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Proteomic Analysis Identifies Dysregulated Proteins in Butanol-Tolerant Gram-Positive *Lactobacillus mucosae* BR0713−33

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ABSTRACT: Butanol can be produced biologically through fermentation of lignocellulosic biomass-derived sugars by Gram-positive *Clostridium* species. For cost-effective production, increased butanol fermentation titers are desired. However, the currently available butanol-fermenting microbes do not tolerate sufficiently high butanol concentrations; thus, new butanol-tolerant strains are desired. One promising strategy is to genetically modify *Clostridium* species by introducing stress tolerance-associated genes. This study was aimed to seek butanol tolerance genes from other Gram-positive species, which might be better suited than those from Gram-negative *E. coli* or eukaryotic *Saccharomyces cerevisiae*. Several butanol-tolerant lactobacilli were reported previously, and *Lactobacillus mucosae* BR0713−33, which showed the most robust anaerobic growth in 4% butanol, was used here for proteomics analyses. Cellular proteins that responded to 2, 3, and 4% butanol were characterized. Twenty-nine proteins that were identified were dysregulated in response to increased concentrations of butanol in *L. mucosae*. Seventeen genes involved in coding for stress-tolerant proteins GroES, GroEL, and DnaK and genes involved in substrate utilization, fatty acid metabolism, and nucleotide synthesis were induced by increased butanol, and 12 genes involving energy production (F_0F_1ATP synthases) and redox balance preservation were repressed by increased butanol. These results can help guide targeted engineering strategies to improve tolerance and production of biobutanol.
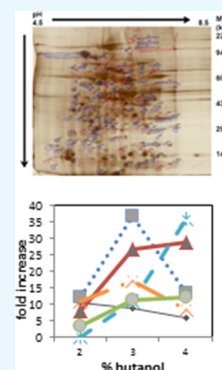
■ INTRODUCTION

Butanol has been an important chemical feedstock in the plastic polymer industry and as a potential drop-in biofuel. This solvent is used as an extractant in the flavor and food industry.[1] Approximately 10 to 12 billion pounds of butanol are produced annually through petrochemical routes.[2] The biological butanol production route has been attributed to solventogenic Clostridia anaerobic fermentation from glucose, predominantly by strains of *C. acetobutylicum* and *C. beijerinckii*.[3] More recently, the utilization of renewable lignocellulosic biomass materials for biobutanol has drawn a great deal of attention.[4] However, most naturally occurring *Clostridium* species can only produce up to 2% butanol because of end-product inhibition.[3] The bottleneck is that the increased accumulation of butanol in the bioreactor becomes toxic to its producing microbes,[5] and it is recently reported that 21 g/L of butanol is the maximum butanol that can be produced by the naturally growing *Clostridium* culture.[4a] Two recent studies reported comprehensive transcriptional profile analyses of *C. beijerinckii* genes in response to butanol shock.[6] The highest butanol-tolerant strains of *C. acetobutylicum*, NT642 and T64, were reported to be tolerant to 2.4 and 3.2% (w/v) butanol, respectively.[7]

Although the butanol synthetic pathway has been introduced into other microbes, including *Escherichia coli*,[8] *Saccharomyces cerevisiae*,[9] *Pseudomonas putida*, and *Bacillus subtilis*,[10] nevertheless, these resulting strains produce much less butanol than naturally occurring clostridial strains. Recently, a different approach has been used for increased tolerance and production

of biobutanol in model organisms. For example, a genomic library enrichment strategy was used in Gram-negative *E. coli*; from 55 genes involved in butanol challenge that were tested by overexpression or deletion, 11 of them endowed enhanced tolerance to butanol when overexpressed and 3 of them endowed increased butanol resistance when deleted.[11] The butanol stress-related genes played roles in iron transport, metabolism, and acid resistance in *E. coli*.[11] A report of overexpression of genes for fatty acid synthesis, iron uptake, and efflux pump resulted in increased butanol tolerance of up to 2% in *E. coli*.[12]

Among the engineered microbes, strains of *E. coli*, *Synechococcus elongatus*, and *S. cerevisiae* that produced butanol displayed low range tolerance (from 0.5 to 2%) to butanol.[1] Therefore, it is critical to develop higher butanol-tolerant microbes for a sustainable butanol fermentation industry. There is a need to seek more tolerant species and improve engineering strategies to reach butanol fermentation titer higher than 20 g per liter; therefore, the feedback inhibition might be avoided. If a higher titer could be achieved, the cost for butanol separation and recovery would be reduced.[13]
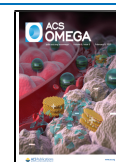
Research studies around the world have worked toward this goal, and recently, chemically induced mutant strains of *C. beijerinckii* NRRL B-598 have been shown to tolerant up to 25 g/L of butanol.[4b] The genetic engineering of a more robust butanol production host relies upon identifying butanol tolerance genes with optimized codons and better understanding of the mechanisms involved in butanol tolerance. However, the existing knowledge of genes from Gram-positive species involved in butanol tolerance is limited.

