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Identification of Enterococci from Fermented Foods

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ABSTRACT : Fifteen strains of gram-positive and catalase negaive cocci in chains isolated from fermented foods were systematically studied. These isolates were included in the genus *Enterococcus* based on phenotypic and chemotaxonomic characteristics. On the basis of fluorometric DNA-DNA hybridization studies, 6 isolates were identified as *E. hirae*, 3 as *E. faecalis*, 3 as *E. faecium* and one as *E. casseliflavus*. Two strains could not be identified. DNA base composition of the strains studied ranged from 37.0 to 44.8 mol% of guanine-plus-cytosine. The most strains tested contained large amounts of strainght-chain fatty acids of $C_{18:1}$, $C_{16:0}$, $C_{16:1}$, and cyclopropane acid of C_{19}. Demethylmenaquinone with nine isoprenes was a major menaquinone in *E. faecalis* strains and menaquinone with seven isoprenes was a major menaquinone in *E. casseliflavus* strain. The strains in *E. hirae* and *E. faecium* had no quinone systems. Detailed characteristics of these bacteria were described.

KEY WORDS: Enterococci, *E. hirae, E. faecalis, E. faecium, E. casseliflavus*, fermented food, identification

INTRODUCTION

Previously, the identification and distribution of pediococci and lactobacilli in fermented foods in Thailand were reported (1-3). Further, the gram-positive and catalase positive cocci were isolated and were identified as *Staphylococcus carnosus* and *S. piscifermentans* (4, 5). In the study of those bacteria, a small number of gram-positive, catalase negative cocci which arranged in pairs and in chains were also isolated from pla- ra (fermented fish), pla-chom (fermented small fish), pla-som (fermented fish), sai-krog-prieo (sour pork sausage), mum (sour beef sausage), phak-gard-dong (pickled mustard), and miang (fermented tea leaves). The related cocci from kecap koji (soybean koji) and kecap moromi (soy sauce mash) in Indonesia were also isolated. In this paper, we identified those cocci in chains based on phenotypic and chemotaxonomic characteristics including fluorometric DNA-DNA hybridization.

MATERIALS AND METHODS

Isolation method and bacterial cultures. The cocci in chains were isolated from fermented foods by the use of GYP-$CaCO_3$ agar (3). GYP-sodium acetate-mineral salt broth (3) adjusted pH to 7.2 was used for working cultures. A total of 15 isolates, each type strain of *Enterococcus* species which mentioned below, and *Lactococcus lactis* NRIC 1149^T were used in this study. The designations and isolation sources are listed in Table 1. All tests were performed by incubating the cultures at 30°C, except for the tolerance of temperature.

Identification methods

Morphological and cultural characteristics. Cell form, cell size, cell arrangement, and colonial appearance were examined on the cells grown on GYP agar incubated for 3 days. Hucker-Conn modification method (6) was used for

gram stain. Spore formation was examined in gram-stained specimens. Motility was detected by the appearance of stab cultures in soft agar (7).

Biochemical and physiological characteristics. Catalase activity, hydrolysis of gelatin, esculin, arginine, and starch, reduction of nitrate, production of gas from glucose, gluconate and citrate, and acid formation from carbohydrates were tested as reported by Tanasupawat *et al.*(3). Hydrolysis of horse blood was performed as described by Cowan and Steel (8). Oxidation and fermentation test was examined in soft agar (9). The reactions in litmus milk (Difco Laboratories, Detroit, Mich., U.S.A.) were investigated after incubating the cultures for 3, 7 and 14 days. Effects of temperature (10°C, 45°C), of different starting pHs (4.0, 4.5, 5.0, 7.5, 9.0 and 9.6) and of diffenent concentrations of NaCl (2, 4, 6, 6.5, 8 and 10%) were detected by the growth in GYP-sodium acetate-mineral salt broth. Vitamin requirements were examined by the method of Kihara and Snell (10) with modification.

Isomer of lactic acid. The tested strains were cultivated in GYP-mineral salt broth (3) for 3 days. Lactic acid was extracted from supernatant of fermentation broth with ether, and was analyzed enzymatically (11).

DNA base composition. DNAs were isolated from cells grown in GYP-sodium acetate-mineral salt broth for a day, and were purified by the method of Saito and Miura (12). The medium was supplemented with 0.5% glycine (13) for the strains not sensitive to lysozyme. The purified DNA was hydrolyzed enzymatically (14), and was analyzed by reversed phase high-performance liquid chromatography (HPLC).

DNA-DNA hybridization. Fluorometric DNA-DNA hybridization was performed in 2 x SSC (saline-trisodium citrate) and 50% formamide solution at 45°C for 3 h as reported by Ezaki *et al.* (15, 16).

