Chromohalobacter salarius sp. nov., a moderately halophilic bacterium isolated from a solar saltern in Cabo de Gata, Almería, southern Spain

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A moderately halophilic, Gram-negative bacterium (strain CG4.1T), which was isolated from a solar saltern at Cabo de Gata, a wildlife reserve located in the province of Almería, southern Spain, was subjected to a polyphasic taxonomic study. This organism was an aerobic, motile rod that produced colonies with a yellow pigment. Strain CG4.1T grew at salinities of 3–25 % (w/v), at 15–45 °C and at pH 5–9. The organism reduced nitrate, hydrolysed starch and had phenylalanine deaminase activity. The major fatty acids were C18:1ω7c, C16:0 and C19:0 cyclo ω8c. The DNA G+C content was 63.6 mol%. On the basis of phenotypic and phylogenetic data, strain CG4.1T appears to be a member of the genus Chromohalobacter and clustered closely with Chromohalobacter species, with 95–96 % similarity between their 16S rRNA gene sequences. However, DNA–DNA relatedness between the isolate and the type strains of Chromohalobacter species was low. Therefore, it is proposed that strain CG4.1T represents a novel species, Chromohalobacter salarius sp. nov. The type strain is strain CG4.1T (= CECT 5903T = LMG 23626T).

Moderately halophilic micro-organisms represent a very heterogeneous group that are distributed extensively in high salinity zones (Rodríguez-Valera, 1988; Ventosa et al., 1998; Oren, 2002; Kaye et al., 2004). Among the bacterial families that form part of the Gammaproteobacteria, the family Halomonadaceae is characterized as being represented by several halophilic, halotolerant and non-halophilic species that belong to different genera. Members of the genus Chromohalobacter, which currently has seven species, form a monophyletic group included in the family Halomonadaceae (Arahal et al., 2002). Ventosa et al. (1989) reclassified ‘Chromobacterium marismortui’ as Chromohalobacter marismortui and other species were subsequently placed in the genus Chromohalobacter as follows: Chromohalobacter canadensis and Chromohalobacter israelensis (Arahal et al., 2001a), Chromohalobacter salseigenes (Arahal et al., 2001b), Chromohalobacter sarecensis (Quillaguamán et al., 2004), Chromohalobacter nigrandesensis (Prado et al., 2006), and Chromohalobacter beijerinckii (Pečonek et al., 2006). All these species are moderately halophilic, aerobic, motile, Gram-negative, heterotrophic rods.

In the screening of halophilic micro-organisms isolated from the solar saltern of Cabo de Gata, a wildlife reserve located in the province of Almería, southern Spain, a moderately halophilic Gram-negative bacterial organism, strain CG4.1T, was characterized by a polyphasic approach, including phylogenetic analysis based on 16S rRNA gene sequences, genomic relatedness and chemotaxonomic and phenotypic properties. The results reported in this study indicate that CG4.1T belongs to the genus Chromohalobacter, but it is clearly distinguishable from all other Chromohalobacter species.

Water samples were spread on MH complex medium supplemented with a balanced mixture of sea salts to give an adequate salts concentration for growth of marine and moderately 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 the balanced mixture of sea salts of Subov (1931). The pH was adjusted to 7.2 with 1 M NaOH.
The medium was solidified with 20 g Bacto agar (Difco) 1\(^{-1}\). The isolate was maintained and routinely grown aerobically on MH complex medium with 7.5 % (w/v) total salts at 35 \(^\circ\)C, except where indicated otherwise.

The methods used for phenotypic characterization have been described previously in detail (Ventosa et al., 1982; Quesada et al., 1983; Prado et al., 1991; Mata et al., 2002). The salts concentrations required for growth of strain CG4.1\(^T\) were determined at 35 \(^\circ\)C. 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, 25 and 30 % (w/v). Each 50 ml medium batch was inoculated with 0.1 ml (approximately 10\(^5\) cells per ml) from an appropriate dilution of a 20 h culture of the microorganism grown in MH medium containing 7.5 % (w/v) salts. The cultures were incubated in a rotary shaker. Viable cells were determined from plate counts on solid MH medium at the appropriate salts concentration. Experiments were performed in triplicate. The pH growth range was determined in a similar way on MH medium by adjusting the final pH to values of 4, 5, 6, 7, 8, 9 and 10 with HCl or NaOH. The temperature range was determined as above by incubating the strain at temperatures of 4, 10, 15, 20, 25, 30, 35, 40, 45 and 50 \(^\circ\)C.

