Lactobacillus equigenerosi sp. nov., a coccoid species isolated from faeces of thoroughbred racehorses

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Two strains of lactic acid bacteria were isolated from faeces of two actively racing thoroughbred horses. The isolates formed a subcluster in the *Lactobacillus reuteri* phylogenetic group, closely related to *Lactobacillus fermentum*, *L. gastricus*, *L. ingluviei* and *L. mucosae*, by phylogenetic analysis based on 16S rRNA gene sequences. Levels of DNA–DNA relatedness revealed that the isolates belonged to the same taxon and were genetically separated from their phylogenetic relatives. Biochemical and physiological characteristics also distinguished the isolates from their phylogenetic relatives. The isolates produced spherical or oval cells, and tetrad-like cells were rarely seen. To the best of our knowledge, this is the first report of this morphological characteristic within the genus *Lactobacillus*. Thus, the isolates represent an atypical novel species of the genus *Lactobacillus*, for which the name *Lactobacillus equigenerosi* sp. nov. is proposed. The type strain is NRIC 0697^T (=JCM 14505^T =DSM 18793^T).

The gastrointestinal tracts of animals harbour complex microbiota, and lactic acid bacteria are common members of the microbiota of many animals. As lactic acid bacteria have been reported to have several health-promoting effects on host animals (Ouwehand *et al.*, 2002), the microbiota of the organisms in faeces and/or gastrointestinal contents have been actively studied for many animals (Brashears *et al.*, 2003; Casey *et al.*, 2004; Walter *et al.*, 2001).

During a study of lactic acid bacteria inhabiting the gastrointestinal tract of thoroughbred racehorses, 35 strains of lactic acid bacteria were isolated from faeces of six thoroughbreds. Thirty of the isolates were identified as

Lactobacillus equi, *L. johnsonii*, *L. kitasatonis*, *L. mucosae*, *L. salivarius*, *Streptococcus bovis*, *S. equinus* or *Weissella confusa*, and the other five strains could not be identified (unpublished data). Two of the five strains were phylogenetic relatives of *Lactobacillus gastricus*, one was a phylogenetic relative of *Lactobacillus salivarius* and the other two belonged to the *Streptococcus* phylogenetic cluster. The phylogenetic relative of *L. salivarius* was recently classified within a novel *Lactobacillus* species, *Lactobacillus hayakitensis* (Morita *et al.*, 2007). This paper deals with the taxonomic study of the two phylogenetic relatives of *L. gastricus*.

Faecal samples were collected from six actively racing thoroughbred horses at the Miho or Ritto Training Center in Ibaraki or Shiga prefecture of Japan in 2003, and the faeces were immediately brought to our laboratory under anaerobic conditions by using anaerobic jars (GasPak System; BBL). Strains NRIC 0696 and NRIC 0697^T were isolated from faeces of different thoroughbreds collected at the Ritto Training Center by using LBS agar (BBL) under

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains NRIC 0696 and NRIC 0697^T are respectively AB288049 and AB288050.

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

anaerobic conditions in anaerobic jars. After isolation, they were maintained on MRS agar (Oxoid) containing 5.0 g calcium carbonate l^{-1} . *L. gastricus* DSM 16045^T was obtained from the DSMZ and the strain was used as a reference strain in the present study. The reference strain was also maintained on MRS agar.

16S rRNA gene sequences of the two isolates were determined by methods described previously (Endo & Okada, 2005). The closest recognized relatives of the isolates were determined by performing DataBase searches, and sequences of closely related species were retrieved from DDBJ. Multiple alignments of the sequences were carried out with the program CLUSTAL_X, version 1.18 (Thompson *et al.*, 1997). Distance matrices for the aligned sequences were calculated by using the two-parameter method of Kimura (1980). The neighbour-joining method was used to construct a phylogenetic tree (Saitou & Nei, 1987). The robustness of individual branches was estimated by bootstrapping with 1000 replicates (Felsenstein, 1985). Phylogenetic trees were also constructed by using the maximum-likelihood (Cavalli-Sforza & Edwards, 1967) and maximum-parsimony (Kluge & Farris, 1969) methods with PHYLIP version 3.65 as described previously (Endo & Okada, 2006). The determined 16S rRNA gene sequences of the isolates were compared with each other, and the sequence of NRIC 0697^T was used to search for sequence similarity with DataBase. Approximately 1450 bp of the 16S rRNA gene sequences of the isolates and related species were used for constructing phylogenetic trees. The sequence similarity between NRIC 0696 and NRIC 0697^T was 99.9%. The highest sequence similarity of NRIC 0697^T to the type strains of known species of lactic acid bacteria was found with *L. gastricus*, *L. mucosae*, *L. ingluviei* and *L. fermentum* (97.9, 95.8, 95.2 and 93.4%, respectively). The DataBase search also showed that the partial sequence of NRIC 0697^T was identical to the sequence of the

