

Genetic analysis around aminoalcohol dehydrogenase gene of *Rhodococcus erythropolis* MAK154: a putative GntR transcription factor in transcriptional regulation

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Abstract NADP⁺-dependent aminoalcohol dehydrogenase (AADH) of *Rhodococcus erythropolis* MAK154 catalyzes the reduction of (*S*)-1-phenyl-1-keto-2-methylaminopropane ((*S*)-MAK) to *d*-pseudoephedrine, which is used as a pharmaceutical. AADH is suggested to participate in aminoalcohol or aminoketone metabolism in this organism because it is induced by the addition of several aminoalcohols, such as 1-amino-2-propanol. Genetic analysis of around the *aadh* gene showed that some open reading frames (ORFs) are involved in this metabolic pathway. Four of these ORFs might form a carboxysome-like polyhedral organelle, and others are predicted to encode aminotransferase, aldehyde dehydrogenase, phosphotransferase, and regulator protein. OrfE, a homologous ORF of the FadR subfamily of GntR transcriptional regulators, lies downstream from *aadh*. To investigate whether or not *orfE* plays a role in the regulation of *aadh* expression, the gene disruption mutant of *R. erythropolis* MAK154 was constructed. The Δ *orfE* strain showed higher AADH activity than wild-type strain. In addition, a transformed strain, which harbored multi-*orfE*, showed no AADH activity even in the induced condition

with 1-amino-2-propanol. These results suggest that OrfE is a negative regulator that represses *aadh* expression in the absence of 1-amino-2-propanol.

Keywords *Rhodococcus erythropolis* · Aminoalcohol dehydrogenase · Aminoalcohol metabolism · GntR family transcriptional regulator

Introduction

NAD(P)⁺-dependent L-1-amino-2-propanol dehydrogenase (EC 1.1.1.75) is found in several microorganisms, such as *Escherichia coli*, *Pseudomonas* sp., and *Bacillus subtilis* (Turner 1967; Turner and Willetts 1967; Pickard et al. 1968; Willetts and Turner 1971; Faulkner and Turner 1974). These enzymes might be involved in the metabolism of aminoacetone, which is formed from threonine in the case of *B. subtilis* (Willetts and Turner 1971). On the other hand, NAD⁺-dependent D-1-amino-2-propanol dehydrogenase, which is also involved in threonine metabolism via aminoacetone, was found in *E. coli* cells (Dekker and Swain 1968; Lowe and Turner 1970; Campbell and Dekker 1973; Campbell et al. 1978; Kelley and Dekker 1984, 1985). These enzymes might play important roles in the metabolism of 1-amino-2-propanol, aminoacetone, or threonine, but biochemical and enzymological analyses of their enzyme properties have been insufficient, especially for L-1-amino-2-propanol dehydrogenase.

We have recently discovered NADP⁺-dependent L-1-amino-2-propanol dehydrogenase activity in *Rhodococcus erythropolis* MAK154 and cloned the gene encoding this enzyme (hereafter referred to as aminoalcohol dehydrogenase (AADH)) (Kataoka et al. 2006, 2008). AADH also catalyzes the reduction of (*S*)-1-phenyl-1-keto-2-methyl-

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aminopropane ((*S*)-MAK) to *d*-pseudoephedrine with an optical purity of >99% *e.e.*, accompanied by stereo-position recognition of the methylamino group (α -carbon) of (*S*)-MAK in racemic MAK (Kataoka et al. 2006). Many enzymes have been found to catalyze the asymmetric reduction of prochiral carbonyl compounds, but AADH exhibits a unique stereospecificity: the formation of a double chiral compound. Because of its unique and high stereoselectivity, AADH might have strong potential for practical application to chiral aminoalcohol production.

This enzyme activity was induced by the addition of any of several aminoalcohols, such as 1-amino-2-propanol, during the growth of *R. erythropolis* MAK154. However, the expression level of this enzyme was quite low even in induced condition. Therefore, mutant strains showing higher AADH activity are needed when this microorganism is used as a biocatalyst of *d*-pseudoephedrine production. In *R. erythropolis* PR4, the whole genomic sequence has been determined (GenBank accession no. AP008957), and no gene showed high homology with *aadh*. Therefore, AADH seemed to be a unique enzyme in *R. erythropolis* MAK154, and it is interesting what role AADH plays in this bacterium. Here, we report the genetic analysis of the gene around *aadh* and the construction of a mutant *R. erythropolis* MAK154 strain that has higher AADH activity with the disruption of the putative regulator gene.

