Halorubrum chaoviator Mancinelli *et al.* 2009 is a later, heterotypic synonym of *Halorubrum ezzemoulense* Kharroub *et al.* 2006. Emended description of *Halorubrum ezzemoulense* Kharroub *et al.* 2006

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Abbreviations: ANI, Average Nucleotide Identity; DDH, DNA-DNA hybridization; GGDC, Genome-to-Genome Distance Calculator; GTR, General Time Reversible; HPTLC, High-Performance Thin Layer Chromatography; JTT, Jones, Taylor, Thornton model; MLSA, MultiLocus Sequence Analysis; OGRI, Overall Genome Relatedness Indexes; TIM, Transitional Model.

Abstract

A polyphasic comparative taxonomic study of *Halorubrum ezzemoulense* Kharroub *et al.* 2006, *Halorubrum chaoviator* Mancinelli *et al.* 2009 and eight new *Halorubrum* strains related to these haloarchaeal species was carried out.

The MLSA study using the five concatenated housekeeping genes *atpB*, *EF-2*, *glnA*, *ppsA* and *rpoB'*, and the phylogenetic analysis based on the 757 core protein sequences obtained from their genomes showed that *Hrr. ezzemoulense* DSM 17463^T, *Hrr. chaoviator* Halo-G*^{T}/DSM 19316^T and the eight *Halorubrum* strains formed a robust cluster, clearly separated from the rest of species of the genus *Halorubrum*. The orthoANI and digital DDH, calculated by the Genome-to-Genome Distance Calculator (GGDC), showed percentages among *Hrr. ezzemoulense* DSM 17463^T, *Hrr. chaoviator* DSM 19316^T and the eight *Halorubrum* strains ranging from 99.4 to 97.9 %, and 95.0 to 74.2 %, respectively, while these values for those strains and the type strains of the most closely related species of *Halorubrum* were 88.7 to 77.4 %, and 36.1 to 22.3 %, respectively. Although some differences were observed, the phenotypic and polar lipids profiles were quite similar for all these strains studied. Overall, these data show that *Hrr. ezzemoulense, Hrr. chaoviator* and the eight new *Halorubrum* isolates constitute a single species. Thus, *Halorubrum chaoviator* should be considered as a later, heterotypic synonym of *Halorubrum ezzemoulense*. We propose an emended

description of *Halorubrum ezzemoulense*, including the features of *Halorubrum chaoviator* and those of the eight new isolates.

The genus *Halorubrum* is classified within the family *Halorubraceae*, order *Haloferacales*, class *Halobacteria* [1,2]. Currently this genus includes 37 species with validly published names, isolated from diverse hypersaline habitats, such as saline and soda lakes, salterns or saline soils, as well as from rock salt and salted food [3,4]. Divergence patterns leading to speciation of *Halorubrum* populations have been previously studied based on phylogenetic, genomic and fingerprinting analyses [5,6]. Recently, we carried out a study of 25 isolates obtained from different hypersaline environments, belonging to the genus *Halorubrum* and they were compared with the type strains of species of *Halorubrum* by using several taxonomic approaches: 16S rRNA gene sequence comparative analysis, MLSA based on the comparison of *atpB*, *EF-2*, *glnA*, *ppsA* and *rpoB'* housekeeping genes, ANI, conventional DNA-DNA hybridization, and polar lipid profiles [7]. This study showed that several *Halorubrum* isolates, designated as phylogroup 1, clustered together and showed common features with the two species *Halorubrum ezzemoulense* and *Halorubrum chaoviator* [7]. *Hrr. ezzemoulense* was described by Kharroub *et al.* in 2006 [8] on the basis of the features of a single strain (designated as strain 5.1^T), isolated from a water sample of Ezzemoul sabkha in Algeria, while *Hrr. chaoviator* was described by Mancinelli *et al.* in 2009 [9], based on the features of strain Halo-G*^{T}, isolated from an evaporitic salt crystal from the coast of Baja California, Mexico, and two additional strains isolated from a salt pool in Western Australia and a salt lake on the island of Naxos in Greece, respectively. Our recent comparative study on the new isolates and the species of *Halorubrum* indicated that *Hrr. ezzemoulense* DSM 17463^T and *Hrr. chaoviator* Halo-G*^{T}/DSM 19316^T constitute a single species together with eight of those new isolates. In this paper we have compared in detail the type strains of both species of *Halorubrum* as well as eight representative strains of our previous study that were closely related to these species, in

order to carry out a comprehensive polyphasic taxonomic study, which supports that *Hrr. chaoviator* should be considered as a later heterotypic synonym of *Hrr. ezzemoulense,* and that the new eight isolates are members of the species *Hrr. ezzemoulense,* for which we propose an emended description.

