Teth137, a Conserved Factor of Unknown Function from *Thermoanaerobacter ethanolicus* JW200, Represses the Transcription of the *adhE* Gene In Vitro

Qingqing Jing · Jingkai Wang · Guogan Wu

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Abstract Teth137, a 13.7 kD protein of unknown function from *Thermoanaerobacter ethanolicus* JW200, is encoded by 360 nucleotides and has been obtained by DNA-coupled column previously. However, no function study of Teth137 has been published. Homologous modeling of Teth137 shows the protein is comprised of a helix-turn-helix motif which is a typical DNA-binding domain. Therefore, it is speculated Teth137 is a DNA-binding protein and involved in transcription of the *adhE* gene (encodes alcohol dehydrogenase E). To investigate the function of Teth137, recombinant Teth137 is overexpressed in *Escherichia coli* JM109 and purified by DEAE column. Purified Teth137 exhibits the affinity with the *adhE* promoter region in gel electrophoresis mobility shift assay (GEMSA). Teth137 at the concentration of 48 μ M retards the migration of 5 nM of probe in the presence of the competitor DNA.

Mutant analysis indicates that S69, T70, P71 and T72 are critical to protein–DNA interface; Gly substitutions at these residues results in the loss of the binding ability with the *adhE* promoter region. Moreover, *T. ethanolicus* JW200 RNA polymerase, σ subunit and template plasmid are prepared for in vitro transcription assay to detect the regulation function of Teth137. The results of the in vitro transcription show that the transcription of 5 nM of the template plasmid is inhibited by 48 μ M of Teth137.

Keywords *Thermoanaerobacter ethanolicus* · AdhE · GEMSA · In vitro transcription

Introduction

Due to scarcity of fossil fuel, more and more attempts have been focusing on alternate energy produced by microbial fermentation from renewable sources. The anaerobic thermophile, *Thermoanaerobacter ethanolicus* JW200, is a potential biofuel fermentation strain, thanks to its ability of producing ethanol from a wide range of carbon source and the convenience of separating ethanol in continuous cultures during the thermophilic growth stage [1, 2]. However, the ethanol productivity of this strain is low (below 3 %, w/v), which restricts its industrial application [3].

In the ethanol conversion of *T. ethanolicus*, three important enzymes are involved, which are an alcohol dehydrogenase AdhA, and two bifunctional alcohol dehydrogenases, AdhB and AdhE, encoded by the *adhA*, *adhB* and *adhE*, respectively [4–7]. AdhA is generally deemed to be responsible for the consumption of ethanol and AdhB is a critical enzyme during ethanol formation [4]. Recently, it is reported that the AdhE exhibits the highest activity of acetyl-CoA reduction in the simulated physiological

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Q. Jing
Modeling and Simulation Group, Qingdao Institute of Bioenergy and Bioprocess Technology, Chinese Academy of Sciences, Qingdao 266061, China
e-mail: jqj1985@gmail.com

J. Wang · G. Wu (✉)
Jiangsu Key Laboratory for Biodiversity and Bio-resources, College of Life Sciences, Nanjing Normal University, Nanjing 210046, China
e-mail: guoganwu@gmail.com

J. Wang
e-mail: wajingkai@163.com

G. Wu
College of Chemistry and Chemical Engineering, Southeast University, Nanjing 210096, China

conditions which is the first ethanol formation step whereas AdhB exhibits little activity in the same conditions [8]. And in the same study, the transcription of *adhE* is activated at ethanol concentration of 0.25 % but repressed at the ethanol concentration of 0.75 %. These findings imply that AdhE also plays an important role in ethanol formation of *T. ethanolicus* JW200 and its expression is regulated by some factors. These results may be one of reasons why the ethanol yield of *T. ethanolicus* is normally below 3 %.

