Proteomic Profiling of the Acid Stress Response in Lactobacillus plantarum 423

ARTICLE *in* JOURNAL OF PROTEOME RESEARCH · JULY 2014

Impact Factor: 4.25 · DOI: 10.1021/pr500353x · Source: PubMed

4 AUTHORS:

Tiaan Heunis
Stellenbosch University
10 PUBLICATIONS **112** CITATIONS
SEE PROFILE

Shelly M Deane
Stellenbosch University
30 PUBLICATIONS **553** CITATIONS
SEE PROFILE

Salome Smit
Anatech
7 PUBLICATIONS **50** CITATIONS
SEE PROFILE

Leon M.T. Dicks
Stellenbosch University
203 PUBLICATIONS **5,057** CITATIONS
SEE PROFILE

Journal of proteome research

Article

pubs.acs.org/jpr

Proteomic Profiling of the Acid Stress Response in *Lactobacillus plantarum* 423

Tiaan Heunis,[†,∥] Shelly Deane,[†] Salome Smit,[‡,§] and Leon M. T. Dicks*[,†]

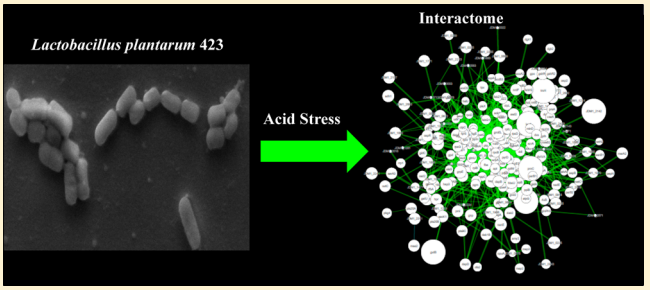
[†]Department of Microbiology, Stellenbosch University, Private Bag X1, 7602 Matieland, South Africa

[‡]Division of Molecular Biology and Human Genetics, Stellenbosch University, Francie van Zijl Drive, Tygerberg 7505, South Africa

[§]MS Unit, Proteomics lab, Central Analytical Facility, Stellenbosch University, Private Bag X1, 7602 Matieland, South Africa

Supporting Information

ABSTRACT: Acid tolerance is considered an important characteristic of probiotic bacteria. *Lactobacillus plantarum* 423 tolerates acidic pH and is the ideal candidate in which to study molecular mechanisms that acid-tolerant lactic acid bacteria employ to survive such conditions. In this study we recorded changes in the protein profile of *L. plantarum* 423 when exposed to pH 2.5 by using a gel-free nanoLC−MS/MS proteomics approach. In total, 97 proteins were detected as more abundant, and 12 proteins were detected solely when strain 423 was exposed to pH 2.5. General stress response proteins, the utilization of a variety of carbohydrate sources in a glucose rich environment, altered pyruvate metabolism, increased lysine biosynthesis, and a significant oxidative stress response was observed in acid-stressed cells. The accumulation of basic compounds also seemed to play an integral role in the response to acid stress. We observed a marked decrease in proteins involved in cell wall and phospholipid biosynthesis, transcription, translation, and cell division. The most abundant protein detected was an uncharacterized protein, JDM1_2142. Functional analysis revealed that this protein plays a role in survival during acid stress. Our results contribute to the growing body of knowledge on the molecular mechanisms employed by lactobacilli, in particular *L. plantarum*, to ensure survival in acidic conditions.



KEYWORDS: *Probiotic, Lactobacillus plantarum, acid stress, gel-free shotgun nanoLC−MS/MS proteomics*

INTRODUCTION

Lactobacillus plantarum is a versatile lactic acid bacterium capable of occupying a variety of niches and is a common commensal in the human gastrointestinal tract (GIT).[1] Some probiotic strains of *L. plantarum* alleviate symptoms associated with irritable bowel syndrome, exhibit beneficial immunomodulatory activity, and reduce colonization by *Clostridium difficile*.[2−4] It is thus no surprise that *L. plantarum*, among other lactobacilli, are commonly found in various commercially available products marketed as probiotics and functional foods.

The high acidity of the stomach is believed to suppress microbial colonization, prevent overgrowth, and pose a major stress on microorganisms during GIT transit. Certain microorganisms have, however, developed various mechanisms to ensure survival in acidic environments. Mechanisms utilized by Gram-positive bacteria include maintenance of intracellular pH (pHi) homeostasis with F_1F_0-ATPase proton pumps, action of glutamate decarboxylases and arginine deiminases, repair of damaged DNA and proteins, cell envelope changes, and altered metabolism.[5,6] It is of utmost importance that probiotic organisms survive transit through the hostile environment of the GIT to exert a beneficial effect on the host, as probiotics are regarded as live microorganisms. Acid tolerance is thus considered a vital probiotic trait and is commonly used in screening for new probiotic strains.

Proteomics has extensively aided our understanding of several probiotic traits and has facilitated the elucidation of underlying molecular mechanisms involved.[7] Mechanisms employed by probiotic lactobacilli and bifidobacteria to survive acidic conditions have also been studied using proteomics, and the data generated has shed some light on survival mechanisms in these two genera.[8−13] However, most proteomic studies investigating acid stress in lactobacilli use two-dimensional gel electrophoresis (2-DE)-based systems, and few differentially expressed proteins are usually detected. Thus, some of the molecular mechanisms involved in acid tolerance/survival are still poorly understood on a global level, and in-depth analysis of the "acid stress proteome" is needed to elucidate these mechanisms.

L. plantarum has intrinsically high acid tolerance and is the ideal species in which to study acid survival strategies.[14] In this study we aimed to gain insight into, and contribute knowledge to, the mechanisms employed by *L. plantarum* to survive

Received: April 11, 2014
Published: July 17, 2014

dx.doi.org/10.1021/pr500353x | *J. Proteome Res.* 2014, 13, 4028−4039

extreme acid challenge by using a gel-free nanoLC−MS/MS proteomics approach.

MATERIALS AND METHODS

Bacterial Culture and Growth Conditions

Lactobacillus plantarum 423[15] cultures were maintained in glycerol at −80 °C. *L. plantarum* 423 was streaked onto MRS (de Man, Rogosa and Sharpe) agar plates, MRS broth supplemented with 2% (w/v) agar (Biolab Diagnostics), and incubated at 37 °C for 48 h. Three colonies from this plate were inoculated, separately, into 5 mL of MRS broth and incubated at 37 °C for 24 h. These cultures were suspended into 200 mL of MRS broth, at 1% (v/v), and cultured for 12 h ($OD_{600nm} \approx 8.0$) to reach the onset of stationary phase. The cells were harvested (8 000*g*, 15 min, 4 °C) and resuspended in 10 mL prewarmed MRS broth. Five milliliters of the cell suspension was subsequently inoculated into 100 mL of MRS broth (pH 6.4) and 5 mL into 100 mL of MRS broth, adjusted to pH 2.5 with HCl. The media was prewarmed to 37 °C before resuspension of the cultures. The cultures were incubated at 37 °C for 1 h, harvested (8 000*g*, 15 min, 4 °C), and washed twice with sterile saline (0.85% w/v NaCl, prepared with double distilled water). Cell pellets were stored at −80 °C until further use.

Sample Preparation and Protein Extraction

Cell pellets were suspended in lysis buffer (7 M urea, 2 M thiourea, 50 mM DTT, and 4% w/v CHAPS), containing a bacterial protease inhibitor cocktail (Sigma-Aldrich). Cells were disrupted with glass beads (0.5 mm) using a vortex on maximum speed for 7 min, kept on ice for 1 min, and vortexed for another 7 min. The lysate was centrifuged (12 000*g*, 15 min, 25 °C), the supernatant transferred to a new microfuge tube and centrifuged for a further 15 min (12 000*g*, 25 °C) to remove cellular debris. The protein concentration in the cell-free extract was determined using a 2D Quant kit (GE Healthcare, Life Sciences), according to the instructions provided by the manufacturer.

Filter-Aided Sample Preparation (FASP)

Protein samples (10 μg) were mixed in a ratio of 5 μL of sample to 45 μL of SDT lysis buffer (4% w/v SDS, 100 mM Tris-HCl pH 7.6 and 100 mM DTT added just before use). The 50 μL sample was mixed with 150 μL of UA buffer (8 M urea and 100 mM Tris-HCl, pH 8.5), placed on an Amicon ultra 0.5 centrifugal filter with a cutoff of 30 kDa (Millipore) and centrifuged (14 000*g*) for 15 min. An additional 200 μL of UA was added to the filter, followed by a further 15 min of centrifugation (14 000*g*). The proteins were alkylated by 100 μL of 50 mM iodoacetamide in UA and kept in the dark for 20 min. The proteins were collected by centrifugation (14 000*g*, 10 min). To each sample 100 μL of UB (8 M urea and 100 mM Tris-HCl, pH 8.0) was added and centrifuged (14 000*g*, 10 min) and repeated once more. After centrifugation, 100 μL of a 50 mM ammonium bicarbonate solution was added, the sample was centrifuged (14 000*g*, 10 min), and this process was repeated once more. The proteins were digested with 40 μL of a 100 ng/μL trypsin solution, at 37 °C for 17 h in a wet chamber. The next day the filter was placed in a clean microcentrifuge tube and centrifuged (14 000*g*, 10 min), followed by the addition of 40 μL of a 0.5 M NaCl solution and another centrifugation step (14 000*g*, 10 min). The solution was acidified by the addition of 4 μL of formic acid. The filtrate was desalted using C18 StageTips; the desalted solution was dried *in vacuo* and then stored at −20 °C. Dried peptides were dissolved in 5% (v/v) acetonitrile in 0.1% (v/v) formic acid, and 10 μL injections were made for nano-LC chromatography.

