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Impact of High-Level Expression of Heterologous Protein on *Lactococcus lactis* Host

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The impact of overproduction of a heterologous protein on the metabolic system of host *Lactococcus lactis* was investigated. The protein expression profiles of *L. lactis* IL1403 containing two near-identical plasmids that expressed high- and low-level of the green fluorescent protein (GFP) were examined via shotgun proteomics. Analysis of the two strains via high-throughput LC-MS/MS proteomics identified the expression of 294 proteins. The relative amount of each protein in the proteome of both strains was determined by label-free quantification using the spectral counting method. Although expression level of most proteins were similar, several significant alterations in metabolic network were identified in the high GFP-producing strain. These changes include alterations in the pyruvate fermentation pathway, oxidative pentose phosphate pathway, and de novo synthesis pathway for pyrimidine RNA. Expression of enzymes for the synthesis of dTDP-rhamnose and *N*-acetylglucosamine from glucose was suppressed in the high GFP strain. In addition, enzymes involved in the amino acid synthesis or interconversion pathway were downregulated. The most noticeable changes in the high GFP-producing strain were a 3.4-fold increase in the expression of stress response and chaperone proteins and increase of caseinolytic peptidase family proteins. Characterization of these host expression changes witnessed during overexpression of GFP was might suggested the metabolic requirements and networks that may limit protein expression, and will aid in the future development of lactococcal hosts to produce more heterologous protein.

Keywords: Comparative proteomics, green fluorescent protein, label-free quantification, *Lactococcus lactis*, systems biology, protein overexpression

Introduction

Lactic acid bacteria (LAB) have long been considered beneficial to human health [1]. Currently, LAB are employed in numerous probiotic products that are considered "functional foods," or foods that provide a health benefit beyond satisfying traditional nutrient requirements [2]. Recently, engineered strains of LAB have been employed as oral vectors for delivery of therapeutic proteins [3], and even in situ secretion of IL-10 by engineered *Lactococcus lactis* [4].

The approach of using engineered LAB is attractive for a number of reasons. LAB are consumed frequently in numerous fermented products and therefore possess a Generally Regarded As Safe designation. Some LAB strains are also members of the human gastro-intestinal tract (GIT) microbiota and are able to survive passage through, and in some cases proliferate within, the GIT. Finally, owing to their prevalent use in commercial food and beverage fermentations, there is a wealth of knowledge of LAB fermentation characteristics, strain preservation systems, and shelf-life behavior(s). These latter advances position engineered LAB as an ideal route for creation of stable oral protein therapeutics that can be delivered without injection

and withstand non-refrigerated transport, an important goal for health agencies seeking to ameliorate numerous diseases that predominantly afflict populations in third world countries.

L. lactis is one of the most commonly used LAB for heterologous protein production [5]. However, expression to date has typically been orders of magnitude lower than current commercial processes utilizing *Escherichia coli*, *Saccharomyces*, *Pichia*, or mammalian cell systems, which commonly reach or exceed 0.5 to 2 g target protein/L. To increase the recombinant protein production, research has focused on optimization of the fermentation process, including induction strategies, environmental conditions (*e.g.*, pH and temperature), media, and expression vector changes [6–16]. Despite these efforts, few studies have examined the host response to protein overproduction. A comprehensive understanding of the bacterial host response to heterologous protein production is key to the rational development of novel production hosts that can deliver a maximum level of desired protein.

High-throughput identification and quantitation of whole-cell proteomes is an essential tool for the systematic study of cellular behavior [17–19]. Along with other "omics-," whole-cell proteomics characterizes all protein expression at a moment in time, thereby providing a systematic view of a bacterial response to different biological or environmental conditions [20–22]. Moreover, changes in protein expression observed in two or more different conditions can be readily accessed by various quantification tools available for comparative proteomics [23–28].

L. lactis strains HR279 [14] and JHK24 [10] are derived from *L. lactis* IL1403 [29] and harbor plasmids pHR086 and pJH24, respectively. The plasmid pHR086 is an *E. coli-L. lactis* shuttle vector that produces green fluorescent protein (GFP) under the strong inducible promoter of *nisA*. The plasmid pJH24 was designed to improve the GFP production by increasing the copy number using interruption of the copy number control region of pHR086. In a previous work, the comparative study of protein expression using these high- and low-copy vectors showed that the expression of GFP on JHK24 is 5.0-fold higher than that in HR279 [10]. In this study, we examined global changes in the protein expression pattern of *L. lactis* IL1403 with the heterologous protein expression by low- and high-copy number plasmids. Whole-cell proteomes were analyzed by LC-MS/MS followed by tryptic digestion and identified by a database-driven search using X! Tandem. Then, the relative change of protein expression level between JHK24 and HR279 was calculated by the label-free approach using a spectral counting method.

Materials and Methods

Cell Culture

L. lactis HR279 and *L. lactis* JHK24 were cultivated in M17 medium (BD, USA) containing 3% (w/v) of glucose (M17-G) with the supplement of 5 μg/ml of erythromycin (Sigma, USA). Fermentation was initiated by the inoculation of 5 ml of seed culture into 300 ml of M17-G medium with an initial pH of 6.5. The temperature was controlled at 30°C without shaking. The pH was not controlled during the fermentation. The optical density (OD) was measured by a Beckman DU 7400 spectrophotometer (Beckman, USA) at 600 nm. The expression of GFP was induced by the addition of nisin at a final concentration of 25 ng/ml when the cell OD reached 0.7. The expression of GFP was monitored by measuring the fluorescence from the cells. To do this, cell pellets were washed three times in phosphate-buffered saline (PBS) and normalized to an OD_{600nm} of 0.1. The fluorescence from 100 μl of cells was measured in an ABI770 real-time thermocycler using excitation and emission wavelengths of 488 and 520 nm, respectively [14].

Sample Preparation and Protein Identification

Fifty milliliters of sample was taken at the late exponential phase of cell growth. The initial cell mass of each sample was normalized to an OD_{600nm} of 1.0 by dilution or concentration to a final volume of 25 ml. After centrifugation, the cell pellets were washed three times with PBS and then resuspended in 1 ml of lysis buffer containing 100 mM of Tris and 8.0 M of urea. The initial pH of the lysis buffer was 9.0. With addition of 300 μg of silica beads (Sigma-Aldrich, USA), the cells were disrupted using a bead beater (FastPrep; QBiogen, USA) for six 30 sec pulses each with a 30 sec interval on ice in between pulses. Beads and cell debris were removed by centrifugation and the soluble fraction was kept at −80°C for further analysis. Protein concentration was measured using a protein assay kit (BioRad, USA).

For reduction, 4 μl of 450 mM dithiothreitol (Sigma-Aldrich) was added to 200 μg of whole-cell lysate and incubated for 45 min at 55°C. Without alkylation, the reduced protein was digested with 2.5 μg of mass spectrometry grade trypsin (Promega, USA) overnight at 37°C. The tryptic peptides were purified by C18 Ziptip (Millipore, USA) according to the manufacturer's manual. The Ziptip was prepared by washing with 50% acetonitrile (ACN)/H_2O followed by 0.1% (v/v) trifluoroacetic acid (TFA) in H_2O. The tryptic peptide solution was then loaded onto the Ziptip and washed with 0.1% (v/v) TFA in H_2O. The peptides were eluted with 50% ACN in H_2O. The purified sample was dried prior to mass spectrometry analysis.

