**Halomonas andesensis** sp. nov., a moderate halophile isolated from the saline lake Laguna Colorada in Bolivia

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A moderately halophilic, motile, Gram-negative, rod-shaped bacterium, strain LC6T, was isolated from a water sample of lake Laguna Colorada in the Bolivian Andes. The major cellular fatty acids were C18:1\(\Delta^7\)c, iso-C16:1\(\Delta^7\)c 2-OH, C16:0 and C12:0 3-OH. The respiratory ubiquinones found in strain LC6T were Q-9 (97 %) and Q-8 (3 %). Strain LC6T was aerobic, heterotrophic, and able to utilize various carbohydrates and other substrates as carbon source. The G+C content of the genomic DNA of strain LC6T was 52.5 mol%. The organism was able to grow at pH 6.0–11.0 (optimum, pH 7.0–8.0), at 4–45 °C (optimum, 30–35 °C) and in the presence of 0.5–20 % (w/v) NaCl (optimum, 1–3 %, w/v). Based on 16S rRNA gene sequence analysis, strain LC6T was most closely related to *Halomonas hydrothermalis* DSM 15725T and *Halomonas venusta* DSM 4743T (98.8 % similarity), followed by *Halomonas axioides* DSM 15723T and *Halomonas meridiana* DSM 5425T (98.4 %). However, levels of DNA–DNA relatedness between strain LC6T and the above type strains were low (<31 %). Strain LC6T resembled recognized *Halomonas* species with respect to various physiological, biochemical and nutritional characteristics. Combined phenotypic data and DNA–DNA hybridization data supported the conclusion that strain LC6T represents a novel species of the genus *Halomonas*, for which the name *Halomonas andesensis* is proposed. The type strain is LC6T (=CCUG 54844T=LMG 24243T=DSM 19434T).

Micro-organisms requiring salt for growth, the halophiles, are found among all the domains of life, the *Archaea*, *Eucarya* and *Bacteria* (Oren, 2002). The different branches of the phylum *Proteobacteria* have various halophilic representatives with close relatives that are non-halophilic (Oren, 2002). Among the bacterial families that form part of the class *Gammaproteobacteria*, the family *Halomonadaceae* is mainly represented by halophilic and halotolerant species belonging to different genera. *Halomonas* is the largest genus in this family with, at the time of writing, more than 50 recognized species. Identification of *Halomonas* species requires data from polyphasic studies owing to the phenotypic heterogeneity of the genus.

This paper describes the phylogenetic and phenotypic characterization of a moderate halophile, strain LC6T, isolated from a liquid sample collected from the saline lake Laguna Colorada located in the south-western part of Bolivia (22° 12’ S 67° 49’ W) at 4300 m above sea level. Laguna Colorada owes its name to the bright red coloration provided by algae and halophilic micro-organisms.

Strain LC6T was isolated by using HM medium (Quillaguaman et al., 2004), based on a medium described by Ventosa et al. (1982), containing 5 % (w/v) NaCl. The isolation procedure consisted of suspending 1 ml lake water into a 250-ml Erlenmeyer flask containing 100 ml medium and incubating for 2 days at 30 °C with shaking at 200 r.p.m. The enriched bacterial medium was diluted (104 fold) with sterile liquid medium, and then surface-inoculated onto solid HM medium with 2 % (w/v) agar and incubated for 3 days at 30 °C.

HM medium was used for bacterial growth and characterization unless indicated otherwise. Reference strains *Halomonas venusta* DSM 4743T, *Halomonas meridiana* DSM 5425T,
Halomonas aquamarina DSM 30161T, Halomonas axialensis DSM 15723T and Halomonas hydrothermalis DSM 15725T were cultured under similar conditions as those used for characterization of strain LC6T.

Growth and tolerance to salt concentration, temperature and pH were studied by culturing strain LC6T in 12 ml HM medium in 50-ml screw-capped bottles (with shaking at 200 r.p.m.). For these studies, cells were grown in the presence of 0, 0.5, 1, 2, 3, 5, 8, 15, 20, 25 and 30 % (w/v) NaCl for 10 days at pH 7.5 and 30 °C; at 0, 4, 25, 30, 35, 45 and 50 °C for 14 days at pH 7.5 and with 3 % (w/v) NaCl; and at pH 4, 5, 6, 7, 8, 9, 10 and 11 (adjusted with 5 M NaOH or 2 M HCl) for 10 days at 30 °C and with 3 % (w/v) NaCl. Growth rates were determined by monitoring the optical density (OD) of the culture broth at 600 nm during cultivation compared with sterile HM medium as the reference by using an Ultrospec 3000 spectrophotometer (Pharmacia Biotech). OD values were plotted to determine optimal conditions for growth.

