Chryseobacterium oranimense sp. nov., a psychrotolerant, proteolytic and lipolytic bacterium isolated from raw cow's milk

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A Gram-negative, rod-shaped, oxidase-positive, aerobic, non-motile and orange-pigmented bacterial strain, containing flexirubin-type pigments, designated H8^T, was isolated from raw cow's milk in Israel. 16S rRNA gene sequence analysis indicated that the isolate should be placed in the genus *Chryseobacterium* (family *Flavobacteriaceae*, phylum *Bacteroidetes*). The levels of 16S rRNA gene sequence similarity between strain H8^T and the type strains of described *Chryseobacterium* species were 97.5 % or lower. Strain H8^T grew at 5–37 °C and with 0–3.0 % NaCl. The dominant cellular fatty acids were iso-15:0, iso-17:0 3-OH, iso-17:1 ω9c and summed feature 3 (comprising iso-15:0 2-OH and/or 16:1 ω7c). On the basis of phenotypic properties and phylogenetic distinctiveness, the milk isolate H8^T is classified as a member of a novel species in the genus *Chryseobacterium*, for which the name *Chryseobacterium oranimense* sp. nov. (type strain H8^T = LMG 24030^T = DSM 19055^T) is proposed.

The genus *Chryseobacterium* (Vandamme *et al.*, 1994) is a member of the family *Flavobacteriaceae*, phylum *Bacteroidetes*, and currently consists of 25 species, with *Chryseobacterium gleum* as the type species. Strains belonging to this genus occur in a variety of ecological niches such as water, soil, plants and the clinical environment (Bernardet *et al.*, 2002, 2005, 2006; Park *et al.*, 2006). They are also a part of the psychrotolerant and proteolytic bacterial population that causes a variety of defects in food products such as milk, meat, poultry and fish (González *et al.*, 2000; Hugo *et al.*, 1999, 2003; de Beer *et al.*, 2005, 2006; Hantsis-Zacharov & Halpern, 2007a, b; Hantsis-Zacharov *et al.* 2008).

Strain H8^T was isolated in April 2004 in the course of a study on the diversity of culturable psychrotolerant bacteria in raw milk (Hantsis-Zacharov & Halpern, 2007b). Raw milk 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. A comparative analysis of 16S rRNA gene sequences indicated that strain H8^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). PCR products (approx. 1.5 kb) were purified using an AccuPrep PCR purification kit (Bioneer; <http://www.bioneer.co.kr>). 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 the primers 8f (5'-GGATCCAGAC TTTGATYMTGGCTCAG-3'), 534r (5'-ATTACCGCGGC TGCTGG-3'), 968f (5'-AACGCGAAGAACCTTAC-3') and 1512f (5'-GTGAAGCTTACGGYTACCTTGTTACG ACTT-3'). The newly determined sequence was compared to those available in GenBank (<http://www.ncbi.nlm.nih.gov>) using the BLAST program (version 2.0). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura *et al.* 2007).

The 1453 bp sequence of strain H8^T shared highest sequence similarity of 97.5 % with *Chryseobacterium joostei* LMG 18212^T, 97.2 % with *Chryseobacterium shigense*

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain H8^T is EF204451.

Fig. 1. Neighbour-joining dendrogram of 16S rRNA gene sequences obtained from strain H8^T and related species and genera. The 16S rRNA gene sequence of *Bacteroides fragilis* ATCC 25285^T was used as an outgroup. Bootstrap values (>50 %) resulting from 1000 replicates are indicated at branching nodes. Bar, 0.02 substitutions per nucleotide position.

GUM-Kaji^T and <97.0 % with the type strains of all other *Chryseobacterium* species (Fig. 1).

DNA–DNA hybridization studies between strain H8^T and strains of other *Chryseobacterium* species were not carried out, as strains exhibiting a difference of 2.5 % or more at the 16S rRNA gene sequence level are unlikely to share more than 70 % DNA–DNA relatedness (Stackebrandt & Goebel, 1994). Moreover, a new threshold (of 1.3 % difference) has recently been suggested by Stackebrandt & Ebers (2006).

For electron microscopy, bacteria grown on SPC agar (48 h, 32 °C) were suspended in saline, fixed to a carbon-coated grid, stained with 2 % uranyl acetate and photographed using a JEM-1200EX electron microscope (JEOL).

