Short communication: Global transcriptome analysis of *Lactococcus lactis* ssp. *lactis* in response to gradient freezing

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ABSTRACT

Lactic acid bacteria are often preserved as starter cultures by freezing to extend shelf stability as well as maintain cell viability and acidification activity. Previous studies showed that the endocyte extracted from gradient-freezing pretreated cells could act as lyoprotectant in the lyophilization process of *Lactococcus lactis* ssp. *lactis*. In this study, the molecular mechanisms of *L. lactis* in response to gradient freezing exposure are described using high-throughput sequencing. Nineteen of 56 genes were upregulated after gradient freezing, whereas 37 genes were downregulated. Further validation results of quantitative real-time PCR experiments were consistent with the RNA sequencing. Gene Ontology (<http://www.geneontology.org/>) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG; <https://www.genome.jp/kegg/>) pathway were used to analyze the differentially expressed genes. Several pathways, such as glutathione metabolism, ATP-binding cassette transport, metabolism of cell wall and cell membrane components, and stress response-related pathways, were affected by gradient freezing. Six genes relevant to freezing stress response were selected for quantitative real-time PCR, including 3 upregulated genes (*hisK*, *eutD*, *dukA*) and 3 downregulated genes (*als*, *yedF*, *pepN*). The Gene Ontology enrichment and KEGG pathway analyses showed these genes may influence stress response-related pathways, improving the survival of the *L. lactis* under freezing stress. The identification of these genes deepened an understanding about their response under freezing stress, helping us find potential genes or pathways related to gradient freezing for further research on lyoprotectants.

Key words: global transcriptome, gradient freezing, *Lactococcus lactis* ssp. *lactis*, pathways

Short Communication

Lactic acid bacteria (LAB) are generally regarded as a safe starter culture in food industry (Corcoran et al., 2008; Konkitt et al., 2014). During industrial production, LAB often experience freezing stress (Song et al., 2014). Freezing and thawing could impair the cell structure and physiological metabolic process, or lead to cell apoptosis in some serious cases. Therefore, the freezing-adaption mechanisms of *Lactococcus lactis* ssp. *lactis* have gained more attention in recent years.

As a starter culture, *L. lactis* is usually produced through lyophilization and stored under refrigeration conditions (Broadbent and Lin, 1999). Many studies have reported that sublethal stress pretreatment on LAB can increase the survival rate of LAB under freezing stress. In previous research, we found that the endocyte extracted from gradient freezing pretreated cells could act as lyoprotectant in the lyophilization process of *L. lactis* and significantly improve the cell viability (Lu et al., 2017). The gradient freezing pretreatment involved incubating the cells at the temperatures of 4, –20, and –80°C for 2 h in turn. The intracellular components were water soluble and mainly consisted of cytoplasmic proteins, carbohydrates, lipids, small molecules, and other metabolites without cell wall debris and unbroken cells. Furthermore, proteomics and bioinformatics methods, including high-throughput sequencing, Gene Ontology (GO; <http://www.geneontology.org/>) enrichment analysis, Kyoto Encyclopedia of Genes and Genomes (KEGG; <https://www.genome.jp/kegg/>) metabolic pathway analysis, and protein function and analysis were employed to elucidate the protein changes of *L. lactis* in response to gradient freezing (Kim et al., 1998; Song et al., 2014). In our study, profile analysis by RNA deep-sequencing technology was conducted to investigate the effects of gradient freezing stress on *L.*

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lactis and potential related mechanisms. As far as we know, this work represents the first transcriptomic data on the gradient freezing stress response of *L. lactis*.

To investigate the response mechanism of *L. lactis* under gradient freezing stress, we profiled all the genes affected by the freezing stress using RNA deep sequencing after the RNA quality check (Supplemental Figure S1; <https://doi.org/10.3168/jds.2018-15972>). By searching the annotated *L. lactis* IL1403 RNA sequence database (<https://www.ncbi.nlm.nih.gov/nuccore/15671982?report=genbank>), 1,857 freezing stress-related genes were identified. The relative expression levels of these genes after quantification were classified into 2 categories: genes with quantitative ratio over 2 and $P < 0.05$ were considered as upregulated, whereas genes with quantitative ratio below 0.5 and $P < 0.05$ were considered as downregulated. The number of differentially expressed genes (DEG) and the specific information were summarized (Supplemental Table S1; <https://doi.org/10.3168/jds.2018-15972>). Fifty-six genes were significantly regulated by gradient freezing stress conditions. Of these DEG, 37 genes were found to be downregulated, whereas the other 19 genes were upregulated compared with the controls.

