

Bifidobacterium lactis DSM 10140: Identification of the *atp* (*atpBEFHAGDC*) Operon and Analysis of Its Genetic Structure, Characteristics, and Phylogeny

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The *atp* operon is highly conserved among eubacteria, and it has been considered a molecular marker as an alternative to the 16S rRNA gene. PCR primers were designed from the consensus sequences of the *atpD* gene to amplify partial *atpD* sequences from 12 *Bifidobacterium* species and nine *Lactobacillus* species. All PCR products were sequenced and aligned with other *atpD* sequences retrieved from public databases. Genes encoding the subunits of the F₁F₀-ATPase of *Bifidobacterium lactis* DSM 10140 (*atpBEFHAGDC*) were cloned and sequenced. The deduced amino acid sequences of these subunits showed significant homology with the sequences of other organisms. We identified specific sequence signatures for the genus *Bifidobacterium* and for the closely related taxa *Bifidobacterium lactis* and *Bifidobacterium animalis* and *Lactobacillus gasseri* and *Lactobacillus johnsonii*, which could provide an alternative to current methods for identification of lactic acid bacterial species. Northern blot analysis showed that there was a transcript at approximately 7.3 kb, which corresponded to the size of the *atp* operon, and a transcript at 4.5 kb, which corresponded to the *atpC*, *atpD*, *atpG*, and *atpA* genes. The transcription initiation sites of these two mRNAs were mapped by primer extension, and the results revealed no consensus promoter sequences. Phylogenetic analysis of the *atpD* genes demonstrated that the *Lactobacillus atpD* gene clustered with the genera *Listeria*, *Lactococcus*, *Streptococcus*, and *Enterococcus* and that the higher G+C content and highly biased codon usage with respect to the genome average support the hypothesis that there was probably horizontal gene transfer. The acid inducibility of the *atp* operon of *B. lactis* DSM 10140 was verified by slot blot hybridization by using RNA isolated from acid-treated cultures of *B. lactis* DSM 10140. The rapid increase in the level of *atp* operon transcripts upon exposure to low pH suggested that the ATPase complex of *B. lactis* DSM 10140 was regulated at the level of transcription and not at the enzyme assembly step.

Bifidobacteria and lactobacilli are gram-positive bacteria, and these groups include large numbers of different taxa that colonize various environments. Bifidobacteria and lactobacilli are important residents of the gastrointestinal microflora and have been the subjects of intense and growing interest due to their possible role in the maintenance of gastrointestinal health (4). The ability of these microorganisms to grow in this environment is also linked to their ability to resist its harsh conditions; the gastric pH is less than 2.0 in healthy humans (29). Changes in pH in the environment have been reported to influence the expression of many genes (34), most of which are involved in maintaining the pH at values around 7.0 (21). It has been demonstrated that in *Lactobacillus acidophilus* NCK 56 the F₁F₀-ATPase is an important element in the response and tolerance to low pH (21). A similar situation has been found in *Enterococcus faecalis* (17), in *Salmonella enterica* serovar Typhimurium (9, 10), and in *Clostridium* (7). The F₁F₀-ATPase is encoded by the *atp* operon, which in many bacteria consists of eight genes (*atpB*, *atpE*, *atpF*, *atpH*, *atpA*, *atpG*, *atpD*, and

atpC, encoding the α , β , δ , γ , ϵ , δ , α , γ , β , and ϵ subunits, respectively). The *atp* operon of *Escherichia coli* includes an additional gene, designated *atpI*, whose function is unknown and which precedes the other eight *atp* genes (14, 15). The F₁F₀-ATPase consists of two subcomplexes, a membrane-extrinsic F₁ part and a membrane-intrinsic F₀ part. In organisms with a respiratory chain, the primary role of this enzyme is to couple the electrochemical potential difference for H⁺ across the inner membrane to synthesis of ATP from ADP and phosphate. Conversely, in bacteria that lack a respiratory chain, its role is to create a proton gradient, and this process is then driven by ATP hydrolysis. This is the case for streptococci (3, 20), *E. faecalis* (17), *L. acidophilus* (21), and *Lactococcus lactis* (18, 19). In all these bacteria the activity of the F₁F₀-ATPase increases as the pH of the growth media decreases. However, the regulation of this pH-inducible phenotype has not been clearly established at the molecular level. In fact, while in *L. acidophilus* transcription of the *atp* operon is pH regulated, this is not the case for *Streptococcus faecalis* and other streptococci. In addition, in *S. faecalis*, the control seems to act at the level of ATPase enzyme assembly (2). The *atp* genes are included in the category of housekeeping genes; in fact, their presence in the bacterial genome is considered essential for the survival of these microorganisms. Especially due to its ubiquitous distri-

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TABLE 1. Strains, origins, and *atpD* and 16S rDNA sequence accession numbers

Species	Strain ^a	Origin	<i>atpD</i> accession no. ^b	16S rDNA accession no. ^b
<i>Bifidobacterium lactis</i>	DSM 10140 ^T	Yoghurt	AY487153	X89513
<i>Bifidobacterium animalis</i>	ATCC 25527 ^T	Rat feces	AY487152	X70971
<i>Bifidobacterium coryneforme</i>	ATCC 25911 ^T	Honeybee hindgut	AY487148	M58733
<i>Bifidobacterium breve</i>	ATCC 15700 ^T	Intestine of infant	AY487154	AB006658
<i>Bifidobacterium adolescentis</i>	ATCC 15703 ^T	Intestine of adult	AY487144	M58729
<i>Bifidobacterium choerinum</i>	ATCC 27686 ^T	Swine feces	AY487147	D86186
<i>Bifidobacterium longum</i>	NCC 2705	Intestine of adult	NC_004307	NC_004307
<i>Bifidobacterium infantis</i>	ATCC 15697 ^T	Intestine of infant	AY487150	D86184
<i>Bifidobacterium suis</i>	ATCC 27533 ^T	Swine feces	AY487151	M58743
<i>Bifidobacterium bifidum</i>	ATCC 29521 ^T	Infant feces	AY487145	M84777
<i>Bifidobacterium dentium</i>	ATCC 27534 ^T	Dental caries	AY487149	D86183
<i>Bifidobacterium catenulatum</i>	ATCC 27539 ^T	Intestine of adult	AY487146	M58732
<i>Lactobacillus gallinarum</i>	ATCC 33199 ^T	Chicken crop	AY487160	AJ242968
<i>Lactobacillus crispatus</i>	NCTC 4	Human vagina	AY487158	AJ242969
<i>Lactobacillus amylolyticus</i>	DSM 11614 ^T	Beer wort	AY487155	Y17361
<i>Lactobacillus gasserii</i>	DSM 20243 ^T	Human	AY487156	M58820
<i>Lactobacillus johnsonii</i>	ATCC 33200 ^T	Human blood	AY487162	AJ002515
<i>Lactobacillus acidophilus</i>	ATCC 4356 ^T	Human	AY487161	M58802
<i>Lactobacillus amylovorus</i>	DSM 20531 ^T	Cattle waste corn fermentation	AY487157	M58805
<i>Lactobacillus rhamnosus</i>	DSM 20021	Unknown	AY487159	M58815
<i>Lactobacillus hilgardii</i>	DSM 20051	Wine	AJ508914	M58821
<i>Lactobacillus helveticus</i>	NCDO 2712 ^T	Cheese	AY487163	X61141
<i>Lactobacillus brevis</i>	DSM 2647	Human feces	AJ508913	D37785
<i>Lactobacillus plantarum</i>	WCFS1	Human oral cavity	NC_004567	NC_004567
<i>Lactococcus lactis</i> subsp. <i>lactis</i>	IL-1403	Unknown	NC_002662	NC_002662
<i>Oenococcus oeni</i>	IOB84.13	Wine	AJ491851	NA
<i>Oenococcus oeni</i>	JCM 6125	Wine	NA	AB022924
<i>Leuconostoc mesenteroides</i>	NCDO 523 (= DSM 20343)	Fermenting olives	AJ508912	X95978
<i>Streptococcus thermophilus</i>	ATCC 19258	Cheese	NA	AY188354
<i>Streptococcus thermophilus</i>	CHCC2136	Unknown	AY090612	NA
<i>Streptococcus pyogenes</i>	M1 GAS	Nasal cavity	NC_002737	NC_002737
<i>Listeria monocytogenes</i>	EGD-e	Food	NC_003210	NC_003210
<i>Pediococcus parvulus</i>	JCM 5889 (= DSM 20332)	Silage	AJ515143	D88528
<i>Pediococcus damnosus</i>	DSM 20331	Beer	AJ515142	AJ318414
<i>Mycobacterium leprae</i>	TN	Human	NC_002677	NC_002677
<i>Escherichia coli</i>	K-12		NC_000913	NC_000913
<i>Streptomyces coelicolor</i>	A3		NC_003888	NC_003888
<i>Bacterioides thetaiotaomicron</i>	VP1 5482	Human feces	NC_004663	NC_004663
<i>Enterococcus faecalis</i>	V583		NC_004668	NC_004668
<i>Clostridium perfringens</i>	Str. 13	Human	NC_003366	NC_003366