For phenotypic characterization, Luria–Bertani (LB) agar was used as the basal growth medium. Salt tolerance was determined at 30 °C on LB agar containing 0–5 % (w/v) NaCl at 1 % intervals. Growth was measured on SPC agar at 5, 7, 10, 14, 17, 25, 30, 33, 37, 40 and 45 °C. The pH range for growth was determined using LB broth adjusted to pH 4.0–10.0 at 2 pH 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 a BBL GasPak anaerobic jar (Becton Dickinson) with approximately 4–10 % CO₂ on LB 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 detected 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). Growth was tested on MacConkey agar (Difco) and cetrimide agar (Himedia). Hydrolysis of casein and tributyrin was determined using 1 % skimmed milk agar supplemented with 0.5 % yeast extract and tributyrin agar (Hylabs), respectively. Hydrolysis of starch was determined using plates containing soluble starch (1 %), beef extract (0.3 %) and agar (2 %). DNase activity was tested on DNase test agar with methyl green (Difco). The phenotypic traits of the isolate are given in the species description and in Table 1.

The cell mass of strain H8^T was subjected to the KOH test as described by Fautz & Reichenbach (1980) and Bernardet *et al.* (2002). A colour change was observed, demonstrating that flexirubin-type pigments are produced. As two type strains of the genus *Chryseobacterium* were found to possess carotenoids when grown under light conditions (Hantsis-Zacharov & Halpern, 2007a; Hantsis-Zacharov *et al.*, 2008), the production of carotenoid-type pigments of strain H8^T was also investigated. The yellow pigments were extracted and investigated according to Peterson *et al.*

