Epilithonimonas lactis sp. nov., isolated from raw cow's milk

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A Gram-staining-negative, rod-shaped, oxidase-positive, aerobic, non-motile and yellow-pigmented bacterial strain containing flexirubin type pigments, designated H1^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 *Epilithonimonas* (family *Flavobacteriaceae*, phylum *Bacteroidetes*). The level of 16S rRNA gene sequence similarity between strain H1^T and the type strain of *Epilithonimonas tenax* was 97.6%. Strain H1^T grew at 5–33 °C and with 0–2.0% NaCl. The dominant cellular fatty acids of strain H1^T were iso-C_{15:0}, iso-C_{17:0} 3-OH and summed feature 3 (comprising iso-C_{15:0} 2-OH and/or C_{16:1}ω7c), and the DNA G+C content was 38.0 mol%. On the basis of phenotypic properties and phylogenetic distinctiveness, the milk isolate is classified as a new species in the genus *Epilithonimonas*, for which the name *Epilithonimonas lactis* sp. nov. (type strain H1^T = LMG 24401^T = DSM 19921^T) is proposed.

The genus *Epilithonimonas* (O'Sullivan *et al.*, 2006) is a member of the family *Flavobacteriaceae*, phylum *Bacteroidetes*. At the time of writing, it only consists of the type species, *Epilithonimonas tenax*, which was isolated from river epilithon (River Taff, Cardiff, UK).

Strain H1^T was isolated in April 2004, in the course of a study on the diversity of culturable psychrotolerant bacteria in raw cow's milk (Hantsis-Zacharov & Halpern, 2007). Raw milk samples were serially diluted and plated on sterile standard plate count agar (SPC, Oxoid CM0463). Plates were incubated at 7 °C for 10 days and colonies displaying distinct morphologies were subcultured to obtain pure cultures. A comparative analysis of 16S rRNA gene sequences indicated that the strain was a member of the genus *Epilithonimonas*. 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 PCR products (approx. 1.5 kb) were purified by means of an AccuPrep PCR purification kit (Bioneer). Purified PCR products were sequenced at the 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 use of the 8f (5'-GGATCCAGACTTTGATYMTGGCTCAG-3'), 534r (5'-ATTACCGCGGCTGCTGG-3'), 968f (5'-AACGCGAAGAACCCTTAC-3') and 1512f (5'-GTGAAGCTTACGGYTAGCTTGTTACGACTT-3') primers.

Phylogenetic neighbours were initially identified by the BLAST (Altschul *et al.*, 1997) and FASTA (Pearson & Lipman, 1988) programs against the database of type strains with valid prokaryotic names (Chun *et al.*, 2007). The 50 sequences with the highest scores were then selected for the calculation of pairwise sequence similarity using the global alignment algorithm, which was implemented at the EzTaxon server (<http://www.eztaxon.org/>; Chun *et al.*, 2007). Sequence alignment was performed with the CLUSTAL W program, and a phylogenetic tree (Fig. 1) was generated by the neighbour-joining and by maximum-parsimony methods in the MEGA version 4.1 software (Tamura *et al.*, 2007). The 1497 bp sequence of strain H1^T shared the highest sequence similarity of 97.6% with *Epilithonimonas tenax* DSM 16809^T, 96.0% sequence similarity with *Chryseobacterium arothri* DSM 19326^T and *Chryseobacterium hominis* CCUG 52711^T, and <95.9% similarity with the type strains of all other type species.

Together with *Epilithonimonas tenax*, strain H1^T formed a branch distinctly separate from the *Chryseobacterium/Sejongia* cluster (Fig. 1). This result suggests that strain H1^T may represent a separate species within the genus *Epilithonimonas*. As previous studies have reported DNA-

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain H1^T is EF204460.

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic positions of strain H1^T, *Epilithonimonas tenax* and representatives of related taxa. Bootstrap values (>50%) resulting from 1000 replicates are indicated at branching nodes. *Bacteroides fragilis* was used as an outgroup. Asterisks indicate branches of the tree that were also formed by using the maximum-parsimony method. Bar, 0.02 substitutions per nucleotide position.

DNA reassociation values well below 70% between *Chryseobacterium* strains sharing high values of 16S rRNA gene sequence similarity [e.g. 28% (Weon *et al.*, 2006), 33.9% (Kim *et al.*, 2005) and 27.1% (Young *et al.*, 2005)], we refrained from performing DNA hybridization experiments and considered strain H1^T to represent a distinct genospecies (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 under a JEM-1200EX electron microscope (JEOL, Japan). No flagella were observed.

