Proteome Analysis of *Streptococcus thermophilus* Grown in Milk Reveals Pyruvate Formate-Lyase as the Major Upregulated Protein

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We investigated the adaptation to milk of *Streptococcus thermophilus* LMG18311 using a proteomic approach. Two-dimensional electrophoresis of cytosolic proteins were performed after growth in M17 medium or in milk. A major modification of the proteome concerned proteins involved in the supply of amino acids, like the peptidase PepX, and several enzymes involved in amino acid biosynthesis. In parallel, we observed the upregulation of the synthesis of seven enzymes directly involved in the synthesis of purines, as well as formyl-tetrahydrofolate (THF) synthetase and serine hydroxy-methyl transferase, two enzymes responsible for the synthesis of compounds (THF and glycine, respectively) feeding the purine biosynthetic pathway. The analysis also revealed a massive increase in the synthesis of pyruvate formate-lyase (PFL), the enzyme which converts pyruvate into acetyl coenzyme A and formate. PFL has been essentially studied for its role in mixed-acid product formation in lactic acid bacteria during anaerobic fermentation. However, formate is an important methyl group donor for anabolic pathway through the formation of folate derivates. We hypothesized that PFL was involved in purine biosynthesis during growth in milk. We showed that PFL expression was regulated at the transcriptional level and that *pfl* transcription occurred during the exponential growth phase in milk. The complementation of milk with formate or purine bases was shown to reduce *pfl* expression, to suppress PFL synthesis, and to stimulate growth of *S. thermophilus*. These results show a novel regulatory mechanism controlling the synthesis of PFL and suggest an unrecognized physiological role for PFL as a formate supplier for anabolic purposes.

The thermophilic bacteria *Streptococcus thermophilus* is one of the most widely used lactic acid bacteria (LAB) in the dairy fermentation industry for yoghurt and cheese production. In the industrial implementation of *S. thermophilus*, fast-growing capacity is crucial to enable rapid and intense acidification of milk. Identification of functions activated during growth in milk should help to understand the molecular adaptation of this bacterium to milk and provide the basis for targeted strain selection.

In contrast with other LAB, the only environment from which *S. thermophilus* has been isolated is milk (38). In line with this restricted ecological niche, lactose, not glucose, is the preferred carbon source for *S. thermophilus* (31). The capacity to ferment lactose, the main sugar of milk, into lactic acid is essential for growth in milk and depends on a non-phosphotransferase system lactose permease (LacS) and a beta-galactosidase (LacZ) (40). Concerning the capacity of *S. thermophilus* to fulfill its amino acid needs during growth in milk, the species displays only a few amino acid auxotrophies compared to the model LAB, *L. lactis* (29, 33). The growth of *Lactococcus lactis* in milk depends largely on the activity of a cell-wall-bound proteinase (PrtP) that is responsible for casein hydrolysis, the main source of amino acids in milk. The presence of such a cell-wall-bound proteinase is, however, exceptional among *S. thermophilus* strains (37). This situation is likely explained by the amino acid biosynthetic capacities of *S. thermophilus* and its frequent association with *Lactobacillus delbrueckii* subsp. *bulgaricus*. This latter species possesses a very efficient cell-wall-bound proteinase whose activity is beneficial to both species (14). Several genetic and biochemical studies have focused on the characterization of *S. thermophilus* cytoplasmic peptidases (for examples, see references 1, 13, 28, 29, 34, 35, and 39), and the genome sequences revealed the presence of more than 20 potential cytoplasmic peptidases in the two strains analyzed (12). However, the role of these enzymes in nitrogen metabolism during growth in milk has not yet been established. More recently, transposon mutagenesis was used to isolate mutants of *S. thermophilus* deficient for growth in milk (17, 18). The genes inactivated in the mutants were associated with the peptide transport, branched-chain amino acid, and purine base biosynthetic capacities.

In this work, we investigated the adaptation of *S. thermophilus* to milk by comparing its proteomic profile after growth in synthetic medium or in milk. The analysis of the main variations of the proteome has shown that the physiology *S. thermophilus* is subjected to important changes during growth in milk that concern both nitrogen and carbon metabolism. A surprising finding was the strong upregulation of the pyruvate formate-lyase (PFL). The key role of this enzyme in the routing of the fermentation pathway prompted us to further study the regulation of its expression. We observed that *pfl* expres-

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sion was upregulated in milk and found that formate or purine nucleotide starvation was responsible for *pfl* transcriptional activation, leading to the increase in the PFL level. This result established a novel link between a key fermentative enzyme and the nutritional resources of the environment.

MATERIALS AND METHODS

Bacterial strains, cultures, and media. *S. thermophilus* LMG18311 (BCCM collection) was isolated from yogurt. Stock cultures were prepared in reconstituted low-heat 10% (wt/vol) Nilac skim milk (Nilac low-heat milk powder, NIZO, Ede, The Netherlands) that was autoclaved at 110°C for 12 min. At a pH value of 5.4 to 5.6, milk cultures were shock frozen in liquid nitrogen and maintained at −80°C. For M17-Lac cultures, precultures were inoculated with a colony isolated on M17 agar. Four other strains of *S. thermophilus*, LMD9, CNRZ385, CNRZ1585, and ATCC 19258, were used to generalize some of the results found with LMG18311. Like the majority of *S. thermophilus* strains, strain LM18311, chosen for the proteomic analysis, does not express a cell wall proteinase (PrtS⁻). For proteomic and transcript analysis, *S. thermophilus* was grown at 42°C either in M17 broth (Difco, Sparks, MD) supplemented with 1% (wt/vol) lactose (M17-Lac) or in skim Marguerite milk (La Laiterie, Villefranche sur Saône, France). Marguerite milk is a commercial milk sterilized by microfiltration. Before use, it was skimmed by centrifugation at 4°C at 5,000 × *g* for 30 min. The sterilization of milk by microfiltration avoids the chemical modifications and proteolysis induced by classical heat treatment. All cultures were performed by inoculating M17-Lac or milk with approximately 5 × 10⁶ CFU/ml of stock cultures and incubating it without shaking. The pH of the culture was monitored. After disruption of cell chains by a 40-s treatment with a mechanical blender (Turax X620; Labo-Moderne, France) bacterial CFU were determined by plating dilutions on M17-Lac agar with an automatic spiral plater (AES Laboratoires, Combourg, France). The cell number was measured with the EC1 colony counter software (AES Laboratoires) after overnight incubation in anaerobic jars (Anaerocult A; Merck, Darmstadt, Germany). To study the effects of formate and purine on growth, milk was supplemented with 10 mM sodium-formate or 50 μg/ml of adenine and guanine.