In this study we used the following type strains obtained from culture collections: *Hrr. ezzemoulense* DSM 17463^T and *Hrr. chaoviator* DSM 19316^T, as well as *Hrr. chaoviator* Halo-G*^{T} and the *Halorubrum* sp. strains C191, Ec15, Fb21, G37, Ga2p, Ga36, SD612 and SD683. The former six strains were isolated from the hypersaline lake Aran-Bidgol, Iran, and the latter two were obtained from water samples of a saltern in the Namibia desert as previously described [7]. They were routinely cultured in modified SW20 medium [10] with 20 % (w/v) total salts, prepared using a salt mixture designated as SW 30 % (w/v) stock solution [11] which consists of (per litre): 234 g NaCl, 39 g $MgCl_2 \cdot 6H_2O$, 61 g $MgSO_4 \cdot 7H_2O$, 1 g $CaCl_2$, 6 g KCl, 0.2 g $NaHCO_3$ and 0.7 g NaBr. This solution was supplemented with 0.5 % (w/v) yeast extract (Difco) and 0.5 % (w/v) casamino acids. The pH was adjusted to 7.2 with 1 M KOH and the cultures were incubated at 37 ºC. For solid media 2.0 % (w/v) agar was used when necessary. The strains were maintained on the same medium in slant tubes, and for long term preservation they were prepared as cryotubes for freezing at -80 ºC as suspensions with 15 % glycerol [7].

The 16S rRNA and MLSA phylogenetic analyses were carried out as previously described [7]. The 16S rRNA gene nucleotide sequence of the strains was assembled with ChromasPro software version 1.5 and aligned using ARB 6.0.5 software package [12]. Sequence similarities were analyzed by comparing the 16S rRNA gene sequence

of *Hrr. ezzemoulense* CECT 7099^T and *Hrr. chaoviator* Halo-G*^{T} as well as the eight *Halorubrum* sp. isolates with the known sequences of the *Halorubrum* species shown in Table S1, using ARB 6.0.5 and the EzBioCloud tool [http://www.ezbiocloud.net/eztaxon; 13]. The analysis based on the almost complete 16S rRNA gene sequences showed the percentages of similarity (Table S2). The 16S rRNA gene sequences of the type strains of *Hrr ezzemoulense* CECT 7099^T and *Hrr. chaoviator* Halo-G*^{T} showed a percentage of similarity of 99.7 %; besides, these two strains and all the eight new isolates showed percentages of similarity in the range 99.6 to 100 %. Similarities equal or lower than 99.4% were obtained between those strains with the type strains of other species of *Halorubrum* and other haloarchaeal genera. The phylogenetic study based on the 16S rRNA gene sequence comparison was performed by constructions of trees using the algorithms neighbour-joining [14], maximum-parsimony [15] and maximum-likelihood [16] with the ARB program package version 6.0.5 [12]. Maximum-likelihood analysis was performed using the Transitional Model 2 of nucleotide substitution with invariable sites, rate variation among sites and unequal base frequencies (TIM2+I+G+F) [17]. Base-frequency filters were applied in the sequence comparison analysis and the effects on the results were evaluated. To evaluate the robustness of the tree, a bootstrap analysis (1000 replications) was performed [18]. The inferred tree based on the 16S rRNA gene constructed by maximum-likelihood showed that the eight *Halorubrum* sp. strains clustered with *Hrr. ezzemoulense* CECT 7099^T, *Hrr. chaoviator* Halo-G*^{T}, as well as with *Halorubrum californiense* SF3-213^T (Fig. 1). The bootstrap values were low in all cases. The topologies of the trees reconstructed using the neighbour-joining and maximum-parsimony algorithms were highly similar to that of the tree constructed by maximum-likelihood. As previously indicated the comparison of the 16S rRNA gene sequences does not permit to determine

in depth the phylogenetic relationships within the genus *Halorubrum* and thus, a MLSA approach based on the comparison of partial sequences of the *atpB* (ATP synthase subunit B), *EF-2* (elongation factor 2), *glnA* (glutamine synthetase), *ppsA* (phosphoenolpyruvate synthase) and *rpoB′* (RNA polymerase subunit B') housekeeping genes (Table S1) has been recently recommended for this genus [7]. PCR cycling conditions and amplification and sequencing primers for these genes are described elsewhere [6,7]. Lengths of the resulting multiple alignments for each gene were 496, 507, 526, 514 and 522 bp for the *atpB*, *EF2*, *glnA*, *ppsA* and *rpoB'* genes, respectively, with the concatenation of the five genes yielding a final alignment of 2565 pb. Fig. 2 shows the phylogenetic tree obtained by concatenation of these five housekeeping genes, constructed by the maximum-likelihood algorithm using the GTR+I+G substitution model, as implemented in PhyML version 3.1 [19]. This tree shows a better phylogenetic separation of the species of *Halorubrum* and, on the other hand, here the eight *Halorubrum* isolates constitute a cluster with the type strains of *Hrr. ezzemoulense* and *Hrr. chaoviator*. The percentage of similarity of the five concatenated gene sequences between *Hrr. ezzemoulense* and *Hrr. chaoviator* is 99.7 % and those of these two species and the other eight related strains varied from 98.8 to 99.8 % and 98.9 to 99.8 %, respectively. Overall, the percentages of MLSA similarity of the two *Halorubrum* species and the eight isolated strains that constitute a single cluster ranged from 98.8 to 99.8 % (Table S2).