Studies reported that Gram-positive lactic acid bacteria (LAB) are found to be naturally more adaptive and tolerant to higher butanol concentrations. Strains of *L. brevis, L. delbrueckii,* and *L. diolivorans* sustained growth in 2.5 and 3% butanol.[14] A recent review of omics studies associated with butanol tolerance suggested that LAB and extremophiles might be used as potential hosts or a genetic pool for butanol tolerance studies.[7a] Continuous adaptive laboratory evolution of *Lactococcus lactis* culture against gradually increased isobutanol resulted in a strain-tolerant 28 g/L isobutanol.[15] We have used this adaptive evolution strategy to select butanol-tolerant lactic acid bacteria strains. Ten lactic acid bacteria strains from several species including *L. amylovorus, L. crispatus, L. mucosae, Pediococcus parvulus,* and *Weissella confusa* demonstrated increased butanol tolerance after long-term adaptation, and some selected strains are found to be capable of growing anaerobically in media containing 3−4% butanol.[16] The stress assay of 4% butanol exposure suggested that *L. mucosae* strains BR0713−30 and *L. mucosae* BR0713−33 appeared to be the most robust butanol-tolerant strains.[16]

The molecular mechanisms that lead to butanol tolerance in lactic acid bacteria are not well understood yet, although it has been known that lactic acid bacteria can tolerate various stress conditions.[17] Thus far, there are no reported studies linking changes in expression of specific proteins with butanol stress and/or tolerance in Gram-positive lactic acid bacteria. Taking advantage of the available genome sequences of *L. mucosae,*[18] we chose to use the butanol-tolerant *L. mucosae* strain BR0713−33, a strain isolated in our laboratory, to identify genes that are differentially expressed under increased butanol exposure. In this study, a proteomic approach was used to examine changes in proteins, with which the expression levels were altered during growth in media supplemented with 2, 3, and 4% butanol. This study will aid our understanding of major cellular events behind butanol stress and/or tolerance. Results will help in identifying unique genes responsible for butanol tolerance and further aid in improving natural producers by introducing genes responsible for butanol tolerance traits.

■ MATERIALS AND METHODS

Bacterial Growth. High butanol-tolerant strain of *L. mucosae* strain BR0713−33 was isolated and described previously in our laboratory.[16] This strain is maintained in DeMan Rogosa and Sharpe (MRS) (Becton Dickinson, Sparks, MD) under an anaerobic condition at 37 °C.[16] Single colonies were grown in 3 mL MRS broth and incubated overnight at 37 °C in a sealed anaerobic jar with an AnaeroGen sachet (OXOID Ltd., Basingstoke, England). Bacterial growth was monitored by measuring the optical absorbance at 600 nm. For butanol-induced protein expression studies, 0, 2, 3, and 4% (v/v) of 1-butanol (Sigma-Aldrich, St. Louis, MO) was added to MRS broth to a final volume of 5 mL in 14 mL sterile disposable culture tubes. The tubes were vortexed briefly and inoculated with fresh overnight cultures in MRS at a 5% ratio.

After 8 h of growth under an anaerobic condition, BR0713−33 cells were collected by centrifugation. For each butanol concentration, three duplicated cultures were carried out. Cell pellets from duplicated samples were collected and combined for protein isolation. Total cellular proteins were extracted by the Fast Protein blue kit, utilizing a bead-beating technology with a benchtop FastPrep machine (MP Biomedicals, Santa Ana, CA), and protein concentrations were determined by a BioRad Bradford assay (Hercules, CA).

Two-Dimensional Electrophoresis. Two-dimensional electrophoresis (2D-PAGE) protein gel analyses were performed by Kendrick Labs, Inc. (Madison, WI) using the carrier ampholine method of isoelectric focusing (IEF).[19] Briefly, the IEF electrophoresis was carried out in glass tubes of inner diameter 2.3 mm using 2% pH 4−8 mix Servalytes (Serva, Heidelberg Germany). 50 ng of an IEF internal standard, tropomyosin, was added to each sample prior to loading. Tropomyosin produces two polypeptide spots of a similar isoelectric point (pI); the lower spot of molecular weight (MW) 33,000 and pI 5.2 was marked on the silver-stained gels following standard protocols.[19b,20] The pH gradient plot was determined using a surface pH electrode. SDS slab gel electrophoresis was carried out according to the published protocol.[19b,21] Briefly, after equilibrium in SDS sample buffer (10% glycerol, 50 mM dithiothreitol, 2.3% SDS, and 62.5 mM tris, pH 6.8), each tube gel was sealed to the top of a stacking gel that overlays a 10% acrylamide slab gel. The SDS slab gel electrophoresis was conducted at 15 mA/gel for 4 h, as previously described.[19b,21] The molecular markers obtained from Sigma Chemical Co (St. Louis, MO) were used as size references: myosin (220,000), phosphorylase A (94,000), catalase (60,000), actin (43,000), carbonic anhydrase (29,000), and lysozyme (14,000). The gels were dried between cellophane sheets with the acid end to the left, as described in refs.[19b,21]

Computerized Comparisons. Digital analyses and gel spots comparisons were carried out as described previously.[21] Duplicate gels were generated from each sample, and the dried gels were scanned with a laser densitometer (Model PDSI, Molecular Dynamics Inc., Sunnyvale, CA). The scanner was checked for linearity prior to scanning with a calibrated Neutral Density Filter Set (Melles Griot, Irvine, CA). The images were analyzed using Progenesis Same Spots software (version 4.5, 2011, Nonlinear Dynamics, Durham, NC) and Progenesis PG240 software (version 2006, Nonlinear Dynamics, Durham, NC), following published protocols.[19b,21] The method of computerized analysis for these pairs included image warping followed by spot finding, background subtraction (average on boundary), spot position matching, spot intensity quantification, and final detailed manual checking. The spot percentage is equal to spot-integrated density above background (volume), expressed as a percentage of total density above background of all spots measured.[19b,21] The protein expression level (spot intensity) difference is defined as fold-change of spot percentages.[19b] When the corresponding protein spots from control versus butanol have the same spot percentage, the difference field will show 1.0; if the spot percentage from butanol is twice as large as control, the difference field will display 2.0, indicating twofold upregulation.

