Chromohalobacter nigrandesensis sp. nov., a moderately halophilic, Gram-negative bacterium isolated from Lake Tebenquiche on the Atacama Saltern, Chile

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A total of 52 strains of moderately halophilic bacteria isolated from hypersaline sediment of Lake Tebenquiche on the Atacama Saltern, Chile, were subjected to a taxonomic study. The morphological, physiological, biochemical and nutritional characteristics of the strains matched those described for the genus *Chromohalobacter*. Cells were Gram-negative, non-spore-forming, rod-shaped and motile. A black pigmentation was produced. One strain, designated LTS-4N^T, grew optimally at 32 °C. Growth occurred in media containing 0.5–25 % (w/v) total salts; the optimum was 7.5 % (w/v) total salts. The pH range for growth was 5–10. The G + C content of the DNA of strain LTS-4N^T was 59.8 mol%. Analysis of 16S rRNA gene sequence similarity revealed that strain LTS-4N^T was closely related to *Chromohalobacter* species; however, DNA–DNA hybridization of representative strain LTS-4N^T failed to associate the strain with any species of the genus *Chromohalobacter* with validly published names. Therefore, the name *Chromohalobacter nigrandesensis* sp. nov. is proposed. The type strain is LTS-4N^T (= CECT 5315^T = DSM 14323^T).

Moderately halophilic micro-organisms have been the subject of a variety of taxonomic and ecological studies (Ventosa *et al.*, 1998; Oren, 2002). Moderate halophiles grow best at NaCl concentrations between 0.5 and 2.5 M. They represent a very heterogeneous group distributed extensively in high salinity zones (Rodríguez-Valera, 1988; Kaye *et al.*, 2004). The different branches of the phylum *Proteobacteria* have various halophilic representatives with close relatives that are non-halophilic. Among the bacterial families that form part of the largest subgroup, the class *Gammaproteobacteria*, the family *Halomonadaceae* is characterized as being represented by several halophilic, halotolerant and non-halophilic species that belong to different genera. *Halomonas* is the largest genus in this family, containing more than 30 species,

followed by the genus *Chromohalobacter*, which presently has five species. *Cobetia marina*, *Carnimonas nigrificans* and *Zymobacter palmae* are also included in the family *Halomonadaceae*.

'*Chromobacterium marismortui*', isolated from the Dead Sea, was originally described as a moderately halophilic bacterium by Elazari-Volcani (1940). Later, Ventosa *et al.* (1989) reclassified '*Chromobacterium marismortui*' as *Chromohalobacter marismortui*. Other species were subsequently placed in the genus *Chromohalobacter*, as *Chromohalobacter canadensis* and *Chromohalobacter israelensis* (Arahal *et al.*, 2001a), *Chromohalobacter salexigens* (Arahal *et al.*, 2001b) and the recently described species *Chromohalobacter sarecensis* (Quillaguamán *et al.*, 2004). All these species are moderately halophilic, aerobic, motile, Gram-negative, heterotrophic rods. The species of the genus *Chromohalobacter* form a monophyletic group within the family *Halomonadaceae* (Arahal *et al.*, 2002).

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain LTS-4N^T is AJ277205.

In the present study, the characteristics of strain LTS-4N^T, which was selected as a representative from a group of 52 moderately halophilic strains isolated from a hypersaline lake on Atacama Saltern, Chile, were examined in detail and strain LTS-4N^T was compared with other halophilic, Gram-negative rods. Based on its morphological and physiological characteristics, as well as on its phylogenetic position determined by 16S rRNA gene sequence analysis and DNA–DNA relatedness data, strain LTS-4N^T should be classified as representing a novel species within the genus *Chromohalobacter*.

