

Halomonas boliviensis sp. nov., an alkalitolerant, moderate halophile isolated from soil around a Bolivian hypersaline lake

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Halomonas boliviensis sp. nov. is proposed for two moderately halophilic, psychrophilic, alkalitolerant bacteria, LC1^T (=DSM 15516^T = ATCC BAA-759^T) and LC2 (=DSM 15517 = ATCC BAA-760), both of which were isolated from a soil sample around the lake Laguna Colorada, located at 4300 m above sea level in the south-west region of Bolivia. The bacteria are aerobic, motile, Gram-negative rods that produce colonies with a cream pigment. Moreover, they are heterotrophs that are able to utilize various carbohydrates as carbon sources. The organisms reduce nitrate and show tryptophan deaminase activity. The genomic DNA G + C contents were 51.4 mol% for isolate LC1^T and 52.6 mol% for isolate LC2. Based on 16S rDNA sequence analysis, isolates LC1^T and LC2 were identified as members of the genus *Halomonas* and clustered closely with *Halomonas variabilis* DSM 3051^T and *Halomonas meridiana* DSM 5425^T. However, DNA–DNA relatedness between the new isolates and the closest related *Halomonas* species was low.

During the last decade, extensive studies of hypersaline environments in different geographical locations have led to the isolation and characterization of a large number of moderately halophilic species (Ventosa *et al.*, 1998; Oren, 2002).

Many Gram-negative, halotolerant or halophilic species are currently included in the family *Halomonadaceae*, which belongs to the γ -subclass of the *Proteobacteria*. Among the genera that comprise this family, *Halomonas* covers the major number of species (>20) with heterogeneous features and has recently been distinguished into two phylogenetic groups, based on 16S and 23S rDNA sequences

of the different species (Arahal *et al.*, 2002). It has also been noted that the genus *Halomonas* has an unusually wide range of DNA G + C content, of about 52–68 mol% (Arahal *et al.*, 2002). We have isolated a number of moderately halophilic organisms from the south-west region of Bolivia, which lies between the eastern and western ranges of the South American Andes. Much of this region lies >4000 m above sea level, allowing the survival of limited and unique flora and fauna. A number of lakes that have been formed because of evaporation exceeding rainfall and the presence of undrained, intravolcanic basins (Risacher, 1992) contain salt concentrations in the range of 10–103 g l⁻¹ (Hurlbert & Chang, 1988).

This study describes the characterization and identification of two strains, LC1^T and LC2, that belong to the genus *Halomonas* and were isolated from a soil sample around Laguna Colorada (red-coloured lake), a large, shallow, hypersaline lagoon that is located 4300 m above sea level (22° 13' 7" S 67° 48' 28" E) (Jones, 1993). The name of the lake is attributed to the red colour provided by algal communities and halophilic micro-organisms that are present in the waters. The area is of volcanic origin and the lagoon is partly fed by geothermal springs. Other sources of

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The GenBank/EMBL/DDBJ accession numbers for the 16S rDNA sequences of isolates LC1^T and LC2 are AY245449 and AY245450, respectively.

A table showing differential characteristics of *H. boliviensis* from other *Halomonas* species and micrographs of *H. boliviensis* strains are available as supplementary material in IJSEM Online.

water include the inflow of two rivers, Río Sulor and Río Aguaditas. The lagoon is surrounded by thin, sandy, volcanic soils with high concentrations of sodium, potassium, magnesium, boron, arsenic, lead, borax, gypsum, sulphates and phosphates (Jones, 1993). The environmental temperature varies between -15 and 20 °C during the year.

The medium used for isolation and maintenance of the bacteria was that described by Ventosa *et al.* (1982). The medium contained (% w/v): NaCl, 17.8; MgSO₄·7H₂O, 0.1; CaCl₂·2H₂O, 0.036; KCl, 0.2; NaHCO₃, 0.006; NaBr, 0.023; proteose/peptone (Difco), 0.5; yeast extract (Difco), 1.0; glucose, 0.1; and (for solid medium) granulated agar, 2.0. The pH was adjusted to 7.5 by using 3 M NaOH, to make it similar to that of the soil sample. The isolation procedure consisted of mixing about 500 mg soil sample from the shore of Laguna Colorada, Bolivia, with 0.5 ml medium by gentle vortexing, inoculating 0.2 ml mixture into a 250 ml Erlenmeyer flask that contained 100 ml medium and incubating for 12 days at 15 °C with shaking at 200 r.p.m. The enriched bacterial medium was diluted by using sterile liquid medium and then surface-inoculated onto solid agar medium for 10 days further incubation at 18 °C. Colonies were isolated, taking their morphological differences into consideration.