MW and pI Measurements. The pI measurements are based on the pH gradient plot generated with 9 M urea at 22 °C. The MW and pI values for each spot are determined from

algorithms applied to the reference image, as previously described.[19b]

Protein Digestion and Peptide Extraction. Proteins that were separated by 2D-PAGE and stained by Coomassie dye were individually excised, and the proteins from each gel slice were treated individually according to the published protocols.[22] Briefly, each gel piece corresponding to individually marked spots was washed separately in high-performance liquid chromatography (HPLC) grade water, and each gel piece was dehydrated. The gel slices were destained stepwise in 50 mM ammonium bicarbonate, 50 mM ammonium bicarbonate/50% acetonitrile, and 100% acetonitrile with moderate shaking. The gel slices were dried by using a speed-vac concentrator and rehydrated in 50 mM ammonium bicarbonate as described.[22b] This procedure was repeated twice. The gel slices were then rehydrated in 50 mM ammonium bicarbonate containing 10 mM DTT and incubated at 56 °C for 45 min and then transferred in 50 mM ammonium bicarbonate containing 100 mM iodoacetamide and incubated for 45 min in the dark, with occasional shaking. The gel pieces were then rewashed again in 50 mM ammonium bicarbonate/50% acetonitrile and 100% acetonitrile with moderate shaking, followed by drying in speed-vac.[22b] The dried gel pieces were then rehydrated using 50 mM ammonium bicarbonate containing 10 ng/mL trypsin and incubated overnight at 37 °C with gentle shaking, allowing peptide digestion reactions to be complete. The resulting fragmented peptides were extracted twice with 5% formic acid/50 mM ammonium bicarbonate/50% acetonitrile and once with 100% acetonitrile. The purified peptide mixture was dried again in a speed-vac and solubilized in 20 μL of 0.1% formic acid and 2% acetonitrile in HPLC grade water.

LC with Tandem Mass Spectrometry. The peptides mixture from each individual protein spot was analyzed by reversed-phase LC and LC with tandem mass spectrometry (LC−MS/MS) using a NanoAcuity UPLC (Micromass/Waters, Milford, MA) coupled to a Q-TOF Micro MS (Micromass/Waters, Milford, MA), according to published procedures.[22] Briefly, the peptides were loaded onto a 100 μm × 10 mm NanoAquity BEH130 C18 1.7 μm UPLC column (Waters, Milford, MA) and eluted over a gradient of 2−80% organic solvent (ACN containing 0.1% FA) at a flow rate of 400 nL/min for 150 min. The column was coupled to a Picotip Emitter Silicatip nano-electrospray needle (New Objective, Woburn, MA), and the aqueous solvent used was 0.1% FA in HPLC water.[22b]

MS data acquisition involved survey MS scans and automatic data-dependent analysis of the top three ions of the highest intensity and the charge of 2+, 3+, or 4+.[22b] The MS/MS was triggered when the MS signal intensity exceeded 10 counts/second. In survey MS scans, the three most intense peaks were selected for collision-induced dissociation and fragmented until the total MS/MS ion counts reached 10,000 or for up to 6 s each; these parameters were same as previously published.[22b] Calibration for precursor and product ions was conducted with 1 pmol of the standard peptide GluFib (Glu1-Fibrinopeptide B), which has the amino acid sequence EGVNDNEEGFFSAR, and the monoisotopic doubly charged peak with an m/z value of 785.84, and the condition was previously published.[23]

Data Processing and Protein Identification. The raw MS data were processed using ProteinLynx Global Server (PLGS, version 2.4) software, as previously described.[22] The following standard parameters were used: background subtraction of polynomial order 5 adaptive with a threshold of 30%, two smoothings with a window of three channels in Savitzky−Golay mode, and centroid calculation of top 80% of peaks based on a minimum peak width of 4 channels at half height, as described.[23]

The resulting pkl files were initially submitted to the Mascot database (version 2.2.1, Matrix Science, www.matrixscience.com, London, UK) for protein identifications using the following parameters: databases from NCBI (all organisms), parent mass error of 1.3 Da, product ion error of 0.8 Da, enzyme used: trypsin, one missed cleavage, propionamide as cysteine fixed modification and methionine oxidized as variable modification; these guidelines are followed exactly as published.[23] To eliminate false negatives, further parameters including additional databases and a strict organism choice, with a narrower window for the parent mass error (1.2 and then 0.2 Da) and product ion error (0.6 Da), plus up to two missed cleavage sites for trypsin were used. Furthermore, the pkl files were also used to search against PLGS database version 2.4 (www.waters.com) with similar parameters as used for Mascot search.[22b] The Mascot and PLGS searches through NCBI and Swiss-Prot databases were cross-checked, and only proteins identified by two or more peptides and with a MASCOT score > 50 were used for the final identifications of corresponding proteins. For proteins identified by one peptide or a Mascot score lower than 25, their MS/MS spectra were manually inspected to confirm the identity of the protein. The final check-up of all identified proteins was conducted by blast search against published *L. mucosae* genome.[18]

