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Postgenomic Analysis of *Streptococcus thermophilus* Cocultivated in Milk with *Lactobacillus delbrueckii* subsp. *bulgaricus*: Involvement of Nitrogen, Purine, and Iron Metabolism▽†

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***Streptococcus thermophilus* is one of the most widely used lactic acid bacteria in the dairy industry, in particular in yoghurt manufacture, where it is associated with *Lactobacillus delbrueckii* subsp. *bulgaricus*. This bacterial association, known as a proto-cooperation, is poorly documented at the molecular and regulatory levels. We thus investigate the kinetics of the transcriptomic and proteomic modifications of *S. thermophilus* LMG 18311 in response to the presence of *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 during growth in milk at two growth stages. Seventy-seven different genes or proteins (4.1% of total coding sequences), implicated mainly in the metabolism of nitrogen (24%), nucleotide base (21%), and iron (20%), varied specifically in coculture. One of the most unpredicted results was a significant decrease of most of the transcripts and enzymes involved in purine biosynthesis. Interestingly, the expression of nearly all genes potentially encoding iron transporters of *S. thermophilus* decreased, whereas that of iron-chelating *dpr* as well as that of the *fur* (*perR*) regulator genes increased, suggesting a reduction in the intracellular iron concentration, probably in response to H_2O_2 production by *L. bulgaricus*. The present study reveals undocumented nutritional exchanges and regulatory relationships between the two yoghurt bacteria, which provide new molecular clues for the understanding of their associative behavior.**

Streptococcus thermophilus is one of the most widely used lactic acid bacteria (LAB) in the dairy fermentation industry. This bacterium is traditionally used in combination with *Lactobacillus delbrueckii* subsp. *bulgaricus* for the manufacture of yoghurt. When both bacteria gain a mutual benefit, this association is known as proto-cooperation (57), and it often results in higher acidification rates (2, 7, 8, 25, 46, 50), a lower final pH (46, 53), a more abundant *S. thermophilus* population (10, 46, 53), stimulation of aromatic compound production (10, 15, 24, 30), improved stability of the final product (24), and an increase of exopolysaccharide production (11) compared to monocultures. This cooperation effect improves the yield of fermentation and is therefore of industrial interest. The characterization of this phenomenon so far evidenced that it depends on the strains of each species which is associated and is, at least partly, based on nutritional exchanges. In milk, the amounts of several free amino acids (AA) are growth limiting for LAB such as *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*, which have to degrade caseins into peptides and AA in order to fulfill their AA requirements. *L. delbrueckii* subsp. *bulgaricus* has a cell wall proteinase, PrtB, enabling the degradation of caseins, whereas only a few strains of *S. thermophilus* exhibit PrtS, an extracellular protease ortholog of PrtB (55). The peptides and AA released from caseins by *L. delbrueckii* subsp. *bulgaricus* (1, 7, 25, 50, 53) stimulate the growth of *S. thermophilus*, and PrtS does not play a significant role in the presence of PrtB$^+$ *L. delbrueckii* subsp. *bulgaricus* (16). In turn, *S. thermophilus* stimulates the growth of *L. delbrueckii* subsp. *bulgaricus* by the production of formic acid under anaerobic conditions (10, 64) and of carbon dioxide (22). Carbon dioxide results from the decarboxylation of urea catalyzed by urease, present and widely distributed only in *S. thermophilus* among LAB, which are generally recognized as safe (36, 47). This association has thus mainly been studied in terms of nutritional exchanges, but it remains poorly documented at the global level.

It is only recently and in a very limited number of studies that postgenomic approaches were used for the analysis of simple ecosystems such as bacterial cocultures. Initial work on *Pseudomonas aeruginosa* revealed that, in the presence of *Staphylococcus aureus*, the transcription of iron-regulated genes decreased in coculture, indicating that the presence of *S. aureus* increased iron availability for *P. aeruginosa* in this environment (45). A transcriptomic analysis of the hyperthermophilic bacterium *Thermotoga maritima* demonstrated that 15.5% of its genome was differentially expressed in coculture with *Methanococcus jannaschii* compared to monoculture (35). More recently, a DNA microarray analysis identified *Streptococcus gordonii* genes regulated in response to coaggregation with *Actinomyces naeslundii* (34). The only study of a dual bacterial culture by combining transcriptomics and proteomics reported up to now described the interactions of two dental

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plaque bacteria, *Streptococcus mutans* and *Veillonella parvula* (37). This study pointed out that growth in a biofilm together with a nonpathogenic bacterium such as *V. parvula* changes the physiology of *S. mutans* and gives this bacterium an advantage in surviving antimicrobial treatment.

The recent completion of the genome sequences of *S. thermophilus* CNRZ 1066, LMG 18311 (9), and LMD-9 (44) and *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 (62) allows us to reexamine the association between these bacteria at a molecular level. We recently investigated the physiology of *S. thermophilus* LMG 18311 during the late stage of milk fermentation (32), using proteomic and transcriptomic approaches. We revealed the upregulation of nitrogen metabolism (transport and biosynthetic pathways, notably for sulfur AA) and of the metabolism of various sugars. In the present work, we explored the physiology of the same strain of *S. thermophilus* (LMG 18311) cultivated in the presence of its yoghurt partner, *L. delbrueckii* subsp. *bulgaricus* (ATCC 11842), during growth in milk, by identifying the proteome and transcriptome modifications that were specific for the coculture. This study revealed an ambiguous relationship providing evidence that *S. thermophilus* benefits but also protects itself from compounds produced by *L. delbrueckii* subsp. *bulgaricus*. In addition, undocumented nutritional exchanges and hints of regulators altered during the coculture growth were evidenced, providing new molecular clues for the understanding of this dairy ecosystem.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. *S. thermophilus* LMG 18311 was obtained from the BCCM collection (Belgium), and *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 from ATCC (USA). Stock cultures were prepared in reconstituted 10% (wt/vol) Nilac skim milk (NIZO, Ede, The Netherlands) as described previously (32). Cocultures of *S. thermophilus* with *L. delbrueckii* subsp. *bulgaricus* (ratio of 1:1 CFU/ml) were grown at 42°C in Marguerite milk (La Laiterie, Villefranche sur Saône, France) and sterilized by microfiltration. Before use, the milk was skimmed by centrifugation (4°C, 5,000 × *g*, 30 min). A total of 500 ml of milk was then inoculated with 10^6 CFU/ml of stock cultures of each strain and incubated at 42°C. For coculture assays in the presence of catalase, bovine catalase (Sigma) was added to cultures after 2 h 30 min of growth at a final concentration of 1,000 U/ml.

The pH was measured until it reached a value of 4.9; the acidification rate was calculated as $\Delta pH/\Delta t$ between pH 6.2 and 5.2, i.e., before milk coagulation. Every 20 min, cell chains of *S. thermophilus* were disrupted by a 40-s treatment with a mechanical blender (Turax X620, Labo-Moderne, France), and culture dilutions were plated on M17 agar lactose (1%) (for *S. thermophilus* counts) or MRS agar lactose (2%) acidified to pH 5.2 (for *L. delbrueckii* subsp. *bulgaricus*) with an automatic spiral platter (AES Laboratoires, Combourg, France). Colonies were counted after 16 h of incubation (*S. thermophilus*) or 36 h (*L. delbrueckii* subsp. *bulgaricus*) in anaerobic conditions (Anaerocult A; Merck, Darmstadt, Germany) at 42°C. All cultures were prepared in three independent experiments.

Transcriptomic analysis. Total RNA was extracted from Marguerite milk cocultures with the Trizol method as described previously (32). RNA was extracted at 2 h 30 min (as a control) and at 5 h 30 min (with or without catalase).

