Emended description of *Salinivibrio proteolyticus*, including *Salinivibrio costicola* subsp. *vallismortis* and five new isolates

Clara López-Hermoso, Rafael R. de la Haba, Cristina Sánchez-Porro and Antonio Ventosa

Department of Microbiology and Parasitology, Faculty of Pharmacy, University of Sevilla, 41012 Sevilla, Spain

Correspondence: Antonio Ventosa (ventosa@us.es)

Subject category: New Taxa-Proteobacteria

Running title: Emended description of *Salinivibrio proteolyticus*

Keywords: *Salinivibrio proteolyticus*, *Salinivibrio costicola* subsp. *vallismortis*, *Vibrionaceae*, moderately halophilic bacteria, emendation of species description

Abbreviations:

ANI, Average Nucleotide Identity

DDH, DNA-DNA Hybridization

GGDC, Genome-to-Genome Distance Calculator

GTR, General Time Reversible

ML, Maximun likelihood

MLSA, MultiLocus Sequence Analysis

Abstract

We carried out a comparative taxonomic study of *Salinivibrio proteolyticus* and *Salinivibrio costicola* subsp. *vallismortis*, as well as five halophilic strains (IB574, IB872, PR5, PR919 and PR932), isolated from salterns in Spain and Puerto Rico that were closely related to these bacteria. Multilocus Sequence Analysis (MLSA) of concatenated *gyrB*, *recA*, *rpoB* and *rpoD* housekeeping genes showed that they constituted a single cluster separate to the rest of species and subspecies of *Salinivibrio*. Experimental and *in silico* DNA-DNA hybridization (GGDC) studies indicated that they are members of the same species, with relatedness of 100 to 74 % and 100 to 70.4 %, respectively. Besides, the Average Nucleotide Identity (ANI) determined for these strains was 100 to 95.5 % for ANIb and 100 to 95.7 % for OrthoANI. However, the ANI values for *Salinivibrio costicola* subsp. *vallismortis* DSM 8285^T with respect to *S. costicola* subsp. *costicola* DSM 11403^T and *S. costicola* subsp. *alcaliphilus* DSM 16359^T were 82.0 and 82.3 % (ANIb) and 79.4 and 79.4 % (OrthoANI), respectively. The phylogenomic tree based on 1,072 concatenated orthologous single-copy core genes confirmed that *Salinivibrio proteolyticus*, *Salinivibrio costicola* subsp. *vallismortis* and the five new isolates constitute a coherent single phylogroup, separated from the other species and subspecies of *Salinivibrio*. All these data indicate that *Salinivibrio costicola* subsp. *vallismortis* is a heterotypic synonym of *Salinivibrio proteolyticus* and we propose the emended description of this species.

The genus *Salinivibrio* belongs to the family *Vibrionaceae* within the class *Gammaproteobacteria*. It was proposed by Mellado et al. [1] to accommodate Gram-stain-negative, facultatively anaerobic, motile, curved-rods halophilic bacteria, previously named as *Vibrio costicola* [2, 3]. These bacteria have developed cellular mechanisms to thrive in harsh environmental conditions, such as high salinities, UV and arsenic tolerance [4, 5]. Currently, this genus includes four species, one of them with three subspecies: *Salinivibrio costicola*, with the subspecies *S. costicola* subsp. *costicola* [1-3, 6], *Salinivibrio costicola* subsp. *vallismortis* [6], and *Salinivibrio costicola* subsp. *alcaliphilus* [7], *Salinivibrio proteolyticus* [8], *Salinivibrio siamensis* [9] and *Salinivibrio sharmensis* [10]. The type species of this genus is *Salinivibrio costicola* subsp. *costicola* which is considered a representative model of studies on moderately halophilic bacteria, in which the osmoregulatory and other physiological mechanisms have been elucidated [4, 11, 12].

In 2000 Huang *et al*. [6] described taxonomically the features of strain DV^T, isolated from a hypersaline pond located in the Death Valley, California, USA. The 16S rRNA gene sequence analysis showed that this strain was most closely related to *Salinivibrio costicola* (97.7 % similarity) and DNA-DNA hybridization (93 % relatedness) indicated its close relationship to the type species of *Salinivibrio*, *S. costicola*. However, phenotypic characteristics such as its halotolerance, gas production from glucose, or utilization of different organic compounds and its 16S rRNA secondary structure were sufficiently different from *S. costicola* to warrant designating this strain as a new subspecies of *S. costicola*, as *Salinivibrio costicola* subsp. *vallismortis*, which automatically created *S. costicola* subsp. *costicola* for the existing species. In 2008 Amoozegar *et al*. [8] described the new species *Salinivibrio proteolyticus*, isolated from a hypersaline lake in Iran. Phylogenetic analysis based on 16S rRNA gene sequence

comparisons showed that its closest relatives were *S. costicola* subsp. *vallismortis* (99.0 % sequence similarity), *S. costicola* subsp. *costicola* (97.0 %) and *S. costicola* subsp. *alcaliphilus* (96.8 %). However, DNA-DNA hybridization (DDH) experiments were performed only between *Salinivibrio proteolyticus* and *S. costicola* subsp. *costicola* but not with the other two subspecies of this species. The low DDH percentage between *Salinivibrio proteolyticus* and *S. costicola* subsp. *costicola* (10 % relatedness) supported their proposal for a separate species for *S. proteolyticus*.

