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Comparative Sequence Analysis of the *tuf* and *recA* Genes and Restriction Fragment Length Polymorphism of the Internal Transcribed Spacer Region Sequences Supply Additional Tools for Discriminating *Bifidobacterium lactis* from *Bifidobacterium animalis*

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The relationship between *Bifidobacterium lactis* and *Bifidobacterium animalis* was examined by comparative analysis of *tuf* and *recA* gene sequences and by restriction fragment length polymorphism analysis of their internal 16S-23S transcribed spacer region sequences. The bifidobacterial strains investigated could be divided into two distinct groups within a single species based on the *tuf*, *recA*, and 16S-23S spacer region sequence analysis. Therefore, all strains of *B. lactis* and *B. animalis* could be unified as the species *B. animalis* and divided into two subspecies, *Bifidobacterium animalis* subsp. *lactis* and *Bifidobacterium animalis* subsp. *animalis*.

Bifidobacterium lactis and *Bifidobacterium animalis* are closely related, representing a relatively small number of known strains. *B. lactis* includes strains employed as probiotics for dairy products and infant formula. Despite their high industrial importance, the taxonomy of *B. lactis* and *B. animalis* is still unclear. Historically, the species annotation of *B. animalis* (12, 15) was introduced first, followed by the isolation of a highly oxygen-tolerant *Bifidobacterium* organism from yogurt, which was classified in 1997 as the new species *B. lactis* (11). The definition criteria for *B. lactis* and *B. animalis* were based on phenotypic characteristics such as carbohydrate fermentation patterns and high tolerance of oxygen stress. However, due to the high levels of DNA relatedness and 16S ribosomal DNA (rDNA) sequence similarity between *B. lactis* and *B. animalis*, Cai et al. (1). proposed the rejection of the name *B. lactis* and suggested that *B. lactis* should be considered a junior subjective synonym of *B. animalis*. The nomenclature of the species *B. lactis* and *B. animalis* is still ambiguous, and a taxonomic decision by the International Committee on Systematic Bacteriology to maintain them as two separate species has been pending since 1999 (6).

Recently, we have demonstrated that analysis of 16S rDNA sequences, analysis of the 16S-23S spacer region, and enterobacterial repetitive intergenic consensus-PCR have powerful potential for tracing *B. animalis* or *B. lactis* (20–23). The *tuf* and *recA* genes have been proposed as useful markers in inferring bacterial phylogeny (2, 10, 18) and have recently been successfully used to differentiate species and subspecies within various bacterial genera (3, 7, 8, 17). Our results show that the sequence analysis of *tuf* and *recA* as well as restriction fragment length polymorphism (RFLP) analysis of the internal transcribed spacer (ITS) sequences are relatively simple and rapid methods by which *B. lactis* and *B. animalis* can be identified without resorting to the use of species-specific PCR primer sets.

***tuf* and *recA* sequence analysis.** Bacterial strains used in this study were either obtained from culture collections or isolated from human or animal fecal samples (Table 1) and grown as described previously (19). The DNA of all bifidobacterial strains was prepared as described by Ventura and Zink (19). The 940-bp *tuf* fragment sequences and the 690-bp *recA* sequences were amplified with the oligonucleotides tuf1 (5′-GAGTACGACTTCAACCAG-3′) (22) and tuf2 (5′-CAGGCGAGGATCTTGGT-3′) (22) and the oligonucleotides rec1 (5′-TCGAGGTGATTCCCACC-3′) and rec2 (5′-GAACCAAGAACCGGACTTC-3′), respectively. Each PCR mixture (50 μl) contained a reaction cocktail of 20 mM Tris-HCl at pH 8.0, 50 mM KCl, a 200 μM concentration of each deoxynucleoside triphosphate, 50 pmol of each primer, 1.5 mM $MgCl_2$, and 1 U of *Taq* DNA polymerase (Gibco BRL, Paisley, United Kingdom). Each PCR cycling profile consisted of an initial denaturation step of 3 min at 95°C, followed by amplification for 30 cycles as follows: denaturation (30 s at 95°C), annealing (30 s at 52°C), and extension (2 min at 72°C). The PCR was completed with an elongation phase (10 min at 72°C). PCR fragments were purified with a PCR purification kit (Qiagen, West Sussex, United Kingdom) and were subsequently cloned in the pGEM-T Easy plasmid vector (Promega, Southampton, United Kingdom) following the supplier's instructions. Subsequently, the sequence of the inserted DNA fragment was determined by sequencing three randomly selected clones on both strands for each bacterial species to ensure that no sequencing errors were attributable to misincorporation by the *Taq* polymerase.