Protein identification was performed using a Nano LC 2-D system (Eksigent, USA) coupled to an LTQ ion-trap mass spectrometer (Thermo-Fisher, USA) through a Picoview Nano-spray source. Peptides were loaded onto a nanotrap device (Zorbax 300SB-C18;

Agilent Technologies, USA) at a loading flow rate of 5.0 µl/min. Peptides were then eluted from the trap and separated by a nano-scale 75 µm × 15 cm New Objectives picofrit column packed in-house with Michrom Magic C18 AQ packing material. Peptides were eluted using a 90 min gradient of 2–80% buffer B (Buffer A = 0.1% formic acid, Buffer B = 95% acetonitrile/0.1% formic acid). The top 10 ions in each survey scan were subjected to automatic low-energy CID.

Database Searching, False Discovery Rate, and Quantitation

Tandem mass spectra were extracted and charge state deconvoluted by BioWorks ver. 3.3. Deisotoping was not performed. All MS/MS samples were analyzed using X! Tandem. X! Tandem was set up to search against the *L. lactis* whole proteome with the supplement of the proteins expressed from the plasmid. X! Tandem was searched with a fragment ion mass tolerance of 0.60 Da. Oxidation of methionine was specified as a variable modification.

A preliminary protein search was performed using the false discovery rate (FDR) determined by independent searching of MS/MS spectra against the forward (target) and the reversed database (decoy) of *L. lactis* IL1403, including plasmid proteins. The FDR was calculated as $R/(F+R)$ where R and F are the number of proteins from the decoy and target database, respectively. Initial protein identification was obtained at a FDR level of 1.5%. Among the proteins obtained, those having a number of unique peptides greater than 2 ($P_{uniq} \geq 2$) and a probability score lower than -7 (log(E) ≤ -7) were considered to be expressed and were used for further quantitative analysis.

The relative quantitation of protein was performed by the spectral counting method [30–32]. Briefly, a spectral abundance factor of each protein was calculated from the length of a protein (a number of amino acids: L_k) and the number of spectra used to identify the protein (SpC_k). After normalization of each spectral abundance factor in a sample, the value of a normalized spectral abundance factor (NSAF) was used to determine the relative amount of each protein within a sample. The relative amount of a protein in *L. lactis* JHK24 was obtained by the comparison of the two NSAF values for strains JHK24 and HR279. For an accurate calculation, the number of total spectra (SpC_k) of each protein in JHK24 and HR279 had to be greater than 4.

Bioinformatics

The genomic information of the *L. lactis* IL1403 genome were obtained from NCBI (http://www.ncbi.nlm.nih.gov) and JGI (http://img.jgi.doe.gov) [29]. The functional information of enzymes was obtained from JGI (http://img.jgi.doe.gov) and EXPASY (http://www.expasy.org).

Results

Performance of LC-MS/MS and Protein Expression in *L. lactis* JHK24 and HR279

Samples were taken in the late exponential phase of

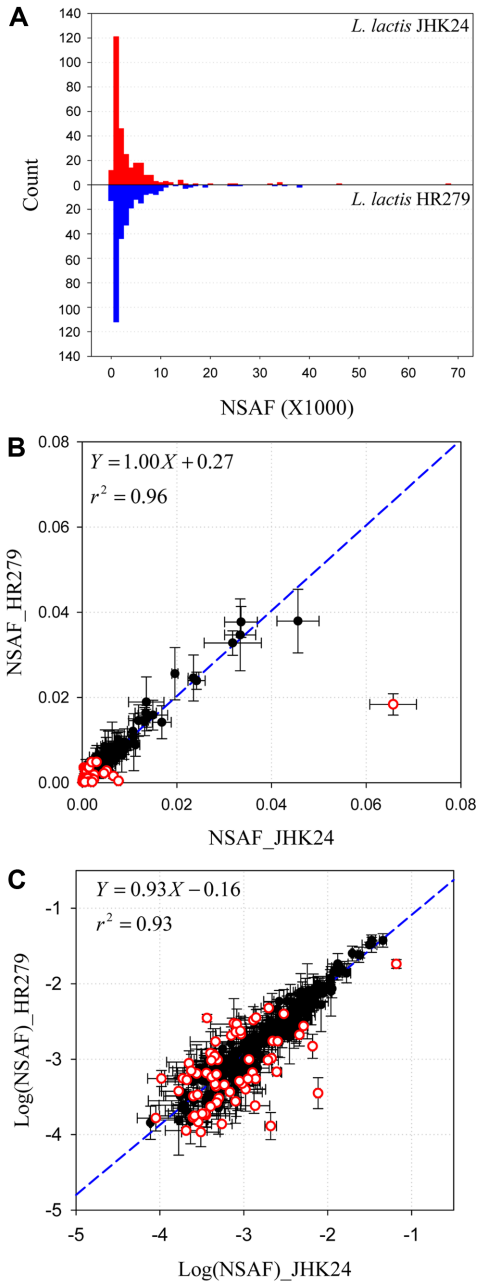

Fig. 1. Analysis of the whole-cell proteomes of *L. lactis* HR279 and *L. lactis* JHK24 by LC-MS/MS.
Quantitation of protein abundance was obtained by the spectral counting method. (**A**) Expression of each protein in *L. lactis* HR279 and JHK24. Protein abundance in any sample is represented by a normalized spectral abundance factor (NSAF) and plotted in a linear scale. Open circles indicate the protein exhibited more than 2- fold changes between JHK24 and HR279. (**B**) NSAF values for each protein in HR279 and JHK24 plotted on a log-log scale to visualize the low abundance proteins. (**C**) NSAF distribution in a sample. Proteomes of *L. lactis* JHK24 and HR279 are presented in the upper and lower graph, respectively. Highly expressed proteins are listed separately in Table 1.

growth and the experiments were performed in triplicates. A total of 294 proteins were identified in the *L. lactis* strains and, among these, 279 proteins were commonly found in both strains. Proteins uniquely found in either JHK24 or HR279 suggested that the expression of protein in the cognate strain was below the detection limit of MS but does not necessarily indicate no expression. For example, NisR and NisK encoded on the low-copy vector pHR082 are essential for the expression of GFP, but were not found in the proteome of HR279. The expression of those two proteins was observed in the whole-cell proteome of JHK24 with increase of the copy number of plasmid.

The quantitative protein expression profile was determined using the NSAF. As presented in Fig. 1A, most proteins exhibited similar NSAF values in JHK24 and HR279, showing a range from 65.7 to 0.11 (expressed as $\times 10^3$ fold value). Most proteins had NSAFs less than 10.0 and only 60 proteins had NSAF values larger than 5.0, which corresponds to 0.5% fraction of the total proteome obtained from a sample. Table 1 lists the 20 most expressed proteins in *L. lactis* JHK24. Not surprisingly, GFP was the most highly expressed protein, occupying 6.6% of the intracellular JHK24 proteome. Interestingly, GFP was also strongly expressed in HR279. Although GFP was encoded on pHR082, which is the low-copy variant of pJH24, it was the 9th highly expressed protein in HR279. Whereas proteomic analysis indicated that the GFP expression from JHK24 was 3.57-fold higher than that from HR082, the fluorescence measurement suggested an increase of 4.00±0.62 folds, which correlated with the proteomic analysis. Two stress response proteins, CspE and DnaK, were strongly induced in both *L. lactis* JHK24 and HR279 proteomes (Table 1). CspE was the second most expressed protein, reaching up to 4.6% and 3.8% of the proteome of JHK24 and HR279, respectively. CspE and DnaK were expressed at a higher level in JHK24 than in HR279. Other highly expressed proteins were glycolytic enzymes and transcription/translation-related proteins. Most of these proteins, in particular glycolytic proteins, exhibited similar NSAF values in *L. lactis* JHK24 and HR279, suggesting constitutive expression in

Table 1. The twenty most highly expressed proteins in *L. lactis* JHK24.

