Chromohalobacter sarecensis sp. nov., a psychrotolerant moderate halophile isolated from the saline Andean region of Bolivia

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A moderately halophilic, aerobic, motile, Gram-negative, rod-shaped bacterium (strain LV4^T) was isolated from saline soil around the lake Laguna Verde in the Bolivian Andes. The organism is a heterotroph, able to utilize various carbohydrates as a carbon source. It showed tryptophan deaminase, oxidase and catalase activity, but was unable to produce indole or H_2S; nitrate was not reduced. The G+C content of the genomic DNA was 56·1 mol%. The pH range for growth was 5–10, temperature range was 0–45 °C and the range of NaCl concentrations was 0–25 % (w/v). On the basis of 16S rRNA gene sequence analysis, strain LV4^T was found to be closely related to *Chromohalobacter canadensis* DSM 6769^T and *Pseudomonas beijerinckii* DSM 7218^T; however, its DNA–DNA relatedness with these type strains was low. Strain LV4^T resembled other *Chromohalobacter* species with respect to various physiological, biochemical and nutritional characteristics but also exhibited differences. Thus, a novel species, *Chromohalobacter sarecensis* sp. nov., is proposed, with LV4^T (=CCUG 47987^T=ATCC BAA-761^T) as the type strain.

Micro-organisms thriving in saline and hypersaline habitats are spread across the domains *Archaea*, *Eucarya* and *Bacteria* (Oren, 2002). The domain *Bacteria* comprises several representatives that form part of a large number of phylogenetic groups, with distinctive characteristics. The different branches of the *Proteobacteria* group have various halophilic representatives with close relatives that are non-halophilic (Oren, 2002). Among the bacterial families that form part of the largest subgroup, i.e. the *γ-Proteobacteria*, the family *Halomonadaceae* is characterized as being mainly represented by several halophilic and halotolerant species that belong to different genera. *Halomonas* is the largest genus in this family, containing more than 20 reported species, and is followed by the genus *Chromohalobacter*, which presently has four species: *Chromohalobacter canadensis* (Arahal *et al.*, 2001a), *Chromohalobacter israelensis* (Arahal *et al.*, 2001a), *Chromohalobacter marismortui* (Ventosa *et al.*, 1989) and *Chromohalobacter salexigens* (Arahal *et al.*, 2001b). All these species are moderately halophilic, aerobic, motile, Gram-negative, heterotrophic rods.

This report describes the phylogenetic and phenotypic characterization of an isolate, LV4^T, obtained from a soil sample collected around the saline lake Laguna Verde located in the south-western part of Bolivia (22° 47′ 16″ S, 0·67° 49′ 55·1″ W) at 4300 m above sea level. Laguna Verde has a typical green coloration (hence its name) owing to the high levels of magnesium in the water. The area around the lagoon is of volcanic origin with environmental temperatures ranging between −15 and 20 °C during the year.

The medium for isolation and maintenance of strain LV4^T was modified from that described by Ventosa *et al.* (1982), being composed of as follows (%, w/v): NaCl, 13·4; $MgSO_4.7H_2O$, 0·075; $CaCl_2.2H_2O$, 0·03; KCl, 0·15; $NaHCO_3$, 0·005; NaBr, 0·017; proteose-peptone (Difco), 0·5; yeast extract (Difco), 1·0; glucose, 0·1; and granulated agar, 2·0 for solid medium. The pH of the medium was adjusted to 7·5 using 3 M NaOH, to make it similar to that of the soil sample. The isolation procedure consisted

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene of strain LV4^T is AY373448.

