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BIOENERGY AND BIOFUELS

Transcriptional analysis of adaptation to high glucose concentrations in *Zymomonas mobilis*

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Abstract The ethanologenic bacterium *Zymomonas mobilis* is usually tolerant to high concentrations of glucose. The addition of sorbitol decreases the lag phase and increases ethanol yield and productivity of the bacteria in high glucose concentrations. The molecular mechanisms of adaptation to high glucose concentrations and the effect of sorbitol are still unclear. In this study, microarray analysis was used to study the global transcriptional adaptation responses of *Z. mobilis* to high glucose concentrations. A total of 235 genes were differentially expressed when 220 g/L glucose was added with or without 10 mM sorbitol. These genes are involved in diverse aspects of cell metabolism and regulation, including membrane transporters, nitrogen metabolism, and plasmid-encoded genes. However, most differentially expressed genes were downregulated when sorbitol was added. Notably, the transcription of almost all genes involved in the Entner-Doudoroff and ethanol production pathways was not significantly affected. In addition, a prophage and a nitrogen-fixation cluster were significantly induced. These results revealed that *Z. mobilis* cells responded to high glucose concentrations by regulating the transcriptional levels of genes related to membrane channels and transporters, stress response mechanisms, and metabolic pathways. These data provide insight into the intracellular adaptation responses to high glucose concentrations and reveal strategies to engineer efficient ethanol fermentation in *Z. mobilis*.

Keywords *Zymomonas mobilis* · Transcription · High concentrations of glucose · Sorbitol · Microarray

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Introduction

Zymomonas mobilis, a facultative anaerobic Gram-negative bacterium, is a promising ethanologenic strain due to its unique metabolic traits (Rogers et al. 1979, 2007; Swings and De Ley 1977). Wild *Z. mobilis* strains can rapidly ferment glucose, fructose, and sucrose to produce ethanol via the Entner-Doudoroff (ED) pathway and two important enzymes: pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) (Conway 1992; Rogers et al. 2007; Viikari and Berry 1988). The enzymes involved in the ED pathway constitute up to 50 % of the total soluble protein, and each glycolytic gene is expressed at high levels (Algar and Scopes 1985; An et al. 1991). Therefore, *Z. mobilis* can synthesize up to 97 % of the theoretical ethanol yield.

The fermentation industry requires substrates to be utilized rapidly and efficiently, to enable higher yield and productivity. However, high concentrations of glucose lower ethanol production when glucose is used as the sole substrate (Swings and De Ley 1977). In addition, fermentation efficiency is also reduced due to the formation of levan and sorbitol when cells are grown on sucrose or mixtures of glucose and fructose (Viikari 1984). For example, the ethanol yield was only 70 % of the theoretical maximum value when sucrose is used as the fermentation material (Lyness and Doelle 1981). Furthermore, osmotic stress resulting from high glucose

concentrations inhibits cell growth and extends the lag period in *Z. mobilis* (Struch et al. 1991). Therefore, it is necessary to elucidate the mechanism by which high glucose concentrations are tolerated in *Z. mobilis*.

In most bacteria, glucose is transported by three types of carrier systems: phosphoenolpyruvate-dependent phosphotransferase systems (PTSs), ATP-binding cassette (ABC) transporters systems, and H^+/Na^+ sugar symport systems (Baldwin and Henderson 1989). However, unlike other bacteria, *Z. mobilis* cells take up glucose by a facilitated diffusion system without consuming metabolic energy (Barnell et al. 1990; DiMarco and Romano 1985; Parker et al. 1995; Weisser et al. 1995). In addition, this transporter also has the following traits: low affinity, high velocity, and preferential glucose absorption (Parker et al. 1997). Because of long-term adaptation to sugar-rich niches, *Z. mobilis* has a higher capacity for growth on sugar-containing materials. For example, it can grow on high concentrations of glucose up to 2.22 M (40 % *w*/*v*), albeit with a long lag phase of 4 to 20 days (Swings and De Ley 1977). Sorbitol or ectoine can be used as compatible solutes to overcome toxic osmotic pressure. The lag period can be significantly shortened when sorbitol or ectoine is added to media containing high concentrations of glucose, suggesting that the adaptation of *Z. mobilis* cells can be enhanced (Loos et al. 1994; Zhang et al. 2008). However, sorbitol cannot be synthesized by glucose-fructose oxidoreductase (GFOR) when glucose is the sole carbon source (Loos et al. 1994).

The mechanism of adaptation to high sugar concentrations in *Z. mobilis* is still unclear. Previous studies observed that the facilitated diffusion system rapidly generates an equilibration between external and internal glucose concentrations (Struch et al. 1991). Notably, the intracellular glucose concentration in *Z. mobilis* cells is much lower than the extracellular concentration. Furthermore, lipid composition and the enzymatic activities of glucokinase (GK), glucose-6-phosphate dehydrogenase (G-6-P), PDC, and ADH, which are required for glucose metabolism, were not significantly altered when *Z. mobilis* cells were shifted from a low (2 %) to a high (11 %) glucose concentration (Douka et al. 1999). Analysis of a *Z. mobilis* mutant with a longer lag period revealed that expression of the *glc* operon, which encodes four putative genes (ORF1, ORF2, ORF3, and ORF4), was regulated by glucose concentration, and the promoter of the *glc* operon was osmo-induced by the ORF4 product (Christogianni et al. 2005).

The initial sequencing and subsequent improved annotation of the *Z. mobilis* ZM4 genome (Seo et al. 2005; Yang et al. 2009a) have highlighted novel avenues to elucidate the physiology and metabolism as well as gene function and regulation in this microorganism. Knowledge of the genome sequence has facilitated microarray-based profiling of the *Z. mobilis* transcriptome under ethanol and furfural stress (He et al. 2012a, b; Yang et al. 2013) as well as aerobic and anaerobic fermentations (Yang et al. 2009b); furthermore, phenotypic changes in a flocculent mutant strain (ZM401) have been examined using this technology (Jeon et al. 2012). The differentially expressed genes are diverse and associated with the respective tolerance and/or cell motility pathways.

In this study, microarray analysis was performed to identify the genes involved in the adaptation of *Z. mobilis* to high glucose concentrations. These results revealed that over two hundred genes were differentially expressed when *Z. mobilis* cells were transferred from a low (2 %) to a high (22 %) glucose concentration. The roles of these genes involved in the adaptive response to high glucose concentrations are discussed.

