Chromohalobacter japonicus sp. nov., a moderately halophilic bacterium isolated from a Japanese salty food

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A Gram-negative, non-spore-forming, rod-shaped, motile bacterium, designated strain 43^T, was isolated from a Japanese salty food and then subjected to a polyphasic taxonomic study. Strain 43^T is moderately halophilic, growing at NaCl concentrations in the range 5–25% (w/v), with optimum growth between 7.5 and 12.5% (w/v) NaCl. Growth occurs at temperatures from 15 to 42 °C (optimally at 28–37 °C) and at pH 5.5–9.0 (optimally at pH 7.0–8.0). A phylogenetic analysis based on 16S rRNA gene sequence comparisons revealed that strain 43^T belongs to the genus *Chromohalobacter*. The closest relatives were *Chromohalobacter canadensis* ATCC 43984^T (99.3% 16S rRNA gene sequence similarity), *Chromohalobacter beijerinckii* ATCC 19372^T (99.1%), *Chromohalobacter sarecensis* LV4^T (98.3%), *Chromohalobacter nigrandesensis* LTS-4N^T (97.9%) and *Chromohalobacter marismortui* ATCC 17056^T (97.9%). The DNA G+C content was 62.9 mol%, which is within the range described for the genus *Chromohalobacter*. DNA–DNA hybridization studies between strain 43^T and *C. canadensis* CECT 5385^T and *C. beijerinckii* DSM 7218^T showed 38 and 49% relatedness, respectively; lower DNA–DNA hybridization percentages were obtained with respect to other related *Chromohalobacter* species. The major fatty acids of strain 43^T were C_{16:0}, C_{19:0} cyclo ω 8c and C_{12:0} 3-OH. Overall, the phenotypic, genotypic and phylogenetic results demonstrated that strain 43^T represents a novel species within the genus *Chromohalobacter*. The name *Chromohalobacter japonicus* sp. nov. is proposed, with strain 43^T (=CECT 7219^T =CCM 7416^T) as the type strain.

The family *Halomonadaceae* includes the genera *Halomonas*, *Carnimonas*, *Chromohalobacter*, *Cobetia* and *Zymobacter*. These genera are members of the *Gammaproteobacteria*, within the order *Oceanospirillales* (Arahal & Ventosa, 2006; Garrity *et al.*, 2005). The genus *Chromohalobacter* was proposed by Ventosa *et al.* (1989) with a single species, *Chromohalobacter marismortui*, in order to include a halophilic bacterium previously described as ‘*Chromobacterium marismortui*’ (isolated from the Dead Sea by Elazari-Volcani, 1940) and several isolates from a saltern in Spain. Currently, the genus *Chromohalobacter* includes the species *Chromohalobacter canadensis* (Arahal *et al.*, 2001a), *C. israelensis* (Arahal *et al.*, 2001a), *C. salexigens* (Arahal *et al.*, 2001b), *C. sarecensis* (Quillaguamán *et al.*, 2004) and *C. nigrandesensis* (Prado *et al.*, 2006). Recently, Peçonek *et al.* (2006) proposed the

reclassification of *Pseudomonas beijerinckii* (Hof, 1935) as *Chromohalobacter beijerinckii* and emended the description of this species. The genus *Chromohalobacter* includes Gram-negative, straight or slightly curved, motile rods that are moderately halophilic, i.e. with optimum salt concentrations for growth between 8 and 10% NaCl. They are aerobic, catalase-positive and (in most species) oxidase-negative. They have been isolated from hypersaline environments such as the Dead Sea, salterns and saline soils (Ventosa, 2005). This genus represents a coherent phylogenetic cluster closely related to the genus *Halomonas* (Mellado *et al.*, 1995; Arahal *et al.*, 2002).

In this study, we have determined the taxonomic position of strain 43^T, which was isolated from a Japanese salty food in Shiokara (Onishi *et al.*, 1980), as a novel member of the genus *Chromohalobacter*.

