Proteome analysis of *Lactobacillus helveticus* H9 during growth in skim milk

Y. F. Chen,^{*†1} W. J. Zhao,^{*‡1} R. N. Wu,^{†§} Z. H. Sun,^{*†} W. Y. Zhang,^{*} J. C. Wang,^{*} M. Bilige,^{*} and H. P. Zhang^{*†2}

^{*}Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education, Inner Mongolia Agricultural University, Huhhot, P. R. China, 010018

[†]Synergetic Innovation Center of Food Safety and Nutrition, Jiangnan University, Wuxi, P. R. China, 214122

[‡]The Institute of Products Quality Inspection and Research Inner Mongolia Autonomous Region, Huhhot, P. R. China, 010070

[§]College of Food Science, Shenyang Agricultural University, Shenyang, P. R. China, 110866

ABSTRACT

Lactobacillus helveticus H9 was isolated from traditionally fermented yak milk in Tibet (China) with the ability to produce the antihypertensive peptides Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP) during milk fermentation. To understand the changes in the protein expression of *L. helveticus* H9, proteome analysis was performed at 3 different growth stages, lag phase (pH 6.1), log phase (pH 5.1), and stationary phase (pH 4.5) using 2-dimensional electrophoresis (2-DE). Further analysis showed that 257 differential protein spots were found and 214 protein spots were identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS). The cellular functions of the differentially expressed proteins were complex. Interestingly, the proteolytic system-related proteins aminopeptidase N (PepN), aminopeptidase E (PepE), endopeptidase O2 (PepO2), and oligopeptide transport system permease protein (OppC) were observed only on the maps of pH 5.1 and pH 4.5, which was consistent with the presence of angiotensin I-converting enzyme (ACE)-inhibitory peptides VPP and IPP during these 2 growth stages (log phase and stationary phase). These results, combined with a previous study of gene expression of the proteolytic system, led us to conclude that the Opp transport system, pepE, and pepO2 are likely related to the production of ACE-inhibitory peptides.

Key words: proteomics, *Lactobacillus helveticus* H9, proteolytic system

INTRODUCTION

Lactobacillus helveticus is widely distributed in dairy fermentation worldwide (Sadat-Mekmene et al., 2013; Strahinic et al., 2013). Some isolates, such as *Lactobacillus helveticus* strains DPC4571, CP790, LBK16H,

and CNRZ32, are regarded as probiotic bacteria and have been used in functional fermentation production due to their high proteolytic activity and production of bioactive peptides (Nakamura et al., 1995; Yamamoto et al., 1999; Kilpia et al., 2007). Therefore, much attention has been focused on *L. helveticus* in China recently (Pan et al., 2005; Sun et al., 2009; Tian et al., 2009; Zhang et al., 2013). *Lactobacillus helveticus* H9 was isolated from traditionally fermented yak milk in Tibet by our group (Chen et al., 2014). Previous experiments demonstrated that *L. helveticus* H9 has higher proteolytic activity than other isolated *Lactobacillus* strains when incubated in skim milk and, during the fermentation process of skim milk, it produces the antihypertensive peptides Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP), which can reduce blood pressure.

Thus, in *L. helveticus*, not only is the essential amino nitrogen produced for growth in milk through the proteolytic system, but the proteolytic system probably also contributes to the formation of VPP and IPP. Developments in molecular technology provide the tools to further study the mechanisms involved in *L. helveticus* proteolytic systems.

Generally, proteolytic systems involved in lactic acid bacteria contain 2 enzyme parts, an extracellular Prt serine proteinase that can degrade casein and the main milk globular protein to oligopeptides, and peptidases that are capable of hydrolyzing oligopeptides to peptides. *Lactobacillus helveticus* strains exhibit great biodiversity of 1–4 cell-envelope proteinase (*CEP*) gene, including *prtH1*, *prtH2*, *prtH3*, *prtM*, and *prtP* (Yamamoto et al., 1994; Scolari et al., 2006; Broadbent et al., 2011). Moreover, Manso et al. (2005) reported that 5 peptidases (PepN, PepC, PepDA, PepV, PepQ) and 2 endopeptidases (PepO and PepO3) were observed in stationary growing cells of *L. helveticus* ITG LH1 using 2-dimensional electrophoresis (2-DE).

Great improvements in our understanding of proteolytic systems have been made by comparative genomic analysis. The complete genome sequences of *L. helveticus* (with its characteristic rapid lysis and high proteolytic activity) show many genes responsible for key

Received June 24, 2014.

Accepted August 28, 2014.

¹Yongfu Chen and Wenjing Zhao contributed equally to this paper.

²Corresponding author: hepingdd@vip.sina.com

metabolic functions such as proteolysis, lipolysis, and cell lysis (Callanan et al., 2008; Prajapati et al., 2011; Zhao et al., 2011; Tompkins et al., 2012). Cremonesi et al. (2013) hypothesized a pathway for VPP and IPP formation in *L. helveticus*, which was based on microarray analysis of gene expression in *L. helveticus* CM4 of the proteolytic enzymes involved in the processing of VPP and IPP by Wakai et al. (2012). The proteolytic action consists of the hydrolysis of β -casein by cell-wall proteinase; then, the generated peptides are transferred into the cell by oligopeptide transporter, where endopeptidases, such as PepO2, PepC2, and PepX, can act on at the C- and N-terminal sequences to produce VPP and IPP. Besides protease PrtM, proteins involved in Opp peptide transport system and the intracellular peptidases PepE, PepN, and PepQ were found in the probiotic strain *L. helveticus* M92 when cultivated in de Man, Rogosa, and Sharpe medium using SDS-PAGE and liquid chromatography-tandem MS analysis (Beganović et al., 2013). However, more data are needed.

In the current study, proteome analysis of *L. helveticus* H9 cultivated in skim milk was performed using 2-DE and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS). Protein expression profiles were compared in 3 different growth stages in an attempt to reveal comprehensive metabolism changes in *L. helveticus* H9 during fermentation of skim milk.

MATERIALS AND METHODS

Bacterial Strain, Medium, and Growth Conditions

The *L. helveticus* strain H9 (LABCC IMAU60208) was isolated from traditionally fermented yak milk in Tibet (China) and was preserved in the Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education, Inner Mongolia Agricultural University, China. Strain H9 was incubated in sterilized skim milk for 48 h at 37°C. The pH value was measured using a pHSJ-3F pH meter (Leici, Shanghai, China). The concentrations of VPP and IPP were determined using liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS/MS), and the analyses were performed in triplicate (Chen et al., 2010). Samples of the bacterial cells were collected in the lag, log and stationary phase during growth in milk for 1, 5, and 7 to 8 h, and the corresponding pH values were 6.1, 5.1, and 4.5.

Preparation of the Protein Extraction

Two different methods were used to collect the bacterial cells as described by Wang et al. (2013). Before cur-

dling at pH 6.1, cultures were harvested by centrifugation at $3,000 \times g$ for 7 min at 4°C. Then, 0.5 M ice-cold EDTA was added to the precipitate and centrifuged at $3,000 \times g$ for 7 min at 4°C. The precipitate was then washed with 0.5 M ice-cold EDTA in 30% (vol/vol) ice-cold ethanol, centrifuged, and washed again with 30% ice-cold ethanol and centrifuged at $3,000 \times g$ for 7 min at 4°C. The isolated cells were frozen in liquid nitrogen and preserved at -80°C . After curdling at pH 5.1 and pH 4.5, cultures were harvested by centrifugation at $200 \times g$ for 10 min at 4°C, and the corresponding supernatants were filtered and centrifuged at $3,000 \times g$ for 10 min at 4°C to collect the cells. The cell pellets were frozen in liquid nitrogen and preserved at -80°C .

