Lactobacillus kitasatonis sp. nov., from chicken intestine

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Four strains isolated from chicken small intestine and strains JCM 1038 and JCM 1039 (designated as *Lactobacillus acidophilus*) were characterized by phenotypic and molecular taxonomic methods. They were Gram-positive, catalase-negative, facultatively anaerobic rods that did not produce gas from glucose. These strains had similar phenotypic characteristics and exhibited intergroup DNA relatedness values of >77%, indicating that they comprised a single species. The 16S rRNA gene sequence of a representative strain, JCM 1039^T (designated as type strain in this study), was determined and aligned with those of other *Lactobacillus* species. JCM 1039^T was placed in the *Lactobacillus delbrueckii* cluster of the genus *Lactobacillus* on the basis of phylogenetic analysis and formed an independent cluster that was distinct from its closest neighbours, *Lactobacillus amylovorus*, *Lactobacillus crispatus*, *Lactobacillus gallinarum*, *L. acidophilus* and *Lactobacillus helveticus*. Results of DNA–DNA hybridization experiments and whole-cell protein profiles clearly indicated that these strains represent a novel *Lactobacillus* species, for which the name *Lactobacillus kitasatonis* sp. nov. is proposed; the type strain of this species is JCM 1039^T.

Hansen & Møcquot (1970) described the properties of *Lactobacillus acidophilus* in detail. As later revealed by Johnson *et al.* (1980) and others (Lauer *et al.*, 1980; Sarra *et al.*, 1980), six genome clusters (A1–A4, B1 and B2) exist within the *L. acidophilus* group. Johnson *et al.* (1980) designated homology group A1 as *L. acidophilus*. Later, group B1 was designated as *Lactobacillus gasseri* by Lauer & Kandler (1980) and group A2 was demonstrated to be synonymous with *Lactobacillus crispatus* by Cato *et al.* (1983). Furthermore, Fujisawa *et al.* (1992) proposed that groups A4 and B2 were novel species (*Lactobacillus gallinarum* and *Lactobacillus johnsonii*, respectively) and that group A3 should be considered as *Lactobacillus amylovorus*.

Although six genome clusters of the *L. acidophilus* group have been designated as separate species with validly published names, they are difficult to distinguish solely on the basis of phenotypic characteristics. Consequently, many strains besides *L. acidophilus* (group A1) are still catalogued in culture collections as '*L. acidophilus*', as suggested by Fujisawa *et al.* (1996). It has been reported that S-layers,

which are composed of a single protein species with a relative molecular mass of 43 000–46 000, commonly occur in *Lactobacillus* isolates that belong to genome clusters A1–A4 within the *L. acidophilus* group, but are absent from isolates that belong to genome clusters B1 and B2 (Masuda, 1992; Mukai & Arihara, 1994; Boot *et al.*, 1996). Our previous studies showed that cell-surface protein profiles of *L. acidophilus* JCM 1038 and JCM 1039 from chicken intestine were apparently different from those of reference cells that belonged to *L. acidophilus* group A (Mukai & Arihara, 1994). Based on our preliminary results, we suspected that these strains do not belong to *L. acidophilus* (group A1). Furthermore, data from a recent study by Ryu *et al.* (2001), who used a ribotyping technique, confirmed the results of a previous study that showed that the *L. acidophilus* group could be divided into two major clusters. Noticeably, the results also showed that the *L. acidophilus* group could be further divided by ribotyping into 14 genotypes (A1–A11 and B1–B3) with similarity levels of 50% and that JCM 1038 and JCM 1039 belong to an independent genotype among the 14 genotypes. These ribotyping results, along with those of our previous study, suggest strongly that strains JCM 1038 and JCM 1039 should be reclassified as a different species. We have recently isolated homofermentative *Lactobacillus* strains from the mucosal surface of chicken small intestine that show cell-surface protein profiles similar to those observed in strains JCM 1038 and JCM 1039. In this paper, we describe the

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A figure showing SDS-PAGE analysis of whole-cell proteins is available as supplementary material in IJSEM Online.

characteristics of JCM 1038, JCM 1039 and their related *Lactobacillus* strains isolated from the mucosa of chicken small intestine and propose that this novel species should be named *Lactobacillus kitasatonis* sp. nov.

