Thermotolerant *Zymomonas mobilis*: Comparison of Ethanol Fermentation Capability with that of an Efficient Type Strain

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Abstract: *Zymomonas mobilis* is an alternative microorganism to *Saccharomyces cerevisiae* for ethanol production. To find a thermotolerant *Z. mobilis* strain, the growth and ethanol production of four isolates in Thailand were compared with those of the efficient strain ZM4 (NRRL B-14023) at different temperatures. One of the selected strains, TISTR 405, was found to grow and produce ethanol even at 39˚C to an extent similar to that at 30˚C, and the growth and ethanol productivity at 39˚C were better than those of ZM4 at 30˚C, suggesting that TISTR 405 is suitable for ethanol fermentation at high temperatures. Analysis of genes directly related to ethanol formation or degradation, *adhA*, *adhB* and *pdc,* encoding alcohol dehydrogenase (Adh) A, AdhB and pyruvate decarboxylase, respectively, revealed that these genes were highly conserved in both strains. Comparison of their gene expression and activity of the products in both TISTR 405 and ZM4 at different temperatures or growth phases indicated that there was not a great difference at the transcriptional level, but the total activity of AdhA and AdhB in TISTR 405 was higher than that in ZM4. Both strains showed a significant increase in AdhB activity in the stationary phase.

Keywords: Thermotolerant, *Zymomonas mobilis*, ethanol production.

INTRODUCTION

Unlike mesophilic microorganisms, the use of thermotolerant microorganisms enables fermentation to be performed at high temperatures, which may have several advantages: a) reduction in cost for cooling fermentation units, which maintain optimal conditions for the fermentation reaction, b) reduction in contamination of mesophilic microorganisms, c) increase in the speed of catalytic reactions related to fermentation, for example, saccharification of starch for ethanol fermentation, and d) thereby, reduction in total costs including costs of facilities or labor. The Gram-negative and facultative anaerobe *Zymomonas mobilis* is known to be a potent ethanol producer [1], but no thermotolerant strain of the organism that is thought to be beneficial for ethanol fermentation at high temperatures has been reported.

In *Z. mobilis*, the ethanol production route from glucose, which consists of the Entner-Doudoroff (ED), glyceraldehyde-3-phosphate-to-pyruvate (GP) and pyruvate-to-ethanol (PE) pathways, provides most of the ATP required for cell activities, about one mole of ATP per mole of glucose [2,3], a much lower level than that in *Saccharomyces cerevisiae*, the traditional ethanol producer. The organism thus appears to maintain a high level of glucose flux through the pathways to compensate its low ATP yield [4], for which large amounts of enzymes related to the pathways are expressed, constituting 30-50% of total soluble proteins of cells [3]. As a consequence, it performs less biomass formation and efficient production of ethanol compared to *S. cerevisiae* [1]. *Z. mobilis*, which has a relatively compact genome with a small number of genes, about 2,000, possesses incomplete Embden-Meyerhof-Parnas pathway and incomplete TCA cycle due to a lack of genes for 6-phosphofructokinase, 2-oxoglutarate dehydrogenase complex and malate dehydrogenase [3-5] but possesses strong activities of ED-GP pathways [6]. Therefore, it has been thought that *Z. mobilis* is one of the promising microorganisms for fermentative production of various materials from pyruvate or other intermediates.

Many experiments on ethanol production by using *Z. mobilis* or by introducing its genes into other bacteria have been carried out [7-10], but ethanol production at high temperatures has not been attempted yet. We previously found that magnesium and sugar alcohols protected *Z. mobilis* from heat stress and promoted cell growth and ethanol production at high temperatures [11]. In this study, we selected and characterized relatively thermotolerant *Z. mobilis* strains from isolates in Thailand. The genes directly related to ethanol fermentation and their expressions in one of the strains at 30-37˚C were compared with those of an efficient strain for ethanol production. We also discussed the regulation of expression of the genes.

