

Mixed-Culture Transcriptome Analysis Reveals the Molecular Basis of Mixed-Culture Growth in *Streptococcus thermophilus* and *Lactobacillus bulgaricus*^{∇†}

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Received 10 May 2010/Accepted 21 September 2010

Many food fermentations are performed using mixed cultures of lactic acid bacteria. Interactions between strains are of key importance for the performance of these fermentations. Yogurt fermentation by *Streptococcus thermophilus* and *Lactobacillus bulgaricus* (basonym, *Lactobacillus delbrueckii* subsp. *bulgaricus*) is one of the best-described mixed-culture fermentations. These species are believed to stimulate each other's growth by the exchange of metabolites such as folic acid and carbon dioxide. Recently, postgenomic studies revealed that an upregulation of biosynthesis pathways for nucleotides and sulfur-containing amino acids is part of the global physiological response to mixed-culture growth in *S. thermophilus*, but an in-depth molecular analysis of mixed-culture growth of both strains remains to be established. We report here the application of mixed-culture transcriptome profiling and a systematic analysis of the effect of interaction-related compounds on growth, which allowed us to unravel the molecular responses associated with batch mixed-culture growth in milk of *S. thermophilus* CNRZ1066 and *L. bulgaricus* ATCC BAA-365. The results indicate that interactions between these bacteria are primarily related to purine, amino acid, and long-chain fatty acid metabolism. The results support a model in which formic acid, folic acid, and fatty acids are provided by *S. thermophilus*. Proteolysis by *L. bulgaricus* supplies both strains with amino acids but is insufficient to meet the biosynthetic demands for sulfur and branched-chain amino acids, as becomes clear from the upregulation of genes associated with these amino acids in mixed culture. Moreover, genes involved in iron uptake in *S. thermophilus* are affected by mixed-culture growth, and genes coding for exopolysaccharide production were upregulated in both organisms in mixed culture compared to monocultures. The confirmation of previously identified responses in *S. thermophilus* using a different strain combination demonstrates their generic value. In addition, the postgenomic analysis of the responses of *L. bulgaricus* to mixed-culture growth allows a deeper understanding of the ecology and interactions of this important industrial food fermentation process.

Fermented dairy products are typically produced using mixed cultures of lactic acid bacteria, a prominent group of Gram-positive bacteria. Yogurt is milk fermented by the lactic acid bacteria *Streptococcus thermophilus* and *Lactobacillus bulgaricus* (basonym, *Lactobacillus delbrueckii* subsp. *bulgaricus*). These bacteria stimulate each other's growth and acid production. This mutual stimulation is based on the exchange of growth enhancing metabolites (for a recent review, see reference 30). *S. thermophilus* is suggested to provide *L. bulgaricus* with formic acid (12), folic acid (10, 36), and carbon dioxide (14), compounds that are all associated with purine biosynthe-

sis either as precursors or as cofactors. Other metabolic interactions exist at the level of nitrogen metabolism. Typically, the nonproteolytic *S. thermophilus* profits from the proteolytic action of the membrane-resident protease PrtB of *L. bulgaricus* (8, 29, 32). *L. bulgaricus* was reported to be stimulated by long-chain fatty acids (LCFA) such as oleic acid and lauric acid (24), but it remains to be established whether *S. thermophilus* plays a role in improving fatty acid availability in mixed culture.

Two recent postgenomic studies addressed the global response of *S. thermophilus* LMG18311 to growth in milk in monoculture or mixed with *L. bulgaricus* ATCC 11842 (15, 16). These studies revealed several additional metabolic responses to mixed culture growth. The downregulation of genes associated with purine metabolism and the upregulation of *stu0336*, a xanthine/uracil permease, suggested that purine (precursors) were provided by *L. bulgaricus* and consumed by *S. thermophilus*. In addition, genes in the pathways for the biosynthesis of arginine and branched-chain amino acids (BCAA) were upregulated in mixed culture. Finally, in response to H₂O₂ produced by *L. bulgaricus*, *S. thermophilus* showed multiple responses that may lead to lower intracellular iron concentrations (15), minimizing damage by reactive oxygen species (ROS) that are generated by the Fenton reaction.

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† Supplemental material for this article may be found at <http://aem.asm.org/>.

[∇] Published ahead of print on 1 October 2010.

Since the postgenomic analyses described above were only performed in *S. thermophilus*, the responses of *L. bulgaricus* to mixed-culture growth remain to be established. In the present study we sought to (i) analyze the regulatory responses to cocultivation in milk in both strains simultaneously, (ii) extend analyses performed by Hervé-Jimenez et al. (15, 16) to another strain combination in order to explore the generic value of specific responses identified by these authors, and (iii) validate hypotheses derived from postgenomic studies with cultivation experiments using candidate interaction compounds. The combination of the regulatory response identified with transcriptomics and results acquired from population dynamics studies with supplementation of candidate interaction compounds showed that *S. thermophilus* provides *L. bulgaricus* with (precursors for) purines and that *L. bulgaricus* LCFA biosynthesis genes are downregulated in mixed cultures despite a higher growth rate. The results also show that the proteolytic activity of *L. bulgaricus* is insufficient to meet the demands for BCAA and sulfur amino acids by both strains.

MATERIALS AND METHODS

Strains and culture conditions. *S. thermophilus* CNRZ1066 (2) and *L. delbrueckii* subsp. *bulgaricus* ATCC BAA-365 (21) were maintained as frozen stocks in M17 broth and MRS broth (both Oxoid, Basingstoke, England), respectively, containing 22% (vol/vol) glycerol (Scharlau, Sentmenat, Spain) at -80°C . These strains were chosen because their genomes were annotated and publicly available at the start of the present study. Moreover, applying a transcriptomics study on different strains than those in reference 15 shows the generic relevance of the obtained results. Cultures were made by inoculating prewarmed ultrahigh-temperature-treated 10% (wt/vol) reconstituted skim milk (Nilac; NIZO food research, Ede, Netherlands) in unstirred 250-ml Scott bottles with 1×10^5 CFU/ml for *S. thermophilus* and 2×10^4 CFU/ml for *L. bulgaricus* and grown at 42°C , i.e., at an optical density at 600 nm (OD_{600}) of 0.005 per strain. OD_{600} was determined after mixing 1 volume of culture with 9 volumes of a solution comprising 0.2% (wt/vol) sodium hydroxide and 0.2% sodium EDTA acid (both from Merck, Darmstadt, Germany). Colony counts were acquired by spread plating onto M17 agar (*S. thermophilus*) and MRS agar (*L. bulgaricus*), followed by incubation at 42°C , the *S. thermophilus* in aerobic conditions and the *L. bulgaricus* in anaerobic conditions. The pH was recorded with a porotrode (Metrohm, Herisau, Switzerland) connected to a Cinac device (Alliance Instruments, Frepillon, France).

Effect of candidate interaction compounds on growth. Cultures of *S. thermophilus*, *L. bulgaricus*, and the mixed culture were prepared supplementing 4 volumes of milk with 1 volume of water containing compounds for which it has been established or hypothesized that they influence the interactions (30). These represented compounds related to purine and pyrimidine metabolism, including sodium pyruvate (1.82 mM) and sodium formate (1.47 mM) (both from Merck) and folic acid (1 mM) and nucleobases (10 mg/liter each) (both from Sigma-Aldrich, Steinheim, Germany). Proteolysis was represented by Casitone (4 g/liter) to abolish all effects of proteolysis, including that on low abundant amino acids and histidine (650 μM) and fatty acid metabolism using Tween 20 (105.9 μM) (13, 17) and Tween 80 (110 mg/liter) (all from Sigma). Tween 20 and Tween 80 were used as a supply of lauric acid and oleic acid since these free fatty acids are poorly soluble (24). Similarly, L-ornithine monohydrochloride (590 μM) and putrescine (1.13 mM) (both from Sigma) were used since both are involved in arginine metabolism and the urea cycle (6, 30, 38). The effect of each component on growth and acidification was tested in a single-addition and a single-omission strategy. Acidification by cultures of 250 μl was measured in quadruplicate in hydroplates (PreSens; Precision Sensing GmbH, Regensburg, Germany) both aerobically and anaerobically at 37°C because this temperature allowed comparable population densities of *S. thermophilus* and *L. bulgaricus*. After 19 h of culture, the CFU counts were determined by using a rapid miniplating method (31), incubating the plates at 37°C . Differences in acidification rates were determined by comparing the maximal and minimal acidification rates (Fig. 1B) using a two-tailed Student *t* test ($P = 0.05$). Differences between the final pH values and between the final viable counts were determined in a similar manner. Compounds showing significant effects were confirmed at the conditions used for transcriptomic analysis. A higher cell count, lower final pH, higher acidification

