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Lactobacillus equi sp. nov., a predominant intestinal *Lactobacillus* species of the horse isolated from faeces of healthy horses

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***Lactobacillus equi* sp. nov. is described on the basis of 18 strains isolated as one of the predominant intestinal lactobacilli from horse faecal specimens. These 18 strains were isolated from 10 horses of 6 different farms out of 20 horses of 10 farms examined. They were Gram-positive, facultatively anaerobic, catalase-negative, non-spore-forming, non-motile, lactic-acid-homofermentative rods. The DNA G+C content was 38·9±0·8 mol%. DNA–DNA hybridization failed to associate these strains closely with any of the validly described type strains used. Analysis of the 16S rRNA gene sequence of representative strain YIT 0455^T revealed that the new isolates represent a new *Lactobacillus* species, for which the name *Lactobacillus equi* is proposed. The type strain is YIT 0455^T (= ATCC BAA-261^T = JCM 10991^T).**

Keywords: *Lactobacillus equi* sp. nov., horse intestinal microflora, taxonomy, phylogeny, lactic acid bacteria

INTRODUCTION

Lactobacilli are common members of the gastrointestinal tracts of man and mammals. In our study examining microbial colonization of the intestinal tract in newborn foals (Sakaitani *et al*., 1999), we noticed a dense layer of Gram-positive rods on the stratified squamous epithelium in the non-secreting area of the stomach of the horse. These bacteria have been identified as *Lactobacillus salivarius*, *Lactobacillus crispatus*, *Lactobacillus reuteri* and *Lactobacillus agilis* by DNA–DNA hybridization and 16S rRNA gene sequencing techniques (Yuki *et al*., 2000). As part of a study of the intestinal microflora in horses, this work reports the isolation of a dominant, indigenous, new *Lactobacillus* species of horses, for which the name *Lactobacillus equi* is proposed.

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An expanded version of Fig. 1 is available as supplementary data in IJSEM Online (http://ijs.sgmjournals.org/).

The DDBJ accession number for the 16S rRNA gene sequence of strain YIT 0455^T (= ATCC BAA-261^T = JCM 10991^T) is AB048833.

METHODS

Isolation of strains. Bacterial strains were isolated from faeces of 20 thoroughbreds, bred and reared on 10 farms in Hokkaido, Aomori, Chiba and Kagoshima prefectures, Japan. Fresh faeces were collected and transferred to the laboratory within 48 h under anaerobic conditions (AnaeroPack; Mitsubishi Gas Chemical) at 4 °C. Initial processing and subsequent weighing and dilution of the specimens were carried out under strictly anaerobic conditions by using a modification of the method of Hungate (Holdeman *et al*., 1977). Then, each dilution was spread on selective medium (LBS agar; Becton Dickinson) and incubated anaerobically (AnaeroPack) at 37 °C for 3 d. All further cultivation was done at 37 °C in MRS broth (Difco Laboratories) unless stated otherwise.

Biochemical characterization. Sugar fermentation patterns were determined using the API 50 CH system (BioMérieux), according to the manufacturer's instructions. Results were recorded after 48 h at 37 °C. Isomers of lactate formed from glucose were determined enzymically with the F kit (Boehringer Mannheim). Other biochemical tests, such as motility, growth at a fixed temperature and gas production from glucose, were performed by the methods described by Mitsuoka (1969).

RAPD fingerprinting. Bacteria were grown in MRS broth overnight at 37 °C. Chromosomal DNA to be used as

Table 1. Origin of strains of *Lactobacillus equi* isolated from faeces of healthy horses

Horse no.	Area (prefecture)	Farm	No. of strains
1	Hokkaido	A	2
2	Hokkaido	A	3
3	Hokkaido	A	3
4	Aomori	B	1
5	Aomori	B	2
6	Chiba	C	2
7	Chiba	D	1
8	Kagoshima	E	1
9	Kagoshima	E	1
10	Kagoshima	F	2

template for RAPD PCR and 16S rDNA amplification was prepared from bacterial strains by the method of Zhu *et al.* (1993). PCR-based RAPD fingerprinting was carried out by the method of Akopyanz *et al.* (1992) using two primers (GAGGACAAAG and GGCGTCGGTT). The PCR products were electrophoresed in 2% agarose gels and photographed under UV light.

