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Differential Expression of Proteins and Genes in the Lag Phase of *Lactococcus lactis* subsp. *lactis* Grown in Synthetic Medium and Reconstituted Skim Milk†

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We investigated protein and gene expression in the lag phase of *Lactococcus lactis* subsp. *lactis* CNRZ 157 and compared it to the exponential and stationary phases. By means of two-dimensional polyacrylamide gel electrophoresis, 28 highly expressed lag-phase proteins, implicated in nucleotide metabolism, glycolysis, stress response, translation, transcription, cell division, amino acid metabolism, and coenzyme synthesis, were identified. Among the identified proteins, >2-fold induction and down-regulation in the lag phase were determined for 12 proteins in respect to the exponential phase and for 18 proteins in respect to the stationary phase. Transcriptional changes of the lag-phase proteins in *L. lactis* were studied by oligonucleotide microarrays. Good correlation between protein and gene expression studies was demonstrated for several differentially expressed proteins, including nucleotide biosynthetic enzymes, adenylosuccinate synthase (PurA), IMP dehydrogenase (GuaB), and aspartate carbamoyl transferase (PyrB); heat-shock protein DnaK; serine hydroxymethyl transferase (GlyA); carbon catabolite control protein (CcpA); elongation factor G (FusA); and cell division protein (FtsZ).

Intracellular events occurring in bacteria just after introduction to a new environment provide the first evidence about physiological changes in the cells. When lactic acid bacteria are used as starter cultures, these intracellular changes may ensure a rapid prediction of the overall physiological status of the culture, which in turn defines the activity of the culture. Upon inoculation, the culture is exposed to massive environmental changes including nutrient availability, temperature, and pH. To adapt to new environments, bacteria have developed various metabolic responses. Recent research revealed a number of responses in *Lactococcus lactis* caused by exhaustion of essential nutrients such as purines (1), carbohydrates (7, 18, 36), and branched amino acids (10, 29), as well as responses to the temperature shift (6, 42), low pH (4, 23, 26, 31), and a combination of these factors (13, 25). However, only a few papers describe the intracellular changes occurring early in the cultivation upon transfer of the culture to a new rich medium. For example, proteomic research carried out with *Lactobacillus delbrueckii* subsp. *bulgaricus* showed that several proteins were strongly expressed in the lag phase, although only one protein (phosphoryl carrier protein HPr) was positively identified (30). Expression studies of *Saccharomyces cerevisiae* revealed about 20 proteins induced in the lag phase, which were primarily involved in protein synthesis, biosynthesis of amino acids, and carbohydrate metabolism (2, 3). A recent transcriptional study of *Bacillus licheniformis* based on 250 DNA probes exposed at least 15 differentially expressed genes of diverse functions in the lag phase (14).

In this work, we investigated protein and gene expression in *Lactococcus lactis* subsp. *lactis* CNRZ 157 in the lag phase in comparison to the exponential and stationary growth phases to achieve a better understanding of the effect of the intracellular changes in the lag phase on subsequent fermentation. Proteins identified in this research as highly induced in the lag phase may find an application in dairy fermentations as biological markers for prediction of culture performance.

MATERIALS AND METHODS

Chemicals. Chemicals were purchased from Sigma-Aldrich (Denmark) or Merck (Damstadt, Germany), unless otherwise stated.

Bacterial strains, media, and growth conditions. The strain of *Lactococcus lactis* subsp. *lactis* CNRZ 157 was obtained from the CNRZ collection of the Institut National de la Recherche Agronomique, Jouy-en-Josas, France. As distinct from the fully sequenced parental strain of *L. lactis* IL 1403, strain *L. lactis* CNRZ 157 contains nine plasmids, one of them encoding lactose-degrading enzymes. Two different growth media were used throughout the experiments: the chemically defined synthetic amino acid medium (SA) supplemented with 1.0% (wt/vol) glucose (15) and reconstituted skim milk (RSM; 10% [wt/vol]) skim milk powder in demineralized water). Growth of the culture in RMS was stimulated by addition of 0.05% Casamino Acids. CFU were determined after duplicate spreading of 0.1 ml of serial dilutions of the culture onto LM17 agar plates (M17 agar containing 0.5% [wt/vol] lactose), followed by overnight incubation at 30°C. Optical density was measured at 600 nm (OD_{600}) with a spectrophotometer (Shimadzu UV-1201). Stock cultures were prepared by inoculation of 100 ml of either SA or RSM with a single colony grown on LM17 agar plates. After incubation overnight at 30°C, the cultures were split into 1-ml aliquots and kept at −80°C until use. Aliquots from SA were supplemented with 15% (vol/vol) glycerol solution, while the aliquots from RSM were stored without addition of cryoprotectants. *L. lactis* NCRZ 157 was transferred from the frozen stocks to 100 ml SA or RSM to a final concentration of 5.5×10^7 CFU/ml and incubated at 30°C. Cell growth was followed by measurements of OD and CFU.

