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Effect of Transient Acid Stress on the Proteome of Intestinal Probiotic *Lactobacillus reuteri*

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Abstract—We report the acid tolerance response and changes in the level of protein expression of probiotic *Lactobacillus reuteri* subjected to transient (1.5 h) acid stress at pH 3.0. Sixteen acid-responsive proteins were identified by peptide mass fingerprinting including members of five broad functional categories: metabolism, transcription/translation, DNA replication/repair, transport and binding proteins, and pH homeostasis and stress responses. This work can provide some new and relevant information on the inducible mechanisms underlying the capacity of probiotic *L. reuteri* to tolerate acid stress.

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Probiotics are health-promoting bacteria that, when supplied in sufficient quantities, can confer direct benefits to human and animal hosts through enhancement of intestinal barrier function, modulation of immune response, and antagonism of pathogens. The ability of bacteria to survive passage through the human and animal gastrointestinal tracts is key to their ability to function as a probiotic [1]. To date, several genera have been identified as having probiotic activity; among them, the lactobacilli are the largest group of probiotic bacteria in the intestine [1-3]. The present study examines the acid-stress tolerance of *Lactobacillus reuteri* that is a resident of the human and animal gastrointestinal tracts and has been suggested to possess probiotic activity [4, 5]. When probiotic strains such as *Lactobacillus* enter the gastrointestinal tracts, they are exposed to acidic pH. Acid stress has been shown to disturb bacterial growth and survival; thus, a successful probiotic must adapt to acid stress. This often occurs through protective mechanisms that help maintain various bacterial cell structures and functions, as well as assisting in the protection and/or repair of cytoplasmic proteins and DNA. Such physiological changes should be accompanied by corresponding changes in gene expression, protein expression, and enzyme activity.

Recent comparative genomic analyses of *Lactobacillus* species provide a framework for elucidating their physiological responses to various conditions through examination of their global mRNA expression patterns (transcriptomes) [6, 7]. To gain additional insight into the molecular mechanisms underlying a given response, global protein expression patterns (proteomics) can be studied to provide additional information beyond that obtained from transcriptomics [8-10]. Recent advances in 2-DE and mass spectrometry can be used to support high-resolution protein analysis and quantification, while progress in genome sequence analysis allows for good functional annotation of many identified proteins [11, 12]. Thus, proteomics offers a comprehensive approach for identifying proteins involved in the acid response. However, prior studies on this matter have been performed primarily in a particular *Lactobacillus* strain [13-15].

Here, a strain of *L. reuteri* grown at pH 6.8 was transiently acid-shocked at pH 3.0 for 1.5 h, and the protein expression profiles of stressed cells were compared. We

Abbreviations: IEF, isoelectric focusing; IPG, immobilized pH gradient; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry; MRS, DeMan–Rogosa–Sharpe medium; PMF, proton motive force.

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employed 2-DE coupled with peptide mass fingerprinting to identify proteins commonly involved in the acid-stress responses of *L. reuteri*. Our findings provide new insight into cellular events occurring under acid conditions, and can improve our understanding of the molecular mechanisms through which *L. reuteri* adapts to the gastrointestinal tracts to function as a probiotic bacterium.

MATERIALS AND METHODS

Bacterial strain and culture condition. *Lactobacillus reuteri* (ATCC 23272) was grown in DeMan–Rogosa–Sharpe (MRS) broth (Difco Laboratories, USA) at constant pH of 6.8 ± 0.1. For 2-DE, precultures were grown overnight at 37°C with shaking in MRS medium, and the precultures were used to inoculate 200 ml of culture medium (initial A_{600} = 0.1). These cultures were incubated until they reached the mid-exponential growth phase (A_{600} ~ 0.6). For acid shocks, cells were harvested by centrifugation (10,000*g*, 10 min, 4°C) and then resuspended to A_{600} ~ 0.6 in fresh low-pH MRS medium previously adjusted to pH 3.0 using hydrochloric acid for acid-stress responses. Control cells were resuspended in fresh medium at pH 6.8 (non-acid-shock cells).

Sample preparations for two-dimensional electrophoresis. After incubation at 37°C for 1.5 h, control and acid-shocked cells were harvested by centrifugation and washed with 100 mM Tris-HCl, pH 7.5. The resulting cell pellets were resuspended in 1 ml lysis buffer (62.5 mM Tris-HCl, pH 6.8), and the cells were disrupted by ultrasonication (Cole-Parmer (USA) sonifier, 4 bursts, 20 sec each). Unbroken cells were removed by centrifugation (10,000*g* for 10 min at 4°C), and the protein content of the supernatant was measured using a BCA protein assay kit (Pierce, USA), with bovine serum albumin used as the standard. The isolated proteins were stored at –80°C and resuspended in a rehydration solution prior to electrophoresis.