Nucleotide sequencing of both strands from cloned DNA was performed with a fluorescence-labeled primer cycle sequencing kit (Amersham Buchler, Braunschweig, Germany) following supplier's instructions. The primers used were tuf1,

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TABLE 1. Bacterial strains used in this study

Species	Strain[a]	Origin
B. lactis	DSM 10140^T	Yogurt
	NCC 363	Human feces
	NCC 311	Human feces
	NCC 239	Human feces
	NCC 383	Yogurt
	NCC 402	Yogurt
B. animalis	ATCC 25527^T	Rat feces
	ATCC 27672	Rat feces
	ATCC 27673	Sewage
	ATCC 27674	Rabbit feces
	ATCC 27536	Chicken feces

[a] T, type strain. NCC strains were isolated at our institute (Nestlè Culture Collection). All other strains were obtained from the American Type Culture Collection.

tuf2, rec1, and rec2 labeled with IRD800 (MWG Biotech, Germany). The *tuf* and *recA* sequences of all *Bifidobacterium* strains determined here as well as those available in the GenBank database were used for comparison. The partial nucleotide sequences of the *tuf* and *recA* genes from 11 *Bifidobacterium* strains belonging to *B. lactis* and *B. animalis* were determined, and phylogenetic trees based on these data as well as those retrieved from GenBank databases were constructed. Phylogenetic trees were constructed with the programs Clustal X, DNAML (maximum likelihood), and DNAPARS (parsimony) (PHYLIP [Phylogeny Inference Package], version 3.5c; J. Felsenstein, University of Washington, Seattle, Wash.).

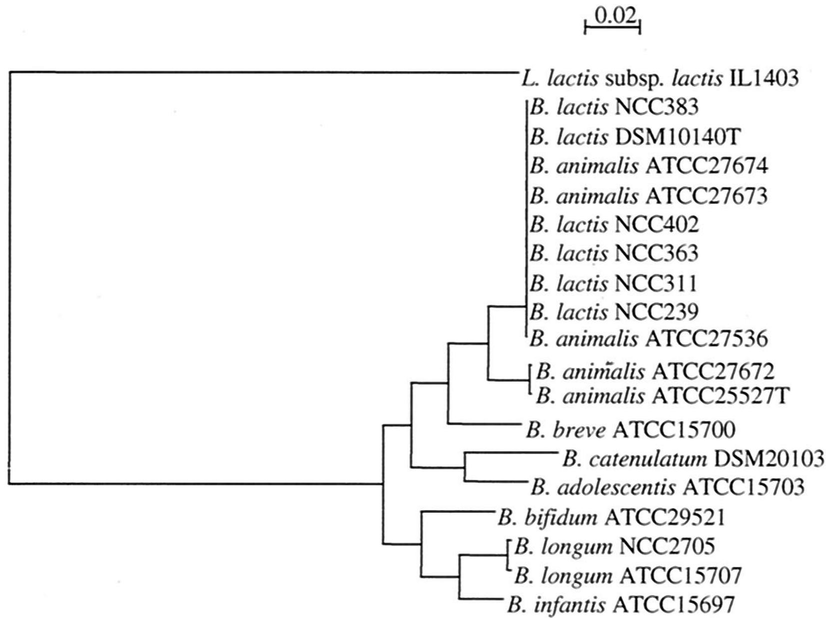
The topologies of the *recA*- and *tuf*-based trees were comparable (Fig. 1). In these trees, *B. lactis* and *B. animalis* strains were grouped into two clusters; the first one contained nine strains, including all *B. lactis* strains as well as *B. animalis* ATCC 27673, ATCC 27674, and ATCC 27536, while the second one contained only the type strain of *B. animalis* and *B. animalis* ATCC 27672. The phylogenetic distances among strains of the *B. lactis* are virtually zero (Fig. 1). The individuality of each of these strains was supported by various molecular typing tools, such as pulsed-field gel electrophoresis, random amplified polymorphic DNA PCR, and triplicate arbitrarily primed PCR (19; data not shown).