[^{35}S]methionine labeling of proteins and protein extraction. Labeling of proteins in the cells grown in SA was carried out with lag, exponential, and stationary growth phases as follows. Cells were harvested from the frozen stocks by centrifugation (10,000 × *g* for 3 min at 4°C), transferred into SA medium with reduced L-methionine content (0.1 mg/ml) to a starting OD_{600} of 0.20 ± 0.02,

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

and incubated at 30°C. Samples, containing a number of cells equivalent to that of a 1-ml sample at an OD_{600} of 1.0, were removed just after inoculation (lag-phase cells), after 3 h of incubation (exponentially growing cells), and after 8 h of cultivation (stationary-phase cells). The samples were subsequently incubated separately for 40 min with 100-μCi/ml L-[^{35}S]methionine (>1,000 Ci/mmol; AG1594; Amersham Biosciences, Sweden). The labeling experiments with RSM were performed essentially as above, with the exception that they were limited to lag-phase and exponential-phase proteins because of difficulties in harvesting the cells in the stationary phase where milk coagulated due to low pH. Protein synthesis was halted by the addition of chloramphenicol solution to a final concentration of 100 μg/ml. Cells were harvested by centrifugation (10,000 × *g* for 3 min at 4°C). The pellet was washed first in 500 μl of phosphate-buffered saline (100 mM; pH 7.0) and then washed twice in 500 μl of Tris-HCl buffer (32 mM; pH 7.5). Washing buffers were supplemented with chloramphenicol (100 μg/ml). The pellet was recovered after each washing by centrifugation (8,000 × *g* for 3 min at 4°C). The washed pellet was resuspended in 100 μl of protease inhibitor cocktail solution (one Mini tablet[Roche Diagnostics Corp.] dissolved in 5 ml Tris-EDTA buffer). Acid-washed glass beads (100 μm) were added into the cell slurry, and the cells were disrupted with FastPrep FP120 (Bio101 Savant Instruments, Inc., Holbrook, NY) at a setting of 6.5 for 45 s. Nucleic acids were degraded by incubation of the cell suspension in the presence of DNaseI (20 U/ml; Amersham Biosciences, Sweden) and RNase I (40 U/ml; Amersham Biosciences, Sweden) at 37°C for 30 min. The proteins were then solubilized by the addition of denaturing agents and detergents, including urea (9.5 M), dithiothreitol (100 mM), 4% (wt/vol) 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS; Amersham Biosciences, Sweden), and 2% (vol/vol) Pharmalyte, pH 3 to 10 (Amersham Biosciences, Sweden). The cell suspension was incubated at 30°C for 2 h, followed by centrifugation (8,000 × *g* for 10 min at room temperature). The supernatant-containing protein extract was removed and stored at −80°C.

Protein separation by 2D-PAGE. Protein extracts were resolved by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) according to the manufacturer's instructions with the use of the reagents and equipment provided by the manufacturer (Amersham Biosciences, Sweden). Briefly, Immobiline Dry Strips, ranging from pH 4 to 7 were applied for isoelectric focusing (the first dimension). Proteins were then separated by size with ExcelGel XL SDS 12-14 (second dimension). Protein concentration was determined with the PlusOne 2-D Quant Kit, and the minimum amount of protein loaded per gel was 25 μg. Proteins were visualized by silver staining (PlusOne Silver staining kit), and afterwards the gels were stored for 2 weeks in phosphor screens (Molecular Dynamics). The resulting autoradiograms of the gels were scanned using a phosphorimager (STORM 840). Protein spots in the autoradiograms were quantified in the ImageMaster-2D software (version 3.1; Amersham Biosciences, Sweden) by the measurements of the spot volumes with the use of non-spot mode of background subtraction. Data sets obtained in four independent cultivations were analyzed for each growth medium. Each data set included the autoradiograms of the proteome obtained from cells in lag, exponential, and stationary phases from the same growth experiment. The individual spot volumes were normalized to the total volume of the matched spots. Induction folds were estimated by averaging the ratios of normalized spot volumes in the lag phase to those in exponential and stationary phases.

Protein identification. Protein extracts from the lag-phase cells grown in SA were separated by 2D-PAGE and visualized by Coomassie R-350 staining (Amersham Biosciences, Sweden). Protein identification was performed as a contract work by Alphalyse A/S (Odense, Denmark). In brief, proteins were subjected to matrix-assisted laser desorption ionization–time of flight mass spectrometry peptide mass fingerprinting and identified by matches across the peptide sequences in the National Center for Biotechnology Information database (34).