Recently, López-Hermoso *et al.* [13] carried out a study of 70 new isolates belonging to the genus *Salinivibrio* as well as the type strains of the current species and subspecies of this genus by a comparison of 16S rRNA gene sequence analysis, Multilocus Sequence Analysis (MLSA), based on the housekeeping genes *gyrB, recA, rpoB* and *rpoD*, experimental DDH and *in silico* DDH. These studies showed that five new isolates clustered together with *S. proteolyticus* and *S. costicola* subsp. *vallismortis*, and clearly supported their single species assignment. The aim of the present study was the comparison of the five new isolates and these two previously described taxa. The 16S rRNA and MLSA phylogenetic relationships, phenotypic and chemotaxonomic data and features based on the comparison of their genomes support that *S. proteolyticus* and *S. costicola* subsp. *vallismortis* constitute a single species. Besides, the taxonomic characterization of the five new isolates that were also shown to be members of this single taxon, permitted to determine the intra-species relationship and an emended description of the species.

The five new strains used in this study were isolated from water ponds of salterns from two different locations: strains IB574 and IB872 from Isla Bacuta salterns, Huelva, Spain, and strains PR5, PR919 and PR932 from salterns in Cabo Rojo, Puerto Rico. Details about their isolation and source habitats are shown in López-Hermoso *et al.*

[13]. Besides, type strains of the following species and subspecies were obtained from culture collections: *S. costicola* subsp. *costicola* DSM 11403^T, *S. costicola* subsp. *alcaliphilus* DSM 16359^T, *S. costicola* subsp. *vallismortis* DSM 8285^T, *S. proteolyticus* DSM 19052^T, *S. sharmensis* DSM 18182^T and *S. siamensis* JCM 14472^T and used as reference strains for comparison purposes in the present study. The strains were cultivated on SW medium [13] at 37 ºC during 24-48 h.

The cellular morphology and motility were examined by phase-contrast microscopy (Olympus CX41) from exponentially growing cultures. The morphology, size and pigmentation of the colonies were observed on SW solid medium after 24 h of incubation at 37 ºC. Growth range and optimum were determined on SW medium with different salt concentrations (0.5, 1, 2, 3, 4, 5, 7.5, 10, 12.5, 15, 17.5, 20, 21, 22, 23, 24 and 25 %, w/v) at pH 7.2-7.4. To determine the optimal and range of temperature and pH supporting the growth of the strains, SW broth cultures were incubated at 5-35 ºC at intervals of 5 ºC and from 35-50 ºC in increments of 1 ºC and at pH 4-11 at intervals of 0.5 pH units with the addition of the appropriate buffering capacity to each medium [14]. Growth was determined by monitoring the optical density at 600 nm using a spectrophotometer. Catalase activity was determined by the addition of 1 % (v/v) H_2O_2 solution to colonies on SW medium. Oxidase activity was examined using 1 % (w/v) tetramethyl-*p*-phenylenendiamine [15]. Hydrolysis of aesculin, casein, DNA, gelatin, starch or Tween 80, Voges-Proskauer and methyl red tests, production of indole, phenylalanine deaminase, phosphatase, nitrate and nitrite reduction and Simmons' citrate were determined as described by Cowan and Steel [16] with the addition of 7.5 % (w/v) total salts to the medium [17, 18]. Growth under anaerobic conditions (with H_2/CO_2) was determined by incubation of the strains in an anaerobic jar using Anaerogen (Oxoid) to generate an anaerobic atmosphere, and an anaerobic indicator

(Oxoid), on SW solid medium during one week. Acid production from carbohydrates was determined using a phenol red base supplemented with 1 % (w/v) carbohydrate and SW medium; as described elsewhere [17]. For determination of the range of substrates used as carbon and energy sources or as carbon, nitrogen and energy sources, the classical medium of Koser [19] as modified by Ventosa *et al.* [17] was used. This medium contained (per liter): 75 g NaCl, 2 g KCl, 0.2 g $MgSO_4 \cdot 7\ H_2O$, 1 g KNO_3, 1 g $(NH_4)_2HPO_4$, 0.5 g KH_2PO_4 and 0.05 g yeast extract (BD). 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}.

Phenotypically, strains *S. proteolyticus* DSM 19052^T and *S. costicola* subsp. *vallismortis* DSM 8285^T along with the five new isolates have very similar features: they are Gram-stain-negative, non-endospore forming curved rods, motile, facultative anaerobic and catalase-, oxidase-, and phosphatase-positive. The optimum temperature growth was 37 ºC, near neutral pH (7.0-7.5) and 7.5 % (w/v) NaCl. They hydrolyzed DNA. Voges-Proskauer was positive, but phenylalanine deaminase and indole production tests were negative. Acid production from fructose, D-glucose, maltose, mannitol, ribose and D-trehalose were positive, while from aesculin, D-galactose, lactose, raffinose and D-xylose were negative. Utilization of D-glucose, D-maltose, raffinose, ribose, sucrose and D-trehalose as sole carbon and energy source were positive (Table 1).