■ RESULTS AND DISCUSSION

2D-PAGE Profile. There are a total of 1998 protein coding genes with 1623 functional COG categories of the whole *L. mucosae* genome.[18] In this study, 603 protein spots (30% of total proteins) were analyzed for either fold of induction or reduction detected by 2D gels. Total cellular proteins were isolated and compared when growing for 8 h in MRS with addition of 2, 3, and 4% butanol vs 0% butanol control. 8 h of butanol exposure was chosen here corresponding to mid-exponential and late exponential growth phases for proteomic analyses because our previous studies[16] suggested that the protein contents after 8 h of growth might be better in reflecting and representing long-term butanol stress responses (habituation adaptation) in contrast to short-term butanol shock (transient adaptation). It is not well understood yet how lactic acid bacteria switch from transient to habituation adaptation to a specific stress; these two states are not completely identical, but may overlap to a certain degree.[17] This is our reasoning of using a higher butanol-tolerant *L. mucosae* strain BR0713−33 to identify its butanol tolerance trait-associated genes.[16] The changes of individual polypeptides in responses to butanol were shown on 2D-PAGE gels. Figure 1A,B shows protein profile changes in 4% butanol (**1A**) in contrast to the 0% butanol (**1B**) growth condition. Figure S1 A-B shows protein profile changes in 2% butanol (**A**) in contrast to the 0% butanol (**B**) growth condition, and Figure S2A-B shows protein profile changes in 3% butanol (**A**) in contrast to the 0% butanol (**B**) growth condition; these analyses demonstrated significant changes of protein expression levels, the changes vary with environmental butanol concentrations, and these changes are more pronounced with increased butanol from 2 to 4%, indicating the global and
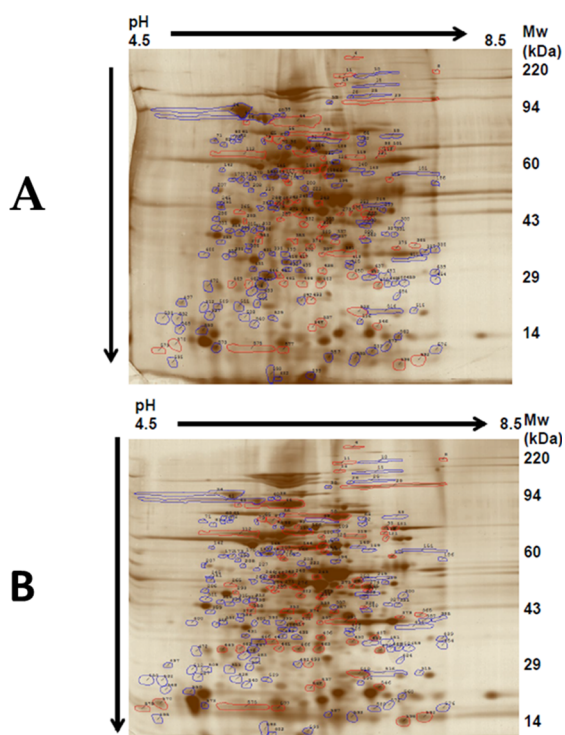
Figure 1. (A) 2D Gel Difference Image (2835 #8) of averaged 4% butanol (gels 2835 #7−8) versus averaged control (gels 2835 #1−2). Polypeptide spots increased in 4% butanol vs control are outlined in blue, while spots decreased in 4% butanol vs control are outlined in red. (B) 2D gel difference image (2835 #2) of control to show spots increased in averaged control (gels 2835 #1−2) versus averaged 4% butanol (gels 2835 #7−8). Polypeptide spots increased in 4% butanol vs control are outlined in blue, while spots decreased in 4% butanol vs control are outlined in red.

complex nature of cellular responses to increased butanol in the growth medium.

LC−MS/MS Analyses. A total of 53 out of 603 spots characterized above with detectable changes in response to butanol concentrations were subjected for LC−MS/MS analyses. Among the 53 spots, 39 proteins showed from 2.3- to 18.7-fold (p value of T-test less than 0.05 for at least one of the tested butanol concentrations) of induction (Table S1) and 14 proteins showed from −1.9- to −20.8-fold (p value of T-test less than 0.05 for at least one of the tested butanol concentrations) of reduction (Table S2). Results from **LC−MS/MS** analyses of the polypeptide peaks were identified by using bioinformatic analysis tools as described in the experimental section. False positives were eliminated manually, and a total of 29 protein spots were identified. Based on detected segments of positive peptide sequences, the corresponding genes were identified after searching known protein databases and protein sequences were translated from *L. mucosae* genome. Among those identified, 17 protein spots shown increased expression (Table 1), and 12 protein spots shown reduced expression in the presence of 2 or 3 or 4% butanol (Table 2). It is noted that two protein spots 311 and 337 showed increased expression in the presence of butanol in this study, but have not yet been identified by genome annotations and thus presented as hypothetical proteins (Table 1).

Butanol-Stimulated Gene Expression. For induced expression of proteins, the following categories including

protein degradation, protein folding and synthesis, substrate utilization, fatty acid degradation, and nucleotide synthesis are described below.

Protein Degradation. Among the increased protein induction (Table 1, Table S1), the identification of spot 71 (Dipeptidae pepV), spot 73 (molecular chaperone DnaK), spot 318 (GroES, 345 aa), 511 (GroEL), and 599 (GroES, 102 aa) (Table 1) suggested that growth in butanol facilitated the degradation of mis-folded proteins by proteinase pepV. The induced production of this group of enzymes in butanol growth conditions suggested that *L. mucosae* cells responded to ensure correct folding of proteins. It is noted that the predicted MWs of spot 511 and 599 (Table 1) are more than twofold that detected on 2D gel (Table S1), which is expected because most chaperones are multimeric. The fact that spot 318 and 511 both belong to the GroES family confirmed the important role of protein folding in cellular response to butanol.

