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Cloning and Expression of the Structural Gene for Alcohol Dehydrogenase of *Zymomonas mobilis* in *Escherichia coli*

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Zymomonas mobilis 알코올 탈수소 효소 유전자의 Cloning 과 *Escherichia coli* 에서의 발현

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A genomic library of *Zymomonas mobilis* DNA was constructed in *Escherichia coli* using plasmid pUC9. Allyl alcohol was used to screen a genomic clone expressing alcohol dehydrogenase. The plasmids isolated from two clones, which were sensitive to allyl alcohol, were found to be related and to share a common 2.6 kb fragment encoding alcohol dehydrogenase II identified as one of two isozymes in *Z. mobilis* by staining for alcohol dehydrogenase activity on polyacrylamide gel and spectrophotometric analysis of several substrate oxidations.

The two isozymes of alcohol dehydrogenase (ADH) responsible for the final step during alcoholic fermentation were each purified from *Z. mobilis* strains able to make a more rapid and efficient conversion of glucose to ethanol than yeasts (1,2). Two isozymes of *Z. mobilis* alcohol dehydrogenase (ZADH) were separately isolated as two bands of activity by staining for the enzyme on starch gels (2) despite of their unknown functions. The isozyme with faster electrophoretic mobility (ZADH-II) was found to be the iron-activated enzyme unlike most other alcohol dehydrogenases. Conway *et al.* (3) recently reported an isolation procedure for the alcohol dehydrogenase gene from *Z. mobilis* by using an aldehyde indicator plate.

This being the case, the two isozymes of ZADH were reported to have the ability to convert allyl alcohol to its poisonous aldehyde acrolein (4), though their specific activities were different with allyl alcohol. In this work we have therefore used a suicide substrate, allyl alchol, to clone the structural gene for *Z. mobilis* ADH in *E. coli.*

Materials and Methods

Bacterial strains and plasmids

Z. mobilis ATCC 10988 is the wild type strain used as a source of the ADH gene. *E. coli* JM83 (*ara, Δ(lac proA,B), rspL, φ* 80, *lacZ Δ M15*(r_k^+, m_k^+)) and JM103 (*Δ(lac proA,B), thi, strA, supE, endA, sbcB, hsdR*$^-$, F′*traD*36, *proAB, lacI*^{q}*Z Δ M15*) served as hosts for transformation. Plasmid pUC9 was used as a vector for cloning and subcloning of the gene.

Media and growth conditions

Z. mobili was grown in RM broth consisting of 20 g glucose, 10g yeast extract and 2g KH_2PO_4 per

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Table 1. Allyl alcohol resistance of organisms.

Strains	Resistance level* (mM)
Z. mobilis ATCC 10988	0.5
E. coli JM83	>300
E. coli JM83 (pADS93)	5

*Resistance level is defined as the concentration of allyl alcohol which completely inhibits the survival of organisms on complex agar plate.

liter, pH 5.5 (5) without shaking at 30°C. *E. coli* was cultured in LB broth (10g tryptone, 5g yeast extract, 10g NaCl, per liter, pH 7.0). *E. coli* transformants were grown in medium containing 50 μg of ampicillin/m*l*.

Preparation of DNA and gel electrophoresis

The pUC9 DNA from *E. coli* was prepared by cesium chloride – ethidium bromide centrifugation of cleared lysates (6). For rapid isolation of plasmids from the bacteria, the alkaline lysis method described by Birnboim and Doly (7) was employed. Plasmid DNAs and their restriction digests were analyzed on horizontal 0.7 to 1.2% agarose gels (8). *Zymomonas* chromosomal DNA was isolated from exponentially growing cells according to the preparative method described by Rodriguez and Tait (9).

Construction of *Z. mobilis* gene bank

Fifty micrograms of the purified *Z. mobilis* chromosomal DNA was partially digested with *Sau*3AI, and DNA fragments ranging from 2 to 10 kb were isolated by sucrose gradient centrifugation for 20 h at 25,000 rpm in a Beckman SW40 rotor. The *Sau*3AI-generated chromosomal DNA fragments (3 μg) were ligated to 1 μg of *Bam*HI-digested, dephosphorylated pUC9 DNA in a 50-μl volume as recommended by the manufacturer. The ligation mixture was used to transform *E. coli* JM83 (10).

