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*irr*E, an Exogenous Gene from *Deinococcus radiodurans*, Improves the Growth of and Ethanol Production by a *Zymomonas mobilis* Strain Under Ethanol and Acid Stresses

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During ethanol fermentation, bacterial strains may encounter various stresses, such as ethanol and acid shock, which adversely affect cell viability and the production of ethanol. Therefore, ethanologenic strains that tolerate abiotic stresses are highly desirable. Bacteria of the genus *Deinococcus* are extremely resistant to ionizing radiation, ultraviolet light, and desiccation, and therefore constitute an important pool of extreme resistance genes. The *irrE* gene encodes a general switch responsible for the extreme radioresistance of *D. radiodurans*. Here, we present evidence that IrrE, acting as a global regulator, confers high stress tolerance to a *Zymomonas mobilis* strain. Expression of the gene protected *Z. mobilis* cells against ethanol, acid, osmotic, and thermal shocks. It also markedly improved cell viability, the expression levels and enzyme activities of pyruvate decarboxylase and alcohol dehydrogenase, and the production of ethanol under both ethanol and acid stresses. These data suggest that *irrE* is a potentially promising gene for improving the abiotic stress tolerance of ethanologenic bacterial strains.

Keywords: *Zymomonas mobilis*, *irr*E expression, abiotic stress tolerance, ethanol productivity

The hyper-ethanologenic bacterium *Zymomonas mobilis* has attracted considerable interest as a candidate for industrial ethanol production. This organism converts glucose into ethanol very efficiently *via* the Entner–Doudoroff (ED) pathway [4], while producing less biomass than yeast [10]. However, various environmental stresses adversely affect both its growth and its ability to produce ethanol. High ethanol concentrations, osmotic pressure, and oxidative stresses are major stress factors that retard the specific growth rate and viability of *Z. mobilis* cells, as well as its ethanol production [21, 24, 30]. Acids produced in the hydrolysis of hemicellulose or lignocellulose or generated during fermentation are also major potential inhibitors [16, 18, 36]. To address these limitations, a variety of approaches have been used to develop mutant strains with improved stress tolerance, such as chemical mutagenesis [8, 14, 29]. More recently, Alper and Stephanopoulos [3] used global transcription machinery engineering (gTME) to improve the ethanol tolerance of *Saccharomyces cerevisiae* by modifying transcription factor Spt15p. This method provides a route for identifying transcription factor mutants that tolerate various stresses. However, little progress has been made on the genetic improvement of stress tolerance in ethanologenic strains.

Deinococcus radiodurans is one of the most radioresistant organisms known. This bacterium can survive extreme cold and dehydration as well as exposure to a vacuum and acidic conditions [20]. *D. radiodurans irr*E encodes a regulatory protein, IrrE (DR0167, also named PprI), and plays a critical role in the regulation of *rec*A and *ppr*A genes expression following γ-irradiation [7, 13]. The heterologous expression of *irr*E in *Escherichia coli* promotes DNA repair and oxidative damage protection [9]. Interestingly, the *irr*E gene protects *E. coli* cells against salt shock and other abiotic stresses such as oxidative, osmotic, and thermal shocks, and confers significantly enhanced salt tolerance in plants [25]. In the present study, we showed that *D. radiodurans irr*E, which encodes a regulatory protein, improved cell viability, abiotic stress tolerance, and ethanol production in *Z. mobilis*. Compared with the

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control strain, the expression levels and enzyme activities of pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) in IrrE-expressing *Z. mobilis* were enhanced under stress conditions. The results indicate that *irr*E plays a protective role to improve the fermentation performance of *Z. mobilis*.

MATERIALS AND METHODS

Strains and Growth Conditions

The wild type *Zymomonas mobilis* 10232 (China Center of Industrial Culture Collection) and the IrrE-expressing *Z. mobilis* were grown on RM medium (20 g/l glucose, 10 g/l Bacto yeast extract, 2 g/l potassium phosphate, pH 6.0) at 30°C for 1–2 days under anaerobic condition. The *E. coli* BL21 strain was grown at 37°C in Luria–Bertani medium (LB). When required, the medium was supplemented with 50 μg kanamycin (Km)/ml.