Isolation of RNA. RNA was isolated from *L. lactis* NCRZ 157 grown in SA with the use of the RNeasy Mini kit (QIAGEN, Inc.; Merck Eurolab A/S). The medium was inoculated with frozen stocks to an OD_{600} value of 0.20 ± 0.02 and incubated at 30°C. Samples of the culture in the amount, corresponding to an OD_{600} value of 1.0, were collected in the lag phase after 40 min, in the exponential phase after 3.5 h (OD_{600} = 0.65 ± 0.02), and in the stationary phase after 8.5 h (OD_{600} = 1.10 ± 0.05). Cells were harvested by centrifugation (10,000 × *g* for 3 min at 4°C) and frozen immediately in liquid nitrogen. The cells were mixed with acid-washed glass beads at 50 μg per sample (1-mm diameter; Struers Kebolab, Denmark) and lysis buffer (450 μl RLT buffer supplied with the RNeasy Mini kit, added to 10 μl β-mercaptoethanol) and then homogenized in FastPrep FP120 (Bio101 Savant Instruments, Inc., Holbrook, NY) at a setting of 6.5 for 45 s. The supernatant was removed and mixed with 250 μl of 96% ethanol. Afterwards, the procedure was continued according to the RNeasy Mini protocol (QIAGEN, Inc.; Merck Eurolab A/S). According to this protocol, we routinely isolated 150 to 250 μg of total RNA with an A_{260}/A_{280} ratio of 1.8 to 2.2 as measured with a UV-VIS spectrophotometer (Shimadzu, Inc., Japan). The integrity of RNA was controlled by gel electrophoresis of the RNA preparation (5 μg per lane) on a 0.8% (wt/vol) agarose gel. RNA was stored at −80°C until use.

Preparation of target-labeled cDNA. cDNA was synthesized from the total RNA using priming by random hexamers (Amersham Biosciences, Sweden) essentially as recommended in the protocol for *Escherichia coli* by Khodursky et al. (16). Each cDNA sample was obtained from 20 μg of the total RNA. The procedure included primer annealing, cDNA synthesis with incorporation of amino-allyl dUTP (aa-dUTP-cDNA), RNA hydrolysis, and purification of aa-dUTP-cDNA. Coupling reaction of monofunctional dyes Cy3 and Cy5 to aa-dUTP-cDNA was carried out according to the instructions with the CyDye Post-Labeling Reaction kit (Amersham Biosciences, Sweden). Preparations of labeled cDNA were combined and purified with the CyScribe GFX Purification kit (Amersham Biosciences, Sweden). To avoid false positives and to eliminate the effect of the dye, each sample of target DNA was separately labeled with the fluorescence dye Cy3 or Cy5 and afterwards hybridized to the different microarrays.

Production of microarrays. Oligonucleotide DNA probes of 50 bp each with 5′ amino linker modification were designed and synthesized by MWG-BIOTECH (Ebersberg, Germany). The probes were complementary to 40 selected genes of *L. lactis* subsp. *lactis*, encoding the proteins identified by 2D-PAGE, stress-related proteins, metabolic enzymes, and proteins implicated in transcription and energy conversion. The Pan Epoxy slides for microarrays were provided by the same manufacturer. Oligomers at a concentration of 50 μM were spotted on the slides by VersArray ChipWriter Pro (Bio-Rad Laboratories, Ltd., Ontario, Canada) in triplicate. Postprocessing of the arrays was performed according to the MWG Array Application guide. Shortly, printed arrays were kept overnight at 42°C and 50% humidity, washed with 0.2% (wt/vol) sodium dodecyl sulfate (SDS) solution at room temperature, and afterwards incubated for 20 min at 50°C in distilled water. Arrays were dried and stored in a desiccator until use. Just before use, the arrays were blocked by incubation in a 2% (wt/vol) water solution of bovine serum albumin at 42°C for 45 min.

Hybridization conditions. Target cDNA was mixed with 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) buffer, 0.3% (wt/vol) SDS, 0.5-μg/μl yeast tRNA (Invitrogen A/S), and distilled water to a total volume of 30 μl. The mixture was heated at 90°C for 2 min, cooled down on ice for a few seconds, and loaded on the array under a LifterSlip coverslip (no. 221X25-2-4635; Erie Scientific Company, Portsmouth, NH) for overnight hybridization at 60°C. The slides were washed at room temperature in the solutions of buffer 1 (2× SSC–0.1% [wt/vol] SDS), buffer 2 (1× SSC–0.1% [wt/vol] SDS), and buffer 3 (0.5× SSC) for 5 min in each buffer. The slides were then dried by centrifugation (5,000 × *g* for 3 min at room temperature) and scanned immediately.

Microarrays image processing and data analysis. The microarrays were scanned on ArrayWoRx white-light charge-coupled device-based scanner (Applied Precision, Issaquah, WA) with a 60-s exposure time in both the Cy3 and the Cy5 channels. Dye swapping did not reveal significant differences in dye incorporation. The mean signal intensity of each oligomer was estimated by averaging the counts in triplicates with the use of ArrayWoRx software. Spots without oligomers were used as negative controls. Quantification of gene expression was performed by normalizing the mean intensities of the individual spots to the total intensity of the array in each channel (Cy3 and Cy5) and comparing the ratios of normalized signals in the lag phase to those in the exponential and stationary phases. Each gene was assigned an induction value, calculated by averaging the ratios obtained in labeling experiments of four independent biological replicates.