Rank	gi[a]	Gene	Name	NSAF × 1,000 (AVE ± SD)[b]	
				pJH24	pJHR082
1 (9)			GFP	65.7 ± 4.9	18.4 ± 2.5
2 (1)	15672150	*cspE*	Cold shock protein E	45.6 ± 4.4	37.9 ± 7.5
3 (2)	15674228	*gapB*	Glyceraldehyde 3-phosphate dehydrogenase	33.6 ± 3.5	37.7 ± 3.6
4 (3)	15673843	*tuf*	Elongation factor Tu	33.4 ± 3.3	34.7 ± 8.4
5 (4)	15672484	*hslA*	HU like DNA-binding protein	31.8 ± 6.0	32.8 ± 2.9
6 (7)	15672626	*eno*	Phosphopyruvate hydratase	24.1 ± 1.8	23.9 ± 2.0
7 (6)	15673250	*rplL*	50S ribosomal protein L7/L12	23.5 ± 1.1	24.6 ± 5.4
8 (5)	15673665	*malE*	Maltose ABC transporter substrate binding protein	19.6 ± 0.7	25.6 ± 6.1
9 (15)	15674173	*rpsF*	30S ribosomal protein S6	16.8 ± 2.0	14.2 ± 3.9
10 (11)	15674077	*rpsS*	30S ribosomal protein S19	14.9 ± 3.1	15.8 ± 3.5
11 (12)	15672227	*pgk*	Phosphoglycerate kinase	13.6 ± 0.7	15.1 ± 3.0
12 (8)	15674007	*dpsA*	Non-heme iron-binding ferritin	13.5 ± 3.7	18.9 ± 5.9
13 (10)	15673891	*fbaA*	Fructose-bisphosphate aldolase	13.4 ± 2.2	16.4 ± 0.9
14 (14)	15672820	*rpsA*	30S ribosomal protein S1	13.2 ± 0.6	14.3 ± 3.3
15 (13)	15672266	*rpsD*	30S ribosomal protein S4	11.9 ± 2.1	14.6 ± 3.7
16 (25)	15672936	*dnaK*	Molecular chaperone DnaK	11.2 ± 0.9	8.8 ± 2.6
17 (20)	15673061	*rplU*	50S ribosomal protein L21	10.9 ± 0.3	9.5 ± 6.7
18 (17)	15673578	*rpmE*	50S ribosomal protein L31	10.8 ± 1.1	10.8 ± 0.4
19 (16)	15673116	*tpiA*	Triosephosphate isomerase	10.7 ± 1.2	12.0 ± 2.9
20 (24)	15674134	*tsf*	Elongation factor Ts	9.7 ± 1.1	8.9 ± 1.2

Numbers in parentheses represent the rank in *L. lactis* HR279.

[a]gi: Gene identification number as per NCBI.

[b]The average and standard deviation of the normalized spectral abundance factor (NSAF). Experiments were performed in triplicates.

both strains.

The relative amount of proteins in JHK24 was examined by comparing the two NSAF values of each protein in JHK24 and HR297. As shown in Figs. 1B and 1C, the quantitative expression profiles were generally well correlated between JHK24 and HR297 with a linear correlation (r^2) of 0.96 in normal and 0.93 in log-log plot, respectively. Among 279 quantifiable proteins, only 66 proteins showed more than ±2.0-fold increase in JHK24 in comparison with HR279 (open circles in Figs. 1B and 1C).

Sugar Nucleotide and Amino Sugar Metabolism

One of the main differences in metabolism between JHK24 and HR279 was found in sugar metabolism. The enzymes

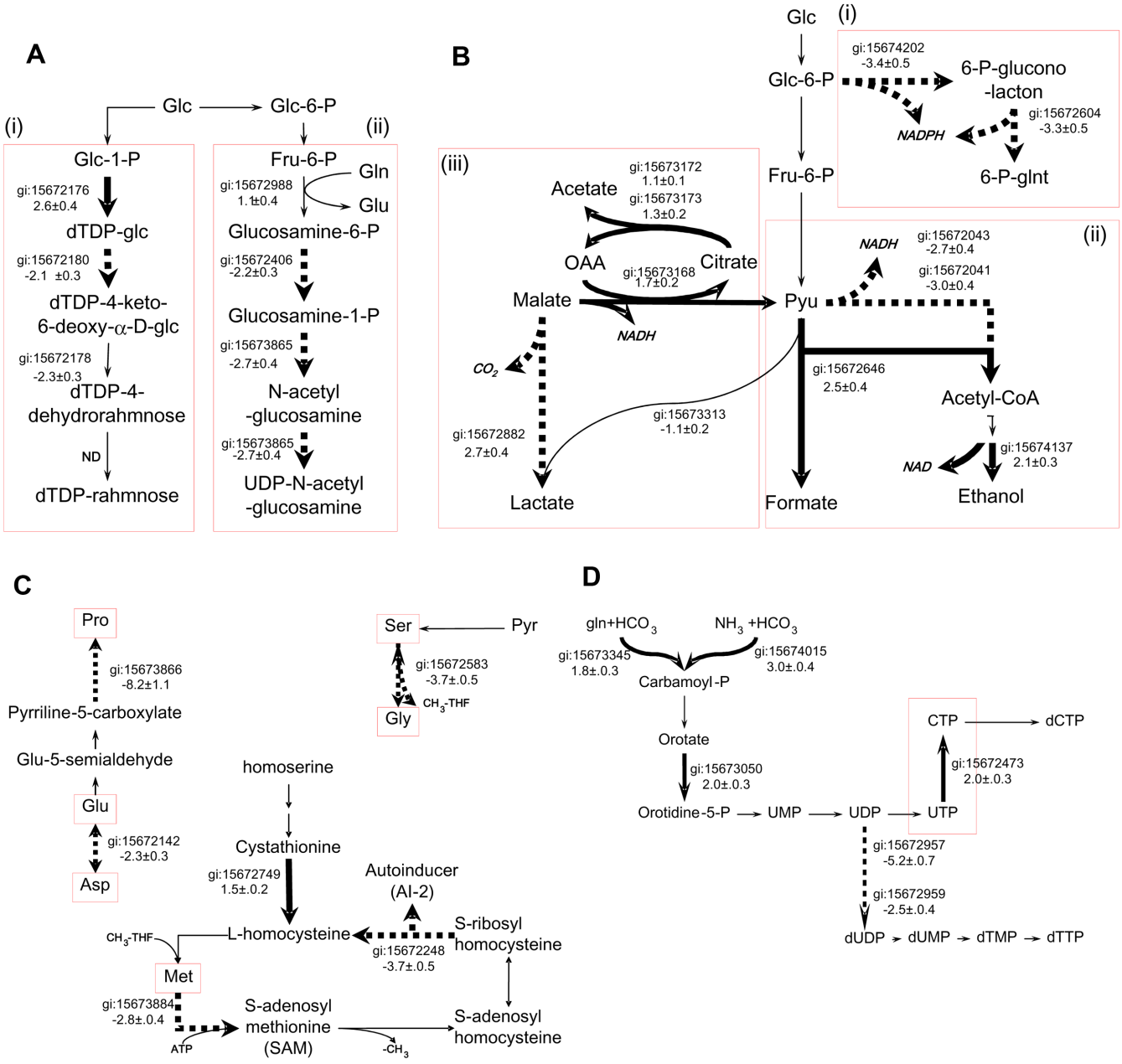

Fig. 2. The metabolic pathways that exhibited significant changes in *L. lactis* JHK24 by comparison with strain HR279.
The relative abundance of each enzyme in JHK24 compared with HR279 is presented under the Gene Identification (gi) numbers. Bold and dotted arrows represent the increased or decreased expression in *L. lactis* JHK24, respectively. (**A**) Synthesis pathway for (i) dTDP-rhamnose and (ii) GluNAc. (**B**) (i) Oxidative pentose phosphate pathway, (ii) pyruvate fermentation, and (iii) malate conversion. (**C**) De novo synthesis pathway for amino acids. (**D**) Pyrimidine synthesis pathway.