Genome-wide expression profiles were established using a commercial DNA microarray (EGT-K40C, Eurogentec) containing 92% of *S. thermophilus* LMG 18311 genes (spotted in duplicate), according to the method described by Hervé-Jiménez et al. (32). The cross-hybridization of *L. delbrueckii* subsp. *bulgaricus* ATCC 11842-labeled cDNA with the *S. thermophilus* LMG 18311 microarray was expected to be minimal, since *L. delbrueckii* subsp. *bulgaricus* RNA comprised a small fraction of the coculture total RNA (less than 10%, checked by real-time quantitative reverse transcription-PCR [RT-qPCR]). RNA from *L. delbrueckii* subsp. *bulgaricus* was labeled using the protocols described above, and by hybridizing the labeled samples to the *S. thermophilus* microarrray, we verified that *L. delbrueckii* subsp. *bulgaricus* RNA did not cross-hybridize.

A total of 10 μg of total RNA was reverse transcribed by random priming, using the Pronto! plus direct system (Corning-Promega, United States) and labeled by incorporation of Cy3- or Cy5-dCTP nucleotides (Amersham Biosciences, United Kingdom). A total of 100 pmol of each labeled cDNA was used for overnight hybridization at 42°C. The arrays were scanned on a microarray scanner (Agilent, United States). The statistical analysis was based on dye swap. For each array, the raw data comprised the logarithm of the median feature pixel intensity at wavelengths of 635 and 532 nm. No background was subtracted. Arrays were normalized with the Anapuce package (http://cran.r-project.org/web/packages/anapuce/), using general loess and a block effect correction. In order to determine differentially expressed genes, we used the Varmixt method, which is based on a variance mixture analysis (19). Finally, the raw *P* values were adjusted by the Bonferroni method, which controls the family-wise error rate. We considered genes with both a *P* value of ≤0.05 and a ratio higher than 2 to be differentially expressed.

RT-qPCR was carried out using cDNA synthesized from 3 μg of RNA samples by PowerScript reverse transcriptase (ClonTech, Saint-Quentin-en-Yvelines, France) according to the supplier's protocol. All gene-specific primers were designed using Primer3 software (54) and are reported in Table S1 in the supplemental material. For each condition, the measures were done in triplicate, with cDNAs synthesized from two independent RNA samples. Data were recorded as threshold cycles (C_T), expressed as means ± standard deviations, and computed using the comparative critical threshold ($2^{-\Delta\Delta C_T}$) method (43). The results were normalized using stu1254 for *S. thermophilus* and *gmk1* for *L. delbrueckii* subsp. *bulgaricus* as references, as they were expressed at a constant level under our conditions (microarray data, present work).

Proteomic analysis. Cytoplasmic proteins were extracted from 300 ml of milk cocultures at two different growth stages (early [2 h 30 min] and late exponential [5 h 30 min]) and separated by two-dimensional gel electrophoresis (2-DE) as described previously (32). Briefly, 300 μg of proteins was precipitated with the 2-DE clean-up kit in 10% trichloroacetic acid (GE Healthcare, Saclay, France) and loaded on 24-cm (pH 4 to 7) IPG strips (Bio-Rad, Hercules, CA), which were rehydrated at 50 V for 12 h, and isoelectric focusing was carried out for 60,000 V/h at a maximum of 10,000 V using an Ettan IPGphor (GE Healthcare); 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used for the second migration. Gels were stained with BioSafe colloidal Coomassie blue (Bio-Rad); they were digitized using an Epson Expression 1640XL scanner set (at 256 gray levels) controlled by the Silver Fast software and analyzed using the Phoretix 2-DE software package (GE Healthcare).

The 2-DE images obtained at the two growth stages and from three independent experiments were compared and an analysis of variance was performed using the statistical software R as described previously (32). Only differences with *P* values of <0.05 and at least twofold volume variations between the two conditions were further analyzed. When a value was missing for one of the triplicate experiments, it was set to the mean value of the two other values, as proposed in reference 14. Proteins that were significantly altered in abundance between the two growth stages were identified by mass spectrometry analyses using a Voyager-DE-STR mass spectrometer (Applied Biosystems, Framingham, MA) on our proteomic platform (http://www.jouy.inra.fr/unites/proteines/papss/), according to Guillot et al. (29), except that the monoisotopic mass lists were searched against local *S. thermophilus* LMG 18311 (9) and *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 (62) databases using a local version of the MS-FIT program (http://prospector.ucsf.edu).

The codon adaptation index was calculated for all open reading frames of the *S. thermophilus* LMG 18311 genome with the synonymous codon usage analysis program with Codonmixture 1.0 (P. Nicolas, personal communication).

Determination of the E_h and partial pressure of dissolved O_2. Monocultures of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* were cultivated in the Biostat Q fermentor (B. Braun Biotech International, Melsunge, Germany) in 1-liter vessels with a working volume of 500 ml skim Marguerite milk. The temperature was set at 42°C, and the cultures were stirred at 22 rpm. The E_m (redox electrode, Einstabmesskelte, EasyFerm plus K8RX; Hamilton, Switzerland), partial pressure of dissolved O_2 (Oxyferm O_2 sensor FDA 160; Hamilton, Switzerland), pH (pH sensor EasyFerm Plus K8; Hamilton, Switzerland), and temperature were continuously monitored as previously reported (39) with the software MFCS Shell/win 2.0 (B. Braun Biotech, International Sartorius Group). Corrections of the pH and temperatures of E_m measures were performed with the hydrogen electrode as a reference. The final value of E_h (mV) was expressed as $E_h = E_m + E_{ref}$, where $E_{ref} = +198$ mV at 37°C.

RESULTS

The coculture in milk of *S. thermophilus* LMG 18311 and *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 favors *S. thermophilus* growth. We first characterized the growth of both LAB in