MATERIALS AND METHODS

Materials

Restriction enzymes and T4 DNA ligase were purchased from Takara Shuzo (Kyoto, Japan) and New England Biolabs (Beverly, MA). A DNA sequencing kit (ABI PRISMP® BigDye® Terminator v3.1 Cycle Sequencing Kit) was purchased from Applied Biosystems Japan. Oligonucleotide

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primers were synthesized by Proligo Japan K. K. (Tokyo, Japan). Alcohol dehydrogenase (Adh) purified from *Gluconobacter suboxydans* IFO 12528 [12] was used for determination of the concentration of ethanol produced. All other chemicals were of analytical grade and obtained from commercial sources.

Bacterial Strains and Culture Conditions

Z. mobilis strains, TISTR 405, TISTR 548, TISTR 550 and TISTR 551, were obtained from the TISTR Culture Collection Bangkok MIRCEN, and ZM4 (NRRL B-14023) was provided by E. Yanase. These strains were cultivated in YPD medium consisting of 0.3% (w/v) yeast extract, 0.5% (w/v) peptone and 3% (w/v) glucose [13] at the temperature indicated with a shaking speed of 100 rpm [14]. Cells were harvested and suspended in 10 mM phosphate buffer (pH 7.0) and then passed twice through a French press at 16,000 psi, followed by a low-speed centrifugation for removing unbroken cells, then centrifuged at 86,000 g for 90 min. The supernatant was used as a soluble fraction. *E. coli* was grown in LB medium consisting of 1% (w/v) Bactotryptone, 0.5% (w/v) yeast extract and 0.5% (w/v) NaCl. Fifty μg of ampicillin per ml was added to the medium if necessary.

Cloning and Analysis of *adh* and *pdc* Genes

Conventional recombinant DNA techniques were applied [15]. The genomic DNA of *Z. mobilis* was prepared by the standard method [15]. The *adh* and *pdc* genes including about 500 bp upstream from the initiation codon and coding region from *Z. mobilis* were cloned after PCR amplification using primers (Table **1**) and the genomic DNA as a template. The specific primer sets for *adhA, adhB* and *pdc* were designated according to the nucleotide sequences of the genomic sequence of *Z. mobilis* ZM4. Each amplified DNA fragment was inserted into the multi-cloning site of pUC119 and then subjected to nucleotide sequencing [16]. PCR was performed on Takara PCR thermal cycler MP (Takara Biomedicals, Japan). Nucleotide sequencing was analyzed by using ABI PRISM™ 310 Genetic analyzer (The Perkin-Elmer Corp., USA). Primers used for the sequencing are M13 universal primers and also listed in Table **1**. The determined nucleotide sequences were deposited in DDBJ and the accession numbers are AB359061, AB359062 and AB359063 for *adhA, adhB* and *pdc*, respectively.

RT-PCR Analysis

Cells were grown at 30˚C or 37˚C in YPD medium until exponential (8 h) and stationary (20 h) phases, and then subjected to RNA isolation. For determining the effect of glucose, glycerol or ethanol on the expression of the three genes, cells were first grown for 14 h at 37˚C in YPD medium, washed twice with YP medium, transferred to YPD, YPG and YPE medium containing 3% (w/v) glucose, 3% (w/v) glycerol and 2% (v/v) ethanol, respectively, and further incubated for 2 h at 37˚C. Total RNA from the cells was isolated by the hot phenol method [17]. The concentration of RNA was estimated spectrophotometrically at 260 nm. RT-PCR analysis was performed using an mRNA Selective RT-PCR Kit (Takara Shuzo, Kyoto Japan) with 0.1 μg of total RNA as a template and primer sets. For detection of 16S rRNA, 0.001 μg of total RNA was used as a template. RT-PCR was performed on Takara PCR thermal cycler MP (Takara Biomedicals, Japan). After RT reaction had been performed at 40˚C for 15 min, PCR consisting of denaturing at 82˚C for 1 min, annealing at a fixed temperature, 5 degrees lower than T_m, which was calculated by the rule of thumb

