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Membrane-Bound Alcohol Dehydrogenase Is Essential for Glyceric Acid Production in *Acetobacter tropicalis*

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Abstract: ***Acetobacter tropicalis*** **NBRC16470 can produce highly enantiomerically pure D-glyceric acid (D-GA; >99 % enantiomeric excess) from glycerol. To investigate whether membrane-bound alcohol dehydrogenase (mADH) is involved in GA production in *A. tropicalis*, we amplified part of the gene encoding mADH subunit I (*adhA*) using polymerase chain reaction and constructed an *adhA*-disrupted mutant of *A. tropicalis* (Δ*adhA*). Because Δ*adhA* did not produce GA, we confirmed that mADH is essential for the conversion of glycerol to GA. We also cloned and sequenced the entire region corresponding to *adhA* and *adhB*, which encodes mADH subunit II. The sequences showed high identities (84–86 %) with the equivalent mADH subunits from other *Acetobacter* spp.**

Key words: glycerol use, glyceric acid, alcohol dehydrogenase, *Acetobacter tropicalis*, acetic acid bacteria

1 INTRODUCTION

Glycerol is a renewable feedstock derived largely from natural sources such as vegetable oils and animal fats. It is produced as a by-product in biodiesel fuel (BDF) production and the oleochemical industry. As glycerol production expands owing to increased use of BDF, the price of glycerol is expected to drop. The efficient use of glycerol may be a key factor in both BDF and oleochemical manufacturing processes.

The production of glyceric acid (GA) from glycerol using biotechnological processes has been investigated as a potential use of glycerol[1-7]. GA has reported biological functions as a liver stimulant and cholesterolytic agent in dogs[8] and an inducer of ethanol and acetaldehyde oxidation in rats[9]. These properties suggest that GA is a promising chemical, but it is expensive as a reagent for investigational use. Hence, the development of a cheaper GA manufacturing process is important.

Previously, we investigated the biotechnological production of GA using acetic acid bacteria (AAB) and examined the abilities of 162 AAB strains to produce GA from glycerol. We found that the patterns of productivity and enantiomeric GA composition among the AAB strains differed significantly[4]. For example, under optimal cultivation conditions in a jar fermentor, *Gluconobacter frateurii* accumulated 136.5 g/L of GA with a 72% D-GA enantiomeric excess (ee), whereas *Acetobacter tropicalis* produced 101.8 g/L D-GA with a 99% ee. To the best of our knowledge, *A. tropicalis* NBRC16470 is the only species that can produce highly enantiomerically pure D-GA (>99% ee)[1,4].

At the same time, gene disruption analysis using *Gluconobacter oxydans* revealed that the membrane-bound alcohol dehydrogenase (mADH)-encoding gene *adhA* is required for GA production; moreover, purified mADH from *G. oxydans* catalyzed the oxidation of glycerol to glyceraldehyde[4]. These results suggest that mADHs are also involved in GA production in *G. frateurii* and *A. tropicalis*. No direct evidence shows that mADH is essential for GA production in *A. tropicalis*, however. Additionally, studies of mADH from *Acetobacter* spp., *Gluconobacter* spp., and *Gluconacetobacter* spp. have shown that mADH usually consists of three subunits (I, II, and III; for an excellent recent review, see Yakushi and Matsushita, 2010)[10]; however, no sequences of the respective mADH components from *A. tropicalis* have been available to date.

Subunit I contains pyrroloquinoline quinone (PQQ) and

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one heme *c* moiety as a prosthetic group, and functions as the actual catalytic site for glycerol oxidation as well as ethanol oxidation, whereas subunit II works as the electron mediator from subunit I, and subunit III seems to be a molecular chaperone for the folding of subunit I[10]. Among these mADH subunits, the sequence of subunit I is the most important for us to know as a first step in understanding how *A. tropicalis* produces enantiomerically pure D-GA.

In this study, we first investigated the involvement of mADH in GA production in *A. tropicalis* (Fig. 1). We then cloned and sequenced the DNA region containing the two genes encoding mADH subunits.

2 EXPERIMENTAL PROCEDURES

2.1 Materials

All reagents and solvents were commercially available and of the highest purity (Sigma-Aldrich, St. Louis, MO; Kanto Chemical, Tokyo, Japan; Wako Pure Chemical, Osaka, Japan; Nacalai Tesque, Kyoto, Japan).