To increase the resolution, we carried out a phylogenetic analysis based on the 757 core protein sequences obtained from the available genomes of *Hrr. ezzemoulense* DSM 17463^{T}, *Hrr. chaoviator* DSM 19316^{T}, the eight *Halorubrum* strains and the type strains of other related *Halorubrum* species (Table S1). All predicted protein sequences NCBI-

155 annotated from each available genome were compared using an all-versus-all BLAST
156 search by using the enveomic tool [20]. This analysis identified reciprocal best matches
157 (defined as > 40 % amino acid identity) in all pairwise genome comparisons of the ten
158 *Halorubrum* strains and the related *Halorubrum* type species. From all those pairwise
159 reciprocal best match proteins, the 757 shared proteins present in all the analyzed
160 genomes were selected to constitute the core orthologues. These core orthologous
161 proteins were individually aligned using MUSCLE [21]. The resulting protein
162 alignments were concatenated to create a core-protein alignment consisting of 250,398
163 amino acids, and the phylogenomic tree was reconstructed by neighbour-joining method
164 with the JTT model of amino acid substitution [22], as implemented in MEGA 5 [23].
165 As shown in Fig. 3, the overall topology of the phylogenetic tree was in agreement with
166 the MLSA tree. The two *Halorubrum* species, *Hrr. ezzemoulense* DSM 17463^T, *Hrr.*
167 *chaoviator* DSM 19316^T, and the eight *Halorubrum* strains formed a well-defined
168 cluster, separate from the rest of species of *Halorubrum*.
169
170 Currently, it has been recommended the use of Overall Genome Relatedness Indexes
171 (OGRI), such as the ANI and digital DDH, for delineation of prokaryotic species [24-
172 29] and minimal standards have been recently reported [29]. The orthoANI percentages,
173 determined according to Lee *et al.* [30] on the basis of the comparison of the genome
174 sequences of *Hrr. ezzemoulense* DSM 17463^T, *Hrr. chaoviator* DSM 19316^T, and the
175 new eight *Halorubrum* isolates, indicate that the cluster formed by these strains possess
176 a range of 99.4 % to 97.9 %, while the range with respect to the type strains of the
177 related species of *Halorubrum* was 88.7 % to 77.4 % (Table 1). The threshold of 95-96
178 % defined for species delineation [24,25,29] clearly supports the placement of these
179 strains within a single species.

On the other hand, we also calculated the digital DNA–DNA hybridizations, determined online (http://ggdc.dsmz.de/distcalc2.php) using the Genome-to-Genome Distance Calculator (GGDC) version 2.0 as described by Meier-Kolthoff *et al*. [27]. The estimated digital DDH values were calculated using formula two at the GGDC website, originally described by Auch *et al*. [26] and updated by Meier-Kolthoff *et al*. [27]. The GGDC among *Hrr. ezzemoulense* DSM 17463^{T}, *Hrr. chaoviator* DSM 19316^{T}, and the new eight *Halorubrum* strains ranged from 95.0 % to 74.2 %, but the values among these strains and the type strains of the related species of the genus *Halorubrum* were 36.1-22.3 % (Table 1). These percentages are lower than the 70 % cut-off established for species delineation [27,29], and thus, showing unequivocally that the strains under study constitute a single species of *Halorubrum*, clearly separated from the rest of species of this genus. These data are in agreement with our recent study [7], showing an experimental DDH percentage of relatedness between *Hrr. ezzemoulense* DSM 17463^{T} and *Hrr. chaoviator* Halo-G*^{T} of 79 %, in contrast to the previously reported percentage of 39 % [9], using in both cases the same DDH competition procedure of the membrane filter method [7,9].

The phenotypic characterization was carried out using the standard taxonomic methods following the proposed minimal standards for *Halobacteria* recommended by Oren *et al.* [31]. Cell morphology and motility was examined in liquid medium after 7 days of growth by optical and phase-contrast microscopy (BX41; Olympus). Gram staining was performed using acetic acid-fixed samples, as described by Dussault [32]. The growth and optimum requirements for NaCl, Mg^{2+}, pH and temperature were determined in the routine modified SW20 medium, changing the recipe for testing growth at different

concentrations [33]. The range of NaCl (5–30 %, w/v) was tested at intervals of 5 units. Magnesium range was tested using $MgCl_2$ (0-10 %, w/v) at intervals of 1 % (w/v). Routine cultivation was performed at 37 °C and pH 7.5. The pH range for growth was assayed at pH 5.5–10.0, at intervals of 0.5 pH units in liquid modified SW20 medium with various pH buffers: MES (pH 5.5-6.0), PIPES (pH 6.5–7.0), Tricine (pH 7.5–8.5), CHES (pH 9.0–9.5) or CAPS (pH 10.0), at a concentration of 50 mM. The range and optimum temperatures were determined incubating at 4, 10, 20, 30, 37 and 45 ºC in modified SW20 medium with optimal NaCl and Mg^{2+} concentrations and pH.