^a T = type strain. ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen; NCC, Nestlé Culture Collection; JCM, Japanese Collection of Microorganisms; NCDO, National Collection of Dairy Organisms; NCTC, National Collection of Type Cultures; VPI, Virginia Polytechnic Institute.

^b For the strains whose genome sequences are available the *atpD* gene and 16S rDNA sequences were retrieved from the complete bacterial genome, and the accession numbers are indicated. NA, not available.

bution, functional constancy, and conservation, the gene encoding the β -subunit of the *atp* operon (*atpD*) is considered to be a suitable molecular marker for bacterial phylogenetic investigations (22, 23).

So far, the evolutionary relationships among lactic acid bacteria (LAB) have been determined by comparing the sequences of rRNA genes, mainly 16S rRNA genes, because of their ubiquity and their resistance to evolutionary changes. 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. The rates of evolutionary substitution in protein-encoding genes are an order of magnitude higher than those in 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 at the 16S rRNA level yet (11). Therefore, the importance of comparing the sequences of several genes to evaluate a comprehensive bacterial phylogeny has been stressed (35). Conse-

quently, in the present study we investigated the value of another gene, *atpD*, which is recognized as a valid molecular marker (22, 23), in order to supplement the classification scheme for some bifidobacteria and LAB species. To date, little is known about the *atp* genes of bifidobacterial species. In this report we describe the *atp* locus whose genes code for various subunits of the F_1F_0 -ATPase in *Bifidobacterium lactis* DSM 10140. We tested the pH inducibility of this operon by Northern blot hybridization and primer extension experiments. Moreover, we partially sequenced of the gene coding for the β subunit of the *atp* operon from 12 bifidobacterial species and nine *Lactobacillus* species in order to provide a new molecular marker for phylogenetic analysis of bifidobacteria and LAB species.

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 (Difco, Detroit, Mich.). In order to preserve the anaerobic conditions all media were supplemented with 0.05% L-cysteine-HCl and incubated at 37°C for 16 h. *Lactobacillus* strains were grown aerobically in MRS (Difco) and incubated at 37°C for 16 h.

DNA isolation. Genomic DNA was extracted by using the protocol described in a previous study (50).

DNA amplification and cloning of the *atpD* gene. PCR was used to amplify the *atpD* gene in all *Bifidobacterium* strains investigated. DNA fragments that were approximately 1,100 bp long and corresponded to the *atpD* gene were amplified by using oligonucleotides atBIF-1 (5'-CACCTCGAGGTCGAAC-3') and atBIF-2 (5'-CTGCATCTTGTGCCACTTC-3'), while the *atpD* fragment of *Lactobacillus* strains was amplified by employing oligonucleotides AT-1 (5'-CTNGAAGTTCNCTNGAAC-3') and AT-2 (5'-ACGGAANGCATCTTCTGG-3').

Each PCR mixture (50 µl) contained 20 mM Tris-HCl, 50 mM KCl, each deoxynucleoside triphosphate at a concentration of 200 µM, 50 pmol of each primer, 1.5 mM MgCl₂, 1 U of Taq DNA polymerase (Gibco BRL, Paisley, United Kingdom), and 50 ng of DNA template. Each PCR cycling profile consisted of an initial denaturation step of 3 min at 95°C, followed by amplification for 30 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 50°C, and extension for 2 min at 72°C. The PCR was completed with an elongation phase consisting of 10 min at 72°C. The resulting amplicons were separated on a 1% agarose gel, and this was followed by ethidium bromide staining. PCR fragments were purified by using a PCR purification kit (Qiagen, West Sussex, United Kingdom) and were subsequently cloned in the pGEM-T Easy plasmid vector (Promega, Southampton, United Kingdom) by following the supplier's instructions.

DNA sequencing and phylogeny study. DNA was extracted from the clones with a Quiaprep kit by following the instructions of the supplier (Promega). Nucleotide sequencing of both strands of cloned DNA was performed with a fluorescence-labeled primer cycle sequencing kit (Amersham Buchler, Braunschweig, Germany) by following the supplier's instructions. The primers used were atBIF-1, atBIF-2, AT-1, and AT-2, labeled with IRD800 (MWG Biotech, Ebersberg, Germany). The *atpD* genes of all *Bifidobacterium* and *Lactobacillus* strains examined in this study, as well as those available in the GenBank database, were used for comparison. Sequence alignments were performed by using the MultiAlign program and Clustal W (45). Phylogenetic trees were constructed by using the neighbor-joining program from the PHYLIP software package (version 3.5c) (8). Dendrograms from gene sequences were also drawn by using the Clustal X program (National Center for Biotechnology Information) and were visualized with the TreeView program.

Reference sequences used. The genome accession numbers for the sequences of the *atp* operons used in this study were as follows: *Bifidobacterium longum* NCC 2705, NC_004307; *Lactobacillus plantarum* WCFS1, AL_004567; *L. acidophilus* NCK56, AF098522; *L. lactis* subsp. *lactis* IL-1403, NC_002662; and *E. coli* K-12, NC_000913.

RNA isolation and Northern blot analysis. *B. lactis* DSM 10140 was grown to an optical density at 560 nm of 0.7, and then the pH of culture was adjusted to 6.0, 5.5, 4.0, or 3.5 with concentrated HCl. RNA was isolated from *B. lactis* at 100 min after exposure to pH 6.0, 5.5 and 4.0 or at 0, 10, 20, and 100 min after exposure to pH 3.5. Total RNA was isolated by resuspending bacterial cell pellets in TRIzol (GibcoBRL), adding 106-µm-diameter glass beads (Sigma, St. Louis, Mo.), and shearing the slurry with a Mini-Beadbeater-8 cell disruptor (Biospec Products, Bartlesville, Okla.) as described by Ventura et al. (49). The initial Northern blot analysis of *Bifidobacterium atpD* gene activity was carried out with 15-µg aliquots of RNA isolated from 10-ml portions of *Bifidobacterium* strains collected after 8 or 18 h of growth under the conditions described above. The RNA was separated in a 1.5% agarose-formaldehyde denaturing gel, transferred to a Zeta-Probe blotting membrane (Bio-Rad, Hertfordshire, United Kingdom) as described by Sambrook and Russell (39), and fixed by UV cross-linking by using a Stratallinker 1800 (Stratagene, La Jolla, Calif.). PCR amplicons obtained with primers atBIF-1 and atBIF-2 were radiolabeled (39). Prehybridization and hybridization were carried out at 65°C in a mixture containing 0.5 M NaHPO₄ (pH 7.2), 1.0 mM EDTA and 7.0% sodium dodecyl sulfate (SDS). Following 18 h of hybridization, the membrane was rinsed twice for 30 min at 65°C in 0.1 M NaHPO₄ (pH 7.2)–1.0 mM EDTA–1% SDS and twice for 30 min at 65°C in 0.1 M NaHPO₄ (pH 7.2)–1.0 mM EDTA–0.1% SDS and exposed to X-OMAT autoradiography film (Eastman Kodak, Rochester, N.Y.). Autoradiographs were analyzed with ImaGene 5.1 (BioDiscovery).