Materials and methods

Bacterial strains and culture conditions

Z. mobilis ZM4 (ATCC31821) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). This strain was grown at 30 °C for 24–36 h without shaking in rich medium (RM) containing the following (per liter): glucose, 20 g; yeast extract, 10 g; $(NH_4)_2SO_4$, 1 g; K_2HPO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; and pH 6.2 (Goodman et al. 1982). Fresh cultures were subcultured in 200 mL of RM in 500-mL flasks using 5 % inoculums. These cultures were continuously cultivated without pH control, and 6-h culture samples were collected as controls. Subsequently, glucose (44 g) was added to a final concentration of 22 % (*w*/*v*). Cell samples were then collected after 2 h (8 h postinoculation) and 14 h (20 h postinoculation). In a parallel treatment, sorbitol (1.82 g, 10 mM) and glucose (44 g) were added simultaneously, and cell samples were collected after 12 h (18 h postinoculation). Samples were immediately immersed in liquid nitrogen for RNA extraction. The cell density at 600 nm was measured using a UV-2450 spectrophotometer (SHIMADZU EMIT CO., Tokyo, Japan). Fermentation broth was centrifuged at 12,000 rpm for 2 min; the resulting supernatants were filtered using a 0.2-μm membrane (Millipore Co., Bedford, MA, USA) and then used to determine the glucose and ethanol concentrations. The residual glucose level was measured by the 3,5-dinitrosalicylic acid method (Miller 1959). Ethanol was assayed using GC122 gas chromatography (Shanghai analysis instrument factory, Shanghai, China) 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 butyl acetate was added as an internal control (He et al. 2012b). All experiments were performed in triplicate.

RNA extraction and preparation of fluorescently labeled cDNA

The total RNA was isolated independently from the 12 cell samples using a Bacterial RNA Extraction Kit (Kangwei Century Biotech Co., Ltd., Beijing, China). Each RNA sample was purified using a NucleoSpin® RNA cleanup kit (Macherey-Nagel, Düren, Germany) to eliminate trace DNA. The RNA quality was examined by agarose gel electrophoresis and by obtaining the ratio of OD_{260} to OD_{280} using a UV-2450 spectrophotometer.

The purified RNA was amplified using the Ambion MessageAmp II bacterial RNA amplification kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, the qualified RNA was polyadenylated using poly(A) polymerase, and the resulting products were used to generate double-stranded complementary DNA (ds-cDNA) by a two-step reverse transcription reaction. The ds-cDNA was transcribed in vitro to produce complementary RNA (cRNA). The purified cRNA was used as a template to synthesize cDNA with random primers. Each cDNA sample was labeled with Cy3-dCTP (GE Healthcare UK Limited, Buckinghamshire, UK) using the Klenow enzyme and random primers (Takara Biotechnology-Dalian Co., Ltd., Dalian, China).

Microarray hybridization, scanning, image quantification, and data analysis

Microarrays were performed as described by He et al. (2012b). Microarray probes were designed using coding sequences predicted by The Institute for Genomic Research (TIGR, http://www.tigr.org/). Generation of cDNA, fluorescent labeling, microarray hybridization, washing, scanning, and data analysis were performed by CapitalBio Corporation (Beijing, China) according to the Roche NimbleGen Expression user guide (Roche NimbleGen Inc., Madison, WI, USA). Gene expression analysis was performed with three biological replicates.

Image signals were transformed into digital signals as raw data using the NimbleScan 2.6 software. The Pearson's correlation coefficients were calculated to assess the uniformity of biological replicates in each group. All raw data were corrected using robust multichip analysis (RMA) normalization method to remove variations. Significance Analysis of Microarray (SAM, ver3.02) was used to identify significantly differentially expressed genes between the treated and control groups. Fold changes were expressed as $\log_2$ values. Genes were considered to be significantly differentially expressed with a selection threshold of false discovery rate (FDR) <0.05 and fold change ≥ 1.00 (significant induction) or ≤ -1.00 (significant repression). The Friedman test was applied to analyze the influence of glucose supplementation on gene transcription among different samples, and the independent *t* test was used to analyze the significance of differences between two groups. K-mean clustering was performed with Cluster 3.0 and visualized by the software TreeView. In addition, these differentially expressed genes were classified for the convenience of description. Comparison of functional enrichment analysis for these differentially expressed genes was performed between the MG-RAST Function Catalog (http://metagenomics.anl.gov/) (Meyer et al. 2008) and GO (Gene Ontology, www.geneontology.org) (Ashburner et al. 2000).

Real-time PCR confirmation

Real-time quantitative PCR (qPCR) analysis was performed to validate the differential expression of 12 genes selected from the microarray data. The same RNA samples were reverse-transcribed by the PrimeScript® RT Enzyme Mix I according to the manufacturer's instructions (Takara Biotechnology-Dalian Co., Ltd., Dalian, China) after eliminating trace genomic DNA contamination. The sequences of the primer pairs were listed in Table 1. The gene *rrsA* (ZMOr009), encoding the 16S ribosomal RNA, was used as a control gene. Real-time PCR was performed using an iQ5 Real-time PCR System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with reaction mixtures (total volume 20 μL) containing 10 μL 2× TaKaRa SYBR Green Real-Time PCR Master Mix (Takara Biotechnology-Dalian Co., Ltd., Dalian, China), 1.6 μL forward and reverse primers (10 μM stock), and 2 μL cDNA. The PCR conditions were as follows: step 1, 95 °C for 30 s (hot-start activation); step 2, 95 °C for 5 s (denaturation); step 3, 60 °C for 30 s (annealing and extension) and 60 °C for 30 s (extension), 40 cycles of step 2 and 3; step 4, 95 °C for 1 min (denaturation of the PCR product); step 5, 55 °C for 1 min (annealing of PCR product); and step 6, heat from 55 to 95 °C with a ramp speed of 0.5 °C per 10 s; the resulting melting curves were used to confirm the specificity of the primer pairs in each experiment. The data were analyzed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Results

Establishment of a model for adaptation to high glucose concentrations

Because the length of the *Z. mobilis* lag period depends on the initial glucose concentration in the RM, the effect of high glucose concentrations (up to ~250 g/L) on the growth of *Z. mobilis* ZM4 was examined first. Growth of the bacterium was significantly delayed with increasing glucose concentrations (Fig. S1 in the Supplementary Material). The rate of glucose consumption was roughly consistent with the cell-

Table 1 The primer pairs and the oligonucleotide sequences used for RT-qPCR

Locus tag number	Function	Forward and reverse primer	Product size (bp)	Array[a]	qPCR[a]
ZMO0253	Type I secretion outer membrane protein	TGACGAACCCGCTTATTTG CGCATCGGTGATAGAAGGA	121	2.08±0.09a	6.88±0.32
ZMO0255	Type I secretion membrane fusion protein	AACGGCAGGTAAGCGAAA CGGGCGAAGTAAAGGTCA	164	1.32±0.22a	4.96±0.24
ZMO0749	RNA polymerase factor sigma-32	AGGAACCGCTACAAGACCA CTTCCAGTGTTTTCGGGTTA	150	1.31±0.25a	6.72±0.28
ZMO0789	TonB-dependent receptor	GGCATACCTCGGTTCGTG CCTGCGGATAAACAGCGTA	99	1.33±0.20b	1.37±0.09
ZMO1237	D-Lactate dehydrogenase	CCTCGTCAATACCAGTCGC AAACATCCGCCGCATAAC	95	1.72±0.26b	5.44±0.25
ZMO0293	Sugar transporter	TTCACGCTGCGACTTTGT AAGCATTCAGACCGCCATA	114	3.73±0.30b	7.49±0.56
ZMO1828	Nitrogen fixation protein NifX	GTATTCACCCCGTCAAACTG CATCCCCATAGCCTTCCTTA	113	−1.16±0.36c	−1.81±0.19
ZMO0213	Outer membrane autotransporter barrel domain-containing protein	ATCTGCGGTTGTTAATGTCG GAGAAGGTATCTTGTGCCTGACTA	140	−1.32±0.42c	−4.55±0.37
ZMO0374	Levansucrase	GCGGATGCTATGAAAGTGC CCAAGTGTCCCAAACCCA	105	1.34±0.20c	2.78±0.29
ZMO1449	Mannitol-dehydrogenase domain-containing protein	CCAAGGTGACGGCAGGTA CGGTCTCCGAGGCTAATG	136	2.02±0.31d	5.29±0.41
pzmob1_p07	Hypothetical protein	CCGATGGAAATTGGAAGC ATCTGCGGTTGCGTCATC	99	−1.21±0.24e	−2.78±0.18
ZMO0252	Major intrinsic protein	GGCGTTTACGAGCAAGGT GCGGTCAAAGTCGCAGATA	86	1.79±0.09e	4.10±0.55
ZMOr009	16S RNA	AGTTGGGCACTTTAGAGGAAC TCACCGCCATTGTAGCAC	111	1.00	