Transmission electron micrographs of *C. sarecensis* (strain LV4^T), showing that some of the cells become longer and thinner with increasing size, are available as supplementary figures in IJSEM Online.

of mixing 500 mg soil sample with 0·5 ml medium using gentle vortexing, inoculating 0·2 ml suspension in a 250 ml Erlenmeyer flask containing 100 ml medium, then incubating this at 15 °C with agitation at 200 r.p.m. for 10 days. The enriched bacterial broth was diluted in the sterile liquid medium and then surface-inoculated on solid medium and incubated for 7 days at 18 °C. Finally, the microbial colonies were isolated, taking their morphological differences into consideration.

For taxonomic characterization, strain LV4^T was grown in a Casamino acids-based (CAS) medium supplemented with 8 % (w/v) NaCl (Vreeland, 1987) at 30 °C and pH 7·5 (adjusted after sterilization), unless otherwise stated. CAS medium was also previously utilized to characterize three of the species of the genus *Chromohalobacter* (Huval *et al.*, 1995, Vreeland *et al.*, 1980). Gram staining was performed using a Difco Gram stain set. Colony morphology was analysed according to Smibert & Krieg (1994), after growth for 30 h at 30 °C on CAS solid medium. Bacterial flagella were observed using a JEM-123 (HC) transmission electron microscope by using staining with 2 % uranyl acetate according to Vreeland *et al.* (1980). Cell size and morphology were examined from 30 h cultures of bacteria using a Nikon optiphot-2 microscope at ×1000 magnification. Cell morphology was also observed using a JSM-5600 LV scanning electron microscope (JEOL). For this purpose, cells of strain LV4^T were extracted from liquid cultures, washed twice with water and dehydrated through a graded series of ethanol and isopropyl alcohol aqueous solutions. Cells were then mounted on 12 mm cover-slips, dried in a vacuum desiccator overnight and then coated with gold/palladium (80 : 20, w/w).

Acid production by strain LV4^T from different carbon sources was analysed as reported by Smibert & Krieg (1994) in a medium containing 1 % (w/v) of a carbon source, 0·3 % (w/v) yeast extract and 9 % (w/v) sea salts (Sigma). Sugar utilization was determined for strain LV4^T and *C. canadensis* using the Biolog GN (for Gram-negative bacteria) MicroPlates system inoculated with 130 µl cell suspension per well. For this purpose, cells were grown in CAS medium for 30 h at 30 °C and suspended in sterile medium containing 5 % (w/v) sea salts (Sigma).

Hydrolysis of starch, Tween 80 and DNA was determined as described previously by Sánchez-Porro *et al.* (2003). Other biochemical characteristics were screened by using conventional methods according to Smibert & Krieg (1994). In all cases, 9 % (w/v) sea salts (Sigma) was supplied in the medium.

Growth at, and tolerance of, various salt concentrations, temperatures and pH values, respectively, were studied by culturing strain LV4^T in 12 ml CAS medium in 50 ml screw-capped bottles (shaken at 200 r.p.m.). For these studies, cells were grown in 0, 5, 8, 10, 15, 25 and 33 % (w/v) NaCl for 10 days, at 0, 4, 15, 25, 30, 35, 45 and 50 °C for 14 days, and at pH 4, 5, 6, 7, 7.5, 8, 9, 10 and 11 (adjusted with 2 M KOH or 2 M HCl) for 10 days. Optical density at 600 nm was monitored, using sterile CAS medium as the reference, with an Ultrospec 3000 spectrophotometer (Pharmacia Biotech). Slopes of optical density against salt concentration, temperature and pH were plotted and the optimal conditions for growth were determined.

Sensitivity to antibiotics was determined using the standard disc assay method (Smibert & Krieg, 1994) in CAS medium supplemented with 8 % (w/v) NaCl, as reported earlier (Huval *et al.*, 1995). The resistance and degree of sensitivity were determined by measuring the sizes of inhibition zones after 30 h incubation at 30 °C in the presence of different amounts of each antibiotic.