2.2 Apparatus

The concentration of D-GA in culture broth was analyzed using high-performance liquid chromatography (HPLC) with an LC-20AD HPLC pump (1.0 mL/min flow rate) and an RID-10A detector (Shimadzu) equipped with a Shodex® SH1011 column (Showa Denko) for GA. Mobile phases of 5 mM H_2SO_4 was used for the column. During the analysis, the columns were maintained at 60℃.

2.3 Bacterial strains

A. tropicalis NBRC16470 was originally obtained from the National Institute of Technology and Evaluation (NITE), Japan[1, 4]. Stock cultures were routinely cultivated at 30℃ on agar medium containing 5 g/L polypeptone (Nihon Pharmaceutical, Tokyo, Japan), 5 g/L yeast extract (Difco Laboratories, Detroit, MI), 5 g/L glucose, and 1 g/L $MgSO_4 \cdot 7H_2O$ (pH 6.5). When necessary, kanamycin (Km) was added to the media at a final concentration of 50 μg/mL. *Escherichia coli* strain JM109[11] was cultivated at 37℃ using Luria-Bertani broth, 2× YT medium, or Terrific broth, as described by Sambrook and Russell (2001). Ampicillin and Km were added to the media when necessary at final concentrations of 50 μg/mL.

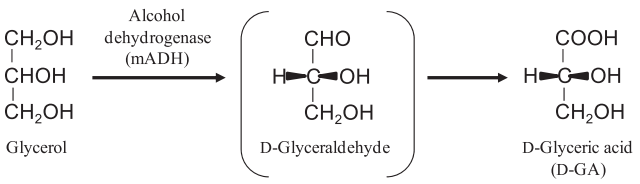

Fig. 1 Pathways for the conversion of glycerol to D-glyceric acid by *Acetobacter tropicalis*.

2.4 DNA manipulations

Total DNA extraction, plasmid isolation, restriction endonuclease digestion, cloning, and the transformation of *E. coli* were carried out using standard protocols[11].

2.5 PCR amplification of partial *adhA*

To amplify the DNA region within *adhA* using polymerase chain reaction (PCR), we used a primer set (AtADH-F1, 5′-GCTGACAAAGGCTGCTGTGA-3′, and AtADH-R1, 5′-AGGCTTCATGCTGGTGTCGA-3′) designed according to information from the draft genomic sequence of *A. tropicalis* SKU1100 (NBRC 101654; Matsutani *et al.*, submitted for publication; the data will be available under GenBank accession nos. BABS01000001-BABS01000773). After an initial denaturation at 94℃ for 2 min, three cycles at 94℃ for 1 min, at 65℃ for 1 min, and at 72℃ for 2 min were run with the above primers. The program ended with a 10-min extension at 72℃. The product (approximately 1.5 kb) was purified using agarose gel electrophoresis, cloned into pT7Blue (R) (Novagen, Madison, WI), and sequenced (designated pT7-adhA).

2.6 Disruption of *adhA* and GA productivity of Δ*adhA*

The DNA region containing the Km^r cassette (~0.9 kb) from pTKm[12] was excised via *Eco*RV digestion and ligated to the *Hpa*I site in the partial *adhA* gene of pT7-adhA. The resultant plasmid, pT7-AtADH::Km, was introduced into *A. tropicalis* NBRC16470 with electroporation (electroporator settings: 200 Ω, 25 μF, and 1.9 kV). Next, the *adhA* disruptant, Δ*adh*A, was screened on YPG (5 g/L yeast extract, 2 g/L peptone, and 30 g/L glucose) plates[13] containing Km.

Wild-type *A. tropicalis* and its Δ*adhA* mutant were precultivated in 5 mL of media consisting of 5 g/L glucose, 20 g/L glycerol, 10 g/L yeast extract, 10 g/L polypeptone, and 4 g/L potato extract (Difco Laboratories) at 30℃ for 1 day, after which the seed cultures (1.5 mL) were transferred to 300-mL Erlenmeyer flasks containing 30 mL of media consisting of 150 g/L glycerol, 0.9 g/L KH_2PO_4, 0.1 g/L K_2HPO_4, 20 g/L yeast extract, and 1 g/L $MgSO_4 \cdot 7H_2O$ (pH 6.5). The flasks were incubated for 4 days at 30℃ on a rotary shaker (200 rpm). After removal of the cells with centrifugation, the respective supernatants were analyzed using HPLC.