2-DE and image analysis. The proteins were subjected to electrophoretic separation. Briefly, samples were diluted into isoelectric focusing (IEF) buffer containing 8 M urea, 2 M thiourea, 4% w/v CHAPS, 0.5% v/v carrier ampholyte (pH 4-7), 1% w/v dithiothreitol, and a trace of bromophenol blue to yield the desired protein amount (40 μg) in a volume that could be adsorbed by the pH 4-7 IPG (7-cm long; Pharmacia Biotech, Sweden). The diluted samples were used to rehydrate immobilized pH gradient (IPG) strips at room temperature for 12 h at 50 V. IEF was performed using the Protean® IEF Cell (Bio-Rad, USA) and its 7-cm IEF tray. After rehydration, the following voltage program was applied: a linear ramp to 250 V over 15 min, followed by a linear ramp to 4000 V over 2 h, and then a consistent 4000 V for 5 h, for a total of 24,000 V·h. Focused strips were stored at –80°C before equilibration and application to SDS-polyacrylamide gel. The IPG strips were equilibrated first in 6 M urea, 50 mM Tris-HCl, pH 8.0, 30% glycerol, 2% SDS, and 30 mM dithiothreitol for 10 min followed by 6 M urea, 50 mM Tris-HCl, pH 8.0, 30% glycerol, 2% SDS, and 5% iodoacetamide for 10 min. The IPG strips were then embedded in hot 0.5% agarose (approximate 70°C) containing bromophenol blue and laid on top of 10% Tris-Tricine-polyacrylamide gels. The two-dimensional (2D) SDS-PAGE was run for 2.5 h at 50 mA/gel (constant setting) with external cooling, until the tracking dye migrated to within 0.5 cm of the bottom of the gel. Upon completion of 2D SDS-PAGE, the gels were silver stained as previously described [16], and stained protein spots were scanned on a GS-800 calibrated densitometer (Bio-Rad). The 2D gels were calibrated using 2D SDS-PAGE standard (Bio-Rad). Gels were run in triplicate, and spots that appeared consistently in all three runs were selected for analysis. Spot detection and analysis was performed using the PDQuest version 8.0.1 software (Bio-Rad).

MALDI-TOF mass spectrometric analysis and protein identification. For protein identification, spots were excised from the gels and subjected to *in situ* digestion with trypsin as described previously [17]. The digested supernatant fluid was mixed with MALDI matrix (α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% trifluoroacetic acid) and spotted onto an MTB AnchorChip TM 600/384 MALDI plate (Bruker Daltonics, Germany), and peptide masses were determined using a matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometer (Bruker Daltonics). Calibration was carried out based on the internal mass of trypsin. Peptide masses were matched with the theoretical peptides of all lactic acid bacteria proteins in the NCBI database, using the MASCOT software and the MS-Fit software of Protein Prospector (website: http//prospector.ucsf.edu). The peptide mass fingerprint search included all *Lactobacillus* species and other related species of lactic acid bacteria.