Table 1. Primers Used in this Study

Name	Sequences	Usage
AdhA-F	5′-ACACTGCAGAAGCATAGCCGGACATCATACC-3′	Cloning of *adhA* gene
AdhA-R	5′-CTCGAATTCCCGTTTATGCTTCCGCCTTCAC-3′	Cloning of *adhA* gene
AdhB-F	5′-ACAGTCGACGAGGAAAGCCTGATCTGCCATT-3′	Cloning of *adhB* gene
AdhB-R	5′-CGCGAATTCGCCGGTGTCTTGATCTTGTCTA-3′	Cloning of *adhB* gene
Pdc-F	5′-AGAGCATGCGGCTTTGTCAGTGTTGCGGTAT-3′	Cloning of *pdc* gene
Pdc-R	5′-ACTGAATTCAAAAGCCCGCCCGGTAAAACCG-3′	Cloning of *pdc* gene
AdhART-F	5′-CATGAAAGCAGCCGTCA-3′	RT-PCR and sequencing of *adhA* gene
AdhART-R	5′-TACACCCGCGCAAGTGA-3′	RT-PCR and sequencing of *adhA* gene
AdhBRT-F	5′-GTCAACGAAATGGGCGA-3′	RT-PCR and sequencing of *adhB* gene
AdhBRT-R	5′-GTGACGGTCAACAATGG-3′	RT-PCR and sequencing of *adhB* gene
PdcRT-F	5′-GACTACAACCTCGTCCT-3′	RT-PCR and sequencing of *pdc* gene
PdcRT-R	5′-CAGGGCATGGGAGCAAT-3′	RT-PCR and sequencing of *pdc* gene
M4	5′-GTTTTCCCAGTCACGAC-3′	Sequencing of all 3 genes
RV	5′-CAGGAAACAGCTATGAC-3′	Sequencing of all 3 genes
AdhA-S	5′-TCGCCGTTGGTCTGCCTCCT-3′	Sequencing of *adhA* gene
AdhB-S	5′-TATCGAGACCCATAGCAACA-3′	Sequencing of *adhB* gene
Pdc-S	5′-AATTCATCGCCAACCGCGAC-3′	Sequencing of *pdc* gene

method [18], for 1 min and extension at 72˚C for 1 min was carried out using the two primers for each gene. The PCR products after 20, 25, 30 and 35 cycles for each gene were analyzed by 0.9% agarose gel electrophoresis, and the relative intensity of the products was densitometrically estimated by using a Bio-Rad molecular imager [19,20]. Under our conditions, the RNA-selective RT-PCR was able to specifically detect mRNA, because no band was observed when reverse transcriptase was omitted.

Comparison of Nucleotide Sequences

Published nucleotide sequences were obtained from the DDBJ database. Comparison and alignment of nucleotide sequences were conducted by using GENETYX (Software Development, Tokyo, Japan).

Analytical Procedures and Enzyme Assays

Alcohol concentration in the medium was measured by an enzymatic method (ferricyanide reductase activity) with purified *G. suboxydans* Adh [12]. Protein content was determined by the Dulley and Grieve method [21] using bovine serum albumin as a standard. Adh activity was determined by measuring the alcohol-dependent reduction of NAD^+ using ethanol or butanol as described previously [22]. One unit of activity is defined as µmole of NADH formed per minute per mg of proteins. For detection of Adh activity by staining, soluble fraction (100 µg of proteins) was separated on 7.5% polyacrylamide gel with Tris-glycine buffer (pH 8.5). NAD^+-dependent Adh activity was detected by soaking the gel in a solution containing 100 mM Tris-HCl (pH 8.0), 0.1 mM NAD^+, 0.1 M ethanol or butanol, 0.2 mM nitroblue tetrazolium (NBT) and 0.2 mM phenazine methosulfate. After incubation in the dark at 25˚C for 2-5 min, the reaction was stopped by transferring the gel into 7% acetic acid solution [23]. NADH-dependent Adh activity was detected as described previously [24] under UV light by using NADH as a cofactor. The reduction reaction was performed in 100 mM MES (pH 6.0) with 0.1 M acetaldehyde as a substrate.