Strain 43^T was isolated in SGC medium containing 4 M NaCl (Onishi *et al.*, 1980). The strain was cultivated

The GenBank/EMBL/DBJ accession number for the 16S rRNA gene sequence of strain 43^T is AB105159.

in SW10 medium with 10 % (w/v) total salts (8.1 % NaCl, 0.7 % MgCl₂, 0.96 % MgSO₄, 0.036 % CaCl₂, 0.2 % KCl, 0.006 % NaHCO₃, 0.0026 % NaBr, 0.5 % yeast extract; Difco) (Ventosa *et al.*, 1982). The pH was adjusted to 7.2 with 1 M KOH. When necessary, solid media were prepared by adding 2.0 % (w/v) Bacto agar (Difco).

Optimal conditions for growth were determined by growing strain 43^T in SW medium at 0, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20, 25 and 30 % (w/v) total salts at temperatures of 4, 15, 20, 25, 30, 37, 40, 42 and 45 °C. The pH range for the isolate was tested in SW10 medium adjusted to pH 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 using the appropriate buffers. The cells were cultivated with constant agitation (180 r.p.m.) and growth was monitored by measuring optical density at 600 nm. The cells of strain 43^T were found to be motile, Gram-negative, non-spore-forming rods. The cells were 1.2–4.5 µm long and 0.4 µm wide at the exponential phase of growth in SW10 medium at 37 °C. Optimal growth occurred at 7.5–12.5 % (w/v) total salts, 28–37 °C and pH 7.0–8.0; the ranges for growth for strain 43^T are shown in Table 1.

To characterize the isolate phenotypically, standard tests (including those for determining the Gram reaction, cell morphology, motility and catalase and oxidase activities) were performed, along with other tests shown in Table 1 or included in the species description. Macroscopic properties were determined using the classical characterization of colony appearance. Biochemical tests were carried out at 10 % total salts and 37 °C, unless stated otherwise. Catalase activity was determined by adding a 1 % (w/v) H₂O₂ solution to colonies on SW10 agar medium. An oxidase test was performed using the Dry Slide (Difco). Hydrolysis of starch, gelatin and Tween 80 and the production of urease were determined as described by Cowan & Steel (1965) with the addition of 10 % total salts to the medium. Citrate utilization was determined on Simmons' citrate medium supplemented with SW10. Acid production from carbohydrates was determined using phenol red base supplemented with 1 % of the carbohydrate and SW10. Growth under anaerobic conditions was determined by incubating strain 43^T in SW10 medium in an anaerobic chamber. Tests for sugar fermentation and enzymes were carried out using API 20NE and API ID 32E kits

Table 1. Characteristics that serve to distinguish strain 43^T from recognized species of the genus *Chromohalobacter*

Strains: 1, strain 43^T; 2, *C. marismortui* ATCC 17056^T; 3, *C. canadensis* ATCC 43984^T; 4, *C. beijerinckii* DSM 7218^T; 5, *C. israelensis* ATCC 43985^T; 6, *C. salexigens* DSM 3043^T; 7, *C. sarecensis* LV4^T; 8, *C. nigrandesensis* DSM 5349^T. Data are from Arahal *et al.* (2001a, b), Peçonek *et al.* (2006), Prado *et al.* (2006), Quillaguamán *et al.* (2004) and this study. +, Positive, –, negative, +/–, weak reaction; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8
Pigmentation	Cream	Brown–yellow	White	Light yellow	Cream	White–cream	Brown	Black
NaCl range for growth (% w/v)	5–25	2–30	3–25	0.35–25	3.5–20	0.9–25	0–25	0.5–25
Optimum NaCl concentration (% w/v)	7.5–12.5	10	8	8–10	8	7.5–10	8	7.5
Temperature range (°C)	15–42	5–45	15–45	4–42	15–45	4–45	0–45	15–45
Optimum temperature (°C)	28–37	37	30	30	30	37	30–35	32
pH range	5.5–9	5–10	5–9	4.5–8	5–9	5–9	5–10	5–10
Optimum pH	7–8	7.5	ND	7.5	ND	7.5	7.5–8.0	ND
Oxidase activity	–	+	–	+	–	–	+	–
Acid production from:								
Arabinose	–	–	+	+	+	+	–	+
Maltose	+	+	–	–	+	+	–	–
Sucrose	+	+	–	–	+	+	–	+
Trehalose	–	+	–	–	–	–	–	ND
Urease	–	–	–	–	–	+	–	+
Simmons' citrate	+	–	+	+	ND	+	–	+
Nitrate reduction	+	–	+	+	+	+	–	–
H ₂ S production	–	–	–	–	–	+	–	+
Indole production	–	–	+	–	+	–	–	ND
Hydrolysis of:								
Casein	–	–	–	–	–	+	–	ND
Aesculin	–	–	+	–	+	+/–	–	–
Gelatin	+	–	–	–	–	+/–	–	–
Lysine decarboxylase	–	–	+	–	+	–	ND	ND
Ornithine decarboxylase	+	–	+	–	+	–	ND	ND
DNA G + C content (mol%)	62.9	62.3	62	60.7	65	64.2	56.1	59.8