Mass Spectrometry

A total of 1 μg of peptide mixture was used for mass spectrometry. All experiments were performed on a Thermo Scientific EASY-nLC II connected to a LTQ Orbitrap Velos mass spectrometer (Thermo Scientific) equipped with a nanoelectrospray source. For liquid chromatography, separation was performed on an EASY-Column (2 cm, ID 100 μm, 5 μm, C18) precolumn, followed by separation on a XBridge BEH130 NanoEase column (15 cm, ID 75 μm, 3.5 μm, C18) with a flow rate of 300 nL/min. Solvent A was 100% water in 0.1% formic acid, and solvent B was 100% acetonitrile in 0.1% formic acid. The gradient used was from 5 to 8% B in 5 min, 8−40% B in 270 min, 40−60% B in 10 min, 60−80% B in 5 min, and 80% B for 10 min. The mass spectrometer was operated in data-dependent mode to automatically switch between Orbitrap-MS and LTQ-MS/MS acquisition. Data were acquired using the Xcalibur software package. The precursor ion scan MS spectra (m/z 400−2000) were acquired in the Orbitrap with resolution $R = 60\,000$ with the number of accumulated ions being 1×10^6. The 20 most intense ions were isolated and fragmented in the linear ion trap (number of accumulated ions 1.5×10^4), using collision induced dissociation (CID). The lock mass option (polydimethylcyclosiloxane; m/z 445.120025) enabled accurate mass measurement in both the MS and MS/MS modes. In data-dependent LC−MS/MS experiments, a CID energy of 35% and dynamic exclusion with 60 s exclusion duration was used. Mass spectrometry conditions were 1.8 kV and capillary temperature of 250 °C, with no sheath and auxiliary gas flow. The ion selection threshold was 500 counts for MS/MS and an activation Q-value of 0.25 and activation time of 10 ms were applied for MS/MS.

Data Analysis

MaxQuant 1.2.2.5[16] was used to identify proteins via automated database search of all tandem mass spectra against the *L. plantarum* JDM1 database (CP001617). Carbamidomethyl cysteine was set as fixed modification, and oxidized methionine, *N*-acetylation, and deamidation (NQ), Pyro-Gln and Pyro-Glu, were used as variable modifications. The precursor mass tolerance was set to 20 ppm, and fragment mass tolerance was set to 0.8 Da. Two missed tryptic cleavages were allowed. Proteins were considered positively identified when they were identified with at least 1 unique tryptic peptide per protein and protein and peptide false discovery rate (FDR) of less than 0.01 (Supplementary Table 1). A label-free approach was followed during data analysis using MaxQuant,[16] and the LFQ values assigned were used for statistical analysis using Perseus, as part of MaxQuant. Data was transformed (log 2) and imputated, and subsequent two sample ANOVA testing was performed using p-value with threshold of 0.05. Differentially abundant proteins were only considered if they were detected in all three replicates, $p < 0.05$, had a fold regulation of at least two-times, and %CV (coefficient of variation) of less than 5% (See Supplementary Table 1 and Supplementary Table 2). For protein quantification a minimum of 2 unique peptides per protein (with an average of 7 peptides per protein) was used for quantification, and stringent criteria of PEP < 0.01 and FDR < 0.01 were implemented. For the differentially abundant

dx.doi.org/10.1021/pr500353x | *J. Proteome Res.* 2014, 13, 4028−4039

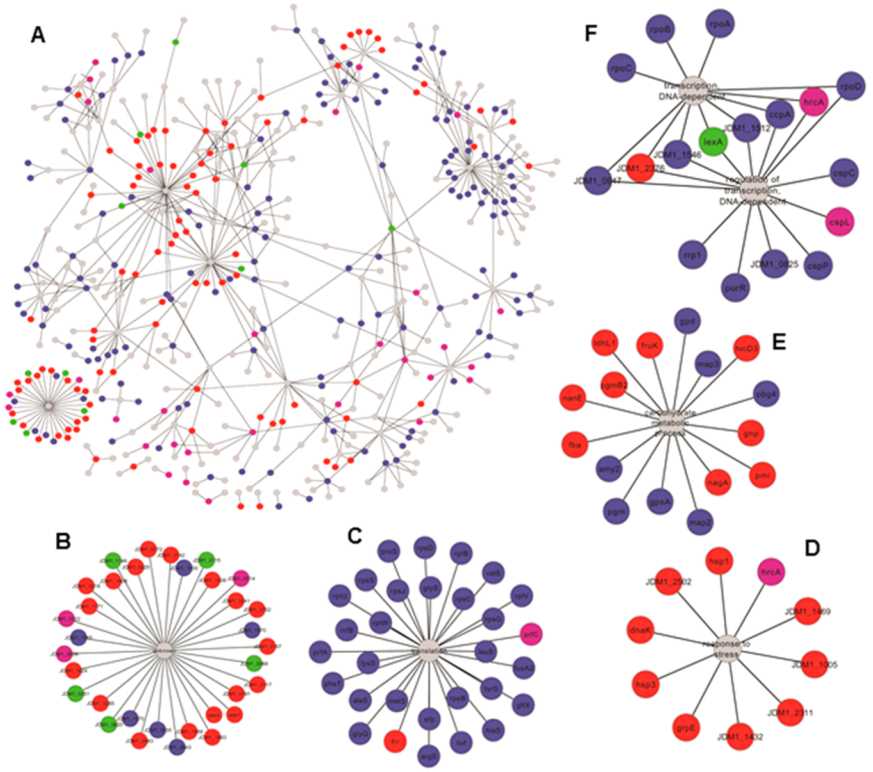


Figure 1. Functional annotation network of proteins detected in this study. Overview of all proteins detected as differentially expressed during this study are shown in A, whereas specific GO biological processes affected by acid stress are shown as subnetworks, drawn from first neighbors of selected GO biological process nodes, in B–F. These include proteins with unknown functions (B) and proteins involved in translation (C), response to stress (D), carbohydrate metabolic processes (E), and transcription (F). Red nodes indicate proteins detected as more abundant in acid-stressed *L. plantarum* 423, whereas blue nodes indicate proteins detected as less abundant. Green nodes indicate proteins detected solely in acid-stressed *L. plantarum* 423, whereas purple nodes indicate proteins detected solely in control cells. Gray nodes indicate GO biological processes.

proteins, 255 out of 289 proteins had 3 or more peptides associated with the quantified protein, and 63 of these proteins had more than 10 unique peptides per protein (Supplementary Table 2).

The Kyoto Encyclopedia of Genes and Genomes (KEGG)[17] was used to determine the position of the identified proteins in respective pathways. LocateP[18] was used to determine the subcellular localization of the identified proteins. Protein–protein interactions were predicted using STRING v9.1[19] set at high confidence. Cytoscape[20] was used for network visualization, and gene ontology (GO) enrichment was performed with Gene Ontology Enrichment Analysis Software Toolkit (GOEAST).[21]

Determination of Reactive Oxygen Species Formation

L. plantarum 423 cells (cultured and stressed as previously described) were harvested by centrifugation, resuspended in 500 μL sterile saline, lysed, and cellular debris removed as previously described. Cell free supernatants were stained with 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Life Technologies). Briefly, 150 μL of the lysate was added to each well of a 96-well plate, followed by the addition of 10 μL H_2DCFDA and sterile saline to reach a final volume of 200 μL. Negative controls (blank) contained only saline and H_2DCFDA, whereas the positive control contained cell lysates of cells incubated in MRS at pH 6.4 to which H_2O_2 was added to reach a final concentration of 2.5%. The reaction was performed in the dark at 25 °C for 1 h and fluorescence recorded using the In Vivo Imaging System (IVIS 100, Caliper Life Sciences/PerkinElmer) set to fluorescence and using the GFP bkg Excitation and GFP emission filter. Images were acquired for 0.2 s on efficiency mode. Two technical repeats were performed per biological replicate (n = 6).

Detection of Sugars and Metabolites in Supernatants

The pH of the supernatants, obtained after pelleting of acid-stressed and control *L. plantarum* 423 cells used for the proteomics analysis, was adjusted to pH 7.0 and subsequently filter-sterilized with a 0.22 μm filter. The samples were stored at −80 °C until further use. Acetic acid, malic acid, D-lactic acid, L-lactic acid, and residual glucose and fructose were analyzed using Enzytec Fluid kits (AEC Amersham) and an Arena enzyme robot, 20XT (Thermo Scientific) at the Central Analytical Facility, Stellenbosch University, South Africa.

Knockout of *JDM1_2142*

Primers 2142Fwd and 2142Rev based on *L. plantarum* WCFS1 (NC_004567) and JDM1 (NC_012984) sequences were used to amplify (FastStart High Fidelity Taq) an approximately 1.4 kb region of *L. plantarum* 423 containing the *JDM1_2142* gene and flanking region (Supplementary Figure 8). This product was cloned in pGEM-T (Promega) and its identity confirmed by partial sequencing using M13 primers. The *Staphylococcus aureus cat* gene, coding for chloramphenicol (*Cm*) resistance, was similarly amplified from pSK236 (generous gift from Dr. S Khan) using primers catFwd and catRev and cloned in pGEM-T-Easy (Promega). The *cat* gene was cloned into *JDM1_2142* at the Bsp119I sites, and the resulting pGEM-T-based construct was used for transformation of electro-competent *L. plantarum* 423.[22] Transformants were selected by enrichment culturing in

dx.doi.org/10.1021/pr500353x | *J. Proteome Res.* 2014, 13, 4028–4039

5 mL of MRS media containing 5 μg/mL *Cm* for 2 days at 37 °C, followed by selection on solid MRS containing 15 μg/mL *Cm*. Since the pGEM-T based plasmid is nonreplicating in *L. plantarum* 423, all transformants were likely to be the result of a double-crossover event. Colony PCR screens were performed with 2142Fwd and 2142flankRev primers to verify integration into the chromosomal *JDM1_2142* gene using TaKara Ex taq. Genomic DNA was isolated from selected transformants by treating the *L. plantarum* 423 cells with lysozyme (1 mg/mL), and the cells were subsequently lysed with 1% (w/v) SDS in the presence of proteinase K (1 mg/mL). Proteins were precipitated by centrifugation in the presence of 2 M ammonium acetate before DNA was precipitated with ethanol according to standard procedures. Genomic DNA from selected transformants was PCR screened with appropriate primer combinations (Supplementary Figure 8) to verify orientation of the *cat* gene on the chromosome of *L. planatarum* 423. Primers used for PCR are as follows with restriction enzymes in bold:

2142Fwd CG**GAATTC**GATACTAATACCTCGTAA-TACCG,

2142Rev GC**TCTAGA**ATATGAAATGGCCTATGATGG,

2142flankRev GC**TCTAGA**TTTATTGTATTTCGATCT-TACCTTCG,

catFwd G**TTCGAA**GGATCCATTCTAATGAAG,

catRev A**TTCGAA**GCTTAACGTCAATAAAGC.