[a] The data from microarray and qPCR were transformed with $\log_2$ and shown as mean±S.D. a, b, c, d, and e indicate the data derived from the comparison group T/C, L/C, L/T, S/C, and S/L, respectively. C, T, and L represent ZM4 cells treated by 220 g/L glucose for 0, 2, and 14 h, respectively. S represents ZM4 cells treated by 220 g/L glucose and 10 mM sorbitol for 6 h

growth status. Addition of 10 mM sorbitol to the medium significantly shortened the growth lag period (data not shown). Based on these results, 220 g/L glucose was used to study the transcriptional responses of ZM4 cells to high glucose concentrations.

ZM4 cells were initially cultivated in RM (20 g/L glucose), and their growth was monitored throughout the experimental period (Fig. 1). When cultures were at the middle log phase (6 h postinoculation; group C), cell samples were collected as control (C). At this point, the residual glucose and ethanol concentrations were 1.93±0.05 and 8.93±0.23 g/L, respectively, indicating that 90.35 % of the glucose in the medium was consumed by ZM4. In parallel, 220 g/L of glucose was added to induce adaptation of ZM4 cells to a high glucose concentration, resulting in a period of growth arrest. After 2 h, the cultures were still in stationary phase with no increase in cell density. Samples collected subsequently were designated as the adaptation treatment group (T). After prolonged incubation, the cells recovered gradually and were able to grow. Cell samples were collected 14 h after treatment and used as the adaptation treatment (L) (group G). When sorbitol was added with glucose, the cells resumed growth more quickly and vigorously. Therefore, cell samples were collected 6 h after treatment (12 h postinoculation) and designated as treatment S (group GS). The time points for C, S, and L were roughly chosen at the middle of exponential phase; meanwhile, ZM4 cells were highly homogeneous during this phase. At the end of the experimental phase (52 h postinoculation), 22.7 and 16.8 % of the added glucose remained in the medium when added without and with sorbitol, respectively; the ethanol yield was approximately 64.3 and 76.2 % of the theoretical value in the absence and presence of sorbitol, respectively.

The RNA samples isolated from the above cell samples were applied to the next microarray analysis. Customized microarrays were designed using ZM4 genomic information (GenBank Accession number NC_006526.2) with probes covering 1918 genes. After scanning and data transformation, the expression signal for each probe was deposited at the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE49620) under accession number GSE49620. The comparison result showed that the classification number (203) of the MG-RAST system is higher than that (168) of the GO analysis for 235 differentially expressed genes. Therefore, the classification result obtained from MG-RAST was used in this study. In addition to the classification by MG-RAST, some differentially expressed genes with obviously similar functions were grouped into specific categories by manual searches.

The microarray data were analyzed to identify genes involved in adaptation to high glucose concentrations. The results obtained from Friedman test revealed that gene transcription of ZM4 cells was significantly influenced by addition of glucose and sorbitol (Friedman chi-squared=46.6317, p value =4.163e-10). The Pearson's correlation coefficients showed good correlation among biological replicates (the minimum r value ≥0.80, p<0.01) (Fig. S2 in the Supplementary

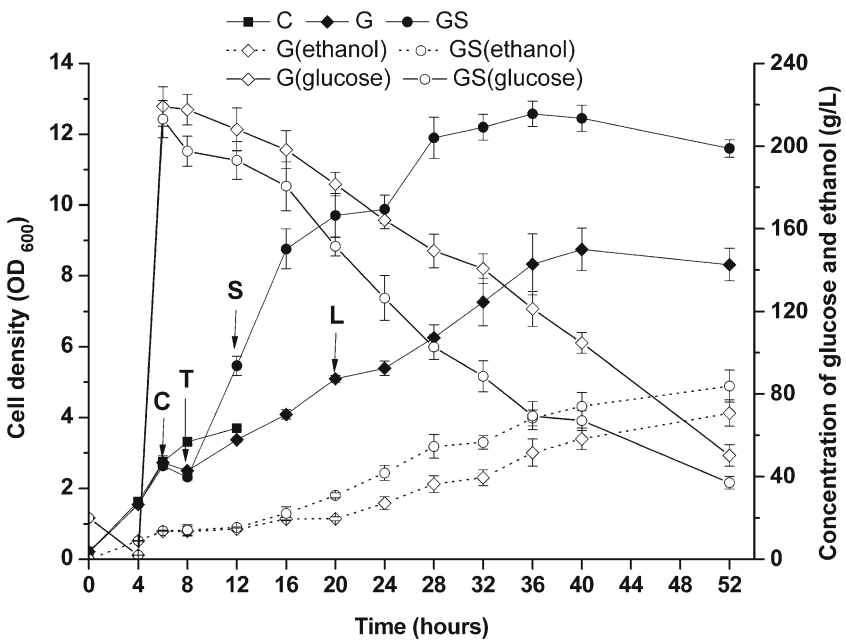

Fig. 1 *Zymomonas mobilis* ZM4 fermentation in 220 g/L glucose in the absence and presence of 10 mM sorbitol. *Z. mobilis* ZM4 was cultured in normal rich medium (20 g/L glucose, *C*). When the culture reached the mid-exponential phase (6 h postinoculation), glucose and sorbitol were added to the indicated final concentrations. *Squares* indicate 20 g/L glucose (*C*), *diamonds* indicate 220 g/L glucose (*G*), and *circles* indicate 220 g/L glucose plus 10 mM sorbitol (*GS*). *Filled symbols with solid lines* represent the cell growth curves, *open symbols with solid lines* represent glucose consumption processes, and *open symbols with dotted lines* represent the processes of ethanol production. The data from three independent experiments are expressed as the means±S.D. *Arrows* (*C*, *T*, *S*, and *L*) indicate the sampling time points

Material). A total of 235 genes were significantly differentially expressed during different phases of adaptation to a high concentration of glucose (Fig. S3 in the Supplementary Material). Among these genes, 150 and 68 were differentially expressed 2 h (T/C) and 14 h (L/C) after treatment with the high glucose concentration, respectively. During the restoration phase, 93 genes were differentially expressed (L/T). When sorbitol was added with glucose, 60 and 56 genes were differentially expressed compared with the transcription levels before (S/C) and after (S/L) treatment, respectively. In addition, 158 genes were filtered because their hybridization signals were below the cutoff value (400) in normally growing cells, which were mainly plasmid-encoded genes (data not shown).

