APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 2003, p. 6908–6922 Vol. 69, No. 11
0099-2240/03/$08.00+0 DOI: 10.1128/AEM.69.11.6908–6922.2003

Analysis, Characterization, and Loci of the *tuf* Genes in *Lactobacillus* and *Bifidobacterium* Species and Their Direct Application for Species Identification

Marco Ventura,[1]* Carlos Canchaya,[1] Valèrie Meylan,[1] Todd R. Klaenhammer,[2] and Ralf Zink[1]†

Nestlé Research Center, 1000 Lausanne 26, Switzerland,[1] and Department of Food Science, College of Agriculture and Life Sciences, North Carolina State University, Raleigh, North Carolina 27695-7624[2]

Received 16 June 2003/Accepted 19 August 2003

We analyzed the *tuf* gene, encoding elongation factor Tu, from 33 strains representing 17 *Lactobacillus* species and 8 *Bifidobacterium* species. The *tuf* sequences were aligned and used to infer phylogenesis among species of lactobacilli and bifidobacteria. We demonstrated that the synonymous substitution affecting this gene renders elongation factor Tu a reliable molecular clock for investigating evolutionary distances of lactobacilli and bifidobacteria. In fact, the phylogeny generated by these *tuf* sequences is consistent with that derived from 16S rRNA analysis. The investigation of a multiple alignment of *tuf* sequences revealed regions conserved among strains belonging to the same species but distinct from those of other species. PCR primers complementary to these regions allowed species-specific identification of closely related species, such as *Lactobacillus casei* group members. These *tuf* gene-based assays developed in this study provide an alternative to present methods for the identification for lactic acid bacterial species. Since a variable number of *tuf* genes have been described for bacteria, the presence of multiple genes was examined. Southern analysis revealed one *tuf* gene in the genomes of lactobacilli and bifidobacteria, but the *tuf* gene was arranged differently in the genomes of these two taxa. Our results revealed that the *tuf* gene in bifidobacteria is flanked by the same gene constellation as the *str* operon, as originally reported for *Escherichia coli*. In contrast, bioinformatic and transcriptional analyses of the DNA region flanking the *tuf* gene in four *Lactobacillus* species indicated the same four-gene unit and suggested a novel *tuf* operon specific for the genus *Lactobacillus*.

The members of the genera *Lactobacillus* and *Bifidobacterium* are gram-positive organisms considered to belong to the general category of lactic acid bacteria (LAB), even though the genus *Bifidobacterium* is phylogenetically unrelated and has a unique mode of sugar fermentation (44). These organisms are inhabitants of a wide range of environments, including the gastrointestinal and urogenital tracts of humans and animals. Many LAB strains have a worldwide industrial use as starters in the manufacture of fermented foods. Moreover, some *Lactobacillus* and *Bifidobacterium* strains have been shown to have beneficial effects on human and animal health (45).

The evolutionary relationships among LAB have been determined by comparing rRNA gene sequences (mainly 16S rRNA) because of their ubiquity and their resistance to evolutionary changes. Several new genetic approaches for the identification of *Lactobacillus* and *Bifidobacterium* species have been used in recent years, including the sequencing of rRNA genes (2, 46, 49, 50, 51, 53), restriction endonuclease fingerprinting (51, 52), analysis with oligonucleotide probes (13, 33, 35), analysis of plasmid content (41), analysis of sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis patterns of whole-cell proteins (13, 33), and comparisons of *tuf* sequences (4, 26, 27). Now, with the advent of the genomics era, this rRNA-based view of bacterial phylogeny is being critically examined. Indeed, many microbial genome sequencing projects are providing phylogenetic markers that supply alternatives for the widely accepted small-subunit rRNA marker.

Many studies emphasize that the present LAB phylogeny, deriving almost entirely from the analysis of only a single gene, may be unsatisfactory; a critical reevaluation of phylogenetic relationships is needed (11, 25). A highly conserved protein, such as RecA, was proposed as an alternative phylogenetic marker for comparative phylogenetic analysis of the genus *Bifidobacterium* (22) and the *Lactobacillus plantarum* group (47). Alternative molecules, such as 23S rRNA (26), ATPase subunits (26), RNA polymerase (25), and other proteins (16, 36), recently were used to examine whether phylogenies derived from comparative analysis of 16S rRNA reflect the evolution of microorganisms in general or only their own history. In addition, the significance of 16S rRNA genes as molecular markers sometimes has been questioned, as in the genus *Helicobacter*, where a large insertion of DNA could change the overall evolutionary scenario. The low rate of 16S rRNA evolution is responsible for the failure of this molecule to provide multiple diagnostic sites for closely related but ecologically distinct taxa. Rates of evolutionary substitution in protein-coding genes are 1 order of magnitude greater than those for 16S rRNA genes. Thus, some pairs of ecologically distinct taxa may have had time to accumulate neutral sequence divergence at rapidly evolving loci but not yet at the 16S rRNA level (11, 30). The highly conserved function and ubiquitous distribution of the gene encoding elongation factor Tu (EF-Tu) may render this gene a valuable phylogenetic marker for eubacteria; this

* Corresponding author. Mailing address: Department of Microbiology, National University of Ireland, Cork, Western Road, Cork, Ireland. Phone: 353 21 4901365. Fax: 353 21 4903101. E-mail: m.ventura@ucc.ie.

† Present address: Cognis, Dusseldorf, Germany.

gene already has given satisfying results for enterococcal species (18, 19) and some eubacterial species (27).

EF-Tu is a GTP binding protein playing a central role in protein synthesis. It loads the amino-acyl tRNA molecule onto the ribosome during the translation process. The EF-Tu protein is encoded by the *tuf* gene in eubacteria and is present in various copy numbers per bacterial genome. The *tuf* gene belongs to a large transcriptional unit, the *str* operon, which encodes many ribosomal proteins and related regulatory proteins (5, 21). The *str* operon of *Escherichia coli* is composed of four genes: *rpsL* (coding for ribosomal protein S12), *rpsG* (ribosomal protein S7), *fus* (elongation factor G), and *tufA* (EF-Tu). The order of these genes in this transcriptional unit is similar to that described for many species, including *Enterococcus* spp., *Bacillus subtilis*, and *Neisseria meningitidis* (24). In myxobacteria, EF-Tu is genetically organized in the tRNA-*tufB* operon, where the *tuf* gene is preceded by four tRNA genes which are cotranscribed with the *tuf* gene (3).

In this study, short *tuf* gene sequences of different LAB strains were obtained and used to analyze the phylogeny of many *Lactobacillus* and *Bifidobacterium* species. We also describe the genomic locations of the *tuf* genes in some *Lactobacillus* and *Bifidobacterium* species and their transcription patterns. Moreover, species-specific primers for the identification of members of the *L. casei* group were designed based on available genome sequences and used successfully in a multiplex PCR assay.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and their origins are summarized in Table 1. All *Bifidobacterium* strains were grown anaerobically in MRS medium (Difco, Detroit, Mich.) supplemented with 0.05% L-cysteine–HCl and incubated at 37°C for 16 h. *Lactobacillus* strains were grown aerobically in MRS medium and incubated at 37°C for 16 h.

DNA amplification and cloning of the *tuf* gene and its locus. PCR was used to amplify the *tuf* gene in all investigated *Lactobacillus* strains. A DNA fragment corresponding to the *tuf* gene was amplified by using oligonucleotides TUF-1 (5′-GATGCTGCTCCAGAAGA-3′) and TUF-2 (5′-ACCTTCTGGCAATTCA ATC-3′). The *tuf* fragment sequence of *Bifidobacterium* strains was amplified by using oligonucleotides BIF-1 (5′-GAGTACGACTTCAACCAG-3′) and BIF-2 (5′-CAGGCGAGGATCTTGGT-3′). In order to amplify DNA sequences located upstream of the *tuf* gene in *L. delbrueckii* subsp. *bulgaricus* ATCC BAA-365, we used primers rp (5′-ATAAGACCTTTAGAAGCAGC-3′) and Tu-inv (5′-CACGAGTTTGTGGCATAG-3′), targeting the *rpsT* gene and the 5′ end of the *tuf* gene, respectively.

Each PCR mixture (50 μl) contained a reaction cocktail of 20 mM Tris-HCl, 50 mM KCl, 200 μM each deoxynucleoside triphosphate (dNTP), 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 (3 min at 95°C), followed by amplification for 30 cycles as follows: denaturation for 30 s at 95°C, annealing for 30 s at 52°C, and extension for 2 min at 72°C. PCR was completed with an elongation phase (10 min at 72°C). The resulting amplicons were separated on 1% agarose gels, followed by ethidium bromide staining. PCR fragments were purified by using a PCR purification kit (Qiagen, West Sussex, United Kingdom) and then cloned in the pGEM-T Easy plasmid vector (Promega, Southampton, United Kingdom) by following the supplier's instructions.

DNA sequencing and phylogeny study. Nucleotide sequencing of both strands from cloned DNA was performed by using a fluorescence-labeled primer cycle sequencing kit (Amersham Buchler, Braunschweig, Germany) by following the supplier's instructions. The primers used were TUF-1, TUF-2, BIF-1, and BIF-2 labeled with IRD800 (MWG Biotech, Ebersberg, Germany). The sequences determined for the *tuf* genes of all *Lactobacillus* and *Bifidobacterium* strains used in this study and those available in the GenBank database were compared. Sequence alignments were done by using the MultiAlign program and the Clustal-W package. Phylogenetic trees were constructed by using the neighbor-joining program from the PHYLIP software package, version 3.5c (10). Calculation of distance matrices was carried out by using the DNADIST and PROTDIST programs (10) for nucleotide and putative amino acid sequences, respectively, and by using the default models. Dendrograms from gene sequences were also drawn by using the Clustal-X, DNAML (maximum likelihood), and DNAPARS (parsimony) programs (10). The numbers of synonymous substitutions between all possible pairs of *tuf* genes were determined by applying the method of Nei and Gojobori (29) and by using the MEGA computer program (23). The correction for multiple substitutions was made by using the Jukes-Cantor formula (17).

Reference sequences used. *tuf* gene sequences from the following bacteria (GenBank accession numbers) were used for our phylogenetic analysis: *L. helveticus* ATCC 15009 (AJ418903), *L. acidophilus* ATCC 4356 (AJ418902), *L. amylovorus* DSM 20531 (AJ418904), *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 (AJ418910), *L. delbrueckii* subsp. *delbrueckii* ATCC 9649 (AJ418911), *L. delbrueckii* subsp. *lactis* (ATCC 12315), *L. reuteri* ATCC 23272 (AJ418925), *L. fermentum* ATCC 14931 (AJ418939), *L. rhamnosus* ATCC 11443 (AJ459828), *L. rhamnosus* ATCC 11981 (AJ459829), *L. casei* NCDO 173 (AJ459390), *L. paracasei* subsp. *paracasei* ATCC 27216 (AJ418937), *L. paracasei* subsp. *paracasei* ATCC 335 (AJ459399), *L. lactis* ATCC 11154 (AF274745), *Enterococcus faecalis* ATCC 29212 (AF124221), *E. gallinarum* ATCC 49573 (*tufA*) (AF124223), *E. gallinarum* ATCC 49573 (*tufB*) (AF274725), *E. faecium* ATCC 19434 (*tufA*) (AF124222), *E. faecium* ATCC 19434 (*tufB*) (AF274724), *Streptococcus pyogenes* ATCC 19615 (AF274743), and *S. mutans* ATCC 25175 (AF274741).

We extracted the genes surrounding the *tuf* gene from the *Bifidobacterium longum* NCC 2705 genome (GenBank accession number NC004307) and from the *L. plantarum* WCFS1 genome (GenBank accession number AL935263). Preliminary sequence data for the *L. gasseri* ATCC 33323 genome (Genbank accession number NZAAAB00000000), the *L. casei* ATCC 334 genome, and the *L. delbueckii* subsp. *bulgaricus* ATCC BAA-365 genome were obtained from the U.S. Department of Energy Joint Genome Institute at http://www.jgi.doe.gov/JGI_microbial/html/index.html.