Primer extension analysis. The 5' ends of the RNA transcripts were determined as follows. Separate primer extension reactions were conducted with 15-µg aliquots of RNA isolated as described above and mixed with 1 pmol of primer labeled with IRD800 (MWG Biotech) and 2 µl of buffer H [2 M NaCl, 50 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES); pH 6.4]. The mixture

was denatured at 90°C for 5 min and then hybridized for 60 min at 42°C. After 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 MgCl₂, 20 µl of a mixture containing each deoxynucleoside triphosphate at a concentration of 2.5 mM, 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 adding 250 µl of an ethanol-acetone mixture (1:1), and the reaction mixture was incubated at –70°C for 15 min and then centrifuged at 10,000 × g for 15 min. The pellets were dissolved in 4 µl of distilled water and mixed with 2.4 µl of loading buffer from a sequencing kit (ThermoSequenase, fluorescence labeled; Amersham, Piscataway, N.J.). The cDNA was separated on an 8% polyacrylamide-urea gel along with mixtures from sequencing reactions which were conducted with the same primers that were used for the primer extension reactions and detected with a LiCor sequencer machine (MWG Biotech). The synthetic oligonucleotides used were met-prom (5'-GTCTCGTCTCGATCTTCTTC-3'), atp-B-prom (5'-CACGAAGATGATGCGGTTGATTG-3'), and atp-A-prom (5'-CAAAGTCCAGCAGTT CGTTG-3').

Factorial correspondence analysis. Factorial correspondence analysis was performed with the assistance of the GCUA software (version 1.0) (28).

Nucleotide sequence accession numbers. The GenBank accession numbers for partial *atpD* gene sequences generated in this study are as follows: *Lactobacillus gallinarum* ATCC 33199, AY487160; *Lactobacillus crispatus* NCTC 4, AY487158; *Lactobacillus amylolyticus* DSM 11614, AY487155; *Lactobacillus gasseri* DSM 20243, AY487156; *Lactobacillus johnsonii* ATCC 33200, AY487162; *Lactobacillus amylovorus* DSM 20531, AY487157; *Lactobacillus rhamnosus* DSM 20021, AY487159; *Lactobacillus helveticus* NCDO 2712, AY487163; *L. acidophilus* ATCC 4356, AY487161; *Bifidobacterium bifidum* ATCC 29521, AY487145; *Bifidobacterium infantis* ATCC 15697, AY487150; *Bifidobacterium catenulatum* ATCC 27539, AY487146; *Bifidobacterium adolescentis* ATCC 15703, AY487144; *Bifidobacterium breve* ATCC 15700, AY487154; *Bifidobacterium animalis* ATCC 25527, AY487152; *B. lactis* DSM 10140, AY487153; *Bifidobacterium suis* ATCC 27533, AY487151; *Bifidobacterium choerinum* ATCC 27686, AY487147; *Bifidobacterium corneiforme* ATCC 25911, AY487148; and *Bifidobacterium dentium* ATCC 27534, AY487149. The nucleotide sequence data for the *atp* operons reported in this paper have been deposited in the GenBank database under accession numbers AY488175 (*B. lactis* DSM 10140) and AY488174 (*B. breve* NCIMB 8807).

RESULTS

Analysis of the protein sequences from a random clone library of *B. lactis* DSM 10140 revealed the presence of a clone with sequences which exhibited significant amino acid homology with the gene products of *atpD* of *B. longum* NCC 2705 (41) and *L. acidophilus* NCK 56 (21). By using a PCR-based chromosome-walking strategy we cloned the regions surrounding the *atpD* gene. The complete nucleotide sequence of the *atp* operon was determined, and the proposed organization of the operon is shown in Fig. 1. The *atp* operon was composed of eight open reading frames (ORFs) corresponding to a 7.3-kb DNA segment. The gene order of the *atp* operon was *atpBEFH AGDC*, which was identical to that observed previously in other bacteria (21, 40). The deduced amino acid sequences encoded by the *B. lactis* DSM 10140 *atp* operon were aligned with those of *B. longum* NCC 2705, *B. breve* NCIMB 8807, *L. acidophilus* NCK 56, *L. plantarum* WCFS1, *L. lactis* subsp. *lactis* IL-1403, and *E. coli* K-12 (Fig. 1). Protein comparison (Fig. 1) showed that the proteins most similar to the *B. lactis* ATPase subunits were those from *B. breve* and *B. longum*. Levels of identity of >21% were observed for the eight subunits and the subunits of lactobacilli, while the comparison of the *atp* operon genes of *B. lactis* with the genes of the phylogenetically distant taxa (*L. lactis* subsp. *lactis* and *E. coli*) revealed consistent identities for only a few *atp* genes. In general, greater homology was observed for the *atpA* and *atpD* gene products corresponding to the cytoplasmic domain. The con-