TEM and SEM were used for morphological studies of cells from a 20 h culture of strain CG4.1\(^T\) grown on the surface of MH agar plates covered with MH liquid medium (7.5 %, w/v, salts). Cells for TEM were stained with 2 % (w/v) phosphotungstic acid and observed by using a Zeiss model EM 902 TEM. Samples for SEM 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 SEM.

Whole-cell fatty acids of strain CG4.1\(^T\) were analysed by the Analytical Service Microbial Identification System (DSMZ, Braunschweig, Germany) using the MID/Hewlett Packard Microbial Identification System (MIS), which relies upon high-resolution GC to obtain the fatty acid profile. A moist pellet of cells obtained from cultivation on MH complex medium supplemented with 7.5 % (w/v) sea salts for 2 days at 35 \(^\circ\)C was used and the relative amount of each fatty acid was expressed as a percentage of the total fatty acids.

DNA was isolated and purified by the method of Lind & Ursing (1986). The G + C content (mol%) 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 using 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. Phylogenetic analysis based on the 16S rRNA gene sequence of strain CG4.1\(^T\) was made as described previously (Prado et al., 2006). The sequence obtained was compared with other publicly available 16S rRNA sequences deposited in the EMBL database. Sequences were aligned by using CLUSTAL W 1.8 (Thompson et al., 1994). Phylogenetic trees were constructed with the aid of the MEGA 3.1 software package (Kumar et al., 2004) using the neighbour-joining and maximum-parsimony algorithms.

Table 1 provides a comparison of the taxonomic features of strain CG4.1\(^T\) and Chromohalobacter species. Cells of strain CG4.1\(^T\) were Gram-negative straight rods, 2.5–3.2 \(\mu\)m long and 1.2–1.3 \(\mu\)m wide. They were motile by means of subpolar flagella. No spores were observed. Colonies of strain CG4.1\(^T\) on MH medium containing 7.5 % (w/v) salts were circular/slightly irregular, convex and yellow pigmented. Optimum growth of strain CG4.1\(^T\) was observed in 7.5–10.0 % (w/v) total salts in MH complex medium, and at pH 7.2 and 35 \(^\circ\)C. The isolate was able to grow at 35 \(^\circ\)C in 3–25 % (w/v) total salts; it was unable to grow in the absence of NaCl. This result indicated that the isolate could be assigned to the group of moderately halophilic bacteria (Kushner & Kamekura, 1988). With respect to its nutritional and biochemical characteristics, strain CG4.1\(^T\) showed some similarity to reference Chromohalobacter species, but differences were also noted. Strain CG4.1\(^T\) was able to hydrolyse starch and produce phenylalanine deaminase, activities that have not yet been described in Chromohalobacter species.

The major cellular fatty acids (up to 96 %) of strain CG4.1\(^T\) were C\(_{18:1}\)ω7c (43.0 %), C\(_{16:0}\) (32.6 %), C\(_{19:0}\) cyclo ω8c (11.1 %), and C\(_{16:1}\)ω7c and/or iso-C\(_{15:0}\) 2-0H (10.1 %). The minor fatty acids of strain CG4.1\(^T\) were C\(_{17:0}\) cyclo (1.7 %), C\(_{18:1}\)ω7c 11-methyl (0.5 %), C\(_{12:0}\) 3-0H (0.4 %), C\(_{18:0}\) (0.3 %), C\(_{12:0}\) (0.2 %) and C\(_{14:0}\) (0.2 %). The variation in relative amounts in the fatty acid profile of strain CG4.1\(^T\) in relation to that reported for other Chromohalobacter species (Vargas et al., 2005; Mutnuri et al., 2005; Peçonek et al., 2006) can be explained by the different NaCl concentrations that were used in the growth media.

The DNA G + C content of strain CG4.1\(^T\) was 63.6 mol%. Strain CG4.1\(^T\) was placed within the genus Chromohalobacter based on its 16S rRNA gene sequence and shared 96.4 % similarity with C. canadensis ATCC 43984\(^T\), 95.9 % similarity with C. marismortui ATCC 17056\(^T\), 95.8 % similarity with C. israelensis ATCC 43985\(^T\), 95.6 % similarity with C. salexiens DSM 3043\(^T\), C. beijerinckii ATCC 19372\(^T\) and C. sarecensis LV4\(^T\) and 95.5 % similarity with C. nigrandesinis LST-4N\(^T\). 16S rRNA gene sequence similarity between strain CG4.1\(^T\) and Halomonas desiderata FB2\(^T\) was 93 %, the highest value observed with species of the genus Halomonas. The phylogenetic tree constructed using the neighbour-joining algorithm showed that strain CG4.1\(^T\) exhibited closest phylogenetic affinity to the Chromohalobacter cluster (Fig. 1). The maximum-parsimony algorithm gave a similar result (see Supplementary Fig. S1 available in IJSEM Online).