Cell size and morphology were examined from bacterial cultures grown for 20 h by using a Nikon Optiphot-2 phase-contrast microscope at 1000× magnification. Gram staining was performed by using a Difco Gram stain set.

Acid production by strain LC6T from different carbon staining was performed by using a Difco Gram stain set. Characterization of strain LC6T.

Growth of strain LC6T and the five reference Halomonas strains on different carbon sources was analysed as described by Smibert & Krieg (1994) in medium containing 1 % (w/v) carbon source, 0.3 % (w/v) yeast extract and 9 % (w/v) sea salts (Sigma) (Sánchez-Porro et al., 2003).

Growth of strain LC6T and the five reference Halomonas strains on different carbon sources was analysed in medium containing (w/v): 0.01 % yeast extract, 3.0 % NaCl, 0.025 % MgSO4•7H2O, 0.009 % CaCl2•2H2O, 0.05 % KCl, 0.006 % NaBr and 0.5 % carbon source. As a reference, each strain was grown in the same medium without carbon source.

Hydrolysis of gelatin, casein, starch, Tween 80 and DNA was determined as described 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 added to the medium.

Susceptibility to antibiotics was determined by using the standard disc assay method (Smibert & Krieg, 1994). Resistance and degree of susceptibility were determined by measuring the size of inhibition zones after 30 h incubation in HM medium containing 3 % (w/v) NaCl at 30 °C in the presence of different concentrations of the various antibiotics.

Colony morphology was analysed according to Smibert & Krieg (1994) after growth for 30 h at 30 °C on solid HM medium. Bacterial flagella were observed by using a JEM-123 (HC) transmission electron microscope after staining with 2 % (v/v) uranyl acetate, according to Vreeland et al. (1980).

Genomic DNA was extracted and purified according to Arahal et al. (2002), and its purity was assessed from A260/A280 and A260/A230 ratios (Johnson, 1994). The 16S rRNA gene of strain LC6T was amplified, purified and sequenced as described by Quillaguamán et al. (2004). The GenBank and RDP databases were used to search for similar 16S rRNA gene sequences (Maidak et al., 2000). Phylogenetic analysis based on 16S rRNA gene sequences was performed via the MEGA2 software package (Kumar et al., 2001). For tree construction, only sequences from the type strains of recognized species were taken into account. The 16S rRNA gene sequence of strain LC6T comprised 1408 bp.

Determination of DNA G+C content and DNA–DNA hybridization experiments were performed by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). For determination of the DNA G+C content, DNA of strain LC6T was hydrolysed with P1 nuclease and nucleotides were dephosphorylated with bovine alkaline phosphatase (Mesbah et al., 1989). The resulting deoxyribonucleosides were analysed by HPLC (Shimadzu) with chromatography conditions adapted from Tamaoka & Komagata (1984). The DNA G+C content was calculated from the ratio of deoxyguanosine (dG) and deoxythymidine (dT) according to the method of Mesbah et al. (1989). For these experiments, strain LC6T was grown in HM medium containing 3 % (w/v) NaCl. DNA–DNA hybridization experiments between strain LC6T and the five Halomonas reference strains were performed. The organisms were grown in HM medium. DNA was isolated by chromatography on hydroxyapatite according to the procedure of Cashion et al. (1997). DNA–DNA hybridization was carried out as described by De Ley et al. (1970) with the modifications described by Huß et al. (1983) and Escara & Hutton (1980) by 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 Jahnine (1992).

The cellular fatty acids of strain LC6T were analysed by GC at the Belgian Coordinated Collections of Microorganisms (BCCM) after growth at 28 °C on tryptcase soy agar for 24 h. Inoculation and harvesting of cells were performed according to the recommendations of the commercial identification MIDI system (Microbial Identification System, Inc.), except that cells were harvested from the whole plate (QALL) and from three plates (3PL) to obtain a sufficient concentration of fatty acids in the extract. Extraction and analysis were performed according to recommendations provided with the MIDI system. Respiratory quinone analysis was carried out using HPLC by the Indentification Service and Dr B. J. Tindall, DSMZ, Braunschweig, Germany.