Cytoplasmic protein extract preparation for proteome analysis. The cellular protein extracts were prepared from 400-ml cultures. Before centrifugation, a 0.33 volume of 1 M trisodium citrate and a 0.13 volume of buffered saline solution (0.145 M sodium chloride, 0.016 M sodium β-glycerophosphate, 0.1% Tween 80, pH 7) were added to cultures. This procedure, described by Guimont et al. (23), avoids the precipitation of caseins at the time of centrifugation. Cells were harvested by centrifugation (8,000 × *g* for 10 min at 4°C), and the cell pellet was washed twice with ice-cold extraction buffer, pH 7.0 (5 mM sodium phosphate, 1 mM EDTA, 2 mM β-mercaptoethanol), and resuspended in 20 mM Na phosphate buffer, pH 6.4. Protease Inhibitor Cocktail diluted 20 times (Sigma-Aldrich, St Louis, Mo.), 40 U/ml catalase (Sigma), and 10 mM tributylphosphine (Applied Biosystems) were added. The cells were mechanically disrupted in an FP120 FastPrep cell disruptor (Bio 101 Systems, Qbiogen, Inc., Irvine, CA) by 2 30-s cycles of homogenization at maximum speed with 1-min intervals in ice. The suspension was centrifuged at 5,000 × *g* for 15 min at 4°C to remove unbroken cells and large cellular debris. The supernatant was next collected and ultracentrifuged at 50,000 × *g* for 30 min at 4°C to remove cell envelope components. The total protein concentration was determined by the Coomassie protein assay reagent (Pierce, Rockford, Ill.) using bovine serum albumin as a standard.

Analytical 2-DE. The procedures were essentially performed as described previously (22). Benzonase (1% [vol/vol], 10,000 U/ml; Merck, Darmstadt, Germany) and 1% (vol/vol) 1 M MgSO₄ were added to a volume of cytosolic fraction corresponding to 50 μg (7-cm two-dimensional gel electrophoresis [2-DE]) or 350 μg (24-cm 2-DE) of protein. The reaction mixture was incubated at 37°C for 30 min and precipitated with 3 volumes of ice-cold methanol. The protein pellet was solubilized in 155 or 500 μl of isoelectric focusing buffer {7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 100 mM dithiothreitol, 0.5% pH 4 to 7 immobilized pH gradient (IPG) buffer (Amersham-Pharmacia Biotech, Uppsala, Sweden)} and loaded onto a 7-cm or 24-cm pH 4 to 7 IPG strip (Bio-Rad, Hercules, CA) using the in-gel rehydration technique (32).

Isoelectric focusing (IEF) of the 7-cm IPG strip was carried out at 15°C using the Protean II IEF cell (Bio-Rad). For 7-cm strips, IEF was performed for 30 min at 50- to 175-V ramp, 45 min at 175- to 2,000-V ramp, and 30 min at 2,000 V (total = 1.868 kVh). Second-dimension electrophoresis was performed on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis using precast 4 to 12% Bis-Tris ZOOM gel (Invitrogen, Carlsbad, CA). For the 24-cm strip, IEF

was performed with the Ettan IPGphor system (Amersham Pharmacia Biotech, Uppsala, Sweden) with the following conditions: 11 h at 50 V, 50 to 300 V in 1 h, 300 to 1,500 V in 1 h, 1,500 to 8,000 V in 5 h, 5 h at 8,000 V (total = 68 kVh). After IEF was completed, the IPG strip was positioned onto SDS-polyacrylamide gel electrophoresis gels using 1% low-melting-point agarose in 150 mM Tris-HCl, pH 8.8. The second dimension was performed on 12% polyacrylamide gels (24 by 20 by 0.1 cm) in 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3, using the Ettan-Dalt II apparatus. Electrophoresis was run at 1 W/gel for 16 h at 4°C. The gels were stained with BioSafe colloidal Coomassie blue (Bio-Rad) for 1 h and destained with three successive washes in deionized water.

Gels were digitized using an Epson Expression 1640XL scanner controlled by the Silver Fast software. Image files were recorded at 256 gray levels. Images of 2-DE were edited and matched using the Image Master 4.01 software package (Amersham-Pharmacia).

The comparative analysis was performed by analyzing images from three independent cultures. The normalized intensity (NI) of each spot was calculated as the ratio between its intensity and the sum of the intensities of all spots in the gel. The mean intensity values of each spot were calculated on at least three gels and used to calculate the ratio (relative [*n*-fold] change) of expression level of the corresponding protein between the two experimental conditions (M17 versus milk). A protein was included in the list of up- or downregulated proteins with the following criteria: (i) it was present in the three duplicate biological experiments, (ii) a minimum of a twofold change in NI was observed between the two conditions. The average NI for the three independent experiments was used to calculate the ratios.

MALDI-TOF (mass spectrometry) and database searching. Mass spectrometry analyses were performed using a matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) Voyager-DE-STR mass spectrometer (Applied Biosystems), operated in positive-ion reflector mode. Protein spots of interest were cut out and digested with porcine trypsin (Promega, Madison, Wis.) as described previously (22). Peptide mixtures were analyzed using an α-cyano-4-hydroxycinnamic acid matrix prepared at 4 mg/ml in 50% acetonitrile containing 0.1% trifluoroacetic acid. The trypsin autolytic peptides (842.5 and 2,211.1 Da) were used as internal calibrants. Peptides were selected in the mass range of 840 to 4,000 Da. A local copy of the MS-FIT program, developed by the University of California at San Francisco, was used to search the *S. thermophilus* proteome database deduced from the genome sequence (12). Search parameters were as follows: monoisotopic masses, maximum allowed peptide mass error of 30 ppm, consideration of one incomplete cleavage per peptide, and carbamidomethylation of cysteines and oxidation of methionines. To assign an identification we used the following criteria: (i) a minimum of four matching peptides, (ii) a sequence coverage of at least 20%, (iii) conformity between theoretical and experimental PM and pI. The 2-DE images and information on the protein spots can be obtained by downloading the PARIS software http://www.inra.fr/bia/J /imaste/paris/ (41).