The medium (HM) for bacterial growth and characterization was based on the isolation medium and had the following composition (% w/v): NaCl, 4.45; MgSO₄·7H₂O, 0.025; CaCl₂·2H₂O, 0.009; KCl, 0.05; NaBr, 0.006; proteose/peptone (Difco), 0.5; yeast extract (Difco), 1.0; glucose, 0.1; and (for solid medium) granulated agar, 2.0%; the pH was adjusted to 7.5 by using 1 M KOH.

Cells of strains LC1^T and LC2 were grown in HM medium at 25 °C and pH 7.5 unless otherwise stated. Cell size and morphology were examined from 20 h bacterial cultures by using a Nikon Optiphot-2 phase-contrast microscope at magnification $\times 1000$. Gram staining was performed by using a Difco Gram stain set.

Colony morphology was analysed after growth for 30 h at 30 °C on solid medium, according to Smibert & Krieg (1994). Bacterial flagella were observed by using a JEM-123 (HC) transmission electron microscope after staining with 2% uranyl acetate, according to Vreeland *et al.* (1980). Cells were also observed under a JSM-5600 LV scanning electron microscope (JEOL). For this, cells were harvested from liquid cultures during their exponential-growth phase, 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 overnight in a vacuum desiccator and then coated with gold/palladium (80:20).

Sugar assimilation was determined by using the API 50 CHB system (bioMérieux). Other biochemical characteristics were screened by the API 20E system, according to Logan & Berkeley (1984).

Growth at and tolerance to salt concentration, temperature and pH of isolates LC1^T and LC2 were studied in 12 ml HM medium in 50 ml screw-capped bottles. For these studies, cells were respectively grown at 0, 5, 10, 15, 25 and 30% (w/v) NaCl for 10 days; at temperatures of 0, 4, 15, 25, 35, 45 and 50 °C for 14 days; and at initial pH values of 4, 5, 6, 7, 8, 9, 10 and 11 (adjusted with 2 M KOH or 2 M HCl) for 10 days. OD₆₀₀ was measured in an Ultrospec 3000 spectrophotometer (Pharmacia Biotech) by using sterile medium as the reference. Slopes of OD₆₀₀ versus salt concentration, temperature and pH were plotted for determination of optimal conditions for growth.

Sensitivity to antibiotics was determined by the standard disc assay method, according to Smibert & Krieg (1994). Resistance and degree of sensitivity were determined by measuring the size of inhibition zones from different amounts of each antibiotic after 24 h incubation at 30 °C.

Genomic DNA was extracted and purified according to Marmur (1961) and its purity was assessed from A₂₆₀/A₂₈₀ and A₂₆₀/A₂₃₀ ratios (Johnson, 1994). Universal primers that corresponded to positions 8–27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1422R (5'-GGTTACCTTGTTACGACTT-3') were used to amplify the 16S rDNA region of isolates LC1^T and LC2 (Weisburg *et al.*, 1991). PCR products were purified by using a QIAquick PCR Purification kit (Qiagen) and were then resuspended in 40 µl sterile water. DNA sequencing of 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 supplied by the manufacturer. GenBank and the RDP database were used to search for 16S rDNA sequence similarities (Maidak *et al.*, 2000). Phylogenetic analysis of 16S rDNA sequences was performed with the aid of the MEGA2 software package (Kumar *et al.*, 2001) by using the neighbour-joining method (Saitou & Nei, 1987). For phylogenetic trees, only sequences from the type strains of species whose names have been validly published were taken into account. Almost-complete 16S rDNA sequences (about 1460 bp) of isolates LC1^T and LC2 were used in the analysis.

Genomic DNA G+C content was determined and DNA–DNA hybridization was performed by Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). For these assays, isolates LC1^T and LC2 were grown in HM medium and reference strains *Halomonas variabilis* DSM 3051^T and *Halomonas meridiana* DSM 5425^T were grown according to the conditions given by DSMZ.

Among the strains isolated from the Laguna Colorada soil sample, two isolates, namely LC1^T and LC2, were placed within the genus *Halomonas* by their 16S rDNA sequence (Fig. 1). 16S rDNA sequence similarity between LC1^T and LC2 was 99.7%. They were placed in a parallel branch with *H. variabilis* DSM 3051^T (with 98.7% similarity) and