A solution of 250 mM sucrose (pH 7.0) and 10 mM Tris was added to the frozen bacterial pellets and centrifuged at $6,000 \times g$ for 10 min at 4°C, and the collected precipitate was suspended in lysis buffer, which contained 8 M urea, 2 M thiourea, 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS; wt/vol), 2% ampholytes, 1% dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. Subsequently, 50 $\mu\text{g}/\text{mL}$ of DNaseI was added to the solution, which was then sonicated with a sonicator (Hielscher, Stuttgart, Germany) 4 times (ultrasonic: 30 s, pulse duration: 30 s), and the proteins in the supernatant were obtained through centrifugation at $14,000 \times g$ for 30 min at 4°C and preserved at -80°C . The protein concentrations were quantified using the Bradford protein assay.

Two-Dimensional Electrophoresis

The protein extract was first separated using first-dimension isoelectric focusing (IEF). Seventeen-centimeter immobilized pH gradient (IPG) strips, with a nonlinear range of pH 4 to 7 (Bio-Rad, Hercules, CA), were rehydrated in buffer containing 8 M urea, 2 M thiourea, 0.5% CHAPS (wt/vol), 1% dithiothreitol, 0.52% (vol/vol) IPG buffer, and 0.02% bromophenol blue for 12 h. A total of 0.8 g of the protein was diluted with the above buffer to a volume of 170 μL and loaded on the IPG strips. The IEF was performed in a Multiphor II Electrophoresis system (Bio-Rad, Hercules, CA) according to the following conditions: 0–500 V for 500 V·h, 500 V for 2,500 V·h, 500–3,500 V for 10,000 V·h, 3,500 V for 50,000 V·h, and 3,500–500 V for 8,000 V·h.

Following the IEF program, the strips were equilibrated immediately in 2 steps in buffers I and II, respectively, which contained 50 mM Tris-HCl (pH 6.8), 6 M urea, 30% glycerol, 2% SDS, 0.02% bromophenol blue, and 2% dithiothreitol (buffer I) or 2.5% iodoacetamide (buffer II); the equilibration time for each step was 15 min.

The equilibrated strips were loaded on a 12% SDS-PAGE gel, and the second-dimension SDS-PAGE was performed in a Protean II xi Cell (Bio-Rad) at 15 mA/gel and then at 45 mA/gel. The gels were silver stained and scanned using the Powerlook 2100XL apparatus (UMAX, Taiwan).

The images of the gels were assayed with PDQuest 7.0 software (Bio-Rad). After the automatic detection of the matched protein spots, manual collection was performed using the Edit Spot Tools and Match Tools from Bio-Rad. Three gels were produced for each growth stage, and the protein spots present on at least 2 of the gels were used for the subsequent assays. The protein spots were identified as being differential if they met the following conditions: the average ratio of the spot intensities showed at least a 2-fold difference (ratio ≥ 2.0 or ≤ 0.5) in the 3 growth stages, and the protein spots were produced only at the pH 6.1 (1 h) growth stage (lag phase) or at both the pH 5.1 (5 h) and pH 4.5 (7–8 h) growth stages (log and stationary phases, respectively).

Mass Spectrometry Analyses

The differential protein spots were excised manually from the 2-DE gels and washed 3 times, destained 3 times (15 min each) with a mixture (equal volume) of 30 mM potassium ferricyanide and 100 mM sodium thiosulfate, dehydrated 5 min with 30 μ L of acetonitrile (100%), and incubated in 8 μ L of a trypsin solution (0.05 mg/mL) for 16 h at 37°C. This reaction solution was also used for the mass spectrometry analysis.

An equal volume of the above reaction solution was mixed with a matrix solution, containing 25 mM ammonium bicarbonate, 0.1% trifluoroacetic acid, 50% acetonitrile, and 10 mg/mL of α -cyano-4-hydroxycinnamic acid, and was used for the mass spectrum. The peptide mass fingerprinting was performed on a 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA). All of the differential proteins were identified using MALDI-TOF/MS.

The GPS Explorer v3.5 software (Applied Biosystems) was used to assay the mass spectrum data under the following parameters: the peptide fragment mass tolerance was ± 0.1 Da, the fixed modification was carbamidomethylation, and the variable modification was oxidation. The complete genome sequence of *L. helveticus* H9 (LBH CP002427) was used as the database to search the proteins. Generally, protein identification was regarded as successful when the protein score confidence interval (MALDI-TOF) was greater than 95%. If more than one protein was successfully identified for one protein spot, we selected the protein with the higher score.

Theoretical molecular weights and isoelectric points of the identified proteins and all of the genome-annotated proteins were calculated using the ExPASy ProtParam tool (us.expasy.org/tools/pi_tool.html). The functional classifications of the proteins were also analyzed through clusters of orthologous groups (COG; <http://www.ncbi.nlm.nih.gov/COG/>).

RESULTS AND DISCUSSION

Generation Information of *L. helveticus* H9 on 2-DE Maps

The theoretical 2-DE map of *L. helveticus* H9 showed that the protein points were bimodally distributed between an isoelectric point (pI) of 4 and 10 (Figure 1). Similar to other lactobacilli, the major proteins were observed between pI 4 and 7. Hence, the present study constructed 2-DE maps of *L. helveticus* H9 during growth in milk using IPG pI 4 to 7 (Figure 2).

The study further compared the proteome profiles of *L. helveticus* at 3 growth stages. The numbers of corresponding detected protein spots on maps were approximately 729 (pH 6.1, lag phase), 1,020 (pH 5.1, log phase), and 925 (pH 4.5, stationary phase; Figure 2). The obvious variation between pH 6.1, pH 5.1, and pH 4.5 was probably related to the beginning growth of bacteria and the different methods used in protein extraction. We identified 214 differentially expressed protein spots using MALDI-TOF/MS, including spots that were only observed in one growth stage, as well as protein spots that were up- or downregulated between pH 6.1 and pH 5.1, and between pH 6.1 and pH 4.5, respectively (Table 1 and Supplementary Figure S1 and Tables S1 and S2; <http://dx.doi.org/10.3168/jds.2014-8520>). In detail, 35 protein spots were observed only

Figure 1. The theoretical 2-dimensional electrophoresis (2-DE) map of *Lactobacillus helveticus* H9. MW = molecular weight; pI = isoelectric point.

Figure 2. Two-dimensional electrophoresis (2-DE) maps of *Lactobacillus helveticus* H9 during growth in skim milk at 3 different stages and the differential expressed proteins found in the study: (AA) 2-DE map of lag phase (pH 6.1) and the protein spots expressed only on the map of pH 6.1; (BB) 2-DE map of log phase (pH 5.1) and the protein spots also expressed at the growth stages of stationary phase (pH 4.5) and the protein spots both expressed at the growth stages of pH 4.5; (BC) 2-DE map of stationary phase (pH 4.5) and the protein spots also expressed at the growth stages of log phase (pH 5.1). M = molecular weight; pI = isoelectric point.

on the map of pH 6.1 (Supplementary Table S1), and 150 protein spots were found on the maps of pH 5.1 and 4.5 (Table 1), as well as 29 up- or downregulated protein spots between pH 6.1 and both pH 5.1 and 4.5 (Supplementary Table S2), respectively.

Furthermore, several proteins were identified in multiple spots and, in most cases, with different forms. Posttranslational modifications appeared to be common in *L. helveticus* H9, such as elongation factor Ts (spots 2159, 4303, 4324, 4423), serine hydroxymethyltransferase (spots 4121, 6623, 7236, 7509), and phosphoglycerate kinase (spots 2305, 2416, 2512, 3419, 3421, 3407). Posttranslational modifications are known to play an important role in eukaryotes, whereas less is known about their role in bacterial physiology (Yuan et al., 2006).

Differentially Expressed Proteins in *L. helveticus* H9

Based on the analysis of 214 identified protein spots, the metabolism involved in the growth of *L. helveticus* H9 in skim milk at different stages is complex. Based on the COG results, cellular roles of both theoretical and identified proteins are presented in Figure 3. Proteins belonging to translation, ribosomal structure, and biogenesis comprised the majority of identified proteins, followed by proteins related to nucleotide transport and metabolism, carbohydrate transport and metabolism, and amino acid transport and metabolism.

Considering the detection of ACE-inhibitory peptides VPP and IPP only in the log phase (pH 5.1) and stationary phase (pH 4.5; Table 2), the following discussion focuses particularly on the proteins found on the maps of both pH 4.5 and pH 5.1, but not on the map of pH 6.1 (Table 1).

