***Lactobacillus casei* asp23 gene contributes to gentamycin resistance via regulating specific membrane-associated proteins**

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ABSTRACT

Lactobacillus casei Zhang is a probiotic strain originally isolated from koumiss. Previously, we showed that an alkaline shock protein (encoded by *asp23*) was involved in the adaptation of *L. casei* Zhang to gentamycin. In the present study, we compared the proteomes of the *asp23* mutant and its parent strain grown in the presence of gentamycin. The results showed that 22 and 21 proteins were significantly up- and downregulated, respectively (>1.5-fold difference). By parallel reaction monitoring analysis, we further validated that specific membrane-associated proteins were important in regulating the antibiotics-induced cell wall stress. The findings provide insight into the physiological role of the *asp23* gene in the growth response of *L. casei* when exposed to antibiotic stress.

Key words: *Lactobacillus casei* Zhang, proteomics, gentamycin, membrane-associated protein, *asp23* gene

INTRODUCTION

Lactobacillus casei is one of most studied species in the largest lactic acid bacteria genus, *Lactobacillus*. Members of this species commonly are present in various food environments, such as pickles, cheeses, and yogurt, reflecting their versatility (Bao et al., 2016). The first *L. casei* genome was decoded in 2009 and has revealed the genomic features contributing to the broad ecological adaptability and evolution of this species (Cai et al., 2009). Particularly, the *L. casei* species has high flexibility in sugar utilization, as revealed by comparative genomic analysis of several strains, including *L. casei* Zhang, BL23, and ATCC334 (Zhang et al., 2010). The real breakthrough of the genomic studies on *L. casei* is driven by the development of highly cost-effective and high-throughput DNA sequencing techniques. Using these techniques, we recently investigated

the adaptive evolution of the genomes of 50 *L. casei* Zhang strains isolated at different stages of prolonged cultivation in an antibiotics-containing environment. This studied probiotic strain was found to have high genome stability even under long-term antibiotic stress (Wang et al., 2017).

Although the genomes of several *L. casei* strains are now available, we are only starting to gain some understanding of the physiology of this species. Further work is required to deepen our knowledge of their cell biology and function. In the last decade, much progress has been made in the study of *L. casei* strain genetics. One classical study performed in *L. casei* BL23 created several genetic mutants of the *iolTABCDG1G2EJK* cluster. The work deciphered the mechanism of myo-inositol utilization of this bacterium (Yebra et al., 2007). With the help of a gene-modifying system derived from a conditionally replicating vector, several interesting genes have been characterized in the same strain (e.g., genes that code for the enzyme L-lactate dehydrogenase, a global catabolite regulator protein, a fibronectin-binding protein, and a 2-component system; Viana et al., 2005; Rico et al., 2008; Muñoz-Provencio et al., 2010; Alcántara et al., 2011). Most of these characterized genes are likely involved in regulating bacterial metabolic capacity or stress response.

The *L. casei* alkaline shock protein, encoded by *asp23*, is a stress-responsive protein (Zhang et al., 2017). However, its exact cellular function is unknown. Previously, we observed an increase in the sensitivity of gentamycin in the *L. casei* Zhang *asp23* knockout mutant (Zhang et al., 2017). In the present study, we compared the proteomes of the *asp23* knockout mutant and its wild-type strain to characterize the biological role of the *L. casei* *asp23* gene at protein level.

MATERIALS AND METHODS

Bacterial Isolates and Culture Conditions

The *asp23* gene disruption mutant and the wild-type strain (*L. casei* Zhang-G-1200) were obtained from our

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previous studies (Wang et al., 2017; Zhang et al., 2017). The minimum inhibitory concentrations of the wild-type strain and the *asp23* mutant were 32 and 16 µg/mL, respectively. For proteomic analysis, these bacteria were grown in lactic acid bacteria susceptibility test medium (LSM) supplemented with 8 µg/mL of gentamycin (Klare et al., 2005). At this antibiotic concentration level, the bacterial growth would be inhibited by 50%, as determined by optical density measurement. Growth curves were constructed in relation to optical density, pH, and the number of viable counts based on readings taken every 2 h during 30 h of fermentation. All analyses were performed in triplicate.