Twenty-seven nucleotide substitutions were observed between the *tuf* gene sequences of *B. lactis* DSM 10140 and *B. animalis* ATCC 25527, but only four contributed to an amino acid substitution. In parallel, only 13 synonymous nucleotide substitutions were noticed among the *recA* gene sequences in the same set of strains. Interestingly, many of the base differences observed between the *tuf* and *recA* sequences of the two taxa are guanine or cytosine in *B. lactis* and adenine and thymine in *B. animalis*. The spontaneous deamination of cytosine leading to a thymine is a frequent event, and it has already been described in relation to the species identification of *Lactobacillus delbrueckii* (4). Because of the similar G+C content of the organisms under consideration, sequence differences are likely due to real evolutionary divergence. Therefore, we focused our interest on the *tuf* and *recA* nucleotide sequences. When we aligned the *tuf* and the *recA* sequences of all *B. lactis* and *B. animalis* strains investigated, we noticed that these 27- and 13-nucleotide differences could be directly used to distinguish all *B. lactis* from *B. animalis* strains (Fig. 2). Some of these sequence signatures are typical of either *B. lactis* strains or *B. animalis* strains but are not exhibited in all other microorganism investigated here (Fig. 1b and d). These sequence signatures could be directly used for designing PCR-specific primers, or they could be a target for specific restriction enzymes, providing species-specific RFLP patterns. In fact, theoretical restriction profiles with different restriction enzymes were obtained from the *tuf* and *recA* sequences. The restriction enzymes *Hin*dII, *Bam*HI, and *Cla*I were found to give the clearest and most reliable distinction in theoretical RFLP patterns in order to differentiate *B. lactis* and *B. animalis* strains (Fig. 2c and d).

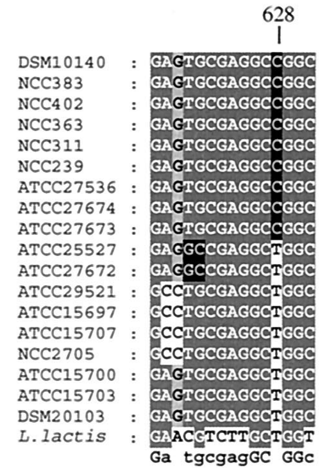
The synonymous distances calculated by the method of Nei et al. (13) from the nucleotide substitution ratios at synonymous positions in *tuf* and *recA* were examined for all possible combinations of these 22 *Bifidobacterium* gene sequences. A significant correlation between the synonymous distances in the *tuf* genes and those in the *recA* genes were observed, with a correlation coefficient of 0.99. This result was not unexpected, because it has been demonstrated before that a synonymous substitution rate is constant for many chromosomal genes in various organisms and can thus serve indeed as a molecular clock of their evolution (9). The evolutionary distances calculated for *tuf*, *recA*, ITS, and 16S rDNA sequences for strains of *B. lactis* and *B. animalis* (Table 2) clearly underline the inadequacy of the 16S rDNA sequences for distinguishing these closely related taxa. From these data, it appears that the K_{nuc} values (average extent of sequence change at any position in two homologous sequences [5]) calculated for 16S rDNA sequences are at least threefold lower than those calculated for the other taxonomic molecular markers (*tuf* and *recA* gene sequences) and sevenfold lower than those of the ITS. Hence, *tuf*, *recA*, and ITS sequences provide different complementary phylogenetic information. While *tuf* and *recA* sequences are excellent tools for inferring interspecific relationships, 16S-23S rDNA spacer sequence comparisons provide information concerning intraspecific links (e.g., characterization of strains).

