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GENOMICS, TRANSCRIPTOMICS, PROTEOMICS

Transcriptome profiling of *Zymomonas mobilis* under furfural stress

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Abstract Furfural from lignocellulosic hydrolysates is the prevalent inhibitor to microorganisms during cellulosic ethanol production, but the molecular mechanisms of tolerance to this inhibitor in *Zymomonas mobilis* are still unclear. In this study, genome-wide transcriptional responses to furfural were investigated in *Z. mobilis* using microarray analysis. We found that 433 genes were differentially expressed in response to furfural. Furfural up- or down-regulated genes related to cell wall/membrane biogenesis, metabolism, and transcription. However, furfural has a subtle negative effect on Entner–Doudoroff pathway mRNAs. Our results revealed that furfural had effects on multiple aspects of cellular metabolism at the transcriptional level and that membrane might play important roles in response to furfural. This research has provided insights into the molecular response to furfural in *Z. mobilis*, and it will be helpful to construct more furfural-resistant strains for cellulosic ethanol production.

Keywords Furfural · Lignocellulosic hydrolysates · Bioethanol · *Zymomonas mobilis* · Microarray

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Introduction

Although lignocellulosic biomass provides an abundant and renewable source for the fermentative production of liquid fuels and valuable chemicals, biomass pretreatments are necessary to make carbohydrates available for enzymatic hydrolysis and further fermentation. As a result, by-products such as furfural and 5-hydroxymethylfurfural (HMF), organic acids, vanillin and lignin monomers will be produced during the pretreatment process of acid-catalyzed hydrolysis of lignocelluloses, which will have a negative effect on the consequent enzymatic hydrolysis and ethanol fermentation (Almeida et al. 2007; Liu 2006). One approach to overcome the inhibition caused by pretreatment processes is to remove the inhibitor from the hydrolyzate by physical, chemical, or biochemical method (Zhang et al. 2010), which will increase costs, add complexity to the process, and generate extra waste products of manufacturing cellulosic ethanol (Liu 2006). Engineered ethanologenic strains' tolerance to hydrolysate by-products is another attractive method for improving lignocellulosic bioethanol production based on the mechanisms of stress response. Recently, extensive reviews were concentrated on inhibitors formed by the pretreatment of lignocellulosic materials and their inhibition of ethanol production in yeast and bacteria (Pienkos and Zhang 2009).

Furfural is one of the major inhibitors in lignocellulosic hydrolysates. Although the mechanism of furfural stress has been studied intensively on DNA damage (Barciszewski et al. 1997; Khan et al. 1995), metabolite analysis, enzyme activity analysis, metabolic flux analysis, kinetic analysis, genomics, and comparative proteomic in *Saccharomyces cerevisiae* or *Escherichia coli* (Lin et al. 2009a, b; Palmqvist and Hahn-Hagerdal 2000), the molecular mechanism involved in the tolerance and adaptation of ethanologenic

strains to inhibitors represented in lignocellulosic hydrolysate is still unclear.

S. cerevisiae, *E. coli*, and other microorganisms have been shown to contain enzymes that catalyze the reduction of furfural to the less toxic product (e.g., furfuryl alcohol, etc.) (Almeida et al. 2008; Gutiérrez et al. 2006; Koopman et al. 2010; Liu et al. 2008; Zaldivar et al. 2000). Previous studies (Koopman et al. 2010) at the gene level showed that furfural tolerance in *S. cerevisiae* requires a functional pentose phosphate pathway (PPP) and induces the accumulation of reactive oxygen species (Allen et al. 2010; Gorsich et al. 2006). Comparative proteomics also indicated that central carbon metabolism, levels of alcohol dehydrogenases, the redox balance, and other general stress response (such as unfolded protein response, oxidative stress, osmotic and salt stress, DNA damage, and nutrient starvation) may be related to the tolerance of ethanologenic yeast for and its adaptation to furfural (Lin et al. 2009a). These insights into the response of yeast to the presence of furfural will benefit the design and development of inhibitor-tolerant ethanologenic yeast by metabolic engineering or synthetic biology. For example, overexpression of *zwf*1 of PPP pathway and *Lsm* protein allowed *S. cerevisiae* to grow at furfural concentrations that are normally toxic (Gorsich et al. 2006; Yang et al. 2010).

Cellulosic hydrolysate toxicity and tolerance mechanisms in *E. coli* have also been reviewed by Ryan T. Gill (Mills et al. 2009). Previous studies in *E. coli* suggested that furfural may detrimentally affect multiple glycolytic enzymes essential to central metabolism (Zaldivar et al. 1999). Furan tolerance in *E. coli* primarily depends on lower expression of *yqhD* and *dkgA*. Furan tolerance was also increased by adding plasmids encoding a NADPH/NADH transhydrogenase (*pntAB*). Together, Ingram LO proposed a hypothesis that the NADPH-dependent reduction of furan by *yqhD* and *dkgA* inhibits growth by competing with biosynthesis for this limiting cofactor (Miller et al. 2010). This hypothesis was verified by deleting *yqhD* and *dkgA* gene (Miller et al. 2009). Another study revealed that overexpression of NADH-dependent oxidoreductase *FucO* could also improve furfural tolerance in *E. coli* (Wang et al. 2011). These extensive studies on furfural stress response to yeast and *E. coli* will play an important role in strain improvement by metabolic engineering or synthetic biology.

