Metabolic Engineering 12 (2010) 420–428

Contents lists available at ScienceDirect

Metabolic Engineering

journal homepage: www.elsevier.com/locate/ymben

Thermoanaerobacter spp. control ethanol pathway via transcriptional regulation and versatility of key enzymes

Jianjun Pei [a,1], Qing Zhou [b,1], Yu Jiang [b,d,1], Yilin Le [a,1], Huazhong Li [a], Weilan Shao [a,b,*], Juergen Wiegel [c,**]

[a] School of Biotechnology, Jiangnan University, Wuxi 214122, PR China
[b] College of Life Sciences, Nanjing Normal University, Nanjing 210046, PR China
[c] Department of Microbiology, and Department of Biochemistry & Molecular Biology, University of Georgia, Athens, GA 30602, USA
[d] Key Laboratory of Synthetic Biology, Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200032, China

ARTICLE INFO

Article history:
Received 14 January 2010
Received in revised form
11 May 2010
Accepted 7 June 2010
Available online 12 June 2010

Keywords:
Acetaldehyde dehydrogenase
Ethanol formation
Physiological function
Thermoanaerobacters
Transcriptional regulation

ABSTRACT

Ethanologenic *Thermoanaerobacter* species produce ethanol from lignocellulose derived substrates at temperatures above 70 °C. In the final steps of ethanol formation, two bifunctional acetaldehyde/alcohol dehydrogenases, AdhB and AdhE, and an alcohol dehydrogenase, AdhA, catalyze redox reactions between acetyl-CoA and ethanol via an acetaldehyde intermediate. DNA cloning and analysis revealed that the dehydrogenase genes and their transcriptional regulatory regions were highly conserved in these species. As determined by real-time PCR, the transcription of *adhE* was activated by ethanol, while *adhB* was transcribed without ethanol; however, all of their transcription was reduced at higher ethanol concentrations. Under imitating physiological conditions, AdhE played a crucial role in ethanol formation, and AdhB favored ethanol consumption when ethanol concentration was high e.g. 1%. Thus, the ethanol titer of fermentation is controlled via transcriptional regulation and the properties of specific enzymes in *Thermoanaerobacter*. These results provide evidence for an ethanol balance model and offer the possibility to raise the ethanol titer by metabolic engineering.

1. Introduction

As concerns about climate change increase and fossil fuels become scarcer, it is becoming urgent to develop highly efficient techniques to produce biofuels from renewable sources, which include hydrogen, hydrocarbons, short chain alcohols, and microbial fatty acid derivatives. Metabolic engineering of fermentation strains has emerged as a powerful tool for the improved production of these biofuels (Jiang et al., 2009; Taylor et al. 2009; Zha et al., 2009). However, it is very fundamental to investigate the rate-limiting elements in a fermentation pathway, and thus the metabolic engineering strategy can be successfully applied to elevate the productivity of a biofuel (Liu et al., 2010; Willquist and van Niel, 2010).

The genus *Thermoanaerobacter* is comprised of thermophilic anaerobic bacteria, mostly heterotrophs (Onyenwoke and Wiegel, in press). Three strains in this genus have been of great interest: *Thermoanaerobacter ethanolicus* JW200 (*Tet*) is known for its ability to produce ethanol from a wide range of hexoses and pentoses as well as starch and xylan (Wiegel and Ljungdahl, 1981; Wiegel, 1982; Carreira et al., 1983); *Thermoanaerobacter pseudethanolicus* 39E (*Tps*, renamed from *T. ethanolicus* 39E, Onyenwoke and Wiegel, in press) is a strain in which the ethanol production pathway has been extensively investigated (Burdette and Zeikus, 1994; Burdette et al., 1996); and *T. tengcongensis* (*Tte*) is the first species of this genus in which the genome sequence was published (Xue et al., 2001; Bao et al., 2002) before genomic data for *Tps* and another strain, *T.* sp. X514 (*Tsp*) are available in the GenBank (Accession nos. NC_010321 and NC_010320).

Fermentation by *Tet*, *Tps*, or *Tsp* offers the potential to produce ethanol from lignocellulose and to separate ethanol in continuous cultures during thermophilic growth (Wiegel, 1982; Bryant et al., 1988, 1992; Burdette and Zeikus, 1994). The practical application of this strategy, however, has been hindered by the fact that ethanol fermentation by these strains is limited to relatively low final ethanol concentrations (below 3.0%, w/v), although *Tet* and *Tps* can be readily adapted to grow at 8% (v/v) supplemented ethanol concentrations by serial transfers into media with increasing ethanol concentrations (Carreira et al., 1983; Ljungdahl and Carriera, 1982; Burdette et al., 2002). Thus, it is important to elucidate at the molecular level how ethanol metabolism is regulated in these systems so that strategies can be developed to increase the final ethanol concentration (or ethanol titer) for an economical fermentation process.

* Corresponding author at: School of Biotechnology, Jiangnan University, Wuxi 214122, PR China. Fax: +86 25 85891836.
** Corresponding author. Fax: +1 706 542 2651.
E-mail addresses: jianjunpei@hotmail.com (J. Pei), shaoweilan@njnu.edu.cn (W. Shao), jwiegel@uga.edu (J. Wiegel).
[1] These authors contributed equally to this work.

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doi:10.1016/j.ymben.2010.06.001

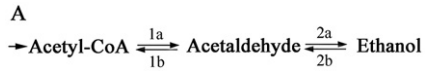

Fig. 1. Possible reactions in the final steps of the ethanol pathway in anaerobes.

The final steps of ethanol fermentation in anaerobes such as *Tet* are two reversible redox reactions, from acetyl-CoA to acetaldehyde and from acetaldehyde to ethanol (Fig. 1). Researchers have been focussing their efforts on acetaldehyde dehydrogenase (ALDH) (EC 1.2.1.10) and alcohol dehydrogenase (ADH) since 1988. A primary alcohol dehydrogenase (P-ADH) (EC 1.1.1.1) and a secondary alcohol dehydrogenase (S-ADH, EC 1.1.1.2.) were purified and characterized from *Tet*, and S-ADH was proposed to be largely responsible for ethanol formation because it was produced early in the growth phase and had a high affinity for acetaldehyde (Bryant et al., 1988). A few years later, ALDH, P-ADH, and S-ADH were purified and characterized from *Tps*, and S-ADH was found to have reductive thioesterase activity, suggesting that S-ADH catalyzed the production of ethanol from acetyl-CoA and acetaldehyde released by ALDH (Burdette and Zeikus, 1994).