Table 1. Differentiating characteristics of *Chryseobacterium* species

Strains: 1, *C. oranimense* sp. nov. H8^T; 2, *C. bovis* (n=3); 3, *C. haifense* LMG 24029^T; 4, *C. joostei* (n=11); 5, *C. indoltheticum* LMG 4025^T; 6, *C. scopthalmum* (n=7); 7, *C. defluvii* DSM 14219^T; 8, *C. gleum* (n=12); 9, *C. daecheongense* DSM 15235^T; 10, *C. formosense* CCUG 49271^T; 11, *C. taichungense* CCUG 50001^T; 12, *C. shigense* DSM 17126^T; 13, *C. vrystaatense* (n=36); 14, *C. soldanellicola* KCTC 12382^T; 15, *C. taeanense* KCTC 12381^T; 16, *C. piscium* (n=4); 17, *C. hispanicum* CECT 7129^T; 18, *C. wanjuense* DSM 17724^T; 19, *C. taiwanense* BCRC 17412^T; 20, *C. caeni* DSM 17710^T; 21, *C. daeguense* JCM 14362^T; 22, *C. balustinum* LMG 8329^T; 23, *C. indologenes* (n=13); 24, *C. luteum* (n=3); 25, *C. flavum* KCTC 12877^T; 26, *C. hominis* (n=11). The number of strains examined, when greater than 1, is shown in parentheses. Data were taken from Yabuuchi *et al.* (1983), Holmes *et al.* (1984), Hugo *et al.* (2003), Kämpfer *et al.* (2003), Kim *et al.* (2005), Shen *et al.* (2005), Shimomura *et al.* (2005), Young *et al.* (2005), de Beer *et al.* (2005, 2006), Gallego *et al.* (2006), Park *et al.* (2006), Tai *et al.* (2006), Weon *et al.* (2006), Quan *et al.* (2007), Yoon *et al.* (2007), Hantsis-Zacharov & Halpern (2007a), Behrendt *et al.* (2007), Zhou *et al.* (2007), Vaneechoutte *et al.* (2007), Hantsis-Zacharov *et al.* (2008) and this study. +, 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	21	22	23	24	25	26
Type of pigment*	F	F(w), C†	F(w), C†	F	F	F	NA	F	F	F	F	F	F	NA	NA	F	F(w)	F	F	F	NA	F	F	F	F	–
Growth on/at:																										
3 % NaCl	+	–	–	NA	W	W	–	NA	NA	NA	NA	NA	–	+	+	+	–	–	+	+	–	W	+	NA	+	–
Cetrimide agar	–	–	–	+	–	–	–	–	NA	NA	NA	NA	+	NA	NA	–	NA	NA	NA	NA	NA	–	+	NA	+	–
MacConkey agar	+	–	–	+	+	–	–	+	–	–	–	–	–	–	–	–	–	W	NA	–	–	+	+	–	+	–
5 °C	+	–	+	+	+	+	–	–	–	–	–	+	+	+	+	+	+	+	+	+	–	–	–	+	+	–
37 °C	+	+	+	–	–	–	+	+	+	+	+	–	–	+	+	–	–	+	+	+	+	+	+	–	+	+
42 °C	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	+	–	+	–
Hydrolysis of casein	+	v	+	+	+	+	+	+	+	NA	NA	+	NA	NA	NA	+	+	+	+	NA	+	+	+	v	+	NA
Nitrate reduction	–	v	–	–	–	–	–	NA	+	–	–	–	–	–	–	+	+	–	–	–	–	+	v	–	–	v
Urease activity	–	–	–	v	–	+	–	v	–	–	–	–	+	–	–	+	–	–	–	+	–	–	–	–	–	–
β-Galactosidase activity	+‡	v	+	–	–	+	NA	v	–	–	W	NA	NA	–	–	NA	+	+	–	+	–	–	–	+	+	–
Production of:																										
H ₂ S	–	–	–	–	+	–	–	–	–	–	–	–	v	–	–	–	–	NA	–	–	–	–	–	NA	–	–
Indole	+	–	+	+	+	–	+	+	–	+	W	+	+	–	–	+	W	NA	+	–	–	+	+	+	+	+
Acid production from:																										
Glucose	–	v	+	+	NA	–	+	+	–	W	W	+	NA	NA	NA	NA	+	+	–	–	+	+	+	+	–	+
L-Arabinose	–	–	–	–	–	–	–	+	–	–	–	–	NA	–	–	NA	NA	–	+	–	W	NA	v	+	–	–
D-Fructose	–	–	+	+	–	–	NA	+	+	NA	NA	+	NA	–	–	NA	+	–	v	–	+	+	+	+	+	NA
Lactose	–	v	+	–	–	–	NA	–	–	NA	–	–	NA	–	–	NA	–	–	–	–	+	–	–	–	–	–
Maltose	–	v	+	+	+	–	+	+	–	–	+	–	NA	–	–	NA	+	–	–	–	+	–	+	+	+	+
D-Mannitol	–	–	–	v	NA	–	–	–	–	–	–	–	NA	–	–	NA	–	–	–	–	–	–	v	–	–	–
Trehalose	–	–	–	+	–	–	+	+	+	+	+	–	NA	–	–	NA	–	+	–	–	+	–	+	+	+	–
D-Xylose	–	–	–	–	–	–	–	v	+	W	+	–	NA	–	–	NA	+	–	NA	–	W	–	–	–	+	–

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

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

‡Detected only in API 20NE strips.

(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 400 to 700 nm was performed on the acetone extract. Strain H8^T did not contain carotenoid-type pigments.

For cellular fatty acid analysis, strain H8^T was cultured on tryptic soy agar (Difco) for 24 h at 28 °C and the fatty acids were extracted and methylated (Ben-Ze'ev *et al.*, 2005). 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 H8^T were iso-15:0 (39.6 %), iso-17:1ω9c (20.6 %), iso-17:0 3-OH (18.5 %) and summed feature 3 (comprising iso-15:0 2-OH and/or 16:1ω7c; 10.8 %). The following fatty acids were present as minor components: iso-15:0 3-OH (3.5 %), iso-17:0 (1.5 %), 16:0 (1.2 %), unknown ECL 16.582 (1.1 %) and 16:0 3-OH (1.0 %).