Phylogenetic analysis with the neighbour-joining method based on 16S rRNA gene sequences placed strain LC6T into the Halomonadaceae.
within the genus *Halomonas* (Fig. 1). Strain LC6<sup>T</sup> was related most closely to *H. hydrothermalis* DSM 15725<sup>T</sup> and *H. venusta* DSM 4743<sup>T</sup> (98.8 % 16S rRNA gene sequence similarity), followed by *H. aquamarina* DSM 30161<sup>T</sup>, *H. axialensis* DSM 15723<sup>T</sup> and *H. meridiana* DSM 5425<sup>T</sup> (98.4 %). Phylogenetic trees based on the maximum-parsimony and minimum-evolution methods showed a similar relationship between strain LC6<sup>T</sup> and the *Halomonas* species examined in Fig. 1 (see Supplementary Fig. S1a and b in IJSEM Online).

Cells of strain LC6<sup>T</sup> were motile by means of lophotrichous flagella. Cells were Gram-negative rods that divided in duplets during the exponential phase of growth. Colonies of strain LC6<sup>T</sup> were circular.

Strain LC6<sup>T</sup> was able to grow in the presence of 0.5–20 % (w/v) NaCl (optimal growth at 1–3 %, w/v). It grew at temperatures from 4 °C (the lowest temperature tested was 0 °C) to 45 °C (optimal growth at 30–35 °C). A minimum growth temperature of 4 °C has been reported for *H. meridiana*, *H. venusta* and *H. hydrothermalis* (Table 1). Strain LC6<sup>T</sup> was able to grow over a broad pH range (Table 1).

Table 1 provides a comparison of various features of strain LC6<sup>T</sup> with phylogenetically most closely related *Halomonas* species. Strain LC6<sup>T</sup> was aerobic and heterotrophic and was able to assimilate a diverse range of carbon sources (as with the other *Halomonas* species investigated); however, strain LC6<sup>T</sup> showed various differences from the *Halomonas* reference strains with respect to certain nutritional and biochemical characteristics (Table 1).

Strain LC6<sup>T</sup> was unable to grow in minimal medium containing a single defined carbon source. It was therefore not possible to characterize this novel strain by using kits such as Biolog, as previously performed for other reference strains (Kaye et al., 2004; Mata et al., 2002). Strain LC6<sup>T</sup> and the five reference strains were thus grown in medium...
containing yeast extract. Under these conditions, strain LC6\textsuperscript{T} showed broader ability to utilize various carbon sources than the type strains of \textit{H. venusta} and \textit{H. hydrothermalis} (Table 1).

Growth of strain LC6\textsuperscript{T} was inhibited by ampicillin, erythromycin, nalidixic acid, chloramphenicol and streptomycin (all at 10 \text{mg}).

The G + C content of the genomic DNA of strain LC6\textsuperscript{T} was 52.5 \text{mol\%}, similar to that for \textit{H. venusta} DSM 4743\textsuperscript{T} but considerably lower than those for the other four reference \textit{Halomonas} type strains (Table 1). Levels of DNA–DNA relatedness between strain LC6\textsuperscript{T} and \textit{H. hydrothermalis} DSM 15725\textsuperscript{T}, \textit{H. venusta} DSM 4743\textsuperscript{T}, \textit{H. aquamarina} DSM 30161\textsuperscript{T}, \textit{H. axialensis} DSM 15723\textsuperscript{T} and \textit{H. meridiana} DSM 5425\textsuperscript{T} were 30.2, 32.3, 23.3, 27.3 and 24.1 \text{\text{\%}}, respectively. These values are lower than the recommended 70 \text{\text{\%}} for the delineation of novel species (Wayne \textit{et al.}, 1987).

Based on taxonomic differences and low levels of DNA–DNA relatedness with respect to its closest phylogenetic relatives, strain LC6\textsuperscript{T} is considered to represent a novel species of the genus \textit{Halomonas}, for which we propose the name \textit{Halomonas andesensis} sp. nov.

\textbf{Description of \textit{Halomonas andesensis} sp. nov.}

\textit{Halomonas andesensis} (an.de.sen’sis. N.L. fem. adj. ande-sensis pertaining to the Andes).