Materials and methods

Chemicals and reagents

(*RS*)-1-Phenyl-1-keto-2-ethylaminopropane (EAM) and 2-ethylamino-1-phenyl-1-propanol (EPE) were donated from Daiichi Fine Chemical (Toyama, Japan). Glucose dehydrogenase was purchased from Amano Enzyme (Aichi, Japan). *Rhodococcus*–*Escherichia* shuttle vector pK4 (Hashimoto et al. 1992) was kindly gifted from Mitsubishi Rayon (Tokyo, Japan). All restriction enzymes and DNA modification enzymes were purchased from Takara-Bio (Shiga, Japan). All other chemicals used in this study were of analytical grade, commercially available, and used without further purification.

Bacterial strains and culture conditions

R. erythropolis MAK154 (FERM BP-7451; International Patent Organism Depository, National Institute of Advanced Industrial Science and Technology, Japan) was cultured at 28°C in medium (pH 7.0) containing 10 mg/ml glucose, 15 mg/ml peptone, 3 mg/ml KH₂PO₄, 2 mg/ml NaCl, 1 mg/ml yeast extract, and 0.2 mg/ml MgSO₄·7H₂O. DL-1-Amino-2-propanol and/or kanamycin was added into

the medium for final concentrations of 0.1% (*v/v*) and/or 75 µg/ml, respectively. *E. coli* JM109 was employed as a regular cloning host. *E. coli* XL1-Blue MRF' was used as a host for propagating cosmid clones. All *E. coli* transformants were grown in Luria–Bertani (LB) medium (10 mg/ml tryptone, 5 mg/ml yeast extract and 10 mg/ml NaCl). Antibiotics were included as appropriate at the following concentrations: ampicillin, 50 µg/ml; kanamycin 25 µg/ml.

General DNA technique and DNA sequence analysis

All basic DNA procedures, such as genomic or plasmid DNA preparation, restriction enzyme digestion, ligation of DNA, and transformation of *E. coli*, were followed as standard protocols (Sambrook and Russell 2001). PCR was performed using a PTC-200 thermal cycler (MJ Research, Watertown, MA, USA) and Ex-Taq DNA polymerase (Takara-Bio). The nucleotide sequence was determined by the dideoxy chain termination method (Sanger et al. 1977) using a CEQ dye terminator cycle sequencing kit (Beckman Coulter, Fullerton, CA, USA) with an automated sequencer DNA analysis system, CEQ2000XL (Beckman Coulter).

Construction and screening of genomic library

The total DNA of *R. erythropolis* MAK154 was partially digested with *Mbo*I and ligated with *Bam*HI/*Xba*I-treated SuperCos I (Stratagene, La Jolla, CA, USA). The *in vitro* packaging process was carried out using a Gigapack III XL packaging extract (Stratagene). The packaging phages were transfected into *E. coli* XL1-Blue MRF'. A library of cosmid clones was screened by the colony hybridization method using the AlkPhos Direct Labeling and Detection System (GE Healthcare Bio-Sciences, Buckinghamshire, UK), and a full-length *aadh* gene was used as a hybridization probe, which was amplified with PCR using primers AADH-N (5'-GAATTCATGTTCAACTCCATTGAAGGTCGTT-3') and AADH-C (5'-CCCGGGTACAGTTCGCCGAGCGC-3'). A cosmid vector obtained from a positive clone was named pMAK4000 and used for further analysis.

Construction of disruption mutant of *orfE*

A 3-kb *Sac*II-digested fragment that was derived from pMAK4000 and that contained partial *aadh* and *orfE* and the entire *orfE* was blunt-ended and then ligated with *Sma*I-digested pBluescript II SK+ (Stratagene). The resulting plasmid pBMAK300 was digested with *Sma*I. A larger fragment, from which most of *orfE* had been removed, was recovered. This fragment was then ligated with an *Eco*RV-digested PCR fragment containing the kanamycin resis-

tance gene (*Km^r*) obtained by PCR using pK4 as template and primers Km-f (5'-GGGATATCTAGCGGGTCTACAGAGTTC-3'; *EcoRV* site was underlined) and Km-r (5'-GGGATATCCAGCTGGCGTAATAGCGAAG-3'; *EcoRV* site was underlined). The resulting plasmid, pBMAK360, was used for the construction of an *orfE* disruption mutant (see Fig. 2).

