Salinivibrio kushneri sp. nov., a moderately halophilic bacterium isolated from salterns

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Abbreviations: MLSA, Multilocus Sequence Analysis; ANI, Average Nucleotide Identity, DDH, DNA-DNA hybridization; GGDC, Genome-to-Genome Distance Calculator.

Abstract

Ten Gram-strain-negative, facultatively anaerobic, moderately halophilic bacterial strains, designated AL184^T, IB560, IB563, IC202, IC317, MA421, ML277, ML318, ML328A and ML331, were isolated from water ponds of five salterns located in Spain. The cells were motile, curved rods and oxidase and catalase positive. All of them grew optimally at 37 ºC, at pH 7.2-7.4 and in the presence of 7.5 % (w/v) NaCl. Based on phylogenetic analyses of the 16S rRNA, the isolates were most closely related to *Salinivibrio sharmensis* DSM 18182^T (99.6-98.2 % 16S rRNA gene sequence similarity) and *Salinivibrio costicola* subsp. *costicola* DSM 11403^T (99.0-98.1 %). According to the MLSA analyses based on four (*gyrB*, *recA*, *rpoA* and *rpoD*) and eight (*ftsZ*, *gapA*, *gyrB*, *mreB*, *pyrH*, *recA*, *rpoA* and *topA*) concatenated gene sequences, the most closely relatives were *S. siamensis* JCM 14472^T (96.8-95.4 % and 94.9-94.7 %, respectively) and *S. sharmensis* DSM 18182^T (94.0-92.6 % and 92.9-92.7 %, respectively). *In silico* DNA-DNA hybridization (GGDC) and Average Nucleotide Identity (ANI) showed values of 80-100 % and 97.7-100 %, respectively with the related species demonstrating that the ten isolates constituted a single novel species of the genus *Salinivibrio*. Its pangenome and core genome consist of 6,041 and 1,230 genes, respectively. The phylogeny based on the concatenated orthologous core genes revealed that the ten strains form a coherent phylogroup well separated from the rest of the species of the genus *Salinivibrio*. The major cellular fatty acids of strain AL184^T were $C_{16:0}$ and $C_{18:1}$. The DNA G+C content range was 51.9-52.5 mol% (T_m) and 50.2-50.9 mol% (genome). Based on the phylogenetic-phylogenomic, phenotypic and chemotaxonomic data, the ten isolates represent a novel species of the genus *Salinivibrio*, for which the name *Salinivibrio kushneri* sp. nov. is proposed. The type strain is AL184^T (= CECT 9177^T = LMG 29817^T).

Keywords: *Vibrionaceae*, *Salinivibrio*, moderately halophilic bacteria, new species, salterns, genomic analysis.

Introduction

In 1938 Smith **[44]** described the species *Vibrio costicolus* isolated from rib bones of bacon. This species was one of the few moderately halophilic bacteria included on the Approved Lists of Bacterial Names **[43]**, correctly named as *V. costicola*. Early studies on water of ponds from marine salterns showed that halophilic vibrios were easily isolated **[50]** and lately they were characterized taxonomically as members of *V. costicola* **[11,14]**, permitting an amended description of this species. This bacterium has been used as a model microorganism for many physiological studies **[19,22,33,49**], due to the fact that it is able to grow over a wide range of salt concentrations. In 1996 Mellado et al. **[31]** proposed a reclassification of *V. costicola* into a new genus, named as *Salinivibrio costicola*, on the basis of the 16S rRNA gene sequence comparison, showing that it constitutes a separate phylogenetic clade with respect to the genus *Vibrio*, as well as on the phenotypic differences with the most closely related taxa. This genus belongs to the family *Vibrionaceae* within the class *Gammaproteobacteria* and includes Gram-stain-negative, curved motile rods, facultatively anaerobic, able to grow on a NaCl range from 0.5 to 20 % (w/v), and catalase and oxidase positive. The DNA G+C content ranges from 49.0 to 51.0 mol% **[16,31]**. Currently, the genus *Salinivibrio* comprises four species, one of them with three subspecies: *Salinivibrio costicola* subsp. *costicola* **[11,16,31,44]**, *Salinivibrio costicola* subsp. *vallismortis* **[18]**, *Salinivibrio costicola* subsp. *alcaliphilus* **[38]**, *Salinivibrio proteolyticus* **[2]**, *Salinivibrio siamensis*

[5] and *Salinivibrio sharmensis* **[39]**. Members of this genus inhabit salted meats, brines and hypersaline environments.

The evolutionary history of several vibrios (including the type species of the genus *Salinivibrio*) was reconstructed in 2007 by means of multilocus sequence analysis (MLSA) of nine genes (*ftsZ*, *gapA*, *gyrB*, *mreB*, *pyrH*, *recA*, *rpoA*, *topA* and 16S rRNA), proposing the *Salinivibrio* clade **[41]**. Later in 2013, the vibrio clades were updated by MLSA based on the eight protein-coding housekeeping genes previously mentioned, delineating a super-clade grouping the genera *Salinivibrio*, *Grimontia* and *Enterovibrio* **[42]**. Recently, López-Hermoso et al. **[27]** carried out a study in which 70 new isolates belonging to the genus *Salinivibrio* and the type strains of the species and subspecies of this genus were analyzed by 16S rRNA gene sequence comparison and MLSA. The data showed that the 16S rRNA gene was not able to properly differentiate the new isolates or assign them to the previously described species of *Salinivibrio* but alternative MLSA analyses allowed to clearly distinguish well-defined phylogroups. They validated the MLSA, based on the concatenation of *gyrB, recA, rpoA* and *rpoD* housekeeping genes, in order to replace the DNA-DNA hybridization (DDH) assays in the genus *Salinivibrio*, establishing a cut-off value for species delineation of 97 % concatenated MLSA similarity. Besides, in this study they observed clearly that some of the isolates (which formed a single phylogroup) could constitute a new species within this genus.

