RESEARCH ARTICLE

Physiology of *Streptococcus thermophilus* during the late stage of milk fermentation with special regard to sulfur amino-acid metabolism

Luciana Herve-Jimenez[1, 2], Isabelle Guillouard[2], Eric Guedon[2], Céline Gautier[2], Samira Boudebbouze[2], Pascal Hols[3], Véronique Monnet[1], Françoise Rul[1] and Emmanuelle Maguin[2]*

[1] INRA,UR477 Biochimie Bactérienne, Jouy-en-Josas, France
[2] INRA, UR895 Génétique Microbienne, Jouy-en-Josas, France
[3] Unité de Génétique, Institut des Sciences de la Vie, Université Catholique de Louvain, Louvain-la-Neuve, Belgium

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Streptococcus thermophilus is a thermophilic lactic acid bacterium widely used as starter in the manufacture of dairy products in particular in yoghurt manufacture in combination with *Lactobacillus delbrueckii* ssp. *bulgaricus*. However, in spite of its massive use, the physiological state of *S. thermophilus* in milk has hardly been investigated. We established the first map of the cytosolic proteome of *S. thermophilus* LMG18311 grown in milk. It comprises 203 identified proteins corresponding to 32% of theoretical proteome. In addition, using proteomic and transcriptomic approaches, we analyzed the physiology of LMG18311 during its late stage of growth in milk (between 2h30 and 5h30). It revealed the up-regulation of (i) peptides and AA transporters and of specific AA biosynthetic pathways notably for sulfur AA and (ii) genes and proteins involved in the metabolism of various sugars. These two effects were also observed in LMG18311 grown in milk in coculture with *L. bulgaricus* although the effect on sugar metabolism was less pronounced. It suggests that the stimulatory effect of *Lactobacillus* on the *Streptococcus* growth is more complex than AA or peptides supply.

Keywords:
Lactobacillus bulgaricus / Proteomic reference map / Protocooperation / Sugar metabolism / Sulfur amino acids

1 Introduction

Streptococcus thermophilus is a thermophilic lactic acid bacterium (LAB) widely used as starter in the manufacture of dairy products; it is the second most important industrial dairy starter after *Lactococcus lactis*. For cheese-making, *S. thermophilus* is used alone or in combination with several lactobacilli or mesophilic starters while in yoghurt it is always cocultured with *Lactobacillus delbrueckii* ssp. *bulgaricus* (*L. bulgaricus*) [1]. *S. thermophilus* is closely related to *L. lactis*, but it is even more closely related to other streptococcal species including several pathogens [2]. Recently, the complete genome sequence of three strains became available [3, 4]. Comparison of *S. thermophilus* genome sequences with published genomes of streptococcal pathogens reveals that the most important determinants for pathogenicity are either absent or present as pseudogenes and that *S. thermophilus* has followed an evolutionary path divergent to that of pathogenic species due to its adaptation to milk [3].

S. thermophilus is highly adapted to grow on lactose, the main carbon source in milk and rapidly converts it into lac-

Correspondence: Dr. Françoise Rul, Unité de Biochimie Bactérienne, INRA, 78350 Jouy-en-Josas, France
E-mail: françoise.rul@jouy.inra.fr
Fax: +33-1-3465-2163

Abbreviations: **AA**, amino acid(s); **LAB**, lactic acid bacterium

* Present address: Danone Vitapole, Palaiseau, France.

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tate during growth. Lactose is transported into the cell by a lactose permease (LacS), which operates as a galactoside-proton symport system or as a lactose-galactose antiporter [5]. Lactose is efficiently transported into the cell and subsequently hydrolyzed by an intracellular β-galactosidase. The vast majority of *S. thermophilus* strains only metabolized the glucose moiety of lactose, while galactose is excreted into the medium [6].

The milk is poor in free amino acids (AA) and short peptides [7], therefore for optimal growth, *S. thermophilus* requires either hydrolysis of caseins followed by the internalization and the degradation of the resulting peptides or *de novo* AA biosynthesis [8]. For many LAB including *S. thermophilus,* the hydrolysis of milk caseins (*i.e.* the AA supply) mostly depends on the activity of a cell-wall-anchored proteinase. Few strains of *S. thermophilus* possess this proteinase [9]; they grow rapidly in milk and reach up to 10^9 cell/mL, when they are proteinase-minus, they only reach a cell density of about 10^8 cell/mL [10]. For the internalization and degradation of peptides, *S. thermophilus* posses an efficient oligopeptide transport system, Ami [11] and a set of intracellular peptidases [12]. Although, some strains of *S. thermophilus* are prototroph for AA, others are auxotrophs for given AA. For example, the analysis of LMG18311 genome sequence (the strain used in this work) suggests auxotrophies for His, Lys, and Glu or Gln while all other biosynthesis pathways appear functional. The importance of AA biosynthesis for the growth in milk was established [8] and the branched-chain AA biosynthesis pathway was identified as a key pathway for optimal growth of *S. thermophilus* in milk [13].

Only few proteomic studies were so far performed on *S. thermophilus* and most of them were done on bacteria grown in rich laboratory medium (M17). An *S. thermophilus* reference map was established from exponential phase culture in M17 [14] and stress-responsive proteins were identified in various conditions (heat, cold, acid or oxidative stress and starvation) [15–20]. The first proteomic study of *S. thermophilus* in milk demonstrated that in comparison with M17, the main differences observed after the first 1h30 of fermentation affected the pyruvate formate lyase and proteins involved in the supply and biosynthesis of AA and purines [21]. The objective of the present study was to investigate the physiology of *S. thermophilus* during late stage of growth in milk. By combining proteomic and transcriptomic approaches, we revealed a diversification of carbon metabolism as well as an up-regulation of nitrogen uptake and AA biosynthesis and thus evidenced factors that may hamper *S. thermophilus* late growth in milk.

2 Materials and methods

2.1 Strains and culture conditions

S. thermophilus LMG18311 was obtained from the BCCM collection (Belgium), and *L. bulgaricus* ATCC11842 from ATCC (USA). Bacteria stocks were prepared in 10% w/v Nilac skim milk (NIZO, Ede, The Netherlands) autoclaved 15 min at 112°C. At a pH value of 5.4–5.5, cultures were frozen in liquid nitrogen and stored at −80°C. Before use, the micro-filtrated Marguerite® milk (La Laiterie, Villefranche sur Saône, France) was skimmed by centrifugation (4°C, 5000 × *g,* 30 min). Milk (500 mL) was inoculated at 10^6 CFU/mL with each bacteria stock (monoculture and coculture) and incubated at 42°C. The pH and cell counts were monitored every 20 min. For enumeration, the bacterial chains were disrupted with a mechanical blender (Turax X620, Labo-Moderne, France) for 40 s (for *S. thermophilus*) and dilutions were then plated with an automatic spiral platter (AES Laboratoires, Combourg, France) on M17 agar lactose (1%) or MRS agar lactose (2%) acidified to pH 5.2 (for *L. bulgaricus*). Colonies were counted after 16 h incubation (*S. thermophilus*) or 36 h (*L. bulgaricus*) at 42°C in anaerobiosis (Anaerocult A, Merck, Darmstadt, Germany). Cultures were prepared in three independent experiments. When required, the medium was supplemented with AA as previously described [22].

2.2 Extraction of proteins and 2-DE

Proteins were extracted from 300 mL cultures in milk at early (2h30) and late exponential (5h30) phases according to [21] with modifications: cells were harvested by centrifugation at 20°C and the cell pellet was washed three times with extraction buffer. Cell lysates were centrifuged (5000 × *g,* 15 min, 4°C), the supernatant collected and centrifuged (200 000 × *g,* 30 min, 4°C) to remove cell debris.