RESULTS

Comparison of Growth and Ethanol Production

In order to obtain thermotolerant *Z. mobilis* strains, which are suitable for fermentation at high temperatures, growth at high temperatures of four isolates in Thailand, TISTR 405, TISTR 548, TISTR 550 and TISTR 551, was compared with that of a type strain, ZM4 (NRRL B-14023), which is known to be efficient for ethanol production. When grown in YPD plates at different temperatures from 30˚C to 40˚C, TISTR 405, TISTR 548 and TISTR 550 grew well at temperatures up to at 39˚C, but the other strains did not. No strains, however, were able to grow well at more than 39˚C. Their abilities for growth and ethanol production in YPD medium were then compared (Fig. **1**). TISTR 405 showed the highest optical density at 30˚C and 39˚C. TISTR 405 and

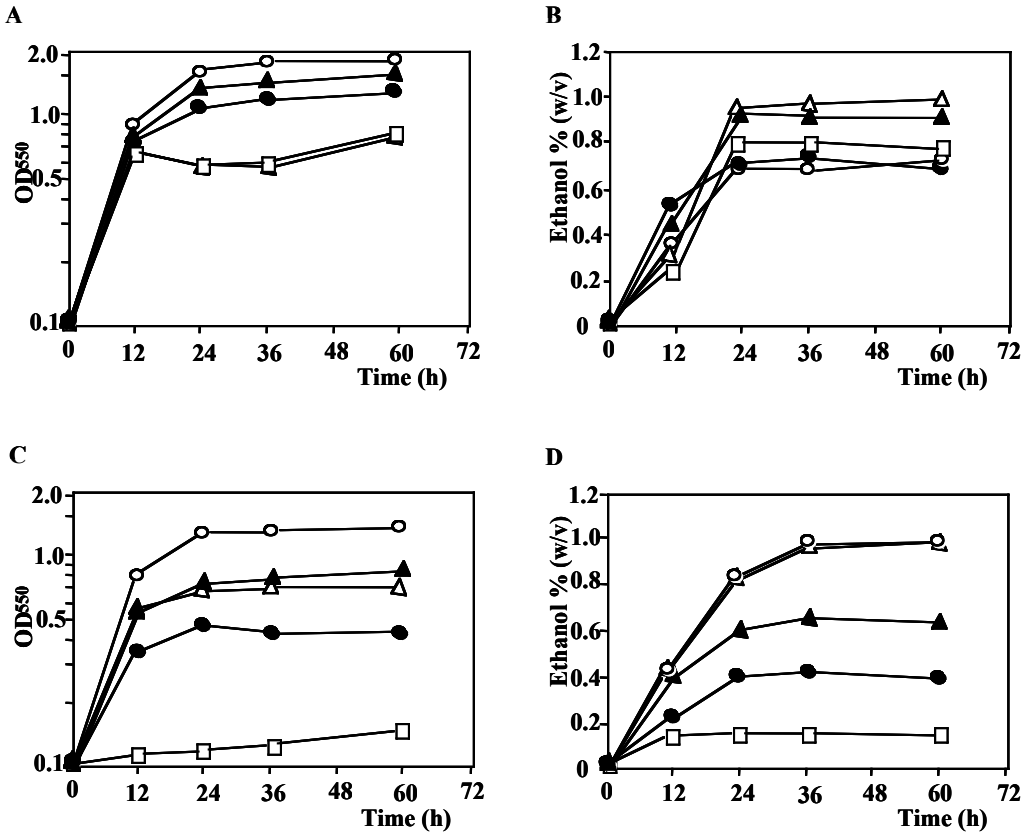

Fig. (1). Comparison of growth and ethanol production of *Z. mobilis* strains from Thailand collection with the efficient type strain at 30˚C and 39˚C. *Z. mobilis* TISTR 405 (○), TISTR 550 (Δ), TISTR 551 (□), TISTR 548 (▲) and the type strain ZM4 (●) were grown in YPD medium at 30˚C (**A**) or 39˚C (**C**) under shaking condition at 100 rpm and the ethanol concentration in the medium at 30˚C (**B**) or 39˚C (**D**) were measured. The experiments were repeated three times and the patterns were confirmed to be reproducible. Data from one such representative experiment are shown.