Transcription of membrane-transport genes

Addition of a high glucose concentration to the medium can significantly affect the osmotic pressure and the balance of membrane potential in ZM4 cells. To respond to hyperosmotic conditions, the activities of transporters or channels might be altered as the first line of defense. When glucose was added to the medium of ZM4 cells, 30 genes involved in membrane transport systems were significantly differentially expressed, including 15 genes of the Ton transport systems, 4 genes encoding ABC transporter-related proteins, and 4 genes of the Type I secretion system (ZMO0252–ZMO0255) (Table 2 (T/C) and Table S1 in the Supplementary Material). In addition, other membrane channel-related genes such as ZMO0293 (sugar transporter), ZMO0547 (chloride channel protein), ZMO1766 (Mg^{2+} transporter protein, CorA family protein), and ZMO0916 (heavy metal transport/detoxification protein) were also upregulated in response to the high glucose concentration, although these genes were "unclassified" in the RAST subsystem. In contrast, only two genes ZMO0688 (auxin efflux carrier) and ZMO0800 (ABC transporter-like protein) were downregulated in response to the high glucose concentration.

Notably, most of the above genes returned to their original expression status and were not significantly differentially expressed when ZM4 cells resumed growth (14-h adaptation). Only six genes (ZMO0031, ZMO0547, ZMO0789, ZMO0916, ZMO0293, and ZMO1815) were still transcribed at higher levels compared to the control after resumption of growth (Table 2 (L/C) and Table S2 in the Supplementary Material). In particular, the transcriptional level of ZMO0293 was further upregulated by more than 3.7-fold during the growth recovery stage. ZMO0688 and ZMO0800 were further downregulated after a 14-h glucose-adaptation period.

Compared to the transcription levels 2 h after treatment with the high glucose concentration, only three genes (ZMO0031, ZMO0688, and ZMO0293) remained upregulated and 25 genes remained downregulated after a 14-h adaptation period (Table 2 (L/T) and Table S3 in the Supplementary Material). Furthermore, nine genes were significantly downregulated by ≥1-fold, including four genes related to the TonB-dependent receptor and three genes of the Type I secretion system (except ZMO0252).

Transcription of carbohydrate- and nitrogen-metabolism genes

The osmotic stress caused by a high glucose concentration might affect carbohydrate metabolism. Consistent with this hypothesis, five genes related to carbohydrate metabolism were differentially expressed (Tables S1–S3 in the Supplementary Material). ZMO1237 encodes D-lactate dehydrogenase, and its transcription was first increased and then decreased. Both ZMO0215 and ZMO0454 function in one-carbon metabolism and encode 5-formyltetrahydrofolate cyclo-ligase and formate-tetrahydrofolate ligase, respectively.

In addition, ZMO1747, which encodes 5,10-methylenetetrahydrofolate reductase, was downregulated during growth resumption (approximately −1.2-fold). Expression of the gene ZMO0889, which encodes aldose 1-epimerase, showed no significant change after glucose addition but was upregulated by ~1.6-fold during growth resumption. Notably, the upregulation of ZMO0889 is consistent with its function, which catalyzes the conversion between the α- and β-anomers of hexose sugars such as glucose and galactose (Tables S2–S3 in the Supplementary Material).

Based on the ZM4 genome annotation (Seo et al. 2005), at least 25 genes (ZMO01816 to ZMO01840) comprise a canonical gene cluster for nitrogen fixation. Among these genes, 15 (ZMO1816, ZMO1817, ZMO1823–ZMO1829, ZMO1832–ZMO1835, ZMO1837, and ZMO1838) were upregulated in response to the osmotic pressure induced by a high glucose concentration; however, during normal growth and after the 14-h adaptation period, these transcript levels were generally low or even undetectable. Furthermore, most of these genes were downregulated compared to their levels 2 h after glucose treatment. Only two genes, ZMO1823 and ZMO1824, were transcribed at higher levels after ZM4 cells resumed growth (Tables S1–S3 in the Supplementary Material).

Transcription of DNA and protein metabolism and stress response genes

The transcription of several genes (ZMO0354, ZMO0892, ZMO1401, ZMO1450, ZMO1588, and ZMO1699), possibly involved in DNA metabolism, increased by ~1-fold in response to the high glucose concentration and subsequently decreased or returned to normal levels during the growth recovery stage (Tables S1–S3 in the Supplementary Material). These genes encode nucleases that potentially function in DNA repair during stress. Increased glucose concentrations

Table 2 Differentially expressed genes related to membrane transporter in the presence of high glucose concentration without sorbitol

Locus tag number	Function	Fold change		
		T/C[a]	L/C[a]	L/T[a]
ZMO0001	TonB-dependent receptor plug	1.23±0.23**	0.25±0.32	−0.97±0.18**
ZMO0031	TonB-dependent receptor	0.96±0.16**	1.22±0.20**	0.26±0.07*
ZMO0128	TonB-dependent receptor	−0.69±0.28*	−1.08±0.34**	−0.39±0.29
ZMO0213	Outer membrane autotransporter barrel domain-containing protein	2.08±0.14**	0.76±0.36*	−1.33±0.42**
ZMO0252	Major intrinsic protein	1.24±0.10**	0.26±0.20*	−0.97±0.19**
ZMO0253	TolC family type I secretion outer membrane protein	2.08±0.09**	0.62±0.29*	−1.47±0.27**
ZMO0254	Type I secretion system ATPase	1.48±0.25**	0.23±0.35	−1.25±0.23**
ZMO0255	HlyD family type I secretion membrane fusion protein	1.32±0.22**	0.07±0.20	−1.25±0.08**
ZMO0293	Sugar transporter	3.71±0.29**	3.73±0.16**	0.02±0.14
ZMO0547	Chloride channel protein	1.32±0.07**	1.09±0.03**	−0.23±0.09*
ZMO0688	Auxin efflux carrier	−1.47±0.18**	−1.04±0.14**	0.44±0.05*
ZMO0789	TonB-dependent receptor	2.19±0.07**	1.33±0.20**	−0.86±0.27**
ZMO0795	TonB-dependent receptor plug	1.23±0.22**	0.14±0.40	−1.10±0.24**
ZMO0800	ABC transporter-like protein	−0.71±0.10**	−1.11±0.10**	−0.40±0.11**
ZMO0902	TonB-dependent receptor	2.74±0.24**	0.63±0.56	−2.12±0.35**
ZMO0916	Heavy metal transport	1.42±0.33**	1.30±0.09**	−0.12±0.24
ZMO1040	TonB-dependent receptor	1.70±0.02**	0.92±0.30**	−0.79±0.32**
ZMO1260	TonB-dependent receptor	1.02±0.14**	0.38±0.38	−0.65±0.24**
ZMO1298	TonB-dependent receptor plug	1.94±0.28**	0.92±0.38**	−1.03±0.17**
ZMO1475	TonB-dependent receptor plug	1.64±0.21**	0.69±0.36*	−0.95±0.17**
ZMO1522	TonB-dependent receptor	1.20±0.09**	0.66±0.23**	−0.54±0.15**
ZMO1631	TonB-dependent receptor	1.24±0.44**	0.71±0.19*	−0.54±0.40
ZMO1766	Mg^{2+} transporter protein	1.49±0.10**	0.62±0.09**	−0.87±0.06**
ZMO1815	TonB-dependent receptor	3.10±0.18**	1.06±0.26**	−2.05±0.19**
ZMO1822	TonB-dependent receptor	1.05±0.11**	0.08±0.35	−0.98±0.25**
ZMO1846	ABC transporter-like protein	1.44±0.24**	0.80±0.37*	−0.65±0.36*
ZMO1859	Carbohydrate-selective porin OprB	1.06±0.12**	0.11±0.29	−0.95±0.16**
ZZM4_0134	ABC transporter-related protein	1.06±0.15**	0.27±0.12	−0.79±0.24**
ZZM4_0136	ABC transporter-related protein	1.14±0.36**	0.01±0.02	−1.14±0.37**
ZZM4_0137	TonB-dependent receptor	1.18±0.11**	0.26±0.31	−0.94±0.23**