In this study, we carried out the taxonomic characterization of ten moderately halophilic bacteria from five salterns located in different places in Spain selected from the previous study of López-Hermoso et al. **[27]**, and the data suggest that they constitute a novel species of the genus *Salinivibrio*. Their characterization was achieved by following a polyphasic approach. The phylogenetic, genotypic and phenotypic

100 characteristics of these strains, including 16S rRNA gene sequence analysis, MLSA,
101 DDH, ANI, GGDC and chemotaxonomic features, have been carried out in order to
102 define their taxonomic status.

103 **Materials and methods**

104 *Bacterial strains and growth conditions*

105 The strains used for this study were isolated from water samples collected from
106 different ponds of salterns of Spain: strain AL184^T was isolated from Santa Pola,
107 Alicante, strains IB560 and IB563 from Isla Bacuta, Huelva, strains IC202 and IC317
108 from Isla Cristina, Huelva, strain MA421 from La Malahá, Granada, and strains
109 ML277, ML318, ML328A and ML331 were isolated from Es Trenc, Mallorca. The
110 salinity of the samples was determined to be between 5 and 21 % salts **[27].** The strains
111 were isolated by plating 0.1 ml of the water samples on SW medium with the pH
112 adjusted between 7.2-7.4 with 1 M KOH and incubated at 37 ºC. After isolation, the
113 strains were subsequently purified three times by plating on the same medium. The
114 composition of SW medium is the following: (g l^{-1}): NaCl, 58.5; $MgCl_2 \cdot 6H_2O$, 9.75;
115 $MgSO_4 \cdot 7H_2O$, 15.25; $CaCl_2$, 0.25; KCl, 1.5; $NaHCO_3$, 0.05; NaBr, 0.175; and yeast
116 extract, 5.0, and solidified with 1.8 % (w/v) agar when necessary. The strains were
117 routinely grown on SW medium and were maintained on the same medium in slant
118 tubes, and for long term preservation at -80 ºC in SW broth with 20 % (v/v) glycerol. *S.*
119 *costicola* subsp. *costicola* DSM 11403^T, *S. costicola* subsp. *alcaliphilus* DSM 16359^T,
120 *S. costicola* subsp. *vallismortis* DSM 8285^T, *S. proteolyticus* DSM 19052^T, *S.*
121 *sharmensis* DSM 18182^T and *S. siamensis* JCM 14472^T were used as reference strains
122 for comparison purposes in the present study (Supplementary Table S1).

123 *Phenotypic characterization*

Cell morphology and motility were examined by phase-contrast microscopy (Olympus CX41) from exponentially growing cultures. The morphology and pigmentation of 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, 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 5-35ºC at intervals of 5 ºC and from 35-50 ºC in increments of 1 ºC and at pH 4-10 at intervals of 0.5 pH units with the addition of the appropriate buffering capacity to each medium **[40]**. Growth was determined by monitoring the optical density at 600 nm using a spectrophotometer. Catalase activity was determined by bubble production in 3 % (w/v) H_2O_2 solution. Oxidase activity was examined using 1 % (v/v) tetramethyl-*p*-phenylenendiamine **[21]**. Hydrolysis of aesculin, casein, DNA, gelatin, starch and Tween 80, Voges-Proskauer and methyl red tests, production of indole, phenylalanine deaminase, phosphatase, nitrate and nitrite reduction and Simmon's citrate were determined as described by Cowan and Steel **[6]** with the addition of 7.5 % total salts to the medium **[36,50]**. Growth under anaerobic conditions (with H_2/CO_2) was determined by incubation 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 % carbohydrate and SW medium; this medium was a modification of the original described elsewhere **[50]**. 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 **[20]** as modified by Ventosa et al. **[50]** 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}.

Phylogenetic analysis

The almost-complete 16S rRNA gene and four protein-coding housekeeping genes were previously sequenced and analyzed by López-Hermoso et al. **[27]**. The almost-complete 16S rRNA gene sequence of strains AL184^T, IB560, IB563, IC202, IC317, MA421, ML277, ML318, ML328A and ML331 had a length of 1435, 1468, 1467, 1478, 1435, 1480, 1483, 1479, 1431 and 1445 bp, respectively; whereas housekeeping genes had a length: *gyrB* (623 bp), *recA* (701 bp), *rpoA* (820 bp) and *rpoD* (734 bp) **[27]**. The sequences obtained from the 16S rRNA gene and the four housekeeping genes were assembled by using ChromasPro software (Technelysium Pty) and edited to solve ambiguous positions. Multiple sequence alignments were made using CLUSTAL_X 2.1 **[23]** and corrected by visual inspection using BioEdit **[15]** taking into account the corresponding amino acid alignments for protein-coding genes. Phylogenetic trees were constructed using MEGA 5 **[47]** for neighbour-joining and maximum-parsimony methods and PhyML **[13]** for the maximum-likelihood (ML) **[9]** method. Neigbour-joining analyses were performed using Jukes-Cantor parameter model **[18]**. 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 **[10]**. The sequence accession numbers used in this study are shown in Supplementary Table S1. Additionally, an eight gene (*ftsZ*, *gapA*, *gyrB*, *mreB*, *pyrH*, *recA*, *rpoA* and *topA*) MLSA was also carried out as previously described **[1,41,42]**, retrieving the housekeeping

genes from the available genomes **[26]**, using the methodology indicated above for sequence alignment and tree construction.

DNA G+C content and DNA–DNA hybridization

The G+C content of the genomic DNA of the ten strains was determined from the midpoint value (T_m) of the thermal denaturation profile **[29]** by using the equation of Owen and Hill **[34]**, obtained with a Perkin-Elmer UV-Vis Lambda 20 spectrophotometer at 260 nm equipped with a PTP-1 peltier system programmed with an increasing temperature of 1 ºC min^{-1}. DNA–DNA hybridization studies were performed using the competition procedure of the membrane method **[17]**, as described in detail by Arahal et al. **[3,4]**. The hybridization temperature was 52.2 ºC, and the percentage of hybridization was calculated according to Johnson **[17]**. The experiments were carried out in triplicate and the percentages shown are the mean values. Besides, the genomic G+C content calculation based on the draft genome sequences was performed with the tool enveomics **[28]**.

