Enterococcus camelliae sp. nov., isolated from fermented tea leaves in Thailand

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A Gram-positive and catalase-negative coccus that formed chains, strain FP15-1^T, isolated from fermented tea leaves ('miang'), was studied systematically. The strain was facultatively anaerobic and produced L-lactic acid from glucose. Demethylmenaquinone (DMK-7) was the major menaquinone. Straight-chain unsaturated fatty acids C_{16:1} and C_{18:1} were the dominant components. The DNA G + C content was 37.8 mol%. On the basis of 16S rRNA and RNA polymerase α subunit (*rpoA*) gene sequence analysis, strain FP15-1^T was closely related to *Enterococcus italicus* KCTC 5373^T, with 99.2 and 93.8 % similarity, respectively. The strain could be clearly distinguished from *E. italicus* ATCC 5373^T by low DNA–DNA relatedness (≤ 33.8 %) and phenotypic characteristics. Therefore, this strain represent a novel species of the genus *Enterococcus*, for which the name *Enterococcus camelliae* sp. nov. is proposed. The type strain is FP15-1^T (=KCTC 13133^T =NBRC 101868^T =NRIC 0105^T =TISTR 932^T =PCU 277^T).

The enterococci comprise an important group of lactic acid bacteria found ubiquitously in the environment, the gastrointestinal tract, traditional fermented foods and dairy products. The classification of the genus *Enterococcus* has undergone considerable changes as a consequence of the increasing number of species and also improvements in methods to discriminate between species (Baele *et al.*, 2000; Merquior *et al.*, 1994; Naser *et al.*, 2005). At the time of writing, 33 *Enterococcus* species names have been validly published (Euzéby, 1997; last full update 1 February 2007). Four species groups, the *Enterococcus faecium*, *E. avium*, *E. gallinarum* and *E. cecorum* groups, and other ungrouped species, including *Enterococcus faecalis*, *E. saccharolyticus*, *E. sulfureus* and *E. dispar*, were revealed by comparative 16S rRNA gene sequence analysis (Hardie & Whiley, 1997). Recently, *Enterococcus saccharominimus*, represented by isolates from contaminated pasteurized cow's milk

(Vancanneyt *et al.*, 2004), was reclassified as a later synonym of *Enterococcus italicus*, isolated from artisanal Italian cheeses (Fortina *et al.*, 2004), by comparison of partial sequences for three housekeeping genes, phenylalanyl-tRNA synthase α subunit (*pheS*), RNA polymerase α subunit (*rpoA*) and the α subunit of ATP synthase (*atpA*), and as confirmed by DNA–DNA hybridization (Naser *et al.*, 2006). In the present paper, we describe a novel *Enterococcus* strain from fermented tea leaves ('miang') based on phenotypic and chemotaxonomic characteristics, DNA–DNA relatedness and 16S rRNA and *rpoA* gene sequence analysis.

Samples of fermented tea leaves were collected from Chiangmai province, in the northern part of Thailand. Cocci in chains were isolated from the samples using GYP-CaCO₃ agar (Tanasupawat *et al.*, 1992). GYP-sodium acetate-mineral salt broth (Tanasupawat *et al.*, 1992) (pH 7.2) was used for working cultures. All tests were performed by incubating the cultures at 30 °C. Cell shape, size and arrangement and colony appearance were examined using cells grown on GYP agar for 3 days. Gram staining was done as described by Hucker & Conn (1923). Spore formation was examined in the Gram-stained specimen. Results of the oxidation/fermentation test and motility were examined in soft agar (Whittenbury, 1963). Catalase activity,

Abbreviation: DMK, demethylmenaquinone.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *rpoA* gene sequences of strain FP15-1^T are respectively EF154454 and EF197993.

16S rRNA gene sequence-based maximum-likelihood and maximum-parsimony trees are available as supplementary material with the online version of this paper.