Genomic DNA was extracted and purified according to Arahal *et al.* (2002); its purity was assessed from the A_{260}/A_{280} and A_{260}/A_{230} ratios (Johnson, 1994). Universal primers 8-27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1422 (5′-GGTTACCTTGTTACGACTT-3′) were used to amplify the 16S rRNA gene (Weisburg *et al.*, 1991). The PCR products were purified using the QIAquick PCR purification kit (Qiagen) and then resuspended in a final volume of 40 µl. DNA sequencing on both strands was performed by the dideoxy chain-termination method with an ABI prism 3100 DNA analyser, using an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (PE Biosystems) according to the protocol provided by the manufacturer. GenBank and Ribosomal Database Project databases were used to search for 16S rRNA gene similarities (Maidak *et al.*, 2000). Phylogenetic analysis based on the 16S rRNA gene was performed with the aid of the DNAMAN 4.03 software package, using the neighbour-joining and Jukes–Cantor distance correction methods (Saitou & Nei, 1987). For constructing a phylogenetic tree, only sequences from the type strains of species whose names have been validly published were taken into account. An almost-complete sequence (1450 bp) of the 16S rRNA gene of strain LV4^T (GenBank/EMBL/DDBJ accession no. AY373448) was used in the analysis.

Genomic DNA G+C content and DNA–DNA hybridization between strain LV4^T and reference strains *C. canadensis* DSM 6769^T and *Pseudomonas beijerinckii* DSM 7218^T, respectively, were determined by the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). For these assays, DNA was isolated by chromatography on hydroxyapatite by the procedure of Cashion *et al.* (1977). DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970), with the modification described by Huss *et al.* (1983) and Escara & Hutton (1980), using a model 2600 spectrophotometer equipped with a model 2527-R thermoprogrammer and plotter (Gilford Instrument Laboratories). Renaturation rates were computed with the TRANSFER.BAS program of Jahnke (1992). For the determination of G+C content, DNA was hydrolysed with P1 nuclease and the nucleotides dephosphorylated with bovine alkaline phosphatase (Mesbah *et al.*, 1989). The resulting

deoxyribonucleosides were analysed by HPLC (Shimadzu equipment) using chromatography conditions adapted from those of Tamaoka & Komagata (1984). The G+C content was calculated from the ratio of deoxyguanosine (dG) and thymidine (dT) according to the method of Mesbah *et al.* (1989). For these experiments, strain LV4^T was grown in CAS medium; reference strains *C. canadensis* DSM 6769^T and *P. beijerinckii* DSM 7218^T were grown according to the conditions given by the DSMZ.

The results of 16S rRNA gene analysis are shown in Fig. 1. Strain LV4^T was seen to bear the closest affiliation to *C. canadensis* DSM 6769^T, with sequence similarity of the order of 98·5 %, which is within the mean range reported previously for the genus *Chromohalobacter* (Arahal *et al.*, 2002).

The next closest level of similarity shown by strain LV4^T was with *P. beijerinckii* DSM 7218^T (97·6 %), followed by other *Chromohalobacter* species. This observation seems to support an earlier suggestion that *P. beijerinckii* DSM 7218^T does not belong to the family *Pseudomonadaceae*, but is instead more closely related to the *Halomonadaceae* group (Kersters *et al.*, 1996; Anzai *et al.*, 2000). Because of the close phylogenetic relationship between *P. beijerinckii* and the genus *Chromohalobacter*, its position as another member of this genus should be considered.