The 2-DE was performed as described in [23] with modifications: 300 μg of proteins was precipitated with the 2-D Clean-Up Kit at 10% TCA (GE Healthcare, Saclay, France), IEF was carried out for 60 000 V/h at a maximum of 10 000 V using an Ettan IPGphor (GE Healthcare), and 10% SDS PAGE was used for the 2nd migration. Gels were digitized using an Epson Expression 1640XL scanner set (at 256 grey levels) controlled by the Silver Fast software and analyzed using the Phoretix 2D software package (GE Healthcare). The gel images were calibrated for MW (molecular weight) and p*I* using the theoretical values of a set of identified proteins: DnaK, Eno, Fba, Ldh, Pfl, GapA. The relative volume of each spot was obtained from its spot intensity and normalized with the intensity of all spots. Three independent samples were analyzed for each condition. An analysis of variance was performed using R (http://www.R-project.org) after logarithmic transformation of the data. Only differences with p-value <0.05 and at least twofold volume variations between the two conditions were further analyzed. When a data was missing for one of triplicates, it was set to the mean value of the two other values, as proposed in [24]. MS analyses were performed using a Voyager-DE-STR (Applied Biosystems, Framingham, USA) on our PAPSS proteomic platform (http://www.jouy.inra.fr/unites/proteines/papss/) [23]. The proteins were identified using MS-FIT (http://prospector.ucsf.edu).

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2.3 RNA isolation and transcriptomics

Bacteria were harvested from 300-mL culture. The RNA extraction was carried out as described in [21] with modifications: cells were harvested by centrifugation (10 000 × *g*, 10 min, room temperature) and frozen pellets were resuspended in 400 µL of Tris (10 mM pH 7) and 60 µL EDTA (0.5 M pH 8). The protocol of Sperandio *et al*. [25] was then followed.

The expression profiles were obtained using the EGT-K40C microarray (Eurogentec, Liège, Belgium) containing 92% of the LMG18311 genes. RNA from *L. bulgaricus* was labeled using the protocols described above and, by hybridizing the labeled samples to the *S. thermophilus* microarray, we verified that *L. bulgaricus* RNA did not cross-hybridize.

Of total RNA, 10 µg was reverse-transcribed by random priming, using the Pronto!™ Plus Direct System (Corning-Promega, USA), and labeled by incorporation of Cy3- or Cy5-dCTP nucleotides (Amersham Biosciences, UK). Of each labeled cDNA, 100 pmoles was used for overnight hybridization at 42°C. The arrays were scanned on a microarray scanner (Agilent, USA). The statistical analysis was based on one dye-swap. For each array, the raw data comprised the logarithm of median feature pixel intensity at wavelengths 635 and 532 nm. No background was subtracted. Arrays were normalized with the Anapuce package (http://cran.r-project.org/web/packages/anapuce/) in 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 [26]. Finally, the raw *p*-values were adjusted by the Bonferroni method and we considered as being differentially expressed the genes with a *p*-value $\leq$0.05 and a difference higher than 2 (up-regulated) or lower than 0.5.

The quantitative RT-PCR (RT-qPCR) was carried out using cDNA synthesized from 3 µg of RNA samples by the PowerScript reverse transcriptase (Clontech, Saint-Quentin-en-Yvelines, France) according to the supplier protocol. All gene-specific primers (Table S1) were designed using primer3 [27]. PCR was carried as described in [28]. For each condition, the measures triplicated with cDNA synthesized from two independent RNA samples. The results were normalized using *stu1254* (*murE*) as reference.

2.4 Analysis of milk compounds

Free AA were quantified in milk supernatant at various times (means of the three values obtained for three independent cultures). Supernatants were recovered after precipitation with 3% sulfosalycilic acid, 15 min on ice and centrifugation (13 000 × *g*, 10 min, 4°C). AA were then analyzed by ion-exchange chromatography, followed by ninhydrin post-column derivatization and detected at 570 and 440 nm on a Waters 2487 dual absorbance detector [29]. A PCX5200 post-column derivatizer (Pickering Laboratories, USA) was connected to a Waters 626 HPLC system (Waters, USA).

3 Results and discussion

3.1 Growth of LMG18311 in milk

In agreement with previous work [21], LMG18311 presented two growth phases in milk (Fig. 1). The first growth phase was characterized by a generation time of 31.6 $\pm$ 0.9 min that lasted until 2 h. It corresponded to a rapid growth phase during which both the LMG18311 monoculture and the LMG18311-*L. bulgaricus* ATCC11842 coculture exhibited similar acidification curves and numerations (Hervé *et al*., personal communication). After 2h30, the growth phase slowed down with a generation time of 314 $\pm$ 25 min. At this stage, a clear difference between the mono- and cocultures existed, suggesting that, in the monoculture, LMG18311 encountered condition(s) that hampered its growth whereas in the coculture *L. bulgaricus* overcame directly or indirectly this effect. We therefore analyzed by transcriptomic and proteomic approaches the physiological state of LMG18311 at 2h30 and 5h30 of growth, *i.e.* during the late phase of growth.

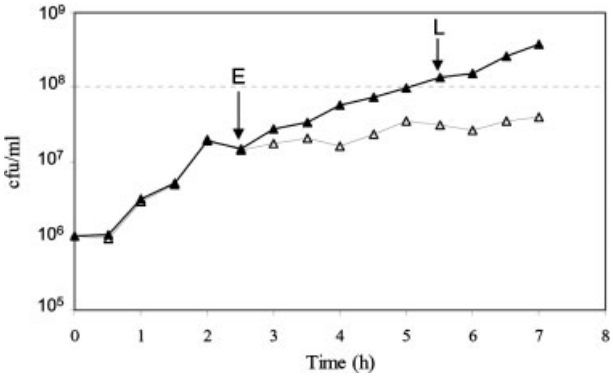

Figure 1. Enumeration of *S. thermophilus* viable counts (CFU/mL) during growth in milk either in mono-(Δ) or in coculture (▲) with *L. bulgaricus* ATCC11842. The curves are representative of three independent experiments. At early (E, 2h30), and late (L, 5h30) exponential phases (arrows), bacteria were harvested for proteomic and transcriptomic analysis.

3.2 Transcriptome analysis during growth in milk

Statistical analysis of the transcriptome data revealed that 60 genes (~3.4% of the tested genes) varied at least twofold between 2h30 and 5h30 of growth; 3 and 57 genes were down-regulated and up-regulated, respectively. For 15 of these genes, we also performed specific RT-qPCR measurements; the modifications of expression were confirmed for 11 of them.

For 42 genes out of 60 (70%), a consistent pattern of expression was observed for several genes clustered next to each other in the chromosome, suggesting the existence of 15 operons (Table 1). The location of rho-independent terminators in the LMG18311 genome sequence fitted the expression cluster in nine cases. An additional RT-qPCR was

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Table 1. Gene differentially expressed during the late phase of LMG18311 growth in milk