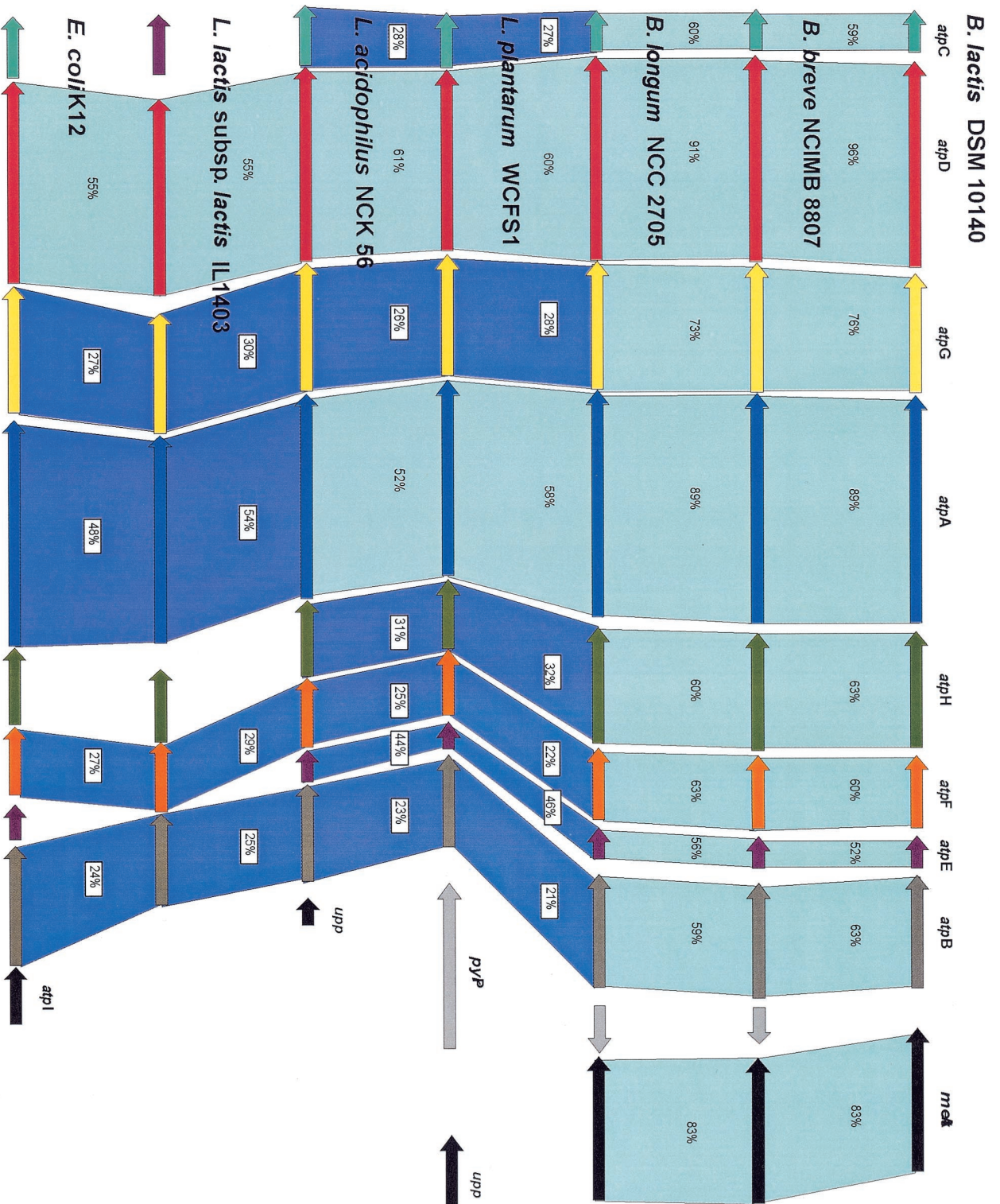


FIG. 1. Comparison of the *atp* operon of *B. lactis* DSM 10140 with the corresponding operons of different bacteria. The putative function of the protein is indicated above each arrow. Related proteins are linked by light blue shading ($\geq 52\%$) and dark blue shading ($\leq 51\%$) according to the different levels of amino acid similarity; the levels of amino acid identity (expressed as percentages) are indicated. The length of each arrow is proportional to the length of the predicted ORF. Corresponding genes are indicated by arrows that are the same color.

sensus nucleotide-binding domains, Walker motifs A (GXXX XGKT) and B (L-hydrophobic-hydrophobic-hydrophobic-D) (1, 51), were also conserved in the deduced sequences of the α and β subunits. Significantly lower levels of homology were observed for the *atpB*, *atpE*, and *atpF* gene products comprising the membrane-binding domain, F_0 . The *atpG* and *atpF* genes appeared to use UUG and GTG, respectively, as the initiation codons instead of the more frequently used AUG start codon; it was reported previously that *L. lactis* and *L. acidophilus* each use this alternative start codon in one of the genes of the *atp* operon (19, 21).

Notably, the first gene of the *atp* operon of *B. lactis*, *B. breve*, and *B. longum* was preceded by an ORF which exhibited homology to bacterial *metA* coding for homoserine *O*-succinyltransferase, while in *L. acidophilus* the initial *atpB* gene of the *atp* operon is preceded by a homologue of the *upp* gene, which codes for uracil phosphoribosyltransferase (21). Interestingly, in the *L. plantarum* genome a gene encoding for the pyrophosphate hydrolase is inserted between the *atpB* and *upp* genes. The *atp* operons of bifidobacteria and lactobacilli (21) do not contain the *atpI* gene, which is not present in any of the bacteria investigated so far (42, 43).

The gene order of all of the *Lactobacillus* regions investigated was found to be identical to that of the *B. longum* NCC 2705 region. The *atpC* gene of *B. lactis* DSM 10140 is preceded by two ORFs, ORF1 and ORF2, while the *metA* gene is followed by two other ORFs, ORF3 and ORF4 (Fig. 2). BLAST analysis of ORF1 and ORF2 gave positive matches with a conserved hypothetical protein of *B. longum* (accession number AE014654_4; 72% identity) and with a putative drug-binding lipoprotein of *B. longum* (accession number AE014654_3; 53% identity), respectively. ORF3 showed 51% identity with a multidrug transporter of *L. lactis* subsp. *lactis* (accession number AL646AE006394_2), whereas ORF4 exhibited 66% identity with an inosine-uridine nucleoside *N*-ribohydrolase of *Corynebacterium glutamicum* (accession number BAB98757.1).

Transcriptional analysis of the *atp* operon. In order to verify that the genes belonging to the *atp* operon are expressed, we probed total RNA extracted from *B. lactis* DSM 10140 cells with DNA probes targeting different genes of the *atp* operon. It appeared that initially long transcripts were synthesized, which were subsequently degraded into smaller products, as indicated by the presence of smears in the blots (Fig. 2). In the background of the blots hybridized to the *atpC*, *atpD*, *atpG*, and *atpA* probes (corresponding to the genes encoding the F_1 subunit of the ATP synthase) we found one pronounced band of a smaller transcript corresponding to a 4.5-kb mRNA (Fig. 2). The maximum size of the transcripts was approximately 7.3 kb, suggesting that all eight genes are transcribed into one single polycistronic mRNA (Fig. 2). The presence of a background in Northern blots of *atp* mRNA has also been reported for *E. coli* and is probably due to specific endonucleolytic cleavage of the transcripts of the corresponding genes (26, 27). When the Northern blots were probed with the *metA* and *recB* genes, no transcripts or 700-bp mRNA species were detected, suggesting that the *metA* and the *recB* genes are not part of the *atp* operon (Fig. 2). An inverted repeat was observed in the region immediately downstream of the *atpC* gene, which may serve as the terminator sequence. A similar palindromic sequence was noticed in the intergenic region downstream of the

metA gene. Other secondary structures that might act as weak terminator sequences were also found within the operon (Fig. 2).

Primer extension analysis showed that the *metA* gene does not possess a promoter region and confirmed that this gene is not included in the *atp* operon. Primer extension analysis was attempted for the *atp* operon in order to elucidate the transcriptional start site of the two transcripts identified. The transcriptional start sites were identified upstream of the assumed start codons of the *atpB* and *atpA* genes (Fig. 3a). Once the start sites were identified, sequence analysis of the regions immediately upstream suggested that there was a putative Pribnow box, while no canonical -35 region sequences were observed (Fig. 3a). Primer extension experiments were conducted with RNA extracted from cultures in which the medium pH was shifted from pH 6 to 3.5. While the abundance of transcripts appeared to increase in response to pH 3.5, the transcription start site did not change (data not shown).

Induction of ATPase activity under acidic growth conditions. Previous data have shown that in other LAB the ATPase is up-regulated under acidic conditions (21). Maximal induction of activity was seen at pH 3.5. To examine the issue more directly, RNA was isolated from acid-treated cultures of *B. lactis* and used as a target in an RNA slot blot analysis in which the *atpD*, *atpH*, and *atpB* genes were used as probes (Fig. 3b and data not shown). The level of the mRNAs increased upon exposure to pH 3.5 and was almost 15-fold greater after 100 min.

Sequencing and phylogenetic analysis of the *atpD* gene. The *atpD* sequences from a number of selected bacterial species were aligned and compared. Two highly conserved regions were identified, and a pair of primers (AT-1 and AT-2) amplifying a 1,114- to 1,120-bp region was designed. These primers allowed amplification of *atpD* sequences from a wide variety of bacteria, including 12 *Bifidobacterium* species and nine *Lactobacillus* species.

