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ORIGINAL ARTICLE

Transcriptome analysis of *Bacillus coagulans* P38, an efficient producer of L-lactic acid from cellulosic hydrolysate, in response to 2-furfural stress

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Abstract A comparative transcriptome analysis was conducted to study the expression profiles of the lactic acid-producing strain *Bacillus coagulans* P38 cultivated in the presence and absence of 2-furfural stress. In response to 7 g l^{-1} 2-furfural stress, the cell growth rate of *B. coagulans* P38 was reduced, and a total of 740 genes were upregulated and 540 genes downregulated. KEGG pathway enrichment analysis revealed that nine pathways were significantly affected by 2-furfural stress ($P<0.01$). The downregulation of glycolysis/gluconeogenesis and upregulation of the tricarboxylic acid cycle cycle revealed that L-lactic acid fermentation was negatively affected by 2-furfural stress. Gene Ontology assignments showed that five transporters and one transcriptional regulator were upregulated by more than sixfold under 2-furfural stress. The expression of seven alcohol dehydrogenase genes and eight short-chain dehydrogenase/reductase genes, all of which may be involved in the mediation of furfural tolerance, were induced under 2-furfural stress. Our findings offer new insights into the response of *B. coagulans* to 2-furfural stress. In addition, our RNA-seq dataset constitutes a robust database that can be used to investigate the genetic functions induced by 2-furfural stress.

Keywords *Bacillus coagulans* · 2-Furfural · Stress · Transcriptome

Lactic acid is a valuable chemical used in the polymerization of lactic acid to polylactic acid. Current production methods rely on the fermentation of carbohydrate feedstocks that have alternative uses as foods (Wang et al. 2013a). As lactic acid is also one of the top 30 biomass building blocks, the search for cheap raw materials as lactate sources is an important economic objective. Cellulosic biomasses are inexpensive, abundant, renewable, and readily available sources of sugar that may help meet the large market demand for lactic acid (Xue et al. 2012). However, acid treatment of lignocelluloses to release fermentable sugars also yields unwanted side products, such as furfural and hydroxymethylfurfural (HMF), which retard fermentation and increase production costs. Furfural causes DNA strand breaks, damages membranes, and reacts with other cellular components (Geddes et al. 2014). Thus, it is important to develop biocatalysts with the capacity to resist furan stress.

Furfural and HMF resistance mechanisms have been investigated in yeasts and *Escherichia coli* since the advent of cellulosic ethanol production. In *Saccharomyces cerevisiae*, overexpression of the Yap1 transcription factor increases furfural and HMF resistance; in contrast, disruption of the pentose phosphate pathway increases sensitivity to furfural and

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HMF (Kim and Hahn 2013). In *E. coli*, NADPH-dependent YqhD mediates furan tolerance, and blocking the functional expression of *yqhD* increases resistance to furfural. Increased expression of the NADH-dependent furfural reductase gene (*fucO*) also improves furfural tolerance (Wang et al. 2011).

The lactic acid-producing strain *Bacillus coagulans* P38 tolerates up to 10 g furfural l^{-1} in lactic acid production systems, but lactate production is sharply inhibited at furfural concentrations exceeding 6–7 g l^{-1} (Peng et al. 2013). This is the highest furfural tolerance reported to date. *B. coagulans* P38 can produce 185 g l^{-1} L-lactic acid, with a product yield of 0.98 g g^{-1} total reducing sugars. Its high productivity and tolerance to inhibitors make *B. coagulans* P38 a promising candidate for polymer-grade L-lactic acid production from cellulosic biomass (Peng et al. 2013). However, the mechanisms of the response of this *Bacillus* sp. to such high concentrations of 2-furfural have never been reported.

In this study, we conducted a comparative transcriptome analysis to study differential gene expression in *B. coagulans* P38 cultured in the presence and absence of 2-furfural stress.

Materials and methods

Bacterial strains and growth conditions

Strain *B. coagulans* P38 was isolated from the sludge of a sewage treatment plant by using a high concentration of cellulosic hydrolysate as the sole carbon source and subsequently deposited in the China General Microbiological Culture Collection Center (CGMCC) as CGMCC No. 7312 (Peng et al. 2013, 2014). Seed cultures were cultivated at 42 °C without agitation in medium containing 50 g l^{-1} glucose, 10 g l^{-1} yeast extract, and 30 g l^{-1} $CaCO_3$ until the glucose was exhausted. The culture was then inoculated into the same medium with or without 7 g l^{-1} 2-furfural. The inoculum volume was 10 % (v/v), and the cells were incubated at 42 °C with shaking at 150 rpm. Three repeated runs were conducted, and one batch was selected for analysis. Growth was monitored, and cells were harvested at the early exponential growth phase. The mixtures of each of the three samples harvested under 2-furfural stressed and nonstressed conditions were used for total RNA isolation.