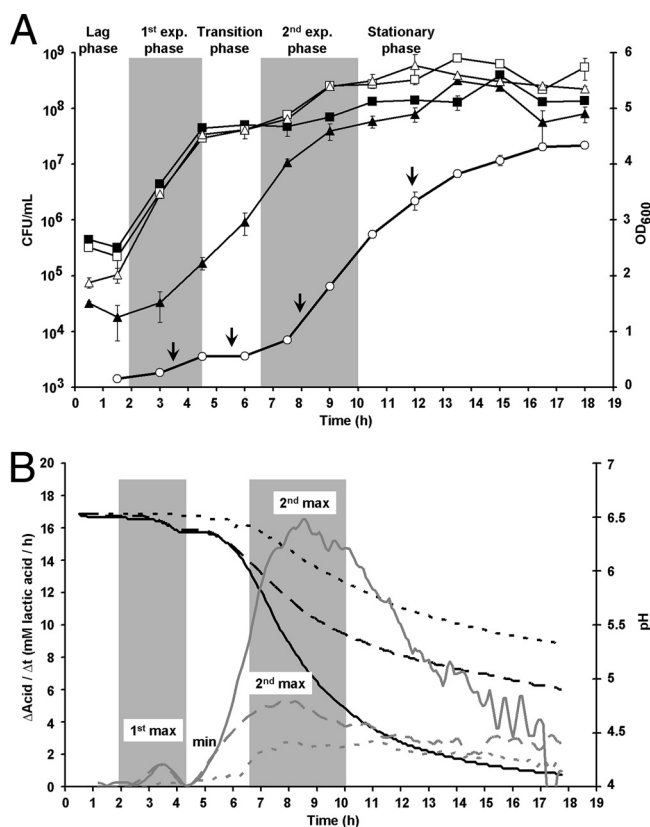


FIG. 1. Growth and acidification of monocultures and mixed cultures grown in 10% reconstituted skim milk at 42°C . (A) CFU per ml of *S. thermophilus* in monoculture (■) and mixed culture (□) and *L. bulgaricus* in monoculture (▲) and mixed culture (△), and the OD_{600} of the mixed culture (○). Error bars indicate the standard deviations of triplicate measurements. Gray and white boxes indicate the five distinct growth phases of the mixed culture. Arrows indicate sampling points for transcription profiling. (B) pH (black lines) and acidification (mM lactic acid/h) (gray lines) of typical milk fermentations of *S. thermophilus* (dashed lines), *L. bulgaricus* (dotted lines), and the mixed culture (solid lines). In the *S. thermophilus* and mixed culture, the two exponential phases display a maximal acidification rate (1st and 2nd max). In between, at the transition phase at $t = 4.5$ h, there is a point at which the acidification rate drops almost to zero.

rate, and a reduced time needed to reach this rate were considered stimulatory compared to the control.

Metabolite analyses. The free amino acid content was determined by high-pressure liquid chromatography from the cultures used for transcription profiling as described previously (18). To calculate the concentration of lactic acid produced by the cultures, a calibration curve was constructed by acidifying milk to various pH values with lactic acid. This procedure was followed instead of determining titratable acidity of each sample since it allowed a semiautomated determination of acid production rates in large numbers of samples for the compound screening.

EPS isolation. Similar cultures as for the transcription profiling study were grown at 37°C for 24 h. An 80-g portion of these cultures was used for exopolysaccharide (EPS) isolation. The samples were incubated at 55°C for 2 h to release all EPS from the cells. After 5.3 ml of 60% trichloroacetic acid (Merck) was added, the mixture was stirred at room temperature for 1 h. After centrifugation for 30 min at $6,000 \times g$ and 4°C , the pH of the supernatant was adjusted to 4.0 with 10 M sodium hydroxide. Dialysis tubes (Medicell International, Ltd., London, England) were boiled for 5 to 10 min in water containing 1 g of sodium carbonate (Merck)/liter, rinsed, and boiled in water for 5 min. The supernatants were applied to the tubes, and dialysis was performed in flowing tap water for 24 h and twice in Milli-Q water for 3 h. Dialyzed samples were freeze-dried in an IIShin freeze dryer (IIShin, Kteongki-do, South Korea).

RNA isolation, cDNA synthesis, labeling, and hybridization. Aliquots of 125 ml (after 3.5 and 5.5 h) or 50 ml (after 8 and 12 h) of the yogurt cultures was quenched in 3 volumes 60% glycerol at -40°C , leading to immediate arrest of the cellular processes (27), and kept at -20°C for 0.5 h. Subsequently, the pH was adjusted to 6.5 to 7.0 with 1 M sodium hydroxide. The medium was cleared by adding 4 ml of 25% (wt/vol) trisodium citrate (Merck) per 100 ml. The sample was gently mixed each 5 min for 0.5 h, while keeping it at -20°C . Cells were harvested by centrifugation at -20°C and $23,000 \times g$ for 16 min. In order to remove residual protein, the cell pellet was resuspended in a solution comprised of 50% (wt/vol) guanidine thiocyanate (Sigma), 0.5% (wt/vol) *N*-laurylsarcosine (Sigma), and 2.5% (vol/vol) of a 1 M trisodium citrate solution, adjusted to pH 7.0. After centrifugation, the cells were resuspended in $500 \mu\text{l}$ of $1 \times \text{TE}$ ($10 \times \text{TE}$ contains 10 mM Tris [pH 8.0; Merck] and 1 mM EDTA [pH 8.0]) and transferred to a screw-cap tube. RNA was isolated by using a phenol-chloroform extraction, followed by a column purification as described earlier (28), with the modification that cells were disrupted by shaking three times for 45 s each time in a Fast-Prep apparatus (MP Biomedicals, Illkirch, France) at 5.5 m/s separated by a period of 1 min on ice. The quantity of RNA was measured with a ND-1000 photospectrometer (Nanodrop Technologies, Wilmington, DE), and the quality was determined with a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). RNA samples with a A_{260}/A_{280} ratio of >1.9 and a clear two-peak (16S and 23S rRNA) pattern were included for transcriptome analysis. RNA samples were stored at -80°C .

Portions (5 to 7 μg) of RNA were used for cDNA synthesis and labeling with a Cyscribe Post-Labeling kit (Amersham Biosciences, Amersham, England) as described earlier (33). For each array, 0.3 μg of cDNA labeled with either cyanine 3 (Cy3) or cyanine 5 (Cy5) was hybridized using the solutions and according to the protocol supplied by Agilent (version 5.5) for 8x15K slides. A hybridization scheme was designed that allowed duplicate comparisons between different stages within a fermentation experiment, as well as between monocultures and mixed cultures (see Fig. S1 in the supplemental material).

Microarray design. Microarrays for the 8x15K platform containing *in situ*-synthesized 60-mer oligomers were produced by Agilent Technologies (Santa Clara, CA), according to a custom probe design based on the genome sequences of *S. thermophilus* CNR21066 (released by NCBI, GenBank accession no. NC_006449) and *L. bulgaricus* ATCC BAA-365 (released by JGI, NC_008529). The oligomers were designed using the algorithm described previously (28). With respect to the genome of *S. thermophilus*, 55 genes were represented by one probe, 115 were represented by two probes, and 1,724 were represented by three or more probes. For *L. bulgaricus*, 77 genes were represented by one probe, 94 were represented by two probes, and 1,507 were represented by three or more probes. Only 5 genes of *S. thermophilus* and 31 genes of *L. bulgaricus* were not represented on the array because no unique probe satisfying the criteria could be selected. Selectivity of the probes was tested by hybridization with *S. thermophilus* RNA labeled with Cy5 and *L. bulgaricus* RNA labeled with Cy3. This showed cross-hybridization only for probes representing tRNAs (data not shown).

Microarray analysis. Slides were scanned using an Agilent microarray scanner (G2565BA). Lasers of wavelengths at 532 and 635 nm were used to excite Cy3 and Cy5 dyes, respectively. Fluorescent images were captured in a multi-image-tagged image file format and analyzed using Imagen software (Axon) (BioDiscovery, Marina del Rey, CA). The extent of hybridization was derived from a median value of pixel-by-pixel ratios. *S. thermophilus* and *L. bulgaricus* spots were normalized by using Lowess (39) separately since a varying ratio between both organisms would impact the determined expression of genes in each species. This implies that a direct comparison of gene expression levels between both organisms cannot be done. Differential regulation was determined by false-discovery rate (FDR) from the Cyber-*T* *P* values by means of multiple testing corrections (1, 40). The confidence value for the Bayesian variance estimate was calculated for each comparison of two treatments (conditions) by determining the lowest number of replicates in both treatments and multiplying that value by 3. To account for the two technical replicates for each sample, due to that each mRNA preparation was labeled with both Cy3 and Cy5, the confidence value was divided by 2. Differential regulation was defined as a 2-fold or higher differential expression with a FDR cutoff value of 0.05 or lower. In addition, ≥ 1.5 -fold unidirectional regulation of genes cotranscribed in operon structures was also scored as significant. Regulated genes were divided into functional classes as described by the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/sutils/coxik.cgi?gi=529> for *S. thermophilus*) and <http://www.ncbi.nlm.nih.gov/sutils/coxik.cgi?gi=20019> for *L. bulgaricus*). Using hierarchical clustering and principal-component analysis (34), the reproducibility of replicates of the same condition and the relation between different conditions was verified. Finally, results were visualized by plotting onto KEGG maps, Simpheny (Genomatica, Inc., San Diego, CA), metabolic maps (25, 37), and Minomics (5).