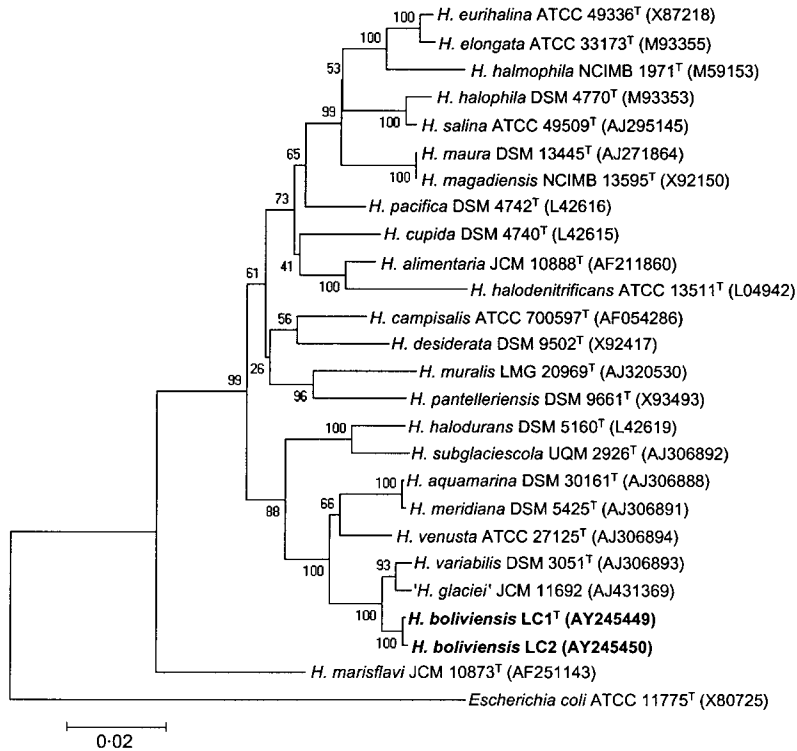


Fig. 1. Phylogenetic tree constructed from comparison of the 16S rDNA sequences of isolates LC1^T and LC2 with those of members of the genus *Halomonas*. GenBank accession numbers of the type strains used in the phylogenetic analysis are given in parentheses. *Escherichia coli* was taken as the outgroup. Bar, 2 substitutions in 100 nt.

with *H. meridiana* DSM 5425^T, with which they displayed 97 % similarity.

Several morphological and taxonomic features of isolates LC1^T and LC2 were investigated and compared with those of *H. variabilis* and *H. meridiana* (see Supplementary Table, available in IJSEM Online). In this comparison, '*Halomonas glaciei*' MTCC 4321, a novel species that was reported recently (Reddy *et al.*, 2003), was also added. Furthermore, type strains of the genus *Halomonas* that belong to other clusters were also included.

In previous studies, Tindall (1994) and Baumgarte *et al.* (2001) showed that biochemical and chemical data sometimes contradicted the conclusion derived from 16S rDNA sequences of the organisms. In the present work, the Supplementary Table (available in IJSEM Online) shows several taxonomic and physiological differences of isolates LC1^T and LC2 from the closest related species, *H. variabilis*. Indeed, there are more differences than similarities, although the new isolates shared some characteristics with *H. meridiana*, such as their wide range of salt tolerance and growth temperature.

Isolates LC1^T and LC2 were motile by means of lophotrichous flagella (Supplementary Fig. A, available in IJSEM Online) and were Gram-negative, rod-shaped organisms that were found both singly and in pairs [Supplementary Figs B and C (left), available in IJSEM Online] and occasionally as cellular chains of three bacteria during their exponential phase. Cells exhibited varying lengths during

the exponential phase [Figs B and C (right), available in IJSEM Online]; however, they became shorter and more uniformly long when reaching the end of their exponential phase. Furthermore, both isolates showed binary fission in short cells [Supplementary Fig. C (left), available in IJSEM Online], whereas apical and transverse fission was observed in elongated cells, as a result of cell division (Supplementary Fig. B, available in IJSEM Online). Two principal differences were seen in the cellular morphology of the two isolates: cells of strain LC2 were longer than those of LC1^T (see Supplementary Table in IJSEM Online) and their shape became slightly curved in short rods, whereas isolate LC1^T maintained a straight rod shape during its exponential-growth phase.

Both isolates showed similar tolerance and optimal growth conditions with respect to pH, temperature and salinity. The organisms grew optimally at an NaCl concentration of 5 % (w/v) and tolerated up to 25 % (w/v) NaCl, hence they can be considered as moderately halophilic bacteria (Ventosa *et al.*, 1998). These characteristics were in contrast to those of *H. variabilis* and *H. meridiana* (see Supplementary Table in IJSEM Online). Nevertheless, salt requirement and tolerance may vary according to growth temperature and nature of nutrients available (Ventosa *et al.*, 1998). Optimum temperature for growth was about 25–30 °C; the isolates were able to grow at 0 °C (the lowest temperature tested) and up to 45 °C and could be classified as psychrophiles [according to the definition of Morita (1975)], whereas *H. variabilis* is a mesophilic bacterium

that can only grow at 15–37 °C (Fendrich, 1988). Finally, cells were able to grow in a broad pH range of 6–11 with an optimal pH for growth between 7.5 and 8, and can therefore be considered to be alkalitolerant (like other *Halomonas* species), although with varying pH limits for growth (see Supplementary Table in IJSEM Online).