Zymomonas mobilis is a candidate microorganism for converting cellulosic biomass into ethanol or other valuable chemicals for its special Entner–Doudoroff (ED) pathway and desirable industrial characteristics. By using ED pathway to metabolize glucose, 1 mol of ATP is produced per molecule of glucose (Swings and Deley 1977). Different engineered *Z. mobilis* strains have also been successfully constructed by introducing desirable genes including C5 (arabinose and xylose) metabolic pathway (including five arabinose-metabolized related genes—*araA*, *araB*, *araD*, *talB*, and *tktA*, four xylose-metabolized related genes—XI, XK, *tkt*, and *tal*, respectively) (Deanda et al. 1996; Zhang et al. 1995) and cellulolytic enzymes (Linger et al. 2010) to convert cellulosic biomass into ethanol. Seo et al. reported the first genome sequence for *Z. mobilis* ZM4 (Seo et al. 2005). The complete genome of *Z. mobilis* ZM4 contains a 2,056,416-bp circular chromosome and five circular plasmids. To date, the US Department of Energy's Joint Genome Institute (JGI) has also published the complete genome sequence of *Z. mobilis* NCIMB11163, 29192, and 10988 (Kouvelis et al. 2009, 2011; Pappas et al. 2011). The other strains of this organism have been hitherto sequenced for comparative genome analysis (http://www.ncbi.nlm.nih.gov/sites/entrez?db=bioproject&cmd=Retrieve&dopt=Overview&list_uids=12354). With the completed genome from different *Z. mobilis* strains on hand, comparative genomics or global expression analysis should reveal ways to improve the performance of *Z. mobilis*, and more approaches to strain improvement will certainly be identified in the future (Jeffries 2005). In addition, recent achievements to improve transformation efficiency by modifying DNA restriction–modification systems (Kerr et al. 2010), as well as the genome-scale modeling and in silico analysis were completed (Widiastuti et al. 2011). These extensive studies will also aid future metabolic engineering and synthetic biology in strain improvement.

However, few studies have examined the response of *Z. mobilis* to various stresses. For example, furfural was examined for toxicity against CP4 (pZB5) for the first time in 1997 (Ranatunga et al. 1997). The influence of furfural on the recombinant *Z. mobilis* strain CP4 (pZB5) was also investigated in 2005, which showed that furfural led to decrease by 30 and 60 % (*w*/*v*) in biomass production and 19 and 76 % (*v*/*v*) in ethanol production in *Z. mobilis* CP4 (pZB5) when 0.475 and 1.9 g/l furfural was added in the fermentation media, respectively (Gutierrez-Padilla and Karim 2005). Zhang's group at National Renewable Energy Laboratory, USA, have also presented an effective high-throughput method to evaluate the impact of inhibitory compounds from lignocellulosic hydrolysate on the growth of *Z. mobilis* (Franden et al. 2009). However, only one research has examined response to furfural stress at the gene level. Steven D. Brown et al. showed that *Z. mobilis Hfq* (ZMO0347) plays important roles in resisting multiple lignocellulosic pretreatment inhibitors including acetate, vanillin, furfural, and HMF (Yang et al. 2010). It offers the possibility to manipulate *hfq* for *Z. mobilis* strain improvement. However, the physiological basis and genetic mechanisms involved in furfural tolerance for *Z. mobilis* are poorly understood.

In this study, microarray technology was used to investigate the expression profiling of the ethanologenic *Z. mobilis* in response to furfural stress. The results showed that 433 genes were expressed as up- or down-regulated. These data will help us to understand the molecular mechanisms and provide a global insight into strain improvement by metabolic engineering or synthetic biology.

Materials and methods

Bacterial strains and fermentation conditions

Z. mobilis ZM4 (ATCC31821) was cultured in rich media (Goodman et al. 1982) at 30 °C without shaking. Cultures were maintained on glucose agar (20.0 g/l glucose, 10.0 g/l yeast extract, and 15.0 g/l agar). The organism was subcultured to fresh inoculum media for 24 h at 30 °C before being inoculated into the fermentation medium. The inoculum medium (g/l) consisted of 10.0 g yeast extract, 1.0 g $MgCl_2$, 1.0 g $(NH_4)_2SO_4$, 1.0 g KH_2PO_4, and 20.0 g glucose. The concentration of furfural in lignocellulosic hydrolysates was measured in the range from 0.5 to 11 g/l (Almeida et al. 2007). Our primary experiment showed that *Z. mobilis* ZM4 was not able to grow on the medium added with 1.5–2.0 g/l of furfural (initially added). Therefore, the final concentration of furfural was set up at 1.0 g/l for the study of the response of *Z. mobilis* to the presence of furfural under an extreme set of conditions. The optical density was measured with a spectrophotometer at 600 nm with an initial OD_{600} of 0.05 when the inoculum was added to each flask (with or without 1.0 g/l furfural).

Cell growth, glucose, and ethanol analysis

Cell growth was determined by monitoring the optical density at 600 nm by using Multi Scanner Spectrometer (Thermo Inc.) at 4-h intervals. Fermentation supernatant was prepared by passing through a 0.2-μm membrane (Millipore) and used to determine the concentrations of glucose and ethanol. Ion chromatography (Metrohm Bio-Scan 871, Switzerland) was applied to measure the concentration of glucose with sodium hydroxide (0.1 M) as mobile phase at a flow rate of 1 ml/min. Sugars were quantified by comparing their peak areas with standard sugar of known concentrations. Ethanol was assayed using GC122 gas chromatography with a glass column (0.26×200 cm) filled with Porapak Type QS (80–100 mesh, Waters, Milford, MA, USA) at 150 °C and a FID detector at 80 °C. N_2 was used as the carrier gas (30 ml/min), and butylacetate was added as inner reference.