Type strains. The type strains used in the study were from our collection (Yakult Institute Tokyo, YIT), originally obtained from the American Type Culture Collection (ATCC), Japan Collection of Microorganisms (JCM), Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM) and NODAI Research Institute Culture Collection (NRIC). These were: *Lactobacillus acidophilus* YIT 0070^T (ATCC 4356^T), *Lactobacillus agilis* YIT 0253^T (JCM 1187^T), *Lactobacillus amylovorus* YIT 0211^T (JCM 1126^T), *Lactobacillus animalis* YIT 0256^T (JCM 5670^T), *Lactobacillus brevis* YIT 0076^T (ATCC 14869^T), *Lactobacillus buchneri* YIT 0077^T (ATCC 4005^T), *Lactobacillus casei* YIT 0180^T (ATCC 334^T), *Lactobacillus coryniformis* subsp. *coryniformis* YIT 0237^T (JCM 1164^T), *Lactobacillus crispatus* YIT 0212^T (JCM 1185^T), *Lactobacillus fermentum* YIT 0081^T (ATCC 14931^T), *Lactobacillus gasseri* YIT 0192^T (DSM 20243^T), *Lactobacillus graminis* YIT 0260^T (NRIC 1775^T), *Lactobacillus johnsonii* YIT 0219^T (JCM 2012^T), *Lactobacillus murinus* YIT 0239^T (JCM 1717^T), *Lactobacillus plantarum* YIT 0102^T (ATCC 14917^T), *Lactobacillus reuteri* YIT 0197^T (JCM 1112^T), *Lactobacillus rhamnosus* YIT 0105^T (ATCC 7469^T), *Lactobacillus ruminis* YIT 0221^T (JCM 1152^T), *Lactobacillus salivarius* subsp. *salicinius* YIT 0089^T (ATCC 11742^T) and *Lactobacillus salivarius* subsp. *salivarius* YIT 0104^T (ATCC 11741^T).

DNA base composition and DNA–DNA hybridization. The G+C content was determined by hydrolysing the DNA enzymically and quantifying the nucleosides by HPLC according to the method of Ezaki *et al.* (1990). Fluorometric DNA–DNA hybridization in microdilution wells was carried out by the method of Ezaki *et al.* (1989). Details of culture conditions, isolation and purification of DNA were as described by Yuki *et al.* (2000).

16S rRNA gene sequencing. *In vitro* PCR amplification of

Table 2. Differential characteristics of *Lactobacillus equi* sp. nov. and closely related lactobacilli

+, 90% or more strains positive; −, 90% or more strains negative; D, 11–89% strains positive. *Lactobacillus equi* is also positive for acid production from galactose, glucose, fructose, maltose, sucrose, raffinose, *N*-acetylglucosamine (14 of 18 strains), D-turanose (7 of 18), arbutin (3 of 18), inulin (3 of 18), L-arabinose (2 of 18) and D-xylose (1 of 18). There was no fermentation of glycerol, erythritol, D-arabinose, L-xylose, adonitol, β-methyl-D-xyloside, sorbose, dulcitol, inositol, α-methyl-D-mannoside, α-methyl-D-glucoside, amygdalin, cellobiose, trehalose, melezitose, starch, glycogen, xylitol, β-gentiobiose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate and 5-ketogluconate. No gas was produced from glucose. D- and L-lactate were produced by all strains. Eight of the 18 strains hydrolysed aesculin. Catalase activity was negative in all strains.

Character	*L. equi*	*L. animalis**	*L. salivarius**	*L. ruminis**	*L. agilis**	*L. murinus**	*L. aviarius*†
G+C (mol%)	38–40	41–44	34–36	44–47	43–44	43–44	39–43
Stereo isomer of lactic acid	DL	L	L	L	L	L	DL
Fermentation of:							
Amygdalin	−(0/18)	D	−	+	+	D	D
Arabinose	−(0/18)	D	−	−	−	+	−
Cellobiose	−(0/18)	+	−	+	+	+	+
Galactose	+(18/18)	+	+	+	+	+	D
Lactose	+(18/18)	+	+	D	+	+	−
Mannitol	+(18/18)	−	+	−	+	D	−
Mannose	D(8/18)	+	+	+	+	+	+
Melezitose	−(0/18)	−	−	−	+	−	−
Melibiose	+(18/18)	+	+	+	+	+	D
Rhamnose	D (10/18)	−	D	−	−	−	−
Ribose	D (8/18)	−	−	−	+	+	−
Salicin	D (3/18)	+	D	+	+	D	+
Sorbitol	D (11/18)	+	+	+	D	−	−
Trehalose	−(0/18)	−	+	−	+	D	+

* According to Kandler & Weiss (1986).