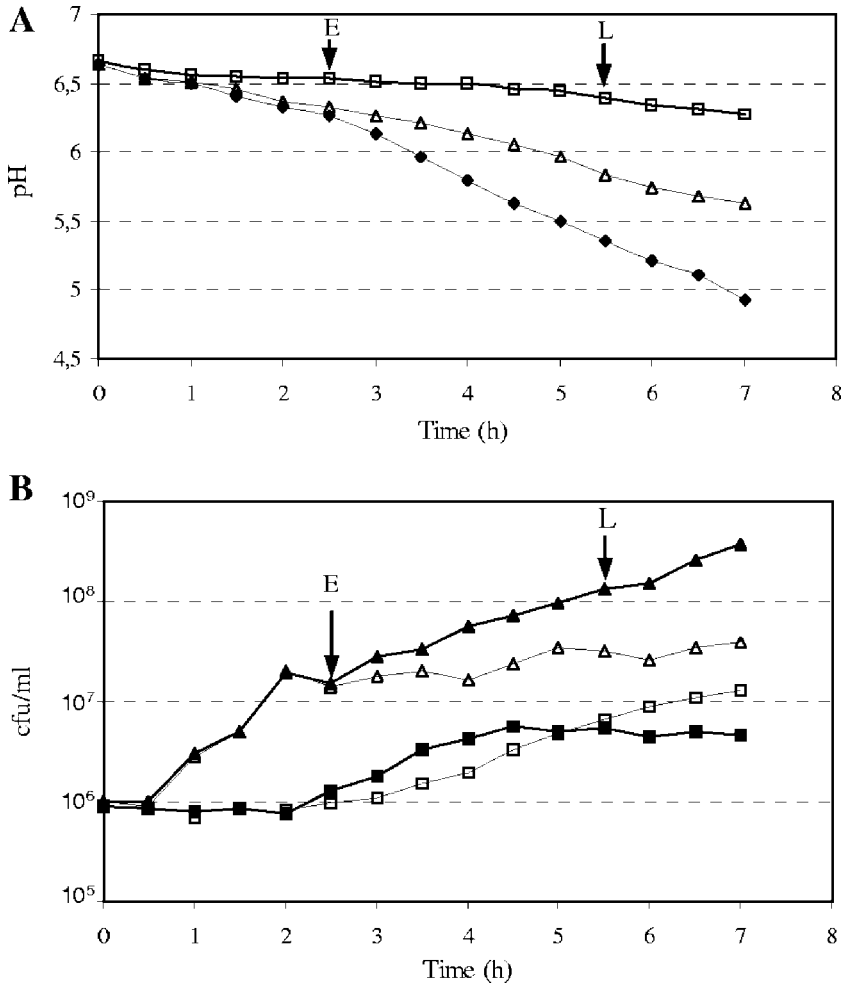

FIG. 1. Milk growth of *S. thermophilus* LMG 18311 in mono- and cocultures with *L. delbrueckii* subsp. *bulgaricus* ATCC 11842. (A) pH evolution of monocultures of *S. thermophilus* LMG 18311 (Δ) and *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 (□) and of coculture (◆). (B) Evolution of bacterial counts of *S. thermophilus* (Δ) and *L. delbrueckii* subsp. *bulgaricus* (□) in monoculture and of *S. thermophilus* (▲) and *L. delbrueckii* subsp. *bulgaricus* (■) in coculture. These graphs are representative of the curves obtained in three independent experiments. At early (E) (2 h 30 min) and late (L) (5 h 30 min) exponential phases (arrows), bacteria were harvested for proteomic and transcriptomic analysis.

mono- and cocultures in milk by monitoring the pH and species-specific bacterial counts. Compared to results with the monocultures, the acidification of milk was enhanced when the two LAB were cultivated together (Fig. 1A). The coculture presented significantly higher ΔpH values (0.38 ± 0.02) (between 0 and 4 h) than *S. thermophilus* (0.31 ± 0.02) and *L. delbrueckii* subsp. *bulgaricus* (0.11 ± 0.02) monocultures. Similarly, the acidification rate ($\Delta pH/\Delta t$) of the coculture (0.31 h^{-1} ± 0.02) was 1.8- and 1.3-fold higher than that of *S. thermophilus* (0.17 h^{-1} ± 0.02) and of *L. delbrueckii* subsp. *bulgaricus* (0.23 h^{-1} ± 0.02) monocultures, respectively.

Consistently, species-specific bacterial counts differed between the mono- and the cocultures (Fig. 1B). During the first 2 h to 2 h 30 min, the growth curves of the mono- or cocultures superimposed. After 2 h 30 min, the cocultures resulted in higher bacterial counts than each of the monocultures. The bacterial counts revealed that the stimulatory effect was transitory for *L. delbrueckii* subsp. *bulgaricus*, which stopped growing after 4 h 30 min, but lasted until the end of fermentation for *S. thermophilus*. For *L. delbrueckii* subsp. *bulgaricus*, the coculture resulted in a threefold decrease of the final bacterial counts (6.0×10^6 CFU/ml, compared to 2.0×10^7 CFU/ml in monoculture). For *S. thermophilus*, the association with *L. delbrueckii* subsp. *bulgaricus* resulted in a 10-fold-higher final population (3.7×10^8 CFU/ml) than that of the monoculture (3.9×10^7 CFU/ml). At the end of fermentation (5 h 30 min), LMG 18311 significantly dominated *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 in the coculture, with 60-fold more cells.

Transcriptome and proteome analysis of LMG 18311 cultivated in milk with ATCC 11842. The effects of *L. delbrueckii* subsp. *bulgaricus* on the physiology of LMG 18311 were further analyzed using transcriptomics and proteomics on samples harvested at two stages of growth, where *S. thermophilus* development was stimulated by the presence of *L. delbrueckii* subsp. *bulgaricus*: early (2 h 30 min) and late (5 h 30 min) exponential phase. The evolution of the gene expression and protein abundance of *S. thermophilus* at these two stages was thus established and compared to that of *S. thermophilus* monoculture

(32). In mono- as well as in coculture, we observed the upregulation of (i) peptides, AA transporters, and specific AA biosynthetic pathways, notably for sulfur AA, and (ii) genes and proteins involved in the metabolism of various sugars, although the effect on sugar metabolism was less pronounced in coculture. The variations that were obtained strictly in coculture were thus considered specifically related to the presence of *L. delbrueckii* subsp. *bulgaricus* and are reported in the present work (Tables 1 and 2).

First, to identify *S. thermophilus* genes differentially expressed during the coculture in milk, the expression of 1,759 coding sequences (92% of the genome) was assessed with LMG 18311 microarrays before and during the stimulatory effect of *L. delbrueckii* subsp. *bulgaricus*, i.e., at 2 h 30 min and 5 h 30 min of coculture, respectively. According to our statistical analysis, the levels of expression of 67 genes of *S. thermophilus* LMG 18311 (3.8% of the spotted genes) varied in the presence of *L. delbrueckii* subsp. *bulgaricus* at 5 h 30 min of growth compared to 2 h 30 min. A total of 43 and 24 genes corresponding to 48 putative transcriptional units were upregulated and downregulated, respectively. Of these, approximately 47% showed more than a fourfold alteration in transcript levels. In order to independently confirm microarray results, the transcript levels of 21 genes, representatives of the main pathways modulated during *S. thermophilus* growth with *L. delbrueckii* subsp. *bulgaricus*, were measured by RT-qPCR. For 13 of them, RT-qPCR results confirmed microarray data (underlined in Table 1), while for the others, RT-qPCR data showed significant variations not detected by microarrays; *feoC* was not spotted on the microarray, and expression of *mntH* did not significantly vary in RT-qPCR. These discrepancies probably reflect differences in the relative sensitivities and specificities of the two methods.

In parallel, a proteomic analysis based on 2-DE was performed on the same samples as those used for transcriptomics. The proteomes of three independent cultures at 2 h 30 min and 5 h 30 min of growth in milk were compared. The statistical analysis revealed that 21 proteins were significantly altered between the two growth stages (P value of <0.05 and at least twofold volume variations): 8 and 13 became more and less abundant, respectively (Table 2 and Fig. 2). Of these proteins, approximately 35% (8/21 [in bold in Table 2]) varied in the same way at the RNA level, indicating a correlation between transcriptomic and proteomics data.

Taken together, the proteomic and transcriptomic analyses revealed 77 different genes or proteins (4.1% of total coding sequences) that significantly varied during the growth of *S. thermophilus* in coculture with *L. delbrueckii* subsp. *bulgaricus*. These variations triggered mostly nitrogen metabolism (24% of the altered genes/proteins), nucleotide base metabolism (21%), and iron metabolism (20%) (see below). The other modifications affected nucleic acid metabolism (9%), translation (6%), stress responses (6%), and carbon metabolism (mannose metabolism) (3%).

Main modifications in LMG 18311 during growth with ATCC 11842. Nitrogen metabolism. In the coculture between 2 h 30 min and 5 h 30 min, the AA transport systems and biosynthesis pathways were upregulated. Although the specificities of the four polar AA transporters which were overexpressed were not experimentally established, one of them, encoded by stu0158-0159 and sharing homologies with a *Streptococcus sanguinis* Arg/His transporter, could be involved in the transport of arginine.

Regarding the AA biosynthesis, the branched-chain AA (BCAA) and Arg pathways were massively induced, while the overexpression of the *thrB* gene indicated that the Thr biosynthesis may also be modified. Concerning the BCAA, four enzymes (IlvBN, IlvC, IlvD1, and BcaT) are needed to produce Val and Ilv from pyruvate, while to produce Leu, the LeuA, LeuD, and LeuB enzymes are required. The transcription of *ilvC* and *ilvD1* and the abundance of IlvC, IlvB, and LeuB increased between 2 h 30 min and 5 h 30 min, suggesting a higher production of BCAA in the cells. Interestingly, we observed a concomitant increase of IleS and ValS proteins (tRNA synthetases), necessary to load Ilv and Val AA on their respective tRNAs during translation.