Microarray accession numbers. The microarray design and hybridization data are available at the Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo>) under accession number GSE21593.

RESULTS

Population dynamics of *S. thermophilus* and *L. bulgaricus*. In order to study the interaction of *S. thermophilus* and *L. bulgaricus* in milk, growth and acid production in monoculture and mixed culture were compared (Fig. 1). Although the *L. bulgaricus* monoculture showed only one exponential phase, a typical complex growth and acid production behavior, following the lag phase, was observed in the *S. thermophilus* and in the mixed cultures, comprising four steps: (i) a first exponential phase, (ii) a transition phase at which the growth rate decreased, (iii) a second exponential phase, and (iv) a stationary phase (9, 19).

Effect of candidate interaction compounds on growth. Several compounds are known or hypothesized to influence the interaction between the two yogurt bacteria. We reasoned that the addition of these compounds to milk is likely to influence the interactions. The decrease or elimination of nutritional dependencies would thereby affect acidification and outgrowth in monocultures and mixed cultures. In order to establish these effects, each of the interaction compounds was added in a single addition strategy, and the effect on maximal acidification rate, final pH, and final colony count was scored. In parallel, a series of growth experiments was performed in which all but one interaction compounds were added in a single omission strategy that allows the identification of combined effects. The results are summarized in Table 1 and Fig. 1B. Clearly, acidification by *S. thermophilus* was stimulated by the following compounds in decreasing order with respect to the magnitude of the effect: formic acid, Casitone, pyruvic acid, folic acid, and Tween 20. *L. bulgaricus* acidification was stimulated most strongly by formic acid and nucleobases, whereas pyruvic acid, folic acid, Tween 20, and Tween 80 had a smaller stimulatory effect. In the mixed cultures, acidification was stimulated by pyruvic acid, formic acid, and Casitone, but less so than in the monocultures. Only formic acid and Casitone led to a higher final cell count of *S. thermophilus*. In *L. bulgaricus* monocultures, the cell counts were higher when pyruvic acid or Tween 20 was supplied. These effects were less evident in mixed cultures. Taken together, these studies highlight the potential importance of purine, amino acid, and LCFA acquisition in the interactions between the two strains.

Transcriptome analysis of monocultures and mixed cultures. We next reasoned that potential interactions would also be revealed by differential expression of the genes and pathways involved in monocultures and mixed cultures. In order to identify genes that are differentially expressed in both species upon cocultivation, we performed transcriptome profiling on mixed cultures at four different growth phases (Fig. 1)—i.e., the first exponential phase (3.5 h after starting the fermentation), transition phase (5.5 h), second exponential phase (8 h), and stationary phase (12 h)—and compared these to monocultures. Similarly, these four distinct growth phases were compared within a culture. These studies allowed analysis of global regulatory responses and development of the interactions throughout the fermentation.

TABLE 1. Stimulation of growth and acidification by compounds potentially involved in the interactions between *S. thermophilus* and *L. bulgaricus* in milk^a

| Component | Culture | Final cell count | | End pH | Acidification pattern ($\Delta\text{acid}/\Delta t$) | | |
|------------------------------------|---------|------------------|----|--------|--------------------------------------------------------|-----|-------|
| | | S | L | | Max 1 | Min | Max 2 |
| Sodium-pyruvic acid | S | | NA | + | | | |
| | L | | + | | | | |
| | M | | | | + | | |
| Sodium-formic acid | S | + | NA | + | | + | + |
| | L | NA | + | | | | + |
| | M | | + | | | | |
| Folic acid | S | | NA | | | | + |
| | L | NA | | | | | |
| | M | + | | | | + | |
| Nucleobases | S | | NA | + | | | + |
| | L | NA | | | | N | N |
| | M | | + | | | + | |
| Tween 20 | S | | NA | + | | | + |
| | L | NA | + | | | | |
| | M | | | | | + | |
| Tween 80 | S | | NA | | | | |
| | L | NA | | | | | |
| | M | | | | | + | |
| Casitone | S | + | NA | | + | | + |
| | L | NA | + | | | N | N |
| | M | | + | | | + | |
| Histidine | S | | NA | + | | | + |
| | L | NA | - | | | N | N |
| | M | | | | | | + |
| L-Ornithine monohydrochloride | S | | NA | | | | |
| | L | NA | | | | | |
| | M | | | | | | |
| Putrescine | S | | NA | | | | |
| | L | NA | - | | | | |
| | M | | | | | + | |
| All components | S | | NA | + | + | + | + |
| | L | NA | + | | | | |
| | M | + | | | | N | N |
| All: sodium-pyruvic acid | S | + | NA | + | | | + |
| | L | NA | | | | N | N |
| | M | | | | | N | N |
| All: sodium-formic acid | S | + | NA | - | - | - | - |
| | L | NA | | | | N | N |
| | M | | | | | N | N |
| All: folic acid | S | | NA | | - | | - |
| | L | NA | + | | | N | N |
| | M | | | | | N | N |
| All: nucleobases | S | | NA | | | | - |
| | L | NA | | | | + | - |
| | M | | | | | N | N |
| All: Tween 20 and Tween 80 | S | + | NA | | - | | - |
| | L | NA | | | | N | N |
| | M | | | | | N | N |
| All: Casitone | S | - | NA | - | - | | - |
| | L | NA | | | | N | N |
| | M | | | | | N | N |
| All: histidine | S | + | NA | | | | - |
| | L | NA | | | | N | N |
| | M | + | | | | N | N |
| All: L-ornithine monohydrochloride | S | + | NA | | | | + |
| | L | NA | | | | + | |
| | M | + | | | | N | N |
| All: putrescine | S | + | NA | | | | - |
| | L | NA | | | | N | N |
| | M | + | | | | N | N |
| None | S | | NA | | | | |
| | L | NA | | | | N | N |
| | M | | | | | | |

^a Cultures with single additions were tested versus cultures without additions. Cultures containing all components excluding one were compared to the cultures containing all components (single omissions). The pH was monitored for 19 h upon which dilutions were plated for cell enumeration. The final populations presented were determined from duplicate plating of quadruplicate cultures. The acidification rates and final pH values were calculated from the same cultures. Abbreviations and symbols: S, *S. thermophilus*; L, *L. bulgaricus*; M, mixed culture; +, positive effect (higher cell count, lower end pH, higher acidification rate, earlier maximal rate); -, negative effect; N, not present; NA, strain not added in the culture.

Global transcriptional responses in *L. bulgaricus*. The presence of *S. thermophilus* stimulated *L. bulgaricus* growth already in the early stages of the fermentation (Fig. 1A), which was reflected by a high portion of differentially expressed genes in *L. bulgaricus* in the two early growth phases (17 to 24% of all genes, see Table S1 in the supplemental material), in particular genes related to growth. The functional groups with the highest fraction of differentially expressed genes were “amino acid transport and metabolism” (21 to 36% of the genes in the category), “inorganic ion transport and metabolism” (20 to 27%), and “nucleotide transport and metabolism” (20 to 45%). In the *L. bulgaricus* monoculture many pathways were downregulated in the two later phases of fermentation compared to the two early phases, particularly those associated with the biosynthesis of folic acid, purines, LCFA, and amino acids and genes directly related to growth. In the mixed culture there was a clear lower expression in the transition phase of genes encoding folic acid, purine, and LCFA biosynthesis and sulfur amino acid metabolism compared to the first exponential phase. This may be due to the lower growth rate in the transition phase. In the second exponential phase, however, the expression of purine and LCFA biosynthesis genes remained at a low level despite the higher growth rate compared to the transition phase, indicating that there was no shortage of these compounds. In addition, genes involved in polysaccharide and sulfur amino acid metabolism were upregulated in the second exponential phase compared to the transition phase.

Global transcriptional responses in *S. thermophilus*. Cocultivation affected *S. thermophilus* gene expression mainly in the second exponential phase (24% of all genes were more than 2-fold differentially expressed, see Table S2 in the supplemental material), which is in agreement with the observation that only at this growth phase is *S. thermophilus* profoundly stimulated by *L. bulgaricus* (Fig. 1A) (15). The functional groups displaying the greatest differential expression were “amino acid transport and metabolism” (18 to 43% of the genes in the category), “inorganic ion transport and metabolism” (14 to 32%) and “nucleotide transport and metabolism” (12 to 51%). In the *S. thermophilus* monoculture, the gene *pflA* (4.6-fold), coding for pyruvate-formate lyase (PFL) activating enzyme, necessary for the production of formic acid from pyruvic acid, and the pathway for purine biosynthesis were upregulated in the transition phase compared to the first exponential phase despite the lower growth rate. Similarly, BCAA import and production genes were 2.9- to 3.0-fold more highly expressed in the transition phase, whereas genes encoding biosynthesis of other amino acids were mostly downregulated. This suggests that a shortage of BCAA occurs relatively early in the fermentation. There was little difference in the second exponential phase compared to the transition phase except for the upregulation of sulfur amino acid metabolism, as was also described by Hervé-Jimenez et al. (16). These trends were also observed in mixed culture, with the exception that the higher expression of BCAA acquisition genes did not occur in the mixed culture. In the second exponential phase in mixed culture, purine biosynthesis genes were downregulated compared to the transition phase, but many pathways involved in amino acid acquisition were upregulated, especially those for BCAA (2- to 3.1-fold) and sulfur amino acids (2.2- to 61.5-fold), suggesting a limitation in the availability of these amino acids from the

medium. EPS biosynthesis genes of *S. thermophilus* were upregulated in the second exponential phase and stationary phase compared to the earlier growth phases in mixed culture, but not in monoculture.