RNA isolation, library construction, and sequencing

Cells were collected from 20 ml of the cultured cells by centrifugation (4000 *g*, 10 min, 4 °C). Total RNA was extracted with the RNAprep Pure Cell/Bacteria kit (TIANGEN BIOTECH Co., Ltd., Beijing, China) and treated with DNaseI. RNA quality and quantity were determined by Nanovue Plus™ spectrophotometry (GE Healthcare, Chalfont, UK). mRNA was enriched by using a Ribo-Zero™ Magnetic kit (Gram-positive bacteria) (EpiCentre Biotechnologies, Madison, WI), following which it was fragmented by ultrasonication and converted into an RNA-seq library with an RNA-Seq Library Preparation kit (Gnomegen, San Diego, CA) according to the manufacturer's instructions. RNA 2×150 bp paired-end sequencing was performed using an ultra-high-throughput sequencing system (Hiseq2500; Illumina, San Diego, CA) according to the manufacturer's protocols at the Chinese National Human Genome Center in Shanghai, China.

Transcriptome analysis

Sequencing reads (150 nt) were cleaned using the Sickle trimming tool and then mapped to the draft *B. coagulans* P38 genome using the aligning tool Bowtie2 under default parameters. Reads that did not align to the genome were discarded (Langmead et al. 2009). Raw read counts of each gene in the two samples were imported into DEGseq. The MARS model (MA-plot-based method with the random sampling model) was used to calculate the relative abundances of each transcript (Wang et al. 2010). Thefalse discovery rate (FDR) was used to determine the threshold *P* value for this analysis. In both sets of comparisons (cells cultivated without 2-furfural stress vs. cells grown with 2-furfural), we identified differentially expressed genes as those that were significant at a FDR of<0.001 and a |Normalized Fold_change| of>2. To identify the pathways in which the differentially expressed genes (DEGs) were likely to participate, we mapped all DEGs to terms in the KEGG database and searched for significantly enriched terms.

Nucleotide sequence accession numbers

All RNA-seq data for both conditions (2-furfural stressed and 2-furfural nonstressed) have been deposited in the NCBI Sequence Read Archive (SRA, http://www.ncbi.nlm.nih.gov/Traces/sra) under accession numbers SRX744513 [cultivation in medium containing glucose, without 2-furfural (G)] and SRX744514 [cultivation in medium containing glucose and 2-furfural (GF)]. The Whole Genome Shotgun sequence of *B. coagulans* P38 has been deposited at DDBJ/EMBL/GenBank under accession number JSVI00000000 (Peng et al. 2015).

Results and discussion

Growth of strain *B. coagulans* P38 under 2-furfural stress

Compared with conventional strains, *B. coagulans* has a number of advantages: high fermentation temperature, no need for sterilization of culture media, and high optical purity of products (Peng et al. 2014; Wang et al. 2014). All of these features

satisfy the production requirements for low energy consumption and high quality. *Bacillus coagulans* strain P38 tolerates a high concentration of 2-furfural; lactate production is significantly inhibited only at 2-furfural concentrations exceeding 7 g l^{-1} (Peng et al. 2013). In our study also, when this strain was cultured in the presence of 7 g l^{-1} 2-furfural, growth was inhibited when glucose was the sole carbon source, especially during the initial 10 h of culture (Fig. 1). Thus, for our analysis, we prepared total RNA from cultured cells harvested at the early exponential growth phase (arrows in Fig. 1). In this study, the cell growth differences were induced by 2-furfural stress. Therefore, the differences in gene expression due to the altered cell growth rates and 2-furfural stress were investigated together.

Overview of the transcriptional profiles

After removing the low-quality reads and trimming off the adapter sequences, we generated 54,288,160 reads with an average length of approximately 150 bp. There were 11,435,648 paired-end sequencing reads for glucose cultivation without 2-furfural stress (G) and 16,784,034 reads for glucose cultivation with 2-furfural (GF). The ratios of clean reads for the two test conditions were 95.39 % (G) and 96.57 % (GF), and the gene-mapping rates were 88.13 % (G) and 93.26 % (GF).