Expression of *irr*E in *Z. mobilis*

The enolase promoter (P*eno*) was amplified from *Z. mobilis* genomic DNA by PCR with primers 5'-CCGCGGATCTGCTGTCCTATATGAA-3' and 5'-GTTGGCACTGGGCACAT-CGAAACCTTTCTTA-3' (*Sac*I site is underlined). The *irr*E gene was amplified from *D. radiodurans* R1 genomic DNA by PCR with primers 5'-TAAGAAAGG TTTCGAT-GTGCCCAGTGCCAAC-3' and 5'-GAGCTCAGATCTC CAGTTCACTGTG-3' (*Sac*II site is underlined). The P*eno*::*irr*E fusion fragment was amplified by the overlap extension PCR method. The final PCR product digested with *Sac*I and *Sac*II was ligated into the *Sac*I and *Sac*II sites of pBBR1MCS-1 (from Professor Sandra K. Armstrong, U.S.A.). The resulting plasmid pBBR-IrrE was used for IrrE expression under control of the *eno* promoter of *Z. mobilis*. The plasmid pBBR-IrrE was transformed into the wild-type strain to generate the IrrE-expressing *Z. mobilis* strain. The *Z. mobilis* strain carring only the pBBR1MCS-1 was used as the control strain.

Western Blot Analysis

Purified proteins were run on 10% SDS–PAGE gel and transferred electrophoretically onto a PVDF membrane and probed with anti-his antibodies raised in rabbits, followed by chemiluminescent detection (Tiangen Biotech Co., Ltd., China).

Abiotic Stress Resistance Assays

Z. mobilis strains were grown in RM medium at 30°C to an OD_{600nm} of 0.8. Cells were extracted from 1-ml cultures by centrifugation to remove the growth medium before transferring to fresh RM medium in the presence of 3.0 M NaCl, 18% mannitol, or 20% ethanol. Cell suspensions were incubated at 30°C for 4 h. For heat stress, the cell suspensions were incubated at 43°C for 2 h; for acid shock, the cell suspensions were incubated in the medium of where the pH was adjusted to 2.0 at 30°C for 30 min. After incubation, serial dilutions of 10 times were made. Each dilution (10 μl) was spotted onto RM solid plates. All experiments were performed in triplicate.

Morphological Observation

Z. mobilis strains were incubated at a pH of 3.8 conditions or in the presence of 12% ethanol. The cells were centrifuged and resuspended in 50 mM potassium phosphate buffer (pH 6.5) and stained with fluorescence staining solutions of Hoechst 33342 and PI (Meck, U.S.A.) for 15 mins, as recommended by the manufacturer. The morphology of the stained cells was observed under a Nikon E600 microscope with fluorescence capability (Nikon, Tokyo, Japan).

Ethanol Fermentation Under Stress Conditions

Z. mobilis and the IrrE-expressing *Z. mobilis* were grown on RM medium containing 5% glucose at 30°C to the mid-log phase. Ten ml of the culture was tranferred to 400 ml of the medium at pH 3.8 or the medium containing 12% of ethanol in a 500-ml flask. Cells were grown at 30°C for 2–3 days under the anaerobic condition. The growth of the strain was monitored by measuring the optical density at 600 nm with a HITACHI 3010 spectrophotometer. The ethanol was analyzed by gas chromatography (Shimadzu, Model 14B; Japan; equipped with a Parapak Q column). Total reducing sugars were measured using HPLC (Agilent 1100).

Real-Time PCR (qPCR)

Total RNA was isolated by using a RNA extraction kit (Introgen). The RNA samples were reverse-transcribed by using the Protoscript First Strand cDNA Synthesis Kit (New England Bio-Labs) as described in the manufacturer's protocol. The expressions of representative identified genes (*abhB* and *pdc*) from different treatments were quantified by quantitative real-time PCR (qPCR) using a BIO-RAD Real-Time PCR System. Optimized primers were designed using primer software (length from 19–24 nucleotides and predicted annealing temperatures ranging from 59 to 61°C) to amplify about 100–300 bp at the 3' end of the target genes. First-strand cDNA was synthesized using a cDNA synthesis kit (New England Bio-Labs). 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). A dilution series of genomic DNA was used as the PCR template to construct a standard curve to quantify the expression levels of the tested genes. The *rrsA* gene, encoding the 16S RNA, served as an endogenous control to normalize for differences in total RNA quantity [17].