Determination of Acid Survivability of Wild Type and *JDM1_2142* Knockout *L. plantarum* 423

Precultures were prepared as previously described, inoculated at 1% (v/v) into 20 mL of MRS broth, and cultured for 12 h ($OD_{600nm} \approx 8.0$) at 37 °C to reach the onset of stationary phase. One milliliter of the cells were harvested (8000*g*, 3 min, 25 °C) and resuspended in 1 mL of prewarmed (37 °C) MRS broth adjusted to pH 2.5 with HCl. The cultures were incubated at 37 °C for 1 h to induce HCl stress. Acid-stressed cells were subsequently diluted in sterile saline (0.85% w/v NaCl), plated onto MRS agar plates, and incubated at 37 °C for 36 h before colonies were counted. CFU/mL was also determined at the start of the experiment. The experiment was repeated on two separate days ($n = 5$).

RESULTS AND DISCUSSION

L. plantarum 423 was cultured until the cells reached early stationary phase (Supplementary Figure 1) as more pronounced immunomodulatory activity has been associated with *L. plantarum* WCFS1 cells in this phase.[23] Early stationary phase cells will also undergo an acid tolerance response, or acid adaptation, as a result of natural acidification by lactic acid during growth, and various stress response proteins have been observed during entry into the stationary phase by *L. plantarum* WCSF1.[24]

The highest number of proteins was identified with MaxQuant 1.2.2.5 when using the total proteome of *L. plantarum* JDM1 as search database. In total, 294 proteins were detected as differentially expressed (≥2.0-fold, $p < 0.05$, FDR < 0.01) at pH 2.5. Identified proteins could be grouped to their different subcellular localizations and into all of the major functional categories that influence the physiological state of the cells (Figure 1 and Supplementary Figure 2). Gene ontology (GO) enrichment analysis (Supplementary Figures 3 and 4) revealed overrepresented GO terms during acid stress. However, further analysis was needed to elucidate the mechanisms involved.

Lipid and Phospholipid Metabolism, and Cell Wall Biogenesis

Dak2 and Dak1B (subunit DhaK) were the only proteins involved in phospholipid metabolism more abundant in acid-stressed *L. plantarum* 423. Dak2 and Dak1B convert dihydroxyacetone to dihydroxyacetone phosphate (DHAP) during glycerol metabolism to generate intermediates either used in glycolysis or glycerophospholipid metabolism. The former is more likely, as the majority of other proteins involved in phospholipid and fatty acid biosynthesis were less abundant. Furthermore, a large number of proteins involved in carbohydrate metabolism were more abundant (discussed below).

Fatty acid biosynthesis was negatively affected during acid stress in *L. plantarum* 423. A marked decrease in proteins playing a role in fatty acid biosynthesis, including FabZ1 and 2, FabH2, FabG1, FabD, AccA2, AccD2, AccB2, and AcpA2, was observed. Decreased fatty acid metabolism was also observed in acid-stressed *S. aureus*,[26] and there was no reported increase in the abundance of proteins involved in cell wall and membrane biogenesis in acid-stressed *L. casei* Zhang.[11] In contrast are the results reported for *L. rhamnosus* GG cultured at pH 4.8 and acid-stressed *L. reuteri*.[10,27] Furthermore, changes in membrane fatty acid composition seems to play a role in the survival of *S. mutans* under acidic conditions.[28,29] These contrasting results might be explained by the fact that modified fatty acid metabolism might be restricted to an early acid stress response, as *L. reuteri* was only stressed for up to 15 min, as opposed to an hour for *L. plantarum* 423. Reduced fatty acid biosynthesis may also be due to decreased growth under acidic conditions, as observed for *S. aureus*.[26]

Proteins playing a role in peptidoglycan biosynthesis (Ddl, MurE1, and GlmU) were less abundant in acid-stressed *L. plantarum* 423. Furthermore, MurA2, which feeds monomers into peptidoglycan biosynthesis, was only detected in control cells. An extracellular protein (JDM1_2414), involved in cell wall macromolecule catabolic processes, was only detected in control *L. plantarum* 423 that usually plays a role in the rearrangement of peptidoglycan during growth.[30,31] It is a possibility that decreased peptidoglycan biosynthesis may be due to decreased growth observed under acidic conditions, as previously mentioned. However, this does not explain the higher abundance of NanE, NagA, and Gnp that indicate utilization of cell wall components from *L. plantarum* 423 itself or those present in the growth media. Interestingly, NagA and NagB (= NanE) were also more abundant during acid stress in *L. casei* Zhang.[11] Utilization of cell wall components leads to the formation of ammonia, which could alleviate lowering of pH_i.

Ion Transporters

A cobalt transporter (JDM1_0884), a stress-induced DNA binding protein (JDM1_2502, playing a role in iron ion homeostasis), and a protein involved in copper ion homeostasis (CutC) were more abundant in acid-stressed *L. plantarum* 423. Maintaining copper homeostasis in acidic conditions is in agreement with a previous report on *L. bulgaricus*[32] indicating the importance of copper, probably as cofactor for enzymes, to ensure survival of bacterial cells upon exposure to acidic conditions. Ion transporters have previously been shown to

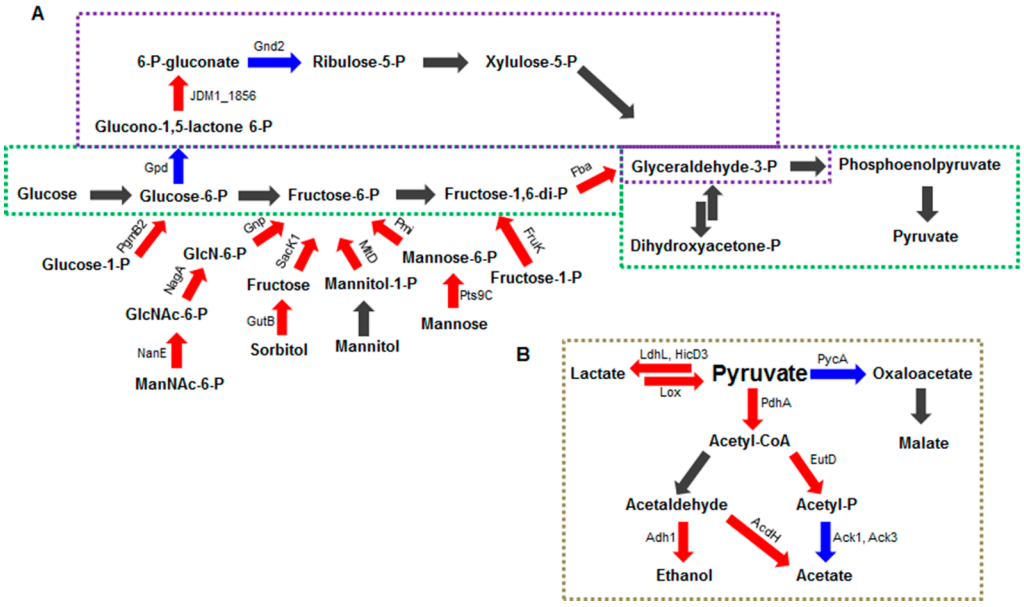


Figure 2. Overview of proteins involved in (A) glycolysis and the pentose phosphate pathway as well as (B) pyruvate metabolism as detected in acid-stressed *L. plantarum* 423. Red arrows indicate more abundant proteins or proteins detected solely in acid-stressed *L. plantarum* 423, while blue arrows indicate less abundant proteins or proteins detected solely in control *L. plantarum* 423. Black arrows indicate proteins that were not detected as differentially expressed in this study. The purple box represents the pentose phosphate pathway, the green box, glycolysis, and the gold box, pyruvate metabolism. Gnd2 = 6-phosphogluconate dehydrogenase, JDM1_1856 = 6-phosphogluconolactonase, Gpd = glucose-6-phosphate 1-dehydrogenase, PgmB2 = beta-phosphoglucomutase, NanE = *N*-acetylmannosamine-6-phosphate 2-epimerase, NagA = *N*-acetylglucosamine-6-phosphate deacetylase, Gnp = glucosamine-6-phosphate isomerase, GutB = L-iditol 2-dehydrogenase, SacK1 = fructokinase, MtlD = mannitol-1-phosphate 5-dehydrogenase, Pts9C = mannose-specific IIC component phosphotransferase system, Pmi = mannose-6-phosphate isomerase, FruK = 1-phosphofructokinase, Fba = fructose-bisphosphate aldolase, LdhL1 = L-lactate dehydrogenase, HicD3 = L-2-hydroxyisocaproate dehydrogenase, Lox = lactate oxidase, PycA = pyruvate carboxylase, PdhA = pyruvate dehydrogenase E1 component subunit alpha, EutD = phosphotransacetylase, Ack1,3 = acetate kinases, AcdH = acetaldehyde dehydrogenase, Adh1 = alcohol dehydrogenase.