hydrolysis of gelatin, aesculin, arginine and starch, nitrate reduction, production of gas from glucose, gluconate and citrate and acid formation from carbohydrates were tested as reported by Tanasupawat *et al.* (1992). Additional biochemical characteristics were recorded after 2 days incubation in API 50 CH galleries. Hydrolysis of horse blood was assessed as described by Barrow & Feltham (1993). Growth on Slanetz–Bartley agar (Oxoid) and on kanamycin aesculin azide agar (Merck) was tested. The reaction in litmus milk (Difco) was investigated after incubating cultures for 3, 7 and 14 days. Effects of temperature (10–45 °C), initial pH (pH 4.0, 4.5, 5.0, 7.5, 9.0 and 9.6) and NaCl concentration (2, 4, 6, 6.5, 8 and 10 %, w/v) were observed by examining growth in GYP-sodium acetate-mineral salt broth. Vitamin requirements were examined by the method of Kihara & Snell (1960) with modifications. The isomer of lactic acid was analysed enzymically (Okada *et al.*, 1978). Methyl esters of fatty acids were prepared as described by Ikemoto *et al.* (1978) and cellular fatty acid compositions were analysed by GLC [model GC-14A (Shimadzu), equipped with a CBP1 (OV-1) type capillary column, 25 m by 0.25 mm inside diameter, at 180–220 °C, and a flame ionization detector]. Gas-liquid chromatograms were calculated by the Chromatopac C-R 4A data-processor (Shimadzu). Quinones were extracted from freeze-dried cells and purified as described by Collins *et al.* (1977) and Collins & Jones (1979). Purified quinones were analysed by HPLC (Tamaoka *et al.*, 1983).

DNA was isolated and purified by the method of Saito & Miura (1963). The DNA base composition was determined by reversed-phase HPLC (Tamaoka & Komagata, 1984). PCR amplification and sequencing of the 16S rRNA and *rpoA* genes were performed as described by Lane (1991) and Naser *et al.* (2005), respectively. The sequences were assembled and compared with deposited type strain sequences available in GenBank/EMBL/DBJ by using BioEdit version 7.0.1 (Hall, 1999) and CLUSTAL_X version 1.83 (Thompson *et al.*, 1997). Phylogenetic trees were constructed based on the neighbour-joining method (Saitou & Nei, 1987) by the NJPlot program (Perrière & Gouy, 1996) and on the maximum-likelihood and the maximum-parsimony methods using MEGA version 3.1 (Kumar *et al.*, 2004). Confidence values of branches of the phylogenetic tree were determined using bootstrap analysis (Felsenstein, 1985) based on 1000 resamplings. DNA–DNA hybridization experiments were performed at 40 °C for 16 h, as reported by Ezaki *et al.* (1989).

Cells of strain FP15-1^T were Gram-positive, facultatively anaerobic, non-motile, non-spore-forming, spherical or ovoid and arranged in pairs or in chains. The detailed morphological, cultural, physiological and biochemical properties, including chemotaxonomic characteristics, are listed in the species description. Demethylmenaquinones with seven to nine isoprene units [DMK-7, 94.7 %; DMK-8, 4.2 %; DMK-9, 1.1 %] were found. The strain contained the straight-chain unsaturated fatty acids C_{16:1} (30.5 %) and C_{18:1} (20.9 %) as the dominant components. The

remainder of the fatty acid profile consisted of C_{14:1} (4.6 %), C_{14:0} (5.4 %), C_{16:0} (5.0 %), C_{18:0} (3.6 %), C_{20:1} (6.7 %), cyclopropane acids of C₁₇ (3.4 %) and C₁₉ (9.1 %), a trace of C_{12:0} and unidentified components (7.2 %). Strain FP15-1^T contained roughly the same fatty acid pattern as its closest relatives, *E. italicus* KCTC 5373^T and *E. saccharolyticus* ATCC 43076^T. The amount of C_{16:1} was a significant marker to differentiate strain FP15-1^T from *E. italicus* KCTC 5373^T and *E. saccharolyticus* ATCC 43076^T, in which it has been reported to be present in small amounts. The cyclopropane acid of C₁₇ was absent in both *E. italicus* KCTC 5373^T and *E. saccharolyticus* ATCC 43076^T (Fortina *et al.*, 2004). The DNA G + C content of the strain was 37.8 mol%. The almost-complete 16S rRNA gene sequence (1325 bp) of strain FP15-1^T indicated that the strain was closely related to *E. italicus* KCTC 5373^T (99.2 %), *E. saccharolyticus* ATCC 43076^T (98.3 %), *E. sulfureus* ATCC 49903^T (98.1 %) and *Enterococcus casseliflavus* NCIMB 11449^T (97.0 %) (Fortina *et al.*, 2004). Lower sequence similarities (<97 %) were found to other described species of the genus *Enterococcus* (Fig. 1). Trees constructed by the maximum-likelihood and maximum-parsimony methods are available as Supplementary Figs S1 and S2 in IJSEM Online. The *rpoA* gene sequence (615 bp) of strain FP15-1^T showed 93.8, 88.3 and 88.1 % similarity, respectively, to those of *E. italicus* KCTC 5373^T, *E. sulfureus* ATCC 49903^T and *E. saccharolyticus* ATCC 43076^T (Fig. 2). The application of multilocus sequence analysis (MLSA) for rapid identification of *Enterococcus* species based on the *rpoA* gene (Naser *et al.*, 2005, 2006) confirmed the separation of strain FP15-1^T from related species. Strain FP15-1^T showed low DNA–DNA relatedness to *E. italicus*

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences showing the relationships between strain FP15-1^T and related bacterial species. The branching pattern was generated by the neighbour-joining method. Based on 1000 replications, bootstrap percentages above 52 % are shown. Bar, 2 substitutions per 100 nucleotide positions.