Proteins Related to the Proteolytic System

Interestingly, proteins involved in the proteolytic system were not expressed at pH 6.1 (Supplementary Tables S1 and S2; <http://dx.doi.org/10.3168/jds.2014-8520>). However, 4 proteins related to the proteolytic system were found on the maps of pH 5.1 and 4.5 (Table 1): aminopeptidase N (**PepN**, spot 1909) and aminopeptidase E (**PepE**, spots 1512 and 2509), endopeptidase O2 (**PepO2**, spots 2804 and 2806) and oligopeptide transport system permease protein (**OppC**, spots 8227 and 8314). The function of aminopeptidase is to hydrolyze AA from the N terminus of a polypeptide chain. General aminopeptidases include PepN and aminopeptidase C (**PepC**); PepN has wide substance specificity and plays important roles in the hydrolysis of peptides derived from milk proteins. Previous studies demonstrated that PepN was important for the growth

of *L. helveticus* in milk (Christensen and Steele, 2003) and that PepN was related to the inhibitory activity of ACE in fermented milk (Kilpi et al., 2007). Endopeptidase E is a cysteine proteinase that may play a role in peptide hydrolysis during cheese ripening (Fenster et al., 1997). The results were similar to those described by that also found PepE and PepN in *L. helveticus* M92 when cultivated in de Man, Rogosa, and Sharpe medium using SDS-PAGE and LC-MS/MS analysis (Beganović et al., 2013).

The endopeptidase pepO2, which is a post-proline endopeptidase, has a different substrate specificity compared with endopeptidase O (**PepO**). It has a preference for small, uncharged AA and AA with a proline residue on the carboxyl-side bond. It may play a central role in reducing the bitterness of cheese (Sridhar et al., 2005). Casein-derived peptides have been observed to contain relatively large amounts of Pro; thus, PepO2 may play an important role in hydrolyzing the peptides derived from milk proteins, which has been reported by Cremonesi et al. (2013).

The oligopeptide transport system (**Opp**) transports the oligopeptides derived from the hydrolysis of milk proteins to the intracellular space; the system includes the oligopeptide-binding protein OppA, 2 transmembrane proteins (OppB and OppC), and the nucleotide-binding proteins OppD and OppF. We identified one component of the Opp system, OppC, revealing that the Opp system exists in *L. helveticus* H9 and is expressed at growth stages pH 5.1 and pH 4.5, and could be involved in transport of oligopeptides. Previous research showed that the cell wall-associated proteinase (**PrtP**) and the Opp transport system are necessary for the growth of the strain in milk because they participate in the first step of casein hydrolysis and in the utilization of generated peptides (Tynkkynen et al., 1993; Juillard et al., 1995a,b). Although proteinase PrtP was not identified in the present study, expression of *prtP* was found upregulated in the log and stationary phases, and *prtM* was upregulated in log phase and then downregulated in stationary phase (Zhang et al., 2014). Additionally, *PepE*, *PepO2*, and *OppC* were highly expressed in both log and stationary phases. This might indicate that the Opp transport system, PepE, and PepO2 are essential for growth of the strain *L. helveticus* H9 in milk to produce ACE-inhibitory peptides from milk proteins.

In addition, we identified several proteins related to amino acid transport and metabolism, such as aspartate aminotransferase (Ast, spot 5522), serine hydroxymethyltransferase (GlyA, spots 4121, 6623, 7236, 7509), dihydrodipicolinate synthase (DapA, spot 3314) and glutamine synthetase (GlnA, spot 7217). Among the proteins, GlyA catalyzes the conversion of serine into glycine, which can then provide a methyl group for

Table 1. Proteins expressed on 2-dimensional electrophoresis (2-DE) gels in the stationary phase (pH 4.5) and log phase (pH 5.1) of *Lactobacillus helveticus* H9 cultured in milk, but not expressed in the lag phase (pH 6.1)¹

No.	Spot	Protein name	Protein MW (Da)	Protein pI	Peptide count	COG	Fold-change pH 4.5/ pH 5.1
1	1512	Aminopeptidase E	49,365.7	5.2	6	AA transport and metabolism	1.014
2	1621	Peptidase T	47,168	4.8	9	AA transport and metabolism	1.200
3	1909	Aminopeptidase N	95,821.5	4.8	32	AA transport and metabolism	0.589
4	2509	Aminopeptidase E	49,365.7	5.2	15	AA transport and metabolism	0.762
5	4121	Serine hydroxymethyltransferase	45,107	5.8	5	AA transport and metabolism	2.085
6	5522	Aspartate aminotransferase	42,525.9	5.5	7	AA transport and metabolism	0.729
7	6623	Serine hydroxymethyltransferase	45,107	5.8	5	AA transport and metabolism	0.599
8	6808	5-Methyltetrahydropteroyltriglutamate—homocysteine methyltransferase	87,450.5	5.9	22	AA transport and metabolism	0.567
9	7217	Glutamine synthetase	50,856	5.1	6	AA transport and metabolism	0.314
10	7236	Serine hydroxymethyltransferase	45,107	5.8	4	AA transport and metabolism	0.457
11	7509	Serine hydroxymethyltransferase	45,107	5.8	15	AA transport and metabolism	0.368
12	8227	Oligopeptide ABC superfamily ATP binding cassette transporter permease protein	120,288	7.3	12	AA transport and metabolism	0.833
13	3314	Dihydrodipicolinate synthase	34,522.7	5	8	AA transport and metabolism	0.773
14	8314	Oligopeptide ABC superfamily ATP binding cassette transporter permease protein	120,288	7.3	19	AA transport and metabolism	0.763
15	1318	Citrate lyase β -chain	32,180	4.8	15	Carbohydrate transport and metabolism	0.963
16	1620	Enolase 1	46,895.2	4.8	11	Carbohydrate transport and metabolism	0.360
17	2305	Phosphoglycerate kinase	44,476.3	5.5	7	Carbohydrate transport and metabolism	1.684
18	2416	Phosphoglycerate kinase	44,476.3	5.5	7	Carbohydrate transport and metabolism	0.906
19	2512	Phosphoglycerate kinase	44,476.3	5.5	5	Carbohydrate transport and metabolism	1.235
20	3318	6-Phosphogluconolactonase	160,504	7.3	13	Carbohydrate transport and metabolism	0.411
21	3419	Phosphoglycerate kinase	44,476.3	5.5	5	Carbohydrate transport and metabolism	0.531
22	3421	Phosphoglycerate kinase	44,476.3	5.5	6	Carbohydrate transport and metabolism	0.701
23	3615	Phosphoglucomutase	64,364.7	5.4	13	Carbohydrate transport and metabolism	1.656
24	7224	Galactose-1-phosphate uridylyltransferase	55,814.3	5.7	5	Carbohydrate transport and metabolism	0.398
25	7501	Galactose-1-phosphate uridylyltransferase	55,814.3	5.7	24	Carbohydrate transport and metabolism	0.581
26	3815	Pyruvate kinase	103,272	5.1	15	Carbohydrate transport and metabolism	0.394
27	5317	UTP-glucose-1-phosphate uridylyltransferase	33,634.7	6.1	6	Cell envelope biogenesis, outer membrane	0.697
28	6717	Glutamine-fructose-6-phosphate transaminase (Isomerizing)	66,131.2	5.5	10	Cell envelope biogenesis, outer membrane	1.879
29	7220	UDP-glucose 4-epimerase (fragment)	36,391.5	6.1	8	Cell envelope biogenesis, outer membrane	2.440
30	7223	UDP-glucose 4-epimerase (fragment)	36,391.5	6.1	7	Cell envelope biogenesis, outer membrane	1.509
31	7229	UTP-glucose-1-phosphate uridylyltransferase	33,878	6.7	4	Cell envelope biogenesis, outer membrane	0.724
32	7323	UTP-glucose-1-phosphate uridylyltransferase	33,634.7	6.1	16	Cell envelope biogenesis, outer membrane	0.231
33	7325	UDP-glucose 4-epimerase (fragment)	36,391.5	6.1	3	Cell envelope biogenesis, outer membrane	0.479
34	7608	UDP-N-acetylmuramoyl-l-alanyl-d-glutamyl-l-lysine ligase	58,157.9	5.8	15	Cell envelope biogenesis, outer membrane	0.310
35	7615	UDP-N-acetylmuramoyl-l-alanyl-d-glutamyl-l-lysine ligase	58,157.9	5.8	18	Cell envelope biogenesis, outer membrane	0.237
36	8315	UDP-glucose 4-epimerase (fragment)	36,391.5	6.1	17	Cell envelope biogenesis, outer membrane	1.085
37	8316	UDP-glucose 4-epimerase (fragment)	36,391.5	6.1	14	Cell envelope biogenesis, outer membrane	0.372
38	8317	UDP-glucose 4-epimerase (fragment)	36,391.5	6.1	15	Cell envelope biogenesis, outer membrane	1.602
39	8318	UDP-glucose 4-epimerase (fragment)	36,391.5	6.1	20	Cell envelope biogenesis, outer membrane	1.460
40	4225	NAD-dependent epimerase/dehydratase	23,699.2	5.3	4	Cell envelope biogenesis, outer membrane	0.531
41	5417	Probable tRNA sulfurtransferase	45,866.8	6.4	8	Coenzyme metabolism	0.663
42	6414	Probable tRNA sulfurtransferase	45,866.8	6.4	12	Coenzyme metabolism	0.380
43	7218	Bifunctional protein folD	30,762.4	7.8	8	Coenzyme metabolism	0.333
44	7231	Bifunctional protein folD	30,762.4	7.8	8	Coenzyme metabolism	0.479
45	8213	Bifunctional protein folD	30,762.4	7.8	9	Coenzyme metabolism	0.217
46	8223	Bifunctional protein folD	30,762.4	7.8	8	Coenzyme metabolism	2.782