It has been known that DnaK is needed to maintain protein stability and correct folding, which are ensured by molecular chaperones (GroEL) and cochaperones (GroES).[24] It has also been reported that increased expression of DnaK and GroESL are essential when butanol accumulates in high concentrations during fermentation by using *C. beijerinckii* NCIMB 8052[25] A recent study reported improved butanol titers of *C. acetobutylicum* ATCC824 after overexpressing GroESL and DnaK from an extremophilic bacterium[26] The activation of heat-shock proteins has also been identified in the upregulated gene clusters via transcriptional profiling studies of *C. beijerinckii* NRRL B-598 to a butanol shock [5]. DnaK was also reported during acid stress responses.[27]

Substrate Utilization. The identification of spot 168 as aldose 1-epimerase (Table 1) confirmed the role of this enzyme in butanol-induced carbohydrate utilization. While spot 385, identified as phosphoglyceromutase, and spot 536, identified as 6-phospho 3-hexuloisomerase (Table 1), were increased in response to 2 to 4% butanol, it also suggested that carbohydrate metabolisms dealing with glycolysis and substrate phosphorylation are important in supplying energy in butanol stress-responsiveness. *Lactobacillus rhamnosus* GG was found modifying the metabolism of carbon sources in response to acid stressed conditions by altering the expression of enzymes and substrate metabolites.[27]

Fatty Acid Degradation. Fatty acid degradation and synthesis are also found in butanol growth responses (Table 1). Spot 207 (cholylglycine hydrolase), and spot 251 for 2-oxoisovalerate dehydrogenase that involves branched-chain fatty acid metabolism and degradation of amino acid (valine, leucine, and isoleucine). Spot 548 (cyclopropane-fatty-acyl-phospholipid synthase) plays a role in fatty acid biosynthesis and lipid metabolism.

Butanol as a short chain alcohol can partition into the cytoplasmic membranes and changes the membrane structures and thus interrupts membrane functions. Studies showed that when exposed to 1% butanol, *C. acetobutylicum* adapted to 20−30% increase of membrane fluidity.[28] Similarly, a chemically induced butanol-tolerant strain of *C. acetobutylicum* ATCC 824-SA-2 showed an increase in longer acyl chain fatty acids and increase in membrane fluidity.[29] The high solvent-tolerant Gram-positive strain of *Staphylococcus haemolyticus* showed an increase of anteiso fatty acids from 25.8 to 33.7% and decrease of 20:0 straight-chain fatty acids from 19.3 to 10.1%.[30] The importance of fatty acid metabolism in butanol tolerance has been evident in recently reported omics analyses.[4a,6b,7a]

Table 1. LC−MS/MS Identification of Proteins from *L. mucosae* with Increased Expression in Response to Butanol (17 Spots).

Spot #	Increased Proteins Identified	Mascot Score	NCBI Reference Sequence Accession#	MW
10	Alanyl-tRNA synthase [Lactobacillus mucosae]	146	WP_006500230.1	101295
26	Inosine 5'-monophosphate dehydrogenase [Lactobacillus mucosae]	228	WP_006499311.1	39784
60	mannose-1-phosphate guanylyltransferase/ guanylyltransferase/ [Variovorax paradoxus B4]	97	WP_021007855.1	54246
68	GMP synthase [Lactobacillus mucosae]	148	WP_006500536.1	57894
71	Dipeptidase PepV [Lactobacillus mucosae]	404	WP_006500122.1	51008
73	molecular chaperone DnaK [Lactobacillus mucosae]	176	WP_006499794.1	67170
168	aldose 1-epimerase [Lactobacillus mucosae]	62	WP_006500547.1	39362
207	Cholylglycine hydrolase [Lactobacillus mucosae]	PLGS DB	WP_006499363.1	37046
251	2-oxoisovalerate dehydrogenase subunit beta [Lactobacillus mucosae]	146	WP_006499818.1	35501
311	Hypothetical protein [Lactococcus garvieae]	56	WP_003136551.1	101845
318	molecular chaperone GroES [Lactobacillus mucosae]	64	WP_003713822.1	36906
337	Hypothetical protein [Lactobacillus mucosae]	230	WP_006500133.1	32920
385	Phosphoglyceromutase [Lactobacillus reuteri DSM 20016]	98	WP_003665449.1	26176
511	molecular chaperone GroEL [Lactobacillus mucosae]	189	WP_006500434.1	56830
536	6-phospho 3-hexuloisomerase [Lactobacillus mucosae]	71	WP_006500786.1	19010
548	Cyclopropane-fatty-acyl-phospholipid synthase [Lactobacillus buchneri NRRL B-30929]	49	WP_006499331.1	44868
599	molecular chaperone GroES [Lactobacillus mucosae]	259	WP_006500435.1	10824

Nucleotide Synthesis. Spot 26 (Inosine 5′-monophosphate dehydrogenase) is used for biosynthesis of guanine nucleotides, which plays a role in the guanine nucleotide level. Along with spot 60 (mannose-1-phosphate guanylyltrasferase) and spot 68 (GMP synthase), these enzymes are responsible for DNA and RNA synthesis, signal transduction, glycoprotein synthesis, and energy transfer, which appeared important in butanol tolerance (Table 1). The levels of guanine nucleotides and cellular accumulation of alarmones guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp) have been recognized as critical triggers for bacterial stress responses; this response is called the stringent response.[31] This highly conserved stress response was originally found as a response to amino acid starvation, but has been recognized as a response to many different environmental stress conditions[32] as well as low temperature growth stress[33] and heat shock.[34] Recently, a master regulator of almost all aspects of bacterial physiology, including growth control, growth phase transition, the production of toxin, development of antibiotics resistance, and virulence associations, was reported.[35] Our study linked butanol tolerance to the stringent response conditions, and further study is essential to elucidate the roles of alarmones to butanol tolerance.