RNA isolation and preparation of fluorescein-labeled cDNA

Total RNA was isolated essentially as described previously (Chomczynski 1993). Briefly, two samples from *Z. mobilis* 24-h culture were harvested by centrifugation and TRIzol reagent (Invitrogen) was used to extract total cellular RNA. Each total RNA preparation was further purified using NucleoSpin® RNA clean-up (MACHEREY-NAGEL, Germany) according to the manufacturer's instructions. The RNA quality was assessed by formaldehyde agarose gel electrophoresis and quantitated at OD_{260} and OD_{280} by a spectrophotometer, respectively. The purified RNA from each sample was used as the template to generate cDNAs while labeled with either Cy3-dUTP or Cy5-dUTP (CapitalBio). In a duplicate set of cDNA synthesis reactions, the fluorescent dyes were reversed for each sample so that the effects of a specific dye were minimized.

Microarray hybridization, scanning, image quantification, and data analyses

Z. mobilis microarrays were constructed by CapitalBio Corporation (Beijing, China) using coding sequences predicted by The Institute for Genomic Research (TIGR,http://www.tigr.org/). Microarray hybridization, washing, scanning, and data analysis were carried out according to NimbleGen's Expression user's guide. Gene expression analysis was performed using six independent microarray experiments (two dye reversal reactions × three biological replicates) with each microarray containing one to two probes each per predicted coding sequence.

Hierarchical clustering with the average linkage method was performed with Cluster 3.0 software and the cluster result was visualized through the Treeview software. In a comparison analysis, two-class unpaired method in the Significant Analysis of Microarray software (SAM, version 3.02) was performed to identify significantly differentially expressed genes between furfural-treated and control (furfural-untreated) groups. Genes were determined to be significantly differentially expressed with a selection threshold of false discovery rate, FDR <5 % and fold change ≥1.2 (significant induction) or ≤0.8 (significant repression). Raw data were $\log_2$-transformed and imported.

Quantitative-PCR analysis

Real-time quantitative-PCR (RT-qPCR) was carried out to assess the microarray expression results for the selected genes as described earlier. Total RNA was isolated by using TRIzol reagent (Invitrogen). The RNA samples were reverse-transcribed by using Protoscript First Strand cDNA Synthesis Kit (MBI, Fermentas Inc.) as described in the manufacturer's protocol. Sixteen genes representing

different functional categories and a range of gene expression values, based on microarray hybridizations, were analyzed using qPCR (iQ5 Real-Time PCR System, Bio-Rad) from cDNAs derived from different treatment samples. Optimized primers were designed using Beacon Designer 7 software (length from 18 to 20 nucleotides and predicted annealing temperatures ranging from 55 to 57 °C) to amplify about 90–120 bp of the target genes, and the oligonucleotide sequences of the 16 genes selected for qPCR analysis are listed in Table 1. First-strand cDNA was synthesized using a cDNA synthesis kit (MBI, Fermentas Inc.). PCR conditions were 10 min at 94 °C, followed by 40 cycles of heating at 94 °C for 20 s and 60 °C for 30 s, and final extension at 72 °C for 5 min. PCR amplification was detected by SYBR fluorescence dye (Takara). The *rrsA* gene (ZMOr009), encoding the 16 S ribosomal RNA gene, served as an endogenous control to normalize for differences in total RNA quantity.

Results

Profiling of cell growth, glucose utilization, and ethanol yield under furfural stress

The presence of furfural in the medium led to negative impacts on cell growth, glucose consumption, and ethanol production of *Z. mobilis* ZM4 (Fig. 1). In the furfural-untreated culture, maximal cell density (OD_{600}) reached to 4.5 approximately 16 h post-inoculation, while the time needed for *Z. mobilis* to reach its highest cell density of 3.62 (OD_{600}) was delayed until 24 h after initial inoculation under furfural stress conditions.

Z. mobilis also consumed glucose more slowly under furfural stress conditions, with more than half of the initial aerobic glucose concentration (10.8 g/l) remaining after 12 h of incubation. In opposite, 99.4 % of glucose has been utilized at this time point under normal conditions. When

