Rapid Identification, Differentiation, and Proposed New Taxonomic Classification of *Bifidobacterium lactis*

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Identification of *Bifidobacterium lactis* and *Bifidobacterium animalis* is problematic because of phenotypic and genetic homogeneities and has raised the question of whether they belong to one unique taxon. Analysis of the 16S-23S internally transcribed spacer region of *B. lactis* DSM10140^T, *B. animalis* ATCC 25527^T, and six potential *B. lactis* strains suggested two distinct clusters. Two specific 16S-23S spacer rRNA gene-targeted primers have been developed for specific detection of *B. animalis*. All of the molecular techniques used (*B. lactis* or *B. animalis* PCR primers, enterobacterial repetitive intergenic consensus PCR) demonstrated that *B. lactis* and *B. animalis* form two main groups and suggest a revision of the strains assigned to *B. animalis*. We propose that *B. lactis* should be separated from *B. animalis* at the subspecies level.

Bifidobacterium lactis is used as a probiotic in dairy products (e.g., yoghurt) or supplied in infant food (7, 10). This species was previously described by Meile et al. (8) and characterized by its high oxygen resistance and production of considerable amounts of formate. The phylogenetic position of *B. lactis* DSM 10140, defined by sequence similarity and sequence analysis of the *ldh* gene (13), revealed that *B. animalis* ATCC 25527 is the most closely related strain. Recently, Cai et al. (2) reinvestigated the taxonomic position of *B. lactis* DSM 10140 and proposed that *B. lactis* should be considered a junior subjective synonym of *B. animalis*.

In view of the widespread interest in *B. lactis* and *B. animalis* because of the expanding application of these species as probiotics, it is highly desirable to develop a molecular approach to clear and reliable species identification. The rRNA gene (rDNA) has been used widely to infer phylogenetic relationships among bacteria (23). However, as evolutionary distances decrease, the diversity found in the 16S rDNA is often insufficient and genetic relationships of closely related species cannot be accurately defined (1, 11). It has been recently suggested that sequencing of the internally transcribed spacer (ITS) region could overcome this problem since ITS regions might be under less evolutionary pressure and could provide greater genetic variation. Indeed, analysis of this region has already successfully differentiated strains and species of many bacterial groups (1, 3, 4, 9, 17).

We investigated 106 bifidobacterial strains isolated from different environments (Table 1) with recently published *B. lactis* species-specific primers (20). Six strains gave a specific *B. lactis* PCR amplicon. By applying the species-specific amplified ribosomal DNA restriction analysis method to all of the strains, it was possible to allocate them only to the *B. animalis*-*B. lactis* cluster (19). The 16S-23S ITS region from each strain was amplified by using primers that annealed to conserved regions of the 16S and 23S genes (primer 16S-for, 5'-GCTAGTAAT

CGCGGATCAG-3'; primer 23Si, 5'-CATTCGGACACCCTGGGATC-3'). Chromosomal DNA was PCR amplified in accordance with the manufacturer's (Gibco BRL) instructions. Amplicons were purified with the QIAquick PCR Purification Kit (Qiagen). Nucleotide sequences of PCR products were determined by using the fluorescently labeled primer cycle-sequencing kit (Amersham) and the LI-COR sequencer (MWG). Sequence alignments were done with the Multi-align program, and the ClustalW dendrogram was drawn with the ClustalX program.

For the enterobacterial repetitive intergenic consensus (ERIC) PCR, primers ERIC-1 (5'-ATGTAAGCTCCTGGG GATTAC-3') and ERIC-2 (5'-AAGTAAGTGACTGGGG TGAGCG-3') (21) were used. The total reaction mixture of 25 µl contained 10 mM Tris-HCl, 50 mM KCl, 2 mM MgCl₂, 200 µM each deoxynucleoside triphosphate (Gibco BRL), 1 µM each primer, 2.5 U of *Taq* DNA polymerase (Gibco BRL), and 25 ng of template DNA. Amplifications were carried out with a Perkin-Elmer Cetus 9700 thermal cycler (1 cycle of 94°C for 3 min; 35 cycles of 94°C for 30 s, 48°C for 30 s, and 72°C for 4 min; and 1 cycle of 72°C for 6 min). PCR products were electrophoresed in 2% (wt/vol) agarose gels at a constant voltage of 7 V/cm. Gels were stained with ethidium bromide (0.5 µg/ml) and photographed under UV light at 260 nm.