Sample Preparation

The *asp23* gene disruption mutant and its wild-type strain were collected after 24 h of cultivation in LSM supplemented with 8 µg/mL of gentamycin. Three biological replicates were prepared for the 2 tested strains. Samples were lysed using SDT lysis buffer (Invitrogen, Carlsbad, CA). Supernatants were collected after centrifuging the samples for 15 min at $14,000 \times g$ at 25°C. The Pierce BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA) was used to estimate the sample protein content.

Tandem Mass Tags Labeling and High-pH Reverse-Phase Peptide Fractionation

Isobaric labels of tandem mass tag (TMT) reagents with varying molecular weights (126–131; Thermo Fisher Scientific) were applied to the digested samples of the wild-type strain and *asp23* mutant according to the manufacturer's protocols. Briefly, every sample was labeled with 100 µg of TMT tag dissolved in acetonitrile. The labeled peptide mixtures were then fractionated using high-pH reverse-phase chromatography. For each sample, 10 fractions were collected, desalted, and lyophilized.

Liquid Chromatography–MS/MS Analysis

We performed liquid chromatography (LC)–MS/MS analysis using the UltiMate 3000 Nano LC System (Thermo Fisher Scientific) coupled to a Q-Exactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific) with a nanoelectrospray ionization source. The Orbitrap mass spectrometer was set at a data-dependent mode. Each full MS scan (60,000 resolving power) was followed by 6 MS/MS scans. Based on the scans, the 3 most abundant molecular ions were chosen to be fragmented by collision-induced

dissociation (normalized collision energy of 35%) before scanning by higher energy collisional dissociation-MS/MS (collision energy of 45%).

Database Search and Quantitative Data Analysis

The original map file was first generated by TMT quantitative proteomics analysis using the high-resolution Q-Exactive mass spectrometer (Thermo Scientific). Then, the file was converted to the .mgf format by Proteome Discoverer 1.4 software (Thermo Scientific). Data were submitted to the MASCOT2.2 server for database retrieval via built-in software tools (Colaert et al., 2011). Afterward, the library file on the MASCOT server was imported into Proteome Discoverer 1.4 software again. Data screening was then performed with a stringent false discovery rate cut-off value of <0.01 to ensure the reliability of the qualitative results.

The protein sequence was qualitatively analyzed by combining the protein databases available for *L. casei* Zhang (Zhang et al., 2010, 2015). Mascot 2.2 was used for database searching and quantitative analysis. Proteome Discoverer 1.4 was used to extract the peptide ion peak intensity, and the quantitative values were normalized. Differential expression of individual proteins between the 2 strains was evaluated with *t*-test (at a cut-off value of an adjusted false discovery rate *P*-value <0.05). All differentially expressed proteins were distributed over clusters of orthologous genes (COG) and were subjected to the Kyoto Encyclopedia of Genes and Genomes database search (Tatusov et al., 1997).

Validation of Protein Expression by Parallel Reaction Monitoring

To validate the protein expression data obtained by TMT label-free analysis, LC–parallel reaction monitoring (PRM) MS analysis was used to quantify the expression of selected proteins (Peterson et al., 2012). Briefly, peptides were prepared according to the TMT label-free protocol, and an AQUA stable isotope peptide was spiked in each sample as an internal standard reference. Tryptic peptides were desalted on C18 stage tips before being separated by reverse-phase chromatography on an Easy nLC-1200 system (Thermo Scientific). One-hour LC gradients with acetonitrile ranging from 5 to 23% in 42 min were used. The PRM analysis was carried out with a Q-Exactive Plus mass spectrometer (Thermo Scientific). The optimal parameters such as collision energy, charge state, and retention times were determined experimentally for the most significantly regulated peptides based on conditions developed from high-intensity and confidence-unique peptides of each target protein. The mass spectrometer was set at the

positive ion mode. The full MS1 scan was acquired with resolution at 60,000 [at a mass:charge ratio (m/z) of 200], an automatic gain control target value of 3.0×10^{-6} , and a maximum ion injection time of 250 ms. Full MS scans were followed by 20 PRM scans at resolution 30,000 (at m/z 200), an automatic gain control target value of 3.0×10^{-6} , and a maximum injection time of 200 ms. The targeted peptides were isolated with a 1.6 Thomson (Th) window. Ion activation and dissociation took place in a higher energy collisional dissociation collision cell with normalized collision energy of 27. Skyline, developed by the MacCoss Lab at the University of Washington in Seattle, was used to analyze the raw data (MacLean et al., 2010). The peptide signal intensities detected from unique peptides of the differentially expressed proteins were normalized to the respective sample and to the standard reference.