PCR-RFLP analysis of the 16S-23S ITS region. Theoretical restriction profiles for the ITS region of *B. lactis* and *B. animalis* with different enzymes were obtained by using the Webcutter online analysis tool (http://users.unimi.it/~camelot/tools/cut2.html). The enzyme *Sau*3AI gave the clearest and most reliable theoretical PCR-RFLP pattern, in order to differentiate between the *B. animalis* and *B. lactis* type strains. PCR was used to amplify the 16S-23S ITS region of all *B. lactis* and *B. animalis* strains with the primers 16S-for (5′-GCTAGTAATCGCGGATCAG-3′) (19) and 23Si (5′-CATTCGGACACCCTGGGATC-3′) (19). Before a restriction digestion of all PCR products was done, amplicons were purified with the QIAquick PCR purification kit (Qiagen, Valencia, Calif.). In order to achieve complete digestion, reactions were carried out for 3 h at 37°C in a 10-μl volume of incubation buffer containing 2 U of *Sau*3AI (Boehringer-Mannheim, Mannheim, Germany) and 1 μg of the purified PCR product. The restriction products were loaded on 3% (wt/vol) agarose gel (NuSieve; FMC Bioproducts, Rockland, Maine) and separated at 7 V/cm, followed by ethidium bromide staining (UV at 260 nm). PCR-amplified 16S-23S rDNA ITS sequences of all *B. lactis* and *B.*

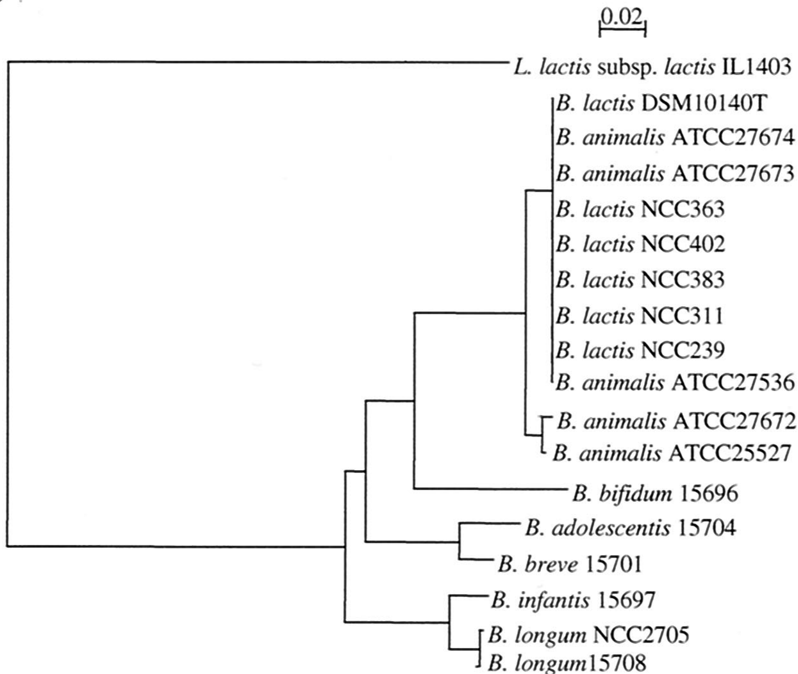
a)



c)



b)



d)

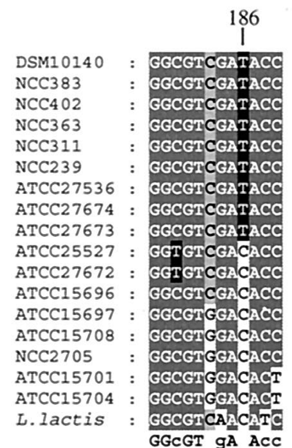


FIG. 1. (a and b) Phylogenetic trees obtained with *tuf* gene sequences (a) and *recA* gene sequences (b). The scale indicates a genetic distances of 2%. (c and d) Alignment showing the sequence signatures for the *tuf* gene sequences (c) and *recA* gene sequences (d). Sequence signatures are shaded in black; residues shaded in light grey have ≤75% identity, while residues in dark grey have ≥76% identity.

