Effect of bile salts stress on protein synthesis of *Lactobacillus casei* Zhang revealed by 2-dimensional gel electrophoresis

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ABSTRACT

Lactobacillus casei Zhang, isolated from koumiss in Inner Mongolia of China, is known from previous findings to be tolerant to bile salts. Bile salts secreted by mammals act as a natural antibacterial barrier and may serve as a component of innate immunity, as they have limited antagonistic effect against resident microflora. In this work, we compared the growth and protein expression patterns of *L. casei* Zhang with and without bile salts. Twenty-six proteins were found to be differentially expressed using 2-dimensional gel electrophoresis. Peptide mass fingerprinting was used to identify these proteins. Further verification by using real-time, quantitative reverse transcription-PCR and bioinformatics analysis showed that the implicated pathways are involved with a complex physiological response under bile salts stress, particularly including cell protection (DnaK and GroEL), modifications in cell membranes (NagA, GalU, and PyrD), and key components of central metabolism (PFK, PGM, CysK, LuxS, PepC, and EF-Tu). These results provide insight on the protein expression pattern of *L. casei* under bile salts stress and offer a new perspective for the molecular mechanisms involved in stress tolerance and adaptation of bacteria. **Key words:** bile salts tolerance, *Lactobacillus casei* Zhang, 2-dimensional gel electrophoresis, quantitative reverse transcription-PCR

INTRODUCTION

Lactobacillus spp. have achieved popularity in the manufacture of probiotic products because of their convincing beneficial effects on human health. However, before providing benefit to human health, probiotic bacteria have to survive the difficult journey through

the human gastrointestinal tract in sufficient densities in the presence of bile salts (Dunne et al., 2001). Therefore, tolerance to bile salts is an important criterion for selection of probiotics and their application in value-added foods.

Proteomics analysis is currently considered a powerful tool for global evaluation of protein expression, and proteomics has been widely applied in analysis of molecular mechanisms involved in bile salts tolerance of probiotic bacteria. Two-dimensional electrophoresis (2-DE) is an effective technique for the investigation of protein expression of various microorganisms. Matrix-assisted laser desorption and ionization time-of-flight (MALDI-TOF) mass spectrometry, based on the bombardment of the molecules with a laser light for sample ionization, is a useful tool for rapid protein identification. The combination and application of these 2 techniques in proteomics research have contributed greatly to the current understanding of the mechanisms of bile salts resistance of probiotics. Previous research showed that up to 34 proteins were up- or downregulated when *Bifidobacterium longum* or *Lactobacillus reuteri* were grown in bile salts (Sanchez et al., 2005; Lee et al., 2008). Changes in expression of proteins in *Enterococcus faecalis* and *Bifidobacterium animalis* ssp. *lactis* when exposed to bile salts, indicated that exposure of probiotics to sublethal levels of bile salts allows cells to adapt to the environment and protects them against subsequent exposure to normally lethal levels (Flahaut et al., 1996a; Sanchez et al., 2007).

In general, gram-positive bacteria seem to be more sensitive to the deleterious effects of bile than are gram-negative bacteria (Begley et al., 2005). The research of van Velkinburgh and Gunn (1999) indicated that the MIC of *Salmonella typhimurium* and *Salmonella typhi* at stationary phase in oxgall (bovine bile) were 18% and 12% (wt/vol), respectively. In another study, Jacobsen et al. (1999) showed that most *Lactobacillus* strains examined (31 of 47) did not replicate in broth supplemented with 0.3% oxgall (wt/vol). However, lactobacilli have been exhibited strain-to-strain variation in their tolerance to bile salts. According to the study

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of Margolles et al. (2003), 7 bifidobacteria showed MIC for oxgall of between 1.0 and 2.0%, whereas 12 other tested strains had MIC below 1.0%.

Lactobacillus casei Zhang, isolated from traditional fermented koumiss in Inner Mongolia, has displayed the highest resistance to bile salts among lactobacilli isolated from samples. Our previous study showed that *L. casei* Zhang could tolerate 1.5% (wt/vol) oxgall (Wu et al., 2009a). Because of its desirable probiotic properties, *L. casei* Zhang has already been identified as a new probiotic strain and is used for fermented food (Zhang et al., 2007; Ya et al., 2008; Wang et al., 2009). However, until now, little is known about the mechanism of bile salts stress response of *L. casei* from the standpoint of proteomics and transcriptomics.

