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Cloning, Sequencing, and Characterization of the Gene Encoding the Smallest Subunit of the Three-Component Membrane-Bound Alcohol Dehydrogenase from *Acetobacter pasteurianus*

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The membrane-bound alcohol dehydrogenase (ADH) of *Acetobacter pasteurianus* NCI1452 consists of three different subunits, a 78-kDa dehydrogenase subunit, a 48-kDa cytochrome *c* subunit, and a 20-kDa subunit of unknown function. For elucidation of the function of the smallest subunit, this gene was cloned from this strain by the oligonucleotide-probing method, and its nucleotide sequence was determined. Comparison of the deduced amino acid sequence and the NH_2-terminal sequence determined for the purified protein indicated that the smallest subunit contained a typical signal peptide of 28 amino acids, as did the larger two subunits. This gene complemented the ADH activity of a mutant strain which had lost the smallest subunit. Disruption of this gene on the chromosome resulted in loss of ADH activity in *Acetobacter aceti*, indicating that the smallest subunit was essential for ADH activity. Immunoblot analyses of cell lysates prepared from various ADH mutants suggested that the smallest subunit was concerned with the stability of the 78-kDa subunit and functioned as a molecular coupler of the 78-kDa subunit to the 48-kDa subunit on the cytoplasmic membrane.

Acetic acid fermentation, in which ethanol is oxidized to acetic acid by acetic acid bacteria, is the most characteristic process in vinegar production (10). This ethanol oxidation is catalyzed by two membrane-bound enzymes, alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) (1–3, 6, 11, 27). The ADHs of *Acetobacter* spp., a genus of acetic acid bacteria (10), are classified into two types, two component and three component. The former in *Acetobacter polyoxogenes* consists of a 72- to 78-kDa dehydrogenase subunit (subunit I) and a 44- to 48-kDa cytochrome *c* subunit (subunit II), and the latter type in *Acetobacter aceti* and *Acetobacter pasteurianus* has one more subunit, a 20-kDa subunit of unknown function (subunit III). We recently purified both types of ADHs and cloned the genes encoding the larger two subunits of both types of ADHs. These studies revealed that both subunit I and subunit II were essential for ADH activity (25–27). The genes encoding subunits I and II were clustered (25, 26), but the gene encoding subunit III was not present close to the cluster (25). It had thus been postulated that only the two larger components were required for ADH activity.

In subsequent investigations, we found that some spontaneous mutants of *A. pasteurianus* NCI1452 which lost subunit III showed an ADH activity-deficient phenotype. We therefore began to study how subunit III of the three-component ADH participates in the enzymatic activity and to clarify its function. In the present study, we cloned the gene encoding subunit III from *A. pasteurianus* NCI1452 and analyzed various ethanol oxidation-defective mutants derived from the parental strain genetically and immunologically. This paper provides evidence that this subunit is indispensable for ADH activity. The function of subunit III in the three-component-type ADH is also discussed.

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MATERIALS AND METHODS

Bacterial strains and plasmids. All the bacterial strains and plasmids used are listed in Table 1.

Media and culture conditions. YPG medium (pH 6.5) consisted of 5 g of yeast extract (Wako Pure Chemicals, Osaka, Japan), 2 g of polypeptone (Wako Pure Chemicals), and 30 g of glucose in 1 liter of water. *Acetobacter* strains were first cultured in a 50-ml test tube containing 5 ml of YPG medium with shaking for 24 to 40 h at 30°C. From 1 to 5 ml of the both was inoculated into 100 ml of YPG medium or YPG medium supplemented with ethanol (1 to 3%) in a 500-ml shaking flask and further cultured with shaking at 30°C. *Escherichia coli* was routinely cultured in Luria broth (20). Ampicillin and kanamycin were used at a final concentration of 50 mg/ml when necessary to maintain plasmids.

Isolation of mutants deficient in ADH activity. ADH-deficient mutants were obtained by positive selection on YPG agar medium supplemented with allyl alcohol (0.002%). Allyl aldehyde formed from allyl alcohol by oxidation by ADH was lethal to the cells (14). Allyl alcohol-resistant colonies were randomly selected, and the enzyme activities of the mutants were examined. The ADH subunits of mutant strains were also examined by immunoblot hybridization, described below.

DNA complementation of ADH-deficient mutants. ADH-deficient mutants were transformed by the genes encoding the ADH subunits on pMV24, described below. Colonies of transformants were inoculated on YPG agar medium supplemented with $CaCO_3$ (0.1%) and ethanol (3%). After 16 to 24 h at 30°C, transformants which complemented the ADH activity made halos around colonies. Parent and mutant strains harboring pMV24 were used as positive and negative controls, respectively.

Enzyme assays. Cell lysates were prepared by ultrasonic treatment (Branson Sonifier cell disrupter 250) or passage through a French pressure cell (15,000 lb/in^2) from mid-logarithmic-phase growth or stationary-phase growth. Cells were suspended in 10 mM potassium phosphate buffer (pH 6.0). The activities of ADH and ALDH were measured by the ferricyanide method of Ameyama and Adachi (4, 5).