Protein Synthesis. The induced expression of spot 10 (alanyl-tRNA synthase) (Table 1) suggested that butanol facilitated protein synthesis especially in adding alanine, and this may involve production of peptidoglycan that seems needed during butanol growth. The Gram-positive cell wall peptidoglycan is the first line of defense against high environmental butanol that these cells encounter. Peptidoglycan is a complex polymer matrix with amino acids and sugars, and alanine is one of the major amino acids. Genetic engineering of the bacterial lipid membrane and cell wall components was proposed as one of the new promising strategies for increased butanol tolerance.[36] Further studies to confirm the role of the alanyl-tRNA synthase gene and its impact of cell wall synthesis in butanol response would provide new insights to enhance butanol tolerance.

Dynamics of Butanol-Induced Expression. In order to understand the dynamics of protein production in response to

Table 2. LC−MS/MS Identification of Proteins from _L. mucosae_ with Decreased Expression in Response to Butanol (12 Spots).

Spot #	Decreased Proteins Identified	Mascot Score	NCBI Reference Sequence Accession #	MW
4	DNA-directed RNA polymerase subunit beta [Lactobacillus mucosae]	598	WP_006500631.1	132836
33	transketolase [Lactobacillus mucosae]	53	WP_006500262.1	73310
46	aspartate carbamoyltransferase [Lactobacillus mucosae]	282	WP_006499951.1	35664
65	F0F1 ATP synthase subunit alpha [Lactobacillus mucosae]	323	WP_006500288.1	55633
111	6-phosphogluconate dehydrogenase [Lactobacillus mucosae]	186	WP_006500812.1	53644
113	F0F1 ATP synthase subunit beta [Lactobacillus mucosae]	74	WP_006500286.1	61508
121	D-lactate dehydrogenase [Lactobacillus mucosae]	MS Tag	WP_006499141.1	37067
270	ribose-phosphate pyrophosphokinase [Lactobacillus mucosae]	184	WP_006499720.1	36603
302	UTP–glucose-1-phosphate uridylyltransferase [Lactobacillus mucosae]	108	WP_006500415.1	33770
462	Peptide deformylase [Lactobacillus mucosae]	PLGS DB	WP_006499727.1	21074
463	Asparagine synthetase [Lactobacillus mucosae]	PLGS DB	WP_039946159.1	60173
466	Cysteine synthase [Lactobacillus mucosae]	PLGS DB	WP_006499568.1	32521

butanol, graphic plots of the fold induction of individual spot (y-axis) versus butanol concentrations in the growth medium (X-axis) were presented (Figure 2). These panels were grouped based on fold increases, highest on the top (Figure 2A) and lowest on the bottom panel (Figure 2C). It is shown here that the following proteins, spots **71**, **337**, and 551 (Figure 2A), spots **168**, **207**, and **385** (Figure 2B), and spots 10, 251, 536, 548, and **599** (Figure 2C), demonstrated stable fold increases in response to increased butanol contents from 2 to 4%. The spot number highlighted in bold are more pronounced than others.

Spot **60** (mannose-6-phosphate isomerase) and spot 73 (DnaK, Figure 2A), spot **68** (GMP synthase), spot **318** (GroES, Figure 2B), and spot 311 (hypothetical, Figure 2B) showed greater fold induction in response to 3% butanol but reduced fold induction in response to 2 and 4% butanol. As reported in this work, results suggested that bacterial cellular responses to butanol are not a simple linear curve corresponding to the concentration of butanol from 2, 3, and 4% increases. This could be attributed to multilayered differences in cellular metabolic flux and bacterial cell growth stage/life cycle variations. Previously, a proteomics study of ethanol-tolerant _Lactobacillus buchneri_ B-30929 strain identified 20 key ethanol-responsive genes,[19b] and some of these genes have been confirmed for their roles to endow ethanol tolerance traits in recombinant _E. coli_.[37] Although bacterial tolerance mechanisms to ethanol and butanol are very different because these two alcohols have unique chemical properties and certain tolerance traits might be strain-specific, it was found that the alcohol tolerance is closely associated with common function-related proteins/enzymes such as proteinases and GroES molecular chaperones, as well as dehydro-

genases for cofactor regeneration/redox balance maintenance.[19b]

Overall, the increased expression of the detected proteins in Figure 2 suggested their important roles in butanol-responsiveness by _L. mucosae_, particularly in dealing with substrate utilization, energy production, and general stress responses. Our study demonstrated the important function of molecular chaperones in cellular responses to butanol stress. Global responses to butanol by clostridial species including cell envelope modification, general stress protein formation, efflux stimulation, and accumulation of cellular protective compounds have been documented.[4a]

One protein spot 26 (inosine 5′-monophosphate dehydrogenase, Figure 2A) showed that fold induction gradually reduced from 10.5- to 8.9- and 5.8-folds corresponding to the increased butanol concentration from 2 to 3% and 4%; this might indicate that _L. mucosae_ cells switched from an active carbohydrate metabolism to an energy-saving mode, and it is possible that the adaptation of butanol through increased dehydrogenase activity will thus regenerate NAD(P)H to balance redox potential.