In this study, we attempted to explore the changes in protein profile in the strain of *L. casei* Zhang under high bile salts stress by using the 2-DE technique, and to use real-time, quantitative reverse transcription-PCR (qRT-PCR) to confirm differential expression of proteins at the transcriptional level. The goals of this work were 1) to understand the effect of bile salts on the expression of proteins and genes in *L. casei*, 2) to determine how *L. casei* adapts to the stressful situation through regulation of proteins and genes, and 3) to provide a useful reference to reveal the molecular mechanisms of this physiological process.

MATERIALS AND METHODS

Strains and Growth Conditions

The bacterial strain used in the study was *L. casei* Zhang, obtained from traditionally homemade koumiss in Inner Mongolia of China, and subsequently identified using biochemical tests and 16S rDNA genetic analysis (Wu et al., 2009a). This strain was cultured at 37°C in De Man, Rogosa, and Sharpe (MRS) broth and stored at -80°C.

For proteomic analysis, 300 mL of fresh MRS broth with or without 1.5% oxgall (Oxoid, Hampshire, UK) were inoculated with 1.0% (vol/vol) overnight suspensions of *L. casei* Zhang (absorbance_{600nm} = 0.06), and incubated until the density at 600 nm reached 0.7 (mid-log phase, approximately 3×10^8 cfu/mL). Then, appropriate cultures were collected by centrifugation at $3,000 \times g$ at 4°C for 10 min for further analysis.

Two-Dimensional Gel Electrophoresis

The collected culture samples were ground into fine powder in the presence of liquid nitrogen. The powdered samples were suspended in cold acetone containing 10% TCA to remove acetone-soluble materials and washed

3 times with cold acetone. The lyophilized powder was suspended in lysis buffer containing 7.0 M urea, 2.0 M thiourea, 4.0% [(3-cholanidopropyl) dimethylammonio]-1-propanesulfonate, 2.0% Pharmalyte pH 3-10 (Bio-Rad Inc., Hercules, CA), 1.0 mM phenylmethylsulfonyl fluoride, 2.0 mM EDTA, and 65 mM dithiothreitol. The solution was sonicated for 5 min and then centrifuged at $35,000 \times g$ at 4°C for 30 min. Soluble proteins in the supernatant were collected, and 0.02 U/ μ L of DNase (Fermentas, Burlington, Canada) and 25 μ g/ μ L of RNase (Fermentas) were added for overnight digestion at 4°C and stored at -80°C.

Before electrophoresis, 100 μ g of protein was mixed in 350 μ L of rehydration buffer. The first dimension of electrofocusing was done with immobilized pH gradient (IPG) strips (18 cm with a nonlinear range of pH 4-7, Amersham Biosciences, Uppsala, Sweden) in an IPGphor at 20°C for 65 kV·h. Prior to the second dimensional electrophoresis, IPG strips were equilibrated with dithiothreitol and iodoacetamide. Then, the equilibrated strips were run on 12% SDS acrylamide gels (26 cm $\times$ 20 cm, Ettan DALT Twelve System, Amersham Biosciences).

Gel Image Analysis and Protein Identification

The gels were stained with silver according to the method described by Lauber et al. (2001). Gel images were acquired with an Image Scanner (Amersham Biosciences) in a transmission mode and analyzed by ImageMaster 2D Elite (version 5.0, Amersham Biosciences and Geneva Bioinformatics S.A., Geneva, Switzerland). At least 2 independent cultures from each time point were prepared, and proteins subjected to further analysis were visible in at least 2 gels under each condition. Proteins displaying at least 2.5-fold variation (volume %) were considered in the present work for statistical analysis ($P < 0.05$, Student's *t*-test).

Selected 2-DE gel spots were excised and digested by using modified trypsin solution (10 ng/mL in 25 mM ammonium bicarbonate) overnight at 37°C. The supernatant of stopped digestion was placed onto the Bruker anchor chip, mixed with the matrix solution α -cyano-4-hydroxycinnamic acid (12 mg/mL) in 70% acetonitrile with 0.1% trifluoroacetic acid, and air-dried. The sampled anchor chip was delivered to an AutoFlex MALDI-TOF/MS with the Flexcontrol 2.4 package using default parameters (Bruker Daltonics, Karlsruhe, Germany) for protein identification.