Table 1 Primer pairs used for q-PCR analysis with target gene information

Primary locus	Gene	Function	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Product size (bp)	Array[a]	qPCR[b]	Array/qPCR
ZMO0055		predicted permease	AATTGTTGGTTCCGATAT	CAATAAAGAAATCAGCATC	106	2.4	1.8	1.3
ZMO1936		hypothetical protein	CTCTACCCTGATGCCGAT	CCGTCATGGATCAGATGT	86	1.8	5.8	0.3
ZMO1813	*rnfB*	NADH: ubiquinone oxidoreductase subunit	CACAAGCGGAAGATATTG	ATAATGGCATCACTGGAA	100	1.8	5.5	0.3
ZZM4_0037		"phage portal protein, PBSX family"	AATGGTTCAAGACTTCCT	TCTCCGAGTGTAAATAGC	109	1.6	7.6	0.2
ZMO1524		hypothetical protein	GCAATGGTTCTATCTGTTAA	CAAAGCGATGGTATTAGC	100	1.6	5.6	0.3
ZMO1669		ubiquinone biosynthesis protein COQ7	AGATGATTGCCGAATATC	AACCCATATAAAACAGGAT	100	1.6	8.1	0.2
ZMO1064	*pspB*	phage shock protein B	TTAGTCTGCCTTATTCTG	CTCATAAAGCTCTTCAATC	120	1.6	4.0	0.4
ZMO1760		extracellular ligand-binding receptor	GCCTCTTCAATCAACAAT	AATAACTGCTACCGAATG	120	1.6	6.0	0.3
pzmob1_p05		hypothetical protein	TTCCAATCGGTTCAATTAGT	CAGCCATAGTATCGGTAAG	100	1.5	4.9	0.3
ZMO1300		hypothetical protein	ATGGTGAAGGTCAGAATT	GCAGCAAAGAGATGAAAT	104	−0.6	−1.4	0.4
ZMO1301		hypothetical protein	TCGTCATAGACATCATAGT	GCTCTGGATATTGCTTTT	113	−0.5	−2.3	0.2
ZMO1966		hypothetical protein	GTTCTCATAGTTGGAGGAAG	TCTTTCTTGTGCTGTATCG	120	−0.3	−2.1	0.1
ZMO1700		hypothetical protein	AAACCCGTTGTTAATCAG	ATAGGTTCTCCGAATATCA	102	−0.5	−2.1	0.2
ZMO2014		hypothetical protein	TTATTGGTGATGCTAATGGT	CGACAAATACTCCCTACAAA	100	−0.3	−1.6	0.2
ZMO1959		hypothetical protein	CAATGAAGCCAAGGAAGA	ACAAGAGACGGGTGATAT	113	−0.5	−1.1	0.5
ZZM4_0169		hypothetical protein	ATAACTGACCACCAATCC	TGATCTTACTCTTCACCAA	112	−0.5	−1.6	0.3
Endogenous control								
ZMOr009	*rrsA*	16s RNA	TCAACTATAGACCAGTAAGT	AGAACATAGAAGAGGTAAGT	101		1.0	

[a] Microarray ratio of gene expression (furfural stress/normal)

[b] qPCR ratio of gene expression (furfural stress/normal)

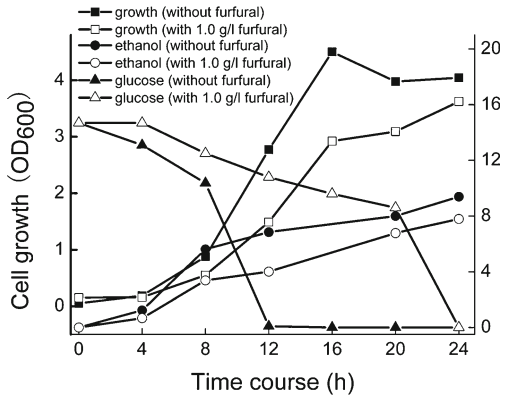

Fig. 1 *Z. mobilis* fermentations under normal and furfural stress conditions. The data come from mean values of triplicate experiments

Z. mobilis growth reached its peak after 24 h under stress conditions, 14 % of glucose remained in the culture. As a result, the yield of ethanol was also decreased by 20.5 % under furfural stress conditions (Fig. 1).

Global gene expression patterns in response to furfural

Since the growth and other phenotypes of *Z. mobilis* were significantly affected by addition of furfural in the medium, we may ask how it happened in the transcriptome. In this study, microarray technology was adopted to answer this question. Based on the genome data of *Z. mobilis* (Seo et al. 2005), 1,800 gene fragments were amplified by PCR and spotted onto the glass slide. With the sophisticated microarray, the global transcriptional response of *Z. mobilis* ZM4 to furfural stress was examined at 24 h post-inoculation under normal (media with no furfural) and stress conditions (media with 1.0 g/l furfural). Of the 1,800 genes examined by microarray analysis, 433 genes (24 % of the total number of open reading frames represented on the array) were identified as being significantly up- or down-regulated ($P \leq 0.05$) during furfural stress condition (Figs. 2 and 3; Tables S1 and S2). Two hundred sixteen genes were up-regulated 24 h post-inoculation under furfural stress condition and 217 genes were down-regulated (Fig. 3; Tables S1 and S2). Figure 4 provides a summary of the percentage of differentially expressed genes grouped by functional categories according to TIGR's annotation of the *Z. mobilis* genome (Seo et al. 2005). We have deposited the entire microarray data at Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE37848) database so interested parties can conduct their analyses. Approximately two thirds of the genes down-regulated in the presence of furfural were related to metabolism. In the presence of furfural, about 49.53 % of the genes related to regulation, cell processes, transport, and unknown function showed greater expression as compared to normal conditions. Nearly 25 % of the genes including plasmid-encoding genes showing greater expression under stress condition remain uncharacterized. However, the genes in ED pathway such as *glk*, *zwf*, *pgl*, *pgk*, and *eno*, as well as ethanol fermentation-related genes like *pdc* and *adh*B, were shown to be little differentially expressed under stress conditions, indicating that furfural yielded, at most, a subtle negative effect on ED pathway under low concentration of furfural.

To confirm the microarray results, 16 genes involved in metabolism and information transfer, plasmid-encoding genes, and hypothetical proteins were chosen for the qPCR analysis. The data showed that qPCR was more sensitive with greater differences in comparison to the microarray results (Table 1; Fig. 5), which was in keeping with previous reports (Yang et al. 2009). On the other hand, genes involved in ED and pyruvate pathways were also chosen for the qPCR analysis, which showed the same results as in the microarray (data not shown).