FIG. 2. (a and b) Pairwise alignment of the *tuf* sequences (a) and *recA* sequences (b) of *B. lactis* DSM 10140^{T} and *B. animalis* ATCC 25527^{T}. Nucleotides which are different between *B. lactis* strains and *B. animalis* ATCC 25527 are shaded in black. (c and d) Theoretical RFLP profiles from *B. lactis-B. animalis tuf* sequences (c) and from *B. lactis-B. animalis recA* sequences (d).

TABLE 2. K_{nuc} values for *tuf*, *recA*, ITS, and 16S rDNA sequences of *B. lactis* DSM 10140 with respect to *B. lactis* and *B. animalis* strains

Strain	K_{nuc}[a] for:			
	tuf	ITS[b]	16S rDNA	*recA*
NCC 402	0	0.009	0	0
NCC 383	0	0.001	0	0
NCC 311	0	0.006	0	0
NCC 363	0	0.005	0	0
ATCC 27536	0	0.012	0	0
ATCC 25527	0.03	0.077	0.01	0.03

[a] $K_{nuc} = -3/4\ln(1 - 4/3D)$, where D is the fractional difference in compared sequences (5).
[b] Data are from reference 19.

animalis strains were digested with *Sau*3AI, leading to polymorphic patterns and hence allowing direct species-level identification (Fig. 3). The clustering from these patterns consistently grouped all restriction fragment patterns into two distinct subsets. One shared the restriction pattern of the type strain of *B. lactis* and includes *B. lactis* DSM 10140, *B. animalis* ATCC 27536, *B. animalis* ATCC 27673, *B. animalis* ATCC 27674, and all other *B. lactis* isolates, and the other includes the type strain of *B. animalis*, ATCC 25527, and *B. animalis* ATCC 27672. In all experiments, we obtained clearly distinguishable and always reproducible RFLP patterns of the 16S-23S ITS region, confirming all theoretically expected restriction profiles.