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|c|c|c|c|}
\hline
\textbf{Characteristic} & \textbf{1} & \textbf{2} & \textbf{3} & \textbf{4} & \textbf{5} & \textbf{6} \\
\hline
Temperature range (°C) & 4–45 & 4–45 & 4–45 & 4–35 & 0–35 & 4–35 \\
Total salts (%, optimum) & 1–3 & 0.5–5 & 1–3 & 8 & 5 & 5–8 \\
PH range & 6–11 & 5–11 & 5–11 & 5–10 & 5–11 & 5–11 \\
NO\textsubscript{3} reduction & + & + & – & + & + & + \\
Pigmentation & Cream & Cream & White & Cream & Cream & Cream \\
Hydrolysis of: & & & & & & \\
Starch & + & – & + & + & – & – \\
TWEEN 80 & – & – & + & + & – & – \\
Acid production from: & & & & & & \\
Lactose & + & + & + & + & – & + \\
l-\text{-Arabinose} & + & + & – & + & + & + \\
d-Mannitol & – & + & + & + & – & – \\
l-Rhamnose & – & + & + & + & + & + \\
Trehalose & + & + & + & + & + & + \\
Growth on: & & & & & & \\
l-\text{-Arabinose} & + & – & – & – & – & – \\
Citric acid & – & – & – & – & – & – \\
d-Fructose & W/+ & + & – & + & – & + \\
d-Galactose & + & + & – & – & – & + \\
d-Gluconic acid & + & + & – & + & + & + \\
Itaconic acid & – & + & – & – & – & + \\
l-Lactic acid & + & + & – & – & – & + \\
Lactose & – & – & w/+ & – & – & – \\
Maltose & + & + & – & + & + & + \\
myo-Inositol & + & + & – & w/+ & – & – \\
Propionic acid & + & + & + & + & – & – \\
Raffinose & + & + & w/+ & – & – & – \\
l-Rhamnose & w/+ & w/+ & – & – & – & – \\
Starch & + & + & + & + & + & + \\
d-Xylose & + & – & – & w/+ & – & – \\
Growth in minimal medium & + & + & + & + & + & + \\
DNA G + C content (mol\%)\textsuperscript{*} & 52.5 & 52.3 & 59.5 & 58 & 57.6 & 56.3 \\
\hline
\end{tabular}
\caption{Differential characteristics between strain LC6\textsuperscript{T} and the type strains of related \textit{Halomonas} species}
\end{table}

*Data for reference strains were obtained from Kaye \textit{et al.} (2004) and Mata \textit{et al.} (2002).
Cells are aerobic, Gram-negative rods (1.3 × 1.8–1.3 × 3 μm) that occur primarily singly. Cells are motile with lophotrichous flagella. Colonies are circular with entire margins, convex, smooth and cream-coloured. Growth occurs at 4–45 °C (optimum, 30–35 °C), at pH 6–11 (optimum, pH 7–8) and in the presence of 0.5–20 % (w/v) NaCl (optimum, 1–3 %, w/v). Poly-β-hydroxyalkanoate is produced. Production of exopolysaccharide is negative. Oxidase- and catalase-positive. Negative for facultative anaerobic growth. Reduces nitrate to nitrite. Respiration on nitrate and nitrite is negative. Does not produce gas from nitrate. H₂S production from L-cysteine is negative. Starch is hydrolysed, but ascorbin, casein, gelatin and Tween 80 are not. Negative for phenylalanine deaminase, Voges-Proskauer reaction, methyl red, indole production, lysine and ornithine decarboxylase. Urease activity is positive. Acid is produced from L-arabinose, D-fructose, D-galactose, lactose, sucrose and trehalose, but not from D-mannitol or L-cell-wall fatty acids are C₁₈ : 1ω₁. The quantitative determination of DNA base ratio is positive. Acid is produced from L-arabinose, D-fructose, D-galactose, lactose, myo-inositol, maltose, sucrose and trehalose, but not from D-mannitol or L-rhamnose. The following compounds are utilized as sole carbon sources: acetate, L-arabinose, cellobiose, D-fructose, galactose, D-gluconic acid, D-glucose, L-lactic acid, maltose, myo-inositol, propionic acid, raffinose, L-rhamnose, sucrrose, starch and D-xyllose. The following carbon sources are not utilized: citric acid, itaconic acid and lactose. Sensitive to ampicillin, erythromycin, nalidixic acid, chloramphenicol and streptomycin (at 10 μg). The DNA G+C content of the type strain is 52.5 mol%. The major cell-wall fatty acids are C₁₈ : 1ω₇c, iso-C₁₆ : 1ω₇c 2-OH, C₁₆ : 0 and C₁₂ : 0 3-OH. Respiratory ubiquinones are Q-9 (97 %) and Q-8 (3 %).

The type strain, LC6T (＝CCUG 54844T＝LMG 24243T＝DSM 19434T), was isolated from a water sample of the lake Laguna Colorada in the Bolivian Andes.

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References