To further understand the functions and features of the transcriptome genes, GO annotation enrichment was used to describe the functions of the identified DEG involved in molecular functions, cellular components, and biological processes among the 2 groups (Figure 1, and the Materials and Methods are shown in the Supplemental Material, <https://doi.org/10.3168/jds.2018-15972>). The identified DEG were mainly related to generation of precursor metabolites and energy, glycolytic process, cellular AA catabolic process, single-organism carbohydrate catabolic process, monosaccharide metabolic and catabolic process, hexose metabolic and catabolic process, glucose metabolic and catabolic process, and the biological processes part showed these genes were involved in GO terms. The molecular components, which were involved in the gradient freezing treatment, were mainly related to GO terms including ligase activity, queuine transfer RNA (tRNA)-ribosyltransferase activity, sulfate transmembrane transporter activity, aminotransferase activity, secondary active sulfate transmembrane transporter activity, 1,4- α -glucan branching enzyme activity, 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase activity, pyruvate kinase activity, inosine 5'-monophosphate dehydrogenase activity, phosphate acetyltransferase activity, glucose-6-phosphate dehydrogenase activity, alkali metal ion binding, histidinol-phosphatase activity, and protein serine/threonine/tyrosine kinase activity (Figure 1).

To identify the biological pathway that contributed to the different protective effects among gradient freezing and control groups, a pathway enrichment analysis was conducted by KEGG for DEG between the 2 groups. The primary pathways identified as significantly influenced by gradient freezing were glutathione metabolism, ATP-binding cassette (ABC) transport system, metabolism of cell wall and cell membrane components, stress response-related pathways, carbohydrate metabolism, nucleic acid metabolism, biosynthesis of AA, fatty acid metabolism, ion transport, and translation-related metabolism.

Figure 2 showed the transcriptome results, which were verified using the quantitative real-time PCR (qRT-PCR) method. Six related genes (Supplemental Table S2; <https://doi.org/10.3168/jds.2018-15972>) were selected for qRT-PCR quantification, including 3 upregulated genes (*hisK*, *eutD*, *dukA*) and 3 downregulated genes (*als*, *yedF*, *pepN*). The gene *hisK* encodes histidinol-phosphatase or relevant hydrolase of the PHP family (AA transport and metabolism). The gene *als* is a key acetolactate synthase that catalyzes the first reaction step of branched-chain amino acid synthesis. The gene *pepN* is an aminopeptidase gene, participating in the degradation of major milk proteins. The gene *yedF* can act as binding protein participating in protein, peptide, and AA transporting, which is an important part of ABC transport system or phosphotransferase system. The gene *eutD* has been reported to be responsible for the synthesis of phosphotransacetylase, which is required for ethanolamine catabolism in *Salmonella* (Brinsmade and Escalante-Semerena, 2004). Last, the gene *dukA* is involved in the nucleotide transport and metabolism. The results by qRT-PCR were consistent with the RNA deep-sequencing results at the transcription level. Several significant pathways related to freezing stress response exist, including the glutathione metabolism, ABC transport system, metabolism of cell wall, and cell membrane components. Other pathways, such as carbohydrate metabolism, nucleic acid metabolism, biosynthesis of AA, fatty acid metabolism, ion transport, and some translation-related metabolisms, were also found to be involved.

In the pathway of glutathione metabolism, *pepN*, an aminopeptidase gene, participates in the degradation of major milk proteins. The degradation products, including small peptides and free AA, are essential for the metabolism of the fastidious lactococci (van Alen-Boerriqter et al., 1991; Bhosale et al., 2013). In addition, 4 downregulated genes (*yahG*, *choQ*, *optD*, and *yedF*) were found to be related to the ABC transporter system for transportation of osmo- and cryoprotective substances. This system has significant regulatory ef-

