APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1989, p. 171–176
0099-2240/89/010171-06$02.00/0

Vol. 55, No. 1

Cloning of the Membrane-Bound Aldehyde Dehydrogenase Gene of *Acetobacter polyoxogenes* and Improvement of Acetic Acid Production by Use of the Cloned Gene

MASAHIRO FUKAYA, KENJI TAYAMA, TOSHIMI TAMAKI, HARUKO TAGAMI, HAJIME OKUMURA, YOSHIYA KAWAMURA, AND TERUHIKO BEPPU*

Nakano Biochemical Research Institute, Nakano Vinegar Co., Ltd., Handa, Aichi-ken 475, and Department of Agricultural Chemistry, Faculty of Agriculture, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113, Japan*

Received 21 July 1988/Accepted 17 October 1988

A genomic clone bank of *Acetobacter polyoxogenes* NBI1028 constructed in *Escherichia coli* by use of the expression vector pUC18 was screened with antibody raised against membrane-bound aldehyde dehydrogenase (ALDH; 75 kilodaltons [kDa]) from *A. polyoxogenes* NBI1028. A clone that synthesized a 41-kDa protein cross-reactive with anti-ALDH antibody was isolated. For cloning of the full-length ALDH structural gene, a cosmid gene bank was screened by Southern blot hybridization with the cloned DNA as a probe, and subcloning from the positive cosmid clone was performed with shuttle vector pMV24. Plasmid pAL25, containing the full-length ALDH structural gene, was isolated and expressed in both *E. coli* and *Acetobacter aceti* to produce a fused protein (78 kDa) with a short NH_2-terminal β-galactosidase peptide. pAL25 conferred ALDH production on a mutant of *A. aceti* lacking the enzyme activity. Transformation of *A. aceti* subsp. *xylinum* NBI2099 with pAL25 caused 2- and 1.4-fold increases in the production rate and in the maximum concentration of acetic acid in submerged fermentation, respectively.

Ethanol-oxidizing ability, the most crucial property of acetic acid bacteria, is catalyzed by the two membrane-bound enzymes alcohol dehydrogenase (ADH) (1, 3) and aldehyde dehydrogenase (ALDH) (2, 9). Both of these enzymes possess pyrroloquinolin quinone (PQQ) as a prosthetic group and are purified as the complex form composed of several subunits as well as cytochrome *c* (4, 5). Many reports have described the genetic instability of acetic acid bacteria, which yield mutants deficient in the ability to oxidize ethanol (11, 22, 28). A loss of one or both of these enzymes was observed in such mutants of *Acetobacter aceti* (25). For elucidation of the genetic background of acetic acid bacteria and the molecular constitution of these key enzymes, cloning of their genes is required. Cloning and amplification of the key enzyme genes may also improve the vinegar productivity of the strains.

This paper describes the cloning of the structural gene of ALDH from the industrial vinegar producer *Acetobacter polyoxogenes* NBI1028. Improved acetic acid fermentation by use of a strain transformed with the cloned ALDH gene on a multicopy plasmid is also described.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. pMV24, a shuttle vector for *Escherichia coli* and *Acetobacter* species, was constructed by inserting *A. aceti* plasmid pMV329 (16) between the *Aat*II and *Nde*I sites of pUC18 (see Fig. 1). Plasmid pMV24 allows translation of the cloned sequence as a fused protein with a short NH_2-terminal peptide of *E. coli* β-galactosidase.

Media and culture conditions. *E. coli* was routinely grown in Luria broth (LB) (14). YPG medium and growth conditions for *A. aceti* subsp. *aceti* 10-812 and *A. polyoxogenes* NBI1028 were described previously (15, 27). *A. aceti* subsp. *xylinum* NBI2099 was cultivated under the same conditions as those used for *A. aceti* subsp. *aceti* 10-812. All *Acetobacter* strains were grown at 30°C. Ampicillin and tetracycline were added, if necessary, at final concentrations of 100 and 10 μg/ml, respectively. LB containing 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was used for induction of the *lac* promoter in *E. coli*.

DNA preparation and manipulation. Total DNA and plasmid DNA were prepared by previously described methods (26). Restriction endonucleases, T4 DNA ligase, and bacterial alkaline phosphatase were used in accordance with the instruction manuals of the manufacturers. Agarose gel electrophoresis was carried out as described previously (26).

Transformation. *E. coli* JM109 was transformed as described by Hanahan (19). *Acetobacter* strains were transformed as described previously (17).