2.7 Genomic library screening and sequence analysis

A genomic fosmid library of *A. tropicalis* was constructed as described previously[14]. To screen for positive clones carrying the amplified DNA fragment described above, colony PCR experiments were carried out using 96-well deep-well plates and the primers AtADH-F1 and AtADH-R1. One of the positive fosmid clones, pCC1FOSAT1, was used in our subsequent analyses. Sequencing of pCC1FOSAT1 was performed at the Takara Dragon Ge-

nomics Center (Mie, Japan). To identify open reading frames (ORFs), the nucleotide sequence was analyzed with Genetix software (version 8.0; Genetix, New Milton, UK). Homology searches were carried out using the SWISS-PROT protein sequence database or DNA DataBank of Japan (DDBJ), European Molecular Biology Laboratory (EMBL), and GenBank nucleotide sequence databases with the BLAST program[15]. All automated ORF predictions and functional assignments were controlled manually for the entire contiguous DNA sequence.

2.8 Nucleotide sequence accession number

The nucleotide sequence of the 9.9-kb DNA region in the pCC1FOSAT1 insert was deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession no. AB623210.

3 RESULTS AND DISCUSSION

3.1 PCR amplification of the internal DNA region of *adhA* from *A. tropicalis* NBRC16470

As a preliminary step toward analyzing the sequences of mADH genes from *A. tropicalis* NBRC16470, we attempted to amplify the internal region of *adhA* with PCR using the primer set described above. The amplified DNA fragment was cloned into pT7Blue (R) to construct pT7-adhA and then sequenced. The partial *adhA* homolog from *A. tropicalis* NBRC16470 was similar to the *adhA* sequences from *Acetobacter pasteurianus*[16] and *Acetobacter aceti*[17]; the sequence of the translated polypeptide was 86% identical to AdhA from *A. pasteurianus* and 85% identical to that from *A. aceti*. Usually, AdhA contains PQQ and one heme *c*, and the amino acid residues involved in both PQQ and heme *c* binding have been identified in ADH based on genetic and structural information[18]. Within the sequenced region of the AdhA homolog, amino acid residues involved in PQQ binding were also identified (alignment not shown).

3.2 Involvement of mADH in GA production

To investigate the involvement of *adhA* in GA production, the *adhA* homolog was disrupted via homologous recombination. For this purpose, pT7-AtADH::Km was first constructed using both pT7-adhA and the Km^r cassette from pTKm[12] and was subsequently introduced into *A. tropicalis* NBRC16470 to isolate the recombinant. The resultant mutant, Δ*adhA*, was examined for its GA productivity. As shown in Fig. 2, the wild-type strain produced 11.8 g/L GA, whereas Δ*adhA* produced only 0.09 g/L GA after a 4-day incubation. This indicates that the *adhA* homolog is a component of mADH and that mADH is involved in GA production in *A. tropicalis* NBRC16470.

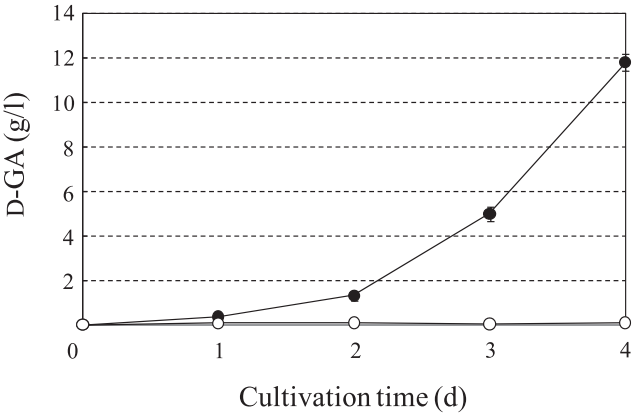

Fig. 2 Time course of D-glyceric acid production from glycerol by *Acetobacter tropicalis* NBRC16470 and Δ*adhA*. Black circles, wild-type *A. tropicalis* NBRC16470; white circles, Δ*adhA*.

3.3 Isolation of fosmid clones carrying *adhA*

A fosmid library of genomic DNA from *A. tropicalis* was constructed. In the initial screen for positive clones, 576 of the resultant fosmid clones were screened with colony PCR using 96-well deep-well plates (six clones per well), and in nine wells, the amplification of an approximately 1.5-kb DNA fragment was observed. Next, using the 54 clones from the nine wells, a second round of screening was performed. Amplification of the expected DNA fragment was observed in three clones. One of them, pCC1FOSAT1, was selected for further sequence analysis.