To specifically detect *B. animalis* strains, we applied a PCR amplification strategy for the ITS regions with primers based on specific *B. animalis* sequences (see Fig. 5). All PCRs were in a total of 50 µl of a solution containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, 200 µM each deoxynucleoside triphosphate (Gibco BRL), 10 pmol (each) of Lm3 and Lm26 (6), 50 pmol of *B. animalis*-specific primer Ban2 (5'-CATAT TGGATCACGGTTCG-3'), 50 pmol of primer 23Si, 25 ng of template DNA, and 2.5 U of *Taq* DNA polymerase (Gibco BRL). Amplifications were performed with a DNA thermocycler (Perkin-Elmer Cetus 9700) as follows: 1 cycle of 95°C for 5 min; 30 cycles of 95°C for 30 s, 54°C for 1 min, and 72°C for 1.5 min; and 1 cycle of 72°C for 7 min. Amplicons were analyzed by 1.5% (wt/vol) agarose gel electrophoresis in Tris-acetate-EDTA buffer at a constant voltage of 7 V/cm and

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TABLE 1. Bacterial strains used to evaluate the specificity of *B. animalis*-specific PCR primers by a Multiplex-PCR approach

Species	Strain ^a	PCR result		Origin
		Ban2-23SI	Lm3-Lm26	
<i>B. animalis</i>	ATCC 25527 ^T	+	+	Rat feces
<i>B. animalis</i>	ATCC 27672	+	+	Rat feces
<i>B. lactis</i>	DSM 10140 ^T	—	+	Yoghurt
<i>B. lactis</i>	NCC 363	—	+	Human feces
<i>B. lactis</i>	NCC 311	—	+	Human feces
<i>B. lactis</i>	NCC 239	—	+	Human feces
<i>B. lactis</i>	NCC 387	—	+	Infant feces
<i>B. lactis</i>	NCC 383	—	+	Yoghurt
<i>B. lactis</i>	NCC 402	—	+	Yoghurt
<i>B. animalis</i>	ATCC 27673	—	+	Sewage
<i>B. animalis</i>	ATCC 27674	—	+	Rabbit feces
<i>B. animalis</i>	ATCC 27536	—	+	Chicken feces
<i>B. adolescentis</i>	ATCC 15703 ^T	—	+	Adult intestine
<i>B. adolescentis</i>	ATCC 15704	—	+	Adult intestine
<i>B. bifidum</i>	ATCC 29521 ^T	—	+	Infant feces
<i>B. bifidum</i>	ATCC 15696	—	+	Infant intestine
<i>B. breve</i>	ATCC 15700 ^T	—	+	Infant intestine
<i>B. breve</i>	ATCC 15701	—	+	Infant intestine
<i>B. catenulatum</i>	ATCC 27539 ^T	—	+	Adult intestine
<i>B. coryneforme</i>	DSM20216 ^T	—	+	Honeybee hindgut
<i>B. cuniculi</i>	ATCC 27916 ^T	—	+	Rabbit feces
<i>B. dentinum</i>	ATCC 27534 ^T	—	+	Dental caries
<i>B. infantis</i>	ATCC 15697 ^T	—	+	Infant intestine
<i>B. infantis</i>	ATCC 25962	—	+	Infant intestine
<i>B. angulatum</i>	DSM 20098 ^T	—	+	Human feces
<i>B. longum</i>	LMG 13197 ^T	—	+	Adult intestine
<i>B. magnum</i>	ATCC 27540 ^T	—	+	Rabbit feces
<i>B. pseudocatenulatum</i>	DSM 20438 ^T	—	+	Infant feces
<i>B. pseudolongum</i>	DSM 20099 ^T	—	+	Swine feces
<i>B. pullorum</i>	DSM 20433 ^T	—	+	Chicken feces
<i>B. merycicum</i>	DSM 6492 ^T	—	+	Bovine rumen
<i>B. minimum</i>	DSM 20102 ^T	—	+	Sewage
<i>B. ruminantium</i>	DSM 6489 ^T	—	+	Bovine rumen
<i>B. saeculare</i>	DSM 6531 ^T	—	+	Rabbit feces
<i>B. subtilis</i>	DSM 20096 ^T	—	+	Sewage
<i>B. thermophilum</i>	DSM 20210 ^T	—	+	Swine feces
<i>B. asteroides</i>	DSM 20089 ^T	—	+	Honeybee hindgut
<i>B. boum</i>	DSM 20432 ^T	—	+	Cattle rumen
<i>B. gallicum</i>	DSM 20093 ^T	—	+	Human feces
<i>B. gallinarum</i>	DSM 20670 ^T	—	+	Chicken cecum
<i>B. inopinatum</i>	DSM 10107 ^T	—	+	Dental plaque
<i>B. choerinum</i>	ATCC 27686 ^T	—	+	Swine feces
<i>B. suis</i>	ATCC 27533 ^T	—	+	Swine feces