Below, the responses in the major metabolic pathways that were affected, and the possible physiological consequences are discussed in more detail.

Purine metabolism. In a proteome study of *S. thermophilus*, Dezelle et al. (12) found that PFL was highly abundant during growth in milk. In our study we found that the PFL-encoding gene, *pfl*, and the gene encoding for PFL-activating enzyme, *pflA*, were higher expressed in mixed culture, especially in the first exponential phase (3.0- and 4.1-fold, respectively) compared to monocultures. Expression of genes in the biosynthetic pathway for folic acid was not affected, but folic acid cycling genes (C1 pool) were upregulated in mixed culture. In *L. bulgaricus*, the (incomplete) folate biosynthetic pathway was downregulated, especially at the first two growth stages (Table 2). Genes encoding the purine biosynthesis pathway in *S. thermophilus* were upregulated in the mixed culture in the two earlier growth phases (Table 2), but in accordance to the study by Hervé-Jimenez et al. (15), downregulated in the second exponential phase despite the higher growth rate. Purine metabolism in *L. bulgaricus* was downregulated throughout the fermentation in mixed culture despite its higher growth rate (Table 2).

Amino acid and carbon dioxide metabolism. Nitrogen metabolism was poorly affected in *L. bulgaricus* with few exceptions. In mixed culture there was a considerably higher expression level of the *prtB* gene, *LBUL_1105*, encoding the extracellular protease that is responsible for the first step in the proteolytic degradation of caseins (8.9-fold in the second exponential phase). Since the presence of *S. thermophilus* is likely to lead to lower levels of available peptides and amino acids in the medium, *L. bulgaricus* may increase the expression of *prtB*. Genes involved in the biosynthesis of the sulfur containing amino acids cysteine and methionine were markedly upregulated in mixed culture (Table 2). Also, in *S. thermophilus* in mixed culture there was a slightly higher expression of genes for the conversion of serine into cysteine and methionine (Table 2).

In *L. bulgaricus* in mixed culture, *LBUL_0431*, encoding a BCAA permease, was 2.3-fold higher expressed during the second exponential phase, which may be attributed to by the much lower BCAA availability in mixed culture. In *Lactococcus lactis*, the transcriptional repressor CodY inhibits peptidases and BCAA and methionine transporter expression in the presence of sufficient amounts of free isoleucine (11). In *S. thermophilus*, CodY acts as a pleiotropic regulator regulating the expression of many peptidases and peptide transporters (20). The expression of *codY* was slightly lowered in mixed culture (1.3- to 1.7-fold in the transition phase and the second exponential phase), which was reflected in the upregulation of peptide import and peptidolysis genes, exemplified by the 2.5- to 2.8-fold higher expression of the ABC transport system encoded by *amiC*, *amiD*, *amiE*, and *amiF1* and the 2.4-fold higher expression of the peptidase *pepN* in the second exponential phase. In addition, genes encoding BCAA biosynthesis were 2-fold upregulated in *S. thermophilus* in mixed culture (Table 2).

TABLE 2. Expression ratio values of the genes showing largest regulatory responses to growth in mixed culture^a

| Category and locus tag | Gene | COG name | Expression ratio (mixed culture/ monoculture) | | | |
|------------------------------|--------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------|-------------|-------------|--------------|
| | | | 3.5 h | 5.5 h | 8 h | 12 h |
| Folic acid metabolism | | | | | | |
| <i>LBUL_0208</i> | | Dihydroneopterin aldolase | 0.88 | 0.43 | 1.28 | 2.25 |
| <i>LBUL_0209</i> | | GTP cyclohydrolase I | 0.40 | 0.32 | 1.35 | 1.64 |
| <i>LBUL_0210</i> | | Folypolyglutamate synthase | 0.50 | 0.31 | 1.41 | 1.44 |
| <i>LBUL_0211</i> | | Dihydropteroate synthase and related enzymes | 0.69 | 0.39 | 1.39 | 2.30 |
| <i>LBUL_0212</i> | | NTP pyrophosphohydrolases including oxidative damage repair enzymes | 0.63 | 0.31 | 1.26 | 2.88 |
| <i>LBUL_0660</i> | | Folypolyglutamate synthase | 0.69 | 0.37 | 0.78 | 4.82 |
| Purine metabolism | | | | | | |
| <i>LBUL_0156</i> | | Phosphoribosylpyrophosphate synthetase | 2.09 | 1.16 | 0.44 | 0.50 |
| <i>LBUL_0246</i> | | IMP dehydrogenase/GMP reductase | 0.24 | 0.30 | 1.27 | 0.05 |
| <i>LBUL_0251</i> | | IMP dehydrogenase/GMP reductase | 2.63 | 0.68 | 1.33 | 0.91 |
| <i>LBUL_0252</i> | | GMP synthase PP-ATPase domain/subunit | 2.32 | 0.94 | 1.57 | 1.60 |
| <i>LBUL_0305</i> | | Phosphoribosylpyrophosphate synthetase | 0.78 | 0.66 | 0.57 | 1.32 |
| <i>LBUL_1330</i> | | Phosphoribosylamine-glycine ligase | 0.99 | 0.37 | 1.18 | 0.06 |
| <i>LBUL_1331</i> | | AICAR transformylase/IMP cyclohydrolase PurH (only IMP cyclohydrolase domain in Aful) | 1.21 | 0.43 | 1.23 | 0.05 |
| <i>LBUL_1332</i> | | Folate-dependent phosphoribosylglycinamide formyltransferase PurN | 1.20 | 0.58 | 1.22 | 0.06 |
| <i>LBUL_1333</i> | | Phosphoribosylaminoimidazole (AIR) synthetase | 1.29 | 0.34 | 1.19 | 0.04 |
| <i>LBUL_1334</i> | | Glutamine phosphoribosylpyrophosphate amidotransferase | 1.25 | 0.37 | 1.28 | 0.05 |
| <i>LBUL_1335</i> | | Phosphoribosylformylglycinamide (FGAM) synthase synthetase domain | 1.66 | 0.46 | 1.18 | 0.07 |
| <i>LBUL_1336</i> | | Phosphoribosylformylglycinamide (FGAM) synthase glutamine amidotransferase domain | 0.96 | 0.44 | 0.92 | 0.04 |
| <i>LBUL_1337</i> | | Phosphoribosylformylglycinamide (FGAM) synthase PurS component | 0.89 | 0.61 | 0.81 | 0.03 |
| <i>LBUL_1338</i> | | Phosphoribosylaminoimidazolesuccinocarboxamide (SAICAR) synthase | 0.83 | 0.64 | 0.88 | 0.03 |
| <i>LBUL_1339</i> | | Adenylosuccinate lyase | 1.79 | 1.78 | 0.85 | 0.17 |
| <i>LBUL_1340</i> | | Phosphoribosylaminoimidazole carboxylase (NCAIR synthetase) | 2.05 | 1.90 | 0.92 | 0.10 |
| <i>LBUL_1341</i> | | Phosphoribosylcarboxyaminoimidazole (NCAIR) mutase | 1.96 | 1.55 | 0.57 | 0.14 |
| <i>LBUL_1754</i> | | GMP synthase-glutamine amidotransferase domain | 1.21 | 0.59 | 1.52 | 1.78 |
| <i>str0030</i> | <i>purC</i> | Phosphoribosylaminoimidazole-succinocarboxamide synthase | 2.74 | 1.15 | 0.27 | 0.10 |
| <i>str0031</i> | <i>purL</i> | Phosphoribosylformylglycinamide synthase II (FGAM synthetase) | 1.94 | 1.50 | 0.40 | 0.31 |
| <i>str0031</i> | <i>purL</i> | Phosphoribosylformylglycinamide synthase II (FGAM synthetase) | 1.94 | 1.50 | 0.40 | 0.31 |
| <i>str0032</i> | <i>purF</i> | Amidophosphoribosyltransferase | 1.59 | 1.61 | 0.45 | 0.43 |
| <i>str0033</i> | <i>purM</i> | Phosphoribosylaminoimidazole synthetase | 1.54 | 1.64 | 0.48 | 0.47 |
| <i>str0034</i> | <i>purN</i> | Phosphoribosylglycinamide formyltransferase | 1.47 | 1.60 | 0.50 | 0.48 |
| <i>str0035</i> | <i>purH</i> | Bifunctional phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase | 1.86 | 2.18 | 0.47 | 0.70 |
| <i>str0040</i> | <i>purD</i> | Phosphoribosylamine-glycine ligase | 4.90 | 2.56 | 0.50 | 0.15 |
| <i>str0041</i> | <i>purE</i> | Phosphoribosylaminoimidazole carboxylase catalytic subunit | 5.76 | 4.05 | 0.79 | 0.20 |
| <i>str0042</i> | <i>purK</i> | Phosphoribosylaminoimidazole carboxylase | 5.41 | 3.90 | 0.77 | 0.31 |
| <i>str0045</i> | <i>purB1</i> | Adenylosuccinate lyase | 2.23 | 2.08 | 0.53 | 0.37 |
| <i>str1640</i> | <i>pflA</i> | Pyruvate-formate lyase activating enzyme | 4.06 | 0.79 | 0.66 | 0.47 |
| <i>str1657</i> | <i>pfl</i> | Pyruvate formate-lyase | 3.03 | 1.38 | 0.97 | 0.76 |
| BCAA metabolism | | | | | | |
| <i>LBUL_0077</i> | | L-2-Hydroxyisocaproate dehydrogenase | 0.64 | 0.86 | 1.78 | 1.48 |
| <i>LBUL_0392</i> | | Aspartate/tyrosine/aromatic aminotransferase | 1.33 | 1.02 | 3.31 | 0.62 |
| <i>LBUL_0431</i> | | Branched-chain amino acid permease | 0.71 | 0.58 | 2.34 | 1.44 |
| <i>LBUL_0659</i> | | Valyl-tRNA synthetase | 1.08 | 0.43 | 1.27 | 6.46 |
| <i>LBUL_0681</i> | | Isoleucyl-tRNA synthetase | 0.94 | 0.62 | 1.74 | 4.51 |
| <i>LBUL_1180</i> | | Aspartate/tyrosine/aromatic aminotransferase | 1.15 | 1.21 | 0.51 | 1.13 |
| <i>LBUL_1219</i> | | BCAA aminotransferase/4-amino-4-deoxychorismate lyase | 0.64 | 0.63 | 0.56 | 4.74 |
| <i>LBUL_1451</i> | | Leucyl-tRNA synthetase | 0.86 | 0.41 | 0.87 | 3.54 |
| <i>LBUL_2034</i> | | Thiamine pyrophosphate-requiring enzymes [acetolactate synthase pyruvate dehydrogenase (cytochrome) glyoxylate carbonylase phosphonopyruvate decarboxylase] | 0.44 | 0.24 | 0.80 | 11.11 |
| <i>str0220</i> | <i>leuS</i> | Leucyl-tRNA synthetase | 2.13 | 1.95 | 2.17 | 1.22 |
| <i>str0359</i> | <i>livJ</i> | BCAA ABC transporter substrate-binding protein | 1.37 | 0.80 | 1.30 | 0.60 |
| <i>str0360</i> | <i>livH</i> | BCAA ABC transporter permease protein | 1.18 | 0.76 | 1.33 | 0.79 |
| <i>str0361</i> | <i>livM</i> | BCAA ABC transporter permease protein | 1.09 | 0.67 | 1.34 | 1.22 |
| <i>str0362</i> | <i>livG</i> | BCAA ABC transporter ATP binding protein | 2.00 | 1.14 | 1.03 | 1.49 |

