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Salimicrobium salexigens sp. nov., a moderately halophilic bacterium from salted hides☆

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ABSTRACT

Two Gram-positive, moderately halophilic bacteria, designated strains 29CMI^T and 53CMI, were isolated from salted hides. Both strains were non-motile, strictly aerobic cocci, growing in the presence of 3–25% (w/v) NaCl (optimal growth at 7.5–12.5% [w/v] NaCl), between pH 5.0 and 10.0 (optimal growth at pH 7.5) and at temperatures between 15 and 40 °C (optimal growth at 37 °C). Phylogenetic analysis based on 16S rRNA gene sequence comparison showed that both strains showed a similarity of 98.7% and were closely related to species of the genus *Salimicrobium*, within the phylum *Firmicutes*. Strains 29CMI^T and 53CMI exhibited 16S rRNA gene sequence similarity values of 97.9–97.6% with *Salimicrobium album* DSM 20748^T, *Salimicrobium halophilum* DSM 4771^T, *Salimicrobium flavidum* ISL-25^T and *Salimicrobium luteum* BY-5^T. The DNA G+C content was 50.7 mol% and 51.5 mol% for strains 29CMI^T and 53CMI, respectively. The DNA–DNA hybridization between both strains was 98%, whereas the values between strain 29CMI^T and the species *S. album* CCM 3517^T, *S. luteum* BY-5^T, *S. flavidum* ISL-25^T and *S. halophilum* CCM 4074^T were 45%, 28%, 15% and 10%, respectively, showing unequivocally that strains 29CMI^T and 53CMI constitute a new genospecies. The major cellular fatty acids were anteiso-$C_{15:0}$, anteiso-$C_{17:0}$, iso-$C_{15:0}$ and iso-$C_{14:0}$. The main respiratory isoprenoid quinone was MK-7, although small amounts of MK-6 were also found. The polar lipids of the type strain consist of diphosphatidylglycerol, phosphatidylglycerol, one unidentified phospholipid and one glycolipid. The peptidoglycan type is A1γ, with *meso*-diaminopimelic acid as the diagnostic diamino acid. On the basis of the phylogenetic analysis, and phenotypic, genotypic and chemotaxonomic characteristics, we propose strains 29CMI^T and 53CMI as a novel species of the genus *Salimicrobium*, with the name *Salimicrobium salexigens* sp. nov. The type strain is 29CMI^T (=CECT 7568^T = JCM 16414^T = LMG 25386^T).

Introduction

The genus *Salimicrobium* was proposed by Yoon et al. [32] in order to transfer two previously described species, *Marinococcus albus* [9] and *Bacillus halophilus* [28], as *Salimicrobium album* and *Salimicrobium halophilum*, respectively, and to accommodate a new species, *Salimicrobium luteum* [32]. The genus *Salimicrobium* includes Gram-positive, strictly aerobic rods and cocci which are within the *Firmicutes*; they are catalase- and oxidase positive, moderately halophilic bacteria which require NaCl for growing. The predominant menaquinone is MK-7 and its cell wall peptidoglycan contains *meso*-diaminopimelic acid as the diagnostic diaminoacid. The DNA G+C content is 44.9–51.5 mol%. The type species of this genus is *S. album*, which was originally isolated from a solar saltern in Spain [31]. The other two species, *S. halophilum* and *S. luteum* were isolated from marine solar salterns in Spain and Korea, respectively [28,32]. More recently, another species has been described within this genus: *Salimicrobium flavidum* isolated from a marine solar saltern of the Yellow Sea in Korea [33].

Recent studies focused on the determination of the microbial diversity of salted hides permitted us to isolate a new species of the genus *Thalassobacillus*, *Thalassobacillus pellis* [23]. The aim of this study was to determine the taxonomic position of two new isolates, strains 29CMI^T and 53CMI, using a polyphasic approach. Our results show that these isolates represent a novel species of the genus *Salimicrobium*, for which we propose the new designation *Salimicrobium salexigens* sp. nov.

☆ *Note*: Nucleotide sequence data for the 16S rRNA gene are available in the GenBank/EMBL/DDBL databases under the accession numbers: FR714935 (strain 29CMI^T) and FR714936 (strain 53CMI).

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Materials and methods

Isolation and bacterial strains

Strains 29CMI^T and 53CMI were isolated from salted hides obtained from Australia on SW15 medium which contained a mixture of 15% salts [30] after incubation at 37 °C for 7 days. For routine growth the strains were cultivated in SW10 medium with 10% (w/v) total salts (8.1% NaCl, 0.7% $MgCl_2$, 0.96% $MgSO_4$, 0.036% $CaCl_2$, 0.2% KCl, 0.006% $NaHCO_3$, 0.0026% NaBr, 0.5% yeast extract [Difco]) [30]. The pH was adjusted to 7.5 with 1 M KOH. When necessary, solid media were prepared by adding 2.0% (w/v) Bacto-agar (Difco). These cultures were maintained at −80 °C in SW10 medium containing 50% (v/v) glycerol. The type strains of *S. album* CCM 3517^T, *S. halophilum* CCM 4074^T, *S. luteum* BY-5^T and *S. flavidum* ISL-25^T were used for comparative purposes. They were cultivated under the same conditions than strains 29CMI^T and 53CMI.