play a role in the acid response of *Listeria monocytogenes*, *S. mutans*, and *Escherichia coli*.[33−35]

Carbohydrate Metabolism

Various proteins that play a role in carbohydrate utilization were differentially abundant in acid-stressed cells. This could influence energy production and intracellular redox potential. Altered carbohydrate metabolism has also been observed in other proteomic studies on cells exposed to acidic environments.[8−11,13,36−38] These alterations observed could result in increased flux through glycolysis and, combined with altered pyruvate metabolism, lead to the production of ATP and NAD^+, as well as the production of more basic compounds that may ease acid stress and enhance the survival of *L. plantarum* 423 (Figure 2). It seems as if acid-stressed cells were relieved from catabolite repression, the catabolite control protein A (CcpA) was less abundant. This gives the cells the ability to utilize any available sugars, even in a glucose rich environment. The phosphocarrier protein HPr that plays a role in the phosphorylation of sugars as they enter the cells through the phosphotransferase system was more abundant. However, a phosphoenolpyruvate-protein phosphotransferase (PtsI) was less abundant. PtsI is a general component of the phosphoenolpyruvate-dependent sugar phosphotransferase system and was shown to be more abundant in acid-stressed *L. rhamnosus* GG[10] and *L. casei* Zhang.[11] It is possible that the phosphate needed for sugar phosphorylation is not being obtained solely from the conversion of phosphoenolpyruvate to pyruvate during acid stress in *L. plantarum* 423, and other molecules (possibly acetyl phosphate) might serve as phosphate donor during acid stress. Furthermore, it is also possible that the PTS is negatively influenced in acidic conditions and that secondary transport systems, such as uniport or cotransport, could play a role in sugar transport during acid stress in *L. plantarum* 423.

Glycolysis and Pathways Feeding into Glycolysis

Results obtained in this study suggest that various carbon sources are used in a glucose rich environment to increase flux toward pyruvate formation. For an overview of glycolysis, see Figure 2A. Furthermore, a maltose O-acetyltransferase (Maa2), also known as maltose transacetylase, was more abundant in acid-stressed cells. It has been proposed that the maltose transacetylase and thiogalactoside transacetylase prevent the accumulation of free sugars to high internal levels and might have a detoxifying role.[39,40] A phosphoglycerate dehydrogenase (SerA3) was also more abundant, which could lead to carbon flux being diverted from glycolysis to serine and glycine metabolism. This indicates that even in severe stress conditions equilibrium is still being maintained with regards to intracellular sugar levels as well as intermediates in glycolysis.

Pyruvate Metabolism

Altered pyruvate metabolism was observed in *L. plantarum* 423 upon exposure to acid stress, as compared to control cells (Figure 2B). Strangely, acetate kinase 1 (Ack1) was less abundant in acid-stressed cells, and acetate kinase 3 (Ack3) was only detected in control *L. plantarum* 423. This was rather unexpected as ATP would be generated by the dephosphorylation of acetyl phosphate to acetate via the acetate kinases. This leads to the postulation that the acetyl phosphate pool might be increased during acid stress in *L. plantarum* 423 and that acetyl phosphate could potentially serve as phosphate

dx.doi.org/10.1021/pr500353x | *J. Proteome Res.* 2014, 13, 4028−4039

Table 1. Sugars Consumed and Metabolites Produced (in g/L) by *L. plantarum* 423 in MRS Broth under Acid Stress and Control Conditions

	glucose[a,c]	fructose[a,c]	malic acid[a]	acetic acid	D-lactic acid[b]	L-lactic acid[b]
MRS pH 6.4	12.9 ± 0.02	0.14 ± 0.006	0.01 ± 0.006	2.3 ± 0.035	2.8 ± 0.07	2.7 ± 0.73
MRS pH 2.5	19.0 ± 0.80	0.11 ± 0.008	0.05 ± 0.0006	2.3 ± 0.036	0.6 ± 0.04	0.7 ± 0.04

[a]*$p < 0.05$. [b]**$p < 0.0001$. [c]Residual sugars in supernatant.

donor during acidic conditions. Acetyl phosphate has previously been shown to serve as global signaling molecule in *E. coli* and to phosphorylate intracellular glucose in certain streptococcal strains.[41,42]

Metabolites measured after 1 h of incubation at pH 2.5 and pH 6.4 are shown in Table 1. More glucose (6.1 g/L) was consumed by control *L. plantarum* 423, whereas slightly more fructose (0.03 g/L) was consumed by acid-stressed cells, which correlates with higher abundance of proteins involved in fructose utilization identified in the proteomic data. Lower levels of both D- and L-lactic acid were produced by acid-stressed *L. plantarum* 423, with 4.7- and 3.8-fold lower levels of L- and D-lactic acid being detected, respectively. Interestingly, similar levels of acetic acid were produced by both acid-stressed and control *L. plantarum* 423, even though there was a difference in the amount of glucose consumed. Taken together these results indicate that pyruvate metabolism was altered to produce less acidic compounds (i.e., lactic acid), which could further decrease extracellular pH as well as pHi.

Purine and Pyrimidine Metabolism

The majority of proteins playing a role in purine and pyrimidine metabolism were less abundant (Figure 1 and Supplementary Table 2) and is in agreement with other proteomic studies.[10,26] Proteins more abundant included Xtp1, NrdF, Adk, PurE, PyrD, GuaC, and a ribonucleoside hydrolase RihC (JDM1_0325). Xtp1 catalyzes the hydrolysis of (d)ITP, (d)GTP, (d)UTP, and XTP to their respective monophosphates and could have a detoxifying role as noncanonical nucleotides are formed as a result of the conversion of amino groups on bases to keto groups, which can be caused by oxidative stress, chemical mutagenesis, and oxidative deamination of nucleotides.[43,44]

A nonspecific ribonucleoside hydrolase RihC (JDM1_0325), which catalyzes the irreversible hydrolysis of purine and pyrimidine nucleosides to ribose and their respective nucleobases,[45] was more abundant in acid-stressed *L. plantarum* 423. The ribose moiety could thus potentially be utilized as carbon source under severe acidic conditions and has previously been shown to be an effective fermentable substrate for *L. sakei*.[46] Ribose catabolism has also previously been linked to nucleoside catabolism.[47] Prs1 was less abundant in acid-stressed *L. plantarum* 423 and plays a major role in feeding phosphoribosyl diphosphate into purine, pyrimidine, and histidine biosynthesis and supports the idea that ribose can be utilized as carbon source and not for nucleotide biosynthetic processes under acidic conditions.

Transcription, Translation, Protein Synthesis and Protein Degradation

The majority of proteins playing a role in transcription and regulation of transcription were less abundant or detected solely in control cells (Figure 1 and Supplementary Table 2). Similarly, the majority of proteins involved in RNA processing, modification, and degradation were less abundant in acid-stressed *L. plantarum* 423. These results correlate well with previous reports that show decreased transcription during acid stress[9] and could be as a result of reduced growth under stress conditions.

Only a few proteins involved in protein synthesis were more abundant in acid-stressed *L. plantarum* 423. A ribosome-recycling factor (Frr) was more abundant, as well as a peptidylprolyl isomerase (PpiB) that plays a role in protein folding and stabilization, potentially having a protective role. Numerous proteins involved in ribosome biosynthesis, ribosome binding and assembly, protein biosynthesis, protein processing (post-translational modification), and protein secretion and export were significantly less abundant in acid-stressed *L. plantarum* 423 (Figure 1 and Supplementary Figures 5−7). Our results correlate well with previous reports demonstrating the lower abundance of proteins involved in protein synthesis during acid stress in *L. rhamnosus* GG and *B. longum* 8809.[9,10]

Amino Acid Metabolism

Proteins playing a role in the biosynthesis of certain amino acids were significantly more abundant during acid stress, especially those involved in lysine biosynthesis (Asd2, DapA1, DapB, and DapD). Intermediates formed during lysine biosynthesis are usually required for peptidoglycan biosynthesis. However, peptidoglycan biosynthesis was severely affected during acid stress in *L. plantarum* 423, and these intermediates could now possibly be directed toward lysine biosynthesis. A protein involved in branched chain amino acid (BCAA) biosynthesis (a branched-chain-amino-acid aminotransferase, BcaT) was more abundant in acid-stressed *L. plantarum* 423. Correspondingly, it was recently shown that proteins involved in branched-chain amino acid biosynthesis were upregulated during acid stress in *S. mutants* and that a branched-chain amino acid aminotransferase (IlvE) is involved in acid tolerance of *S. mutans*.[48,49]

Only a few proteins playing a role in amino acid biosynthesis were significantly less abundant during acid stress. A glutamate dehydrogenase (Gdh) was less abundant, and the one identified in our study utilizes $NADP^+$, suggesting that formation of glutamate will be favored under normal conditions.[50] Lower abundance of this protein could thus lead to decreased ammonia assimilation and potential accumulation of ammonia. A glutamine synthetase (GlnA) was less abundant in acid-stressed *L. plantarum* 423, similar to that reported for *L. rhamnosus* GG.[10] Taken together, these results indicate a potential accumulation of ammonia in the cytoplasm, which may lead to an increase in the pHi. Lower abundance of these proteins might also be due to decreased growth under extreme acidic conditions.