Cellular fatty acid composition. Methyl esters of fatty acids were prepared as described by Ikemoto *et al.* (17). Fatty acids were analyzed by gas-liquid chromatography system, a model GC-14 A (Shimadzu Corp., Kyoto, Japan) equipped with a CBP 1 (OV-1 type) capillary column [25 m by 0.25 mm (inside diameter)] at 180-220°C and a flame ionized detector. Gas-liquid chromatograms were calculated by Chromatopac C-R 4 A data-processor (Shimadzu Corp.)

Quinone systems. Quinones were extracted from freeze-dried cells and purified as described by Collins *et al.* (18, 19). The purified quinones were analyzed by high - performance liquid chromatography (20). The abbreviation (DMK-7, DMK-8, DMK-9, etc.) used for demethylmenaquinones indicated the number of isoprene units in a side chain.

Table 1 Strain designations and isolation sources.

Strain	Other designation			Source
	JCM	NRIC	TISTR	Fermented product
Str 01		0102	928	Pla-chom (fermented small fish)
Str 02	8718		956	Pla-chom (fermented small fish)
Str03	8712	0103	927	Pla-ra (fermented fish)
F30-2	8717	0108	929	Pla-som (fermented fish)
A 30-1		0101		Pla-som (fermented fish)
F 34-1	8719	0109	930	Sai-krog-prieo (sour pork sausage)
FP 48-3		0107	933	Mum (sour beef sausage)
FP 13	8720	0104	931	Phak-gard-dong (pickled mustard)
FP 15-1		0105	932	Miang (fermented tea leaves)
FP 17-2	8716	0106	957	Miang (fermented tea leaves)
SB 1-1	8713	0110		Kecap koji (soybean koji in Indonesia)
SB 1-3	8714	0111		Kecap koji (soybean koji in Indonesia)
SB 3-2		0112		Kecap koji (soybean koji in Indonesia)
SB 8-2		0113		Kecap moromi (soy sauce mash in Indonesia)
SB 9-1	8715	0114		Kecap moromi (soy sauce mash in Indonesia)

JCM, Japan collection of Microorganisms, RIKEN, Saitama, Japan.
NRIC, NODAI Research Institute Culture Collection, Tokyo University of Agriculture, Tokyo, Japan.
TISTR, Thailand Institute of Scientific and Technological Research, Bangkok, Thailand.

RESULTS

Phenotypic characteristics

Morphological and cultural characteristics. All isolates were gram-positive, nonmotile, nonsporing cocci which arranged in pairs and in chains. Cells are spherical or ovoid, 0.5-1 μm in diameter. Colonies on GYP agar plate were circular, raise or low convex with entire margins, and nonpigmented.

Biochemical and physiological characteristics. They hemolyzed horse blood slightly. All grew at pH 6.0 to 9.6, at 15°C to 45°C, and in 2 to 6% NaCl. Most of isolates hydrolyzed arginine, grew at 10°C, pH 5.0, and in 6.5 to 8% NaCl. They were negative to catalase, hydrolysis of gelatin, reduction of nitrate, production of gas from glucose and gluconate, and growth at pH 4.0. Variable characteristics are shown in Table 2.

All isolates were facultatively anaerobic and split glucose fermentatively. They produced acid aerobically from D-glucose, D-fructose, D-Cellobiose, esculin, D-mannose, and D-ribose but failed to produce acid from α-methylglucoside, raffinose, L-rhamnose, and inulin. Variable productions of acid from carbohydrates are shown in Table 2.

The strains tested required calcium-pantothenate and niacin but did not require *p*-aminobenzoic acid for growth. Vitamins which gave variable for growth are shown in Table 3.

Isomer of lactic acid. The isolates Str 01, F 30-2, A 30-1, F 34-1, FP 13, Str 03, FP 17-2, FP 15-1, and FP 48-3 produced L+DL lactic acid from glucose.

Chemotaxonomic characteristics

DNA base composition. DNA base composition of the strains studied ranged from 37.0 to 44.8 mol% of guanine-plus-cytosine as shown in Table 4.