All phenotypic tests were carried out using the modified SW20 medium prepared at 20 % (w/v) total salts, pH 7.5 and at 37 ºC. The type strain of the type species of *Halorubrum*, *Halorubrum saccharovorum* JCM 8865^T was used as a reference for comparative purposes. Anaerobic growth was tested in the presence of nitrate and L-arginine by adding to the medium 3 % (w/v) KNO_3 or 4 % L-arginine, respectively, in filled stoppered tubes, as well the plates of cultures incubated for 10 days at 37 ºC in an anaerobic jar [31]. Catalase activity was determined by adding a 1 % (v/v) H_2O_2 solution to colonies on solid medium. The oxidase test was performed using a DrySlide assay (Difco). The hydrolysis of starch, gelatin, aesculin, casein, DNA and Tween 80 were carried out as described by Barrow & Feltham [34]. Test for indole production from tryptophan and urea hydrolysis were performed as described by Gerhardt *et al.* [35]. The methyl red, Voges-Proskauer and Simmons citrate tests were performed as described by Oren *et al.* [31]. H_2S formation was determined by monitoring the production of a black sulfide precipitate in modified SW20 medium containing 0.5 % (w/v) sodium thiosulfate, and the reduction of nitrate was detected by using sulfanilic acid and α-naphthylamine reagents [36]. To determine the utilization of different

organic substrates such as carbohydrates, alcohols, amino acids and organic acids as the only source of carbon and energy, a medium containing 0.05 % (w/v) yeast extract and supplemented with 1 % (w/v) of the tested substrate (sterilized separately) was assessed as described by Ventosa *et al.* [37]. *Hrr. ezzemoulense* DSM 17463^T, *Hrr. chaoviator* DSM 19316^T, and the new eight *Halorubrum* isolated strains were Gram-stain-negative motile rods, producing red pigmented colonies. They were catalase and oxidase positive; not able to produce indole, nor hydrolyze gelatin, casein, DNA, aesculin or Tween 80. Voges-Proskauer, methyl red and urease tests were negative. The phenotypic features that showed variable results for the strains studied and their differential characteristics with respect to the type species of the genus *Halorubrum*, *Hrr. saccharovorum* are shown in Table 2. Other phenotypic features are included on the emended description of the species.

For polar lipid analyses cell biomass of the strains was obtained after 10 days of aerobic incubation in modified SW20 liquid medium under optimal conditions: 20 % (w/v) NaCl, 37 ºC and pH 7.5. Polar lipids were extracted with chloroform/methanol following the method for extraction of membrane polar lipids of halophilic archaea previously described by Corcelli *et al.* [38]; the extracts were carefully dried using a SpeedVac Thermo Savan SPD111V before weighing and then dissolved in chloroform to obtain a concentration of 10 mg/ml of lipid dissolved in $CHCl_3$. The total lipid extracts were analyzed by one dimensional High-Performance Thin Layer Chromatography (HPTLC) on Merck silica gel plates crystal back (Merck 10×20 cm; Art. 5626), the plates were eluted in the solvent system chloroform/methanol 90 %/acetic acid (65:4:35, v/v) [39,40]. To detect all polar lipids, the plate was sprayed with sulfuric acid 5 % (v/v) in water and charred by heating at 160 ºC [41]. The

glycolipids appear as purple spots and the rest of polar lipids as brown spots after prolonged heating; alternatively, the polar lipids were developed by spraying the plate with a solution of primuline and detecting the lipids upon excitation by UV light (336 nm) [42]. Furthermore, the following stainings were performed in order to identify the chemical nature of the lipids present in the HPTLC bands: (a) molybdenum-blue Sigma spray reagent for phospholipids [41]; (b) azure-A/sulfuric acid for sulfatides and sulfoglycolipids [43]; (c) ninhydrin in acetone/lutidine (9:1) for free amino groups. To analyze the whole profiles of the strains studied the universal staining was performed with phosphomolybdic acid (PMA) solution 20% (w/v) in ethanol and charred by heating at 160 ºC. The high sensitivity of this staining allows detecting all lipids even in smaller amounts.

The polar lipids HPTLC (Fig. S1) revealed that *Hrr. ezzemoulense* DSM 17463^{T}, *Hrr. chaoviator* Halo-G*^{T} and the eight *Halorubrum* strains possessed a similar polar lipids profile, showing the major lipids: phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (PGP-Me), phosphatidylglycerol sulfate (PGS) and one glycolipid chromatographically identical to sulfated mannosyl glycosyl diether (S-DGD-3). Biphosphatidylglycerol (BPG) is also found as minor component and minor phospholipids are also detected. The polar lipid profile of all these strains possesses all major lipids described for neutrophilic species of the genus *Halorubrum* [44,45], although some minor differences were observed on minority polar lipids for the strains investigated which could be related to their different isolation habitats.