RESULTS

Growth of *L. lactis* subsp. *lactis* CNRZ 157 in SA and RSM. The growth of *L. lactis* in SA and RSM was followed by measurements of optical density at 600 nm and CFU, as presented in Fig. 1. Duration of the lag phase was, on average, 1.7 h in SA and about 1 h in RSM, as estimated with the program MicroFit, version 1.0 (Institute of Food Research, United Kingdom). In both media, transition to the stationary phase occurred after 8 h of cultivation, when the viable counts stabilized.

Protein expression in the lag phase studied by 2D-PAGE. Protein extracts isolated from the cells of *L. lactis* subsp. *lactis* CNRZ 157 in the lag, exponential, and stationary phases were

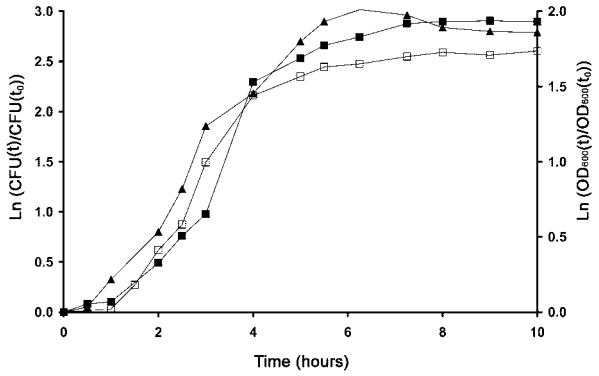

FIG. 1. Growth of *L. lactis* subsp. *lactis* CNRZ 157 in synthetic medium by CFU (■) and OD_{600} (□) and in reconstituted skim milk by CFU (▲).

analyzed by 2D-PAGE. Figure 2 presents autoradiograms of the lag-phase proteomes obtained from SA and RSM, showing that most of the spots have molecular weights ranging from 20,000 to 80,000 and pI values of 4.4 to 5.6. As seen from Fig. 2, a variety of proteins comprising up to 217 spots were synthesized in the first 40 min after inoculation. A total of 28 proteins that were strongly and differentially expressed in the lag phase were identified and mapped as shown in Fig. 2. Proteins involved in carbohydrate metabolism (pyruvate kinase [Pyk], glyceraldehyde-3-phosphate dehydrogenase (GapA), phosphoglycerate kinase (Pgk), bisphosphate aldolase (FbaA), EnoA, CitE, and PtsI) comprised the largest group and accounted for 21 to 23% of the total radioactivity of the spots. Proteins participating in translation (FusA, Tuf, methionyl-tRNA formyltransferase [Fmt], and RpsB) accounted for 10 to 13%, while proteins involved in nucleotide metabolism (GuaB, adenylosuccinate synthase [PurA], aspartate carbamoyl transferase [PyrB], and PurH) accounted for another 3.5 to 4% of total radioactivity readings. Comparison of the relative positions of the proteins in this study with the 2D-PAGE map of *L. lactis* recently published by Guillot et al. (12) showed a good match of the majority of the abundant proteins, such as GapA, EnoA, Pyk, Pgk, Tuf, FbaA, RpsB, and GuaB. A slight mismatch in the positions was observed for a few less-expressed proteins, including MurC, PyrB, and FtsZ, and was possibly caused by the differences in the conditions of separation procedures. As far as we know, the proteins NadE and Fmt identified in this study have not been located in 2D gel maps of *L. lactis* before.

Protein and gene expression in the lag phase compared to the exponential phase. Table 1 presents the changes in protein expression in the lag phase compared to the exponential phase, determined as averages of induction (fold) for four biological replicates obtained from SA and RSM. The total number of analyzed spots was between 195 and 210 for SA and between 178 and 190 for RSM, depending on the replicate. As shown in Table 1, 9 proteins of 28 proteins identified were induced >2-fold in the lag phase in relation to the exponential phase in SA. They included nucleotide biosynthetic enzymes, PurA, PyrB; stress-related proteins GroEL and DnaK; proteins involved in translation, namely, Fmt and 30S ribosomal protein (RpsB);