To study the transcriptional regulation mechanism of the *adhE* gene in *T. ethanolicus* JW200, proteins binding with the column coupled with the *adhE* promoter region have been isolated and sequenced in our lab [9]. Based on one of the results of the N-terminal amino acid sequencing, Teth137, a 13.7 kD conserved protein without any function annotations in GenBank, has been obtained. Homologous modeling predicts the model of Teth137 is similar to the helix-turn-helix (HTH) domain of the regulator of polyketide synthase expression from *Bifidobacterium adolescentis* (PDB ID 3ONQ). We speculate that Teth137 is a DNA-binding protein and may be involved in transcription of the *adhE* gene. Revealing the function of the protein will benefit further understanding on the regulation mechanism of AdhE expression and promote strategies to increase the final ethanol yield. Therefore, the recombinant Teth137 is overproduced in *Escherichia coli* and purified, and the binding ability and the regulation function are investigated by GEMSA and in vitro transcription assay. The study illustrates that Teth137 is a transcriptional repressor of the *adhE* gene.

Materials and Methods

Strains

T. ethanolicus JW200 (ATCC 31550) was gifted by Dr. Juergen Wiegel (University of Georgia, USA) and cultured in the medium as described previously [10]. *E. coli* strain JM109 and BL21 (DE3) were used for cloning and expression, respectively.

Plasmids

All primers are listed in Table 1. A fragment carrying the *teth137* gene was amplified using the genomic DNA of *T. ethanolicus* JW200 as template and sequenced. Based on the sequencing result, the plasmid pTeth137 was created by amplifying the *teth137* gene from the genomic DNA and cloning it into the expression vector pET-28a(+). To express the Teth137 mutants S69G, Y70G, P71G and T72G, plasmids pS69G, pY70G, pP71G and pT72G were constructed by inverse PCR using pTeth137 as template. For the in vitro transcription assay, the template plasmid pTmpl was produced by cloning the fragment of sequence –223 to +77 relative to the *adhE* start codon (GenBank accession number GU586297.1 and DQ836061.1) into the pUC19 vector. To express the σ subunit of *T. ethanolicus* RNA polymerase (RNAP), the plasmid pRpoD was created by ligating the coding region *rpoD* (GenBank accession number ZP_08212133.1) fused with His-tag into pET-28a(+).

Table 1 Primers used in this study

	Primer pairs
Fragment carrying <i>teth137</i>	5'-AAGCACCTCAAGCAGCAGG-3' 5'-GACAAAGTTGCAAGCCAAACG-3'
pTeth137	5'-CCCCCATGGGGAATGAAGTAATAGGTA-3' 5'-CCCCTCGAGCTATTCTTTTAAAGTTTC-3'
pS69G	5'-TGTAGGATATTCTAAGCCTAATCTTTTTC-3' 5'-ATTAGAAACAAATTGGACAATT-3'
pY70G	5'-TGTAGGTTTCAGATAAGCCTAATCTTTTTC-3' 5'-ATTAGAAACAAATTGGACAATT-3'
pP71G	5'-TGTTTCATAAGATAAGCCTAATCTTTT-3' 5'-ATTAGAAACAAATTGGACAATT-3'
pT72G	5'-TTCAGGATAAGATAAGCCTAATTC-3' 5'-ATTAGAAACAAATTGGACAATT-3'
pRpoD	5'-CCCCCATGGGGAACAACGAAGAATTGTCT-3' 5'-CCCCTCGAGATCAAGGAAATCTTTTAATT-3'
pTmpl	5'-CCC ^{GG} ATCCAATCGTTTCATATCGAT-3' 5'-CCC ^{GA} ATTCTGTCTTTTAACATCCAGC-3'
Probe <i>adhE</i> promoter region	5'-AATCGTTTCATATCGAT-3' 5'-TTCTCTTCTTTCTTGTA-3' (DIG labeled)
Primer <i>adhE</i> -RT	5'-TGTCTTTTAACATCCAGC-3' (DIG labeled)

The underlined sequences are artificial restriction enzyme recognition sites

Homologous Modelling

Homologous modeling of Teth137 was built using the Phyre2 server (<http://www.sbg.bio.ic.ac.uk/phyre2/index.cgi>) [11].