Several evidences indicated that the requirement for de novo Arg biosynthesis increased during LMG 18311 growth in coculture. From glutamate, four enzymes, ArgJ, ArgB, ArgC, and ArgD, are involved in the biosynthesis of ornithine, which is then transformed into Arg by three enzymes, ArgF, ArgG, and ArgH. Note that the last enzyme coproduces fumarate and Arg from arginosuccinate. During the growth of LMG 18311, the transcription of *argCJBD* and *argH* increased, and RT-qPCR confirmed a 57-fold overexpression of *argH*. Consistently, fumarate was produced in the coculture medium; we measured 45 μM of fumarate at time zero and 131 μM after 5 h of growth. We also observed a 3.7-fold increased expression of *argR*, the putative Arg regulator, suggesting an activator role for ArgR.

Nucleic base metabolism. In all organisms, nucleotides are essential; they are substrates for RNA and DNA synthesis and are the main energy donors for cellular processes. In milk, purine nucleotides are growth limiting for *S. thermophilus* and the purine biosynthesis pathway is essential for its optimal growth (27). Thus, one of the most unexpected results of our analysis was the downregulation of the vast majority of the genes and corresponding enzymes involved in purine biosynthesis. In silico analysis of LMG 18311 indicated that the purine nucleotides are synthesized from PRPP (5-phosphoribosyl-α-1-pyrophosphate) to IMP via nine different enzymes; the pathway then split in two, leading to AMP or GMP, thanks to two enzymes for each of them (Fig. 3). Between 2 h 30 min and 5 h 30 min of growth, 10 genes and seven proteins involved in this pathway were downregulated. Importantly, we also observed a decrease in the transcription of *prsA1* and *fhs* and in the level of the Fhs protein, which are required for the purine biosynthesis for the supply of PRPP and formyl groups, respectively. All together, these results show that during the growth of LMG 18311 with *L. delbrueckii* subsp. *bulgaricus* in milk, the biosynthesis of purines was switched off at the transcription level. Consistently, a 14-fold increase of the putative purine repressor PurR was observed. It is noteworthy that the expression of stu0336, a homolog of the nucleobase transporter *pbuX* of *Lactococcus lactis*, increased 2.4-fold.

Iron metabolism. The transcription of the following five of the seven genes of LMG 18311 potentially involved in iron transport was downregulated between 2 h 30 min and 5 h 30 min of growth: an ABC transporter of iron complexes (*fatA*, *B*,

TABLE 1. Changes in *S. thermophilus* mRNA levels in coculture with *L. delbrueckii* subsp. *bulgaricus* between early and late exponential phases

Gene name or locus tag (stu no.)[d]	Functional category	Description	Fold change L vs E[a]		Putative operon structure[b]
			Microarray	RT-qPCR	
0158	AA and peptide transporter	Polar AA ABC transporter, ATP binding protein	3.9		stu0159-0158
0159		Polar AA ABC transporter, permease	2.4[c]		stu0159-0158
0605		Polar AA ABC transporter, permease	4.6		stu0605-0606
1579		Polar AA ABC transporter, substrate binding protein	2.7[c]		stu1582-1581-1580-1579
1580		Polar AA ABC transporter, ATP binding protein	4.4		stu1582-1581-1580-1579
1652		Polar AA ABC transporter, ATP binding protein	3.5		stu1653-1652
1653		Polar AA ABC transporter, permease	3.9		stu1653-1652
argR	AA metabolism	Arginine repressor	3.7		*argR*-stu0049-*mutS1*
argC		*N*-Acetyl-gamma-glutamyl-phosphate reductase	2.7[c]		*argCJBD*
argJ		Bifunctional ornithine acetyltransferase/*N*-acetylglutamate synthase protein	6.3		*argCJBD*
argB		Acetylglutamate kinase	4.5		*argCJBD*
argD		Acetylornithine aminotranferase	4.9		*argCJBD*
ilvD		Dihydroxy-acid dehydratase	3.0[c]	2.0 ± 0.2	*ilvD*
argH		Arginosuccinate lyase	3.2[c]	57.5 ± 10	*argH*
ilvC		Ketol-acid reductoisomerase	5.6	2.1 ± 0.4	*ilvC*
ilvB		Acetolactate synthase, large subunit	NV	NV	*ilvBN*
leuB		3-Isopropylmalate dehydrogenase	NV	NV	*leuAB*
thrB		Homoserine kinase		2.8[c]	*thrB-rarD*
aldB		Alpha-acetolactate decarboxylase	NV	NV	*als-aldB*
1413		Bifunctional glutamate-cysteine ligase	6.6		stu1413
0336	Purine and pyrimidine metabolism	Putative MFS transporter, xanthine/uracil permease	2.4[c]	1.8 ± 0.40	stu0336
prsA1		Ribose-phosphate pyrophosphokinase	0.24	0.14 ± 0.05	*prsA1*
purC		Phosphoribosylaminoimidazole-succinocarboxamide synthase	0.12	0.39 ± 0.10	*purCLFMNH*
purL		Phosphoribosylformylglycinamidine synthase II	0.14	0.03 ± 0.01	*purCLFMNH*
purF		Amidophosphoribosyltransferase	0.19		*purCLFMNH*
purM		Phosphoribosylformylglycinamide cyclo-ligase	0.36[c]		*purCLFMNH*
purN		Phosphoribosylformylglycinamide formyl transferase	0.3[c]		*purCLFMNH*
purH		Phosphoribosylaminoimidazole carboxamide formyl transferase	0.26[c]		*purCLFMNH*
purD		Phosphoribosylamine-glycine lyase	0.3[c]		*purDEK*
purE		Phosphoribosylaminoimidazole carboxylase I	0.36[c]		*purDEK*
purB		Adenylosuccinate lyase	0.59	0.22 ± 0.08	*purB*
purA		Adenylosuccinate synthetase	NV	0.40 ± 0.14	*purA*
purR		Purine operon repressor	NV	1.90 ± 0.40	*purR*
pyrC		Dihydroorotase	5.8		*ung-pyrC*
feoA	Metal ion metabolism	Ferrous iron uptake transporter, protein A	NV	0.33 ± 0.15	*feoABC*
feoB		Ferrous iron uptake transporter, protein B	NV		*feoABC*
feoC		Hypothetical protein	NP	0.43 ± 0.2	*feoAB*
mntH		Mn^{2+} transporter	3.6	1.7 ± 0.2	*mntH*
dpr		Peroxide resistance protein, non-heme-containing ferritin	18.3	13.7 ± 4.0	*dpr-fur*
fur		Ferric transport regulator protein	8.1		*dpr-fur*
tatA		Sec-independent protein translocase	NV	0.14 ± 0.02	stu1024-1023-1022-*tatC-tatA*
tatC		Sec-independent protein translocase	0.4[c]		stu1024-1023-1022-*tatC-tatA*
1022		Predicted membrane protein of the lead (Pb^{2+}) uptake porter (PbrT) family	0.3		stu1024-1023-1022-*tatC-tatA*
1023		Hypothetical protein, putative iron-dependent peroxidase	0.4[c]		stu1024-1023-1022-*tatC-tatA*
1024		Hypothetical protein (predicted lipoprotein)	0.4[c]		stu1024-1023-1022-*tatC-tatA*
fatB		Iron complex ABC transporter, substrate binding protein	0.4[c]		*fatDCAB*
fatA		Iron complex ABC transporter, ATP binding protein	0.2		*fatDCAB*
fatC		Iron complex ABC transporter, permease	0.4[c]		*fatDCAB*
hrcA	Stress	Heat-inducible transcription repressor	NV	0.16 ± 0.07	*hrcA-grpE-dnaK*
grpE		Heat shock protein, chaperonine	0.3		*hrcA-grpE-dnaK*
dnaK		Molecular chaperone	NV		*hrcA-grpE-dnaK*
htpX		Heat shock protein	4.5		*htpX*
htrA		Exported serine protease	4		*htrA*
0808	C metabolism	Simple sugar transport system, substrate-binding protein	0.3	0.11 ± 0.01	stu0806-0807-0808-0809-0810-0811
manN		Mannose PTS system, component IID	4.4		*manLMN*-stu0330
manM		Mannose PTS system, component IIC	NV		*manLMN*-stu0330
manL		Mannose PTS system, component IIAB	3.6		*manLMN*-stu0330
mutS1	DNA and RNA metabolism	DNA mismatch repair protein	2.4[c]		*argR*-stu0049-*mutS1*
polC		DNA polymerase III	5.0		*polC*
ung		Uracyl DNA glycosylase	8.7		*ung-pyrC*
rhe		ATP-dependent RNA helicase	6.0		*rheA*
ssbB		Single-strand binding protein	3.5		*rpsF-ssbB-rpsR*
mutY		A/G-specific adenine glycosylase	13.2		*mutY*
radA		DNA repair protein	0.3		*radA*