The identification of spot 337 (estimated MW of 28,428 Da from the 2D gel analysis) as a hypothetical protein is closer to the predicted MW of 32,920 Da from its GenBank entry. Spot 337 showed 10.9- and 36.1-folds of induction with 3 and 4% butanol, respectively (Table S1, Figure 2A), but this 32,920 Da protein is a hypothetical protein with unknown functions (Table 1). In contrast, spot 311 as a hypothetical protein with a predicted MW of 101 kDa was originally detected in 2D gel of MW 30 kDa; this could be fragmentation of a large protein during butanol stress, and this observed disagreement needs to be addressed with follow-up studies. Spot 311 showed 9.1- and
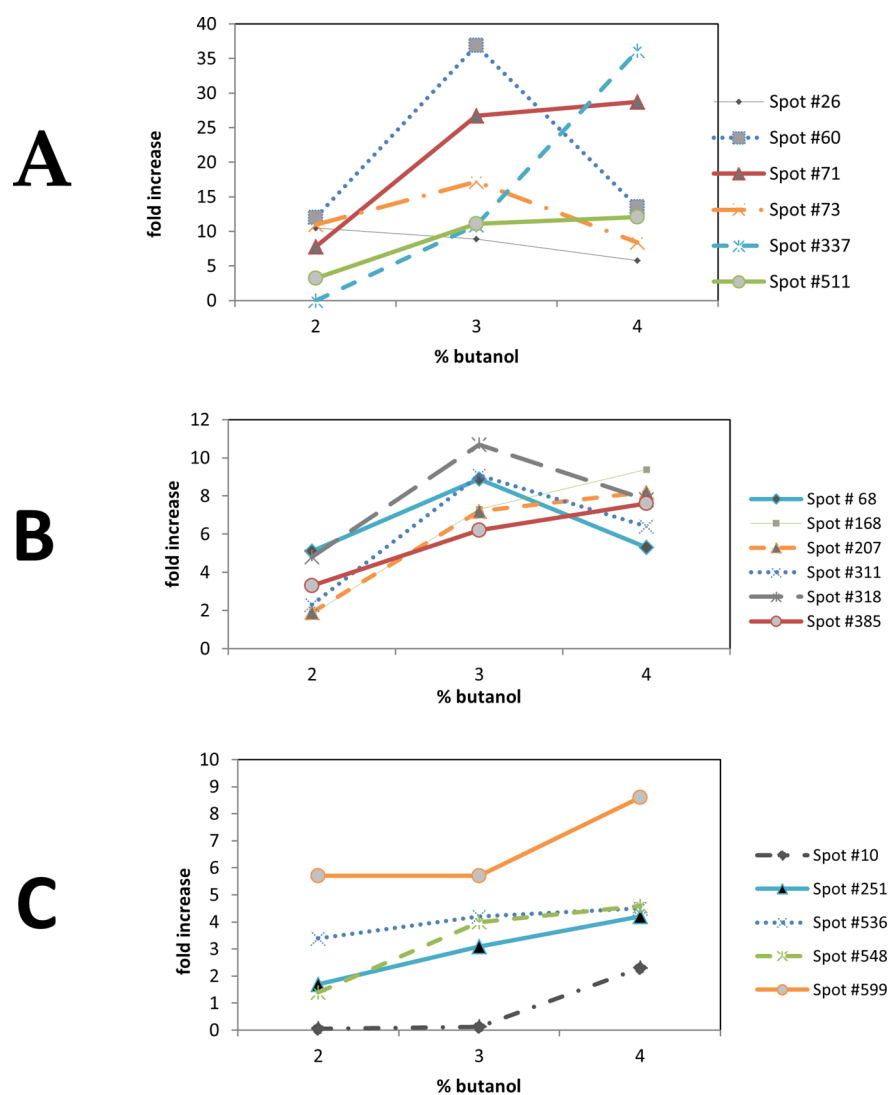
Figure 2. Panels A, B, and C. Graphic plots of the fold induction of individual spot (y-axis) versus butanol concentrations in the growth medium (x-axis). The fold changes were relative to the expression levels in the control of 0% butanol sample. The corresponding accession NCBI Reference Sequence numbers from *L. mucosae* genome for individual spots are listed in Table 1.

6.4-folds of induction with 3 and 4% butanol, respectively (Table S1) Further investigation and confirmation of these two genes (corresponding to spots 337 and 311) and their specific roles in response to butanol that were uncovered in this study will be necessary.

Butanol-Repressed Expression. Among the most decreased expression in response to increased butanol contents in the growth condition (Table 2, Table S2), spots including 113 (F_0F_1ATP synthase subunit beta, from −4.7 to −19.5), 65 (F_0F_1ATP synthase subunit alpha, from −4.1 to −9.3), 111 (6-phospohogluconate dehydrogenase, from −1.5 to −20.8), 121 (D-lactate dehydrogenase, from −6.7 to −27.7), 270 (ribose-phosphate pyrophosphokinase, from −2.2 to −11.4), 462 (peptide deformylase, from −1.8 to −13.6), 463 (asparagine synthetase, from −1.2 to −7.1), and 466 (cysteine synthase from −2.4 to −7.4) showed higher fold of reduction. The F_0F_1-ATPase is a well-known mechanism that lactic acid bacteria use for protection against acid stress conditions.[27,38] The genome sequence of higher butanol-producing mutants showed mutations in genes encoding ATPase, efflux pump regulators, and stress responses, and changes in the cell membrane structure were significant in the butanol stress response.[4b]

Our results suggested that the pentose phosphate pathway and amino acid (Asn and Cys) syntheses were slowed down during growth with increased butanol. Asp plays an important role in glycoprotein synthesis, Cys as a sulfur-containing amino acid is critical for redox reactions, and the formation of a disulfide bond between two cysteine residues of a protein is important to maintain a stable 3D structure.