Pathway functional enrichment analysis of the DEGs

A total of 1280 DEGS were identified under the glucose cultivation conditions, of which 740 were upregulated and 540 were downregulated by 2-furfural stress and the reduced cell growth rate (FDR<0.001). Pathway functional enrichment analysis showed the most significantly regulated pathways under 2-furfural stress included the tricarboxylic acid (TCA) cycle, glycolysis/gluconeogenesis, amino acid biosynthesis, and fatty acid metabolism (P<0.0001) (Table 1). We also observed differences in the expression profile of the pyruvate metabolism and the phosphotransferase system (PTS) (P<0.001).

The schematic overview of the metabolic pathways associated with the differentially expressed genes identified in *B. coagulans* P38 cells exposed to 2-furfural stress is provided in Fig. 2. We found that 2-furfural stress had a negative effect on the expression of 'glycolysis/gluconeogenesis' genes but a positive effect on the expression of 'citrate cycle (TCA cycle)' genes. The significantly downregulated genes involved in 'glycolysis/gluconeogenesis' included 6-phosphofructokinase 1 (−1.52 fold change in expression), glyceraldehyde 3-phosphate dehydrogenase (−1.38), phosphoglycerate kinase (−1.56), enolase (−1.29), and L-lactate dehydrogenase (−2.72). The significantly upregulated genes involved in the 'citrate cycle (TCA cycle)' included citrate synthase (3.05), aconitate hydratase (2.04), isocitrate dehydrogenase (2.81), succinyl-CoA synthetase alpha subunit (3.41), and fumarate hydratase, class II (3.23). These results reveal the mechanism of 2-furfural stress on L-lactate fermentation by *B. coagulans* P38 and explain why L-lactate production is sharply inhibited with 7 g l^{-1} furfural (Peng et al. 2013).

Identification and comparison of DEGs with large differences (log_2>6)

Genes (n=11) upregulated by more than sixfold under the 2-furfural stress culture condition were considered to be potential targets for genetic engineering (Table 2); no downregulated gene showed more than a sixfold change. Among the upregulated genes, five were transporters or symporters, including the

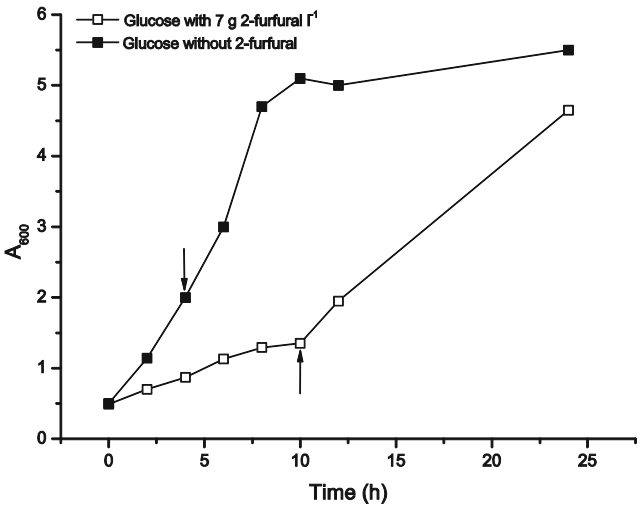

Fig. 1 Growth of *Bacillus coagulans* P38 with and without 2-furfural stress in the presence of glucose as the sole carbon source. Total RNA was isolated from cultures harvested at early exponential growth, as indicated by the *arrows*

Table 1 Results of the pathway functional enrichment analysis[a]

Metabolic pathway	P value
C5-Branched dibasic acid metabolism [PATH: ko00660]	0
Citrate cycle (TCA cycle) [PATH: ko00020]	2.34E–06
Glycolysis/gluconeogenesis [PATH: ko00010]	7.83E–05
Valine, leucine and isoleucine biosynthesis [PATH: ko00290]	7.83E–05
Pyruvate metabolism [PATH: ko00620]	0.000129
Ribosome [PATH: ko03010]	0.00035
Propanoate metabolism [PATH: ko00640]	0.000372
Butanoate metabolism [PATH: ko00650]	0.00092
Arginine and proline metabolism [PATH: ko00330]	0.002056

TCA, Tricarboxylic acid

[a] Differentially expressed genes (DEGs) were those that were significantly different at a false discovery rate (FDR) of<0.001 and a |Normalized Fold_change| of>2