Purification of Teth137 and Its Mutants

To express Teth137 and its mutants, corresponding plasmids were transformed into *E. coli* BL21 (DE3), respectively. Cells were grown at 37 °C to OD₆₀₀ of 0.6, and induced at 30 °C for 6 h with 1 mM isopropylthio- β -D-galactoside (IPTG). Then cells were harvested, resuspended in 20 ml of buffer A (25 mM Tris-HCl, 5 % glycerol, 0.1 mM EDTA, 1 mM dithiothreitol, pH 8.5) and disrupted by French Press (Thermo, USA) at 1500 PSI. After being heated at 69 °C for 30 min and centrifuged at 10,000 $\times g$ for 15 min, the supernatant was loaded onto HiTrap DEAE column (Amersham Biosciences, USA) equilibrated with buffer A. The protein was eluted from the column with a 100 ml linear gradient of 0–500 mM NaCl in buffer A at a flow rate of 0.5 ml/min. Fractions containing the pure protein were pooled and dialyzed against TGED buffer (25 mM Tris-HCl, 5 % glycerol, 0.1 mM EDTA, 1 mM dithiothreitol, pH 7.5).

GEMSAs

The probe DNA, 5' Digoxin (DIG) labeled *adhE* promoter region (−223 to +30), was prepared by PCR with the primers listed in Table 1. GEMSAs were performed according to the modified protocol [12]. The assays were carried out with 5 nM of labeled probe and different amounts of Teth137 (0, 3, 6, 12, 24 and 48 μ M) in the binding buffer (25 mM HEPES, 0.2 mM EDTA, 1 mM dithiothreitol, 160 mM NaCl, 5 % glycerol, 50 ng/ μ l sheared salmon sperm DNA, pH 7.5). Mixtures were incubated at 69 °C for 15 min and separated by 5 % polyacrylamide bis-acrylamide gels (40:1) in 0.5 $\times$ TBE buffer (44.5 mM Tris-HCl, 44.5 mM boric acid, 1 mM EDTA, pH 8.3) with 20 mA conducting current, the labeled probe was electro-blotted onto an N⁺ Hybond nylon membrane and detected by DIG Nucleic Acid Detection kit (Roche, USA). In the mutant analysis, 7 μ M of each mutant (S69G, Y70G, P71G or T72G) was used, respectively, in the reaction with 5 nM of the labeled *adhE* promoter region.

Purification of RNAP Core Enzyme and σ Subunit

8 l of *T. ethanolicus* JW200 cells were cultured and harvested in stationary phase (OD₆₀₀ of 1.8). The RNAP core enzyme was purified by the modified protocol [13], except

that the DNA-cellulose column was employed. Cells were suspended in 20 ml of TGED buffer, disrupted by French Press at 2,000 PSI and centrifuged at 10,000 $\times g$ for 15 min. Then 80 % saturated ammonium sulfate was applied in the extract. Following centrifugation at 6,000 $\times g$ for 15 min, the final pellet was dissolved with 2 ml of TGED buffer and dialyzed against the same buffer. Sample was loaded on the HiTrap Heparin HP column (Amersham Biosciences, USA) carried out in the TGED buffer, and eluted with a 50 ml linear gradient of 0–700 mM NaCl in TGED buffer. Pools containing RNAP were dialyzed against TGED buffer, and separated on DEAE-Sephacrose column and eluted with a 100 ml linear gradient of 0–500 mM NaCl in TGED buffer. Purified enzyme was collected and dialyzed against TGED buffer containing 200 mM KCl.

Recombinant σ subunit of *T. ethanolicus* RNAP was grown in 1 l of *E. coli* cells harboring the plasmid pRpoD, and induced with 1 mM IPTG for 6 h when OD₆₀₀ reached 0.6. *E. coli* was harvested and purified by His-tag affinity column (Novagen, USA) according to the manufacturer's instructions. The fractions containing pure σ subunit was collected and dialyzed against TGED buffer.

RNAP holoenzyme was reconstituted by adding a fourfold molar of pure σ subunit to the purified RNAP core enzyme in TGED buffer containing 200 mM KCl and incubated at 30 °C for 30 min [14].

