Halomonas indalinina 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 bacterium, strain CG2.1^T, 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, Gram-negative rod that produced orange-pigmented colonies. Strain CG2.1^T was able to grow at salinities of 3–25 % (w/v) and at temperatures of 15–40 °C. The pH range for growth was 5–9. Strain CG2.1^T was a heterotroph capable of utilizing various carbohydrates as carbon sources. The organism reduced nitrate and showed phenylalanine deaminase activity. The major fatty acids were $C_{18:1}\omega7c$, $C_{16:0}$ and $C_{19:0}$ cyclo $\omega8c$. The DNA G+C content was 60.9 mol%. On the basis of the phenotypic and phylogenetic data, strain CG2.1^T appeared to be a member of the genus *Halomonas* and clustered closely with *Halomonas marisflavi* (97.1 % 16S rRNA gene sequence similarity). However, the level of DNA–DNA relatedness between the novel isolate and the most closely related *Halomonas* species was low. On the basis of these data, strain CG2.1^T represents a novel member of the genus *Halomonas*, for which the name *Halomonas indalinina* is proposed. The type strain is CG2.1^T (=CECT 5902^T=LMG 23625^T).

The family *Halomonadaceae* belongs to the class *Gammaproteobacteria*, formerly known as the γ-subgroup of the *Proteobacteria* (Franzmann *et al.*, 1988), and is characterized as being mainly represented by several halophilic, halotolerant and non-halophilic species belonging to different genera. *Halomonas* is the largest genus in this family; it includes more than 30 species, most of which have been isolated from saline environments and some of which have been recognized as having potential applications in biotechnology (Ventosa & Nieto, 1995; Calvo *et al.*, 1998; Margesin & Schinner, 2001; Martínez-Checa *et al.*, 2002; Arias *et al.*, 2003). Moderately halophilic micro-organisms have been subjected to a variety of taxonomical and ecological studies (Oren, 2002) and the genus *Halomonas* has been considered as a model system among the moderate halophiles and has been used extensively for investigations concerning both osmoregulatory mechanisms and physiological adaptations (Ventosa *et al.*, 1998; Nieto *et al.*, 2000). Moreover, this genus is a taxon that comprises strains that are metabolically versatile (García *et al.*, 2004). The genus *Halomonas* encompasses species with an unusually wide range of DNA G+C contents (about 52–68 mol%) and has been divided into two phylogenetic groups on the basis of 16S and 23S rRNA gene sequences (Arahal *et al.*, 2002).

In the course of screening halophilic micro-organisms isolated from the solar salterns of Cabo de Gata, a wildlife reserve located in the province of Almería, southern Spain, a moderately halophilic Gram-negative bacterial organism, strain CG2.1^T, was characterized by using a polyphasic approach. This approach included a phylogenetic analysis based on 16S rRNA gene sequences, an analysis of genomic relatedness and the determination of various chemotaxonomic and phenotypic properties. The results obtained in this study indicated that strain CG2.1^T is a member of the genus *Halomonas* and that it is clearly distinguishable from the other recognized species in the genus.

Water samples were spread on MH complex medium supplemented with a balanced mixture of sea salts to give an adequate salts concentration for the 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 was

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CG2.1^T is AJ427627.

supplemented with the balanced mixture of sea salts described by Subov (1931). The pH was adjusted to 7.2 with 1 M NaOH. The medium was solidified with Bacto agar (Difco) at 20 g 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 were as 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 the growth of strain CG2.1^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 batch of medium was inoculated with 0.1 ml (approx. 10^5 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 on 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 range for growth was determined in a similar way on MH medium by adjusting the final pH to values between 5 and 10, using HCl or NaOH. The temperature range was determined as above by incubating the strain at temperatures from 4 to 45 °C.

Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) were used for morphological studies of cells from a 20 h culture of strain CG2.1^T grown on the surface of MH agar plates covered with MH liquid medium with a salt concentration of 7.5 % (w/v). The cells used for TEM (model EM 902; Zeiss) were stained with 2 % (w/v) phosphotungstic acid. The samples used for SEM (model DSM950; Zeiss) were fixed in a 2 % (v/v) glutaraldehyde solution (pH 7.2), dehydrated in an acetone series, critical-point dried and then coated with gold.