The 52 strains used in this study were moderately halophilic, Gram-negative, motile, rod-shaped bacteria that were isolated in 1991 from sediments of the hypersaline Lake Tebenquiche at the Atacama Saltern, Chile. The following halophilic, Gram-negative micro-organisms were used as reference strains: *C. marismortui* ATCC 17056^T, *C. salexigens* CECT 5384^T, *C. canadensis* CECT 5385^T, *C. israelensis* CECT 5287^T, *C. sarecensis* CCUG 47987^T, *Halomonas elongata* ATCC 33173^T, *Halomonas halophila* DSM 4770^T, *Halomonas halmophila* ATCC 19717^T, *Halomonas venusta* DSM 4743^T, *Halomonas pacifica* DSM 4742^T, *Halomonas salina* ATCC 49509^T and *Halomonas eurihalina* ATCC 49336^T. MH complex medium was used as the maintenance medium for halophilic strains. This medium contained (l⁻¹) 10 g yeast extract (Difco), 5 g proteose peptone no. 3 (Difco) and 1 g glucose (Ventosa *et al.*, 1982) and it was supplemented with a balanced mixture of sea salts (Subov, 1931) to give an adequate salts concentration for growth of marine and moderately halophilic strains. The medium was solidified with 20 g Bacto agar (Difco) l⁻¹ and the pH was adjusted to 7.2 with 1 M NaOH.

One hundred and nineteen phenotypic characters including morphological, physiological, biochemical, nutritional and antimicrobial susceptibility tests were determined for each strain. Details of the procedures have been described previously (Prado *et al.*, 1991). The results from the 52 strains and the data for reference strains were coded in a binary form (0, negative; 1 positive; 9, missing or non-comparable data). Strain similarities were estimated by simple matching coefficients (S_{SM}) (Sokal & Michener, 1958) and clustered by the unweighted pair-group method of association (UPGMA) (Sneath & Sokal, 1973). Cophenetic correlation was also obtained for each method. The test error was estimated by examining 10 strains in duplicate. The computation was performed by the MINT program using an Eclipse model MV/10000 computer at the Computer Centre, University of Granada.

The colonial morphology and pigmentation of strain LTS-4N^T, which was chosen as a representative of the group, were observed in mid-exponential phase cultures (20 h) and old cultures (3 days) grown on solid MH medium supplemented with 7.5 % (w/v) total salts. The optimal salts concentration for growth of strain LST-4N^T was determined at different incubation temperatures. The strain was cultured in MH medium containing the following concentrations of a

balanced mixture of sea salts (Subov, 1931): 0, 0.5, 3, 5, 7.5, 10, 15, 20 and 25 % (w/v). Each 50 ml batch of medium was inoculated with 0.1 ml (approx. 10⁵ cells ml⁻¹) from an appropriate dilution of a 20 h culture of the micro-organism grown in MH medium containing 7.5 % (w/v) salts. The cultures were incubated at 15, 22, 32 or 42 °C in a rotary shaker. Viable cells were determined from plate counts on solid MH medium at the appropriate salt concentration. Experiments were performed in triplicate.

Transmission electron microscopy and scanning electron microscopy were used for morphological studies of cells from a 20 h culture of strain LST-4N^T grown on the surface of MH agar plates covered with MH liquid medium (7.5 % w/v total salts). Cells for transmission electron microscopy were stained with 2 % (w/v) phosphotungstic acid and observed by using a Zeiss model EM 902 transmission electron microscope. Samples for scanning electron microscopy were fixed in 2 % (v/v) glutaraldehyde solution (pH 7.2), dehydrated in an acetone series, critical-point-dried, coated with gold and scanned in a Zeiss model DSM950 scanning electron microscope.