Southern hybridization. Ten micrograms of bacterial DNA was digested to completion with restriction endonuclease *Hin*dIII as recommended by the supplier (Roche, Sussex, United Kingdom). This restriction enzyme was chosen because no restriction sites were observed within the amplified *tuf* gene fragments. Southern blots of agarose gels were performed with Hybond N+ membranes (Amersham, Little Chalfont, United Kingdom) as described by Sambrook and Russell (37). The filters were hybridized with a PCR-generated probe obtained with primer pairs TUF-1–TUF-2 and BIF-1–BIF-2 and labeled with α-^{32}P by using a random-primer DNA labeling system (Roche) (37) and DNA templates extracted from *B. longum* NCC 2705 and *L. johnsonii* NCC 533. Subsequent prehybridization, hybridization, and autoradiography were carried out as described by Sambrook and Russell (37).

RNA isolation and Northern blot analysis. Total RNA was isolated by resuspending bacterial cell pellets in TRIzol (Gibco BRL), adding 106-μm glass beads (Sigma), and shearing the slurry with a Mini-Beadbeater cell disruptor (Biospec Products) as described by Walker et al. (55). An initial Northern blot analysis of the *tuf* activity of lactobacilli was carried out with 15-μg aliquots of RNA isolated from 10 ml of *Lactobacillus* strains collected after 8 or 18 h under the growth conditions described above. The RNA was separated in 1.5% agarose–formaldehyde denaturing gels, transferred to Zeta-Probe blotting membranes (Bio-Rad, Hemel Hempstead, United Kingdom) as described by Sambrook and Russell (37), and fixed by UV cross-linking with a Stratalinker 1800 (Stratagene). PCR amplicons obtained with primers TUF-1 and TUF-2 were radiolabeled (37). Prehybridization and hybridization were carried out at 65°C with 0.5 M $NaHPO_4$ (pH 7.2)–1.0 mM EDTA–7.0% SDS. Following 18 h of hybridization, the membranes were rinsed twice for 30 min each time at 65°C in 0.1 M $NaHPO_4$ (pH 7.2)–1.0 mM EDTA–1% SDS and twice for 30 min each time at 65°C in 0.1 mM $NaHPO_4$ (pH 7.2)–1.0 mM EDTA–0.1% SDS and then exposed to X-Omat autoradiography film (Eastman-Kodak). The sizes of the transcripts were estimated by direct comparison to a molecular RNA ladder (Life Technologies).

Primer extension analysis. The 5′ ends of the RNA transcripts were determined in the following manner. Separate primer extension reactions were conducted with 15-μg aliquots of RNA isolated as described above and mixed with 1 pmol of primer (IRD800 labeled) and 2 μl of buffer H (2 M NaCl, 50 mM PIPES [pH 6.4]). The mixture was denaturated at 90°C for 5 min and then hybridized for 60 min at 42°C. After the addition of 5 μl of 1 M Tris-HCl (pH 8.2), 10 μl of 0.1 M dithiothreitol, 5 μl of 0.12 M $MgCl_2$, 20 μl of 2.5 mM dNTP mixture, 0.4 μl (5 U) of reverse transcriptase (Sigma), and 49.6 μl of double-distilled water, the enzymatic reaction mixture was incubated at 42°C for 2 h. The reaction was stopped by the addition of 250 μl of ethanol-acetone (1:1), and the mixture was incubated at −70°C for 15 min and centrifuged at 12,000 × *g* for 15

TABLE 1. Bacterial strains used

Species	Strain[a]	PCR results obtained with *L. casei* group-specific primers[b]	Origin
L. acidophilus	ATCC 4356^T		Human
L. amylovorus	DSM 20531^T		Cattle waste (corn silage)
L. crispatus	DSM 20584^T		Unknown
	NCDO A4		Unknown
L. gallinarum	ATCC 33199^T		Chicken crop
L. helveticus	ATCC 15009^T		Cheese
	CNRZ 303		Cheese
L. delbrueckii subsp. *bulgaricus*	ATCC 11842^T		Yogurt
L. delbrueckii subsp. *delbrueckii*	ATCC 9649^T		Sour grain mash
L. delbrueckii subsp. *lactis*	ATCC 12315^T		Cheese
L. gasseri	DSM 20243^T		
	ATCC 19992		Feces
L. johnsonii	ATCC 33200^T		Human blood
	NCC 533		Human feces
L. reuteri	DSM 20016^T		Human feces
L. fermentum	ATCC 14931^T		Unknown
L. rhamnosus	ATCC 11443	*L. rhamnosus*	Unknown
	ATCC 11981	*L. rhamnosus*	Unknown
	NCC 2504^T	*L. rhamnosus*	Unknown
L. casei	NCDO 173	*L. casei*	Unknown
	NCC 2508^T	*L. casei*	Cheese
L. paracasei subsp. *paracasei*	ATCC 27216	*L. paracasei* subsp. *paracasei*	Saliva of child
	NCC 989^T	*L. paracasei* subsp. *paracasei*	Unknown
	NCC 2461	*L. paracasei* subsp. *paracasei*	Infant feces
B. longum	ATCC 15707^T		Intestine of adult
	NCC 2705		Infant feces
B. infantis	ATCC 15697^T		Intestine of infant
B. bifidum	ATCC 29521^T		Infant feces
B. lactis	DSM 10140^T		Yogurt
B. catenulatum	DSM 20103^T		Intestine of adult
B. adolescentis	ATCC 15703^T		Intestine of adult
B. breve	ATCC 15700^T		Intestine of infant
B. animalis	ATCC 25527^T		Rat feces
L. casei group	NCC 400	*L. paracasei* subsp. *paracasei*	Unknown
	NCC 438	*L. paracasei* subsp. *paracasei*	Unknown
	NCC 476	*L. paracasei* subsp. *paracasei*	Yogurt
	NCC 1002	*L. paracasei* subsp. *paracasei*	Milking machine
	NCC 2548	*L. paracasei* subsp. *paracasei*	Adult feces
	NCC 2556	*L. paracasei* subsp. *paracasei*	Adult feces
	NCC 171	*L. paracasei* subsp. *paracasei*	Pizza
	NCC 617	*L. casei*	Unknown
	NCC 596	*L. rhamnosus*	Unknown
	NCC 587	*L. rhamnosus*	Unknown
	NCC 2488	*L. rhamnosus*	Infant feces
	NCC 534	*L. rhamnosus*	Unknown
	NCC 2455	*L. rhamnosus*	Infant feces
	NCC 511	*L. paracasei* subsp. *paracasei*	Wine
	NCC 500	*L. paracasei* subsp. *paracasei*	Wine
	NCC 443	*L. paracasei* subsp. *paracasei*	Wine
	NCC 588	*L. paracasei* subsp. *paracasei*	Unknown
	NCC 1813	*L. paracasei* subsp.*paracasei*	Unknown
	NCC 2501	*L. paracasei* subsp. *paracasei*	Unknown
	NCC 558	*L. paracasei* subsp. *paracasei*	Fermented drink
	NCC 2472	*L. paracasei* subsp. *paracasei*	Adult feces
	NCC 159	*L. paracasei* subsp. *paracasei*	Pizza
	NCC 179	*L. paracasei* subsp. *paracasei*	Pizza
	NCC 174	*L. paracasei* subsp. *paracasei*	Pizza
	NCC 177	*L. paracasei* subsp. *paracasei*	Pizza
	NCC 2511	*L. paracasei* subsp. *paracasei*	Unknown
	NCC 2537	*L. paracasei* subsp. *paracasei*	Panenone
	NCC 108	*L. casei*	Unknown
	NCC 546	*L. casei*	Italian cheese

[a] ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen; NCDO, National Collection of Dairy Organisms; CNRZ, Centre National de Recherches Zootechniques; NCC, Nestlé Culture Collection. *L. casei* group strains were used for species-specific detection.

[b] Identification by the multiplex PCR assay described in this study.

TABLE 2. Comparison of nucleotide and amino acid sequence identities for EF-Tu among different *Lactobacillus* strains[a]

Strain no.	Strain	% Sequence identity for strain no.:																						
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	ATCC 11443		80	79	100	79	93	82	82	79	80	81	81	79	100	96	81	80	93	96	79	93	79	79	78
2	ATCC 15009	87		80	80	96	79	89	89	95	99	84	88	95	80	80	88	97	79	80	96	79	88	88	87
3	ATCC 14931	86	83		79	80	80	82	82	80	80	89	82	81	79	81	82	80	80	81	81	80	80	80	80
4	ATCC 11981	108	87	86		79	93	82	82	79	80	81	81	79	100	96	81	80	93	96	79	93	79	79	78
5	ATCC 33199	78	90	76	80		78	88	88	95	98	83	88	96	79	78	87	96	78	78	96	78	87	87	87
6	ATCC 27216	98	85	86	96	76		82	82	79	79	80	81	78	93	92	81	80	100	92	78	100	79	79	79
7	ATCC 19992	87	91	84	87	84	86		99	88	88	87	97	88	82	81	97	88	82	81	89	82	85	85	85
8	DSM 20243	88	91	84	88	84	87	100		88	88	87	97	89	82	81	97	89	82	81	89	82	85	85	85
9	ATCC 4356	85	97	83	85	89	85	91	92		95	84	87	94	79	79	87	96	79	79	94	79	87	87	87
10	CNRZ 303	87	100	83	87	90	85	91	91	97		84	88	95	80	80	88	96	79	80	96	79	87	87	87
11	DSM 20016	88	85	94	88	78	86	86	85	84	85		87	84	81	81	87	84	80	81	84	80	81	81	81
12	ATCC 33200	85	87	82	85	82	84	95	94	87	87	88		88	81	81	100	88	81	81	88	81	86	86	85
13	DSM 20584	100	87	86	100	78	98	87	88	85	87	88	89		79	79	88	95	78	79	100	78	87	87	87
14	NCC 2504	85	96	82	85	90	83	90	90	95	96	84	85	85		96	81	80	93	96	79	93	79	79	78
15	NCC 2508	100	88	85	100	78	97	87	87	86	87	88	84	100	97		81	80	92	100	79	92	79	79	79
16	NCC 533	88	90	85	88	82	87	98	98	90	90	87	97	88	89	87		88	81	81	88	81	85	85	85
17	DSM 20531	85	97	83	85	90	85	91	92	98	97	85	87	85	96	86	90		80	80	96	79	88	88	88
18	NCC 2461	98	85	86	98	76	100	86	87	85	85	86	84	98	83	97	87	85		92	78	100	79	79	79
19	NCDO 173	100	88	85	100	78	97	87	87	86	87	88	84	100	85	100	87	86	97		79	92	79	79	79
20	NCDO A4	80	91	77	80	89	78	85	85	89	91	79	84	80	92	80	84	91	78	80		78	87	87	87
21	NCC 989	98	85	86	98	76	100	86	87	85	85	86	84	98	83	97	87	85	100	97	79		79	79	79
22	ATCC 9649	85	91	83	85	84	84	89	89	91	91	85	86	85	91	85	89	91	84	85	85	84		99	99
23	ATCC 12315	85	91	84	85	84	84	88	88	91	91	85	85	85	90	85	89	91	84	85	85	84	100		99
24	ATCC 11842	85	90	83	85	83	84	88	88	91	90	85	85	85	90	85	88	91	84	85	84	84	99	100	

[a] Data in the upper right triangle represent DNA sequence identities of the *tuf* genes in *Lactobacillus* strains, while data in the lower left triangle represent deduced amino acid sequence identities of the corresponding EF-Tu proteins.

min. The pellets were dissolved in 4 μl of distilled water and mixed with 2.4 μl of loading buffer from the sequencing kit (Thermosequenase, fluorescence labeled). The cDNA was separated on 8% polyacrylamide–urea gels. Sequencing reactions were conducted with the same primers as those used for the primer extension reactions and detected by using a LiCor sequencer (MWG Biotech). The synthetic oligonucleotides used (designed in this study) were tuf-a (5′-CA AAACAGTAGTAATAGCTGC-3′) and tgf-1 (5′-CGAGAAACGTGACCTTT AC-3′).