predominant DNA (GenBank accession no. AB250240) derived from faeces of thoroughbreds by using *Lactobacillus*-group-specific denaturing gradient gel electrophoresis analysis in our other study (Endo *et al.*, 2007). This probably shows that the isolates represent one of the predominant *Lactobacillus* species in faeces of thoroughbreds. The isolates formed a subcluster with *Lactobacillus fermentum*, *L. gastricus*, *Lactobacillus ingluviei* and *L. mucosae* in the *Lactobacillus reuteri* phylogenetic group (Schleifer & Ludwig, 1995; Hammes & Hertel, 2006) on the basis of neighbour-joining analysis (Fig. 1). Identical tree topologies were obtained by using the maximum-parsimony and maximum-likelihood analysis (see Supplementary Figs S1 and S2 in IJSEM Online). *L. gastricus*, *L. ingluviei* and *L. mucosae* were originally isolated from the gastrointestinal contents of animals (Roos *et al.*, 2000, 2005; Baele *et al.*, 2003) and *L. fermentum* has been isolated from various environmental samples including animal faeces (Dellaglio *et al.*, 2004). This suggests that the major habitat of members of this subcluster is the animal gastrointestinal tract.

Levels of DNA–DNA relatedness between the isolates and *L. gastricus* DSM 16045^T and the G+C contents of the isolates were determined by methods described previously (Endo & Okada, 2006). Extraction and isolation of bacterial DNAs were performed by the method of Marmur (1961) as modified by Ezaki *et al.* (1983). Strains of *L. fermentum*, *L. ingluviei* and *L. mucosae* were not used for the determination of DNA–DNA relatedness because the sequence similarities between the isolates and the type strains of these three species were lower than the recommended value for species differentiation described by Stackebrandt & Goebel (1994). Recently, a revised recommended value for species differentiation was suggested, at 98.7–99% (Stackebrandt & Ebers, 2006). The isolates showed a high level of DNA–DNA relatedness

Fig. 1. Phylogenetic relationships of strains NRIC 0696 and NRIC 0697^T (*L. equigenerosi* sp. nov.) to related species based on 16S rRNA gene sequences. The tree was constructed by the neighbour-joining method. *L. delbrueckii* ATCC 9649^T was used as an outgroup. Bootstrap percentages above 70% are given at branching points. Bar, evolutionary distance (K_{nuc}) of 0.01.

Table 1. Differential characteristics between *L. equigenersi* sp. nov. (strains NRIC 0696 and NRIC 0697^T) and closely related species

Species: 1, *L. equigenersi* sp. nov.; 2, *L. gastricus* (unless indicated, data from Roos *et al.*, 2005); 3, *L. fermentum* (Dellaglio *et al.*, 2004); 4, *L. ingluviei* (Baele *et al.*, 2003); 5, *L. mucosae* (Roos *et al.*, 2000). +, Positive; –, negative; v, variable; w, weakly positive.

Characteristic	1	2	3	4	5
Cell morphology	Round	Round*	Rod	Rod	Rod
Acid from:					
D-Xylose	+	–	v	+	v
D-Fructose	w	+	+	+	–
Cellobiose	–	+	v	–	–
Melibiose	+	+	+	–	v
D-Salicin	–	+	–	–	–
Trehalose	–	+	v	–	–
Growth at 15/45 °C	–/+	–/–	+/+	–/+	–/+
G+C content (mol%)	42	41.3	52–54	49	46.5

*Determined in the present study.

