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ARTICLE

Quantitative Proteogenomics and the Reconstruction of the Metabolic Pathway in *Lactobacillus mucosae* LM1

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Abstract

Lactobacillus mucosae is a natural resident of the gastrointestinal tract of humans and animals and a potential probiotic bacterium. To understand the global protein expression profile and metabolic features of *L. mucosae* LM1 in the early stationary phase, the QExactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer was used. Characterization of the intracellular proteome identified 842 proteins, accounting for approximately 35% of the 2,404 protein-coding sequences in the complete genome of *L. mucosae* LM1. Proteome quantification using QExactive™ Orbitrap MS detected 19 highly abundant proteins (> 1.0% of the intracellular proteome), including CysK (cysteine synthase, 5.41%) and EF-Tu (elongation factor Tu, 4.91%), which are involved in cell survival against environmental stresses. Metabolic pathway annotation of LM1 proteome using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database showed that half of the proteins expressed are important for basic metabolic and biosynthetic processes, and the other half might be structurally important or involved in basic cellular processes. In addition, glycogen biosynthesis was activated in the early stationary phase, which is important for energy storage and maintenance. The proteogenomic data presented in this study provide a suitable reference to understand the protein expression pattern of lactobacilli in standard conditions.

Keywords: *Lactobacillus mucosae*, QExactive^{TM} Orbitrap, mass spectrometry, proteome, metabolic pathway

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Introduction

Lactobacillus mucosae is a *Lactobacillus* species recently discovered to be a natural resident of the human and animal gastrointestinal tract. *L. mucosae* is a Gram-positive, heterofermentative bacterium found on the mucosal surfaces of pigs, cows and humans (Bleckwedel *et al*., 2014; Lee *et al*., 2012; London *et al*., 2014; Roos *et al*., 2000). In addition, *L. mucosae* is a potential probiotic bacterium, with efficient competitive exclusion activity against pathogens *in vitro* (Valeriano *et al*., 2014), and specificity for blood group antigen receptors (Watanabe *et al*., 2010). Furthermore, some strains of *L. mucosae* are able to produce mannanase and exopolysaccharides (Bleckwedel *et al*., 2014; London *et al*., 2014), which are desirable for industrial applications. Currently, three draft genomes of *L. mucosae* are available, containing operons comprising putative genes associated with adhesion (Lee *et al*., 2012). However, the mechanisms of *L. mucosae* adaptation, colonization and survival under various environmental conditions have not yet been delineated.

Several studies have been conducted using genomic and transcriptomic techniques, as well as 2-D and labeled proteomic analysis, to assess the physiological and functional characteristics of lactobacilli under different environmental conditions, including bile, oxidative, starvation and acid stresses, revealing important insights to their potential as probiotic bacteria (Al-Naseri *et al*., 2013; Heunis *et al*., 2014; Lee *et al*., 2013; Lee *et al*., 2015). However, the stand-alone data generated by each of these studies is often limited (Kucharova and Wiker, 2014).

In this study, the high-throughput QExactive™ Orbitrap MS was used in tandem with ultra high-pressure liquid chromatography (UHPLC) to analyze and quantify the intracellular proteome of *L. mucosae* LM1 in the early stationary phase. We also cross-referenced high-quality proteome data with the complete genome of *L. mucosae* LM1, to confirm gene expression through detection of

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corresponding gene products. In addition, bioinformatics analysis was employed to identify novel biosynthetic gene clusters in *L. mucosae* LM1.

Materials and Methods

Bacterial strain, growth conditions, and microscopic observation

The *Lactobacillus mucosae* strain LM1 used in this experiment was isolated from the intestine of a piglet (Lee *et al*., 2012). The strain is a non-spore-forming, facultative anaerobic Gram-positive rod. Optimal growth was observed at 30-37°C on de Man, Rogosa and Sharpe (MRS) medium (Difco Laboratories, USA) (Valeriano *et al*., 2014). Bacterial cells were grown at 37°C into the early stationary phase, and harvested by centrifugation, and only one out of seven biological replicates was used as a representative for microscopic observation. After centrifugation, the pH of the cell-free supernatants of each replicate were measured. Cells were washed with TE buffer [50 mM Tris-Cl (pH 7.8)], and then fixed with 8% (v/v) paraformaldehyde and 3% (v/v) glutaraldehyde in 0.1 N sodium cacodylate buffer (pH 8.0) overnight. The cell sample was coated with gold after washing and dehydration, according to established methods (Jan *et al*., 2001). Cells were viewed with a scanning electron microscope (XL30CP; Philips, Netherlands).

Preparation of the intracellular proteome

To prepare the intracellular proteome of *L. mucosae* strain LM1, an overnight cell culture was inoculated into 200 mL MRS broth and grown at 37°C into the early stationary phase, as indicated by achieving an optical density (OD) at 600 nm of approximately 1.3 units. At this growth phase, the viable cell count was approximately $1.0\text{-}1.5\times10^8$ colony-forming units (CFU)/mL. The cells were harvested by centrifugation at 10,000 *g* for 10 min at 4°C, then washed with phosphate-buffered saline (PBS) buffer (pH 7.0) and re-dissolved in lysis buffer (10 mM Tris-EDTA and 0.5% Triton X-100, pH 7.8) supplemented with Pierce' Protease Inhibitor (Thermo Fisher Scientific, USA). Cells were disrupted by sonication using a Vibra-Cell™ VCX 750 (Sonics & Materials, Inc. USA) at 35% amplitude for 20 min (at 2 s pulse on/ 15 s pulse off). Seven biological replicates (cell culture) were prepared for sonication, protein extraction and proteome analysis. After sonication, crude lysates were cleared by centrifugation at 10,000 *g* for 20 min at 4°C. Protein concentrations were determined by Bradford protein assay (Bio-Rad, USA).