(bioMérieux) inoculated according to the manufacturer's instructions, using the inoculated fluid at 10 % NaCl and with incubation at 37 °C.

The nutritional requirements of the isolate were determined using Biolog microplates. Strain 43^T was grown on SW10 medium at 37 °C for 48 h and suspended in a 10 % NaCl (w/v) solution, within the density range specified by the manufacturer (model 21101 photometer; Biolog). Immediately after the cells had been suspended in the saline solution, the suspensions were transferred into sterile multichannel pipette reservoirs (Biolog) and the Biolog GN microplates were inoculated with 125 µl cell suspension per well by means of an eight-channel repeating pipette. The inoculated Biolog plates were incubated at 37 °C for 7 days and the results were read with a MicroPlate Reader using Microlog 3.59 software to perform automated readings. Antibiotic susceptibility was determined according to the conventional Kirby–Bauer method (Bauer *et al.*, 1966). The phenotypic characteristics of strain 43^T are included in the species description.

Chromosomal DNA was isolated and purified according to the method described by Marmur (1961). The 16S rRNA gene was amplified using the universal primers 16F27 and 16R1488, as described by Mellado *et al.* (1995). The almost-complete nucleotide sequence was determined by NBT-Newbiotechnics (Seville, Spain) using an automated DNA sequencer (model 3100; Applied Biosystems) and was compared with reference 16S rRNA gene sequences retrieved from the GenBank and EMBL databases by BLAST searching. The subsequent sequence analysis was conducted using the ARB program package (Ludwig *et al.*, 2004). Several treeing methods (maximum parsimony, distance matrix and maximum likelihood) were performed on the basis of the recommendations of Ludwig *et al.* (1998). A comparison based on the 16S rRNA gene sequences from the databases revealed that the sequence (1501 bp) of strain 43^T displays the highest levels of similarity with those from

Chromohalobacter species. The closest relatives were *C. canadensis* ATCC 43984^T (99.3 % sequence similarity), *C. beijerinckii* ATCC 19372^T (99.1 %), *C. sarecensis* LV4^T (98.3 %) and *C. marismortui* ATCC 17056^T (97.9 %). Sequence similarities with respect to the other *Chromohalobacter* species were ≤96.6 %. The phylogenetic tree obtained by using the maximum-parsimony method shows strain 43^T within the branch constituted by the *Chromohalobacter* species (Fig. 1). These results were consistent when other algorithms were used to construct phylogenetic trees.

Fatty acids were analysed at the BCCM/LMG (Ghent, Belgium) using GC (Kämpfer & Kroppenstedt, 1996; Miller, 1982). Cells were cultured on TSA supplemented with 10 % NaCl (w/v) at pH 7.0 and 37 °C for 24 h. The predominant fatty acids of strain 43^T were C_{16:0}, C_{19:0} cyclo ω8c and C_{12:0} 3-OH. This composition is very similar to those described for recognized *Chromohalobacter* species, except that the C_{18:1}ω7c content is lower than for *C. beijerinckii* and *C. marismortui* (Table 2).