Whole-cell fatty acids of strain CG2.1^T were analysed by the Analytical Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) using the MIDI/Hewlett Packard Microbial Identification System, 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 for analysis and the relative amount of each fatty acid was expressed as percentage of the total fatty acids.

DNA was isolated and purified by using the method of Lind & Ursing (1986). The G+C content (mol%) was determined by using the thermal denaturation method (T_m) (Marmur & Doty, 1962) with a Perkin Elmer Lambda 3B spectrophotometer fitted with a temperature-programme accessory. DNA–DNA hybridization studies were performed with 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 a nick-translation kit (Boehringer Mannheim). Phylogenetic analyses based on the 16S rRNA gene sequence of strain CG2.1^T were performed as described previously (Prado *et al.*, 2006). 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). The phylogenetic tree was constructed by using the neighbour-joining method with the MEGA3 software package (Kumar *et al.*, 2004).

Table 1 provides a comparison of the taxonomic features of strain CG2.1^T and the most closely phylogenetically related species of the genus *Halomonas*. Cells of strain CG2.1^T were Gram-negative rods and were 1.1–1.3 × 0.8 μm in size. They were motile by means of polar flagella. No spores were observed. On MH medium containing 7.5 % (w/v) salts, colonies of strain CG2.1^T were circular with entire margins, flat/slightly convex, smooth and orange-pigmented. Optimum growth occurred at pH 7.2 and 35 °C in MH complex medium containing 7.5–10 % total salts. The range of total salts concentrations in which the bacterium grew at 35 °C was 3–25 % (w/v). Strain CG2.1^T did not grow in the absence of NaCl, which indicated that the isolate could be assigned to the group of moderately halophilic bacteria (Kushner & Kamekura, 1988).

The major cellular fatty acids (i.e. comprising up to 85 %) of strain CG2.1^T were $C_{18:1}\omega7c$ (36.9 %), $C_{16:0}$ (26.8 %), $C_{19:0}$ cyclo $\omega8c$ (15.1 %) and $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH (6.9 %). The DNA G+C content was 60.9 mol% (determined with the T_m method).

The 16S rRNA gene sequence of strain CG2.1^T indicated placement within the family *Halomonadaceae* (Fig. 1). The sequence showed 97.1 % similarity with that of *Halomonas marisflavi* and less than 93 % similarity with those of related species of the genus *Halomonas* (92.7 % with *Halomonas ventosae*, 91.6 % with *Halomonas anticariensis*, 91.6 % with *Halomonas desiderata* and 91.6 % with *Halomonas organivorans*).

Strain CG2.1^T produced phenylalanine deaminase, an enzyme that has been previously described only in *H. desiderata*, *Halomonas salina* and *H. ventosae* (Mata *et al.*, 2002; Martínez-Cánovas *et al.*, 2004a). Moreover, strain CG2.1^T shows several taxonomic and physiological differences with respect to *H. marisflavi* (the most closely related species), such as the reduction of nitrate, the hydrolysis of aesculin, anaerobic growth and acid production from sugars (Table 1). DNA–DNA hybridization between strain CG2.1^T and *H. marisflavi* JCM 10873^T was low (33.1 %).

The genus *Halomonas* includes species that are, in some cases, too different to be considered as belonging to the same genus and thus the genus is currently recognized as being very heterogeneous (Arahal *et al.*, 2002). The 16S rRNA gene sequence similarity for strain CG2.1^T and *H. marisflavi* JCM

Table 1. Differential phenotypic characteristics of strain CG2.1^T and related type strains of species of the genus *Halomonas*

Taxa: 1, strain CG2.1^T; 2, *H. marisflavi*; 3, *H. ventosae*; 4, *H. anticariensis*; 5, *H. desiderata*; 6, *H. organivorans*; 7, *Halomonas taeanensis*; 8, *Halomonas muralis*; 9, *Halomonas pantelleriensis*; 10, *Halomonas campisalis*. Data are from Berendes *et al.* (1996), García *et al.* (2004), Heyrman *et al.* (2002), Lee *et al.* (2005), Mata *et al.* (2002), Martínez-Cánovas *et al.* (2004a, b), Mormile *et al.* (1999), Romano *et al.* (1996), Yoon *et al.* (2001) and this study. +, Positive; −, negative; ND, not described.