Northern blot analysis of *pfl* mRNA. *S. thermophilus* total RNA was prepared from 200-ml cultures. Before harvesting the cells, a 0.33 volume of 1 M trisodium citrate and a 0.13 volume of buffered saline solution (0.145 M sodium chloride, 0.016 M sodium β-glycerophosphate, 0.1% Tween 80, pH 7) were added to the media (23). Cells were collected by centrifugation for 5 min at 4°C at 10,000 × *g*, washed with 100 ml ice-cold extraction buffer, pH 7.0 (5 mM sodium phosphate, 1 mM EDTA, 2 mM β-mercaptoethanol), shock frozen, and stored at −80°C. Bacterial pellets were resuspended in 500 μl of 25 mM Tris-HCl (pH 7.6) with 60 mM EDTA and transferred into tubes containing 500 μl of acid phenol (pH 4.5) and 0.5 g of 0.1-mm-diameter glass beads (Sigma). Cells were mechanically broken with a Fastprep apparatus (2 30-s cycles of homogenization at maximum speed with 1-min intervals on ice). RNA was then extracted and purified with the TRIzol reagent kit (Gibco-BRL) as previously described (15). RNA samples were resuspended in 100 μl of TE (10 mM Tris, 1 mM EDTA, pH 7.6) and quantified by measuring A_{260} and A_{280}. Both RNA purity and integrity were controlled by separating a sample on agarose gel to ensure that mRNA, tRNA, and rRNA precursors could be seen. For Northern blot analysis, 20 μg of glyoxylated RNA samples were separated by electrophoresis in 1% (wt/vol) agarose gel containing 10 mM sodium iodoacetic acid in 10 mM sodium phosphate buffer (pH 7) (15). RNA was transferred to Hybond N nylon membranes (Amersham) and fixed by UV treatment. Blots were stained with bromophenol blue to ensure that equivalent amounts of total RNA were loaded in each sample. RNA extraction and hybridization experiments were carried out on two independent blots with similar results. A 690-bp PCR-amplified *pfl* gene probe obtained with primers pfl1 (5′-GAACGTGACCTTGCTCGTGG-3′) and pfl3 (5′-GCCATTTGAACNGCTTCRTAG-3′) was used for hybridization. The probe was labeled with the ECL Direct Nucleic Acid Labeling and Detection

systems (Amersham Biosciences, Little Chalfont, United Kingdom) according to the manufacturer's procedures.

Measurements of formic acid. Skim milk cultures of *S. thermophilus* LMG18311 were performed as described above. Cells were harvested at different times after inoculation, and deproteinization of cultures supernatants was performed with trichloroacetic acid at a final concentration of 1.2% (vol/vol). Samples were allowed to stand for 10 min on ice and centrifuged at $5,000 \times g$ for 10 min at 4°C. Supernatants were then filtrated on Centriplus filter devices (YM-30,000 MWCO) (Millipore Corporation) according to the manufacturer's recommendations. Immediately prior to formate dosage, neutralization of filtrates to a final pH of 7 was carried out with 1 M NaOH and samples were briefly centrifuged. Formate was measured enzymatically by using formate dehydrogenase with the Test combination kit for determination of formic acid (r-Biopharm, Saint Didier au Mont d'Or, France) according to the manufacturer's protocol and using a known formic acid dilution as the standard.

RESULTS

Proteome profiles of *S. thermophilus* LMG18311 grown in M17 and milk. The proteome of *S. thermophilus* has been studied on protein extracts from cells cultivated in M17-Lac and microfiltrated skim milk. For the proteome analysis, bacteria were harvested during the exponential growth phase at a cell density corresponding to 5.0×10^7 CFU/ml in milk (pH 6.35) and 7.5×10^7 CFU/ml (pH 6.7) for M17-Lac. Cytoplasmic proteins were separated by 2-DE in the pH range 4 to 7 (Fig. 1). More than 350 protein spots were detected on this gel system, and 150 unique proteins were identified by peptide mass fingerprinting using MALDI-TOF mass spectrometry. We found that the glycolytic enzymes represent almost 20% of the total spot volume detected on pH 4 to 7 2-DE. This property of the cytoplasmic acidic proteome of *S. thermophilus* is reminiscent of that found for *L. lactis* (22); it thus seems that this is a common property of the proteome of the lactic acid bacteria, for which the central metabolism is based on homolactic fermentation. All modifications observed in protein level between the two media are summarized in Table 1.

Among the 23 proteins downregulated in milk, 16 belong to three functional classes: purine and pyrimidine salvage, translation, and fatty acid biosynthesis. While the levels of two peptide chain release factors (PrfA and PrfC) and two acidic ribosomal proteins (L10 and S2) decreased in milk, the amount of the elongation factors EF-Tu, EF-Ts, and EF-G, which are abundant proteins, stayed at similar levels in the two media. The fatty acid biosynthesis group contains two enzymes involved in the elongation of the fatty acid chain (FabG at FabK) and the enzyme cyclopropane fatty acid synthase, which has been shown to be responsible for the in situ synthesis of cyclopropane fatty acids (20).

On the other hand, most of the upregulated proteins in milk were associated with amino acid and purine base metabolism. A major modification of the proteome concerned enzymes involved in the synthesis or supply of amino acids. The amounts of two enzymes, CysD (*O*-acetylhomoserine sulfhydrylase) and CysM1 (cysteine synthase), were found to increase in milk. These proteins are potentially responsible for cysteine synthesis from acetyl serine. Most of the dairy strains of *S. thermophilus* have the ability to synthesize branched-chain amino acids (BCAA) (Leu, Ile, Val), and mutants in which *ilvB* or *ilvC* genes are inactivated present a strong growth deficit in milk (18, 19). The present proteomic analysis showed that BcaT (BCAA aminotransferase) and IlvC (ketol-acid reduc-

toisomerase) were produced at higher levels during growth in milk. We identified several peptidases by 2-DE (PepC, PepN, PepQ, PepXP), and only one them, the X-prolyl dipeptidyl peptidase PepXP was found to be upregulated during growth in milk. This is in contrast with the situation found for *L. lactis*, where *pepX* was shown to belong to the same regulon as *pepC* and *pepN* (see Discussion).