Enzyme Assays and Protein Electrophoresis

PDC activity was assayed by monitoring the pyruvic-acid-dependent oxidation of NADH with ADH as a coupling enzyme at pH 6.5, as previously described [6]. The reaction was carried out at 25°C in 50 mM sodium citrate buffer (pH 6.5) containing 0.15 mM NADH, 5 mM $MgCl_2$, 0.1 mM TPP, 5 mM pyruvate, and 10 μl (10 U) of ADH. The reaction was started by the addition of 10 μl of cell-free extract. The rate of NADH oxidation was measured at 340 nm.

ADHB activity was determined by measuring the alcohol-dependent reduction of NAD^+ at pH 6.5. Cells were permeabilized for enzyme assays as previously described [19, 23], and the cell lysate (10–30 μl) was added to a final volume of 1 ml containing 333 mM ethanol and 8.3 mM NAD^+ in 50 mM sodium phosphate buffer, pH 6.5. The production of NADH was assayed from the change in absorbance at 340 nm. One unit of PDC/ADH activity was defined as the generation of 1 μmol NAD^+/NADH per minute under the conditions specified. Enzyme activities were reported as international units per milligram of total cell protein. Protein was measured by the Lowry method with bovine serum albumin as a standard.

Protein molecular masses were estimated by SDS–PAGE using 12% (v/v) polyacrylamide gels that were subsequently stained by heating in the presence of Coomassie Blue R-250.

Results

Effects of Abiotic Stresses on the Growth of IrrE-Expressing *Z. mobilis*

The expression of the *irr*E gene from *D. radiodurans* in *Z. mobilis* or *E. coli* under the control of the *eno* promoter from *Z. mobilis* was confirmed using Western blot analysis (Fig. 1A). To study the effect of IrrE in *Z. mobilis*, we compared the stress response of the IrrE-expressing *Z. mobilis* strain with a control strain carrying only pBBR1MCS-1. According to RM plate assays, *irr*E protected *Z. mobilis* cells against ethanol, acid, osmotic (salt and mannitol), and thermal shocks (Fig. 1B). To further investigate how IrrE expression provides increased ethanol- and acid-stress tolerance, the growth of the IrrE-expressing strain was determined under ethanol and acid stresses. The IrrE-expressing strain reached a maximum OD_{600} of 1.57 after fermentation for 60 h in the presence of 12% ethanol, whereas the control strain displayed significantly impaired growth (Fig. 1C). A similar result was observed at pH 3.8 (Fig. 1D). These data indicate that IrrE protects *Z. mobilis* cells against various abiotic stresses.

Morphology of the IrrE-Expressing Strain Under Ethanol and Acid Stresses

The effects of *irr*E expression on the morphology of *Z. mobilis* under stress conditions were observed (Fig. 2). IrrE expression enhanced the survival of *Z. mobilis* cells under ethanol and acid stresses. The survival rate of IrrE-expressing *Z. mobilis* was twice that of the control strain (Fig. 2).

Ethanol Fermentation by the IrrE-Expressing Strain

During ethanol fermentation, *Z. mobilis* may encounter ethanol and acid stresses that adversely affect the ability of cells to perform efficient and consistent conversion of sugars to ethanol. Therefore, we examined the influence of *irr*E expression on the fermentation ability of *Z. mobilis* under stress conditions (Fig. 3). After fermentation for 96 h in the presence of 12% ethanol, net ethanol production by IrrE-expressing *Z. mobilis* was 14.59 g/l versus only 8.75 g/l for the control strain (Fig. 3A). When the initial pH of the fermentation medium was 3.8, the final ethanol concentration in cultures of the IrrE-expressing and control strains was 18.8 and 14.2 g/l, respectively (Fig. 3B).