[a] C, T, and L represent ZM4 cells treated by 220 g/L glucose for 0, 2, and 14 h, respectively

*Statistically significant at $p<0.05$

**Statistically significant at $p<0.01$

may also impair amino acid and protein metabolism. Several genes (ZMO0192, ZMO0216, ZMO0794, ZMO1291, ZMO1704, and ZMO1776) involved in proteolytic function were initially upregulated and then downregulated when ZM4 cells resumed growth (Tables S1–S3 in the Supplementary Material).

Bacteria have evolved different stress-responsive systems to survive changes in their natural habitat. Many genes are involved in the response to stresses such as cold shock, heat shock, detoxification, osmotic stress, and oxidative stress. The glucose-induced osmotic stress caused a minor increase in the transcription of several genes including ZMO0749 (RNA polymerase sigma factor 32), ZMO0918 (catalase), and ZMO1061 (sigma 54-specific transcriptional activator PspF) (Table S1 in the Supplementary Material). In addition, other differentially expressed genes assigned into RAST subsystems as unclassified or other subsystems, such as ZMO0191 (DSBA oxidoreductase), ZMO0305 (stress responsive alpha-beta barrel domain-containing protein), ZMO0740 (CsbD family protein), ZMO0989 (heat shock protein Hsp20), ZMO1069 (heat shock protein DnaJ domain-containing protein), ZMO1545 (heat shock protein DnaJ domain-containing protein), ZMO1705 (thioredoxin domain-containing protein), and ZMO1690 (chaperone DnaJ domain-containing protein), may be involved in the response of ZM4 cells to high glucose concentrations. ZMO1063 (phage shock protein A, PspA) and

ZMO1064 (phage shock protein B, PspB) were also upregulated after glucose addition and during growth recovery (Tables S1–S3 in the Supplementary Material).

Transcription of regulatory and cell signaling genes

Intricate regulatory mechanisms mediated by regulatory genes coordinate cellular responses to environmental changes. Upon glucose addition, transcription of four regulatory genes, ZMO0050 (LysR-family transcriptional regulator), ZMO1438 (AsnC-family transcriptional regulator), ZMO1697 (HxlR-family transcriptional regulator), and ZMO2033 (XRE-family transcriptional regulator), was upregulated by ~1-fold. However, their transcription was downregulated after cells resumed growth (Tables S1–S3 in the Supplementary Material).

Transcription of other genes involved in regulating amino acid and nitrogen metabolism, such as ZMO0037 (putative PTS IIA-like nitrogen regulatory protein PtsN), ZMO1854 (GntR-family transcriptional regulator with an aminotransferase domain), and ZMO1944 (GntR-family transcriptional regulator with aminotransferase domain), was upregulated after glucose addition and then downregulated during growth recovery. In addition, transcription of ZMO1748 (ArsR-family transcriptional regulator), ZMO0190 (RpiR-family transcriptional regulator), ZMO1438 (AsnC-family transcriptional regulator), and ZMO1697 (HxlR-family transcriptional regulator) was decreased at the glucose-adaptation stage.

Transcription of plasmid-encoded genes

Z. mobilis ZM4 contains five circular plasmids pZZM401–pZZM405 (Yang et al. 2009a). Under normal growth conditions, most plasmid-encoded genes were not transcribed (data not shown). When cells were subjected to high osmotic stress, 22 plasmid-encoded genes were differentially expressed 2 h after treatment, including 20 upregulated genes and 2 downregulated genes. After adaptation of ZM4 cells to the high glucose concentration for 14 h, 25 genes were significantly induced. Among them, 17 genes were also upregulated 2 h after adaptation. Compared to the transcription levels of differentially expressed genes 2 h after glucose treatment, the expression of ten genes continued to increase, and pzmob1_p06 and pzmob1_p18 were initially upregulated and then downregulated after growth resumption. Furthermore, other six genes were differentially expressed (Tables S1–S3 in the Supplementary Material), and their expression differed from chromosomal genes whose transcription generally increased in response to osmotic pressure and decreased after cell growth resumed. Remarkably, 24 genes, ZZM4_0014 to ZZM4_0037, most likely formed a prophage cluster, and transcription of the entire cluster increased during growth resumption.

Resumption of cell growth is promoted by addition of sorbitol after treatment with a high glucose concentration

When sorbitol was added to the culture treated with a high glucose concentration, ZM4 cells resumed growing more quickly and had a shorter adaptation phase (Fig. 1). Furthermore, several genes that were significantly differentially transcribed in the presence of a high glucose concentration were transcribed at baseline levels (C) (Tables S4 and S5 in the Supplementary Material). In particular, sorbitol had the greatest impact on the transcriptional levels of genes related to membrane transport and nitrogen metabolism. Only nine genes involved in membrane transport were detected after sorbitol addition, accounting for 30 % of the corresponding genes without sorbitol. In addition, most of the genes involved in nitrogen metabolism in the absence of sorbitol addition were not differentially expressed after sorbitol addition, with the exception of three genes (ZMO1823–ZMO1825). Such situations also applied to others genes involved in carbohydrate, DNA, protein and amino acid metabolism, etc. In relation to stress responses, only ZMO0573 (glutaredoxin 2), ZMO2032 (DNA repair protein RadC), and ZMO1063-1065 (PspA, PspB, PspC) were found to be significantly differentially expressed, and no one gene was detected to be involved with heat shock protein, sigma factor, and peptidase.