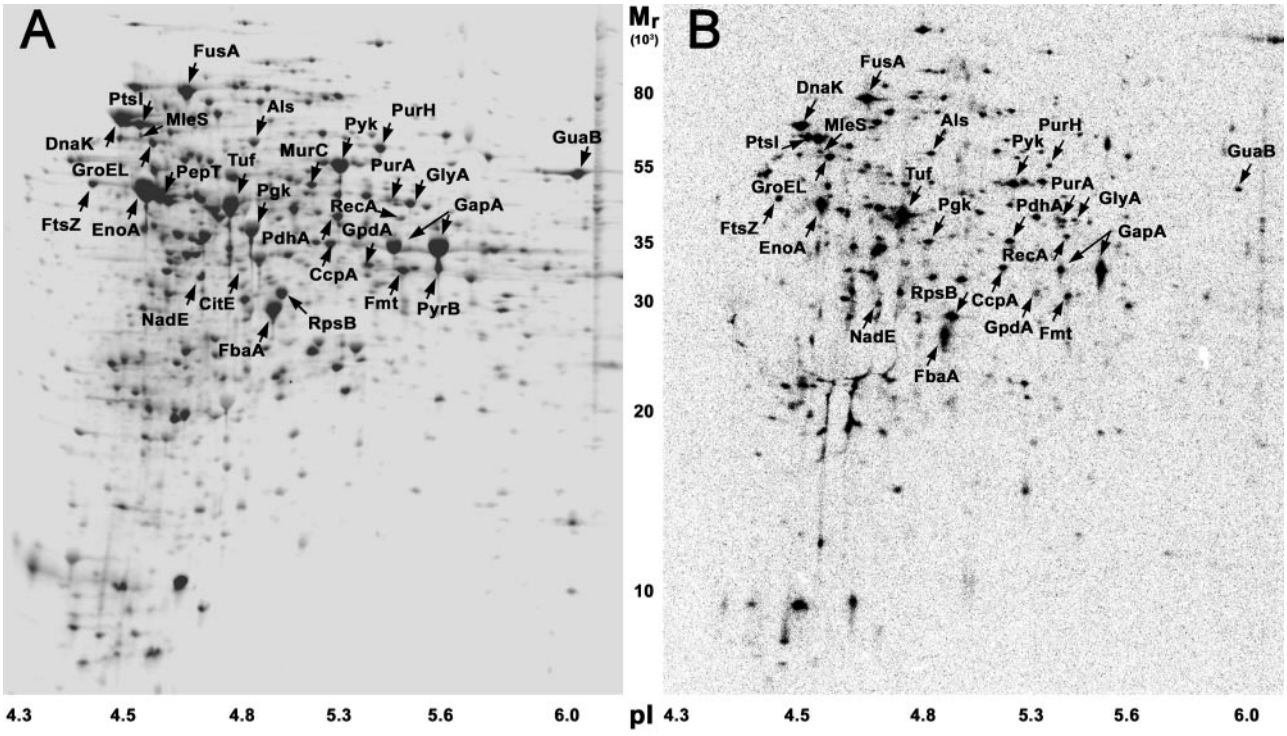

FIG. 2. Autoradiograms of [^{35}S]methionine-labeled protein extract obtained from the lag-phase cells of *L. lactis* subsp. *lactis* CNRZ 157 grown in SA medium (A) and RSM (B) and separated by 2D-PAGE. Proteins identified by matrix-assisted laser desorption ionization–time of flight mass spectrometry are indicated. Images were digitally contrast enhanced.

TABLE 1. Changes in expression of the lag-phase proteins and genes in *L. lactis* subsp. *lactis* CNRZ 157 grown in synthetic medium and reconstituted skim milk as determined by 2D-PAGE and by microarrays[a]

COG functional category and protein(s)	Protein induction (Lag/Exp), *n*-fold ± SD[b]		Gene induction (Lag/Exp), *n*-fold ± SD[b]
	RSM	SA	SA
Nucleotide transport and metabolism			
Adenylosuccinate synthase (PurA)	**4.2 ± 1.6**	**4.0 ± 1.7**	**2.8 ± 0.7**
Inosine monophosphate dehydrogenase (GuaB)	**3.1 ± 0.1**	3.0 ± 2.2	1.2 ± 0.2
Aspartate carbamoyltransferase (PyrB)	Not matched	**3.7 ± 1.2**	1.1 ± 0.3
Bifunctional purine biosynthesis protein (PurH)	0.8 ± 0.4	1.2 ± 0.7	**2.5 ± 0.5**
Posttranslational modification			
Chaperonin GroEL 60-kDa protein (GroEL)	**2.7 ± 0.6**	**12.2 ± 4.5**	1.8 ± 0.7
Heat shock 70-kDa protein (DnaK)	**5.7 ± 2.2**	**2.4 ± 0.4**	**3.0 ± 1.0**
Carbohydrate transport and metabolism			
Phosphoglycerate kinase (Pgk)	0.5 ± 0.2	**0.3 ± 0.1**	1.1 ± 0.4
Pyruvate kinase (Pyk)	0.8 ± 0.2	**0.4 ± 0.1**	0.7 ± 0.2
Phosphotransferase system, enzyme I (PtsI)	1.9 ± 0.5	1.6 ± 0.3	1.2 ± 0.5
Glyceraldehyde 3-phosphate dehydrogenase (GapA)	0.7 ± 0.3	1.0 ± 0.2	**7.4 ± 2.9**
Fructose-bisphosphate aldolase (FbaA)	0.6 ± 0.2	**0.4 ± 0.2**	**0.4 ± 0.1**
Citrate lyase beta chain (CitE)	Not matched	1.0 ± 0.5	1.0 ± 0.2
Enolase (EnoA)	1.0 ± 0.2	0.9 ± 0.1	1.2 ± 0.3
Amino acid transport and metabolism			
Peptidase T (PepT)	0.7 ± 0.2	1.0 ± 0.5	1.2 ± 0.3
Serine hydroxymethyl-transferase (GlyA)	0.7 ± 0.3	0.9 ± 0.4	1.9 ± 0.6
α-Acetolactate synthase (Als)	1.0 ± 0.3	0.6 ± 0.2	0.9 ± 0.2
Translation			
Elongation factor G (FusA)	1.4 ± 0.8	1.1 ± 0.8	1.1 ± 0.1
Elongation factor Tu (Tuf)	1.5 ± 0.9	1.2 ± 0.3	1.0 ± 0.0
Methionyl-tRNA formyltransferase (Fmt)	**4.7 ± 2.7**	**24.3 ± 10.9**	1.3 ± 0.3
30S ribosomal protein S2 (RpsB)	**2.7 ± 0.8**	**2.8 ± 0.8**	0.9 ± 0.2
Cell division			
Cell division protein (FtsZ)	1.3 ± 0.3	1.4 ± 0.5	1.0 ± 0.2
UDP-*N*-acetylmuramate-alanine synthase (MurC)	Not matched	**5.7 ± 3.3**	0.9 ± 0.5
Energy production and conversion			
Glycerol-3-phosphate dehydrogenase (GpdA)	1.5 ± 0.4	**23.2 ± 8.4**	0.7 ± 0.2
PDH E1 component alpha subunit (PdhA)	1.8 ± 0.8	1.8 ± 0.3	1.7 ± 0.2
Malolactic enzyme (MleS)	1.2 ± 0.3	0.9 ± 0.2	0.8 ± 0.3
Transcription			
Carbon catabolite control protein (CcpA)	2.8 ± 1.8	**2.7 ± 0.8**	1.2 ± 0.7
Coenzyme transport and metabolism			
NH_3-dependent NAD^+ synthase (NadE)	**3.1 ± 0.2**	**4.9 ± 1.3**	1.4 ± 0.6
DNA recombination			
Recombinase A (RecA)	1.5 ± 0.5	1.1 ± 0.3	1.1 ± 0.2