3.4 Nucleotide sequence analysis and identification of the genes encoding mADH subunits

Sequencing of part of the fosmid clone gave a 9.9-kb contiguous sequence. In this region, we found six ORFs, and among them, ORF5 and ORF6 were homologous to *adhA* (mADH subunit I) and *adhB* (mADH subunit II), respectively (Fig. 3; Table 1). Upstream of the *adhAB* genes, we identified ORFs homologous to the genes encoding cytochrome *o* ubiquinol oxidase subunits I, II, and III (Fig. 3;

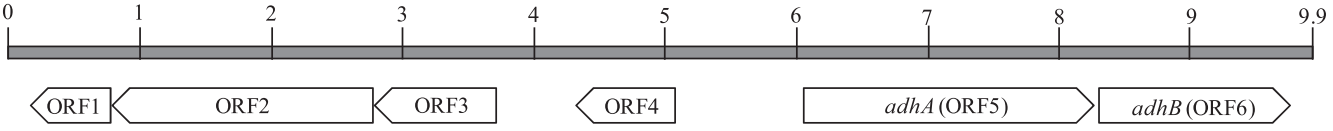

Fig. 3 Diagram indicating the positions of open reading frames present in the fosmid insert (9.9 kb). Blocks indicate the size, location, and direction of transcription of the genes.

Table 1 Annotated open reading frames (ORFs) of the DNA region analyzed (9.9 kb).

ORF			Homologous protein			
Name	Length (aa)	Probable Function	Protein	Source	Identity (aa %)	Accession No.
ORF1*	201	Cytochrome *o* ubiquinol oxidase, subunit III	Cytochrome *o* ubiquinol oxidase, subunit III	*Acetobacter pomorum* DM001	92	EGE47621
ORF2*	663	Cytochrome *o* ubiquinol oxidase, subunit I	Cytochrome *o* ubiquinol oxidase subunit I	*Acetobacter pasteurianus* IFO3283-01	93	YP_003186627
ORF3*	307	Cytochrome *o* ubiquinol oxidase, subunit II	Cytochrome *o* ubiquinol oxidase subunit II	*Acetobacter pasteurianus* IFO3283-01	94	YP_003186627
ORF4*	258	Unknown	Hypothetical protein	*Acetobacter pomorum* DM001	79	EGE47618
ORF5	742	Membrane-bound alcohol dehydrogenase, subunit I	AdhA	*Acetobacter pasteurianus* IFO3283-01	86	YP_003186627
ORF6	144	Membrane-bound alcohol dehydrogenase, subunit II	AdhB	*Acetobacter pasteurianus* IFO3283-01	85	YP_003186627

*ORFs are encoded in the complementary strand.