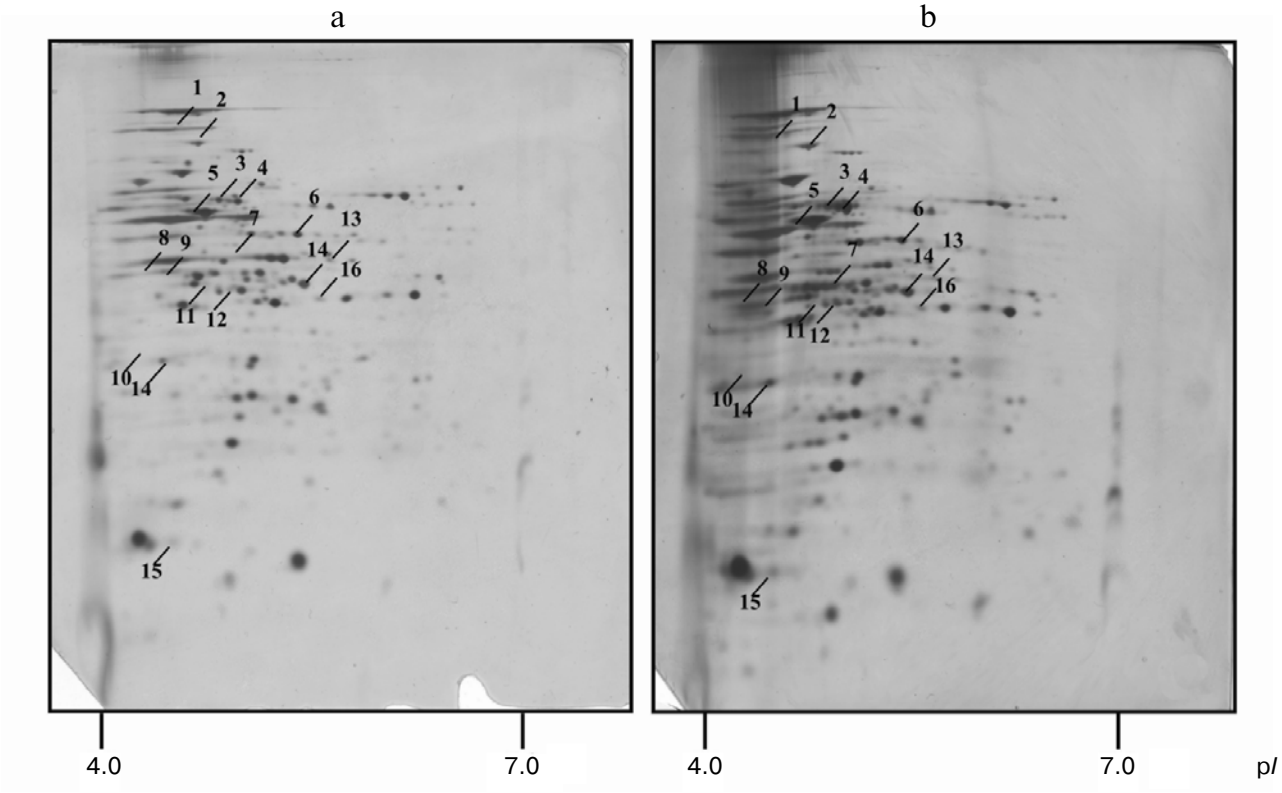
RESULTS AND DISCUSSION

Lactobacillus species are microorganisms whose growth is hindered by lactic acid, which acidifies the medium to pH of less than 3.0. Below pH 3.0, the bacteria begin to die, with different strains showing different capacities to resist lethal acid stress [18]. To investigate metabolic adaptation for *L. reuteri* survival and its physiological activity, the proteome of *L. reuteri* under transient (1.5 h) acid stress from pH 6.8 to 3.0 was studied. We chose the time of mid-exponential growth phase to harvest the cells for acid-shock experiment. Within 1.5 h after being acid-shocked at pH 3.0, the *L. reuteri* stopped growing and resumed growing at a reduced rate after 60 min (data not shown), perhaps indicating that at this

time bacterial cells begin to synthesize different proteins and other components, thereby leading to more resistance to the low pH conditions.

To identify some proteins that can be induced by acid shock in *L. reuteri*, we compared the proteomes of *L. reuteri* using 2-DE with a linear gradient of p*I* in the range 4-7. The cell extracts obtained after a transient pH downshift demonstrated the differential protein synthesis of *L. reuteri* (figure). The total number of protein spots on 2D gels detected after culture at pH 6.8, and after the strong acid shock at pH 3.0 was 383 and 539, respectively. Interestingly, expression levels and sequence information are provided for 16 proteins that were differentially expressed in acid-shocked cells grown at pH 3.0 compared to control cells (pH 6.8). To gain a better understanding of the adaptation of *L. reuteri* under acid-shock stress, we used MALDI-TOF MS to identify these 16 acid-responsive proteins and performed database searches to assign the identified proteins to their relevant functional categories (table). The 16 identified acid-responsive proteins included proteins involved in energy metabolism (one protein), nucleotide metabolism (one protein), coenzyme metabolism (one protein), transcription/translation (two proteins), DNA replication/repair (two proteins), transport and binding protein (two proteins), amino acid transport/metabolism (three proteins), and pH homeostasis and stress (one protein). There were also three proteins of unknown function.