(93 %). Therefore, we concluded that the isolates belonged to the same taxon. In contrast, the isolates showed low levels of DNA–DNA relatedness to *L. gastricus* DSM 16045^T (18–23 %). The G+C contents of the isolates were 42 mol%.

Biochemical and physiological characteristics of the isolates were determined by methods described previously (Endo & Okada, 2005). The detailed characteristics of the isolates are given in the species description, and the characteristics

were compared with those of phylogenetic relatives *L. fermentum*, *L. gastricus*, *L. ingluviei* and *L. mucosae* (Table 1). The isolates were obligately heterofermentative lactic acid bacteria, and produced DL-lactic acid, carbon dioxide and ethanol or acetic acid from D-glucose. The isolates were distinguished from *L. gastricus* by their acid production patterns from carbohydrates and growth at 45 °C (Table 1). Furthermore, values of DNA G+C content of the isolates were very different from those of *L. fermentum*, *L. ingluviei* and *L. mucosae* (Table 1).

Morphological characteristics of NRIC 0697^T and *L. gastricus* DSM 16045^T were determined by using a scanning electron microscope (SEM). The type strains were cultured for 1 to 2 days in MRS broth at 37 °C under anaerobic conditions. After culturing, cells were washed with 0.2 M phosphate buffer (pH 7.0), fixed with 2 % glutaraldehyde for 2 h at 4 °C, post-fixed with 1 % OsO₄ for 3 h at 4 °C, dehydrated with a series of increasing ethanol concentrations (70, 80, 90 and 95 % and twice at 100 %, for 15 min each) and soaked in 3-methyl butyl acetate for 1 day. The prepared cells were subsequently critical-point-dried in a critical-point drying unit (model HCP-2; Hitachi), sputtered with Pt/Pd (model E-102; Hitachi) and observed with an SEM (model S-4000; Hitachi). Scanning electron micrographs of NRIC 0697^T and *L. gastricus* DSM 16045^T are shown in Fig. 2. The micrographs showed that the cells of both strains were spherical or oval cocci and occurred singly or in pairs. Tetrad-like coccoid cells were rarely seen for either strain. *L. gastricus* has been reported to produce rod-shaped cells (Roos *et al.*, 2005), but we were unable to observe rod-shaped cells for either type strain in various growth phases and/or in various media (data not shown). We then determined the morphological characteristics of

Fig. 2. Scanning electron micrographs of cells of *L. equigenersi* NRIC 0697^T (a, b) and *L. gastricus* DSM 16045^T (c, d). Bars, 1 μm.

the original type strain of *L. gastricus*, Kx156A7^T, preserved at the Swedish University of Agricultural Sciences. In our analysis, this strain formed coccoid cells that occurred in pairs or in tetrads, but not rod-shaped cells. Therefore, we concluded that *L. gastricus* is a coccus. The species of the genus *Lactobacillus* are well known as producing rod-shaped cells (Rogosa, 1974; Hammes & Vogel, 1996), but the phylogenetic cluster of the genus *Lactobacillus* comprises bacilli with cocci, which are the tetrad-forming cocci in the genus *Pediococcus* (Collins *et al.*, 1991; Schleifer & Ludwig, 1995). Almost all members of the genus *Pediococcus* formed a subcluster within the *Lactobacillus* cluster (Liu *et al.*, 2006) but, exceptionally, *Pediococcus dextrinicus* did not belong to the *Pediococcus* subcluster and formed a subcluster with *Lactobacillus* species (Liu *et al.*, 2006; Tong & Dong, 2005). As *P. dextrinicus* was a tetrad-forming coccus (Garvie, 1986), the species was classified as a member of the genus *Pediococcus* (Simpson & Taguchi, 1996; Garvie, 1986). However, more recently, classification of lactic acid bacteria at the genus level has been based on phylogenetic position using 16S rRNA gene sequences (Schleifer & Ludwig, 1995) rather than morphological characteristics. The genus *Weissella*, one of the genera of lactic acid bacteria, also comprises bacilli and cocci (Collins *et al.*, 1993). As mentioned above, as the new isolates and *L. gastricus* formed a subcluster with *Lactobacillus* species by phylogenetic analysis (Fig. 1), the isolates and *L. gastricus* should be regarded as coccoid species in the genus *Lactobacillus* based on the current classification method of lactic acid bacteria (Schleifer & Ludwig, 1995). With application of this classification method, *P. dextrinicus* should also be a member of the genus *Lactobacillus* rather than the genus *Pediococcus*, although this is not discussed further in the present study.

