Chryseobacterium haifense sp. nov., a psychrotolerant bacterium isolated from raw milk

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Strain H38^T was isolated during a study on the diversity of culturable psychrotolerant bacteria in raw milk. Comparative 16S rRNA gene sequence analysis showed that strain H38^T belongs to the genus *Chryseobacterium* (family *Flavobacteriaceae*, phylum *Bacteroidetes*), with the highest similarity (96.7 %) to *Chryseobacterium hispanicum* VP48^T. Strain H38^T grows aerobically, at 4–41 °C, with 0–2.5 % NaCl and at pH 6.5–10.5. Light induces the formation of yellow carotenoid pigments. The dominant cellular fatty acids are 15:0 iso (41.6 %), 15:0 anteiso (16.6 %) and 17:0 iso 3-OH (10.3 %). The G + C content of the DNA is 37.8 mol%. On the basis of its phenotypic properties and phylogenetic distinctiveness, strain H38^T (=LMG 24029^T =DSM 19056^T) is classified in the genus *Chryseobacterium* as the type strain of a novel species, for which the name *Chryseobacterium haifense* sp. nov. is proposed.

The genus *Chryseobacterium* is a member of the family *Flavobacteriaceae*, phylum *Bacteroidetes*, and currently consists of 19 species, with *Chryseobacterium gleum* as the type species (Vandamme *et al.*, 1994). Two additional species were recently transferred to the new genus *Elizabethkingia* (Kim *et al.*, 2005b). Most members of the genus *Chryseobacterium* are widely distributed in aquatic and soil environments. Other *Chryseobacterium* species are pathogenic to humans and animals (Bernardet *et al.*, 2002, 2005, 2006). *Chryseobacterium* strains are also part of the psychrotolerant and proteolytic bacterial population that causes a variety of defects in food products such as processed meats, chilled fish and shellfish and dairy products (González *et al.*, 2000; Hugo *et al.*, 1999, 2003).

Strain H38^T was isolated in the course of a study on the diversity of culturable psychrotolerant bacteria in raw milk. Raw milk from four farms in Israel was sampled every other month from April 2004 to January 2005. The samples were serially diluted and plated on sterile standard plate count (SPC) agar (Oxoid CM0463). Plates were incubated at 7 °C for 10 days and colonies that displayed distinct morphologies were subcultured to obtain pure cultures. Among about 300 pure cultures isolated in this study, strain H38^T was isolated on SPC agar during the sampling of April 2004. A comparative analysis of 16S rRNA gene sequences indicated that strain H38^T was a member of the genus *Chryseobacterium*. In order to determine its exact taxonomic position, a polyphasic taxonomic study was carried out.

The universal bacterial primers 8f and 1512r (based on *Escherichia coli* positions) were used to amplify internal fragments of the 16S rRNA gene according to Felske *et al.* (1997). The amplified PCR product (approx. 1.5 kb) was purified using a Wizard PCR product purification kit (Promega). Purified PCR products were sequenced at Technion Medical School (Haifa, Israel) directly by the dideoxynucleotide chain-termination method using a DNA sequencer (ABI PRISM 3100) with BigDye terminator reagents (Applied Biosystems) according to the manufacturer's instructions. Sequencing was performed by using primers 8f (5'-GGATCCAGACTTTGATYMTGGCTCAG-3'), 534r (5'-ATTACCGCGGCTGCTGG-3'), 968f (5'-AACGCGAAGAACCCTTAC-3') and 1512f (5'-GTGAAGCT-TACGGYTAGCTTGTTACGACTT-3'). The newly determined sequence (1480 bp) was compared to those available in EMBL (<http://www.ebi.ac.uk>) using the Washington University BLAST program (version 2.0). Sequence alignment was performed using the CLUSTAL W program and a phylogenetic tree (Fig. 1) was generated using the neighbour-joining method in the MEGA3 software (Kumar *et al.*, 2004).

Strain H38^T was closely related to representatives of the genus *Chryseobacterium*. The highest sequence similarity was 96.7 %, with *Chryseobacterium hispanicum* VP48^T, and similarity values were less than 96 % with the type strains of other *Chryseobacterium* species. The similarity between strain H38^T and members of the genera *Riemerella* and *Elizabethkingia* was less than 93.8 % (Fig. 1).