DNA preparation and manipulation. Total DNA from *Acetobacter* species was prepared as described by Okumura et al. (17). DNA-DNA hybridization was performed by the standard method (20, 24) with a nylon membrane (Hybond-N^+; Amersham International plc). Restriction endonucleases, T4 polynucleotide kinase, and T4 DNA ligase (all from Takara Shuzo Co., Kyoto, Japan) were used. *E. coli* was transformed as described by Hanahan (13). *Acetobacter* strains were transformed by the electroporation method described by Wong et al. (29). Nucleotide sequences were determined by the dideoxy chain termination method (21) combined with the M13 cloning system (30) on a DNA sequencer (DSQ-1; Shimadzu Co., Kyoto, Japan). For hybridization analyses and cloning of the gene

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Reference
A. pasteurianus		
NCI1452	*met* Sm^r, thermophilic	25
m00-09	Reduced ADH activity (subunit III deficient); spontaneous mutant of NCI1452	This work
m00-21	Reduced ADH activity (subunit I- and subunit II-deficient mutant); spontaneous mutant of NCI1452	This work
m00-27	Reduced ADH activity (subunit II-deficient mutant); spontaneous mutant of NCI 1452	This work
m36-05	Reduced ADH activity (subunit I-deficient mutant); spontaneous mutant of NCI1452	This work
A. aceti		
10-8S2	*pro* Sm^r, thermophilic	17
Δ1	*pro* Sm^r *adhS*::*neo*	This work
E. coli JM109	*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*lac-proAB*)/F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM*15*]	30
Plasmids		
pMV24	Ap^r *lacZ′*; *Acetobacter-E. coli* shuttle vector	12
pADS1892	Ap^r, containing *adhS* on pMV24	This work
pADS1991	Ap^r containing *adhS* in the opposite orientation from that of pADS1892 on pMV24	This work
pADS1411	Ap^r *adhS′* (truncated *adhS* gene) on pMV24	This work
pADL1912	Ap^r, containing dehydrogenase and cytochrome *c* subunit genes on pMV24	This work
pADL182	Ap^r, containing dehydrogenase subunit gene on pMV24	This work
pADL191	Ap^r, containing cytochrome *c* subunit gene on pMV24	This work
pUC18	Ap^r, *lacZ′*	30
pADS141	Ap^r, containing *adhS*	This work
pADSΔ1	Ap^r *adhS′*::*neo*	This work

[a] Ap^r, ampicillin resistance; Sm^r, streptomycin resistance.

(*adhS*) encoding subunit III, the oligonucleotides shown in Fig. 1 were synthesized on a Milligen/Biosearch Cyclon Plus DNA synthesizer.

Computer-aided analysis of nucleotide sequence. The DNA sequence was analyzed by using the Genetyx sequence analysis program (Software Development Co., Ltd., Tokyo, Japan).

Immunoblot hybridization. Antisera raised against each of the ADH subunits (25) were used for immunoblot hybridization of electrophoresed proteins by the method of Burnette (8). A polyvinylidine difluoride (PVDF) membrane (Immobilon Transfer; 0.45-μm pore size; Millipore) was used for blotting. The blotted membrane was cut into three pieces corresponding to the mobility of each subunit and then hybridized with the respective antibodies.

Fractionation of membrane and soluble fractions. The cell lysates prepared as described above were centrifuged at 100,000 × *g* for 1 h at 4°C. The supernatants were used as the soluble fractions. The pellets were resuspended in 10 mM potassium phosphate buffer (pH 6.0) and used as the membrane fractions.

Nucleotide sequence accession number. The *adhS* nucleotide sequence data have been registered in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession number D31730.

RESULTS

Cloning and nucleotide sequence of the *adhS* gene. In our previous work (25), we purified the three-component ADH from *A. pasteurianus* NCI1452 and determined the amino acid sequence of the NH_2-terminal region of each subunit. In order to clone the gene encoding subunit III of ADH, we synthesized two sets of oligonucleotides for hybridization probes (Fig. 1). Chromosomal DNA from *A. pasteurianus* digested with several restriction endonucleases was hybridized with the ^{32}P-labeled probes. An approximately 5-kb *Eco*RV fragment showing positive hybridization against two probes was recovered from gel slices. After a synthetic *Bam*HI linker had been attached to the *Eco*RV ends, they were inserted into the *Bam*HI site of pUC18. One colony among about 1,000 ampicillin-resistant transformants gave a positive signal by colony hybridization (20) with the two probes, and the recombinant plasmid, named pADS141 (Fig. 2), was recovered from the transformant.