[a] Expression changes are presented as the averages of induction (fold in the lag phase to the exponential phase [Lag/Exp] ± standard deviation [SD]) obtained for four biological replicates. COG, cluster of orthologous groups.
[b] Induction values of <0.5-fold and >2-fold are denoted in boldface type.

MurC, involved in cell division; glycerol-3-phosphate dehydrogenase (GpdA), implicated in glycerol metabolism; carbon catabolite control protein (CcpA); and NAD^+ synthase (NadE), catalyzing coenzyme biosynthesis. Glycolytic enzymes Pgk, Pyk, and fructose-bisphosphate aldolase (FbaA) were repressed by about twofold in the lag phase.

Comparison of the lag-phase protein expression in the laboratory medium (SA) and milk (RSM) showed a good agreement between the media for most of the proteins (Fig. 2 and Table 1). Lack of correlation was only observed for GpdA, glycolytic enzymes (Pyk and FbaA), and for CcpA, probably caused by the differences between the media in composition and availability of carbon sources.

Changes in gene expression of the identified lag-phase proteins were studied by microarrays and are presented in Table 1. Induction (fold) of other genes included in microarrays and not identified in 2D gels can be found in Table SA1 in the supplemental material. Up-regulation in the lag phase at the level of transcription was determined for PurA and DnaK, confirming the proteomics results. Additionally, genes *purH*

TABLE 2. Changes in expression of the lag-phase proteins and genes in *L. lactis* subsp. *lactis* CNRZ 157 grown in synthetic medium as determined by 2D-PAGE and by microarrays[a]