We performed a phylogenetic analysis based on the *atpD* sequences of bifidobacteria and lactobacilli using the neighbor-joining method (Fig. 4). For completeness we included in our analysis the *atpD* DNA sequences of other strains belonging to different genera representing the LAB group (e.g., *Lactococcus*, *Streptococcus*, *Pediococcus*, *Oenococcus*, *Leuconostoc*, and *Enterococcus*) and other gram-positive and gram-negative bacteria with high and low G+C contents, including some *Actinomycetales* (e.g., *Corynebacterium*, *Streptomyces*, and *Mycobacterium*). A phylogenetic tree was also generated by using the 16S ribosomal DNA (rDNA) sequences from the same set of strains that was used for the *atpD* gene-based tree (except for *Streptococcus thermophilus*, *Oenococcus oeni*, and *L. acidophilus*) (Fig. 4a). The reliability of the trees generated was supported by bootstrap analysis. Both trees showed that there was a clear division between the low-G+C-content bacteria (*Lactobacillus*, *Lactococcus*, *Streptococcus*, *Pediococcus*, *Oenococcus*, *Leuconostoc*, and *Enterococcus*) and the high-G+C-content bacteria (*Bifidobacterium*, *Mycobacterium*, and *Streptomyces*). Interestingly, all bifidobacterial strains clustered together at the right end of both trees (Fig. 4), whereas an apparent shuffling of lactobacilli in the *atpD* gene-based tree was observed (Fig. 4b). In fact, *Lactococcus*, *Streptococcus*, *Enterococcus*, and *Listeria* clustered within the genus *Lactoba-*

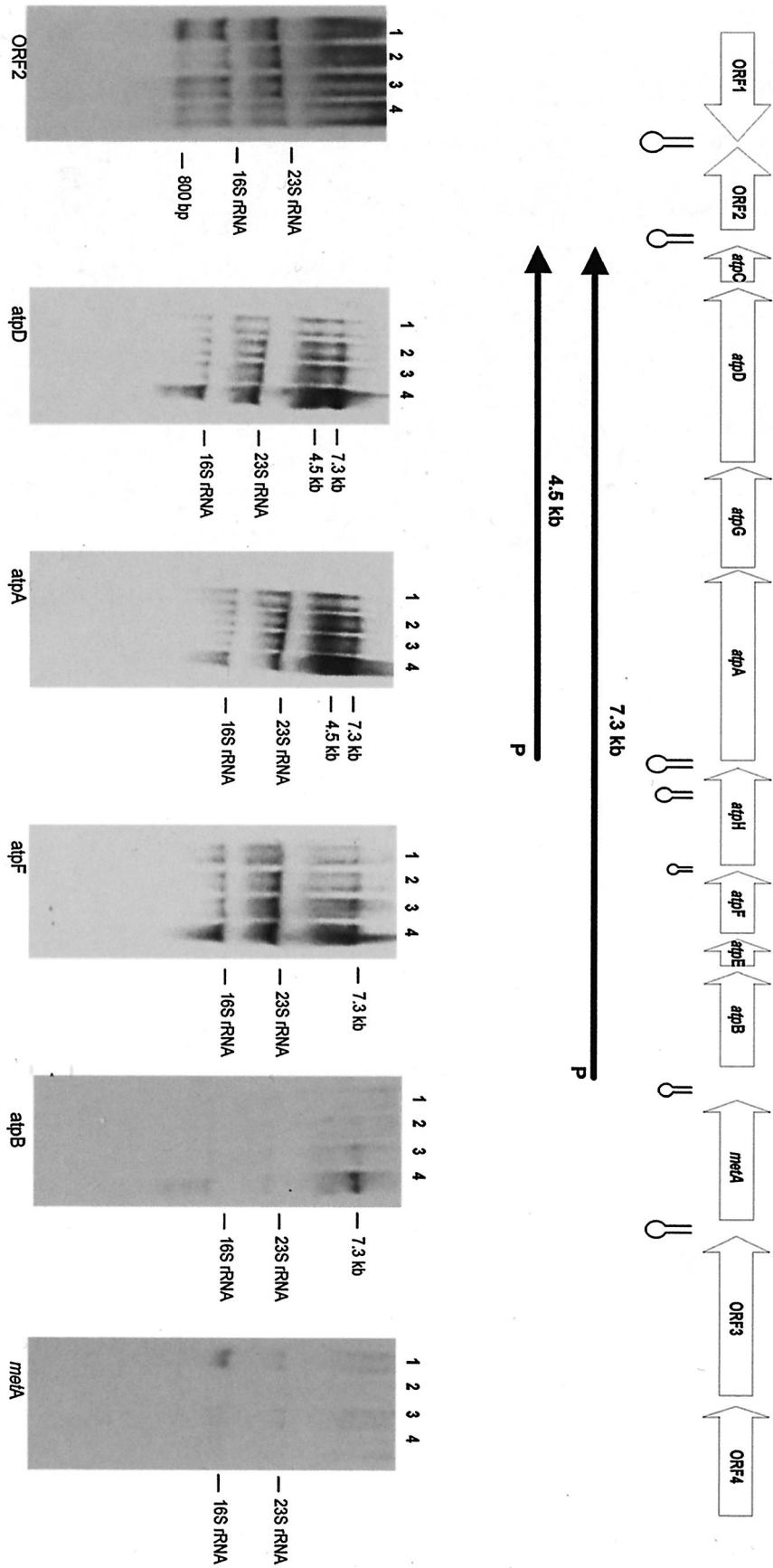


FIG. 2. Northern hybridization analysis of *B. lactis* RNA and transcription unit mapping of the *atp* operon. All predicted ORFs are indicated and are annotated with their database matches. The transcripts are indicated by arrows, and the arrows point toward the 3' end of the mRNA. The estimated size of the mRNA is indicated. Hairpins indicate possible rho-independent terminators. The transcripts are positioned with respect to the genome map shown at the top. The gels show the results of Northern blot hybridization of RNA isolated from *B. lactis* DSM 10140 upon exposure to low pH at 100 min. Lane 1, RNA isolated from a culture after exposure to pH 6.0; lane 2, RNA isolated from a culture after exposure to pH 3.5; lane 3, RNA isolated from a culture after exposure to pH 4.0; lane 4, RNA isolated from a culture after exposure to pH 3.5. Hybridization was performed by using the probes corresponding to the ORFs shown in the gene map at the top. The molecular size calculated for the hybridization signal is indicated next to each autoradiograph.