Fig. 2. Phylogenetic tree based on *rpoA* gene sequences showing the relationships between strain FP15-1^T and related bacterial species. The branching pattern was generated by the neighbour-joining method. Based on 1000 replications, bootstrap percentages above 53 % are shown. Bar, 2 substitutions per 100 nucleotide positions.

KCTC 5373^T (21.6 %), with 33.8 % relatedness in the reciprocal reaction. In addition, strain FP15-1^T could be differentiated from closely related *Enterococcus* species by growth in 6.5 % NaCl and at 10 °C, acid production and DNA G+C content (Table 1). Therefore strain FP15-1^T should be classified in the genus *Enterococcus* as the type strain of a novel species, *Enterococcus camelliae* sp. nov.

Description of *Enterococcus camelliae* sp. nov.

Enterococcus camelliae [ca.mel'li.ae. N.L. gen. n. *camelliae* of *Camellia*, referring to the isolation of the type strain from fermented tea leaves (*Camellia sinensis*)].

Cells are Gram-positive, facultatively anaerobic, non-motile, non-spore-forming, spherical or ovoid, 0.5–1 µm in diameter and arranged in pairs or in chains. Colonies on GYP agar plates are circular, raised or low-convex with entire margins, and non-pigmented. Small, red colonies appear on Slanetz–Bartley agar. No growth is observed on kanamycin aesculin azide agar. Positive for hydrolysis of aesculin but weak reactions for starch hydrolysis and blood haemolysis. Negative for catalase, hydrolysis of arginine and gelatin, reduction of nitrate and production of gas from glucose, gluconate and citrate. Utilizes glucose

Table 1. Differential characteristics of FP15-1^T and related *Enterococcus* species

Strains: 1, FP15-1^T; 2, *E. italicus* (seven strains); 3, *E. saccharolyticus* DSM 20726^T; 4, *E. sulfureus* DSM 6905^T. Data for reference strains were obtained from Ennahar & Cai (2005), Fortina *et al.* (2004) and Manero & Blanch (1999). +, Positive; d, strain-dependent; –, negative; w, weak. All taxa are negative for acid production from L-arabinose.

Characteristic	1	2	3	4
Growth in 6.5 % NaCl	–	–	+	+
Growth at 10 °C	–	w	+	+
Acid production from:				
D-Galactose	–	+	+	+
Lactose	–	+	+	+
D-Melezitose	–	–	+	+
D-Melibiose	–	–	+	+
Raffinose	–	–	+	+
D-Ribose	–	–	+	+
D-Sorbitol	–	d	+	–
DNA G+C content (mol%)	37.8	39.9–41.1	37.2	38.4

fermentatively. No acidification, coagulation, reduction or liquefaction in litmus milk. Grows at pH 5.0–9.6, at 15–45 °C and in 2–6 % NaCl. Acid is produced from D-glucose, D-fructose, D-cellobiose, aesculin, D-mannose, maltose, mannitol, N-acetylglucosamine, trehalose, sucrose and salicin, but not from glycerol, erythritol, D- or L-arabinose, D-ribose, D- or L-xylose, adonitol, methyl β-xyloside, D-galactose, D-sorbose, rhamnose, dulcitol, inositol, methyl α-D-mannoside, methyl α-D-glucoside, D-amygdalin, arbutin, lactose, inulin, D-melibiose, D-melezitose, D-sorbitol, raffinose, starch, glycogen, xylitol, β-gentiobiose, D-turanose, D-lyxose, D-tagatose, D- or L-fucose, D- or L-arabitol, gluconate or 2- or 5-ketogluconate. Riboflavin, niacin and calcium pantothenate are required for growth. DMK-7 is the major menaquinone. C_{16:1} is the dominant component of the fatty acid profile. The DNA G+C content of the type strain is 37.8 mol%.

The type strain, FP15-1^T (=KCTC 13133^T =NBRC 101868^T =NRIC 0105^T =TISTR 932^T =PCU 277^T), was isolated from fermented tea leaves ('miang') produced in Thailand.

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