Induction and repression of cell envelope components under furfural stress

As expected, genes involved in cell motility and cell wall/membrane biogenesis were showed to be down-regulated under furfural stress, such as flagellar-related types (*flhA*, *fliE*, *fliG*, *flgH*, *flgL*, and ZMO0619), lipoproteins (ZMO0285, ZMO0780, ZMO1525, and *oprM*), capsule polysaccharide biosynthesis protein *kpsC* (ZMO0307), efflux protein (ZMO0779), porin protein *oprB* (ZMO0064), cell division protein *ftsQ* (ZMO0835), penicillin-binding

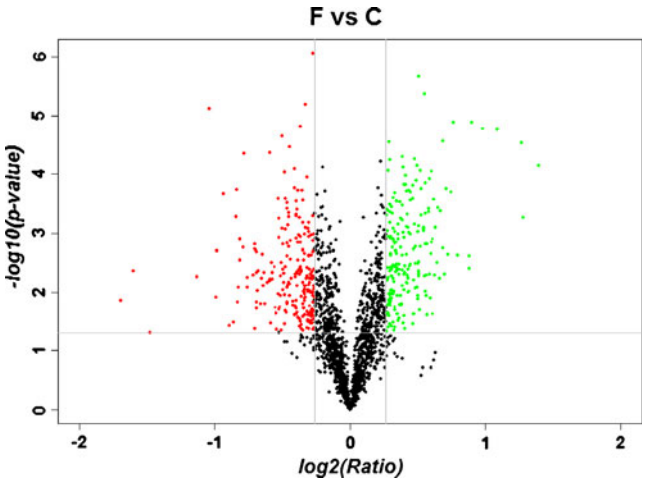

Fig. 2 Volcano plot result from JMP genomics analysis showing significantly differentially expressed genes under furfural stress condition. *Red dots* indicate down-regulated genes and *green dots* indicate up-regulated genes. *Black-colored dots* were not considered as significantly differentially expressed. The *X*-axis shows the difference values between furfural stress and normal conditions based on a $\log_2$ scale. The *Y*-axis shows statistical significance values for expression values, based on a−log10 *p*-value. The *gray line* shows the statistical significance cutoff used in this study

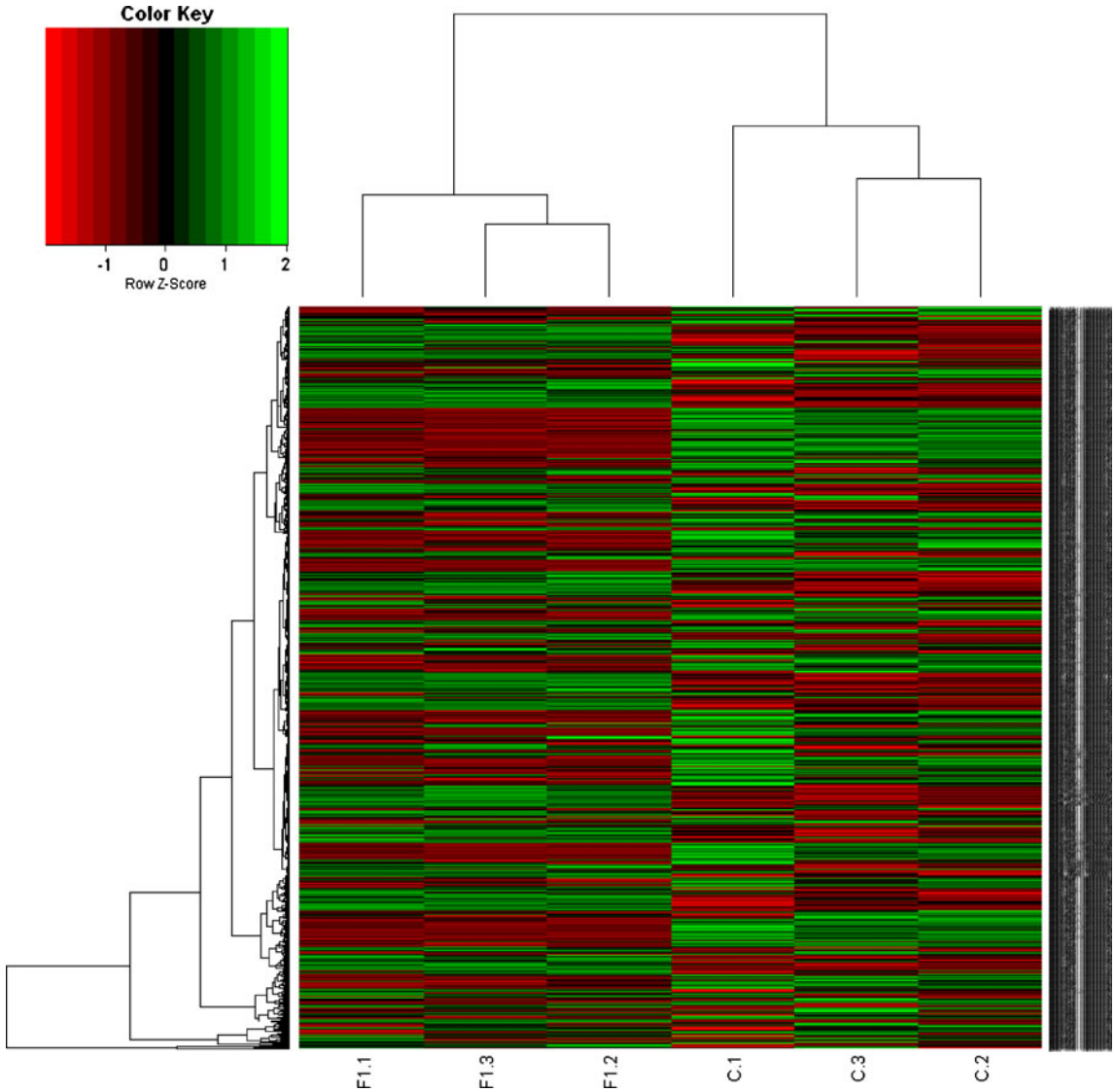

Fig. 3 Hierarchical cluster analysis of significantly differentially expressed ZM4 genes for normal and furfural stress condition at 24 h. Gene expression values were clustered based on their $\log_2$-based expression values. *Negative numbers* (colored *red*) indicates less relative gene expression under normal conditions and positive numbers (colored *green*) indicate greater relative gene expression under furfural stress