The proteome of *Streptococcus thermophilus* LMG18311 cultivated in milk was also characterized by an increase in the level of seven enzymes involved in the de novo purine biosynthetic pathway. These proteins participate in the biosynthesis of GMP and AMP. In parallel, we observed an increase of Fhs (formyl-tetrahydrofolate synthetase), which is involved in the formation of folic acid derivatives, and of GlyA (serine hydroxymethyl transferase) responsible for the conversion of serine into glycine. Both enzymes are implied in the supply of the methyl group in various anabolic pathways, including purine biosynthesis.

One of the most impressive changes in the proteome pattern concerned PFL. While the enzyme was barely detectable in M17-grown cells, PFL was one of the 10 most abundant proteins in cells cultivated in milk. This intriguing result prompted us to further analyze the role and control of the synthesis of this protein responsible for the conversion of pyruvate into formate and acetyl-coenzyme A.

Transcriptional activation of *S. thermophilus* *pfl* gene in milk. Previous studies performed on the LAB *S. bovis* and *L. lactis* have shown that the expression of PFL is controlled at the transcriptional level (3, 5). The expression of the *S. thermophilus* *pfl* gene was studied in cells grown in M17-Lac and after transfer to milk. Northern blot analysis with an internal fragment of the *pfl* gene as a probe allowed us to detect a single transcript of 2.3 kb under both conditions (Fig. 2). The size of the transcript agreed with the length of the transcriptional unit of *pfl* deduced from the nucleotide sequence (2,309 bp) and shows that *pfl* gene from *S. thermophilus* is monocistronic. The presence of a potential stem-loop structure (12.2 Kcal mol^{-1}) in the downstream region of the *pfl* gene is indicative of a potential rho-independent transcription terminator. Similar organization of the *pfl* gene has been demonstrated in *S. bovis* (7) and *L. lactis* (3). When *S. thermophilus* cultures grown exponentially in M17-Lac medium were shifted to milk for 15 min, a strong increase in the abundance of the *pfl* mRNA was observed (Fig. 2). This result indicated that the transcriptional activation of *pfl* in milk took place rapidly, suggesting that a yet unknown intrinsic property of the medium has triggered this response. We then measured the levels of *pfl* mRNA during a complete growth cycle in milk by Northern blot analysis (Fig. 3A). The *pfl* transcript abundance was maximal during the exponential phase (time points, 45 and 90 min) but drastically decreased at the very start of stationary phase (time, 2 h, 30 min and later). The proteome analysis performed in parallel showed that the same trend was observed at the protein level (Fig. 3B).

Formate and purine bases as repressors of *S. thermophilus* *pfl* gene expression. The proteome and transcriptional data suggested that PFL was important for the development of *S. thermophilus* in milk. Previous studies on *S. bovis* and *L. lactis* have shown that activation of PFL lead to excretion of formate in the medium (5, 7, 9, 25, 26). To determine if the
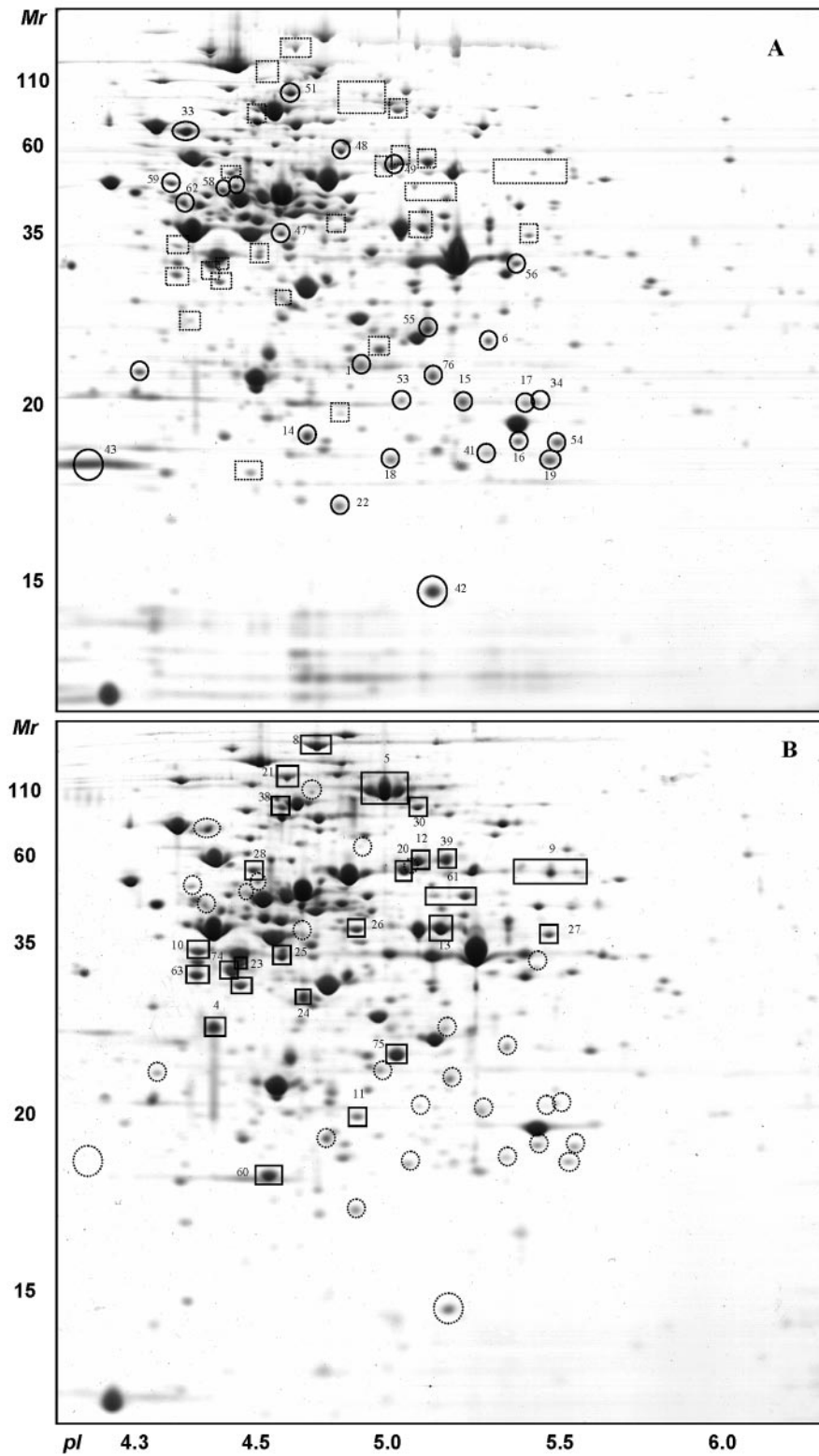
FIG. 1. 2-DE (pH range, 4 to 7) of intracellular extracts of *S. thermophilus* LMG18311 after growth in M17-Lac (A) or milk (B). Proteins (350 µg) were loaded on an IPG strip and stained with colloidal Coomassie blue. The proteins differentially expressed are indicated by circles (higher level in M17) or squares (higher level in milk).