Chemotaxonomic analysis

The fatty acid composition was determined for cells of strain AL184^T, selected as representative of this bacterial group, grown for 24 h at 35 ºC in the medium 1 described by Romano et al. **[39]** containing (g l^{-1}): yeast extract, 10.0; NaCl, 100.0; Na_3-citrate, 3.0; Na_2CO_3 3.0, KCl, 2.0; $MgSO_4 \cdot 7H_2O$, 1.0; $MnCl_2 \cdot 4H_2O$, 0.00036; $FeSO_4$, 0.05. The resulting pH was 9.0. The fatty acid profile was obtained with an Agilent 6850 gas chromatograph using the database TSBA6 (MIDI, 2008) **[32]**. These analyses were carried out at the Spanish Type Culture Collection (CECT), Valencia, Spain.

Genome sequencing, assembly and annotation

Draft genomes of the ten isolates and the type strains of the species and subspecies of *Salinivibrio* were previously sequenced by López-Hermoso et al. **[26]**. We also used the graft genome sequence of *Salinivibrio costicola* subsp. *costicola* LMG 11651^{T} as described by Gorriti et al. **[12]**. The accession numbers of the draft genome sequences used in this study are shown in Supplementary Table S1.

Calculation of in silico *DDH and ANI values*

In silico DDH was calculated by the Genome-to-Genome Distance Calculator (GGDC 2.0) using the BLAST+ method **[30]**. Results were based on recommended formula 2 (identities/HSP length), which is independent of genome length and is thus robust against the use of incomplete draft genomes. Calculation of the average nucleotide identity (ANI) from the draft genome sequences used previously for *in silico* DDH was performed with JSpecies **[37]**, whereas OrthoANI percentages were calculated as described by Lee et al. **[24]**.

Core genome phylogenetic reconstruction

All predicted protein-coding genes and amino acids annotated from each available genome were compared using an all-versus-all BLAST search **[28]**. This analysis identified shared reciprocal best matches (defined as > 70 % nucleotide identity or > 40 % amino acid identity) in all pairwise genome comparisons (core orthologues) of the ten *Salinivibrio* strains and the related taxa of the genus *Salinivibrio*, as well as the reference strain *Vibrio cholerae* N16961. The core orthologous genes were individually aligned using MUSCLE **[8].** The resulting nucleotide alignments were concatenated to create a core-genome alignment, and the phylogenomic tree was reconstructed by neighbour-joining method with MEGA 5 **[47]**. Additionally, the core

orthologous and unique proteins were used to construct a Venn diagram with the VennPainter tool **[25]**.

Results and discussion

Phenotypic characterization

Cells of the ten new isolates AL184^T, IB560, IB563, IC202, IC317, MA421, ML277, ML318, ML328A and ML331 were motile, slightly curved rods, Gram-stain-negative and facultative anaerobes. They were moderately halophilic, growing at 2-20 % (w/v) NaCl, with optimal growth at 7.5 % (w/v) NaCl; they were not able to grow in the absence of NaCl. The temperature range for growth was 17-49 ºC, with optimal growth at 37 ºC. The pH range for growth was at pH 5-10 and optimal growth was at pH 7.2-7.4. Other morphological, physiological, biochemical and nutritional characteristics of the strains are given in the species description and Supplementary Table S2. The differential characteristics of these strains with respect to those of the species and subspecies of the genus *Salinivibrio* are shown in Table 1.

Phylogenetic analysis based on the 16S rRNA gene sequence

The 16S rRNA gene sequence analysis showed that all isolated strains were closely related to members of the genus *Salinivibrio*. The calculation of pairwise 16S rRNA gene sequence similarities was achieved using the EzBioCloud tool **[51]**, showing 98.6-100 % similarity among the new strains. Additionally, according to the EzBioCloud server, strains AL184^T, IB560, IB563, IC202, IC317, MA421, ML277, ML318, ML328A and ML331 were most closely related to *S. sharmensis* DSM 18182^T (99.6-98.2 % sequence similarity) and *S. costicola* subsp. *costicola* DSM 11403^T (99.0-98.1 %). The 16S rRNA gene sequence similarity between the mentioned strains and the type strains of species of other related genera was lower than 92.7 %. Phylogenetic

analysis using the neighbour-joining algorithm revealed that seven of the ten strains form an independent lineage whereas three remaining strains form a monophyletic group with the species *S. sharmensis* DSM 18182^T and *S. siamensis* JCM 14472^T (Fig. 1). In a previous study López-Hermoso et al. **[27]** concluded that the 16S rRNA gene is not a good molecular marker for the delineation of species on the genus *Salinivibrio* and they proposed the MLSA approach as alternative.

Multilocus sequence analysis

Concatenation of the sequences of the four (*gyrB*, *recA*, *rpoA* and *rpoD*) and eight (*ftsZ, gapA, gyrB, mreB, pyrH, recA, rpoA* and *topA*) housekeeping genes yielded an alignment of 2981 and 3817 nt, respectively. The resulting trees (Fig. 2 and Supplementary Fig. S1) confirmed the taxonomic affiliation of the new strains to the genus *Salinivibrio*, being most closely related to *S. siamensis* JCM 14472^T (96.8-95.4 % based on four gene MLSA, and 94.9-94.7 % based on eight gene MLSA) and *S. sharmensis* DSM 18182^T (94.0-92.6 % and 92.9-92.7 %, respectively). According to the 97 % cut-off value proposed by López-Hermoso et al. **[27]** these strains may constitute a new species of the genus *Salinivibrio* (Supplementary Table S3). On the other hand, the new strains constitute a separate phylogroup sharing concatenated similarity values ranging from 99.9 to 96.9 % (four gene MLSA) and from 100 to 99.0 % (eight gene MLSA) (Supplementary Table S3), which are above the cut-off value proposed by López-Hermoso et al. **[27]** for species delineation within the genus *Salinivibrio*; thus, according to this criterion this phylogroup constitutes a new species of this genus.