Amplification with species-specific primers. Amplification reactions were performed with a 50-μl (total volume) solution containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each dNTP (Gibco BRL), 10 pmol each of primers PAR (5′-GACGGTTAAGATTGGTGAC-3′), CAS (5′-ACTGAAGGCGACA AGGA-3′), and RHA (5′-GCGTCAGGTTGGTGTTG-3′), 50 pmol of primer CPR (5′-CAANTGGATNGAACCTGGCTTT-3′), 25 ng of template DNA, and 2.5 U of *Taq* DNA polymerase. Amplification reactions were performed by using a thermocycler (Perkin-Elmer Cetus 9700) with the following temperature profiles: 1 cycle at 95°C for 5 min; 30 cycles at 95°C for 30 s, 54°C for 1 min, and 72°C for 1.5 min; and 1 cycle at 72°C for 7 min. Primers CAS, PAR, RHA, and CPR were all designed in this study. For routine identification, cells were lysed by using a rapid DNA extraction protocol and were used as direct PCR templates. PCR amplicons were analyzed by 2% (wt/vol) agarose gel electrophoresis in Tris-acetate-EDTA buffer at a constant voltage of 7 V/cm, visualized with ethidium bromide (0.5 μg/ml), and photographed under UV light at 260 nm.

Nucleotide sequence accession numbers. The GenBank accession numbers for the partial *tuf* gene sequences generated in this study are as follows: *L. gallinarum* ATCC 33199 (AY372032), *L. helveticus* CNRZ 303 (AY372033), *L. crispatus* DSM 20584 (AY372034), *L. crispatus* NCDO 4 (AY373256), *L. gasseri* ATCC 19992 (AY372035), *L. johnsonii* ATCC 33200 (AY372036), *L. johnsonii* NCC 533 (AY372049), *L. rhamnosus* NCC 2504 (AY372037), *L. casei* NCC 2508 (AY372038), *L. paracasei* subsp. *paracasei* NCC 989 (AY372039), *L. paracasei* subsp. *paracasei* NCC 2461 (AY372040), *B. bifidum* ATCC 29521 (AY372041), *B. longum* ATCC 15707 (AY372042), *B. infantis* ATCC 15697 (AY372043), *B. catenulatum* DSM 20103 (AY372044), *B. adolescentis* ATCC 15703 (AY372045), *B. breve* ATCC 15700 (AY372046), *B. animalis* ATCC 25527 (AY370920), and *B. lactis* DSM 10140 (AY370919). Since the *L. gasseri tuf* sequence extracted from the ongoing genome sequencing of *L. gasseri* ATCC 33323 (NZAAAB00000000) contained various reading errors, we decided to sequence this *tuf* gene again and deposited it in GenBank under accession number AY372047). The DNA region located upstream of the *tuf* gene of *L. delbueckii* subsp. *bulgaricus* ATCC BAA-365 and reported here was deposited in GenBank under accession number AY372048. The GenBank accession number for the *tuf* locus sequence of *L. johnsonii* NCC 533 is AY372049.

RESULTS

Identification and alignment of *tuf* sequences. The *tuf* sequences from selected bacterial species for which genome sequences are publically available were aligned and compared. Four conserved regions were identified, and two pairs of primers (BIF-1–BIF-2 and TUF-1–TUF-2) for amplifying regions of 800 bp were designed. These primers allowed the amplification of *tuf* sequences from different *Bifidobacterium* and *Lactobacillus* species. All PCR products were cloned into the vector system pGEMT-Easy. Subsequently, the nucleotide sequence of the inserted DNA fragment was determined by sequencing of three randomly selected clones on both strands for each bacterial species.

A multiple alignment of the *tuf* sequences determined in our laboratory with those retrieved from databases revealed regions which were conserved in all strains from the same species but which were variable in other species. A similarity comparison of the *tuf* sequences for lactobacilli and for bifidobacteria demonstrated that the *tuf* genes were highly conserved among all *Lactobacillus* species investigated here, with identities ranging from 78 to 98% for DNA (reaching a value of 100% between strains belonging to the same species) and from 76 to 100% for translated gene products (Table 2). Identities among the *tuf* genes of the bifidobacteria ranged from 89 to 97% (reaching a value of 100% for strains belonging to the same species) for DNA and from 91 to 99% for amino acid sequences (Table 3). Many of the differences observed in DNA

TABLE 3. Comparison of nucleotide and amino acid sequence identities for EF-Tu among different *Bifidobacterium* strains[a]

Strain no.	Strain	% Sequence identity for strain no.:								
		1	2	3	4	5	6	7	8	9
1	DSM 10140		90	90	97	91	91	95	89	91
2	ATCC 15697	92		97	90	95	91	91	96	09
3	ATCC 15707	92	99		90	94	90	91	100	89
4	ATCC 25527	99	93	93		91	91	95	89	91
5	ATCC 29521	95	96	96	95		90	91	94	89
6	ATCC 15703	93	93	93	93	91		91	90	94
7	ATCC 15700	97	95	94	98	93	94		91	91
8	NCC 2705	93	99	100	93	96	93	94		89
9	DSM 20103	93	91	91	93	92	93	93	91	

[a] Data in the upper right triangle represent DNA sequence identities of the *tuf* genes in *Bifidobacterium* strains, while data in the lower left triangle represent deduced amino acid sequence identities of the corresponding EF-Tu proteins.

sequences among species were silent in terms of their effects on the encoded amino acid sequences. Thus, there were many identical protein sequences, even though their encoding DNAs exhibited substantial divergence.

Alignment of the amino acid sequences deduced from the *tuf* genes of lactobacilli and bifidobacteria with other EF-Tu sequences available in databases demonstrated that their gene products are conserved and carry conserved amino acid residues typically found in prokaryotic EF-Tu (18). The portion of the *tuf* genes of lactobacilli and bifidobacteria described in this study encodes the portion of the EF-Tu protein from residues 104 to 335, according to the numbering for *E. coli* (42). A secondary structure prediction for this portion included the last four α helices and two β sheets of domain I, the entire domain II, and the N-terminal portion of domain III, on the basis of the experimentally determined structure of EF-Tu of *E. coli* (42). These domains have been determined to play a crucial role in the correct folding of the protein (42); consequently, the corresponding sequences have remained highly conserved among eubacterial species.

Phylogenetic analysis. Phylogenetic analysis of the *tuf* DNA sequences within the genera *Lactobacillus* and *Bifidobacterium* by neighbor-joining and maximum-parsimony methods showed clear distinct positions of the two genera (Fig. 1). These data were supported by the reported bootstrap values. For completeness, we included in our analysis the *tuf* DNA sequences of other strains belonging to different genera representing the LAB group (e.g., *Lactococcus*, *Streptococcus*, and *Enterococcus*). The tree shows two major clusters representing the low-G+C-content gram-positive bacteria (genera *Lactobacillus*, *Lactococcus*, *Streptococcus*, and *Enterococcus*) and the high-G+C-content gram-positive bacteria (genus *Bifidobacterium*). Moreover, a further subdivision into three groups corresponding to *Lactobacillus*, *Lactococcus-Streptococcus*, and *Enterococcus* was identified.

In order to improve the accuracy of our phylogenetic estimation, we traced trees using different methods. The tree topologies obtained showed similar hierarchical arrangements (data not shown).

The different *Lactobacillus* and *Bifidobacterium* species under investigation were unambiguously differentiated by a comparative sequence analysis of a fragment of the *tuf* gene, as indicated by the phylogenetic tree in Fig. 1. A phylogenetic tree was also constructed on the basis of 16S ribosomal DNA (rDNA) sequences available in databases. The tree topologies obtained with the 16S rDNA sequences showed a phylogenetic arrangement very similar to that of the *tuf*-based tree (data not shown). A striking feature of the *tuf* phylogeny is that *L. delbrueckii tuf* sequences clustered closely with *L. acidophilus* group A *tuf* sequences, while *L. acidophilus* group B *tuf* sequences clustered more distantly. Interestingly, closely related strains with nearly identical 16S rRNA sequences, e.g., the *L. casei* group (*L. casei*, *L. paracasei* subsp. *paracasei*, and *L. rhamnosus*), the *L. acidophilus* B group (*L. gasseri* and *L. johnsonii*), *B. animalis-B. lactis*, and *B. longum-B. infantis*, clearly branched separately in the *tuf*-based tree (Fig. 1).

Most of the base substitutions in the *tuf* genes were synonymous, i.e., did not result in amino acid changes. The synonymous distances calculated from the nucleotide substitution ratios at synonymous positions in the *tuf* genes were examined for all possible combinations of *Lactobacillus* and *Bifidobacterium tuf* genes. The relationships between the pairwise distances for the 16S rRNAs and the synonymous distances for the *tuf* genes are shown in Fig. 2. There was a significant correlation between the genetic distances for the16S rDNA sequences and those for the *tuf* sequences. In fact, lactobacilli and bifidobacteria showed a correlation coefficient (r) of 0.94 (Fig. 2). The two groups of dots depicted in Fig. 2 represent the *Lactobacillus* and *Bifidobacterium* species in accordance with the different G+C contents of their 16S rRNAs and *tuf* genes. Therefore, it can be concluded that the base substitutions occurring in the *tuf* sequences during the evolutionary process render the *tuf* gene a reliable molecular evolutionary clock.

Presence of *tuf* genes in the *Lactobacillus* and *Bifidobacterium* genomes. We surveyed all available genomic data for the presence of the *tuf* gene and its genomic location in various *Lactobacillus* and *Bifidobacterium* species and strains (Fig. 3). In *B. longum* NCC 2705, the *tuf* gene is directly downstream of the *fusA* gene (translation elongation factor G), the *rpsG* gene (30S ribosomal protein S7), and the *rpsL* gene (30S ribosomal protein S12) and directly upstream of an unidentified open reading frame (Fig. 3a). PCR amplification with a primer targeting a conserved region of the *fus* gene and the *tuf* gene yielded the expected amplicons for all nine bifidobacteria tested, indicating the presence of the conserved *fus-tuf* organization in the *Bifidobacterium* species tested here (data not shown). The overall organization of the *tuf* gene of bifidobac-

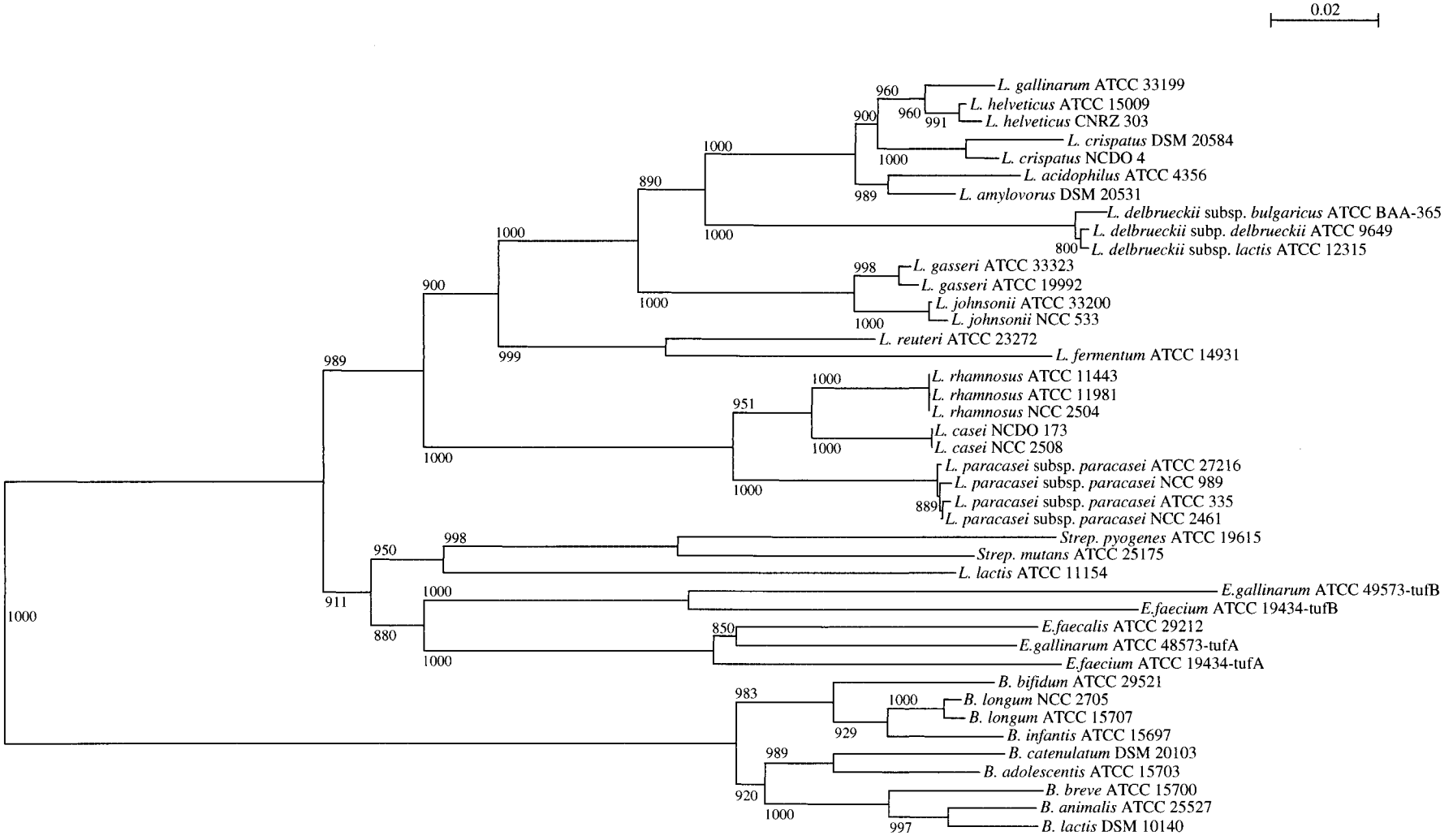


FIG. 1. Phylogenetic tree of *Lactobacillus* EF-Tu and *Bifidobacterium* EF-Tu based on nucleotide sequence homology. The phylogenetic tree was constructed by the neighbor-joining method. The scale bar represents sequence divergence. In this tree topology, the phylogenetic distance between organisms is the sum of the horizontal segments. *L. acidophilus* group A includes the species *L. acidophilus*, *L. amylovorus*, *L. gallinarum*, and *L. crispatus*; *L. acidophilus* group B includes only *L. gasseri* and *L. johnsonii*. Bootstrap values are reported for a total of 1,000 replicates. tufA and tufB indicate the *tufA* and *tufB* genes, respectively.