The P-ADH-encoding gene *adhA* from *Tet* (Holt et al., 2000) and the S-ADH-encoding gene *adhB* from *Tps* (Burdette et al., 1996) have been cloned and sequenced, but the gene for ALDH, one of the key enzymes in ethanol formation, was not reported until the genomic sequences of *Tps* and *Tsp* became available in the GenBank (Peng et al., 2007). The ALDH is encoded by an alcohol dehydrogenase gene, *adhE*, which also exhibits both acetaldehyde and alcohol dehydrogenase activities (Peng et al., 2008). Therefore, the ethanol fermentation pathway of *Thermoanaerobacter* comprised of 3 key enzymes encoded by *adhA*, *adhB*, and *adhE*; gene products AdhA, AdhB, and AdhE are corresponding to P-ADH, S-ADH, and ALDH, respectively.

The aims of this study were to determine the roles and regulation of the alcohol dehydrogenase AdhA and the two bifunctional acetaldehyde/alcohol dehydrogenases, AdhB and AdhE, in ethanol formation and consumption in ethanologenic *Thermoanaerobacter* species. In particular, how the function and regulation of these enzymes affect the final concentration of ethanol was addressed. This paper reports the cloning and analysis of *adhB* and *adhE* and their regulatory elements from *Tet*, and the biochemical and physiological properties of the recombinant enzymes, AdhB and AdhE. In addition, the *in vivo* transcription of *adhA*, *adhB*, and *adhE* was examined in the presence of their substrates or products. The results are discussed in the context of the plausibility of using genetically modified *Thermoanaerobacter* as a venue in which to raise ethanol titer.

2. Materials and methods

2.1. Bacterial strains and plasmids

T. ethanolicus JW200 (*Tet*) was grown anaerobically in 8 g/L glucose medium as described previously (Wiegel and Ljungdahl, 1981). The cell density in this medium can reach an OD_{660} of 1.1 in stationary growth phase. Intracellular and extracellular pH of *Tet* was monitored during cell growth by using culture supernatants, or washed and sonicated cell extracts.

Escherichia coli JM109 and JM109 (DE3) (Promega) were used as hosts for cloning and expression of genes in plasmids pET-20b (Novagen), pTrc99a (Phamacia), or pUC19 (Phamacia). *E. coli* cells were routinely grown aerobically in Luria-Bertani (LB) medium at 37 °C, and ampicillin (100 μg/ml) or kanamycin (30 μg/ml) was added in LB medium for selective cultures.

2.2. Molecular cloning and sequencing

Genomic DNA was prepared from *Tet* cells according to the method described by Sambrook and Russell (2001). Genes *adhB* and *adhE* were amplified by using *Pyrobest* DNA polymerase (Takara) with primers designed on the basis of the genes coding for S-ADH and a putative AdhE (GenBank Accession no. ZP_00778525.1) of *Tps* (Table 3). PCR amplified *adhB* was inserted in pET-20b at the *EcoR* I and *EcoR* V sites to yield pET-*adhB*. The DNA fragment containing *adhE* along with its wild-type promoters was amplified combined with primers for TRR_{adhE} and *adhE* (Table 3), and inserted into pUC19 at the *Sma*I/*Xba*I sites to generate pUC-*PP-adhE*, which carried two putative promoters.

The flanking sequences of *adhB* and *adhE* were cloned and sequenced in pMD19-T (Takara) by using the RSD-PCR protocol (Jiang et al., 2007). The specific primers (Table 3) for walking upstream and downstream from *adhB* and *adhE* were paired with RSD-primers for gene amplifications.

2.3. Preparation of cell-free extracts and recombinant proteins

The *Tet* cells were grown for 5 h (OD_{660}=0.58), harvested, resuspended in buffer A [20 mM MOPS, pH 6.8, 1 mM dithiothreitol (DTT), 0.02% (w/v) NaN_3], and disrupted by passing twice through a French press cell (Thermo) at 1.25×10^5 kPa. The cell-free extracts were obtained by centrifugation (14,000*g*, 30 min) to remove cell debris.

E. coli JM109 (DE3) cells harboring pET-*adhB* were grown at 37 °C to an OD_{600} of 0.6–0.8, and then cultured at 30 °C for 2–3 h after adding 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for gene expression induction. AdhE was expressed for 4 h in *E. coli* JM109 cells harboring pUC-*PP-adhE* without particular induction of gene expression. The cell-free extracts were prepared from these *E. coli* cells using the same procedure as described for *Tet* cells, and were incubated in a 70 °C water bath for 30 min. After the denatured protein was removed by centrifugation, the supernatants were applied onto a Cibacron Blue-3 GA column (Sigma) equilibrated with buffer A containing 150 mM NaCl, and the bound proteins were eluted with 4 mM NAD^+ in the same buffer. The enzyme in active fractions was collected into a dialysis bag, concentrated by embedding the dialysis bag in PEG 20,000, dialyzed against buffer A, and stored in buffer A with the addition of 0.1 mM $ZnCl_2$, 0.02% (w/v) NaN_3, and 20% (v/v) glycerol. Protein concentration was determined by using the Bradford method with bovine serum albumin as the standard.

2.4. Dehydrogenase activity assay and ethanol assay

All ALDH and ADH activities were measured by monitoring the changes of NAD(P)H by following the absorbance at 320 nm. One unit of enzyme activity was defined as the amount of enzyme producing or consuming 1 μmol of NAD(P)H per min. For standard assays, substrate concentrations were 1 mM NAD(P) or NAD(P)H, 2 mM acetyl-CoA or CoASH, and 20 mM acetaldehyde or ethanol, respectively, at optimal pH and temperature as indicated for each reaction (Table 1). The concentration of ethanol was measured by Shimadzu GC-2010 gas chromatograph (Shimadzu, Japan). Separation took place in the Rtx-Was column (length, 30 m; 0.25 mm ID) at 65–130 °C with N_2 as the carrier gas. The injector temperature was 200 °C, and the detector temperature was 200 °C.

Table 1
Catalytic properties of AdhB and AdhE.