Overall, the polyphasic taxonomic study shows that *Hrr. ezzemoulense* and *Hrr. chaoviator* constitute a single species, having the name *Hrr. ezzemoulense* priority

according to the Code of Nomenclature of Prokaryotes [46] and thus, *Hrr. chaoviator* should be considered a later heterotypic synonym of *Hrr. ezzemoulense*. Besides, the eight new isolated strains are members of this species and thus we propose the emended description of the species *Hrr. ezzemoulense*, including the features of *Hrr. chaoviator* and those of the forementioned eight isolates.

Emended description of *Halorubrum ezzemoulense* Kharroub *et al.* 2006

Halorubrum ezzemoulense (ez.ze.mou.len'se. N.L. neut. adj. *ezzemoulense* pertaining to Ezzemoul sabkha, where the type strain was isolated).

The description is that of Kharroub *et al.* [8] with the following modifications: aerobic growth occurs at 15-30 % (w/v) NaCl, pH 6.5-9.0 and 20-45 ºC. Optimum NaCl concentration, pH and temperature for growth are 20-25 % (w/v), pH 7.5, and 37-40 ºC. Nitrate is generally reduced to nitrite, but nitrite is not reduced. Starch is generally not hydrolysed. Voges-Proskauer and methyl red tests are negative. Casein and DNA are not hydrolysed. D-arabinose, D-fructose, D-galactose, D-mannose, maltose, melezitose, lactose, salicin, glycerol, m-inositol, methanol, acetate, citrate, succinate are not generally utilized as sole carbon and energy source. Sucrose, D-mannitol and fumarate are generally utilized as sole carbon and energy source. Xylose, butanol, ethanol, methanol, propanol, sorbitol, benzoate, hippurate, propionate, succinate, valerate, and tartrate are not utilized as sole carbon and energy source. The polar lipids profile includes: phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (PGP-Me), phosphatidylglycerol sulfate (PGS) and one glycolipid chromatographically identical to sulfated mannosyl glycosyl diether (S-DGD-3), the main glycolipid of the

genus *Halorubrum*. Biphosphatidylglycerol (BPG) is also found as minor component, and minor phospholipids are also detected.

The G+C content of the genomic DNA is 66.0-70.1 mol% (genome).

The type strain 5.1^T (= CECT 7099^T = DSM 17463^T), was isolated from Ezzemoul sabkha in Algeria. The DNA G+C content of this strain is 66.6 mol% (genome).

The 16S rRNA gene sequence and complete genome sequence of the type strain Halo-G*^{T} are AB663412 and NEDJ00000000, respectively.

Halorubrum chaoviator strain Halo-G*^{T} (= DSM 19316^T = NCIMB 14426^T = ATCC BAA-1602^T) is an additional strain of *Halorubrum ezzemoulense*, and *Halorubrum chaoviator* a later heterotypic synonym of *Halorubrum ezzemoulense.* Strains C191, Ec15, Fb21, G37, Ga2p, Ga36 (isolated from the hypersaline lake Aran-Bidgol in Iran), SD612 and SD683 (isolated from a saltern in Namibia) are additional strains of this species.

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336 **Conflict of interest**

337 The authors declare that there are no conflicts of interest.
338

339 **Ethical statement**

340 No experimental work with animals or humans has been carried out in this study.
341

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480 **Legends to figures**

481

482 **Fig. 1.** Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences
483 comparison showing the relationship between *Hrr. ezzemoulense* CECT 7099^{T}, *Hrr.*
484 *chaoviator* Halo-G*^{T}, the new eight *Halorubrum* strains and other related species of the
485 genus *Halorubrum* and other haloarchaea. The accession numbers of the sequences used
486 are shown in parentheses after the strain designation. Bootstrap values (%) based on
487 1000 replicates are shown for branches with more than 70 % bootstrap support. The
488 species *Haloarcula vallismortis*, *Haloferax volcanii* and *Halobacterium salinarum* were
489 used as outgroups. The scale bar represents 0.05 substitutions per nucleotide position.

490

491 **Fig. 2.** Maximum-likelihood phylogenetic tree based on the five-housekeeping gene
492 (*atpB*, *EF-2*, *glnA*, *ppsA* and *rpoB'*) concatenated sequences showing the relationship
493 between *Hrr. ezzemoulense*, *Hrr. chaoviator*, the new eight *Halorubrum* strains and
494 other related species of the genus *Halorubrum* and other haloarchaea. The accession
495 numbers of the sequences used are shown in Table S1. Bootstrap values >70 % are
496 indicated. The species *Haloarcula vallismortis*, *Haloferax volcanii* and *Halobacterium*
497 *salinarum* were used as outgroups. The scale bar represents 0.05 substitutions per
498 nucleotide position.