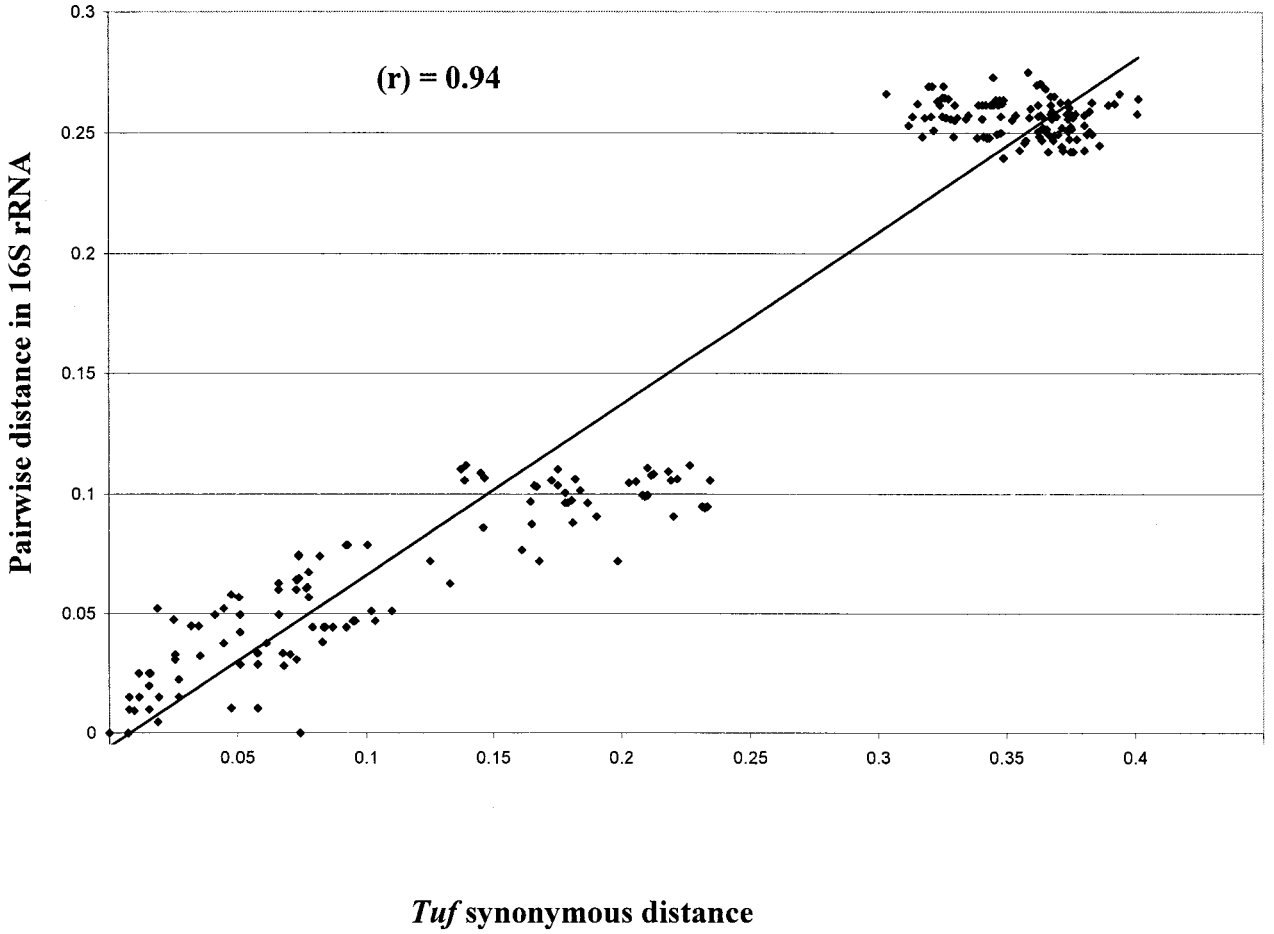


FIG. 2. Comparison between genetic distances estimated from 16S rRNA and synonymous distances estimated from *tuf* genes. The synonymous distances were obtained from the nucleotide sequences of the *tuf* genes by applying the method of Nei and Gojobori (29). r, coefficient of correlation.

teria displayed extensive homology with that of the *str* operon of *E. coli* (15) and enterobacteria (19).

Screening of the sequence data from the ongoing genome sequencing projects for *L. gasseri* ATCC 33323, *L. casei* ATCC 334, *L. johnsonii* NCC 533, *L. delbrueckii* subsp. *bulgaricus* ATCC BAA-365, and *L. plantarum* WCFS1 revealed a similar *tuf* genomic location (Fig. 3). Surprisingly, this *tuf* arrangement does not resemble any other so far described for *tuf* loci (3, 5, 6, 14, 15, 19, 20, 21, 38, 54). In *L. gasseri* ATCC 33323 and *L. johnsonii* NCC 533, the *tuf* gene is located downstream of a metallo-beta-lactamase gene, the *rpsO* gene (30S ribosomal protein S15), and the *rpsT* gene (30S ribosomal protein S20), while directly downstream of the *tuf* gene is a transcription regulator trigger factor gene (*tig* gene), which is followed by genes encoding a Clp protease (*clp* gene), a GTP binding protein, and a phosphotyrosine protein phosphatase. Both strains showed high sequence identity (from 82 to 96%) within a 9-kb genome fragment. The consensus nucleotide binding domain, Walker motif A (GXXXXGKT), was conserved in the deduced sequences of the *tuf* and *clp* genes. An examination of the immediate neighborhood of the Walker sequence indicates that this region is preceded by a β strand and followed by an α helix, an arrangement which complies with the rules for Walker motif A (34). A comparison with various other *Lactobacillus* species showed a very similar genetic organization of the *tuf* region. Analysis of the *tuf* region of *L. casei* ATCC 334 revealed similar corresponding genes (Fig. 3b). Interestingly, between the *tuf* gene and the *tig* gene is an insertion of a 4-kb DNA segment that bears a high resemblance to a mobile element. It contains four genes, the first of which encodes a predicted protein sharing 51% identity with a transposase of *Leuconostoc mesenteroides*. The next gene encodes a protein which matches an ATP binding cassette transporter. The following genes encode proteins that are identical to a putative transposase of *L. rhamnosus* and to an ATP binding protein.

Since the ongoing genome sequencing project for *L. delbrueckii* subsp. *bulgaricus* ATCC BAA-365 provided incomplete DNA sequences for the *tuf* gene, these sequences were completed for this study. The DNA sequence located upstream of the *tuf* gene of *L. delbrueckii* subsp. *bulgaricus* ATCC BAA-365 was generated by PCR with two primers targeting conserved regions in the *tuf* and *rpsT* genes. The *tuf* region of *L. delbrueckii* subsp. *bulgaricus* ATCC BAA-365 showed the same gene order as that identified for the *L. johnsonii*, *L. gasseri*, and