G+C content and DNA–DNA hybridization

The DNA G+C content of strains AL184^T, IB560, IB563, IC202, IC317, MA421, ML277, ML318, ML328A and ML331, determined by the T_m method, was

estimated to be in the range 51.9 to 52.5 mol%; the DNA G+C content for strain AL184[4] was 52.5 mol% (Table S2). The DNA G+C content based on the drafts genomes was in the range 50.2 to 50.9 mol%, with a value of 50.2 mol% for strain AL184^T (Table S2). These percentages are close to the range described for members of the genus *Salinivibrio* (49.0 to 51.0 mol%) (Table 1). The level of DNA–DNA hybridization between strain AL184^T and strains IB560, IB563, IC202, IC317, MA421, ML277, ML318, ML328A and ML331 was 75, 98, 98, 96, 95, 90, 100, 81 and 74 %, respectively, indicating that the ten strains are members of the same species **[45,46]**. However, the percentage of DNA–DNA hybridization between strain AL184^T and *S. sharmensis* DSM 18182^T, *S. costicola* subsp. *costicola* DSM 11403^T, *S. proteolyticus* DSM 19052^T, *S. costicola* subsp. *alcaliphilus* DSM 16359^T, *S. costicola* subsp. *vallismortis* DSM 8285^T and *S. siamensis* JCM 14472^T was 8, 17, 21, 28, 44, and 47 %, respectively. These levels of DNA–DNA hybridization with respect to the type strains of the phylogenetically most closely related species and subspecies of *Salinivibrio* are significantly lower than the 70 % threshold value recommended for the delineation of novel species **[45,46]**. These experimental data clearly show that the new isolates constitute a new genospecies.

Cellular fatty acid analysis

The major fatty acids of strain AL184^T were $C_{16:0}$ (23.7 %), $C_{18:1}$ (14.2 %), $C_{12:0}$ (3.5 %), $C_{18:0}$ (2.6 %) and $C_{14:0}$ (2.1 %). This fatty acid composition is similar to those of the type strains of the related species and subspecies of the genus *Salinivibrio*, being $C_{16:0}$ the most abundant fatty acid (Supplementary Table S4). This feature is in agreement with previous studies on members of *Salinivibrio,* for which the predominant fatty acids were always $C_{16:0}$ and $C_{18:1}$ **[2,5,38,39]**.

293 *Whole-genome sequencing and ANI and GGDC calculations*

294 The mainly characteristic of the draft genomes of the ten new isolates have been
295 previously reported **[26]**. Comparison among the ten strains showed *in silico* GGDC
296 values higher than 70 % (80-100 %) (Table 2). According to Thompson *et al*. **[48]**,
297 strains from the same species share 70 % or higher *in silico* GGDC values, confirming
298 the taxonomic position of the new strains as a single species. Values obtained from
299 OrthoANI and ANIb (100-97.7 % for both) (Table 2 and Supplementary Table S5) were
300 also consistent and above the proposed cut-off value for species boundary (95-96 %)
301 **[37]**. All OrthoANI and ANIb values between strains in the new phylogroup and the
302 existing taxa were higher than 95-96 %, thus supporting the proposal for a new species.
303 As expected, according to Lee et al. **[24]**, OrthoANI percentages were slightly higher
304 than the ANIb values (Table 2 and Supplementary Table S5).

305 *Core genome phylogenetic reconstruction*

306 An all-versus-all BLAST protein-coding gene comparison indicated that the
307 pangenome of strains of the new phylogroup formed by the ten isolates and the closely
308 related species of the genus *Salinivibrio* comprised 6,041 genes. Of these, 1,230 genes
309 were shared by all strains (core orthologous genes), and phylogenetic reconstruction
310 based on their concatenated alignment revealed that the new phylogroup was well
311 separated from the rest of existing species and subspecies of the genus *Salinivibrio* (Fig.
312 3).

313 *Insights from the genome sequences*

314 The draft genomes of the ten strains of *Salinivibrio kushneri* sp. nov. ranged
315 from 3,3 to 3,6 Mbp (Supplementary Table S6), similarly to other *Salinivibrio* genomes
316 available in the public databases **[12,26]**, with the exception of one of the two released

draft genomes of *S. costicola* subsp. *costicola*, which has 4,8 Mbp **[7]** probably due to a misassembly. The sequenced genomes have quality enough, with N50 values equal or higher than 21,208, contig number equal or lower to 236, almost 100 % completeness and ≤ 0.72 % contamination (estimated using several markers as implemented in CheckM software **[35]**) (Table 3). Among the most than 3,100 genes in each genome, more than 72 % of the predicted protein-coding genes (CDS) were classified according to functional categories, being the most representatives those related to the flagellum (probably due to the motility by means of a polar flagellum characteristic of the strains of this genus), RNA methylation, methionine biosynthesis, bacterial cytoskeleton, serine-glyoxylate cycle, DNA repair and phosphate metabolism. All the *Salinivibrio kushneri* genomes presented, at least, five rRNA operons (Table 3).

Genomes of the type strains of the *Salinivibrio* species are genetically coherent, sharing 2,375 proteins (core orthologous proteins) (Fig. 4), while this number is dramatically reduced to 1,893 core proteins when including the *Vibrio cholerae* N16961 genome into the analysis. Since there is a large number of proteins common to *Salinivibrio* representatives that are not present in *V. cholerae*, we could expect that the latter possessed a large amount of unique proteins which could not be identified in *Salinivibrio*. Surprisingly, only 19 proteins were specific of *V. cholerae*, while the *Salinivibrio* genomes contained up to 113 unique proteins. When considering just the five type strains of the genus, *S. costicola* subsp. *costicola* possessed the higher number of exclusive proteins (149), followed by *S. proteolyticus* (121). The new proposed species, *S. kushneri* also contained a considerable number of unique proteins (59) (Fig. 4).