protein (*pbp* and ZMO0197), and ion channel protein *mscS* (ZMO1331). However, the transcripts encoding flagellin domain-containing protein *fliC* (ZMO0629), rod shape-determining protein MreC (ZMO0356), ion channel protein *MscS* (ZMO0996), membrane protein (ZMO0216, ZMO1174), organic solvent tolerance protein *ostA* (ZMO1311), and lipoprotein *lgt* (ZMO0291) were shown to be more abundant under stress condition by microarray analysis (see Table S1). This suggests that *Z. mobilis* might minimize furfural-induced damage by altering or modifying the composition and structure of the cell membrane. Especially, gene encoding putative organic solvent tolerance protein *ostA* may play an important role under stress conditions. Further work may be performed by overexpression of this gene to get more tolerant *Z. mobilis* strains for producing valuable chemicals.

Repression of protein synthesis under furfural stress

In this study, about 39 genes related to protein synthesis were down-regulated in response to furfural. They belong to ribosomal proteins (such as *rpsD*, *rpsF*, *rplI*, *rbsR*, *frr*, and *rbfA*), tRNA synthetases (*proS*, *alaS*, *leuS*, *glyS*, *pheT*, and *valS*), and amino acid metabolism-related genes (*glnA*, *trpA*, *trpB*, *argG*, *gltB*, *ilvE*, *glnB*, *serA*, and *serC*). Only one amino acid biosynthetic gene (*leuC*) was induced by 1.5-fold under furfural stress (Tables S1 and S2). Further work about metabolomics data of detected amino acids may be verified by these array

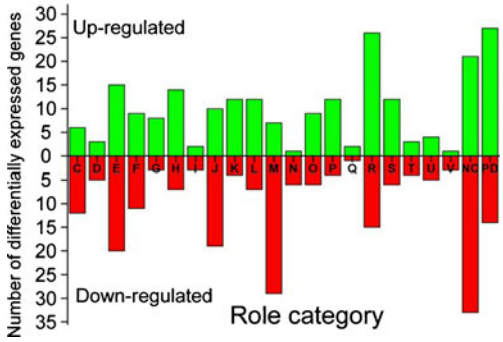

Fig. 4 Number of differentially expressed genes according to the *Z. mobilis* genome database. Columns: *C* energy production and conversion, *D* cell cycle control, mitosis, and meiosis; *E* amino acid transport and metabolism, *F* nucleotide transport and metabolism, *G* carbohydrate transport and metabolism, *H* coenzyme transport and metabolism, *I* lipid transport and metabolism, *J* translation, *K* transcription, *L* replication, recombination, and repair, *M* cell wall/membrane biogenesis, *N* cell motility, *O* post-translational modification, protein turnover, chaperones, *P* inorganic ion transport and metabolism, *Q* secondary metabolites biosynthesis, transport, and catabolism, *R* general function prediction only, *S* function unknown, *T* signal transduction mechanisms, *U* intracellular trafficking and secretion, *V* defense mechanisms, *NC* not in COGs, *PD* plasmid encoding genes. *Columns above 0* represent the genes whose expression increased in response to 1.0 g/l furfural; *columns below 0* represent the genes whose expression decreased in response to furfural stress

results. The repression of these genes may indicate an arrest of overall protein synthesis presumably contributing to the diminished growth, while cells apparently redirected energy toward the increased expression of genes more directly involved in the protective response to an external environment.

Repression of terpenoid biosynthesis under furfural stress

Hopanoids are a class of pentacyclic triterpenoid lipids that occur in a wide range of Gram-negative and Gram-positive bacteria. Squalene–hopene cyclase (*shc*) is the only enzyme involved in the biosynthesis of hopanoid lipids that has been characterized on the genetic level in *Z. mobilis*. There are five open reading frames (designated as *hpnA-E*) in close arrangement with the *shc* gene (ZMO0872) (Perzl et al. 1998). In this study, the genes such as *hpnD* (ZMO0870) and *hpnE* (ZMO0871) involved in hopanoid biosynthesis pathway were shown to be down-regulated (0.7-fold) in the presence of 1.0 g/l furfural after 24 h of incubation. Other terpenoid biosynthesis-related genes, such as *dxs* (ZMO1234 and ZMO1598) and *dxr* (ZMO1150), also exhibited a down-regulated expression pattern. The importance of hopanoids for ethanol tolerance of *Z. mobilis* was firstly demonstrated by an increased growth inhibition of cells with reduced hopanoid content by ethanol (Horbach et al. 1991). Recently, a further study indicated that hopanoids play an important role in maintaining membrane integrity and pH homeostasis in *Rhodopseudomonas palustris* TIE-1 (Welander et al. 2009). Taken together, these data suggest that furfural has a negative effect on terpenoid biosynthesis and then may damage the cell membrane of *Z. mobilis*.