Continued on following page

TABLE 1—*Continued*

Gene name or locus tag (stu no.)[d]	Functional category	Description	Fold change L vs E[a]		Putative operon structure[b]
			Microarray	RT-qPCR	
rplU	Ribosome and translation	50S ribosomal protein L21	4.2		*rplU-rpmA*
rpmA		50S ribosomal protein L27	5.8		*rplU-rpmA*
gatB		Glutamyl-tRNA Gln amidotransferase, subunit B	10.5		*gatCAB*
rpsF		30S ribosomal protein S6	3.4		*rpsF-ssbB-rpsR*
0049	Other	Hypothetical protein	5.6		*argR*-stu0049-*mutS1*
0103		Hypothetical protein	3.6		stu0103
0110		Hypothetical protein poly-gamma glutamate synthesis (capsule biosynthesis)	0.2		stu0110-0112-0113
0161		Hypothetical protein	5.0		stu0161
rr01		Response regulator	NV	0.26 ± 0.18	*rr01-hk01*
rarD		Chloramphenicol sensitivity protein	3.5		*thrB-rarD*
fhs		Formate-tetrahydrofolate ligase	0.44[c]		*fhs*
0800		Methyl transferase	0.3		stu0800-0801
0912		Hypothetical protein	4.1		stu0912
oxlT		Oxalate/formate antiporter	0.2		*oxlT*
ksgA		Dimethyladenosine transferase	4.9		*tatD*-stu1800-*ksgA*
1896		Predicted membrane protein	4.5		stu1896

[a] E, early exponential phase (2 h 30 min); L, late exponential phase (5 h 30 min); L versus E, ratio between the relative mRNA levels at 5 h 30 min and 2 h 30 min; NV, no variation; NP, not spotted on the microarray.
[b] Determined with ProFinder software (http://www.softberry.com/all.htm).
[c] Significantly differentiated genes without Bonferroni correction belonging to the putative operons identified.
[d] Underlined genes are genes with similar variations, as determined by microarray and RT-qPCR analyses.

and *C*), a ferrous iron transporter (*feoA*), and a putative high-affinity iron permease (stu1022). Interestingly, the *dpr* gene, which codes for an intracellular ferritin (i.e., an iron chelator) and is involved in H_2O_2 tolerance in streptococci (52), was the highest induced gene between 2 h 30 min and 5 h 30 min, with an induction factor of ~18. In LMG 18311, *dpr* forms a putative operon with a *perR*-like gene homolog (annotated *fur*), the putative regulator of iron transport, which was induced ~eightfold. All together, our data establish that at the late growth stage, LMG 18311 induced several systems to limit its intracellular iron concentration. It was previously reported that *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 can produce H_2O_2 during its growth in M17 (61). We therefore wondered whether the observed modification of the LMG 18311 iron metabolism could be a coordinated response aimed at limiting the production of damageable reactive oxygen species by the Fenton reaction, which happens with H_2O_2 in the presence of an iron catalyst.

Stress. It is obvious that among the genes or proteins altered during the LMG 18311 growth in coculture, several are involved in stress adaptation. Intriguingly, several were upregulated, while others were downregulated. It is therefore difficult to conclude whether LMG 18311 is at least partly stressed or not at 5 h 30 min of growth in the presence of *L. delbrueckii* subsp. *bulgaricus*. Beyond the upregulation of *dpr*, the upregulation of *htrA* (serine protease) and *htpX* (heat shock protein) could be related to stress, as is the upregulation of the *mutS*, *mutY*, and *ung* genes, coding for DNA glycosylases involved in DNA repair relative to oxidative stress damages (18, 66). However, we observed a downregulation of Gor, the glutathione reductase, and *radA*, which belong to the oxidative stress response. Moreover, the transcription of *hrcA* and *grpE*, coding for stress responsive proteins, decreased by threefold between 2 h 30 min and 5 h 30 min. In many *Firmicutes*, HrcA is the repressor of the *hrcA-grpE-dnaK* and *groEL-groES* operons, which encode chaperonins involved in different stress responses (heat shock, acid stress, salt stress, and oxidative stress) (5, 63). The expression of *rr01* also decreased ~3.8-fold, whereas the corresponding protein increased 2.2-fold. This two-component response regulator (RR01) is homologous to the CovR protein described for other streptococci (28, 41) and thus may be involved in the regulation of general stress responses.

Additional data which strengthen the hypothesis of H_2O_2 production by *L. delbrueckii* subsp. *bulgaricus*. We hypothesized that *L. delbrueckii* subsp. *bulgaricus* produces H_2O_2 during growth in milk. Since the production of H_2O_2 by ATCC 11842 was probably too low (<100 μM) to be directly measured in milk by classical means ($TiCl_4$ or peroxydase methods, data not shown), we monitored the oxido-reduction potential (E_h) of monocultures in milk. In contrast to the *S. thermophilus* monoculture, the *L. delbrueckii* subsp. *bulgaricus* monoculture presented an increase of E_h from 2 to 5 h, indicating the production of a molecule more oxidant than O_2, which is likely to be H_2O_2 (data not shown). The expression of the *L. delbrueckii* subsp. *bulgaricus* genes potentially involved in H_2O_2 production (from in silico analysis, *pox1*, *pyrD1*, and *pyrD2*) was determined by qPCR in cocultures at 2 h 30 min and 5 h 30 min of growth. While the pyruvate oxidase (pyruvate + phosphate + O_2 ⇒ acetyl phosphate + CO_2 + H_2O_2) *pox1* expression did not vary during the growth of *L. delbrueckii* subsp. *bulgaricus* in coculture, those of the two dihydroorotate dehydrogenase (dihydroorotate + O_2 ⇒ orotate + H_2O_2)-encoding genes *pyrD1* and *pyrD2* increased by factors of 6.4 (± 0.3) and 7.7 (± 0.43), respectively. Furthermore, we observed an increase (by a factor of 13.2 ± 0.6) in the expression of the gene *pyrF*, coding for an orotidine-5′-phosphate decarboxylase involved in the following steps of orotate metabolism. These results demonstrated that the H_2O_2 production pathway of *L. delbrueckii* subsp. *bulgaricus* is activated in coculture.