Locus *(stu #)*	Gene name	Description	Fold change L *vs.* E		Putative operon structure[b]
			μArray[a]	RT-qPCR	
AA and peptide transporter					
0296	*atmA*	Polar AA ABC transporter, substrate binding protein	5.4		*stu0296-297-fragmented gene- stu0301-0302-dctA*
0297	*atmB*	Methionine ABC transporter, substrate binding protein	4.6	3.4 ± 1	*stu0296-297-fragmented gene- stu0301-0302-dctA*
0301	*atmD*	Methionine ABC transporter, ATP-binding protein	6.9		*stu0296-297-fragmented gene- stu0301-0302-dctA*
0302	*atmE*	Methionine ABC transporter, permease	5.5		*stu0296-297-fragmented gene- stu0301-0302-dctA*
0303	*dctA*	Serine-threonine:Na+symporter	2.6[c]		*stu0296-297-fragmented gene- stu0301-0302-dctA*
0359	*livJ*	Branched-chain AA transporter, substrate binding protein	np[d]	2.4 ± 0.2	*livJHMGF*
0360	*livH*	Branched-chain AA transporter, permease	3.0		*livJHMGF*
0361	*livM*	Branched-chain AA transporter, permease	3.1[c]		*livJHMGF*
0362	*livG*	Branched-chain AA transporter, ATP-binding protein	3.8		*livJHMGF*
0363	*livF*	Branched-chain AA transporter, ATP-binding protein	3.3[c]		*livJHMGF*
1161		Polar AA transporter, ATP-binding protein	3.5	2.6 ± 0.4	*stu1161-1162-1163-1164*
1162		Polar AA transporter, substrate binding protein	nv[e]		*stu1161-1162-1163-1164*
1163		Polar AA transporter, permease	2.7[c]		*stu1161-1162-1163-1164*
1164		Polar AA transporter, permease	2.7[c]		*stu1161-1162-1163-1164*
0970	*dtpT*	Di/tripeptide transporter	3.5	2.7 ± 1	
1438	*amiF*	Oligopeptide ABC transporter, ATP-binding protein	13.2	13 ± 4	*amiA$_1$CDEF*
1439	*amiE*	Oligopeptide ABC transporter, ATP-binding protein	10.2		*amiA$_1$CDEF*
1440	*amiD*	Oligopeptide ABC transporter, permease	9		*amiA$_1$CDEF*
1441	*amiC*	Oligopeptide ABC transporter, permease	7.8		*amiA$_1$CDEF*
1442	*amiA$_1$*	Oligopeptide ABC transporter, substrate binding protein pseudogene	3.5		*amiA$_1$CDEF*
1445	*amiA$_3$*	Oligopeptide ABC transporter, substrate binding protein	3.6		
0125	*amiA$_2$*	Oligopeptide ABC transporter, substrate binding protein	nd[f]	8.89 ± 5	
AA metabolism					
1527	*serA*	D-3-phosphoglycerate dehydrogenase hypoxanthine	3.3	2.8 ± 0.1	
0366	*cysM$_1$*	Cysteine synthetase	10.7		
0352	*metB$_1$*	Cystathionine gamma-synthase	nv[e]	4.7 ± 0.9	*metB$_1$-stu0353*
0353		Aminotransferase (class II)	3.9	2.3 ± 0.3	*metB$_1$-stu0353*
0846	*cysM$_2$*	Cysteine synthetase	3.7	2.8 ± 1	*cysM$_2$-metB$_2$-cysE$_2$*
0847	*metB$_2$*	Cystathionine beta-lyase	3.3		*cysM$_2$-metB$_2$-cysE$_2$*
0848	*cysE2*	Serine acetyltransferase, putative	nv[e]	nv[e]	
0785	*metE*	5-Methyltetrahydropteroyltriglutamate	4.3	2.6 ± 0.1	*metE-metF*
0786	*metF*	5,10-Methyltetrahydrofolate reductase	3.4		*metE-metF*
0987	*cysD*	*O*-acetyl homoserine sulfydralase	4.5		
0378	*lysC*	Aspartate kinase	5.1		
1172	*metK*	*S*-adenosylmethionine synthetase	3.2[c]	nv[e]	
1588	*trpB*	Tryptophan synthase, beta subunit	2.6[c]		*stu1594-trpEGDCFBA*
1593	*trpE*	Anthranilate synthase, component I	6.9		*stu1594-trpEGDCFBA*
0463	*aspC$_3$*	Aminotransferase	6.4		

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Table 1. Continued

Locus *(stu #)*	Gene name	Description	Fold change L *vs.* E		Putative operon structure[b]
			μArray[a]	RT-qPCR	
tRNA synthetase					
1043	*tyrSE*	Tyrosyl-tRNA synthetase	7.5	nv[e]	
Protease					
0721		Peptidase family S1	0.3		
Ribosome and translation					
0592	*rpsA*	30S ribosomal protein S1	3.8		
1817	*rplA*	50S ribosomal protein L1	3.1[c]		*rplK-rplA*
1818	*rplK*	50S ribosomal protein L11	3.5		*rplK-rplA*
C-metabolism					
0163	*rgpG*	Glycosyl transferase	3.6		*mecA-rgpG*
0400	*fruB*	Fructose 1-phosphate kinase	7.9		
1657	*pfl*	Pyruvate formate lyase	0.2		
1797	*rpe*	Ribulose phosphate 3-epimerase	8.7		
Purine & pyrimidine metabolism					
0355	*upp*	Uracile phosphoribosyl transferase	4.2	nv[e]	
1270	*nrdE*	Ribonucleoside-diphosphate reductase, alpha chain	3.6		
Fe-S cluster metabolism					
0164		Fe-S cluster assembly, ATP binding protein	3.9	6.5 ± 0.8	*stu0164-sufD-nifS$_1$-nifU-sufB*
0165	*sufD*	Fe-S cluster assembly protein	4.2		
Trancription regulator					
1044		Transcriptional regulator MutR family	3.6		
DNA metabolism					
1761	*polA*	DNA polymerase I	8.5		*polA-mutS2*
1762	*mutS2*	DNA mismatch repair protein	9.5		*polA-mutS2*
1845	ssbA	Single-strand binding protein	3.7		
Other					
0162	*mecA*	Protease adaptor protein	4.1		*mecA-rgpG*
0292	*pps*	Non-ribosomal peptide synthetase	3.0[c]		*pps-stu0293-stu0294*
0293		Hypothetical protein	4.5		*pps-stu0293-stu0294*
0294		Drug:H+ antiporter-3 family	3.4		*pps-stu0293-stu0294*
0651		Hypothetical protein	5.9		*mip-stu0651-eetB*
0652	*mip*	Mutative macrophage infectivity potentiator-related protein	3.4		*mip-stu0651-eetB*
0748		Hypothetical protein	0.3		
1283		Hypothetical protein	nv[e]		
1284	*pdxK*	Pyridoxine kinase	3.0[c]		*stu1285- pdxK-stu1283*
1285		Predicted membrane protein	3.1[c]		*stu1285- pdxK-stu1283*
1633		Hypothetical protein (putative lipoprotein)	4.3		
1634	*entB*	Pyrazinamidase/nicotidanimase	4.0		*entB*

a) L/E ratio: ratio between the relative mRNA levels at 5h30 and 2h30.
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) np: Not spotted on the microarray.
e) nv: No variation.
f) nd: Not detected.

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performed on *livJ* (not spotted on the microarray) to confirm that it belongs to the same expression cluster and operon than the other *liv* genes. For the other five clusters, further analyses would be required to define the operons. (i) The mRNA of *eetB* (stu0650) supposedly in operon with *stu0652-stu0651* was not detected on microarrays, (ii) the *stu0164-sufD* and the downstream *stu0166-nifS1-nifU-sufB* genes displayed different expression patterns despite the absence of a putative terminator, (iii) the mRNA of *stu1283* and *cysE2*- supposedly in operon with *stu1285-pdxK* and *cysM2-metB2,* respectively - did not vary on microarrays, and (iv) the expression of only two genes over eight in the *trp* putative operon increased. In these cases, either the genes do not really belong to the same transcriptional units or additional regulation mechanisms decreased the levels of some transcripts.

The 60 genes over- or down-expressed during the growth of LMG18311 belong to various functional classes: AA and peptides transport (19 genes), AA biosynthesis (13), functions dependent on the availability of AA (5), carbon metabolism (4), DNA metabolism (3), base metabolism (2), iron homeostasis (2), transcriptional regulation (1), protease adaptor protein (*mecA*) and other or unknown functions (10). It is obvious from these expression data that nitrogen metabolism and more specifically AA supplying pathways were highly regulated during the growth in milk.

3.3 Reference map and proteomic analysis during growth in milk

The total number of different protein spots in all 2-DE gels was 336. They were all excised and characterized by MS analysis allowing the identification of 203 proteins (Supporting Information Table S2).

The 1.8-Mb genome sequence of LMG18311 contains 1889 ORF corresponding to 1529 putative proteins (pseudogenes excluded) [3]. According to predictions (http://www.jvirgel.de/index.html, [30]), 852 of these proteins have a p*I* between 4 and 7 and a MW ranging from 15 to 120 kDa, *i.e.* visible in our 2-DE conditions. Considering that 183 of these proteins have at least one transmembrane domain and that 34 exhibited a peptide signal, our LMG18311 theoretical proteome comprised 635 proteins, and we identified 32% of this proteome (Supporting Information Table S2). High codon adaptation indexes (CAI) often indicate genes that are highly expressed [31, 32]. For 89% of the proteins identified by 2-DE, the corresponding genes presented a CAI above >0.5. The genes coding for glycolytic enzymes and elongation factors presented the highest values (>0.675) and they corresponded to the major proteins in the 2-DE map. In a previous study [21], 46 proteins of LMG18311 whose abundance varied between exponential growth in milk and in a rich laboratory medium were identified. Most of them were included in the 203 proteins that constitute the first reference map of *S. thermophilus* in milk (Fig. 2 and http://migale.jouy.inra.fr/herve-paris/). This map gives an overview of the functions required for the growth in milk; the main categories are AA transport and metabolism (20%), translation (14%), nucleotides transport and metabolism (12%), unknown functions (9%), energy production (8%), and carbohydrate transport and metabolism (7%).