Quantitative RT-PCR

For transcriptional analysis, total RNA of *L. casei* Zhang grown in MRS with or without 1.5% oxgall at

Table 1. Information for validation of 2-dimensional electrophoresis data by real-time reverse transcription-PCR

Protein	Gene	Locus	Primer	Sequence 5' to 3'	Product (bp)
GAPDH	<i>gapdh</i>	LCAZH_0910	Sense	GGCTATCGGTTTGGTTATCC	164
			Antisense	TGTTTTCGGTGTGCTTCTTG	
DnaK	<i>dnaK</i>	LCAZH_2207	Sense	TGACGAATTGCACAAGATTAGC	192
			Antisense	GCATCCCTTCAACAACAGAGAG	
GroEL	<i>groEL</i>	LCAZH_2811	Sense	CCTGGCATTGAGAAGCAAAATA	158
			Antisense	TTGGCAGCCGATAACTACGACT	
EF-Tu	<i>tuf</i>	LCAZH_1321	Sense	ACGTTGGTGTCTTGCTTCGT	198
			Antisense	GGTGTGGTGACTACAATGGC	
6-Phosphofructokinase	<i>pfk</i>	LCAZH_1351	Sense	TGGCTTTGATACTGCCGTTAATA	175
			Antisense	TACAGTAGTAAGGTCTTGTGCTG	
Phosphoglycerate mutase 1	<i>pgm</i>	LCAZH_2094	Sense	TCAGGCTTACACCTCAGTTT	161
			Antisense	TCTTCCTTTGGCGACTATTTA	
N-Acetylglucosamine-6-phosphatideacetylase	<i>nagA</i>	LCAZH_1799	Sense	ATGGATGCTTCCTTGTGTTGAT	179
			Antisense	CAGTCTTCGGTGTGCTGTTTA	
UDP-glucose pyrophosphorylase	<i>galU</i>	LCAZH_2896	Sense	GCTTATGGCGTTATTG ACCCT	161
			Antisense	TTTCGTTACCCTTACCAGGTT	
Conserved hypothetical protein		LCAZH_0885	Sense	TTAGCTTCCAAGTACCAAACA	197
			Antisense	ACTCTCGATCATGACACCATC	
Dioxygenase	<i>pyrD</i>	LCAZH_2076	Sense	AGTTGTCGATGCGGTTGATA	160
			Antisense	GGCGTCCTTGAGTTCTGGT	
Cysteine synthase	<i>cysK</i>	LCAZH_0511	Sense	GCCGGGTGCGATTAAAAAA	157
			Antisense	AACGAAGGCGTCTGGGGTG	
S-Ribosylhomocysteinase	<i>luxS</i>	LCAZH_0709	Sense	TATTGATTGTTACCATTTCGGC	156
			Antisense	AGGGACCACAACGTAGTTCTC	
Peptidase C	<i>pepC</i>	LCAZH_2303	Sense	GACGACAAGCCATACAACCAT	159
			Antisense	CAAACCAAGCCAAGACTACAA	
Acetyltransferase, GANT family		LCAZH_0695	Sense	CCCGAGAAGATATTCCTCGC	161
			Antisense	CTGCCCATCAACGACCAAA	

mid-log phase was extracted from cells with the EZNA Bacterial RNA Kit (Omega Bio-Tek, Norcross, GA), and the genomic DNA was digested with RNase-free DNase-I (Omega Bio-Tek). For reverse transcription, 100 ng of total RNA was added to the 10-μL reaction mixture from PrimeScript RT reagent kit (TaKaRa Bio-Inc., Otsu, Shiga, Japan), containing 1 × PrimeScript buffer, 50 pmol of random 6-mer primer and 0.5 μL of PrimeScript RT Enzyme Mix I. Real-time PCR was performed with 1 μL of cDNA template, 1 × reaction buffer, 0.2 mM dNTP mix, 0.4 μM each primer, 0.6 × SYBR Green-I dye (Generay Biotech, Shanghai, China), and 0.3 U of rTaq DNA polymerase (TaKaRa Bio-Inc.), and subjected to 40 cycles of amplification at 94°C for 30 s, 63°C for 30 s, and 72°C for 30 s using the Mastercycler ep realplex system (Eppendorf, Hamburg, Germany).

The PCR primers were designed based on genomic data of *L. casei* Zhang (W. Zhang, D. Yu, Z. Sun, R. Wu, X. Chen, W. Chen, H. Meng, S. Hu, and H. Zhang; unpublished data) and targeted for PCR products of 150 to 200 bp in length (Table 1). Previously, an experiment to determine amplification efficiency had been conducted, showing that these primers were all suitable for comparative analysis based on CT value. Therefore, the 2^{-ΔΔCT} value method was used to compare the expression of the genes from different samples (Livak

and Schmittgen, 2001; Meng et al., 2007); GAPDH was used as an internal control in PCR amplification. Melting curves for each PCR were carefully monitored to avoid nonspecific amplifications. The expression level in the control culture was arbitrarily set to 1, and the expression levels in the samples from the 1.5% oxgall-culture were calculated relative to the control.