Characteristic	1	2	3	4	5	6	7	8	9	10
Cell morphology	Short rod	Rod	Short rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Colony pigmentation	Orange	Yellow	Cream	Cream	Cream	Cream	Cream	Cream	Cream	White
Oxidase	−	−	+	+	+	−	+	+	+	+
NaCl range (%, w/v)	3–25	0.5–27	3–15	0.5–15	0–20	1.5–30	1–25	0–15	1–15	0.5–15
NaCl optimum (%, w/v)	7.5–10	0.5–12	6–9	7.5	1–5	7.5–10	10–12	2.5–10	10	5
pH range	5–9	5–10	6–10	6–9	7–11	6–10	7–10	5–10	6–11	8–11
Temperature range (°C)	15–40	4–37	15–50	20–45	10–45	15–45	10–45	10–35	10–45	4–50
Anaerobic growth	−	+	−	−	−	−	−	−	−	−
Nitrate reduction	+	−	+	+	+	ND	+	+	+	+
Acid production from:										
L-Arabinose	+	+	−	−	−	ND	+	ND	+	−
D-Galactose	+	ND	−	−	−	ND	ND	ND	+	−
D-Glucose	+	+	−	−	−	ND	+	ND	+	−
Lactose	−	+	−	−	−	ND	+	ND	−	−
Maltose	−	+	−	−	−	ND	+	ND	+	−
D-Mannose	−	+	−	−	−	ND	+	ND	+	−
D-Salicin	−	+	−	−	−	ND	−	ND	+	−
Sucrose	−	+	−	−	−	ND	ND	ND	+	−
D-Trehalose	−	+	−	−	−	ND	+	ND	+	−
Hydrolysis of:										
Aesculin	−	+	−	−	−	+	−	+	+	−
Gelatin	+	+	−	−	−	−	−	−	−	−
Urea	−	−	−	+	+	ND	+	ND	−	−
Tween 20	+	−	+	+	+	ND	ND	ND	+	+
Tween 80	−	−	−	−	+	+	−	ND	+	−
Phenylalanine deaminase	+	−	+	−	+	ND	ND	ND	−	−
H_2S production	−	−	+	−	+	+	ND	ND	+	−
DNA G+C content (mol%)	60.9	59.0	74.3	61.4	66.0	61.0	65.0	62.4	65.0	66.0

10873^T compared with other species of the genus *Halomonas* suggests that these two micro-organisms could be placed within a novel genus. However, the results obtained in the phenotypic and chemotaxonomic analyses make it possible to assign strain CG2.1^T to the genus *Halomonas*. The placement of strain CG2.1^T and *H. marisflavi* within the genus *Halomonas* might have to be changed if the genus is taxonomically re-evaluated, but this would have to be given very careful consideration in order to avoid excessive and unnecessary renaming.

On the basis of the morphological, phenotypic and genotypic data, strain CG2.1^T represents a novel species within the genus *Halomonas*, for which the name *Halomonas indalinina* sp. nov. is proposed.

Description of *Halomonas indalinina* sp. nov.

Halomonas indalinina (in.da.li.ni′na. N.L. n. *indalo -inis* a prehistoric magical symbol; L. suff. *-inus -a -um* suffix used in the sense of 'belonging to'; N.L. fem. adj. *indalinina* pertaining to the Indalo, the symbol of the province of Almería, Spain, from where the type strain was isolated).

Cells are straight or slightly curved short rods 1.1–1.3 × 0.8 μm in size. Cells stain Gram-negative and are motile by means of polar flagella. Non-spore-forming. On MH complex solid medium containing 7.5 % (w/v) total salts (mixture of sea salts), the bacteria grow in orange, flat/slightly convex, smooth and circular/slightly irregular colonies. Moderately halophilic; growth occurs in total salts concentrations of 3–25 % (w/v), with optimum growth at 7.5–10 % (w/v). No growth in the absence of salt. Growth occurs at 15–40 °C and at pH 5–9. Strictly aerobic. Catalase is produced and gelatin and Tween 20 are hydrolysed. Negative in tests for oxidase and indole and in methyl red and Voges–Proskauer tests. Casein, starch, aesculin, DNA, tyrosine and Tween 80 are not hydrolysed. Phosphatase and phenylalanine deaminase are produced, but urease is not produced. Nitrate is reduced to nitrite. H_2S is not produced