COG functional category and protein(s)	Protein induction (Lag/Stat) *n*-fold ± SD[b]	Gene induction (Lag/Stat) *n*-fold ± SD[b]
Nucleotide transport and metabolism		
Adenylosuccinate synthase (PurA)	**>20**	**7.9 ± 3.7**
Inosine monophosphate dehydrogenase (GuaB)	**3.1 ± 0.1**	**8.9 ± 4.2**
Aspartate carbamoyltransferase (PyrB)	**2.9 ± 0.4**	**7.2 ± 3.3**
Bifunctional purine biosynthesis protein (PurH)	**4.0 ± 1.2**	1.1 ± 0.3
Posttranslational modification		
Chaperonin GroEL 60-kDa protein (GroEL)	1.0 ± 0.6	0.8 ± 0.1
Heat shock 70-kDa protein (DnaK)	**0.4 ± 0.1**	**0.4 ± 0.1**
Carbohydrate transport and metabolism		
Phosphoglycerate kinase (Pgk)	**7.5 ± 2.3**	1.3 ± 0.4
Pyruvate kinase (Pyk)	**7.9 ± 3.2**	1.3 ± 0.5
Phosphotransferase system, enzyme I (PtsI)	1.1 ± 0.3	1.6 ± 0.7
Glyceraldehyde 3-phosphate dehydrogenase (GapA)	1.8 ± 0.7	1.7 ± 0.9
Fructose-bisphosphate aldolase (FbaA)	1.6 ± 0.5	0.6 ± 0.2
Citrate lyase beta chain (CitE)	Not matched	**0.4 ± 0.1**
Enolase (EnoA)	1.9 ± 0.6	0.9 ± 0.3
Amino acid transport and metabolism		
Peptidase T (PepT)	**5.4 ± 2.6**	0.9 ± 0.3
Serine hydroxymethyl-transferase (GlyA)	**5.0 ± 1.7**	**2.6 ± 0.6**
α-Acetolactate synthase (Als)	1.4 ± 0.3	**0.2 ± 0.1**
Translation		
Elongation factor G (FusA)	**>20**	**3.2 ± 0.9**
Elongation factor Tu (Tuf)	**5.3 ± 1.1**	1.3 ± 0.3
Methionyl-tRNA formyltransferase (Fmt)	**5.8 ± 3.6**	0.5 ± 0.2
30S ribosomal protein S2 (RpsB)	**5.2 ± 1.3**	1.1 ± 0.4
Cell division		
Cell division protein (FtsZ)	**4.5 ± 1.8**	**2.3 ± 0.3**
UDP-*N*-acetylmuramate-alanine synthase (MurC)	1.8 ± 0.7	0.9 ± 0.4
Energy production and conversion		
Glycerol-3-phosphate dehydrogenase (GpdA)	**7.4 ± 3.8**	**3.5 ± 0.9**
PDH E1 component alpha subunit (PdhA)	**3.3 ± 1.2**	0.8 ± 0.1
Malolactic enzyme (MleS)	Not matched	1.7 ± 0.8
Transcription		
Carbon catabolite control protein (CcpA)	**7.9 ± 3.3**	**2.4 ± 0.4**
Coenzyme transport and metabolism		
NH_3-dependent NAD^+ synthase (NadE)	>20	0.7 ± 0.2
DNA recombination		
Recombinase A (RecA)	Not matched	0.8 ± 0.3

[a] Expression changes are presented as the averages of induction (fold in the lag phase to the stationary phase (Lag/Stat) ± standard deviation [SD]) obtained for four biological replicates. COG, cluster of orthologous groups.
[b] Induction values of <0.5-fold and >2-fold are denoted in boldface type.

and *gapA* were up-regulated in the lag phase; however, this result was not shown by 2D-PAGE.

Protein and gene expression in the lag phase compared to the stationary phase. Expression changes of the proteins and the corresponding genes identified in the lag phase in relation to the stationary phase in *L. lactis* grown in SA are shown in Table 2. The microarrays results obtained for the genes not included in Table 1 are presented in Table SA1 in the supplemental material. The number of analyzed protein spots in the 2D gels varied between 90 and 115. At least a twofold differential expression in the lag phase in respect to the stationary phase was demonstrated for 18 identified proteins (Table 2). Further, expression changes of nine of these proteins were confirmed by microarray results.

A large group of the proteins differentially induced in the lag phase comprised the enzymes of purine and pyrimidine biosynthetic pathways: PurA, GuaB, and bifunctional protein (PurH). These proteins, with exception of PurH, were also induced at the level of translation by >7-fold (Table 2).

Another major subset of proteins up-regulated in the lag phase by >5-fold consisted of elongation factors G (FusA) and Tu (Tuf) and two proteins involved in translation, Fmt and 30S ribosomal protein (RpsB). Among these proteins, induction at the level of transcription was demonstrated for FusA.

The enzymes of carbohydrate metabolic pathways, Pgk and Pyk, were up-regulated in the lag phase by >7-fold (Table 2). Induction of the corresponding genes (*pgk* and *pyk*) was not shown by the gene expression studies. The enzymes involved in amino acid metabolism, GlyA and tripeptidase (PepT), were induced by about fivefold in the lag phase. Additionally, up-regulation of *glyA* was determined by microarrays. The other differentially induced lag-phase proteins were GpdA and CcpA, each induced by 7-fold; cell division protein (FtsZ), induced by >4-fold, and pyruvate dehydrogenase PDH E1 alpha subunit (PdhA), induced by >3-fold. Proteins GpdA, CcpA, and FtsZ were also up-regulated in the lag phase at the level of transcription by >2-fold. Down-regulation in the lag phase in relation to the stationary phase was established for a heat shock protein (DnaK) by both 2D-PAGE and microarrays.

DISCUSSION

Protein expression in *L. lactis* subsp. *lactis* CNRZ 157 cultured in SA and RSM was studied by 2D-PAGE with the purpose of identifying proteins highly and differentially expressed in the lag phase compared to the exponential and stationary growth phases. Furthermore, expression changes in the lag phase of *L. lactis* cultured in SA were examined at the level of transcription by oligonucleotide microarrays. The study was focused on the genes encoding the proteins differentially expressed in the lag phase of cultivation.

Although RSM is a natural habitat for *L. lactis*, its application had a number of experimental drawbacks, such as the difficulty of separating cells from milk coagulum, resulting in a large amount of milk proteins isolated together with protein extracts from the cells, which affected the 2D-PAGE separation of bacterial proteins. The 2D-PAGE results indicate that SA medium presents a good alternative to RSM without the experimental difficulties and providing 2D patterns of protein expression similar to those obtained for RSM.