RESULTS

Proteomic Analysis of Bile Salts Stress During Growth

The cytosolic proteins of *L. casei* Zhang in MRS with or without 1.5% oxgall at mid-log phase were extracted and compared by 2-DE in an isoelectric point (pI) range of 4 to 7 (Figure 1). The number of protein spots quantified from the gels was about 509 ± 6 (control) and 512 ± 10 (1.5% oxgall).

Further analysis of protein patterns of the samples revealed that the presence of bile salts resulted in differential expression >2.5-fold of 26 spots. The results of MALDI-TOF/MS are shown in Table 2, and 19 spots were overexpressed in 1.5% bile salts, including proteins DnaK (spot 1), GroEL (spot 2), conserved hypothetical protein LCAZH_0885 (spot 3), N-acetylglucosamine-6-phosphate (NagA, spot 7), elongation

Figure 1. Two-dimensional electrophoresis gels of whole cytoplasmic proteins of *Lactobacillus casei* Zhang grown in De Man, Rogosa, and Sharpe medium with or without bile salts at 37°C (isoelectric point of 4–7). One hundred micrograms of protein extract was loaded in the first dimension. Gels were stained with silver. A) control, B) 1.5% oxgall. Arrows indicate identified proteins and correspond to spot numbers listed in Table 2. pI = isoelectric point; Mr = molecular mass.

factor Tu (**EF-Tu**, spot 8), adenylate kinase (spot 9), UDP-glucose pyrophosphorylase (**GalU**, spot 10), 30S ribosomal protein S2 (spot 13), cysteine synthase (**CysK**, spots 14, 21), phosphoglycerate mutase 1 (**PGM**, spot 15), putative NADH-flavin reductase (spot 16), saccharopine dehydrogenase related protein (spot 17), acetyltransferase (spot 18), universal stress protein related nucleotide-binding protein (**UspA**, spot 19), 6-phosphofructokinase (**PFK**, spot 22), S-ribosyl-

homocysteinase (**LuxS**, spot 24), peptidase subunit of TP-dependent protease HslVU (**ClpYQ**, spot 26), and 1 unidentified protein (spot 11).

According to Gene Ontology (GO; <http://www.ebi.ac.uk/ego/>) and Kyoto Encyclopedia of Genes and Genomes (KEGG; <http://www.genome.jp/kegg/>), the identified proteins could be grouped into categories including carbohydrate and energy metabolism (4 proteins), AA biosynthesis (2 proteins), stress proteins (3 proteins), nucleotide metabolism (1 protein), translation (1 protein), ribosomal protein (1 protein), hypothetical protein (1 protein), and proteins with unclear function (4 proteins).

The expression of 6 proteins decreased at the presence of bile salts and 5 of these were identified as the B subunit of Asp-tRNA-Asn/Glu-tRNA-Gln amidotransferase (spot 4) belonging to amino acid metabolism, NAD (FAD)-dependent dehydrogenase (spot 6) of unclear function, dioxygenase (PyrD, spot 12) involved in fatty acid metabolism, and 2 peptidases as proline dipeptidase (Pep Q, spot 23) and peptidase C (PepC, spot 25). The unidentified protein (spot 20) was detected only in the bile salts-induced bacterial cultures.

Transcriptional Expression of Genes by qRT-PCR

The bile-inducible characteristics of DnaK (LCAZH_2207; LCAZH number refers to the genomic locus of *L. casei* Zhang), GroEL (LCAZH_2811), CysK (LCAZH_0511), PFK (LCAZH_1351), PGM (LCAZH_2094), EF-Tu (LCAZH_1321), conserved hypothetical protein (LCAZH_0885), acetyltransferase (LCAZH_0695), GalU (LCAZH_2896), NagA (LCAZH_1799), LuxS (LCAZH_0709), PepC (LCAZH_2303), and PyrD (LCAZH_2076) were investigated further using real-time qRT-PCR.

Nine of 13 of these proteins (DnaK, CysK, conserved hypothetical protein, acetyltransferase, GalU, NagA, LuxS, PepC, and PyrD) showed varying expression levels with exposure to 1.0% (wt/vol) oxgall for 30 min, whereas GroEL, PFK, PGM, and EF-Tu did not change extensively (data not shown).