TABLE 1. List of identified proteins of *S. thermophilus* LMG18311 varying between M17 and milk[a]

Level in milk and spot no.	Gene	pI$_{th}$	$M_{r\,th}$ (10³)	pI$_{exp}$	$M_{r\,exp}$ (10³)	Sequence coverage (%)	Accession no.	Fold change	Protein identification
Higher level in milk									
									Carbohydrate metabolism
4	tpi	4.7	26.6	4.7	32.2	71	YP_139013.1	2.3	Triose phosphate isomerase
5	pfl	5.2	87	5.1	78.5	53	YP_140075	>100	Pyruvate formate-lyase
									Nucleotide biosynthesis
13	purB	5.2	49.5	5.2	45.6	77	YP_138590.1	4.5	Adenylosuccinate lyase
11	purC	5.0	27	5.0	24.7	69	YP_138575.1	4.4	SAICAR synthetase
10	purD	4.7	45.1	4.7	41.9	30	YP_138585.1	5.9	GAR synthetase
9	purF	5.4	56.5	5.5	52.6	49	YP_138577.1	10	PRPP amidotransferase
12	purH	5.4	58.5	5.2	58.8	48	YP_138580.1	5.5	IMP cyclohydrolase
8	purL	5.0	136	4.9	94.8	50	YP_138576	5.6	FGAM synthetase
74	purM	4.7	36.3	4.7	39.1	31	YP_138578.1	>30	AIR synthetase
									Amino acids biosynthesis
25	aspC3	4.9	42.8	4.9	41.4	43	YP_138988.1	3.5	Aspartate aminotransferase
24	bcaT	4.9	37.5	4.9	35.7	27	YP_139111.1	3.7	Branched-chain amino acid aminotransferase
26	cysD	5.0	47.5	5.0	45.4	70	YP_139462.1	3.2	*O*-acetylhomoserine sulfhydrylase
75	cysM1	5.2	32.3	5.1	30.1	25	YP_138901.1	2.7	Cysteine synthase serine
27	glyA	5.4	44.6	5.5	45.1	55	YP_139261.1	2	Hydroxymethyltransferase
23	ilvC	4.7	37.4	4.7	40	49	YP_140285.1	>10	Ketol-acid reductoisomerase
28	thrC	4.8	53.9	4.8	56.9	30	YP_140292.1	2	Threonine synthase
									Translation
39	lysS	5.3	58.2	5.2	59.2	37	YP_139201.1	2.7	Lysyl-tRNA synthetase
38	metS	4.8	76.2	4.8	73.2	51	YP_138976.1	4.1	Methionyl-tRNA synthetase
									Miscellaneous
20	fhs	5.2	59.8	5.1	57	47	YP_139285.1	8.3	Formate-tetrahydrofolate ligase
30	pepXP	5.2	85.5	5.2	73.3	46	YP_140089.1	2.5	X-prolyl dipeptidyl peptidase
63	ppaC	4.6	33.8	4.7	38.6	66	YP_138906.1	2.3	Manganese-dependent inorganic pyrophosphatase
60	sodA	5.1	24.7	4.8	21.1	52	YP_139228.1	3.7	Superoxide dismutase (Mn)
Lower level in milk									
									Nucleotide biosynthesis and salvage
14	deoD	4.9	26.2	4.9	23.4	32	YP_139567.1	0.35	Purine nucleoside phosphorylase
15	punA	5.3	25.7	5.3	28.9	45	YP_139571.1	0.2	Purine nucleoside phosphorylase
19	pyrE	5.6	25.7	5.5	21.9	38	YP_139445.1	0.14	Orotate phosphoribosyltransferase
18	pyrF	5.2	25.2	5.1	22.1	40	YP_139444.1	0.23	Orotidine-5-phosphate decarboxylase
17	pyrH	5.6	26.3	5.4	25.6	52	YP_138966.1	0.05	Uridylate kinase
16	upp	5.4	23	5.4	23.1	53	YP_138890.1	0.38	Uracil phosphoribosyltransferase
									Translation
49	argS	5.1	62.9	5.1	58.1	59	YP_138592.1	0.33	Arginyl-tRNA synthetase
41	defB	5.4	22.7	5.3	22.4	72	YP_138691.1	0.15	Polypeptide deformylase
47	prfA	4.9	40.6	4.9	44.7	51	YP_139258.1	0.46	Peptide chain release factor 1
48	prfC	5.0	58.4	5.0	61.7	34	YP_139992.1	0.04	Peptide chain release factor 3
42	rplJ	8.6	20.8	5.2	16.8	60	YP_139058.1	0.16	50S ribosomal protein L10
43	rpsB	5.2	28.3	4.3	31.8	57	YP_138617.1	0.01	30S ribosomal protein S2
51	typA	4.9	68.7	4.9	78.2	38	YP_139237.1	0.09	GTP-binding protein TypA/BipA (tyrosine phosphorylated protein A)
									Fatty acid biosynthesis
56	cfa	5.4	45.1	5.4	40	45	YP_138668.1	0.22	Cyclopropane-fatty-acyl-phospholipid synthase
54	fabG	5.6	25.7	5.5	23.1	44	YP_138920.1	0.13	Beta-ketoacyl-ACP reductase
55	fabK	5.2	33.7	5.2	32.2	48	YP_138918.1	0.12	*trans*-2-enoyl-ACP reductase II
									Cell division protein
58	ftsA	4.8	49.9	4.8	52.6	55	YP_139242.1	0.24	Cell division protein
59	ftsZ	4.6	46.5	4.7	54.2	46	YP_139243	0.16	Cell division protein
									Miscellaneous
62	nox	4.7	50.1	4.7	50.1	30	YP_139728.1	0.32	NADH oxidase (H$_2$O forming)
33	ptsl	4.7	62.9	4.7	66.6	49	YP_139711.1	0.4	Phosphoenolpyruvate-sugar phosphotransferase system enzyme I
53	rr01	5.0	26.6	5.2	25.8	32	YP_138854.1	0.35	Response regulator (homolog to *csrR/covR* Spy)
1	scrK	5.1	32.3	5.1	28.6	24	YP_140147.1	0.31	Fructokinase
22	ssbB	5.0	18.7	5.0	19.8	50	YP_140167.1	0.19	Single-stranded binding protein