PCR amplification and sequencing of the 16S rRNA and the four housekeeping genes (*gyrB, recA, rpoB* and *rpoD*) of the strains and phylogenetic analysis were performed and described previously [13]. Phylogenetic trees were constructed using MEGA 5 [20] for neighbour-joining and maximum-parsimony methods and PhyML [21] for the maximum-likelihood (ML) [22] method. Neighbour-joining analyses were performed

using Jukes-Cantor parameter model [23]. Maximum-parsimony analyses were carried out using a heuristic search option. For ML analysis, the GTR model was selected and the base frequencies, the rate matrix, the proportion of invariable sites and the gamma distribution were estimated via likelihood. Bootstrap analyses were based on 1000 replications [24]. The 16S rRNA and housekeeping genes sequence accession numbers used in this study are shown in Suppl. Table S1. The phylogenetic trees based on the 16S rRNA gene sequences showed that the three subspecies of *S. costicola* do not form a monophyletic group (Fig. 1). While *S. costicola* subsp. *costicola* ATCC 35508^T and *S. costicola* subsp. *alcaliphilus* 18AG^T clustered together, *Salinivibrio proteolyticus* AF-2004^T clustered with *S. costicola* subsp. *vallismortis* DSM 8285^T and four of the new isolates. Recent studies by López-Hermoso *et al.* [13] indicated that the 16S rRNA gene was not an adequate phylogenetic marker for the genus *Salinivibrio*, due to the slow evolutionary rate and relative lack of resolving informative characters of this gene in *Salinivibrio*. For that reason we have carried out a MLSA study in order to clarify the phylogenetic relationships of these strains. Fig. 2 shows the phylogenetic tree based on the concatenated *gyrB*, *recA*, *rpoB* and *rpoD* gene sequences for the five isolates and the species and subspecies of *Salinivibrio*, obtained by the maximum-likelihood. *S. proteolyticus* DSM 19052^T, *S. costicola* subsp. *vallismortis* DSM 8285^T and the five isolates from salterns form a robust cluster, with a bootstrap of 100 % and clearly separated from the rest of members of the genus *Salinivibrio*. Besides, *S. costicola* subsp. *costicola* DSM 11403^T and *S. costicola* subsp. *alcaliphilus* DSM 16359^T cluster together, as could be expected on the basis of previous 16S rRNA gene sequence analyses (Fig. 2). Lopez-Hermoso *et al*. [13] proposed a MLSA scheme based on these four housekeeping genes as a replacement for DDH assays in *Salinivibrio*; they carried out a comparative MLSA-DDH study in this genus and determined a cut-off value for

171 species delineation of 97 % for the concatenated MLSA gene sequences. The
172 percentages of similarity determined in this study for the four concatenated
173 housekeeping genes in the phylogroup constituted by *S. proteolyticus* DSM 19052^T, *S.*
174 *costicola* subsp. *vallismortis* DSM 8285^T and the five isolates ranged from 97.0 to 98.8
175 %, corroborating the species level status for all these strains. However, the percentages
176 between *S. costicola* subsp. *costicola* DSM 11403^T and *S. proteolyticus* DSM 19052^T or
177 *S. costicola* subsp. *vallismortis* DSM 8285^T were 88.2 and 88.3 %, respectively, and
178 between *S. costicola* subsp. *alcaliphilus* DSM 16359^T and *S. proteolyticus* DSM 19052^T
179 or *S. costicola* subsp. *vallismortis* DSM 8285^T were 85.8 and 85.4 %, respectively.
180 These values are low enough for considering *S. proteolyticus* DSM 19052^T and *S.*
181 *costicola* subsp. *vallismortis* DSM 8285^T as separate members of the species *S.*
182 *costicola*.

183 In this study, we have determined the G+C content of the DNA from these strains by the
184 midpoint value (T_m) of the thermal denaturation profile [25] as well as from their draft
185 genomes by using the tool enveomics [26]. The values obtained for the five strains and
186 the two reference strains were 49.5-52.0 mol% by the T_m method while the range when
187 these determinations were based on the draft genomes was from 49.7 to 49.9 mol%
188 (Table 1). These values are similar to those determined for the other members of the
189 genus *Salinivibrio* (Table 1).

190 To verify the species status of the five strains and the two species and subspecies of the
191 genus *Salinivibrio*, DNA-DNA hybridization studies were performed among these
192 strains and other related *Salinivibrio* species. The DNA was extracted and purified by
193 the method of Marmur [27], and the DDH experiments were carried out by the
194 competition procedure of the membrane method [28], described in detail elsewhere [29,
195 30]. The hybridization experiments were carried out under optimal conditions, at a

196 temperature of 51.9 ºC, which is within the limits of validity for the filter method [31].
197 The percentage of hybridization was calculated as described by Johnson [28]. Our
198 results revealed a high level of DNA–DNA hybridization among the five new isolates
199 and the type strains of *S. proteolyticus* DSM 19052^T and *S. costicola* subsp. *vallismortis*
200 DSM 8285^T, ranging from 74 to 100 %. The percentages of DDH determined between
201 *S. costicola* subsp. *vallismortis* DSM 8285^T and *S. proteolyticus* DSM 19052^T or *S.*
202 *costicola* subsp. *costicola* DSM 11403^T were 74 % and 22 %, respectively.
203 Alternatively, digital DNA–DNA hybridizations were determined online
204 (http://ggdc.dsmz.de/distcalc2.php) using the Genome-to-Genome Distance Calculator
205 (GGDC) version 2.0 as described by Meier-Kolthoff *et al.* [32]. The estimated digital
206 DDH values were calculated using formula two at the GGDC website, originally
207 described by Auch *et al.* [33] and updated by Meier-Kolthoff *et al.* [32]. The draft
208 genomes of the five new isolates and the species and subspecies of *Salinivibrio* were
209 recently sequenced by López-Hermoso *et al.* [34] except that of *S. costicola* subsp.
210 *costicola* LMG 11651^T, that was sequenced by Gorriti *et al.* [5] and their accession
211 numbers are shown in Suppl. Table S1. The GGDC similarity of strains belonging to the
212 genus *Salinivibrio* is shown in Table 2. Our results reveal high values of *in silico* DDH
213 among the five new strains, *S. proteolyticus* DSM 19052^T and *S. costicola* subsp.
214 *vallismortis* DSM 8285^T, ranging from 70.4 to 100 %. However, the percentages of the
215 strains conformed by the phylogroup represented by *S. proteolyticus* DSM 19052^T, *S.*
216 *costicola* subsp. *vallismortis* DSM 8285^T and the five new isolates with respect to the
217 other species or subspecies of *Salinivibrio* were always lower than 70 % (22.3 to 24.2
218 %) (Table 2). These results correlated with those obtained by experimental DNA-DNA
219 hybridization experiments and supported that all these seven strains constitute a single
220 species [32, 35, 36].