Continued

Table 1 (Continued). Proteins expressed on 2-dimensional electrophoresis (2-DE) gels in the stationary phase (pH 4.5) and log phase (pH 5.1) of *Lactobacillus helveticus* H9 cultured in milk, but not expressed in the lag phase (pH 6.1)¹

No.	Spot	Protein name	Protein MW (Da)	Protein pI	Peptide count	COG	Fold-change pH 4.5/ pH 5.1
47	8225	Bifunctional protein fold	30,762.4	7.8	9	Coenzyme metabolism	1.334
48	8418	Probable tRNA sulfurtransferase	45,866.8	6.4	5	Coenzyme metabolism	0.238
49	8525	Nicotinate phosphoribosyltransferase	120,210	9.1	14	Coenzyme metabolism	1.023
50	4116	Transposase IS607 family	84,479.1	8.7	9	DNA replication, recombination, and repair	1.056
51	4230	Transposase IS607 family	84,479.1	8.7	10	DNA replication, recombination, and repair	3.593
52	4611	DNA replication protein DnaD	85,946.7	5.4	17	DNA replication, recombination, and repair	0.164
53	2219	Glycerol-3-phosphate dehydrogenase [NAD(P)+]	37,267.2	5.4	6	Energy production and conversion	0.850
54	6224	Citrate lyase ligase	39,249.1	5.6	6	Energy production and conversion	0.171
55	9613	Pyridine nucleotide-disulphide oxidoreductase family protein	49,741.9	7.1	10	Energy production and conversion	0.325
56	3019	Pyridoxine 5'-phosphate oxidase V related favin-nucleotide-binding protein	14,679.7	6.3	4	General function prediction only	0.800
57	3020	Pyridoxine 5'-phosphate oxidase V related favin-nucleotide-binding protein	14,679.7	6.3	4	General function prediction only	0.626
58	4217	Permease	69,668.1	7.3	9	General function prediction only	1.513
59	5523	ATP-grasp superfamily protein	50,645.4	5.8	5	General function prediction only	0.459
60	7235	Cystathionine β -synthase (CBS) domain protein	26,519.7	6.2	10	General function prediction only	0.593
61	8712	Methionyl-tRNA synthetase	108,138	5.6	11	General function prediction only	0.225
62	1149	Acetyl-CoA biotin carboxyl carrier	17,377.8	4.9	7	Lipid metabolism	1.890
63	1152	Acetyl-CoA biotin carboxyl carrier	17,377.8	4.9	5	Lipid metabolism	1.022
64	3221	Enoyl-[acyl-carrier-protein] reductase (NADH)	27,186	5.5	6	Lipid metabolism	0.773
65	4223	Enoyl-[acyl-carrier-protein] reductase (NADH)	27,186	5.5	7	Lipid metabolism	0.519
66	5528	Phosphate acyltransferase	36,032.8	5.5	6	Lipid metabolism	3.834
67	6013	Carrier protein	13,471	5.7	4	Lipid metabolism	0.986
68	3218	Phosphoribosylformylglycinamide synthase 1	24,733.5	5.1	4	Nucleotide transport and metabolism	5.143
69	5217	Phosphoribosylaminoimidazolecarboxamide formyltransferase	79,401.4	6.6	6	Nucleotide transport and metabolism	1.997
70	5415	Phosphoribosylamine-glycine ligase	46,386.4	5.4	13	Nucleotide transport and metabolism	0.651
71	6219	Adenylate kinase (fragment)	21,098.8	6	8	Nucleotide transport and metabolism	0.327
72	6520	Inosine-5-monophosphate dehydrogenase	36,822.8	5.8	10	Nucleotide transport and metabolism	0.462
73	7113	Purine/pyrimidine phosphoribosyltransferase	20,374.8	5.6	5	Nucleotide transport and metabolism	0.786
74	7114	Purine/pyrimidine phosphoribosyltransferase	20,374.8	5.6	5	Nucleotide transport and metabolism	1.074
75	7238	Guanylate kinase	23,030.9	5.7	4	Nucleotide transport and metabolism	0.521
76	7613	Bifunctional GMP synthase/glutamine amidotransferase protein	58,939.6	5.7	18	Nucleotide transport and metabolism	0.643
77	7614	Formate-tetrahydrofolate ligase (fragment)	51,279.6	5.9	7	Nucleotide transport and metabolism	0.502
78	8111	Purine/pyrimidine phosphoribosyltransferase	20,374.8	5.6	4	Nucleotide transport and metabolism	1.748
79	8112	Adenine phosphoribosyltransferase	20,124.6	5.8	9	Nucleotide transport and metabolism	6.373
80	8419	Phosphoribosylaminoimidazole carboxylase ATPase subunit	42,597.7	6.3	4	Nucleotide transport and metabolism	0.286
81	8528	Phosphoribosylaminoimidazole carboxylase ATPase subunit	42,597.7	6.3	7	Nucleotide transport and metabolism	0.193
82	8530	Phosphoribosylaminoimidazole carboxylase ATPase subunit	42,597.7	6.25	9	Nucleotide transport and metabolism	0.560
83	8716	Phosphoribosylaminoimidazolecarboxamide formyltransferase	79,401.4	6.6	21	Nucleotide transport and metabolism	0.238
84	9208	Adenylate kinase (fragment)	21,098.8	6	8	Nucleotide transport and metabolism	0.192
85	9506	Phosphoribosylaminoimidazole carboxylase ATPase subunit	42,597.7	6.3	8	Nucleotide transport and metabolism	0.643
86	9507	Phosphoribosylaminoimidazole carboxylase ATPase subunit	42,597.7	6.3	5	Nucleotide transport and metabolism	0.115
87	9804	Phosphoribosylaminoimidazolecarboxamide formyltransferase	79,401.4	6.6	17	Nucleotide transport and metabolism	0.369
88	6322	Ribose-phosphate pyrophosphokinase	36,519	5.7	11	Nucleotide transport and metabolism	0.525
89	7221	Ribose-phosphate pyrophosphokinase 1	35,600.7	6.5	8	Nucleotide transport and metabolism	1.138
90	7222	Ribose-phosphate pyrophosphokinase 1	35,600.7	6.5	8	Nucleotide transport and metabolism	0.478
91	7322	Ribose-phosphate pyrophosphokinase	36,519	5.7	12	Nucleotide transport and metabolism	0.394
92	8216	Ribose-phosphate pyrophosphokinase 1	35,600.7	6.5	7	Nucleotide transport and metabolism	0.195