[a] pI$_{th}$, theoretical isoelectric point; pI$_{exp}$, experimental isoelectric point.
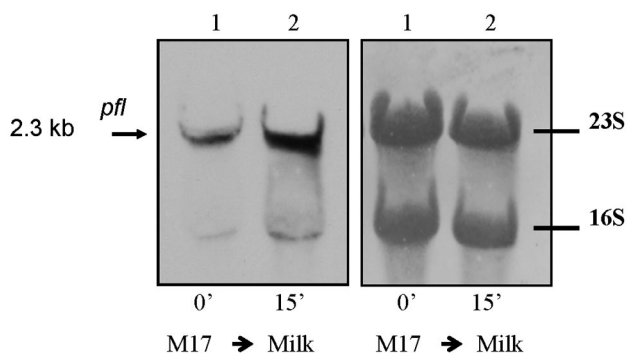
FIG. 2. Northern blot of RNA extracted after exponential growth in M17 (1) and after 15-min transfer in milk (2) and hybridized with a *pfl* probe. The size of the transcript (in kilobases) is shown on the left. On the right, the same membrane is shown stained with bromophenol blue.

increase in *S. thermophilus* PFL was followed by secretion of formate in the medium, formate concentration was measured in the supernatant of milk cultures at several growth times (see Materials and Methods). The concentration was quite constant during the growth and did not significantly differ from that of noninoculated milk (about 4.3 mg/liter) (see Discussion). This result indicated either that the increase in PFL was not associated with a higher formate production or that the formate produced was not excreted by the cells.

The proteomic results showed the activation of de novo purine biosynthesis in milk. Because this pathway requires formate as a 1-carbon unit, we hypothesized that the upregulation of PFL synthesis was driven by the cell formate needs. In agreement with this hypothesis, we next showed that formate could act as a repressor of PFL synthesis. Transcriptional and proteome analysis of *S. thermophilus* LMG18311 grown in milk supplemented with formate (5 mM) or adenine plus guanine

(50 μM each) were performed (Fig. 4). When formate was supplied to milk, the *pfl* mRNA abundance sharply decreased (Fig. 4A) and the amount of PFL became almost undetectable (Fig. 4B). When purines, but not formate, were added to milk, a similar strong reduction in the level of PFL was observed (Fig. 4B). In addition, the amounts of PurM, PurH, and Fhs proteins were also strongly diminished in the presence of purines but not formate. This result strongly suggested that PFL synthesis and the purine biosynthetic pathway are linked in *S. thermophilus*.

Formate supply improves *S. thermophilus* growth in milk. Interestingly, it was also noticed during formate and purine supplementation experiments that the addition of both compounds to milk enhanced the growth of *S. thermophilus* and acidification of the medium (Fig. 5). While similar exponential growth rates were monitored for all cultures, the final biomass reached by the LMG18311 strain was about threefold higher in supplemented media than in milk. This growth-stimulating effect of formate in milk, which was also observed for other *S. thermophilus* strains (see Materials and Methods; data not shown), suggested that the supply of formate might be a limiting step for the metabolism of *S. thermophilus* growing in milk. Curiously, we observed for strain LMG18311 a transient cell susceptibility to the mechanical treatments used for cell chain disruption before plating. This was observed at the end of the exponential growth in milk (lasting between 2 and 4 h after milk inoculation) and was abolished when milk was supplemented with formate or purines (Fig. 5). This peculiar growth characteristic of the strain studied was not further investigated.

DISCUSSION

To ensure basic cellular functions and rapid multiplication, bacteria must take into account the physicochemical charac-
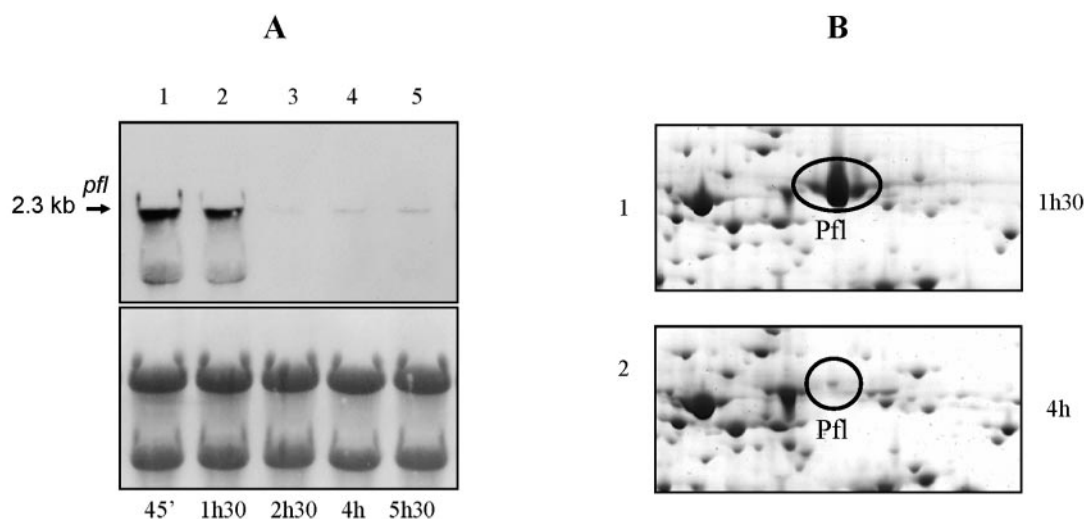