Strain H8^T was isolated when psychrotolerant bacterial communities in raw milk and their proteolytic and lipolytic traits were studied (Hantsis-Zacharov & Halpern, 2007b). Five per cent of the isolates were members of the genus *Chryseobacterium* (GenBank accession numbers

EF204446–EF204459). Four of these isolates have already been described as representatives of two novel *Chryseobacterium* species, *Chryseobacterium haifense* and *Chryseobacterium bovis* (Hantsis-Zacharov & Halpern, 2007a; Hantsis-Zacharov *et al.*, 2008). Strain H8^T exhibited lipolytic and proteolytic activities. Heat-stable proteolytic and lipolytic enzymes originating from psychrotolerant bacteria are a limiting factor in maintaining the flavour quality of fluid milk and its products. However, the impact of *Chryseobacterium* isolates and in particular the role of strain H8^T on milk quality has yet to be determined.

Being non-motile rods, catalase- and oxidase-positive and containing flexirubin-type pigments, with iso-15:0 and iso-17:0 3-OH as the dominant fatty acids, the new isolate shared the main characteristics of *Chryseobacterium* strains. However, it differed from described *Chryseobacterium* species in several phenotypic traits listed in Table 1. Strain H8^T can be differentiated from its closest relative, *Chryseobacterium joostei*, which has also been isolated from raw milk (Hugo *et al.*, 2003), by the following characteristics: ability to grow at 37 °C, inability to produce acid from sugars including glucose, production of large amounts of the fatty acid summed feature 3 (comprising iso-15:0 2-OH and/or 16:1 ω 7c; 10.8%) and lack of summed feature 4 (iso-17:1 I and/or anteiso-17:1 B). Combinations of phenotypic characteristics also differentiate strain H8^T clearly from the other *Chryseobacterium* species isolated from raw milk (*C. haifense* and *C. bovis*; Table 1).

On the basis of phenotypic characterization and phylogenetic analysis, we propose that isolate H8^T be classified as the type strain of a novel species, for which the name *Chryseobacterium oranimense* sp. nov. is proposed.

Description of *Chryseobacterium oranimense* sp. nov.

Chryseobacterium oranimense (o.ra.ni.men'se. N.L. neut. adj. *oranimense* pertaining to Oranim campus, where the type strain was characterized).

Cells are Gram-negative, non-motile rods, 0.7–1.2 μ m wide and 1.2–2.0 μ m long. After 48 h of incubation on SPC agar at 32 °C in the dark, colonies are circular with entire edges, opaque, mucoid and orange-coloured. Flexirubin-type pigments are produced but carotenoid-type pigments are not produced. Growth is not observed under anaerobic conditions. Grows at 5–37 °C (optimum, 30–33 °C), with 0–3.0% NaCl (optimum, 0–1.0% NaCl) and at pH 4.0–8.0 (optimum, pH 6.0–8.0). Growth occurs on nutrient, trypticase soy and MacConkey agars but not on cetrimide agar. Catalase and oxidase activities are present. Starch, casein and tributyrin are hydrolysed. DNase activity is present. The following results are obtained from API 20NE strips after 48 h of incubation at 32 °C: indole is produced; aesculin and gelatin are hydrolysed; glucose, arabinose, mannose, maltose and citrate are assimilated; adipic and malic acids are assimilated weakly; β -galactosidase activity is present. The following results are obtained from API 20E strips after 24 h

of incubation at 32 °C: indole is produced; gelatin is hydrolysed; nitrate is reduced to N₂; H₂S and acetoin are not produced; urea is not hydrolysed; citrate is not utilized; activities of β -galactosidase, arginine dihydrolase, lysine and ornithine decarboxylases and tryptophan deaminase are absent. In API 50CH strips incubated for 48 h at 32 °C, acid is produced from aesculin. In API ZYM strips incubated for 4.5 h at 32 °C, activities of alkaline and acid phosphatases, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, naphthol-AS-BI-phosphohydrolase and β -glucosidase are present. The dominant cellular fatty acids are iso-15:0, iso-17:1 ω 9c, iso-17:0 3-OH and summed feature 3 (comprising iso-15:0 2-OH and/or 16:1 ω 7c). The following fatty acids are present as minor components: iso-15:0 3-OH, iso-17:0, 16:0, unknown ECL 16.582 and 16:0 3-OH. Traces (<1%) of iso-13:0, 14:0, anteiso-15:0, summed feature 4 (comprising iso-17:1 I and/or anteiso-17:1 B) and 18:1 ω 5c are also present.

The type strain is H8^T (=LMG 24030^T =DSM 19055^T), isolated from raw cow's milk in Israel.

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