Purification of membrane-bound ALDH. Cells (wet weight, 9 g) of *A. polyoxogenes* NBI1028 harvested at the mid-log phase were suspended in 50 ml of 10 mM sodium phosphate buffer (pH 6.0) and sonicated at 10 kHz for 10 min. The sonicates was further treated by passage through a French pressure cell (20,000 lb/in^2). The extract was centrifuged at 100,000 × *g* for 1 h at 4°C. The precipitate was solubilized with 25 ml of 1.5% Triton X-100 and 1% *N*-lauroylsarcosine in 10 mM sodium phosphate buffer (pH 6.0) containing 10 mM benzaldehyde to avoid rapid inactivation of the enzyme throughout purification. The solubilized enzyme was precipitated with ammonium sulfate up to 60% saturation. The precipitate was dissolved in 10 ml of the same buffer and dialyzed against 1 liter of the buffer. The dialyzed enzyme was applied to a DEAE–Sepharose CL-6B column (1.7 by 7 cm) previously equilibrated with buffer P (10 mM sodium phosphate buffer [pH 6.0] containing 0.3% Triton X-100 and 0.1% cetyl pyridinium chloride) and eluted with a linear gradient of 10 to 100 mM phosphate. Active fractions appearing at around 50 mM phosphate were collected, dialyzed, loaded onto a hydroxyapatite column (1.7 by 3 cm)

* Corresponding author.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
E. coli HB101	F⁻ *hsdS20 recA13 ara-14 proA2 lacY1 galK2 rps120 xyl-5 mtl-1 gyrA96 supE44 leuB6 thi-1*	12
E. coli JM109	*recA1 lac-pro endA1 gyrA96 thi-1 hsdR17 relA1* F′ *traD36 proAB lacI*^{q}gZ M15	29
A. aceti subsp. *aceti* 10-8	*pro* Eth$^+$ Ace^r	27
A. aceti subsp. *aceti* 10-812	*Pro* Eth$^-$ Ace^s; spontaneous mutant from 10-8	27
A. polyoxogenes NBI1028	Wild type	15
A. aceti subsp. *xylinum* NBI2099	Wild type	This study
Plasmids		
pUC18	Ap^r *lacZ′*	26
pHC79	Ap^r Tc^r *cos*	21
pMV24	Ap^r	This study

[a] Eth$^+$, Ethanol-oxidizing ability, resulting in the production of more than 6% acetic acid; Eth$^-$, no ethanol-oxidizing ability; Ace^r, resistance to 5% acetic acid; Ace^s, sensitivity to 1.5% acetic acid; Ap^r, ampicillin resistance; Tc^r, tetracycline resistance.

previously equilibrated with buffer P, and eluted with a linear gradient of 10 to 100 mM phosphate. Active fractions eluting at around 50 mM phosphate were collected, dialyzed, and used as the purified ALDH preparation. The specific activity of the final preparation was about 800 U/mg of protein. The purified preparation produced a single protein band in native polyacrylamide gel electrophoresis (3) when gels were stained with Coomassie brilliant blue, but sodium dodecyl sulfate-polyacrylamide gel electrophoresis (27) revealed that it was composed of two proteins of 75 and 19 kilodaltons (kDa) in an equimolar ratio. Judging from the comparison of the fluorescence spectrum (6) of each component with authentic PQQ, a protein of 75 kDa is ALDH containing PQQ as a prosthetic group. Enzyme activity was assayed as described below. Protein concentration was measured by the method of Lowry et al. (23) with bovine serum albumin as the standard.

Antibody preparation. The purified ALDH preparation was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the protein band of 75 kDa was eluted from the gel slices by electroelution. About 0.05 mg of the eluted 75-kDa protein was mixed with 0.5 ml of incomplete Freund adjuvant and injected into a male albino rabbit intradermally at multiple sites. A booster injection of 0.05 mg of the 75-kDa protein in 0.5 ml of incomplete Freund adjuvant was given 1 month later at the same sites. Three weeks after the booster injection, whole serum was prepared by a standard method and fractionated by ammonium sulfate up to 20 to 35% saturation.

Construction of the clone bank and the cosmid bank of *A. polyoxogenes*. The clone bank was constructed by use of vector pUC18, which allows expression of the cloned coding sequence as a fused protein with an NH_2-terminal 10-amino-acid peptide of *E. coli* β-galactosidase. pUC18 was digested with *Pst*I and dephosphorylated with bacterial alkaline phosphatase. Total DNA of *A. polyoxogenes* NBI1028 was digested with *Pst*I and fractionated by agarose gel electrophoresis. Fragments of more than 2 kilobases (kb) were eluted from the agarose gel by electroelution and ligated with the linear pUC18 DNA. *E. coli* JM109 was transformed with the ligation mixture by use of ampicillin resistance (Ap^r) as a selection marker.