Ultra-high-pressure Liquid Chromatography and QExactive™ Orbitrap mass spectrometry

In-solution digestion and sample preparation were performed as described previously (Wiśniewski *et al*., 2009). Peptides were separated using an UHPLC Dionex UltiMate® 3000 (Thermo Fisher Scientific, USA) instrument. Tryptic digests were separated by reversed-phase chromatography. Fractions were reconstituted in solvent A (water/acetonitrile, 98:2 v/v; 0.1% formic acid). The UHPLC was equipped with a Acclaim PepMap 100 trap column (100 μm × 2 cm, nanoViper C_{18}, 5 μm, 100 Å) to trap the sample, which was subsequently washed with 98% solvent A for 6 min at a flow rate of 6 μL/min, and then continuously separated on a Acclaim PepMap 100 capillary column (75 μm × 15 cm, nanoViper C18, 3 μm, 100 Å) at a flow rate of 400 nL/min. The LC analytical gradient was run at 2% to 35% solvent B over 90 min, then 35% to 95% over 10 min, followed by 90% solvent B for 5 min, and finally 5% solvent B for 15 min. Resulting peptides were electro-sprayed through a coated silica tip (PicoTip emitter, New Objective, USA) at an ion spray voltage of 2,000 eV.

The UHPLC was coupled with a heated electrospray ionization source (HESI-II) to the quadrupole-based mass spectrometer QExactive™ Orbitrap High Resolution Mass Spectrometer (Thermo Fisher Scientific, USA). The MS spectra were acquired at a resolution of 70,000 (200 m/z) in a mass range of 350-1,800 m/z. A maximum injection time was set to 100 ms for ion accumulation. Eluted samples were used for MS/MS events, measured in a data-dependent mode for the 10 most abundant peaks (Top10 method), in the high mass accuracy Orbitrap after ion activation/dissociation with Higher Energy C-trap Dissociation (HCD) at 27 collision energy in a 100-1650 m/z mass range.

Data processing and analysis

All raw proteomic data from seven biological replicates were cross-referenced against the *L. mucosae* LM1 genome database in NCBI (Accession Number SAMN02470226) and UniProt (taxon identifier 1130798). The false discovery rate (FDR) of all peptide identifications was set at less than 1%. All proteins with at least two unique peptides were taken into consideration. All UniProt identifiers of the *L. mucosae* LM1 dataset were used, and the results were acquired to extract the best hits for proteome analysis.

To quantify the proteins, label-free approach based on the spectra counts was used. All spectra of modified peptides after the full data scan were processed in Xcalibur Qual Browser curated by Protein Discoverer software (version 1.4). Each row contains the GenBank and UniProt ID, functional property, amino acid sequence and protein intensity level. For Gene Ontology enrichment analysis, an *e*-value of 0.0001 was used as a cutoff. Genome and proteome data were sorted according to the annotation in the cluster of orthologous groups (COG) and the count of proteins per functional category were visualized as a bar graph. The percent abundance of each identified protein was calculated from the total count of proteins in the proteome of each replicate sample. Proteins were arranged from most to least abundant, and a graphical output was created using a scatter plot method. For pathway analysis, the complete genome sequence (.fasta) file was uploaded to the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/) Automatic Annotation Server (KAAS) for full annotation of functional genes by BLAST or GHOST comparisons, parallel to the manually curated KEGG GENES database. The bi-directional best-hit (BBH) method was used to assign orthologs. The result contains KEGG Orthology (KO) assignments and automatically generated KEGG pathways. An overview of reconstructed KEGG pathways is shown as superimposed schematic diagrams. The specific metabolic and biosynthetic pathways that were activated in *L. mucosae* LM1 were further illustrated using KO assignments based on the curated proteome data.

Results and Discussion

A recent study reported the probiotic potential of *L. mucosae* LM1, in the context of its adhesion ability, carbohydrate specificity, antimicrobial activity and pH and bile tolerance (Valeriano *et al*., 2014). The study described here included high-resolution and -throughput quantification of cytosolic proteins produced by *L. mucosae* LM1, in order to identify the species- or strain-specific factors for adaptation, and delineate important metabolic pathways involved in the potential probiotic mechanisms of *L. mucosae* LM1. The cells were grown in MRS medium to determine its growth rate until the early stationary phase. Because the standard growth medium, MRS broth, was used for the cultivation of lactobacilli, these results might be used as a reference for future comparative studies and/or proteomic analysis under different conditions. Furthermore, the bacterial stationary phase provides the maximum number of bacterial proteins, compared with other growth phases (Cohen *et al*., 2006; Hussain *et al*., 2009). In addition, the microscopic image of *L. mucosae* LM1 cells suggests that cell morphology remains intact and undamaged in its early stationary phase, even at highly acidic conditions (pH=4.31±0.04). In this study, UHPLC, together with a QExactive™ Orbitrap MS instrument was used for the characterization of all functional proteins in the early stationary phase. Quantification of differentially abundant proteins enabled the identification of important survival mechanisms and activated metabolic pathways in *L. mucosae* LM1. These data contribute towards a better understanding of the potential of *L. mucosae* LM1 as a probiotic supplement for humans and other animals.