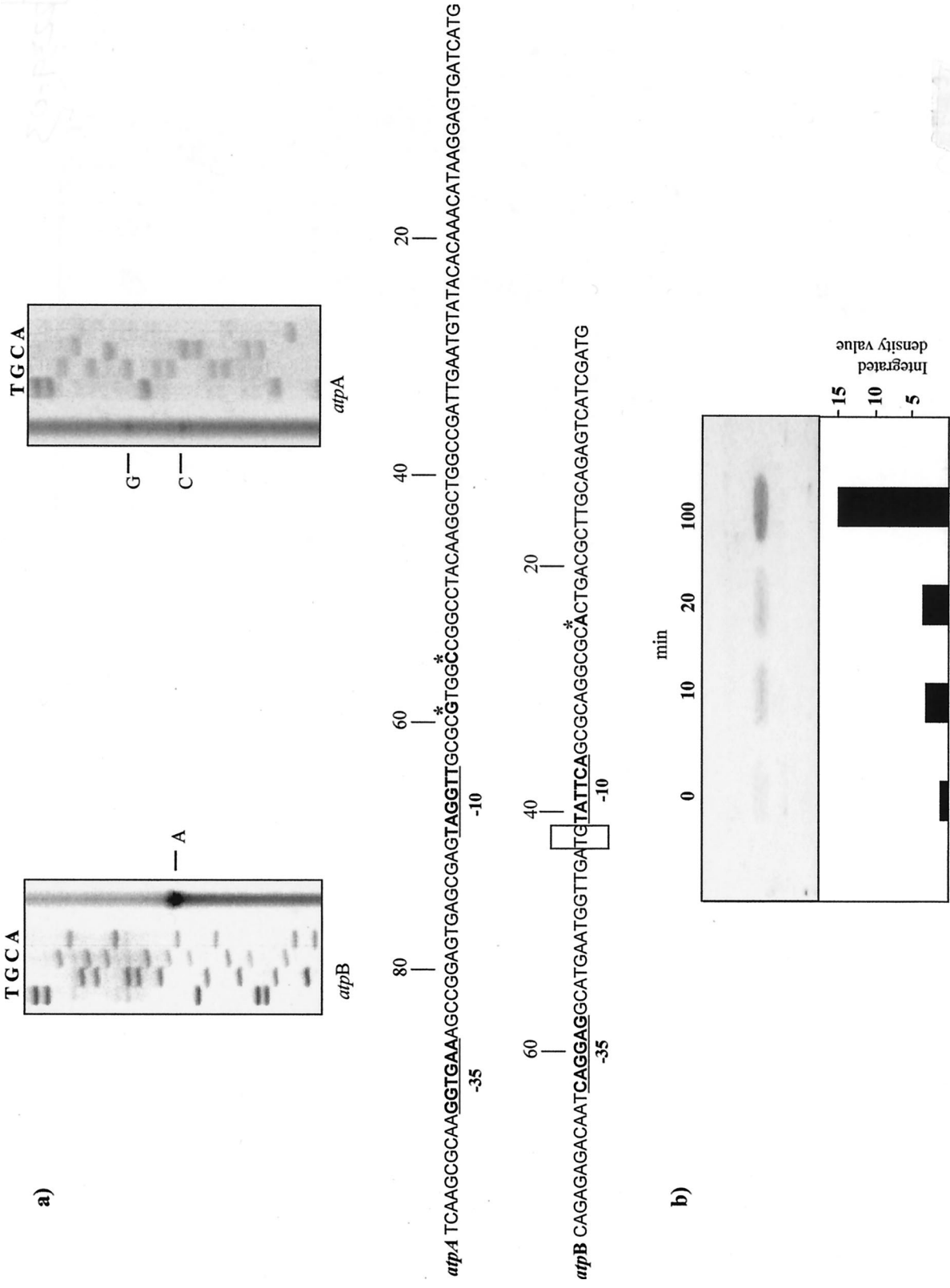


FIG. 3. (a) Primer extension analysis and comparison of the putative promoter sequences for the *atp* operon. Boldface type and underlining indicate -10 and -35 putative hexamers; the box indicates a TG doublet of an extended -10 sequence; and the asterisks indicate transcription start points. The start codon is at the right end of the sequence. (b) Slot blot hybridization of mRNA from cells incubated at pH 3.5 for various times. Each slot contained 20 μ g of RNA, and all slots were probed with 32 P-labeled PCR products corresponding to the *atpD* gene. The graph shows the results of two different experiments. The numbers above the slot blot indicate the times of exposure of the *B. lactis* DSM 10140 culture at pH 3.5.

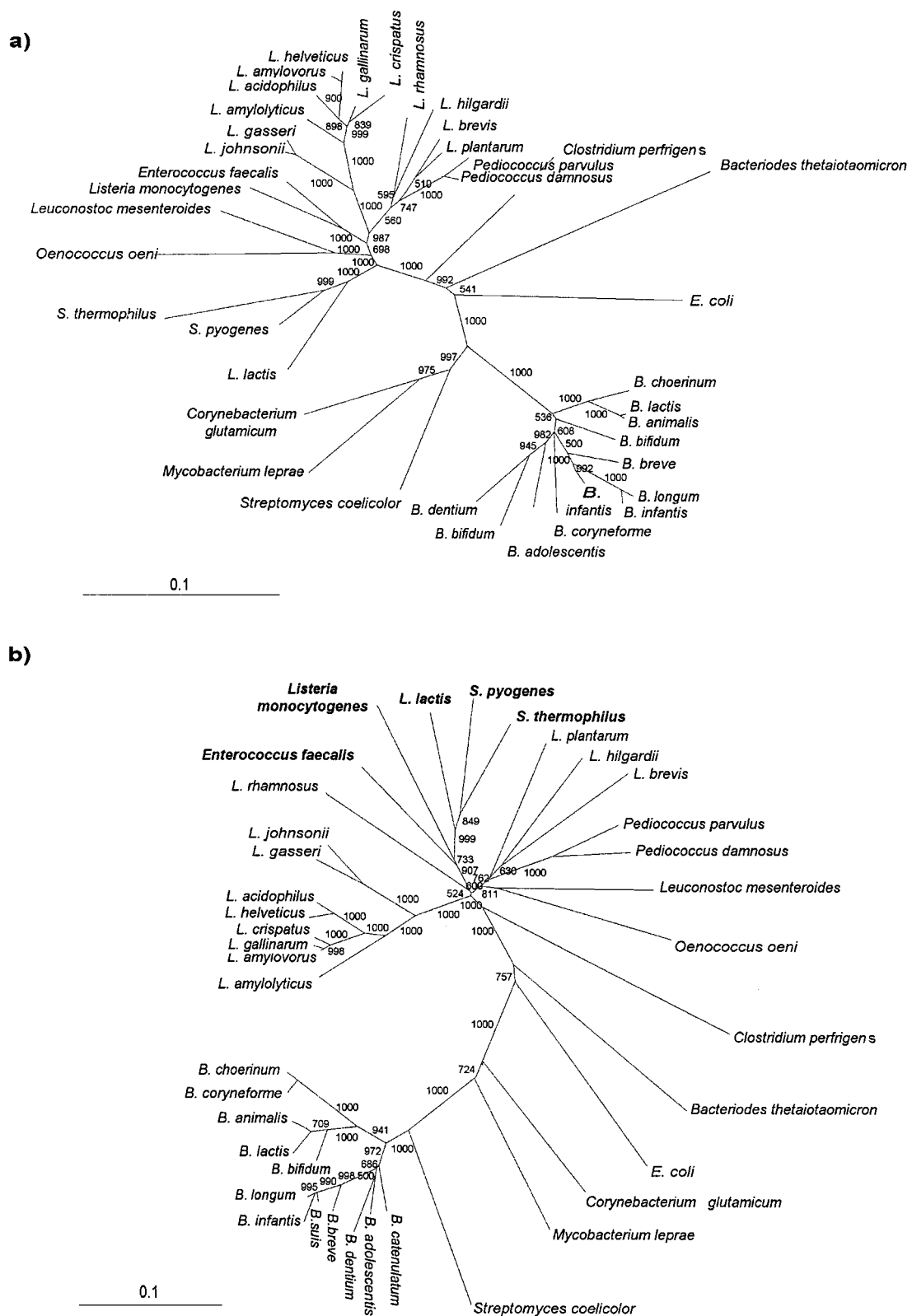


FIG. 4. Neighbor-joining phylogenetic trees obtained by using the 16S rRNA genes (a) and the *atpD* genes (b). The scale bars indicate phylogenetic distances. Bootstrap values are indicated at the nodes for a total of 1,000 replicates. Boldface type indicates species in which horizontal gene transfer of the *atpD* gene is suspected. The strains and the accession numbers of the *atpD* genes and 16S rDNA sequences are shown in Table 1.

cillus. This could have been due to extensive gene transfer among bacteria and/or independent sequence divergence. In addition, the G+C contents of the *Lactococcus*, *Streptococcus*, *Enterococcus*, and *Listeria atpD* genes were between 3.12- and 6.8-fold higher than the average G+C contents of the genomes of these organisms, whereas the G+C content of the *atpD* gene of *Lactobacillus* was nearly identical to that of the rest of the genome. Moreover, the factorial correspondence analysis of codon usage in *Lactococcus*, *Streptococcus*, and *Listeria* ORFs revealed a very different codon usage bias for the *atpD* gene (Fig. 5). In fact, as shown in Fig. 5, the dots corresponding to the *atpD* genes of *Lactococcus*, *Streptococcus*, and *Listeria* are located at some distance from the rest of the cloud of dots representing all of the rest of the genomic genes. Notably, closely related strains which have nearly identical 16S rRNA sequences, like the *L. acidophilus* B group (*L. gasseri* and *L. johnsonii*), as well as *B. animalis* and *B. lactis*, clearly branch separately in the *atpD* sequence-based tree (Fig. 4b).