The cosmid bank was constructed by use of pHC79 as a cosmid vector essentially as described by Goldberg and Ohman (18). Total DNA of *A. polyoxogenes* NBI1028 was partially digested with *Bam*HI to yield fragments predominantly larger than 15 kb. The digested total DNA was ligated to pHC79 DNA which had been digested with *Bam*HI and dephosphorylated previously. The ligation mixture was packaged into phage lambda particles by use of an in vitro packaging kit (Promega Biotec) in accordance with the instruction manual. *E. coli* HB101 cells grown in LB containing 0.2% maltose at 30°C were centrifuged and suspended in a 0.5 volume of LB containing 10 mM $MgSO_4$. The packaging mixture was added to the cell suspension, and the mixture was incubated for 30 min at room temperature to allow infection. The mixture was transferred to LB and incubated at 37°C with shaking for 1 h. A portion of the culture was spread onto plates of LB agar containing ampicillin and incubated at 37°C overnight. The Ap^r colonies examined were all tetracycline sensitive (Tc^s).

Subcloning of the ALDH gene. Cosmid pHV473, which was screened by Southern blot hybridization (described below) with the inserted DNA of pAL18 (see Fig. 1) as a probe, was digested with *Eco*RI and ligated to pMV24 digested with *Eco*RI. The ligation mixture was introduced by transformation into *E. coli* JM109. One of the recombinant plasmids containing a 10.5-kb *Eco*RI fragment in which the total inserted DNA of pAL18 was present was designated pAL22 (see Fig. 1). For subcloning of the ALDH gene, pAL22 partially digested with *Ava*I was subjected to agarose gel electrophoresis, and a 3.6-kb fragment was electroeluted from the gel. The recovered fragment was ligated to the linear DNA of pMV24 digested with *Ava*I. The recombinant plasmid thus constructed was designated pAL25. Recombinant plasmid pAL31 was also constructed and contained the same 3.6-kb fragment in the opposite orientation as that in pAL25. The 3.6-kb *Ava*I fragment contained part of the polylinker region derived from pUC18 on pAL22 adjacent to the right end of the inserted DNA of pAL25 (see Fig. 3). pAL1, pAL2, and pAL3 were constructed by ligation of the inserted DNA of pAL22 digested with restriction endonucleases (see Fig. 3) to the linear DNA of pMV24.

Antibody screening. The clone bank was screened with antibody raised against ALDH as described by Helfman et al. (20) with the following modifications. Transformed cells were spread on LB agar plates containing ampicillin and grown overnight at 30°C. Colonies were transferred by replica plating onto nitrocellulose membrane filters, and the filters were placed on LB agar plates containing ampicillin. The filters were incubated at 37°C for several hours to allow the formation of tiny colonies. The filters with colonies were

placed on filter papers previously soaked in 10 mM IPTG and were incubated at 37°C for 3 to 4 h to induce the synthesis of the hybrid proteins. The filters were exposed to chloroform vapor for 10 min and incubated overnight in buffer A (150 mM NaCl, 50 mM Tris hydrochloride [pH 7.5]) containing lysozyme (40 μg/ml), DNase I (5 μg/ml), $MgCl_2$ (5 mM), and 3% bovine serum albumin at room temperature to lyse the *E. coli* cells. After being washed twice with buffer A, the filters were incubated for several hours at room temperature with anti-ALDH antibody which had been preincubated for 1 h with cell lysates of *E. coli* JM109 to remove any antibodies cross-reactive with the *E. coli* extracts. The filters were washed five times with buffer A and incubated with peroxidase-conjugated goat anti-rabbit immunoglobulin G (Bio-Rad Co., Ltd.) for 2 h at room temperature. Clones containing ALDH protein were visualized with 4-chloro-1-naphthol as specified by the manufacturer.

Western blot (immunoblot) analysis. Western blotting of ALDH was performed as described by Burnette (13) with some modifications. Proteins (70 to 100 μg) separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis were transferred electrically overnight to a nitrocellulose filter. The filter was treated with anti-ALDH antibody and developed with peroxidase-conjugated goat anti-rabbit immunoglobulin G as a secondary antibody as described above.