In addition, average nucleotide identities (ANI) were determined for these strains by the JSpecies for the ANIb [37], whereas OrthoANI percentages were calculated as described by Lee *et al.* [38]. The results of these determinations are shown in Table 2. They are in agreement with the DDH data, showing pairwise ANI values equal or higher than 95.5 % (for ANIb) or 95.7 % (for OrthoANI) for the five new isolates, *S. proteolyticus* DSM 19052^T and *S. costicola* subsp. *vallismortis* DSM 8285^T, which is well above the threshold of 95 % defined for species delineation. On the other hand, when they were compared to strains of another species or subspecies of *Salinivibrio* the ANIb and OrthoANI values ranged between 82.0 and 85.1 %, and 79.2 and 87.1 %, respectively, showing that the seven strains constituted a separate taxon at the species level.

Since the draft genomes of all the species and subspecies of *Salinivibrio* and the five new isolates are already available [5, 34], we carried out a core genome phylogenetic reconstruction. All predicted protein-coding genes annotated from each available genome were compared using an all-versus-all BLAST search [39] This analysis identified shared reciprocal best matches in all pairwise genome comparisons (core orthologous genes) of the five *Salinivibrio* strains and the related taxa of the genus *Salinivibrio*. The core orthologous genes were individually aligned using MUSCLE [20] with diagonal optimization and adjusting to 1 the maximum number of iterations (default values for the other parameters). The resulting alignments were concatenated to create a core-genome alignment, and the phylogenomic tree was reconstructed by neighbour-joining method with Jukes-Cantor correction as implemented in MEGA 5 [37]. Table 3 shows the genomic features of the draft genomes of *S. proteolyticus* DSM 19052^T, *S. costicola* subsp. *vallismortis* DSM 8285^T and the five isolates. The sequenced genomes have quality enough, with N50 values ranging from 33,984 to

196,230, almost 100 % completeness and 0 to 0.27 contamination. Their genome sizes ranged from 3.6 to 3.4 Mb. The pangenome of the seven strains that constituted the phylogroup and the related species and subspecies of the genus *Salinivibrio* comprised 5,750 genes. Of these, 1,072 single-copy genes were shared by all strains (core orthologous), and phylogenetic reconstruction based on their concatenated alignment revealed the phylogroup conformed by *S. proteolyticus* DSM 19052^T, *S. costicola* subsp. *vallismortis* DSM 8285^T and the five isolates was well separated, with a bootstrap of 100 %, from the rest of related species and subspecies of the genus *Salinivibrio* (Figure 3).

These results demonstrate that the seven strains studied: the new strains IB574, IB872, PR5, PR919 and PR932 and the type strains of *S. proteolyticus* DSM 19052^T and *S. costicola* subsp. *vallismortis* DSM 8285^T represent a single species. Consequently, *S. costicola* subsp. *vallismortis* is a heterotypic synonym of *Salinivibrio proteolyticus*. The data also support the emended description of *S. proteolyticu*s. The resulting emended description is based also on the features of the five new isolates.

Emended description of *Salinivibrio proteolyticus* Amoozegar *et al.* 2008

Salinivibrio proteolyticus (pro.te.o.ly´ti.cus. N.L. masc. adj. *proteolyticus* proteolytic).

The description is the same as given by Amoozegar *et al*. [8], with the following amendments. Colonies are creamy-white to cream pigmented. Hydrolysis of DNA and phosphatase are positive. Methyl red and phenylalanine deaminase are negative. Acid production from mannitol and ribose positive. Utilization of fructose is generally negative, while of lactose, mannose or D-xylose is generally positive. The DNA G+C range is 49.7 to 49.9 mol% (genome) 49.5-52.0 mol% (HPLC/T_m).

The type strain is strain AF-2004^T (= DSM 19052^T = CIP 109598^T) which was isolated from Bakhtegan, a hypersaline lake in southern Iran. The G+C content of DNA of the type strain is 49.8 mol% (genome) and 49.5 mol% (HPLC). The species includes *Salinivibrio costicola* subsp. *vallismortis*, which is a heterotypic synonym of *Salinivibrio proteolyticus*. Strains IB574, IB872, PR5, PR919 and PR932 are additional isolates of this species.

Funding information

This study was supported by the Spanish Ministry of Economy and Competitiveness (MINECO) through projects CGL2013-46941-P and CGL2017-83385-P, which included European (FEDER) funds, and Junta de Andalucía (Spain).

Acknowledgements

C.L-H. was recipient of a fellowship from the Spanish Ministerio de Educacion, Cultura y Deporte. We thank Aharon Oren for helpful nomenclature advice and Rafael "Tito" Montalvo for the samples from Puerto Rico salterns.

Conflict of interest

The authors declare that there are no conflicts of interest.

Ethical statement

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

References

1. Mellado E, Moore ERB, Nieto JJ, Ventosa A. Analysis of 16S rRNA gene sequences of *Vibrio costicola* strains: description of *Salinivibrio costicola* gen. nov., comb. nov. *Int J Syst Bacteriol* 1996;46:817-821.