For electron microscopy, bacteria were suspended in saline, fixed to a carbon-coated grid, stained with 2 % uranyl

The GenBank/EMBL/DBJ accession number for the 16S rRNA gene sequence of strain H38^T is EF204450.

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic positions of strain H38^T, other species of the genus *Chryseobacterium* and related taxa. Bootstrap values (>50%) resulting from 1000 replicates are indicated at branching nodes. Bar, 0.01 substitutions per nucleotide position.

acetate and photographed using a JEM-1200EX electron microscope (JEOL). Electron microscopy showed that the cells were non-flagellated rods, 0.6–0.9 µm wide and 1.3–2.5 µm long.

For phenotypic characterization, SPC agar was used as the basal growth medium. Salt tolerance was determined on SPC agar containing varying concentrations of NaCl (0–5% w/v at 0.5% intervals) at 30 °C. Growth at 4, 7, 18, 22, 25, 30, 32, 37, 40, 42, 43 and 45 °C was measured on SPC agar. The pH range for growth was determined using SPC adjusted to various pH values (pH 3.5–10.5 at 0.5 unit intervals). The pH was adjusted prior to sterilization by the addition of HCl or NaOH and measured again after sterilization. Growth under anaerobic conditions was determined after incubation in an anaerobic chamber on SPC agar supplemented with 0.5% (w/v) glucose or 0.1% (w/v) potassium nitrate. Biochemical tests were performed by using API 20E, API 20NE, API 50CH and API ZYM identification systems (bioMérieux) according to the manufacturer's instructions, except that the incubation temperature was 32 °C. Catalase activity was examined by bubble production in a 3% (v/v) hydrogen peroxide solution. Oxidase activity was determined using 1% *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride (Sigma-Aldrich; T3134). Growth was tested on MacConkey agar (Difco) and cetrimide agar (Himedia). Haemolytic activity was determined using blood agar base supplemented with 5% sheep blood (Hylabs). Hydrolysis of casein and tributyrin was determined using 1% skimmed milk agar supplemented with 0.5% yeast extract and tributyrin agar (Hylabs), respectively. The phenotypic traits of strain H38^T are given in the species description and in Table 1.

As all *Chryseobacterium* species tested so far produce flexirubin pigments, cell mass of strain H38^T was subjected to the KOH test as described by Fautz & Reichenbach (1980) and Bernardet *et al.* (2002). A very weak colour

change was observed, demonstrating that minor amounts of flexirubin-type pigments are produced. The yellow pigments were extracted and investigated according to Peterson *et al.* (1954). Briefly, the isolate was grown on LB agar at room temperature under light and dark conditions for 48 h. Cells were transferred to acetone, vortexed for 1 min, allowed to stand for 10 min and then centrifuged for 3 min at 13 000 r.p.m. Spectral analysis from 250 to 700 nm was performed on the acetone extract. A broad peak with two shoulder peaks at 455 and 482 nm typical of carotenoids was observed. Hence, the yellow colour of strain H38^T mostly results from the production of carotenoid pigments under light conditions.

For cellular fatty acid analysis, cells were cultured on tryptic soy agar (Difco) for 24 h at 28 °C and then the fatty acids were extracted and methylated (Ben-Ze'ev *et al.*, 2005). The fatty acid methyl esters were analysed by gas chromatography using the MIDI/Hewlett Packard microbial identification system (Analytical Services Inc.). The major fatty acids (>10%) of strain H38^T were 15:0 iso (41.6%), 15:0 anteiso (16.6%) and 17:0 iso 3-OH (10.3%).

For determination of the DNA G+C content, genomic DNA of strain H38^T was prepared according to a modification of the procedure of Wilson (1987). The DNA G+C content was determined using HPLC analysis of hydrolysed DNA according to Mesbah *et al.* (1989). The analysis was performed by the BCCM/LMG Bacteria Collection Identification Service (Laboratory of Microbiology, Ghent University, Belgium). The G+C content of strain H38^T was 37.8 mol%.