Differences between protein expression patterns between the lag and stationary phase were most apparent. Thus, among the proteins identified in the lag phase, 18 were >2-fold differentially expressed in relation to the stationary phase, while 12 proteins were differentially expressed in respect to the exponential phase. A comparison between the lag phase and the exponential phase reflects the intracellular changes between the stage of adaptation at the start of cultivation and the optimal conditions of exponential growth. Comparison between the lag phase and the stationary phase is relevant for industrial fermentation, because inoculated cells will often derive from a stationary-phase starter culture.

The enzymes of nucleotide de novo pathways, including PurA, and PyrB, were induced by >3-fold in the lag phase in respect to both exponential and stationary phases. Up-regulation of PurA, GuaB, and PyrB in relation to the stationary phase and PurA in relation to the exponential phase was confirmed by microarrays. Induction of the enzymes of purine and pyrimidine biosynthesis early in cultivation was most probably associated with the increased requirements for nucleotides during growth of the culture. Similarly, the higher expression in the lag phase of the proteins involved in the nucleotide metabolic pathways was recently demonstrated with *S. cerevisiae* (3) and *B. licheniformis* (14). Induction of GlyA in the lag phase compared to the stationary phase was in accordance with prior findings showing the correlation between the expression of GlyA and purine metabolic enzymes (1, 39).

Another group of proteins up-regulated in the lag phase was implicated in translation. Ribosomal protein RpsB and Fmt, which catalyzes the reaction of formylation of initiator methionyl-tRNA, were up-regulated in respect to the exponential and stationary phases. Fmt was previously reported to be important for the initiation of protein synthesis (11, 27). Induction of elongation factors FusA and Tuf was observed in relation to the stationary phase. Higher levels of FusA and Tuf at the conditions of growth initiation and their down-regulation at low pH are in accordance with previous findings (20, 33, 37, 41).

The stress-related protein DnaK was down-regulated in the lag phase in respect to the stationary phase and up-regulated in respect to the exponential phase, probably reflecting the adaptation of the culture to the decrease in pH and changes in nutrient composition (9, 13, 17, 30, 32).

Induction of the glycolytic enzymes Pyk and Pgk in the lag phase compared to the stationary phase and down-regulation in respect to the exponential phase were demonstrated by the proteomic approach, although not confirmed by gene expression studies. Additionally, GapA was found to be highly up-regulated in the lag phase compared to the exponential phase at the level of transcription; however, this was not proved by 2D-PAGE results. Theoretically, higher expression levels of the catabolic enzymes, such as Pyk, Pgk, and GapA, during initiation of growth and log phase could be expected, taking into account the positive effect of these enzymes on glucose consumption and biomass increase (5, 18). These speculations are supported by up-regulation of CcpA, determined in the lag phase in relation to the stationary phase at the levels of both transcription and translation. CcpA is a regulatory protein, which activates transcription of the *las* operon, encoding a number of glycolytic enzymes, including pyruvate kinase (19, 42). The lag-phase up-regulation of Pyk, Pgk, and GapA established in this research is in accordance with the recently presented expression studies of batch fermentations of *L. lactis* MG1363 in response to acid stress (8). Reduced expression of *citE*, encoding citrate lyase, in the lag phase in respect to the stationary phase was probably provoked by lactate accumulation at the end of cultivation. Induction of the *cit* operon is proposed to facilitate lactate outflux, enhancing cell resistance to the inhibitory effect of lactate accumulation (21, 22). Higher levels of the *als* gene, encoding acetolactate synthase, in the stationary phase in relation to the lag phase were possibly caused by accumulation of pyruvate and lowering of pH (24, 35, 38). Up-regulation of the *als* gene at lower pH values, although not determined by 2D-PAGE, is consistent with the recent transcriptomics research with *B. licheniformis* (14).

Among the proteins implicated in cell division, FtsZ was induced in the lag phase in respect to the stationary phase, while MurC was up-regulated compared to the exponential phase. Induction of these proteins early in cultivation might facilitate initiation of growth and cell division. Higher levels of FtsZ and MurC at the beginning of the exponential phase were documented previously for several bacteria species (28, 40).

In summary, 28 proteins, highly expressed in the lag phase of cultivation, were identified. Most of the proteins were involved in biosynthetic reactions and the process of translation. As

shown by 2D-PAGE, 12 of the identified proteins were >2-fold differentially expressed in the lag phase in relation to the exponential phase. Among them, PurA and DnaK showed the same expression pattern at the level of transcription. Differential expression in relation to the stationary phase was determined for 18 lag-phase proteins. Furthermore, correlation of protein and gene expression results was demonstrated for PurA, GuaB, PyrB, FusA, FtsZ, GpdA, CcpA, GlyA, and DnaK. Differential expression of a number of identified proteins was not verified by gene expression results, demonstrating that other factors, besides levels of mRNA, had an influence on protein synthesis. Earlier studies (2, 8) have reported the absence of correlation between expression patterns of the early induced proteins and the corresponding genes in other microorganisms.

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