2. Smith, F.B. An investigation of a taint in rib bones of bacon. The determination of halophilic vibrios (n. spp.). *Proc R Soc Queensland* 1938,49,29–52.

3. Garcia, MT, Ventosa, A, Ruiz-Berraquero, F, Kocur, M. Taxonomic study and amended description of *Vibrio costicola*. *Int J Syst Evol Microbiol* 1987;37,251–256.

4. Kushner, DJ, Kamekura, M. Physiology of halophilic eubacteria. In: Rodríguez-Varela. F. (editor). *Halophilic Bacteria*. Boca Raton: CRC Press, 1988; pp. 109–138.

5. Gorriti MF, Dias GM, Chimetto LA, Trindade-Silva AE, Silva BS *et al*. Genomic and phenotypic attributes of novel salinivibrios from stromatolites, sediment and water from a high altitude lake. *BMC Genomic* 2014;15:473

6. Huang CY, García JL, Patel BKC, Cayol JL, Baresi L *et al*. *Salinivibrio costicola* subsp. *vallismortis* subsp. nov., a halotolerant facultative anaerobe from Death Valley, and emended description of *Salinivibrio costicola. Int J Syst Evol Microbiol.* 2000;50:615–622.

7. Romano I, Gambacorta A, Lama L, Nicolaus B, Giordano A. *Salinivibrio costicola* subsp. *alcaliphilus* subsp. nov., a haloalkaliphilic aerobe from Campania Region (Italy). *Syst Appl Microbiol.* 2005;28:34–42.

8. Amoozegar MA, Schumann P, Hajighasemi M, Fatemi AZ, Karbalaei-Heidari HR. *Salinivibrio proteolyticus* sp. nov., a moderately halophilic and proteolytic species from a hypersaline lake in Iran. *Int J Syst Evol Microbiol* 2008;58:1159–1163.

9. Chamroensaksri N, Tanasupawat S, Akaracharanya A, Visessanguan W, Kudo T *et al.* *Salinivibrio siamensis* sp. nov., from fermented fish (pla-ra) in Thailand. *Int J Syst Evol Microbiol* 2009;59:880–885.

10. Romano I, Orlando P, Gambacorta A, Nicolaus B, Dipasquale L *et al.* *Salinivibrio sharmensis* sp. nov., a novel haloalkaliphilic bacterium from a saline lake in Ras Mohammed Park (Egypt). *Extremophiles* 2011;15;213-20.

11. Ventosa, A., Nieto, J.J., Oren, A. Biology of moderately halophilic aerobic bacteria. *Microbiol Mol Biol Rev* 1998;62,504-544.

12. Oren A. *Halophilic Microorganisms and their Environments*, London: Kluwer Academic Press, 2002.

13. López-Hermoso, C, de la Haba, RR, Sánchez-Porro, C, Papke, RT, Ventosa, A. Assessment of MultiLocus Sequence Analysis as a valuable tool for the classification of the genus *Salinivibrio*. *Front Microbiol* 2017;8,1107.

14. Sánchez-Porro, C, Amoozegar, MA, Rohban, R, Hajighasemi, M, Ventosa, A. *Thalassobacillus cyri* sp. nov., a moderately halophilic Gram-positive bacterium from a hypersaline lake. *Int J Syst Evol Microbiol* 2009;59,2565–2570.

15. Kovacs, N. Identification of *Pseudomonas pyocyanea* by the oxidase reaction. *Nature* 1956;178,703.

16. Cowan, ST, Steel, KJ. *Manual for the Identification of Medical Bacteria*. Cambridge: Cambridge University Press, 1977.

17. Ventosa, A, Quesada, E, Rodríguez-Valera, F, Ruiz-Berraquero, F, Ramos-Cormenzana, A. Numerical taxonomy of moderately halophilic Gram-negative rods. *J Gen Microbiol* 1982;128,1959–1968.

18. Quesada, E, Ventosa, A, Ruiz-Berraquero, F, Ramos Cormenzana, A. *Deleya halophila*, a new species of moderately halophilic bacteria. *Int J Syst Bacteriol* 1984;34,287–292.

19. Koser, SA. Utilization of the salts of organic acids by the colon-aerogenes group. *J Bacteriol* 1923;8,493–523.

20. Tamura, K, Peterson, D, Peterson, N, Stecher, G, Nei, M *et al*. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 2011;8, 2731–2739.

21. Guindon, S, Gascuel, O. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol* 2003;52,696–704.

22. Felsenstein, J. Evolutionary trees from DNA sequences: a maximum likelihood approach. *J Mol Evol* 1981;17,368–376.

23. Jukes, TH, Cantor, CR. Evolution of protein molecules. In: Munro, HN (editor), *Mammalian Protein Metabolism*. New York: Academic Press; 1969, pp. 21-132.

24. Felsenstein J. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 1985;39,783–791.

25. Marmur, J, Doty, P. Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J Mol Biol* 1962;5,109–118.

26. Luo, C, Walk, ST, Gordon, DM, Feldgarden, M, Tiedje, JM *et al.* Genome sequencing of environmental *Escherichia coli* expands understanding of the ecology and speciation of the model bacterial species. *Proc Natl Acad Sci USA* 2011;108,7200–7205.

27. Marmur J. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. *J Mol Biol* 1961;3:208–218.

28. Johnson JL. Similarity analysis of DNAs. In: Gerhardt P, Murray, RGE, Wood, WA, Krieg, NR (editors), *Methods for General and Molecular Bacteriology*. Washington, DC: American Society for Microbiology; 1994. pp. 655–681.