The restriction map of the cloned 5.0-kb *Eco*RV fragment is shown in Fig. 2. The nucleotide sequence of the 0.7-kb *Sph*I fragment in the inserted DNA fragment showing positive hybridization in the second cycle of Southern hybridization revealed the presence of an open reading frame (ORF) containing the NH_2-terminal amino acid sequence described above. Because this 0.7-kb *Sph*I fragment lacked the COOH-terminal portion, we again subcloned the 1.1-kb *Sph*I partially digested fragment containing the NH_2-terminal region from pADS141 and determined its nucleotide sequence. The complete nucleotide sequence of the region encoding subunit III of ADH is shown in Fig. 3. We named this gene *adhS*. The restriction map around the *adhS* gene revealed that *adhS* was not located very close to the ADH gene cluster encoding the dehydrogenase subunit (78 kDa, subunit I) and the cytochrome *c* subunit (48 kDa, subunit II). The ORF corresponding to subunit III of ADH started with ATG at nucleotides (nt) 173 to 175 and terminated with TAA (nt 788 to 790). A possible ribosome-binding sequence (22), AGGGAGA, was present 6 nt upstream of the ATG codon. The NH_2-terminal amino acid sequence determined with the purified subunit III (25) is present in the ORF at positions 30 to 39. Gln-29 appears to be modified into pyroglutamate in vivo, because the NH_2-terminal sequence was obtained only after treatment with pyroglutamate aminopeptidase. The extra 28 amino acids at the NH_2 terminus of this ORF show features typical of a signal peptide (28), i.e., positively charged residues at the NH_2 terminus followed by a stretch of hydrophobic residues. Subunits I and II also contain typical signal peptides (25).

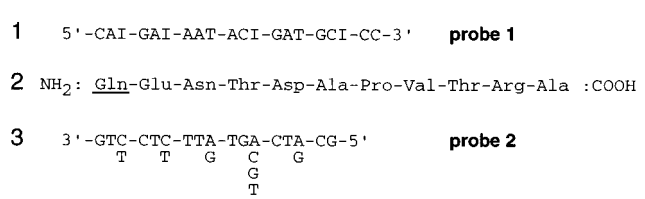

FIG. 1. Designs of the oligonucleotide probes (line 1, probe 1; line 3, probe 2) on the basis of the NH_2-terminal amino acid sequence of subunit III (line 2) previously described (24). The underlined glutamine seems to be converted into pyroglutamate in the mature enzyme.

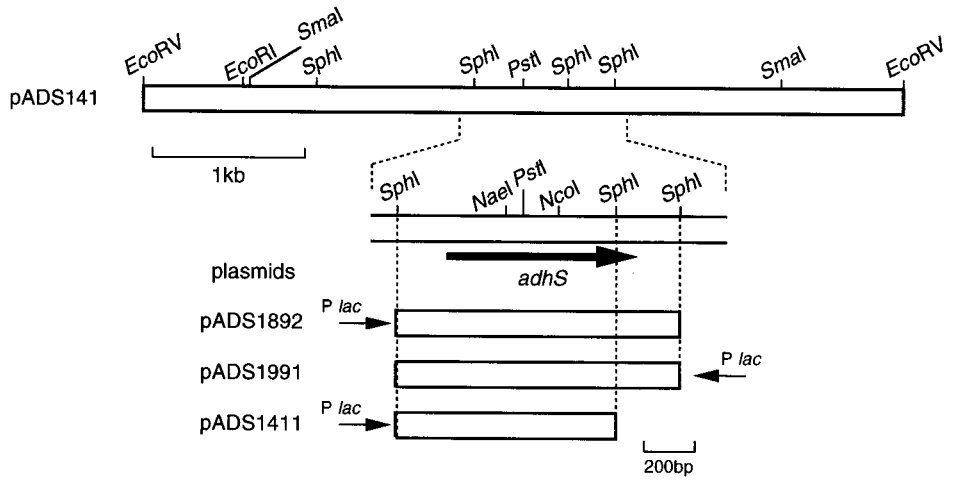

FIG. 2. Restriction maps of pADS141 and derived plasmids. pADS141 contains a 5-kb *Eco*RV fragment at the *Bam*HI site of pUC18. pADS1892, pADS1991, and pADS1411 contain the subcloned *Sph*I fragment at the *Sph*I site of pMV24. The heavy arrow indicates the extent and direction of *adhS*. P *lac* indicates the *lac* promoter, and the accompanying thin arrow represents its direction.

Complementation of ADH activity by the *adhS* gene. In order to test for the ability of the cloned *adhS* gene to complement the ADH-deficient phenotype of strain m00-09, we placed the *adhS* gene on *E. coli-Acetobacter* shuttle vector plasmid pMV24 (Fig. 2) and introduced the resulting plasmids, pADS1892 and pADS1991, into the mutant by the electroporation method (29). Mutant strain m00-09 lacked subunit III, as determined by immunoblotting (Fig. 4, lane 2). In this mutant, the amount of subunit I was greatly reduced, which will be discussed later in relation to the function of subunit III. Introduction of pADS1892 caused the appearance of subunit III in the mutant strain (lane 3) and restored ADH activity to the same level as in the parental strain. Plasmid pADS1991 also caused the production of subunit III in strain m00-09 at a level

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                                                                      100
GCATGCGCTTATGCCCCGGCCAAGGCCAGTCCGCGCCGGGGCATCTGGCACGCTGTGGGGAAAAACCCTGATATATCTGTGGTCTGCCATGTGTGGGGTG