Continued

Table 1 (Continued). Proteins expressed on 2-dimensional electrophoresis (2-DE) gels in the stationary phase (pH 4.5) and log phase (pH 5.1) of *Lactobacillus helveticus* H9 cultured in milk, but not expressed in the lag phase (pH 6.1)¹

No.	Spot	Protein name	Protein MW (Da)	Protein pI	Peptide count	COG	Fold-change pH 4.5/ pH 5.1
93	2621	Trigger factor	49,428.4	5.1	9	Posttranslational modification, protein turnover chaperones	0.465
94	2720	Trigger factor	49,428.5	5.11	8	Posttranslational modification, protein turnover chaperones	1.051
95	2721	60 kDa chaperonin	57,603.2	5.2	15	Posttranslational modification, protein turnover chaperones	0.527
96	2804	Endopeptidase O2	73,619.4	5	14	Posttranslational modification, protein turnover chaperones	6.998
97	2806	Endopeptidase O2	73,619.4	5	12	Posttranslational modification, protein turnover chaperones	0.376
98	3731	60 kDa chaperonin	57,603.2	5.2	8	Posttranslational modification, protein turnover chaperones	0.989
99	5319	Predicted: similar to caseinolytic peptidase X	46,811.6	6	10	Posttranslational modification, protein turnover chaperones	0.898
100	7324	60 kDa chaperonin	57,603.2	5.2	13	Posttranslational modification, protein turnover chaperones	1.302
101	2415	Central glycolytic gene regulator	82,055.7	5.5	15	Transcription	0.882
102	2422	Central glycolytic gene regulator	82,055.7	5.5	6	Transcription	1.895
103	3316	Central glycolytic gene regulator	82,055.7	5.5	12	Transcription	0.636
104	3919	DNA-directed RNA polymerase subunit β	135,777	5.5	15	Transcription	0.212
105	5212	Central glycolytic gene regulator	82,055.7	5.5	8	Transcription	2.230
106	5222	YyaA-like protein	31,851.9	6.9	7	Transcription	0.570
107	112	50S ribosomal protein L10	18,617.8	4.8	8	Translation, ribosomal structure and biogenesis	0.875
108	308	30S ribosomal protein S2	28,736.7	4.8	14	Translation, ribosomal structure and biogenesis	0.303
109	313	30S ribosomal protein S2	28,736.7	4.8	14	Translation, ribosomal structure and biogenesis	0.982
110	1212	Elongation factor P	21,045.7	5.5	4	Translation, ribosomal structure and biogenesis	0.611
111	1414	Elongation factor Tu	44,762.97	4.99	8	Translation, ribosomal structure and biogenesis	0.835
112	1626	Elongation factor Tu	44,763	5	11	Translation, ribosomal structure and biogenesis	3.007
113	1627	Elongation factor Tu	44,763	5	8	Translation, ribosomal structure and biogenesis	0.863
114	2159	Elongation factor Ts	37,710.4	5.4	6	Translation, ribosomal structure and biogenesis	0.645
115	2316	Elongation factor Tu	44,763	5	14	Translation, ribosomal structure and biogenesis	2.037
116	2417	Elongation factor Tu	44,763	5	16	Translation, ribosomal structure and biogenesis	1.120
117	2420	Elongation factor Tu	44,763	5	12	Translation, ribosomal structure and biogenesis	0.814
118	2421	Elongation factor Tu	44,763	5	17	Translation, ribosomal structure and biogenesis	2.077
119	2508	Elongation factor G	76,703.8	5	6	Translation, ribosomal structure and biogenesis	0.934
120	2520	Elongation factor P	66,022.6	4.9	8	Translation, ribosomal structure and biogenesis	0.476
121	2622	Elongation factor Tu	44,763	5	10	Translation, ribosomal structure and biogenesis	0.631
122	2630	Elongation factor Tu	44,763	5	4	Translation, ribosomal structure and biogenesis	0.430
123	2809	Glycyl-tRNA synthetase β subunit	78,396.1	5.1	17	Translation, ribosomal structure and biogenesis	0.778
124	3224	TrmH family RNA methyltransferase	28,281.6	5.3	6	Translation, ribosomal structure and biogenesis	0.637
125	3723	Alanine-tRNA ligase	100,466	5.4	11	Translation, ribosomal structure and biogenesis	0.256
126	3810	Proline-tRNA ligase	63,289.4	5.4	8	Translation, ribosomal structure and biogenesis	0.608
127	4303	Elongation factor Ts	37,710.4	5.4	13	Translation, ribosomal structure and biogenesis	1.898
128	4324	Elongation factor Ts	37,710.4	5.4	4	Translation, ribosomal structure and biogenesis	0.625
129	4421	Sua5 family translation factor	36,992.4	5.5	5	Translation, ribosomal structure and biogenesis	2.779
130	4423	Elongation factor Ts	37,710.4	5.4	13	Translation, ribosomal structure and biogenesis	0.603
131	5416	Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit B	48,933.9	6.2	13	Translation, ribosomal structure and biogenesis	1.355
132	6009	30S ribosomal protein S4	23,372.2	9.8	8	Translation, ribosomal structure and biogenesis	0.028
133	6014	30S ribosomal protein S4	23,372.2	9.8	8	Translation, ribosomal structure and biogenesis	0.227

Continued

Table 1 (Continued). Proteins expressed on 2-dimensional electrophoresis (2-DE) gels in the stationary phase (pH 4.5) and log phase (pH 5.1) of *Lactobacillus helveticus* H9 cultured in milk, but not expressed in the lag phase (pH 6.1)¹

No.	Spot	Protein name	Protein MW (Da)	Protein pI	Peptide count	COG	Fold-change pH 4.5/ pH 5.1
134	6616	Glutamyl-tRNA synthetase	57,580.4	5.9	24	Translation, ribosomal structure and biogenesis	0.275
135	6620	Glutamyl-tRNA synthetase	57,580.4	5.9	7	Translation, ribosomal structure and biogenesis	0.275
136	6928	Leucyl-tRNA synthetase	92,436.6	5.8	19	Translation, ribosomal structure and biogenesis	0.291
137	7112	50S ribosomal protein L1	24,760	8.9	7	Translation, ribosomal structure and biogenesis	1.921
138	7118	D-tyrosyl-tRNA(Tyr) deacylase	16,022.3	5.8	3	Translation, ribosomal structure and biogenesis	0.834
139	7215	50S ribosomal protein L1	24,760	8.9	7	Translation, ribosomal structure and biogenesis	0.846
140	7216	50S ribosomal protein L1	24,760	8.9	9	Translation, ribosomal structure and biogenesis	0.560
141	7612	Glutamyl-tRNA(Gln) amidotransferase subunit A	51,814.2	5.6	7	Translation, ribosomal structure and biogenesis	1.598
142	8617	Glutamyl-tRNA(Gln) amidotransferase subunit A	51,814.2	5.6	11	Translation, ribosomal structure and biogenesis	1.621
143	1410	Cse4 family CRISPR-associated protein (fragment)	249,981	5.7	12	Translation, ribosomal structure and biogenesis	0.665
144	2320	MutT/NUDIX family protein	59,361.4	5.1	14	Translation, ribosomal structure and biogenesis	1.198
145	3216	MutT/NUDIX family protein	59,361.4	5.1	16	Translation, ribosomal structure and biogenesis	1.545
146	3621	Conserved protein	165,699	5.3	12	Translation, ribosomal structure and biogenesis	0.995
147	5320	Cof family protein	56,946.9	5	7	Translation, ribosomal structure and biogenesis	0.502
148	6223	Putative uncharacterized protein	119,441	5.8	5	Translation, ribosomal structure and biogenesis	0.313
149	7321	Cof family protein	56,946.9	5	12	Translation, ribosomal structure and biogenesis	1.098
150	7708	Putative uncharacterized protein	104,157	8.6	14	Translation, ribosomal structure and biogenesis	0.266

¹MW = molecular weight; pI = isoelectric point; COG = clusters of orthologous groups.

other synthesis pathways (Derzelle et al., 2005); GlnA is a key enzyme that allows a strain to grow in milk cultures (Hornbaek et al., 2004).