In addition, we compared the PDC and ADH activities of IrrE-expressing *Z. mobilis* under stress conditions with that of the control strain (Fig. 4). The activities of both enzymes were significantly higher than that in the control

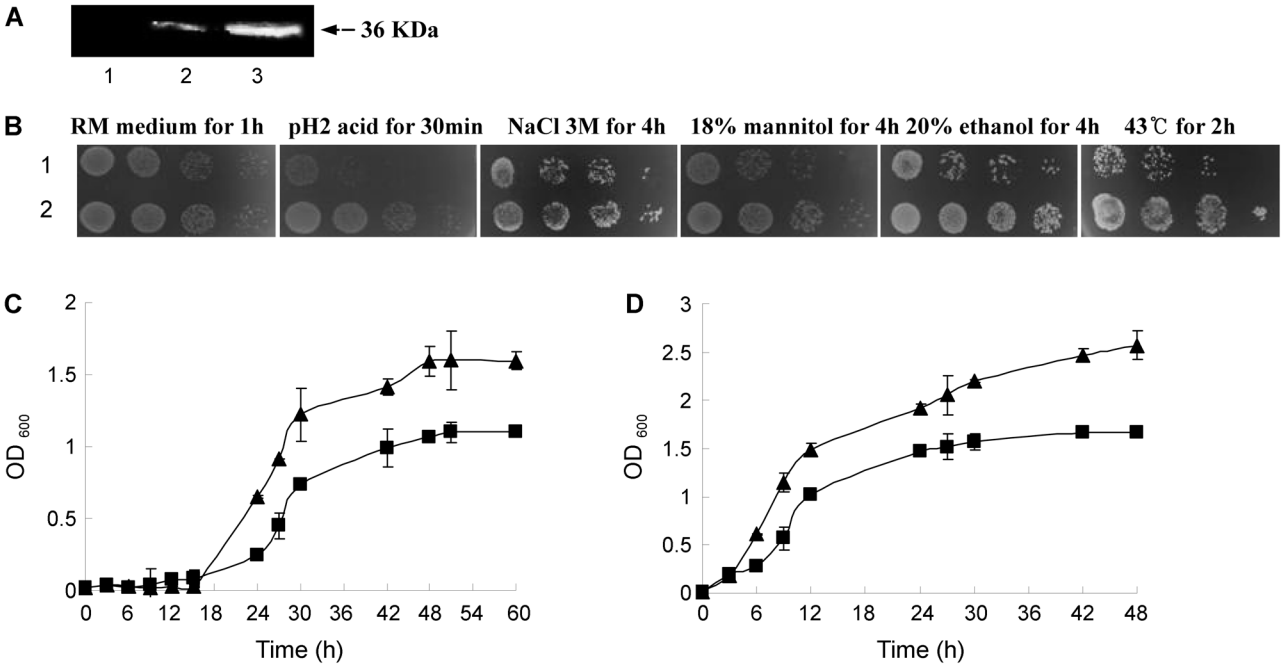

Fig. 1. Effects of abiotic stresses on growth of *Z. mobilis* strains.
A. Western blot analyses of IrrE in *Z. mobilis* (lane 1), *E. coli* BL21 harboring irrE-pet28a plasmid (lane 2), and the IrrE-expressing *Z. mobilis* strain (lane 3). Molecular mass is indicated on the right. **B**. Growth of the control strain carrying only the empty vector (1) and the IrrE-expressing strain (2) in RM medium after exposure to various abiotic stresses. **C**, **D**. Cells were grown in RM medium with 12% ethanol and at pH 3.8, respectively. Symbols: *Z. mobilis* strains (-■-); the IrrE-expressing *Z. mobilis* (-▲-).