Activity assay and electrophoretic analysis of ADH enzyme

Whole cell extracts were prepared from cells grown in 20 m*l* of culture volume with or without ampicillin for 12 h. Harvested cells were washed with 50 mM phosphate buffer containing 10% glycerol (pH 6.8), pellets were suspended in 5 m*l* of the same buffer, and sonicated for 2 to 5 min with a Branson Sonifier Model 350 at 40% output. Cell debris was removed from the extract by centrifugation (20 min, 9000 x g); the supernatants were assayed for activity. ADH activity was assayed according to Das *et al.* (11) and substrate specificities were measured under the same condition with 333 mM concentration of the substituted alcohol and 1 mM NAD^+ (2). The electrophoresis system used for electrophoretic analysis of ADH enzymes was adopted from the Tris – glycine system, and staining of ADH activity on polyacrylamide gels was carried out according to Young *et al.* (12).

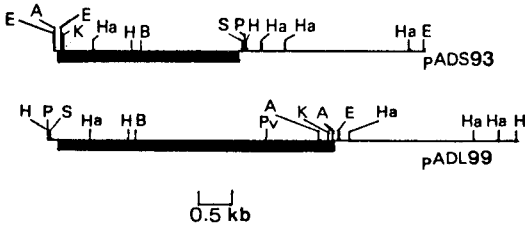

Fig. 1. Restriction endonuclease maps of pADS93 and pADL99.

The heavy lines correspond to the chromosomal fragments carrying ADH gene derived from *Z. mobilis* and thin lines to pUC9 DNA. The cleavage sites of restriction enzymes are designated as follows:
A; *Ava*I, B; *Bal*I, E; *Eco*RI, Ha; *Hae*II, H; *Hin*dIII, P; *Pst*I, Pv; *Pvu*II, S; *Sal*I, K; *Kpn*I.

Southern hybridization

The chromosomal insert of plasmid construct was isolated and labelled by nick – translation using *E. coli* polymerase I in the presence of [α -^{32}P]dATP (800 Ci/mmole) as described by Maniatis *et al.* (6). The technique used for blotting and hybridization of DNA digests was described by Southern (13).

Results

Isolation of the *Z. mobilis* alcohol dehydrogenase II (*zadh*II) gene in *E. coli*

The system used to identify *E. coli* cells with expressed ADH enzyme was based on the inability to grow in the presence of allyl alcohol. A library of *Z. mobilis Sau*3AI DNA fragments ranging in size from 2 to 10 kb was constructed in the vector pUC9, and transformed into *E. coli* JM83. Approximately 7,000 white clones obtained on MacConkey agar containing ampicillin were picked on to selective plates. Although the *E. coli* host cell could grow on LB agar plates containing 50 mM allyl alcohol, two ADH

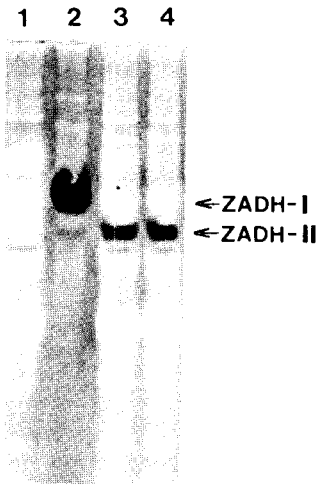

Fig. 2. Electrophoretic analysis of ADH enzymes showing that *E. coli* cells transformed with the cloned *Z. mobilis* chromosomal DNA of pADS93 and pADL99 contain enzymes identical to *Z. mobilis* ADH.
Total cell extracts of *E. coli* JM83 (pADS93) (lane 1), *Z. mobilis* (lane 2), *E. coli* JM83 (pADS93) (lane3), and *E. coli* JM83 (pADL99) (lane 4) were electrophoresed on a non-denaturing 5% polyacrylamide stacking gel, and stained for ADH activity as previously described (12).

Table 2. ADH activities of *E. coli* JM83 cells transformed with plasmids.

Plasmids	ADH activity[a] (mU[b]/mg protein)
pUC9	ND[c]
pADS93	1,030
pADL99	570

[a]ADH activities were determined after growing cells overnight on LB broth. Each value represents the average of 3 to 5 determinants.
[b]One unit of enzyme has been taken as 1 μmole NADH produced per min.
[c]ND, not detectable activity.

positive clones which did not grow, were obtained as described in Table 1.

One, named pADS93, of two recombinant plasmids from two clones has a 2.6 kb insert and the other, named pADL99, a 4.0 kb insert. Their restriction sites (Fig. 1) were mapped indicating that these plasmids share a common fragments of 2.6 kb. To determine whether these insert DNA fragments have the structural gene for *Z. mobilis* ADH, the enzyme products of these *E. coli* clones were investigated. Electrophoretical analysis of cell extracts on a polyacrylamide gel indicates that two clones produced an enzyme displaying the same band of activity co – migrating with ZADH-II band of the two isozymes from the *Z. mobilis* donor strain. Control *E. coli* host cells produced no such band as shown in Fig. 2.