DNA homologies. Strains Str 01, Str 02, F 30-2, A 30-1, F 34-1, and FP 13 showed more than 75% homologies with *E. hirae* NRIC 1140^T, and Str 03, SB1-1, and SB 3-2 showed more than 87% with *E. faecalis* NRIC 1142^T. Strains SB1-3, SB 8-2, and SB 9-1 had more than 71% homologies with *E. faecium* NRIC 1145^T, and a strain FP 17-2 had 80% with *E. casseliflavus* GIFU 12717^T. The strains FP 15-1 and FP 48-3 showed low homologies (4-14%) with the four type strains (Table 4), and showed less than 13% with *E. avium* GIFU 12714^T, *E. dispar* GIFU 12713^T, *E. durans* GIFU 12715^T, *E. gallinarum* GIFU 8809 ^{T}, *E. malodoratus* GIFU 11735^T, *E. mundtii* GIFU 11736^T, *E. pseudoavium* GIFU 12712^T, *E. raffinosus* GIFU 12711^T, *E. seriolicida* GIFU 12716^T, and *Lactococcus lactis* NRIC 1149^T.

Cellular fatty acid composition. Most strains tested contained straight-chain fatty acids of $C_{18:1}$, $C_{16:0}$, $C_{16:1}$, and cyclopropane acid of C_{19} as dominant fatty acids. The other fatty acid profiles are shown in Table 5.

Quinone systems. Strains Str 03, SB 1-1 and SB 3-2 contained DMK-9, and the strain FP 15-1 had DMK-7 as a major demethylmenaquinone. Strain FP 17-2 had MK-7 as a dominant menaquinone. Other strains had not any quinones. Minor menaquinones detected are shown in Table 6.

Table 2. Characteristics of enterococcal strains.

Strain	Hydrolysis of Arginine	Esculin	Starch	Litmus milk: Gas from citrate	Litmus milk: Acid	Litmus milk: Coagulation	Litmus milk: Reduction	Litmus milk: Liquefaction	Growth: 10°C	Growth: pH 4.5	Growth: pH 5.0	Growth: 6.5% NaCl	Growth: 8.0% NaCl	Growth: 10.0% NaCl	Acid production from: D-Amygdalin	L-Arabinose	D-Galactose	Gluconate	Glycerol	Lactose	D-Maltose	D-Mannitol	D-Melibiose	D-Melezitose	Salicin	D-Sorbitol	Sucrose	D-Trehalose	D-Xylose
Str 01	+	-	-	-	+	+	+	-	+	-	+	+	+	+	-	-	+	-	-	-	+	-	+	-	+	-	+	-	-
Str 02	+	-	-	-	+	+	+	-	+	-	+	+	+	+	-	-	+	-	-	-	+	-	+	-	+	-	+	-	-
F 30-2	+	-	-	-	-	-	-	-	+	w	+	+	+	-	+	-	+	-	-	-	+	-	-	-	+	-	+	-	-
A 30-1	+	+	-	-	+	+	-	-	+	w	+	+	+	+	+	-	+	-	-	+	+	-	+	-	+	-	+	-	+
F 34-1	+	-	-	-	w	-	-	-	+	w	+	+	+	+	+	-	+	-	-	+	+	-	+	-	+	-	+	-	-
FP 13	+	-	-	-	-	-	-	-	+	-	+	+	+	w	-	-	+	-	-	-	+	-	-	-	+	-	+	-	-
Str 03	+	w	-	+	+	+	+	-	+	-	+	+	+	w	+	-	+	+	+	-	+	+	-	+	+	+	-	-	-
SB 1-1	+	w	-	+	-	-	+	+	+	-	+	w	w	-	+	-	+	+	+	-	+	+	-	+	+	+	-	-	-
SB 3-2	+	w	-	-	-	-	+	+	+	-	+	w	w	-	+	-	+	+	+	-	+	+	-	+	+	+	-	-	-
SB 1-3	+	-	-	-	w	-	-	-	+	-	+	w	-	-	-	-	+	-	-	-	+	+	+	-	+	-	+	-	-
SB 8-2	+	-	-	-	+	+	+	-	+	-	+	w	-	-	-	+	+	-	-	+	+	-	+	-	+	-	+	-	-
SB 9-1	+	-	-	-	+	+	+	-	+	-	+	-	-	-	+	+	+	-	-	+	+	-	+	-	+	-	+	-	-
FP 17-2	+	+	-	+	w	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FP 15-1	-	w	w	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	+	-	-	+	-	+	+	-
FP48-3	+	-	-	-	-	-	-	-	-	-	+	+	w	-	+	-	+	+	+	+	-	-	-	-	+	-	+	-	-
E. hirae NRIC 1140^T	+	-	-	-	w	-	-	-	+	-	-	+	+	-	+	-	-	-	-	+	-	+	+	-	-	+	+	+	-
E. faecalis NRIC 1142^T	+	+	-	+	+	-	+	-	+	-	+	w	-	-	+	-	+	-	+	+	+	+	-	+	+	+	-	+	-
E. faecium NRIC 1145^T	+	w	-	-	w	-	-	-	+	w	+	w	-	-	-	+	+	+	-	+	+	+	-	-	+	-	+	-	-
E. casseliflavus GIFU 12717^T	+	w	-	-	+	+	+	-	+	-	-	+	-	-	-	+	-	+	-	-	-	+	+	-	-	-	+	+	+

+, positive reaction; w, weak reaction;

-, negative reaction. GIFU, Gifu University School of Medicine, Gifu, Japan.

