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 CG4.1^T), 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 CG4.1^T 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 C_{18:1}ω7c, C_{16:0} and C_{19:0} cyclo ω8c. The DNA G+C content was 63.6 mol%. On the basis of phenotypic and phylogenetic data, strain CG4.1^T 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 CG4.1^T represents a novel species, *Chromohalobacter salarius* sp. nov. The type strain is strain CG4.1^T (=CECT 5903^T=LMG 23626^T).

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 salexigens* (Arahal *et al.*, 2001b), *Chromohalobacter sarecensis* (Quillaguamán

et al., 2004), *Chromohalobacter nigrandesensis* (Prado *et al.*, 2006), and *Chromohalobacter beijerinckii* (Peçonek *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 CG4.1^T, 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 CG4.1^T 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 GenBank/EMBL/DDBJ accession number for the 16S rRNA sequence of strain CG4.1^T is AJ427626.

A phylogenetic tree based on the maximum-parsimony algorithm showing relationships between strain CG4.1^T, *Chromohalobacter* species and other related taxa is available as supplementary material with the online version of this paper.

The medium was solidified with 20 g Bacto agar (Difco) l⁻¹. The isolate was maintained and routinely grown aerobically on MH complex medium with 7.5 % (w/v) total salts at 35 °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 °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 10⁵ cells per ml) 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 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 °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 °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 µm long and 1.2–1.3 µm wide. They were motile by means of sub-polar 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 °C. The isolate was able to grow at 35 °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-OH (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-OH (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. salaxigens* DSM 3043^T, *C. beijerinckii* ATCC 19372^T and *C. sarencensis* LV4^T and 95.5 % similarity with *C. nigrandesensis* 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

Table 1. Differential characteristics of strain CG4.1^T and other *Chromohalobacter* species

Strains/species: 1, CG4.1^T; 2, *C. nigrandesensis*; 3, *C. marismortui*; 4, *C. canadensis*; 5, *C. israelensis*; 6, *C. salexigens*; 7, *C. sarecensis*; 8, *C. beijerinckii*. Data from Ventosa *et al.* (1989), Arahall *et al.* (2001a, b, 2002), Quillagumán *et al.* (2004), Peçonek *et al.* (2006), Prado *et al.* (2006) and this study. +, Positive; –, negative; NR, not reported.

Characteristic	1	2	3	4	5	6	7	8
Ranges for growth								
NaCl (% w/v)	3–25	0.5–25.0	2–30	3–25	3.5–20.0	0.9–25.0	0–25	0.3–25.0
pH	5–9	5–10	5–10	5–9	5–9	5–9	5–10	4.5–8.0
Temperature (°C)	15–45	15–45	5–45	15–45	15–45	4–45	0–45	4–42
Flagellar arrangement	Subpolar	Subpolar	Peritrichous	Polar	Polar	Subpolar	Polar	Polar
Pigmentation	Yellow	Black	Brown–yellow	White	Cream	White–cream	Brown	Yellow
Oxidase	–	–	+	–	–	–	+	+
Phenylalanine deaminase	+	–	–	–	–	–	NR	NR
Phosphatase	+	–	–	+	–	NR	NR	–
Acid production from:								
Arabinose	–	+	–	+	+	+	–	+
Maltose	–	–	+	–	+	+	–	–
Sucrose	–	+	+	–	+	+	–	–
Nitrate reduction	+	–	–	+	+	+	–	+
Indole production	–	–	–	+	+	–	–	–
H ₂ S production	+	+	–	–	–	+	–	+
Hydrolysis of:								
Aesculin	–	–	–	+	+	–	–	–
Starch	+	–	–	–	–	–	–	–
Growth on:								
Cellobiose	–	–	–	+	+	–	+	NR
Citrate (Simmons')	+	+	+	–	–	+	NR	+
DNA G + C content (mol%)	63.6	59.9	62–65	62	65	62.4–66.0	56.1	60.4–60.7

of *Chromohalobacter* showed DNA–DNA relatedness values below 25 % with *C. marismortui* ATCC 17056^T, *C. canadensis* CECT 5385^T, *C. israelensis* CECT 5287^T, *C. salexigens* CECT 5384^T, *C. sarecensis* CCUG 47987^T, *C. nigrandesensis* CECT 5315^T and *C. beijerinckii* ATCC 19372^T.

On the basis of morphological, phenotypic and genotypic data, it is proposed that strain CG4.1^T 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 aesculin, 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, D-salicin, D-sorbitol, sucrose, D-trehalose or D-xylose. Acetate, citrate, formate, fumarate, gluconate, lactate, malate, malonate, propionate, pyruvate, succinate, D-mannitol, D-fructose, 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-xylose 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 C_{18:1}ω7c, C_{16:0}, C_{19:0} cyclo ω8c and C_{16:1}ω7c and/or iso-C_{15:0} 2-OH.

Fig. 1. Phylogenetic relationship between strain CG4.1^T and all species of the genus *Chromohalobacter*, several species of the genus *Halomonas* and the related species *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 of the sequences used in the phylogenetic analysis are given in parentheses. *Escherichia coli* ATCC 11775^T was used as the outgroup. Only bootstrap values greater than 50% are shown (1000 replications). Bar, 0.02 substitutions per nucleotide position.

The type strain is CG4.1^T (=CECT 5903^T=LMG 23626^T), isolated from a solar saltern in Cabo de Gata (Almería, Spain). The DNA G+C content of the type strain is 63.6 mol% (T_m method).

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