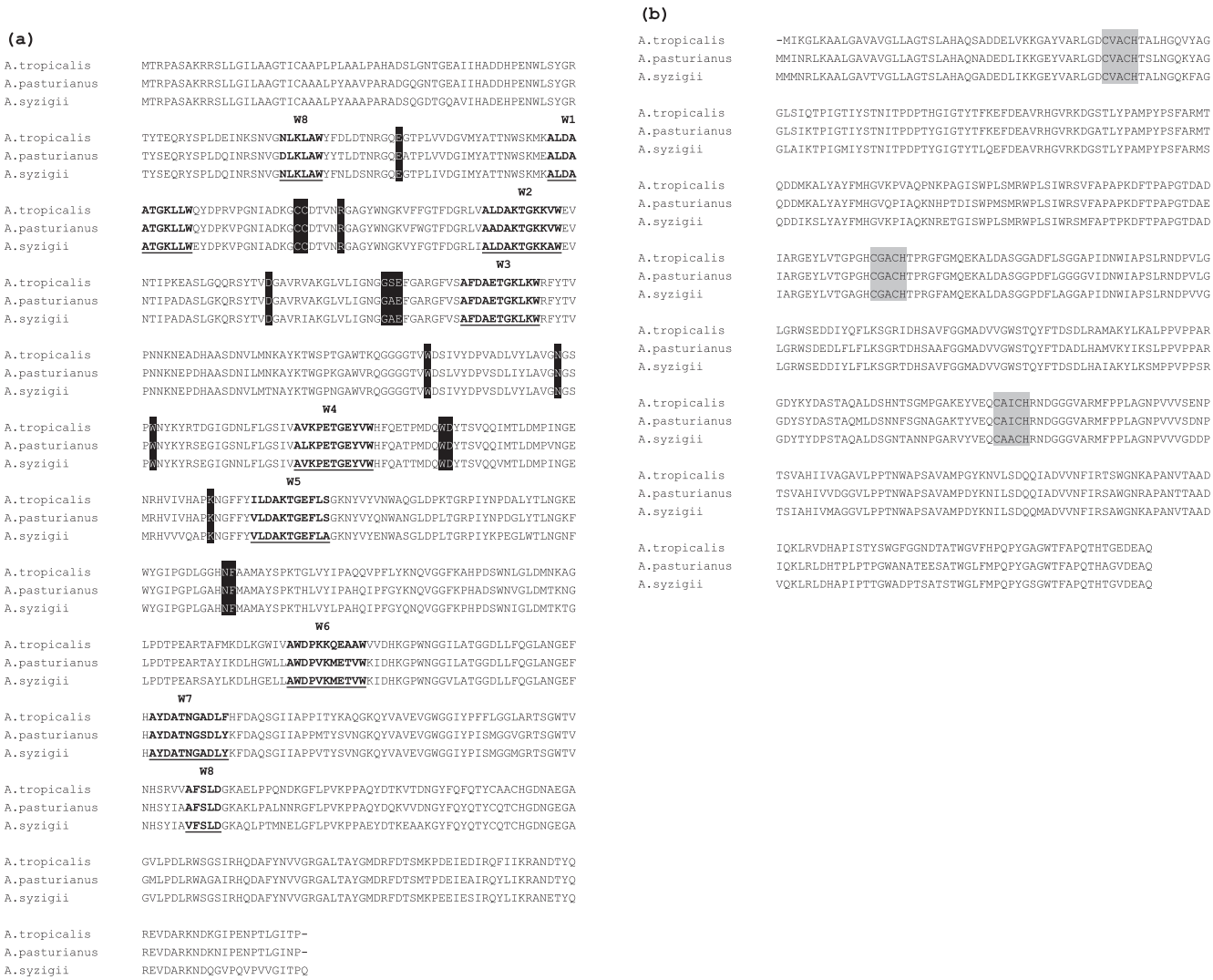

Fig. 4 Alignment of the amino acid sequences of the respective membrane-bound alcohol dehydrogenase subunits. (a) Subunit I (AdhA), (b) subunit II (AdhB). W-motifs (W1-W8) are underlined with bold letters. Amino acid residues involved in pyrroloquinoline quinone (PQQ)-binding and heme *C* binding are indicated by highlighted letters and shaded in grey, respectively.

see Table 1).

(i) AdhA. The nucleotide sequence of *adhA* is 2,229 bp, and the deduced amino acid sequences of *adhA* shared the highest identity with AdhA from *A. pasteurianus* IFO3283-01 (86%)[16], *A. aceti* (86%)[17], and *A. syzygii* (84%; accession no. BAE97418). In the AdhA sequence from *A. tropicalis*, amino acid residues involved in PQQ and heme *c* binding were highly conserved (Fig. 4a).

PQQ-containing quinoproteins have a common basic structure called a propeller fold superbarrel made up of eight four-stranded anti-parallel β-sheets called W motifs because they look like the letter W[18, 19]. In the AdhA sequence, W-motifs (W1-W8) were also conserved (see Fig. 4a).

(ii) AdhB. The nucleotide sequence of *adhB* is 1,416 bp, and the deduced amino acid sequences of *adhB* shared the highest homology with AdhB from *A. pasteurianus* IFO3283-01 (85%)[16], *Acetobacter pomorum* DM001 (85%; EGE47615), and *Acetobacter syzygii* (84%; accession no. BAE97419). In the AdhB sequence from *A. tropicalis*, amino acid residues involved in heme *c* binding (three regions) were conserved (Fig. 4b).

The results of our sequence comparison of mADH subunit-encoding genes suggest that these two genes, which are highly homologous to *adhA* and *adhB* from *Acetobacter* spp., are functional in *A. tropicalis* NBRC16470. The strong homologies among *A. tropicalis*, *A. pasteurianus*, and *A. syzygii* suggest that mADH is also involved in GA production in *A. pasteurianus* and *A. syzygii*.

4 CONCLUSIONS

This study showed that mADH is essential for GA production in *A. tropicalis* NBRC14670, which produces enantiomerically pure D-GA, because the disruption of *adhA*, which encodes a dehydrogenase subunit (subunit I) of mADH, eliminated GA production. We also isolated and sequenced the genes encoding the subunits of mADH (*adhA* and *adhB*). The strong sequence similarities to the corresponding mADH components from other *Acetobacter* spp. suggest that these subunits are functional in *A. tropicalis* NBRC16470. To understand the detailed mechanism of enantiomerically pure D-GA production by mADH, purification and crystallization of the enzyme complex, including subunit III, to determine the three-dimensional structure will be necessary.

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