However, other genes were significantly differentially expressed compared to the control. These genes were roughly assigned into two groups based on their transcriptional responses to the high glucose concentration in the presence of sorbitol (Table S6 in the Supplementary Material). In the first group, gene transcription was always downregulated after addition of sorbitol, and transcript levels were maintained at that level during the entire process of adaptation to glucose. The operon containing genes ZMO0632, ZMO0633, and ZMO0634, which encode flagellar structural proteins, was downregulated by <1-fold when only glucose was added. Transcription of this operon decreased further by >1-fold after addition of sorbitol. In addition, transcription of ZMO1019 and ZMO1020 was reduced when glucose was added, irrespective of the presence of sorbitol. In the second group, gene transcription was significantly upregulated when sorbitol was added with glucose; moreover, transcription of these genes was increased upon addition of glucose alone. For example, transcription of the operon ZMO0252–ZMO0254 was upregulated in the presence of the high glucose concentration and increased further upon addition of sorbitol. However, transcription of this operon decreased after cells resumed growth without the addition of sorbitol (Table 2). Furthermore, transcription of the plasmid-encoded genes (ZZM4_0014 to ZZM4_0037) was upregulated upon addition of the high glucose concentration and further increased upon addition of sorbitol (Tables S4 and S5 in the Supplementary Material). Most notably, plasmid-encoded genes comprised 44 and 31 % of

differentially expressed genes in the presence of sorbitol in the S/C and S/L groups, respectively. These genes were upregulated upon sorbitol addition.

The distribution of differentially expressed genes was analyzed using Venny (http://bioinfogp.cnb.csic.es/tools/venny/index.html). The results demonstrated that 61 % (143) of the total differentially expressed genes were specific to the adaptation to a high concentration of glucose in the absence of sorbitol, while 10 % (24) were specific to sorbitol addition. Another 68 genes existed simultaneously and were not specific (Fig. 2a). Further analysis illustrated that only nine genes occurred together in three phases of glucose adaptation (T/C, L/C, and L/T), involving nitrogen metabolism (ZMO1823, ZMO1824), lactic acid formation (ZMO1237), and an unknown function (ZMO1936, ZMO1937, ZMO1849, ZMO1943) (Fig. 2b). When sorbitol was added, 19 genes coexisted in both phases (S/C and S/L) and mainly included 4 genes of the Type I secretion system and 11 plasmid encoding genes (Fig. 2c).

Validation of microarray gene expression data using RT-qPCR

RT-qPCR was performed to quantify the transcription levels of 12 differentially expressed genes selected from microarray analysis. These genes belonged to different functional categories and were differentially expressed at various magnitudes during different experimental phases. The same RNA samples applied for microarray analysis were used for reverse transcription of cDNA. RT-qPCR analysis displayed a wider range in the fold change of gene expression (Table 1), and a strong correlation was observed between the RT-qPCR and microarray data (R^2=0.86).

Discussion

High glucose concentrations are optimal for very-high-gravity (VHG) ethanol fermentation but severely affect the growth and metabolism of ZM4 cells (Struch et al. 1991; Zhang et al. 2008). In this study, microarray analysis was performed to identify genes that were differentially expressed under high glucose concentrations in the presence and absence of sorbitol, and this analysis revealed several important genes involved in high-sugar tolerance.

Microorganisms have evolved different strategies to survive fluctuations in environmental osmotic pressure. Exposure of cells to high osmolarity often leads to a rapid efflux of water and solutes and immediate cell shrinkage. Ultimately, a balance is reached between intracellular and extracellular osmolarity, which enables the cells to maintain turgor and prevents lysis or plasmolysis. Previous studies in a variety of microorganisms have demonstrated that the primary response of bacteria to osmotic stress involves the activation of transporters (Poolman et al. 2002). *Z. mobilis* cells appear to have evolved the same strategy because 30 genes related to different membrane channels and transporters were upregulated in response to osmotic stress induced by a high glucose concentration (Table 2). Among these genes, the operon encoding the TolC Type I secretion system was significantly induced. However, other independent studies using microarray-based transcriptome analysis did not observe differential expression of this operon in response to ethanol, furfural, and aerobic growth (He et al. 2012a, b; Yang et al. 2009b). In other bacteria, this secretion system is involved in the secretion of various substrates such as proteases, lipases, and hemolysin (Delepelaire 2004; Holland et al. 1990; Zgurskaya et al. 2011). Although the cellular functions of these genes are yet

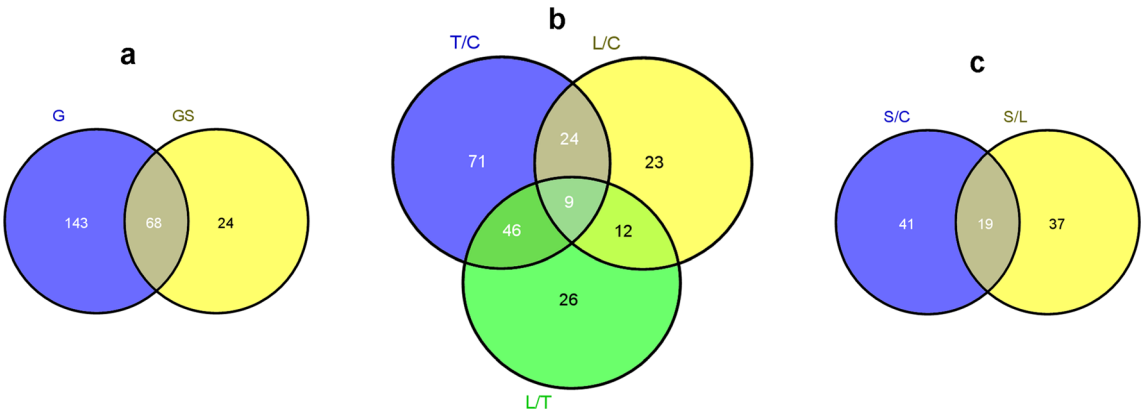

Fig. 2 Distribution of differentially expressed genes between different groups. Distribution maps of significantly differentially expressed genes were performed using Venny. **a** *G* (*blue*) represents the differentially expressed genes in response to 220 g/L glucose, and *GS* (*yellow*) represents the differentially expressed genes in response to 220 g/L glucose and 10 mM sorbitol. **b** *T/C* (*blue*), *L/C* (*yellow*), and *L/T* (*green*) represent the corresponding genes of different phases when 220 g/L glucose was added. **c** *S/C* (*blue*) and *S/L* (*yellow*) represent the corresponding genes before and after treatment with 10 mM sorbitol and 220 g/L glucose. *C* represents control, without treatment, *T* and *L* represent ZM4 cell samples that were treated with 220 g/L glucose for 2 and 14 h, and *S* represents ZM4 cell samples that were treated with 220 g/L glucose and 10 mM sorbitol for 6 h (color figure online)

unclear in *Z. mobilis*, this secretion system might be specific to high concentrations of glucose in ZM4 cells.

Another group of differentially expressed membrane-related genes mainly included TonB-dependent transporters (Table 2), which are usually not expressed or are expressed at undetectable levels under normal growth conditions (data not shown). TonB-dependent transporters are present in most bacteria and are required for active uptake of iron complexes, nickel, vitamin B_{12}, polypeptides, and carbohydrates (Schauer et al. 2008). Expression of these transporters is broadly regulated by metal-dependent regulators, σ/anti-σ factor systems, and small RNAs. *Z. mobilis* cells do not produce siderophores (high-affinity iron-chelating compounds), which are transported by specific TonB-dependent transporters in other bacteria (Noinaj et al. 2010; Wandersman and Delepelaire 2004). Because iron assimilation is essential in bacteria, these differentially expressed TonB-dependent transporter genes in ZM4 may be involved in transporting unknown molecules, some of which may contribute to osmotic pressure resistance. In addition, the gene *nhaA*, which encodes a sodium-proton antiporter that enhances sodium acetate tolerance when overexpressed in ZM4 cells, was also not differentially expressed (Yang et al. 2010a).