Southern blot hybridization. Standard Southern blot hybridization was performed (24). Probe DNA was labeled with the biotin labeling kit (Bethesda Research Laboratories), and the membranes were treated in accordance with the instructions of the manufacturer.

Assay of ADH and ALDH. Cells of *E. coli* or the *Acetobacter* strains were disrupted by passage through a French pressure cell (20,000 lb/in^2), and the cellular extracts were centrifuged at 100,000 × *g* for 1 h at 4°C to separate the soluble fraction and the membrane fraction. The activities of ADH and ALDH in the fractions were measured by the methods of Adachi et al. (2, 3).

Acetic acid fermentation. *A. aceti* subsp. *xylinum* NBI2099 and its transformant carrying the cloned ALDH gene were cultivated at 30°C in YPG medium (27) containing appropriate amounts of ethanol and acetic acid in a 5-liter jar fermentor (Marubishi Bioengineering Co., Ltd.) with an agitation speed of 500 rpm and aeration of 0.2 vvm. Acetic acid was determined by titration. Bacterial growth was determined by monitoring the A_{660}.

Enzymes and chemicals. Restriction endonucleases, bacterial alkaline phosphatase, and T4 DNA ligase were purchased from Takara Shuzo Co., Ltd. Lysozyme and DNase I were obtained from Sigma Co., Ltd. PQQ was the product of Ube Kosan Co., Ltd. DEAE–Sepharose CL-6B and hydroxyapatite were obtained from Pharmacia Co., Ltd., and Bio-Rad Co., Ltd., respectively. Other chemicals were of analytical grade.

RESULTS

Cloning of part of the ALDH gene. *Pst*I-digested and dephosphorylated pUC18 DNA was ligated to *Pst*I-digested total DNA of *A. polyoxogenes* NBI1028, and the ligated mixture was introduced into *E. coli* JM109. More than 90% of the Ap^r transformants harbored a plasmid containing some inserts. About 5,000 colonies of the Ap^r transformants were screened for positive reactivity with anti-ALDH antibody as described in Materials and Methods, and two positive clones were detected. These two transformants possessed an identical plasmid, pAL18 (Fig. 1), which was composed of pUC18 and two *Pst*I fragments of approximately 1.1 kb, both of which hybridized with genomic DNA of *A. polyoxogenes* NBI1028 (data not shown). A cellular extract of one of the transformants carrying pAL18 was analyzed by Western blot analysis. A protein of 41 kDa was cross-reactive with anti-ALDH antibody (Fig. 2). The 41-kDa protein was produced only when the transformant was induced with IPTG. When the inserted DNA was ligated to pUC18 in the opposite orientation, no cross-reactive protein was detected. These results indicate that the 41-kDa protein is a fused protein with a portion of β-galactosidase coded for

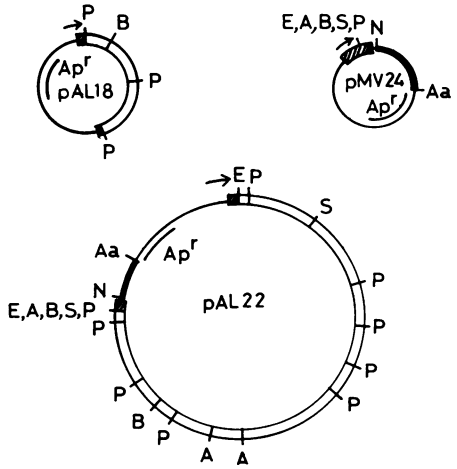

FIG. 1. Restriction maps of pAL18, pMV24, and pAL22. The thin lines and thick lines represent pUC18 and pMV329, respectively. The shaded boxes represent the fragment of *lacPO-lacZ'* with the polylinker for *Ava*I, *Bam*HI, *Eco*RI, *Kpn*I, *Pst*I, *Sac*I, *Sal*I, *Sma*I, and *Xba*I. The open boxes represent the cloned fragment. Restriction sites: P, *Pst*I; E, *Eco*RI; N, *Nde*I; Aa, *Aat*II; A, *Ava*I; B, *Bam*HI; and S, *Sal*I. The arrows indicate the direction of transcription.