Conclusion

Results obtained from this polyphasic taxonomic study, including the phylogenetic analyses, DDH, ANI, GGDC, core-genome, chemotaxonomic analysis and phenotypic tests demonstrated that the ten strains investigated constitute a novel species within the genus *Salinivibrio*, for which the name *Salinivibrio kushneri* sp. nov. is proposed, with strain AL184^T (= CECT 9177^T = LMG 29817^T) designated as the type strain.

Description of *Salinivibrio kushneri* sp. nov.

Salinivibrio kushneri (kush´ner.i. N.L. gen. n. of Kushner, in honour of Professor Donn J. Kushner, for his pioneer work and his contributions to halophile microbiology).

Cells are Gram-strain-negative; non-endospore-forming, motile singly curved rods, 0.5 x 2.5-3.2 μm**.** Colonies on SW medium are circular, with entire edges, bright, and creamy-pigmented (0.9–2.1 mm). Facultative anaerobic. Grows at 17-49 ºC (optimally at 37 ºC), at pH values of 5-10 (optimally at pH 7.2-7.4). Moderately halophilic, growing at 2-20 % (w/v) NaCl (optimally at 7.5 %, w/v NaCl). No growth occurred in the absence of sodium chloride in the culture media. KCl cannot substitute NaCl. Positive for catalase, oxidase, nitrate reduction, Voges-Proskauer, phosphatase and hydrolysis of DNA. Negative for the hydrolysis of Tween 80, methyl red test and nitrite reduction. Most strains are able to hydrolyze casein and gelatin, while most strains are not able to hydrolyze aesculin or starch. Acid is produced from fructose, D-glucose, maltose, ribose and D-trehalose, but not from aesculin, lactose, raffinose and D-xylose. Production of indole is generally negative. Ribose and sucrose are utilized as sole carbon and energy source, and alanine is used as sole carbon, nitrogen and energy source. Utilization of D-trehalose and glutamine are positive for most strains as sole source of carbon and energy or carbon, nitrogen and energy, respectively. $C_{16:0}$ and $C_{18:1}$ are the predominant cellular fatty acids. The DNA G+C content range is: 50.2-50.9

366 mol% (genome) and 51.9-52.5 mol% (T_m). The estimated genome size is 3.3-3.6 Mb.
367 Isolated from marine salterns.

368 The type strain is AL184^T (= CECT 9177^T = LMG 29817^T), isolated from water
369 pond of a solar saltern in Santa Pola, Alicante, Spain. The DNA G+C content of the
370 type strain is 50.2 mol% (genome) and 52.5 mol % (T_m). Strains IB560, IB563, IC202,
371 IC317, MA421, ML277, ML318, ML328A and ML331 are additional strains of the
372 species. The Digital Protologue taxonumber is TA00236.

373

374

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382

383 **Appendix A. Supplementry data**

384 Supplementary data associated with this article can be found, in the online
385 version at XXXXXX.

386

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Legends of figures

Fig. 1. Neighbour-joining tree based on the 16S rRNA gene sequence comparison, showing the phylogenetic position of strains AL184^T, IB560, IB563, IC202, IC317, MA421, ML277, ML318, ML328A and ML331 and their relationship with other species and subspecies of the genus *Salinivibrio*. The GenBank/EMBL/DDBJ accession number of each sequence is shown in parenthesis. Filled circles indicate nodes that were also obtained in maximum-parsimony and maximum-likelihood trees based on the same sequences. Bootstrap values over 70 % are shown at the nodes. Bar, 0.01 substitutions per nucleotide position. The species *Vibrio cholerae* CECT 514^T was used as outgroup.

Fig. 2. Neighbour-joining tree based on the concatenated *gyrB, recA, rpoA* and *rpoD* gene sequence comparison, showing the phylogenetic position of strains AL184^T, IB560, IB563, IC202, IC317, MA421, ML277, ML318, ML328A and ML331 and their relationship with other species and subspecies of the genus *Salinivibrio*. Filled circles indicate nodes that were also obtained in maximum-parsimony and maximum-likelihood trees based on the same sequences. Bootstrap values over 70 % are shown at the nodes. Bar, 0.05 substitutions per nucleotide position. The species *Vibrio cholerae* N16961 was used as outgroup.

Fig. 3. Core phylogenomic tree of the 16 available genomes of the genus *Salinivibrio*. This tree was constructed based on the neighbour-joining distance calculated from the alignment of 1,230 shared orthologous genes of these genomes. All genomes were sequenced by López-Hermoso et al. **[26]** and Gorriti et al. **[12]**. Filled circles indicate nodes that were also obtained in maximum-parsimony and maximum-likelihood trees based on the same sequences. Bootstrap values over 70 % are shown above the branch. Bar, 0.01 substitutions per nucleotide position. The species *Vibrio cholerae* N16961 was used as outgroup.

Fig. 4. Venn diagram showing the core orthologous and unique proteins for the type strains of species of the genus *Salinivibrio*.

573 **Table 1.** Differential characteristics between *Salinivibrio kushneri* sp. nov. and related
574 species and subspecies of the genus *Salinivibrio*.

575 1, *Salinivibrio kushneri* sp. nov. (10 strains); 2, *S. siamensis* JCM 14472^T; 3, *S. sharmensis*
576 DSM 18182^T; 4, *S. costicola* subsp. *alcaliphilus* DSM 16359^T; 5, *S. costicola* subsp. *costicola*
577 DSM 11403^T; 6, *S. proteolyticus* DSM 19052^T; 7, *S. costicola* subsp. *vallismortis* DSM 8285^T.
578 All strains were curve rods. They were positive for catalase, oxidase, hydrolysis of DNA,
579 Voges-Proskauer and phosphatase, and utilization of ribose as sole carbon and energy source.
580 All strains produced acid from fructose, D-glucose, maltose and D-trehalose. All strains were
581 negative for acid production from lactose, raffinose and D-xylose. All data are from this study
582 unless otherwise indicated. +, positive, -, negative.