Enzyme	Property	Reaction				
		1a. Acetyl-CoA reduction	2a. Acetaldehyde reduction	1b. Acetaldehyde oxidation	2b. Ethanol oxidation	
					20 mM	1% oxidation
AdhB	Optimal pH	8.7	8.7	N/A	8.3	8.3
	$T_{maximal\ activity}$, 2 min	85 °C	80 °C	N/A	70 °C	70 °C
	Specific activity (U/mg)[a]	6.64 ± 1.33	4.61 ± 0.43	ND	1.05 ± 0.01	4.41 ± 0.24
AdhE	Optimal pH	6.6	8.0	8.4	N/A	N/A
	$T_{maximal\ activity}$, 2 min	55 °C	60 °C	60 °C	N/A	N/A
	Specific activity (U/mg)[a]	224.82 ± 5.23	2.12 ± 0.39	156.43 ± 3.17	ND	ND

N/A, not applicable; ND, no detectable activity.

[a] Activities were determined with a single nucleotide of NAD(P) or NAD(P)H under the optimal pH and temperature for each reaction; substrate concentrations: 1 mM nucleotide and 2 mM acetyl-CoA or CoASH, 20 mM acetaldehyde, and 20 mM or 1% ethanol (as indicated).

2.5. Cell incubation, RNA isolation and cDNA preparation

All procedures were performed in an anaerobic chamber under N_2-atmosphere. Exponentially growing cells were harvested by centrifugation at 3000*g* for 10 min at 4 °C when the cell density reached an A_{660} of approximately 0.32. Cells were washed with 2 volumes of reduced PM buffer (11 mM KH_2PO_4, 11.5 mM Na_2HPO_4, 1 mM $MgCl_2$, 1 mM Na_2S, pH 7.6) and resuspended in a 0.33 volume of basic medium, which contained 9.3 mM NH_4Cl, 3 mM cysteine HCl, 0.05% (v/v) vitamin solution, 0.5% (v/v) modified Wolfe's mineral elixir and 4 μM resazurin in PM buffer (Wiegel and Ljungdahl, 1981). The cell suspension was incubated at 69 °C for 1 h in Hungate tubes, and the incubation was continued at 69 °C for up to 2 h after it was aliquoted into the pre-warmed basic medium with or without the addition of glucose, or ethanol. Samples were withdrawn at indicated time points, and the cells were immediately harvested by centrifugation at 5000*g* for 5 min at 4 °C, rapidly frozen in liquid nitrogen, and stored at −80 °C for gene transcription analyses.

Total RNA was isolated from each aliquot of cells by using PureLink™ Micro-to-Midi Total RNA purification kit (Invitrogen), and trace DNA was removed with Trizol (Invitrogen) according to the manufacturer's protocol. The quantity and quality of purified RNA were examined spectrophotometerically and by formaldehyde agarose gel electrophoresis, and verified by a 40-cycle PCR of 16 S rRNA and target genes.

First-strand cDNA was synthesized from high-quality and intact RNA by using the SuperScript™ III Platinum Two-step qRT-PCR Kit with SYBR Green (Invitrogen) following the manufacturer's directions. Five micrograms of RNA was reverse transcribed per 100 μl of reaction mixture, and the resulting cDNA samples were aliquoted and stored at −20 °C for further use.

2.6. Real-time PCR

Primers listed in Table 4 were designed using Primer Premier 5.0 software with T_m values between 55 and 65 °C, and an amplification length between 100 and 250 bp. The specificity of the primers to the target genes was confirmed by BLAST analysis against sequences in GenBank. Quantitative PCR was performed by using the SuperScript™ III Platinum Two-step qRT-PCR Kit with SYBR Green (Invitrogen). The sample cDNA (1 μl, if necessary diluted 1:10–1:100 in DEPC water) was used in PCR reactions prepared with appropriate primers (0.1 μM, final concentration) and the master mix in a final volume of 25 μl following the manufacturer's protocol. The PCRs were carried out on an iCycler Real Time Detection System (Bio-Rad), using the following amplification parameters: an initial step at 50 °C for 2 min, denaturation at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at the indicated temperature (Table 4) for 1 min, and elongation at 72 °C for 30 s. After the cycles were completed, the melting curves were analyzed to confirm the specificity of the PCR products.

RNA quantification was performed employing a relative standard curve method. The standard threshold cycle (C_T) values were obtained from the PCRs by using 10–10^5 times diluted *Tet* genomic DNA as the template and the same oligonucleotides used in the cDNA analysis as primers. The relative standard curve was plotted with C_T values against input quantity (log dilution factor) for calibration and each target gene in every PCR assay. In this study, the 16 S rRNA gene was used as the standard. Possible variability in the initial amount of total RNA in each sample was controlled by this calibration procedure. The PCR assay for each target gene was performed simultaneously with a series of diluted *Tet* genomic DNA and non-template controls in the same plate. The relative concentrations of standard and the target genes of each sample were then calculated from the standard curves. The ratio of the relative concentration of amplified target gene to that of the standard represented the relative abundance of the mRNA from a target gene of each sample. All measurements were performed in triplicate.

3. Results

3.1. Gene cloning and analysis

Gene cloning and sequence analysis focused on *adhB* and *adhE* and their regulatory elements from *Tet*. The *adhA* gene and transcription region were not included in this study as they have already been characterized by Holt et al. (2000). The nucleotide sequence of a 3 kb fragment containing *adhB* and its flanking sequence was obtained from *Tet* genomic DNA (GenBank Accession no. DQ323135). The gene product, *Tet*-AdhB, had a putative amino acid sequence of 97.7% identical to that of a putative *Tte* threonine dehydrogenase and Zn-dependent dehydrogenase (GenBank Accession no. NP_622353), and 96.9% identical to that of a *Tps* S-ADH (Burdette et al., 1996). Analysis of the genomic organization revealed that the *adhB* genes in all of these strains are followed by a gene encoding a flavodoxin oxidoreductase related 2-polyprenylphenol hydroxylase with no obvious transcriptional terminator between them. However, different genes were located upstream of *adhB* in the different

species: genes coding for N-acetylglutamate semialdehyde dehydrogenase in *Tet*, integrase in *Tps*, and ADH in *Tte* and *Tsp*.