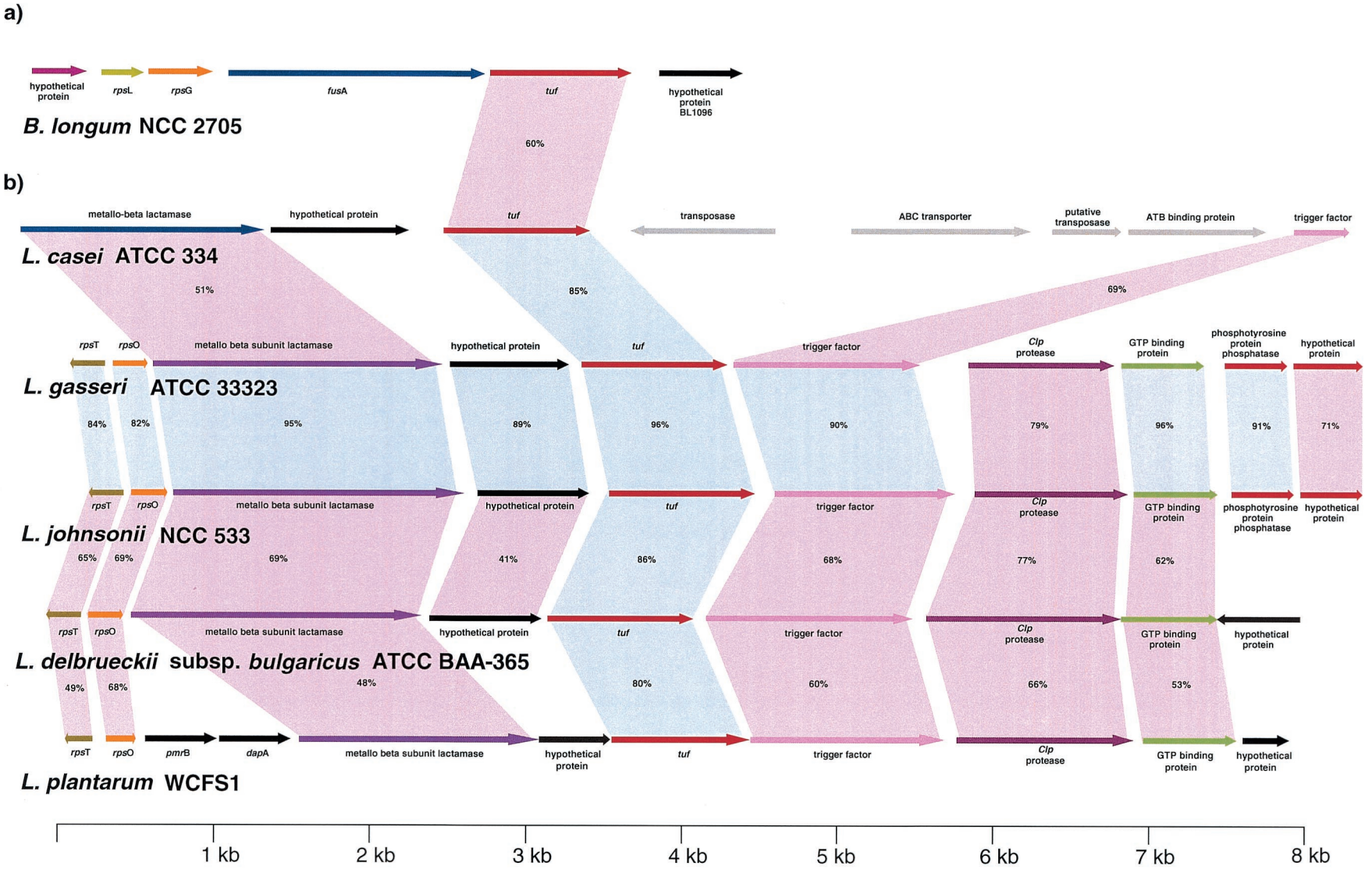
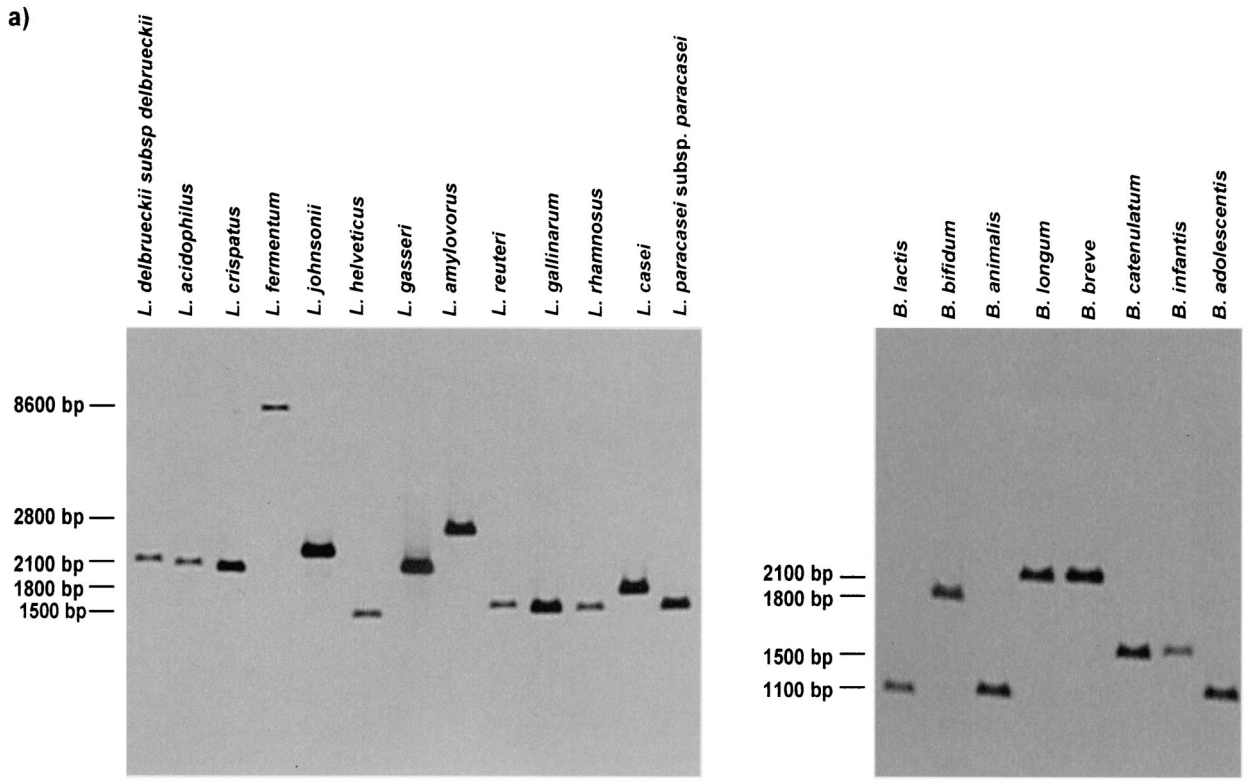
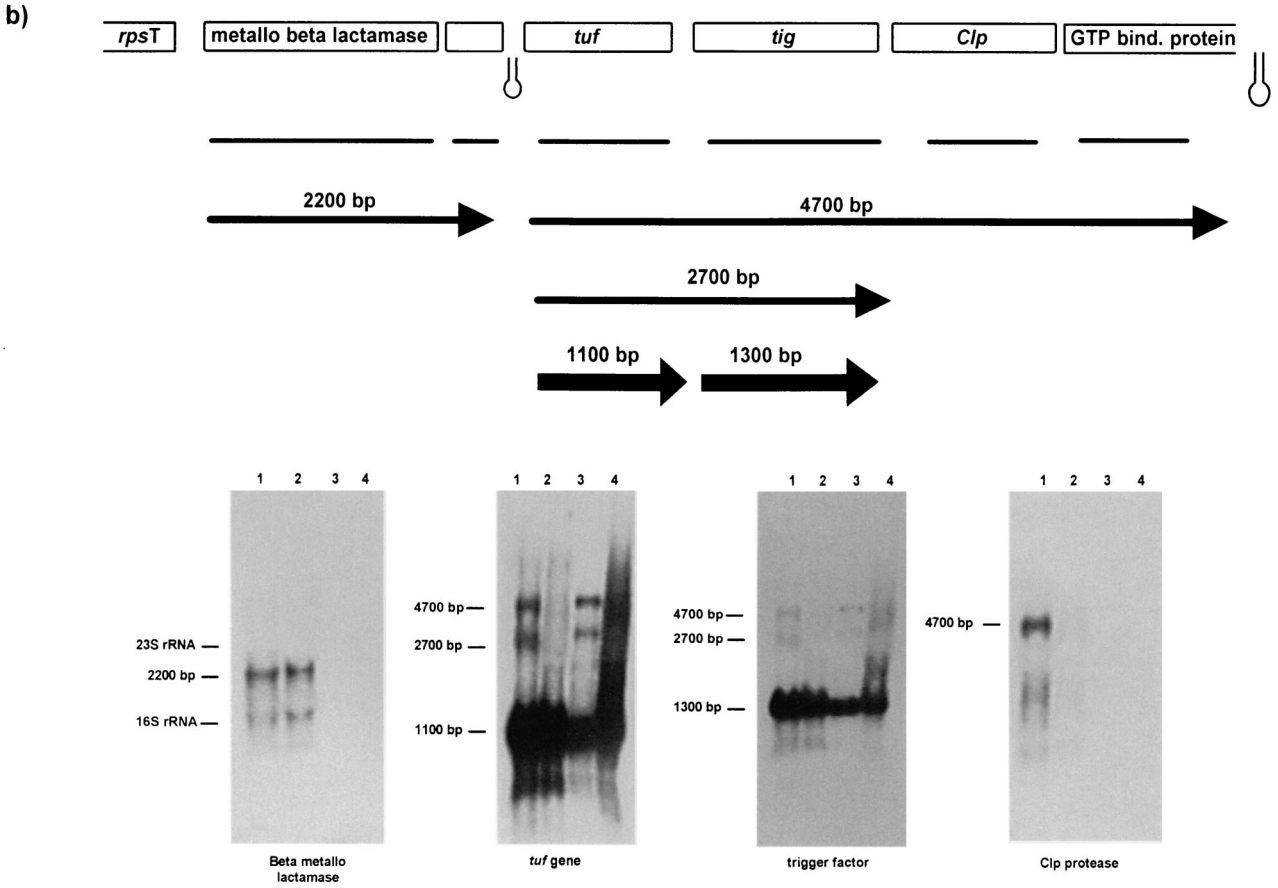


FIG. 3. Comparison of the organization of the *tuf* loci in bifidobacteria (a) and in lactobacilli (b). (a) *B. longum* NCC 2705. The DNA sequence fragments of *Bifidobacterium* represented here are 4.8 kb. (b) *L. casei* ATCC 334, *L. gasseri* ATCC 33323, *L. johnsonii* NCC 533, *L. delbrueckii* subsp. *bulgaricus* ATCC BAA-365, and *L. plantarum* WCFS1. The putative function of a protein is indicated above or below each arrow. Related proteins are linked by blue shading (≥80%) and pink shading (<80%) according to different amino acid similarities; percent amino acid identities are shown. The length of an arrow is proportional to the length of the predicted open reading frame. Corresponding genes are indicated by the same colors of arrows.


a)
L. delbrueckii subsp delbrueckii
L. acidophilus
L. crispatus
L. fermentum
L. johnsonii
L. helveticus
L. gasseri
L. amylovorus
L. reuteri
L. gallinarum
L. rhamnosus
L. casei
L. paracasei subsp. paracasei
8600 bp
2800 bp
2100 bp
1800 bp
1500 bp
B. lactis
B. bifidum
B. animalis
B. longum
B. breve
B. catenulatum
B. infantis
B. adolescentis
2100 bp
1800 bp
1500 bp
1100 bp


b)
rpsT
metallo beta lactamase
tuf
tig
Clp
GTP bind. protein
2200 bp
4700 bp
2700 bp
1100 bp
1300 bp
1 2 3 4
23S rRNA
2200 bp
16S rRNA
Beta metallo lactamase
4700 bp
2700 bp
1100 bp
tuf gene
4700 bp
2700 bp
1300 bp
trigger factor
4700 bp
Clp protease

L. casei strains examined (Fig. 3b). In *L. plantarum* WCFS1, except for two gene insertions (*pmrB* gene, encoding a multidrug resistance efflux pump, and *dapA* gene, encoding dihydrodipicolinate synthase) upstream of the open reading frame encoding the metallo-beta-lactamase, the gene order surrounding the *tuf* gene was conserved (Fig. 3b). The degree of sequence conservation in the *tuf* region of *Lactobacillus* species reflects the evolutionary distance separating these different species. Bioinformatic analysis suggested a highly conserved DNA module among the *Lactobacillus* strains investigated here, consisting of the *tuf*, *tig*, *clp*, and GTP binding protein genes.

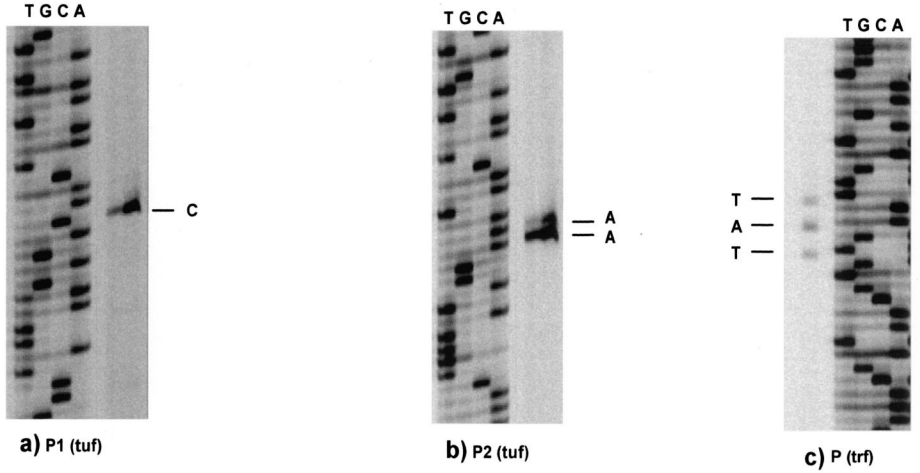
Estimation of the numbers of *tuf* genes in the *Lactobacillus* and *Bifidobacterium* genomes. In a Southern hybridization analysis, *Hin*dIII-digested genomic DNAs from 13 *Lactobacillus* species and from 8 *Bifidobacterium* species were probed with the *tuf* gene (Fig. 4a). All investigated strains of lactobacilli and bifidobacteria yielded single bands of different sizes (ranging from 1,500 to 8,600 bp in lactobacilli and from 1,100 to 2,100 bp in bifidobacteria), suggesting that only one *tuf* gene is present in all of the genomes. All bacterial DNAs were also digested with *Bam*HI, and the resulting hybridization patterns again yielded only one band for each bacterial species, confirming the presence of a single *tuf* gene(data not shown). This result was also confirmed by analysis of the incomplete lactobacillus and bifidobacterium genomes. Sequence analysis of the entire genomes of *L. gasseri* ATCC 33323, *L. johnsonii* NCC 533, and *B. longum* NCC 2705 (39) reveals a unique copy of the *tuf* gene, whereas other eubacterial taxa (e.g., enterobacteria) have a duplication of EF-Tu.

