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

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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 °C, 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* BAG^T (99.6–98.2% 16S rRNA gene sequence similarity) and *Salinivibrio costicola* subsp. *costicola* ATCC 35508^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 23.3–44.8% and 80.2–91.8%, 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 6041 and 1230 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).

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

Abbreviations: MLSA, multilocus sequence analysis; ANI, average nucleotide identity; DDH, DNA–DNA hybridization; GGDC, Genome-to-Genome Distance Calculator.

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

Materials and methods

Bacterial strains and growth conditions

The strains used for this study were isolated from water samples collected from different ponds of salterns of Spain: strain AL184^T was isolated from Santa Pola, Alicante; strains IB560 and IB563 from Isla Bacuta, Huelva; strains IC202 and IC317 from Isla Cristina, Huelva; strain MA421 from La Malahá, Granada; and strains ML277, ML318, ML328A and ML331 were isolated from Es Trenc, Mallorca. The salinity of the samples was determined to be between 5 and 21% salts [27]. The strains were isolated by plating 0.1 ml of the water samples on SW medium with the pH adjusted between 7.2–7.4 with 1 M KOH and incubated at 37 °C. After isolation, the strains were subsequently purified three times by plating on the same medium. The composition of SW medium is the following: (g l^{-1}): NaCl, 58.5; $MgCl_2{\cdot}6H_2O$, 9.75; $MgSO_4{\cdot}7H_2O$, 15.25; $CaCl_2$, 0.25; KCl, 1.5; $NaHCO_3$, 0.05; NaBr, 0.175; and yeast extract, 5.0, and solidified with 1.8% (w/v) agar when necessary. The strains were routinely grown on SW medium and were maintained on the same medium in slant tubes, and for long term preservation at −80 °C in SW broth with 20% (v/v) glycerol. *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 were used as reference strains for comparison purposes in the present study (Supplementary Table S1).

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 °C. 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 5–35 °C at intervals of 5 °C (and additionally at 17 °C) and from 35 to 50 °C in increments of 1 °C and at pH 4–11 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 $MgSO_4{\cdot}7H_2O$, 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 the following 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

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