The DNA was isolated and purified by the method of Lind & Ursing (1986). The DNA G + C content was determined by the thermal denaturation method (T_m) (Marmur & Doty, 1962) with a Perkin Elmer Lambda 3B spectrophotometer fitted with a temperature program accessory. DNA–DNA hybridization studies were performed by the non-radioactive method described by Ziemke *et al.* (1998). Reference DNA was double-labelled using DIG-11-dUTP and biotin-16-dUTP (Boehringer Mannheim). The labelling was carried out using the Boehringer Mannheim nick-translation kit. The fragment of the 16S rRNA gene was obtained from purified genomic DNA by direct PCR amplification. Primers 16F27 (5'-AGAGTTTGATCMTGGCTC-3') and 16R1525 (5'-AAGGAGGTGWTCCARCC-3') were used for amplification and the sequencing primers used were 16F357, 16R519 and 16F945 (Fox *et al.*, 1992). The oligonucleotides were produced by Pharmacia Biotech and were then diluted to 100 pmol µl⁻¹. The PCR was performed in a thermal cycler (480 model; Perkin Elmer) with 30 cycles of 1 min denaturation at 94 °C, followed by 1 min annealing at 55 °C and 2 min elongation at 72 °C. The reaction mixture in a total volume of 100 µl contained 2 µl genomic DNA, 10 µl buffer (100 mM Tris/HCl, pH 8.3, 500 mM KCl; Perkin Elmer), 4 µl MgCl₂ (25 mM; Perkin Elmer), 1 µl dNTP mixture (dATP, dCTP, dGTP and dTTP each at 10 mM; Ultrapure dNTPs set from Pharmacia Biotech) and 1 µl *Taq* DNA polymerase (Ampli^{Taq} DNA polymerase; Perkin Elmer). Each primer was used at a concentration of 20 pmol µl⁻¹. PCR products were analysed by electrophoresis in 1.7 % agarose gels in TAE buffer, using a phage λ *Pst*I digest as the size marker. The products were purified with Microcon 100 concentrators (Amicon). Purified PCR products were sequenced directly using a Applied Biosystems ABI 373 Stretch DNA sequencer and the manufacturer's protocols for Ampli^{Taq} FS with fluorescent dye-labelled

dideoxynucleotides (ABI PRISM BigDye terminator cycle sequencing ready reaction kit; Perkin-Elmer). The sequences were analysed with ABI PRISM 373 xl Collection 2.0 and Sequencing Analysis 3.3 programs for Macintosh G3.

The sequence obtained was compared with other publicly available 16S rRNA gene sequences deposited in the EMBL database. The sequences were aligned by using CLUSTAL W 1.8 (Thompson *et al.*, 1994). Phylogenetic trees were constructed by the neighbour-joining method with the MEGA3 program package (Kumar *et al.*, 2004).

The 52 strains isolated from the Atacama saltern clustered in a single phenon at 83 % similarity. The cophenetic correlation value was 0.94 %, and the estimated test error was less than 3 %. None of the reference strains clustered with the environmental isolates. Phenotypically, the strains studied constitute a homogeneous group. Strain LTS-4N^T was selected as the type strain and representative of the 52 isolates.

Table 1 provides a comparison of the taxonomic features of strain LTS-4N^T with other *Chromohalobacter* species. Cells were Gram-negative rods, 2.3–4.2 × 0.35–0.5 µm. They were motile by means of subpolar flagella. No spores were observed. Colonies of all strains on MH medium containing 7.5 % (w/v) salts were circular with entire margins, convex, smooth and characteristically black-pigmented. On liquid medium, all the strains produced a brown, diffusible pigment. Optimum growth of strain LTS-4N^T was at a concentration

of 7.5 % total salts in MH complex medium, at pH 7.5 and 32 °C. The range of total salts concentration in which the bacterium grew at 32 °C was 0.5–25 % (w/v). At 15 °C, the micro-organism grew in the balanced mixture of sea salts (Subov, 1931) at concentrations between 0.5 and 20 % (w/v) and 7.5 % salts was optimal for growth; however, the strain only grew in a total salts concentration of 0.5–15 % (w/v) when it was incubated at 42 °C and it grew optimally at 5 % total salts (w/v) at this temperature.

A nearly complete 16S rRNA gene sequence was obtained for strain LTS-4N^T and comparative analysis of the sequence confirmed the affiliation of this strain to the genus *Chromohalobacter*, with the highest value of 98.3 % sequence similarity. The phylogenetic tree showed that strain LTS-4N^T exhibited the closest phylogenetic affinity to the *Chromohalobacter* cluster (Fig. 1). The DNA G + C content in strain LTS-4N^T was 59.8 mol% (determined by T_m method). DNA–DNA hybridization experiments between strain LTS-4N^T and previously described species of *Chromohalobacter* showed values of DNA–DNA relatedness of 48.7, 56.1, 40.9, 51.4 and 56.9 % with *C. marismortui* ATCC 17056^T, *C. canadensis* CECT 5385^T, *C. israelensis* CECT 5287^T, *C. salexigens* CECT 5384^T and *C. sarecensis* CCUG 47987^T, respectively.

On the basis of morphological, phenotypic and genotypic data, we propose that strain LTS-4N^T should be classified as representing a novel species within the genus *Chromohalobacter*, *Chromohalobacter nigrandesensis* sp. nov.

