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 LC6^T, was isolated from a water sample of lake Laguna Colorada in the Bolivian Andes. The major cellular fatty acids were C_{18:1}ω7c, iso-C_{16:1}ω7c 2-OH, C_{16:0} and C_{12:0} 3-OH. The respiratory ubiquinones found in strain LC6^T were Q-9 (97 %) and Q-8 (3 %). Strain LC6^T 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 LC6^T 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 LC6^T was most closely related to *Halomonas hydrothermalis* DSM 15725^T and *Halomonas venusta* DSM 4743^T (98.8 % similarity), followed by *Halomonas aquamarina* DSM 30161^T, *Halomonas axialensis* DSM 15723^T and *Halomonas meridiana* DSM 5425^T (98.4 %). However, levels of DNA–DNA relatedness between strain LC6^T and the above type strains were low (<31 %). Strain LC6^T 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 LC6^T represents a novel species of the genus *Halomonas*, for which the name *Halomonas andesensis* is proposed. The type strain is LC6^T (=CCUG 54844^T=LMG 24243^T=DSM 19434^T).

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.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain LC6^T is EF622233.

Maximum-parsimony and minimum-evolution phylogenetic trees based on 16S rRNA gene sequences showing the position of strain LC6^T among some members of the genus *Halomonas* are available as supplementary material with the online version of this paper.

This paper describes the phylogenetic and phenotypic characterization of a moderate halophile, strain LC6^T, 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 LC6^T was isolated by using HM medium (Quillaguamán *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 4743^T, *Halomonas meridiana* DSM 5425^T,

Halomonas aquamarina DSM 30161^T, *Halomonas axialensis* DSM 15723^T and *Halomonas hydrothermalis* DSM 15725^T were cultured under similar conditions as those used for characterization of strain LC6^T.

Growth and tolerance to salt concentration, temperature and pH were studied by culturing strain LC6^T 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 LC6^T from 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 LC6^T 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 LC6^T 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 LC6^T 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 LC6^T 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 LC6^T was grown in HM medium containing 3 % (w/v) NaCl. DNA–DNA hybridization experiments between strain LC6^T 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 thermoprogammer and plotter (Gilford Instrument Laboratories). Renaturation rates were computed with the TRANSFER BAS program of Jahnke (1992).

The cellular fatty acids of strain LC6^T were analysed by GC at the Belgian Coordinated Collections of Microorganisms (BCCM) after growth at 28 °C on trypticase 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 Identification Service and Dr B. J. Tindall, DSMZ, Braunschweig, Germany.

Phylogenetic analysis with the neighbour-joining method based on 16S rRNA gene sequences placed strain LC6^T

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

Cells of strain LC6^T 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^T were circular.

Strain LC6^T 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^T was able to grow over a broad pH range (Table 1).

Table 1 provides a comparison of various features of strain LC6^T with phylogenetically most closely related *Halomonas* species. Strain LC6^T 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^T showed various differences from the *Halomonas* reference strains with respect to certain nutritional and biochemical characteristics (Table 1).

Strain LC6^T 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^T and the five reference strains were thus grown in medium

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strain LC6^T (=DSM 19434^T) among some members of the genus *Halomonas*. *Escherichia coli* ATCC 11775^T was used as an outgroup. Numbers at branch points are bootstrap values (percentages of 500 resamplings). Bar, 2 substitutions per 100 nt.

Table 1. Differential characteristics between strain LC6^T and the type strains of related *Halomonas* species

Strains: 1, LC6^T (*H. andesensis* sp. nov.); 2, *H. venusta* DSM 4743^T; 3, *H. meridiana* DSM 5425^T; 4, *H. aquamarina* DSM 30161^T; 5, *H. axialensis* DSM 15723^T; 6, *H. hydrothermalis* DSM 15725^T. +, Positive; –, negative; w+, weakly positive. All stains are Gram-negative, motile rods. All are negative for hydrolysis of aesculin and gelatin. Colonies of all strains have entire margins.

Characteristic	1	2	3	4	5	6
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 ₃ [–] reduction	+	+	–	+	+	+
Pigmentation	Cream	Cream	White	Cream	Cream	Cream
Hydrolysis of:						
Starch	+	–	+	+	–	–
Tween 80	–	–	+	+	–	–
Acid production from:						
Lactose	+	+	+	+	–	+
L-Arabinose	+	+	–	+	+	+
D-Mannitol	–	+	+	+	+	–
L-Rhamnose	–	+	+	+	+	+
Trehalose	+	+	+	+	+	–
Growth on:						
L-Arabinose	+	–	–	–	–	+
Cellobiose	+	–	–	–	–	–
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%)*	52.5	52.3	59.5	58	57.6	56.3

*Data for reference strains were obtained from Kaye *et al.* (2004) and Mata *et al.* (2002).

containing yeast extract. Under these conditions, strain LC6^T showed broader ability to utilize various carbon sources than the type strains of *H. venusta* and *H. hydrothermalis* (Table 1).

Growth of strain LC6^T was inhibited by ampicillin, erythromycin, nalidixic acid, chloramphenicol and streptomycin (all at 10 µg).

The G + C content of the genomic DNA of strain LC6^T was 52.5 mol%, similar to that for *H. venusta* DSM 4743^T but considerably lower than those for the other four reference *Halomonas* type strains (Table 1). Levels of DNA–DNA relatedness between strain LC6^T and *H. hydrothermalis* DSM 15725^T, *H. venusta* DSM 4743^T, *H. aquamarina* DSM

30161^T, *H. axialensis* DSM 15723^T and *H. meridiana* DSM 5425^T were 30.2, 32.3, 23.3, 27.3 and 24.1 %, respectively. These values are lower than the recommended 70 % for the delineation of novel species (Wayne *et al.*, 1987).