Genomic comparison and proteome profile of *L. mucosae* LM1

The genomic data of *L. mucosae* LM1 contain 2,288 and 116 (2,404 total) protein-encoding genes in chromosomal (2.3 Mb) and plasmid (0.1 Mb) DNA, respectively (Lee *et al*., 2012). In addition, 24 rRNA genes, 92 tRNA genes, and 1,464 functional genes were detected in the chromosomal DNA. In our study, 842 proteins were identified from the intracellular proteome of *L. mucosae* LM1, using the UHPLC-MS/MS method, accounting for 35% coverage (842 proteins out of 2,404 genes) of the proteome. This is significantly greater than the number of proteins detected using 1- and 2-DE gel electrophoresis in previous studies of other lactobacilli (Cohen *et al*., 2006; Hussain *et al*., 2009; Koistinen *et al*., 2007), which identified less than 25% of the total proteome. In addition, the number of proteins detected in the stationary phase of *L. mucosae* LM1 was higher than other lactobacilli in the same growth phase (Cohen *et al*., 2006; Hussain *et al*., 2009). This demonstrates that the QExactive™ Orbitrap MS is an efficient instrument for the accurate quantification and identification of both high- and low-molecular-weight cytosolic proteins in bacterial proteomic studies (Krauss *et al*., 2010).

The LM1 proteome comprises 668 functional and 174 hypothetical proteins (data not shown). Genes and proteins were categorized based on the cluster of orthologous groups (COG). In the complete genome of *L. mucosae* LM1, many genes for general functions, including replication, recombination and repair, amino acid transport and metabolism were detected (Fig. 1). On the other hand, the LM1 proteome was abundant with enzymes for protein synthesis (translation, ribosomal structure and biogenesis) (Fig. 1). These results imply that cells in this phase

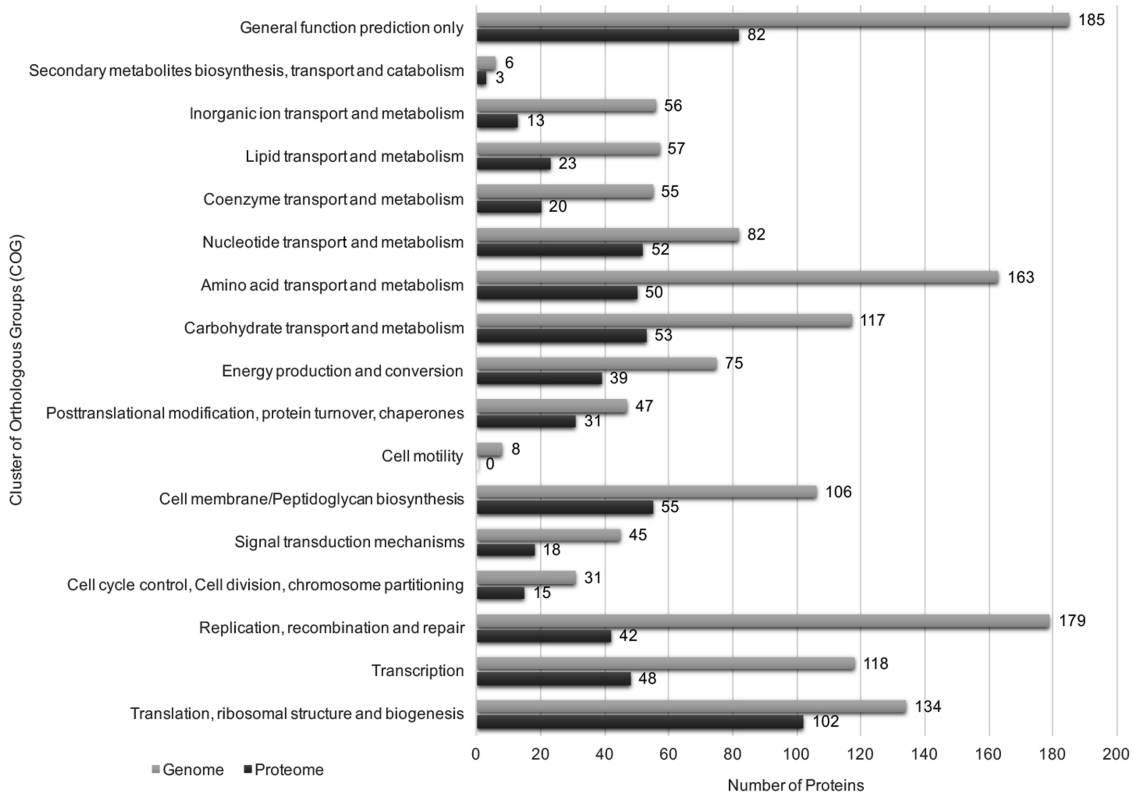

Fig. 1. Functional categories of protein-encoding genes of *L. mucosae* LM1. Genes and proteins detected in the genome (gray) and proteome (black) were identified and grouped according to the cluster of orthologous groups (COG), respectively.

focus primarily on regeneration of damaged intracellular components and maintenance of ribosomal integrity, since bacterial cells encounter acidic conditions (pH=4.31±0.04) and growth is diminished in the stationary phase (Lee *et al*., 2013). In addition, proteins that are involved in the synthesis and repair of the peptidoglycan layer were detected (Fig. 1). Activation of cell membrane biosynthesis suggests that one key to survival is protection against toxic substances generated in a stressful environment (Lee *et al*., 2013). The peptidoglycan layer acts as a thick protective layer against external stresses that may damage intracellular components and cytosolic enzymes that are important for maintenance of normal metabolic activity. The strengthening of the protective layer and repair of intracellular components might be the primary and immediate responses of the probiotic bacterium to the increasing acidity, oxidative stress, increasing toxicity resulting from accumulating metabolites, and other stresses in the stationary phase.