Proteins Related to Carbohydrate Metabolism and Peptidoglycan Assembly

The proteins related to carbohydrate metabolism contained enolase (Eno, spot 1620), phosphoglycerate kinase (Pkg, spots 2305, 2416, 2512, 3419, 3421, 3407), and pyruvate kinase (Pyk, spots 6715, 3815, 5711, 5713, 3711) (Table 1, Supplementary Tables S1 and S2; <http://dx.doi.org/10.3168/jds.2014-8520>). Moreover, citrate lyase was expressed at both the pH 5.1 and pH 4.5 growth stages (Table 1) and may be related to the accumulation of lactate in the fermentation process. Previous research revealed that citrate lyase could be induced under acidic conditions, making cells resistant to the inhibitory effects of the fermentation product (lactate; Martín et al., 2004). Two galactose degradation pathways have been identified in the lactic acid bacteria: the Leloir pathway (which converts galactose to glucose-6-phosphate) and the tagatose-6-phosphate pathway (which converts galactose to glyceraldehyde-3-phosphate and dihydroxyacetone phosphate; Poolman, 1993; de Vos, 1996). Several proteins that participate in galactose metabolism were also detected, including uridine diphosphate (UDP)-glucose-1-uridylyltransferase (GalU, spots 5317, 7229, 7323), UDP-glucose-4-epimerase (GalE, spots 7220, 7223, 7325, 8615, 8616, 8617, 8618), glucosylphosphomutase (Pgm, spots 3615, 4609), and β -galactosidase (lacL, spot 4704), which revealed that *L. helveticus* H9 could utilize galactose through either of the 2 pathways. The expression of these proteins can increase the level of UDP-glucose, which is the precursor of glycolipids and teichoic acids. Because previous research has shown that glycolipids are related to the stability of the cell membrane, our results indicated that the expression of these proteins at the 2 growth stages (pH 5.1 and pH 4.5) may be related to the strengthening of the cell wall, thus leading to the strong resistance of the strain.

Table 2. Angiotensin I-converting enzyme inhibitory (ACEI) activity and the concentrations of VPP and IPP of *Lactobacillus helveticus* H9 grown in skim milk at different growth stages¹

pH	ACEI (%)	VPP (μ M)	IPP (μ M)
6.1 $\pm$ 0.01	20.9 $\pm$ 2.7	ND ²	ND
5.1 $\pm$ 0.03	62.4 $\pm$ 3.7	0.469 $\pm$ 0.019	0.195 $\pm$ 0.024
4.5 $\pm$ 0.00	79.6 $\pm$ 3.6	2.923 $\pm$ 0.037	1.732 $\pm$ 0.083

¹VPP = Val-Pro-Pro; IPP = Ile-Pro-Pro.

²ND = not detected.

Figure 3. Schematic of the cellular role categories (COG; clusters of orthologous groups) of the theoretical (open bars) and identified (black bars) proteins on a 2-dimensional electrophoresis (2-DE) gel in *Lactobacillus helveticus* H9. C = energy production and conversion; D = cell division, chromosome partitioning; E = amino acid transport and metabolism; F = nucleotide transport and metabolism; G = carbohydrate transport and metabolism; H = coenzyme metabolism; I = lipid and metabolism; J = translation, ribosomal structure and biogenesis; K = transcription; L = DNA replication, recombination, and repair; M = cell envelope biogenesis, outer membrane; N = cell motility and secretion; O = posttranslational modification, protein turnover, chaperones; P = inorganic ion transport and metabolism; Q = secondary metabolites biosynthesis, transport and catabolism; R = general function prediction only; S = function unknown; T = signal transduction mechanisms; U = intracellular trafficking, secretion, and vesicular transport; and V = defense mechanisms.

Two key enzymes, UDP-N-acetylmuramoylalanine D-glutamate ligase (MurD, spots 6504, 6511) and UDP-MurNac-tripeptide synthetase (MurE, spots 7603, 7608, 7615) involved in the synthesis of a cytoplasmic peptidoglycan precursor, were identified in Table 1 and Supplementary Table S2 (<http://dx.doi.org/10.3168/jds.2014-8520>). Peptidoglycans are important components of the cell wall, thus, the expression of MurD and MurE might indicate the cell division during the growth or an increase of the peptidoglycan content.

Proteins Related to Nucleotide Transport and Metabolism

We identified 10 protein spots related to nucleotide transport and metabolism. The enzymes were grouped into purine biosynthesis pathways (Table 1 and Supplementary Table S2; <http://dx.doi.org/10.3168/jds.2014-8520>), and included phosphoribosylamine-glycine ligase (PurD, spot 5415), phosphoribosylaminoimidazole carboxamide formyltransferase/IMP cyclohydrolase (PurH, spots 5217, 8716, 9804, 8615), phosphoribosylaminoimidazole carboxylase NCAIR mutase subunit (PurK, spots 8419, 8528, 8530, 9506, 9507) and phosphoribosylformylformylglycinamide synthase (PurL, spot 3218); pyrimidine metabolism were found in Supplementary Table S1 (<http://dx.doi.org/10.3168/jds.2014-8520>), including CTP synthase (PyrG, spot

6612), uracil phosphoribosyltransferase/pyrimidine operon regulatory protein (pyrR, spot 9101), uracil phosphoribosyltransferase (Upp, spot 9205), cytidylate kinase (Cmk, spot 9206), and Ser/Thr phosphatase family protein (spot 9611).

Lactobacilli can produce nucleotides through de novo biosynthesis or acquire them from the surroundings (Kilstrup et al., 2005). Milk contains a substantial amount of the pyrimidine precursor orotate but is very poor in purine bases (Beyer et al., 2003). Recent studies also showed that proteins related to the nucleotide metabolism pathway were expressed in the lag stage of *Lactococcus lactis* and *Lactobacillus casei* Zhang (Beyer et al., 2003; Gitton et al., 2005; Wang et al., 2013). The findings might indicate faster metabolism of pyrimidine than purine in *L. helveticus* H9.

The current study also identified formic acid tetrahydrofolic acid synthetase (Fhs, spot 7614), which can produce a cofactor of the biosynthesis of inosine monophosphate (IMP), the intermediate product of purine metabolism. Brejning et al. (2003) demonstrated that Fhs was induced in the absence of purine using a proteomic method. Gitton et al. (2005) and Smeianov et al. (2007) revealed that the expression of the protein Fhs and the corresponding gene *fhs* were regulated when the strain was grown in a milk culture. Our results agreed with these conclusions, showing that the absence of purine bases in milk.

Proteins Related to Translation and Transcription

Proteins related to the synthesis of DNA and RNA, such as transfer (t)RNA synthetases (aspartyl/glutamyl-tRNA, arginyl-tRNA synthetase, glycyl-tRNA synthetase β subunit glutamyl-tRNA synthetase, leucyl-tRNA synthetase, and methionyl-tRNA synthetase) were found on all 3 maps, whereas transcription elongation factors (EF-T; EF-Tu, EF-G, and EF-P) were identified on maps of pH 5.1 and pH 4.5 (Table 1). The primary function of elongation factors is to deliver aminoacyl-tRNA to the ribosome, and research has revealed that they may be related to protein folding or stress protection. In the current study, EF-Tu was expressed at growth stages pH 5.1 and pH 4.5, which might indicate that EF-Tu has a protective role in the synthesis of new proteins or it might be related to the tolerance of the strain.