The plasmid pBMAK360 was introduced into *R. erythropolis* MAK154 by electroporation, as follows. A mid-exponential culture of *R. erythropolis* MAK154 cells was harvested and washed with cold demineralized water. Cells were then concentrated 20-fold in cold demineralized water (compared with culture volume) and kept on ice. Ice-cold cells (200 μ l) were mixed with 200 ng of pBMAK360 in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) in a 2-mm gapped electrocuvette, and subjected to a 2.5-kV electric pulse from a Gene Pulser (Bio-Rad Laboratories, Hercules, CA, USA) connected to a pulse controller (25 μ F capacitor; external resistance, 400 Ω). Pulsed cells were diluted immediately with 1 ml of MYP medium (Hashimoto et al. 1992) and incubated for 2 h at 28°C. They were then spread on LB medium containing 75 μ g/ml kanamycin. Kanamycin resistance clones were cultured, and total the DNA was isolated. The homologous recombination was checked with PCR using primers *orfE*-N (5'-GGGATATCTATGGGTGCGCAAAGTCTGAGG-3') and *orfE*-C (5'-GGGATATCTCAGGATTCGGAGTCGGTGC-3'), with which the entire region of *orfE* was amplified.

Construction of strain bearing multi-copy numbers of *orfE*

A 2.2-kb *EcoRV/ScaI* fragment of pBMAK300, which contained 70-bp upstream to 1,200-bp downstream of *orfE* (Fig. 2) was ligated with *FspI*-digested *Rhodococcus*–*Escherichia* shuttle vector pK4. *R. erythropolis* MAK154 was transformed with the resulting plasmid pKMAK309 using the electroporation method as described above.

AADH activity analysis

AADH activity was measured with a bioconversion rate of (*RS*)-EAM to EPE. EAM and EPE are corresponding analogs of MAK and *d*-pseudoephedrine, and EAM is a good substrate for AADH. The wild-type or Δ *orfE* strain of *R. erythropolis* MAK154 was cultured in medium with or without 0.1% (*v/v*) 1-amino-2-propanol for 24 h. Cells were harvested, frozen at –80°C, and lyophilized. The reaction was carried out in 500 μ l of reaction mixture comprised of 2 mg/ml each of lyophilized cells, 100 mM sodium phosphate (pH 8.0), 0.6 mg/ml NADP⁺, 125 mM glucose, 20 mg/ml (*RS*)-EAM, and 15 units/ml glucose dehydrogenase. Incubation was performed at 30°C with shaking for 24 h. After centrifugation, the resulting supernatant was

analyzed by HPLC on an Inertsil Ph-3 column (4.6 \times 75 mm; GL Science, Tokyo, Japan) at 40°C. Then, 3% (*v/v*) acetonitrile containing 50 mM NaH₂PO₄ was used as a mobile phase, and the flow rate was 1.2 ml/min. The absorbance of the eluent was monitored at 220 nm.

RT-PCR

After the wild-type or Δ *orfE* strain of *R. erythropolis* MAK154 was cultured, 1-amino-2-propanol was added for a final concentration of 0.1% (*v/v*) when OD₆₆₀ reached 0.4, and the cells were further cultivated for 2 h. Total RNA was extracted from these cells using RNeasy Mini Kit (Qiagen, Valencia, CA, USA). The relative amount of *aadh* RNA was quantified with real-time PCR using the LightCycler Quick System 350S (Roche Diagnostics, Mannheim, Germany), LightCycler RNA Amplification Kit SYBR Green I (Roche Diagnostics) and primers *aadh*-f (5'-GAGGACATGGACAGCG-3') and *aadh*-r (5'-GCAAAGAACAGTGGCG-3'). mRNA of the glyceraldehyde-3-phosphate dehydrogenase gene was used as an internal standard amplified with primers *gapdh*-f (5'-CTGCCCTCAACATCGT-3') and *gapdh*-r (5'-GTTGGAGTAGCCCCAC-3').