TISTR 550 showed the largest production of ethanol at 39˚C, the levels being more than and nearly equivalent to, respectively, the level at 30˚C. The growth and ethanol production level of both strains at 39˚C were much higher than those of the efficient strain. Based on these data, we chose TISTR 405 as a thermotolerant strain for further analysis.

Cloning and Characterization of *adhA*, *adhB* and *pdc* Genes Directly Related to Ethanol Synthesis or Degradation

To examine the molecular diversity of *adhA*, *adhB* and *pdc* genes between the thermotolerant TISTR 405 and the type ZM4 strains, the *adhA*, *adhB* and *pdc* genes, each including an approximately 500-bp upstream region, were individually cloned and sequenced. The identities in nucleotide sequence in the coding region and 5'-noncoding region (within 200 bases upstream from the initiation codon) of the three genes are compared in Table **2**. All sequences of the coding and 5'-noncoding regions of the three genes shared identities of more than 96% between the two strains. None of the nucleotide substitutions found in the coding regions of the three genes caused amino acid substitutions; that is, there was complete conservation in primary sequences of AdhA, AdhB and Pdc. Therefore, the three genes directly related to ethanol synthesis or degradation are highly conserved between the two strains. Additionally, since both the coding and 5'-noncoding regions are conserved, these genes are unlikely to be responsible for thermotolerance of TISTR 405.

Table 2. Identity of Nucleotide Sequences of Genes Related to Ethanol Synthesis and Degradation Between *Z. mobilis* Strains and Between *E. coli* Strains

	adhA	*adhB*	*pdc*	*adhP*[a]	*yiaY*[a]
Zymomonas mobilis ZM4	100	100	100		
	(100)	(100)	(100)		
Zymomonas mobilis TISTR 405	99	99	96		
	(100)	(100)	(100)		
Escherichia coli K-12				100	100
MG1655				(100)	(100)
Escherichia coli K-12 W3110				100	100
				(100)	(100)
Escherichia coli 101-1				100	97
				(100)	(99)
Escherichia coli APEC 01				96	98
				(99)	(85)
Escherichia coli O157: H7				98	99
EDL933				(99)	(96)

[a]*adhP* encodes for Adh corresponding to the *Z. mobilis adhA* (74% identity in primary sequence between AdhA and AdhP) and *yiaY* encodes for Adh corresponding to the *Z. mobilis adhB* (75% identity in primary sequence between AdhB and YiaY). Values and values in parentheses represent percentages of identity of nucleotide sequences in the coding region and the 5'-noncoding region (within 200 bases upstream from the initiation codon), respectively. Comparison and alignment of the sequences were performed with the GENETYX software.

When the conservation of genes related to the ethanol formation or degradation among *E. coli* strains, K-12 MG1566, K-12 W3110, 101-1 and APEC 01, O157: H7 EDL1933 was estimated by comparison of their genomic sequences in databases, *adhP* encoding Adh corresponding to *Z. mobilis adhA* showed more than 99% in the 5'-noncoding regions (in 200 bases upstream from the initiation codon) and more than 97% in the coding region (Table **2**). *yiaY* encoding Adh corresponding to *Z. mobilis adhB* showed more than 85% in the 5'-noncoding region and more than 96% in the coding region. Therefore, the extent of conservation at the nucleotide sequence level in *adhA* and *adhB* between the mesophilic and thermotolerant *Z. mobilis* strains seems to be the level in *adhP* and *yiaY* between general *E. coli* strains.

Expression of *adhA*, *adhB* and *pdc* Genes in Exponential and Stationary Phases at 30˚C and 37˚C