Table3. Vitamin requirements of enterococcal strains.

Strain	Thiamine	Riboflavin	Biotin	Folic acid	Pyridoxine
Str 01	-	-	-	+	-
Str 03	-	-	-	-	+
SB 1-1	-	+	w	-	+
SB 1-3	-	-	w	w	-
SB 8-2	-	-	-	w	-
FP 17-2	w	-	w	+	+
FP 15-1	-	+	-	w	-
FP 48-3	-	-	w	+	-
E. hirae NRIC 1140^T	-	-	-	+	-
E. faecalis NRIC 1142^T	-	-	-	-	-
E. faecium NRIC 1145^T	-	+	-	w	-
E. casseliflavus GIFU 12717^T	-	-	w	+	+

+, required; w, stimulative; -, not required.

Strains F 30-2, F 34-1 and FP 13 showed the same reaction as a strain Str 01 did.

Table4. DNA base compositions and DNA relatedness of enterococcal strains.

Strain	G+C content (mol%)	%Homology wigh labeled strains: NRIC 1140^T	NRIC 1142^T	NRIC 1145^T	GIFU 12717^T	Str 01
Str 01	39.6	92	17	5	10	100
Str 02	39.8	92	5	0	6	89
F 30-2	39.6	81	11	14	11	104
A 30-1	39.2	76	6	9	9	76
F 34-1	39.4	75	9	8	10	72
FP 13	39.6	77	2	1	9	74
Str 03	37.9	6	106	2	7	7
SB 1-1	38.4	5	87	5	7	4
SB 3-2	37.0	4	104	8	3	4
SB 1-3	38.3	12	16	72	13	13
SB 8-2	39.1	14	11	82	8	13
SB 9-1	37.5	10	16	71	10	12
FP 17-2	44.8	6	9	10	80	7
FP 15-1	37.8	6	12	10	6	8
FP48-3	37.9	6	10	14	4	14
E. hirae NRIC 1140^T	38.0	100	9	18	10	101
E. faecalis NRIC 1142^T	38.6	5	100	11	6	10
E. faecium NRIC 1145^T	39.0	9	20	100	8	21
E. casseliflavus GIFU 12712^T	43.7	6	5	9	100	8

DNA base compositions of the type strain were cited from reference 24.

Table 5. Cellular fatty acid composition of enterococcal strains and related cocci.

Strain	Straight-chain acids (%) $C_{12:0}$	$C_{14:1}$	$C_{14:0}$	$C_{16:1}$	$C_{16:0}$	$C_{18:1}$	$C_{18:0}$	$C_{20:1}$	Cy 17	Cy 19	Unknown% (no. of peak)
Str 01		1.0	5.5	9.0	19.8	25.3	5.4	11.2		14.9	7.0 (4)
F 30-2		1.0	5.0	8.5	17.2	23.8	6.5	12.9		16.1	8.9 (6)
A 30-1		T	5.2	6.9	20.8	25.5	6.4	11.3		14.3	8.2 (6)
F 34-1		T	4.7	7.3	20.4	24.1	7.3	11.9		15.5	8.4 (5)
FP 13	T	1.3	7.9	9.5	20.2	16.0	7.0	12.1		16.3	9.1(6)
Str 03		T	5.5	6.7	27.8	18.3	9.8	T		11.6	7.5 (5)
SB 1-1		T	6.2	7.1	33.2	18.0	5.2	4.0		20.3	5.3 (6)
SB 1-3		T	4.4	8.8	21.4	21.3	3.8	6.5		28.3	4.5 (5)
SB 9-1		T	5.2	8.9	22.3	20.8	3.5	6.0		28.2	3.9 (4)
FP 17-2	T	T	11.6	11.2	35.9	32.6	2.2				3.9 (1)
FP 15-1	T	4.6	5.4	30.5	5.0	20.9	3.6	6.7	3.4	9.1	7.2(5)
FP 48-3		1.2	10.1	13.3	25.7	45.9	T				2.4 (2)
E. hirae NRIC 1140^T		1.2	5.5	7.6	17.1	26.5	3.4	5.9		28.1	4.2 (4)
E. faecalis NRIC 1142^T			6.4	4.9	34.1	19.1	10.1	7.8		10.4	6.5 (6)
E. faecium NRIC 1145^T		T	3.7	9.8	16.7	30.5	3.1	4.3		26.0	4.5 (5)
E. casseliflavus GIFU 12717^T			6.2	5.9	29.3	52.8	1.3				4.2 (3)
Lactococcus lactis NRIC 1149^T		1.1	4.7	4.4	15.6	9.6	10.1	18.0	1.1	23.9	11.6 (5)

T, Trace of acid less than 1%; Cy, cyclopropane acid.