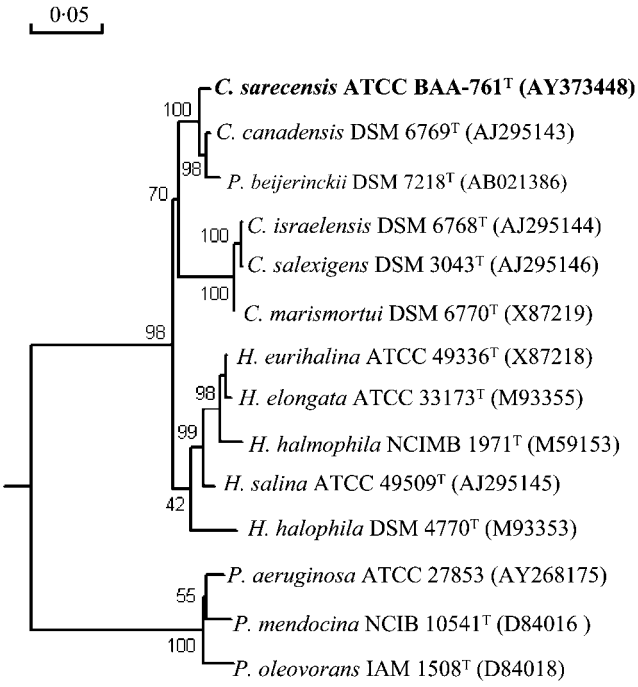

Fig. 1. Phylogenetic tree constructed using 16S rRNA gene sequences and including some members of the genus *Halomonas* and all species of the genus *Chromohalobacter*. *Pseudomonas aeruginosa* ATCC 27853, *Pseudomonas mendocina* NCIB 10541^T and *Pseudomonas oleovorans* IAM 1508^T were used as outgroup bacteria. *P. beijerinckii* DSM 7218^T shows a closer phylogenetic relationship to the genus *Chromohalobacter* than to the genus *Pseudomonas*. Bar, 5 substitutions per 100 nucleotides. Numbers at branching points refer to bootstrap values (100 resamplings).

Most members of the genus *Chromohalobacter* were previously considered to belong to the genus *Halomonas* (Huval *et al.*, 1995; Arahal *et al.*, 2001b), but a re-evaluation of the phylogeny of the species within the family *Halomonadaceae*, using comparative sequence analysis of the 16S rRNA gene, clustered the *Chromohalobacter* species in a separate branch from the genus *Halomonas* (Arahal *et al.*, 2002). In the phylogenetic tree constructed for this report, strain LV4^T was grouped together with *C. canadensis* DSM 6769^T and *P. beijerinckii* DSM 7218^T (Fig. 1), while the *Halomonas* species having a close phylogenetic affiliation with the genus *Chromohalobacter* (Arahal *et al.*, 2002) clustered in a separate group.

Table 1 provides a comparison of the taxonomic features of strain LV4^T with those of other *Chromohalobacter* species. Cells of strain LV4^T are aerobic and motile by means of a polar flagellum. Moreover, strain LV4^T is a Gram-negative, rod-shaped organism and the cells occur singly or occasionally in pairs. Strain LV4^T cells exhibit a wide range of sizes during the exponential phase of growth, ranging from short rods with a coccoid form to sporadically elongated cells that become thinner with increasing size (see supplementary figures in IJSEM Online). With respect to its nutritional and biochemical characteristics, strain LV4^T showed some similarity to the reference *Chromohalobacter* species, but differences were also noted. Like the other *Chromohalobacter* species, strain LV4^T is a heterotrophic bacterium able to assimilate a diverse range of carbon sources; however, the substrates it can assimilate differ from those assimilated by *C. canadensis* (Table 1). The ability of some *Chromohalobacter* species to reduce nitrate was not found in strain LV4^T (Table 1). Furthermore, resistance to lysis in water, the absence of indole production and the presence of oxidase activity are other features that differentiate strain LV4^T from *C. canadensis* DSM 6769^T (Table 1).