The ratio of $NAD(P)H$ /$NAD(P)^+$ might be adjusted by reduced activity of D-lactate dehydrogenase and 6-phospohogluconate dehydrogenase. The reduced expression levels of the F_0F_1ATP synthase subunit alpha beta subunits suggested reduced membrane-bound ATP production, a negative impact of proton transfer coupled with substrate utilization. The reduced expression of this group of proteins appeared more pronounced with 4% butanol content in growth media, except spot 113 (Figure 3A), in which −19.5 reduction was observed with the 3% butanol condition. Additional proteins were more repressed by 3% butanol, but to a less degree by 4% butanol, including spot 4 (DNA-directed RNA polymerase, −4.8, Figure 3B) and spot 33 (transketolase, −10.3, Figure 3A).
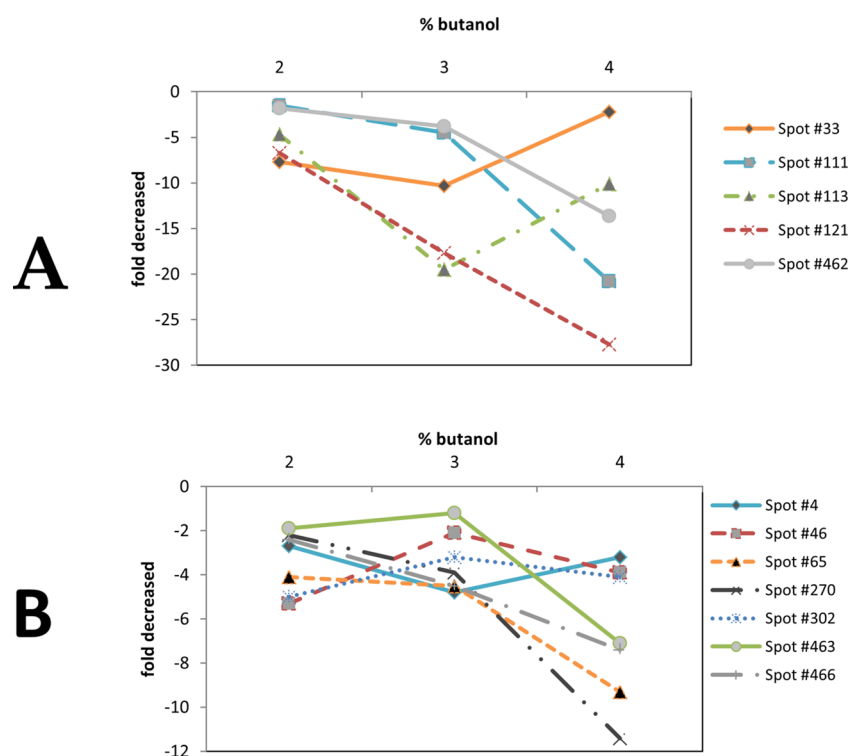
Figure 3. Panels A and B. Graphic plots of the fold reduction of individual spot (y-axis) versus butanol concentrations in the growth medium (x-axis). The fold changes were relative to the expression levels in the control of the 0% butanol sample. The corresponding accession NCBI reference sequence numbers from *L. mucosae* genome for individual spots are listed in Table 1.

This suggested that butanol induced slowdown of essential RNA transcription for cellular activities as well as metabolism through the pentose phosphate pathway. Nevertheless, the highly repressed spots 175 (−26.3 fold) and 252 (−53.5 fold) in 3% butanol vs 0% butanol conditions were not identified.

Graphic charts of butanol-repressed proteins are presented in Figure 3. The upper panel (Figure 3A) showed proteins with higher folds of decreases than the group in the lower panel (Figure 3B). These data indicated that spots 111, 121, and 462 (Figure 3A) and spots 65, 270, and 466 (Figure 3B) showed downhill decrease of protein production corresponding to increased butanol concentrations from 2 to 4%.

Spots 46 (aspartate carbamoytransfeerase), 302 (UTP-glucose-1-phosphate uridylytransferase), and 463 (asparagine synthetase) shown in Figure 3B show a similar response pattern, in that 2 and 4% butanol repressed more than 3% butanol, except that more pronounced repression was observed for spot 463 in the 4% butanol condition (Figure 3B).

In summary, this study used 2D-PAGE and proteomics analyses to identify dysregulated proteins in response to growth in different concentrations of butanol. The proteins identified in the current study will be crucial in identification of the molecular mechanisms responsible for adaptation to growing conditions with high concentrations of butanol. Work is in progress using a recombinant expression approach in an independent *E. coli* system to examine genes identified here for their roles in butanol tolerance without interference of their original genetic background. We anticipated that several key tolerance genes from the current study will be used to engineer butanol-producing strains to enhance butanol tolerance.

■ CONCLUSIONS

The identification of butanol stress-related proteins provided us a glimpse of the dynamic gene expression profile governing tolerance to high butanol concentrations. These proteomic analyses successfully identified 29 proteins involved in environmental butanol stimuli. The results will facilitate the cloning and confirmation of induced or reduced expression in response to butanol conditions that may be most likely due to butanol stress when compared with control culture without butanol. Once confirmed by either genetic knockouts of selected genes leading to butanol sensitivity or recombinant overexpression of selected genes endowing butanol tolerance in *E. coli*, the corresponding butanol-tolerant genes will be used for improving biocatalysts for efficient conversion of biomass to biofuel in butanol-producing strains.

■ FUNDING

■ ASSOCIATED CONTENT

ⓢ **Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c06028.

2D gel difference images and 2D gel computational images (PDF)

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S.L. and K.M.B. designed the experiments. C.D. and N.Q. assisted in data analyses and editing of this manuscript. S.L. wrote and edited the manuscript. All authors approved the final manuscript.

Notes

The authors declare no competing financial interest.

■ DEDICATION

[⊥]The authors dedicate this study to the memory of our colleague Dr. Kenneth Bischoff.

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