Continued on following page

TABLE 2—Continued

| Category and locus tag | Gene | COG name | Expression ratio (mixed culture/ monoculture) | | | |
|------------------------------------------------|--------------|-------------------------------------------------------------------------------------------------------------------|--------------------------------------------------|-------------|--------------|--------------|
| | | | 3.5 h | 5.5 h | 8 h | 12 h |
| <i>str0363</i> | <i>livF</i> | BCAA ABC transporter ATP binding protein | 1.51 | 0.62 | 0.96 | 1.37 |
| <i>str0477</i> | <i>valS</i> | Valyl-tRNA synthetase | 1.03 | 0.83 | 3.59 | 4.89 |
| <i>str0590</i> | <i>bcaT</i> | BCAA aminotransferase | 2.01 | 1.18 | 0.78 | 0.30 |
| <i>str0644</i> | <i>hdhL</i> | L-2-Hydroxyisocaproate dehydrogenase | 1.14 | 1.11 | 1.15 | 3.24 |
| <i>str0741</i> | <i>ileS</i> | Isoleucyl-tRNA synthetase | 0.81 | 0.86 | 2.18 | 2.63 |
| <i>str1357</i> | <i>brnQ</i> | BCAA transport system II carrier protein | 0.51 | 0.59 | 1.44 | 0.28 |
| <i>str1438</i> | <i>amiF1</i> | Oligopeptide ABC transporter ATP-binding protein | 0.49 | 0.39 | 2.75 | 0.72 |
| <i>str1439</i> | <i>amiE</i> | Oligopeptide ABC transporter ATP-binding protein | 0.61 | 0.23 | 2.46 | 0.62 |
| <i>str1440</i> | <i>amiD</i> | Oligopeptide ABC transporter membrane-binding protein | 0.45 | 0.14 | 2.73 | 0.55 |
| <i>str1441</i> | <i>amiC</i> | Oligopeptide ABC transporter membrane-binding protein | 0.50 | 0.30 | 2.79 | 0.39 |
| <i>str1635</i> | <i>codY</i> | Transcriptional repressor CodY | 1.01 | 0.76 | 0.59 | 1.02 |
| Sulfur amino acid metabolism | | | | | | |
| <i>LBUL_0089</i> | | Homoserine <i>trans</i> -succinylase | 0.61 | 0.46 | 0.76 | 0.21 |
| <i>LBUL_0096</i> | | Methionine synthase II (cobalamin-independent) | 2.66 | 0.84 | 2.71 | 1.24 |
| <i>LBUL_0268</i> | | Homocysteine/selenocysteine methylase (S-methylmethionine-dependent) | 2.48 | 0.79 | 3.03 | 0.93 |
| <i>LBUL_0297</i> | | Methionyl-tRNA synthetase | 1.81 | 0.91 | 1.11 | 2.24 |
| <i>LBUL_0685</i> | | Adenosyl homocysteine nucleosidase | 0.23 | 0.35 | 4.17 | 5.42 |
| <i>LBUL_0686</i> | | Cysteine sulfinate desulfinate/cysteine desulfurase and related enzymes | 0.41 | 0.34 | 1.14 | 5.14 |
| <i>LBUL_1103</i> | | Bifunctional PLP-dependent enzyme with beta-cystathionase and maltose regulon repressor activities | 0.32 | 0.75 | 1.54 | 1.97 |
| <i>LBUL_1232</i> | | Methionine synthase II (cobalamin-independent) | 0.50 | 0.55 | 0.94 | 2.23 |
| <i>LBUL_1235</i> | | Cysteine synthase | 9.11 | 2.88 | 23.09 | 2.84 |
| <i>LBUL_1236</i> | | Cystathionine beta-lyases/cystathionine gamma-synthases | 6.30 | 2.85 | 13.94 | 4.31 |
| <i>LBUL_1259</i> | | Homoserine dehydrogenase | 0.93 | 0.93 | 1.84 | 1.02 |
| <i>LBUL_1353</i> | | Homoserine <i>trans</i> -succinylase | 0.50 | 0.27 | 0.59 | 0.08 |
| <i>LBUL_1354</i> | | Cysteine synthase | 0.69 | 0.21 | 0.36 | 0.03 |
| <i>LBUL_1455</i> | | S-Adenosylmethionine synthetase | 1.08 | 0.68 | 1.98 | 0.63 |
| <i>LBUL_1554</i> | | Cysteiny-tRNA synthetase | 0.53 | 0.75 | 0.35 | 1.30 |
| <i>str0083</i> | <i>cysE1</i> | Serine acetyltransferase | 0.69 | 0.76 | 1.59 | 0.56 |
| <i>str0084</i> | <i>cysS</i> | Cysteiny-tRNA synthetase | 0.79 | 0.74 | 1.86 | 0.73 |
| <i>str0352</i> | <i>metB1</i> | Cystathionine gamma-synthase | 0.09 | 0.02 | 1.27 | 0.05 |
| <i>str0366</i> | <i>cysM1</i> | Cysteine synthase | 0.44 | 0.10 | 0.88 | 0.31 |
| <i>str0451</i> | <i>metG</i> | Methionine-tRNA ligase | 1.12 | 0.83 | 1.33 | 0.37 |
| <i>str0566</i> | <i>pfs</i> | 5'-Methylthioadenosine/S-adenosylhomocysteine nucleosidase | 0.49 | 0.94 | 2.91 | 1.59 |
| <i>str0584</i> | <i>mmuM</i> | Homocysteine methyltransferase | 0.40 | 0.45 | 1.42 | 0.32 |
| <i>str0846</i> | <i>cysM2</i> | Cysteine synthase | 1.73 | 0.69 | 1.56 | 0.42 |
| <i>str0847</i> | <i>metB2</i> | Cystathionine beta-lyase | 1.17 | 0.68 | 0.91 | 0.86 |
| <i>str0848</i> | <i>cysE2</i> | Serine acetyltransferase, putative | 1.48 | 0.61 | 1.53 | 1.13 |
| <i>str0987</i> | <i>cysD</i> | O-Acetylhomoserine sulfhydrylase | 0.28 | 0.20 | 3.24 | 0.09 |
| <i>str1172</i> | <i>metK</i> | S-Adenosylmethionine synthetase | 0.40 | 0.57 | 1.52 | 0.06 |
| <i>str1222</i> | <i>metA</i> | Homoserine O-succinyltransferase | 0.28 | 0.26 | 1.49 | 0.13 |
| Other amino acid and carbon dioxide metabolism | | | | | | |
| <i>LBUL_1105</i> | | Subtilisin-like serine protease <i>prtB</i> | 0.45 | 0.42 | 8.89 | 0.42 |
| <i>str1658</i> | <i>cah</i> | Carbonate dehydratase | 15.75 | 3.73 | 0.71 | 0.92 |
| ROS production and iron metabolism | | | | | | |
| <i>LBUL_2034</i> | | Acetolactate synthase pyruvate dehydrogenase, (cytochrome) glyoxylate carboligase phosphonopyruvate decarboxylase | 0.44 | 0.24 | 0.80 | 11.11 |
| <i>LBUL_1241</i> | | Translation initiation factor 2 (IF-2; GTPase) | 2.29 | 2.19 | 3.48 | 1.22 |
| <i>LBUL_1955</i> | | Dihydroorotate dehydrogenase | 0.94 | 1.04 | 2.60 | 1.10 |
| <i>str1025</i> | <i>fatB</i> | Ferrichrome ABC transporter, substrate-binding protein | 0.83 | 0.90 | 4.34 | 0.59 |
| <i>str1026</i> | <i>fatA</i> | Ferrichrome ABC transporter, ATP-binding protein | 0.66 | 1.00 | 4.52 | 0.54 |
| <i>str1027</i> | <i>fatC</i> | Ferrichrome ABC transporter, permease protein | 0.74 | 0.96 | 3.95 | 0.34 |
| <i>str1028</i> | <i>fatD</i> | Ferrichrome ABC transporter, permease protein | 0.77 | 0.95 | 2.74 | 0.09 |
| <i>str0723</i> | <i>dpr</i> | Peroxide resistance protein, non-heme iron-containing ferritin | 1.48 | 0.76 | 0.35 | 1.22 |
| <i>str0724</i> | <i>fur</i> | Ferric transport regulator protein | 1.37 | 1.13 | 0.42 | 1.51 |
| Fatty acid metabolism | | | | | | |
| <i>LBUL_0106</i> | | 1-Acyl- <i>sn</i> -glycerol-3-phosphate acyltransferase | 1.37 | 0.53 | 3.13 | 1.40 |