for sugar nucleotide synthesis, in particular dTDP L-rhamnose, differed in the two strains (Box (i) in Fig. 2A). The expression of glucose-1-phosphate thymidylyltransferase (*rmlA*, gi:15672176), which converts glucose-1-phosphate to dTDP-glucose, increased at 2.6-fold in JHK24 compared with that in HR279. However, dTDP-glucose 4,6-dehydratase (*rmlB*, gi: 15672180), which uses dTDP-glucose as a substrate to form dTDP-4-keto-6-deoxyglucose, showed a decrease of expression by 2.3-fold. Although the expression levels were low in both JHK24 and HR279 (data not shown), the dTDP-4-keto-6-deoxyglucose-3,5-epimerase, which is the next step in dTDP-rhamnose synthesis, was also reduced in expression in JHK24 to 2.4-fold.

With the overexpression of GFP, the synthesis of UDP-*N*-acetyl-D-glucosamine (UDP-GlcNAc) appeared to be suppressed in JHK24 (Box (ii) in Fig. 2A). The initial precursor of UDP-GlcNAc is fructose 6-phosphate. The fructose 6-phosphate amidotransferase transfers an amine group from glutamine to form glucosamine 6-phosphate. Then phosphoglucomutase (*femD*, gi: 15672406) converts the glucosamine 6-phosphate to glucosamine 1-phosphate, which is further converted to UDP-GlcNAc by a multifunctional enzyme, glucosamine 1-phosphate *N*-acetyltransferase/UDP-GlcNAc pyrophosphorylase (*glmU*, gi: 15673865). In JHK24, the expression levels of the mutase and multifunctional enzymes were decreased 2.2- and 2.7-fold, respectively (Table 2).

Sugar Metabolism

The enzymes for the oxidative pentose phosphate pathway (PPP) were reduced in expression in *L. lactis* JHK24 (Box (i) in Fig. 2B). The glucose 6-phosphate 1-dehydrogenase (*zwf*, gi:15674202) and 6-phosphogluconate dehydrogenase (*gnd*, gi: 15672604), which diverts glucose 6-phosphate flux to PPP with the generation of two NADPH, decreased by 3.4- and 3.3-fold in JHK24 compared with HR279.

Pyruvate metabolism was also influenced by high-level expression of GFP (Box (ii) in Fig. 2B). Although the expression level of lactate dehydrogenase (gi: 15673313, *ldh*) was similar in both JHK24 and HR279, the E1 (*pdhD*, gi: 15672041) and E3 beta subunits (*pdhB*, gi: 15672043) of the pyruvate dehydrogenase (PDH) complex decreased in expression 2.7- and 3.0-fold, respectively, in JHK24 (Table 2). Instead of PDH, which requires NAD as a cofactor, the expression of pyruvate-formate lyase (*pfl*, gi: 15672646), which cleaves pyruvate to formate and acetyl-CoA without the regeneration of NAD^+, was significantly increased, showing 2.5-fold increase in JHK24 compared with that in HR279. The alcohol-acetaldehyde dehydrogenase (*adhE*, gi:15674137) also showed remarkable changes in expression between the two strains. In *L. lactis* JHK24, the expression of AdhE increased 2.1-fold compared with that in HR279. The protein abundance of AdhE in JHK24 was high and comparable to the level of LDH as well. The NSAF (×1,000) of LDH was 5.6, making it the 47th most abundant protein in JHK24, whereas the NSAF of AdhE was 4.6, corresponding to the 66th most abundant protein in HR279.

L. lactis IL1403 metabolizes malate, a four-carbon metabolite, through two independent routes. Malate can be converted to pyruvate by NAD-dependent malate oxidoreductase (*mae*, gi: 15673168) with co-conversion of oxaloacetic acid (OAA) to citric acid. Citric acid, which receives a single carbon from malate, is converted to OAA by citrate lyase with the production of acetic acid. An alternative route for malate conversion is malolactic enzyme (*mleS*, gi: 15672882) that converts malate to lactic acid by decarboxylation with liberation of CO_2. In HR279, the low GFP expression variant, the malolactic enzyme (MleS) was one of the major proteins and the expression level of MleS and Mae were similar with an NSAF of 2.47 and 3.02, respectively. However, in JHK24, the expression of MleS was decreased to an NSAF of 0.94, resulting in a reduction by 2.7-fold. Concurrently, the expression of Mae was increased 1.6-fold in JHK24 in comparison with HR279 (Box (iii) in Fig. 2B).

Amino Acid and Nitrogen Metabolism

The expression of several key enzymes of amino acid and nitrogen metabolism were altered by the overexpression of GFP. Enzymes that exhibited a ±2.0-fold or more change in expression and their roles in amino acid metabolism are summarized in Fig. 2C and Table 2. In summary, the de novo synthesis of amino acids and the interconversions between amino acids were suppressed in JHK24 in comparison with HR279. The expression of pyrroline-5-carboxylate reductase (*proC*, gi: 15673866) that is the final step of proline biosynthesis from glutamate was reduced 8.2-fold in *L. lactis* JHK24. The amount of aspartate aminotransferase (*aspC*, gi:15672142), an enzyme that facilitates the interconversion of aspartate and alpha-ketoglutarate to oxaloacetate and glutamate, in JHK24 was 2.3-fold less than that in HR279. Serine hydroxymethyltransferase (SHMT, *glyA*, gi: 15672583) is another enzyme that catalyzes the conversion between two amino acids. Employing a methylenetetrahydrofolate (CH_3-THF) cofactor, this enzyme reversibly converts the glycine to serine. The expression level of SHMT in JHK24 was 3.7-fold lower than that in HR279.

Table 2. List of proteins for which the relative amount in JHK24 to HR279 was larger than ±2.0-fold.