High abundance of proteins for stress tolerance and intestinal colonization of *L. mucosae* LM1

Using QExactive™ Orbitrap MS, proteome quantification detected 19 highly abundant (accounting for > 1.0% of the total proteome, in relative abundance) and 823 less abundant (accounting for < 1.0%, in relative abundance) proteins from the cytosolic fraction (Fig. 2). The high abundance of proteins associated with protein synthesis, ATP-generating pathways and stress alleviation might contribute towards increased stress tolerance and survival of *L. mucosae* LM1 in the stationary phase (Table 1). The induction of many stress-related proteins is believed to occur at the onset of the stationary phase, as described for *L. plantarum* (Cohen *et al*., 2006; Hussain *et al*., 2009). These results suggest that *L. mucosae* LM1 increases the expression of enzymes necessary to maintain its cellular integrity and energy supply. For example, CysK (cysteine synthase; LBLM1_06950) was identified as the most abundant protein (5.41% of the total proteome) in the LM1 proteome (Table 1; Fig. 2). The high abundance of CysK is indicative of the significance of cysteine in growth-dependent states, as well as under stressful conditions. Since cysteine is essential for many enzymatic activities and structural functions, CysK plays an important role in major metabolic activities (i.e., cysteine, selenoamino acid and sulfur metabolism). In Gram-positive

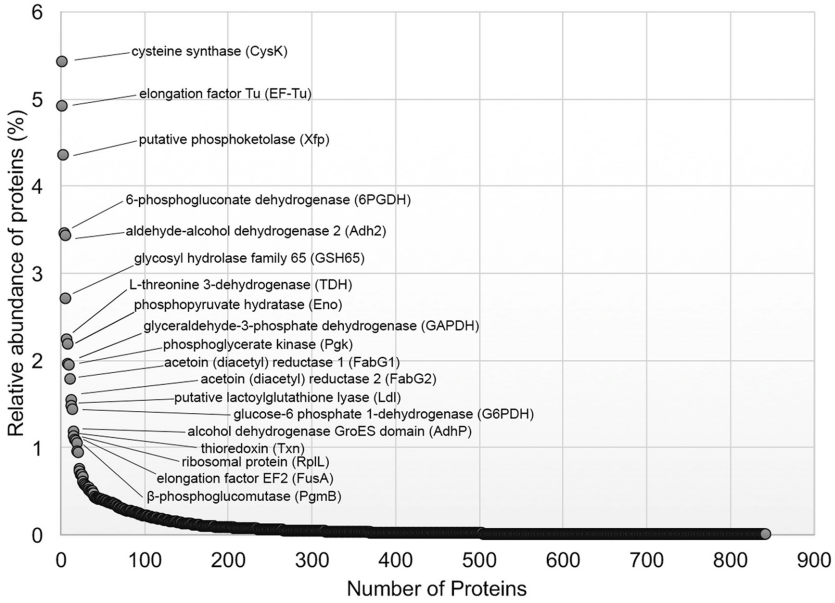

Fig. 2. Relative abundance of proteins (*n*=842) in the intracellular proteome of *L. mucosae* LM1. Highly abundant proteins (> 1.0% in relative abundance, *n*=19) and less abundant protein (0% < *x* < 1.0% in relative abundance, *n*=823) were detected in the intracellular proteome of *L. mucosae* LM1.

Table 1. Highly abundant proteins (> 1.0% of the intracellular proteome) in *L. mucosae* LM1 based on the total mass spectrometry intensity level sorted according to putative COG or KEGG functional categories

Feature name	Protein Name	Protein Symbol	Biological Process[a]	MRA[b]
LBLM1_06950	cysteine synthase	CysK	Protein synthesis	5.41
LBLM1_15720	elongation factor Tu	EF-Tu	Protein synthesis	4.91
LBLM1_00800	putative phosphoketolase	Xfp	Phosphoketolase pathway	4.34
LBLM1_00640	6-phosphogluconate dehydrogenase	6PGDH	Pentose phosphate pathway	3.45
LBLM1_19260	aldehyde-alcohol dehydrogenase 2	Adh2	Pyruvate metabolism	3.43
LBLM1_08520	glycosyl hydrolase family 65 protein	GSH65	Starch, sucrose metabolism	2.70
LBLM1_00200	L-threonine 3-dehydrogenase	TDH	Glycine, serine, and threonine metabolism	2.23
LBLM1_18370	phosphopyruvate hydratase	Eno	Phosphoketolase pathway	2.18
LBLM1_18400	glyceraldehyde-3-phosphate dehydrogenase	GAPDH	Phosphoketolase pathway	1.95
LBLM1_18390	phosphoglycerate kinase	Pgk	Phosphoketolase pathway	1.94
LBLM1_03910	acetoin (diacetyl) reductase 1	FabG1	Butanoate metabolism	1.77
LBLM1_01520	acetoin (diacetyl) reductase 2	FabG2	Butanoate metabolism	1.54
LBLM1_17090	putative lactoylglutathione lyase	Lgl	Pyruvate metabolism	1.47
LBLM1_00650	glucose-6-phosphate 1-dehydrogenase	G6PDH	Pentose phosphate pathway	1.43
LBLM1_02930	alcohol dehydrogenase GroES domain	AdhP	Pyruvate metabolism	1.17
LBLM1_16680	thioredoxin	Txn	Redox signaling	1.13
LBLM1_19280	ribosomal protein L7/L12	RplL	Protein synthesis	1.09
LBLM1_03050	elongation factor EF2	FusA	Protein synthesis	1.07
LBLM1_08530	β-phosphoglucomutase	PgmB	Starch, sucrose metabolism	1.05