Microarray analysis was used to examine 29 genes participating in the ED and ethanol production pathways in *Z. mobilis*. The expression levels of these genes did not fluctuate greatly in the presence and absence of sorbitol except for ZMO1237 (*ldhA*) and ZMO1754 (*ssdA*) (Table 3). This expression pattern is closely related with the unique pattern of glucose metabolism in *Z. mobilis*. Genes in the ED pathway appear to be constitutively expressed. Moreover, almost all these enzymes catalyze glucose near their maximal activities, and their activities were not regulated by feedback inhibition or other regulatory mechanisms (Algar and Scopes 1985; Viikari and Berry 1988). Therefore, the glucose metabolic rate was mainly limited by the maximal capacity of these enzymes. Additionally, four genes *glf*, *zwf*, *edd*, and *glk* comprise an operon that controls glucose uptake and the first three steps of glucose metabolism, which enables rapid glucose utilization (Barnell et al. 1990). Microarray analysis revealed that these genes were transcribed at high levels during the entire adaptation period (Table S7 in the Supplementary Material). In addition, *ldhA* and *ssdA* were upregulated upon glucose addition, which accelerate the conversion of lactate and acetate by-products. These data are consistent with previous studies in which the production of lactate and acetate by *Z. mobilis* increased under aerobic conditions and decreased under anaerobic conditions (Yang et al. 2009b). Moreover, the *ldhA* expression level decreases upon ethanol stress, and the lactic acid yield is reduced by up to 50 % in an *ldhA*-deficient mutant (He et al. 2012a; Lawford and Rousseau 2002). Therefore, redirection of the end product from ethanol to lactate or other by-products can relieve the toxic effects of ethanol. In particular, the gene ZMO0293 (encoding a sugar transporter) was upregulated by 3.7-fold when glucose was added, and its transcription was reduced by ~1-fold upon sorbitol addition (Tables S1, S2, and S4 in the Supplementary Material). Therefore, the inducible gene ZMO0293 is likely involved in adaptation to high glucose concentrations. Lastly, two genes (ZMO1299 and ZMO1300) that contribute to resistance to high osmotic pressure from high concentrations of glucose (Seo et al. 2005) were not significantly differentially expressed in this study.

In addition, a nitrogen-fixation gene cluster (ZMO1823 to ZMO1849) was upregulated in the presence of a high glucose concentration. Other sequenced *Z. mobilis* strains (GenBank accession numbers NC017262, NC018145, NC013355) also encode a nitrogen-fixation gene cluster. Carbon metabolism and nitrogen assimilation can be regulated by the nitrogen regulatory protein PII and the phosphotransferase system (PTS) (Commichau et al. 2006). Most genes in the nitrogen-fixation cluster were activated in the presence of a high glucose concentration, suggesting that *Z. mobilis* is able to fix molecular nitrogen under special conditions; however, the relationship between nitrogen fixation and adaptation of *Z. mobilis* to high glucose concentrations has not been elucidated.

High osmotic stress might impact other aspects of cellular physiology. For example, reactive oxygen species (ROS), which are generated during respiration, can damage DNA, proteins, and lipids within cells (Apel and Hirt 2004). As expected, genes such as ZMO0918 (encoding catalase) and ZMO0573 (encoding glutaredoxin 2), which may be involved in scavenging ROS in response to oxidative stress and redox signals under high osmotic stress, were upregulated in ZM4 (Beer et al. 2004). Similarly, several putative DNA repair genes (ZMO0354, ZMO0892, and others) were significantly upregulated in ZM4 cells. Likewise, several peptidase-encoding genes (ZMO0192, ZMO0216, etc.) were upregulated at the glucose-adaptation stage. In *Bacillus subtilis*, the PapA and PapB peptidases release proline by hydrolysis of proline-containing peptides, and proline can function as a compatible solute in response to high osmolarity (Zaprasis et al. 2013). In addition, two regulatory genes (ZMO1854, ZMO1944) involved in amino acid metabolism were upregulated. Addition of arginine and lysine can lead to increased acid tolerance in *Salmonella* Typhimurium CECT443, and the corresponding genes displayed higher expression levels under acid stress (Alvarez-Ordóñez et al. 2010). It remains to be elucidated whether free amino acids are involved in the response to high glucose concentrations.

Transcriptional changes may be caused by transcriptional regulators, which might respond to various stresses. In this study, several genes encoding putative regulatory proteins were upregulated (Table S1 in the Supplementary Material).

Table 3 The transcriptional levels of the total genes involving in Entner-Doudoroff and pyruvate metabolic pathways under different phases

Locus tag number	Gene	Function	Fold change		
			T/C[a]	L/C[a]	L/T[a]
ZMO0366	*glf*	Sugar transporter	−0.17±0.18	0.09±0.04	0.26±0.22
ZMO0369	*glk*	Glucokinase	−0.14±0.12	0.10±0.15	0.25±0.2*
ZMO0367	*zwf*	Glucose-6-phosphate 1-dehydrogenase	−0.16±0.18	0.14±0.22	0.30±0.22*
ZMO1649	*gnl*	Gluconolactonase	−0.44±0.17**	0.11±0.22	0.55±0.24*
ZMO1757	*gntk*	Thermoresistant glucokinase family carbohydrate kinase	−0.72±0.04**	−0.35±0.09**	0.37±0.08**
ZMO1478	*pgl*	6-Phosphogluconolactonase	−0.47±0.26**	0.02±0.06	0.51±0.21**
ZMO0368	*edd*	Phosphogluconate dehydratase	0.06±0.15	0.26±0.31	0.19±0.23
ZMO0997	*eda*	2-Dehydro-3-deoxyphosphogluconate/4-hydroxy-2-oxoglutarate aldolase	−0.24±0.13*	0.17±0.15	0.41±0.21*
ZMO0177	*gap*	Glyceraldehyde-3-phosphate dehydrogenase, type I	0.05±0.18	0.13±0.26	0.07±0.13
ZMO0178	*pgk*	Phosphoglycerate kinase	−0.20±0.19	0.20±0.22	0.39±0.12**
ZMO1240	*gpm*	Phosphoglyceromutase	−0.16±0.14	0.16±0.22	0.32±0.10**
ZMO1608	*eno*	Phosphopyruvate hydratase	0.07±0.21	0.26±0.27	0.19±0.2
ZMO0152	*pyk*	Pyruvate kinase	−0.17±0.11	0.12±0.16	0.29±0.22*
ZMO1496	*ppc*	Phosphoenolpyruvate carboxylase	−0.26±0.06**	−0.04±0.08	0.22±0.11*
ZMO1237	*ldhA*	D-Lactate dehydrogenase	2.72±0.49**	1.72±0.26**	−1.00±0.23**
ZMO1360	*pdc*	Thiamine pyrophosphate protein TPP binding domain-containing protein	−0.23±0.33	0.11±0.24	0.35±0.28*
ZMO1236	*adhA*	Alcohol dehydrogenase	0.78±0.14**	0.92±0.11**	0.14±0.06*
ZMO1596	*adhB*	Iron-containing alcohol dehydrogenase	0.13±0.11	0.25±0.21*	0.12±0.18
ZMO1722	*adhC*	Class III alcohol dehydrogenase	0.15±0.07*	−0.29±0.05**	−0.44±0.05**
ZMO0544	*CitC*	Isocitrate dehydrogenase	−0.66±0.12**	−0.35±0.09**	0.30±0.11**
ZMO0487	–	Hpch/hpai aldolase	−0.36±0.04**	−0.14±0.12	0.22±0.08*
ZMO0569	*SdhC*	Succinate dehydrogenase (b subunit)	0.55±0.12**	−0.06±0.12	−0.61±0.07**
ZMO0705	*BudB*	Regulatory protein RecX	−0.34±0.06**	0.14±0.01*	0.48±0.07**
ZMO1955	*YqkJ*	Malate dehydrogenase	−0.43±0.09**	−0.37±0.11**	0.06±0.17
ZMO1307	*FumA*	Hydrolyase, Fe-S type, tartrate	−0.75±0.20**	−0.64±0.2**	0.11±0.06
ZMO1963	*GltA*	Citrate synthase I	−0.49±0.08*	−0.46±0.17**	0.03±0.22
ZMO0543	*AcnA*	Aconitate hydratase	−0.83±0.09**	−0.6±0.09**	0.24±0.11*
ZMO1570	*pfl*	Formate acetyltransferase	0.13±0.14	0.37±0.25*	0.23±0.11*
ZMO1754	*ssdA*	Aldehyde dehydrogenase (NAD^+)	1.57±0.40**	0.82±0.25**	−0.75±0.17**