Characteristic	1	2	3	4	5	6	7
Colony pigmentation	Cream	Cream[a]	Cream[b]	Cream-pink[c]	Cream[d]	Cream-white[e]	Cream-white[f]
NaCl range (%, w/v)	2- 20	1-22[a]	6-16[b]	2-25[c]	0.5-20[d]	1-17[e]	0-12.5[f]
NaCl optimum (%, w/v)	7.5	10[a]	10[b]	10[c]	10[d]	5[e]	2.5[f]
Temperature range (ºC)	17-49	10-47[a]	25-40[b]	10-40[c]	5-45[d]	10-45[e]	20-50[f]
Temperature optimum (ºC)	37	37[a]	35[b]	30[c]	37[d]	35[e]	37[f]
pH range	5-10	5-9[a]	6-10[b]	7-10.5[c]	5-10[d]	5-9.5[e]	5-10[f]
pH optimum	7.2-7.4	8[a]	9[b]	9[c]	7.5[d]	8[e]	7.3[f]
Anaerobic growth	+	+	+	-	+	+	+
Nitrate reduction	+	+	+	+	-	+	-
Nitrite reduction	-	-	-	+	-	+	-
Hydrolysis of Tween 80	-	+	-	-	-	-	+
Acid production from ribose	+	+	+	-	-	+	+
Utilization of sucrose as sole carbon and energy source	+	-	-	+	+	+	+
Utilization of alanine as sole carbon, nitrogen	+	+	+	+	+	-	-

Characteristic and energy source	1	2	3	4	5	6	7
DNA G+C content (*Tm*) (mol%)	51.9-52.5	49.0[a]	51.0[b]	49.3[c]	50.0[d]	49.5[e]	50.0[f]
DNA G+C content (genome) (mol%)	50.2-50.9	50.3	50.4	49.1	49.2	49.8	49.7

583

584 [a] Data taken from Chamroensaksri et al. **[5]**.

585 [b] Data taken from Romano et al. **[39]**.

586 [c] Data taken from Romano et al. **[38]**.

587 [d] Data taken from Mellado et al. **[31]**.

588 [e] Data taken from Amoozegar et al. **[2]**.

589 [f] Data taken from Huang et al. **[16].**

590

Table 2. OrthoANI (upper triangle in bold) and GGDC (lower triangle) values among the genomes of the ten strains of *Salinivibrio kushneri* and the type strains of the species and subspecies of the genus *Salinivibrio*.

Strain	1	2	3	4	5	6	7	8	9	10
1. *S. kushneri* AL184^{T}	**100** / 100	**98.4**	**98.2**	**98.2**	**98.4**	**98.1**	**98.3**	**98.4**	**97.7**	**98.3**
2. *S. kushneri* IB560	84.7	**100** / 100	**98.4**	**98.2**	**98.2**	**98.4**	**98.1**	**98.3**	**98.4**	**97.7**
3. *S. kushneri* IB563	83.3	81.9	**100** / 100	**99.4**	**98.2**	**97.8**	**98.1**	**98.2**	**98.2**	**98.1**
4. *S. kushneri* IC202	85.4	81.7	95.6	**100** / 100	**98.2**	**97.8**	**98.1**	**98.1**	**98.2**	**98.0**
5. *S. kushneri* IC317	85.8	85.0	83.4	83.0	**100** / 100	**98.1**	**98.3**	**99.9**	**99.4**	**98.4**
6. *S. kushneri* MA421	83.0	82.4	80.3	80.0	83.1	**100** / 100	**98.5**	**98.2**	**98.1**	**97.9**
7. *S. kushneri* ML277	85.0	84.5	82.4	82.1	83.5	86.7	**100** / 100	**98.4**	**98.3**	**98.2**
8. *S. kushneri* ML318	86.0	85.0	83.3	83.1	99.7	83.1	85.4	**100** / 100	**98.2**	**98.3**
9. *S. kushneri* ML328A	86.0	87.5	83.7	83.2	95.7	83.8	85.2	95.9	**100** / 100	**98.3**
10. *S. kushneri* ML331	85.7	84.0	83.2	82.3	85.3	81.8	84.3	85.5	85.1	**100** / 100
11. *S. costicola* subsp. *alcaliphilus* DSM 16359^{T}	**81.9** / 24.5	**81.9** / 24.5	**82.0** / 24.9	**81.9** / 24.8	**81.9** / 24.7	**81.9** / 24.7	**81.8** / 24.6	**81.8** / 24.5	**81.8** / 24.5	**82.0** / 24.4
12. *S. costicola* subsp. *costicola* LMG 11651^{T}	**81.8** / 24.5	**81.7** / 24.6	**82.2** / 25.1	**82.0** / 25.1	**81.8** / 24.8	**81.8** / 24.7	**81.9** / 24.7	**81.8** / 24.5	**82.0** / 24.5	**81.8** / 24.6
13. *S. costicola* subsp. *vallismortis* DSM 8285^{T}	**80.4** / 23.7	**80.5** / 23.5	**80.5** / 23.5	**80.2** / 23.5	**80.6** / 24.0	**80.5** / 23.4	**80.5** / 23.3	**80.5** / 23.6	**80.3** / 23.5	**80.6** / 23.7
14. *S. proteolyticus* DSM 19052^{T}	**80.7** / 23.6	**80.5** / 23.5	**80.4** / 23.6	**80.5** / 23.6	**80.6** / 24.0	**80.5** / 23.5	**80.4** / 24.4	**80.5** / 23.6	**80.6** / 23.5	**80.8** / 23.6
15. *S. sharmensis* DSM 18182^{T}	**91.1** / 42.7	**91.1** / 42.9	**91.0** / 42.6	**90.9** / 42.6	**91.3** / 42.9	**91.0** / 43.0	**91.2** / 43.0	**91.1** / 42.9	**91.0** / 42.8	**91.1** / 42.8
16. *S. siamensis* JCM 14472^{T}	**91.6** / 44.5	**91.8** / 44.8	**91.6** / 44.6	**91.6** / 44.6	**91.6** / 44.6	**91.8** / 45.0	**91.7** / 44.8	**91.6** / 44.6	**91.5** / 44.5	**91.5** / 44.6