For phenotypic characterization, Luria–Bertani agar (LB) was used as the basal growth medium unless otherwise stated. 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 in 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 (approx. 4–10% CO₂) was determined after incubation in a BBL GasPak anaerobic jar (Becton Dickinson) on LB agar supplemented with 0.5% (w/v) glucose or 0.1% (w/v) potassium nitrate. Biochemical tests were performed in parallel on strains H1^T and *Epilithonimonas tenax* DSM 16809^T 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 25 °C. Catalase activity was evidenced by bubble production in a 3% (v/v) hydrogen peroxide solution. Oxidase activity was determined with 1% *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride (Sigma-Aldrich, T3134). Growth was tested on MacConkey agar (Difco) and cetrimide agar

(Himedia). Hydrolysis of casein and tributyrin was determined on 1 % skimmed milk agar supplemented with 0.5 % yeast extract and tributyrin agar (Hylabs), respectively. For detection of flexirubin-type pigments, the cell mass of strain H1^T was subjected to the KOH test as described by Fautz & Reichenbach (1980) and Bernardet *et al.* (2002). The phenotypic traits of the isolate are given in the species description and in Table 1.

For cellular fatty acid analysis, strain H1^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). The fatty acid methyl esters were analysed by gas chromatography with the MIDI/Hewlett Packard microbial identification system (Analytical Services). The major fatty acids (>20 %) of strain H1^T were iso-C_{15:0} and summed feature 3 (comprising iso-C_{15:0} 2-OH and/or C_{16:1}ω7c) (Table 2).

For determination of the DNA G+C content, genomic DNA of strain H1^T was prepared according to a modified version of the procedure of Wilson (1987). The G+C content of the DNA sample was determined in three independent analyses using the HPLC technique (Mesbah *et al.*, 1989) by the BCCM/LMG Bacteria Collection Identification Service (Laboratory of Microbiology, Ghent University, Ghent, Belgium). The DNA G+C content of strain H1^T was 38.0 mol%.

Strain H1^T was strictly aerobic, catalase- and oxidase-positive and non-motile; iso-C_{15:0} and summed feature 3 (comprising iso-C_{15:0} 2-OH and/or C_{16:1}ω7c) were the dominant fatty acids, the presence of Na⁺ was not required and some carbohydrates could be utilized for growth. Hence, strain H1^T shared all characteristics listed in the description of the genus *Epilithonimonas* (O'Sullivan *et al.*, 2006). A colour change of the cell mass was observed when the KOH test was performed, demonstrating that flexirubin-type pigments are produced, in accordance with *Epilithonimonas tenax* (O'Sullivan *et al.*, 2006). However, strain H1^T differed from *Epilithonimonas tenax* in several phenotypic traits that are listed in Tables 1 and 2.

On the basis of phenotypic characterization and phylogenetic analysis we propose that isolate H1^T be classified as a new species in the genus *Epilithonimonas*, for which the name *Epilithonimonas lactis* sp. nov. is proposed.

Description of *Epilithonimonas lactis* sp. nov.

Epilithonimonas lactis (lac'tis. L. gen. n. lactis of milk, referring to the isolation of the type strain from raw milk).

Cells are Gram-staining-negative rods, 0.7–0.9 µm in width and 1.1–2.2 µm in length, devoid of flagellar motility. After 48 h of incubation on standard plate count agar at 30 °C in the dark, colonies are circular with entire edges, opaque, smooth and orange-coloured. Flexirubin-type pigments are produced. Good growth occurs under aerobic conditions. Growth is not observed after five days under anaerobic conditions on LB agar supplemented

Table 1. Differentiating characteristics of species of the genus *Epilithonimonas*

Strains: 1, *Epilithonimonas lactis* sp. nov. H1^T; 2, *E. tenax* EP105^T. Data for both strains are from the current study except where indicated. Both strains are positive for the following characteristics: production of flexirubin-type pigments; presence of alkaline and acid phosphatases, esterase (C4), esterase lipase (C8), leucine and valine arylamidases and naphthol-AS-BI-phosphohydrolase activities; hydrolysis of aesculin; assimilation of glucose and maltose; acid production from glucose, amygdalin, aesculin, maltose, trehalose, starch and gentiobiose. Both strains are negative for the following characteristics: presence of α-galactosidase, β-glucuronidase, β-glucosaminidase, α-mannosidase and α-fucosidase activities; assimilation of L-arabinose, mannitol, N-acetylglucosamine, potassium gluconate, capric acid, adipate, malate, citrate and phenylacetic acid; acid production from glycerol, erythritol, D- and L-arabinose, D-ribose, D- and L-xylose, D-adonitol, methyl β-D-xylopyranoside, D-galactose, D-fructose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, N-acetylglucosamine, melibiose, inulin, melezitose, raffinose, xylitol, turanose, D-lyxose, D-tagatose, D- and L-fucose, D- and L-arabitol, potassium gluconate, potassium 2-ketogluconate and potassium 5-ketogluconate. +, Positive; w, weakly positive; –, negative.