Stress Response

F_0F_1-ATP Synthase. The F_0F_1-ATP synthase (F_0F_1-ATPase) is an important multimeric enzyme that plays a role in ATP synthesis-associated influx of H^+ ions in fermentative bacteria.[51] It can, however, also play a role in the acid stress response where the opposite occurs, namely, the export of H^+

dx.doi.org/10.1021/pr500353x | J. Proteome Res. 2014, 13, 4028−4039

ions out of the cell by using the energy generated from the hydrolysis of ATP.[5] The gamma chain of the F_0F_1-ATPase (AtpG) was less abundant in acid-stressed *L. plantarum* 423, which was unexpected as F_0F_1-ATPase has been shown to play a role in acid adaptation in bacteria.[37,49,52] Our results are in contrast to other proteomic studies on acid stress in *L. rhamnosus* GG, *B. longum* NCIMB 8809, and *S. mutans* LT11.[9,10,49] These studies were, however, all conducted at milder pH stresses, ranging from pH 4.5−5.0. Other reports are, however, in agreement with our study and reported that some subunits of the F_0F_1-ATPase are either down regulated or nondifferentially expressed during acid stress, as in *S. aureus* 50583 and *L. plantarum* WCFS1.[25,26] F_0F_1-ATPases were also not identified during a recent screen for potential biomarkers of acid resistance in *L. plantarum*.[12] It thus seems that the role of F_0F_1-ATPases in acid stress is more elusive than anticipated and that it might only play a significant role during mild acid stress, whereas a more dramatic approach is needed to survive extreme acidic conditions. The ATP requirements under such extreme conditions might be too high to increase pH_i drastically over a short period using this system. This hypothesis is corroborated by results reported in *L. reuteri,* where the highest increased fold change of *atpA* was 14.1-fold at pH 5.0. Significantly lower fold changes were observed at pH 4.5 (0.01-fold) and pH 4.0 (2.94-fold).[13] Even though none of the F_0F_1-ATPase synthase subunits were more abundant in wild type *L. casei* Zhang stressed at pH 2.5, increased levels of ATPase activity were observed at pH 5.0, whereas ATPase activity was significantly diminished at pH 3.5.[11] These results thus support our hypothesis of limited usage of F_0F_1-ATPase synthase at extreme acidic conditions when a more severe approach may be required to counteract low pH_i.

General Stress Response. Some general stress response proteins were detected as more abundant in acid-stressed *L. plantarum* 423, including small heat shock proteins (Hsp1 and 3) and chaperonins (DnaK, GrpE, GroEL, and GroES), which play a role in protein folding. The negative regulator of the *grpE-dnaK-dnaJ* and *groELS* operons, HrcA, was only detected in control *L. plantarum* 423, which correlates well with the above-mentioned result. Our results are in agreement with various other studies that have shown the importance of general stress proteins during acid stress in lactobacilli.[6,11,13,37,38,53] Universal stress proteins (UspAs, JDM1_1005, JDM1_1432, JDM1_2311, and JDM1_1469) were also more abundant in acid-stressed cells. UspAs are induced by different stress conditions and were identified in the acid stress response of *L. casei* Zhang and shown to influence survival of *L. monocytogenes* during acid stress.[11,54] Alkaline shock proteins (Asp 1 and 2) were also detected in acid-stressed *L. plantarum* 423. Correspondingly, a gene encoding an alkaline shock protein was shown to be upregulated in *S. aureus* during acid stress.[26]

Oxidative Stress Response. Numerous proteins playing a role in oxidative stress were more abundant in acid-stressed *L. plantarum* 423, including two glutathione reductases (GshR2 and 4), a glutathione peroxidase (Gpo), a methionine sulfoxide reductase (MsrA2), and a thiol peroxidase (Tpx). It has previously been shown that acid stress induced a major oxidative stress response in *Bacillus cereus*.[55] Oxidative stress response proteins, including superoxide dismutase (SodA), an alkyl hydroperoxide reductase (AhpC), and a thiol peroxidase (Tpx), have also been detected in *L. lactis* MG1363 when exposed to acid stress.[36,56] As far as we could determine no other major oxidative stress response has been reported in other lactobacilli upon exposure to an acid stress. Glutathione reductases reduce glutathione disulfide (GSSG) to the sulfhydryl form (GSH) that serves as a well-known antioxidant.[57] The protective role of GSH during acid stress has previously been reported in *L. lactis* strains that either import GSH or are capable of synthesizing GSH.[58] Similarly, GSH supplementation has shown protective effects against acid stress in *L. salivarius*[59] and *L. mesenteroides*.[60] A thiol peroxidase (Tpx) was also detected as more abundant in acid-stressed *L. plantarum* 423 that plays a role in oxidative stress and use reducing counterparts from thioredoxin and thioredoxin reductase to reduce hydroperoxides.[61] Furthermore, thioredoxin (TrxH) was only detected in acid-stressed cells, indicating a role for this system in the acid stress response of *L. plantarum.* Similarly, three thioredoxin genes were upregulated in acid-stressed *S. aureus*.[26] A methionine sulfoxide reductase (MsrA2), which plays a role in the repair of oxidized proteins,[62] was more abundant in acid-stressed *L. plantarum* 423. As far as we could determine, no other reports indicate a role for methionine sulfoxide reductases in acid stress. It has previously been shown, however, that *L. reuteri msrB* mutants showed reduced ability to inhabit the murine gut.[63] Msr has also been shown to play an important role in the virulence of pathogenic bacteria, as well as their ability to adhere to epithelial cells.[64,65] The Msr identified in our study thus could not only protect *L. plantarum* 423 against oxidative damage but could also prime the cells for further GIT transit.

To further investigate oxidative stress in acid-stressed *L. plantarum* 423, we employed fluorescent staining of reactive oxygen species in cell lysates from the different treatments. Staining the cells directly did not result in a fluorescent signal, even with the addition of H_2O_2 to the cells (data not shown), and thus, cell lysates were stained. A statistically significant increase in reactive oxygen species levels in acid-stressed *L. plantarum* 423 was observed (Figure 3). Our results, and other reports, thus clearly indicate a link between the acid- and oxidative-stress responses of bacteria that seems widespread and not only confined to a specific genus.[26,66,67]

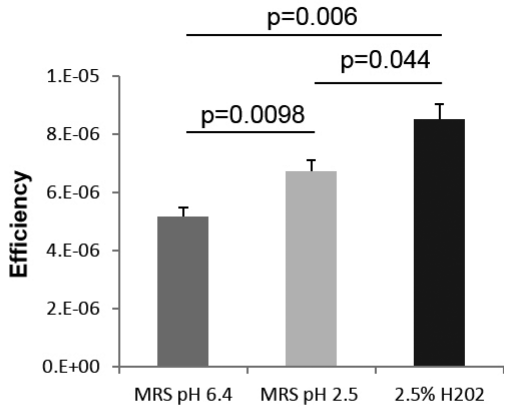


Figure 3. Formation of reactive oxygen species by *L. plantarum* 423 when exposed to acid stress, as compared to control conditions. ROS formation was determined by staining for reactive oxygen species at different pH conditions by using 2′,7′-dichlorodihydrofluorescein diacetate. Statistical significance was determined by Student's *t* test.

dx.doi.org/10.1021/pr500353x | *J. Proteome Res.* 2014, 13, 4028−4039

Table 2. Putative Identifications of Some of the Uncharacterized Proteins Detected in This Study

gene	putative identification/descriptions	organism	accession
More Abundant in Acid-Stressed *L. plantarum* 423			
JDM1_1893	extracellular protein	*L. plantarum* WCSF1	YP_004889982
JDM1_2157	atypical short-chain dehydrogenase/reductase	*L. plantarum* WCSF1	YP_004890327
JDM1_1856	6-phosphogluconolactonase	*L. plantarum* ZJ316	YP_007414890
JDM1_0285	RNA-binding protein	*L. plantarum*	WP_003643742
Only Detected in Acid-Stressed *L. plantarum* 423			
JDM1_0003	RNA-binding S4 protein	*L. plantarum* P-8	YP_007985680
JDM1_1824	cysteine desulfurase associated protein	*L. plantarum* ZJ316	YP_007414856
JDM1_1438	CsbD-like protein	*L. plantarum* ATCC 14917	EFK30312
JDM1_1662	glutaminyl-tRNA synthase b	*L. plantarum* P-8	YP_007987203
Less Abundant in Acid-Stressed *L. plantarum* 423			
JDM1_1477	RNA methyltransferase	*L. plantarum* WCFS1	YP_004889562
JDM1_1366	DAK2 domain containing protein	*L. plantarum* ST-III	YP_003924604
JDM1_1281	GTPase YqeH family protein	*L. plantarum* ZJ316	YP_007414219
Only Detected in Control *L. plantarum* 423			
JDM1_2016	SAM-dependent methyltransferase	*L. plantarum* WCFS1	YP_004890186
JDM1_0434	cystathionine beta-synthase domain containing protein	*L. plantarum* P-8	YP_007986095