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

Description of *Halomonas andesensis* sp. nov.

Halomonas andesensis (an.de.sen'sis. N.L. fem. adj. *andesensis* pertaining to the Andes).

Cells are aerobic, Gram-negative rods (1.3×1.8 – $1.3 \times 3 \mu\text{m}$) 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. H_2S production from L-cysteine is negative. Starch is hydrolysed, but aesculin, 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, 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, sucrose, starch and D-xylose. 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 $\text{C}_{18:1}\omega 7\text{c}$, iso- $\text{C}_{16:1}\omega 7\text{c}$ 2-OH, $\text{C}_{16:0}$ and $\text{C}_{12:0}$ 3-OH. Respiratory ubiquinones are Q-9 (97 %) and Q-8 (3 %).

The type strain, LC6^T (=CCUG 54844^T=LMG 24243^T=DSM 19434^T), was isolated from a water sample of the lake Laguna Colorada in the Bolivian Andes.

Acknowledgements

We are grateful to the Swedish International Development Cooperation Agency (Sida) for supporting this work.

References

- Arahal, D. R., Ludwig, W., Schleifer, K. H. & Ventosa, A. (2002). Phylogeny of the family Halomonadaceae based on 23S and 16S rDNA sequence analyses. *Int J Syst Evol Microbiol* **52**, 241–249.
- Cashion, P., Holder-Franklin, M. A., McCully, J. & Franklin, M. (1997). A rapid method for base ratio determination of bacterial DNA. *Anal Biochem* **81**, 461–466.
- De Ley, J., Cattoir, H. & Reynaerts, A. (1970). The quantitative measurement of DNA hybridisation from renaturation rates. *Eur J Biochem* **12**, 133–142.
- Escara, J. F. & Hutton, J. R. (1980). Thermal stability and renaturation of DNA in dimethylsulphoxide solutions: acceleration of renaturation rate. *Biopolymers* **19**, 1315–1327.
- HuB, V. A. R., Festl, H. & Schleifer, K. H. (1983). Studies on the spectrophotometric determination of DNA hybridization from renaturation rates. *Syst Appl Microbiol* **4**, 184–192.
- Jahnke, K. D. (1992). BASIC computer program for evaluation of spectroscopic DNA renaturation data from GILFORD SYSTEM 2600 spectrophotometer on a PC/XT/AT type personal computer. *J Microbiol Methods* **15**, 61–73.
- Johnson, J. L. (1994). Similarity analysis of DNAs. In *Methods for General and Molecular Bacteriology*, pp. 655–682. Edited by P. Gerhardt, R. G. E. Murray, W. A. Wood & N. R. Krieg. Washington, DC: American Society for Microbiology.
- Kaye, J. Z., Márquez, M. C., Ventosa, A. & Baross, J. A. (2004). *Halomonas neptunia* sp. nov., *Halomonas sulfidaeris* sp. nov., *Halomonas axialensis* sp. nov. and *Halomonas hydrothermalis* sp. nov.: halophilic bacteria isolated from deep-sea hydrothermal-vent environments. *Int J Syst Evol Microbiol* **54**, 499–511.
- Kumar, S., Tamura, K., Jakobsen, I. B. & Nei, M. (2001). MEGA2: molecular evolutionary genetic analysis software. *Bioinformatics* **17**, 1244–1245.
- Maidak, B. L., Coloe, J. R., Lilburn, T. G., Parker, C. T., Jr, Saxman, P. R., Stredwick, J. M., Garrity, G. M., Li, B., Olsen, G. J. & other authors (2000). The RDP (Ribosomal Database Project) continues. *Nucleic Acids Res* **28**, 173–174.
- Mata, J. A., Martínez-Cánovas, J., Quesada, E. & Victoria, B. (2002). A detailed phenotypic characterisation of the type strains of *Halomonas* species. *Syst Appl Microbiol* **25**, 360–375.
- Mesbah, M., Premachandran, U. & Whitman, W. (1989). Precise measurement of the G + C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.
- Oren, A. (2002). Diversity of halophilic microorganisms: environments, phylogeny, physiology, and applications. *J Ind Microbiol Biotechnol* **28**, 56–63.
- Quillaguamán, J., Hatti-Kaul, R., Mattiasson, B., Alvarez, M. T. & Delgado, O. (2004). *Halomonas boliviensis* sp. nov., an alkalitolerant, moderate halophile isolated from soil around a Bolivian hypersaline lake. *Int J Syst Evol Microbiol* **54**, 721–725.
- Sánchez-Porro, C., Martín, S., Mellado, E. & Ventosa, A. (2003). Diversity of moderately halophilic bacteria producing extracellular hydrolytic enzymes. *J Appl Microbiol* **94**, 295–300.
- Smibert, R. M. & Krieg, N. R. (1994). Phenotypic characterization. In *Methods for General and Molecular Bacteriology*, pp. 611–651. Edited by P. Gerhardt, R. G. E. Murray, W. A. Wood & N. R. Krieg. Washington, DC: American Society for Microbiology.
- Tamaoka, J. & Komagata, K. (1984). Determination of DNA base composition by reversed-phase high-performance liquid chromatography. *FEMS Microbiol Lett* **25**, 125–128.
- Ventosa, A., Quesada, E., Rodríguez-Valera, F., Ruiz-Berraquero, F. & Ramos-Cormenzana, A. (1982). Numerical taxonomy of moderately halophilic Gram-negative rods. *J Gen Microbiol* **128**, 1959–1968.
- Vreeland, R. H., Litchfield, C. D., Martin, E. L. & Elliot, E. (1980). *Halomonas elongata*, a new genus and species of extremely salt-tolerant bacteria. *Int J Syst Bacteriol* **30**, 485–495.
- Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E. & other authors (1987). International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.