the family *Halomonadaceae*. Previously, only the genera *Cobetia* and
242 *Kushneria* were proposed on the basis of more than a single gene phylogeny (16S and
243 23S rRNA) [2, 28], but this work is the first one describing a new genus of this family
244 following the MLSA scheme proposed by de la Haba *et al.* [9], and recently endorsed
245 by the ICSP-Subcommittee on the taxonomy of *Halomonadaceae* [24]. On the other
246 hand, the comparison of the sequences of the housekeeping genes analyzed for the two
247 new isolates may give indications about their differences. The two strains were isolated
248 from two samples obtained from different ponds of a marine saltern and thus, they
249 might belong to a single clonal line. However, our data indicate that they have
250 differences on the sequences of the housekeeping genes *atpA*, *rpoD* and *secA*, showing
251 5, 26, and 14 different nucleotides and percentages of similarity of 99.2 %, 97.4 % and
252 97.5 %, respectively, that is a clear indication of their different clonal origin. They also
253 show some differential phenotypic features (Table 1). A fingerprinting study based on
254 BOX-PCR and $(GTC)_5$-PCR also supported these data and showed that isolates M1-18^T
255 and L1-16 were representatives of two different strains (Supplementary Fig. S2).

256 In order to determine if the two new isolates constituted a single species, we
257 carried out DNA-DNA hybridization studies between the two strains, and also between
258 strain M1-18^T, which was selected as the type strain of the new taxon,and the type
259 strains of the most closely related species of the genera *Chromohalobacter* and
260 *Kushneria*. The percentage of DNA-DNA hybridization (DDH) between strain M1-18^T

261 and strain L1-16 was 82 %; which is higher than 70 %, currently accepted as the cut-off
262 value for species delineation [32,33]. The DDH relatedness of strain M1-18^T and the
263 type strains of the species *Chromohalobacter salexigens* DSM 3043^T,
264 *Chromohalobacter beijerinckii* DSM 7218^T, *Chromohalobacter israelensis* CECT
265 5287^T and *Chromohalobacter canadensis* ATCC 43984^T were 32 %, 30 %, 25 % and
266 14 %, respectively, and with respect to *Kushneria avicenniae* Mw2a^T a 48 % DDH
267 relatedness was obtained. These levels of DNA-DNA hybridization permit to consider
268 the new strains as a genotypically distinct taxon [32,33].

269 The G+C content of the DNA for strains M1-18^T and L1-16 was 54.5 and 55.9
270 mol %, respectively. These values are within the range for species of the family
271 *Halomonadaceae* [1] and close to those of related species of the genera
272 *Chromohalobacter* and *Kushneria* (Table 1).

273 The cellular fatty acid profile of strain M1-18^T was characterized by the fatty
274 acids $C_{18:1}\omega7c/C_{18:1}\omega6c$ (32.9 %), $C_{16:0}$ (29.5 %), and $C_{16:1}\omega7c/C_{16:1}\omega6c$ (20.5 %) as
275 the major fatty acids (Table 2). Some differences were observed with respect to the
276 cellular fatty acid composition of this new strain with respect to those found in the
277 species of the genera *Kushneria* and *Chromohalobacter*, in which $C_{16:1}\omega7c/C_{16:1}\omega6c$
278 were absent or in low percentages, as well as the absence of $C_{19:0}$cyclo$\omega8c$ in the new
279 isolate while it was always found as a major fatty acid for the species of *Kushneria* and
280 *Chromohalobacter* (Table 2). The cell-wall peptidoglycan analysis showed the presence
281 of *meso*-diaminopimelic acid as diagnostic diamino acid of the peptidoglycan in strain
282 M1-18^T, indicating that it has a peptidoglycan type A1γ, in accordance with the type
283 reported for other species of the *Halomonadaceae* (*Gammaproteobacteria*) [1]. The
284 only respiratory quinone of strain M1-18^T was ubiquinone 9 (Q-9), which is also present

285 in the members of the family *Halomonadaceae* [1]. The TLC pattern of the total polar
286 lipids of strain M1-18^T is shown in Supplementary Fig. S3. Strain M1-18^T possessed
287 phosphatidylglycerol, phosphatidylethanolamine, a phospholipid, a phosphoglycolipid,
288 a phosphoglycoaminolipid, a glycolipid, and a glycoaminolipid. These results contrast
289 with those reported for the genera *Chromohalobacter* and *Kushneria*, which besides
290 phosphatidylglycerol and phosphatidylethanolamine also have diphosphatidylglycerol
291 [1].