† According to Fujisawa *et al.* (1984).

Table 3. DNA relatedness among *Lactobacillus equi* sp. nov. and phylogenetically closely related *Lactobacillus* species

Reassociation values are means of three determinations.

Strain	Reassociation (%) to DNA from strain:						
	YIT 0455^T	YIT 0456^T	YIT 0457^T	JCM 1187^T	JCM 1152^T	JCM 5670^T	JCM 1717^T
YIT 0455^T	(100)	98	94	20	12	8	26
YIT 0456	105	(100)	87	21	18	21	21
YIT 0457	102	91	(100)	22	7	15	21
L. agilis JCM 1187^T	18	28	16	(100)	5	14	21
L. ruminis JCM 1152^T	8	15	10	11	(100)	22	18
L. animalis JCM 5670^T	5	16	1	3	1	(100)	24
L. murinus JCM 1717^T	19	23	8	18	8	20	(100)

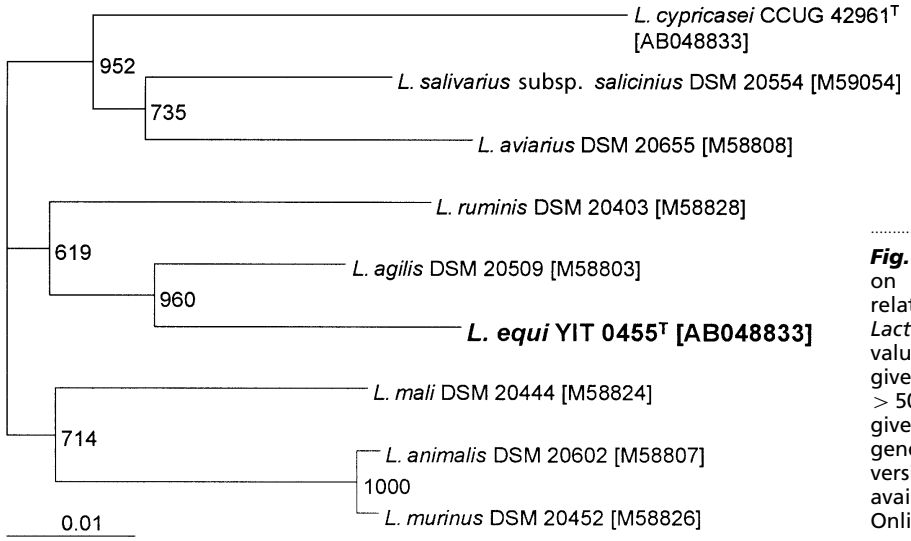

Fig. 1. Unrooted phylogenetic tree based on 16S rRNA comparisons showing the relationships of strain YIT 0455^T to other *Lactobacillus* strains. Bootstrap confidence values obtained with 1000 resamplings are given at the branch points (only values >500 are shown). Accession numbers are given in parentheses. The bar indicates a genetic distance of 0·01. An expanded version of this tree with more taxa is available as supplementary data in IJSEM Online (http://ijs.sgmjournals.org/).

16S rRNA genes and direct sequencing of the amplified DNA fragments were performed. Details of the procedures, except the sequence of primer 8F (5′-AGAGTTTGATCMTGGCTCAG-3′), have been described previously (Miyake *et al*., 1998). Primers 8F and 15R were used for PCR, and 8F, 520F, 930F, 1100F, 15R, 520R, 800R and 1100R were used for 16S rRNA gene sequencing.

Phylogenetic analysis of the sequence data. Phylogenetic analyses were performed using data from the DNA Data Bank of Japan (DDBJ, http://www.ddbj.nig.ac.jp). The alignment and the stability of relationships were assessed by bootstrapping using CLUSTAL W (Thompson *et al*., 1994).