The G + C content of the genomic DNA was determined from the mid-point value (*T_m*) of the thermal denaturation profile (Marmur & Doty, 1962) using the equation of Owen & Hill (1979), as previously described in detail by Ventosa *et al.* (1999). The DNA G + C content of strain 43^T was found to be 62.9 mol%, which is within the range for species belonging to the genus *Chromohalobacter* (Ventosa, 2005).

DNA–DNA hybridization studies were performed by using the competition procedure of the membrane method (Johnson, 1994), described in detail by Mormile *et al.* (1999). The hybridization temperature was 58.1 °C, which is within the limit of validity for the filter method (De Ley & Tijtgat, 1970), and the percentage of hybridization was calculated according to Johnson (1994). The experiments were carried out in triplicate. The percentage of DNA–DNA hybridization between strain 43^T and *C. canadensis*

Fig. 1. Maximum-parsimony phylogenetic tree, based on 16S rRNA gene sequences of strain 43^T and closely related species. Sequence accession numbers used are shown in parentheses. Bootstrap values >50 % are indicated at branch-points. *Zymobacter palmae* T109^T was used as an outgroup. Bar, 1 % sequence divergence.

Table 2. Cellular fatty acid compositions (%) of strain 43^T, *C. beijerinckii* DSM 7218^T and *C. marismortui* DSM 6770^T

Data for *C. marismortui* DSM 6770^T and *C. beijerinckii* DSM 7218^T were taken from Peçonek *et al.* (2006). ND, Not detected; ECL, equivalent chain-length.

Fatty acid	Strain 43 ^T	<i>C. beijerinckii</i> DSM 7218 ^T	<i>C. marismortui</i> DSM 6770 ^T
C _{10:0}	3.3	2.1	2.2
C _{12:0}	ND	ND	0.2
Unknown ECL 11.799	0.2	ND	0.4
C _{12:0}	4.3	3.1	3.6
C _{12:0} 3-OH	10.4	7.1	8.2
C _{14:0}	0.6	0.4	ND
Summed feature 3*	1.8	5.0	6.0
C _{16:0}	34.6	27.6	18.0
C ₁₇ cyclo	7.5	10.4	3.6
C _{18:1} ω7c	5.0	11.0	29.8
C _{18:0}	1.4	0.4	0.8
C _{18:1} ω7c 11-methyl	0.4	1.0	1.2
C ₁₉ cyclo ω8c	29.8	31.4	25.5
C _{20:2} ω6,9c	0.3	0.6	0.7

*Summed features are groups of fatty acids that could not be resolved chromatographically; summed feature 3 consists of C_{16:1}ω7c and/or C_{15:0} iso 2-OH.

CECT 5385^T was 38 %, while those between strain 43^T and *C. beijerinckii* DSM 7218^T, *C. marismortui* ATCC 17056^T, *C. sarecensis* LV4^T and *C. nigrandesensis* LTS-4N^T were 49, 33, 32 and 28 %, respectively. These levels of DNA–DNA hybridization are low enough to justify classifying strain 43^T as a member of a genotypically distinct species within the genus *Chromohalobacter* (Stackebrandt & Goebel, 1994; Stackebrandt *et al.*, 2002). In addition, these genotypic differences are supported by the presence of phenotypic features that can be used to differentiate strain 43^T from related species of the genus *Chromohalobacter* (Table 1).

Overall, the phenotypic, phylogenetic and genotypic results presented in this study demonstrate that strain 43^T represents a novel species within the genus *Chromohalobacter*, for which the name *Chromohalobacter japonicus* sp. nov. is proposed.

Description of *Chromohalobacter japonicus* sp. nov.