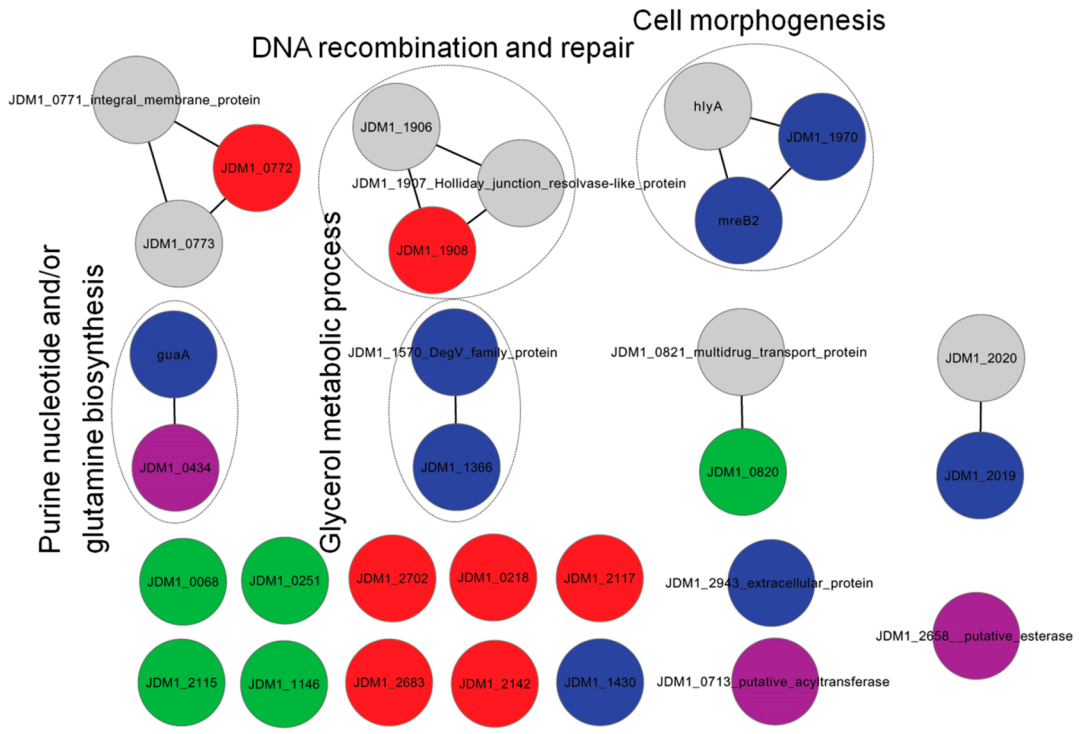


Figure 4. Predicted protein−protein interaction network, using STRING v9.1, of proteins to which no putative identifications could be assigned, with unknown functions, detected in this study. Red nodes indicate proteins detected as more abundant in acid-stressed *L. plantarum* 423, whereas blue nodes indicate proteins detected as less abundant. Green nodes indicate proteins detected solely in acid-stressed *L. plantarum* 423, whereas purple nodes indicate proteins detected solely in control cells. Gray nodes indicate proteins not detected in this study.

Miscellaneous

Proteins MreB1 and B2 determining cell shape were less abundant in acid-stressed *L. plantarum* 423. Furthermore, the rod-shape determining protein, RodA2, was only detected in control cells. Changes in cell morphology have also been observed in *P. freudenreichii* SI 41 upon exposure to acidic conditions.[68]

NadE, a NH_3-dependent NAD^+ synthetase, was less abundant in acid-stressed *L. plantarum* 423. Ammonia may be utilized during NAD^+ synthesis, and decreased abundance of NadE could thus reduce the consumption of ammonia, which might help alleviate acid stress in *L. plantarum* 423.

Uncharacterized Proteins

Some of the uncharacterized proteins detected in this study had gene ontology (GO) annotations, and putative identifications could be assigned to some of the other uncharacterized proteins using PSI-Blast[69] (see Table 2). We constructed protein−protein interaction networks, using proteins with unknown functions and to which no putative identifications could be assigned as query in STRING v9.1, to elucidate the potential role of these proteins (Figure 4). Most of the proteins did not show any predicted interaction, as would be expected. However, some proteins did have predicted interaction with proteins playing a role in cell morphogenesis, DNA

dx.doi.org/10.1021/pr500353x | J. Proteome Res. 2014, 13, 4028−4039

recombination, glycerol metabolic processes, and purine nucleotide biosynthetic processes and/or glutamine metabolic processes.

Functional Analysis of JDM1_2142 during Acid Stress

Of the most abundantly expressed proteins under acid stress conditions, protein JDM1_2142 showed the highest fold increase (373-fold). However, no known function could be assigned to this protein. It is coded for by a 339 bp open reading frame (ORF), closely followed by a second ORF of 417 bp that codes for a small heat shock protein (Hsp2) (Supplementary Figure 8). A *JDM1_2142* knockout strain was generated and compared to wild type *L. plantarum* 423 with respect to viability under acid stress. The mutant showed significantly lower viability as compared to wild type *L. plantarum* 423, indicating a potential role for this protein during acid stress survival (Figure 5). Furthermore, this protein was not detected in bile-stressed *L. plantarum* 423 (unpublished data) and therefore putatively named acid stress response protein (ASRP).

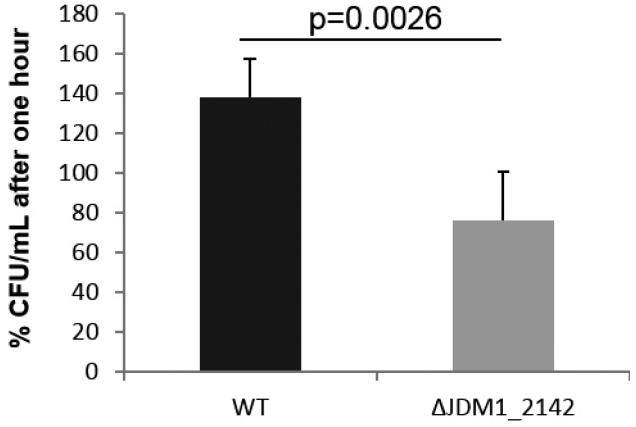


Figure 5. Colony forming units per mL of wild type (WT) *L. plantarum* 423 and a *L. plantarum* 423 *JDM1_2142* knockout (ΔJDM1_2142) after 1 h exposure to MRS equilibrated to pH 2.5 with hydrochloric acid. The data represents the number of viable CFU/mL detected after 1 h as compared to that at the start of the experiment. Statistical significance was determined by Student's *t* test.

CONCLUSIONS

In this study we employed a gel-free nanoLC−MS/MS proteomics approach to gain insight into the intricate mechanisms employed by *L. plantarum* 423 to survive an extreme acid challenge. Our approach resulted in the identification of 294 differentially abundant proteins during acid stress that covered all of the major processes present in a bacterial cell. We observed a marked decrease in proteins involved in cell wall and phospholipid biosynthesis, transcription, translation, cell division, and cell shape that indicates reduced growth upon exposure to severe acid stress. It appeared that acid-stressed *L. plantarum* 423 were relieved from catabolite repression and that cells could utilize a large array of carbon sources, even in a glucose-rich environment, to produce basic compounds and to supply the cell with energy to ensure survival. Production and/or accumulation of basic compounds, more specifically ammonia, seem to be a central theme for survival. Proteins involved in lysine biosynthesis were also found to be more abundant, and a major oxidative stress response was observed in acid-stressed *L. plantarum* 423. An uncharacterized protein, JDM1_2142, was the most abundant protein detected in this study, and functional analysis revealed a possible role for this protein in HCl acid stress survival in *L. plantarum* 423. Elucidation of the exact role of this acid stress response protein is thus required. The other uncharacterized proteins identified in this study also warrant further investigation for their role in acid stress or, in the general stress response, of lactobacilli. We managed to identify proteins and mechanisms involved in the acid stress response of *L. plantarum* 423, not previously described in lactobacilli, and this is, as far as we could determine, the most comprehensive proteome profile of the acid stress response of any lactic acid bacterium to date.

ASSOCIATED CONTENT

Supporting Information

All peptides and proteins detected in this study. Growth curve and acidification profile of *L. plantarum* 423 before induction of acid stress. Predicted subcellular localization of proteins detected as differentially abundant in this study. Gene ontology enrichment analyses of proteins detected as more or less abundant or detected solely in acid stressed or control *L. plantarum* 423. KEGG pathway analyses of proteins involved in ribosome biogenesis and translation, aminoacyl t-RNA biosynthesis, or involved in protein export. Diagrammatic representation of JDM1_2142 gene region in *L. plantarum* 423. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*(L.M.T.D.) E-mail: lmtd@sun.ac.za. Phone: +27-21-808 5849.

Present Address

‖(T.H.) Department of Science and Technology/National Research Foundation Centre of Excellence for Biomedical Tuberculosis Research, Department of Biomedical Sciences, Division of Molecular Biology and Human Genetics, Stellenbosch University, Tygerberg 7505, South Africa.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

T.H. was supported by a Stellenbosch University Subcommittee B Postdoctoral fellowship.

ABBREVIATIONS

AccA2, acetyl-CoA carboxylase, carboxyl transferase subunit alpha; AccB2, acetyl-CoA carboxylase biotin carboxyl carrier protein; AccD2, acetyl-CoA carboxylase carboxyl transferase subunit beta 2; AcpA2, acyl carrier protein; Adk, adenylate kinase; Amy, α-amylase; Asd2, aspartate-semialdehyde dehydrogenase; ATP, adenosine triphosphate; Dak1B, glycerone kinase; Dak2, dihydroxyacetone kinase phosphatase domain-containing protein; DapA1, dihydrodipicolinate synthase; DapB, dihydrodipicolinate reductase; DapD, 2,3,4,5-tetrahydropyridine-2,6-dicarboxylate *N*-acetyltransferase; Ddl, D-alanine-D-alanine ligase; DnaK, chaperone protein DnaK; FabD, malonyl CoA-acyl carrier protein transacylase; FabG1, 3-oxoacyl-(acyl-carrier-protein) reductase; FabH2, 3-oxoacyl-(acyl-carrier-protein) synthase; FabZ1, 3-hydroxyacyl-(acyl-

carrier-protein) dehydratase FabZ; FabZ2, (3R)-hydroxymyristoyl-(acyl-carrier-protein) dehydratase; Fba, fructose-bisphosphate aldolase; FruK, 1-phosphofructokinase; GlmU, bifunctional protein GlmU; Gnd2, 6-phosphogluconate dehydrogenase; Gnp, glucosamine-6-phosphate deaminase; Gpd, glucose-6-phosphate 1-dehydrogenase; GroEL, 60 kDa chaperonin; GroES, 10 kDa chaperonin; GrpE, protein GrpE; GuaC, guanosine 5′-monophosphate oxidoreductase; GutB, L-iditol 2-dehydrogenase; ITP, inosine triphosphate; Map2, maltose phosphorylase; MreB, cell shape determining protein MreB; MurA2, UDP-N-acetylglucosamine 1-carboxyvinyltransferase; MurE1, UDP-*N*-acetylmuramoyl-L-alanyl-D-glutamate-2,6-diaminopimelate ligase; $NADP^+$, nicotinamide adenine dinucleotide phosphate; NagA, *N*-acetylglucosamine-6-phosphate methyltransferase; NanE, putative *N*-acetylmannosamine-6-phosphate 2-epimerase; NrdF, ribonucleotide-diphosphate reductase subunit beta; PgmB2, beta-phosphoglucomutase; Pmi, mannose-6-phosphate isomerase; Prs1, ribose-phosphate pyrophosphokinase; Pts9C, mannose PTS EIIC; PurE, N5-carboxyaminoimidazole ribonucleotide mutase; PyrD, dihydroorotate dehydrogenase; SacK1, fructokinase; UTP, uridine triphosphate; Xtp1, noncanonical purine NTP pyrophosphatase

REFERENCES

(1) Ahrné, S.; Nobaek, S.; Jeppsson, B.; Adlerberth, I.; Wold, A.; Molin, G. The normal Lactobacillus flora of healthy human rectal and oral mucosa. *J. Appl. Microbiol.* **1998**, *85* (1), 88−94.