Results

Sequence analysis of the gene around *aadh*

A genomic cosmid library of *R. erythropolis* MAK154 was constructed in SuperCos I. About 2,000 cosmid clones were screened using the colony hybridization method. With the resulting positive clone of pMAK4000, the nucleotide sequence of an approximately 14-kb DNA region around the *aadh* gene was determined (GenBank accession no. AB573180). Overall, the ORFs were identified using the BLAST program. The computer-aided analysis of the 14-kb sequence revealed the presence of 14 complete ORFs (Fig. 1). Features of the sequence and selected alignments with other genes are listed in Table 1. Recently, whole or draft genomic sequences of some *Mycobacterium* strains, i.e., *Mycobacterium* sp. MCS, *Mycobacterium* sp. KMS, *Mycobacterium* sp. JLS, *Mycobacterium flavescens* PYR-GCK, and *Mycobacterium vanbaalenii* PYR-1, each of which can degrade pyridine or polycyclic aromatic hydrocarbon, were determined at the US DOE Joint Genome Institute. In most of these microorganisms, the homologous genes of 11 ORFs (*orfV*, *-W*, *-X*, *-Y*, *-Z*, *-A*, *-B*, *-C*, *-D*, *-E*, and *aadh*) exist in a cluster and in the same order with *R. erythropolis* MAK154 (gene ID Mmcs_0199 to 0189, in the case of *Mycobacterium* sp. MCS, Fig. 1). In *R. erythropolis* PR4, there are no ORFs showing significant identities (over 40% in amino acid identity) with these ORFs.

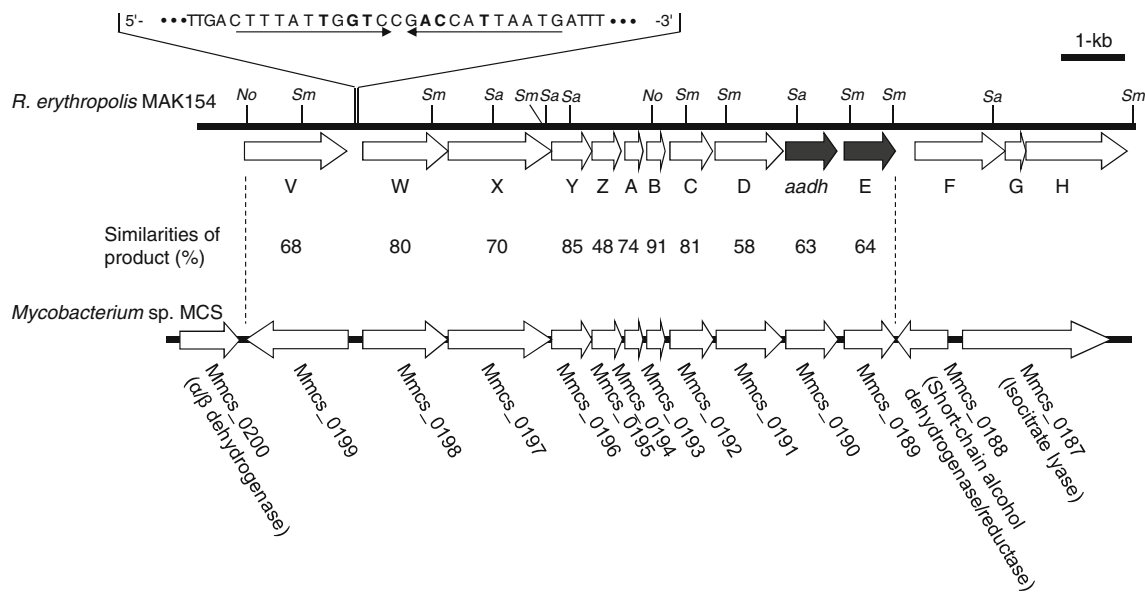


Fig. 1 ORF map of the 14-kb region around *aadh* isolated from *R. erythropolis* MAK154 and its homologous region of *Mycobacterium* sp. MCS. Open arrows indicate the ORFs and their direction of transcription. The *aadh* and *orfE* are shown in solid gray. The similarities of deduced amino acids from each corresponding ORF are

also shown in figure. *NotI*, *SacII*, and *SmaI* restriction enzyme sites are shown with *No*, *Sa*, and *Sm*, respectively. The putative OrfE binding sequences are shown with the text. Arrows under the text indicate the palindromic regions of the OrfE binding sequences