[a]Biological process is based on the annotation from the cluster of orthologous genes (COG) or Kyoto Encyclopedia of Genes and Genomes (KEGG)

[b]Mean realtive abundance (%), calculated from the proteome data of seven biological replicates

bacteria, it was observed that *cysK*-mutants suffered from loss of cell viability, tolerance and survival (Lithgow *et al*., 2004). In addition, recent studies also indicated that CysK exhibits a specific adhesive property and affinity to the dense viscous MUC2-rich polymeric gel layer on the surface of the intestinal epithelium (Johansson *et al*., 2011; McGuckin *et al*., 2011). The MUC2 protein of the outer layers of this coating is abundant in cysteine resi-

dues that are made available to commensal bacteria only when they can penetrate the coating and release anti-inflammatory immune factors into the host (Johansson *et al*., 2014; Marcobal *et al*., 2013).

The second-most abundant protein (4.91% of the total proteome) was identified as EF-Tu (elongation factor Tu; LBLM1_15270) (Table 1; Fig. 2). Many lactobacilli produce large quantities of elongation factors in the early stationary phase and in stress states (Koistinen *et al*., 2007; Lee *et al*., 2013; Wu *et al*., 2011). Under such conditions, the cell envelope and membrane-bound proteins are prone to structural damage and deterioration, leading to loss of cell viability (Lee *et al*., 2013). Elongation factors are necessary for the repair of the damaged structural protein, by incorporating amino acids into the growing or impaired polypeptide chain (Nilsson and Nissen, 2005). On the other hand, EF-Tu, and several cytoplasmic proteins (e.g., DnaK, GroES, GroEL, GAPDH), are considered to be multi-functional (called "moonlighting' proteins), with additional functions usually acquired after modification by glycosylation or phosphorylation (Le Maréchal *et al*., 2015; Zhu *et al*., 2011). In previous studies, EF-Tu was found to play a major role in bacterial colonization of the porcine gut, through its intrinsic property and high affinity for sulfurylated mucus layers (Danielsen *et al*., 2007; Nishiyama *et al*., 2013). It is interesting that *L. mucosae* LM1 cells displayed relatively high affinity to pig ileal mucin (Valeriano *et al*., 2014). In addition, several potentially surfaced expressed (PSE) proteins were identified from the intracellular proteome of LM1 cells (data not shown), which may contribute to the high adhesiveness of *L. mucosae* LM1 in the intestine (Valeriano *et al*., 2014). However, these findings require further study to elucidate the precise mechanisms of adhesion and colonization.

Further analysis revealed that the challenging conditions that might have encountered by the bacterial cells, including oxidative stress, acid toxicity and nutrient starvation, activated group-specific proteins for coping with stress. The high abundance of lactoylglutathione lyase (Lgl; LBLM1_17090) (Table 1) suggests that lactobacilli produce the antioxidant glutathione from methylglyoxal glycolytic intermediates (Kant *et al*., 2010). In *L. plantarum* 423, other enzymes for the production of antioxidants including glutathione were up-regulated under acid stress (Heunis *et al*., 2014). This unique protein is found only in the genomes of the closely related species *L. plantarum*, *L. brevis*, *L. fermentum*, *L. reuteri, L. salivarius* and *L. mucosae*, known as the WCFS group (Kant *et al*., 2010). According to Kant *et al*. (2010), comparative genomics of different *Lactobacillus* groups suggests that lactoylglutathione lyase might be important for the WCFS group when under the stress condition of high acidity. High abundance of lactoylglutathione lyase has not been reported in the proteome of lactobacilli other than LM1. However, the specific mechanisms-of-action of this enzyme need further characterization, because there may be species- or strain-specific functions related to the response to stress. Other highly abundant proteins were FabG1 and FabG2 (diacetyl reductases/acetoin dehydrogenases 1 and 2; LBLM1_03910 and LBLM1_01520) and thioredoxin (Txn; LBLM1_16680) (Table 1). The first two enzymes are involved in the conversion of acidic 2,3-butanediol to neutral acetoin, in response to acid toxicity. The high abundance of FabG in LM1 might lead to the production of ATP and production of more basic compounds that would ease acid stress and promote the survival of *L. mucosae* LM1. The up-regulation of FabG was also observed in *L. casei* strain GCRL163 to promote its survival under carbohydrate-starved conditions (Al-Naseri *et al*., 2013). Furthermore, thioredoxin play a major role in the reduction of hydroperoxides to alleviate the effects of oxidative stress. In a previous study, only acid-stressed *L. plantarum* cells produced thioredoxin indicating a role for this system in response to highly acidic conditions (Heunis *et al*., 2014).