Ribosomal Proteins and Stress Proteins

We identified 5 different ribosomal proteins: 30S ribosomal protein S2 (RpsB, spots 1210, 308, 313, 2315), 50S ribosomal protein L10 (RplJ, spot 112), 30S ribosomal protein S4 (RpsD, spots 6009, 6014), 50S ribosomal protein L1 (RplA, spots 7112, 7215, 7216) and 50S ribosomal protein L32 (RpmF, spot 8713) in the study. Ribosomal proteins are the main component of the ribosome, which is important for protein biosynthesis. They have also been regarded as indicators of heat stress and cold stress (Kawarai et al., 2007). Previous research revealed that ribosomal proteins participate in the growth process of bacteria (VanBogelen and Neidhardt, 1990). In our research, we found that most of ribosomal proteins were expressed in growth stages pH 5.1 and pH 4.5, showing their importance in growth of the strain.

Molecular chaperones or stress proteins play a crucial role in the folding, assembly, transport, and degradation of large biological molecules, especially under different stress conditions (acidic, alkaline, oxidizing, heat or cold stress). In the present study, 4 types of stress proteins, GroEL (spots 2721, 3731, 7324), DnaK (spot 2708), DnaD (spot 4611), and 33 kDa chaperonin (spot 7206), were found. Previous research revealed that the expression of these proteins was regulated in the stationary phase (Haandrikman et al., 1989). In general, when any strain is grown until the stationary phase, nutrients are depleted, leading to the production of stress proteins or molecular chaperone proteins, which then help the cells adapt to the culture conditions (Van De Guchte et al., 2002). Meanwhile, when the strain starts to grow, the chaperone proteins may be induced

to activate the expression of proteins, such as DnaK (spot 2708) in the present study (Table 1).

Other Proteins

We identified several proteins related to fatty acid biosynthesis, including S-malonyltransferase (FabD, spot 4211), enoyl-[acyl-carrier-protein] reductase (FabL, spots 3221, 4223), 3R-hydroxyacyl-acyl-carrier-protein dehydratase (FabZ, spot 6013), biotin carboxyl carrier protein of acetyl-CoA carboxylase (AccB, spots 1149, 1152), biotin carboxylase (AccC, spot 5512), and glycerol-3-phosphate acyltransferase (PlsX, spot 5528). We also identified proteins related to coenzyme metabolism: probable tRNA sulfurtransferase (spots 5417, 6414, 8418, 9404), bifunctional protein (FolD, spots 7218, 7231, 8213, 8223, 8225), nicotinate phosphoribosyltransferase (PncB, spots 8518, 8525), dihydrofolate reductase (FolA, spot 9112), and methionine adenosyltransferase (MetK, spot 3613), as well as some proteins with general or unknown functions. Additional research will be required to understand their physiological roles.

Overall, the growth response of strain *L. helveticus* H9 in milk is complicated. It needs all of the systems to be involved coordinately. During the fermentation of yogurt, ACE-inhibitory peptides were produced increasingly more in the stationary phase than in the log phase. The study showed that the process of VPP and IPP in *L. helveticus* H9 might be directly connected to PepN, PepO2, and OppC. Therefore, considering hypertension is now the most common chronic diseases in the modern circumstance, the production of ACE-inhibitory peptides VPP and IPP by *L. helveticus* H9 might have promising applications; for example, in the treatment of high blood pressure.

ACKNOWLEDGMENTS

This research was supported by the National Natural Science Foundation of China (Beijing, China; Grant No.31101315), the Hi-Tech Research and Development Program of China (863 Planning, Beijing, China; Grant No. 2011AA100902), the Special Fund for Agro-scientific Research in the Public Interest (Beijing, China; Grant No. 201303085, 201203009), and China Agriculture Research System (Beijing, China; Grant No. CARS-37).

REFERENCES

- Beganović, J., K. Blazenka, A. L. Pavunc, K. Uroic, P. Dzidara, and J. Suskovic. 2013. Proteolytic activity of probiotic strain *Lactobacillus helveticus* M92. *Anaerobe* 20:58–64.