gi[a]	Gene	Name	No. of total spectra		NSAF × 1,000 (Ave ± SD)[b]		Relative quantity (JHK24/HR279)
			JHK24	HR279	JHK24	HR279	Ave ± SD
Sugar and pyruvate metabolism							
15672041	*pdhD*	PDH E3 component	8	23	0.3 ± 0.2	1.0 ± 0.2	-3.0 ± 0.4
15672043	*pdhB*	PDHE1 component beta subunit	14	37	0.9 ± 0.4	2.3 ± 0.3	-2.7 ± 0.4
15672646	*pfl*	Pyruvate-formate lyase	55	22	1.4 ± 0.1	0.6 ± 0.2	2.5 ± 0.4
15672541	*gapA*	Glyceraldehyde 3-phosphate dehydrogenase	0	6		0.4 ± 0.2	Repressed
15673653	*arb*	Phospho-beta-glucosidase	2	8	0.1 ± 0.0	0.3 ± 0.2	-4.2 ± 0.6
15672882	*mleS*	Malolactic enzyme	25	66	0.9 ± 0.2	2.5 ± 0.8	-2.7 ± 0.4
15674137	*adhE*	Alcohol-acetaldehyde dehydrogenase	209	97	4.6 ± 0.3	2.2 ± 0.7	2.1 ± 0.3
15674202	*zwf*	Glucose-6-phosphate 1-dehydrogenase	3	10	0.1 ± 0.1	0.4 ± 0.2	-3.4 ± 0.5
15672604	*gnd*	6-Phosphogluconate dehydrogenase	13	42	0.5 ± 0.4	1.8 ± 0.8	-3.3 ± 0.5
Pyrimidine metabolism							
15672473	*pyrG*	CTP synthetase	10	5	0.4 ± 0.1	0.2 ± 0.1	2.0 ± 0.3
15672957	*nrdE*	Ribonucleotide-diphosphate reductase Ib α subunit	4	20	0.1 ± 0.0	0.6 ± 0.1	-5.2 ± 0.7
15672959	*nrdI*	Ribonucleotide-diphosphate reductase Ib gutaredoxin subunit	4	10	1.4 ± 0.6	3.5 ± 1.5	-2.5 ± 0.4
15673050	*pyrE*	Orotate phosphoribosyltransferase	21	10	2.0 ± 0.6	1.0 ± 0.1	2.0 ± 0.3
15673345	*carB*	Carbamoyl-phosphate synthase large subunit	27	15	0.5 ± 0.1	0.3 ± 0.2	1.8 ± 0.3
15674015	*arcC2*	Carbamate kinase	6	2	0.4 ± 0.2	0.1 ± 0.0	3.0 ± 0.4
Amino acid metabolism							
15672142	*aspC*	Aspartate aminotransferase	3	7	0.2 ± 0.0	0.3 ± 0.3	-2.3 ± 0.3
15672248	*ycgE*	*S*-Ribosylhomocysteinase	5	18	0.6 ± 0.3	2.3 ± 1.1	-3.7 ± 0.5
15672583	*glyA*	Serine hydroxymethyltransferase	2	7	0.1 ± 0.0	0.3 ± 0.1	-3.7 ± 0.5
15672975	*ribG*	Riboflavin-specific deaminase	3	9	0.2 ± 0.1	0.5 ± 0.4	-3.0 ± 0.4
15673077	*glyQ*	Glycyl-tRNA synthetase alpha subunit	8	3	0.5 ± 0.2	0.2 ± 0.1	2.6 ± 0.4
15673845	*ileS*	Isoleucyl-tRNA synthetase	3	8	0.1 ± 0.0	0.2 ± 0.1	-2.7 ± 0.4
15673866	*proC*	Pyrroline-5-carboxylate reductase	1	8	0.1 ± 0.0	0.6 ± 0.3	-8.2 ± 1.1
15673884	*metK*	*S*-Adenosylmethionine synthetase	15	41	0.7 ± 0.3	2.1 ± 0.5	-2.8 ± 0.4
Sugar nucleotide synthesis							
15672176	*rmlA*	Glucose-1-phosphate thymidylyltransferase	8	3	0.5 ± 0.3	0.2 ± 0.0	2.6 ± 0.4
15672180	*rmlB*	dTDP-glucose 4,6-dehydratase	5	11	0.3 ± 0.1	0.6 ± 0.3	-2.3 ± 0.3
Cell membrane and wall synthesis							
15672406	*femD*	Phosphoglucosamine mutase	10	22	0.4 ± 0.2	1.0 ± 0.2	-2.2 ± 0.3
15673865	*glmU*	UDP-*N*-acetylglucosamine pyrophosphorylase/ glucosamine-1-phosphate *N*-acetyltransferase	5	13	0.2 ± 0.2	0.6 ± 0.2	-2.7 ± 0.4
15672644	*fadD*	Long-chain acyl-CoA synthetase	11	4	0.4 ± 0.2	0.2 ± 0.0	2.8 ± 0.4
15672755	*fabD*	Acyl-carrier-protein *S*-malonyltransferase	15	5	1.0 ± 0.2	0.3 ± 0.1	3.0 ± 0.4
Energy generation							
15673745	*atpC*	ATP synthase subunit epsilon	6	2	0.7 ± 0.2	0.3 ± 0.0	2.4 ± 0.4
15673750	*atpF*	ATP synthase subunit B	19	10	2.3 ± 0.6	1.2 ± 0.5	1.9 ± 0.3
Transport							
15672395	*ptcB*	Cellobiose-specific PTS system IIB component	14	25	2.6 ± 0.7	4.7 ± 1.3	-1.8 ± 0.2
15672396	*ptcA*	Cellobiose-specific PTS system IIA component	9	4	1.5 ± 1.0	0.7 ± 0.3	2.2 ± 0.3
15672696	*yhcA*	ABC transporter ATP-binding and permease protein	11	2	0.3 ± 0.2	0.1 ± 0.0	5.6 ± 0.8
15673741	*glnP*	Glutamine ABC transporter permease	12	4	0.3 ± 0.1	0.1 ± 0.1	2.9 ± 0.4
15673742	*glnQ*	Glutamine ABC transporter ATP-binding protein	18	2	1.4 ± 0.6	0.2 ± 0.0	8.9 ± 1.3
15674083	*mscL*	Large-conductance mechanosensitive channel	6	2	1.0 ± 0.5	0.3 ± 0.0	2.9 ± 0.4

Table 2. Continued.

gi[a]	Gene	Name	No. of total spectra		NSAF × 1,000 (Ave ± SD)[b]		Relative quantity (JHK24/HR279)
			JHK24	HR279	JHK24	HR279	Ave ± SD
Hypothetical protein							
15672057	*yahB*	Hypothetical protein L1010	4	27	0.5 ± 0.0	3.5 ± 1.9	-6.9 ± 0.9
15672141	*ybgB*	Hypothetical protein L160937	6	2	0.2 ± 0.1	0.1 ± 0.0	3.0 ± 0.4
15672210	*yccJ*	Hypothetical protein L28204	7	2	1.2 ± 0.3	0.3 ± 0.0	3.5 ± 0.5
15672602	*ygaI*	Hypothetical protein L6768	2	8	0.3 ± 0.0	1.2 ± 0.2	-4.0 ± 0.6
15672603	*ygaJ*	Hypothetical protein L7226	19	44	2.0 ± 0.4	4.8 ± 0.8	-2.4 ± 0.3
15672929	*yjhD*	Hypothetical protein L172471	18	7	0.8 ± 0.1	0.3 ± 0.2	2.5 ± 0.4
15673199	*ymdE*	Hypothetical protein L38177	7	1	0.4 ± 0.4	0.1 ± 0.0	6.9 ± 1.0
15673717	*yrjD*	Hypothetical protein L176579	0	7	0.0	0.6 ± 0.4	Repressed
15673815	*ytaA*	Hypothetical protein L84477	7	22	1.0 ± 0.7	3.1 ± 1.5	-3.2 ± 0.4
15673872	*ytgH*	Hypothetical protein L142733	1	29	0.1 ± 0.0	3.5 ± 0.4	-29.0 ± 4.0
15674136	*ywcC*	Hypothetical protein L11851	7	23	0.4 ± 0.1	1.4 ± 0.7	-3.3 ± 0.5
30024037	*yniH*	Hypothetical protein L186490	3	7	0.1 ± 0.1	0.4 ± 0.1	-2.4 ± 0.3
Protease							
15673507	*clpB*	ClpB protein	108	29	2.5 ± 0.3	0.7 ± 0.1	3.6 ± 0.5
15672624	*clpC*	ATP-dependent protease ATP-binding subunit	12	4	0.3 ± 0.1	0.1 ± 0.0	3.0 ± 0.4
15672539	*clpE*	ATP-dependent protease ATP-binding subunit	79	5	2.1 ± 0.3	0.1 ± 0.0	15.4 ± 2.3
15672655	*clpP*	ATP-dependent Clp protease proteolytic subunit	66	15	6.6 ± 0.3	1.6 ± 0.6	4.2 ± 0.6
15672542	*def*	Peptide deformylase	5	12	0.5 ± 0.2	1.2 ± 0.5	-2.5 ± 0.3
15672594	*pepM*	Methionine aminopeptidase	4	12	0.3 ± 0.3	0.8 ± 0.6	-3.0 ± 0.4
Protein synthesis							
15672375	*groES*	GroES	27	18	5.7 ± 1.4	3.9 ± 1.0	1.5 0.2
15672376	*groEL*	GroEL	141	74	5.2 ± 0.6	2.8 ± 0.6	1.9 ± 0.3
15672875	*ppiB*	Peptidyl-prolyl *cis-trans* isomerase	25	12	2.5 ± 0.2	1.2 ± 0.8	2.1 ± 0.3
15672922	*trxB1*	Thioredoxin reductase	18	7	1.2 ± 0.7	0.5 ± 0.3	2.6 ± 0.4
15672935	*grpE*	GrpE	38	21	4.2 ± 0.8	2.4 ± 0.9	1.8 ± 0.3
15674086	*nusG*	Transcription antitermination protein NusG	20	10	2.1 ± 0.1	1.1 ± 0.5	2.0 ± 0.3
15674206	*dnaJ*	DnaJ	22	7	1.2 ± 0.3	0.4 ± 0.1	3.1 ± 0.5
pJH24 related							
		pJH24_GFP	971	264	65.7 ± 4.9	18.4 ± 2.5	3.6 ± 0.5
		pJH24-ErmC	92	3	7.5 ± 0.4	0.1 ± 0.2	74.6 ± 4.4
		pJH24-NisR	42	0	3.9 ± 0.6		Induced
		pJH24-NisK	8	0	0.4 ± 0.1		Induced