Comparison of the proteomes revealed that 27 proteins (44 spots) varied significantly between the two growth stages: 13 and 14 proteins were down- and up-regulated, respectively (Table 2). These proteins are distributed in at least eight functional classes (http://www.biol.ucl.ac.be/gene/genome). Despite the impossibility to detect the AA and peptide transporters by 2-DE, we observed, as in the transcriptome, a strong effect on the AA biosynthesis pathways (11 proteins) or on other processes dependent on AA availability (PepX, Stu0671, HisS, ThrS, and Rrf). The other altered functional classes were carbon metabolism (6), purine-pyrimidine metabolism (1), a response regulator and other or unknown functions (7).

3.4 Comparison of transcriptomic and proteomic data

Comparison of the transcriptome and proteome analysis data revealed a concordance of the observed effect for nine proteins (Table 2, underlined). For the other 21 proteins, the mRNA was either not detected or did not vary in the tested conditions. The expression of 18 genes coding these proteins was re-evaluated using RT-qPCR (Table 2); in 15 cases, it confirmed that the gene expression did not significantly vary between 2h30 and 5h30 in contrast to the protein amounts. These observations evidenced either (i) a transient transcriptional regulation or (ii) a post-transcriptional regulation altering directly the protein turn-over. With respect to these data, it is noteworthy to mention here the fourfold induction of the *mecA* gene, known in *B. subtilis* as an adaptor protein modulating the substrate preference of the ClpCP protease [33].

3.5 Diversification and alteration of the carbon metabolism

Both transcriptomic and proteomic data indicated that *S. thermophilus* diversified its carbon sources during the late stage of growth in milk. Various genes/proteins (*fruB* and *rpe* genes, ScrB and GlmS protein) involved in sugar catabolism as well as in galactose utilization (Leloir pathway; GalE1, GalK proteins) were significantly up-regulated. It is noteworthy that the *gal* genes were expressed in the LMG18311 strain under these conditions, although this strain does not grow on galactose as sole carbon source. One may hypothesize that, at the end of the fermentation, *S. thermophilus* no longer imported lactose and concomitantly no longer exported galactose, and/or re-imports galactose from the medium, as it is the case for several strains of *S. thermophilus* [34]. The intracellular galactose may then be metabolized by the Leloir

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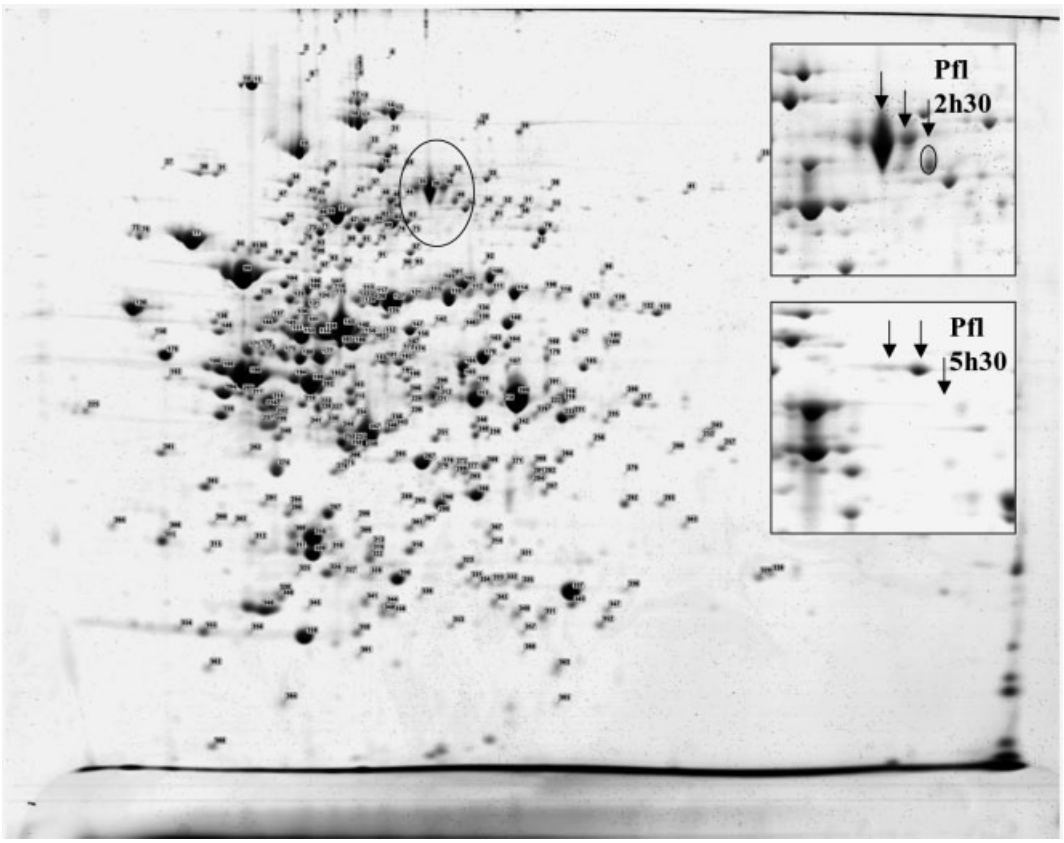

Figure 2. Reference map of LMG18311 cytosolic proteins. Zoom: protein Pfl, the circle indicates the possible active form and the arrows show the different isoforms. Protein (300 μg) was loaded in the first dimension and 2-DE was performed with a pH gradient of 4–7.

pathway involving the *galKTEM* operon. In *L. lactis*, the diversification of carbon sources was shown to be a general response to carbon limitation [35] but also to other stresses such as acidification [36]. At the opposite, all the pyruvate formate lyase isoforms decreased (Fig. 2) including one with a lower MW, probably corresponding, to a derivative of the active form as described for *L. lactis* [37] and *S. bovis* [38].

3.6 Up-regulation of nitrogen uptake and biosynthesis during late growth

Variation of the expression or abundance of 84 genes or proteins was observed between the late and early exponential phase of *S. thermophilus* in milk. Interestingly, 51% of them are involved in AA or peptides transport and metabolism, and functions dependent upon AA availability in the cell (t-RNA synthetases and ribosomal proteins).

Many genes encoding peptide and AA transporters were overexpressed during the late phase of growth (Table 1). They encode the two peptide transporters of *S. thermophilus*: the di-tripeptide transporter DtpT and the Ami oligopeptide transport system (*ami A1CDE F* operon constituted of four genes and a pseudogene *amiA1*) working with two oligopeptides-binding proteins AmiA2 and AmiA3. In addition, four AA transporters were also up-regulated: a putative Ser/Thr:Na^+ symporter (DctA), a polar AA transporter (Stu1161-1164), the only putative branched-chain AA transporter (LivJHMGF), and a probable methionine uptake system (AtmABDE) [39].

Eighteen genes or proteins putatively involved in the AA biosynthesis exhibited altered expression during the growth of LMG18311 (Tables 1 and 2). Interestingly, 55% of them are directly involved in cysteine and methionine metabolism (Fig. 3). The overexpression affected most all steps of these biosynthetic pathways: the synthesis of cysteine from glyceraldehyde 3-P (SerA, CysE2, CysM1), the transsulfuration and sulfhydrylation pathways (MetA, MetB1, Stu0353, CysD), the conversion of homocysteine to methionine (MetE, MetF), and the conversion of methionine in cysteine (MetK, CysM2, MetB2) (Fig. 3). Complementary analysis with RT-qPCR confirmed that pathways leading to threonine (*thrB*) and lysine (*dapA*) biosynthesis were not up-regulated while the complete sulfur AA biosynthetic pathway was fully induced at the transcriptional level except for (i) *mmuM,* which is a putative functional orthologue of *metE,* (ii) genes involved in the SAM recycling pathway (*pfs, luxS*) (Fig. 3). The up-regulation of *metK,* which is involved in the synthesis of S-adenosyl methionine (SAM), the major biological donor

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Table 2. Protein altered during the late phase of growth of LMG18311 in milk