To compare the expression of *adhA*, *adhB* and *pdc* genes in the thermotolerant strain TISTR 405 and that in the type ZM4 strain, RT-PCR was performed with total RNAs from cells that had been grown in YPD medium at 30˚C or 37˚C for 8 h (exponential phase) and 20 h (stationary phase) with glucose still remaining and absent, respectively, in the medium (Fig. **2**). The effect of temperature was tested at 37˚C instead of 39˚C because of the heat sensitivity of the type strain. Band intensities from the *adhA, adhB* and *pdc* genes in both strains were similar at both exponential and stationary phases at 30˚C or 37˚C, though there were minor exceptions that the intensity from *pdc* was reduced 2- to 4-fold and that from *adhB* also slightly reduced at the stationary phase compared with those at the exponential phase. In the type strain, more than 4-fold reduced intensity was found in *adhA* and *pdc* at the stationary phase at both temperatures. These results suggested that the *adhA,* and *adhB* genes are expressed almost similarly at both exponential and stationary phases at 30˚C or 37˚C while *pdc* gene is slightly reduced in expression at stationary phase and that their expressions in both strains are similar, which is consistent with the high identity of the nucleotide sequences of the genes including the 5'-noncoding region.

Next, the effect of glucose, glycerol or ethanol on the expression of the three genes was examined at 37˚C with TISTR 405 (Fig. **3**). Total RNAs from the cells grown in YPD medium, then exposed to glucose, glycerol or ethanol were subjected to RT-PCR. Comparison of band intensities revealed that the expression of all three genes in YPG and YPE media were reduced 2- to 4-fold compared with that in YPD medium. Considering the fact that *Z. mobilis* is unable to utilize glycerol or ethanol as a carbon source, these results suggested that none of these genes are induced by ethanol. The relatively high expression levels of these genes in YPD medium might be due to the vegetative growth but not due to the induction by glucose because the effect of glucose was relatively small.

Enzyme Activities of Gene Products of *adhA*, *adhB* and *pdc* in Exponential and Stationary Phases at 30˚C and 37˚C

To further compare the expression of *adhA* and *adhB* in the thermotolerant strain and that in the type strain, activities of butanol dehydrogenase and ethanol dehydrogenase, which reflect the activity for AdhA and the combined activities for AdhA and AdhB, respectively, were measured with soluble

fraction from cells grown under the same conditions as those used in the experiments for which results are shown in Fig. (**2**) (Table **3**). At both temperatures, activity levels of butanol and ethanol dehydrogenases from cells of both strains in the stationary phase were found to be higher than those in the exponential phase. Notably, both activity levels in the thermotolerant strain at 30˚C in the stationary phase and at 37˚C in both phases were 1.8- to 3.8-fold higher than those of the corresponding samples in the type strain. These significant differences might be related to the thermotolerance of TISTR 405.

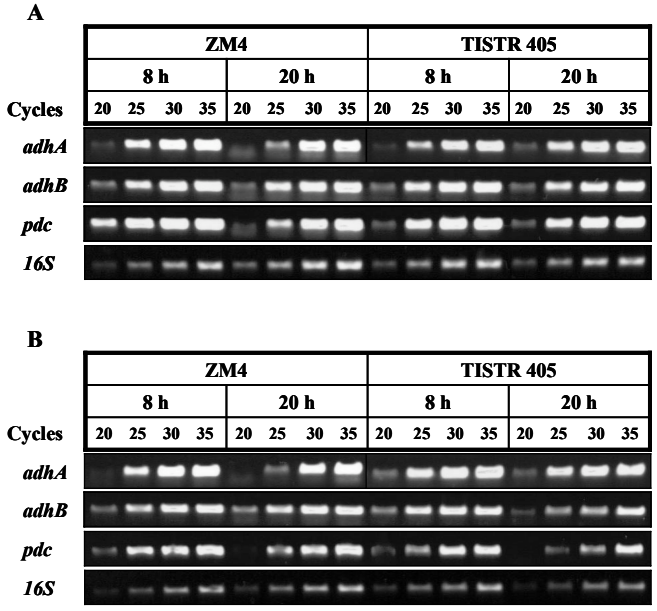

Fig. (2). Expression of *adhA*, *adhB* and *pdc* of TISTR 405 and ZM4 at 30˚C and 37˚C in YPD medium. Cells were grown in YPD medium at 30˚C (**A**) or 37˚C (**B**), and total RNAs from the cells at mid-exponential (8 h) and early stationary (20 h) phases were subjected to RT-PCR. After RT reaction, PCR was performed 20, 25, 30 and 35 cycles and the products were analyzed by agarose gel electrophoresis. Total RNA used was 0.1 μg for detection of the expression of *adhA, adhB* and *pdc*, or 0.001 μg for 16S rRNA.