29. Arahal DR, García MT, Ludwig W, Schleifer KH, Ventosa A. Transfer of *Halomonas canadensis* and *Halomonas israelensis* to the genus *Chromohalobacter* as *Chromohalobacter canadensis* comb. nov. and *Chromohalobacter israelensis* comb. nov. *Int J Syst Evol Microbiol* 2001;51:1443–1448.

30. Arahal, DR, García, MT, Vargas, C, Cánovas, D, Nieto, JJ *et al.* *Chromohalobacter salexigens* sp. nov., a moderately halophilic species that includes *Halomonas elongate* DSM 3043 and ATCC 33174. *Int J Syst Evol Microbiol* 2001;51, 1457–1462.

31. De Ley J, Tijtgat R. Evaluation of membrane filter methods for DNA-DNA hybridization. *Antonie van Leeuwenhoek* 1970;36:461–474.

32. Meier-Kolthoff JP, Auch AF, Klenk HP, Göker M. Genome sequence-based species delimitation with confidence intervals and improved distance functions. *BMC Bioinformatics* 2013;14:60.

33. Auch AF, von Jan M, Klenk HP, Göker M. Digital DNA-DNA hybridization for microbial species delineation by means of genome-to-genome sequence comparison. *Stand Genomic Sci* 2010;2:117–151.

34. López-Hermoso, C, de la Haba, RR, Sánchez-Porro, C, Bayliss, SC, Feil, EJ *et al.* Draft genome sequence of *Salinivibrio proteolyticus*, *Salinivibrio sharmensis*, *Salinivibrio siamensis*, *Salinivibrio costicola* subsp. *alcaliphilus*, *Salinivibrio costicola* subsp. *vallismortis*, and 29 new isolates belonging to the genus *Salinivibrio*. *Genome Announc* 2017;5,e00244-17.

35. Wayne LG, Brenner D.J, Colwell RR, Grimont PAD, Kandler O *et al.* Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* 1987;37:463–464.

36. Stackebrandt E, Goebel BM. Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* 1994;44:846–849.

37. Richter M, Rosselló-Móra R. Shifting the genomic gold standard for the prokaryotic species definition. *Proc Natl Acad Sci U S A* 2009;106:19126–19131.

38. Lee, I, Kim, YO, Park, S, Chun, J. OrthoANI: an improved algorithm and software for calculating average nucleotide identity. *Int J Syst Evol Microbiol* 2016;66,110–113.

39. Edgar, RC. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *BMC Bioinformatics* 2004;5,113.

401

402 **Legends to figures**

403

404 **Figure 1.** Maximum-likelihood phylogenetic tree based on nearly complete 16S rRNA
405 gene sequences of *Salinivibrio* sp. strains IB574, IB872, PR5, PR919 and PR932 and
406 the type strains of species and subspecies of *Salinivibrio*. Circles indicate branches that
407 were supported by neighbour-joining, maximum-parsimony and maximum-likelihood
408 algorithms. Numbers at nodes are bootstrap support values (percentages) based on
409 analyses of 1,000 resampled datasets; only values equal or higher than 70 % are shown.
410 The GenBank/EMBL/DDBJ accession number of each sequence is shown in
411 parentheses. Bar, 0.01 nt changes per position. *Vibrio cholerae* CECT 514^T was used as
412 outgroup.

413

414

415 **Figure 2.** Phylogenetic reconstruction of *Salinivibrio* sp. strains IB574, IB872, PR5,
416 PR919 and PR932 and the type strains of species and subspecies of *Salinivibrio* based
417 on concatenated *gyrB*, *recA*, *rpoA* and *rpoD* gene sequences. The
418 GenBank/EMBL/DDBJ accession numbers of the sequences are shown in Suppl. Table
419 S1. The tree is based on 2,981 nt of common sequence. Analysis was done using
420 maximum-likelihood method. Filled circles indicate branches that were also obtained by
421 neighbour-joining and maximum-parsimony methods. Bar, 0.05 expected nucleotide
422 substitutions per site. Only bootstrap values above 70 % are shown (1,000 replications)
423 at branches points. *Vibrio cholerae* N16961 was used as outgroup.

424

Figure 3. Neighbour-joining core gene phylogenetic tree including 11 genomes of the genus *Salinivibrio*. This tree was based on the Jukes-Cantor distance calculated from the alignment of 1,072 shared orthologous single-copy genes of these genomes. All genomes were retrieved from GenBank (Suppl. Table S1). Maximum-parsimony and maximum-likelihood trees based on the same sequences resulted in identical topology. Bootstrap values over 70 % (based on 1,000 pseudoreplicates) are shown above the branch. Bar, 0.02 substitutions per nucleotide position.

Table 1. Differential phenotypic characteristics between *S. proteolyticus* DSM 19052^T, *S. costicola* subsp. *vallismortis* DSM 8285^T, the five new isolates IB574, IB872, PR5, PR919 and PR932, and related species or subspecies of the genus *Salinivibrio*

1, *S. proteolyticus* DSM 19052^T; 2, *S. costicola* subsp. *vallismortis* DSM 8285^T; 3, strain IB574; 4, strain IB872; 5, strain PR5; 6, strain PR919; 7, strain PR932; 8, *S. costicola* subsp. *costicola* DSM 11403^T; 9, *S. costicola* subsp. *alcaliphilus* DSM 16359^T; 10, *S. sharmensis* DSM 18182^T; 11, *S. siamensis* JCM 14472^T. All strains were positive for catalase, oxidase, phosphatase, hydrolysis of DNA, Voges-Proskauer, acid production from fructose, D-glucose, maltose and D-trehalose, and the utilization of raffinose, ribose and D-trehalose. All strains were negative for production of indole, acid production from aesculin, lactose, raffinose and D-xylose. All data from this study unless otherwise indicated. +, Positive, -, negative.