Table 1. Differential characteristics of strain LTS-4N^T and other *Chromohalobacter* species

Data were taken from Arahal *et al.* (2001a, b, 2002), Quillaguamán *et al.* (2004) and this study. Characters are scored as: +, positive; –, negative; ND, not determined; d, differs among studies.

Characteristic	LTS-4N ^T	<i>C. marismortui</i>	<i>C. canadensis</i>	<i>C. israelensis</i>	<i>C. salexigens</i>	<i>C. sarecensis</i>
NaCl range for growth (% w/v)	0.5–25*	2–30	3–25	3.5–20	0.9–25	0–25
pH range	5–10	5–10	5–9	5–9	5–9	5–10
Temperature range (°C)	15–45	5–45	15–45	15–45	4–45	0–45
Flagellar arrangement	Subpolar	Peritrichous	Polar	Polar	Subpolar	Polar
Pigmentation	Black	Brown–yellow	White	Cream	White–cream	Brown
Oxidase	–	+	–	–	–	+
Acid production from:						
Arabinose	+	–	+	+	+	–
Maltose	–	+	–	+	+	–
Sucrose	+	+	–	+	+	–
Nitrate reduction	–	–	+	+	+	–
Hydrolysis of:						
Gelatin	–	–	–	–	d	–
Aesculin	–	–	+	+	d	–
Growth on:						
Cellobiose	–	–	+	+	–	+
Citrate	+	–	+	ND	+	–
G + C content (mol%)	59.8	62–65	62	65	62.4–66	56.1

*Total salts (refer to the balanced mixture of sea salts of Subov, 1931).

Fig. 1. Phylogenetic relationship between *Chromohalobacter nigrandesensis* LTS-4N^T and all species of genus *Chromohalobacter*, several species of the genus *Halomonas* and related species *Pseudomonas beijerinckii*, *Zymobacter palmae*, *Cobetia marina* and *Carnimonas nigrificans*. The tree was constructed using the neighbour-joining algorithm based on 16S rRNA gene sequences. GenBank accession numbers used in the phylogenetic analysis are given in parentheses. *Escherichia coli* ATCC 11775^T was taken as the outgroup. Only bootstrap values greater than 50% are shown (1000 replications). Bar, 0.02 substitutions per nucleotide position.

Description of *Chromohalobacter nigrandesensis* sp. nov.

Chromohalobacter nigrandesensis (nig.ran.des.en'sis. L. adj. *niger* black; N.L. masc. adj. *andesensis* pertaining to the Andes; N.L. masc. adj. *nigrandesensis* relating to the black pigment and to the Andes, the mountain range of Chile).

Cells are Gram-negative, straight or sometimes slightly curved rods, 2.3–4.2 × 0.35–0.5 µm. Motile by means of subpolar flagella. Non-spore-forming. Colonies are circular, black and very mucous in complex solid medium containing 7.5% (w/v) total salts. Catalase-positive and oxidase-negative. Chemo-organotrophic. Growth occurs in media containing 0.5–25% (w/v) total salts and optimal growth occurs at 7.5% (w/v) total salts. The pH range for growth is 5 to 10. The optimal temperature for growth is 32 °C. Tween 20 is hydrolysed. Citrate-positive. Nitrate is not reduced. Arginine

dihydrolase-positive. Urease-positive. H₂S is produced from L-cysteine. Acids are produced from various sugars (arabinose, xylose, fructose, galactose, glucose and sucrose). A great variety of organic compounds, including arabinose, fructose, galactose, glucose, inulin, maltose, mannose, sucrose, trehalose, xylose, acetate, fumarate, gluconate, lactate, malate, pyruvate, succinate, glycerol and mannitol, are used as sole carbon and energy sources. The following compounds are utilized as sole carbon, nitrogen and energy sources: alanine, arginine, asparagine, aspartate, glutamate, ornithine and serine. Susceptible to ampicillin, chloramphenicol, erythromycin, penicillin G and rifampicin and resistant to tetracycline. The G + C content of the DNA of the type strain is 59.8 mol% (*T_m*).

The type strain is LST-4N^T (= CECT 5315^T = DSM 14323^T), isolated from hypersaline Lake Tebenquiche in the Atacama Saltern, Chile.

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