FIG. 3. mRNA and proteomic analyses of *pfl* of *S. thermophilus* LMG18311 after growth in milk. (A) Northern blot of RNA extracted during exponential growth (lanes 1 and 2) or stationary phase (lanes 3 to 5) in milk and hybridized with a *pfl* probe. The size of the transcript (in kilobases) is shown on the left. On the right, the same membrane is shown stained with bromophenol blue. (B) 2-DE of the intracellular proteins of *S. thermophilus* LMG18311 extracted during exponential growth (1 h, 30 min; lane 1) or stationary phase (4 h; lane 2) in milk. The picture represented a zoom of the Pfl region of a pH 4 to 7 gel.
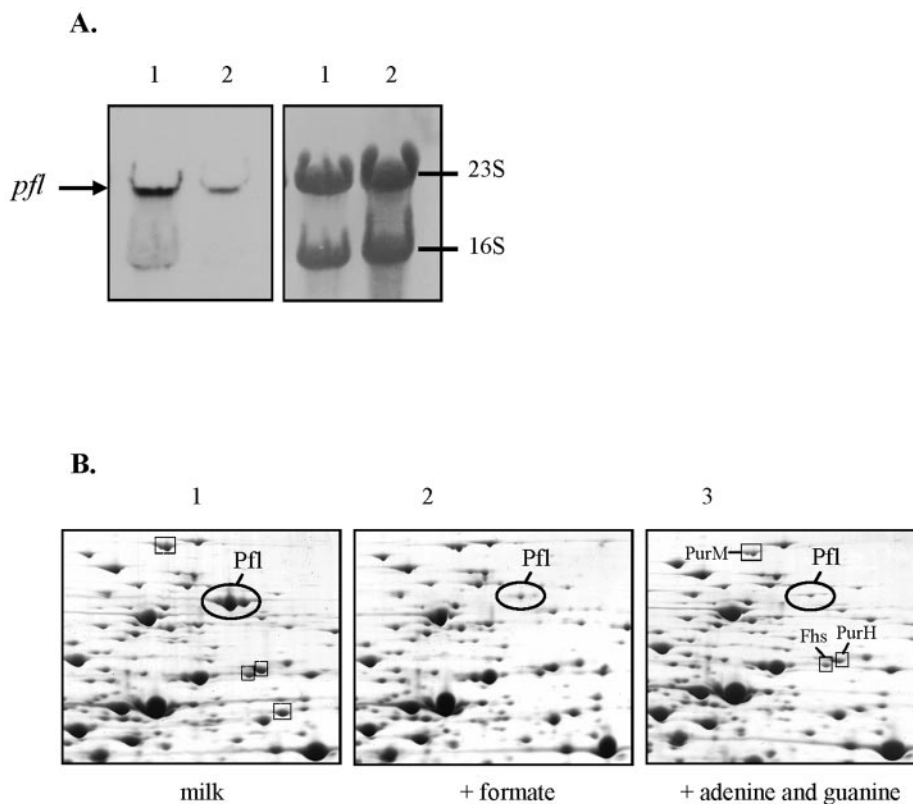
A.

B.

FIG. 4. mRNA and proteomic analyses of *pfl* of *S. thermophilus* LMG18311 after growth in milk supplemented or not with formate or adenine plus guanine. (A) Northern blot of RNA extracted from *S. thermophilus* LMG18311 after exponential growth in milk supplemented (lane 2) or not (lane 1) with 5 mM formate. The arrow indicates the 2.3-kb *pfl* transcript. On the right, the corresponding blots of extracted RNA after bromophenol blue coloration are presented. (B) 2-DE of the intracellular proteins of *S. thermophilus* LMG18311 extracted during exponential growth in milk in the presence (lane 2) or not (lane 1) of formate (5 mM) or adenine plus guanine (50 µM) (lane 3). The picture represented a zoom of the Pfl region of a pH 4 to 7 gel.

teristics of their environment. Milk is known to be poor in purine bases and free amino acids (19, 30). The proteomic data indicate that *S. thermophilus* adapts to these nutritional limitations by the induction of enzymes implicated in the corresponding biosynthetic and conversion pathways.

First, several proteins involved in the supply of amino acids were more abundant in milk-cultivated cells. Caseins that are the main source of amino acid in milk are rich in prolyl residues (5 to 17%). Therefore, the intracellular degradation of peptides issued from the caseins requires enzymes specifically devoted to the hydrolysis of the prolyl peptidic bond. In milk, we observed an increased level of the peptidase PepX (X-prolyl dipeptidyl aminopeptidase), which is responsible for the production of X-Pro dipeptides from the N terminus of peptides. In LAB, PepX is the only peptidase that efficiently cleaves these sequences (27). In *L. lactis*, *pepX* belongs to the CodY regulon, which is activated by a low intracellular level of BCAA (21). In the course of the present study, we identified by 2-DE two other peptidases (PepC, PepN) that also belong to the CodY regulon in *Lactococcus lactis*. However, and in contrast to *L. lactis* (M.-Y. Mistou, unpublished), we did not find evidence for a higher synthesis of these peptidases in *S. thermophilus* grown in milk. As noted above, the expression of these genes is dependent upon the intracellular BCAA concentration. Since *L. lactis* and *S. thermophilus* do not have
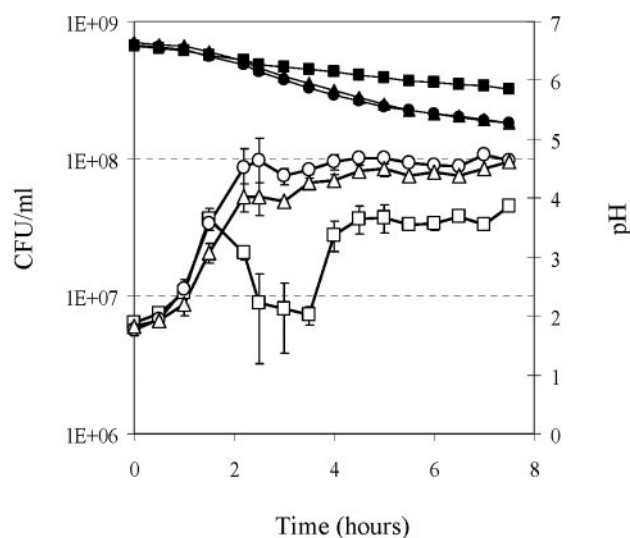

FIG. 5. Growth and acidification curves of *S. thermophilus* LMG18311 in milk supplemented or not with formate or adenine plus guanine. The evolution of CFU (open symbols) and pH (filled symbols) in milk (squares) and milk supplemented with either 5 mM formate (triangles) or 50 µM adenine plus guanine (circles) is shown.

similar requirements for these amino acids, the synthesis of these enzymes is not subjected to the same constraints in the two dairy bacteria cultivated in milk. While *L. lactis* is auxotrophic, *S. thermophilus* possesses a functional BCAA biosynthetic pathway (see below). In addition, two enzymes involved in BCAA biosynthesis, IlvC and BcaT, are synthesized at a higher level in milk in *S. thermophilus*. These results are in good agreement with genetic experiments having shown that inactivation of *ilvC* led to a reduced-growth phenotype of *S. thermophilus* in milk (18). Induction of the BCAA biosynthetic pathway in *S. thermophilus* is thus likely associated with a modification of the transcriptional regulatory mechanisms dependent on the BCAA intracellular concentration. Other enzymes involved in Cys, Met, Asp, and Ser biosynthesis were also more abundant in milk. In this respect, it must be mentioned that the lactococcal AspC, homologous to the streptococcal AspC3 enzymes, was found to have an essential role in the growth of *L. lactis* in milk (16). This result suggests that aspartate aminotransferase, responsible for the synthesis of aspartate from oxaloacetate also plays a key role in the development of *S. thermophilus* in milk.