A single product-specific melting curve was obtained for confirmation of amplicon identities. The fold changes in gene expression are depicted in Figure 2. A gene was defined as differentially expressed if it showed intensity >1.5-fold or <0.5-fold between mid-log cells grown with or without 1.5% oxgall. The expression of the genes of conserved hypothetical protein and acetyltransferase, as well as *dnaK*, *groEL*, and *cysK*, more than doubled compared with that of the uninduced control cultures, especially *groEL*, which was 20 times higher in bacteria grown in the presence of 1.5% oxgall. Although the induction of protein expression was found

Figure 2. Confirmation of differentially expressed proteins by quantitative reverse transcription (qRT)-PCR analysis of gene transcripts produced in *Lactobacillus casei* Zhang cells grown in De Man, Rogosa, and Sharpe medium (MRS) with or without bile salts. The expression of GAPDH was used as an internal control for equal loading. (a) Panels show comparison of protein synthesis patterns of *L. casei* Zhang cultivated in MRS with (bile salts) or without (control) bile salts. (b) Primers specific for genes were used to amplify fragments by qRT-PCR. The x-axis represents gene transcription of *L. casei* Zhang cultivated in MRS with (bile salts) or without (control) bile salts and the y-axis represents the normalized-fold expression of genes. Spot numbers correspond to those listed in Table 2.

Figure 2 (Continued). Confirmation of differentially expressed proteins by quantitative reverse transcription (qRT)-PCR analysis of gene transcripts produced in *Lactobacillus casei* Zhang cells grown in De Man, Rogosa, and Sharpe medium (MRS) with or without bile salts. The expression of GAPDH was used as an internal control for equal loading. (a) Panels show comparison of protein synthesis patterns of *L. casei* Zhang cultivated in MRS with (bile salts) or without (control) bile salts. (b) Primers specific for genes were used to amplify fragments by qRT-PCR. The x-axis represents gene transcription of *L. casei* Zhang cultivated in MRS with (bile salts) or without (control) bile salts and the y-axis represents the normalized-fold expression of genes. Spot numbers correspond to those listed in Table 2.

Table 2. Identification of *Lactobacillus casei* Zhang proteins affected by bile salts exposure

Spot no. ¹	Protein	Variation rate	Theoretical Mr/pI^2	Function taxonomy	Type ³	Gene	Genomic locus
1	Molecular chaperone DnaK	4.65	67,523/4.77	Stress proteins	1	<i>dnaK</i>	LCAZH_2207
2	Molecular chaperone GroEL	2.50	57,393/4.89	Stress proteins	1	<i>groEL</i>	LCAZH_2811
3	Conserved hypothetical protein LCAZH_0885	5.28	53,562/4.99	Unknown	1		LCAZH_0885
4	Asp-tRNA-Asn/Glu-tRNA-Gln amidotransferase B subunit	3.71	53,332/5.25	Translation	2	<i>gatB</i>	LCAZH_1037
6	NAD(FAD)-dependent dehydrogenase	2.72	49,247/4.95	General function	2		LCAZH_0210
7	N-Acetylglucosamine-6-phosphate deacetylase	3.05	42,444/5.14	Carbohydrate metabolism	1	<i>nagA</i>	LCAZH_1799
8	Elongation factor Tu	3.89	43,546/4.87	Translation	1	<i>tuf</i>	LCAZH_1321
9	Adenylate kinase	3.03	19,827/6.31	Nucleotide metabolism	1	<i>adk</i>	LCAZH_2449
10	UDP-glucose pyrophosphorylase	9.41	33,798/6.11	Carbohydrate metabolism	1	<i>galU</i>	LCAZH_2896
12	Dioxygenase	5.55	34,260/5.49	Fatty acid biosynthesis	2	<i>pyrD</i>	LCAZH_2076
13	30S ribosomal protein S2	2.78	29,528/5.30	Ribosomal protein	1		LCAZH_1603
14	Cysteine synthase	4.29	32,698/5.35	AA metabolism	1	<i>cysK</i>	LCAZH_0511
15	Phosphoglycerate mutase 1	3.33	25,964/5.43	Glycolysis	1	<i>pgm</i>	LCAZH_2094
16	Putative NADH-flavin reductase	4.41	23,283/5.78	General function	1		LCAZH_1820
17	Saccharopine dehydrogenase related protein	2.62	23,276/6.30	General function	1		LCAZH_1206
18	Acetyltransferase, GNAT family	10.45	19,191/5.62	General function	1		LCAZH_1095
19	Universal stress protein UspA related nucleotide-binding protein	3.96	18,031/6.13	Stress proteins	1	<i>uspA</i>	LCAZH_1180
21	Cysteine synthase	3.03	32,698/5.35	AA metabolism	1	<i>cysK</i>	LCAZH_0511
22	6-Phosphofructokinase	2.69	34,209/5.74	Glycolysis	1	<i>pfk</i>	LCAZH_1351
23	Proline dipeptidase	2.59	40,550/4.82	Peptidases	2	<i>pepQ</i>	LCAZH_1633
24	S-Ribosylhomocysteine	8.01	17,427/5.93	AA metabolism	1	<i>LuxS</i>	LCAZH_0709
25	Peptidase C	2.64	50,626/5.09	Peptidase	2	<i>pepC</i>	LCAZH_2303
26	ATP-dependent protease HslVU (ClpYQ), peptidase subunit	2.55	18,732/5.01	Peptidase	1	<i>clpYQ</i>	LCAZH_1390
Unidentified							
11		5.71			1		
5		3.00			2		
20					3		