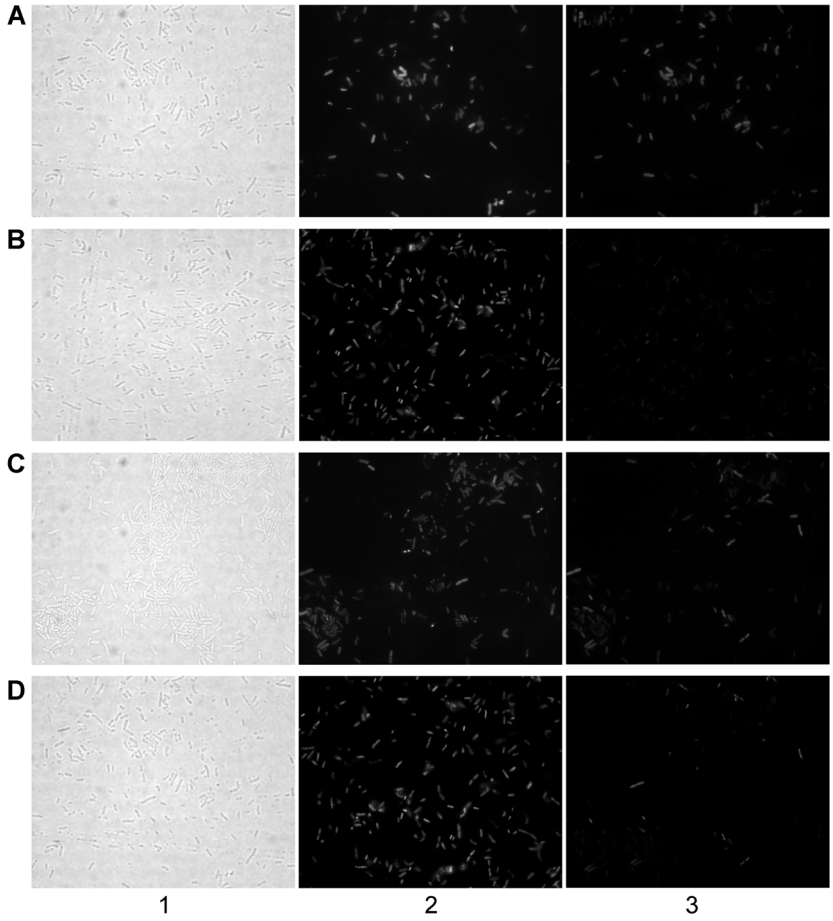

Fig. 2. Morphology of the *Z. mobilis* cells under ethanol- and acid-stress conditions.
The control strain and the IrrE-expressing *Z. mobilis* strain grown in RM medium containing 5% glucose complemented with 12% ethanol (**A** and **B**) or at pH 3.8 (**C** and **D**). The cells are shown unstained (1) and stained with Hoechst (2) and PI (3).

strain under both ethanol (Fig. 4A and 4B) and acid (Fig. 4C and 4D) stresses. We then measured the transcription levels of *adhB* and *pdc* using quantitative RT–PCR (Fig. 5). Under all ethanol and acid stress conditions, the levels of *adhB* and *pdc* mRNA in IrrE-expressing *Z. mobilis* cells were higher than those in the control strain. When IrrE-expressing *Z. mobilis* cells were exposed to ethanol or acid shock for 60 min, the relative expression of *adhB* and *pdc* with *irr*E expression in *Z. mobilis* increased by 7.8- and 4.2-fold, respectively (Fig. 5). These results conclusively demonstrate that *D. radiodurans irr*E protects *Z. mobilis* cells against abiotic stresses during ethanol production.

DISCUSSION

Microbial biocatalysts developed for high ethanol yield and productivity must be able to tolerate adverse environmental conditions, including ethanol stress, low pH, and high temperature. Much effort has been made to increase the tolerance of *Z. mobilis*, *E. coli*, *S. cerevisiae*, and other microbes to ethanol, acid, osmosis, and heat through genetic modifications. Baumler *et al*. [5] reported that the expression of the 24-amino-acid proton-buffering peptide (Pbp) of *E. coli* in *Z. mobilis* increases acid tolerance. Overexpression of trehalase in *E. coli* increases trehalose levels, enhances growth, and augments osmotolerance to both salts and sugars [22, 27]. The overexpression of *S. cerevisiae* tryptophan biosynthesis genes or the *Arabidopsis thaliana FAD2* gene encoding fatty acid desaturase improves tolerance to ethanol [12, 15, 26]. Since tolerance is a multigenic trait [31], genetic modifications of single genes rarely result in a global phenotype optimum [1, 2]. Alper and Stephanopoules [3] used gTME to generate transcription factor mutants with improved tolerance to ethanol and glucose. *D. radiodurans irr*E is a transcriptional regulator in *D. radiodurans* that confers resistance to ionizing radiation, ultraviolet light, and other agents that damage DNA [7, 13]. Expression of the *irr*E gene in *E. coli* promotes DNA repair and confers oxidative damage protection [9] and tolerance to salt and other stresses [25]. Furthermore, a *D. radiodurans* IrrE homolog is present in *D. geothermalis*