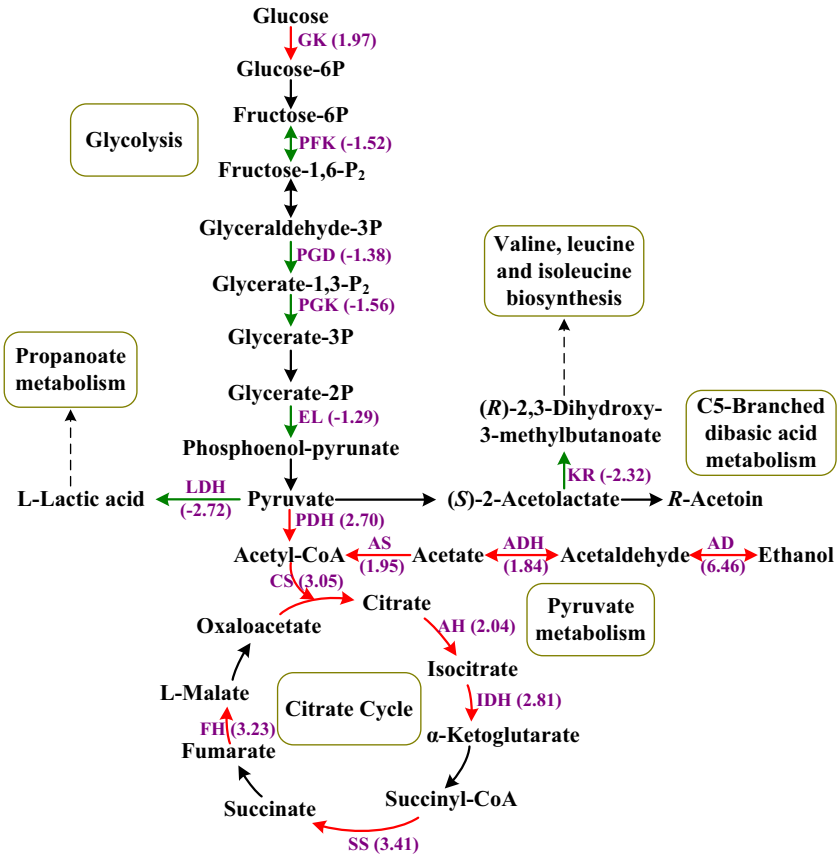

Fig. 2 Schematic overview of the metabolic pathways associated with the differentially expressed genes identified in *B. coagulans* P38 cells exposed to 2-furfural stress. *Red arrows* Upregulated genes, *green arrows* downregulated genes. *GK* Gucokinase [EC:2.7.1.2], *PFK* 6-phosphofructokinase 1 [EC:2.7.1.11], *PGD* glyceraldehyde 3-phosphate dehydrogenase [EC:1.2.1.12], *PGK* phosphoglycerate kinase [EC:2.7.2.3], *EL* enolase [EC:4.2.1.11], *LDH* L-lactate dehydrogenase [EC:1.1.1.27], *PDH* pyruvate dehydrogenase E1 component subunit beta [EC:1.2.4.1], *AS* acetyl-CoA synthetase [EC:6.2.1.1], *ADH* aldehyde dehydrogenase (NAD+) [EC:1.2.1.3], *AD* alcohol dehydrogenase [EC:1.1.1.1], *CS* citrate synthase [EC:2.3.3.1], *AH* aconitate hydratase [EC:4.2.1.3], *IDH* isocitrate dehydrogenase [EC:1.1.1.42], *SS* succinyl-CoA synthetase alpha subunit [EC:6.2.1.5], *FH* fumarate hydratase, class II [EC:4.2.1.2], *KR* ketol-acid reductoisomerase [EC:1.1.1.86]. *Numbers in parenthesis* Fold changes in gene expression

Table 2 Significantly upregulated genes[a] of *Bacillus coagulans* P38 involved in 2-furfural stress

Gene ID	Description	$\log_2$ (GF vs. G)	*P* value
ORF_3097	ABC transporter	8.33	4.22E–42
ORF_3466	Sugar transporter	7.83	0
ORF_3095	TetR family transcriptional regulator	7.59	0
ORF_2605	Hypothetical protein	7.39	0
ORF_3096	Hypothetical protein Bcoa_2038 [*Bacillus coagulans* 36D1]	7.22	0
ORF_0423	Glycerol transporter	7.10	0
ORF_3190	Sugar: proton symporter	7.08	0
ORF_2608	Diacetylchitobiose-6-phosphate hydrolase	7.07	0
ORF_2604	PTS system lactose/cellobiose family transporter subunit IIC	6.64	0
ORF_2184	Hypothetical protein Bcoa_1553 [*Bacillus coagulans* 36D1]	6.46	0
ORF_0425	FAD-dependent oxidoreductase	6.07	0

GF, *B. coagulans* P38 cells cultivated in medium containing glucose and 2-furfural (furfural stressed); G, *B. coagulans* P38 cells cultivated in medium containing glucose, but no 2-furfural (furfual nonstressed)