The above results are suggestive of certain group-specific or potentially unique abilities of *L. mucosae* LM1 related to the colonization of the mammalian gastrointestinal tract and the tolerance of highly oxidative and acidic conditions (Kant *et al*., 2010; Valeriano *et al*., 2014). The remainder of the highly abundant proteins were associated with various metabolic pathways in *L. mucosae* LM1, including the phosphoketolase pathway, pyruvate and starch metabolism (Table 1).

Metabolic pathway annotation and reconstruction in *L. mucosae* LM1

L. mucosae LM1 metabolic pathway annotation and visualization were performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Approximately 45% (1,101 of 2,404 genes) of the LM1 genome and ~50% (418 of 842 proteins) of the LM1 intracellular proteome were involved in metabolic pathways (Fig. 3A). The results suggest that almost half of the proteins expressed in the early stationary phase are important for basic metabolic and biosynthetic processes, and the other half might be structurally important or involved in cellular

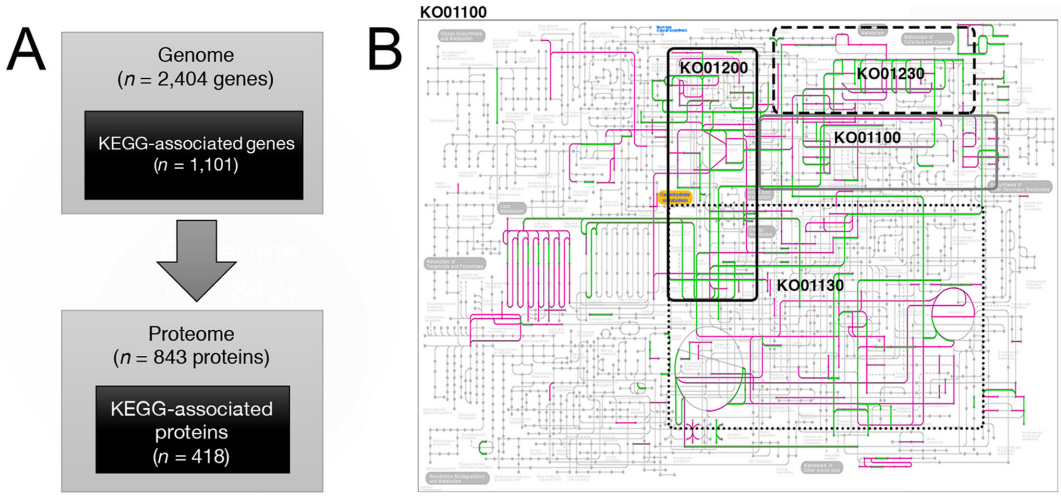

Fig. 3. Overview of the general biosynthetic and metabolic pathways in *L. mucosae* LM1. (A) Annotation of all functionally known proteins identified in the genome and proteome was based on the Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.kegg.jp/). (B) Overview of the KEGG-annotated metabolic pathways of *L. mucosae* LM1. The map shows the presence of genes (red) and proteins (green) in the genomic and proteomic data of *L. mucosae* LM1, respectively. KEGG Orthology (KO) are activated are related to general metabolic pathways (KO01100), carbohydrate metabolism (KO01200), biosynthesis of amino acids (KO01230), secondary metabolites (KO01100) and antibiotic compounds (KO01130).

processes, for example, DNA translation, protein turnover and cell envelope biogenesis, as mentioned above. Enzymes involved in the metabolism of carbohydrates and proteins were the most common and abundant, on the basis of the number of proteins. The presence of KEGG-associated enzymes for breakdown of carbohydrates and proteins indicates that the cells were still thriving in the early stationary phase.

The KEGG pathway reconstruction was mapped through the KEGG Orthology (KO) system (Fig. 3B), which displays all KEGG-associated proteins, classified into 20 functional categories; many enzymes were related to metabolic pathways (KO01100), biosynthesis of secondary metabolites (KO01110) and antibiotics (KO01130). Furthermore, other KEGG-related proteins were connected to carbohydrate metabolism (KO01200) and biosynthesis of amino acids (KO01230). In Fig. 3B, the genomic and proteomic data of *L. mucosae* LM1 coincide in the detection of enzymes linked to the breakdown of carbohydrates for energy (ATP) production. High abundance of carbohydrate-degrading enzymes was observed in the LM1 proteome, particularly GSH65 (glycosyl hydrolase family 65; LBLM1_08520), Xfp (phosphoketolase; LBLM1_00800) and 6PGDH (6-phosphogluconate dehydrogenase; LBLM1_00640) (Table 1).