^a ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen; LMG, Bacteria Collection Universiteit Gent; NCC, Nestlé Culture Collection.

visualized with ethidium bromide (0.5 µg/ml) under UV light at 260 nm.

Furthermore, we investigated the strain individuality of all *B. animalis* and *B. lactis* isolates by pulsed-field gel electrophoresis (PFGE). Agarose-embedded bacterial cells were prepared as described by Walker and Klaenhammer (22). For digestion with restriction endonucleases, cells in agarose blocks were treated with 50 U (each) of *Xba*I and *Spe*I (Roche Molecular). Electrophoresis was carried out as previously reported (18).

Use of the ERIC-PCR technique resulted in clear identification at the bifidobacterial species level (15; data not shown). ERIC-PCR fingerprints demonstrated that *B. lactis* NCC 363, NCC 383, NCC 402, and NCC 311 and *B. animalis* ATCC 27536, ATCC 27673, and ATCC 27674 are not comparable to

any other *B. animalis* strain (e.g., ATCC 25527, ATCC 27672), but they are highly similar to the neotype of the species *B. lactis* (Fig. 1). *B. lactis* strains were characterized by DNA restriction patterns with low-frequency cleavage endonucleases and PFGE. Overall, the size estimates for all *B. lactis* strains were compared with the size estimates for *B. animalis* ATCC 27673, ATCC 27674, and ATCC 27536 (12). These values were clearly different and unique for each of the *B. animalis* strains. Only *B. lactis* NCC 363, NCC 383, NCC 402, and NCC 311 displayed a rather different and unique PFGE profile (Fig. 2). Interestingly, the other two *B. lactis* isolates (NCC 239 and NCC 387) resulted in *Spe*I/*Xba*I PFGE patterns highly similar to that of *B. lactis* DSM 10140 (data not shown). This indicates that these organisms are very closely related. Indeed, it was demonstrated earlier that these strains are different with additional molecular typing assays such as randomly amplified polymorphic DNA PCR and triplicate arbitrary primed PCR (data not shown).

The sequences of the 16S-23S ITS regions of strains NCC 363, NCC 383, NCC 402, NCC 239, NCC 387, and NCC 311 were determined from those amplicons generated with the PCR primers 16S-for and 23Si. The complete fragments were sequenced directly and multiple times on both DNA strands. Comparison of the six spacer sequences obtained with that of *B. lactis* DSM 10140 already deposited in the GenBank database revealed similarity levels ranging from 93.4 to 97% (Fig. 3B). Analysis of most of the *B. lactis* sequences revealed some variable sites, which included base additions, deletions, or substitutions (Fig. 4). The most striking differences were observed in relation to the type strain of *B. animalis*, where two eight-

FIG. 1. DNA fingerprint analysis of *B. lactis* and *B. animalis* strains by ERIC-PCR. Lanes: 1, *B. lactis* DSM 10140; 2, *B. lactis* NCC 363; 3, *B. lactis* NCC 311; 4, *B. animalis* ATCC 25527; 5, *B. animalis* ATCC 27672; 6, *B. animalis* ATCC 27674; 7, *B. animalis* ATCC 27536; 8, *B. animalis* ATCC 27673; 9, *B. lactis* NCC 383; 10, *B. lactis* NCC 402; 11, *B. lactis* NCC 239; M, 1-kb DNA ladder (Gibco-BRL).