Strain JCM 1039^T (=KCTC 3155^T) was designated as the type strain of the novel species in this study. JCM 1038 (=KCTC 3154) and JCM 1039^T originated from chicken intestine; reference strains of *Lactobacillus* were obtained from the Japan Collection of Microorganisms (JCM, Wako, Japan). Strains KM55, KM105, KM132 and KM9212 were isolated from the small intestines of two euthanized White Leghorn hens kept in the same farm; this was performed anaerobically (AnaeroPack; Mitsubishi Gas Chemical) on *Lactobacillus* selective (LBS) agar (Becton Dickinson) after 48 h incubation. All further cultivation was done anaerobically on MRS agar (de Man *et al.*, 1960) or in MRS broth at 37 °C. Cell shape, size and arrangement, Gram-stain and colonial appearance were determined by using cells grown on MRS agar plates for 2 days at 37 °C. Strains were tested for carbohydrate fermentation abilities by using the API 50 CHL system (bioMérieux). Production of gas from glucose and gluconate was examined with Durham tubes. Catalase formation, growth at 15 and 45 °C and motility were tested by previously described methods (Mitsuoka, 1969), except that MRS broth was used as the basal medium. Extraction of whole-cell proteins and their separation by SDS-PAGE were performed as described previously (Mukai & Arihara, 1994).

Chromosomal DNA from *Lactobacillus* strains was prepared as described previously (Luchansky *et al.*, 1991). G+C contents of the DNA preparations were determined by the HPLC method (Kaneko *et al.*, 1986) by using a DNA-GC kit (Yamasa Shoyu). DNA from calf thymus that had a G+C content of 42 mol% was used as a standard. DNA–DNA relatedness was determined by the method of Ezaki *et al.* (1989) by using photobiotin and a microplate. Almost-complete 16S rRNA gene sequences were determined for three representative strains: JCM 1038, JCM 1039^T and KM9212. A fragment of the 16S rRNA gene (corresponding to positions 8–1541 in the *Escherichia coli* numbering system) was amplified by PCR, using conserved primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1522R [5'-AAGGAGGTGATCCA(A/G)CCGCA-3']. The reaction involved 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 2 min. PCR products were purified by using a GFX PCR DNA and Gel Band Purification kit (Amersham Biosciences). Both strands of purified fragments were sequenced directly by using a Dye Terminator Cycle Sequencing FS Ready Reaction kit (Applied Biosystems) under conditions recommended by the manufacturer and using an automated sequence analyser (model 373A; Applied Biosystems). 16S rRNA gene sequences were deposited in, and previously published 16S rRNA gene sequences were obtained from, EMBL/GenBank/DBJ. Nucleotide substitution rates (K_{nuc}) (Kimura, 1980) were calculated and phylogenetic trees were constructed by the

neighbour-joining method (Saitou & Nei, 1987). Topology of the trees was evaluated by bootstrap analysis of the sequence data (1000 replicates) using the CLUSTAL W program (Thompson *et al.*, 1994).

Strains JCM 1038, JCM 1039^T, KM55, KM105, KM132 and KM9212 were Gram-positive, non-motile, non-spore-forming rods. These cells occurred singly, in pairs or occasionally in short chains. Colonies of these strains were circular to slightly irregular, convex, opaque, rough and white. Phenotypic characteristics that differentiate these strains from other reference strains are summarized in Table 1. All strains tested produced DL-lactic acid, did not grow at 15 °C, were catalase-negative and did not produce gas from glucose. The DNA G+C content of the six strains ranged from 37 to 40 mol% (Table 1).