Characteristics	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Colony pigmentation	Cream-white	Cream-white	Cream	Cream	Cream	Cream	Cream	Cream	Cream-pink	Cream	Cream
NaCl range (%, w/v)	1-17[a]	0-12.5[b]	3-20	4-20	3-21	2-20	2-20	0.5-20[c]	2-25[d]	6-16[e]	1-22[f]
Temperature range (ºC)	10-45[a]	20-50[b]	20-55	20-55	20-55	20-55	20-55	5-45[c]	10-40[d]	25-40[e]	10-47[f]
pH range	5-9.5[a]	5-10[b]	5-10	5-10	5-10	5-10	5-10	5-10[c]	7-10.5[d]	6-10[e]	5-9[f]
Anaerobic growth	+	+	+	+	+	+	+	+	-	+	+
Hydrolysis of:											
Aesculin	-	-	+	+	-	-	+	-	+	-	-
Casein	+	+	-	-	-	-	+	+	-	-	+
Gelatin	+	+	-	+	+	-	-	+	-	+	+
Starch	+	+	-	-	+	-	+	-	-	+	-
Tween 80	+	+	-	-	-	-	-	-	-	-	+
Methyl red	-	-	-	+	-	-	+	-	-	-	-
Voges-Proskauer	+	+	+	-	+	+	-	+	+	+	+
Phenylalanine deaminase	-	-	-	-	-	-	-	+	+	-	+
Nitrate reduction	+	-	-	-	-	-	-	-	+	+	+
Nitrite reduction	+	-	-	-	-	-	-	-	+	-	-
Simmons´ citrate	-	-	-	-	-	+	+	-	-	-	-
Acid production from:											
D-Arabinose	-	-	-	+	-	+	-	-	-	-	-
D-Galactose	-	-	-	-	-	-	-	-	+	-	-
Glycerol	-	+	+	+	-	+	-	-	-	-	+
Mannitol	+	+	+	+	+	+	+	+	-	-	+
Mannose	-	-	-	+	+	-	+	-	-	+	+
Ribose	+	+	+	+	+	+	+	-	-	+	+

Characteristics	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Sucrose	+	+	+	+	-	+	-	-	-	-	+
Utilization of:											
Alanine	-	-	+	+	-	+	+	+	+	+	+
D-Arabinose	-	-	-	+	+	+	+	-	+	+	+
Fructose	-	-	-	-	+	-	+	+	-	-	-
D-Galactose	+	+	+	-	+	+	-	+	+	+	+
D-Glucose	+	+	+	+	+	+	+	+	+	-	-
Lactose	+	+	-	+	+	-	-	+	+	+	+
D-Maltose	+	+	+	+	+	+	+	+	+	+	-
Mannose	+	+	+	-	+	+	-	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	-	-
D-Xylose	+	+	+	+	+	-	-	+	+	+	+
DNA G+C content (mol%) (*Tm*)	49.5[a]	50.0[b]	51.9	51.9	51.9	52.0	51.9	50.0[c]	49.3[d]	51.0[e]	49.0[f]
DNA G+C content (%) (genome)	49.8	49.7	49.8	49.8	49.9	49.9	49.9	49.2	49.1	50.4	50.3

446 Data from: [a] [8]; [b] [6]; [c] [1, 3]; [d] [7]; [e] [10]; [f] [9].

447

448

449 **Table 2.** Genomic indexes (ANIb, OrthoANI and GGDC) (%) among the genomes of *S.*

450 *proteolyticus* DSM 19052^T, *S. costicola* subsp. *vallismortis* DSM 8285^T and the five

451 new isolates, and the other type strains of species and subspecies of the genus