Table 1 Features and similarities between the ORFs and known proteins

ORF	Gene		Length (aa size)	Closest similarity to a known protein (organism, accession no.)	Similarity (%) ^a	Putative function
	Start (bp)	Stop (bp)				
<i>orfV</i>	395	1,969	524	Amino acid permease (<i>Streptomyces avermitilis</i> , NP_825720)	58	Permease
<i>orfW</i>	2,199	3,485	428	Ornithine-oxo-acid transaminase (<i>Mesorhizobium loti</i> , NP_107168)	61	Aminotransferase
<i>orfX</i>	3,485	5,047	520	Aldehyde dehydrogenase (<i>Verminephrobacter eiseniae</i> , YP_995461)	46	Aldehyde dehydrogenase
<i>orfY</i>	5,051	5,653	200	Hypothetical protein (<i>Nostoc</i> sp., NP_487297)	47	Shell of microcompartment
<i>orfZ</i>	5,686	6,141	162	Hypothetical protein (<i>Rhodococcus jostii</i> , YP_708763)	36	Unknown
<i>orfA</i>	6,141	6,416	91	Ethanolamine utilization protein eutN (<i>Verminephrobacter eiseniae</i> , YP_995464)	57	Shell of microcompartment
<i>orfB</i>	6,479	6,760	93	Ethanolamine utilization protein eutM (<i>Verminephrobacter eiseniae</i> , YP_995465)	68	Shell of microcompartment
<i>orfC</i>	6,833	7,486	217	Hypothetical protein (<i>Verminephrobacter eiseniae</i> , YP_995466)	58	Shell of microcompartment
<i>orfD</i>	7,520	8,536	338	Aminoglycoside phosphotransferase (<i>Rhodospirillum rubrum</i> , YP_428098)	41	Phosphotransferase
<i>aadh</i>	8,575	9,354	259	3-Ketoacyl-CoA reductase (<i>Mesorhizobium loti</i> , NP_107170)	59	Aminoalcohol dehydrogenase
<i>orfE</i>	9,305	10,228	256	Transcriptional regulator (<i>Bacillus</i> sp., ZP_01171645)	36	Transcriptional regulator
<i>orfF</i>	10,530	11,897	456	Acetylmithine deacetylase (<i>Bacillus clausii</i> , YP_174168)	33	Deacetylase
<i>orfG</i>	11,894	12,193	99	Hypothetical protein (<i>Pseudomonas syringae</i> , NP_792975)	50	Unknown
<i>orfH</i>	12,195	13,715	506	Sodium: solute symporter (<i>Pseudomonas syringae</i> , YP_274404)	55	Symporter

^a Regional (limited) similarity

Construction of *orfE* disruption mutant

OrfE showed homology with many GntR family transcriptional regulator proteins of several microorganisms. Because regulator proteins of prokaryotic cells are frequently located adjacent to genes under their control, we hypothesized that OrfE had some relationship with the expression of *aadh*. Thus, the *orfE* disruption mutant of *R. erythropolis* MAK154 ($\Delta orfE$ strain) was constructed as described in “Materials and methods” (Fig. 2). The gene disruption of mutant *R. erythropolis* MAK154 was confirmed with PCR analysis that amplified the entire region of *orfE* (data not shown). In the $\Delta orfE$ strain, an approximately 1,700-bp fragment (derived from $\Delta orfE::Km^r$) was amplified, and no fragment of about 770 bp (derived from wild-type *orfE*) was detected. Southern hybridization analysis also indicated that the homologous