499

500 **Fig. 3.** Neighbour-joining core protein phylogenetic tree including the genomes of *Hrr.*
501 *ezzemoulense*, *Hrr. chaoviator*, the new eight *Halorubrum* strains and other related
502 species of the genus *Halorubrum*. This tree was based on the JTT distance calculated
503 from the alignment of 757 shared orthologous single-copy genes of these genomes. All

504 genomes were retrieved from GenBank (Table S1). Bootstrap values over 70 % (based
505 on 1,000 pseudoreplicates) are shown above the branch. The scale bar represents 0.05
506 substitutions per nucleotide position.

Table 1. OrthoANI (upper triangle in bold) and GGDC (lower triangle) values among the genomes of *Hrr. ezzemoulense* DSM 17463^T, *Hrr. chaoviator* DSM 19316^T and the new eight *Halorubrum* strains, as well as the type strains of the related species of the genus *Halorubrum*. The main diagonal of the matrix is grey highlighted. Genome accession numbers are shown in Table S1.

Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
1. *Hrr. ezzemoulense*	100	**98. 8**	**98. 8**	**99. 0**	**98. 7**	**99. 0**	**98. 5**	**98. 8**	**98. 2**	**98. 8**	**81. 5**	**81. 0**	**88. 1**	**88. 4**	**88. 0**	**80. 8**	**82. 9**	**82. 7**	**81. 7**	**82. 9**	**82. 6**	**82. 6**	**87. 2**	**86. 0**	**77. 8**	**87. 3**
2. *Hrr. chaoviator*	90. 2	100	**98. 7**	**99. 0**	**98. 7**	**98. 9**	**98. 5**	**98. 9**	**98. 2**	**98. 8**	**81. 4**	**80. 8**	**87. 8**	**88. 3**	**87. 9**	**81. 0**	**83. 0**	**82. 7**	**81. 9**	**82. 7**	**82. 6**	**82. 7**	**87. 3**	**86. 1**	**78. 0**	**87. 6**
3. *Halorubrum* sp. C191	89. 3	88. 5	100	**98. 9**	**99. 4**	**98. 8**	**98. 5**	**99. 0**	**98. 2**	**98. 8**	**81. 4**	**81. 0**	**87. 9**	**88. 5**	**88. 0**	**80. 9**	**83. 0**	**82. 9**	**81. 9**	**82. 6**	**82. 2**	**82. 4**	**87. 1**	**86. 0**	**77. 8**	**87. 4**
4. *Halorubrum* sp. Ec15	91. 1	91. 1	89. 9	100	**98. 7**	**99. 1**	**98. 8**	**99. 1**	**98. 3**	**98. 9**	**81. 5**	**80. 7**	**88. 2**	**88. 6**	**87. 9**	**80. 8**	**82. 7**	**82. 8**	**81. 9**	**82. 8**	**82. 6**	**82. 6**	**87. 1**	**86. 1**	**77. 6**	**87. 5**
5. *Halorubrum* sp. Fb21	89. 1	89. 0	95. 0	89. 4	100	**98. 7**	**98. 4**	**98. 8**	**98. 0**	**98. 6**	**81. 2**	**80. 6**	**88. 0**	**88. 5**	**87. 8**	**80. 8**	**82. 8**	**82. 6**	**82. 2**	**82. 6**	**82. 3**	**82. 2**	**86. 8**	**85. 9**	**77. 8**	**87. 3**
6. *Halorubrum* sp. G37	91. 1	90. 9	90. 4	92. 0	89. 6	100	**98. 7**	**99. 1**	**98. 3**	**98. 7**	**81. 3**	**80. 7**	**87. 9**	**88. 5**	**87. 9**	**80. 9**	**83. 0**	**83. 0**	**82. 1**	**82. 7**	**82. 6**	**82. 7**	**87. 0**	**85. 8**	**77. 7**	**87. 3**
7. *Halorubrum* sp. Ga2p	90. 0	89. 9	89. 1	92. 6	88. 8	91. 3	100	**98. 8**	**97. 9**	**98. 5**	**81. 1**	**80. 5**	**87. 7**	**88. 2**	**87. 4**	**80. 5**	**82. 7**	**82. 7**	**81. 9**	**82. 5**	**82. 1**	**82. 3**	**87. 0**	**85. 6**	**77. 4**	**86. 9**
8. *Halorubrum* sp. Ga36	90. 6	90. 0	90. 8	91. 9	89. 7	92. 6	91. 6	100	**98. 3**	**98. 8**	**81. 6**	**80. 8**	**88. 0**	**88. 5**	**87. 8**	**80. 7**	**83. 0**	**82. 8**	**82. 0**	**82. 9**	**82. 5**	**82. 4**	**87. 3**	**86. 2**	**77. 7**	**87. 4**
9. *Halorubrum* sp. SD612	75. 2	74. 9	75. 1	75. 8	74. 2	75. 6	75. 8	75. 5	100	**98. 5**	**81. 5**	**80. 4**	**88. 2**	**88. 7**	**88. 0**	**80. 9**	**83. 0**	**82. 5**	**82. 0**	**82. 8**	**82. 6**	**82. 7**	**87. 2**	**86. 3**	**77. 7**	**87. 9**
10. *Halorubrum* sp. SD683	89. 4	89. 9	89. 3	90. 6	88. 6	90. 6	89. 5	90. 0	76. 2	100	**81. 2**	**80. 4**	**87. 9**	**88. 7**	**87. 8**	**80. 7**	**82. 7**	**82. 3**	**81. 8**	**82. 7**	**82. 3**	**82. 4**	**87. 1**	**86. 1**	**77. 4**	**87. 4**
11. *Hrr. aidingense*	25. 1	25. 0	25. 1	25. 0	25. 0	25. 2	25. 3	25. 0	25. 6	25. 1	100	**80. 5**	**81. 7**	**81. 4**	**81. 7**	**80. 6**	**83. 4**	**83. 3**	**83. 2**	**84. 0**	**83. 3**	**83. 2**	**81. 6**	**81. 7**	**77. 7**	**81. 7**
12. *Hrr. aquaticum*	25. 1	24. 8	25. 1	24. 8	24. 3	25. 2	25. 1	24. 9	24. 6	24. 3	24. 9	100	**80. 6**	**80. 7**	**80. 9**	**88. 2**	**81. 4**	**81. 3**	**80. 8**	**81. 2**	**81. 2**	**81. 2**	**80. 4**	**80. 9**	**78. 4**	**80. 6**
13. *Hrr. californiense*	34. 9	34. 8	34. 9	34. 9	35. 0	35. 1	35. 1	34. 9	35. 2	34. 8	25. 2	24. 5	100	**88. 1**	**88. 8**	**80. 7**	**83. 0**	**82. 8**	**81. 9**	**82. 8**	**83. 0**	**82. 4**	**87. 5**	**86. 5**	**78. 0**	**88. 4**
14. *Hrr. coriense*	35. 8	35. 9	36. 0	36. 1	35. 5	36. 1	36. 1	35. 9	36. 0	36. 1	25. 3	24. 6	35. 5	100	**87. 3**	**81. 0**	**82. 8**	**82. 8**	**82. 2**	**83. 0**	**82. 7**	**82. 6**	**86. 9**	**86. 3**	**77. 8**	**87. 3**