RESULTS AND DISCUSSION

Isolation and selection of the strains

Among the 178 colonies of Gram-positive rods isolated, 66 different strains distinguished by RAPD DNA fingerprinting were selected. DNA–DNA relatedness of these strains to 20 standard type strains was examined and some of these strains were identified as *Lactobacillus salivarius*, *Lactobacillus johnsonii*, *Lactobacillus crispatus*, *Lactobacillus reuteri*, *Lactobacillus plantarum*, *Lactobacillus amylovorus* and *Lactobacillus coryniformis* (M. Morotomi and others, unpublished data). DNA from 35 strains did not hybridize to the DNA from any of the 20 type strains. DNA–DNA relatedness among these 35 strains was also examined. A group of 18 strains, amongst which the homology was over 80 %, was selected for further identification in this study. The origins of these strains are shown in Table 1.

Cultural and biochemical characteristics

Cultural and biochemical characteristics of the 18 strains isolated are described in Table 2 and the species description below.

DNA base composition and DNA–DNA hybridization

The mean G+C content of the 18 strains was $38{\cdot}9 \pm 0{\cdot}8$ mol %. These strains had DNA homology values of more than 80 % with each other, showing that they belonged to a single species, but less than 30 % with DNA from all the reference strains used. Table 3 summarizes the DNA relatedness among the three strains YIT 0455^T, YIT 0456 and YIT 0457, and phylogenetically closely related *Lactobacillus* species.

16S rRNA gene sequence and phylogenetic analysis

To establish the phylogenetic position of the unknown bacterium, the 16S rRNA genes of the three representative strains, YIT 0455^T, YIT 0456 and YIT 0457 [= JCM 10991^T (ATCC BAA-261^T), JCM 10992 and JCM 10993, respectively], were amplified by PCR and characterized by sequence analysis. The almost complete gene sequences (1369 nt) of the three strains were determined, aligned and found to be identical. A tree depicting the phylogenetic position of strain YIT 0455^T within the *Lactobacillus* genus is shown in Fig. 1. *Lactobacillus agilis* DSM 20509 was the most closely related species in the phylogenetic tree and showed the highest sequence homology (96·3 %) with strain YIT 0455^T.

Table 2 shows the characteristics most useful in distinguishing the strains studied from closely related lactobacilli. Note that the phenotypic data achieved by different methods sometimes generate different results (Lawson *et al*., 2001). Based on its phenotypic and phylogenetic distinctiveness, it is considered that the strains studied represent a new species in the genus *Lactobacillus*, for which we propose the name *Lactobacillus equi* sp. nov.

Description of *Lactobacillus equi* sp. nov.

Lactobacillus equi (e′qui. L. n. *equus* horse; L. gen. n. *equi* of the horse).

Gram-positive, non-motile, non-spore-forming rods. Colonies on MRS agar are white, smooth, convex and approximately 2 mm in diameter. Cells occur singly and in pairs and are 0·7 (0·6–0·8) × 2·7 (1·3–3·5) μm in size. Some strains contain filamentous cells. Facultatively anaerobic, catalase-negative and obligately homofermentative. Most strains grow at 45 °C, but not at 15 °C. D- and L-lactic acid are produced from glucose. Acid is produced from galactose, glucose, fructose, mannitol, maltose, lactose, melibiose, sucrose and raffinose. Acid is not produced from glycerol, erythritol, D-arabinose, L-xylose, adonitol, β-methyl-D-xyloside, sorbose, dulcitol, inositol, α-methyl-D-mannoside, α-methyl-D-glucoside, amygdalin, cellobiose, trehalose, melezitose, starch, glycogen, xylitol, gentiobiose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate and 5-ketogluconate. Most strains produce no acid from L-arabinose, D-xylose, arbutin, salicin and inulin. Reactions of aesculin hydrolysis and acid production from rhamnose, sorbitol, *N*-acetylglucosamine, ribose, mannose and D-turanose are variable. The DNA G+C content of the type strain is 38 % (as determined by HPLC). Isolated from faeces of horses. The type strain is strain YIT 0455^T (= ATCC BAA-261^T = JCM 10991^T)

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