Strain LV4^T cells were able to grow at NaCl concentrations of 0–25 % (w/v), with optimal growth at 8 % (w/v) NaCl: it can therefore be considered as moderately halophilic (Ventosa *et al.*, 1998). In contrast to *C. canadensis* DSM 6769^T, strain LV4^T does not require added NaCl to grow in CAS medium (Huval *et al.*, 1995) (Table 1). Although the composition of the medium may influence the salt tolerance and requirement in halophilic bacteria (Ventosa *et al.*, 1998), growth without NaCl is a characteristic of strain LV4^T not shared with *C. canadensis* under the same culture conditions. Strain LV4^T grows at temperatures from 0 °C (the lowest temperature tested) up to 45 °C and exhibits optimal growth between 30 and 35 °C: thus it can be classified as psychrotolerant according to the definition by Morita (1975). A minimum growth temperature of 4 °C was determined previously for *C. marismortui* and

Table 1. Comparison of characteristics of strain LV4^T with those of type strains of the genus *Chromohalobacter*

Strains: 1, LV4^T; 2, *C. canadensis* DSM 6769^T; 3, *C. marismortui* DSM 6770^T; 4, *C. israelensis* DSM 6768^T; 5, *C. salexigens* DSM 3043^T. Characteristics are scored as follows: +, positive; −, negative; NR, not reported. All strains are Gram-negative, motile, rod-shaped, able to utilize lactose, unable to liquefy gelatin and unable to hydrolyse starch or Tween 80. All the colonies, corresponding to each strain, have entire margins. Data for reference strains were obtained from Arahal *et al.* (2001a, b; 2002), Huval *et al.* (1995), Ventosa *et al.* (1989), Vreeland *et al.* (1980) and this work.

Characteristic	1	2	3	4	5
Cell shape					
Straight rods	+	+	+	+	−
Motility	+	+	+	+	+
Length (μm)	0·8–6·1	2·0–3·8	1·5–4·0	1·5–4·0	2·0–3·0
Width (μm)	0·5–0·8	0·6–1·2	0·6–1·0	0·6–1·0	0·7–1·0
Colony characteristics					
Form	Convex	Convex	Convex	Flat	NR
Smooth surface	+	+	+	−	+
Glistening surface	+	+	NR	−	+
Pigmentation	Brown	White	Brown–yellow	Cream	White–cream
Biochemical characteristics					
NO_3^- reduced to NO_2^-	−	+	−	+	+
Indole production	−	+	−	+	−
Urease activity	−	−	−	−	+
H_2S production	−	−	−	−	+
Oxidase	+	−	−	−	−
Lysis in distilled water	−	+	NR	−	NR
Carbon source utilization					
Trehalose	+	−	+	−	+
Maltose	−	−	+	+	+
Ribose	+	−	+	NR	+
myo-Inositol	−	+	+	NR	+
Xylitol	+	−	NR	NR	NR
L-Alaninamide	+	−	NR	NR	NR
L-Asparagine	+	−	NR	NR	NR
L-Histidine	+	−	NR	NR	NR
L-Threonine	−	+	−	NR	−
Urocanic acid	−	+	NR	NR	NR
Uridine	−	+	NR	NR	NR
Thymidine	−	+	NR	NR	NR
Putrescine	−	+	+	NR	−
Salt, temperature and pH tolerance					
NaCl concn range (%, w/v)	0–25	3–25	2–30	3·5–20	0·9–25
Optimum NaCl concn (%, w/v)	8	8	10	8	7·5–10
Temperature range (°C)	0–45	15–45	4–45	15–45	4–45
Optimum temperature (°C)	30–35	30	37	30	37
pH range	5–10	5–9	5–10	5–9	5–10
Optimum pH	7·5–8·0	NR	7·5	NR	7·5
DNA G+C content (mol%)	56·1	55–57/62*	62·3	65	64·2

*Different values for DNA G+C content have been published in two different reports (Huval *et al.*, 1995; Arahal *et al.*, 2001a, b).

C. salexigens (Table 1); only slight growth was reported for the latter organism at that temperature (Vreeland *et al.*, 1980). Cell growth was observed over a broad range of pH values (5–10), with optimum growth occurring at pH 7·5–8·0.