Proteins noted as "induced" indicate a protein observed only in the whole proteome of *L. lactis* JHK24 but not in HR279. "Repressed" indicates the protein only observed in *L. lactis* HR279.

[a]Gene identification numbers as per NCBI.

[b]The average and standard deviation of the normalized spectral abundance factor. Experiments were performed in triplicates.

Methionine is an important amino acid for translation-initiation of most proteins and, in addition, plays a key role as a methyl group donor molecule (Fig. 2C). *S*-Adenosylmethionine synthase (*metK*, gi: 15673884) that catalyzes the conversion of methionine to *S*-adenosylmethionine reduced its expression level by 2.8-fold in JHK24. The

expression of *S*-ribosylhomocysteinase (*ycgE*, gi: 15672248), which cleaves the *S*-ribosylhomocysteine to an L-homocysteine and a 4,5-dihydroxypentan-2,3-dione, was decreased 3.7-fold as well. Interestingly the by-product of this reaction, 4,5-dihydroxypentan-2,3-dione, spontaneously cyclizes and combines with borate to form an autoinducer (AI-2) that is used for quorum sensing [33]. Finally, cystathionine beta-lyase, which cleaves cystathionine to homoserine and pyruvate, with NH_3 as a by-product, slightly increased its expression to 1.5-fold in *L. lactis* JHK24.

Pyrimidine Metabolism

A portion of the nucleotide synthetic pathway in *L. lactis* was impacted by the increase in GFP expression in JHK24. As shown in Fig. 2D, the expression level of enzymes involved in pyrimidine synthesis, in particular cytidine triphosphate (CTP) and uridine triphosphate (UTP), increased. A key precursor in pyrimidine synthesis is a carbamoyl phosphate, and two routes of synthesis of carbamoyl phosphate are predicted in *L. lactis* IL1403. Carbamate kinase (*arcC2*, gi: 15674015) catalyzes the synthesis of a carbamoyl phosphate from NH_3 and CO_2, and carbamoyl phosphate synthase (*carB*, gi: 15673345) transfers the NH_3 from a glutamine to CO_2 to make carbamoyl phosphate. The relative amount of these enzymes in *L. lactis* JHK24 increased 3.0- and 1.8-fold compared with those in HR279, respectively. The carbamoyl phosphate then converts to orotate with addition of aspartic acid and further to orotidine monophosphate (OMP). The orotate phosphoribosyltransferase (OPRT, *pyrE*, gi: 15673050) that facilitates the conversion of orotate to OMP increased its expression in JHK24 by 2.0-fold compared with that in HR279.

After elimination of CO_2, OMP is converted to UMP and then further to UTP. The expression level of the CTP synthase (*pyrG*, gi: 15672473) that converts UTP to CTP was increased 2.0-fold in *L. lactis* JHK24. Whereas the expression of enzymes involved in synthesis of pyrimidine RNA monomers, UTP and CTP, was increased, the enzymes involved in the synthesis of pyrimidine DNA monomers, dTTP and dCTP, were decreased. In *L. lactis*, uridine diphosphate (UDP) is converted to deoxyuridine diphosphate (dUDP) by ribonucleotide diphosphate reductase. The alpha subunit of ribonucleotide diphosphate reductase Ib (*nrdE*, gi: 15672957) and its glutaredoxin subunit (*nrdI*, gi: 15672959) exhibited a 5.2- and 2.5-fold reduction in expression, respectively, in *L. lactis* JHK24 in comparison with HR279.

Fatty Acid Synthesis

Two enzymes for lipid biosynthesis were overexpressed in *L. lactis* JHK24. The relative amounts of long-chain acyl-CoA synthetase (*fadD*, gi: 15672644) and the acyl-carrier-protein *S*-malonyltransferase (*fabD*, gi: 15672755) in *L. lactis* JHK24 were 2.8- and 3.0-fold of those in *L. lactis* HR279, respectively (Table 2).

Transport Systems

Membrane-bound proteins (including transporters) were not readily identified using shotgun proteomics owing to the low recovery in the soluble fraction. However, it was clear from the shotgun proteomic data that the expression of glutamine transporter was increased in JHK24 when compared with HR279 (Table 2). The relative amount of the glutamine ABC-type permease (*glnP*, gi: 15673741) and its ATP-binding protein (*glnQ*, gi: 15673742) in JHK24 were 2.9- and 9.0-fold higher (respectively) than in HR279.

Table 3. ATP synthase complex proteins observed in the *L. lactis* JHK24 and HR279 proteomes.

gi[a]	Gene	Name	NSAF × 1,000 (Ave ± SD)		Relative quantity (JHK24/HR279)
			JHK24	HR279	AVE ± SD
15673745	*atpC*	ATP synthase subunit epsilon	0.7 ± 0.2	0.3 ± 0.0	2.4 ± 0.4
15673746	*atpD*	ATP synthase subunit beta	3.3 ± 0.5	2.5 ± 0.4	1.3 ± 0.2
15673747	*atpG*	ATP synthase subunit gamma	1.0 ± 0.8	0.8 ± 1.0	1.2 ± 1.1
15673748	*atpA*	ATP synthase subunit alpha	3.1 ± 0.4	2.2 ± 0.6	1.4 ± 0.2
15673749	*atpH*	ATPsynthase subunit delta	0.6 ± 0.2	0.3 ± 0.2	1.7 ± 0.2
15673750	*atpF*	ATP synthase subunit B	2.3 ± 0.6	1.2 ± 0.5	2.0 ± 0.3
30024053	*atpB*	ATP synthase subunit A		ND[b]	
15674249	*atpE*	ATP synthase subunit C		ND[b]	

ATP synthase subunits A and C were not detected owing to their location in the membrane.

[a]Gene identification numbers by NCBI.

[b]Not detected.