¹Spot numbers correspond to those marked in Figure 1.² Mr = molecular mass; pI = isoelectric point.³Protein expression patterns: 1 = upregulated >2.5-fold, 2 = downregulated, 3 = spots only exist on the map of bile salts.

over 2.5-fold, mRNA levels from *galU*, *nagA* and *luxS* in 1.5% oxgall displayed only 1.5- to 2.0-fold increase over control bacteria. The genes of proteins PyrD and PepC were clearly repressed in 1.5% oxgall compared with the control, showing similar expression levels of protein and mRNA. Moreover, the expression levels of *tuf*, *pfk*, and *pgm* were found to be no more than 1.5-fold higher compared with the control.

DISCUSSION

The relevant physiological concentrations of human bile range from 0.3 to 0.5% (wt/vol) (Zavaglia et al., 1998; Dunne et al., 1999). Oxgall (bovine bile) is commonly chosen to assess the in vitro bile tolerance of bacterial strains. Previously, low concentrations of bile salts (0.06 or 0.12%) were used to analyze induced protein fluctuations in probiotic bacteria (Sanchez et al., 2005; Lee et al., 2008). Previous research conducted in our laboratory has shown that *L. casei* Zhang has the ability to grow in 1.5% oxgall and demonstrates greater resistance to bile salts than other lactobacilli isolated from koumiss (Wu et al., 2009a). The results of electrophoresis indicated that multiple metabolic pathways might be involved in the adaptation of *L. casei* Zhang to the stress of bile salts, because the expression of 26 proteins with various functions was influenced by the presence of bile salts. Function annotation analysis showed that these proteins were involved in carbohydrate metabolism, amino acid biosynthesis, transcription and translation, general stress response, fatty acid metabolism, and nucleotide metabolism.

On the other hand, the results of the qRT-PCR experiments indicated that proteins involved in cell protection and modification of the cell membrane as well as key components of central metabolism may play an important role in bile salts resistance of *L. casei* Zhang. However, none of the putative bile salt hydrolase homologs in *L. casei* Zhang reported in previous studies (Zhang et al., 2009) showed differential expression during the bile treatments as revealed by 2-DE in this study.

Differentially Expressed Proteins Under Bile Salts Stress

General Stress Response Proteins. In this study, the increasing expression of heat shock protein DnaK (spot 1, LCAZH_1552) and GroEL (spot 2, LCAZH_2207) was found in *L. casei* Zhang during growth in the presence of 1.5% oxgall bile salts. Heat shock proteins consist of different molecular groups, including the DnaK (70 kDa) and GroEL (60 kDa) families, and are known to function as chaperones, participating in

cellular functions such as protein folding, stabilization, and turnover. Our results indicated that the stimulation of expression of heat shock proteins in *L. casei* Zhang in presence of bile salts might be related to the protection of the proteins of *L. casei* Zhang from misfolding or denaturation by stress. In previous research, the stimulation of expression of DnaK and GroEL by bile salts was reported in several gram-positive bacteria, such as *E. faecalis* and *B. longum* (Flahaut et al., 1996a; Sanchez et al., 2005; Savijoki et al., 2005).