                                                                      200
CGCAATGGGGCTGCTTGATCTTTCGGGCAGATACAGGGCAGTTTGAGGCATATTCCGCTAGGGAGATTCTGAATGAAACTGATTGCCGTACGTGCCCTGT
                                                    SD        M  K  L  I  A  V  R  A  L  S

                                                                      300
CGGCGCTTGCCCTCACCACATCTCTGCTGGCAGGGGCTGCTGTGTCTGCCCATGCGCAGGAAAATACAGATGCGCCCGTAACACGCGCCGGAGATACATC
  A  L  A  L  T  T  S  L  L  A  G  A  A  V  S  A  H  A  Q  E  N  T  D  A  P  V  T  R  A  G  D  T  S

                                                                      400
CCGCCTGACAGATGTAGATCCGTCCGGCTTTGTTGGCTCCATTGATCCGGCAGAAAACGCCGGCCTGCTGAACTACTGTGTGCAGAACGAATACGTTGAT
 R  L  T  D  V  D  P  S  G  F  V  G  S  I  D  P  A  E  N  A  G  L  L  N  Y  C  V  Q  N  E  Y  V  D

                                                                      500
TATGACGACGCTGGCGCACGCTGCAGGAATACAACAAAAAGACCAACGCCGTGCCAGAAGGGCAGGAAGGCAACATGTCTTACGCCAATGGTTCTGCTGG
Y  D  D  A  G  A  R  C  R  N  T  T  K  R  P  T  P  C  Q  K  G  R  K  A  T  C  L  T  P  M  V  L  L  A

                                                                      600
CCTGCTGCACGCCAACAACCACACCTACACCATTGCCATGGCTATTCTGCCAGTGCGCCAGAAAACCTGTAAGGCTGTTCTGGAACGGGCCAAGGCTTCC
  C  C  T  P  T  T  T  P  T  P  L  P  W  L  F  C  Q  C  A  R  K  P  V  R  L  F  W  N  G  P  R  L  P

                                                                      700
CTGTAAGCACAAGGCTGTTATTCCGGTTTTGTTGATAAGGCCGGATCACGCTGAACGCGGCATGATGGAAGGGTCAGTCACACAAGGTGGCTGGCCCTTT
 C  K  H  K  A  V  I  P  V  L  L  I  R  P  D  H  A  E  R  G  M  M  E  G  S  V  T  Q  G  G  W  P  F

                                                                      800
TTCTGTATGCGTGCGGTTTATTTGCGGTGCATGCCGGATCAGGGCTTTCACGCAGGCGTGCAATATGGTCAAACCAGTTCTGTTTCGTAACAAGAACAAC
F  C  M  R  A  V  Y  L  R  C  M  P  D  Q  G  F  H  A  G  V  Q  Y  G  Q  T  S  S  V  S  *

                                                                      900
AGAAGCCGAAAGGGCCAGACAGAGTGGTAGAAGCGTTTTTTCCCGCAACGGATAATCCCGTTTCTGATCGGTATCAGGATCTTGTGCAGCGGGCGCAACG

                                                                     1000
TGATCCGGAGGAGTTCTGGCTGGCTCAGGCGCAACGGCTTGTATGGCATACGCAGCCCATGCAGGCCTCCCGTTCTGACTTTACGGGTGATGTACGTGTT

                                                                     1100
TCGTGGTATGAAGACGGGCGGCTGAATGCAGCCGAAAACTGTCTGGACCGGCATGTTCTGTATCAGCCCGATAATGCGGCCCTGATCTGGCAGGGGCAGG

AAGACGGGCACCGTGAGGTAATCTCATACCGGGAGTTGCATGC
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FIG. 3. Nucleotide and deduced amino acid sequences of *adhS*. The NH_2-terminal amino acid sequence determined for the purified protein by the Edman degradation procedure (24) is underlined. A potential ribosome-binding sequence (SD) is marked. The vertical arrow indicates the signal peptide cleavage site.

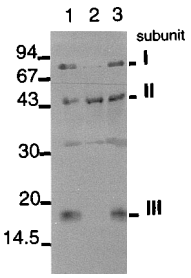

FIG. 4. Detection of ADH subunits in cells. Cell lysates of the parent, mutant strain m00-09, and transformed strains cultured in the presence of ethanol to the stationary phase were prepared as described in Materials and Methods and electrophoresed in an SDS-polyacrylamide gel. The PVDF blot of the gel was cut into three pieces corresponding to the mobility of each subunit and reacted with the respective antibodies individually. Lane 1, cell lysate (30 mg of protein) prepared from the parental strain, *A. pasteurianus* NCI1452; lane 2, cell lysate (30 mg of protein) prepared from mutant strain m00-09; lane 3, cell lysate (30 mg of protein) prepared from strain m00-09 containing pADS1892. Sizes are shown in kilodaltons.

similar to that induced by pADS1892 (data not shown) and restored the ADH activity (Table 2).