To ascertain the phylogenetic relationships of the strains, almost-complete 16S rRNA gene sequences of three representative strains, JCM 1038, JCM 1039^T and KM9212, were determined (>1500 nt) and aligned; they showed high sequence similarities of >99.9%. As shown in Fig. 1, the results of phylogenetic analysis confirmed the association between JCM 1039^T and species that belong to the *Lactobacillus delbrueckii* group of the genus *Lactobacillus* (Collins *et al.*, 1991). Strain JCM 1039^T was included in a monophyletic cluster that contained *L. amylovorus*, *L. crispatus*, *L. gallinarum*, *Lactobacillus helveticus* and *L. acidophilus*, as shown in Fig. 1. High levels of sequence relatedness (97.5–98.6%) were found with *L. amylovorus*, *L. crispatus* and *L. gallinarum*.

We then examined DNA–DNA hybridization of strains JCM 1038, JCM 1039^T, KM55, KM105, KM132 and KM9212 to related reference strains. As shown in Table 2, photobiotin-labelled DNA from JCM 1038, JCM 1039^T and KM9212 reassociated at high levels (77–105%) with unlabelled DNA from each strain and other strains (KM55, KM105 and KM132), indicating that they belong to a single species, whereas photobiotin-labelled DNA from JCM 1038, JCM 1039^T and KM9212 reassociated at low levels with unlabelled DNA from the type strains of *L. crispatus*, *L. gallinarum* and *L. amylovorus* and type strains of other previously described species, indicating that they were different species.

It has been reported that *L. acidophilus* group strains that belong to genome cluster group A (*L. acidophilus*, *L. crispatus*, *L. amylovorus* and *L. gallinarum*) and *L. helveticus*, which are included in a monophyletic cluster as shown in Fig. 1, express S-layer proteins on their cell surface (Masuda, 1992; Mukai & Arihara, 1994; Boot *et al.*, 1996; Callegari *et al.*, 1998). We analysed the cell-surface protein patterns of all six strains in comparison with those of reference strains. SDS-PAGE protein analysis by computer was not possible, due to unavailability of a computer program, and we were therefore not able to construct any relationships. However, differences in the SDS-PAGE patterns between the six strains tested and

Table 1. Differential phenotypic characteristics of *Lactobacillus kitasatonis* sp. nov. and closely related lactobacilli

Taxa: 1, *L. kitasatonis*; 2, *L. acidophilus*; 3, *L. amylovorus*; 4, *L. crispatus*; 5, *L. gallinarum*; 6, *L. gasseri*; 7, *L. johnsonii*; 8, *L. helveticus*. Data for the species of the *L. acidophilus* group are from Kandler & Weiss (1986) and Fujisawa *et al.* (1992). +, Positive; D, strain-dependent; D–, usually negative; –, negative; w, weak reaction. All strains produce acid from glucose, galactose, fructose, mannose and maltose and produce DL-lactic acid. No strains produce acid from arabinose, xylose, rhamnose, melezitose or sorbitol.

Characteristic	1	2	3	4	5	6	7	8
DNA G+C content (mol%)	37–40	32–40	36–38	33–36	36–37	33–34	33–35	38–40
Growth at 15 °C	–	–	–	–	+	–	+	–
Fermentation of:								
Sucrose	+	+	+	+	+	+	+	–
Lactose	DW	+	+	+	D	+	+	+
Mannitol	DW	–	–	D	–	–	–	–
Cellobiose	D	+	D	+	+	+	+	–
Aesculin	+	+	D	+	+	+	+	–
Salicin	D	+	+	+	+	+	+	–
Ribose	–	–	–	D	–	–	–	–
Trehalose	D–	+	–	D	–	+	D	D
Melibiose	–	–	D	+	+	w	D	–
Raffinose	–	+	D	+	+	–	D	–
Amygdalin	–	+	D	+	+	+	+	–

other reference strains were detected visually (see Supplementary Figure available in IJSEM Online). Considerable amounts of multiple protein bands were detected

from the six strains, whereas only single protein bands, corresponding to S-layer proteins, were detected from other reference strains.

On the basis of the results of this study, we propose that JCM 1038 and JCM 1039^T should be reclassified in a novel species and that the four strains isolated from mucosa of chicken small intestine as well as strains JCM 1038 and JCM 1039^T, which also originated in the chicken intestine, are placed in the following novel species of the genus *Lactobacillus*: *Lactobacillus kitasatonis* sp. nov.