[a] C, T, and L represent ZM4 cells treated by 220 g/L glucose for 0, 2, and 14 h, respectively

*Statistically significant at $p<0.05$

**Statistically significant at $p<0.01$

ZMO0749 (encoding the stress-responsive RNA polymerase factor σ^{32}) was upregulated during high osmotic pressure. This gene was also induced significantly under aerobic conditions (Yang et al. 2009b), and its *Escherichia coli* homolog responds to oxidative stress (Kogoma and Yura 1992). Hfq is a bacterial RNA chaperone protein that mediates RNA-RNA interactions and is involved in multiple cellular processes in many bacterial species (Chao and Vogel 2010; Tsui et al. 1994). Yang et al. (2010b) observed that the *Z. mobilis* Hfq protein is important for resisting multiple lignocellulosic pretreatment inhibitors including acetate, vanillin, furfural, and hydroxymethylfurfural (HMF); however, the *hfq* gene (ZMO0347) was not differentially expressed under high glucose concentrations. In addition, in many bacteria, phage shock proteins (Psps) form the core response machinery under stress conditions such as heat, ethanol, osmotic shock, and infection by bacteriophages (Brissette et al. 1990). In the ZM4 genome, the genes ZMO1061, ZMO1063, ZMO1064, and ZMO1065 encode PspF, PspA, PspB, and PspC, respectively. Notably, the transcription levels of these genes were increased by more than 1-fold in response to a high glucose concentration. These genes are also upregulated in response to stresses such as ethanol and furfural, as well as during aerobic growth (He et al. 2012a, b; Yang et al. 2009b). Together, these observations indicate that this operon is important for various stress responses in *Z. mobilis*.

Common compatible solutes such as glycerol, glutamic acid, proline, trehalose, mannitol, and betaine were not detected in the ZM4 cells under high concentrations of glucose (Loos et al. 1994). However, sorbitol can be transported into cells and protects intracellular DNA and proteins from misfolding or other damage. Moreover, the enzymatic activities of GK, G-6-P, and ADH can be maintained at relatively high levels by the compatible solute ectoine under high glucose concentrations (Zhang et al. 2008). The protective mechanism of compatible solutes is that they are strong water-structure formers and function as effective stabilizers of the hydration shell of native proteins at the molecular and whole-cell levels (Galinski 1993). In this study, the role of sorbitol in promoting adaptation to high glucose concentrations was examined. A greater number and type of genes were not differentially expressed in the absence of sorbitol compared to its presence. The differentially expressed genes exclusive to sorbitol addition accounted for 17 % of those genes without sorbitol (Fig. 2a), suggesting that sorbitol can exert a protective effect on ZM4 cells when confronted with the challenge of a high glucose concentration. Recently, the protective effect of sorbitol on protein levels was also confirmed by Sootsuwan et al. (2013) in *Z. mobilis*. The researchers found that the transcription and protein synthesis of *pdc*, *adhA*, and *adhB* were decreased under stress conditions when the gene *gfo* (encoding GFOR) was disrupted; however, the synthesis of almost all of the proteins can be recovered when sorbitol is added to the medium. Together, the combination of our result and functional analysis of the *gfo* knockout can provide useful information for understanding the molecular mechanism underlying the promoting effect of sorbitol on ethanol fermentation and the development of effective ethanol fermentation in the presence of a high glucose concentration.

Lastly, the plasmid pZZM401 contains 24 genes (ZZM4_0014 to ZZM4_0037; approximately 20 kb), which mainly encode phage-related proteins. Transcription of all these genes (except ZZM4_0020 and ZZM4_0021) was induced, especially upon addition of sorbitol (Tables S4 and S5 in the Supplementary Material). In other bacteria, prophages are activated by stress conditions (Waller et al. 2012). Transcription of pzmob1_p06 and pzmob1_p07 is increased under furfural stress and decreased under ethanol stress (He et al. 2012a, b). These plasmid-encoded genes might play an important role in coping with different environmental stresses; however, their function has not been elucidated completely. Therefore, it is important to examine whether this prophage can be activated under certain conditions in *Z. mobilis*.

In conclusion, three possible sites for adaptation to high glucose concentrations in ZM4 cells were proposed: (1) the cell membrane, 14.0 % (33) of the differentially expressed genes (235) were related to membrane channels or transporters including TonB-dependent and ABC transporters, type I secretion system, OprB, etc., suggesting that the cell membrane may be the first site of adaptation to high glucose concentrations; (2) stress response mechanisms, sigma factors, Psp, glutaredoxin, heat shock protein, DNA repair, and protein degradation proteins may constitute a complex mechanism that preserves ZM4 cells during adaptation to high glucose concentrations; and (3) metabolic pathways, although genes involved in ED metabolism were not differentially expressed, they were transcribed at high levels during the entire experimental process, thereby facilitating rapid uptake and conversion of glucose. The two genes *ldhA* and *ssdA* that were upregulated may accelerate shunting of the end product from ethanol to lactate and acetate. When sorbitol was supplemented into the culture medium, most of those genes like membrane-transport, stress response, *ldhA*, *ssdA*, etc., which were significantly influenced by a high glucose concentration, were not differentially expressed, or their transcriptional fluctuations were less than that without sorbitol addition, reflecting the protective role of sorbitol in the adaptation of ZM4 cells to a high glucose concentration.

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Conflict of interest The authors declare that they have no competing interests.

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