Figure 1. Gene Ontology (GO; <http://www.geneontology.org/>) function classification of differently expressed genes and Kyoto Encyclopedia of Genes and Genomes (KEGG; <https://www.genome.jp/kegg/>) pathway enrichment-based clustering analysis. The red histogram represents a significant enrichment of GO-Biological_process, yellow represents a significant enrichment of GO-molecular_function, and blue one represents a significant enrichment of GO-cellular_component. IMP = inosine-5'-monophosphate; tRNA = transfer RNA.

fects on many strains under different stress conditions (Sternes et al., 2017). For example, *choQ* acts as a choline ABC transporter ATP-binding protein, and its substrate can be proline or glycine; proline or glycine can accumulate in the cell to influence the osmotic pressure, so it may be involved in osmoprotection (Cohen et al., 2006). The proteins encoded by both *optD* and *yedF* can act as binding proteins participating in protein, peptide, and AA transporting, which is an important part of ABC transport system or phosphotransferase system (Doerks et al., 2002; Aleksandrak-Piekarczyk et al., 2011). The low temperatures of gradient freezing have an adverse effect on the ABC transporter system because of the temperature-dependent decrease of the cell activity.

An unexpected finding was that the expression of 2 genes related to cell wall synthesis was also downregulated under freezing stress. One is *murC*, encoding UDP-*N*-acetylmuramic acid L-alanine ligase; the other is *murA2*, encoding UDP-*N*-acetylglucosamine 1-carboxyvinyltransferase. A reasonable explanation for this observation is that freezing stress may induce a decrease in cell wall sensitivity. The cytoplasmic membrane is a key target for freezing- or desiccation-induced damage, so maintenance of membrane integrity is central to desiccation tolerance in bacteria. Most cells accomplish this by altering the membrane's fatty acid composition. As growth temperature decreases, for example, cells typically incorporate a higher percentage of lower melting-point (e.g., unsaturated) fatty acids into the

membrane, and this change has been correlated with improved cryotolerance (Rumian et al., 1993; Broadbent and Lin, 1999). Consistent with the observation is that *fadD* was also downregulated; *fadD* encodes long-chain acyl-CoA synthetase. The long-chain acyl CoA synthetase belongs to one part of the fatty acid transport proteins, and it plays an important role in regulating the rate of fatty acid uptake and channeling the imported fatty acids between various metabolic processes within the cell; thus, it may be involved in the cell membrane metabolism under gradient freezing stress (Daniel et al., 2014).

Several upregulated genes identified in our study are also involved in the universal stress responses, such as *napB*, *rlrC*, and *rcfA*. The gene *napB* is a MarR family transcriptional regulator, similar to NapB protein of *Enterococcus hirae*. The gene *rlrC* is a LysR family transcriptional regulator. Last, *rcfA* is a transcription regulator belonging to CRP/FNR family. Two downregulated genes were found in our research, which have been reported in the universal stress response. One is *ptsK* encoding HPr kinase/phosphorylase, catalyzing the phosphorylation of the phosphor carrier protein HPr of the bacterial phosphor transferase system (Nessler, 2005). The other is *recN*, encoding DNA repair protein involved in DNA replication, restriction, modification, recombination, and repair (Cassier-Chauvat et al., 2016) under environmental stress.

Six downregulated genes (*gapB*, *pyk*, *zwf*, *L200009*, *yphD*, and *mapA*) related to carbon metabolism were

Figure 2. Comparison of the expression of 6 genes at the transcription level, including 3 upregulated genes (*hskK*, *eutD*, *dukA*), 3 downregulated genes (*als*, *yedF*, *pepN*), as revealed by quantitative real-time PCR. The y-axis indicates the fold changes in genes. The error bars indicate SD (n = 3). Significant difference from the control group was designated as * $P < 0.05$ and *** $P < 0.001$, respectively.

found in the current study. These genes are involved in GAPDH formation, pyruvate kinase, glucose-6-phosphate 1-dehydrogenase, 1,4- α -glucan branching enzyme, sugar transport symporter, and maltose phosphorylase, respectively. It has been reported that GAPDH is able to interact with proteins participating in the repair process of damaged DNA. Pyruvate kinase can break down the redox balance state of cells and promote the formation of cell biofilm system, which is conducive to intercellular communication and stress response (Vasu et al., 2015; Kosova et al., 2017). Gene *L200009* can transfer a segment of a 1, 4- α -D-glucan chain to a primary hydroxyl group in a similar glucan chain; it plays an essential role in the formation of branched polysaccharides, glycogen, and amylopectin. In addition, *yphD* is able to encode Na⁺/melibiose symporter and related transporters involved in carbohydrate transport and metabolism. Maltose phosphorylase catalyzes the reversible phosphorolysis of maltose to β -D-glucose-1-phosphate and α -D-glucose using orthophosphate as co-substrate (Hüwel et al., 1997).