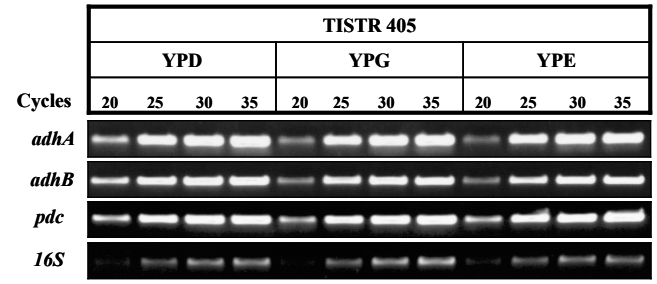

Fig. (3). Expression of *adhA*, *adhB* and *pdc* of TISTR 405 in the presence of glucose, glycerol or ethanol at 37˚C in YPD medium. Total RNAs and RT-PCR were performed described in Materials and Methods and Fig. (**2**).

To detect AdhA and AdhB enzymes in samples from both strains, active staining after polyacrylamide gel electrophoresis was carried out with the same preparation of soluble fraction used in Table **3** (Fig. **4**). When NBT was used as an electron acceptor, a single band was specifically detected in each sample in the presence of butanol, and one band and two bands were observed in exponential and stationary phases, respectively, in the presence of ethanol. Since the position of the upper band of the ethanol-dependent bands corresponded to that of the butanol-dependent band, the upper and lower bands seem to be derived from AdhA and AdhB, respectively. In the procedure to detect the enzyme-substrate-NADH complex, two bands (strong and weak) in exponential phase and two strong bands in stationary phase were detected in the presence of acetaldehyde and NADH. The intensity of bands in stationary phase was stronger than those in the exponential phase, in agreement with enzyme activities shown in Table **3**. Taken together, the results suggested that AdhA are present in both phases at a level corresponding to that of the butanol-dependent activity. On the other hand, the AdhB level appears to be low in the exponential phase compared with that in the stationary phase.

Table 3. Comparison of Adh Activities in the Thermotolerant Strain with those in the Efficient Type Strain

Strain	Growth Temperature	Growth Phase	Adh Activity (U/mg) [a]	
			ButOH	EtOH
ZM4	30ºC	Exponential	0.50±0.1	1.53±0.1
	30ºC	Stationary	0.97±0.3	2.65±0.02
	37ºC	Exponential	0.38±0.04	1.27±0.3
	37ºC	Stationary	1.11±0.1	2.88±0.05
TISTR 405	30ºC	Exponential	0.54±0.1	1.73±0.3
	30ºC	Stationary	1.94±0.2	5.17±0.9
	37ºC	Exponential	1.46±0.1	3.99±0.7
	37ºC	Stationary	2.03±0.1	5.30±0.6

[a]Adh activities were measured using butanol (ButOH) or ethanol (EtOH) as a substrate as described in Materials and Methods. Reported values are the mean (±SD) of three independent experiments.

DISCUSSION

In this study, we attempted to find thermotolerant *Z. mobilis* strains from the Thailand collection with ability for efficient ethanol production. As expected, relatively thermotolerant strains were found to be present in the collection. Out of strains tested, two strains were thermotolerant and able to grow and produce ethanol at 39˚C at a level equivalent to that produced at 30˚C, and they showed higher ethanol productivity at 39˚C than that of the efficient strain ZM4 at 30˚C (close to the optimum temperature for ZM4). Therefore, both strains can be used for ethanol fermentation at high temperatures.