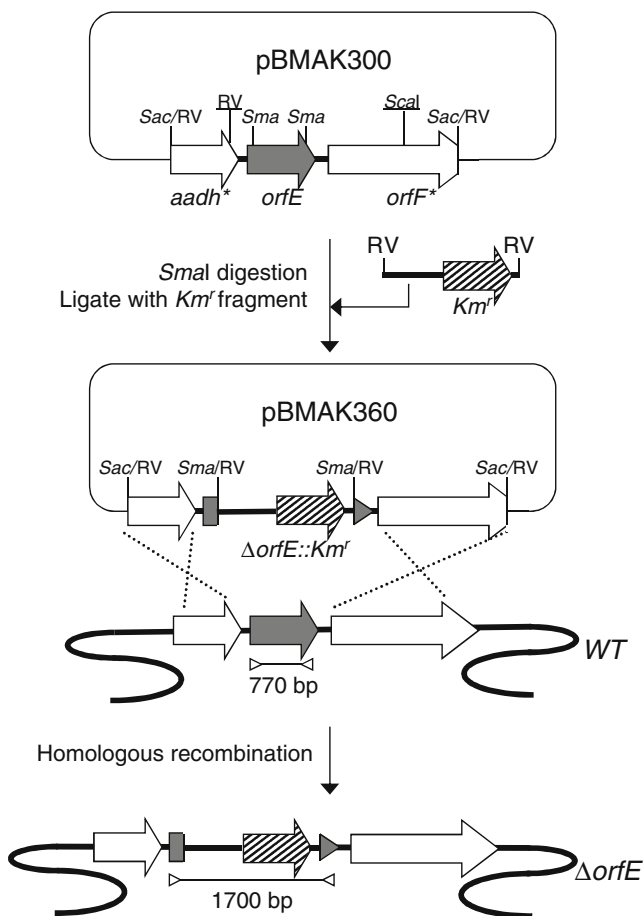


Fig. 2 Construction of *orfE*-disrupted mutant of *R. erythropolis* MAK154. Plasmid pBMAK360 was introduced into *R. erythropolis* MAK154 by electroporation, and gene disruption via a double-crossover homologous recombination was performed. *Sac*II, *Sma*I, and *Eco*RV restriction enzyme sites are shown with *Sac*, *Sma*, and *RV*, respectively. The *aadh** and *orfF** show respectively the partial genes of *aadh* and *orfF*. Open triangles are PCR primers to amplify the entire region of *orfE*. *RV* and *Sca*I are *Eco*RV and *Sca*I restriction enzyme sites, respectively, used for construction of pKMAK309

recombination occurred in the correct position (data not shown). The $\Delta orfE$ strain showed a similar growth rate and morphology to the wild-type strain (data not shown).

Functional analysis of OrfE

To investigate the influence of *orfE* disruption, AADH activities of the wild-type and $\Delta orfE$ strains were analyzed using lyophilized cells grown with or without an inducer (1-amino-2-propanol) (Fig. 3). The non-induced wild-type cells showed little EPE-producing (AADH) activity, whereas the induced wild-type cells produced about 1 mg/ml of EPE. On the other hand, regardless of whether or not 1-amino-2-propanol was added, $\Delta orfE$ strain cells showed higher levels of AADH activity than the wild-type and produced about 7 mg/ml EPE. Non-induced $\Delta orfE$ strain cells showed almost the same AADH activity level as induced cells. The strain transformed with pK4-derived plasmid pKMAK309, a multi-copy plasmid that contained *orfE*, showed no AADH activity regardless of induction (Fig. 3).

The *aadh* expression level of each strain was also analyzed using quantitative RT-PCR (Fig. 4). In the wild-type strain, *aadh* expression was about 100-fold higher when 1-amino-2-propanol was added to the medium than when it was not added. On the other hand, in the $\Delta orfE$ strain, *aadh* expression was not affected by the addition of 1-amino-2-propanol. Furthermore, regardless of the addition of 1-amino-2-propanol, the $\Delta orfE$ strain showed

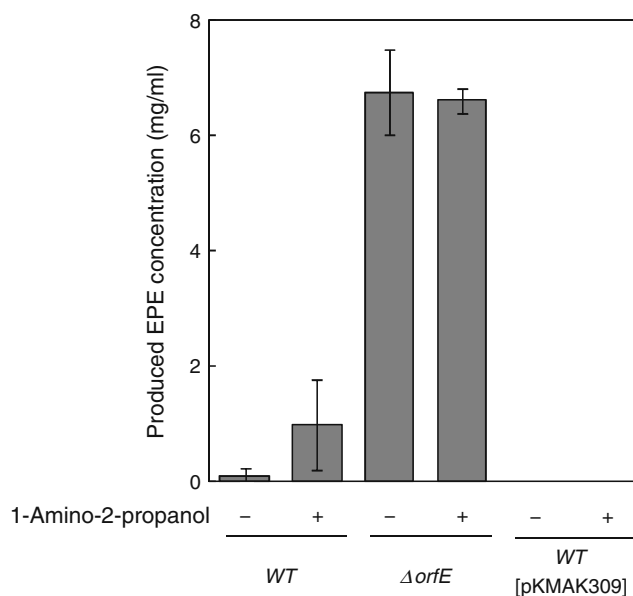


Fig. 3 AADH activities of the wild-type (*WT*) strain, $\Delta orfE$ strain ($\Delta orfE$), and wild-type strain bearing pKMAK309 (*WT* [pKMAK309]) of *R. erythropolis* MAK154 under the conditions with (+) or without (-) 1-amino-2-propanol addition. AADH activities were analyzed by the bioconversion rate of (*RS*)-EAM to EPE using lyophilized cells. Error bars indicate standard deviation (SD) from three independent experiments