Characteristic	1	2
Growth on:		
LB agar with 2 % NaCl	+	–
MacConkey agar	–	+
Growth at 30 °C	+	– (+*)
Nitrate reduction	–	+
Indole production	+	–
Activity of:		
Lipase (C14)	w	–
Cystine arylamidase	w	–
Trypsin	–	w
α-Chymotrypsin	w	–
β-Galactosidase	w	–
α- and β-Glucosidase	+	w
Assimilation of mannose	–	+
Acid production from:		
Arbutin	+	–
Salicin	+	–
Cellobiose	+	–
Lactose	+	–
Glycogen	+	–
D-Mannose	–	+
Sucrose	–	+
DNA G+C content (mol%)	38.0	37.5*

*Data from O'Sullivan *et al.* (2006).

with 0.1 % (w/v) potassium nitrate or 0.5 % glucose. Grows at 5–33 °C (optimum, 25–30 °C), with 0–2.0 % NaCl (optimum, 0–0.5 % NaCl) and at pH 4.0–8.0 (optimum, pH 6.0–8.0). Growth is observed on standard plate count, nutrient and trypticase-soy agars but not on

Table 2. Cellular fatty acid compositions (%) of species of the genus *Epilithonimonas*

Strains: 1, *Epilithonimonas lactis* sp. nov. H1^T; 2, *E. tenax* EP105^T. Cultivation on tryptic soy agar at 28 °C (strain H1^T, this study) or 25 °C (*E. tenax* EP105^T, O'Sullivan *et al.*, 2006), for 24 h. Fatty acids amounting to <1% of the total fatty acids in both strains are not shown. tr, Trace (<1%); ND, not detected.

Fatty acid methyl ester	1	2
C _{14:0}	1.1	ND
C _{16:0}	7.9	8.0
iso-C _{13:0}	1.7	tr
iso-C _{14:0}	tr	4.3
anteiso-C _{14:0}	ND	1.2
iso-C _{15:0}	23.7	20.0
anteiso-C _{15:0}	8.2	14.5
iso-C _{17:1} ω9c	1.1	1.0
C _{13:1}	ND	2.3
C _{16:1} ω5c	1.8	ND
Summed feature 3*	26.7	21.1
C _{15:0} 2-OH	1.1	ND
iso-C _{15:0} 3-OH	3.0	1.6
iso-C _{16:0} 3-OH	1.5	1.9
C _{16:0} 3-OH	8.2	5.9
C _{17:0} 2-OH	1.2	ND
iso-C _{17:0} 3-OH	10.0	9.8
anteiso-C _{17:0} 3-OH	ND	2.5

*Summed features are groups of two or three fatty acids that cannot be separated by GLC using the MIDI system. Summed feature 3 comprised C_{16:1}ω7c and/or iso-C_{15:0} 2-OH.

MacConkey and cetrimide agars. Catalase and oxidase activities are present. The following results were obtained from API 20NE strips after 48 h of incubation at 25 °C: indole is produced; β-galactosidase activity is present; aesculin is hydrolysed; glucose and maltose are assimilated. The following results were obtained from API 20E strips after 24 h of incubation at 25 °C: acetoin and indole are produced; H₂S is not produced; gelatin and urea are not hydrolysed; citrate is not utilized; β-galactosidase activity is present; arginine dihydrolase, lysine and ornithine decarboxylases, and tryptophan deaminase activities are absent. In API 50CH strips incubated for 48 h at 25 °C, oxidative acid production occurs for glucose, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, lactose, trehalose, starch, glycogen and gentiobiose, while oxidative acid production does not occur for glycerol, erythritol, D- and L-arabinose, D-ribose, D- and L-xylose, D-adonitol, methyl β-D-xylopyranoside, D-galactose, D-fructose, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, N-acetylglucosamine, melibiose, sucrose, inulin, melezitose, raffinose, xylitol, turanose, D-lyxose, D-tagatose, D- and L-fucose, D- and L-arabitol, potassium gluconate, potassium 2-ketogluconate and potassium 5-

ketogluconate. In API ZYM strips incubated for 4.5 h at 25 °C, alkaline and acid phosphatases, esterase (C4), esterase lipase (C8), leucine and valine arylamidases, naphthol-AS-BI-phosphohydrolase and α- and β-glucosidase activities are present; lipase (C14), cystine arylamidase, α-chymotrypsin and β-galactosidase activities are weakly present. Trypsin, α-galactosidase, β-glucuronidase, β-glucosaminidase, α-mannosidase and α-fucosidase activities are absent. The dominant (>10%) cellular fatty acids are summed feature 3 (comprising iso-C_{15:0} 2-OH and/or C_{16:1}ω7c), iso-C_{15:0} and iso-C_{17:0} 3-OH. The detailed fatty acid composition of strain H1^T is given in Table 2. The DNA G+C content of the type strain is 38.0 mol%.

The type strain is H1^T (=LMG 24401^T =DSM 19921^T), isolated from raw cow's milk in Israel.

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