Carbohydrate and Nucleotide Metabolism Proteins. Changes in the expression of proteins belonging to carbohydrate metabolism were found in *L. casei* Zhang cultures exposed to 1.5% oxgall, which included proteins PFK (spot 22, LCAZH_1351) and PGM (spot 15, LCAZH_2094). 6-Phosphofructokinase is the most important regulatory enzyme (EC 2.7.1.11) of glycolysis, and it converts fructose 6-phosphate and ATP to fructose 1,6-bisphosphate and ADP. Phosphoglycerate mutase 1 catalyzes the internal transfer of a phosphate group from C-3 to C-2, resulting in the conversion of 3-phosphoglycerate to 2-phosphoglycerate. Sanchez et al. (2007) proved that the changes in the expression of several enzymes, such as xylulose-5-phosphate/fructose-6-phosphate phosphoketolase (Xfp), formyl-CoA transferase, 6-phosphogluconate dehydrogenase and so on, were related to the bile salts adaptation of *B. animalis*. However, PFK and PGM were not observed to be up- or downregulated in other probiotic bacteria, probably suggesting distinctive control among bacteria.

Adenylate kinase (spot 9, LCAZH_2449), an enzyme involved in adenine synthesis and conversion of AMP to ADP (Sanchez et al., 2005), was also overproduced in the presence of 1.5% oxgall. Therefore, adenylate kinase of nuclear metabolism might work together with the glycolytic proteins PFK and PGM to regulate ATP synthesis in *L. casei* Zhang as a response to bile stress.

AA Biosynthesis Proteins. The expression of CysK (spots 14 and 21, LCAZH_0511) was enhanced more than 3.0-fold during growth of *L. casei* Zhang in 1.5% bile salts. Bile salts may cause oxidative stress through the generation of oxygen free radicals (Payne et al., 1998), and CysK was found to play a role in the protection against oxidative stress by bile salts stress (Leverrier et al., 2003). Cysteine-derived proteins, such as thioredoxin or glutathione, have the ability to maintain the reducing state in bacteria (Lechner et al., 2002; Sperandio et al., 2005). However, as in *Lactococcus lactis* and *Lactobacillus plantarum* (Bron, 2004; Sperandio et al., 2005), *cysK* is located with *metC* in *L. casei* Zhang. The mRNA expression of *metC* upon bile salts treatment in *L. casei* Zhang also demonstrated upregulation by qRT-PCR (data not shown). Thus, the *metC-cysK* operon, which takes part in cysteine and methionine

metabolism, may be related to the resistance of *L. casei* Zhang to bile salts stress. Moreover, the upregulation of LuxS (spot 24, LCAZH_0709), which was reported previously in the pathway of *L. casei* for the synthesis of cysteine from methionine (Irmeler et al., 2008), was also observed in this study. Further information is needed to study the relationship between the enzyme CysK and bile salts tolerance.

The results showed that peptidase subunit of ClpYQ (spot 26, LCAZH_1390) was induced by 1.5% oxgall, whereas 2 peptidases—Pep Q (spot 23, LCAZH_1633) and PepC (spot 25, LCAZH_2303)—were downregulated in response to bile stress. Peptidase C is a cysteine aminopeptidase, and was repressed in *L. casei* Zhang when exposed to 1.0% (wt/vol) oxgall for 30 min. Although Zaidi et al. (2008) reported that of the extensive proteolytic system of *L. lactis*, *pepC* exhibited significant repression by cholate stress, the changes of peptidases in lactobacilli have seldom been reported in previous studies.

Cell Membrane-Associated Proteins. In this study, higher inductions of GalU (spot 10, LCAZH_1073) and NagA (spot 7, LCAZH_1799) were found during the growth of *L. casei* Zhang in 1.5% oxgall. As reported by Whitehead et al. (2008), genes that provide protection in bile stress may also protect bacteria against acid stress. Our previous proteomic study of protein expression by *L. casei* Zhang in the exponential phase and stationary phase revealed that these 2 proteins were also induced by acid stress (Wu et al., 2009b).

Genomic and proteomic studies in different species have showed that bile salts are surface-active amphiphatic molecules with potent antimicrobial activity and act as detergents disrupting biological membranes (Lebeer et al., 2008). Uridine diphosphate-glucose pyrophosphorylase catalyzes the formation of UDP-glucose, a precursor of membrane glycolipids and cell envelope teichoic acids (Grundling and Schneewind, 2007). As pointed out by Nesper et al. (2001), *galU* mutants of *Vibrio cholerae* showed much higher sensitivity than wild-type strains to bile salts while demonstrating a reduced ability to colonize the mouse small intestine. N-Acetylglucosamine-6-phosphate deacetylase catalyzes the first committed step in the biosynthetic pathway for the amino-sugar precursors required for cell wall peptidoglycan and teichoic acid biosynthesis (Vincent et al., 2004). Our results implied that *galU* and *nagA* are crucial for the resistance of *L. casei* Zhang to bile salts, probably because of its diverse links with pathways involved in morphogenesis.