- Beyer, N. H., P. Roepstorff, K. Hammer, and M. Kilstrup. 2003. Proteome analysis of the purine stimulon from *Lactococcus lactis*. *Proteomics* 3:786–797.
- Brejning, J., L. Jespersen, and N. Arneborg. 2003. Genome-wide transcriptional changes during the lag phase of *Saccharomyces cerevisiae*. *Arch. Microbiol.* 179:278–294.
- Broadbent, J. R., H. Cai, R. L. Larsen, J. E. Hughes, D. L. Welker, V. G. De Carvalho, T. A. Tompkins, Y. Ardö, F. Vogensen, A. De Lorentiis, M. Gatti, E. Neviani, and J. L. Steele. 2011. Genetic diversity in proteolytic enzymes and amino acid metabolism among *Lactobacillus helveticus* strains. *J. Dairy Sci.* 94:4313–4328.
- Callanan, M., P. Kaleta, J. O'Callaghan, O. O'Sullivan, K. Jordan, O. McAuliffe, A. Sangrador-Vegas, L. Slattery, G. F. Fitzgerald, T. Beresford, and R. P. Ross. 2008. Genome sequence of *Lactobacillus helveticus*, an organism distinguished by selective gene loss and insertion sequence element expansion. *J. Bacteriol.* 190:727–735.
- Chen, Y., W. Liu, J. Xue, J. Yang, X. Chen, Y. Shao, L. Kwok, M. Bilige, L. Mang, and H. Zhang. 2014. Angiotensin-converting enzyme inhibitory activity of *Lactobacillus helveticus* strains from traditional fermented dairy foods and antihypertensive effect of fermented milk of strain H9. *J. Dairy Sci.* 97:6680–6692. <http://dx.doi.org/10.3168/jds.2014-7962>.
- Chen, Y., Z. Wang, X. Chen, Y. Liu, H. Zhang, and T. Sun. 2010. Identification of angiotensin I-converting enzyme inhibitory peptides from koumiss, a traditional fermented mare's milk. *J. Dairy Sci.* 93:884–892.
- Christensen, J. E., and J. L. Steele. 2003. Impaired growth rates in milk of *Lactobacillus helveticus* peptidase mutants can be overcome by use of amino acid supplements. *J. Bacteriol.* 185:3297–3306.
- Cremonesi, P., S. Chessa, and C. Bianca. 2013. Genome sequence and analysis of *Lactobacillus helveticus*. *Front. Microbiol.* 3:435.
- de Vos, W. M. 1996. Metabolic engineering of sugar catabolism in lactic acid bacteria. *Antonie van Leeuwenhoek* 70:223–242.
- 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.
- Fenster, K. M., K. L. Parkin, and J. L. Steele. 1997. Characterization of a thiol-dependent endopeptidase from *Lactobacillus helveticus* CNRZ32. *J. Bacteriol.* 179:2529–2533.
- Gitton, C., M. Meyrand, J. Wang, C. Caron, A. Trubuil, A. Guilhot, and M. Y. Mistou. 2005. Proteomic signature of *Lactococcus lactis* NCDO763 cultivated in milk. *Appl. Environ. Microbiol.* 71:7152–7163.
- Haandrikman, A. J., J. Kok, H. Laan, S. Soemitro, A. M. Ledebor, W. N. Konings, and G. Venema. 1989. Identification of a gene required for maturation of an extracellular lactococcal serine proteinase. *J. Bacteriol.* 171:2789–2794.
- Hornbaek, T., M. Jakobsen, J. Dynesen, and A. K. Nielsen. 2004. Global transcription profiles and intracellular pH regulation measured in *Bacillus licheniformis* upon external pH upshifts. *Arch. Microbiol.* 182:467–474.
- Juillard, V., H. Laan, E. R. Kunji, C. M. Jeronimus-Stratingh, A. P. Bruins, and W. N. Konings. 1995a. The extracellular PI-type proteinase of *Lactococcus lactis* hydrolyzes beta-casein into more than one hundred different oligopeptides. *J. Bacteriol.* 177:3472–3478.
- Juillard, V., D. Le Bars, E. R. Kunji, W. N. Konings, J. C. Gripon, and J. Richard. 1995b. Oligopeptides are the main source of nitrogen for *Lactococcus lactis* during growth in milk. *Appl. Environ. Microbiol.* 61:3024–3030.
- Kawarai, T., S. Furukawa, H. Ogihara, and M. Yamasaki. 2007. Mixed-species biofilm formation by lactic acid bacteria and rice wine yeasts. *Appl. Environ. Microbiol.* 73:4673–4676.
- Kilpi, E. E. R., M. M. Kahala, J. L. Steele, A. M. Pihlanto, and V. V. Joutsjoki. 2007. Angiotensin I-converting enzyme inhibitory activity in milk fermented by wild-type and peptidase-deletion derivatives of *Lactobacillus helveticus* CNRZ32. *Int. Dairy J.* 17:976–984.
- Kilstrup, M., K. Hammer, P. Ruhdal Jensen, and J. Martinussen. 2005. Nucleotide metabolism and its control in lactic acid bacteria. *FEMS Microbiol. Rev.* 29:555–590.
- Manso, M. A., J. Leonil, M. Piot, and V. Gagnaire. 2005. Isolation and characterisation of a *Lactobacillus helveticus* ITG LH1 peptidase-rich sub-proteome. *Int. J. Food Microbiol.* 105:119–129.
- Martín, M. G., P. D. Sender, S. Peiru, D. de Mendoza, and C. Magni. 2004. Acid-inducible transcription of the operon encoding the citrate lyase complex of *Lactococcus lactis* Biovar diacetylactis CRL264. *J. Bacteriol.* 186:5649–5660.
- Nakamura, Y., N. Yamasee, K. Sakai, A. Okubo, S. Vamazak, and T. Takano. 1995. Purification and characterization of angiotensin I-converting enzyme inhibitors from sour milk. *J. Dairy Sci.* 78:777–783.
- Pan, D. D., Y. K. Luo, and M. Tanokura. 2005. Antihypertensive peptides from skimmed milk hydrolysate digested by cell-free extract of *Lactobacillus helveticus* JCM1004. *Food Chem.* 91:123–129.
- Poolman, B. 1993. Energy transduction in lactic acid bacteria. *FEMS Microbiol. Rev.* 12:125–147.
- Prajapati, J. B., C. D. Khedkar, J. Chitra, S. Suja, V. Mishra, V. Sreeja, R. K. Patel, V. B. Ahir, V. D. Bhatt, M. R. Sajani, S. J. Jakhesara, P. G. Koringa, and C. G. Joshi. 2011. Whole-genome shotgun sequencing of an Indian origin *Lactobacillus helveticus* strain, MTCC5463, with probiotic potential. *J. Bacteriol.* 193:4282–4283.
- Sadat-Mekmene, L., R. Richoux, L. Aubert-Frogerais, M. N. Madec, C. Corre, M. Piot, J. Jardin, S. le Feunteun, S. Lortal, and V. Gagnaire. 2013. *Lactobacillus helveticus* as a tool to change proteolysis and functionality in Swiss-type cheeses. *J. Dairy Sci.* 96:1455–1470.
- Scolari, G., M. Vescovo, C. Zacconi, and F. Vescovi. 2006. Extraction and partial characterization of proteolytic activities from the cell surface of *Lactobacillus helveticus* Zuc2. *J. Dairy Sci.* 89:3800–3809.
- Smeianov, V. V., P. Wechter, J. R. Broadbent, J. E. Hughes, B. T. Rodriguez, T. K. Christensen, Y. Ardo, and J. L. Steele. 2007. Comparative high-density microarray analysis of gene expression during growth of *Lactobacillus helveticus* in milk versus rich culture medium. *Appl. Environ. Microbiol.* 73:2661–2672.
- Sridhar, V. R., J. E. Hughes, D. L. Welker, J. R. Broadbent, and J. L. Steele. 2005. Identification of endopeptidase genes from the genomic sequence of *Lactobacillus helveticus* CNRZ32 and the role of these genes in hydrolysis of model bitter peptides. *Appl. Environ. Microbiol.* 71:3025–3032.
- Strahinic, I., J. Lozo, A. Terzic-Vidojevic, D. Fira, M. Kojic, N. Golic, J. Begovic, and L. Topisirovic. 2013. Technological and probiotic potential of BGRA43, a natural isolate of *Lactobacillus helveticus*. *Front. Microbiol.* 4:2.
- Sun, T. S., S. P. Zhao, H. K. Wang, C. K. Cai, Y. F. Chen, and H. P. Zhang. 2009. ACE-inhibitory activity and gamma-aminobutyric acid content of fermented skim milk by *Lactobacillus helveticus* isolated from Xinjiang koumiss in China. *Eur. Food Res. Technol.* 228:607–612.
- Tian, J. Y., D. D. Pan, and Y. X. Guo. 2009. Optimization of enzymatic hydrolysis technology of casein by proteinases from *Lactobacillus helveticus* for ACE-inhibitory peptide production. *Food Sci.* 11:170–174. (In Chinese).
- Tompkins, T. A., G. Barreau, and J. R. Broadbent. 2012. Complete genome sequence of *Lactobacillus helveticus* R0052, a commercial probiotic strain. *J. Bacteriol.* 194:6349.
- Tynkkynen, S., G. Buist, E. Kunji, J. Kok, B. Poolman, G. Venema, and A. Haandrikman. 1993. Genetic and biochemical characterization of the oligopeptide transport system of *Lactococcus lactis*. *J. Bacteriol.* 175:7523–7532.
- van De Guchte, M., P. Serror, C. Chervaux, T. Smokvina, S. D. Ehrlich, and E. Maguin. 2002. Stress responses in lactic acid bacteria. *Antonie van Leeuwenhoek* 82:187–216.
- VanBogelen, R. A., and F. C. Neidhardt. 1990. Ribosomes as sensors of heat and cold shock in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* 87:5589–5593.
- Wakai, T., N. Yamaguchi, M. Hatanaka, Y. Nakamura, and N. Yamamoto. 2012. Repressive processing of antihypertensive peptides, Val-Pro-Pro and Ile-Pro-Pro, in *Lactobacillus helveticus* fermented milk by added peptides. *J. Biosci. Bioeng.* 114:133–137.

- Wang, J. C., R. N. Wu, W. Y. Zhang, Z. H. Sun, W. J. Zhao, and H. P. Zhang. 2013. Proteomic comparison of the probiotic bacterium *Lactobacillus casei* Zhang cultivated in milk and soy milk. *J. Dairy Sci.* 96:5603–5624.
- Yamamoto, N., A. Akino, and T. Takano. 1994. Antihypertensive effect of the peptides derived from casein by an extracellular proteinase from *Lactobacillus helveticus* CP79. *J. Dairy Sci.* 77:917–922.
- Yamamoto, N., M. Maeno, and T. Takano. 1999. Purification and characterization of an antihypertensive peptide from a yogurt-like product fermented by *Lactobacillus helveticus* CPN4. *J. Dairy Sci.* 82:1388–1393.
- Yuan, J., L. Zhu, X. Liu, T. Li, Y. Zhang, T. Ying, B. Wang, J. Wang, H. Dong, and E. Feng. 2006. A proteome reference map and proteomic analysis of *Bifidobacterium longum* NCC2705. *Mol. Cell. Proteomics* 5:1105–1118.
- Zhang, F. M., Z. Li, Z. Liu, L. L. Liu, W. Tian, D. W. Jin, M. S. Li, K. Yang, and Y. M. Han. 2013. The studies on the preparation of antihypertensive peptides of yak milk casein fermentation with *Lactobacillus helveticus*. *J. Northwest University for Nationalities* 34:64–71.
- Zhang, W. Y., Y. F. Chen, W. J. Zhao, L. Y. Kwok, and H. P. Zhang. 2014. Gene expression of proteolytic system of *Lactobacillus helveticus* H9 during milk fermentation. *Ann. Microbiol.* <http://dx.doi.org/10.1007/s13213-014-0902-3>.
- Zhao, W., Y. Chen, Z. Sun, J. Wang, Z. Zhou, T. Sun, L. Wang, W. Chen, and H. Zhang. 2011. Complete genome sequence of *Lactobacillus helveticus* H10. *J. Bacteriol.* 193:2666–2667.