Disruption of the *adhS* gene. We next disrupted the chromosomal *adhS* gene by homologous recombination (19) to examine the function of subunit III in ADH activity. For this purpose, we constructed pADSΔ1 (Fig. 5A), containing a neomycin resistance gene in the coding sequence of *adhS* on pUC18, which was unable to replicate in *A. pasteurianus* strains. Our repeated attempts to obtain a neomycin-resistant colony after introduction of pADSΔ1 failed, probably because of the low recombination ability of strain NCI1452. We then used *A. aceti* 10-8S2 (17) as the host. This strain, closely related to *A. pasteurianus* NCI1452, is thermophilic and has been used for chromosomal gene disruption (18). About 20 neomycin-resistant colonies were isolated, and we randomly picked 5 such colonies (strains Δ1 to Δ5). Insertion of the neomycin resistance gene into the *adhS* gene was confirmed by Southern hybridization analysis by using the *adhS* and *neo* gene sequences as probes (Fig. 5C). All five recombinants thus obtained, which retained the proline auxotrophy of the parental strain, were found to lack ADH activity (Table 2). Consistent with this, the subunit III polypeptide was not produced in these mutant strains, as determined by immunoblot analysis. Figure 5B shows the Western blot of the lysate prepared from mutant Δ1.

TABLE 2. Complementation and disruption analyses of the *adhS* gene

Strain	Plasmid	Enzyme activity[a] (U/mg of protein)	
		ADH	ALDH
A. pasteurianus			
NCI1452	None	2.28	3.67
m00-09	None	<0.01	3.06
m00-09	pMV24	<0.01	2.45
m00-09	pADS1892	1.88	3.50
m00-09	pADS1991	2.32	3.59
A. aceti			
10-8S2	None	2.26	2.29
Δ1[b]	None	<0.01	2.26

[a] Host strains and mutants of *Acetobacter* spp. were cultured for 24 h at 30°C in medium initially containing 3% ethanol. Cells were then harvested, and the ADH and ALDH activities were measured.

[b] Strain Δ1 is an *adhS* null mutant, obtained by gene disruption. Four similar mutants, Δ2 to Δ5, showed almost the same ADH and ALDH activities as strain Δ1.

Localization of the ADH subunits in the cell. We next examined the localization of each subunit of the ADH complex to obtain information on the function of subunit III. For this purpose, we isolated mutant strains deficient in each subunit, in addition to strain m00-09 (Table 1). These mutants were characterized by immunoblotting and DNA complementation testing (Table 3 and Fig. 6). Our attempts to fractionate the cellular sonicate into three fractions (cytoplasmic, periplasmic, and membrane fractions) by the cold osmotic shock method (9, 15), usable for *E. coli*, were unsuccessful, and we fractionated the sonicate into two fractions (cytoplasmic and membrane) in the present study. The soluble fraction thus contained the proteins in both the cytoplasm and the periplasmic space. In the membrane fractions of the parental strain and strain m00-09 containing the *adhS* gene on pADS1892, the three components of ADH appeared to be present at the ratio of 1:1:1 (Fig. 7), although the subunit III bands seemed to be broad, probably because of the unique features of the protein discussed below. Because the sizes of subunit III detected in the membrane and soluble fractions were the same, indicative of the processing of the signal peptide, the subunit III protein fractionated into the soluble fraction supposedly came from the periplasmic space. The protein band with a slightly smaller size than that of subunit I was an artifact due to nonspecific reactivity of the anti-subunit I antibody used, because this band was also detected in mutant m36-05, which is deficient in subunit I (Fig. 7, lane 8S), and in mutant m00-21, which is deficient in subunits I and II (lanes 4S and 9S). This means that the absence of subunit III leads to a decrease in the amount of subunit I to an undetectable level during stationary-phase growth. In logarithmic-phase growth, subunit I was apparently present (Fig. 7, lane 6S). A similar observation was obtained with strain Δ1 containing the disrupted *adhS* gene (data not shown).

In strain m00-27, deficient in subunit II, larger amounts of subunits I and III were present in the soluble fraction than in the membrane fraction (Fig. 7, lane 3S). This abnormal localization of subunits I and III after fractionation was observed in both the logarithmic and stationary phases. These observations suggest that subunit II serves as an anchor for subunits I and III to be fractionated in the membrane fraction. The same function was also suggested by the localization of subunit III in mutant m00-21, which is deficient in subunits I and II; a larger amount of subunit III was detected in the soluble fraction than in the membrane fraction.

The localization of each subunit of ADH in the cellular fractions of various mutant strains is summarized in Table 4. Several points deduced from this summary are as follows: (i) subunit II (cytochrome *c*) is always localized in the membrane fraction, irrespective of the absence of subunits I and III, (ii) the absence of subunit III leads to a decrease in the amount of subunit I in both the membrane and soluble fractions, especially in the stationary phase, and finally (iii) correct localization of each of the three subunits requires the presence of all three subunits, suggesting a close interaction among the three subunits for localization in the membrane.