The *adhE* gene (Peng et al., 2007) amplified from *Tet* (*Tet-adhE*, GenBank Accession no. DQ836061) encodes a protein 97% identical to the putative AdhE from the genomic sequences of *Tps* and *Tsp* but only 52% and 47% identical to the AdhE1 of *Clostridium acetobutylicum* (Nair et al., 1994) and the AdhE of *E. coli* (Kessler et al., 1991), respectively. Amino acid sequence analysis indicated that the domain from residues 30–412 is related to the aldehyde dehydrogenase family (ALDH-SF superfamily) and the domain from residues 551–854 belongs to the iron-containing alcohol dehydrogenase family. The linker between the ALDH and ADH domains is a putative NAD^+ binding motif with a typical GXGXG NAD^+ binding finger (Fontaine et al., 2002). The open reading frames upstream and downstream of *Tet-adhE* (GenBank Accession nos. DQ836060 and DQ836062) were identified as genes encoding a putative methylated-DNA (protein)-cysteine S-methyltransferase and a NLP/P60 protein. The same genes are located upstream and downstream from *adhE* of *Tps* and *Tsp* (*Tps-adhE* and *Tsp-adhE*). No gene similar to *adhE* was found in *Tte*.

In the transcriptional regulation region (TRR) of *adhB* (TRR_{adhB}) from *Tet*, the potential σ^A promoter was identified 70–100 bp upstream of the ribosome binding site (RBS). In comparison, the *adhB* promoters in all four strains have a conserved sequence, but the sequences of the leader RNA from the +1 to the ATG motif were different (Fig. 2A). The sequence of the TRR upstream of the start codon of *adhE* (TRR_{adhE}) was also highly conserved in *Tet*, *Tps*, and *Tsp*, and two sets of potential promoter-RBS elements (P_{197} and P_{63}) were located at bases 197 and 63 upstream from the start codon for *adhE* (Fig. 2B). These conserved bases in different strains indicate that ethanologenic *Thermoanaerobacter* species share the same regulation mechanisms for ethanol metabolism.

3.2. Preparation and biochemical properties of AdhB and AdhE

Four possible reactions can occur in the final steps of ethanol formation from acetyl-CoA (Fig. 1). To determine how many steps each bifunctional dehydrogenase can catalyze, recombinant AdhB and AdhE were expressed in *E. coli*, and purified to homogeneity by gel electrophoresis. In regular enzymology studies, substrate concentration is usually employed at about 10 times higher than the K_m value of the enzyme. Here in this work the biochemical properties of the enzymes were routinely determined by using 1 mM nucleotide and 2 mM acetyl-CoA or CoASH, 20 mM acetaldehyde, and 20 mM or 1% (v/v, about 171 mM) ethanol as substrates.

AdhB was expressed to more than 10% of the total soluble protein in *E. coli*, which was subsequently purified from the cell-free extracts with a recovery of about 90% (data not shown).

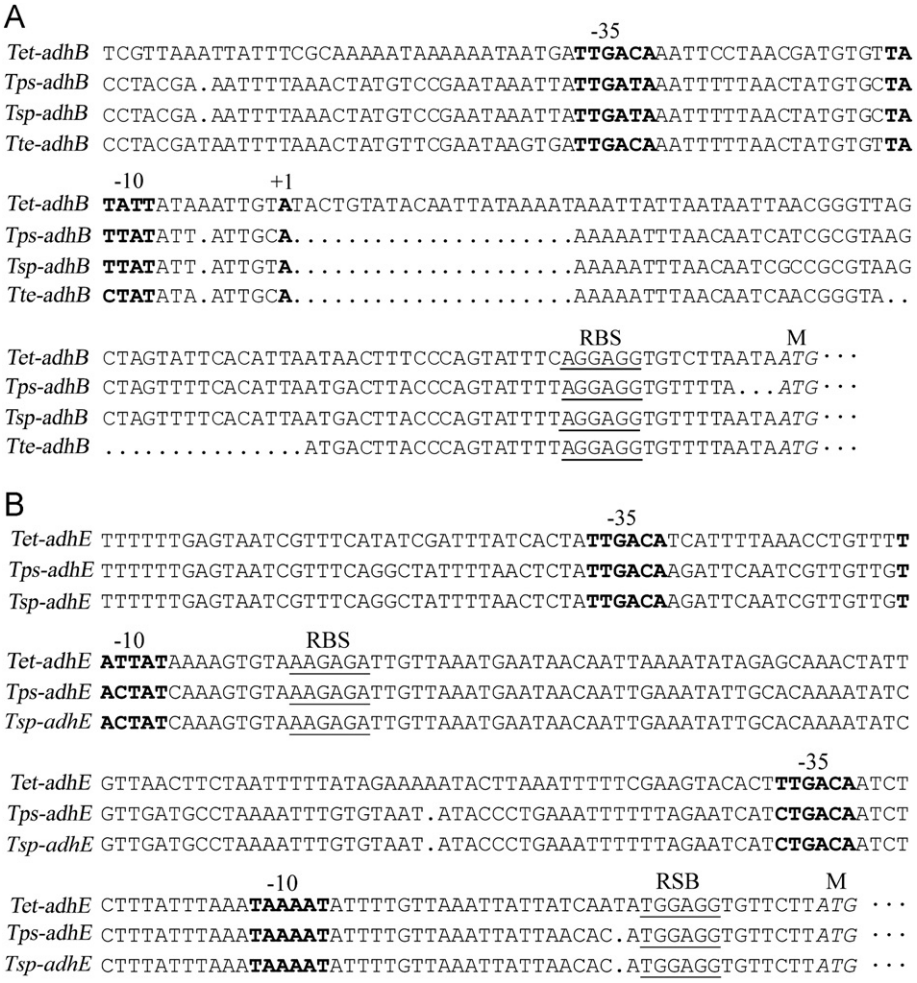

Fig. 2. *DNA sequences upstream from adhB or adhE.* (A) A comparison of the promoter elements for *adhB* of *Tet* with those of *Tps*, *Tsp*, and *Tte*. (B) Analysis of the sequence upstream from *adhE* of *Tet*. Abbreviations and symbols: *Tet*, *T. ethanolicus* JW200; *Tps*, *T. pseudethanolicus 39E*; *Tsp*, *T.* sp. X514; *Tte*, *T. tengcongensis*; Bold-letter bases, putative -35 and -10 sites; RBS, ribosome binding sites; M, start codon for methionine.

The purified AdhB exhibited NADP(H) dependent activities for reactions 1a, 2a, and 2b, but no detectible activity for reaction 1b (Fig. 1), and the highest activities were found at 67, 77, and 84 °C for reactions 2b, 2a, and 1a, respectively (Fig. 3A). AdhB was relatively stable at pH 5.2–6.5, in which its 1-h half-life was at 85 °C for ALDH activity and at 95 °C for ADH activity. However, the maximal activities for reactions 1a, 2a, and 2b were observed at pH 8.7, 8.7, and 8.3 at various temperatures (Fig. 3B). Under optimal pH values and temperatures, purified AdhB exhibited typical bifunctional activity with an ALDH activity (reaction 1a) significantly higher than the ADH activities (reactions 2a and 2b) (Table 1).