292 The main characteristics that differentiate strains M1-18^T and L1-16 from the
293 related species of the genera *Chromohalobacter* and *Kushneria* are summarized in
294 Table 1. On the basis of the phylogenetic, genotypic, chemotaxonomic and phenotypic
295 data, we propose that both strains be classified in a novel genus and species, as *Larsenia*
296 *salina* gen. nov., sp. nov.

297

298 **Description of *Larsenia* gen. nov.**

299 *Larsenia* (Lar.sen'i.a, N. L. fem. n. *Larsenia*, named after Helge Larsen, a
300 pioneering on the study of halophilic microoganisms).

301 Cells are Gram-staining-negative, motile rods. Endospores are not formed.
302 Organotrophic. Strictly aerobic, catalase and oxidase positive. Moderately halophilic.
303 Major fatty acids are $C_{18:1}\omega7c/C_{18:1}\omega6c$, $C_{16:0}$, and $C_{16:1}\omega7c/C_{16:1}\omega6c$. The only
304 respiratory quinone is Q-9 and the major polar lipids are phosphatidylglycerol,
305 phosphatidylethanolamine, phospholipids and glycolipids. The genus *Larsenia* belongs
306 to the family *Halomonadaceae* within the class *Gammaproteobacteria*. The type species
307 is *Larsenia salina*.

Description of *Larsenia salina* sp. nov.

Larsenia salina (sa.li'na. L. fem. adj. *salina,* salted, saline).

Cells are Gram-staining-negative, motile, straight rods, 0.4-0.8 x 0.8-2.1 μm in size. Colonies are circular, entire, smooth, convex, yellow pigmented and 0.7-3.0 mm in diameter on 7.5 % SW agar medium after 48 h incubation at 37 ºC. Strictly aerobic. Moderately halophilic, growing at 3-25 % (w/v) NaCl; with optimal growth at 7.5-10 % (w/v) NaCl. No growth occurs in the absence of NaCl. Grows at 15-40 ºC; showing optimal growth at 37 ºC, and at pH values on the range 4.0-9.0; with optimal growth at pH 7.0. Anaerobic growth on nitrate or arginine negative. Catalase and oxidase positive. Gelatin, DNA, Tween 80 and aesculin are hydrolysed but starch is not hydrolysed. Nitrate is not reduced to nitrite. Acid is produced from D-glucose, D-arabinose, sucrose and D-trehalose but not from D-mannitol, D-amygdaline L-citruline, DL-ethionine, inuline, lactose, melezitose, D-ribose, raffinose, sorbitol or xylitol. Indole or H_2S are not produced. Phosphatase is positive. Methyl red, Voges-Proskauer, Simmons' citrate is variable. Urease, arginine and phenylalanine deaminase tests are negative. The following compounds are utilized as sole sources of carbon and energy: D-galactose, D-glucose, D-ribose, glycerol, salicine, myo-inositol, benzoate, fumarate, hippurate and citrate. The following compounds are not utilized as sole sources of carbon and energy: D-fucose, aesculin, starch, butanol, dulcitol, methanol, formate, malate, propionate and tartrate. The following compounds are utilized as sole sources of carbon, nitrogen and energy: L-isoleucine, L-methionine and L-valine. The following compounds are not utilized as sole sources of carbon, nitrogen and energy: L-arginine, aspartate, L-

threonine and tryptophan. The predominant cellular fatty acids are $C_{18:1}\omega7c/C_{18:1}\omega6c$, $C_{16:0}$, and $C_{16:1}\omega7c/C_{16:1}\omega6c$. The DNA G+C content is 54.5-55.9 mol% (T_m).