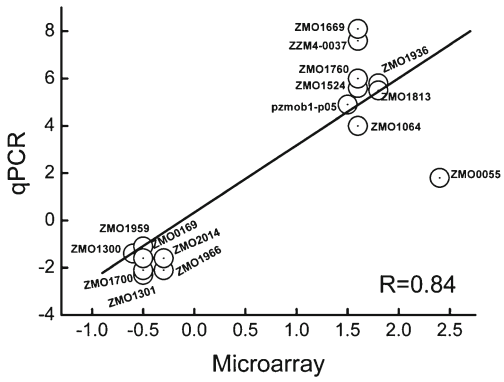

Fig. 5 Comparison of stationary growth phase gene expression measurements by microarray and qPCR. The gene expression ratios for wild-type *Z. mobilis* ZM4 under furfural and normal conditions after 24 h of fermentation were log-transformed in base 2. The microarray ratio values were plotted against the qPCR values. A comparison of the two methods indicated a high level of concordance (R=0.84)

Transcripts of gene related to DNA replication, recombination, and repair

Previous studies have demonstrated that furfural may cause DNA damage (Barciszewski et al. 1997; Khan et al. 1995). As expected, seven genes related to DNA replication, recombination, and repair (putative DNA helicase ZMO0351, double-strand break repair helicase *addA*, double-strand break repair protein *addB*, *rnhB*, uracil-DNA glycosylase *ung*, ZMO1185 and ZMO1562) were revealed as being down-regulated in the presence of 1.0 g/l furfural after 24 h of incubation. However, transcripts encoding the other genes related to DNA replication, recombination, and repair were found to be more abundant under furfural stress (see Table S1), such as *intZ* (ZMO1930), *dnaA* (ZMO1356), DNA repair protein *radC* (ZMO1426), *uvrA* (ZMO1588), *uvrB* (ZMO0362), *recJ* (ZMO1231), *recF* (ZMO1584), DNA mismatch repair enzyme *mutL* (ZMO0354), and *topA* (ZMO1193). This suggests that *Z. mobilis* might minimize furfural-induced DNA damage by inducing some genes related to DNA replication, recombination, and repair.

Transcripts of universal stress response gene under furfural stress

In addition, universal stress genes, such as *groES–groEL* (ZMO1928 and ZMO1929), were not affected significantly under furfural stress (data not shown). However, other universal stress genes, such as chaperone protein *dnaJ* (ZMO0661 and ZMO1545) and ATP-dependent protease

lon (ZMO0376), showed higher expression under furfural stress condition.

Transcripts of respiratory chain genes

Five respiratory chain genes were not differentially expressed under furfural stress condition, including *ndh* (ZMO1113, encoding NADH dehydrogenase), ZMO0022 (putative Fe-S oxidoreductase), ZMO1571 (cytochrome bd-type quinol oxidase subunits 1), ZMO1572 (cytochrome bd-type quinol oxidase subunits 2), and ZMO1844 (oxidoreductase gene). On the other hand, *adhA* (alcohol dehydrogenase I, ZMO1236), *adhB* (alcohol dehydrogenase II, ZMO1596), and *adhC* (ZMO1722) were also not differentially expressed (data not shown). These suggest that the NADH flux from respiration to ethanol synthesis did not redirect under furfural stress (Kalnenieks, 2006). Interestingly, transcripts of the putative respiratory gene *rnfA* and *rnfB* (ZMO1814 and ZMO1813), encoding a putative NADH/ubiquinone oxidoreductase subunit, were illustrated to express more greatly (1.9-fold) under furfural stress condition. Another putative respiratory gene *rnfC* also showed a higher expression level (1.3-fold) under the stress. Previous studies on transcriptomic profiling of *Z. mobilis* during aerobic and anaerobic fermentation have also verified that *rnfA* was expressed more greatly (1.9-fold) under aerobic conditions (Yang et al. 2009). There are six genes encoding the putative NADH/ubiquinone oxidoreductase complex (RnfABCDGE) in the ZM4 genome, most homologous to the *rnf* operon of photosynthetic bacterium *Rhodobacter capsulatus*, which is involved in electron transport to nitrogenase (Schmehl et al. 1993). However, physiological information about the *rnf* gene products of *Z. mobilis* is unavailable (Sootsuwan et al. 2008).

Transcriptional regulation under furfural stress

Eight transcriptional regulators including *Lys*R family (ZMO1336, ZMO0050, ZMO0774 and ZMO0471), *Lyt*R family (ZMO1738), GntR family (ZMO1944), *Tet*R family (ZMO0281), *Lac*I family (ZMO1283), and *rpoD* (ZMO1623) were up-regulated under furfural stress (see Table S1). On the other hand, MerR family (*pbrR*, ZMO0203), Fis family sigma54 specific transcriptional regulator (ZMO0631), and *fliA* (ZMO0626) showed more than 0.6-fold change under stress condition. Actually, the genome sequence shows that *Z. mobilis* has *rpoH* (ZMO0749, sigma-32 factor), *rpoE* (ZMO1404, sigma-24 factor), *rpoN* (ZMO0274, sigma-54 factor), *rpoD*, *rpoA* (ZMO0541), and *fliA* (ZMO0626, sigma F). However, only *rpoD and fliA were differentially expressed in the microarray assay.*