The NAD(H) dependent ALDH activity of AdhE from *Tet* was expressed to 44.2 ± 0.5 U/mg in *E. coli* JM109, although the host cells also contained an *adhE* coding for *E. coli*-AdhE. We found that more than 95% of *E. coli*-AdhE was inactivated after the cell-free extracts were incubated for 5 min at 70 °C (data not shown), thus AdhE encoded by the *Tet* gene was isolated from *E. coli*-AdhE by heat treatment for 30 min at 70 °C. AdhE was much less stable than AdhA (Holt et al., 2000) and AdhB, and lost more than 90% of its activity after it was diluted to 0.3 mg of protein/ml and incubated at 70 °C for 30 min at pH 7.6 (Fig. 3C). However, it was relatively stable at pH 5.2–6.5. The inactivation of AdhE at high pH caused difficulties in protein purification and sequencing in the past when enzyme purification was performed using Tris buffer at pH 7.6–8.0 (Burdette and Zeikus, 1994; Holt et al., 2000; Peng et al., 2007). The AdhE activity was also very sensitive to pH variations; the highest activities were found at pH 6.6 for reaction 1a, but at pH 8.0 for reaction 1b, respectively (Fig. 3D). Under optimized conditions, AdhE exhibited NADH-specific ALDH and NADPH-specific ADH activities, and catalyzed reactions 1a, 1b, and 2a (Fig. 1); however, the ALDH activity of AdhE (224 U/mg) was about one hundred times higher than its ADH activity (Table 1).

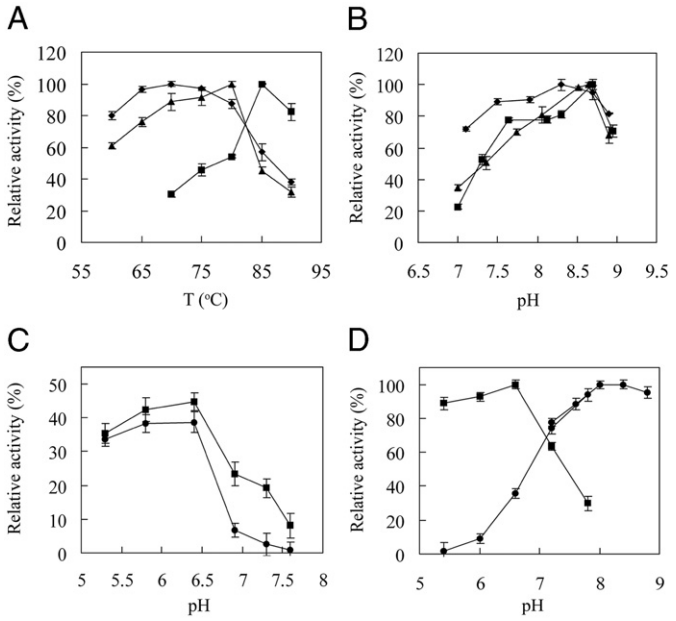

Fig. 3. *The effects of temperature and pH on the stability and activity of recombinant AdhB and AdhE.* Symbols: ■, reduction of acetyl-CoA (1a); ●, oxidation of acetaldehyde (1b); and ▲, reduction of acetaldehyde (2a). (A) The temperature optima of AdhB. Reactions were performed at temperatures from 60 to 90 °C in standard reaction mixtures over a 2 min assay. The highest activity was set at 100%. (B) The optimal pH for the activities of AdhB. The activities were determined in 50 mM Tris–HCl (pH 7.2–8.7) buffer, and the other conditions were the same as those for the standard assays. (C) The pH stability of AdhE. AdhE (0.3 mg/ml) was incubated at 70 °C for 30 min in PI buffer (20 mM phthalate, 20 mM imidazole, and 5 mM DTT) at various pH values. The remaining activities were determined by standard assays. The full activity from the same amount of enzyme kept in an ice bath was set at 100%. (D) The optimal pH for AdhE activity. The activities were determined in 50 mM PI buffer (pH 5.4–7.8) or Tris–HCl buffer (pH 7.2–8.7), and the other conditions were the same as those for the standard assays. The highest activity in the assays for each reaction was set at 100%.

3.3. Enzyme activities under physiological conditions

The pH value, temperature and substrate concentration in the cytoplasm are usually limited to certain levels, which are greatly different from the conditions used in typical enzymology assays. To estimate the contribution of an enzyme to ethanol formation, its specific activities need to be determined under physiological conditions. Under chemostatic conditions, NAD, NADH, NADP, and NADPH concentrations determined for *C. acetobutylicum* cells were 6.8, 0.97, 0.41, and < 0.2 μmol/g of dry cells (Vasconcelos et al., 1994), respectively, which fall in the range observed for *Tet*. Accordingly, the intracellular NAD, NADH, NADP, and NADPH concentrations in *Tet* were about 1.30, 0.19, 0.08, and 0.04 μmol/g of wet cells, respectively. Therefore, the physiological conditions were simulated by using 1.5 mM NAD, 0.25 mM NADH, 0.1 mM NADP, and 0.05 mM NADPH at 70 °C, pH 7.2 or 6.6, and the other substrates were used, respectively: 1a, 2 mM acetyl-CoA; 1b, 2 mM CoASH and acetaldehyde; 2a, 2 mM acetaldehyde; and 2b, 2 mM or 1% (v/v, about 171 mM) ethanol.

Under the simulated physiological conditions, AdhB and AdhE did not act as typical bifunctional aldehyde/alcohol dehydrogenases as observed in enzymology assays. AdhB exhibited only a very weak ALDH activity for reaction 1a, and AdhE had a high ALDH activity with no ADH activity detected for reducing acetaldehyde to ethanol (reaction 2a, Table 2). Furthermore, AdhB was highly active to catalyze reaction 2a (ethanol formation from acetaldehyde) when the substrate concentration was 2 mM. However, its activity for reaction 2b (ethanol consumption) was much higher than that for reaction 2a when the ethanol concentration was increased to 1% (v/v) (Table 2).