DISCUSSION

We cloned the *adhS* gene, encoding the smallest subunit of three-component ADH from *A. pasteurianus*. *adhS* is not located near the other two larger subunit genes (25). This suggests that *adhS* belongs to a transcriptional unit different from

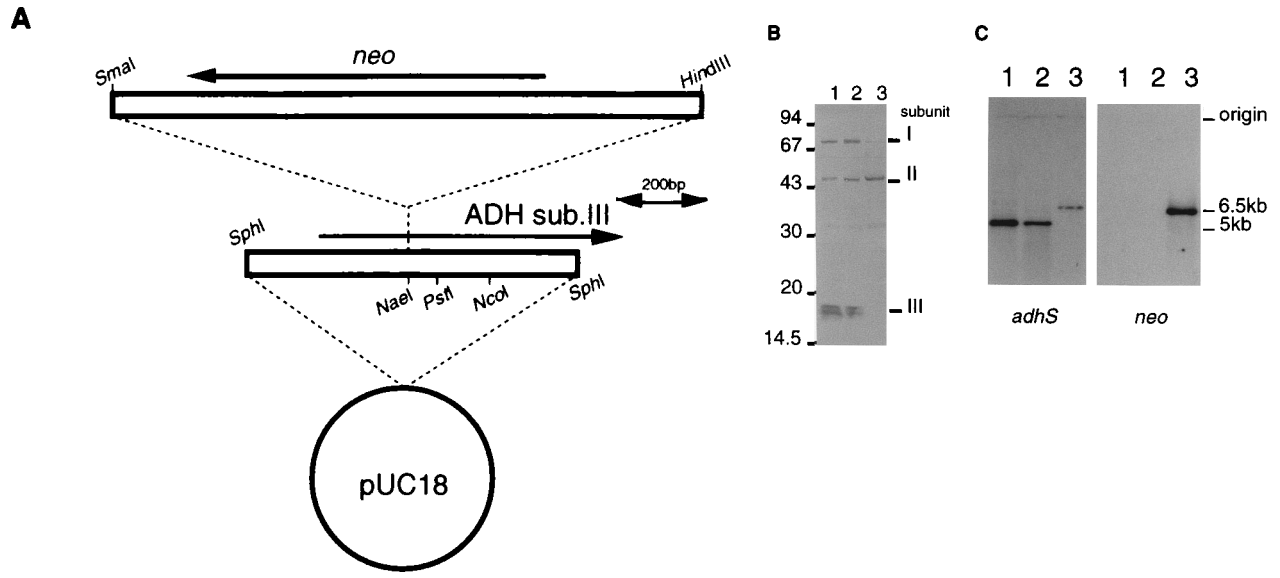

FIG. 5. Disruption of the chromosomal *adhS* gene of *A. aceti*. (A) Structure of pADSΔ1. A 0.7-kb *Sph*I fragment encoding the NH_2-terminal region of the *adhS* product was inserted at the *Sph*I site of pUC18, and a 1.3-kb *Hin*dIII-*Sma*I fragment containing the neomycin resistance gene (*neo*) (7) was inserted at the *Nae*I site of the 0.7-kb fragment. pADSΔ1 was linearized with *Sma*I before transformation for homologous recombination and introduced by the electroporation method. (B) Immunoblot analysis of an *adhS* disruptant. The culture conditions were as described in the legend to Fig. 4. (C) Confirmation of correct disruption of the *adhS* gene by Southern hybridization. Hybridization with the *adhS* probe (left panel) and the *neo* probe (right panel) was done with the *Eco*RV-digested total DNAs from the parental and mutant strains. Lane 1, *A. pasteurianus* NCI1452; lane 2, *A. aceti* 10-8S2 (parental strain of Δ1); lane 3, mutant strain Δ1.

that for the other subunits. The nucleotide sequence of *adhS* shows that the 22-kDa protein is synthesized as a preprotein, with the NH_2-terminal 28 amino acids probably serving as its signal sequence for secretion from cytoplasm to periplasm (28). No homologous proteins have been registered in any protein data bank. The mature subunit III protein contains 11 cysteines (6.2% of the total amino acids of subunit III), and it may be a compact globular form due to possible intramolecular disulfide bonds. This feature makes the molecular mass (15 to 17 kDa) of subunit III, calculated by its mobility in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, smaller than its actual processed size (19.5 kDa). This feature may also explain why the stained subunit III band in SDS-polyacrylamide gels is often broad or sometimes migrates as multiple bands. This effect may be due to imperfect denaturation, especially incomplete cleavage of disulfide bonds.

It is evident that the *adhS* gene product (subunit III) is essentially required for the ADH complex of the three-component type to express its enzyme activity, since mutants deficient in subunit III lack any detectable ADH activity and disruption of the chromosomal *adhS* gene results in the loss of ADH activity. In addition, introduction of the cloned *adhS* gene into such *adhS* disruptants reverses the ADH-deficient phenotype. It was interesting to us to see the effect of subunit III on the enzyme activity of the two-component ADH complex in *Acetobacter polyoxogenes*. The dehydrogenase and cytochrome *c* subunits in *A. pasteurianus* and *A. polyoxogenes* are similar (25, 26). We introduced pADS1892 into *A. polyoxogenes* and determined the ADH activity. However, no change in the enzyme activity was observed (data not shown). We assume that the dehydrogenase and cytochrome *c* subunits of *A. polyoxogenes* assemble to form a stable complex in the absence of an additional small protein. A similar study to determine the localization of each of the two subunits in various mutants is necessary to make this point clear.