To test our hypothesis which links the downregulation of iron transporters and the induction of *dpr* observed in coculture to the presence of H_2O_2 in the medium, the coculture was supplemented with catalase, an enzyme which converts H_2O_2 into O_2 and H_2O. The expression of the *dpr* and *feoA* genes was

TABLE 2. Changes in *S. thermophilus* protein abundance in coculture with *L. delbrueckii* subsp. *bulgaricus* between early and late exponential phases

Gene name or locus tag (stu no.)[d]	Functional category	Description	Spot	Fold change L vs E[b]		
				2-DE	Array	RT-qPCR
ilvC	AA metabolism	Ketol-acid reductoisomerase	232	2.4	5.6	2.1 ± 0.4
			239[a]	1.7		
ilvB		Acetolactate synthase, large subunit	92	4.8	NV	1.6 ± 0.4
leuB		3-Isopropylmalate dehydrogenase	227	2.0	NV	1.4 ± 0.2
purC	Purine and pyrimidine metabolism	Phosphoribosylaminoimidazole-succinocarboxamide	330	0.5	0.12	0.39 ± 0.10
purL		Phosphoribosylformylglycinamidine synthase II	12	NV	0.14	0.03 ± 0.01
			13	NV		
			16	0.48		
			17[a]	0.46		
purH		Phosphoribosylaminoimidazole carboxamide formyl transferase	103	0.24	0.26	
			105[a]	0.18		
purD		Phosphoribosylamine-glycine lyase	205	0.5	0.3	
			204[a]	0.45		
purB		Adenylosuccinate lyase	178	0.26	0.59	0.22 ± 0.08
purA		Adenylosuccinate synthetase	179[a]	0.21		0.40 ± 0.14
			201	NV		
purR		Purine operon repressor[c]	303	14	NV	1.90 ± 0.40
guaA		GMP synthase	106	0.37	NV	
			109[a]	0.5		
			131	0.56		
pyrC		Dihydroorotase	191[a]	1.7	5.8	
			206	NV		
gor	Stress	Glutathione reductase	172	0.35	NV	
gpsA	C metabolism	Glycerol-3-phosphate dehydrogenase [NAD(P)$^+$]	242	0.42	NV	
galU		UDP-glucose pyrophosphorylase	264	0.24	NV	
exoA	DNA and RNA metabolism	3′ exo DNase III	293	3.3	NV	
ileS	t-RNA synthetase	Isoleucyl-tRNA synthetase	22	5.3	NV	
valS		Valyl-tRNA synthetase	28	6.9	NV	
pepT	Peptidase, protease	Peptidase T[c]	156	0.33	NV	
rr01	Transcriptional regulator	Response regulator	341	2.0	NV	0.26 ± 0.18
folP	Other	Dyhydropteroate synthase	327	2.7	NV	
fhs		Formate-tetrahydrofolate ligase	115	0.38	0.44	
			118[a]	0.48		
0113		Hypothetical protein	249[a]	0.13	0.43	
			274	NV		

[a] Most abundant form of the protein.
[b] E, early exponential phase (2 h 30 min); L, late exponential phase (5 h 30 min); L versus E, ratio between the relative mRNA levels at 5 h 30 min and 2 h 30 min; NV, no variation.
[c] Spot of low intensity and missing in one of the three repetitions at the early exponential phase (2 h 30 min).
[d] Boldface type indicates genes with similar variations by 2-DE and transcriptomic analyses. $P < 0.05$ for 2-DE and microarray assays.

measured by RT-qPCR at 2 h 30 min and 5 h 30 min in the presence or absence of catalase. As expected, in the control without catalase, *dpr* and *feoA* were up- and downregulated, respectively, between the two growth stages. However, when catalase was added to the coculture at 2 h 30 min, the transcription of *dpr* and *feoA* stayed constant during growth (ratio between 2 h 30 min and 5 h 30 min equaled 0.92 [±0.2] for *dpr* and 0.8 [±0.5] for *feoA*). This observation established that the modulation of expression of these genes correlated with the presence of H_2O_2, thereby confirming our hypothesis.

DISCUSSION

The proto-cooperation between *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* has been described so far in terms of nutritional exchanges, with *L. delbrueckii* subsp. *bulgaricus* supplying peptides and AA to *S. thermophilus* and, in turn, with *S. thermophilus* producing formic acid and carbon dioxide, which stimulate the *L. delbrueckii* subsp. *bulgaricus* growth. In this work, this phenomenon was analyzed for the first time through global monitoring of transcription and protein abundance at two stages of the growth of the LMG 18311 and ATCC 11842

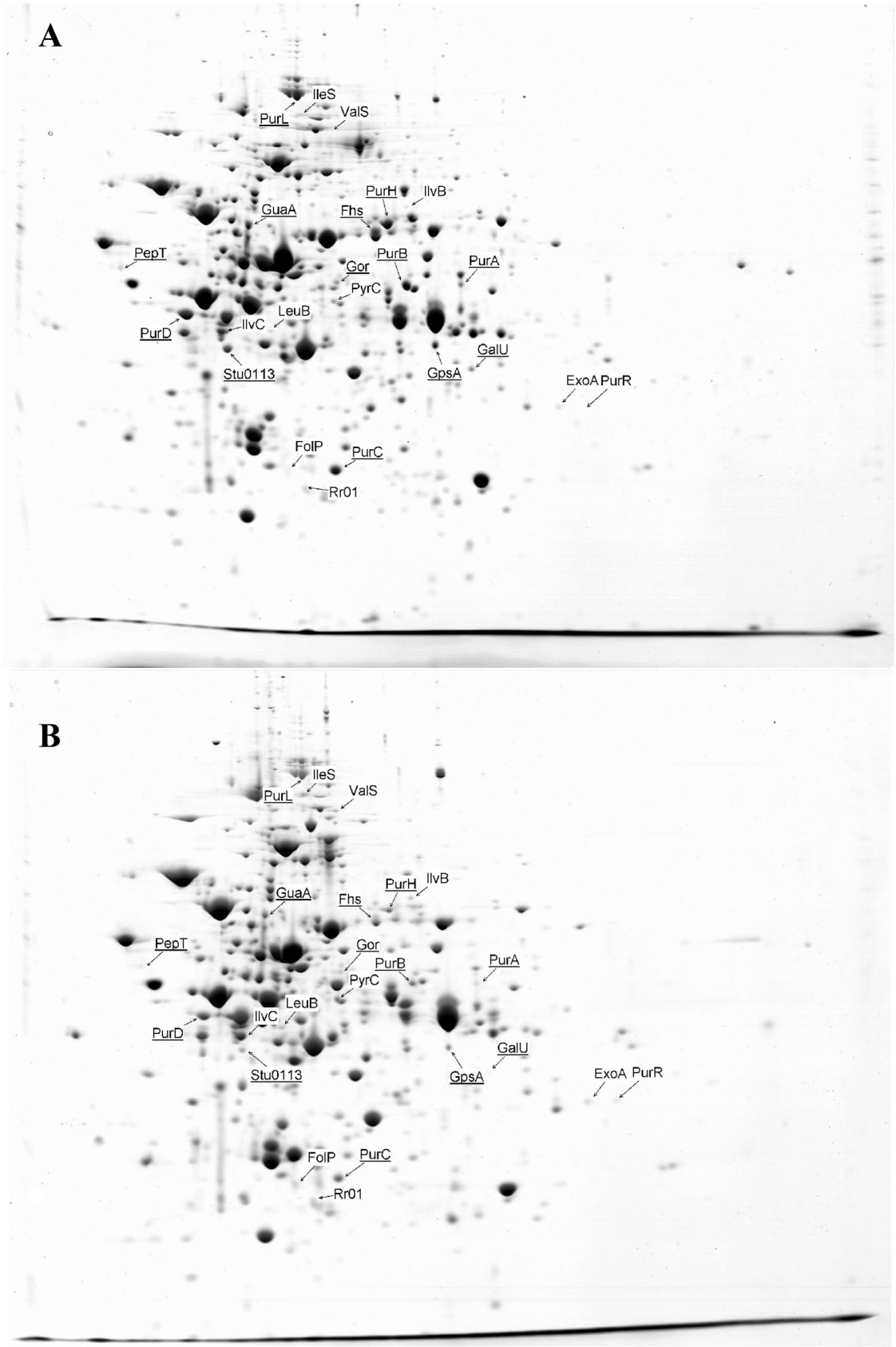

FIG. 2. 2-DE (pH gradient 4 to 7) of cytosolic proteins of *S. thermophilus* LMG 18311 cocultivated in milk with *L. delbrueckii* subsp. *bulgaricus* at early (2 h 30 min) (A) and late (5 h 30 min) (B) exponential phases. A total of 300 μg of proteins were loaded in the first dimension. Proteins whose abundance significantly varied between the two conditions are shown by the arrows as well as the name of their corresponding genes (proteins whose abundance decreases or increases at the early versus late exponential phase are underlined or not underlined, respectively).