The type strain is M1-18^T (= CCM 8464 = CECT 8192^T = IBRC-M 10767^T = LMG 27461^T). The DNA G+C content of the type strain is 54.5 mol% (T_m). This strain is unable to hydrolyze casein and is Simmons' citrate negative. Able to utilize L-cysteine and ethanol as sole carbon and energy source.

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Legends to Figures

Fig. 1. Maximum likelihood phylogenetic tree based on the 16S rRNA gene sequence comparison, showing the phylogenetic position of strains M1-18^T and L1-16 and their relationship with other genera of the family *Halomonadaceae*. Bootstrap values over 70% are shown above the branch. The species *Pseudomonas aeruginosa* DSM 50071^T was used as an outgroup. Bar, 0.02 substitutions per nucleotide position.

Fig. 2. Maximum likelihood phylogenetic tree based on concatenated 16S rRNA, *atpA*, *rpoD* and *secA* gene sequence comparisons, showing the phylogenetic position of strains M1-18^T and L1-16 and their relationship with other genera of the family *Halomonadaceae*. Bootstrap values over 70% are shown above the branch. The species *Pseudomonas aeruginosa* was used as an outgroup. Bar, 0.05 substitutions per nucleotide position.

518 **Table 1.** Differential characteristics of strains M1-18^T and L1-16 and related species of the genera *Kushneria* and *Chromohalobacter.*

519 Strains: 1, M1-18^T; 2, L1-16; 3, *K. aurantia* A10^T; 4, *K. avicenniae* MW2a^T; 5*, C. israelensis* CECT 5287^T; 6, *C. canadensis* ATCC 43984^T; 7,
520 *C. marismortui* ATCC 17056^T; 8, *C. salexigens* DSM 3043^T.

521 All data are from this study, except [a] Sánchez-Porro et al. [28], [b]Arahal et al. [3], [c]Ventosa et al. [36] and [d]Arahal et al. [4].

522 +, Positive, -, negative, w, weakly positive, ND, not determined.

Characteristics	1	2	3	4	5	6	7	8
Cell morphology	Straight rods	Straight rods	Rods	Rods or oval cells	Straight rods	Straight or curved rods	Straight or curved rods	Rods
Colony pigmentation	Yellow	Yellow	Orange	Orange	Cream	White	Brown-yellow	Cream
Cell size (μm)	0.4-0.8x 0.8-2.1	0.4-0.8 x 0.8-2.1	1.0 × 2.0-5.0[a]	0-2.6 × 1.0-2.0[a]	0.6-0.9 × 1.5-4.2[b]	0.6-1.2 × 2.0-3.8[b]	0.6-1.0 × 1.5-4.0[c]	0.7-1.0 × 2.0-3.0[d]
NaCl range (%, w/v)	3-25	3-25	5-17.5[a]	0-25[a]	3.5-20[b]	3-32[b]	1-30[c]	0.9-25[d]
NaCl optimum (%, w/v)	7.5-10	7.5	10[a]	5[a]	8[b]	7,5[b]	10[c]	7.5-10[d]
Temperature range (ºC)	15-40	15-40	20-40[a]	12-40[a]	15-45[b]	5-45[b]	5-45[c]	15-45[d]
Temperature optimum (ºC)	37	37	37[a]	30-35[a]	30[b]	30[b]	37[c]	37[d]
pH range	4.0-9.0	4.0-9.0	5.5-8.5[a]	5.0-9.0[a]	5.0-9.0[b]	5.0-9.0[b]	5.0-10.0[c]	5.0-10.0[d]
pH optimum	7.0	6-7	7.0-8.0[a]	7.0-8.0[a]	7.0-8.0	7.0-8.8	7.5[c]	7.5[d]
Hydrolysis of:								
Aesculin	+	+	+	+	-	-	-	-