Several DEG are involved with biosynthesis of AA, including *als*, *eno*, *gapB*, *gpmA*, *hisK*, *pyk*, *serC*, *trpG*, and *ywiC*. In our study, the observed dramatic down-regulation of *als* (acetolactate synthase) and *trpG* (anthranilate synthase component II) suggested that few hydrophobic AA are needed during peptide and protein synthesis in *L. lactis* under gradient freezing stress. The gene *als* is a key acetolactate synthase that catalyzes the first reaction step of branched-chain AA synthesis (Lloyd Evans and Joshi, 2016). It has been reported that branched-chain AA are required for protein synthesis and turnover, signaling transduction and glucose metabolism, as well as fatty acid oxidation (Deng et al., 2017); they act as hydrophobic AA and can prevent bile salt invasion by constructing hydrophobic regions (Sánchez et al., 2005). One interpretation is that, as cells lose water in the process of gradient freezing, more hydrophilic areas are needed to prevent water loss (Lv et al., 2017). In addition, *hisK*, which encodes histidinol-phosphatase or relevant hydrolase of the PHP family (AA transport and metabolism), is upregulated. This suggests a necessity for cell stimulating protein synthesis in response to environmental stress under gradient freezing treatment.

The pathway of AA modifications on tRNA was found to be suppressed by the gradient freezing treatment. The gene *gatB* encodes aspartyl/glutamyl-tRNA amidotransferase subunit B; it enables the correct formation of charged Asn-tRNA(Asn) and Gln-tRNA(Gln) through the transamidation of misacylated Asp-tRNA(Asn) or Glu-tRNA(Gln) in organisms that lack one or both asparaginyl-tRNA or glutaminyl-tRNA synthetases. The reaction takes place in the

presence of glutamine and ATP through an activated phospho-Asp-tRNA(Asn) or phospho-Glu-tRNA (Huot et al., 2007). The gene *serS* is responsible for encoding seryl-tRNA synthetase, which catalyzes a 2-step reaction: first charging a serine molecule by linking its carboxyl group to the α -phosphate of ATP, followed by the transfer of the aminoacyl-adenylate to the tRNA (Maršavelski et al., 2014). Both reactions are involved in the pathway of AA modifications on tRNA.

Other identified genes are involved in the nucleotide transport and metabolism, such as *guaB*, *carA*, diadenosine polyphosphate hydrolase, and *dukA*. Other downregulated genes indicate that freezing stress induces a decrease in intracellular ability of translation and ion transport. For instance, *fusA* encodes elongation factor G, which promotes GTP-dependent translocation of the ribosome during translation (Yamamoto et al., 2014). Another is *yafB*, which is a sulfate transporter for sulfate and organosulfur compounds (Kertesz, 2001). Furthermore, we found that the expression profiles of 56 ribosomal family genes involved in ribosome pathway are significantly influenced under gradient-freezing conditions by KEGG pathway enrichment analysis (Figure 1); this suggests the possible involvement in gradient freezing stress in *L. lactis*.

In conclusion, we analyzed the global transcriptome of *L. lactis* under gradient freezing stress to identify potential genes involved in the adaptation of this strain to the stress. The regulation of 56 stress-related genes was found to be significantly influenced by gradient freezing. The genes identified were involved in multiple pathways, including glutathione metabolism (*pepN*, *zwf*), ABC transport system (*yahG*, *choQ*, *yedF*, *optD*), metabolism of cell wall and cell membrane components (*murC*, *murA2*, *fadD*, *dfpB*), and stress response (*napB*, *rlrC*, *rcfA*). In addition, other pathways, including carbohydrate metabolism, nucleic acid metabolism, biosynthesis of AA, fatty acid metabolism, ion transport, and translation-related metabolism, were also affected by freezing stress. The identification of these genes deepened our understanding about their response under freezing stress, helping us find potential genes or pathways related to gradient freezing for further research on lyoprotectants.

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