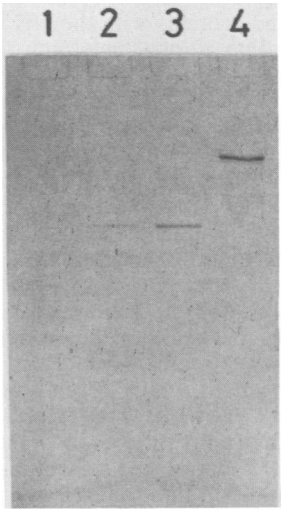

FIG. 2. Detection of the 41-kDa protein cross-reactive with anti-ALDH antibody and coded for by pAL18. Lanes: 1, *E. coli* JM109; 2, uninduced *E. coli* JM109(pAL18); 3, *E. coli* JM109 (pAL18) induced with IPTG; 4, purified ALDH from *A. polyoxogenes* NBI1028.

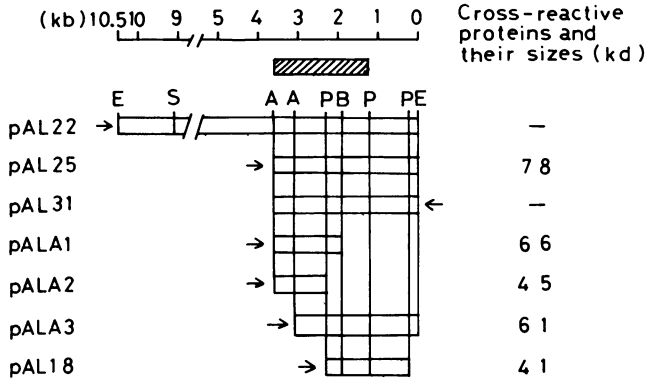

FIG. 3. Synthesis by a set of derivative plasmids in *E. coli* of proteins cross-reactive with anti-ALDH antibody. All the fragments were cloned on pMV24. The arrows indicate the fused *lacPO-lacZ'* and the direction of transcription. The shaded box indicates the putative coding sequence of ALDH estimated from the sizes of the cross-reactive proteins by assuming that the 75-kDa protein corresponds to 2.3 kb of DNA. kD, Kilodaltons. Restriction sites are as defined in the legend to Fig. 1.

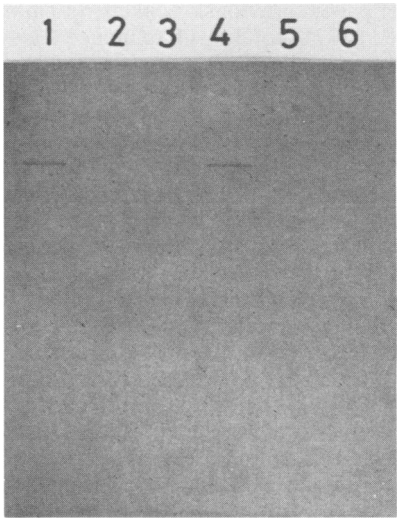

FIG. 4. Detection of the 78-kDa fused ALDH protein in transformants of *E. coli* JM109. Lanes: 1, purified ALDH; 2, JM109 (pAL22) induced with IPTG; 3 and 4, uninduced and induced JM109(pAL25); 5 and 6, uninduced and induced JM109(pAL31).

by pUC18. Since the molecular mass of the fused protein was considerably lower than that of the native ALDH protein (75 kDa), the cloned fragment might be part of the ALDH structural gene, probably lacking its 5'-terminal region.

Cloning of the full-length ALDH gene. For cloning of the full-length ALDH structural gene, a cosmid bank was constructed and screened by Southern hybridization with the inserted DNA of pAL18 as a probe. Of about 1,000 independent clones, 2 produced positive hybridization. A recombinant plasmid (pHV473) carrying a ca. 30-kb *Bam*HI insert was obtained from one of the positive cosmid clones.

Further subcloning was carried out with the *E. coli-A. aceti* shuttle vector pMV24. Recombinant plasmid pAL22 (Fig. 1) was found to carry a 10.5-kb *Eco*RI fragment which contained the total sequence of the pAL18 insert along with the long upstream sequence. Several trimmed fragments from the *Eco*RI fragment were inserted into pMV329 in the form encoding fused proteins with the NH_2-terminal part of β-galactosidase and were introduced into *E. coli*. Although pAL22 carrying the original *Eco*RI fragment failed to produce any protein cross-reactive with anti-ALDH antibody, most of the other plasmids (pAL25, pALA1, pALA2, and pALA3) carrying the shortened fragment directed the synthesis of proteins of various sizes when examined by Western blot analysis (Fig. 3). Among them, the transformant harboring pAL25 synthesized the largest protein, of 78 kDa, which was slightly larger than the 75-kDa native ALDH protein (Fig. 4). The inserted DNA of pAL25 was 3.6 kb, long enough to code for the full-length ALDH structural gene, and the molecular mass of the protein coincided well with the expected size of the fused protein in which full-length ALDH was fused with the NH_2-terminal 10 amino acids of β-galactosidase. This 78-kDa protein was synthesized only when the transformant was induced with IPTG. Furthermore, pAL31, carrying the same insert as pAL25 in the opposite orientation, did not direct the synthesis of the 78-kDa protein. These results indicate that pAL25 contains almost the full-length ALDH structural gene expressed under the control of the *lac* promoter. However, no ALDH activity was detected in the cellular extract of *E. coli* (pAL25) induced with IPTG, even in the presence of PQQ and Mg^{2+}. The fused ALDH protein of 78 kDa was localized in both the membrane and soluble fractions when examined by a similar Western blot analysis.