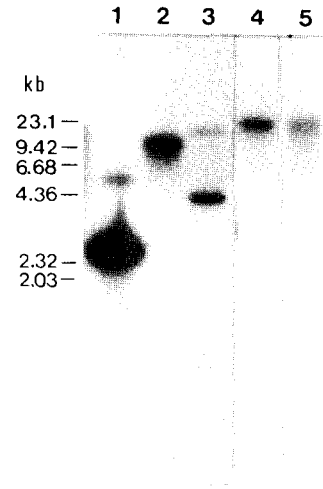

Fig. 3. Southern hybridization of chromosomal DNA purified from *Z. mobilis* ATCC 10988 with the pADS93 insert as a probe.
After digestion of the chromosomal DNA with the indicated restriction enzymes, the DNA fragments were separated on a 0.8% agarose gel, denatured, and transferred to a nitrocellulose filter. The hybridization was performed using the ^{32}P-labelled chromosomal fragment of plasmid pADS93 as a probe at 68°C for 15 h. Lanes 1 to 5 show the autoradiograph of the filter after hybridization. Lane 1, *Pst*I and *Eco*RI-digested fragment of pADS93. The lower band of lane 1 is corresponding to the pADS93 insert containing *zadh*II gene. *Z. mobilis* chromosomal DNA digested with; lane 2, *Eco*RI; lane 3, *Hin*dIII; lane 4, *Pst*I; lane 5, *Bam*HI. Molecular size is indicated to the left side of the gel.

The ADH assay of soluble protein was used to measure the levels of *Z. mobilis* ADH produced by *E. coli* clones. *E. coli* JM83 (pUC9) did not show any ADH activity, but the two clones produced ADH activity as shown in Table 2. It was also found that substrate specificities of ADH obtained from two *E. coli* clones were corresponding to those of ZADH-II presented by Wills *et al.* (2) (data not shown). Since it was confirmed that the cloned genes included the same structural gene (*zadh*II) for ZADH-II, the small recombinant plasmid, pADS93, was selected for the further study.

Source of cloned fragment of pADS93

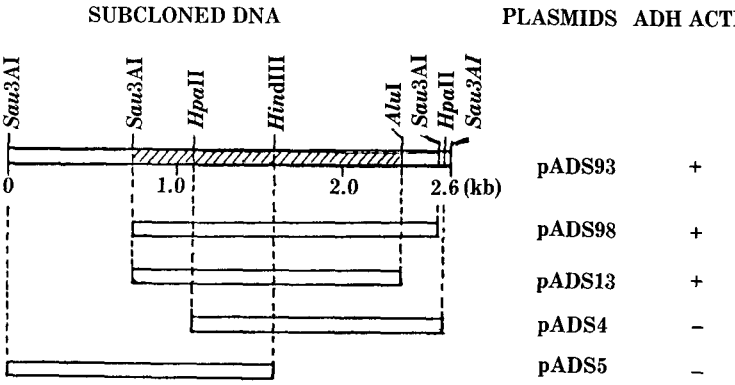

Fig. 4. Localization of *zadh*II. The bars indicate insert fragments corresponding to *Z. mobilis* chromosomal DNA on recombinant plasmids.

The plasmids are pUC9 derivatives containing DNA inserts, respectively. The shaded portion in cloned fragment of pADS93 denotes the fragment on which *zadh*II is located. The restriction sites, which were used for subcloning the *zadh*II gene, are indicated.

To determine whether the cloned fragment of plasmid pADS93 hybridized to a specific region of the *Z. mobilis* genome, Southern blot experiments were performed. When the chromosomal DNA was cut with *Eco*RI, *Bam*HI and *Pst*I, respectively, for which the *zadh*II gene posesses no sites, strong hybridization to only one fragment of *Z. mobilis* was obtained (Fig. 3. lane 2,4, and 5); digestion with *Hin*dIII, for which *zadh*II gene has one site, resulted in two strong hybridization bands (Fig. 3. lane 3). These results indicate that only one copy of the *zadh*II gene exists in the *Z. mobilis* cells.

Localization of the *zadh*II gene

The *Eco*RI and *Pst*I – generated 2.6 kb fragment including the *zadh*II gene of pADS93 was subcloned into pUC9 using *Sau*3AI to reduce the size of cloned gene. The recombinant plasmid pADS98 containing 1.85-kb insert of the *zadh*II gene was obtained. *E. coli* clone carrying pADS98 produced the same level of ADH enzyme as pADS93.

ADH transforming activity was also detected in pADS13 containing 1.6-kb fragment generated by *Alu*I digestion of the subcloned fragment in pADS98 as diagramed in Fig. 4. However, a recombinant plasmid pADS4, which was constructed by ligating the 1.5-kb fragment generated by *Hpa*II partial digestion of the insert of pADS93 to the *Acc*I-digested pUC9, had no ADH complementing activity. It was also found that the structural gene of ADH enzyme was inactivated by digestion with *Hin*dIII (Fig. 4, pADS5).