RESULTS

Growth Performance of the Wild-Type Strain and *asp23* Mutant in the Presence of Gentamycin

As shown in Figure 1, the wild-type strain grew rapidly in the gentamycin-containing LSM broth contrasting to the *asp23* mutant. The cells of the wild-type strain entered into the log phase within 6 h, and cell density reached a high level of 1.07×10^9 cfu/mL after 24 h of cultivation. Consistently, the pH value of the growth medium decreased faster for the wild-type strain.

Differentially Expressed Proteins in the Wild-Type Strain Compared with the *asp23* Mutant

A total of 43 proteins were differentially expressed in the wild-type strain compared with the *asp23* mutant (Table 1). Around one-fourth of the upregulated proteins fell into the COG category of lipid transport and metabolism (27.3%), and around one-third of the downregulated proteins were involved in the process of carbohydrate transport and metabolism (33.3%).

The 6 upregulated proteins that were associated with lipid transport and metabolism were acetyl-CoA carboxylase subunit β (LCAZH_2069), a biotin carboxylase (LCAZH_2070), a 3-hydroxymyristoyl-ACP dehydratase (LCAZH_2071), a 3-oxoacyl-ACP synthase (LCAZH_2078), a 3-hydroxymyristoyl-ACP dehydratase (LCAZH_2080), and an esterase (LCAZH_2891). In the *L. casei* Zhang genome, the former 5 proteins are located within the same gene cluster that is responsible for the de novo synthesis of fatty acids (Zhang et al., 2010). Similarly, 4 of the downregulated carbohydrate utilization-related proteins were clustered, and they

Figure 1. Growth curves of the *asp23* mutant and wild-type strain grown in gentamycin-containing lactic acid bacteria susceptibility test medium based on (a) viable counts, (b) optical density (OD) values, and (c) pH. Error bars represent SD.

were involved in the sorbitol metabolism (Wang et al., 2016). These proteins included the PTS system sorbitol transporter subunits EI_{IA} (LCAZH_2727), EI_{IBC} (LCAZH_2728), and EI_{IC} (LCAZH_2729) and sorbitol-6-phosphate dehydrogenase (LCAZH_2732). Other differentially expressed proteins belonged to the COG categories of AA transport and metabolism [E] and inorganic ion transport and metabolism [P], including tetrahydrodipicolinate *N*-succinyltransferase (LCAZH_0107), the peptidase T (LCAZH_0338), a cation transport ATPase (LCAZH_0107), and a Mn²⁺/Zn²⁺ ABC transporter permease (LCAZH_2388).

Validation of Protein Expression by PRM

To verify the protein expression data obtained by proteomic analysis, 5 differentially upregulated proteins were selected for further PRM validation. Up to 3 peptides were chosen for each protein for the quantification. As shown in Table 2, the upregulation of the 5 proteins ranged from 2.19- to 5.49-fold, which was consistent with the results obtained from proteomic analysis.

DISCUSSION

Based on our previous study, an alkaline shock protein (encoded by the *asp23* gene) was involved in the adaptation of *L. casei* Zhang to gentamycin. The high expression of this protein in the adapted strain is associated with the development of the resistant phenotype, whereas the deletion of this gene from the genome increases the bacterial sensitivity to gentamycin. Thus, to further investigate its cellular role in coping with gentamycin stress, this work compares the proteomes of the *asp23* gene deletion mutant with its parental strain.