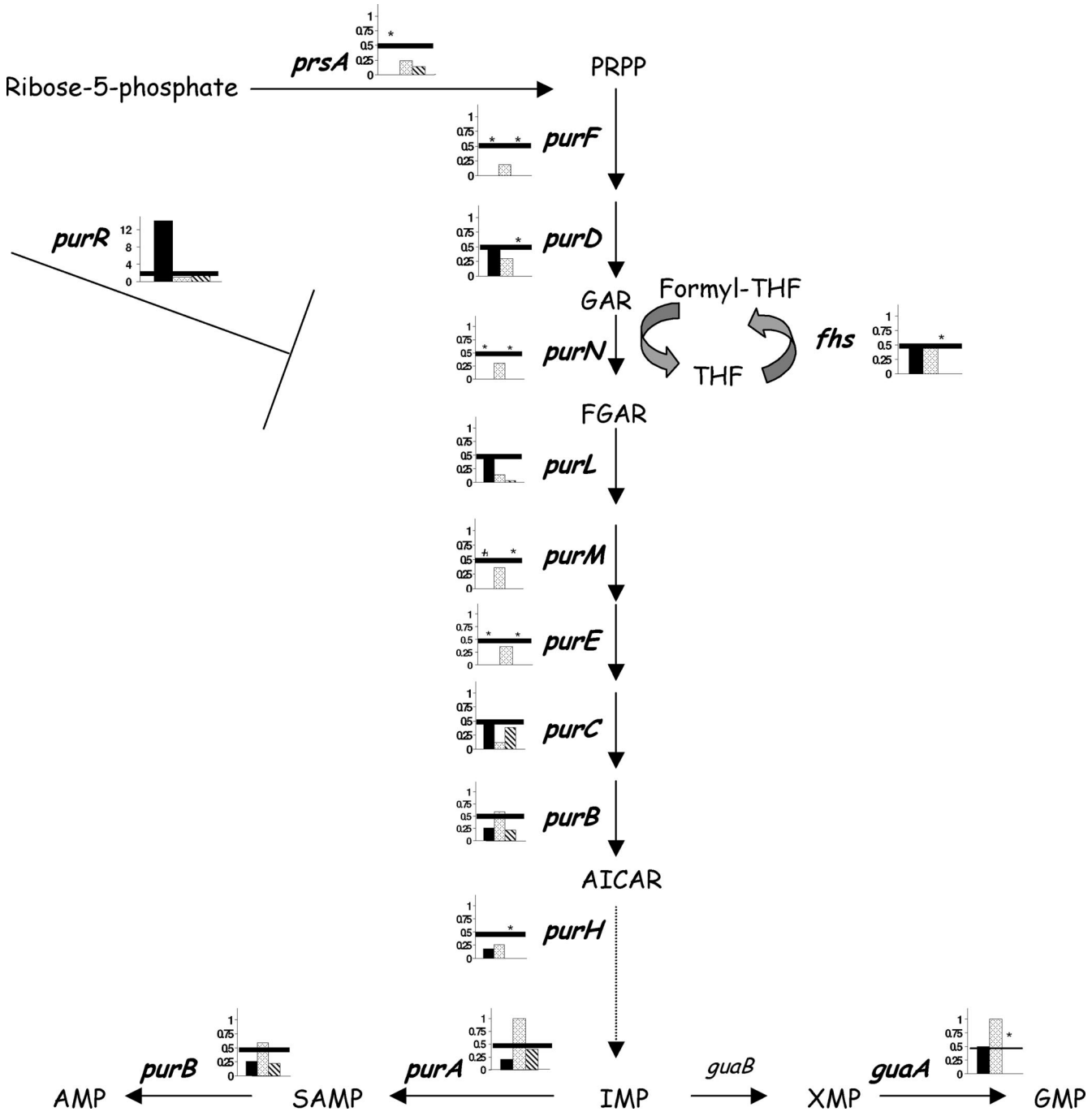

FIG. 3. Comparative analysis of protein abundance (black) and gene expression (dots for microarray analysis and hatching for RT-qPCR analyses) for the purine biosynthesis pathway between 2 h 30 min and 5 h 30 min of growth of *S. thermophilus*. ∗, not detected on 2-DE gels or on microarrays, or not measured by RT-qPCR; #, quantification not possible because of the presence of two proteins in the same spot; bar, a variation ≥ 2. *purA*, adenylosuccinate synthetase; *purB*, adenylosuccinate lyase; *purC*, phosphoribosylaminoimidazole-succinocarboxamide synthetase; *purD*, phosphoribosylamine-glycine ligase; *purE*, phosphoribosylaminoimidazole carboxylase I; *purF*, amidophosphoribosyltransferase; *purH*, phosphoribosylaminoimidazolecarboxamide formyltransferase; *purM*, phosphoribosylformylglycinamide cyclo-ligase; *purN*, phosphoribosylglycinamide (GAR) formyltransferase; *purl*, phosphoribosylformylglycinamidine synthase II; *prsA*, ribose-phosphate pyrophosphokinase; *purR*, purine operon repressor; *guaA*, GMP synthase; *guaB*, IMP dehydrogenase; *fhs*, formate-tetrahydrofolate ligase; PRPP, phosphoribosyl pyrophosphate; GAR, glycinamide ribonucleotide; FGAR, formylglycinamide ribonucleotide; AICAR, aminoimidazole; SAMP, adenylosuccinate.

coculture. It revealed an alteration of 4.1% of the *S. thermophilus* transcriptome and proteome, triggering modifications of specific cellular metabolisms. For each experiment, an LMG 18311 monoculture was grown in parallel to the coculture, and the samples were harvested at the same times and analyzed via the same methods (32). Comparison of the modifications observed in the coculture and in the monoculture revealed common transcriptomic and proteomic modifications. The main

similarity was that sulfur AA metabolism was stimulated in both types of cultures, as was galactose metabolism. Several changes were specific to the coculture and therefore related to the association of *S. thermophilus* with *L. delbrueckii* subsp. *bulgaricus*, among them, the purine biosynthesis pathway, iron metabolism, and two AA biosynthetic pathways (Arg and BCAA).

Upregulation of BCAA and Arg biosynthesis in the presence of *L. delbrueckii* subsp. *bulgaricus*. The upregulation of BCAA and Arg synthesis pathways in coculture is consistent with previous studies establishing that for optimal growth in milk, *S. thermophilus* requires BCAA and arginine (12, 26). These observations also suggest that the AA requirements differed between mono- and coculture. Since we showed that the coculture of *S. thermophilus* LMG 18311 and *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 improved the growth of LMG 18311 (Fig. 1), we propose that the increased requirement for Arg and BCAA observed for LMG 18311 in the coculture reflects a higher level of protein synthesis because of probable growth-limiting free intracellular AA. Indeed, BCAA and Arg are the most abundant AA in the predicted proteins of LMG 18311, as they account for 24.4% and 7.38% of the residues, respectively (http://www.cbs.dtu.dk/services/GenomeAtlas/). On the other hand, we cannot rule out that *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* compete in coculture, in particular for these AA, which would lead to the induction of Arg and BCAA pathways in *S. thermophilus*. In silico analysis of the two sequenced strains of *L. delbrueckii* subsp. *bulgaricus* (including ATCC 11842) indeed indicated that the Arg and BCAA pathways are not present, and the auxotrophy for these AA has been demonstrated for four other strains of *L. delbrueckii* subsp. *bulgaricus* (42). This upregulation of AA biosynthesis and thus of protein biosynthesis possibly attested for the better growth of *S. thermophilus* when associated with *L. delbrueckii* subsp. *bulgaricus*, as well as the overexpression of several genes involved in nucleic acid metabolism (*polC* and *ssbB*) and in translation (ribosomal proteins, tRNA synthetases, and *gatB*, a subunit of the Glu-tRNA^{Gln} amidotransferase).