Continued on following page

TABLE 2—Continued

| Category and locus tag | Gene | COG name | Expression ratio (mixed culture/ monoculture) | | | |
|------------------------|------|---------------------------------------------------------------------------------------------|--------------------------------------------------|-------------|-------------|--------------|
| | | | 3.5 h | 5.5 h | 8 h | 12 h |
| <i>LBUL_0109</i> | | 1-Acyl- <i>sn</i> -glycerol-3-phosphate acyltransferase | 0.63 | 0.62 | 4.76 | 0.91 |
| <i>LBUL_0314</i> | | Phosphopantetheinyl transferase (holo-ACP synthase) | 0.86 | 0.73 | 0.66 | 0.99 |
| <i>LBUL_0818</i> | | 3-Oxoacyl-(acyl-carrier-protein) | 1.67 | 0.37 | 0.26 | 0.20 |
| <i>LBUL_0819</i> | | Acyl carrier protein | 1.49 | 0.84 | 0.32 | 0.41 |
| <i>LBUL_0820</i> | | (Acyl-carrier-protein) <i>S</i> -malonyltransferase | 1.44 | 0.28 | 0.19 | 0.27 |
| <i>LBUL_0821</i> | | Dehydrogenases with different specificities (related to short-chain alcohol dehydrogenases) | 1.66 | 0.33 | 0.26 | 0.20 |
| <i>LBUL_0822</i> | | 3-Oxoacyl-(acyl-carrier-protein) synthase | 1.68 | 0.33 | 0.22 | 0.22 |
| <i>LBUL_0823</i> | | Biotin carboxyl carrier protein | 0.97 | 0.41 | 0.21 | 0.34 |
| <i>LBUL_0824</i> | | 3-Hydroxymyristoyl/3-hydroxydecanoyl-(acyl carrier protein) dehydratases | 1.27 | 0.29 | 0.29 | 0.47 |
| <i>LBUL_0825</i> | | Acetyl/propionyl-CoA ^b carboxylase alpha subunit | 0.98 | 0.30 | 0.13 | 0.39 |
| <i>LBUL_0826</i> | | Acetyl-CoA carboxylase beta subunit | 0.94 | 0.28 | 0.24 | 0.28 |
| <i>LBUL_0827</i> | | Acetyl-CoA carboxylase alpha subunit | 1.36 | 0.26 | 0.60 | 0.37 |
| <i>LBUL_0828</i> | | Enoyl-(acyl-carrier-protein) | 0.90 | 0.30 | 0.10 | 0.32 |
| <i>LBUL_0829</i> | | Biotin-(acetyl-CoA carboxylase) ligase | 0.79 | 0.26 | 0.30 | 0.28 |
| <i>LBUL_1256</i> | | 1-Acyl- <i>sn</i> -glycerol-3-phosphate acyltransferase | 1.09 | 0.35 | 1.55 | 14.99 |
| <i>LBUL_1294</i> | | Acyl carrier protein | 0.88 | 1.00 | 0.29 | 1.78 |
| <i>LBUL_1356</i> | | Cyclopropane fatty acid synthase and related methyltransferases | 0.77 | 0.41 | 0.52 | 0.05 |
| <i>LBUL_1567</i> | | Biotin carboxylase | 1.64 | 0.98 | 3.59 | 0.76 |
| <i>LBUL_1568</i> | | Biotin carboxylase | 0.88 | 0.49 | 1.29 | 0.36 |
| <i>LBUL_1607</i> | | 3-Hydroxymyristoyl/3-hydroxydecanoyl-(acyl carrier protein) dehydratases | 0.60 | 0.44 | 0.71 | 4.36 |

^a Numbers indicate the ratios of differential expression of a gene in the mixed culture compared to the monoculture. Numbers in boldface have a ratio >2 or <0.5 and are significant ($P = 0.05$).

^b CoA, coenzyme A.

The pathways for synthesis of arginine from glutamine and glutamate were more highly expressed in mixed culture. Carbonate dehydratase, encoded by *cah* in *S. thermophilus*, was 3.8- to 15.8-fold upregulated in mixed culture, in particular in the earlier growth phases. By releasing CO₂ from carbonate, this enzyme may play a role in providing the CO₂ required for biosynthesis of aspartate, glutamate, arginine, and nucleotides (3, 22, 41) in both species. This supports the hypothesis that CO₂ production is stimulated in mixed culture, which favors growth of *L. bulgaricus* (14). Although there was no notable difference in urease expression between monoculture and mixed culture, these results are in accordance with the work described by Hervé-Jimenez et al. (15), who argued that BCAA and arginine metabolism in *S. thermophilus* are upregulated in the presence of *L. bulgaricus*.

Iron transport and H₂O₂ resistance. In *S. thermophilus* six of seven genes involved in iron transport were differentially expressed in mixed culture compared to monoculture (Table 2). The iron complex ABC transporter, consisting of *fatA*, *fatB*, *fatC*, and *fatD*, was expressed 2.7- to 4.5-fold higher in mixed culture at the second exponential phase. This observation is in contrast to the earlier findings (15), where downregulation of these genes during mixed culture growth was observed. The putative iron transport regulator *fur* and the iron chelator *dpr* were 2.4- and 2.9-fold downregulated in the mixed culture in this growth phase. In stationary phase, these genes were slightly upregulated (1.5-fold), which is in accordance with the previous study. The expression of these genes is correlated to the expression of *LBUL_2034* (potentially H₂O₂-producing pyruvate dehydrogenase), the homologue of *pox1* in *L. bulgaricus* LMG11842, but not with *LBUL_1421* and

LBUL_1955 (both potentially H₂O₂-producing dihydroorotate dehydrogenase), the homologues of *pyrD2* and *pyrD1* in *L. bulgaricus* strain ATCC 11842 (15). Thus, the intake of iron by *S. thermophilus* appears to be negatively correlated with H₂O₂ production by *L. bulgaricus*, minimizing the damage caused by ROS.

EPS biosynthesis. EPS is mainly produced in the later stages of fermentation by *S. thermophilus* (4). *S. thermophilus* EPS genes (*epsA* to *epsM*) were higher expressed at the second exponential phase and especially at the stationary phase compared to the earlier growth phases. In addition, transcription of these genes was upregulated at these two later phases in the mixed culture compared to the monoculture (Table 2), i.e., 2.1- to 5.1-fold in the second exponential phase and 2.2- to 4.0-fold in stationary phase (the transcriptional activator *epsA*, 2.0- and 1.9-fold). Similarly, many genes in the (poly)saccharide metabolism of *L. bulgaricus* were higher expressed in mixed culture compared to monoculture at the two later growth phases (Table 2). Therefore, we isolated and quantified EPS production in pure and mixed cultures. The amount of EPS increased from 0.80 ± 0.02 g/liter in a *S. thermophilus* monoculture and 1.18 ± 0.12 g/liter in a *L. bulgaricus* monoculture to 1.46 ± 0.04 g/liter in the mixed culture. However, this increase in EPS production in the mixed culture compared to the monocultures (1.3- to 1.8-fold) is less than can be expected based on the increase in total biomass (4.5- to 8.5-fold).