Expression of ALDH activity in *A. aceti* by the cloned ALDH gene. To examine the expression of the cloned ALDH gene in *A. aceti*, we introduced pAL25, pAL31, and the vector pMV24 into *A. aceti* subsp. *aceti* 10-812, which was deficient in both ADH and ALDH activities. The transformants were cultured in YPG medium without the addition of IPTG. The cellular extracts and the membrane and soluble fractions were analyzed by Western blotting. The transformant harboring pAL25 synthesized the cross-reactive 78-kDa protein, which was localized exclusively in the membrane fraction, while those harboring pAL31 or pMV24 did not produce a detectable amount of the protein. These results suggest that the fused ALDH gene is expressed under the control of the *lac* promoter in *Acetobacter* species, even in the absence of IPTG. The specific activities of ADH in *A. aceti* subsp. *aceti* 10-8, 10-812(pMV24), and 10-812(pAL25) were 1.1, 0.05, and 0.05 U/mg of protein, respectively, and those of ALDH were 1.9, 0.05, and 0.43 U/mg of protein, respectively. The specific activity of ALDH in strain 10-812(pAL25) (0.43 U/mg of protein) indicated that the fused ALDH protein had distinct enzymatic activity.

Acetic acid fermentation by *A. aceti* carrying the cloned ALDH gene. Cloning of the ALDH gene in *Acetobacter* species was expected to enhance the production of acetic acid through the gene amplification effect. This was confirmed by use of *A. aceti* subsp. *xylinum* NBI2099, an isolate from a vinegar factory, as the recipient. This strain produces relatively large amounts of acetic acid, but the proteins in the cellular extract are not cross-reactive with anti-ALDH antibody. The strain was transformed with the vector pMV24 and the recombinant plasmid pAL25, and the transformants harboring each plasmid were cultured in YPG medium and examined for their ability to produce acetic acid from ethanol. Western blot analysis revealed that the transformant carrying pAL25 synthesized a large amount of 78-kDa ALDH, which was localized in the membrane fraction (data not shown). The specific ALDH activity of the transformant

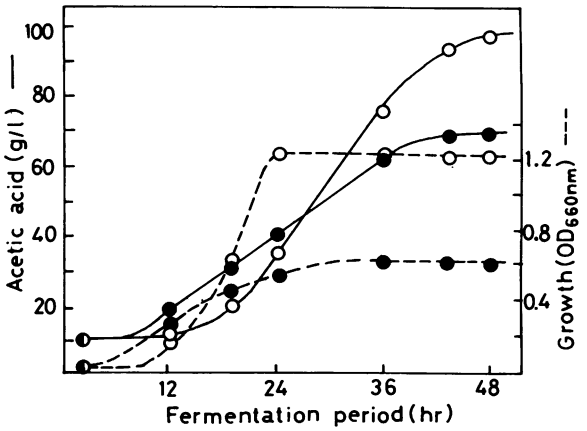

FIG. 5. Acetic acid fermentation by transformants of *A. aceti* subsp. *xylinum* NBI2099. OD_{660nm}, Optical density at 660 nm. Symbols: ●, NBI2099(pMV24); ○, NBI2099(pAL25).

carrying pAL25 (0.44 U/mg of protein) was about two times higher than that of the transformant carrying the vector (0.22 U/mg of protein). The corresponding activities of ADH were 0.68 and 0.52 U/mg of protein, respectively. Fermentation profiles are shown in Fig. 5. The growth rate and the maximum growth of the transformant harboring pAL25 were distinctly higher than those of the transformant harboring the vector; its specific volumetric production of acetic acid at a concentration of 20 g/liter was calculated to be 4.0 g/liter per h, whereas the control, containing only the vector, produced 1.8 g of acetic acid per liter per h. The maximum concentration of acetic acid produced by the transformant harboring pAL25 (96.6 g/liter) was about 1.4 times higher than that in the control (68.4 g/liter). The plasmid was stably maintained during the cultivation period. These results clearly demonstrate that the cloned ALDH gene is effective in improving the production of acetic acid from ethanol.