Nucleotide sequencing revealed that the three genes, *adhA, adhB* and *pdc,* related to ethanol synthesis or degradation are highly conserved in not only the coding region but also in the 5'-noncoding region between one of the thermotolerant strains and the efficient type strain. The conservativeness does not seem to be specific because the degree of identity is similar to those in the corresponding genes of *E. coli* strains. The conservation at the 5'-noncoding region of

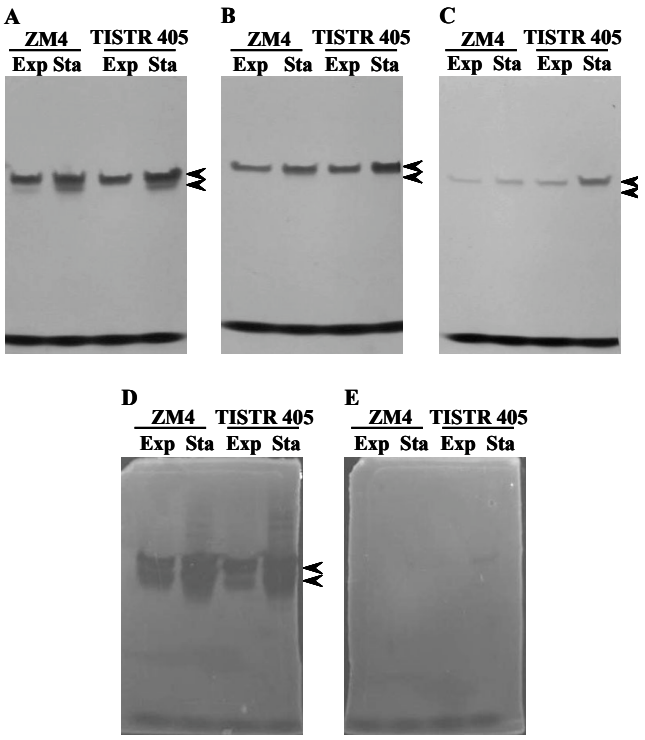

Fig. (4). Detection of AdhA and AdhB activities of TISTR 405 and ZM4. Soluble fractions (100 μg) from cells grown in YPD medium at 37˚C at mid-exponential (8 h; Exp) and early stationary (24 h; Sta) phases were subjected to the active staining using NBT (**A**, **B** and **C**) or the detection of NADH-dependent activity under UV (**D** and **E**) as described in Materials and Methods. The detection was performed in the presence of ethanol and NAD^+ (**A**), butanol and NAD^+ (**B**), NAD^+ (**C**), acetaldehyde and NADH (**D**), and NADH (**E**). Upper and lower arrowheads represent AdhA and AdhB, respectively.

each gene is consistent with the results of RT-PCR, which revealed that the two strains expressed these genes to nearly the same extent at different temperatures or in different growth phases. On the other hand, estimation of AdhA and AdhB activities revealed that both enzyme activities significantly increased in the stationary phase and that at least AdhA activity level of the thermotolerant stain in the exponential phase at 37˚C was about 3-fold higher than that at 30˚C. The latter data agree with the finding that the strain showed higher ethanol productivity at 39˚C than that at 30˚C, indicating suitable characteristics for ethanol production at high temperatures. Therefore, these findings suggest that the expression of AdhA and AdhB is controlled at the translational or post-translational level, not at the transcriptional level. Alternatively, these proteins may be stable and thus accumulate inside cells.

Z. mobilis AdhB has high specificity for ethanol over acetaldehyde [22] as a substrate [22,25,26], thus, it is thought to be involved in ethanol degradation [22] and may be important for lowering the ethanol level inside cells. Active staining experiments indicated that the AdhB activity significantly increased in the stationary phase, where ethanol was accumulated. The increase in AdhB activity is reasonable in respect to its physiological function. However, neither an increase in ethanol nor a decrease in glucose seems to induce the *adhB* expression (Fig. **3**). The regulation of *adhB*, which has not been fully defined, seems to be unique and different from *S. cerevisiae ADH2* [27] or *Kluyveromyces marxianus ADH4* [28], which are repressed by glucose and induced by ethanol, respectively, and proposed to be involved in ethanol degradation. *Z. mobilis* AdhA appears to mainly perform ethanol synthesis [22,25]. The AdhA is like *S. cerevisiae* Adh1 [29] or *K. marxianus* Adh1 [28], which are constitutively expressed and are involved in ethanol formation but might have additional regulations responsible for increase in activity in the stationary phase.

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