15. *Hrr. distributum*	34. 6	34. 5	35. 0	34. 4	34. 3	34. 7	34. 6	34. 6	35. 5	34. 3	25. 4	25. 0	36. 6	33. 6	100	**81. 4**	**83. 6**	**83. 2**	**82. 7**	**83. 6**	**82. 8**	**83. 2**	**88. 5**	**86. 7**	**78. 0**	**89. 3**
16. *Hrr. halodurans*	24. 9	24. 8	25. 0	24. 6	24. 6	24. 8	24. 9	24. 6	24. 8	24. 6	24. 9	34. 9	24. 6	24. 6	25. 1	100	**81. 4**	**81. 1**	**80. 6**	**81. 5**	**81. 2**	**81. 5**	**80. 8**	**81. 0**	**78. 4**	**80. 9**
17. *Hrr. halophilum*	27. 0	26. 9	27. 3	26. 6	26. 6	27. 2	26. 9	26. 7	26. 9	26. 4	27. 5	25. 4	26. 7	26. 3	27. 5	25. 5	100	**87. 9**	**87. 6**	**88. 6**	**88. 0**	**88. 8**	**82. 7**	**83. 1**	**77. 8**	**83. 1**
18. *Hrr. kocurii*	26. 2	26. 3	26. 9	26. 4	26. 3	26. 7	26. 6	26. 6	26. 4	26. 0	27. 7	25. 3	26. 6	26. 7	26. 7	24. 9	34. 7	100	**87. 3**	**89. 4**	**87. 4**	**87. 6**	**82. 6**	**82. 6**	**77. 9**	**83. 2**
19. *Hrr. lacusprofundi*	25. 3	25. 4	25. 5	25. 3	25. 5	25. 4	25. 6	25. 5	25. 6	25. 3	27. 2	24. 4	25. 6	25. 3	26. 0	24. 3	34. 0	33. 4	100	**88. 3**	**86. 9**	**87. 6**	**82. 2**	**82. 0**	**77. 8**	**82. 3**
20. *Hrr. lipolyticum*	26. 3	26. 2	26. 5	26. 4	26. 2	26. 5	26. 7	26. 4	26. 8	26. 2	28. 2	25. 3	26. 6	26. 5	27. 2	25. 1	36. 5	38. 5	34. 9	100	**87. 8**	**88. 6**	**83. 1**	**82. 9**	**78. 1**	**83. 0**
21. *Hrr. persicum*	26. 3	26. 1	26. 3	26. 1	26. 1	26. 2	26. 5	26. 1	26. 6	26. 3	27. 3	25. 1	26. 6	26. 1	26. 6	25. 1	34. 7	33. 8	33. 1	34. 7	100	**87. 8**	**82. 6**	**82. 7**	**78. 1**	**82. 8**
22. *Hrr. saccharovorum*	26. 2	26. 3	26. 2	26. 3	26. 1	26. 2	26. 5	26. 1	26. 7	26. 3	27. 4	25. 0	26. 2	26. 1	26. 8	25. 7	36. 9	34. 7	34. 2	36. 2	34. 5	100	**82. 7**	**82. 9**	**77. 8**	**82. 8**
23. *Hrr. sodomense*	32. 7	32. 8	32. 7	32. 6	32. 4	32. 8	32. 7	32. 8	33. 5	32. 6	25. 1	24. 4	33. 8	32. 5	35. 6	24. 7	26. 3	26. 3	25. 4	26. 6	26. 1	26. 2	100	**86. 5**	**77. 5**	**87. 8**
24. *Hrr. tebenquichense*	30. 9	30. 8	30. 8	30. 7	30. 5	30. 8	30. 8	30. 6	31. 6	30. 9	25. 3	24. 9	31. 3	31. 3	32. 0	24. 8	26. 6	26. 2	25. 5	26. 4	26. 1	26. 2	31. 4	100	**77. 5**	**86. 5**
25. *Hrr. vacuolatum*	22. 7	22. 8	22. 7	22. 5	22. 5	22. 6	22. 9	22. 6	22. 6	22. 3	22. 6	23. 3	22. 8	22. 6	22. 8	23. 2	22. 8	23. 1	23. 3	23. 0	23. 1	23. 0	22. 3	22. 7	100	**77. 8**
26. *Hrr. xinjiangensis*	33. 4	33. 4	33. 6	33. 5	33. 3	33. 8	33. 7	33. 7	34. 6	33. 5	25. 5	24. 6	35. 7	33. 4	37. 2	24. 9	26. 7	26. 7	25. 5	26. 8	26. 4	26. 4	34. 2	31. 5	22. 9	100