Most of the base substitutions in the *atpD* genes were synonymous; i.e., they did not result in amino acid changes. Alignment of the *atpD*-encoded protein sequences of all of the strains investigated led to identification of 16 conserved signatures that appeared to be specific for the genus *Bifidobacterium* (Table 2), whereas no amino acid signatures have been detected for the genus *Lactobacillus*. Interestingly, comparative analysis of the *atpD* nucleotide sequences revealed four amino acid signatures which are found in *B. lactis* or *B. animalis* strains, as well as eight amino acid signatures which are found in the *L. johnsonii* and *L. gasseri* strains, but which are not found in any of the other microorganisms investigated. These sequence signatures can be used directly to design specific PCR primers or as targets for specific restriction enzymes, providing species-specific restriction fragment length polymorphism patterns. Other closely related species, like *B. longum*, *B. suis*, and *B. infantis*, have identical *atpD* sequences, providing new evidence that these bacteria could belong to the same taxon (38).

DISCUSSION

In the present work we genetically characterized the operon encoding the F_1F_0 -ATPase of *B. lactis*, which consists of eight subunits. In contrast to other bacterial *atp* operons, there is no *atpI* gene in *B. lactis* or in *B. breve* or *B. longum*, and the overall order of the genes is *atpB-atpE-atpF-atpH-atpA-atpG-atpD-atpD-atpC*, which is similar to the order in *L. acidophilus* (21) and in *E. faecalis* (17). The inferred amino acid sequences encoded by these genes exhibited homology with the sequences of other ATPases. In particular, there was strong identity between the subunits of *B. lactis* and those of *B. breve*, *B. longum*, *L. plantarum*, and *L. acidophilus*. The operon is transcribed in two separate mRNAs, an mRNA covering all subunits, similar to that reported for the equivalent operon of other bacterial species (12, 42), and a shorter mRNA corresponding to the last four genes of the operon. The transcription start sites of these mRNAs were experimentally determined, and no consensus promoter sequences were identified. The fact that no definitive consensus sequence could be determined from these motifs may have been due to the fact that the RNA polymerase recognition sites can tolerate a considerable amount of degen-

eration or to the fact that the sequences investigated here were not representative of typical -10 and -35 hexamers. So far, there have been only two other reports of use of the primer extension technique for determination of a transcriptional start site in *Bifidobacterium* spp. (24, 37), and neither of them identified the classical recognition sites of the vegetative RNA polymerase.

Interestingly, the last gene of the operon was followed by a long palindromic sequence, which might play the role of a rho-independent terminator for the operon. Other short palindromic sequences were detected in the operon sequences, at positions corresponding to those described in the *L. acidophilus* and *E. coli atp* operons (36, 44). In the ATPases complexes of these bacteria these palindromic sequences have been found to increase the mRNA stability and thus enhance the translational efficiency of the corresponding genes (21).

The bacterial acid response is a very complicated process that involves synthesis of a large arsenal of proteins (33, 34). For many bacteria it has been shown that treatment with acid and a subsequent decrease in the intracellular pH are accompanied by an increase in the amount of the F_1F_0 -ATPase (2, 19, 21, 30, 31). This phenomenon has been studied most extensively in *S. faecalis* (2, 17), in *L. lactis* (18, 19), and in *L. acidophilus* (21), in which the cytoplasmic pH is maintained by the amount and activity of the H^+ -ATPase, which catalyzes the ATP-driven translocation of protons from the cytoplasm. In *S. faecalis* it has been demonstrated that the transcriptional activity of the *atp* operon is not pH regulated and that the increase in the enzyme level is pH regulated at the enzyme assembly step. Conversely, the abundance of *atp* transcripts of *B. lactis* increased upon exposure to a low pH, suggesting that there is regulation at the level of transcription or mRNA degradation. This mechanism of regulation is similar to that described for *L. acidophilus* and *L. lactis* strains.

The discovery of genes whose expression responds to changes in pH is of immense importance for bifidobacteria. The growth of these bacteria is associated with generation of acidic products, which accumulate during fermentation. Similarly, the acidity of the stomach is a strong barrier against passage and survival of bacteria in the gastrointestinal tract. Maus and Ingham (25) showed that a *B. lactis* strain was capable of surviving in synthetic human gastric fluid at a pH of 3.5, especially after application of a sublethal acidic stress during culture production (e.g., exposure to low pH in a yogurt sample). Acids do pass passively through the bifidobacterial membrane and are rapidly dissociated into their impermeable constituent protons and charged derivatives. The maintenance of cytoplasmic pH requires that these protons be neutralized or expelled from the cytosol. In this respect, the proton-translocating ATPase may play an important role in the success of *B. lactis* in environments with low pH values. In this respect, knowledge about the genetic basis of this acid resistance for gastrointestinal or industrially useful bacteria that resist extreme acidic conditions during passage through the human stomach is very important.

Our results indicate that the *atp* operon is essential for growth of bifidobacteria under acidic conditions, which is in agreement with the observation that the activity of the F_1F_0 -ATPase in related anaerobic bacteria is enhanced at a low external pH (19, 21). The genes of the *atp* operon, especially