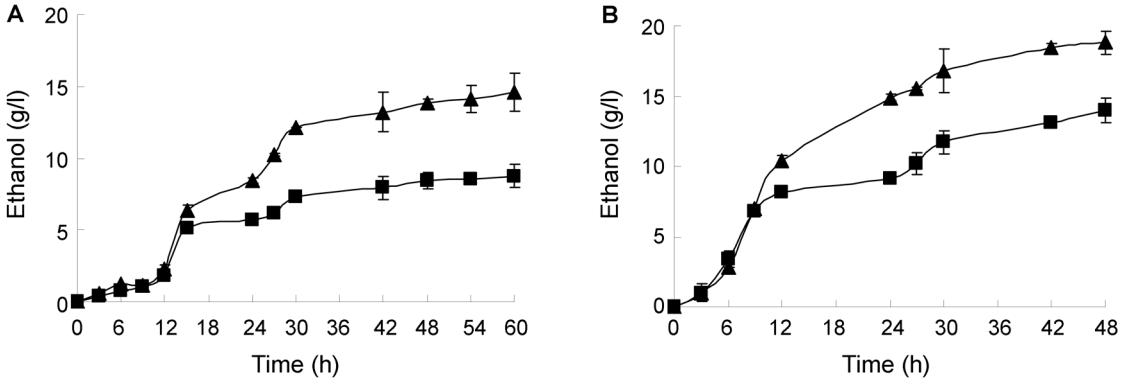

Fig. 3. Protective effect of IrrE protein on cell growth and ethanol production by *Z. mobilis* under ethanol or acid stress .
Cells were grown in RM medium containing 5% glucose and exposed to ethanol or acid stress. Molecular mass is indicated on the left. **A**. Stress of 12% ethanol at an initial concentration. **B**. Stress of pH 3.8 at an initial concentration. Symbols: *Z. mobilis* strain (-■-); the IrrE-expressing *Z. mobilis* (-▲-).

and *D. desert*, suggesting that IrrE is a well-conserved *Deinococcus*-specific regulator [33]. In the present study, we found that *D. radiodurans irr*E protects *Z. mobilis* cells against high levels of salt, acid, ethanol, and heat (Fig. 1A), and improves ethanol production (Fig. 3 and 4). This suggests that the *irr*E gene as a specific regulator may be useful for improving abiotic stress tolerance in ethanologenic bacterial strains.

RpoS is a fundamental regulator of the general response to various stress conditions in *E. coli* [11, 32, 34, 35]. According to a comparative proteomic analysis, the induction of RpoS in IrrE-expressing strains results in a 3-fold