The growth of strain LV4^T cells was inhibited by ampicillin, chloramphenicol and tetracycline, in all cases at a minimum level of 10 μg. However, the organism did not show susceptibility to penicillin G (50 IU), streptomycin (50 μg) or kanamycin (80 μg).

Analysis of strain LV4^T genomic DNA revealed its G+C content to be 56·1 mol%, differing significantly from that of the other *Chromohalobacter* species (Table 1). Nevertheless, it should be pointed out that different values for the G+C content of *C. canadensis* have been reported in different studies (Huval *et al.*, 1995; Arahal *et al.*, 2001a). The DNA–DNA relatedness values obtained for strain LV4^T with respect to *C. canadensis* DSM 6769^T and *P. beijerinckii* DSM 7218^T were 47·9 and 32·8 %, respectively. These DNA similarities are lower than the recommended value of at least 70 % accepted as the definition of a novel species (Wayne *et al.*, 1987).

Because of the taxonomic differences listed, the marked difference in the G+C content, the low DNA–DNA hybridization values for strain LV4^T with respect to the species showing the closest 16S rRNA gene similarity, and the novel characteristics shown by strain LV4^T, we propose that this strain represents a novel member of the genus *Chromohalobacter*, with the name *Chromohalobacter sarecensis* sp. nov.

Description of *Chromohalobacter sarecensis* sp. nov.

Chromohalobacter sarecensis (sa.re.cen′sis. N.L. masc. adj. relating to Sida/SAREC, the institution that supports scientific research in Bolivia).

Aerobic, Gram-negative and motile by means of a polar flagellum. The DNA G+C content is 56·1 mol%. Cells are rod-shaped and 0·5×0·8 to 0·8×6·1 μm in size. Cells occur singly or in pairs and show a wide distribution of sizes during the exponential phase of growth. Colonies are circular with entire margins, convex, smooth with a glistening surface and have a translucent to brown pigment, which is enhanced in old cultures. Acid is produced in medium supplied with fructose, xylose, glucose, rhamnose, galactose or glycerol, but acidification is not detected with trehalose, sucrose, citrate or raffinose. Heterotrophic and able to oxidize L-arabinose, D-arabitol, iso-erythritol, D-galactose, D-mannitol, D-mannose, D-psicose, raffinose, rhamnose, D-sorbitol, xylitol, lactose, cellobiose, glucose, ribose, D-xylose, melibiose, acetic acid, *cis*-aconitic acid, citric acid, formic acid, D-galactonic acid, D-gluconic acid, DL-lactic acid, succinic acid, bromosuccinic acid, L-alanine, D-alanine, L-alaninamide, L-alanylglycine, L-asparagine, L-aspartic acid, L-proline, L-pyroglutamic acid, L-serine and glycerol. Cells are unable to metabolize α-cyclodextrin, dextrin, glycogen, adonitol, L-fucose, gentibiose, *myo*-inositol, turanose, pyruvic acid methyl ester, D-galacturonic acid, D-glucosaminic acid, D-glucuronic acid, itaconic acid, α-ketoglutaric acid, malonic acid, quinic acid, D-saccharic acid, sebacic acid, succinamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, L-phenylalanine, D-serine, L-threonine, urocanic acid, inosine, uridine, thymidine, putrescine, 2-aminoethanol or 2,3-butanediol. Catalase-, oxidase- and tryptophan deaminase-positive. Indole and sulfide are not produced, nitrate is not reduced, caseinase, DNase and urease are not present and gelatin is not liquefied. Tests for hydrolysis of Tween 40, Tween 80, starch and aesculin are negative. Moderately halophilic and psychrotolerant bacterium: grows at 0–25 % (w/v) NaCl in CAS medium, with the optimum at 8 % (w/v) NaCl; grows at 0–45 °C, with the optimum between 30 and 35 °C; grows at pH 5–10, with the optimum between pH 7·5 and 8·0.

The type strain is LV4^T (=CCUG 47987^T=ATCC BAA-761^T).

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