Phenotypic characterization

The proposed minimal standards for describing new taxa of aerobic, endospore-forming bacteria as recommended by Logan et al. [12] were followed. For the determination of cellular morphology and motility, a culture from liquid 10% HM medium was examined by light microscopy under a phase-contrast microscope. The morphology of colonies, their size and pigmentation were observed on the 10% HM solid medium after 48 h of incubation at 37 °C. The composition of the 10% HM medium is (w/v): 8.1% NaCl, 0.7% $MgCl_2$, 0.96% $MgSO_4$, 0.036% $CaCl_2$, 0.2% KCl, 0.006% $NaHCO_3$, 0.0026% NaBr, 0.5% proteose peptone (Difco), 1.0% yeast extract (Difco), 0.1% glucose and 1.5% agar [30]. Optimal conditions for growth were determined by growing the strains in SW medium at 0, 0.5, 3.0, 5, 7.5, 10, 12.5, 15, 20, 25 and 30% (w/v) NaCl, and at temperatures of 4, 15, 20, 28, 30, 37, 40 and 45 °C, respectively. The pH range for the isolates was tested in SW10 medium adjusted to the following pH values: 4.0, 5.0, 6.0, 7.0, 7.5, 8.0, 9.0 and 10.0 with the addition of the appropriate buffering capacity to each medium [22]. All biochemical tests were carried out at 10% NaCl and 37 °C, unless it is stated otherwise. Growth under anaerobic conditions was determined by incubating strains in an anaerobic chamber in SW10 medium. Catalase activity was determined by adding a 1% (w/v) H_2O_2 solution to colonies on SW10 agar medium. Oxidase test was performed using the Dry Slide Assay (Difco). Hydrolysis of aesculin, casein, DNA, gelatin, starch, Tween 80, pullulan and xylan, Voges-Proskauer and methyl red tests, production of indole, arginine, lysine and ornithine decarboxylases, phenylalanine deaminase, phosphatase, urease and nitrate reduction were determined as described by Cowan and Steel [4] with the addition of a 10% total salts to the medium [19,30]. Citrate utilization was determined on Simmon's Citrate medium supplemented with SW10. Acid production from carbohydrates was determined using phenol red base supplemented with 1% of the carbohydrate and SW10 medium [30]. For determining the range of substrates used as carbon and energy sources or as carbon, nitrogen and energy sources, the classical medium of Koser [11] as modified by Ventosa et al. [30] was used: 75 g NaCl l^{-1}, 2 g KCl l^{-1}, 0.2 g $MgSO_4 \cdot 7H_2O$ l^{-1}, 1 g KNO_3 l^{-1}, 1 g $(NH_4)_2HPO_4$ l^{-1}, 0.5 g KH_2PO_4 l^{-1} and 0.05 g yeast extract (Difco) l^{-1}. Substrates were added as filter-sterilized solutions to give a final concentration of 1 g l^{-1}, except for carbohydrates, which were used at 2 g l^{-1}. When the substrate was an amino acid, it was tested as carbon, nitrogen and energy source, and the basal medium was therefore prepared without KNO_3 and $(NH_4)_2HPO_4$.

Phylogenetic analysis

Genomic DNA from strains 29CMI^T and 53CMI was prepared using the method described by Marmur [14]. Their 16S rRNA gene was amplified by PCR with the forward primer 16F27 and the reverse primer 16R1488 [16]. Direct sequence determination of the PCR-amplified DNA was carried out using an automated DNA sequencer (model ABI 3130XL; Applied Biosystems). The 16S rRNA gene sequence analysis was performed with the ARB software package [13]. The 16S rRNA gene sequences were aligned with the published sequences of the closely related bacteria and the alignment was confirmed and checked against both primary and secondary structures of the 16S rRNA molecule using the alignment tool of the ARB software package. Phylogenetic trees were constructed using three different methods: maximum-likelihood [6], maximum-parsimony [8] and neighbour-joining [21], algorithms integrated in the ARB software for phylogenetic inference. Bootstrap test [7] was performed by calculating 1000 replicate trees in order to assess the robustness of the topology. The 16S rRNA gene sequences used for phylogenetic comparisons were obtained from the GenBank database and their strain designations and accession numbers are shown in Fig. 1.

Chemotaxonomic analysis

Fatty acids analysis was performed using the MIDI system (Microbial Identification System). Cells were cultured on Marine agar (MA) medium (Difco) at 37 °C for 24 h. The extraction and analysis of fatty acids were performed according to the recommendations of the MIDI system. This analysis was carried out by the Identification, Characterization and Molecular Typing Service of the BCCM/LMG Bacteria Collection (Gent, Belgium). The peptidoglycan structure of strains 29CMI^T and 53CMI was determined by Dr. Peter Schumann from the Identification Service of the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen), Braunschweig, Germany. The determination was carried out as described by Schleifer [24] and Schleifer and Kandler [25] by thin-layer chromatography on cellulose plates using the solvent system of Rhuland et al. [20].

The analysis of respiratory quinones and polar lipids of strain 29CMI^T was carried out by Dr. Brian Tindall, also from the Identification Service of the DSMZ. The quinones were determined according to the method of Collins et al. [3]. The polar lipids were analysed using the method of Minnikin et al. [17] and Collins and Jones [2].

Determination of the DNA G+C content and DNA–DNA hybridization

The G+C content of the genomic DNA was determined from the midpoint value of the thermal denaturation profile [15] using the equation of Owen and Hill [18]. DNA–DNA hybridization studies were performed by the competition procedure of the membrane filter method [10]. The hybridization temperature was 51.2 °C, which is within the limit of validity for the filter method [5] and the percentage of hybridization was calculated according to Johnson [10]. The experiments were performed in triplicate.

Results and discussion

Strains 29CMI^T and 53CMI were Gram-positive, non-motile and strictly aerobic cocci. They were able to grow in media containing 3–25% (w/v) NaCl and optimally in media containing 7.5–12.5% and 10% (w/v) NaCl, respectively. Both strains were unable to grow in the absence of NaCl. On the basis of the NaCl requirements these bacteria can be considered as moderately halophilic microorganisms [29]. Their optimal temperature and pH were 37 °C and