Fatty acid metabolism in *L. bulgaricus*. In the three later phases of fermentation, the genes encoding for LCFA synthesis by *L. bulgaricus* were 3.3- to 9.6-fold downregulated in mixed culture compared to monoculture (Table 2). However, in the second exponential and the stationary phase

LBUL_0106, *LBUL0109*, and *LBUL_1256* (all encoding 1-acyl-*sn*-glycerol-3-phosphate acyltransferase) were up to 15-fold upregulated in mixed culture. It remains to be established whether in mixed culture this acyltransferase is charged with fatty acids from another source than *de novo* biosynthesis.

DISCUSSION

In this study we report an extensive postgenomic analysis of the cocultivation of *S. thermophilus* and *L. bulgaricus* in milk. The combination of physiological approaches and transcriptome profiling of both pure and mixed cultures allowed the identification of regulatory responses to mixed culture growth in milk in both strains. The responses to cocultivation in *S. thermophilus* in many aspects confirmed findings by Hervé-Jimenez et al. related to amino acid, purine, and iron metabolism (15–16). This emphasizes the generic value of their findings since in our study a different strain combination was used. The differences observed in our study are limited to for instance the downregulation of *fatABCD* genes encoding an iron complex transporter in the second exponential phase. These differences may be explained by strain-specific features of the strains used our study, although it cannot be excluded that differences in cultivation conditions also play a role.

In addition, our study provides a postgenomic analysis of the responses of *L. bulgaricus* to cocultivation in milk. This revealed that the limited availability of purines strongly influences physiological responses in both monocultures and mixed cultures. The high abundance of PFL in *S. thermophilus* in milk (12) and the stimulatory effect of formic acid and folic acid on acidification and growth of both species show that purine availability in milk is too low to sustain optimal growth. This is also exemplified by the relative high expression of the folic acid and purine production pathways in a *L. bulgaricus* monoculture. Both of these pathways are significantly downregulated in mixed culture, whereas genes for folate cycling and purine biosynthesis are upregulated concomitantly in *S. thermophilus* during mixed culture growth (Table 2). These results support a model in which *S. thermophilus* supplies *L. bulgaricus* with crucial components for purine nucleotide biosynthesis, including the precursor formic acid and the cofactor folic acid.

Clearly, the generation of ROS is a second element specific to cocultivation responses inducing specific responses in iron metabolism. Previous findings regarding expression of genes involved in iron uptake by *S. thermophilus* (15) were extended by the discovery that their expression was correlated to growth rate and decreased in the later stages of mixed fermentation, probably as a result of increased production of H₂O₂ by *L. bulgaricus*.

The proteolytic activity of *L. bulgaricus* plays a key role in the mixed culture of *S. thermophilus* and *L. bulgaricus* as was exemplified by the large stimulatory effect of addition of hydrolyzed casein to a *S. thermophilus* culture, whereas the stimulatory effect upon addition to a mixed culture was much smaller. *L. bulgaricus* has a very limited potential for amino acid biosynthesis, which makes the bacterium highly dependent on its proteolytic activity when growing in milk (38). The cleavage of casein into peptides by PrtB and the higher expression of peptidases in *S. thermophilus* support higher growth rates of both species in mixed culture. In casein there is a relatively low

abundance of BCAA (6 to 7% [wt/wt] in casein), arginine (4%), and cysteine (0.35%). The upregulation of the pathways for biosynthesis of BCAA and sulfur amino acid indicates that the proteolytic activity of *L. bulgaricus* does not liberate sufficient amounts of these amino acids from casein to sustain optimal growth. Interestingly, these amino acid residues are relatively abundant in the predicted proteins of *S. thermophilus* (15) and *L. bulgaricus* (<http://www.cbs.dtu.dk/services/GenomeAtlas>), indicating a high biosynthetic demand (35). In fact, the strong limitation in sulfur amino acids in dairy niches may explain why the serine-sulfur amino acid interconversion pathway remained functional in *L. bulgaricus* in contrast to most other amino acid biosynthetic pathways.

Genes coding for extracellular polysaccharide biosynthesis and secretion in both *S. thermophilus* and in *L. bulgaricus* were higher expressed in mixed culture compared to growth in monoculture. This may be a direct response to the lower pH at stationary phase in the mixed culture fermentation. Low pH has been shown to trigger EPS production by *L. bulgaricus* previously (26), increasing acid resistance (23). One of the additional effects may be that it facilitates the exchange of metabolites by forcing close proximities or even physical contact between the two species, as is the case in kefir (7, 30). Moreover, it was found that fatty acid metabolism in *L. bulgaricus* was subject to transcriptional regulation during mixed culture growth, but the mechanistic basis of this observation remains to be established.

Yogurt fermentation is in many aspects the paradigm for microbiologists studying dairy fermentations. Rather than using a laboratory conditions, we conducted our study using a substrate more similar to industrial application. This provided important information on the molecular basis of this mixed-culture fermentation. Apparently, coevolution of *L. bulgaricus* and *S. thermophilus* in a milk environment is at the basis of the strong interactions between both species, which influence both their growth and industrial performance. The approach and methods applied may be extended to other mixed-culture systems since they enable a molecular characterization of responses to mixed cultivation and interactions. Finally, the results may provide prospects for engineering interacting mixed cultures, for instance by introducing nutritional dependencies in dairy systems containing probiotics.

REFERENCES

1. Baldi, P., and A. D. Long. 2001. A Bayesian framework for the analysis of microarray expression data: regularized t-test and statistical inferences of gene changes. *Bioinformatics* 17:509–519.
2. Bolotin, A., B. Quinquis, P. Renault, A. Sorokin, S. D. Ehrlich, S. Kulakauskas, A. Lapidus, E. Goltsman, M. Mazur, G. D. Pusch, M. Fonstein, R. Overbeek, N. Kyprides, B. Purnelle, D. Prozzi, K. Ngui, D. Masuy, F. Hancy, S. Burteau, M. Boutry, J. Delcour, A. Goffeau, and P. Hols. 2004. Complete sequence and comparative genome analysis of the dairy bacterium *Streptococcus thermophilus*. *Nat. Biotechnol.* 22:1554–1558.
3. Bringle, F., and J. C. Hubert. 2003. Extent of genetic lesions of the arginine and pyrimidine biosynthetic pathways in *Lactobacillus plantarum*, *L. paraplantarum*, *L. pentosus*, and *L. casei*: prevalence of CO₂-dependent auxotrophs and characterization of deficient *arg* genes in *L. plantarum*. *Appl. Environ. Microbiol.* 69:2674–2683.
4. Broadbent, J. R., D. J. McMahon, D. L. Welker, C. J. Oberg, and S. Moineau. 2003. Biochemistry, genetics, and applications of exopolysaccharide production in *Streptococcus thermophilus*: a review. *J. Dairy Sci.* 86:407–423.
5. Brouwer, R. W., S. A. F. T. van Hijum, and O. P. Kuipers. 2009. MINOMICS: visualizing prokaryote transcriptomics and proteomics data in a genomic context. *Bioinformatics* 25:139–140.
6. Chattopadhyay, M. K., C. W. Tabor, and H. Tabor. 2003. Polyamines protect