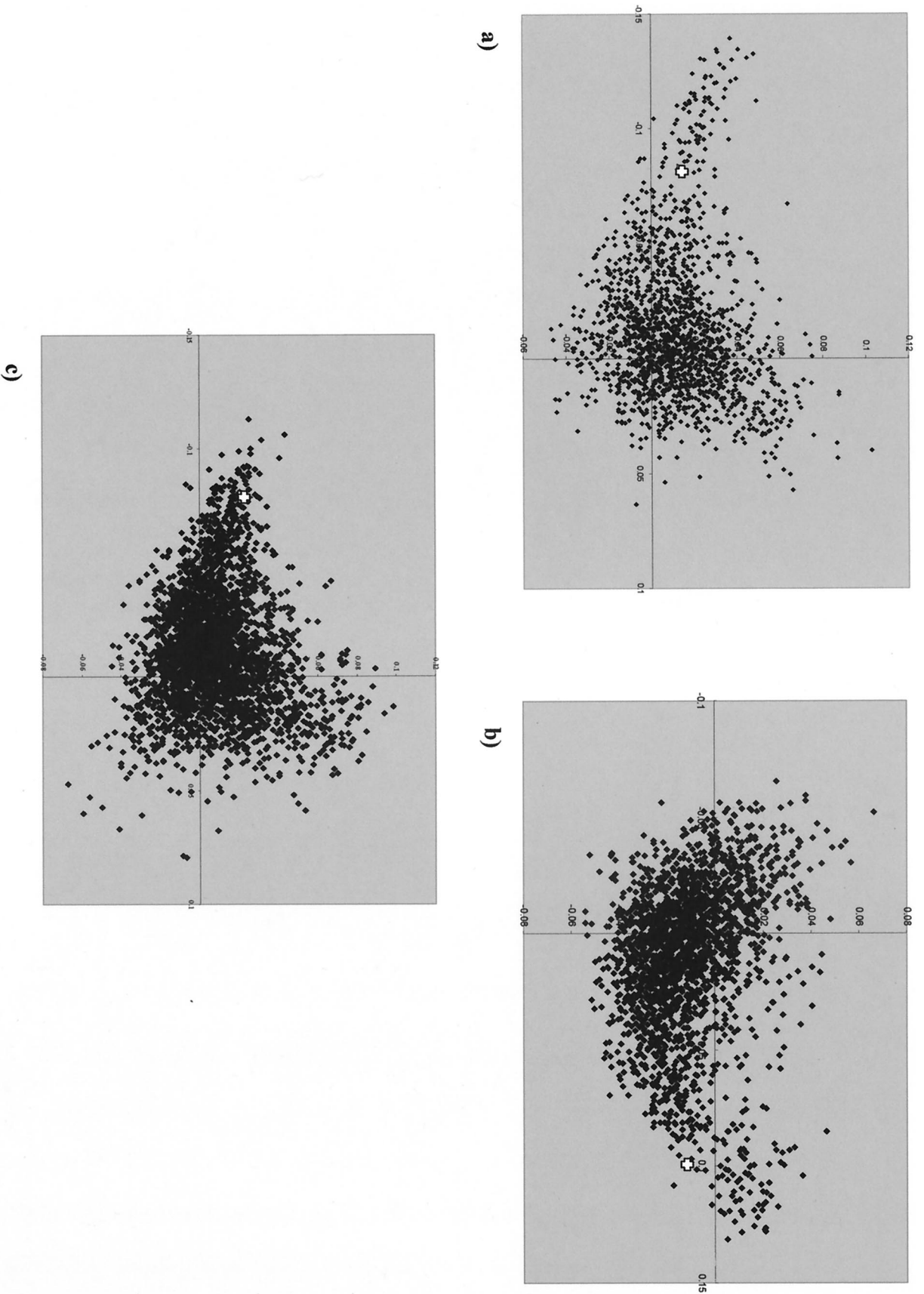


FIG. 5. Factorial correspondence analysis of codon usage in various ORFs in *Streptococcus* (a), *Lactococcus* (b), and *Listeria* (c). The position of *atpD* is indicated by a cross.

TABLE 2. *atpD*-amino acid and nucleotide signatures of *Bifidobacterium* and *Lactobacillus* taxa

Taxon	Position(s) ^a	<i>atpD</i> amino acid signature ^b	<i>atpD</i> nucleotide ^b signature
<i>Bifidobacterium</i>	38	I	ATT
<i>Bifidobacterium</i>	41–42	KK	AAGAAG
<i>Bifidobacterium</i>	58–59	NP	AACCCG
<i>Bifidobacterium</i>	84	Q	CAG
<i>Bifidobacterium</i>	106–107	QR	CAGCGA
<i>Bifidobacterium</i>	137–138	IG	ATCGGC
<i>Bifidobacterium</i>	158	Q	CAG
<i>Bifidobacterium</i>	167	P	CCG
<i>Bifidobacterium</i>	182	N	AAT
<i>Bifidobacterium</i>	304	N	AAC
<i>Bifidobacterium</i>	308	A	GCG
<i>Bifidobacterium</i>	344	Q	CAG
<i>Bifidobacterium</i>	347	G	GGC
<i>Bifidobacterium</i>	351	Y	TAC
<i>Bifidobacterium</i>	355	K	AAG
<i>B. lactis</i> (<i>B. animalis</i>)	12	I (V)	ATC (GTC)
<i>B. lactis</i> (<i>B. animalis</i>)	49	V (E)	GTG (GAG)
<i>B. lactis</i> (<i>B. animalis</i>)	286	A (S)	GCC (TCC)
<i>B. lactis</i> (<i>B. animalis</i>)	337	T (I)	ACA (ATA)
<i>L. johnsonii</i> (<i>L. gasseri</i>)	17	G (A)	GGT (GCT)
<i>L. johnsonii</i> (<i>L. gasseri</i>)	163–164	GK (AT)	GGTAAG(GCCACC)
<i>L. johnsonii</i> (<i>L. gasseri</i>)	252	T (P)	ACA (CCA)
<i>L. johnsonii</i> (<i>L. gasseri</i>)	254–255	LS (PA)	CTTTCA(CCAGCA)
<i>L. johnsonii</i> (<i>L. gasseri</i>)	305	K (Q)	AAG (CAA)
<i>L. johnsonii</i> (<i>L. gasseri</i>)	359	I (L)	ATT (CTT)

^a Position in the amino acid sequence.

^b The residues in parentheses are the residues in the species in parentheses.

the *atpD* gene, have all of the prerequisites to be suitable phylogenetic markers, such as very high genetic stability and a wide distribution (22, 23). This operon has already been used to infer phylogeny in the genera *Salmonella* (6) and *Rhizobium* (13). This alternative molecular marker might corroborate and help complete the evolutionary history of various LAB species. The use of *atpD* genes in LAB species as an alternative or complement to the 16S rDNA marker provides sequence signatures that can be used to distinguish closely related species (e.g., *B. animalis* and *B. lactis*). These sequence signatures can be used directly to design specific PCR primers or might be targets for specific restriction enzymes, providing species-specific restriction fragment length polymorphism patterns. The failure of 16S rDNA sequences to provide multiple diagnostic sites could stem from the unusually low rate of evolution of this molecule. The rates of evolutionary substitution in 16S rRNA genes are an order of magnitude lower than those in protein-encoding genes, such as the *atpD* gene (32, 52). Interestingly, there were some differences in the phylogenies obtained by using *atpD* genes and 16S rRNA genes. These differences are linked to insertion of the genera *Enterococcus*, *Lactococcus*, *Streptococcus*, and *Listeria* into the *Lactobacillus* lineage. Analysis of the G+C contents of these organisms and the bias of their codon usage support the hypothesis that there was probably horizontal transfer of the *atpD* genes. Notably, these organisms showed a reshuffling of the *atp* operon; in fact, the F_0 gene order of *Lactococcus* and *Streptococcus* is *atpEBF*, compared to the most common order, *atpBEF* (18, 20). The *atpD*- and 16S rRNA gene-based trees were generated from the same set of strains with only two exceptions for the 42 strains. This should not decrease the accuracy of the analysis since the differences in the tree topology were observed in more than

one species of the same genus (e.g., *Streptococcus*). The genome sequence analysis showed that horizontal gene transfer is an event that also occurs frequently in housekeeping genes, like the *tuf* gene (16), and in the S14 gene encoding the ribosomal protein (5).

Recently, polyphasic taxonomy (46) has been recognized by the International Committee on Systematic Bacteriology as a new tool for description of species and for revision of the current nomenclature of some bacterial groups. In view of its demonstrated effectiveness, sequence analysis of protein-encoding genes (such as *atpD*) as alternative phylogenetic markers could be added to the arsenal of rRNA sequence databases and to the relatively small *groEL* and *recA* sequence databases.

In conclusion, analysis of *atpD* might enable us to resolve phylogenetic lineages of bifidobacteria at species and subspecies levels. In fact, the comparison of the *atpD* genes of *B. lactis* and *B. animalis* supported previously published data (47, 48, 49, 51) and provided new evidence that *B. lactis* and *B. animalis* are clearly members of two separate lineages. Moreover, analysis of the *atpD* gene also supports the recent decision of the International Committee on Systematic Bacteriology to unify *B. suis*, *B. longum*, and *B. infantis* into one unique taxon since no clear genetic (DNA-DNA hybridization, ribotyping patterns) or physiological differences could be detected (39), whereas caution should be used in any subsequent interpretation of bacterial evolution based on the *atpD* gene, in which horizontal gene transfer events might have occurred (e.g., in the genera *Lactococcus*, *Streptococcus*, *Enterococcus*, and *Listeria*).

Furthermore, the growing picture of pH-regulated catabolism has obvious significance for questions of survival of industrially useful bacteria, like *B. lactis*, in the human body, as well as in fermented foods with low pH values. Our study revealed various avenues of investigation for examining metabolic components of, for example, pH stress responses.

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