The dialyzed cell-free extract from *Tet* cells of mid-log growth phase contained natural enzymes encoded by *adhA*, *adhB*, and *adhE*. In the cell-free extract, NADH-dependent ALDH activity for reaction 1a was found to be much higher than the activities for reactions 1b, 2a, and 2b (Table 2), where reactions 2a and 2b were catalyzed by both AdhA and AdhB in the presence of both NAD(H) and NADP(H). These results confirmed that AdhE has an activity much higher than that previously reported (Holt et al., 2000; Peng et al., 2007). In comparison, AdhE, rather than AdhB, can play a crucial role in the catalysis of the first ethanol formation step from acetyl-CoA in growing cells.

3.4. Effects of glucose and ethanol on transcription of the dehydrogenases

The *in vivo* transcription of *adhA*, *adhB*, and *adhE* genes were examined. Real-time PCR was used to reveal the changes in transcription levels of these genes when 2% glucose (w/v) or 1% ethanol (v/v) was added to the basic medium. The transcription level changes caused by glucose were basically the same as those caused by ethanol, but apparently, they were delayed; implying that ethanol produced from glucose was a direct inducer of these changes (Fig. 4A). This assumption is supported by the observation that the ethanol concentration increased to about 0.3 g/L (6.5 mM) after the cells were incubated for 1.5 h in the presence of 2% glucose. (Fig. 4B).

The relative abundances of mRNAs transcribed from *adhA*, *adhB*, and *adhE* were significantly different in the cells without

Table 2
ALDH/ADH activities determined under physiological conditions.

Enzyme	pH	Specific enzymatic activity (U/mg) for each reaction				
		1a. Acetyl-CoA reduction	2a. Acetaldehyde reduction	1b. Acetaldehyde oxidation	2b. Ethanol oxidation	
					2 mM	1%
AdhB[a]	7.2	<0.01	0.28 ± 0.09	ND	0.11 ± 0.01	1.29 ± 0.07
	6.6	<0.01	0.25 ± 0.01	ND	0.08 ± 0.03	0.62 ± 014
AdhE[a]	7.2	35.61 ± 2.51	ND	45.05 ± 0.93	ND	ND
	6.6	113.35 ± 4.76	ND	29.33 ± 2.09	ND	ND
Cell-free extract[b,c]	7.2	1.50 ± 0.01	0.19 ± 0.01	0.10 ± 0.01	0.07 ± 0.02	0.30 ± 0.01
	6.6	4.23 ± 0.08	0.43 ± 0.06	0.03 ± 0.00	0.02 ± 0.00	0.06 ± 0.01

ND, no detectable activity.

[a] Activities determined under simulated physiological conditions: 70 °C in the mixture of 0.05 mM NADPH, 0.10 mM NADP, 0.25 mM NADH, and 1.50 mM NAD with substrate(s): 1a, 2 mM acetyl-CoA; 1b, 2 mM CoASH and acetaldehyde; 2a, 2 mM acetaldehyde; and 2b, ethanol as indicated.

[b] Cell-free extract from *T. ethanolicun* JW200 cells grown under anaerobic conditions for 5 h at 69 °C.

[c] Activities determined under the same conditions except the nucleotides were used separately to specify the catalytic steps: 0.25 mM NADH for 1a; 1.5 mM NAD for 1b; 0.25 mM NADH and 0.05 mM NADPH for 2a; and 1.50 mM NAD and 0.10 mM NADP for 2b.

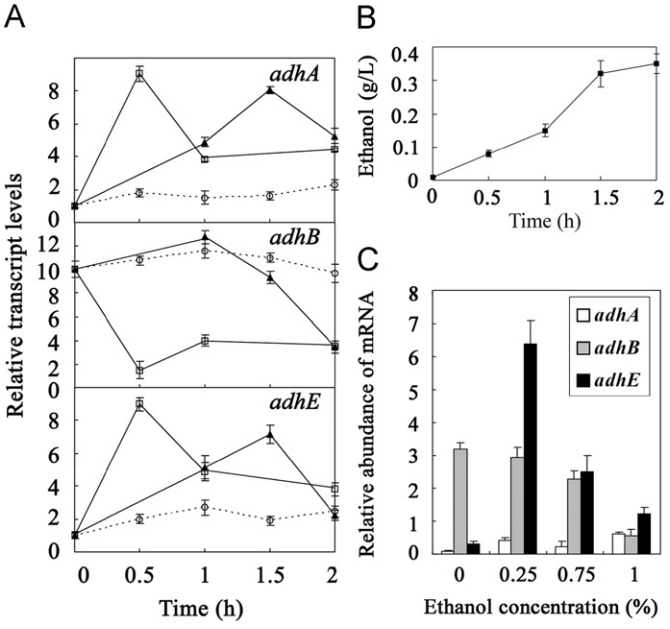

Fig. 4. *Analysis of transcription levels and relative abundance of mRNA from adhA, adhB, and adhE in T. ethanolicus JW200.* (A) The relative transcript levels of *adhA*, *adhB*, and *adhE* in cells incubated in the presence of substrate (2% glucose, ▲) or product (1% ethanol, □), or in the absence of them (○). The starting value at 0 h was set at 1.0 for *adhA* and *adhE*, and 10 for *adhB*. (B) The ethanol concentration incubated in the presence of 2% glucose. (C) Relative abundance of mRNA transcribed from *adhA*, *adhB*, and *adhE*. The cells were incubated under anaerobic conditions at 69 °C for 0.5 h with 0%, 0.25%, 0.75%, and 1% ethanol.

exposure to ethanol, where the amount of mRNA from *adhB* was much higher than those from *adhA* and *adhE* (Fig. 4C). When the cells were exposed to a low ethanol concentration of 0.25% (v/v), the transcription of *adhB* began to decrease, while the transcription of *adhA* and *adhE* was significantly enhanced. It was noticeable that the transcription of *adhE* dramatically increased in the presence of 0.25% (v/v) ethanol, but significantly decreased when the ethanol concentration was increased to 0.75% (v/v). The transcription of both *adhB* and *adhE* was low when 1% ethanol was supplemented to the basic medium.

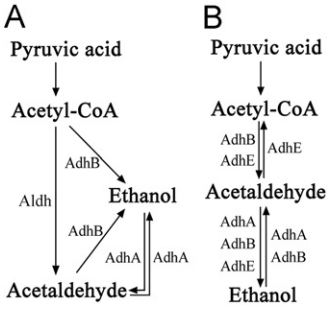

Fig. 5. *Proposed schemes for the pyruvic acid to ethanol pathway in Thermoanaerobacter spp.* (A) The scheme proposed before the identification of *adhE* (Burdette and Zeikus, 1994). (B) The new scheme based on the enzyme activities determined in this study.