Examination of the localization of each subunit of the ADH complex gives an insight into the function of subunit III and the manner of assembly of the three subunits. The present study and our previous study (25) show that all three subunits

TABLE 3. Characterization of mutants by DNA complementation of ADH subunit genes

Strain	Subunit production[a]			DNA complementation for ADH activity[b]				
	I	II	III	I	II	I + II	III	Vector
m00-09	+	+	−	−	−	−	+	−
m00-21	−	−	+	−	−	+	−	−
m00-27	+	−	+	−	+	+	−	−
m36-05	−	+	+	+	−	+	−	−
Δ1	+	+	−	−	−	−	+	−

[a] The subunits produced by the mutants were detected by immunoblotting. +, present; −, absent.

[b] DNA fragments encoding each ADH subunit gene were constructed as described in the legends to Fig. 2 and 6. Transformants were assayed on YPG containing 0.1% $CaCO_3$ and 3% ethanol. Transformants with restored ADH activity have halos surrounding the colonies (+).

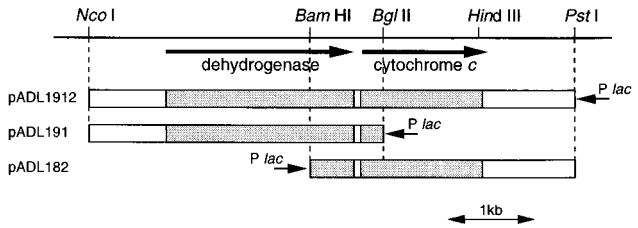

FIG. 6. Plasmid constructions for the DNA complementation test. These fragments were inserted at the *Sma*I site of pMV24. The heavy arrows indicate the extent and direction of the dehydrogenase and cytochrome *c* subunit genes of ADH. P *lac* indicates the *lac* promoter, and the accompanying thin arrow represents its direction.

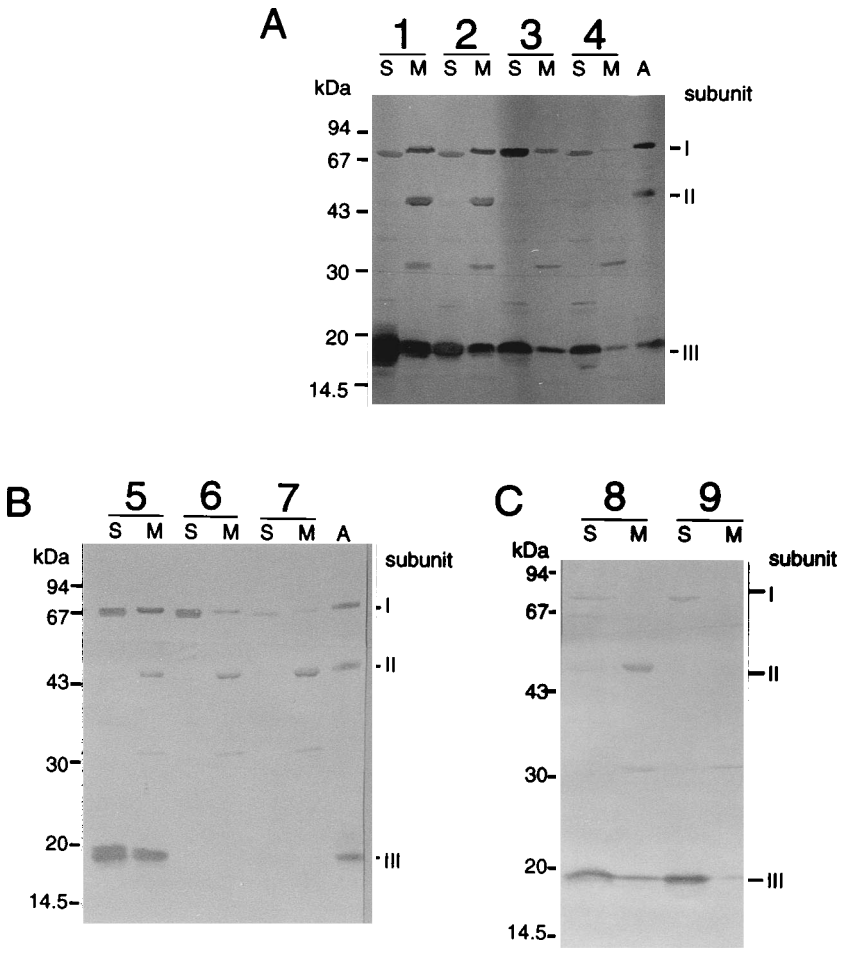

FIG. 7. Localization of ADH subunits. The parental and mutant strains cultured in the presence of ethanol to the stationary phase of growth (except for panel B) were fractionated and electrophoresed in SDS-polyacrylamide gels. In panel B, cells were grown to the mid-logarithmic phase. The PVDF blot of the gel was cut into three pieces according to the mobility of each subunit and probed with the respective antisera. Lanes: A, purified ADH (0.2 mg); S, soluble fraction; M, membrane fraction; 1, mutant strain m00-09 containing pADS1892; 2, parental strain NCI1452; 3, mutant strain m00-27; 4 and 9, mutant strain m00-21; 5, parental strain NCI1452 in the mid-logarithmic phase of growth; 6, mutant strain m00-09 in the mid-logarithmic phase of growth; 7, mutant strain m00-09 in the stationary phase of growth; 8, mutant strain m36-05. Thirty milligrams of protein was applied to every lane.