Other transcriptional regulators such as ZMO1107 (Lrp-like, sharing 40 % identity to *E. coli* global regulator Lrp) and ZMO0347 (sharing 60 % identity to *E. coli* global regulator *Hfq*) (Yang et al. 2009) were not differentially expressed in the studies (nearly 0.8-fold; data not shown). However, previous studies revealed that *E. coli* global regulator *Lrp* affected the expression of at least 10 % of all *E. coli* genes (Hung et al. 2002; Tani et al. 2002). Hfq is an RNA-binding protein that is common to diverse bacterial lineages and has key roles in the control of gene expression. Hfq also affected the translation and turnover rates of specific transcripts, which contributes to complex post-transcriptional networks (Vogel and Luisi 2011; Yang et al. 2009). Hfq could also be associated with *E. coli* motor protein Rho to mediate transcription antitermination via a novel transcription regulatory mechanism (Rabhi et al. 2011). Recently, studies indicated that *Hfq* may play an important role in sRNA network control (Hussein and Lim 2011; Storz et al. 2011). However, the mechanisms of translation, transcriptional, post-transcriptional, or sRNA network control in *Z. mobilis* mediated by Hfq, still remain to be elucidated. Yang et al. (2010) showed that *Z. mobilis hfq* (ZMO0347) contributes to tolerance against multiple lignocellulosic pretreatment inhibitors, which provide a fundamental example for further studies or industrial strain development in the future.

Two phage shock protein A and C (*pspA* and *pspC*) were shown to be more highly differentially expressed. The phage shock protein (Psp) response was originally discovered in P. Model's laboratory at the Rockefeller University while studying filamentous phage f1 infection of *E. coli* (Brissette et al. 1990). Now, *Psp* protein is found out across Gram-positive bacteria, Gram-negative bacteria, and archaea to plants and might perceive cell membrane stress and signal to the transcription apparatus by using an ATP hydrolytic transcription activator to produce effector proteins to overcome the stress (Joly et al. 2010). There are four phage shock proteins in the ZM4 genome (ZMO1061, ZMO1063, ZMO1064, and ZMO1065), which may encode *pspF*, *pspA*, *pspB*, and *pspC* and consist of a *psp* regulon (Seo et al. 2005). However, ZMO1062 is a hypothetical protein, which may be included in the *psp* regulon of *Z. mobilis*. The function of *psp* regulon should be elucidated in response to stress in the future.

Induction of plasmid-encoding genes under furfural stress

Interestingly, 27 genes from ZM4 plasmids were shown to be more abundant (1.3–2.0 fold) under furfural stress condition. However, most of these genes encode hypothetical proteins, such as pzmob1_p05, pzmob1_p15, pzmob1_p16, pzmob1_p18, pzmob1_p19, pzmob1_p29, pzmob1_p33, and pzmob1_p35 (see Table S1).

Discussion

Z. mobilis has a number of positive attributes as an ethanologen for converting cellulosic biomass into ethanol or other valuable chemicals. In the present studies, we confirmed that the presence of furfural will negatively affect cell growth, glucose consumption, and ethanol production in *Z. mobilis* ZM4 fermentation. Furthermore, microarray analysis also revealed that furfural yields multiple effects on various aspects of cellular metabolism at the transcriptional level. This research has provided insights into the molecular response to furfural in *Z. mobilis*, and it will be helpful to construct more furfural-resistant strains for cellulosic ethanol production. Further work should be focused on functions of these more highly differentially expressed genes in response to stress condition.

Unexpectedly, ED pathway mRNAs such as *glk*, *zwf*, *pgl*, *pgk*, and *eno* as well as ethanol fermentation gene transcripts like *pdc* and *adh*B showed less differential expression under furfural stress condition (1.0 g/l) but decreased the yield of ethanol to some extent, which indicated that furfural has a subtle negative effect on ED pathway mRNAs under low concentration. These results were also confirmed by qPCR analysis, which showed no change in the two methods of measurement.

We also observed that two phage shock proteins A and C (*pspA* and *pspC*) were more highly differentially expressed. We deduce that there is a *psp*-like regulon in response to stress in *Z. mobilis*. In *E. coli*, the phage shock protein stress response system is responsible for repairing damage to the inner membrane of the cell and maintenance of the protonmotive force (pmf) across the inner membrane. The Psp stress response is diverse. For example, phage infection, secretin production, blockage of protein export or fatty acid/phospholipid biosynthesis, organic solvents, heat, osmotic, pH, etc., have been characterized in great detail (Huvet et al. 2011). However, function of psp regulon is still unclear, which should be elucidated in the future on a molecular level.

Seo et al. (2005) describe the *Z. mobilis* ZM4 (ATCC31821) genome as consisting of a single chromosome and plasmids (Seo et al. 2005), which we utilized for probe design and microarray fabrication. Therefore, the array data in the present study may fully represent the differences between furfural stress and normal conditions since these plasmid DNA sequences were available. We used a multiplex array format with an average probe length of 36 nucleotides and were able to detect significantly differentially expressed genes. Further work should also be focused on plasmid-encoding gene.

Genes involved in cell motility and cell wall/membrane biogenesis showed differential expression under furfural stress, such as ZMO0629, ZMO0356 ZMO0996, ZMO0216, ZMO1174, ZMO1311, ZMO0291, etc. On the other hand, the terpenoid biosynthesis-related genes, such as ZMO0870, ZMO0871, ZMO0872, ZMO1234, ZMO1598, and ZMO1150, have shown a down-regulated expression pattern, which indicated that furfural has a negative effect on terpenoid biosynthesis and then to make a damage on the cell membrane of *Z. mobilis*. Taken together, we conclude that cell membrane might play important roles in response to furfural stress.

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