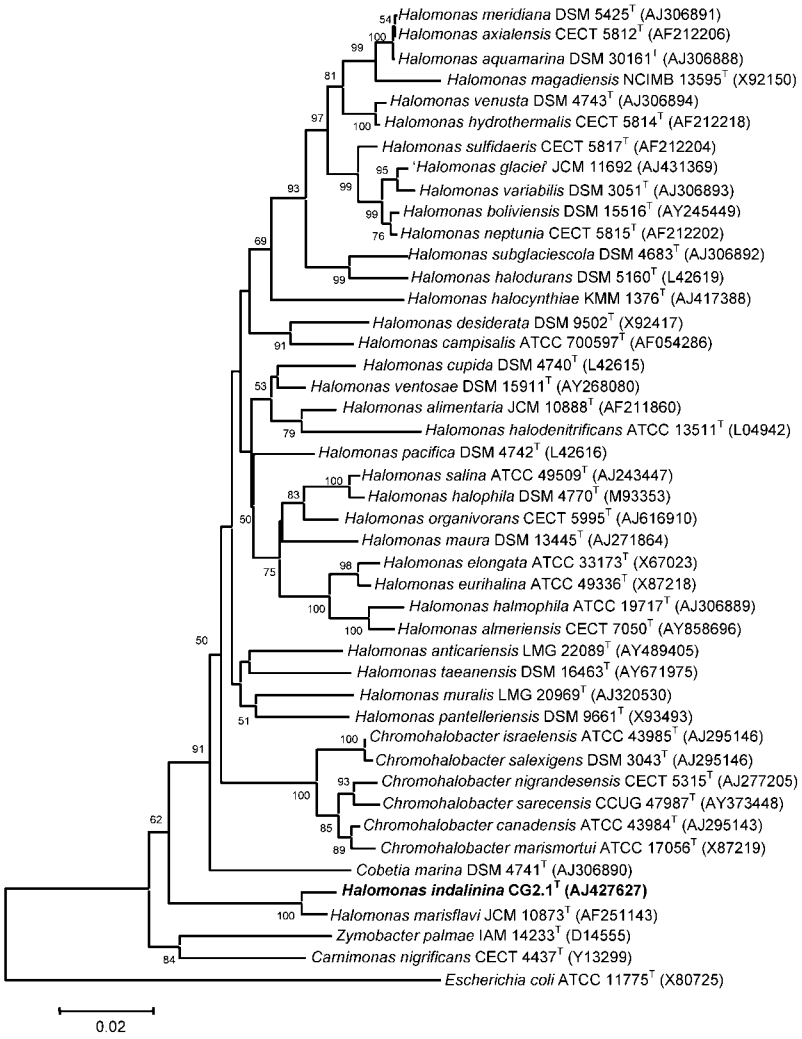

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships between strain CG2.1^T and species of the genera *Halomonas*, *Chromohalobacter*, *Zymobacter*, *Cobetia* and *Carnimonas*. GenBank accession numbers are given in parentheses. *Escherichia coli* was used as the outgroup. Bootstrap percentages greater than 50 % are shown. Bar, 0.02 substitutions per nucleotide position.

from L-cysteine. Acids are produced from L-arabinose, D-galactose and D-glucose, but not from D-cellobiose, lactose, maltose, D-mannose, D-salicin, sucrose, D-trehalose or D-xylose. The following organic compounds are utilized as sole carbon and energy sources: acetate, adonitol, L-arabinose, D-cellobiose, citrate, formate, D-fructose, fumarate, D-galactose, gluconate, D-glucose, lactate, lactose, malate, maltose, D-mannitol, D-mannose, pyruvate and sucrose. *myo*-Inositol, propionate, L-rhamnose, D-sorbitol and D-xylose are not utilized. The following compounds are used as sole carbon, nitrogen and energy sources: L-arginine, isoleucine, L-lysine, L-serine and L-valine. Alanine, L-histidine, L-ornithine and L-tryptophan are not utilized. Susceptible to cefoxitin, chloramphenicol, neomycin, polymyxin B and rifampicin. The major fatty acids are $C_{18:1}\omega7c$, $C_{16:0}$ and $C_{19:0}$ cyclo $\omega8c$ when cells are grown on MH medium containing 7.5 % (w/v) total salts. The DNA G+C content is 60.9 mol% (T_m).

The type strain, CG2.1^T (=CECT 5902^T=LMG 23625^T), was isolated from a water sample from a solar saltern at Cabo de Gata, Almería, Spain.

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