Table 6. Quinone systems of enterococcal strains and related cocci.

Strain	Menaquinone isoprenologue (%)			
	DMK-6	DMK-7	DMK-8	DMK-9
Str 03	-	15.3	32.4	52.3
SB 1-1	0.7	10.3	27.6	61.4
SB 3-2	-	4.9	23.2	71.9
FP 15-1	-	94.7	4.2	1.1
E. faecalis NRIC 1142^T	6.3	10.9	26.2	56.5
	MK-6	MK-7	MK-8	MK-9
FP 17-2	16.8	53.4	28.7	-
E. casseliflavus GIFU 12717^T	12.0	39.4	48.5	-
Lactococcus lactis NRIC 1149^T	5.9	29.1	15.3	49.5

-, not detected.

DISCUSSION

On the basis of phenotypic characteristics, DNA base composition, cellular fatty acid composition, and quinone systems, the isolates were included in the member of genus *Enterococcus* (21, 22). The DNA relatedness studies showed that 13 isolates fell into four species (23-26) and the two isolates FP 15-1 and FP 48-3 could not be identified as mentioned below.

Enterococcus hirae: Str 01, Str 02, F 30-2, A 30-1, F 34-1 and FP 13. These bacteria were isolated from pla-chom, pla-som, sai-krog-prieo and phak-gard-dong.

Enterococcus faecalis: Str 03, SB 1-1 and SB 3-2. These strains produced acid from gluconate, glycerol, D-melezitose and D-sorbitol (Table 2), and contained high amount of DMK-9 and DMK-8 (Table 6) which can distinguish them from other *Enterococcus* species (21, 22). They were found in pla-ra and kecap koji.

Enterococcus faecium: SB 1-3, SB 8-2 and SB 9-1. These strains are similar to *E. hirae* strains in their phenotypic and chemotaxonomic characteristics (22, 23). The acid produced from L-arabinose seem to be useful for separating these two species (22). They were isolated from kecap koji and kecap moromi.

Enterococcus casseliflavus: FP 17-2. This strain contained a high value of DNA base composition and MK-7, but had not the fatty acids of $C_{20:1}$ and cyclopropane acid of C_{19} as reported (22, 23, 27). This strain was found in miang.

Enterococcus species: FP 15-1 and FP 48-3. The strain FP 15-1 was isolated from miang and strain FP 48-3 was isolated from mum. They are different from other enterococcal strains as mentioned in the "Results" and in the Tables 2-6. Therefore, these strains were left unidentified.

In the differentiation of enterococcal strains, the acid production from carbohydrates, production of gas from citrate, folic acid requirement, DNA base composition, cellular fatty acid composition and quinone systems support the identification (21-23, 27) as shown in the Tables. Recently, Knudtson and Hartman (28) reported the usefulness of phenotypic characteristic for identification of enterococci and faecal streptococci. Strains used in this study were mostly identified by their procedure but some strains of the known species could not be identified. Therefore, determination of DNA relatedness is much more correct for the identification of enterococcal species than the phenotypic procedure.

The enterococci have been isolated from plant, soil, animal, human, and raw and prepared food including meat, poultry, fish, egg, milk, cheese and vegetables (29-36). These bacteria were found in a wide variety of fermented foods in Thailand and in Indonesia as mentioned above. Their roles in relation to food is not conclusively proven. The strains of *E. hirae, E. faecalis* and *E. faecium* are common organisms in the intestinal tract of man and animal, and it is difficult to keep them out of foods (23, 36). The presence of them may be used as an indication of faecal pollution and implies inadequate sanitary practices.

The above-mentioned strains have been deposited with Japan Collection of Microorganisms (JCM), RIKEN, Saitama, Japan; NODAI Research Institute Culture Collection (NRIC), Tokyo University of Agriculture, Tokyo, Japan; and Bangkok

MIRCEN, Thailand Institute of Scientific and Technological Research (TISTR), Bangkok, Thailand, and the accession numbers are shown in Table 1.

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