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 1039T (designated as type strain in this study), was determined and aligned with those of other Lactobacillus species. JCM 1039T 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 1039T.

Hansen & Mocquot (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...
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 kitasanotis sp. nov.

Strain JCM 1039T (=KCTC 3155T) was designated as the
type strain of the novel species in this study. JCM 1038
(=KCTC 3154) and JCM 1039T 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
colonial appearance were determined by using cells grown
under conditions recommended by the manufacturer
with unlabelled DNA from each strain and other strains (KM55,
and KM9212 reassociated at high levels (77–105 %) with
Strains JCM 1038, JCM 1039 T, KM55, KM105, KM132
and KM9212. A fragment of the 16S rRNA gene (corresponding
numbering + (Yamasa Shoyu). DNA from calf thymus that had a G+
contents of 42 mol% was used as a standard. DNA–DNA
relatedness (97–98.6 %) were found with L. amylovorus,
L. crispatus and L. gallinarum.

Chromosomal DNA from Lactobacillus strains was prepared
as described previously (Luchansky et al., 1991). G+C
content 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 1039T 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
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/DDBJ.
Nucleotide substitution rates (Kmac) (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 1039T, KM55, KM105, KM132
and KM9212 were Gram-positive, non-motive, non-spore-
forming rods. These cells occurred singly, in pairs or
occasionally in short chains. Colonies of these strains were
regularly 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 1039T 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 1039T and species that belong to
the Lactobacillus delbrueckii group of the genus Lactobacillus
(Collins et al., 1991). Strain JCM 1039T 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 (97–98.6 %) were found with L. amylovorus,
L. crispatus and L. gallinarum.

We then examined DNA–DNA hybridization of strains
JCM 1038, JCM 1039T, KM55, KM105, KM132 and
KM9212 to related reference strains. As shown in Table 2,
photobiotin-labelled DNA from JCM 1038, JCM 1039T
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 1039T 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

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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.so.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 homfermentatively. 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, rhamnose, melezitose or sorbitol. DNA G + C content is 37–40 mol%.

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

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
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<tr>
<td>Growth at 15 °C</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
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<td>Fermentation of:</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Lactose</td>
<td>DW</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>D</td>
<td>+</td>
<td>+</td>
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<td>–</td>
<td>D</td>
<td>–</td>
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<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>D</td>
<td>+</td>
<td>D</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Aesculin</td>
<td>+</td>
<td>+</td>
<td>D</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Salicin</td>
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<td>Trehalose</td>
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<td>–</td>
<td>+</td>
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<td>Melibiose</td>
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<td>D</td>
<td>+</td>
<td>+</td>
<td>W</td>
<td>D</td>
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<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

**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* sub 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.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Reassociation (%) with DNA from strain:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>JCM 1038</td>
</tr>
<tr>
<td>L. kitasatonis:</td>
<td></td>
</tr>
<tr>
<td>JCM 1038</td>
<td>100</td>
</tr>
<tr>
<td>JCM 1039(^T)</td>
<td>105</td>
</tr>
<tr>
<td>KM55</td>
<td>82</td>
</tr>
<tr>
<td>KM105</td>
<td>85</td>
</tr>
<tr>
<td>KM132</td>
<td>87</td>
</tr>
<tr>
<td>KM9212</td>
<td>95</td>
</tr>
<tr>
<td>L. acidophilus JCM 1132(^T)</td>
<td>5</td>
</tr>
<tr>
<td>L. crispatus JCM 1185(^T)</td>
<td>15</td>
</tr>
<tr>
<td>L. amylovorus JCM 1126(^T)</td>
<td>18</td>
</tr>
<tr>
<td>L. gallinarum JCM 2011(^T)</td>
<td>12</td>
</tr>
<tr>
<td>L. helveticus JCM 1120(^T)</td>
<td>3</td>
</tr>
</tbody>
</table>

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

Acknowledgements

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