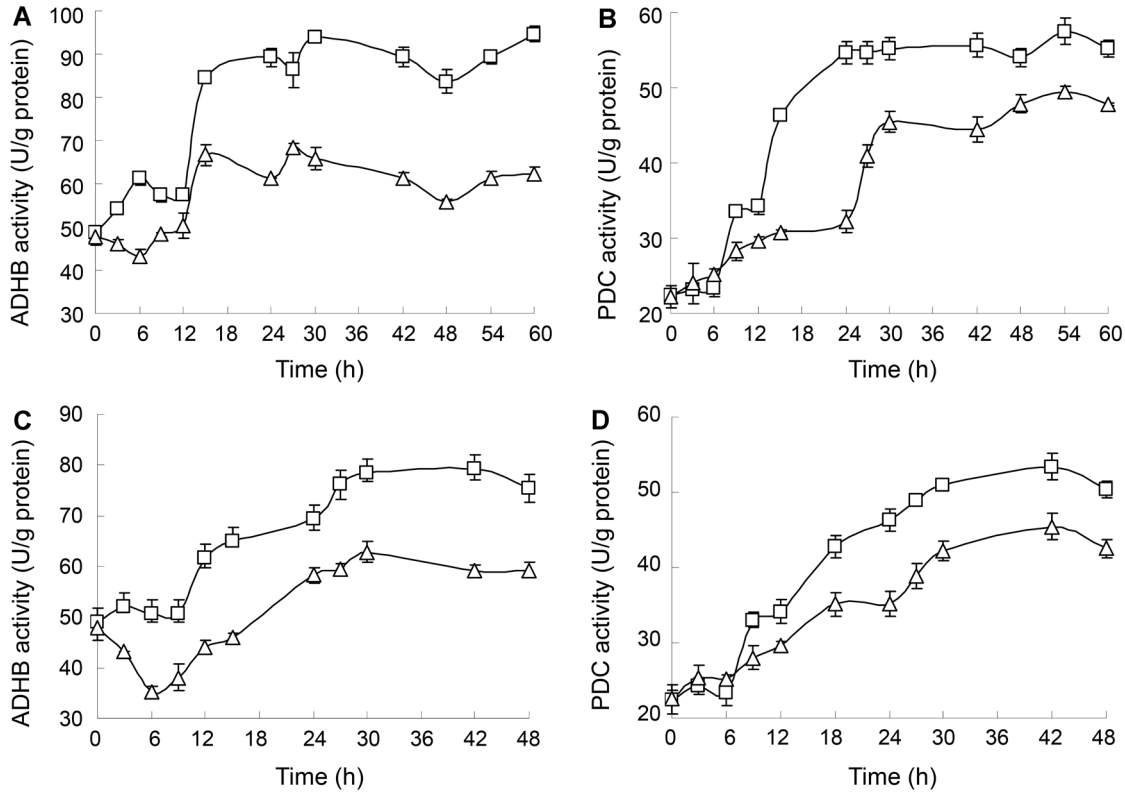

Fig. 4. Specific alcohol dehydrogenase (ADHB) and pyruvate decarboxylase (PDC) activities of crude extracts of *Z. mobilis* strains and the IrrE-expressing *Z. mobilis* under ethanol or acid stress condition.
A. ADHB activities under ethanol (12%) stress; **B**. PDC activities under ethanol (12%) stress; **C**. ADHB activities under acid (pH 3.8) stress; **D**. PDC activities under acid (pH 3.8) stress. Symbols: the IrrE-expressing *Z. mobilis* (-□-); *Z. mobilis* strain (-△-).

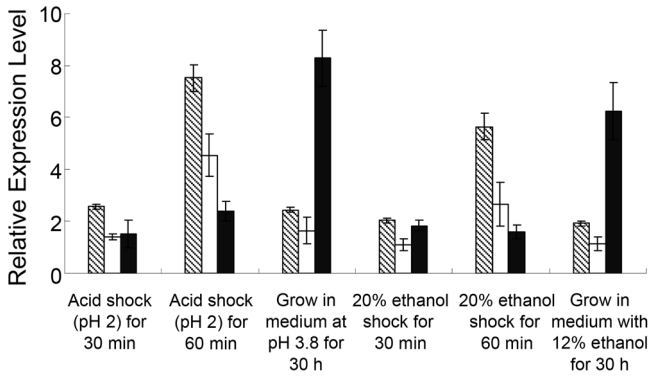

Fig. 5. Quantitative RT–PCR analysis of the relative expression levels of *adh*B(▨), *pdc*(□), and *rpo*E(■) of the IrrE-expressing *Z. mobilis* under different stress conditions (compared with the control strain).

greater expression and the accumulation of a set of proteins usually induced by environmental stress [25]. In *Z. mobilis*, the genome sequence contains no identifiable *rpo*S gene. *rpo*E (sigma-E) is postulated to have RpoS-like functions and to play a key role in resisting high ethanol concentrations in *Z. mobilis* [28]. In the present study, *irr*E enhanced the expression of *rpo*E under ethanol and acid stresses (Fig. 5). In particular, the level of *rpo*E mRNA in IrrE-expressing *Z. mobilis* was 8.2- and 6.0-folds higher than that in the control strain, when incubated in RM medium at pH 3.8 or with 12% ethanol for 30 h, respectively. Although we cannot eliminate the possibility that IrrE has an undiscovered role in regulating different sets of stress-responsive proteins, the significant induction of *rpo*E in IrrE-expressing *Z. mobilis* is a possible pathway that regulates RpoE-dependent genes in response to abiotic stresses. Further studies are required to identify its direct target genes or its interacting partners to elucidate the molecular mechanisms of IrrE in conferring improved abiotic stress tolerance in *Z. mobilis*. Our results provide new insight into the molecular basis of how IrrE contributes to ethanol and acid tolerance.

Acknowledgments

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