4. Discussion

To understand the molecular mechanisms of ethanol production pathway in *Thermoanaerobacter* spp., efforts have been made in cloning and sequencing the genes of key enzymes involved in the ethanol pathway in these species. Heterologously expressed AdhA and AdhB have previously been successfully purified, cloned, and characterized (Burdette and Zeikus, 1994; Burdette et al., 1996; Holt et al., 2000). However, the ALDH has been too labile for a detailed characterization and sequencing, although a scheme was proposed for the pathway from pyruvate to ethanol (Fig. 5A) (Burdette and Zeikus, 1994). The full characterization of ALDH could not be performed until *adhE* in the partial genomic sequence of *Tps* was identified as the ALDH-encoding gene (Peng et al., 2008). The *adhE* gene encodes a protein with the same properties, including NADH-dependence, molecular mass, and unstable state, as ALDH purified from *Tps* and *Tet* (Burdette and Zeikus, 1994; Peng et al., 2007). The ALDH and ADH activities of AdhB and AdhE reveal a scheme (Fig. 5B) in which most reaction steps between acetyl-CoA and ethanol can be catalyzed by two to three of these enzymes.

The roles of AdhA, AdhB, and AdhE in the ethanol production can be to some extent estimated on the basis of their properties. AdhA has been previously characterized, of which the affinity and

catalytic efficiency for the substrate acetaldehyde is about 100 times higher than for ethanol (Burdette and Zeikus, 1994). The study reported here focused mainly on the properties of AdhB and AdhE, and on the reduction or oxidation of ethanol rather than primary, secondary, or long chain alcohols, since the main fermentation product of *Tet*, *Tps*, and *Tsp* during growth on hexoses and pentoses is ethanol.

Enzyme activity is usually dependent on reaction conditions such as pH, temperature, and concentrations of substrate(s) or coenzyme(s). The bifunctions of the enzymes and the involvements of multiple substrates make the analysis of AdhB and AdhE more complex. The ALDH and ADH activities as well as the kinetics of AdhB and AdhE have been determined under various conditions by Bryant et al. (1988), Burdette and Zeikus (1994), and Peng et al. (2008). The work presented here revealed that the highest activities of AdhB for reactions 1a, 2a, or 2b occurred under different pH conditions and temperatures, and AdhE activities for reactions 1a and 1b were strongly affected by pH values (Fig. 3). The AdhE activity for reaction 1a is usually determined at pH above 7.0, and specific activities of 11.0 U/mg for purified enzyme (Peng et al., 2008), and 0.32 U/mg for cell-free extract of *Tsp* (Burdette and Zeikus, 1994) have been reported. However, when AdhE was analyzed at pH 6.6, which was approximately the intracellular pH of the cells growing in middle exponential phase (data not shown), its specific activities were as high as 224 and 4.23 U/mg for the purified enzyme and cell-free extract of *Tet*, respectively (Tables 1 and 2). Furthermore, it was observed that the enzyme was much more stable at pH values below 6.4 than above 6.9 (Fig. 3C), indicating that the enzyme *in vivo* is not as labile as observed during protein purification performed at pH 7.5 or above. Purified AdhE also exhibited a high activity for reaction 1b (this work), implying that the enzyme was ready to catalyze the reverse reaction from acetaldehyde to acetyl-CoA when the acetaldehyde concentration was increased to relatively high levels.

In enzymology assays, both AdhB and AdhE exhibited bifunctional acetaldehyde and alcohol dehydrogenase activities under the optimal pH and temperature for each reaction (Table 1). However, it was important to estimate how these enzymes act under physiological conditions in cells growing at about 70 °C and pH 7.2 or lower. The intracellular nucleotide concentrations calculated from *C. acetobutylicum* (Vasconcelos et al., 1994) are close to 1.5, 0.25, 0.1, and 0.05 mM for NAD, NADH, NADP, and NADPH, respectively, where the concentrations of NADH, NADP, and NADPH are much lower than those used in enzymology assays. The concentrations of cellular acetyl-CoA and acetaldehyde should be relatively low because they are intermediates in metabolic pathways, while ethanol is a fermentative end product, which normally accumulates to 1% (v/v) or higher. Accordingly, these simulated physiological conditions were employed to determine dehydrogenase activities.

Not too surprisingly, the ALDH activity of AdhB was too low to read under imitative physiological conditions because the pH and the concentration of NADPH were much lower than those used in enzymology assays under optimal conditions (Table 2). However, it is interesting to see that AdhB gave a much higher activity for reaction 2b (1.29 U/mg) than for reaction 2a (0.28 U/mg) in the presence of 1% (v/v) ethanol (Tables 1 and 2). This can be explained by the fact that the pH values and temperatures similar to growth conditions favor AdhB to catalyze reaction 2b (Figs. 3A and B), and AdhB has a much higher affinity to NADP (K_m=0.022) than NADPH (K_m=0.36) (Bryant et al., 1988). Thus, AdhB can favor ethanol consumption after a certain amount of ethanol (e.g. 1%, v/v) is produced, and its activity, combined with the activity of AdhA (reaction 2b) may form one of the barriers to limit the formation of high ethanol concentration during fermentation. Gene organization may offer another clue to indicate that AdhB is involved in biosynthesis, i.e., in all of the sequenced strains, the *adhB* gene forms an operon with a flavodoxin oxidoreductase related 2-polyprenylphenol hydroxylase gene.