***tuf* transcription analysis.** Northern hybridization experiments were performed in order to determine whether the *tuf* gene is cotranscribed with its flanking genes. Total RNA was extracted from *L. johnsonii* NCC 533 and *L. gasseri* ATCC 33323 in the exponential and stationary growth phases. A probe corresponding to the *tuf* gene hybridized to transcripts of 4.7, 2.7, and 1.1 kb (Fig. 4b). A second probe, overlapping a gene next to the *tuf* gene that encodes a trigger factor, hybridized to 4.7-, 2.7-, and 1.3-kb transcripts (Fig. 4b). It is highly unlikely that the 1.3-kb transcripts are merely processed products of the 4.7- and 2.7-kb transcripts, since only one band was systematically found with the *clp* gene probe. In fact, a third probe targeting the gene encoding the Clp protease revealed only one transcript, of 4.7 kb, as did a probe targeting the gene encoding the GTP binding protein (data not shown). These results led us to conclude that the transcripts of 4.7 kb correspond to the *tuf* gene cotranscribed with the *tig* and *clp* genes and with the gene encoding the GTP binding protein and that the transcript of 2.7 kb includes the *tuf* and *tig* genes.

The genes located upstream of the *tuf* gene showed transcription patterns independent of those of the *tuf* gene. In fact, two probes corresponding to the genes encoding the metallo-beta-lactamase and a hypothetical protein hybridized to a 2.2-kb transcript (Fig. 4b and data not shown). Moreover, these transcripts were present in both exponential and latent growth phases, while the *tuf*, *tig*, and *clp* genes followed different kinetics and appeared to be transcribed only during the exponential growth phase. The amount of transcript corresponding only to the *tuf* gene is larger than that of the largest transcript species covering the entire *tuf* gene. These results confirmed what was described earlier for the *str* operon of *B. stearothermophilus* and *B. subtilis* (20). The hybridization signals corresponding to lanes loaded with RNA samples extracted from *L. gasseri* ATCC 33323 were absent or were weaker than those of *L. johnsonii* NCC 533 because we used probes for the DNA of *L. johnsonii* NCC 533 which shared variable degrees of similarity with the DNA of *L. gasseri* (ranging from 70 to 80%) (Fig. 3b).

Analysis of the nucleotide sequence of the *tuf* operon revealed several notable features (Fig. 4b). The *tuf* operon was delimited at the border by two strong terminator sequences, one located at the 5′ end of a gene upstream of the *tuf* gene and a second one located at the 5′ end of the GTP binding protein gene. To map precisely the transcription start sites directly upstream of the *tuf* gene, primer extension experiments were carried out with RNA isolated from exponentially growing *L. johnsonii* NCC 533 (Fig. 5). Multiple promoter structures have been found preceding the *tuf* gene. In fact, two transcription start sites were identified at −64 bp (putative promoter P1) and at −119 bp (putative promoter P2) relative to the start site of the coding sequence (Fig. 5a and b). Putative promoter P1 had a −10 region (TATAAT) and a −35 region (TAGGCT), while putative promoter P2 had a −10 box identical to that of putative promoter P1, but no consensus −35 sequences were found (Fig. 5d). Notably, two direct repeats (ATTTTC) were detected in the region upstream of the −10 box for both start sites and could play a role in the recognition of the RNA polymerase. Primer extension experiments confirmed that the gene encoding the trigger factor not only is cotranscribed with the *tuf* gene but also possesses its own promoter. Primer extension experiments located the 5′ end 47 bp upstream of the start codon of the *tig* gene (Fig. 5c). An analysis of the putative promoter regions revealed a potential promoter-like sequence having a putative −10 hexamer (TA

FIG. 4. Southern and Northern hybridization analyses. (a) Southern hybridization analysis of *Hin*dIII-digested genomic DNAs of 13 *Lactobacillus* and 8 *Bifidobacterium* species with the *tuf* gene fragment as a probe. Northern hybridization analysis of *Lactobacillus* RNA and transcription unit mapping of the *tuf* locus. In panel a, the sizes of hybridizing fragments are shown. The tested strain was the type strain (Table 1). In the top portion of panel b, all predicted open reading frames are indicated and are annotated with their database matches. The positions of the probes used in Northern blot experiments are indicated under the gene map. The transcripts are depicted as arrows; the arrows point to the 3′ end of the mRNA. The length of the arrow is proportional to the length of the mRNA derived from the Northern blots. The estimated sizes of the mRNAs are indicated. Hairpins indicate possible rho-independent terminators. The transcripts are positioned with respect to the gene map. The width of an arrow indicates the relative abundance of the mRNA species. bind., binding. The bottom portion of panel b shows Northern blot hybridization of RNA isolated from lactobacilli with probes corresponding to the open reading frames in the gene map. The sizes calculated for the hybridization signals are provided.



d)

tuf GTGCCGATATTGAGCAAAAAAGGTTTACATTTTCTGTGGATCTAGTATAATCAATAAGG
160 140 120
REP -10 P2

tuf ATATTTTCAAAGGGGTACGAGCTAGGCTTTACCTTTGAAAGTAGTATAATAATCAACAGAGAATTATCCGTTACTTACTCAACGGACTTCTTGCAAATTTACAGGAGGGTCATATTAATG
REP -35 P1 -10 P1 RBS
100 80 60 40 20

trf TTAACAAATTTCTCTCTGGATTTGTGTTTTAGCAAGGTTTAGGGTAAGATAGTTAAGTGTGCAATAGCAAACCCAAATTTGACATCGGAGGTATTTAATTAATG
100 80 60 40 20
-35 -10 RBS

FIG. 5. Primer extension analysis and comparison of the putative promoter sequences of the *tuf* and trigger factor genes. (a and b) Results of primer extension experiments with oligonucleotides targeting the 5′ end of the *tuf* gene. P1, putative promoter P1; P2, putative promoter P2. (c) Results of primer extension experiments with an oligonucleotide targeting the 5′ end of the trigger factor gene. The lanes in panels a, b, and c indicate the sequencing products for the *tuf* and trigger factor genes. (d) Overview of experimentally determined 5′ ends of the *tuf* gene (*tuf*) and the trigger factor gene (*trf*). The endpoints of the primer extension products are underlined. Underlined bold type indicates the −10 and −35 boxes and direct repeats (REP); RBS, Shine-Dalgarno sequences. The start codon is given at the right end of the sequence.

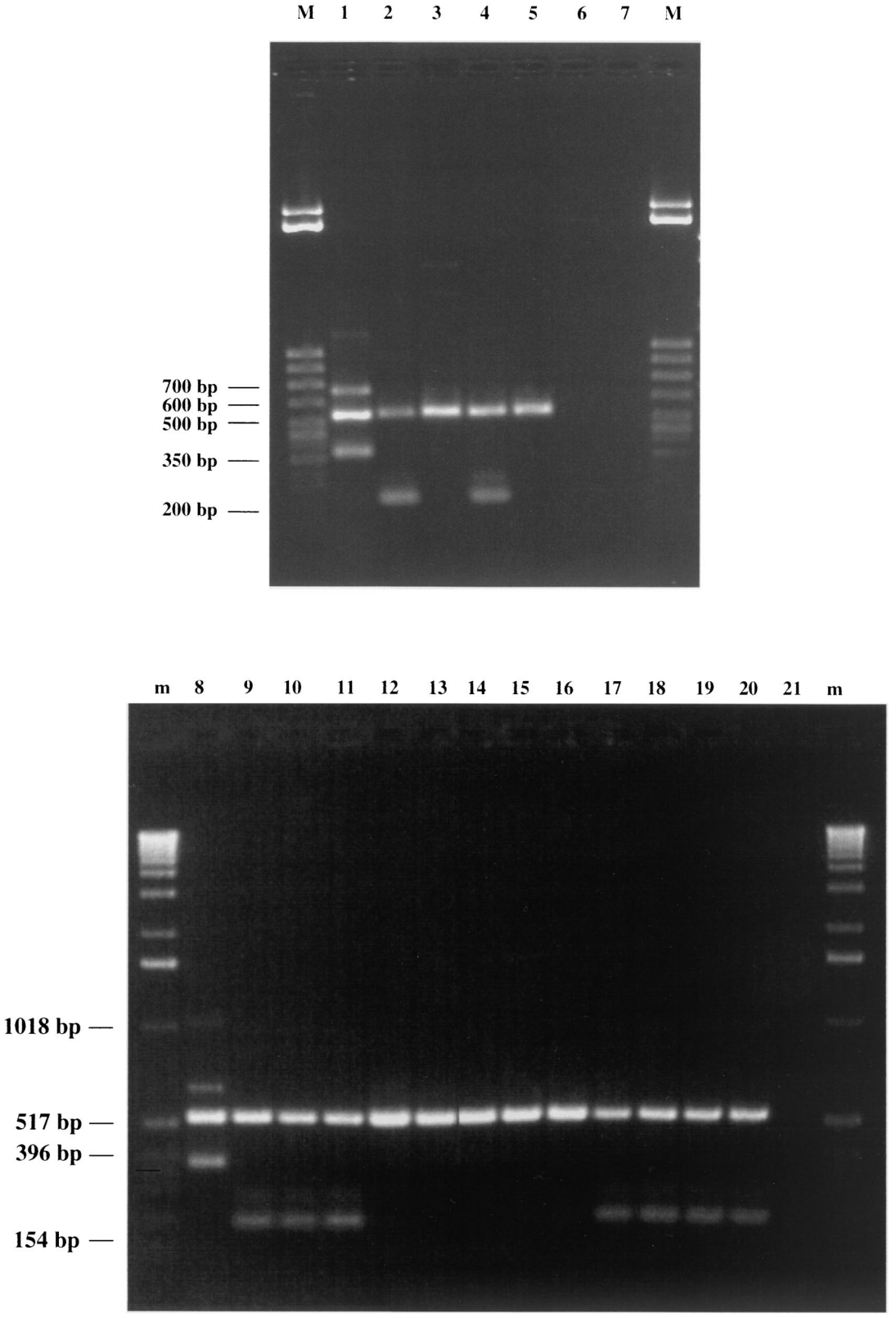


FIG. 6. Amplification products obtained from the *tuf* multiplex assay. Lane M, 50-bp DNA molecular marker (Sigma); lane m, 1-kb DNA ladder (Gibco BRL). Lane 1, *L. casei* NCC 2508; lane 2, *L. paracasei* subsp. *paracasei* NCC 989; lane 3, *L. rhamnosus* NCC 2504; lane 4, *L. paracasei* subsp. *paracasei* NCC 2461; lane 5, *L. rhamnosus* ATCC 11443; lane 6, *L. reuteri* DSM 2006; lane 7, *L. fermentum* ATCC 14931; lane 8, *L. casei* NCC 617; lane 9, *L. paracasei* subsp. *paracasei* NCC 438; lane 10, *L. paracasei* subsp. *paracasei* NCC 476; lane 11, *L. paracasei* subsp. *paracasei* NCC 400; lane 12, *L. rhamnosus* NCC 596; lane 13, *L. rhamnosus* NCC 587; lane 14, *L. rhamnosus* NCC 546; lane 15, *L. rhamnosus* NCC 2488; lane 16, *L. rhamnosus* NCC 2455; lane 17, *L. paracasei* subsp. *paracasei* NCC 1002; lane 18, *L. paracasei* subsp. *paracasei* NCC 2548; lane 19, *L. paracasei* subsp. *paracasei* NCC 2556; lane 20, *L. paracasei* subsp. *paracasei* NCC 171; lane 21, negative control.

AGAT) and −35 box (TTGTGT) (Fig. 5d). The promoter sequences comply with all requirements of *Lactobacillus* promoter sequences necessary for efficient recognition by the σ subunit of the RNA polymerase involved in the transcription of housekeeping genes (7).

Primer design and PCR assay for *Lactobacillus* species identification. We designed a single reverse primer (CPR) and three forward primers (PAR, CAS, and RHA) for the specific detection of *L. paracasei* subsp. *paracasei*, *L. casei*, and *L. rhamnosus*. Application of the CPR-PAR-CAS-RHA oligonucleotide mixture (Fig. 6) resulted in PCR amplicons of 700, 540, and 350 bp with DNA extracted from *L. casei* NCC 2508, amplicons of 540 and 240 bp with DNA derived from *L. paracasei* subsp. *paracasei* NCC 989, but only one amplicon of 540 bp with DNA isolated from *L. rhamnosus* NCC 2504. No PCR product of the above expected sizes was detectable with these primers for any other *Lactobacillus* or *Bifidobacterium* strains listed in Table 1. The amplicon sizes were in agreement with those expected from the analysis of the *tuf* sequences. In fact, the CPR-PAR, CPR-CAS, and CPR-RHA primer pairs must generate amplicons of 240, 350, and 520 bp, respectively. Multiple products are explained by the fact that *L. paracasei* subsp. *paracasei* should generate only two amplicons (240 and 520 bp), *L. casei* should produce two PCR products (350 and 520 bp), and *L. rhamnosus* should generate only one amplicon (520 bp).