(2) Ducrotté, P.; Sawant, P.; Jayanthi, V. Clinical trial: *Lactobacillus plantarum* 299v (DSM 9843) improves symptoms of irritable bowel syndrome. *World J. Gastroenterol.* **2012**, *18* (1), 4012−4018.

(3) De Vries, M. C.; Vaughan, E. E.; Kleerebezem, M.; de Vos, W. M. *Lactobacillus plantarum*: Survival, functional and potential probiotic properties in the human intestinal tract. *Int. Dairy J.* **2006**, *16* (9), 1018−1028.

(4) Klarin, B.; Wullt, M.; Palmquist, I.; Molin, G.; Larsson, A.; Jeppsson, B. *Lactobacillus plantarum* 299v reduces colonisation of *Clostridium difficile* in critically ill patients treated with antibiotics. *Acta Anaesthesiol. Scand.* **2008**, *52* (8), 1096−1102.

(5) Cotter, P. D.; Hill, C. Surviving the acid test: responses of gram-positive bacteria to low pH. *Microbiol. Mol. Biol. Rev.* **2003**, *67* (3), 429−53.

(6) De Angelis, M.; Gobbetti, M. Environmental stress responses in Lactobacillus: a review. *Proteomics* **2004**, *4* (1), 106−122.

(7) Sánchez, B.; Ruiz, L.; Gueimonde, M.; Margolles, A. Omics for the study of probiotic microorganisms. *Food Res. Int.* **2013**, *54* (1), 1061−1071.

(8) Huang, G.; Li, C.; Cao, Y. Proteomic analysis of differentially expressed proteins in *Lactobacillus brevis* NCL912 under acid stress. *FEMS Microbiol. Lett.* **2011**, *318* (2), 177−182.

(9) Sánchez, B.; Champomier-Vergès, M. C.; Collado, M. C.; Anglade, P.; Baraige, F.; Sanz, Y.; de los Reyes-Gavilán, C. G.; Margolles, A.; Zagorec, M. Low-pH adaptation and the acid tolerance response of *Bifidobacterium longum* biotype longum. *Appl. Environ. Microbiol.* **2007**, *73* (20), 6450−6459.

(10) Koponen, J.; Laakso, K.; Koskenniemi, K.; Kankainen, M.; Savijoki, K.; Nyman, T. A.; de Vos, W. M.; Tynkkynen, S.; Kalkkinen, N.; Varmanen, P. Effect of acid stress on protein expression and phosphorylation in *Lactobacillus rhamnosus* GG. *J. Proteomics* **2012**, *75* (4), 1357−1374.

(11) Wu, R.; Zhang, W.; Sun, T.; Wu, J.; Yue, X.; Meng, H.; Zhang, H. Proteomic analysis of responses of a new probiotic bacterium *Lactobacillus casei* Zhang to low acid stress. *Int. J. Food Microbiol.* **2011**, *147* (3), 181−187.

(12) Hamon, E.; Horvatovich, P.; Marchioni, E.; Aoudé-Werner, D.; Ennahar, S. Investigation of potential markers of acid resistance *in Lactobacillus plantarum* by comparative proteomics. *J. Appl. Microbiol.* **2014**, *116* (1), 134−144.

(13) Lee, K.; Lee, H.; Pi, K.; Choi, Y. The effect of low pH on protein expression by the probiotic bacterium *Lactobacillus reuteri*. *Proteomics* **2008**, *8* (8), 1624−1630.

(14) McDonald, L. C.; Fleming, H. P.; Hassan, H. M. Acid tolerance of *Leuconostoc mesenteroides* and *Lactobacillus plantarum*. *Appl. Environ. Microbiol.* **1990**, *56* (7), 2120−2124.

(15) van Reenen, C. A.; Dicks, L. M. T.; Chikindas, M. L. Isolation, purification and partial characterization of plantaricin 423, a bacteriocin produced by *Lactobacillus plantarum*. *J. Appl. Microbiol.* **1998**, *84* (6), 1131−1137.

(16) Cox, J.; Mann, M. MaxQuant enables high peptide identification rates, individualized ppb-range mass accuracies and proteome-wide protein quantification. *Nat. Biotechnol.* **2008**, *26*, 1367−1372.

(17) Kanehisa, M.; Goto, S. KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids Res.* **2000**, *28* (1), 27−30.

(18) Zhou, M.; Boekhorst, J.; Francke, C.; Siezen, R. J. LocateP: genome-scale subcellular-location predictor for bacterial proteins. *BMC Bioinf.* **2008**, *9*, 173.

(19) Franceschini, A.; Szklarczyk, D.; Frankild, S.; Kuhn, M.; Simonovic, M.; Roth, A.; Lin, J.; Minguez, P.; Bork, P.; von Mering, C. STRING v9. 1: Protein−protein interaction networks, with increased coverage and integration. *Nucleic Acids Res.* **2013**, *41*, D808−D815.

(20) Shannon, P.; Markiel, A.; Ozier, O.; Baliga, N. S.; Wang, J. T.; Ramage, D.; Amin, N.; Schwikowski, B.; Ideker, T. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res.* **2003**, *13* (11), 2498−2504.

(21) Zheng, Q.; Wang, X. J. GOEAST: a web-based software toolkit for Gene Ontology enrichment analysis. *Nucleic Acids Res.* **2008**, *36*, W358−W363.

(22) Thompson, K.; Collins, M. A. Improvement in electroporation efficiency *for Lactobacillus plantarum* by the inclusion of high concentrations of glycine in the growth medium. *J. Microbiol. Methods* **1996**, *26* (1−2), 73−79.

(23) van Baarlen, P.; Troost, F.; van der Meer, C.; Hooiveld, G.; Boekschoten, M.; Brummer, R. J.; Kleerebezem, M. Human mucosal *in vivo* transcriptome responses to three lactobacilli indicate how probiotics may modulate human cellular pathways. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108* (Suppl 1), 4562−4569.

(24) Cohen, D.; Renes, J.; Bouwman, F. G.; Zoetendal, E. G.; Mariman, E.; de Vos, W. M.; Vaughan, E. E. Proteomic analysis of log to stationary growth phase *Lactobacillus plantarum* cells and a 2-DE database. *Proteomics* **2006**, *6* (24), 6485−6493.

(25) Pieterse, B.; Leer, R. J.; Schuren, F. H.; van der Werf, M. J. Unravelling the multiple effects of lactic acid stress on *Lactobacillus plantarum* by transcription profiling. *Microbiology* **2005**, *151* (12), 3881−3894.

(26) Bore, E.; Langsrud, S.; Langsrud, Ø.; Rode, T. M.; Holck, A. Acid-shock responses in *Staphylococcus aureus* investigated by global gene expression analysis. *Microbiology* **2007**, *153* (7), 2289−2303.

(27) Wall, T.; Båth, K.; Britton, R. A.; Jonsson, H.; Versalovic, J.; Roos, S. The early response to acid shock in *Lactobacillus reuteri* involves the ClpL chaperone and a putative cell wall-altering esterase. *Appl. Environ. Microbiol.* **2007**, *73* (12), 3924−3935.

(28) Fozo, E. M.; Quivey, R. G., Jr. Shifts in the membrane fatty acid profile of *Streptococcus mutans* enhance survival in acidic environments. *Appl. Environ. Microbiol.* **2004**, *70* (2), 929−936.

(29) Quivey, R. G., Jr.; Faustoferri, R.; Monahan, K.; Marquis, R. Shifts in membrane fatty acid profiles associated with acid adaptation of *Streptococcus mutans*. *FEMS Microbiol. Lett.* **2000**, *189* (1), 89−92.

(30) Margot, P.; Pagni, M.; Karamata, D. *Bacillus subtilis* 168 gene lytF encodes a γ-D-glutamate-meso-diaminopimelate muropeptidase expressed by the alternative vegetative sigma factor, σD. *Microbiology* **1999**, *145* (1), 57−65.

(31) Scheurwater, E.; Reid, C. W.; Clarke, A. J. Lytic transglycosylases: bacterial space-making autolysins. *Int. J. Biochem. Cell Biol.* **2008**, *40* (4), 586−591.