Bacterial cell walls act as a physical barrier to protect the cells from external stress (van de Guchte et al., 2002). In particular, membrane alterations play an important role in response to common stresses exerted from the direct vicinity (Coucheney et al., 2005). For instance, osmotic stress, heat shock, or Tween 80 medium supplementation could induce significant modifications in the membrane fatty acid composition of *Lactococcus lactis* cells (Guillot et al., 2000). The most obvious change was the increase in cyclopropane fatty acid delta C19:0 content while maintaining a stable unsaturated:saturated ratio when the cells were subject to high osmolality (Guillot et al., 2000). Similarly, when *L. casei* was exposed to an acidic environment, a cyclopropane fatty acid synthase-like methyltransferase was induced and expressed constitutively (Wang et al., 2017). The enzyme catalyzed the addition of a methyl-

ene residue across the *cis* double bond of C16:1n(9), C18:1n(9), or C18:1n(11) UFA to form an unsaturated cyclopropane derivative (Wang et al., 2017). Here, we observed the upregulation of 5 proteins in the fatty acid synthetic pathway, among which the acetyl-CoA carboxylase catalyzed the first step of the pathway by carboxylating acetyl-CoA to form malonyl-CoA (Fujita et al., 2007). In the presence of gentamycin, the wild type grew better than the *asp23*-inactivated mutant, suggesting that the *asp23* gene product played a protective role via activating the fatty acid metabolism.

The LCAZH_2891-encoded esterase is another cell wall-associated differentially expressed protein. Although the precise role of this protein in antibiotic resistance is unclear, it is known to be responsive to environmental stress. Our previous proteomic study observed a strong upregulation of an *L. casei* esterase when the cells were subject to acid shock, suggesting that it was likely involved in adaptation to an acidic environment (Wu et al., 2011). Moreover, some esterase homologs were involved in the early response to acid shock and bile stress in *Lactobacillus reuteri*; the inactivation of the corresponding gene from the bacterium significantly decreased its survival rate under such stress (Wall et al., 2007).

During the late stage of growth, *L. casei* tends to alternate the carbon catabolic pathway by increasing the utilization of AA and metal ions to sustain proliferation (Wang et al., 2012), which may explain the upregulation of the carbohydrate, AA, and inorganic ion-related proteins in the gentamycin-adapted strain. Interestingly, trehalose-6-phosphate hydrolase, which functions to take up trehalose, was also upregulated. This protein is a major player in trehalose metabolism, which catalyzes the conversion of trehalose-6-phosphate into glucose and glucose-6-phosphate (Termont et al., 2006). The protein has been shown to protect cells from certain environmental stresses (e.g., cryoprotection in *L. acidophilus*; Duong et al., 2006). However, whether this protein serves a similar role in the case of gentamycin stress still remains to be further explored.

Some sorbitol-related proteins were apparently downregulated in the *asp23* gene deletion mutant. These proteins were associated with carbohydrate transport and metabolism. Several proteomic studies found an increase in the expression of this group of protein when the culture shifted from the exponential growth phase to the stationary phase (Laakso et al., 2011; Wang et al., 2016). Our study observed that the adapted strain (wild type) grew better than the *asp23* gene deletion mutant in the gentamycin-containing medium; thus, such protein expression changes could be regarded as signatures of phase transition of the *asp23* mutant.

Table 1. Differentially expressed proteins in wild-type strain compared with *asp23* mutant in the presence of gentamycin