The identities of the PCR fragments were confirmed by sequence analysis (data not shown). The species-specific primer sets based on the *tuf* gene were also extended to an additional 29 lactobacillus strains (*L. casei* group strains in Table 1). These strains were originally allocated within the *L. casei* group on the basis of their fermentative properties and the results of amplified rDNA restriction analysis for *Lactobacillus* species identification (51). As shown in Table 1 (*L. casei* group strains) and Fig. 5, 21 strains were clearly allocated within the species *L. paracasei* subsp. *paracasei*, 2 strains were identified as belonging to the species *L. paracasei* subsp. *casei*, while the remaining 6 strains were found to belong to the species *L. rhamnosus*. All strains had been previously characterized by ribotyping-hybridization, which produced individual and repeatable profiles for each strain. The heterogeneity among all ribotyping-hybridization patterns clearly demonstrated that all strains investigated with species-specific *tuf*-based primers were different (data not shown).

DISCUSSION

Significant changes have occurred in bacterial taxonomy since the introduction of molecular techniques. The accurate identification of many bacterial species can be accomplished by reference to rRNA gene sequences (mainly the 16S rRNA gene), which is considered an important molecular marker of modern bacterial taxonomy. The use of other highly conserved macromolecules as evolutionary chronometers might have strong applications in the identification, differentiation, and tracing of bacterial species.

In this study, we have investigated the occurrence of the gene encoding EF-Tu in different species of the genera *Bifidobacterium* and *Lactobacillus*. The *tuf* gene product brings aminoacylated tRNA molecules to the ribosome. This gene represents an ideal target candidate for diagnostic purposes because it is highly conserved and ubiquitous in bacteria (26, 27). It has been already applied to infer phylogeny in the genera *Enterococcus* (18), *Mycoplasma* (1), and *Staphylococcus* (28). In addition, in a very recent study, a comparative analysis of partial *tuf* sequences was evaluated for the differentiation of some *Lactobacillus* species (4). It fulfills all prerequisites to server as a suitable phylogenetic marker, such as very high genetic stability and a wide distribution (25). This alternative molecular marker might corroborate and help to complete the evolutionary history of various LAB species. In this report, we demonstrated that there is a high correlation between 16S rDNA sequences and the *tuf* genes of lactobacilli and bifidobacteria. The use of *tuf* genes in LAB species as an alternative or complement to the 16S rRNA marker mainly supports the phylogenetic relationships that are revealed by the 16S rRNA-based determination of bacterial phylogeny but also provides more detail that can be used to distinguish closely related species and that can be helpful for inferring phylogeny in closely related species (e.g., *B. animalis-B. lactis*, *B. longum-B. infantis*, and *L. johnsonii-L. gasseri*).

Recently, polyphasic taxonomy (48) was recognized by the International Committee on Systematic Bacteriology as a new tool for the description of species and for the revision of the present nomenclature of some bacterial groups. In view of its demonstrated effectiveness, sequence analysis of protein-coding genes (e.g., *tuf* genes) as alternative phylogenetic markers could be added to the arsenal of rRNA sequence databases and to the relatively small *groEL* (16) and *recA* (9, 22) sequence databases. It has been shown that species having 70% or greater DNA similarity (at the DNA-DNA hybridization or reassociation level) possess in fact more than 97% 16S rDNA sequence identity (43). Consequently, 16S rDNA sequence analysis might not be an appropriate replacement for DNA reassociation to define closely related taxa. Our results and those of previous studies (4, 18, 19, 26, 27) suggested that *tuf* gene analysis also could be a valid tool for inferring relationships among closely related bacterial species. The use of the *tuf* gene, as well as the *recA* gene, as a phylogenetic marker for LAB has the advantage that the amino acid sequences from these genes can be used to infer bacterial phylogenies, avoiding the problems associated with rRNAs and the likely overestimation of the relatedness of taxa with similar nucleotide differences, nonindependence of substitution patterns at different sites, and bias derived from different G+C contents of microorganisms (8). Moreover, at the nucleotide level, EF-Tu can tolerate mutations that do not or only slightly alter it. These mutations can provide information about recent evolutionary history which is too recent to be fixed in slowly diverging sequences such as 16S rRNAs (31).

In this study, we demonstrated that the *tuf* sequence is a valid molecular marker for inferring interspecies relationships. However, the lack of *tuf* sequence variations in strains within the same species showed its inadequancy for any intraspecific relationship analysis (e.g., as a typing tool at the strain level). The analysis of variable regions within the *tuf* genes of the former *L. casei* group led us to design a set of species-specific PCR primers. We focused our attention on the establishment of a *tuf* PCR-based assay for the identification of closely related microorganims (e.g., within the *L. casei* group), which so

far cannot be differentiated in a reliable manner by traditional approaches (9).

The *tuf* genes have been described to be present in the bacterial genome in various copy numbers. Most gram-positive bacteria carry only one *tuf* gene, and only a few exceptions to this rule have been described (e.g., some *Enterococcus* [19] and *Clostridium* [40] species). Since it has been postulated that the second copy of the *tuf* gene (*tufB*) in enterococci has been generated from a horizontal gene transfer event (19), caution should be exercised in the interpretation of bacterial evolution when such events occurred. This is not the case for *Lactobacillus* and *Bifidobacterium* strains. We have determined that both the low-G+C-content gram-positive bacteria (lactobacilli) and the high-G+C-content gram-positive bacteria (bifidobacteria) investigated here contain only one copy of the *tuf* gene.

The *tuf* genes usually are associated with characteristic flanking genes (5, 24). EF-Tu has been described to be part of either the bacterial *str* operon (20) or the tRNA-*tufB* operon (3, 5). The arrangement of the *tuf* gene in the *str* operon has been described for a variety of bacteria, such as *E. coli* (15), *Bacillus* (20, 21), *Streptomyces* (54), and *Enterococcus* (19). The arrangement of the *tuf* gene in the tRNA-*tufB* operon has been described for *Chlamydia trachomatis* (5), *Thermus thermophilus* (38), and *Aquifex aeolicu*s (6), as has that of the *tufB* gene of *E. coli* (14). It has been postulated that the widespread EF-Tu gene arrangements might argue in favor of their ancient origins (5). All sequenced gram-positive bacteria with a high G+C content (e.g., bifidobacteria) contain only a single copy of the *tuf* gene arranged in a manner similar to that of the *str* operon of *E. coli* (14). This is the case for *B. longum*, *B. lactis* (M. Laloi, personal communication), and all *Bifidobacterium* species tested in this study.

However, in the available *Lactobacillus* genomes, the sequences flanking the *tuf* genes differ from those of any other *tuf* locus described so far. We have found a common genetic map of the *tuf* region in all five investigated *Lactobacillus* species, including *rpsT*, *rpsO*, metallo-beta-lactamase, *tig*, *clp*, and GTP binding protein genes. However, only the EF-Tu-, trigger factor-, Clp protease-, and GTP binding protein-encoding genes seem to constitute a highly conserved module. Functional analysis of these genes seems to corroborate the hypothesis that these genes constitute the same operon. In fact, the trigger factor is a ribosome-associated protein that interacts with the EF-Tu protein and with a wide variety of nascent polypeptides to catalyze their folding (32). Clp ATP-dependent proteases are stress-induced proteins acting to refold or degrade misfolded or denatured proteins (12). The elongation phase of protein synthesis is promoted by two proteins, EF-Tu and elongation factor G, which binds to the ribosome in its GTP form, hydrolyzes GTP to drive tRNA movement on the ribosome, and is released in its GDP form. We might speculate that the GTP binding protein following the Clp protease could play the role of elongation factor G. The *tuf* region of *Lactobacillus* species displays some features that are not found throughout the bacterial world and that could be of great interest from an evolutionary point of view.

We demonstrated that the EF-Tu-, trigger factor-, Clp protease-, and GTP binding protein-encoding genes are cotranscribed and belong to the same transcription unit. Primer extension experiments precisely mapped the start of the transcript species occurring in the *tuf* operon. Transcripts derived from the *tuf* promoter and the readthrough *tig* promoter were present and covered the entire *tuf* operon. A similar situation in which the Ef-Tu gene is cotranscribed with flanking genes has been described already for *Bacillus* (20, 21) and *E. coli* (3). The *tuf* gene of *L. johnsonii* has a multiple-promoter structure, which has been described previously for the *tuf* gene of *B. stearothermophilus* (20, 21). Transcription directed by a multiple *tuf* promoter structure in *Streptomyces ramocissimus* has been demonstrated to be growth phase dependent. Preliminary results regarding the *tuf* gene of other LAB genera (e.g., *Lactococcus* and *Streptococcus*) show a general organization common to their loci but not in common with those of the genus *Lactobacillus*. The analysis of the flanking regions suggests that in general, the genes surrounding the *tuf* gene have coevolved with EF-Tu. The still relatively small number of LAB *tuf* regions available renders attempts to understand the direction of their evolution a challenge. Analysis of a large number of LAB *tuf* regions may provide important clues to better understanding the biology and the evolutionary history of the *tuf* region and LAB phylogeny. The *tuf* locus of *Lactobacillus* should undergo complementary studies to clarify the role of the 5′-proximal region of the locus in the regulation of expression of the genes and the effects of other possible factors (e.g., growth rate phase) on modulation of the promoter activity of the *tuf* gene.

In conclusion, in this study we determined the *tuf* gene sequences of a large number of species of lactobacilli and bifidobacteria, increasing the already existent *tuf* sequence databases of LAB species. We demonstrated a higher distinctness of the *tuf* sequences than of the 16S rRNA sequences and offer a valid molecular marker for inferring phylogeny among closely related taxa (e.g., *L. casei* group). Moreover, we showed for the first time the genetic organization of the *tuf* operon of lactobacilli, which has no counterpart in any other known bacterial genomes so far.

ACKNOWLEDGMENTS

We thank the members of the *L. delbrueckii* subsp. *bulgaricus* ATCC BAA-365, *L. casei* ATCC 364, and *L. gasseri* ATCC 33323 genome sequencing projects, funded by the U.S. Department of Energy Joint Genome Institute, for providing us the sequence of the *tuf* loci. We also thank G. Unnlu and J. Broadbent for making raw genome data for *L. delbrueckii* subsp. *bulgaricus* ATCC BAA-365 and *L. casei* ATCC 334 available for *tuf* locus comparisons (Fig. 3) prior to publication. We are indebted to D. R. Pridmore and M. Laloi (both at NRC) for helpful discussions and comments. Finally, we thank A. Mercenier and F. Arigoni (both at NRC) for constructive and critical reading of the manuscript.

REFERENCES

1. **Berg, S., E. Luneberg, and M. Frosch.** 1996. Development of an amplification and hybridization assay for the specific and sensitive detection of *Mycoplasma fermentans* DNA. Mol. Cell. Probes **10:**7–14.
2. **Bourget, N. L., H. Philippe, I. Mangin, and B. Decaris.** 1996. 16S rRNA and 16S to 23S internal transcribed spacer sequence analyses reveal inter- and intraspecific *Bifidobacterium* phylogeny. Int. J. Syst. Bacteriol. **46:**102–111.
3. **Bremaud, L., C. Fremaux, S. Laalami, and Y. Cenatiempo.** 1995. Genetic and molecular analysis of the tRNA-*tuf*B operon of the myxobacterium *Stigmatella aurantica*. Nucleic Acids Res. **23:**1737–1743.
4. **Chavagnat, F., M. Haueter, J. Jimeno, M. G. Casey.** 2002. Comparison of partial *tuf* gene sequences for the identification of lactobacilli. FEMS Microbiol. Lett. **217:**177–183.
5. **Cousineau, B., C. Cerpa, J. Lefebvre, and R. Cedergren.** 1992. The sequence of the gene encoding elongation factor Tu from *Chlamydia trachomatis* compared with those of other organisms. Gene **120:**33–41.