510 **Table 2.** Differential features among *Halorubrum ezzemoulense* DSM 17463^T, *Halorubrum chaoviator* DSM 19316^T and the eight new strains, as well as the

511 type species of the genus *Halorubrum*, *Hrr. saccharovorum* JCM 8865^T.

512 Taxa: 1*; Halorubrum ezzemoulense* DSM 17463^T; 2, *Halorubrum chaoviator* DSM 19316^T; 3, strain C191; 4, strain Ec15; 5, strain Fb21; 6, strain G37; 7,

513 strain Ga2p; 8, strain Ga36; 9, strain SD612; 10, strain SD683; 11, *Halorubrum saccharovorum* JCM 8865^T.

514 All data are from this study. +, Positive; -, negative; ND, not determined.

Characteristic	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
NaCl (% w/v) range	15-25	20-30	15-30	15-30	20-30	15-30	20-30	15-30	15-30	15-30	10–30
Optimum NaCl (% w/v)	20	20	25	25	25	25	25	25	20	20	25
Range of pH	6.5-9.0	7.0-8.0	7.0-8.0	7.0-8.0	7.0-8.0	7.0-8.0	7.0-8.0	7.0-8.0	6.5- 8.0	6.5- 8.0	6.5-8.0
Optimum pH	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	8.0
Range of temperature (°C)	25-45	25-40	20-40	20-40	20-40	20-40	20-40	20-40	20-40	20-40	30-45
Optimum temperature (°C)	40	37	37	37	37	37	37	37	37	37	40
Mg^{2+} requirement	+	+	-	-	-	-	-	-	+	+	+
Nitrate reduction	+	-	+	+	+	+	+	+	+	+	+
Starch hydrolysis	-	+	-	-	-	-	-	-	-	-	-
Indole production	-	-	-	-	-	-	-	-	-	-	+
Utilization as sole carbon and energy source of:											
D- Arabinose	+	+	-	-	-	-	-	-	+	+	-
D-Fructose	-	+	-	-	-	-	-	-	-	-	-
D-Galactose	-	+	-	-	-	-	-	-	-	-	+
D-Mannose	-	-	-	-	-	-	-	-	+	+	+
Maltose	+	+	-	-	-	-	-	-	+	-	+
Melezitose	+	+	-	-	-	-	-	-	+	+	ND
Lactose	-	+	-	-	-	-	-	-	+	+	+

Salicin	-	-	-	-	-	-	-	-	+	+	-
Sucrose	+	-	+	+	+	+	+	+	+	+	+
Glycerol	+	+	-	-	-	-	-	-	+	+	+
m-Inositol	-	-	-	-	-	-	-	-	+	+	ND
D-Mannitol	+	-	+	+	+	+	+	+	+	+	-
Methanol	+	+	-	-	-	-	-	-	-	-	-
Acetate	+	-	-	-	-	-	-	-	-	-	+
Citrate	+	-	-	-	-	-	-	-	-	-	-
Fumarate	-	+	+	+	+	+	+	+	-	-	-
Succinate	-	-	-	-	-	-	-	-	-	-	+
DNA G+C content (mol%, genome)	66.6	66.5	66.0	67.7	69.3	67.0	67.8	67.7	70.1	69.0	69.9*

515 *Value obtained from the genome of *Halorubrum saccharovorum* DSM 1137^{T}.