In the carbohydrate metabolic pathway, GSH65 is responsible for the breakdown of glycosidic bonds from certain carbohydrate sources, including maltose and trehalose (Fig. 4A). In addition, several hydrolytic enzymes involved in the breakdown of various di-, oligo- and polysaccharides, including cellobiose, 1,4-β-D glucan and glucoside (β-glucosidase; LBLM1_17760), 1,3-β-glucan (glucan 1,3-β-glucosidase; LBLM1_17810) and pectin (pectinesterase; LBLM1_04470) were detected in the LM1 proteome, which may increase the energy yield from alternative carbon sources. Two essential genes, PfkA (6-phosphofructokinase) and DhnA (fructose-bisphosphate aldolase) (Fig. 4A), are absent in all available genomes of *L. mucosae* strains isolated from various mammalian hosts. The primary energy-generating pathway in LM1 is via the phosphoketolase pathway (Fig. 4A), as confirmed by the high abundance of phosphoketolase (Xfp), which promotes the conversion of D-xylulose 5-phosphate to glyceraldehyde 3-phosphate, to stimulate ATP production. Subsequently, the glyceraldehyde 3-phosphate produced by this Xfp enzyme might be converted by GAPDH (glyceraldehyde phosphate dehydrogenase; LBLM1_18400) into glycerate 1,3-bisphosphate, which will undergo a series of conversions and reductions to phosphoenolpyruvate (PEP) and, ultimately, pyruvate. Bacterial cells were forced to convert pyruvate to lactate and ethanol in order to regenerate NAD^+.

Other non-carbohydrate catabolic enzymes enable *L. mucosae* LM1 to metabolize non-carbohydrates substrates, particularly citrate and oxaloacetate, which are present in many substrates used for food fermentation (Torino *et al.*,

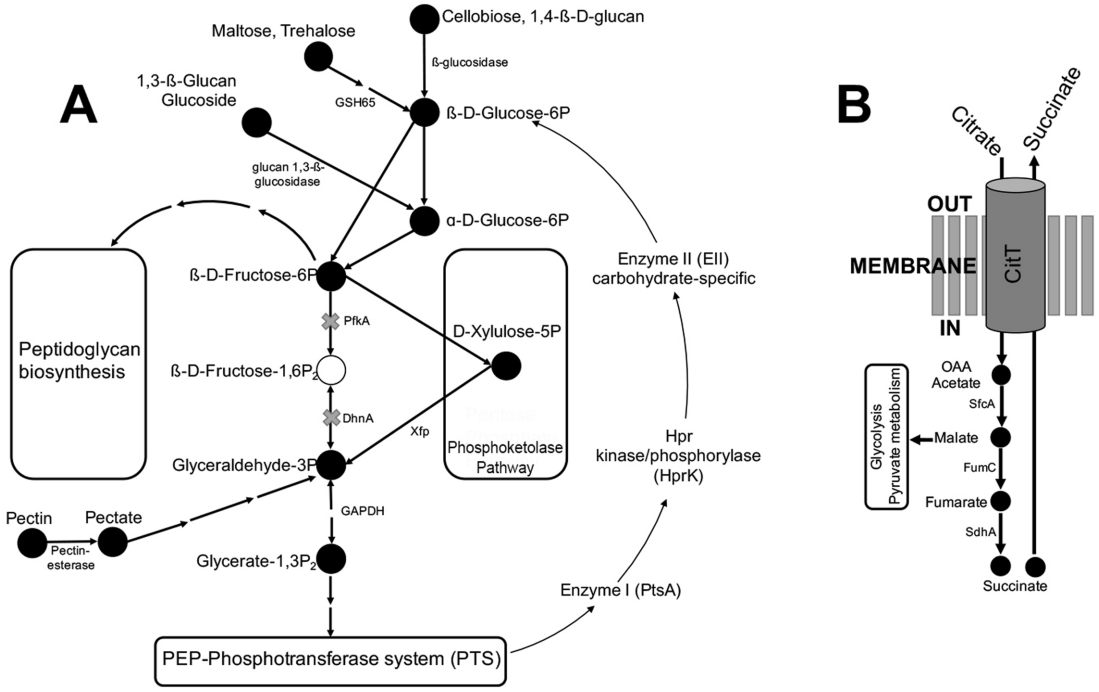

Fig. 4. The proposed pathway for energy production, based on the KEGG-associated proteins found in the proteome of *L. mucosae* LM1. (A) Breakdown of carbohydrate molecules in *L. mucosae* LM1 is via the phosphoketolase pathway. (B) Putative citrate uptake and fermentation pathways in *L. mucosae* LM1. Citrate is converted to malate to be used either as a substrate for energy production or to produce short chain fatty acid, succinate.

2005). LM1 proteome data also demonstrated activation of CitT (citrate/succinate anti-porter carrier protein; LBLM1_00930) for uptake of citrate from the environment (Fig. 4B). After a series of intracellular conversions by SfcA (malate dehydrogenase; LBLM1_11290), FumC (fumarate hydratase; LBLM1_02410) and SdhA (succinate dehydrogenase; LBLM1_02420), *L. mucosae* LM1 may generate essential end-products of citrate fermentation, including ATP, lactate and the short-chain fatty acid, succinate (Fig. 4B). Succinate, which is immediately exported out of the cell via the CitT carrier protein, is an important energy source for host intestinal cells. Previous studies showed that only a few lactobacilli, including *L. plantarum*, *L. casei*, *L. helveticus* and *L. panis*, have the gene set required to catabolize citrate and synthesize succinate (Dudley and Steele, 2005; Kang *et al*., 2013; Torino *et al*., 2005). In a previous study, the reduced availability of carbohydrates stimulated the citrate fermentation pathway in *L. casei* strain GCL163 (Al-Naseri *et al*., 2013). This result suggests that citrate and other non-carbohydrates might serve as the main source of carbon and energy to maintain viability and ensure survival under carbohydrate-depleted environments. Therefore, activating microbial fermentation of non-carbohydrates might be a mechanism by which *L. mucosae* LM1 improves its tolerance to an acidic environment, while concurrently providing an additional energy source for itself and its host (Kang *et al*., 2013).