A second hallmark of the proteome profile of *S. thermophilus* grown in milk is the synthesis of enzymes directly involved in the de novo purine biosynthetic pathway (Table 1). In parallel, glycine synthase (GlyA) and formate-tetrahydrofolate ligase (Fhs), two proteins that are involved in the intracellular production of the methyl group donors glycine and 10-formyltetrahydrofolate, respectively, were also upregulated. Both substrates are feeding the purine biosynthetic pathway. In *L. lactis*, *glyA* and *fhs* have been shown to belong to the PurR regulon that includes proteins upregulated under purine starvation conditions (11). Genes controlled by the transcriptional repressor PurR display a 13-mer sequence called the PurBox located upstream of their promoter. Similar potential Pur boxes (consensus sequence: AWWWCCGAACWWT) were identified upstream of the transcriptional units of *purCLFMNH*, *purDEKB1*, and *fhs* in the strain LMG18311.

Finally, PFL was the strongest overexpressed protein of *S. thermophilus* LMG18311 during exponential growth in milk. On mini 2-DE, we observed a similar increase of PFL for 4 strains (see Materials and Methods) of *S. thermophilus* isolated from various dairy products coming from different geographical locations (data not shown). Upregulation of PFL in milk is thus likely common to the *S. thermophilus* species.

The enzyme catalyzes the nonoxidative transformation of pyruvate into acetyl-coenzyme A and formate. In general, a posttranslational process catalyzed by a PFL-activating enzyme is required to obtain the radical, active form of PFL. In *Streptococcus bovis*, the enzyme has been characterized previously (8); it is encoded by the *act* gene, which is also present under the name *pflA* in the genome of *S. thermophilus*. It is thus likely that a posttranslational activation mechanism of PFL occurs in *S. thermophilus*. Further studies will be required to understand how the system functions and is regulated during growth in milk.

The role of PFL in the modulation of the final fermentation products prompted us to further study the control of its expression. Northern blot analysis indicated (i) that PFL synthesis is regulated at the transcriptional level in *S. thermophilus*, (ii) that the expression of *pfl* is rapidly induced during exponential growth in milk but restricted to this growth phase, and

(iii) that it is strongly reduced in the presence of formate, one of the products of the reaction it catalyzes. The amount of cellular PFL analyzed by 2-DE was in line with the observed changes in mRNA levels, as already observed for *S. bovis* (4).

The regulation of *pfl* expression and PFL activity has been intensively studied in *S. bovis* (4–10) and *L. lactis* (2, 3, 24–26). In anaerobic conditions and in the presence of slowly fermentable sugars, PFL is responsible for the shift from homolactic to mixed-acid fermentation in *L. lactis* (3, 25). In *S. bovis*, it was shown that *pfl* expression is also dependent on the nature of the fermentable carbon source via a CcpA-dependent mechanism (4, 9). In the strain LMG18311, a catabolite-responsive element (cre box) was detected upstream of *pfl* (sequence TGTAAGCGGTTACT, at −92 bp upstream of the putative ATG start codon), suggesting that the nature of the carbon source would also modulate *pfl* expression in *S. thermophilus*.

In *Escherichia coli*, the anaerobic regulation of *pfl* expression is under the control of ArcA and the global regulator FNR (fumarate and nitrate reduction) that controls transcription of genes involved in the adaptation to O_2 limiting conditions. An FNR box (or anaerobox), which is present in the promoter of the FNR-regulated genes, has been characterized upstream of *pfl* in *E. coli* (36) and *L. lactis* (3). No obvious FNR regulator or FNR boxes located upstream of the *pfl* gene were found in the nucleotidic sequence of *S. thermophilus* LMG18311. Further work would nevertheless be required to conclude about the putative role of oxygen tension in the expression of *pfl* in *S. thermophilus*. However, neither the carbon source, the pH, nor aerobic conditions can be considered major determinants of the massive increase in the synthesis of PFL observed during cultivation in milk, since these parameters are similar in the M17-Lac cultures. In milk, the low level of purines suggests that the bacterial ability to synthesize the nucleic acid constituents plays an important role in their capacity to grow at high cell density. In this context, the supply of formate could be a limiting step in the metabolism of LAB growing in milk. Several experimental evidences support this hypothesis: (i) we did not observe an increased production of formate in milk culture supernatants, suggesting that the formate produced by *S. thermophilus* is metabolized by the bacteria rather than excreted, (ii) when purines were added to milk, not only were de novo purine biosynthesis proteins (PurM, PurH) repressed but also Fhs and PFL, indicating a link between these two proteins and purine biosynthetic pathway, (iii) the growth-stimulating effect observed when formate or purines was added to milk. In conclusion, our results indicated that the major factor controlling *pfl* expression in *S. thermophilus* in milk was the simultaneous starvation for formate and purines. Consequently, the regulation of *pfl* seems to involve a sensing mechanism of the product of the PFL activity. To our knowledge, the control of *pfl* expression by formate has not been reported previously and raises questions. Is the control of PFL expression by formate observed in *S. thermophilus* a specific adaptation to milk or does it also exist in nondairy bacteria? The molecular mechanism involved in the regulation of expression of *pfl* would also deserve attention. Finally, *S. thermophilus* is grown in association with *L. delbrueckii* subsp. *bulgaricus* for the production of yogurt. At the moment, the metabolic interactions that could exist between the two species cocultivated in milk are not well defined. A global approach of the proteome of *S. thermophilus* growing in milk provided us novel data on its adaptation to this complex medium. Our future studies

will focus on dairy lactobacilli and on the interactions between these two species in milk.

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