In Vitro Transcription Assay

In vitro transcription assay was performed as described in [9]. RNA was synthesized in 10 μ l reaction mixture containing the transcription buffer (20 mM Tris-HCl, 10 mM MgCl₂, 10 % glycerol, 0.1 mM EDTA, 0.5 mM dithiothreitol, 200 mM KCl, pH 7.5), 5 nM of template plasmid pTmpl, and 0.2 mM each of ATP/CTP/GTP/UTP. In vitro transcription was initiated by adding 20 nM RNAP holoenzyme and terminated by 3 volume of ethanol and 1/10 volume of 4 M LiCl after incubation at 69 °C for 30 min. Following incubation at −70 °C for 30 min, the mRNAs were collected by being centrifuged at 12,000 $\times g$ for 30 min, washed with 500 μ l of 75 % cold ethanol and dried under a vacuum. The corresponding cDNAs were synthesized by using the SuperScript III kit (Invitrogen, USA) with the 5' DIG labeled primer *adhE*-RT (listed in Table 1). The RNA–DNA hybrids were digested with RNase H, and separated by 5 % polyacrylamide bis-acrylamide gels containing 7 M urea and 0.5 $\times$ TBE buffer. After electro-blotted onto an N⁺ Hybond nylon membrane, the cDNAs were detected by DIG Nucleic Acid Detection kit. To analyze the function of Teth137 in transcription reaction, the mixture containing 48 μ M of Teth137 was pre-incubated at 69 °C for 10 min before adding RNAP holoenzyme.

Fig. 1 SDS-PAGE of Teth137, RNAP core enzyme and the σ recombinant subunit. **a** Lane 1, molecular mass protein standards; lane 2, purified Teth137. **b** Lane 1, molecular mass protein standards; lane 2, purified RNAP core enzyme; lane 3, recombinant σ subunit

Results

Sequence Analysis of Teth137

A 2.6 kb fragment carrying the *teth137* coding region was amplified from *T. ethanolicus* JW200 and sequenced. The nucleotide sequence data have been submitted to the GenBank database under accession number JF809614. Sequence analysis displays the *teth137* encodes a 13.7 kD peptide of 119 amino acid residues. The molecular mass of Teth137 on 15 % SDS-PAGE is consistent with the data calculated (Fig. 1a). Amino acid sequence analysis shows that Teth137 is a member of DUF2089 protein family. The homologous proteins possess four conserved amino acid residues (S69, T70, P71 and T72) and the conserved Cys and Pro residues in the N-terminal sequence (Fig. 2a). However, no function description of Teth137 and homologous proteins are found in GenBank. Using the Phyre2

protein structure prediction server, the Teth137 model was built based on the HTH domain (sequence E177 to T248) of the regulator of polyketide synthase expression BAD_0249 from *B. adolescentis* (Fig. 2b). 67 residues of Teth137 (K32 to E98) are modeled with 99.6 % confidence, however, N-terminal sequence (M1–G31) is not included in the predicted model.

Activities of Teth137 and the Mutants

By GEMSAs, the ability of Teth137 binding to the *adhE* promoter region is confirmed. While the labeled *adhE* promoter region was used as the probe in the GEMSAs, Teth137 bound with the probe and retarded the migration of the probe in the presence of 50 ng/ μ l competitor DNA (Fig. 3a). In mutant analysis, the mutants (S69G, T70G, P71G and T72G) were applied in GEMSAs, respectively. The mutants lost their capability of forming detectable protein–DNA complexes (Fig. 3b).

Purification of RNAP

For in vitro transcription assay, purified *T. ethanolicus* RNAP core enzyme and recombinant σ subunit were prepared (Fig. 1b). *T. ethanolicus* RNAP core enzyme includes three subunits α , β and β' , which are estimated to be 150, 130 and 32 kD (lane 2). The purified σ subunit (RpoD) is shown in lane 3, and the molecular weight is about 41 kD.

Repression of the *adhE* Transcription by Teth137

In vitro transcription was carried out in the presence and absence of Teth137. As illustrated in Fig. 4a, the *adhE* promoter region was recognized by the RNAP holoenzyme

Fig. 2 A multiple alignment of Teth137 and the predicted 3D model of Teth137. **a** A multiple alignment of Teth137. Strongly conserved residues are in **bold**. The limits of the sequence are indicated by the residue positions, on each *end* of the sequence. Homologous proteins YP_001396096, YP_003564139, NP_970931 and YP_001620982 are from *Clostridium kluyveri* DSM 555, *Bacillus megaterium* QM

B1551, *Treponema denticola* ATCC 35405 and *Acholeplasma laidlawii* PG-8A, respectively. **b** Model and features of Teth137. Helices are shown in red and labeled with “H”. Conserved residues “SYPT” are shown in *stick* representation and the backbone oxygen atoms are hidden