Strains LC1^T and LC2 utilized a variety of carbohydrates as carbon sources, the latter organism exhibiting wider versatility in choice of substrate. The heterotrophic behaviour and ability of the isolates to utilize different kinds of carbohydrates was similar to that of *H. meridiana*, but differed from *H. variabilis*, which is known for being chemo-organotrophic and for its inability to oxidize carbohydrates (Fendrich, 1988). Even '*H. glaciei*' strains have shown a rather restricted ability for utilization of different carbon sources (Reddy *et al.*, 2003).

Isolates LC1^T and LC2 were comparable with respect to their biochemical characteristics. Some of these characteristics were shared with reference *Halomonas* species, but differences were also noted (see Supplementary Table in IJSEM Online). The attribute that differentiated isolates LC1^T and LC2 from *H. meridiana*, *H. variabilis* and '*H. glaciei*' was their ability to reduce nitrate (see Supplementary Table in IJSEM Online). Furthermore, the presence of urease, which was observed in *H. meridiana* and *H. variabilis*, was not found in cells of strain LC1^T or strain LC2. Many members of the genus *Halomonas* can use nitrate as an alternative electron acceptor (Ventosa *et al.*, 1998). Some type strains that are able to reduce nitrate are included in the Supplementary Table (available in IJSEM Online); they did not, however, bear other significant phenotypic resemblance to isolates LC1^T and LC2.

The DNA G+C contents of isolates LC1^T and LC2 (51.4 and 52.6 mol%, respectively) differed significantly from those of *H. variabilis*, *H. meridiana* and '*H. glaciei*', which have DNA G+C contents of 61, 59 and 57 mol%, respectively. DNA–DNA relatedness between isolates LC1^T and LC2 was 80.4%. DNA–DNA hybridization of the isolates with *H. variabilis* and *H. meridiana* gave 25.4 and 22.8% similarity respectively for isolate LC1^T and 29.7 and 21.4% similarity respectively for isolate LC2. DNA–DNA similarities between the new isolates and reference bacteria were significantly lower than the recommended value of ≥70%, which is accepted as the definition of a novel species (Wayne *et al.*, 1987).

Based on DNA–DNA relatedness shared between isolates LC1^T and LC2, it can be concluded that these are different strains that belong to the same species. Moreover, because of low DNA–DNA hybridization similarities, marked difference in DNA G+C content from the *Halomonas* species with the highest 16S rDNA sequence similarity and the new characteristics shown, we conclude that isolates LC1^T and LC2 constitute a novel species of the genus *Halomonas*, for which the name *Halomonas boliviensis* sp. nov. is proposed.

Description of *Halomonas boliviensis* sp. nov.

Halomonas boliviensis (bo.li.vi.en'sis. N.L. adj. *boliviensis* from Bolivia, relating to the country where the bacteria were isolated).

Aerobic, Gram-negative rods with a size of 0.4 × 1.1–0.6 × 3.4 μm for isolate LC1^T and 0.4 × 1.1–0.5 × 5.5 μm for isolate LC2. Motile by means of lophotrichous flagella. Cells occur singly or in pairs and show a wide size distribution during the exponential phase, but uniform size at the end of their growth. Colonies are circular with undulate margins, convex and have cream pigmentation that is enhanced in old cultures. Moderately halophilic, alkalitolerant and psychrophilic. Growth occurs at 0–25% (w/v) NaCl in complex medium, with an optimum of about 5% (w/v) NaCl. Growth occurs at pH 6–11, with an optimum between 7.5 and 8. Growth occurs at 0–45 °C, with an optimum between 25 and 30 °C. Heterotrophic cells that are able to utilize various carbohydrates as carbon sources. Nitrate reduction- and tryptophan deaminase-positive. No production of indole or sulphide occurs. Arginine dihydrolase-, β-galactosidase-, lysine decarboxylase-, ornithine decarboxylase- and Voges–Proskauer-negative. Gelatin is not liquefied. DNA G+C content is 51.4 mol% for strain LC1^T and 52.6 mol% for strain LC2. Both isolates are sensitive to erythromycin, tetracycline, kanamycin and carbenicillin; isolate LC1^T is also sensitive to chloramphenicol and nalidixic acid.

The type strain is LC1^T (=DSM 15516^T=ATCC BAA-759^T). Reference strain is LC2 (=DSM 15517=ATCC BAA-760).

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