595 **Figure 1**

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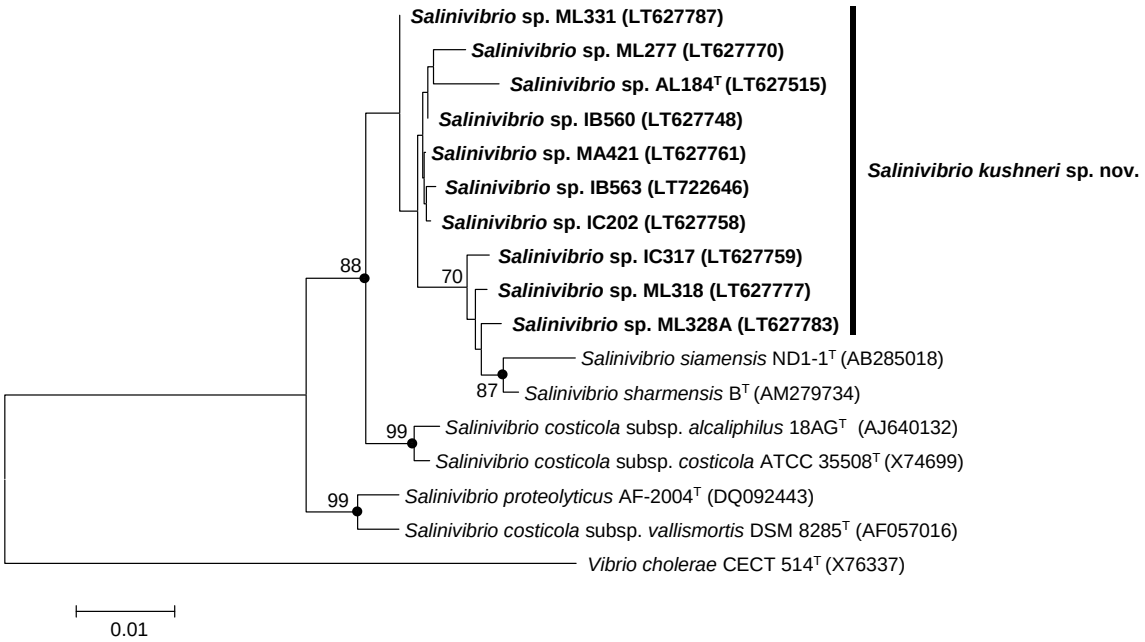

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599 **Figure 2**

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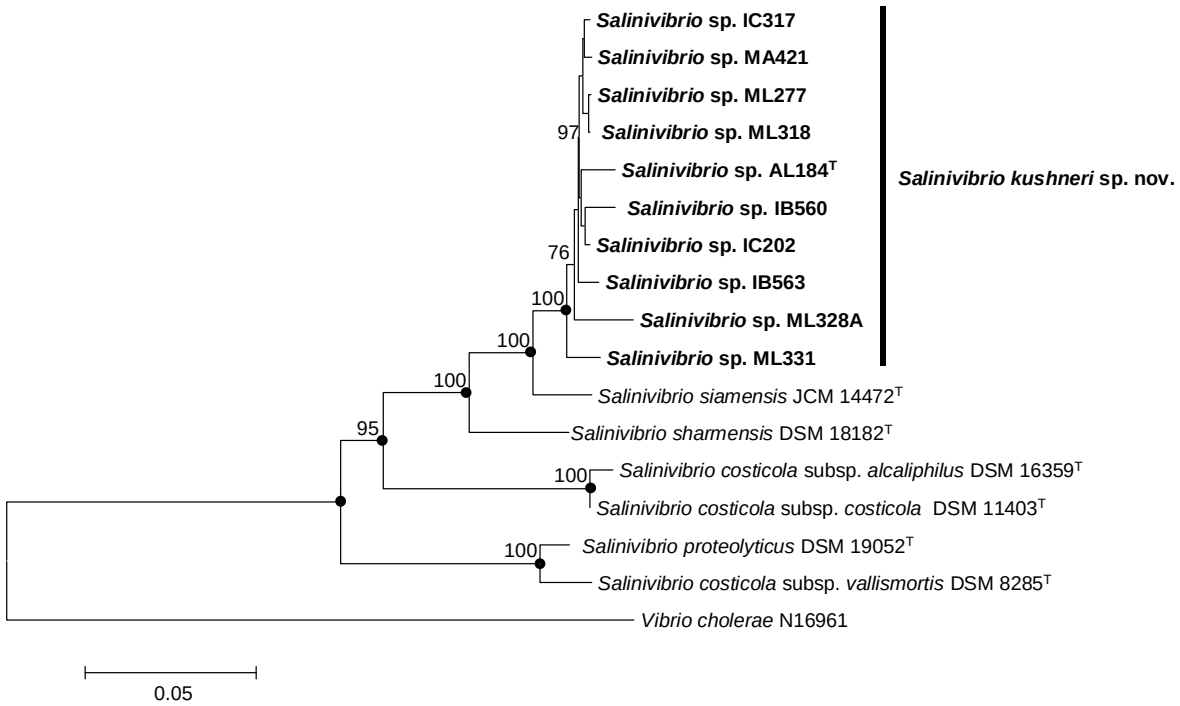