Strain H38^T was isolated in the course of a study of the psychrotolerant bacterial community in raw milk and of its proteolytic and lipolytic activities (unpublished results). Five per cent of the isolates were members of the genus *Chryseobacterium* that possessed a variety of proteolytic and lipolytic activities. Of them, four strains shared 97.5–100.0% 16S rRNA gene sequence similarity with

Table 1. Differentiating characteristics of strain H38^T and other *Chryseobacterium* species

Taxa: 1, strain H38^T (*C. haifense* sp. nov.); 2, *C. gleum* (n=12); 3, *C. balustinum*; 4, *C. indologenes* (n=13); 5, *C. indoltheticum*; 6, *C. scopthalmum* (n=7); 7, *C. defluvii*; 8, *C. joostei* (n=11); 9, *C. daecheongense*; 10, *C. formosense*; 11, *C. taichungense*; 12, *C. shigense*; 13, *C. vrystaatense* (n=36); 14, *C. soldanellicola*; 15, *C. taeanense*; 16, *C. piscium* (n=4); 17, *C. hispanicum*; 18, *C. wanjuae*; 19, *C. taiwanense*; 20, *C. caeni*. Data from Yabuuchi *et al.* (1983), Holmes *et al.* (1984), Hugo *et al.* (2003), Kämpfer *et al.* (2003), de Beer *et al.* (2005, 2006), Kim *et al.* (2005a), Shen *et al.* (2005), Shimomura *et al.* (2005), Young *et al.* (2005), Gallego *et al.* (2006), Park *et al.* (2006), Tai *et al.* (2006), Weon *et al.* (2006), Quan *et al.* (2007) and this study. The number of strains examined is shown in parentheses when greater than one (data for other species are for the type strain only). +, Positive; w, weakly positive; –, negative; v, variable; NA, no data available.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Pigment type(s)*	F (w), C†	F	F	F	F	F	NA	F	F	F	F	F	F	NA	NA	F	F (w)	F	F	F
Growth on/at:																				
3 % NaCl	–	NA	w	+	w	w	–	NA	NA	NA	NA	NA	–	+	+	+	–	–	+	+
Cetrimide agar	–	–	–	+	–	–	–	+	NA	NA	NA	NA	+	NA	NA	–	NA	NA	NA	NA
MacConkey agar	–	+	+	+	+	–	–	+	–	–	–	–	–	–	–	–	–	w	NA	–
5 °C	+	–	–	–	+	+	–	+	–	–	–	+	+	+	+	+	+	+	+	+
37 °C	+	+	+	+	–	–	+	–	+	+	+	–	–	+	+	–	–	+	+	+
42 °C	–	–	–	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	+	–
Nitrate reduction	–	NA	+	v	–	–	–	–	+	–	–	–	–	–	–	+	+	–	–	–
Urease activity	–	v	–	–	–	+	–	v	–	–	–	–	+	–	–	+	–	–	–	+
β-Galactosidase activity	+	v	–	–	–	+	NA	–	–	–	w	NA	NA	–	–	NA	+	+	–	+
Production of:																				
H ₂ S	–	–	–	–	+	–	–	–	–	–	–	–	v	–	–	–	–	NA	–	–
Indole	+	+	+	+	+	–	+	+	–	+	w	+	+	–	–	+	w	NA	+	–
Acid production from:																				
Glucose	+	+	+	+	NA	–	+	+	–	w	w	+	NA	NA	NA	NA	+	+	–	–
L-Arabinose	–	+	NA	v	–	–	–	–	–	–	–	–	NA	–	–	NA	NA	–	+	–
D-Fructose	+	+	+	+	–	–	NA	+	+	NA	NA	+	NA	–	–	NA	+	–	v	–
Lactose	+	–	–	–	–	–	NA	–	–	NA	–	–	NA	–	–	NA	–	–	–	–
Maltose	+	+	–	+	+	–	+	+	–	–	+	–	NA	–	–	NA	+	–	–	–
D-Mannitol	–	–	–	v	NA	–	–	v	–	–	–	–	NA	–	–	NA	–	–	–	–
Trehalose	–	+	–	+	–	–	+	+	+	+	+	–	NA	–	–	NA	–	+	–	–
D-Xylose	–	v	–	–	–	–	–	–	+	w	+	–	NA	–	–	NA	+	–	NA	–
DNA G + C content (mol%)‡	37.8	37.6	33.1	38.5	33.8	34.2	38.8	36.7	36.6	NA	NA	36.6	37.1	28.8	32.1	33.6	34.3	37.8	36.8	38.2

*The type of yellow pigment (when determined) is indicated by F (flexirubin) or C (carotenoid).

†Yellow pigments are produced only when bacteria are grown under light conditions.

‡Data for type strains (where available).