Chromohalobacter japonicus (ja.po'ni.cus. N.L. masc. adj. *japonicus* Japanese).

Cells are Gram-negative, non-spore-forming, straight or sometimes slightly curved, motile rods that are 0.4 × 1.2–4.5 µm in size and occur singly, in pairs and in short chains. Colonies on SW10 medium after 2 days incubation

at 37 °C are 0.5 mm in diameter, cream, circular, regular and convex with an entire margin. Moderately halophilic, growing at NaCl concentrations in the range 5–25 % (w/v), with an optimum between 7.5 and 12.5 (w/v) NaCl. Growth occurs at temperatures from 15 to 42 °C (optimally at 28–37 °C) and at pH 5.5–9.0 (optimally at pH 7.0–8.0). Strictly aerobic. Catalase is produced. Negative for indole production, the Voges–Proskauer test, H₂S production, starch, casein, aesculin, DNA and Tween 80 hydrolysis, urease activity and oxidase activity. Gelatin is hydrolysed. Methyl red-positive. Nitrate is reduced to nitrite but nitrite is not reduced. Positive for the Simmons' citrate test. Acid is produced from D-galactose, mannose, D-glucose, maltose, xylose, galacturonate, adonitol, palatinose, sucrose, D-arabitol, L-rhamnose, inositol, D-cellobiose and D-sorbitol but not from arabinose, D-fructose, glycerol, D-mannitol, trehalose, sorbitol, L-arabitol or 5-ketogluconate. Positive for ornithine decarboxylase and L-aspartic acid arylamidase. Negative for arginine dihydrolase, β-galactosidase, lysine decarboxylase, β-glucosidase, β-glucuronidase, N-acetyl-β-glucosaminidase, α-glucosidase, α-galactosidase and α-maltosidase. The following compounds are used as sole carbon and energy sources (Biolog): L-arabinose, D-fructose, D-galactose, gentiobiose, α-D-glucose, D-mannitol, D-psicose, L-rhamnose, *cis*-aconitic acid, citric acid, D-gluconic acid, D-glucosaminic acid, α-ketoglutaric acid, DL-lactic acid, succinamic acid, D-alanine, L-alanyl glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, L-histidine, L-proline, L-pyroglyutamic acid, L-serine, L-threonine and glycerol. The following compounds are not used as sole carbon and energy sources (Biolog): α-cyclodextrin, dextrin, glycogen, Tween 40, Tween 80, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, adonitol, D-arabitol, cellobiose, i-erythritol, L-fucose, *myo*-inositol, α-D-lactose, lactulose, maltose, D-mannose, D-melibiose, methyl β-D-glucoside, D-raffinose, D-sorbitol, sucrose, trehalose, turanose, xylitol, methyl pyruvate, monomethyl succinate, acetic acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-glucuronic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, *p*-hydroxyphenylacetic acid, itaconic acid, α-ketobutyric acid, α-ketovaleric acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, succinic acid, bromosuccinic acid, glucuronamide, alaninamide, L-alanine, hydroxy-L-proline, L-leucine, L-ornithine, L-phenylalanine, D-serine, DL-carnitine, γ-aminobutyric acid, urocanic acid, inosine, uridine, thymidine, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, DL-α-glycerol phosphate, glucose 1-phosphate and glucose 6-phosphate. Resistant to (µg per disc, unless specified otherwise): penicillin G (10 U), bacitracin (10 U), cephalothin (30), rifampicin (30), streptomycin (10), neomycin (10), erythromycin (15), kanamycin (30), vancomycin (30), nalidixic acid (30), novobiocin (30), polymyxin B (300 U) and chloramphenicol (30). Susceptible to sulfamethoxazole/trimethoprim (23.75/1.25 µg per disc). The major fatty acids are C_{16:0}, C_{19:0} cyclo ω8c and C_{12:0} 3-OH. The DNA G + C content is 62.9 mol%.

The type strain, 43^T (=CECT 7219^T =CCM 7416^T), was isolated from a Japanese salty food.

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