DISCUSSION

The 3.6 kb *AvaI-EcoRI* fragment in pAL25 directed the synthesis of the fused protein which was cross-reactive with anti-ALDH antibody and whose molecular mass coincided with the sum of the full length of ALDH and the NH_2-terminal peptide of β-galactosidase on the vector. pAL25 directed ALDH activity in *A. aceti*. In addition, a comparison of the sequences encoding the cross-reactive proteins of various sizes shown in Fig. 3 indicated that the ALDH-coding sequence might span approximately 2.3 kb starting from the utmost 5′ end of the fragment. However, it seems still possible that a short sequence of a few NH_2-terminal amino acids of ALDH is missing in pAL25. Nucleotide sequencing and amino acid sequencing of ALDH are now being done to solve this problem.

Expression of the cloned ALDH gene on pAL25 in *A. aceti* depended on the *E. coli lac* promoter, but no induction with IPTG was observed. However, pAL22, carrying a longer sequence upstream of the putative coding sequence of ALDH, failed to direct the synthesis of the ALDH protein in both *E. coli* and *A. aceti*. The upstream sequence in pAL22 probably did not contain a promoter for *A. aceti* but contained some sequence interfering with transcription from the *lac* promoter.

The fused ALDH protein of 78 kDa expressed in *A. aceti* was enzymatically active, but the protein in *E. coli* was inactive. Ameyama et al. (7) reported that ALDH of acetic acid bacteria contains PQQ as a prosthetic group. In *E. coli* the PQQ content is very low (10), and the activity of glucose dehydrogenase, a PQQ-requiring enzyme, in this organism was stimulated by adding PQQ and Mg^{2+} to the assay mixture (8). However, no ALDH activity was detected in *E. coli* cells carrying pAL25, even in the culture supplemented with PQQ and Mg^{2+}. The fused ALDH protein in *A. aceti* cells was localized in the membrane fraction, but a considerable part of the protein in *E. coli* was found in the soluble fraction. A different constitution of the cell membrane of *E. coli* might result in an inactive conformation of the protein through an incorrect or weak interaction with the membrane.

The introduction of pAL25 into *A. aceti* subsp. *aceti* 10-812 led to the recovery of only about one-fourth of the ALDH activity of the parent strain, while a twofold increase in ALDH activity was observed upon the introduction of pAL25 into *A. aceti* subsp. *xylinum* NBI2099. *A. aceti* subsp. *aceti* 10-812 is a mutant deficient in both ADH and ALDH and supersensitive to acetic acid. It is possible that ADH or some other component in the cell membrane of *A. aceti* is required for full expression of the enzymatic activity of the ALDH protein.

The work presented in this paper is a good example of improving the vinegar production of industrial strains by recombinant DNA techniques. Cloning of the ADH gene and its effect on acetic acid production are now being explored and will be reported elsewhere.