Table 4. Proteases observed in the *L. lactis* JHK24 and HR279 proteomes.

gi	Gene	Name	NSAF × 1,000 (Ave ± SD)		Relative quantity (JHK24/HR279)
			JHK24	HR279	Ave ± SD
15672655	*clpP*	ATP-dependent Clp protease proteolytic subunit	6.6 ± 0.3	1.6 ± 0.6	4.2 ± 0.6
15673507	*clpB*	ClpB protein	2.5 ± 0.3	0.7 ± 0.1	3.6 ± 0.5
15672624	*clpC*	ATP-dependent protease ATP-binding subunit	0.3 ± 0.1	0.1 ± 0.1	3.0 ± 0.4
15672539	*clpE*	ATP-dependent protease ATP-binding subunit	2.1 ± 0.3	0.1 ± 0.0	15.4 ± 2.3
15672594	*pepM*	Methionine aminopeptidase	0.3 ± 0.3	0.8 ± 0.6	-3.0 ± 0.4
15673540	*pepDB*	Dipeptidase	0.5 ± 0.2	0.9 ± 0.1	-1.9 ± 0.3
15672287	*pepN*	Aminopeptidase N	0.5 ± 0.3	1.0 ± 0.3	-1.8 ± 0.2
15674031	*pepXP*	X-prolyl-dipeptidyl aminopeptidase	0.4 ± 0.1	0.7 ± 0.1	-1.7 ± 0.2
15672824	*pepV*	Dipeptidase	1.4 ± 0.2	1.9 ± 0.4	-1.4 ± 0.2
15673785	*pepO*	Neutral endopeptidase	0.2 ± 0.1	0.3 ± 0.1	-1.2 ± 0.2
15673792	*pepT*	Peptidase T	1.4 ± 0.2	1.5 ± 0.4	-1.1 ± 0.2
15673631	*pepQ*	Proline dipeptidase	0.6 ± 0.4	0.6 ± 0.2	-1.0 ± 0.2
15673860	*pepC*	Aminopeptidase C	1.8 ± 0.2	1.7 ± 0.4	1.1 ± 0.2

F_1F_O ATP Synthase

An increase in ATP synthase expression was observed in *L. lactis* JHK24 (Table 3). The relative amounts of ATP synthase B and epsilon subunits in *L. lactis* JHK24 were 2.0-fold higher than in HR279. Additional subunits of ATP synthase were expressed more in JHK24 as well. The alpha (*atpA*, gi: 15673748), beta (*atpD*, gi: 15673746), and delta (*atpH*, gi: 15673749) subunits increased to 1.4-, 1.3-, and 1.7-fold, respectively. The subunits A and C (F0 portion) could not be identified possibly owing to their localization at the cell membrane. Interestingly, the stoichiometric ratios between subunits indirectly suggest the accuracy of proteomic analysis. The F1 portion of ATP constitutes alpha, beta, and gamma subunits with the α3β3γ structure, and the corresponding concentration ratios of alpha and beta subunits were close to 1, whereas alpha (or beta) and gamma subunits were 5 to 6 (in either JHK24 or HR279 strains).

Hypothetical Proteins

The expression of 34 hypothetical proteins was identified in both *L. lactis* JHK24 and HR279, of which 12 hypothetical proteins showed significant change in their relative expression levels (in the range of -29.0- to +6.9-fold). As shown in Table 2, the expression of most hypothetical proteins was repressed in *L. lactis* JHK24 in comparison with HR279. In particular, the relative amounts of hypothetical proteins L1010 and L142733, which were highly expressed in HR279, were reduced 6.9- and 29.0-fold, respectively, in JHK24.

Proteases

Expression of proteases in *L. lactis* changed dramatically with higher heterologous protein expression. The expression levels of methionine aminopeptidase (*pepM*, gi: 15672594) and peptide deformylase (*def*, gi: 15672542), both involved in the stability of the N-terminal end of proteins, were reduced in *L. lactis* JHK24. The relative amounts of these two enzymes in the JHK24 proteome were 41% (-2.5-fold) and 38% (-3.0-fold) of those in HR279. However, the expression of caseinolytic peptidase (Clp) family increased in *L. lactis* JHK24 noticeably. ClpP, the proteolytic domain of Clp protease (*clpP*, gi: 15672655), was expressed 3.6-fold more in JHK24 than in HR279. Three of the ATPase subunits of the Clp protease also increased in expression in JHK24. The relative amount of ClpB (*clpb*, gi: 15673507), ClpC (*clpc*, gi: 15672624), and ClpE (*clpE*, gi: 15672539) in JHK24 were 4.2-fold, 3.0-fold, and 15.4-fold (respectively) of the amounts observed in HR279. Expression of ClpX (*clpX*, gi: 15673133) was not identified by MS analysis.

Including the Clp family, a total of 13 proteases and peptidases were identified both in *L. lactis* JHK24 and HR279 (Table 4). When GFP expression was low, proteases and peptidases were expressed in a similar and low level, exhibiting an NSAF range (×1,000) between 1.0 and 1.9. However with the increased expression of GFP the expression of caseinolytic proteases increased significantly, whereas other peptidases were maintained at the same level or slightly decreased their expression. For example,

the expression level of ClpP was 5~6 times higher than other peptidases in *L. lactis* JHK24.

Protein Synthesis and Stress Response

The expression of chaperone proteins and stress response proteins was influenced by the overexpression of GFP in *L. lactis* (Table 2). The relative amounts of GroEL (*groEL*, gi: 15672375), GroES (*groES*, gi: 15672376), and thioredoxin reductase (*trxB1*, gi: 15672922), which are involved in protein synthesis and stabilization, increased 1.9-, 1.5-, and 2.6-fold in *L. lactis* JHK24 in comparison with HR279. The expression of transcription antiterminator protein NusG (*nusG*, gi: 15674086) increased in JHK24 as well (2.0-fold). The stress response proteins of GrpE (*grpE*, gi: 15672935) and DnaJ (*dnaJ*, gi: 15674206) were induced in *L. lactis* JHK24 by 1.8- and 3.1-fold (respectively) over the same proteins in HR279. Another stress response protein, the cold shock protein E (*cspE*, gi: 15672150), was highly expressed in both *L. lactis* JHK24 and HR279 (Table 1), being the 1st and the 2nd most expressed protein of *L. lactis* HR279 and JHK24, respectively.

Discussion

The genomics and physiology of LAB have been extensively studied, primarily due to their prominent role in food and beverage fermentations. However, more recently, LAB has been recognized as an attractive model for bacterial-borne delivery of therapeutic proteins to the human GIT. Although numerous studies have examined various expression constructs for protein overproduction in LAB, relatively little work has been done to examine the LAB host response to protein overproduction—a critical step in the rational design of superior production hosts.

The heterologous protein expression systems used in this study were identical twins but differed in plasmid copy number [10]. GFP expression vector pHR082 and its high-copy variant of pJH24 were derived from pIL252 and pIL253, respectively, both of which commonly have the pAMβ1 replicon. Although the pAMβ1 replicon is stable and widely used in LAB, the copy number of its derivatives are low for an industrial protein production, showing 6−9 copies of pIL252 per cell [34, 35]. Plasmid pIL253 was developed to increase the copy number by disrupting the copy control region of cognate WT plasmid pIL252. Despite the ~10-fold increase (45−85 copies), the copy number of pIL253 was not significantly high when compared with *E. coli* vectors that show up to ~1,300 copies per cell [36].