452 *Salinivibrio.*

Strains	1	2	3	4	5	6	7	8	9	10	11
ANIb											
1. *S. proteolyticus* DSM 19052^T	100	96.9	95.8	95.6	96.8	96.8	96.7	78.6	78.6	79.9	80.1
2. *S. costicola subsp. vallismortis* DSM 8285^T	96.8	100	95.9	95.9	98.4	98.4	98.4	78.7	78.9	80.0	80.1
3. *Salinivibrio* sp. IB574	95.7	95.9	100	97.3	95.9	95.9	95.8	78.6	78.6	80.1	79.7
4. *Salinivibrio* sp. IB872	95.6	96.0	97.4	100	96.0	95.9	95.9	79.1	79.1	80.3	79.9
5. *Salinivibrio* sp. PR5	96.8	98.5	95.9	96.0	100	98.5	98.5	78.6	78.7	79.9	80.0
6. *Salinivibrio* sp. PR919	96.8	98.5	95.9	95.8	98.5	100	99.7	78.5	78.6	79.9	79.9
7. *Salinivibrio* sp. PR932	96.7	98.4	95.8	95.8	98.5	99.7	100	78.5	78.6	79.9	79.9
8. *S. costicola* subsp. *costicola* DSM 11403^T	78.7	78.7	78.7	79.2	78.7	78.7	78.7	100	98.4	81.2	81.2
9. *S. costicola* subsp. *alcaliphilus* DSM 16359^T	78.8	79.0	78.9	79.4	78.9	78.8	78.9	98.3	100	81.2	81.5
10. *S. sharmensis* DSM 18182^T	80.0	80.0	80.3	80.3	80.0	80.0	80.1	81.2	81.1	100	91.1
11. *S. siamensis* JCM 14472^T	80.1	80.1	79.9	79.9	80.1	80.0	80.1	81.2	81.3	91.1	100
OrthoANI											
1. *S. proteolyticus* DSM 19052^T	100	97.1	95.9	95.7	96.8	96.9	96.8	79.4	79.4	86.6	80.8
2. *S. costicola* subsp. *vallismortis* DSM 8285^T	97.1	100	96.0	96.0	97.5	98.8	98.5	79.4	79.4	86.5	81.0
3. *Salinivibrio* sp. IB574	95.9	96.0	100	97.4	96.0	96.0	95.9	80.4	79.3	86.4	80.4
4. *Salinivibrio* sp. IB872	95.7	96.0	97.4	100	96.1	96.7	96.0	80.3	79.6	86.4	80.4
5. *Salinivibrio* sp. PR5	96.8	97.5	96.0	96.1	100	96.0	98.5	79.9	79.2	86.9	80.8
6. *Salinivibrio* sp. PR919	96.9	98.8	96.0	96.7	96.0	100	99.7	79.4	79.4	87.1	80.6
7. *Salinivibrio* sp. PR932	96.8	98.5	95.9	96.0	98.5	99.7	100	79.4	79.2	86.4	80.8
8. *S. costicola* subsp. *costicola* DSM 11403^T	79.5	79.4	80.4	80.3	79.9	79.4	79.4	100	98.6	86.5	81.0
9. *S. costicola* subsp. *alcaliphilus* DSM 16359^T	79.3	79.4	79.3	79.6	79.2	79.3	79.2	98.6	100	81.8	81.6
10. *S. sharmensis* DSM 18182^T	86.9	87.0	86.4	86.9	87.1	86.4	86.5	81.7	81.8	100	91.2
11. *S. siamensis* JCM 14472^T	80.8	80.6	80.4	80.4	80.8	80.6	81.0	81.6	81.6	91.2	100
GGDC											

1. *S. proteolyticus* DSM 19052^T	100	73.8	70.5	70.9	73.1	73.1	72.8	23.9	22.8	24.0	24.0
2. *S. costicola* subsp. *vallismortis* DSM 8285^T	73.8	100	72.8	72.5	87.0	86.9	87.0	23.9	22.9	23.9	23.9
3. *Salinivibrio* sp. IB574	70.5	72.8	100	77.3	70.0	70.8	70.0	23.7	23.1	24.0	23.7
4. *Salinivibrio* sp. IB872	70.9	72.5	77.3	100	70.8	70.4	70.0	23.4	23.5	23.7	23.6
5. *Salinivibrio* sp. PR5	73.1	87.0	70.0	70.8	100	87.4	86.8	23.8	22.9	23.6	23.8
6. *Salinivibrio* sp. PR919	73.1	86.9	70.8	70.4	87.4	100	97.8	23.9	22.9	23.8	23.8
7. *Salinivibrio* sp. PR932	72.8	87.0	70.0	70.0	86.8	97.8	100	23.9	22.8	23.8	23.8
8. *S. costicola* subsp. *costicola* DSM 11403^T	23.8	22.3	23.7	23.4	23.8	23.9	23.9	100	88.0	24.0	24.8
9. *S. costicola* subsp. *alcaliphilus* DSM 16359^T	22.8	22.3	23.1	23.5	22.9	22.9	22.8	88.0	100	24.1	24.1
10. *S. sharmensis* DSM 18182^T	23.7	23.7	24.2	24.2	23.9	23.8	23.8	24.1	24.1	100	45.5
11. *S. siamensis* JCM 14472^T	24.0	23.9	23.7	23.6	23.8	23.8	24.0	24.0	24.1	45.5	100

453 **Table 3.** Genomic features of the draft genomes of the seven strains of *S. proteolyticus* DSM 19052^T, *S. costicola* subsp. *vallismortis* DSM 8285^T

454 and the five new isolates.

Characteristics	***S. proteolyticus* DSM 19052^T**	***S. costicola* subsp. *vallismortis* DSM 8285^T**	**Strain IB574**	**Strain IB872**	**Strain PR5**	**Strain PR919**	**Strain PR932**
Genome size (bp)	3,603,496	3,498,876	3,612,537	3,641,359	3,456,024	3,489,646	3,497,261
No. contigs	51	95	69	102	105	176	74
N50	143,067	196,230	106,472	82,702	68,288	33,984	90,599
Largest contig size (bp)	326,199	382,182	286,931	230,174	322,913	88,713	207,403
Sequencing depth	11X	83X	9X	11X	23X	14X	31X
Completeness (%)	99.97	99.97	99.73	100	99.97	100	100
Contamination (%)	0	0	0	0	0	0.27	0
Total genes	3,402	3,278	3,405	3,429	3,217	3,300	3,234
No. CDS	3,298	3,166	3,308	3,327	3,105	3,201	3,130
No. hypotethical proteins	946	869	932	933	826	887	843
5S rRNAs	8	5	8	7	8	8	8
16S rRNAs	4	7	5	4	7	4	4

23S rRNAs	3	3	7	6	7	4	5
tRNAs	85	93	73	81	86	79	83
Pseudo genes	56	36	98	70	33	37	32
GC content (mol%)	49.8	49.9	49.8	49.8	50.0	49.9	49.9
DDBJ/ENA/GenBank accession number	MUFP00000000	MUFQ00000000	MUFN00000000	MUFO00000000	MUFK00000000	MUFL00000000	MUFM00000000

455