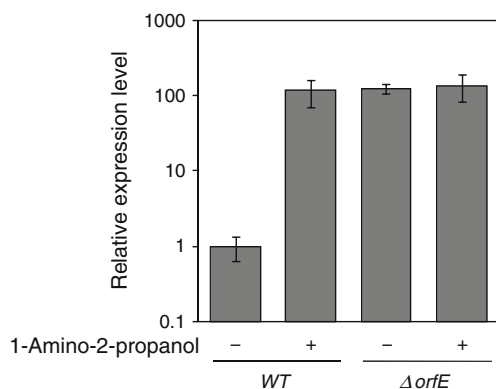


Fig. 4 Relative expression level of *aadh* in *R. erythropolis* MAK154 wild-type (WT) strain and $\Delta orfE$ strain ($\Delta orfE$) under the conditions with (+) or without (-) 1-amino-2-propanol addition. Quantitative RT-PCR was done using LightCycler. The relative amounts of mRNA against non-induced wild-type strain are shown in a semi-logarithmic graph. Error bars indicate SD from five independent experiments

almost the same expression levels as the induced wild-type strain. These results suggested that OrfE was a negative regulator of the expression of *aadh*.

Discussion

AADH of *R. erythropolis* MAK154 is induced with the addition of several aminoalcohols, such as 1-amino-2-propanol, during growth, and catalyzes the reversible conversion of L-1-amino-2-propanol to aminoacetone (Kataoka et al. 2006). These facts suggest that AADH might participate in the metabolic pathway of aminoalcohols or aminoketones, but little is known about the biological role of this enzyme. To elucidate the physiological role of this enzyme, genetic analysis of the region around *aadh* was performed.

An approximately 14-kb sequence around *aadh* of *R. erythropolis* MAK154 was determined. In this region, the existence of 14 putative complete ORFs was indicated. Most of the homologous genes in this region (from *orfV* to *orfE*) are found in several *Mycobacterium* strains. Considering that each non-coding region between *orfW* and *aadh* is quite short, that *orfV* is coded complementary strand in *Mycobacterium* and that OrfE is a putative regulator gene (see Fig. 1 and Table 1), it seems that at least the genes from *orfW* to *aadh* constitute an operon and form a metabolic pathway of some compound.

OrfX, OrfA, and OrfB showed homology with EutE, EutN, and EutM, respectively. EutE, EutN, and EutM together form an ethanolamine utilization (*eut*) operon with the other 14 *eut* genes in *Salmonella enterica*. The biochemistry underpinning ethanolamine catabolism in *S. enterica* is simple. The initial step in this process is catalyzed by an adenosylcobalamin (also known as a

coenzyme form of vitamin B₁₂)-dependent ethanolamine ammonia lyase encoded by the *eutBC* genes; this catalysis converts ethanolamine to acetaldehyde and free ammonia (Faust et al. 1990). Subsequently, acetaldehyde is converted to acetyl coenzyme A (acetyl-CoA) in a single step presumably catalyzed by the acetaldehyde dehydrogenase (EutE) enzyme (Stojiljkovic et al. 1995). Several *eut* genes (i.e., *eutS*, *-M*, *-N*, *-L*, and *-K*) encode proteins with homology to cyanobacterial carboxysomal shell proteins, and these proteins are suggested to form a carboxysome-like polyhedral organelle that is sometimes called a metabolosome (Stojiljkovic et al. 1995; Kofoid et al. 1999). The physiological role of this organelle is poorly understood, but recent studies suggest that the *eut* organelle might have the function of protecting cells against aldehyde toxicity; moreover, it has been suggested that these structures function to concentrate both metabolic enzymes and their substrates to allow high catalytic efficiency while maintaining CoA balance (Brinsmade et al. 2005). OrfA and OrfB showed similarities to EutN and EutM, and OrfY and OrfC contain a BMC (bacterial microcompartment) domain, which is found in the shell protein that form microcompartments such as carboxysomes or *eut* organelles (Kerfeld et al. 2005; Cheng et al. 2008). These observations suggest that in *R. erythropolis* MAK154, the metabolism of aminoalcohol or aminoketone is carried out in carboxysome-like polyhedral organelle.