Table 3 Possible furfural tolerance-related differentially expressed genes

Gene ID	Description	$\log_{2\ (GF\ vs.\ G)}$	Results
ORF_0966	Alcohol dehydrogenase	6.46	Upregulation
ORF_2933	Alcohol dehydrogenase	3.54	Upregulation
ORF_1764	Alcohol dehydrogenase	3.45	Upregulation
ORF_2531	Alcohol dehydrogenase	2.98	Upregulation
ORF_1612	Alcohol dehydrogenase	2.73	Upregulation
ORF_3277	Alcohol dehydrogenase	1.37	Upregulation
ORF_0411	Alcohol dehydrogenase	1.06	Upregulation
ORF_0872	Alcohol dehydrogenase	−1.39	Downregulation
ORF_3232	Short-chain dehydrogenase/reductase	5.86	Upregulation
ORF_1573	Short-chain dehydrogenase/reductase	5.82	Upregulation
ORF_2752	Short-chain dehydrogenase/reductase	4.67	Upregulation
ORF_0818	Short-chain dehydrogenase/reductase	4.41	Upregulation
ORF_2377	Short-chain dehydrogenase/reductase	3.94	Upregulation
ORF_1562	Short-chain dehydrogenase/reductase	3.20	Upregulation
ORF_1022	Short-chain dehydrogenase/reductase	2.00	Upregulation
ORF_2137	Short-chain dehydrogenase/reductase	1.11	Upregulation
ORF_3399	Short-chain dehydrogenase/reductase	−2.18	Downregulation

ABC transporter (ORF_3097), sugar transporter (ORF_3466), glycerol transporter (ORF_0423), sugar: proton symporter (ORF_3190), and the PTS system lactose/cellobiose family transporter subunit IIC (ORF_2604). These results indicate the importance of transporters during 2-furfural stress. A TetR family transcriptional regulator (ORF_3095) was upregulated by 7.59-fold. TetR is a large and important family of one-component signal transduction systems that regulate numerous aspects of bacterial physiology, such as antibiotic production, osmotic stress, efflux pumps, multidrug resistance, metabolic modulation, and pathogenesis (Deng et al. 2013; Cuthbertson and Nodwella 2013). Diacetylchitobiose-6-phosphate hydrolase (ORF_2608), FAD-dependent oxidoreductase (ORF_0425), and three hypothetical proteins (ORF_2605, ORF_3096, and ORF_2184) were also upregulated by more than sixfold under 2-furfural stress.

Identification and comparison of DEGs that may mediate 2-furfural tolerance

2-Furfural is metabolized into the less toxic furfuryl alcohol by NADH-dependent oxidoreductase to confer furfural resistance traits in *E. coli*. Increasing the concentration of NADPH by overexpression of *pntAB* (transhydrogenase for interconversion of NADH and NADPH) or reduction of NADPH depletion (deletion of *yqhD* gene, low K_m for NADPH) will increase the resistance profile of *E. coli* (Wang et al. 2013b). Furfural tolerance is also enhanced by overexpression of an NADH-dependent propanediol oxidoreductase (FucO), which belongs to the alcohol dehydrogenase family of short-chain dehydrogenase/reductases. *Bacillus coagulans* P38 also degrades 2-furfural during lactate fermentation (Peng et al. 2013), possibly via a similar catalytic mechanism. We annotated 20 short-chain dehydrogenase/reductase and 15 alcohol dehydrogenase genes, of which eight alcohol dehydrogenase genes and nine short-chain dehydrogenase/reductase genes were differentially regulated under 2-furfural stress (summarized in Table 3). Most of the genes were upregulated, with only one member each of the alcohol dehydrogenase and short-chain dehydrogenase/reductase groups downregulated. No transhydrogenase gene was identified in this comparative analysis.

Conclusion

In our study, RNAseq technology was used to characterize the transcriptome of *B. coagulans* under stress induced by a high concentration of 2-furfural. KEGG pathway enrichment analysis revealed that nine pathways were significantly affected by 2-furfural stress ($P<0.01$). The downregulation of glycolysis/gluconeogenesis genes and upregulation of genes of the TCA cycle revealed that L-lactic acid fermentation was negatively affected by 2-furfural stress. Five transporters and one transcriptional regulator were upregulated more than sixfold by 2-furfural stress. Eight alcohol dehydrogenase genes and nine short-chain dehydrogenase/reductase genes, which may mediate furfural tolerance, were also differentially expressed. These results provide insight into the responses of *B. coagulans* to 2-furfural stress and suggest potential targets for subsequent genetic engineering to further improve L-lactic acid production by *B. coagulans*.

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