Locus *(stu #)*	Gene name	Description	CAI	Spot #	Fold change L *vs.* E	
					2-DE	RT-qPCR
AA metabolism						
1527[a)]	*serA*	D-3-phosphoglycerate dehydrogenase hypoxanthine	0.567	261	4.8	
0366	*cysM$_1$*	Cysteine synthetase	0.680	289	6.9	
				290[b)]	3.3	
				307	nv[c)]	
0353		Aminotransferase (class II)	0.521	230	4.1	
0987	*cysD*	*O*-acetyl homoserine sulfydralase	0.578	182[b)]	2.7	
				396	18	
				181, 183, 374	nv[c)]	
1222	*metA*	Homoserine *O*-succinyltransferase	0.522	403	5.7	1.1 ± 0.5
0785	*metE*	5-Methyltetrahydropteroyltriglutamate	0.656	21, 56	3.4	
				44	5	
				46	3.1	
				48[b)]	4.2	
				381	33	
				387	24	
				397	31	
0846	*cysM2*	Cysteine synthetase	0.394	256	2.9	
0847	*metB2*	Cystathionine beta-lyase	0.398	222	1.7	2.8 ± 0.8
0463	*acpC3*	Aminotransferase	0.421	223	1.8	
1776	*glnA*	Glutamine synthetase	0.675	177[b)]	0.47	1.71 ± 0.34
				195	nv[c)]	
0641	*aroF*	Chorismate synthase	0.509	217	0.11	1.05 ± 0.26
1873	*ilvB*	Acetolactate synthase, large subunit	0.528	92	2.8	
tRNA synthetase						
1971	*hisS*	Histidyl-tRNA synthetase	0.525	128	0.011	1.5 ± 0.5
				157[b)]	0.48	
0572	*thrS*	Threonyl- tRNA synthetase	0.691	51	0.25	0.93 ± 0.16
Peptidase, protease						
0671		Protease	0.557	259	0.21	0.9 ± 0.5
1672	*pepX*	X-prolyl-dipeptidyl aminopeptidase	0.451	54	0.16	0.89 ± 0.15
Ribosome and translation						
0439	*rrf*	Ribosome releasing factor	0.699	342[b)]	1.8	1.05 ± 0.5
				349	nv	
C-metabolism						
1264	*ptsI*	Phosphoenolpyruvate:sugar phosphotransferase system enzyme I	0.727	84[b)]	2.0	1.21 ± 0.36
				86	0.014	
1735	*scrB*	Sucrose-6-phosphate hydrolase	0.657	119	2.0	0.81 ± 0.32
0873	*glmS*	D-fructose-6-phosphate amidotransferase	0.612	399[b)]	4.7	2.9 ± 0.8
				79, 81	nv[c)]	
1400	*galE1*	UDP-galactose epimerase	0.648	251	2.0	1.48 ± 0.33
1402	*galK*	Galactokinase	0.631	234	2.5	0.82 ± 0.53
1657	*pfl*	Pyruvate formate lyase	0.756	39[b)]	0.001	
				49	0.06	
				36	0.008	
				50, 35	nv[c)]	
Purine & pyrimidine metabolism						
1270	*nrdE*	Ribonucleoside-diphosphate reductase, alpha chain	0.624	58	0.037	

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Table 2. Continued

Locus *(stu #)*	Gene name	Description	CAI	Spot #	Fold change L *vs.* E	
					2-DE	RT-qPCR
Trancription regulator						
1160	*rr05*	Response regulator	0.531	323	0.531	0.47 ± 0.1
Other						
0092	*panE*	Dehydropantoate 2-reductase (ketopantoate reductase	0.498	279	5.0	
		(ketopantoate reductase)		292[b)]	1.8	
0195		Hypothetical protein	0.702	285	0.43	1.18 ± 0.24
0283	*ureC*	Urea amidohydrolase (urease), alpha subunit	0.624	80[b)]	0.16	0.90 ± 0.1
				87	0.52	
0334		Hypothetical protein		318	8.8	
0554	*hipO1*	Aminoacylase/N-acyl-L-AA amidohydrolase/ hippurate hydrolase	0.416	219	3.0	
0735	*ftsZ*	Cell division protein	0.652	138	0.17	0.70 ± 0.03
1820		Hypothetical protein	0.467	363[b)]	0.37	
				365	nv[c)]	

a) Gene and protein were underlined when their expression and amount varied similarly.
b) Most abundant protein.
c) nv: No variation.

of methyl for methylation reactions, was also confirmed at the transcriptional level. Note, however, that at the protein level, the spot intensity did not significantly increase between 2h30 and 5h30. The *pdxK* gene, coding the pyridoxine kinase, which is involved in the synthesis of pyridoxal phosphate, the cofactor of the cysteine β-lyase MetB1 and the cystathionine γ-lyase MetB2 was also overexpressed. These observations confirmed that the complete Met-Cys biosynthesis pathway was fully induced during the late stage of the *S. thermophilus* growth in milk.

3.7 Sulfur AA and growth in milk

Considering the above data, we analyzed the milk composition in free AA (Table 3). It revealed that Asp, Cys, Met and Trp were present at very low amounts at the beginning of the culture and that LMG18311 growth led to undetectable amounts of Ala, Arg, Leu, Ile, Val at 5h30. In order to determine to which extent the AA starvation impaired growth, milk was supplemented and the LMG18311 growth monitored. The addition of Met and Ser had a stimulatory effect on growth (Fig. 4). It indicates that Met and Ser can be transported, possibly *via* the Atm and the Ser/Thr:Na^+ systems and can fulfill the strain requirement for sulfur AA. In contrast, Cys had no effect suggesting that the putative Cys transporter may be inefficient or that an increase of the intracellular Cys pool could lead to a down-regulation of the whole Cys-Met pathway. As expected, the addition of aspartate in milk reduced LMG18311 growth probably through competition with glutamic acid for transport as previously shown [40].

Table 3. Free AA concentrations during growth of *S. thermophilus* LMG18311 in milk

AA	Mean (SD) AA concentration (μM)[a)]		
	0	2h30	5h30
Asp	ND	ND	ND
Glu	666.4 (4.5)	602.8(32.2)	296.8(4.5)
Thr + Ser	58.0 (0.6)	29.6(1.1)	15.2(0.6)
Pro	41	68.4(0.6)	94.4(2.3)
Gly	203.2(2.2)	144.8(6.8)	102.0(1.7)
Ala	61.2(0.6)	30.0(0.6)	ND
Cys	ND	ND	ND
Met	ND	ND	ND
Leu	9.2(1.7)	6.8(2.8)	ND
Ile	6.8(1.7)	5.2(3.9)	ND
Val	22.0	21.2(0.6)	ND
Tyr	6.0	12.4(1.7)	7.2(0.1)
Phe	3.2(1.1)	7.6(0.6)	8.0(1.1)
Trp	ND	ND	ND
His	10.4(0.1)	13.6(2.3)	9.6(2.3)
Arg	98.4[b)]	48.8[b)]	ND
Lys	20.8[b)]	17.6[b)]	8.0[b)]

a) From three independent experiences.
b) Not detected in two of three cultures.
c) ND, below the detection threshold (250 pmoles).

The regulation of the Ser and Cys pathways is not established in *S. thermophilus* but it was shown that MetR is the transcription activator of *metEF* and *atmBDE* [39]. Furthermore, JIM8315, a *metR*-minus mutant of CNRZ1066 [39]