FIG. 2. PFGE patterns of genomic DNAs of *B. lactis* strains after *SpeI* digestion. Lanes: 1, *B. lactis* NCC 383; 2, *B. lactis* NCC 363; 3, *B. lactis* DSM 10140; 4, *B. lactis* NCC 402; 5, *B. lactis* DSM 10140; 6, *B. lactis* NCC 239; 7, *B. lactis* NCC 387; 8, *B. lactis* NCC 311; 9, *B. lactis* DSM 10140; M, λ DNA ladder (Bio-Rad).

nucleotide inserts between bp 319 and 338 were absent in the ITS of any of the *B. lactis* strains studied here (Fig. 4).

The phylogenetic relationships among ITS sequences of the related species *B. animalis* and *B. lactis* (1) are described in Fig. 3A. Consistent with the results of Bourget et al. (1), our phylogenetic tree clearly shows that all of the *B. lactis* strains and *B. animalis* strain ATCC 25527 are in the same cluster, which can be further separated into two subclusters, of which one is formed by *B. lactis* and the other is formed by *B. animalis* ATCC 25527.

Notably, two insertions of eight nucleotides each were identified in *B. animalis* but not in *B. lactis* and provided a PCR target for rapid separation of these two species. The Ban2 primer was designed to anneal to this conserved *B. animalis* region (Fig. 5A). The reverse primer, 23Si, was derived from the sequences encompassing the 3' end of ITS and the 5' end of the 23S rDNA and was complementary overall to all of the ITS sequences of *B. animalis* and *B. lactis* strains. Moreover, because of an often-occurring variability of PCR conditions, the lack of any amplicons must be attributed not only to the absence of any target DNA but also to an overall failure of the amplification reaction. To distinguish between these two events, we included primers Lm3 and Lm26 (6), which target conserved regions within the 16S rDNA of the genus *Bifidobacterium*, in the same PCR mixtures. When a Multiplex-PCR was performed with this mixture of four primers in the same reaction mixture, two amplicons of 467 and 1,350 bp were only detectable in the presence of DNA isolated from *B. animalis* (the sizes of all achievable PCR amplicons were calculated from databases and estimated by gel electrophoresis). The amplification of all other *Bifidobacterium* DNAs produced

only one amplicon of 1,350 bp. The results of these PCR assays, on the basis of the ITS sequence data, are depicted in Fig. 5B. The 467-bp amplicon that occurred was apparent for two *B. animalis* strains but not for any of the *B. lactis* strains. Furthermore, we confirmed the overall absence of PCR amplicons for all other bifidobacterial type strains (data not shown).

B. lactis identification based on molecular sequence data has been hampered so far by the lack of detectable sequence variation between closely related *B. animalis* strains. Genetic information derived from 16S-23S rRNA spacer regions can be used to differentiate closely related organisms (1, 17).

More recently, Cai et al. (2) showed that the DNA of *B. animalis* ATCC 27536 and ATCC 27674 had a high level of overall DNA relatedness with *B. lactis* DSM 10140, ranging from 94.2 to 94.8%, which is significantly higher than their relatedness to *B. animalis* ATCC 25527. Since the DNA-DNA homology between *B. lactis* DSM 10140 and *B. animalis* ATCC

FIG. 3. (A) Dendrogram based on 16S-23S ITS sequences and showing the relationships of members of the genus *Bifidobacterium* related to *B. animalis* and *B. lactis* strains. The bar at the top indicates 0.02% sequence divergence. The 16S-23S ITS sequences used to draw the phylogenetic tree of the following strains were retrieved from the GenBank database: *B. infantis* ATCC 15697 (accession no. U09792), *B. breve* CIP 6470 (accession no. U09521), *B. adolescentis* CIP 6460 (accession no. U09513), *B. adolescentis* CIP 6459 (accession no. U09512), *B. lactis* DSM 10140 (accession no. X89513), *B. animalis* ATCC 25527 (accession no. U09858), *B. choerinum* CIP 103378 (accession no. L36968), *B. pseudolongum* ATCC 25526 (accession no. U09879), and *B. globosum* ATCC 25865 (accession no. U09524). (B) Overall 16S-23S rRNA spacer region sequence similarities of *B. lactis* strains.