Although a molecule of AdhE is comprised of an ALDH and an ADH domain, it was shown to have mainly ALDH activity under either optimal or physiological conditions (Tables 1 and 2). The gene *adhE* was found in three ethanologenic strains of *Thermoanaerobacter*, *Tet*, *Tps*, and *Tsp*, but not in *Tte*, which produced only trace amounts of ethanol. In addition, the over-expression of AdhE in *Tet* results in the rise of ethanol production (Peng et al., 2008). These results have already indicated that AdhE should be crucial for ethanol formation. But the direct evidence to prove the importance of AdhE was obtained from the analysis of ALDH and ADH activities in cell-free extracts prepared from exponentially growing *Tet* cells. Table 2 reveals that NADH dependent ALDH activity from acetyl-CoA to acetaldehyde is much higher than all of the activities for the other steps between acetyl-CoA and ethanol, although the ADH activities were determined for both AdhA and AdhB by simultaneously adding NAD(H) and NADP(H). These results not only demonstrate that the first step for ethanol formation depends highly on the activity of AdhE, but also indicate that the rate limiting step for ethanol formation is reaction 2a, the reduction of acetaldehyde. (Tables 3 and 4)

Cloning and sequencing of *adhB*, *adhE*, and their promoters and potential regulatory regions allowed for analysis of the regulation of these enzymes on the transcriptional level. This report is the first to reveal that the transcription of *adhB* is high in the absence of ethanol, while the transcriptions of *adhA* and *adhE* need to be activated by ethanol. It is also the first to report that the transcription levels of both *adhB* and *adhE* are very low in the presence of 1% (v/v) ethanol. Bryant et al. (1988) reported that AdhA activity occurs later in the growth cycle, while AdhB activity is formed early during high metabolic activity and drastically decreases later on. This phenomenon can now be explained on the molecular level: the transcription levels of *adhA* and *adhE* are a few times higher only when some ethanol has been produced and accumulated, while *adhB* is immediately transcribed before ethanol is formed (Fig. 4). After the transcription of *adhB* is reduced by the presence of ethanol, the enzymatic activity of AdhB remains until the end of cell growth because this enzyme is very stable.

Interestingly, *adhE* transcription was drastically increased in the presence of 0.25% (v/v) ethanol, but gradually decreased when the ethanol concentration increased to e.g., 0.75% (v/v) (Fig. 4). In other words, a strategy exists for reducing the transcription of both *adhB* and *adhE* following an increase in ethanol concentration, while the transcription of *adhE* is simultaneously regulated

Table 3
Primer sequences used in PCR or RSD-PCR.

Target	Primer(s)
adhB	5′-ATGAAAGGTTTTGCAATGCTC-3′
	5′-TTTGAATTCTACTCGAGTATTACAACAGGTTTGATTAGGT-3′
adhE	5′-CCCGATCTTTTTTGAGTAATCGTTTCATATC-3′
	5′-CCC**TCTAGA**TTATTA**GTGGTGGTGGTGGTGGTG**TTCTCCATAGGCTTTTC-3′
adhB-up	5′-TCCTCTTTGCACTTCAGAG-3′
adhB-down	5′-TCTGGTGGAATGCTGGC-3′
adhE-up	5′-TCACTAAGGACGCCGACA-3′
adhE-down	5′-ATTCCAAGAATGCCATTG-3′

Bold letters indicate the addition of His-tag sequence, or restriction sites.

Table 4
Forward (F) and reverse (R) primers for real-time PCR.

Gene (GenBank access. no.)	Sequence	Nucleotide position	PCR product size (bp)	Annealing temp. (°C)
adhA (AF178965)	F: 5′-TTGCCTGTATCCTATGTATGCC-3′	537–558	182	56
	R: 5′-GGTAACGAACTATCAGCCTCACT-3′	696–718		
adhB (DQ323135)	F: 5′-TAACGGAAGGCAAAGGTGT-3′	689–707	154	58
	R: 5′-CATTCAAGACGAGGAACAGG-3′	823–842		
aldhE (DQ836061)	F: 5′-ATGGCTTTGGCTGGTATTG-3′	166–184	241	58
	R: 5′-AAGTAGGGTTTGTAACGGGTG-3′	386–406		
16S rRNA (L09162)	F: 5′-AGGAATACCAGTGGCGAAGG-3′	698–717	107	58
	R: 5′-CGTTTACGGCGTGGACTA-3′	787–804		

by an additional approach, probably through a transcription repressor. The complexity of the transcriptional regulation of *adhE* is also reflected in the structure of two promoter-RSB systems upstream of the open reading frame (Fig. 2).

Although the molecular mechanisms are not yet fully delineated, the effects of ethanol on the transcription of AdhA, AdhB, and AdhE, combined with the properties of the enzymes, can provide unique insights into ethanol metabolism in these bacteria. These include: (1) AdhB catalyzes reactions 1a and 2a to dump reducing power and to produce some ethanol in the early growth phase, therefore, AdhB activity for reaction 2b is enhanced by an increase in ethanol concentration; (2) the dumping of reducing power is slowed down by reaction 2b, and increased reducing power activates the transcription of *adhA* and *adhE*; (3) AdhE then catalyzes reaction 1a with a high activity, and AdhB together with AdhA, or also AdhE, catalyze reaction 2a for ethanol formation; and (4) following the accumulation of produced ethanol, the transcription of both *adhB* and *adhE* is gradually inhibited, and the activities of these enzymes are increased for the reverse reactions to balance the final concentration of ethanol. Wiegel and Ljungdahl, 1981 observed that ethanol formation in some thermophiles was strongly pH dependent. Only if the pH of the culture shifted from above pH 7.2 to below 6.9 was an ethanol to glucose ratio of 1.5–1.6 (mol/mol) obtained (Wiegel and Ljungdahl, 1984). The regulation behind this observation can be explained now: higher pH is desired for AdhB to produce some ethanol which activates the transcription of *adhA* and *adhE*, and lower pH favors AdhE to catalyze reaction 1a for a fast production of ethanol. The limitation of ethanol concentration could be a strategy of self-protection if there are any essential proteins that are sensitive to ethanol. However, *Tet* can be easily adapted to grow at 8% (v/v) supplemented ethanol concentration by serial transfers into media with increasing ethanol concentrations (Burdette et al., 2002). This observation supports a regulation theory, i.e. the limitation of ethanol concentration during fermentation is caused by a systematic regulation through transcriptions and activities of the key enzymes in the ethanol-formation pathway, rather than simply by the tolerance of the cells to ethanol. These results indicate that a high potential exists for the bacteria to produce ethanol above the presently observed concentrations of about 2% (w/v). To be economically competitive, ethanol concentrations around 4–5% (w/v) are achieved. The modifications of the regulatory genes and the enzyme encoding genes for AdhB and AdhE should offer a useful venue to genetically modify *Thermoanarobacter* species to produce ethanol at higher concentrations.

Acknowledgments

We would like to thank Dr. James A. Hoch for his helpful comments. This work was supported by the sub-project of "973" of China (2004CB719600) and the National Natural Science Foundation of China (30770061) and the Key Project from University Natural Science Foundation of Jiangsu, China (09KJA180003).

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