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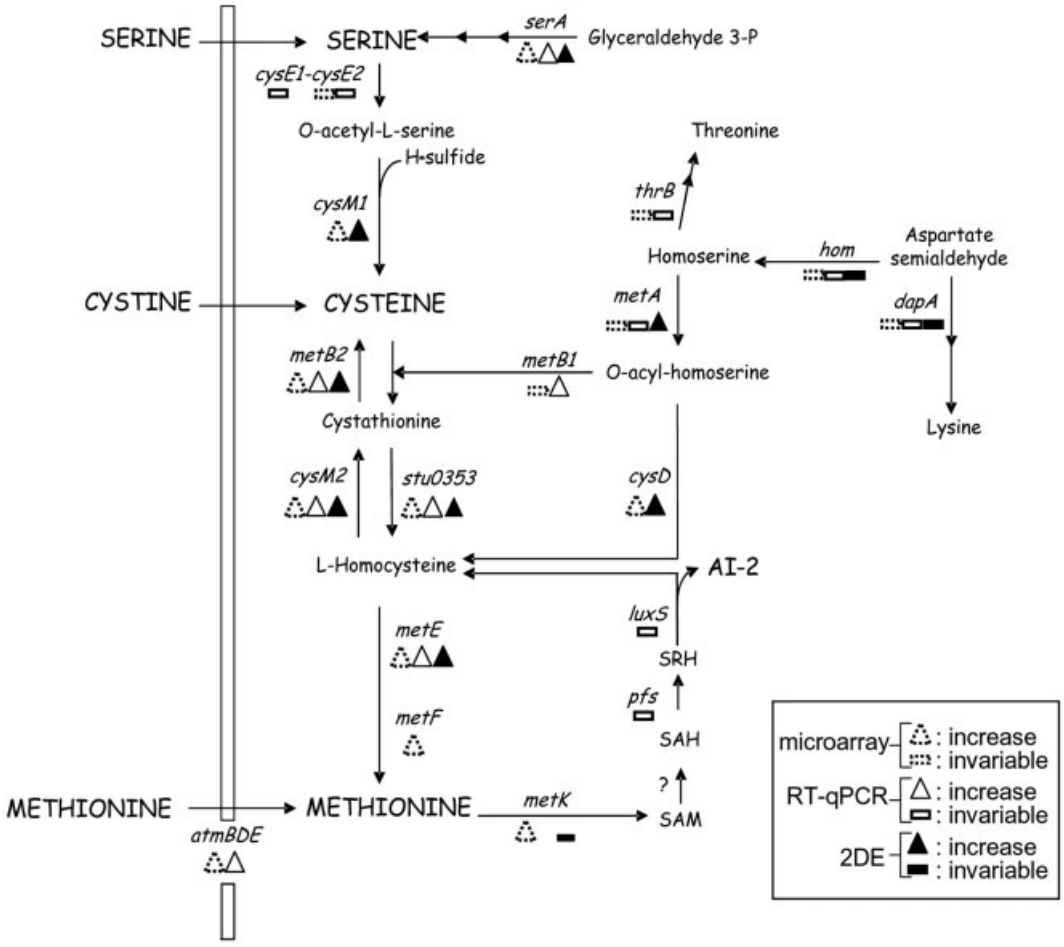

Figure 3. Effect of the late growth stage on the sulfur AA biosynthesis pathway. Variation in gene expression (microarrays, dotted symbols; RT-qPCR, open symbols) and protein abundance (2-DE, black symbols) between 2h30 and 5h30. Triangles and rectangles indicate up- and invariant regulation, respectively. A missing symbol indicates that the measurement was not performed or not possible (*e.g.* protein not detected on 2-DE). The pathway representation is derived from [25]. See Tables 1 and 2 for description of *cysD*, *cysE1*, *cysE2*, *cysM1*, *cysM2*, *metA*, *metB*, *stu0353*, *metE*, *metF*, *metK* and *atmABDE*. *hom*, homoserine dehydrogenase; *luxS*, autoinducer-2 production protein; *pfs*, 5′-methylthioadenosine/S-adenosylhomocysteine nucleosidase; *thrB*, homoserine kinase; *dapA*; dihydrodipicolinate synthase. SAM: S-adenosyl-methionine, SAH: S-adenosylhomocysteine, SRH: S-ribosylhomocysteine.

exhibited a retarded growth in milk compared to the wild-type strain (data not shown), highlighting the importance of methionine for *S. thermophilus* growth in milk.

3.8 Other modifications potentially linked to the slow growth of LMG18311 in milk

During late growth of LMG18311, additional genes/proteins were affected. Some of them may be markers of late exponential phase and/or poor growth conditions. Here, we highlight two of them that would need further attention. (1) The RR05 response regulator, part of a two component system (TC05), decreased during the late phase of growth indicating that a regulatory event took place. The orthologs of TCS05 are conserved in several Firmicutes. They were shown to be (i) essential for cell viability, notably in *L. lactis* [41], (ii) involved in the regulation of cell wall metabolism [42–44], and (iii) involved in cell permeability [45]. In *S. pneumoniae*, this TCS could regulate a set of functions involved in oxidative stress protection [46] and the expression of fatty acid biosynthesis [47]. Although our data are not sufficient for assigning a function to RR05, it is noteworthy that its abundance decrease in condition of harsh growth condition, suggesting that in *S. thermophilus*, the activity of TCS05 may be required for rapid/normal growth. (2) The *stu0292-294* operon is overexpressed at 5h30; it comprises a gene coding a non ribosomal peptide synthase gene potentially involved in the production of antimicrobial peptides. For bacteria, the production of antimicrobials is an efficient response to increase their fitness in harsh environments [48].

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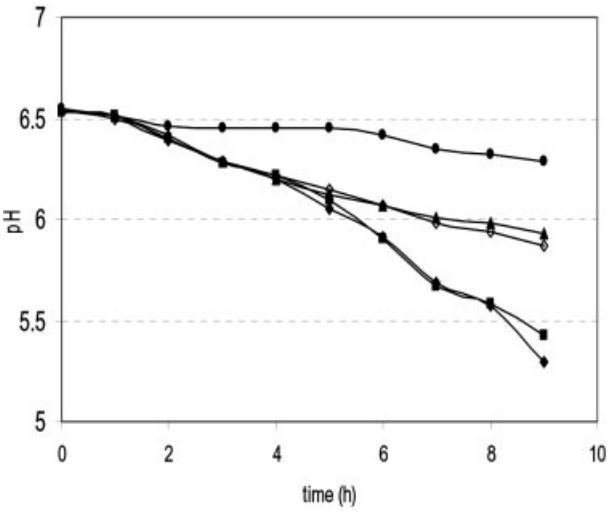

Figure 4. pH evolution in a milk culture of LMG18311 without (◇) and with methionine (31.25 mg/L, ■), serine (56.25 mg/L, ◆), cysteine (62.5 mg/L, ▲) or aspartate (113.75 mg/L, ●). These curves are representative of data obtained in three independent experiments.

3.9 *S. thermophilus* and *L. bulgaricus* association

We observed that *L. bulgaricus* improved the growth of LMG18311 in milk. Comparison of the transcriptomic and proteomic analysis of LMG18311 in monoculture or coculture revealed similar modifications in both conditions (Fig. 5 and Supporting Information Table S3). As detailed below, these common modifications concerned (i) the sugar metabolism, (ii) the biosynthesis of Fe-S clusters and (iii) the AA transport and metabolism.

(i) Regarding the carbon diversification in the monoculture, including two proteins of the Leloir pathway (GalE1, GalK), GlmS and the *rpe* gene (involved in the pentose phosphate pathway) were overexpressed in both conditions whereas *fruB*, ScrB and PtsI were specifically induced in the monoculture (Fig. 5). Taking into account the difference of generation times between the two cultures, this observation may indicate that only the three later genes/proteins respond to slow growth. The other modifications are likely to reflect a physiological modification related to the growth in milk and not involved in the proto-cooperation with *L. bulgaricus*. The Leloir pathway leads to the production of glucose-1 phosphate, the precursor of nucleotide sugars involved in polysaccharides biosynthesis [exopolysaccharides (EPS) and rhamnose polysaccharides (RPS)]. In the same way, GlmS is involved in the biosynthesis of nucleotide sugars and nucleotide aminosugars from fructose-6P. To date, no clear biological function could be assigned to EPS and RPS production in *S. thermophilus* and the production of EPS does not confer any obvious advantage for the growth or survival of *S. thermophilus* in milk [49]. However, it is noteworthy that the induction of these two genes related to EPS or RPS production are not affected by the presence of the *Lactobacillus*.

(ii) The stu0164-*sufD* genes were up-regulated in both conditions. In *S. thermophilus* CNRZ368, *sufD* gene is involved in the biosynthesis of [Fe-S] clusters [50]. The presence of O_2 leads to the production of superoxide, which is harmful for [Fe-S] clusters. The up-regulation of this gene may be related to some level of oxidative stress although other protective functions are not induced in the monoculture. (iii) The AA and peptide transporters and the sulfur AA metabolism were not only induced in the monoculture but also in the coculture. This observation contradicts one of the main hypothesis of the literature concerning the stimulatory effect of *L. bulgaricus* on the *S. thermophilus* growth that is the fulfillment by the lactobacilli of the peptides/AA requirements of *S. thermophilus*. The fact that the sulfur AA biosynthesis pathways was switch on in LMG18311 during the coculture indicates that although *L. bulgaricus* ATCC11842 may supply LMG18311 with peptides/AA, their amounts are not covering all the streptococcal needs for optimal growth. It strongly suggests that the stimulatory effect of *L. bulgaricus* ATCC11842 is likely to result from other and more complex exchanges between the two species.