Chryseobacterium joostei, three shared 98.4 % sequence similarity with *Chryseobacterium scopthalmum* and seven shared less than 97.5 % sequence similarity with all described *Chryseobacterium* species (data not shown). Strain H38^T hydrolysed casein and tributyrin and showed esterase lipase and lipase activities in the API ZYM system. The activities of heat-stable proteolytic and lipolytic enzymes originating from psychrotolerant bacteria are the limiting factor in maintaining the flavour quality of fluid milk and its products. However, the impact of strain H38^T and other *Chryseobacterium* isolates on milk quality has yet to be determined.

Being an aerobic, non-motile rod, catalase- and oxidase-positive, with 15 : 0 iso and 17 : 0 iso 3-OH as the dominant fatty acids, the new isolate shared the main characteristics

of *Chryseobacterium* strains. However, strain H38^T differed from all described *Chryseobacterium* species by several phenotypic traits (Table 1), particularly the presence of carotenoid pigments when grown under light conditions and the large amount of the fatty acid 15 : 0 anteiso (16.6 %) (Bernardet *et al.*, 2006). Strain H38^T could be distinguished from strains of *C. joostei*, also isolated from raw milk, by its ability to grow at 37 °C, its inability to grow on cetrimide and MacConkey agars and its opposite results for β-galactosidase activity and acidification of lactose and trehalose.

On the basis of phenotypic characterization and phylogenetic analysis, we propose that strain H38^T be classified as the type strain of a novel species, for which the name *Chryseobacterium haifense* sp. nov. is proposed.

Description of *Chryseobacterium haifense* sp. nov.

Chryseobacterium haifense [hai.fen'se. N.L. neut. adj. *haifense* pertaining to Haifa, the name of the university (University of Haifa) where this study was conducted].

Cells are aerobic, Gram-negative, non-motile rods, 0.6–0.9 µm wide and 1.3–2.5 µm long, occurring singly, in pairs or in short chains. Growth is not observed under anaerobic conditions. After 48 h incubation on SPC agar at 30 °C, colonies are circular with entire edges, opaque, smooth and cream-coloured. When light is provided during growth, colonies are yellow-coloured due to the production of carotenoid pigments. Flexirubin reaction is weakly positive. Grows at 4–41 °C (optimum 32 °C), with 0–2.5 % NaCl (optimum 0–1.5 % NaCl) and at pH 6.5–10.5 (optimum pH 7.0–9.5). Good growth occurs on SPC and tryptic soy agars, but not on MacConkey or cetrimide agars. No haemolysis occurs on 5 % sheep blood agar. Catalase and oxidase activities are present. Casein and tributyrin are hydrolysed. The following results are obtained from API 20E and API 20NE strips after 48 h of incubation: glucose, arabinose, mannose, maltose and malic acid are assimilated, indole and acetoin are produced, H₂S is not produced, β-galactosidase activity is present, aesculin and gelatin are hydrolysed (gelatin only after 48 h incubation), urea is not hydrolysed, citrate is not utilized and activities of arginine dihydrolase, lysine and ornithine decarboxylases and tryptophan deaminase are absent. In API 50CH strips incubated for 48 h, acid is produced from D-glucose, D-fructose, maltose, D-lactose, sucrose and inulin. In API ZYM strips incubated for 4.5 h, activities of alkaline and acid phosphatases, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, naphthol-AS-BI-phosphohydrolase and β-galactosidase are present. The dominant cellular fatty acids are 15:0 iso (41.6 %), 15:0 anteiso (16.6 %) and 17:0 iso 3-OH (10.3 %). The following fatty acids are present as minor components: summed feature 3 (comprising 15:0 iso 2-OH and/or 16:1ω7c; 8.9 %), 13:0 iso (5.4 %), 16:0 3-OH (3.3 %), 17:1 iso ω9c (2.7 %), 15:0 iso 3-OH (2.2 %), 16:0 (1.9 %), unknown 16.582 (1.6 %), 14:0 (1.1 %), 16:0 iso 3-OH (1.1 %) and 17:0 2-OH (1.0 %). Traces (<1 %) of 13:0 anteiso, 14:0 iso, 15:0 and 15:0 2-OH are also present. The G+C content of the DNA of the type strain is 37.8 mol%.

The type strain is strain H38^T (=LMG 24029^T =DSM 19056^T), isolated from raw milk in Israel.

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