Roy et al. (12, 13) also reported that *B. animalis* ATCC 27536 has an *ldh* nucleotide sequence identical to that of *B. lactis* DSM 10140 and a PFGE pattern more similar to that of the type strain of *B. lactis* than to that of the type strain of *B. animalis*. Analysis of a 60-kDa heat shock protein (HSP60) gene showed that the DNA sequence similarity between *B. lactis* DSM 10140 and *B. animalis* ATCC 25527 is high (98%), as is that among *B. infantis*, *B. longum*, and *B. suis*, but lower than that between strains of the same species (5). Therefore,

the high level of 16S rDNA sequence similarity of 98.6%, (8) between the *B. animalis* and *B. lactis* type strains is additional evidence that these two taxa might, in fact, be combined. Thus, our 16S-23S ITS sequence data and ERIC-PCR data are consistent with above-mentioned data and suggest that these two species might be designated two subspecies of the same species. In fact, 16S rDNA sequence analysis is a good tool with which to explore intergeneric relationships, while 16S-23S rDNA spacer region comparison provides information concerning intraspecific links and allows the detection of recently divergent species, as proposed by Bourget (1). Considering the new *B. lactis* strains described here and comparing them with the few *B. animalis* strains available, we suggest the use of the names *B. animalis* subsp. *lactis*, which includes strains DSM 10140, ATCC 27536, ATCC 27674, and ATCC 27673 and the new

FIG. 5. (A) Primers and amplification systems used to investigate the species *B. animalis*. (B) PCR products of various *Bifidobacterium* species obtained by a Multiplex-PCR approach with *B. animalis* species-specific PCR primers and bifidobacterial genus-specific primers. (A) The position numbering and expected product sizes are given in accordance with the numbering of the *B. animalis* 16S-23S spacer region (1). (B) Lanes: 1, *B. animalis* ATCC 25527; 2, *B. animalis* ATCC 27672; 3, *B. lactis* DSM 10140; 4, *B. animalis* ATCC 27673; 5, *B. animalis* ATCC 27674; 6, *B. animalis* ATCC 27536; 7, *B. lactis* NCC 363; 8, *B. lactis* NCC 387; 9, *B. lactis* NCC 402; 10, *B. lactis* NCC 311; 11, *B. lactis* NCC 383; 12, *B. lactis* NCC 239; 13, *B. bifidum* ATCC 29521; 14, *B. breve* ATCC 15700; 15, *B. longum* ATCC 15707; 16, *B. infantis* ATCC 15679; 17, *B. adolescentis* ATCC 15703; 18, *B. catenulatum* ATCC 27539. m, 1-kb DNA ladder (Gibco-BRL).

strains reported in this study, and *B. animalis* subsp. *animalis*, which includes, e.g., strains ATCC 25527 and ATCC 27672. Notably, all of these strains exhibit individual PFGE and/or randomly amplified polymorphic DNA PCR, and/or triplicate arbitrary primed PCR profiles (12; unpublished results), justifying separate strain designations.

Physiologic characteristics based on their different abilities to grow on milk-based medium give additional support for a potential taxonomic separation of strains of *B. lactis* from

strains of *B. animalis*. In fact, *B. lactis* strains and very few *B. animalis* strains (ATCC 27536, ATCC 27674, and ATCC 27673) are able to grow in pure milk whereas *B. animalis* ATCC 25527 and ATCC 27672 both grow poorly in pure milk. Potentially, for this reason, all strains found in dairy products (e.g., yoghurt and infant formula), including products purported to contain *B. animalis* and/or *B. lactis* strains, do not at all resemble *B. animalis* but are, in fact, only strains of the *B. lactis* taxon. According to *Bergey's Manual of Systematic Bac-*

teriology (16), division of a species into subspecies is based on phenotypic variations (e.g., growth on milk-based medium) or on genetically determined clusters of strains within the species. On the basis of the above criterion, we suggest that *B. lactis* should not be considered a junior subjective synonym of *B. animalis* but rather be reclassified as a subspecies of *B. animalis*.

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