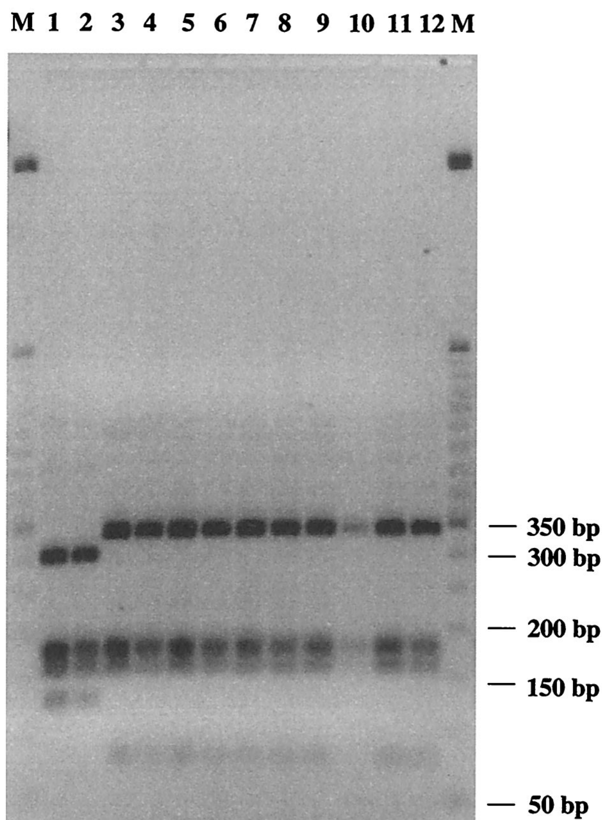


FIG. 3. Restriction patterns of PCR-amplified fragments of 16S-23S rDNA digested with *Sau*3AI. Lane 1, *B. animalis* ATCC 25527; lane 2, *B. animalis* ATCC 27672; lane 3, *B. lactis* DSM 10140; lane 4, *B. animalis* ATCC 27674; lane 5, *B. animalis* ATCC 27673; lane 6, *B. animalis* ATCC 27536; lane 7, *B. lactis* NCC 363; lane 8, *B. lactis* NCC 311; lane 9, *B. lactis* NCC 402; lane 10, *B. lactis* NCC 311; lane 11, *B. lactis* NCC 383; lane 12, *B. lactis* NCC 239; lanes M, 50-bp DNA ladder (Gibco BRL).

Conclusions. Our study shows that comparison of *tuf* and *recA* sequences as well as RFLP analysis of the ITS sequences of *B. lactis* and *B. animalis* strains provides additional and much further detailed practical means to discriminate these closely related taxa. The analysis of *tuf* and *recA* gene sequences and classical molecular species identification tools (rDNA sequencing and species-specific oligonucleotide probes) data should therefore be incorporated in a modern polyphasic approach for bifidobacterial taxonomy. Our results suggest that *tuf* and *recA* gene sequencing and RFLP analysis of the ITS sequences have specific advantages over other molecular tools for tracing *B. lactis*-*B. animalis* strains (19). In fact, *tuf* and *recA* are relatively short sequences that can easily be sequenced on both polynucleotide strands. In addition, it has been shown that bifidobacterial genomes carry only one *tuf* and one *recA* gene (16), which might be advantageous because the interpretation and power of rRNA-based data (e.g., the use of a single gene or operon) in molecular taxonomy appear sometimes to be questionable (24). Finally, the application of *tuf* and *recA* gene sequencing and RFLP analysis of the 16S-23S spacer sequences for our species-specific identification allows simultaneous handling and comparison of many isolates, in contrast to a required repeated PCR amplification with species-specific primers. Establishing PCR primers based on *tuf* and *recA* sequences for quantitative detection of *B. lactis* and *B. animalis* is a task for the future, and research into real-time quantitative detection avoiding the use of multicopy genes (e.g., ribosomal genes) is rapidly proceeding (14).

In the present study, genotypic analysis carried out on *B. lactis* and *B. animalis* strains targeting the *tuf* and *recA* genes showed a consistent similarity between these two taxa. However, taxonomic trees based on *tuf* and *recA* indicate a separate branching of *B. lactis* and *B. animalis* strains. Recently, a reorganization of the *B. animalis* species with the rejection of few strains (ATCC 27673, ATCC 27674, and ATCC 27536) from this species and a reclassification of them as *B. lactis* have been proposed (19). This hypothesis is also validated by the *tuf* and *recA* gene analyses, which suggested a revision of various strains, actually designed to the *B. animalis* species. This study provides a clear image of the genetic variability within the *B. lactis*-*B. animalis* taxa. In fact, the use of genes encoding proteins (e.g., *tuf* and *recA*) instead of ribosomal genes (19) shows that the taxon *B. lactis* is in fact highly homogeneous. In contrast, the analysis of the *tuf* gene in all investigated *B. animalis* strains depicted a significant variability. This might be due to the fact that the taxon *B. animalis*-*B. lactis* was originally a single group that has diverged only recently as a result of different environmental conditions (e.g., growth in different ecological niches). Therefore, based on the distinct phenotypic characteristics (12, 19) of all *B. lactis* strains and further detailed molecular evidence (*tuf* and *recA* sequences as well as their ITS restriction patterns), we proposed that *B. lactis* and *B. animalis* be unified into one species and that this species

should be divided into two subspecies, *Bifidobacterium animalis* subsp. *animalis* and *Bifidobacterium animalis* subsp. *lactis*.

Nucleotide sequence accession numbers. The accession numbers for the *tuf* and *recA* sequences are AY370912 to AY370929 and AY372028 to AY372031.

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