In the intestinal tract, lactic acid bacteria are subjected to several environmental stresses, one of which is acid, which leads to a gradual reduction of cell growth. Intestinal enteric bacteria have developed many different defense strategies including the synthesis of survival-promoting proteins. One protein involved in glycolysis was highly induced in cultures exposed to transient (1.5 h) acid stress from pH 6.8 to 3.0, namely phosphoglycerate kinase. Under acidic conditions bacteria are known to undergo show changes in the glycolytic enzymes [19]. In the present work, we observed that an enzyme that is involved in intermediate carbon metabolism was overexpressed after acid-shock stress. Glucose transport in *L. reuteri* is proton motive force (PMF)-dependent [20], and the dissipation of the PMF by protons reduces glucose uptake. Consequently, the lower intracellular pH can interfere with essential enzyme reactions and PMF-dependent nutrient uptake is likely to be hindered, resulting in cell death [21]. Hence a higher energy yield from glucose metabolism is expected to provide acid tolerance of *L. reuteri* against low pH, helping cells to maintain their intracellular pH during acidification. We observed that glycolytic enzyme is typically present at high levels in living bacterial cells under low pH condi-



Silver-stained 2-DE gels (pH range 4-7) comparing total cellular proteins from *L. reuteri* exposed to transient low pH stress. Shown are 2-DE gels of proteins from bacteria grown at pH 6.8 (control) (a) or grown at pH 6.8 and then grown for 1.5 h in media of pH 3.0 (b). Spot numbers 1-16 represent proteins exhibiting differential expression in response to changes in pH

Biochemical characteristics of the 16 proteins identified as being differentially expressed in *L. reuteri* cultured under acid stress (pH 3.0)

Functional category	Spot number	Protein identification	p*I* observed/ calculated	Molecular mass observed/ calculated (kDa)	Accession number	Sequence coverage*	Expression level**
Energy metabolism	5	phosphoglycerate kinase	4.50/5.18	49.3/43.74	Q5NJY9_LACPL	39	+1.71
Nucleotide metabolism	6	CTP synthase	5.2/5.03	52.5/62.31	Q1WV30	12.6	+1.06
Coenzyme metabolism	3	pyridoxal biosynthesis pdxS lyase	4.70/5.01	54.40/34.40	PDXS_BIFLO	32	0.36
Transcription/ translation	1	isoleucyl-tRNA synthetase	4.40/4.81	73.70/72.70	Q93CY6_LACSK	19.1	+3.81
	4	prolyl-tRNA synthetase	4.80/5.18	53.80/59.83	Q038L9	15.0	+5.34
DNA replication/repair	9	chromosomal replication initiator protein DnaA	4.30/5.73	30.80/57.02	DNAA_LACAC	12.0	+2.64
	2	UvrABC system protein B	4.60/5.10	66.30/71.60	Q1WT70_LACS1	21.2	+2.19
Transport and binding proteins	15	ABC transporter related protein	4.30/5.01	12.70/30.75	Q1U857_LACRE	51	+1.35
	7	multidrug resistance ABC transporter ATP-binding and permease protein	4.80/5.93	35.40/44.79	Q9CHL8	14.6	+4.08
Amino acid transporter/ metabolism	12	cysteine synthase	4.80/4.84	30.90/39.51	A8YVP6_LACH4	62	+2.41
	14	diaminopimelate epimerase	4.50/4.49	23.70/30.24	Q8G7Z7_BIFL0	26	+4.23
	13	3-isopropylmalate dehydrogenase	5.30/5.57	36.80/37.68	LEU3_LACLA	29	+1.59
pH homeostasis and stress	16	ornithine carbamoyl transferase	5.10/5.62	30.40/43.0	Q03NY9	54	+1.20
Unassigned	8	unknown	4.30/4.70	29.90/38.70	–	19.4	+4.07
	11	N(5)-(carboxyethyl)-ornithine synthase	4.80/5.77	29.80/37.37	CEO2_LACLA	15	0.66
	10	UPF0246 protein LJ_0535	4.10/6.85	23.50/29.23	Y535_LACJO	21	+1.56

* Percentage amino acid sequence coverage.

** Average expression level in cultures grown at pH 3.0 compared to cultures at grown at pH 6.8. Expression level in control cultures is set at 1. All values are positive and lie between 0 (expression suppressed completely) and infinity.

tions, where it functions to supply energy essential for cell functions and growth. Wilkins et al. [22] demonstrated that most enzymes converting phosphoglycerate and pyruvate intermediates are highly stimulated by low pH conditions, and these glycolytic enzymes might be needed to efficiently protect the cells from lethal levels of acid (pH 2.0-3.0).

In response to strong acid stress of *L. reuteri*, we also observed upregulation of proteins involved in a nucleotide biosynthesis, including CTP synthase, which is involved in

cytosine production from pyrimidine and is essential for growth. Consistent with the acid stress-related upregulation of CTP synthase observed in this study, a previous study showed that CTP synthase is upregulated during acid stress of *Lactococcus lactis* MG 1363 [23]. Moreover, we identified acid stress-induced upregulation of cysteine synthase, which is involved in regulating amino acid metabolism. Cysteine is an essential amino acid that performs vital functions in the catalytic activity and structure of many proteins. A previous finding that a defect in cysteine biosynthesis led to impaired protein synthesis [24] might suggest that this mechanism could be responsible for the loss of viability in acid-stressed cultures of *L. reuteri*.