LITERATURE CITED

1. **Adachi, O., E. Miyagawa, E. Shinagawa, K. Matsushita, and M. Ameyama.** 1978. Purification and properties of particulate alcohol dehydrogenase from *Acetobacter aceti*. Agric. Biol. Chem. **42**:2331–2340.
2. **Adachi, O., K. Tayama, E. Shinagawa, K. Matsushita, and M. Ameyama.** 1980. Purification and characterization of membrane-bound aldehyde dehydrogenase from *Gluconobacter suboxydans*. Agric. Biol. Chem. **44**:503–515.
3. **Adachi, O., K. Tayama, E. Shinagawa, K. Matsushita, and M. Ameyama.** 1987. Purification and characterization of particulate alcohol dehydrogenase from *Gluconobacter suboxydans*. Agric. Biol. Chem. **42**:2045–2056.
4. **Ameyama, M., and O. Adachi.** 1982. Alcohol dehydrogenase from acetic acid bacteria, membrane-bound. Methods Enzymol. **89**:450–457.
5. **Ameyama, M., and O. Adachi.** 1982. Aldehyde dehydrogenase from acetic acid bacteria, membrane-bound. Methods Enzymol. **89**:491–497.
6. **Ameyama, M., M. Hayashi, K. Matsushita, E. Shinagawa, and O. Adachi.** 1984. Microial production of pyrroquinoline quinone. Agric. Biol. Chem. **48**:561–565.
7. **Ameyama, M., K. Matsushita, Y. Ohno, E. Shinagawa, and O. Adachi.** 1981. Existence of a novel prosthetic group, PQQ, in membrane-bound, electron transport chain-linked, primary dehydrogenases of oxidative bacteria. FEBS Lett. **130**:179–183.
8. **Ameyama, M., M. Nonobe, E. Shinagawa, K. Matsushita, and O. Adachi.** 1985. Purification and characterization of the quinoprotein D-glucose dehydrogenase apoenzyme from *Escherichia coli*. Agric. Biol. Chem. **50**:49–57.
9. **Ameyama, M., K. Osada, E. Shinagawa, K. Matsushita, and O. Adachi.** 1981. Purification and characterization of aldehyde dehydrogenase of *Acetobacter aceti*. Agric. Biol. Chem. **45**: 1889–1890.
10. **Ameyama, M., E. Shinagawa, K. Matsushita, and O. Adachi.** 1984. Growth stimulating substance for microorganisms produced by *Escherichia coli* causing the reduction of the lag phase in microbial growth and identity of the substance with pyrroloquinoline quinone. Agric. Biol. Chem. **48**:3099–3107.
11. **Asai, T.** 1968. Acetic acid bacteria. classification and biochemical activities, p. 59–67. University of Tokyo Press, Tokyo.

12. **Boyer, H. W., and D. Roullard-Dussoix.** 1969. A complementary analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41**:459–472.
13. **Burnette, W. N.** 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. **112**:195–203.
14. **Davis, R. W., D. Botstein, and J. R. Roth (ed.).** 1980. Advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
15. **Entani, E., S. Ohmori, H. Masai, and K. Suzuki.** 1985. *Acetobacter polyoxogenes* sp. nov., a new species of an acetic acid bacterium useful for producing vinegar with high acidity. J. Gen. Appl. Microbiol. **31**:475–490.
16. **Fukaya, M., H. Okumura, H. Masai, T. Uozumi, and T. Beppu.** 1985. Construction of new shuttle vectors for *Acetobacter*. Agric. Biol. Chem. **49**:2083–2090.
17. **Fukaya, M., K. Tayama, H. Okumura, H. Masai, T. Uozumi, and T. Beppu.** 1985. Improved transformation method for *Acetobacter* with plasmid DNA. Agric. Biol. Chem. **49**:2091–2097.
18. **Goldberg, J. B., and D. E. Ohman.** 1984. Cloning and expression in *Pseudomonas aeruginosa* of a gene involved in the production of alginate. J. Bacteriol. **158**:1115–1121.
19. **Hanahan, D.** 1985. Technique for transformation of *E. coli*, p. 109–135. *In* D. M. Glover (ed.), DNA cloning, vol. 1. IRL Press, Oxford.
20. **Helfman, D. M., J. R. Feramisco, J. C. Fiddes, G. P. Thomas, and S. H. Hughes.** 1983. Identification of clones that code chicken tropomyosin by direct immunological screening of a cDNA expression library. Proc. Natl. Acad. Sci. USA **80**:31–35.
21. **Hohn, B., and J. Collins.** 1980. A small cosmid for efficient cloning of large DNA fragment. Gene **11**:291–298.
22. **Leisinger, T., W. Jaggi, P. Weber, and L. Ettlinger.** 1967. Spontane Mutationen bei Essigsauerbakterien. II. Die Bedeutung der Mutationen fur die Systematik. Arch. Mikrobiol. **57**: 76–92.
23. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
24. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
25. **Ohmori, S., T. Uozumi, and T. Beppu.** 1982. Loss of acetic acid resistance and ethanol oxidizing ability in an *Acetobacter* strain. Agric. Biol. Chem. **46**:381–389.
26. **Okumura, H., T. Uozumi, and T. Beppu.** 1985. Construction of plasmid vectors and a genetic transformation system for *Acetobacter aceti*. Agric. Biol. Chem. **49**:1011–1017.
27. **Okumura, H., T. Uozumi, and T. Beppu.** 1985. Biochemical characteristics of spontaneous mutants of *Acetobacter aceti* deficient in ethanol oxidation. Agric. Biol. Chem. **49**:2485–2487.
28. **Schell, J., and J. De Ley.** 1962. Variability of acetic acid bacteria. Antonie van Leeuwenhoek **28**:445–465.
29. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13 mp8 and pUC19 vectors. Gene **33**:103–119.