Description of *Lactobacillus kitasatonis* sp. nov.

Lactobacillus kitasatonis (ki.ta.sa.to'nis. L. gen. n. *kitasatonis* referring to Shibasaburo Kitasato, the founder of Kitasato Institute, the father of Japanese bacteriology).

Cells are Gram-positive, non-motile, non-spore-forming rods that are generally 0.6–1.0 × 2.0–20.0 µm in size and occur singly, in pairs or occasionally in short chains. When the organism is grown on MRS agar at 37 °C for 2 days, colonies are 1.2–2.1 mm in diameter, circular to slightly irregular, convex, opaque, rough and white. There is no growth at 15 °C, but growth occurs at 45 °C. The organism is facultatively anaerobic and produces DL-lactic acid homo-fermentatively. Catalase is not produced. Milk is not curdled. Acid is produced without gas formation from glucose, mannose, maltose, galactose, sucrose, fructose or aesculin. There is no acid formation from arabinose, xylose, ribose, trehalose, melezitose, melibiose, raffinose, amygdalin or sorbitol. DNA G+C content is 37–40 mol%.

Fig. 1. Phylogenetic tree showing the relationship of strain JCM 1039^T (*L. kitasatonis*) to some members of the *Lactobacillus delbrueckii* 16S rRNA gene cluster of the genus *Lactobacillus*. The tree was constructed by using the neighbour-joining method and K_{NUC} values. Numbers indicate bootstrap values for branch-points.

Table 2. DNA relatedness among *Lactobacillus kitasatonis* sp. nov. and phylogenetically closely related *Lactobacillus* species
Reassociation values are means of three determinations.

Strain	Reassociation (%) with DNA from strain:						
	JCM 1038	JCM 1039 ^T	KM9212	JCM 1132 ^T	JCM 1185 ^T	JCM 1126 ^T	JCM 2011 ^T
<i>L. kitasatonis</i> :							
JCM 1038	100	90	82	8	10	8	12
JCM 1039 ^T	105	100	80	4	5	5	10
KM55	82	83	88	10	7	12	7
KM105	85	77	78	7	4	15	10
KM132	87	82	77	12	3	10	18
KM9212	95	80	100	13	5	17	11
<i>L. acidophilus</i> JCM 1132 ^T	5	8	2	100			
<i>L. crispatus</i> JCM 1185 ^T	15	12	18		100		
<i>L. amylovorus</i> JCM 1126 ^T	18	15	10			100	
<i>L. gallinarum</i> JCM 2011 ^T	12	14	8				100
<i>L. helveticus</i> JCM 1120 ^T	3	2	5				

The type strain is JCM 1039^T (= KCTC 3155^T). The habitat of this species is the intestine of chickens.

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References

Boot, H. J., Kolen, C. P. A. M., Pot, B., Kersters, K. & Pouwels, P. H. (1996). The presence of two S-layer-protein-encoding genes is conserved among species related to *Lactobacillus acidophilus*. *Microbiology* **142**, 2375–2384.

Callegari, M. L., Riboli, B., Sanders, J. W., Cocconcelli, P. S., Kok, J., Venema, G. & Morelli, L. (1998). The S-layer gene of *Lactobacillus helveticus* CNRZ 892: cloning, sequence and heterologous expression. *Microbiology* **144**, 719–726.

Cato, E. P., Moore, W. E. C. & Johnson, J. L. (1983). Synonymy of strains of “*Lactobacillus acidophilus*” group A2 (Johnson et al. 1980) with the type strain of *Lactobacillus crispatus* (Brygoo and Aladame 1953) Moore and Holdeman 1970. *Int J Syst Bacteriol* **33**, 426–428.

Collins, M. D., Rodrigues, U., Ash, C., Aguirre, M., Farrow, J. A. E., Martinez-Murcia, A., Phillips, B. A., Williams, A. M. & Wallbanks, S. (1991). Phylogenetic analysis of the genus *Lactobacillus* and related lactic acid bacteria as determined by reverse transcriptase sequencing of 16S rRNA. *FEMS Microbiol Lett* **77**, 5–12.

de Man, J. C., Rogosa, M. & Sharpe, M. E. (1960). A medium for the cultivation of lactobacilli. *J Appl Bacteriol* **23**, 130–135.

Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in micro-dilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.

Fujisawa, T., Benno, Y., Yaeshima, T. & Mitsuoka, T. (1992). Taxonomic study of the *Lactobacillus acidophilus* group, with recognition of *Lactobacillus gallinarum* sp. nov. and *Lactobacillus johnsonii* sp. nov. and synonymy of *Lactobacillus acidophilus* group A3 (Johnson et al. 1980) with the type strain of *Lactobacillus amylovorus* (Nakamura 1981). *Int J Syst Bacteriol* **42**, 487–491.

Fujisawa, T., Yaeshima, T. & Mitsuoka, T. (1996). Lactobacilli in human feces. *Biosci Microflora* **15**, 69–75.

Hansen, P. A. & Mocquot, G. (1970). *Lactobacillus acidophilus* (Moro) comb. nov. *Int J Syst Bacteriol* **20**, 325–327.

Johnson, J. L., Phelps, C. F., Cummins, C. S., London, J. & Gasser, F. (1980). Taxonomy of the *Lactobacillus acidophilus* group. *Int J Syst Bacteriol* **30**, 53–68.

Kandler, O. & Weiss, N. (1986). Genus *Lactobacillus* Beijerinck 1901, 212^{AL}. In *Bergey’s Manual of Systematic Bacteriology*, vol. 2, pp. 1209–1234. Edited by P. H. A. Sneath, N. S. Mair, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Wilkins.

Kaneko, T., Katoh, K., Fujimoto, M., Kumagi, M., Tamaoka, J. & Katayama-Fujimura, Y. (1986). Determination of the nucleotide composition of a deoxyribonucleic acid by high-performance liquid chromatography of its enzymatic hydrolysates: a review. *J Microbiol Methods* **4**, 229–240.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.

Lauer, E. & Kandler, O. (1980). *Lactobacillus gasseri* sp. nov., a new species of the subgenus *Thermobacterium*. *Zentbl Bakteriell Mikrobiol Hyg 1 Abt Orig C* **1**, 75–78.

Lauer, E., Helming, C. & Kandler, O. (1980). Heterogeneity of the species *Lactobacillus acidophilus* (Moro) Hansen and Mocquot as revealed by biochemical characteristics and DNA-DNA hybridization. *Zentbl Bakteriell Mikrobiol Hyg 1 Abt Orig C* **1**, 150–168.

Luchansky, J. B., Tennant, M. C. & Klaenhammer, T. R. (1991). Molecular cloning and deoxyribonucleic acid polymorphisms in *Lactobacillus acidophilus* and *Lactobacillus gasseri*. *J Dairy Sci* **74**, 3293–3302.

Masuda, K. (1992). Heterogeneity of S-layer proteins of *Lactobacillus acidophilus* strains. *Microbiol Immunol* **36**, 297–301.

- Mitsuoka, T. (1969).** Vergleichende Untersuchungen über Laktobazillen aus den Faeces von Menschen, Schweinen und Hünern. *Zentbl Bakteriol Abt 1 Orig* **210**, 32–51 (in German).
- Mukai, T. & Arihara, K. (1994).** Presence of intestinal lectin-binding glycoproteins on the cell surface of *Lactobacillus acidophilus*. *Biosci Biotechnol Biochem* **58**, 1851–1854.
- Ryu, C. S., Czajka, J. W., Sakamoto, M. & Benno, Y. (2001).** Characterization of the *Lactobacillus casei* group and the *Lactobacillus acidophilus* group by automated ribotyping. *Microbiol Immunol* **45**, 271–275.
- Saitou, N. & Nei, M. (1987).** The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Sarra, P. G., Magri, M., Bottazzi, V. & Dellaglio, F. (1980).** Genetic heterogeneity among *Lactobacillus acidophilus* strains. *Antonie van Leeuwenhoek* **46**, 169–176.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994).** CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.