Energy storage for growth and maintenance in *L. mucosae* LM1

Several studies suggest that energy storage is a major factor in the functionality and retention of probiotic bacteria in the gastrointestinal environment. Based on the genomic data of *L. mucosae* LM1 (Lee *et al*., 2012), a polysaccharide for energy storage seems to be produced in the form of glycogen, however, the importance of glycogen in lactobacilli is still unexplored. Study of the LM1 proteome shows that a putative glycogen biosynthetic pathway is activated in the early stationary phase (Fig. 5A). Generally, glycogen biosynthesis occurs in the presence of excess carbon sources under limited growth condition (Preiss, 1984). In addition, the production of glycogen in bacteria allows cells to sense and respond to changing environments, such as starvation and stress (Preiss, 1984). The biosynthetic pathway of glycan in LM1 is encoded by an approximately 10.1 kb gene cluster consisting of the following six genes: *glgB* (putative glycogen-

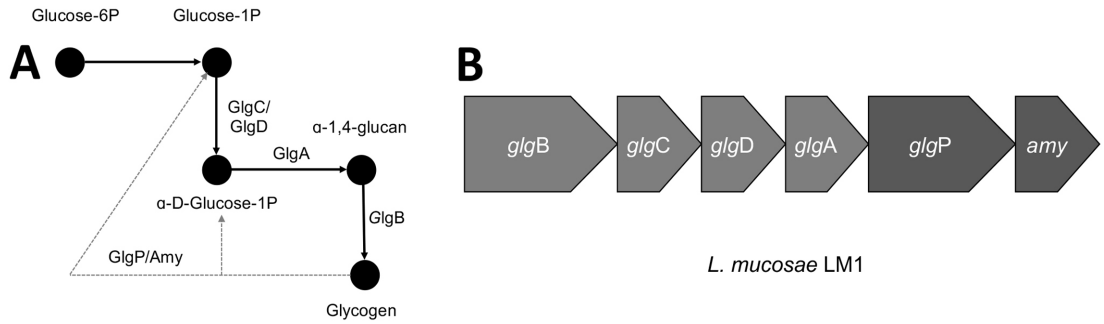

Fig. 5. Putative pathway and gene operon of glycogen synthesis and degradation in *L. mucosae* LM1. (A) Glucose is converted to glucose-6-phosphate then glucose-1-phosphate, which acts as the substrate for GlgC and GlgD to form α-D-glucose phosphate. GlgA creates linear α-1,4-glucan through the transfer of glucosyl moieties, which will be cleaved by GlgB to attach glucan chains and create glycogen. Glycogen biosynthesis is indicated by black arrows, whereas glycogen degradation is indicated by dashed grey arrows. (B) The organization of the 10.1 kb gene operon encoding for the synthesis and degradation of glycan in *L. mucosae* LM1.

branching enzyme; LBLM1_08790), *glgC and glgD* (putative glucose-1-phosphate adenylyltransferase catalytic and regulatory subunits; LBLM1_08800 and LBLM1_08810, respectively), *glgA* (putative glycogen synthase; LBLM1_08820), *glgP* (putative glycogen phosphorylase; LBLM1_08830) and *amy* (putative phosphoglucomutase; LBLM1_08840) (Fig. 5B). In other *Lactobacillus* species, glycogen pathways and gene cluster organization might differ, depending on the bacterial origin (Goh and Klaenhammer, 2014). Interestingly, *L. mucosae* LM1 exhibited higher homology with the *glg* operon of the WCFS group (Kant *et al*., 2010), compared with other bacteria isolated from a similar origin (i.e., mammalian intestines), such as *L. acidophilus* NCFM (Goh and Klaenhammer, 2014). To survive in limiting conditions, the cells need to utilize other carbon sources and minimize energy inefficient processes. It is interesting that enzymes related to the biosynthesis of glycogen are still active in the early stationary phase of *L. mucosae* LM1. In a recent study, glycogen synthesis in *L. acidophilus* NCFM was stably maintained in the stationary phase, under certain conditions (Goh and Klaenhammer, 2014). It is likely that *L. mucosae* LM1 needs to continue glycogen synthesis in the stationary phase for its maintenance and persistence (Goh and Klaenhammer, 2014). However, additional studies are required to elucidate the importance of glycogen in the growth and survival of *L. mucosae* LM1.

Conclusion

We provide the intracellular proteome profile of the probiotic *L. mucosae* in the early stationary phase, using the QExactive™ Orbitrap method. The combination of genomic and proteomic studies provides experimental evidence for the expression of LM1 proteins that had only been predicted by genome annotation. These data will help us understand the probiotic potential of *L. mucosae* LM1, and provide insight into this bacterium's defense against stressful conditions. The detection of several abundant proteins, such as CysK and EF-Tu, that might have important roles in cell survival and colonization in the gastrointestinal tract, as well as the enzymes responsible in energy generation and biosynthesis of glycogen, are suggestive of the strain-specific adaptations and probiotic potential of *L. mucosae* LM1. Given that *L. mucosae* LM1 is autochthonous to the gastrointestinal microbiota, it can be used as a reference organism in host-microbe interaction models. Further characterization of the proteome under intestinal conditions (i.e., bile acid and other stresses) will be needed to better understand the cellular mechanisms and functionality of probiotic lactobacilli under environmental stresses.

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