- Escherichia coli* cells from the toxic effect of oxygen. Proc. Natl. Acad. Sci. U. S. A. **100**:2261–2265.
7. Cheirsilp, B., H. Shimizu, and S. Shioya. 2003. Enhanced kefir production by mixed culture of *Lactobacillus kefiranofaciens* and *Saccharomyces cerevisiae*. J. Biotechnol. **100**:43–53.
 8. Courtin, P., V. Monnet, and F. Rul. 2002. Cell wall proteinases PrtS and PrtB have a different role in *Streptococcus thermophilus*/*Lactobacillus bulgaricus* mixed cultures in milk. Microbiology **148**:3413–3421.
 9. Courtin, P., and F. Rul. 2004. Interactions between microorganisms in a simple ecosystem: yogurt bacteria as a study model. Lait **84**:125–134.
 10. Crittenden, R. G., N. R. Martinez, and M. J. Playne. 2003. Synthesis and utilisation of folate by yoghurt starter cultures and probiotic bacteria. Int. J. Food Microbiol. **80**:217–222.
 11. den Hengst, C. D., M. Groeneveld, O. P. Kuipers, and J. Kok. 2006. Identification and functional characterization of the *Lactococcus lactis* CodY-regulated branched-chain amino acid permease BcaP (Ctra). J. Bacteriol. **188**:3280–3289.
 12. Derzelle, S., A. Bolotin, M. Y. Mistou, and F. Rul. 2005. Proteome analysis of *Streptococcus thermophilus* grown in milk reveals pyruvate formate-lyase as the major upregulated protein. Appl. Environ. Microbiol. **71**:8597–8605.
 13. Donbrow, M., E. Azaz, and A. Pillersdorf. 1978. Autoxidation of polysorbates. J. Pharm. Sci. **67**:1676–1681.
 14. Driessen, F. M., F. Kingma, and J. Stadhouders. 1982. Evidence that *Lactobacillus bulgaricus* in yoghurt is stimulated by carbon dioxide produced by *Streptococcus thermophilus*. Neth. Milk Dairy J. **36**:135–144.
 15. Hervé-Jimenez, L., I. Guillouard, E. Guedon, S. Boudebouze, P. Hols, V. Monnet, E. Maguin, and F. Rul. 2009. Post-genomic analysis of *Streptococcus thermophilus* co-cultivated in milk with *Lactobacillus delbrueckii* subsp. *bulgaricus*: involvement of nitrogen, purine and iron metabolisms. Appl. Environ. Microbiol. **75**:2062–2073.
 16. Hervé-Jimenez, L., I. Guillouard, E. Guedon, C. Gautier, S. Boudebouze, P. Hols, V. Monnet, F. Rul, and E. Maguin. 2008. Physiology of *Streptococcus thermophilus* during the late stage of milk fermentation with special regard to sulfur amino-acid metabolism. Proteomics **8**:4273–4286.
 17. Kerwin, B. A. 2008. Polysorbates 20 and 80 used in the formulation of protein biotherapeutics: structure and degradation pathways. J. Pharm. Sci. **97**:2924–2935.
 18. Krause, I., A. Bockhardt, H. Neckerman, T. Henle, and H. Klostermeyer. 1995. Simultaneous determination of amino acids and biogenic amines by reversed-phase high-performance liquid chromatography of the dansyl derivatives. J. Chromatogr. A **715**:67–79.
 19. Letort, C., M. Nardi, P. Garault, V. Monnet, and V. Juillard. 2002. Casein utilization by *Streptococcus thermophilus* results in a diauxic growth in milk. Appl. Environ. Microbiol. **68**:3162–3165.
 20. Liu, F., L. Du, P. Du, and G. Huo. 2009. Possible promoter regions within the proteolytic system in *Streptococcus thermophilus* and their interaction with the CodY homolog. FEMS Microbiol. Lett. **297**:164–172.
 21. Makarova, K., A. Slesarev, Y. Wolf, A. Sorokin, B. Mirkin, E. Koonin, A. Pavlov, N. Pavlova, V. Karamychev, N. Polouchine, V. Shakhova, I. Grigoriev, Y. Lou, D. Rohksar, S. Lucas, K. Huang, D. M. Goodstein, T. Hawkins, V. Plengvidhya, D. Welker, J. Hughes, Y. Goh, A. Benson, K. Baldwin, J. H. Lee, I. Diaz-Muniz, B. Dosti, V. Smeianov, W. Wechter, R. Barabote, G. Lorca, E. Altermann, R. Barrangou, B. Ganesan, Y. Xie, H. Rawsthorne, D. Tamir, C. Parker, F. Breidt, J. Broadbent, R. Hutkins, D. O'Sullivan, J. Steele, G. Unlu, M. Saier, T. Klaenhammer, P. Richardson, S. Kozyavkin, B. Weimer, and D. Mills. 2006. Comparative genomics of the lactic acid bacteria. Proc. Natl. Acad. Sci. U. S. A. **103**:15611–15616.
 22. McFadden, B. A. 1973. Autotrophic CO₂ assimilation and the evolution of ribulose diphosphate carboxylase. Bacteriol. Rev. **37**:289–319.
 23. Mozzi, F., E. Gerbino, G. Font de Valdez, and M. I. Torino. 2009. Functionality of exopolysaccharides produced by lactic acid bacteria in an *in vitro* gastric system. J. Appl. Microbiol. **107**:56–64.
 24. Partanen, L., N. Marttinen, and T. Alatossava. 2001. Fats and fatty acids as growth factors for *Lactobacillus delbrueckii*. Syst. Appl. Microbiol. **24**:500–506.
 25. Pastink, M. I., B. Teusink, P. Hols, S. Visser, W. M. de Vos, and J. Hugenholtz. 2009. Genome-scale model of *Streptococcus thermophilus* LMG18311 for metabolic comparison of lactic acid bacteria. Appl. Environ. Microbiol. **75**:3627–3633.
 26. Petry, S., S. Furlan, M. J. Crepeau, J. Cerning, and M. Desmazeaud. 2000. Factors affecting exocellular polysaccharide production by *Lactobacillus delbrueckii* subsp. *bulgaricus* grown in a chemically defined medium. Appl. Environ. Microbiol. **66**:3427–3431.
 27. Pieterse, B., R. H. Jellema, and M. J. van der Werf. 2006. Quenching of microbial samples for increased reliability of microarray data. J. Microbiol. Methods **64**:207–216.
 28. Saulnier, D. M., D. Molenaar, W. M. de Vos, G. R. Gibson, and S. Kolida. 2007. Identification of prebiotic fructo-oligosaccharide metabolism in *Lactobacillus plantarum* WCFS1 through microarrays. Appl. Environ. Microbiol. **73**:1753–1765.
 29. Savijoki, K., H. Ingmer, and P. Varmanen. 2006. Proteolytic systems of lactic acid bacteria. Appl. Microbiol. Biotechnol. **71**:394–406.
 30. Sieuwerts, S., F. A. M. de Bok, J. Hugenholtz, and J. E. T. van Hylckama Vlieg. 2008. Unraveling microbial interactions in food fermentations: from classical to genomics approaches. Appl. Environ. Microbiol. **74**:4997–5007.
 31. Sieuwerts, S., F. A. M. de Bok, E. Mols, W. M. de Vos, and J. E. T. van Hylckama Vlieg. 2008. A simple and fast method for determining colony forming units. Lett. Appl. Microbiol. **47**:275–278.
 32. Siezen, R. J. 1999. Multi-domain, cell-envelope proteinases of lactic acid bacteria. Antonie Van Leeuwenhoek **76**:139–155.
 33. Stevens, M. J., D. Molenaar, A. de Jong, W. M. De Vos, and M. Kleerebezem. 2010. σ^{54} -Mediated control of the mannose phosphotransferase system in *Lactobacillus plantarum* impacts on carbohydrate metabolism. Microbiology **156**:695–707.
 34. Sturn, A., J. Quackenbush, and Z. Trajanoski. 2002. Genesis: cluster analysis of microarray data. Bioinformatics **18**:207–208.
 35. Sundararajan, T. A., and P. S. Sarma. 1957. Preparation and amino acid composition of enzymically dephosphorylated casein. Biochem. J. **65**:261–266.
 36. Sybesma, W., M. Starrenburg, L. Tijsseling, M. H. Hoefnagel, and J. Hugenholtz. 2003. Effects of cultivation conditions on folate production by lactic acid bacteria. Appl. Environ. Microbiol. **69**:4542–4548.
 37. Teusink, B., F. H. van Enckevort, C. Francke, A. Wiersma, A. Wegkamp, E. J. Smid, and R. J. Siezen. 2005. In silico reconstruction of the metabolic pathways of *Lactobacillus plantarum*: comparing predictions of nutrient requirements with those from growth experiments. Appl. Environ. Microbiol. **71**:7253–7262.
 38. van de Guchte, M., S. Penaud, C. Grimaldi, V. Barbe, K. Bryson, P. Nicolas, C. Robert, S. Oztas, S. Mangelot, A. Couloux, V. Loux, R. Dervyn, R. Bossy, A. Bolotin, J. M. Batto, T. Walunas, J. F. Gibrat, P. Bessieres, J. Weissenbach, S. D. Ehrlich, and E. Maguin. 2006. The complete genome sequence of *Lactobacillus bulgaricus* reveals extensive and ongoing reductive evolution. Proc. Natl. Acad. Sci. U. S. A. **103**:9274–9279.
 39. van Hijum, S. A. F. T., R. J. Baerends, A. L. Zomer, H. A. Karsens, V. Martin-Requena, O. Trelles, J. Kok, and O. P. Kuipers. 2008. Supervised Lowess normalization of comparative genome hybridization data: application to lactococcal strain comparisons. BMC Bioinformatics **9**:93.
 40. van Hijum, S. A. F. T., A. de Jong, R. J. Baerends, H. A. Karsens, N. E. Kramer, R. Larsen, C. D. den Hengst, C. J. Albers, J. Kok, and O. P. Kuipers. 2005. A generally applicable validation scheme for the assessment of factors involved in reproducibility and quality of DNA-microarray data. BMC Genomics **6**:77.
 41. Wang, H., W. Yu, T. Coolbear, D. O'Sullivan, and L. L. McKay. 1998. A deficiency in aspartate biosynthesis in *Lactococcus lactis* subsp. *lactis* C2 causes slow milk coagulation. Appl. Environ. Microbiol. **64**:1673–1679.