(32) Penaud, S.; Fernandez, A.; Boudebbouze, S.; Ehrlich, S. D.; Maguin, E.; van de Guchte, M. Induction of heavy-metal-transporting

CPX-type ATPases during acid adaptation in *Lactobacillus bulgaricus*. *Appl. Environ. Microbiol.* **2006**, *72* (12), 7445−7454.
(33) Stasiewicz, M. J.; Wiedmann, M.; Bergholz, T. M. The transcriptional response of *Listeria monocytogenes* during adaptation to growth on lactate and diacetate includes synergistic changes that increase fermentative acetoin production. *Appl. Environ. Microbiol.* **2011**, *77* (15), 5294−5306.
(34) Iyer, R.; Iverson, T. M.; Accardi, A.; Miller, C. A biological role for prokaryotic ClC chloride channels. *Nature* **2002**, *419* (6908), 715−718.
(35) Dashper, S. G.; Reynolds, E. C. pH regulation by *Streptococcus mutans*. *J. Dent. Res.* **1992**, *71* (15), 1159−1165.
(36) Budin-Verneuil, A.; Pichereau, V.; Auffray, Y.; Ehrlich, D. S.; Maguin, E. Proteomic characterization of the acid tolerance response in *Lactococcus lactis* MG1363. *Proteomics* **2005**, *5* (18), 4794−4807.
(37) Wu, C.; Zhang, J.; Chen, W.; Wang, M.; Du, G.; Chen, J. A combined physiological and proteomic approach to reveal lactic-acid-induced alterations in *Lactobacillus casei* Zhang and its mutant with enhanced lactic acid tolerance. *Appl. Microbiol. Biotechnol.* **2012**, *93* (2), 707−722.
(38) Fernandez, A.; Ogawa, J.; Penaud, S.; Boudebbouze, S.; Ehrlich, D.; van de Guchte, M.; Maguin, E. Rerouting of pyruvate metabolism during acid adaptation in *Lactobacillus bulgaricus*. *Proteomics* **2008**, *8* (15), 3154−3163.
(39) Brand, B.; Boos, W. Maltose transacetylase of *Escherichia coli*. Mapping and cloning of its structural gene, *mac*, and characterization of the enzyme as a dimer of identical polypeptides with a molecular weight of 20,000. *J. Biol. Chem.* **1991**, *266* (21), 14113−14118.
(40) Andrews, K. J.; Lin, E. C. Thiogalactoside transacetylase of the lactose operon as an enzyme for detoxification. *J. Bacteriol.* **1976**, *128* (1), 510−513.
(41) Keevil, C. W.; Marsh, P. D.; Ellwood, D. C. Regulation of glucose metabolism in oral streptococci through independent pathways of glucose 6-phosphate and glucose 1-phosphate formation. *J. Bacteriol.* **1984**, *157* (2), 560−567.
(42) Klein, A. H.; Shulla, A.; Reimann, S. A.; Keating, D. H.; Wolfe, A. J. The intracellular concentration of acetyl phosphate in *Escherichia coli* is sufficient for direct phosphorylation of two-component response regulators. *J. Bacteriol.* **2007**, *189* (15), 5574−5581.
(43) Lukas, L.; Kuzminov, A. Chromosomal fragmentation is the major consequence of the rdgB defect in *Escherichia coli*. *Genetics* **2006**, *172* (2), 1359−1362.
(44) Zheng, J.; Singh, V. K.; Jia, Z. Identification of an ITPase/XTPase in *Escherichia coli* by structural and biochemical analysis. *Structure* **2005**, *13* (10), 1511−1520.
(45) Miles, R. W.; Tyler, P. C.; Evans, G. B.; Furneaux, R. H.; Parkin, D. W.; Schramm, V. L. Iminoribitol transition state analogue inhibitors of protozoan nucleoside hydrolases. *Biochemistry* **1999**, *38* (40), 13147−13154.
(46) Rimaux, T.; Vrancken, G.; Vuylsteke, B.; De Vuyst, L.; Leroy, F. The pentose moiety of adenosine and inosine is an important energy source for the fermented-meat starter culture *Lactobacillus sakei* CTC 494. *Appl. Environ. Microbiol.* **2011**, *77* (18), 6539−6550.
(47) McLeod, A.; Snipen, L.; Naterstad, K.; Axelsson, L. Global transcriptome response in *Lactobacillus sakei* during growth on ribose. *BMC Microbiol.* **2011**, *11*, 145.
(48) Santiago, B.; MacGilvray, M.; Faustoferri, R. C.; Quivey, R. G., Jr. The branched-chain amino acid aminotransferase encoded by ilvE is involved in acid tolerance in *Streptococcus mutans*. *J. Bacteriol.* **2012**, *194* (8), 2010−2019.
(49) Len, A. C.; Harty, D. W.; Jacques, N. A. Proteome analysis of *Streptococcus mutans* metabolic phenotype during acid tolerance. *Microbiology* **2004**, *150* (5), 1353−1366.
(50) Miñambres, B.; Olivera, E. R.; Jensen, R. A.; Luengo, J. M. A new class of glutamate dehydrogenases (GDH). Biochemical and genetic characterization of the first member, the AMP-requiring NAD-specific GDH of *Streptomyces clavuligerus*. *J. Biol. Chem.* **2000**, *275* (50), 39529−39542.
(51) van de Guchte, M.; Serror, P.; Chervaux, C.; Smokvina, T.; Ehrlich, S. D.; Maguin, E. Stress responses in lactic acid bacteria. *Antonie Van Leeuwenhoek* **2002**, *82* (1−4), 187−216.
(52) Foster, J. W. *Escherichia coli* acid resistance: tales of an amateur acidophile. *Nat. Rev. Microbiol.* **2004**, *2* (11), 898−907.
(53) Wu, R.; Wang, W.; Yu, D.; Zhang, W.; Li, Y.; Sun, Z.; Wu, J.; Meng, H.; Zhang, H. Proteomics analysis of *Lactobacillus casei* Zhang, a new probiotic bacterium isolated from traditional home-made koumiss in Inner Mongolia of China. *Mol. Cell. Proteomics* **2009**, *8* (10), 2321−2338.
(54) Gomes, C. S.; Izar, B.; Pazan, F.; Mohamed, W.; Mraheil, M. A.; Mukherjee, K.; Billion, A.; Aharonowitz, Y.; Chakraborty, T.; Hain, T. Universal stress proteins are important for oxidative and acid stress resistance and growth of *Listeria monocytogenes* EGD-e *in vitro* and *in vivo*. *PLoS One* **2011**, *6* (9), e24965.
(55) Mols, M.; van Kranenburg, R.; Van Melis, C. C.; Moezelaar, R.; Abee, T. Analysis of acid-stressed *Bacillus cereus* reveals a major oxidative response and inactivation-associated radical formation. *Environ. Microbiol.* **2010**, *12* (4), 873−885.
(56) Frees, D.; Vogensen, F. K.; Ingmer, H. Identification of proteins induced at low pH in *Lactococcus lactis*. *Int. J. Food Microbiol.* **2003**, *87* (3), 293−300.
(57) Carmel-Harel, O.; Storz, G. Roles of the glutathione-and thioredoxin-dependent reduction systems in the *Escherichia coli* and *Saccharomyces cerevisiae* responses to oxidative stress. *Annu. Rev. Microbiol.* **2000**, *54*, 439−461.
(58) Zhang, J.; Fu, R. Y.; Hugenholtz, J.; Li, Y.; Chen, J. Glutathione protects *Lactococcus lactis* against acid stress. *Appl. Environ. Microbiol.* **2007**, *73* (16), 5268−5275.
(59) Lee, K.; Pi, K.; Kim, E. B.; Rho, B. S.; Kang, S. K.; Lee, H. G.; Choi, Y. J. Glutathione-mediated response to acid stress in the probiotic bacterium *Lactobacillus salivarius*. *Biotechnol. Lett.* **2010**, *32* (7), 969−972.
(60) Kim, J. E.; Eom, H. J.; Kim, Y.; Ahn, J. E.; Kim, J. H.; Han, N. S. Enhancing acid tolerance of *Leuconostoc mesenteroides* with glutathione. *Biotechnol. Lett.* **2012**, *34* (4), 683−687.
(61) Baker, L. M.; Poole, L. B. Catalytic mechanism of thiol peroxidase from *Escherichia coli*. Sulfenic acid formation and overoxidation of essential CYS61. *J. Biol. Chem.* **2003**, *278* (11), 9203−9211.
(62) Ezraty, B.; Aussel, L.; Barras, F. Methionine sulfoxide reductases in prokaryotes. *Biochim. Biophys. Acta, Proteins Proteomics* **2005**, *1703* (2), 221−229.
(63) Walter, J.; Chagnaud, P.; Tannock, G. W.; Loach, D. M.; Dal Bello, F.; Jenkinson, H. F.; Hammes, W. P.; Hertel, C. A high-molecular-mass surface protein (Lsp) and methionine sulfoxide reductase B (MsrB) contribute to the ecological performance of *Lactobacillus reuteri* in the murine gut. *Appl. Environ. Microbiol.* **2005**, *71* (2), 979−986.
(64) Dhandayuthapani, S.; Blaylock, M. W.; Bebear, C. M.; Rasmussen, W. G.; Baseman, J. B. Peptide methionine sulfoxide reductase (MsrA) is a virulence determinant in *Mycoplasma genitalium*. *J. Bacteriol.* **2001**, *183* (19), 5645−5650.
(65) Wizemann, T. M.; Moskovitz, J.; Pearce, B. J.; Cundell, D.; Arvidson, C. G.; So, M.; Weissbach, H.; Brot, N.; Masure, H. R. Peptide methionine sulfoxide reductase contributes to the maintenance of adhesins in three major pathogens. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93* (15), 7985−7990.
(66) Maurer, L. M.; Yohannes, E.; Bondurant, S. S.; Radmacher, M.; Slonczewski, J. L. pH regulates genes for flagellar motility, catabolism, and oxidative stress in *Escherichia coli* K-12. *J. Bacteriol.* **2005**, *187* (1), 304−319.
(67) Clements, M. O.; Foster, S. J. Stress resistance in *Staphylococcus aureus*. *Trends Microbiol.* **1999**, *7* (11), 458−462.
(68) Jan, G.; Leverrier, P.; Pichereau, V.; Boyaval, P. Changes in protein synthesis and morphology during acid adaptation of *Propionibacterium freudenreichii*. *Appl. Environ. Microbiol.* **2001**, *67* (5), 2029−2036.

(69) Altschul, S. F.; Madden, T. L.; Schäffer, A. A.; Zhang, J.; Zhang, Z.; Miller, W.; Lipman, D. J. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **1997**, *25* (17), 3389−3402.