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603 **Figure 3**

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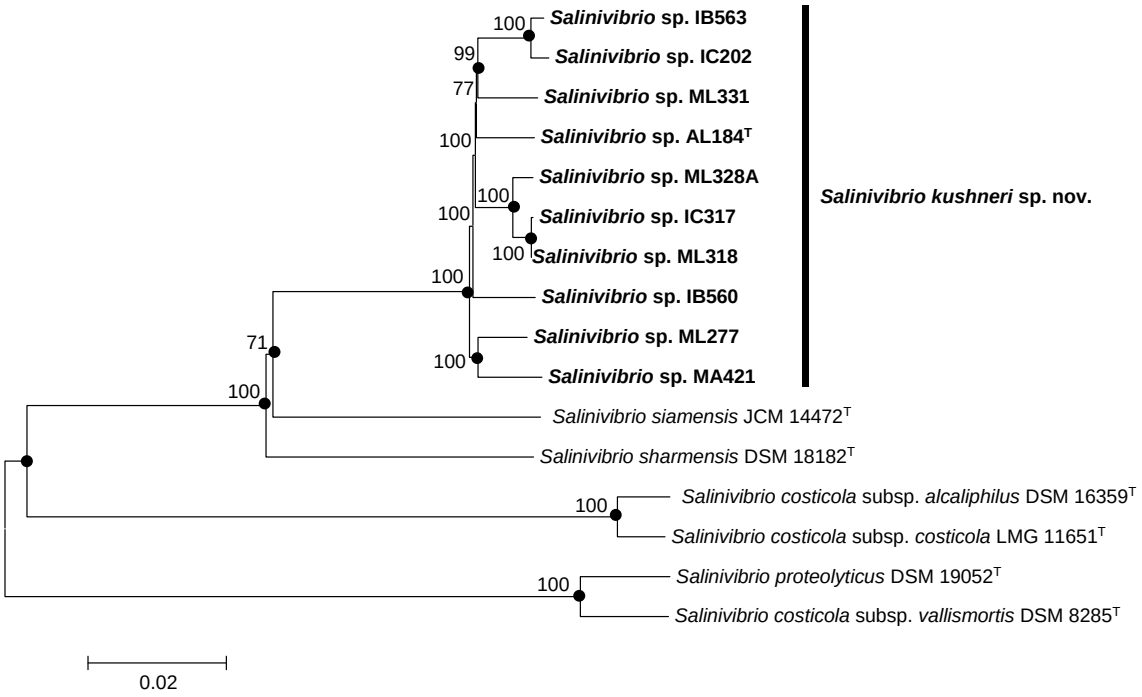

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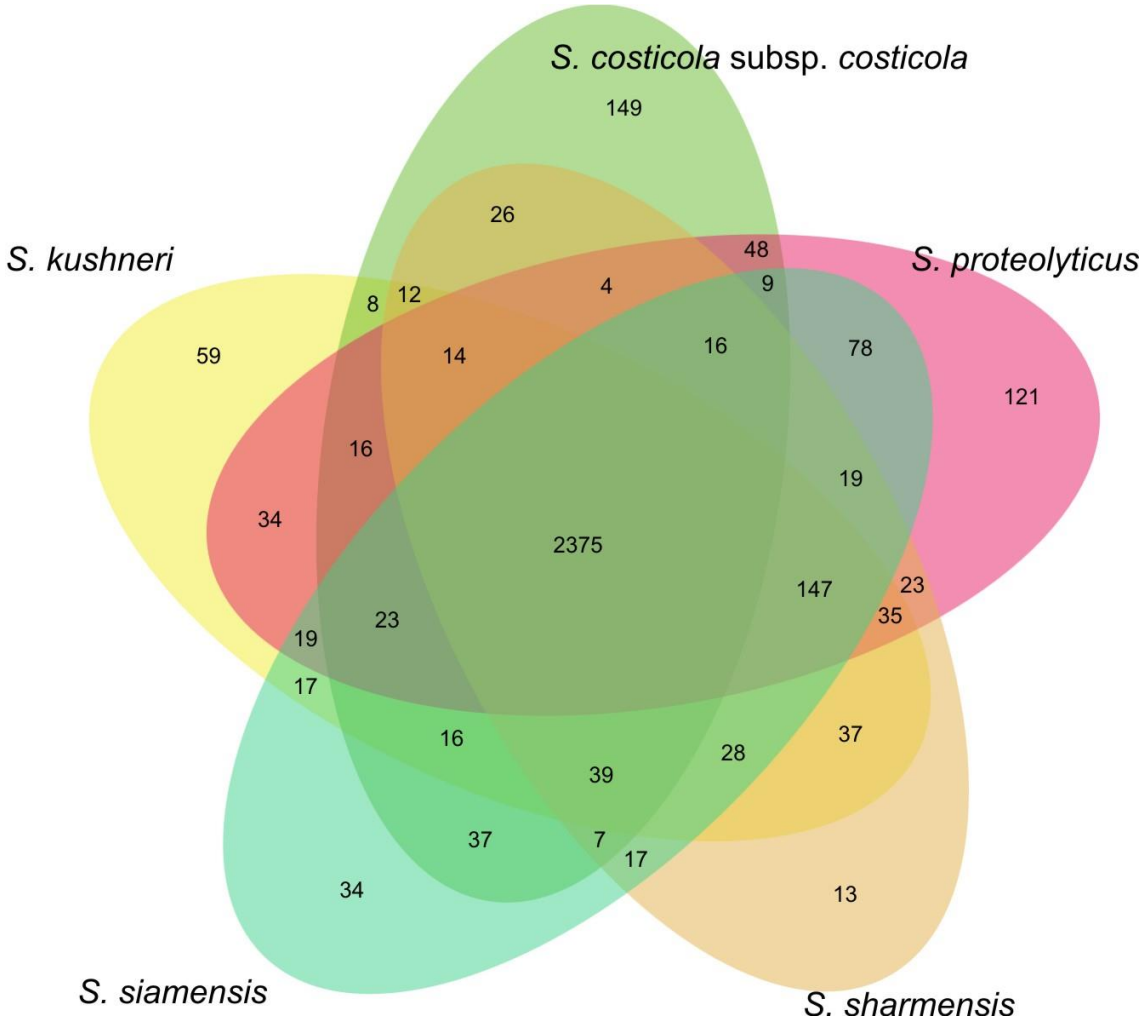
Figure 4


S. costicola subsp. costicola
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S. kushneri
S. proteolyticus
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S. siamensis
S. sharmensis