Discussion

We have cloned the structural gene for *Z. mobilis* ADH into *E. coli* by means of allyl alcohol selection. Since aldehydes in which the keto group is conjugated with a double or triple bond are potent protein-alkylating agents, allyl alcohol is an efficient suicide substrate for ADH (4). The *Z. mobilis* strain could not grow on RM agar plates containing 0.2 mM allyl alcohol but *E. coli* JM83 could grow on LB agar plates containing 100 mM allyl alcohol. However, it was found that the *E. coli* harbouring the cloned *zadh*II gene could not grow on LB agar plates containing 5 mM allyl alcohol as described previously in Table 1. Therefore it is possible that other bacterial ADH genes could be cloned in E. coli using allyl alcohol selection.

By cloning a *Z. mobilis* ADH gene on a high copy number *E. coli* vector, it was possible to increase the ADH production, though ADH activity in *E. coli* transformants containing the *zadh*II gene could not be directly compared with *Z. mobilis* because of the existence of two isozymes in *Zymomonas* as shown in Fig. 2. Furthermore, the *E. coli* clone containing *zadh*II gene produced an enzyme identical to one of the *Z. mobilis* ADH isozymes suggesting that the small component with low specific activity (ZADH-II) is not a degradation product or artifact of the extraction method. Although specific activity of ZADH-II was lower than that of ZADH-I according to intensity of the enzyme bands after activity staining on gels as shown in Fig. 2, the extent of its

specific acticity was increased in the presence of metal ions such as cobaltous or ferrous ion plus dithiothreitol. This effect of metal ions on ZADH – II agreed with those reported by Scopes (14), and Hoppner and Doelle (15).

For digestion with each single restriction endonuclease, such as *Eco*RI, *Pst*I and *Bam*HI, strong hybridization occurred to only one fragment of *Z. mobilis* chromosomal DNA using the *zadh*II gene as a probe, unlike the structural genes of yeast ADH isozymes (16 – 18). This indicates that *zadh*II gene does not have homology to the structural gene for ZADH-I; this was further supported by N-terminal amino acid sequence analysis of two isozymes of ZADH (1). It is therefore assumed that two isozymes of ZADH were each derived from evolutionary diverse sources. It is worth noting that the sequence of iron-activated ZADH-II shows strong homology to the hypothetical *Saccharomyces cerevisiae* ADH4, not being homologous to the other ADH isozymes of *S. cerevisiae* (19). In addition, no hybridization for natural plasmids isolated from *Z. mobilis* was obtained showing that the structural gene of ZADH – II is not homologous with plasmids in *Z. mobilis* in contrast with previous reports (2, 20).

The *Z. mobilis* ADH gene in the three recombinant plasmids, pADS93, pADL99 and pADS98 expressed in *E. coli* regardless of its orientation to be transcribed from the *lac* promoter of the vector and induction of *zadh*II gene with IPTG was barely detected in *E. coli* JM103. This may indicate that *Zymomonas* gene could be transcribed and translated from *Z. mobilis* control system which is recognized by the *E. coli* transcriptional and translational system. The free expression of this *Zymomonas* gene in *E. coli* may indicate that useful genes of *Zymomonas* can be cloned without much difficulty as reported previously (3, 21, 22).

요 약

Zymomonas mobilis ATCC 10988로부터 분리된 chromosomal DNA를 제한효소 *Sau*3AI으로 부분 절단한 후 이를 *Bam*HI으로 완전 절단하여 alkaline phosphatase를 처리한 pUC9과 ligation하여 *Escherichia coli* JM83을 형질전환시키는데 사용하였다. 알코올 탈수소 효소활성을 나타내는 대장균 형질전환체를 선별하기 위해 allyl alcohol을 사용하였는데 이 때 allyl alcohol을 함유한 LB 한천 배지에서 자라지 못하는 두개의 clones을 얻었다. 이들 clones으로부터 분리한 plasmids를 여러가지 제한효소로 처리하여 agarose gel 전기영동으로 분석한 결과 2.6 kb 크기의 동일한 DNA 조각을 공유하고 있음이 밝혀졌으며 이들 plasmids를 함유하고 있는 대장균 형질전환체와 *Z. mobilis*에서 생성된 효소를 각기 polyacrylamide gel 전기영동한 후 효소활성을 염색하고 또한 알코올 기질특이성을 조사한 결과 이들 plasmids가 *Z. mobilis*의 alcohol dehydrogenase II 유전자를 함유하고 있음이 밝혀졌다.

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