In *Erwinia carotovora* N.C.P.P.B.1280, which can grow with 1-amino-2-propanol as a sole nitrogen source, 1-amino-2-propanol is metabolized with phosphorylation and produces 1-amino-2-propanol *O*-phosphate (Jones and Turner 1971; Jones et al. 1973). In *R. erythropolis* MAK154, a similar reaction seemed to occur because OrfD showed homology with homoserine kinases and aminoglycoside phosphotransferases. In *E. carotovora*, 1-amino-2-propanol *O*-phosphate is converted to propionaldehyde and free ammonia catalyzed by aminoalcohol *O*-phosphate phospho-lyase (Jones and Turner 1971; Jones et al. 1973). However, no ORF seemed to catalyze such a reaction around *aadh*. Therefore, a different metabolic pathway might exist in *R. erythropolis* MAK154. OrfW and OrfX bear similarities with various transaminases (aminotransferases) and aldehyde dehydrogenases, respectively. Transaminases catalyze the conversion of amine to aldehyde accompanied by amino transfer to an amino acceptor, and aldehyde dehydrogenases might catalyze the exchange of aldehyde to acyl-CoA. Since the details on this metabolic pathway remain unclear, more detailed examination is required.

OrfE shows similarity with many GntR family transcriptional regulator proteins. The GntR family was first described by Haydon and Guest (1991). This protein family is defined by a 64-amino acid N-terminal DNA-binding helix-turn-helix motif and represses transcription by bind-

ing to operator sequences existing in the promoters of target genes. DNA-binding activity is modulated by an inducer molecule that binds to the C-terminal domain of the regulator protein. In general, when the inducer is present, DNA binding is inhibited, and the repression of target genes is relieved (Rigali et al. 2002). The GntR family proteins can be divided into four subfamilies based on the homology of the C-terminal effector-binding domain (Rigali et al. 2002). A sequence comparison of OrfE with several GntR family regulators showed that OrfE is a member of the FadR subfamily and is most closely related to the archetypal family member, the *B. subtilis* gluconate operon regulator, GntR. FadR subfamily members typically bind metabolites that are substrates of the target enzymes. For example, GntR binds gluconate, thereby relieving repression of the gluconate utilization operon (Miwa and Fujita 1988), and FadR binds fatty acyl-CoA, resulting in the derepression of genes involved in fatty acid degradation (Dirusso and Black 2004). Our results suggested that OrfE was a negative regulator of the expression of *aadh*. The proposed expression mechanism of *aadh* is as follows: When 1-amino-2-propanol does not exist, OrfE binds to operator sequences of *aadh* with N-terminal helix-turn-helix motif and represses transcription of *aadh*. On the other hand, when 1-amino-2-propanol exists, OrfE presumably binds to 1-amino-2-propanol with C-terminal effector-binding domain, then loses DNA-binding activity and leaves operator sequence of *aadh*; thus, the transcription of *aadh* is permitted. In the $\Delta orfE$ strain, the differences of AADH activity or *aadh* expression level were not observed between induced and non-induced cells (Figs. 3 and 4). It might be due to the lack of transcriptional repressor of *aadh* (i.e., OrfE). In the wild-type strain bearing pKMAK309, AADH activity was not detected regardless of induction (Fig. 3). This recombinant strain might produce larger amount of OrfE in the cells as compared with wild-type strain; consequently, *aadh* transcription might be strongly repressed by OrfE. The amount of 1-amino-2-propanol added in the medium might be insufficient to take all OrfE away from operator sequence, and transcription might be restricted completely even on the induced condition. Among FadR subfamily proteins, the deduced sequence (palindromic 5'-N_x-T-N-G-T-N₃-A-C-N-A-N_x-3') is conserved in their binding sites (Rigali et al. 2002). In *R. erythropolis* MAK154, a putative OrfE binding sequence (5'-CTTTATTGGTCCGACCATTAATG-3') exists about 80-bp upstream from the *orfW* start codon (Fig. 1). The *R. erythropolis* MAK154 mutant showing high AADH activity was isolated using a traditional mutagenesis method (data not shown). The genomic region around *aadh* in this mutant strain was also sequenced. In this region, two positions of base substitution were found, one in *orfD* and the other in *orfE*, and both base substitutions caused amino

acid substitutions. Because the amino acid substitution in OrfE occurred in the N-terminal helix-turn-helix motif, this mutant OrfE might lose DNA-binding ability. This result strongly suggests that OrfE is the negative regulator of *aadh*.

The disruption mutant of *orfE* shows higher AADH activity than that of the wild-type strain. AADH might have high potential for practical application to chiral aminoalcohol production by virtue of its unique and high stereoselectivity (Kataoka et al. 2006, 2008). We expect that the mutant obtained in this study will be useful in the biocatalytic process of chiral aminoalcohols and that the obtained insights will be helpful in understanding the aminoalcohol metabolic pathway.

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