Casein	-	+	-	-	-	-	-	-
Starch	-	-	-	-	+	-	-	-
DNA	+	+	-	-	+	+	+	+
Nitrate reduction	-	-	-	-	+	+	-	+
Nitrite reduction	-	-	-	-	+	+	-	+
Simmons' citrate	-	+	+	+	+	+	+	+
Acid production from:								
D-Arabinose	+	+	-	+	+	+	+	+
D-Trehalose	+	+	-	+	-	-	+	-
D-Mannitol	-	-	-	+	-	-	-	-
D-Glucose	+	+	+	+	+	-	+	+
Assimilation of:								
Benzoate	+	+	-	-	-	-	+	+
D,L-Malate	-	-	+	-	-	-	-	-
D,L-Tartrate	-	-	+	-	-	-	-	-
Fumarate	+	+	-	-	+	+	+	+
Hippurate	+	+	-	w	-	+	+	-
Ethanol	+	-	-	-	+w	-	+	+
L-Cysteine	+w	-	+	+	+	+	+	-

L-Methionine	+	+	-	-	-	+	-	-
L-Isoleucine	+	+	-	w	-	+	+	-
L-Valine	+	+	-	-	+	+	+	-
DNA G+C content (mol%)	54.5	55.9	61.7[a]	61.5[a]	65.0[b]	62.0[b]	62.3[c]	64.2[d]

523

524 **Table 2.** Cellular fatty acid content of strain M1-18^T and closely related species of the genera *Kushneria* and *Chromohalobacter.*

525 Strains: 1, M1-18^T; 2, *Kushneria aurantia* A10^T; 3, *Kushneria avicenniae* MW2a^T; 4, *Chromohalobacter marismortui* ATCC 17056^T; 5,
526 *Chromohalobacter israelensis* CECT 5287^T. Values are percentages of total fatty acids; values lower than 0.5 % are not shown. The
527 determination were carried out under the same conditions growing the cells on TSA with 10 % (w/v) NaCl, at 37 ºC for 24 h. Data for *K.*
528 *aurantia* and *K. avicenniae* were previously published by Sánchez-Porro et al. [28] -, Not detected.

Fatty acids[a]	**1**	**2**	**3**	**4**	**5**
$C_{9:0}$	-	-	-	-	1.2
$C_{10:0}$	1.8	1.1	1.0	3.7	3.0
$C_{12:0}$	2.5	0.8	0.8	4.6	4.1
$C_{12:0}$ 2-OH	1.2	0.4	3.2	-	-
$C_{12:0}$ 3-OH	9.8	8.8	11.3	12.9	9.4
$C_{14:0}$	1.0	1.9	0.4	0.6	5.8
Summed feature 3*	20.5	-	-	7.9	7.3
Summed feature 3**	-	3.5	2.6	-	-
$C_{16:0}$	29.5	40.4	37.4	25.7	24.8
$C_{17:0}$ cyclo	-	0.9	3.2	1.0	1.9
Summed feature 8	32.9	-	-	35.5	26.1
$C_{18:1}\omega$7c	-	28.4	16.6	-	-
$C_{18:0}$	0.7	1.1	1.2	2.1	1.1

Fatty acids[a]	**1**	**2**	**3**	**4**	**5**
$C_{18:1}$ ω7c-11-methyl	-	-	-	-	0.8
$C_{19:0}$ cyclo ω8c	-	11.8	22.3	5.6	13.7
$C_{20:2}$ ω6,9c	-	-	-	-	0.5

[a] Summed features represent groups of two or three fatty acids that could not be separated by GCL with the MIDI System. Summed feature 3*: $C_{16:1}\omega$7c/ $C_{16:1}$ω6c; Summed feature 3**: $C_{16:1}$ ω7c/$C_{15:0}$ iso 2-OH; Summed feature 8: $C_{18:1}$ ω7c/$C_{18:1}$ ω6c.