We further identified acid stress-induced upregulation of four proteins involved in transcription/translation and DNA replication/repair (prolyl-tRNA synthetase, isoleucyl-tRNA synthetase, DnaA, UvrABC system protein B). The first two are aminoacyl-tRNA synthetases (ARSs), which are responsible for translating the genetic code by adding amino acids to their cognate transfer RNAs (tRNA). The aminoacylated tRNAs can then be used by the ribosome to decode mRNA [25]. Interestingly, prolyl-tRNA synthetase and isoleucyl-tRNA synthetase, which are essential for protein synthesis and subsequent bacterial growth, were upregulated in *L. reuteri* cultures exposed to low pH. This observation was consistent with similar findings by Wall et al. [26] in *L. reuteri*. In addition, we observed that exposure to strong acid stress was associated with overexpression of DnaA, which is involved in DNA replication. This enzyme is a sequence-specific DNA-binding protein that plays a key role in the initiation of chromosomal DNA replication *in vivo* and *in vitro* [27], and it functions as an activator or a repressor of transcription of many genes, including those in the *ori* region and the *dnaA* gene itself [28]. Interestingly, acid stress was found to trigger upregulation of the DnaA protein in *L. reuteri* cultures exposed to low pH. Although additional work will be required to examine how these changes in DnaA protein levels affect DNA content, origin per cell distributions, and initiation mass, our results might suggest that the additional DnaA protein synthesized in *L. reuteri* at low pH is active for initiation of chromosome replication. We observed significant upregulation of UvrABC system protein B in acid-stressed *L. reuteri*. The function of this protein suggests that UvrB probes one DNA strand for the presence of a lesion [29-32]. Since this protein related to DNA damage is upregulated in *L. reuteri* at low pH, this might suggest that this strain shows acid sensitivities to strong acid conditions. Additional work will be required to determine the mechanism(s) underlying these effects.

Proteomic analysis revealed the acid-induced overexpression of two proteins involved in the transport system (ABC transporter, multidrug resistance ABC transporter ATP-binding and permease protein). The ABC transport system forms a superfamily of diverse membrane proteins (domains) including an extracellular substrate-binding protein, two ATP-binding/hydrolyzing subunits, and two integral membrane subunits [33]. The extracellular substrate-binding protein plays a role in binding sugars, amino acids, minerals, etc. After binding, the extracellular substrate-binding protein complex connects with integral membrane protein subunits, and the ATP-binding/hydrolyzing subunits supply this process with energy (ATP) to fuel the transport of solutes across the cell membrane [34]. The expression of ABC transport system proteins was observed when the yeast *Saccharomyces cerevisiae* was treated with ethanol or weak acids [35]. The acid-induced overexpression of the ABC transporter observed herein suggests that *L. reuteri* increases its uptake of metabolic excretion products, which are essential for organisms to thrive in nutrient-poor environments. Moreover, we identified acid stress-induced overexpression of multidrug resistance ABC transporter ATP-binding and permease protein, which is a component of the multidrug resistance system responsible for allowing bacterial survival in the presence of antibiotics and other cytotoxic drugs [36]. In *Lactococcus lactis*, three toxin excretion systems have been characterized: cationic toxins can be excreted by a toxin/proton antiport system and by an ABC-transporter [37]. Our observation that multidrug resistance ABC transporter ATP-binding and permease protein is overexpressed in acid-stressed *L. reuteri* seems to indicate that induction of the multidrug resistance system is not specific for elimination of toxic metabolites derived from antibiotics and cytotoxic drugs [37], but is also responsive to acid stress. This multidrug resistance ABC transporter ATP-binding and permease protein might contribute to proton export systems generation and maintain the intracellular pH.

We further found a protein involved in pH homeostasis. Exposure to acid stress was associated with overexpression of ornithine carbamoyl transferase. This enzyme, part of the arginine deiminase pathway, contributes to PMF generation and elevates extracellular pH to compensate for acid stress [38]. Finally, we observed acid-related expression changes in proteins lacking good functional annotation. Future work will be required to clarify the role of these unknown proteins in the acid-stress response.

Thus, this study provides some new and relevant information and adds to our understanding of *L. reuteri* adaptation under acid-shock stress. However, this strain needs to be studied further using a combination of proteomics and transcriptomics with regard to its biological functions and mechanisms under acid-shock stress.

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