4 Concluding remarks

The picture of *S. thermophilus* during its late growth phase in milk revealed a laborious late growth in milk probably due to several nutritional limitations. We observed a diversification of carbon sources utilization as observed in other LAB under starvation or stress conditions. Lactose is probably not exhausted in our conditions as lactose is the main C-source in milk and is able to sustain the better growth of LMG18311 in coculture. Therefore, this diversification is likely to indicate the harsh condition encountered in the monoculture. In addition, the AA and peptide transport systems as well as some AA biosynthesis pathways were switched on during growth. This observation is in agreement with the limited concentration of free AA or peptides in milk and the necessity for *S. thermophilus* to synthesize its AA. The reason why the sulfur AA biosynthesis is more affected than the biosynthetic pathways of other AA present at low concentration in milk remains to be further investigated.

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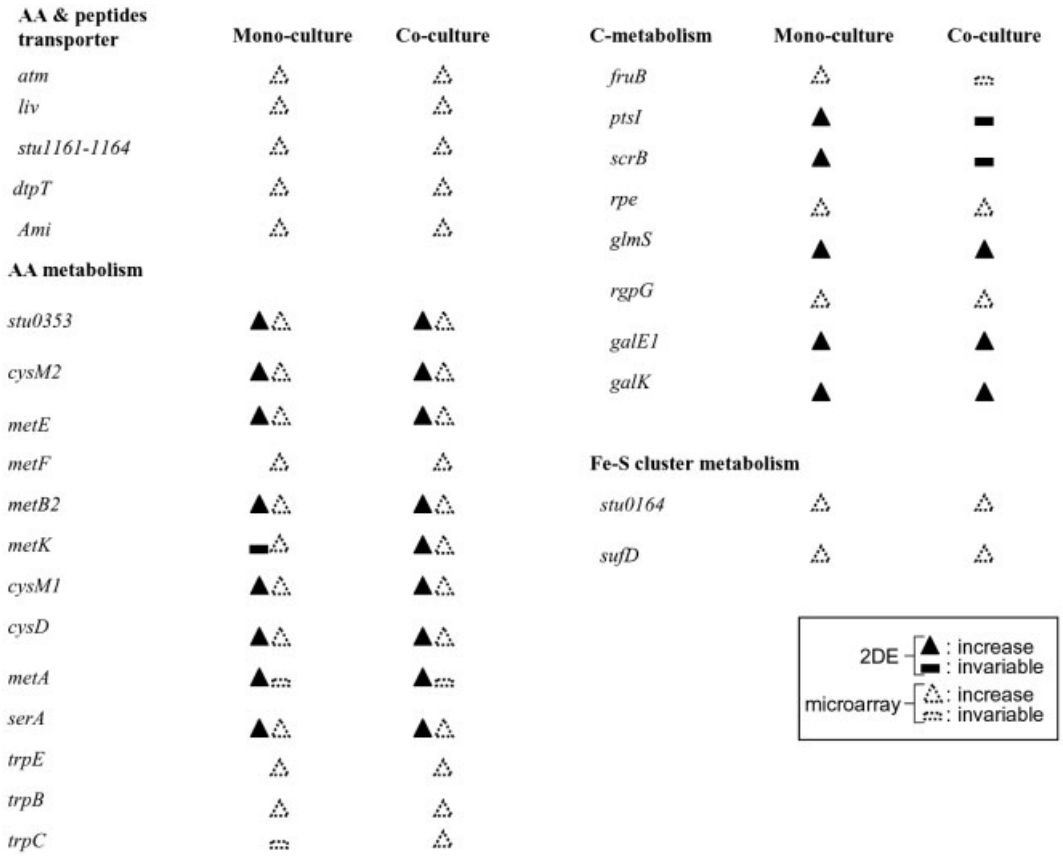

Figure 5. Schematic representation of the common modifications of the LMG18311 transcriptome (dotted symbols) and proteome (black symbols) during the monoculture and the coculture with *L. bulgaricus*. Triangle and rectangle indicate an over-expression or the absence of variation between 2h30 and 5h30, respectively. A missing bar indicates that the measurement was not possible (*e.g.* protein not detected on 2-DE). See Tables 1 and 2 for the description of *atm*, *liv*, *stu1161-1164*, *dtpT*, *Ami*, *metB1*, *stu0353*, *cysM2, metE*, *metF, metB2, metK, cysM1*, *cysD*, *metA*, *serA*, *trpE*, *trpB*, *trpC*, *fruB, rpe, scrB, ptsI, glmS, rgpG, galE1, galK, stu0164, sufD.*

5 References

[1] Tamime, A. Y., Robinson, R. K., *Yoghurt. Science and Technology*. Woodhead publishing limited, Cambridge 1999.

[2] Mitchell, T. J., The pathogenesis of streptococcal infections: from tooth decay to meningitis. *Nat. Rev. Microbiol.* 2003, *1*, 219–230.

[3] Bolotin, A., Quinquis, B., Renault, P., Sorokin, A. *et al.*, Complete sequence and comparative genome analysis of the dairy bacterium *Streptococcus thermophilus*. *Nat. Biotechnol.* 2004, *22*, 1554–1558.

[4] Makarova, K., Slesarev, A., Wolf, Y., Sorokin, A. *et al.*, Comparative genomics of the lactic acid bacteria. *Proc. Natl. Acad. Sci. USA* 2006, *103*, 15611–15616.

[5] Foucaud, C., Poolman, B., Lactose transport system of *Streptococcus thermophilus*. Functional reconstitution of the protein and characterization of the kinetic mechanism of transport. *J. Biol. Chem.* 1992, *267*, 22087–22094.

[6] Hutkins, R., Morris, H.A., McKay, L. L., Galactokinase activity in *Streptococcus thermophilus*. *Appl. Environ. Microbiol.* 1985, *50*, 777–780.

[7] Desmazeaud, M. J., Le lait milieu de culture. *Microbiologie-Aliments-Nutrition* 1990, *8*, 313–325.

[8] Hols, P., Hancy, F., Fontaine, L., Grossiord, B. *et al.*, New insights in the molecular biology and physiology of *Streptococcus thermophilus* revealed by comparative genomics. *FEMS Microbiol. Rev.* 2005, *29*, 435–463.

[9] Shahbal, S., Hemme, D., Desmazeaud, M. J., High cell wall-associated proteinase acticity of some *Streptococcus thermophilus* strains (H-strains) correlated with a high acidification rate in milk. *Lait* 1991, *71*, 351–357.

[10] Letort, C., Nardi, M., Garault, P., Monnet, V. *et al.*, Casein utilization by *Streptococcus thermophilus* results in a diauxic growth in milk. *Appl. Environ. Microbiol.* 2002, *68*, 3162–3165.

[11] Garault, P., Le Bars, D., Besset, C., Monnet, V., Three oligopeptide-binding proteins are involved in the oligopeptide transport of *Streptococcus thermophilus*. *J. Biol. Chem.* 2002, *277*, 32–39.

[12] Rul, F., Monnet, V., Presence of additional peptidases in *Streptococcus thermophilus* CNRZ 302 compared to *Lactococcus lactis*. *J. Appl. Microbiol.* 1997, *82*, 695–704.

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[13] Garault, P., Letort, C., Juillard, V., Monnet, V., Branched-chain amino acid biosynthesis is essential for optimal growth of *Streptococcus thermophilus* in milk. *Appl. Environ. Microbiol.* 2000, *66*, 5128–5133.

[14] Salzano, A. M., Arena, S., Renzone, G., D'Ambrosio, C. *et al.*, A widespread picture of the *Streptococcus thermophilus* proteome by cell lysate fractionation and gel-based/gel-free approaches. *Proteomics* 2007, *7*, 1420–1433.

[15] Perrin, C., Guimont, C., Bracquart, P., Gaillard, J. L., Expression of a new cold shock protein of 21.5 kDa and of the major cold shock protein by *Streptococcus thermophilus* after cold shock. *Curr. Microbiol.* 1999, *39*, 342–0347.

[16] Perrin, C., Gonzalez-Marquez, H., Gaillard, J. L., Bracquart, P. *et al.*, Reference map of soluble proteins from *Streptococcus thermophilus* by two-dimensional electrophoresis. *Electrophoresis* 2000, *21*, 949–955.