Based on the data provided, the isolates can be assigned as members of the genus *Lactobacillus* by their phylogenetic position, but physiological and biochemical characteristics and levels of DNA–DNA relatedness distinguished the isolates from their phylogenetic relatives. Moreover, morphological characteristics demonstrated that the isolates represented an atypical species in the genus *Lactobacillus*. Thus, the isolates represent a novel species, for which the name *Lactobacillus equigenerosi* sp. nov. is proposed. In addition, as the morphological characteristics of *L. gastricus* do not conform to the original description of the species (Roos *et al.*, 2005), an emended description of *Lactobacillus gastricus* is presented below.

Description of *Lactobacillus equigenerosi* sp. nov.

Lactobacillus equigenerosi (e'qui.ge.ne.ro'si. L. n. *equus* a horse; L. adj. *generosus* of noble birth, well-bred; N.L. gen. n. *equigenerosi* of a thoroughbred horse).

Cells are Gram-positive, non-motile and spherical or oval cocci measuring 0.5–0.8 × 0.8–1.5 µm. Cells usually occur singly or in pairs, and tetrad-like cells are uncommon.

Facultatively anaerobic and catalase-negative. Growth in broth is enhanced under anaerobic conditions. Colonies are not formed under aerobic conditions but are formed under anaerobic conditions. Colonies on MRS agar under anaerobic conditions are beige, smooth and approximately 1–2 mm in diameter after incubation for 2 days at 37 °C. Obligately heterofermentative and produces DL-lactic acid, carbon dioxide and ethanol or acetic acid from D-glucose. Nitrate is not reduced. Acid is produced from D-glucose, D-xylose, maltose, melibiose and sucrose and is produced weakly from D-ribose and D-fructose, but acid is not produced from L-rhamnose, cellobiose, D-salicin, trehalose, melezitose, D-mannitol, D-sorbitol or starch. Acid production from L-arabinose, D-galactose, lactose and raffinose is strain-dependent. Cells grow at 30–45 °C and grow slowly at 25 °C, but not at 20 or 50 °C. Both known strains grow at pH 4.0 but not at pH 3.5. Growth is observed in MRS broth containing 2.5 % (w/v) NaCl but not 5.0 % (w/v) NaCl. The DNA G + C content of both known strains is 42 mol%.

The type strain is NRIC 0697^T (=JCM 14505^T =DSM 18793^T). Strains NRIC 0697^T and NRIC 0696 were isolated from faeces of thoroughbred racehorses collected at the Ritto Training Center in Shiga prefecture, Japan, in 2003.

Emended description of *Lactobacillus gastricus* Roos *et al.* 2005

Lactobacillus gastricus (gas'tri.cus. N.L. masc. adj. *gastricus* from Gr. adj. *gastrikos* of the stomach).

The description of *L. gastricus* is as given in detail by Roos *et al.* (2005) with the following change. Cells are spherical or oval cocci measuring 0.6–0.8 × 1.0–2.0 µm. Cells usually occur singly or in pairs, and tetrad-like cells are uncommon. The type strain is Kx156A7^T (=LMG 22113^T =DSM 16045^T =CCUG 48454^T).

Acknowledgements

We thank all concerned parties of the Seishi, Yuasa and Setoguchi stables of the Japan Racing Association and the Muneishi stable of the Kochi Racing Association, as well as M. Iida (Chiyoda Farm), M. Matsumoto (Japan Race Horse Registry), K. Shimamura (KK Bestsellers) and M. Nagayama (Kochi Racing Association) for their help with collection of horse faecal samples. Thanks are also due to J. P. Euzéby (Société de Bactériologie Systématique et Vétérinaire and École Nationale Vétérinaire de Toulouse) for advice on the naming of the novel species, Y. Yaguchi (Electron Microscope Center, Tokyo University of Agriculture) for advice about procedures for SEM observation and C. Obata (NODAI Culture Collection Center) for technical assistance.

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