6. **Deckert, G., P. V. Warren, T. Gaasterland, W. G. Young, A. L. Lenox, D. E. Graham, R. Overbeek, M. A. Snead, M. Keller, M. Aujav, R. Huber, R. A. Felman, J. M. Short, G. J. Olsen, and R. V. Swanson.** 1998. The complete genome of the hyperthermophilic bacterium *Aquifex aeolicus*. Nature **392:** 353–358.
7. **Djordjevic, G., B. Bojovic, N. Miladinov, and L. Topisirovic.** 1997., Cloning and molecular analysis of promoter-like sequences isolated from the chromosomal DNA of *Lactobacillus acidophilus* ATCC 4356. Can. J. Microbiol. **43:**61–69.
8. **Eisen, J. A.** 1995. The *rec*A protein as a model molecule for molecular systematic studies of bacteria: comparison of trees of *rec*As and 16S rRNAs from the same species. J. Mol. Evol. **41:**1105–1123.
9. **Felis, G. E., F. Dellaglio, L. Mizzi, and S. Torriani.** 2001. Comparative sequence analysis of *recA* gene fragment brings new evidence for a change in the taxonomy of the *Lactobacillus casei* group. Int. J. Syst. Evol. Microbiol. **51:**2113–2117.
10. **Felsenstein, J.** 1993. PHYLIP (phylogeny inference package), version 3.5c. Department of Genetics, University of Washington, Seattle.
11. **Fox, G. E., J. D. Wisotzkey, and P. Jurtshuk.** 1992. How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. Int. J. Syst. Bacteriol. **42:**166–170.
12. **Gottesman, S., S. Wickner, and M. R. Maurizi.** 1997. Protein quality control: triage by chaperones and proteases. Genes Dev. **11:**1338–1347.
13. **Hertel, C., W. Ludwig, B. Pot, K. Kersters, and K. H. Schleifer.** 1993. Differentiation of lactobacilli occurring in fermented milk products by using oligonucleotide probes and electrophoretic protein profiles. Syst. Appl. Microbiol. **16:**463–467.
14. **Hudson, H., J. Rossi, and A. Landy.** 1981. Dual function transcripts specifying tRNA and mRNA. Nature **294:**422–427.
15. **Jaskunas, R. S., L. Lindahl, M. Nomura, and R. R. Burgess.** 1975. Identification of two copies of the gene for the elongation factor EF-Tu in *E. coli*. Nature **257:**458–462.
16. **Jian, W., L. Zhu, and X. Dong.** 2001. New approach to phylogenetic analysis of the genus *Bifidobacterium* based on partial HSP60 gene sequences. Int. J. Syst. Evol. Microbiol. **51:**1633–1638.
17. **Jukes, T. H., and C. R. Cantor.** 1969. Evolution of protein molecules, p. 21–132. *In* H. N. Munro (ed.), Mammalian protein metabolism. Academic Press, Inc., New York, N.Y.
18. **Ke, D., F. J. Picard, F. Martineau, C. Menard, P. H. Roy, M. Ouellette, and M. G. Bergeron.** 1999. Development of a PCR assay for rapid detection of enterococci. J. Clin. Microbiol. **37:**3497–3503.
19. **Ke, D., M. Boissinot, A. Huletsky, F. J. Picard, J. Frenette, M. Ouellette, P. H. Roy, and M. G. Bergeron.** 2000. Evidence for horizontal gene transfer in evolution of elongation factor Tu in enterococci. J. Bacteriol. **182:**6913–6920.
20. **Krasny, L., J. R. Mesters, L. N. Tieleman, B. Kraal, V. Fucik, R. Hilgenfeld, and J. Jonak.** 1998. Structure and expression of elongation factor Tu from *Bacillus stearothermophilus*. J. Mol. Biol. **283:**371–381.
21. **Krasny, L., T. Vacik, V. Fucik, and J. Jonak.** 2000. Cloning and characterization of the *str* operon and elongation factor Tu expression in *Bacillus stearothermophilus*. J. Bacteriol. **182:**6114–6122.
22. **Kullen, M., L. J. Brady, and D. J. O'Sullivan.** 1997. Evaluation of using a short region of the *rec*A gene for rapid and sensitive speciation of dominant bifidobacteria in the human large intestine. FEMS Microbiol. Lett. **154:**377–383.
23. **Kumar, S., K. Tamura, and M. Nei.**1993. MEGA: molecular evolutionary genetics analysis, version 1.01. Pennsylvania State University, University Park, Pa.
24. **Lathe, W. C., III, and P. Bork.** 2001. Evolution of *tuf* genes: ancient duplication, differential loss and gene conversion. FEBS Lett. **502:**113–116.
25. **Ludwig, W., and K. H. Schleifer.** 1999. Phylogeny of bacteria beyond the 16S rRNA standard. ASM News **65:**752–757.
26. **Ludwig, W., J. Neumaier, N. Klugbauer, E. Brockmann, C. Roller, S. Jilg, K. Reetz, I. Schachtner, A. Ludvigsen, M. Bachleitner, U. Fischer, and K. H. Schleifer.** 1993. Phylogenetic relationships of bacteria based on comparative sequence analysis of elongation factor Tu and ATP-synthase beta subunit genes. Antonie Leeuwenhoek **64:**285–305.
27. **Ludwig, W., M. Weizenegger, D. Betzl, E. Leidel, T. Lenz, A. Ludvigsen, D. Mollenhoff, P. Wenzig, and K. H. Schleifer.** 1990. Complete nucleotide sequences of seven eubacterial genes coding for the elongation factor Tu: functional, structural and phylogenetic evaluations. Arch. Microbiol. **153:** 241–247.
28. **Martineau, F., F. J. Picard, D. Ke, S. Paradis, P. H. Roy, M. Ouellette, and M. G. Bergeron.** 2001. Development of a PCR assay for identification of staphylococci at genus and species levels. J. Clin. Microbiol. **39:**2541–2547.
29. **Nei, M., and T. Gojobori.** 1986. Simple methods for estimating the numbers of synonymous and non-synonymous nucleotide substitutions. Mol. Biol. Evol. **3:**418–426.
30. **Palys, T., E. Berger, I. Mitrica, L. K. Nakamura, and F. M. Cohan.** 2000. Protein coding genes as molecular markers for ecologically distinct populations: the case of two *Bacillus* species. Int. J. Syst. Bacteriol. **50:**1021–1028.
31. **Palys, T., L. K. Nakamura, and F. M. Cohan.** 1997. Discovery and classification of ecological diversity in the bacterial world: the role of DNA sequence data. Int. J. Syst. Bacteriol. **47:**1145–1156.
32. **Patzelt, H., S. Rudiger, D. Brehmer, G. Kramer, S. Vorderwulbecke, E. Schaffitzel, A. Waitz, T. Hesterkamp, L. Dong, J. Schneider-Mergener, B. Bukau, and E. Deuerling.** 1999. Binding specificity of *Escherichia coli* trigger factor. Proc. Natl. Acad. Sci. USA **98:**14244–14249.
33. **Pot, B., C. Hertel, W. Ludwig, P. Descheemaeker, K. Kersters, and K. H. Schleifer.** 1993. Identification and classification of *Lactobacillus acidophilus*, *L. gasseri* and *L. johnsonii* strains by SDS-PAGE and rRNA targeted oligonucleotide probe hybridization. J. Gen. Microbiol. **139:**513–517.
34. **Ramakrishnan, C., V. S. Dani, and T. Ramasarma.** 2002. A conformational analysis of Walker motif A [GXXXXGKT(S)] in nucleotide-binding and other proteins. Protein Eng. **15:**783–798.
35. **Rodtong, S., and G. W. Tannock.** 1993. Differentiation of *Lactobacillus* strains by ribotyping. Appl. Environ. Microbiol. **59:**3480–3484.
36. **Roy, D., S. Sirois.** 2001. Molecular differentiation of *Bifidobacterium* species with amplified ribosomal DNA restriction analysis and alignment of short regions of *ldh* gene. FEMS Microbiol. Lett. **191:**17–24.
37. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
38. **Satoh, M., T. Tanaka, A. Kushiro, T. Hakoshima, and K. Tomita.** 1991. Molecular cloning, nucleotide sequence and expression of the *tuf*B gene encoding elongation factor Tu from *Thermus thermophilus* HB8. FEBS Lett. **288:**98–100.
39. **Schell, M. A., M. Karmirantzou, B. Snel, D. Vilanova, G. Pessi, M. C. Zwahlen, F. Desiere, P. Bork, M. Delley, and F. Arigoni.** 2002. The genome sequence of *Bifidobacterium longum* reflects its adaptation to the human gastrointestinal tract. Proc. Natl. Acad. Sci. USA **99:**14422–14427.
40. **Sela, S., D. Yogev, S. Razin, and H. Bercovier.** 1989. Duplication of the *tuf* gene: a new insight into the phylogeny of eubacteria. J. Bacteriol. **171:**581–584.
41. **Sgorbati, B., V. Scardovi, and D. J. Leblanc.** 1982. Plasmids in the genus *Bifidobacterium*. J. Gen. Microbiol. **128:**2121–2131.
42. **Song, H., M. R. Parsons, S. Rowsell, G. Leonard, and S. E. V. Phillips.** 1999. Crystal structure of intact elongation factor EF-Tu from *Escherichia coli* in GDP conformation at 2.05A resolution. J. Mol. Biol. **285:**1245–1256.
43. **Stackebrandt, E., and M. Goebel.** 1994. Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int. J. Syst. Bacteriol. **44:**846–849.
44. **Staley, J. T., and N. R. Krieg.** 1986. Classification of procaryotic organisms: an overview, p. 1–4. *In* P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.), Bergery's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore, Md.
45. **Tannock, G. W.**1994. The acquisition of the normal microflora of the gastrointestinal tract, p. 1–16. *In* S. A. Gibson (ed.), Human health: the contribution of microorganisms. Springer, London, United Kingdom.
46. **Tannock, G. W., A. Tilsala-Timisjarvi, S. Rodtong, J. Ng, K. Munro, and T. Alatossava.** 1999. Identification of *Lactobacillus* isolates from the gastrointestinal tract, silage, and yogurt by 16S-23S rRNA gene intergenic spacer region sequence comparison. Appl. Environ. Microbiol. **65:**4264–4276.
47. **Torriani, S., G. E. Felix, and F. Dellaglio.** 2001. Differentiation of *Lactobacillus plantarum*, *L. pentosus*, and *L. paraplantarum* by *recA* gene sequence analysis and multiplex PCR assay with *recA* gene-derived primers. Appl. Environ. Microbiol. **67:**3450–3454.
48. **Vandamme, P., B. Pot, M. Gillis, P. de Vos, K. Kersters, and J. Swings.** 1996. Polyphasic taxonomy, a consensus approach to bacterial systematics. Microbiol. Rev. **60:**407–438.
49. **Ventura, M., and R. Zink.** 2002. Rapid identification, differentiation, and proposed new taxonomic classification of *Bifidobacterium lactis*. Appl. Environ. Microbiol. **68:**6429–6434.
50. **Ventura, M., and R. Zink.** 2002. Specific identification and molecular typing analysis of *Lactobacillus johnsonii* by using PCR-based methods and pulsed-field gel electrophoresis. FEMS Microbiol. Lett. **217:**141–154.
51. **Ventura, M., I. A. Casas, and L. Morelli.** 2000. Rapid amplified ribosomal DNA restriction analysis (ARDRA) identification of *Lactobacillus* spp. isolated from fecal and vaginal samples. Syst. Appl. Microbiol. **23:**504–509.
52. **Ventura, M., M. Elli, R. Reniero, and R. Zink.** 2001. Molecular microbial analysis of *Bifidobacterium* isolates from different environments by the species-specific amplified ribosomal DNA restriction analysis (ARDRA). FEMS Microbiol. Ecol. **36:**113–121.
53. **Ventura, M., R. Reniero, and R. Zink.** 2001. Specific identification and targeted characterization of *Bifidobacterium lactis* from different environmental isolates by a combined multiplex PCR approach. Appl. Environ. Microbiol. **67:**2760–2765.
54. **Vijgenboom, E., L. P. Woudt, P. W. H. Heinstra, K. Rietveld, J. van Haarlem, G. P. van Wezel, S. Schochat, and L. Bosch.** 1994. Three *tuf* like genes in the kirromycin producer *Streptomyces ramocissimus*. Microbiology **140:**983–998.
55. **Walker, D. C., H. S. Girgis, and T. R. Klaenhammer.** 1999. The *groESL* chaperone operon of *Lactobacillus johnsonii*. Appl. Environ. Microbiol. **65:** 3033–3041.