TABLE 4. Localization of each subunit of ADH in various mutant strains[a]

Strain	Subunit production			Localization of subunits											
				Logarithmic growth phase						Stationary growth phase					
				Membrane			Soluble			Membrane			Soluble		
	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
Parent	+	+	+	+	+	+	+	−	+	+	+	+	−	−	+
m00-09(pADS1892)	+	+	+	+	+	+	+	−	+	+	+	+	−	−	+
Δ1(pADS1892)	+	+	+	+	+	+	+	−	+	+	+	+	−	−	+
m00-09	+	+	−	+	+	−	+	−	−	W	+	−	−	−	−
Δ1	+	+	−	+	+	−	+	−	−	W	+	−	−	−	−
m00-21	−	−	+	−	−	−	−	−	+	−	−	−	−	−	+
m00-27	+	−	+	−	−	−	+	−	+	−	−	−	+	−	+
m36-05	−	+	+	−	+	W	−	−	+	−	+	W	−	−	+

[a] Subunit production and localization of subunits were determined by immunoblotting. +, present; −, absent; W, weak signal.

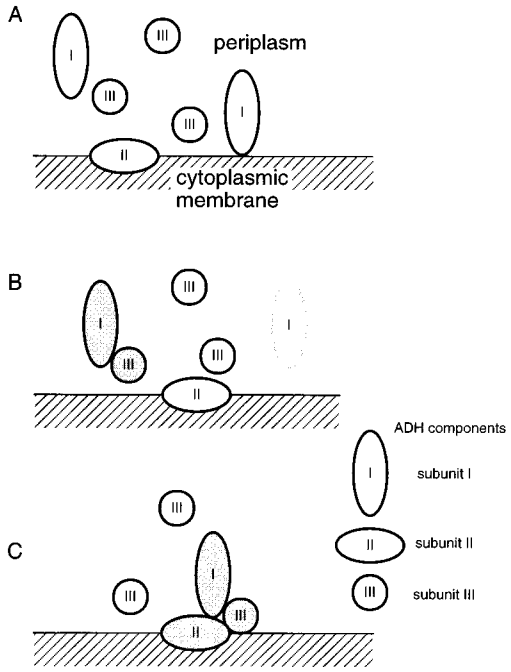

FIG. 8. Speculative model for the formation of the active three-component ADH complex. See the text for details.

are produced as prepeptides with typical signal sequences for secretion, suggesting that they are transported through the cytoplasmic membrane and buried at the periplasmic surface of the membrane or dissolved in the periplasmic space. It is generally believed that cytochromes essential for electron transport are buried in the cytoplasmic membrane (23). Consistent with this, the cytochrome *c* subunit was always detected in the membrane fraction, even in the absence of the other two subunits. The dehydrogenase subunit and subunit III detected in the soluble fraction in various mutants in the present study are supposedly in the periplasmic space, since they are of the same sizes as the corresponding subunits detected in the membrane fraction of the parental strain. The dehydrogenase subunit, probably in the periplasmic space in mutant m00-09, which is deficient in subunit III, was almost invisible by immunoblotting (Fig. 7B), whereas in mutant m00-27, which is deficient in the cytochrome *c* subunit but still contains subunit III, this subunit was detected even in the stationary phase. From these observations, we assume that the dehydrogenase subunit is unstable by itself, probably because of proteolytic degradation, and that subunit III protects the dehydrogenase subunit from such proteolysis by forming a complex (Fig. 8B). The dehydrogenase-subunit III complex seems to be built on the cytochrome *c* subunit embedded in the membrane (Fig. 8C). The stronger interaction between the dehydrogenase and subunit III subunits is implied by the experimental observation that the cytochrome *c* subunit is easily dissociated from the purified ADH complex, but dissociation of the dehydrogenase subunit and subunit III cannot be achieved without extreme conditions in the case of the three-component ADH of *Gluconobacter suboxydans* (14a). The 11 cysteine residues of subunit III may contribute to the tight binding, possibly via intermolecular disulfide bonds. An additional speculative function of subunit III is that it helps the dehydrogenase subunit couple with the cytochrome *c* subunit, thereby keeping the correct conformation of the ADH complex for electron transport on the periplasmic surface of the membrane (Fig. 8C).

The model shown in Fig. 8 is our speculation. It is still not clear to what extent a complex consisting of the larger two subunits, i.e., the dehydrogenase and cytochrome *c* subunits, is enzymatically active. The degree of interaction between the dehydrogenase-subunit III complex and the cytochrome *c* subunit should also be examined. We have not yet succeeded in reconstituting the active enzyme from the individual subunits, because dissociation of the dehydrogenase subunit and subunit III cannot be achieved without damaging either of the two subunits. We expect that these uncertainties will be made clear in the near future.

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