DNA–DNA hybridization experiments between strain CG4.1\(^T\) and the type strains of previously described species.
of *Chromohalobacter* showed DNA–DNA relatedness values below 25% with *C. marismortui* ATCC 17056T, *C. nigrandesensis* CECT 5385T, *C. israelensis* CECT 5287T, *C. salinicola* CECT 5384T, *C. salexigens* CCG 47987T, *C. nigrandesensis* CECT 5315T and *C. beijerinckii* ATCC 19372T.

On the basis of morphological, phenotypic and genotypic data, it is proposed that strain CG4.1T should be classified as a representative of a novel species within the genus *Chromohalobacter, Chromohalobacter salarius* sp. nov.

**Description of Chromohalobacter salarius sp. nov.**

*Chromohalobacter salarius* (sa.la’ri.us. L. masc. adj. salarius of or belonging to salt, because this micro-organism was isolated from brine samples).

Cells are Gram-negative straight rods, 2.5–3.2 × 1.2–1.3 μm, appearing either singly or in pairs. They are motile by one subpolar flagellum and do not form endospores. Colonies are yellow, circular/slightly irregular and convex. Moderately halophilic, growing in a wide range (3–25%, w/v) of salts concentrations (mixture of sea salts) with optimum growth at 7.5–10.0% (w/v) salts; no growth occurs in the absence of salt. Grows at 15–45°C (optimal temperature 35°C) and pH 5–9 (optimal pH 7.0). Catalase is produced but not oxidase. Chemo-organotrophic. Starch and Tween 20 are hydrolysed. Does not hydrolyse ascin, gelatin, casein, tyrosine, Tween 80 or urea. Produces phosphatase and phenylalanine deaminase. H2S is produced from L-cysteine. Positive for nitrate and nitrite reduction. Negative for indole, methyl red, Voges–Proskauer and haemolysis. Acids are produced from D-galactose and D-glucose, but not from L-arabinose, D-adonitol, D-cellobiose, glycerol, *myo*-inositol, lactose, maltose, D-mannitol, D-mannose, D-melezitose, L-raffinose, L-rhamnose, salicin, D-sorbitol, sucrose, D-trehalose or D-xylene. Acetate, citrate, formate, fumarate, gluconate, lactate, malate, malonate, propionate, pyruvate, succinate, D-mannitol, D-fuctose, D-glucose, lactose and D-mannose are used as sole carbon and energy sources, whereas adonitol, *myo*-inositol, sorbitol, D-cellobiose, D-galactose, maltose, L-rhamnose, sucrose and D-xylitol are not. L-Arginine, L-lysine, L-ornithine and L-serine are used as sole carbon and energy sources, whereas adonitol, *myo*-inositol, sorbitol, D-cellobiose, D-galactose, maltose, L-rhamnose, sucrose and D-xylitol are not. L-Arginine, L-lysine, L-ornithine and L-serine are used as sole carbon, nitrogen and energy sources, whereas L-alanine, L-histidine, DL-isoleucine and L-valine are not. Susceptible to amoxicillin (25 μg), ampicillin (10 μg), cefoxitin (30 μg), chloramphenicol (30 μg), nalidixic acid (30 μg), penicillin G (10 U), polymyxin B (300 IU) and rifampicin (30 μg). Resistant to erythromycin (15 μg), streptomycin (10 μg) and tetracycline (30 μg). The major fatty acids (up to 96%) are C18:1ω6c, C16:0, C19:0 cyclo ω8c and C16;1ω7c and/or iso-C15:0 2-OH.
The type strain is CG4.1\textsuperscript{T} (= CECT 5903\textsuperscript{T} = LMG 23626\textsuperscript{T}), isolated from a solar saltern in Cabo de Gata (Almeria, Spain). The DNA G+C content of the type strain is 63.6 mol% (T\textsubscript{m} method).

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References


Fig. 1. Phylogenetic relationship between strain CG4.1\textsuperscript{T} and all species of the genus Chromohalobacter, several species of the genus Halomonas and the related species Zymbacter palmae, Cobetia marina and Carnimonas significans. The tree was constructed using the neighbour-joining algorithm based on 16S rRNA gene sequences. Gen-Bank accession numbers of the sequences used in the phylogenetic analysis are given in parentheses. Escherichia coli ATCC 11775\textsuperscript{T} was used as the outgroup. Only bootstrap values greater than 50% are shown (1000 replications). Bar, 0.02 substitutions per nucleotide position.