Furthermore, dioxygenase (spot 12, LCAZH_2076) in fatty acid metabolism was downregulated for *L. casei* Zhang when growing in 1.5% bile salts. It is known that bile secreted in the small intestine has the ability to

reduce the survival of bacteria by destroying lipids and fatty acids of cell membrane (Gilliland et al., 1984). Previous transcriptome studies with *B. animalis* ssp. *lactis* BB-12 indicated that several genes involved in fatty acid biosynthesis were downregulated in the presence of bile salts (Garrigues et al., 2005). Proteomic analysis of *B. animalis* demonstrated that one long-chain fatty acid-CoA ligase was downregulated in the presence of bile salts (Sanchez et al., 2007). Thus, the downregulation of dioxygenase in *L. casei* Zhang might be connected to bile salts stress.

Transcription and Translation Proteins. In this study, upregulation of EF-Tu (spot 8, LCAZH_1321) was observed during the growth of *L. casei* Zhang with 1.5% oxgall. Elongation factor EF-Tu, which is involved in the delivery of aminoacyl-tRNA to the ribosome, is thought to be implicated in protein folding and protection from stress in *Escherichia coli* (Caldas et al., 1998). In *Campylobacter jejuni* (Fox et al., 2007), EF-Tu appeared to undergo significant modifications at 5% (wt/vol) bile salts, but not in the presence of 2.5% bile salts. Similar results were found in this study, and the expression level of EF-Tu of *L. casei* Zhang was unchanged when exposed to 1.0% oxgall for 30 min (data not shown). Thus, the effect of bile salts on EF-Tu in *L. casei* Zhang might depend on the concentration of bile salts and the exposure time.

In addition, upregulation of 30S ribosomal protein S2 (spot 13, LCAZH_1603), a component of the 70S ribosomal protein complex, was observed, suggesting that ribosomal proteins may play an important role in the cellular response to bile stress conditions. Previous studies demonstrated that some ribosomal proteins were induced in response to bile treatment of *B. animalis*, *L. reuteri* (Sanchez et al., 2005; Lee et al., 2008), and other gram-positive bacteria (Duché et al., 2002).

Verification of Gene Expression

The measurements of mRNA and protein expression levels are complementary and both are necessary for a complete understanding of how the cell works (Hatzi-manikatis et al., 1999). Because of the specificity and sensitivity of transcript detection, qRT-PCR has also been used extensively for verification of differential expression proteins or genes (Sharkey et al., 2004). Quantitative reverse transcription-PCR has been conducted to verify a novel bile-inducible operon encoding a 2-component regulatory system in *Lactobacillus acidophilus* (Pfeiler et al., 2007) and investigate the gene expression levels of stationary-phase and nutrient-starved *L. plantarum* WCFS1 (Marco and Kleerebezem 2008).

In this study, we performed a qRT-PCR analysis of important genes identified by 2-DE to confirm their relative expression levels before and after bile salts stress. From the qRT-PCR results, most of the proteins demonstrated consistent changes compared with 2-DE, including GroEL, DnaK, CysK, LuxS, acetyltransferase, GalU, NagA, PepC, and PyrD. Yet the expression of the *tuf*, *pfk*, and *pgm* genes were virtually unchanged under 1.5% oxgall stress. The discrepancy between protein and gene expression analysis has been reported in other studies (Piuri et al., 2003). This limited predictive value was recognized in later studies and is explained partly by fundamental biological differences between the transcription and translation processes (Hegde et al., 2003), such as the more immediate alterations at the transcriptional level than at the translational level (Wang et al., 2007). Thus, mRNA of *tuf*, *pfk*, and *pgm* of *L. casei* Zhang might be more easily degraded than protein under bile salts stress; this aspect will be further investigated in our research.

CONCLUSIONS

The study provides the first insight into the proteomic information of *L. casei* growing in high concentrations of bile salts. Through the 2-DE method, we found that expression levels of 26 proteins were acutely stimulated and regulated by factor of bile salts. Further protein identification through MALDI-TOF and qRT-PCR analysis showed that a complicated control system was involved in resistance to bile salts stress in *L. casei* Zhang. Several crucial variations in cell protection, cell membranes, and key components of central metabolism were observed. The study provides potentially powerful data about the metabolic processes of the cells under bile salts stress and may help in understanding the response to bile salts of this new probiotic bacterium.

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