The overexpression of GFP clearly has an impact on *L. lactis* physiology. Since P_{nisA} is strong promoter, the amount of GFP in the low-copy expression host HR279 was already significant, reaching up to 1.8% of the total proteome, which made the GFP the 9th most abundant protein. The amount of expressed GFP in the high-copy expression host JHK24 increased 4.0-fold to occupy 6.6% of the total proteome (Table 1), making GFP the most abundant protein in the JHK24 proteome. Considering only 18 proteins were witnessed to be expressed more than 1% of the whole proteome, this increase in GFP expression is significant enough to impact the metabolic activity of *L. lactis*.

Except for the plasmid copy number, *L. lactis* JHK24 and HR279 shared the same conditions during the cell growth and GFP production. Both pJH24 and pHR082 [14] used the same concentration of erythromycin and nisin as a selective agent and an inducer to drive expression of GFP. Thus, both *L. lactis* JHK24 and HR279, which contained pJH24 and pHR082, respectively, received the same level of erythromycin and nisin (a bacteriocin) stress during these comparisons. Therefore, changes occurring in *L. lactis* JHK24 compared with HR279 were solely due to the consequences of increased expression of GFP and possibly by increasing the copy number of the expression vector.

Several changes were noticed in the protein expression of *L. lactis* JHK24 in comparison with HR279. First, the expression of stress response proteins and ATP synthase increased. Cold shock protein E, GroEL, GroES, DnaK, DnaJ, and GrpE were highly expressed in both *L. lactis* JHK24 and HR279, but expression in *L. lactis* JHK24 was higher than in HR279. The expression of ATP synthase subunits also exhibited approximately 2.0-fold increase in JHK24. Since both cells received the same level of erythromycin and nisin, the additional induction of stress response proteins and ATP synthase was likely the result of overexpression of GFP in JHK24 and/or stress derived from the higher plasmid maintenance costs [37]. Changes in the expression pattern of stress response protein can be linked more to the increase of protein stability. GroEL and ES, involved in maintenance of proper protein folding, are upregulated in JHK24. Thioredoxin reductase and peptidyl-prolyl *cis-trans* isomerase, which can modulate the tertiary structure of proteins, increased their expression as well. In addition, both peptide deformylase and methionine aminopeptidase, which hydrolyzes the *N*-terminal end of proteins, were downregulated in JHK24.

The protein expression for the synthesis of pyrimidine ribonucleotides was altered in JHK24. Two carbamate synthesizing pathways, orate kinase and CTP synthase, were upregulated, which likely increased the flux toward

UTP and CTP synthesis. Another interesting change was the decrease in enzymes of the deoxynucleotide synthesis pathway. Expression of the ribonucleotide-diphosphate reductase complex, which converts nucleotides to deoxynucleotides (*i.e.*, UDP to dUDP), was decreased. These results suggest that the synthetic flux for pyrimidine nucleotides toward RNA, which is required for the protein expression, was impacted by protein overexpression. Interestingly, the synthetic pathway for the purine-type nucleotide was not changed significantly.

Several changes were observed in a part of glucose metabolism that regulates the cellular redox balance toward less converting NAD^+/$NADP^+$ to NADH/NADPH (Fig. 2B). *L. lactis* regenerates $NADP^+$ to NADPH via the oxidative pentose phosphate pathway during growth on glucose. A reduction of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase may cause the reduction of NADPH regeneration and the production of pentose phosphate. *L. lactis* also regulates the redox balance of the cell through the fermentation of pyruvate. The primary flux of pyruvate from glucose is converted to lactate by LDH and with the consumption of NADH. The expression level of LDH was similar between *L. lactis* JHK24 and HR279, suggesting this main flux did not change. Instead, pyruvate-formate lyase (Pfl), which splits pyruvate to formate and acetyl-CoA without the use of NAD^+, increased in expression 2.7-fold in *L. lactis* JHK24. Concurrently, expression of the pyruvate dehydrogenase complex, which converts pyruvate to acetyl-CoA with the production of CO_2 and NADH, was reduced more than 2.5-fold. In addition, the alcohol-aldehyde dehydrogenase (AdhE) enzyme, which converts acetyl-CoA to ethanol with the conversion of NADH to NAD^+, was increased to 2.3-fold, whereas acetate kinase, which converts acetyl-CoA to acetate, was consistently expressed in both strains. Overall the pyruvate fermentation pathway appeared to be modulated in the high expression strain for the regeneration of NADH to NAD^+ by the upregulation of Pfl and AdE, and downregulation of Pdh.

Notable changes were observed in the synthesis of amino sugars and sugar nucleotides. The expression levels of key enzymes for the *N*-acetylglucosamine (GlcNAc) and dTDP-rhamnose synthesis were decreased in *L. lactis* JHK24 (Fig. 2A). Since GlcNAc and rhamnose are involved in the cell wall structure, a cell surface polysaccharide in particular, the overexpression of heterologous protein by increasing the copy number may impact on the cell surface properties of *L. lactis* [38].

The most remarkable changes in *L. lactis* JHK24 were the protein folding and the protein hydrolysis systems, likely linked to the overexpression of heterologous protein encoded in pJH24. GroES, GroEL, GrpE, DnaJ, PpiB (peptidyl-prolyl *cis-trans* isomerase), TrxB (thioredoxin reductase), and NusG (transcription antitermination protein) are the enzymes that are involved in the protein folding and stabilization. In *L. lactis* JHK24, expression of these proteins was increased suggesting *L. lactis* has an increased need for protein stabilization concurrent with the increase of the GFP expression. JHK24 exhibited a significant increase in expression of the caseinolytic peptidase (Clp) family as well (Tables 2 and 4). The Clp family proteins are regulated by the global stress response regulator, CtsR, and are involved in the proteolysis of misfolded protein [39]. Increasing the proteolytic activity with the overexpression of heterologous protein suggests that *L. lactis* JHK24 is degrading overexpressed GFP. However, a recent attempt to increase the expression of GFP by suppression of ClpP expression using an antisense approach resulted in the significant reduction of both cell growth rate and GFP production [40]. This suggests that the Clp proteins not only regulate the protein level in the cytosol but also play a critical role in overall cell maintenance of JHK24 when heterologous proteins are overexpressed. For example, the increase of intracellular proteolysis by Clp family proteases in JHK24 may serve to provide enough amino acid by hydrolysis of peptone. Despite the overexpression of GFP, *L. lactis* JHK24 and HR279 exhibited a similar cell growth rate, suggesting that both *L. lactis* strains were provided sufficient energy and cell building blocks, in particular amino acids. Contrary to this growth profile, the expression of several key enzymes for de novo synthesis or the interconversions of amino acids was reduced in *L. lactis* JHK24. Based on these observations, it can be speculated that the demand for amino acids caused by the overexpression of heterologous protein in *L. lactis* JHK24 was satisfied by the hydrolysis of extracellular peptone by Clp proteins instead of their de novo synthesis. Therefore, the knockout of Clp protein limits the amino acid supplement, resulting in disturbing the cell growth and protein production.

In this paper, we investigated the impact of protein overexpression on the proteome profiles in *L. lactis* IL1403. Examining protein expression using identical plasmids with different copy numbers allowed for a clear assessment of metabolism caused by an increase in foreign protein expression only. The protein expression profiling by LC-MS/MS and data-driven protein identification gave a global picture of protein expression of *L. lactis*, and label-free quantification enabled comparison of quantitative

protein expression between high and low GFP-expressing strains. From this systematic approach, we found several changes in the global profile of the *L. lactis* proteome, such as in protein synthesis/stabilization and degradation. The information we observed here is profound and systematic but not as segregated as data on individual proteins or genes. These observations can give a sketch of the metabolic regulation of *L. lactis* IL1403 and also be used for metabolic and genetic engineering to increase protein production.

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