Locus	Function	Fold change	COG ¹
Upregulated			
LCAZH_0107	Tetrahydridipicolinate <i>N</i> -succinyltransferase	1.56	[E]
LCAZH_0279	ADP-Ribosylglycohydrolase	1.64	[O]
LCAZH_0338	Peptidase T	1.68	[E]
LCAZH_1114	Hypothetical protein	1.88	—
LCAZH_1193	Deoxynucleoside kinase	1.65	[F]
LCAZH_1227	Hypothetical protein	1.65	—
LCAZH_1822	Hypothetical protein	2.11	—
LCAZH_1898	Hypothetical protein	1.88	—
LCAZH_2069	Acetyl-CoA carboxylase subunit β	3.28	[I]
LCAZH_2070	Biotin carboxylase	2.08	[I]
LCAZH_2071	3-Hydroxymyristoyl-ACP dehydratase	2.05	[I]
LCAZH_2078	3-Oxoacyl-ACP synthase	1.85	[I]
LCAZH_2080	3-Hydroxymyristoyl-ACP dehydratase	1.97	[I]
LCAZH_2341	Prebacteriocin	1.53	—
LCAZH_2372	Oxidoreductase	1.66	[R]
LCAZH_2373	Short-chain alcohol dehydrogenase	1.78	[R]
LCAZH_2374	Old yellow enzyme family NADH:flavin oxidoreductase	1.51	[C]
LCAZH_2434	Antimicrobial peptide ABC transporter ATPase	1.54	[V]
LCAZH_2435	Hypothetical protein	2.86	—
LCAZH_2653	Trehalose-6-phosphate hydrolase	1.64	[G]
LCAZH_2760	Amidase	1.59	[Q]
LCAZH_2891	Esterase	1.63	[I]
Downregulated			
LCAZH_0079	Cation transport ATPase	1.50	[P]
LCAZH_0938	Sugar ABC transporter permease	1.53	[G]
LCAZH_2806	Hypothetical protein	1.5	[E]
LCAZH_1804	Dipeptide/tripeptide permease	1.55	—
LCAZH_0968	Multidrug ABC transporter ATPase	1.57	[V]
LCAZH_0709	Autoinducer AI2 synthesis LuxS-like protein	1.59	[T]
LCAZH_2286	Dehydrogenase-like protein	1.62	[R]
LCAZH_2388	Mn ²⁺ /Zn ²⁺ ABC transporter permease	1.64	[P]
LCAZH_1179	XRE family transcriptional regulator	1.65	—
LCAZH_1764	Hypothetical protein	1.68	—
LCAZH_0403	PTS system mannose/fructose/ <i>N</i> -acetylgalactosamine-specific transporter subunit IIB	1.71	[G]
LCAZH_0397	Hypothetical protein	1.73	—
LCAZH_0402	LevA protein	1.73	[G]
LCAZH_2104	Glycine/betaine/carnitine ABC transporter membrane-spanning subunit	1.76	[E]
LCAZH_2389	Mn/Zn ABC transporter ATPase	1.78	[P]
LCAZH_2387	Substrate binding protein	1.86	[P]
LCAZH_2725	Transaldolase	2.53	—
LCAZH_2732	Sorbitol-6-phosphate dehydrogenase	2.62	[G]
LCAZH_2729	PTS system sorbitol transporter subunit EIIC	2.88	[G]
LCAZH_2727	PTS system sorbitol transporter subunit EIIA	2.93	[G]
LCAZH_2728	PTS system sorbitol transporter subunit EIIBC	3.43	[G]

¹COG = clusters of orthologous genes. Functional categories: [C] = energy production and conversion; [E] AA transport and metabolism; [F] = nucleotide transport and metabolism; [G] = carbohydrate transport and metabolism; [I] = lipid transport and metabolism; [O] = posttranslational modification, protein turnover, chaperones; [P] = inorganic ion transport and metabolism; [Q] = secondary metabolites biosynthesis, transport and catabolism; [R] = general function prediction only; [T] = signal transduction mechanisms; [V] = defense mechanisms.

Table 2. Parallel reaction monitoring validation of the selected upregulated proteins

Locus	Signature peptides	Ratio
LCAZH_2070	NVVSAAILSGAEAVHPGFGFLSENAEFAK	5.21
LCAZH_2071	DFALALFQAPTVLAKG TLEAQEIQAILPHR	2.66
LCAZH_2078	LIDNAGLGK TAYLGGIK AVYQFAISEVPR	4.24
LCAZH_2080	LVDWHDR	5.49
LCAZH_2891	YPLFYIDHVSSELVPDESILVAEK VPEDVHWETEYR LLPDVDVDR	2.19

CONCLUSIONS

In the present study, we compared the proteomes between the *asp23* gene deletion mutant and its parent strain grown in gentamycin-containing medium. Interestingly, some membrane-associated proteins were significantly upregulated in the parent strain compared with the *asp23* mutant, suggesting that the *asp23*-encoded alkaline shock protein coped with the gentamycin stress via modulating the cell membrane proteins. Our study has provided insight into the physiological role of *asp23* in antibiotic resistance in *L. casei*.

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