Purine downregulation in the presence of *L. delbrueckii* subsp. *bulgaricus*. All the enzymes needed for the purine biosynthesis are present in the genome of *S. thermophilus* (9). However, it is well established that for optimal growth of *S. thermophilus* in milk, supplementation with purines is required (21, 27). In the coculture, LMG 18311 grew better than in the monoculture, but paradoxically, the purine biosynthesis pathway was downregulated. We propose that *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 provided purines or their precursors to LMG 18311. Several evidences support this hypothesis: (i) ATCC 11842 possesses a complete purine biosynthesis pathway and grows in purine-free medium (62); (ii) the LMG 18311 genome encodes putative transporters for purines or purine precursors, such as the gene encoding a putative xanthine/uracil permease (stu0336), which was overexpressed in the coculture, but also the four genes encoding phosphoribosyl transferases (Hpt, Apt, Xpt, and HprT) involved in the phosphorylation of the nucleobases (9); (iii) the addition of purines to a culture of LMG 18311 in milk caused a downregulation of PurM, PurH, and Fhs (21), demonstrating that exogenous purines are internalized and downregulate the corresponding biosynthesis pathway (4); and (iv) we observed an overexpression of PurR and a downregulation of *prsA1* during the growth of LMG 18311 with *L. delbrueckii* subsp. *bulgaricus*.

The increase of PurR is consistent with repressor activity, as in *Bacillus subtilis* (23) and *Streptococcus pneumoniae* (49). In *B. subtilis*, the addition of purines in the medium results in an inhibition of PrsA1, the PRPP synthase (6), leading to a decrease of the intracellular PRPP pool which is perceived by the PurR repressor and leading to the repression of purine biosynthesis (23). The PurR protein of LMG 18311 possesses PRPP and DNA binding domains highly similar to its *B. subtilis* and *L. lactis* homologs (40). We propose that a similar regulatory scheme could be involved in purine metabolism control in *S. thermophilus* and that this regulatory system is operative during the growth of LMG 18311 in coculture. The former hypothesis is strengthened by the presence of the PurR-box motif (AWWWCCGAACWWWT), which is involved in the PurR-dependent activation in *L. lactis* and in the promoter regions of the *S. thermophilus purC*, *purD*, and *fhs* clusters.

Reduction of the intracellular iron in response to H_2O_2 production? We propose that in coculture, LMG 18311 encountered H_2O_2 produced by *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 (as previously reported [61]) possibly via its dihydroorotase dehydrogenase proteins (PyrD1 and PyrD2). Note that the expression of LMG 18311 Nox2 and SodA, the two potential enzymes leading to H_2O_2 production from O_2 in LMG 18311, did not vary at the mRNA and protein level during growth. As *S. thermophilus* (as other streptococci) lacks H_2O_2-degrading enzymes, such as catalase and NADH peroxidase, it probably develops other means of protection against the membrane diffusible H_2O_2, which lead, through the Fenton reaction ($H_2O_2 + Fe^{2+} \Rightarrow Fe^{3+} + {}^{-}OH + {}^{\cdot}OH$), to the production of reactive oxygen species, highly damageable, notably for DNA. Our data show that *S. thermophilus* avoids these damageable reactions by inducing the gene encoding Dpr, which sequesters iron and is involved in bacterial H_2O_2 tolerance (3, 33, 65), and downregulating almost all of the genes potentially involved in iron import in LMG 18311 (*feoA*, *fatABC*, and stu1022). In addition, we established that the modulation in expression of at least *feoA* and *dpr* correlated with the presence of H_2O_2, as it disappeared after the addition of catalase in the coculture medium. Uncommonly among bacteria, *S. thermophilus dpr* constitutes an operon with *fur* (*perR*), the putative ferric transport regulator which was also upregulated during the coculture, as in *E. coli* (67) and in *B. subtilis* (31), in response to H_2O_2. Here, we observed that *S. thermophilus* Fur shared high homologies with PerR from *B. subtilis*, which is the major regulator of the inducible peroxide stress response, in particular of the *dpr* gene (31). In fact, *S. thermophilus* Fur is 55 and 39% identical to PerR from *S. pyogenes* and *B. subtilis*, respectively, with, in particular, a highly conserved 12-AA region (at positions 57 to 68) which could be specific for PerR proteins, as it is not conserved in other Fur proteins (13). The LMG 18311 Fur/PerR protein also potentially contained a structural $Zn(Cys)_4$ site, which is a distinctive feature of the PerR-like metalloregulators in bacteria (60), and we propose that the *S. thermophilus* LMG 18311 Fur-annotated protein is a PerR protein. Interestingly, we compared the upstream regions of the *fatD* and *dpr* (which is in an operon with *fur* [*perR*]) genes with those of genes regulated by *S. aureus*, *Streptococcus pyogenes*, and *B. subtilis perR* and

identified a consensus region (TTANAAWNATTNTWA) (http://weblogo.berkeley.edu/; WebLogo, a sequence logo generator) which could constitute a common PerR box. By using the i-Momi program (51), we showed that 11 *S. thermophilus* LMG 18311 genes possess this consensus sequence; among them, 3 are involved in iron metabolism: *dpr*, *fatD*, and stu0164, which is clustered with genes involved in oxidative stress response via the (Fe-S) cluster formation in *S. thermophilus* (58).

Higher intracellular Mn concentrations have been involved in oxidative stress resistance (4), probably thanks to the H_2O_2-quenching activity of Mn(II) (56). In enteric bacteria, peroxide stress induces the transcription of a Mn(II) transporter, MntH (38), which was also the case for *S. thermophilus mntH* in coculture. In addition, the RR01 protein, a CovR homolog, could be part of the probable *S. thermophilus* H_2O_2 response, as recently demonstrated with *S. mutans* (20).

Finally, we did not observe any upregulation of several genes usually involved in oxidative stress response and in particular in H_2O_2 response (*gor*, *trxAB*, *recA*, *uvrABC*, *clpC*, and *radA*) (48) but, to the contrary, did observe a downregulation of some of them (*radA* and *gor*). We propose that in coculture with *L. delbrueckii* subsp. *bulgaricus*, *S. thermophilus* sets up an adaptative H_2O_2 response rather than a response to an oxidative shock. Indeed, the H_2O_2 production by *L. delbrueckii* subsp. *bulgaricus* is not only most probably very low compared to the concentrations that are usually used for the study of H_2O_2 stress responses in bacteria but also very progressive, occurring during the course of *L. delbrueckii* subsp. *bulgaricus* growth.

Overall, from the present results, one can hypothesize that *S. thermophilus* possesses, as previously suggested (59), an inducible and efficient defense system based on iron homeostasis, which avoids the potential damageable effect of H_2O_2 and would provide an indirect system for this microaerophilic catalase-negative bacterium to tolerate H_2O_2.

In conclusion, the present study revealed specific physiological changes in *S. thermophilus* during growth stimulation due to the presence of *L. delbrueckii* subsp. *bulgaricus*. The combination of transcriptomic and proteomic analyses not only revealed undocumented nutritional effects on the BCAA, Arg, and purine metabolisms with their regulators but also evidenced other unexpected effects, such as the adaptation to H_2O_2, indicating that this bacterial proto-cooperation is more complex and much more ambiguous than previously reported.

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