Fig. 3 GEMSA of Teth137 and its mutants. *Open arrows* indicate unbound probes, and *closed arrows* indicate protein–DNA complexes. **a** Binding ability of Teth137 to the *adhE* promoter region at different concentrations. **b** Mutant analysis of Teth137. 5 nM of probe was applied in the reactions. WT indicates the reaction with 7 μM of Teth137; NC indicates the reaction without Teth137 or mutants

and two transcripts (approx. 240 and 90 nucleotides) were detected (lane 2). However, no detectable transcripts were produced when the transcription reaction was incubated with Teth137 (lane 3).

Discussion

In this study, Teth137, a protein binding with the *adhE* promoter region, is firstly cloned and expressed in *E. coli*, and the function of Teth137 is primarily investigated.

Teth137 is a conserved protein, and the homologous proteins are found in various prokaryotes (Fig. 2a), but no function annotation on these proteins are available in GenBank. Homologous modeling of Teth137 was performed with the aim of further understanding its function (Fig. 2b). The 3D model of Teth137 is comprised of three core helices and a characteristic sharp turn between the second and the third helix which is a typical HTH motif. This motif referred as a common DNA-binding domain can be found in many bacterial regulators (e.g., TrpR, AraC and CRP).

In the Teth137 model, the conserved residues “SYPT” locate in N-terminal of the third helix. It is reported that the third helix of typical HTH motif is suited in the major groove of DNA and responsible for the recognition in the protein–DNA interface [15]. The “SYPT” sequence locating in the recognition helix seems strongly conserved in the homologous protein, underlying the significance of these residues in the protein–DNA interface. And mutant analysis provides evidence that these “SYPT” are involved in the DNA affinity of Teth137. In Fig. 3b, the probe affinity of mutants was lost when these residues were substituted by Gly, respectively. We find that the Gly, whose side chain is only a hydrogen atom, distinguishes itself from Ser, Tyr, Pro and Thr. The side chain of the substituted Gly is too short to form hydrogen bonds to the atoms of nucleotides and the affinity of mutants is weakened. Therefore, the hydrogen bonds between nucleotides and conserved residues SYPT is essential for protein–DNA binding.

In [9], several DNA-binding proteins binding with the *adhE* promoter region are obtained. And the *adhE* transcription has been demonstrated to be regulated by the redox sensing protein in the same study. To avoid the

Fig. 4 In vitro transcription assay of the *adhE* gene and the *adhE* promoter region. **a** In vitro transcription of 5 nM of template plasmid carrying the *adhE* gene in presence and absence of 48 μM of Teth137. The single strand RNA standards are showed on the left with bands.

b The transcriptional elements of the *adhE* promoter region. Putative translational elements, promoter I, promoter II, SD I and SD II, are *underlined*. The consensus sequences of promoter I and promoter II are in *bold*. The translation initial site “A” is marked with “+1”

interference of other regulation factors, in vitro transcription assay was carried out for the function analysis of Teth137. Before application of in vitro transcription, one important question should be replied first: which type of σ subunits is appropriate to initiate the *adhE* gene transcription? Correct σ subunit binding with the RNAP core enzyme is required to start the promoter-specific transcription initiation [16]. Analysis of the *adhE* promoter region is found that both of the two promoters upstream of the *adhE* gene are typical σ^{70} promoters (Fig. 4b). In all available σ subunits from *T. ethanolicus* JW200, RpoD is probably the best σ subunit candidate to recognize and start the σ^{70} promoter, because all conserved domains of RpoD belong to σ^{70} family [17]. Based on the locations of the putative SD I and SD II elements on the *adhE* promoter region, it is speculated that the transcripts from the two promoters are about 90 and 240 nucleotides. In Fig. 4b, the two transcripts of expected lengths were detected (lane 2), suggesting that RpoD could start the transcription from promoter I and promoter II. Different yields of two transcripts also indicate that the two promoters have distinct transcriptional efficiencies, which are consistent with the results described in [18]. When Teth137 was added in the in vitro transcription reaction, the transcription at the two promoters were inhibited. It shows that the factor is the inhibitor repressing the transcription of the *adhE* gene.

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