[17] Wouters, J. A., Rombouts, F. M., de Vos, W. M., Kuipers, O. P. *et al.*, Cold shock proteins and low-temperature response of *Streptococcus thermophilus* CNRZ302. *Appl. Environ. Microbiol.* 1999, *65*, 4436–4442.

[18] Gonzalez-Marquez, H., Perrin, C., Bracquart, P., Guimont, C., A 16 kDa protein family overexpressed by *Streptococcus thermophilus* PB18 in acid environments. *Microbiology* 1997, *143*, 1587–1594.

[19] Auffray, Y., Lecesne, E., Hartke, A., Boutibonnes, P., Basic features of the *Streptococcus thermophilus* heat shock response. *Curr. Microbiol.* 1995, *30*, 87–91.

[20] Arena, S., D'Ambrosio, C., Renzone, G., Rullo, R. *et al.*, A study of *Streptococcus thermophilus* proteome by integrated analytical procedures and differential expression investigations. *Proteomics* 2005, *6*, 181–192.

[21] Derzelle, S., Bolotin, A., Mistou, M. Y., Rul, F., Proteome analysis of *Streptococcus thermophilus* grown in milk reveals pyruvate formate-lyase as the major upregulated protein. *Appl. Environ. Microbiol.* 2005, *71*, 8597–8605.

[22] Letort, C., Juillard, V., Development of a minimal chemical defined medium for the exponential growth of *Streptococcus thermophilus*. *J. Appl. Microbiol.* 2001, *91*, 1023–1029.

[23] Guillot, A., Gitton, C., Anglade, P., Mistou, M. Y., Proteomic analysis of *Lactococcus lactis*, a lactic acid bacterium. *Proteomics* 2003, *3*, 337–354.

[24] Chich, J. F., David, O., Villers, F., Schaeffer, B. *et al.*, Statistics for proteomics: Experimental design and 2-DE differential analysis. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci* 2007, *849*, 261–272.

[25] Sperandio, B., Polard, P., Ehrlich, D. S., Renault, P. *et al.*, Sulfur amino acid metabolism and its control in *Lactococcus lactis* IL1403. *J. Bacteriol.* 2005, *187*, 3762–3778.

[26] Delmar, P., Robin, S., Daudin, J. J., VarMixt: efficient variance modelling for the differential analysis of replicated gene expression data. *Bioinformatics* 2005, *21*, 502–508.

[27] Rozen, S., Skaletsky, H., Primer3 on the WWW for general users and for biologist programmers. *Methods Mol. Biol.* 2000, *132*, 365–386.

[28] Ibrahim, M., Guillot, A., Wessner, F., Algaron, F. *et al.*, Control of the transcription of a short gene encoding a cyclic peptide in *Streptococcus thermophilus*: a new quorum-sensing system? *J. Bacteriol.* 2007, *189*, 8844–8854.

[29] Grunau, J. A., Swiader, J. M., Chromatography of 99 amino acids and other ninhydrin-reactive compounds in the Pickering lithium gradient system. *J. Chromatogr.* 1992, *594*, 165–171.

[30] Hiller, K., Schobert, M., Hundertmark, C., Jahn, D. *et al.*, JVirGel: Calculation of virtual two-dimensional protein gels. *Nucleic Acids Res.* 2003, *31*, 3862–3865.

[31] Ikemura, T., Codon usage and tRNA content in unicellular and multicellular organisms. *Mol. Biol. Evol.* 1985, *2*, 13–34.

[32] Sharp, P. M., Li, W. H., The codon adaptation index – a measure of directional synonymous codon usage bias, and its potential applications. *Nucleic Acids Res.* 1987, *15*, 1281–295.

[33] Schlothauer, T., Mogk, A., Dougan, D. A., Bukau, B. *et al.*, MecA, an adaptor protein necessary for ClpC chaperone activity. *Proc. Natl. Acad. Sci. USA* 2003, *100*, 2306–2311.

[34] de Vin, F., Radstrom, P., Herman, L., De Vuyst, L., Molecular and biochemical analysis of the galactose phenotype of dairy *Streptococcus thermophilus* strains reveals four different fermentation profiles. *Appl. Environ. Microbiol.* 2005, *71*, 3659–3667.

[35] Redon, E., Loubiere, P., Cocaign-Bousquet, M., Transcriptome analysis of the progressive adaptation of *Lactococcus lactis* to carbon starvation. *J. Bacteriol.* 2005, *187*, 3589–3592.

[36] Raynaud, S., Perrin, R., Cocaign-Bousquet, M., Loubiere, P., Metabolic and transcriptomic adaptation of *Lactococcus lactis* subsp. *lactis biovar diacetylactis* in response to autoacidification and temperature downshift in skim milk. *Appl. Environ. Microbiol.* 2005, *71*, 8016–8023.

[37] Melchiorsen, C. R., Jokumsen, K. V., Villadsen, J., Johnsen, M. G. *et al.*, Synthesis and posttranslational regulation of pyruvate formate-lyase in *Lactococcus lactis*. *J. Bacteriol.* 2000, *182*, 4783–4788.

[38] Asanuma, N., Hino, T., Effects of pH and energy supply on activity and amount of pyruvate formate-lyase in *Streptococcus bovis*. *Appl. Environ. Microbiol.* 2000, *66*, 3773–3777.

[39] Sperandio, B., Gautier, C., McGovern, S., Ehrlich, D. S. *et al.*, Control of methionine synthesis and uptake by MetR and homocysteine in *Streptococcus mutans*. *J. Bacteriol.* 2007, *189*, 7032–7044.

[40] Arioli, S., Monnet, C., Guglielmetti, S., Parini, C. *et al.*, Aspartate biosynthesis is essential for the growth of *Streptococcus thermophilus* in milk, and aspartate availability modulates the level of urease activity. *Appl. Environ. Microbiol.* 2007, *73*, 5789–5796.

[41] O'Connell-Motherway, M., van Sinderen, D., Morel-Deville, F., Fitzgerald, G. F. *et al.*, Six putative two-component regulatory systems isolated from *Lactococcus lactis* subsp. *cremoris* MG1363. *Microbiology* 2000, *146*, 935–947.

[42] Szurmant, H., Nelson, K., Kim, E. J., Perego, M. *et al.*, YycH regulates the activity of the essential YycFG two-component system in *Bacillus subtilis*. *J. Bacteriol.* 2005, *187*, 5419–5426.

[43] Bisicchia, P., Noone, D., Lioliou, E., Howell, A. *et al.*, The essential YycFG two-component system controls cell wall metabolism in *Bacillus subtilis*. *Mol. Microbiol.* 2007, *65*, 180–200.

[44] Dubrac, S., Boneca, I. G., Poupel, O., Msadek, T., New insights into the WalK/WalR (YycG/YycF) essential signal transduction pathway reveal a major role in controlling cell

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wall metabolism and biofilm formation in *Staphylococcus aureus*. *J. Bacteriol.* 2007, *189*, 8257–8269.

[45] Martin, P. K., Li, T., Sun, D., Biek, D. P. *et al.*, Role in cell permeability of an essential two-component system in *Staphylococcus aureus*. *J. Bacteriol.* 1999, *181*, 3666–3673.

[46] Echenique, J. R., Trombe, M. C., Competence repression under oxygen limitation through the two-component MicAB signal-transducing system in *Streptococcus pneumoniae* and involvement of the PAS domain of MicB. *J. Bacteriol.* 2001, *183*, 4599–4608.

[47] Mohedano, M. L., Overweg, K., de la Fuente, A., Reuter, M. *et al.*, Evidence that the essential response regulator YycF in *Streptococcus pneumoniae* modulates expression of fatty acid biosynthesis genes and alters membrane composition. *J. Bacteriol.* 2005, *187*, 2357–2367.

[48] Reva, O., Tummler, B., Think big-giant genes in bacteria. *Environ. Microbiol.* 2008, *10*, 768–777.

[49] Broadbent, J. R., McMahon, D. J., Welker, D. L., Oberg, C. J. *et al.*, Biochemistry, genetics, and applications of exopolysaccharide production in *Streptococcus thermophilus*: a review. *J. Dairy Sci.* 2003, *86*, 407–423.

[50] Thibessard, A., Borges, F., Fernandez, A., Gintz, B. *et al.*, Identification of *Streptococcus thermophilus* CNRZ368 genes involved in defense against superoxide stress. *Appl. Environ. Microbiol.* 2004, *70*, 2220–2229.

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