APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1997, p. 1131–1138 Vol. 63, No. 3
0099-2240/97/$04.00+0

Characterization of the Genes Encoding the Three-Component Membrane-Bound Alcohol Dehydrogenase from *Gluconobacter suboxydans* and Their Expression in *Acetobacter pasteurianus*

KOICHI KONDO† AND SUEHARU HORINOUCHI*

Department of Biotechnology, Graduate School of Agriculture and Life Sciences, University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113, Japan

Received 16 September 1996/Accepted 3 December 1996

The three-component membrane-bound alcohol dehydrogenase (ADH) of *Gluconobacter suboxydans* IFO12528 was purified, and the NH_2-terminal amino acid sequence of each subunit was determined. On the basis of the amino acid sequences, the genes *adhA*, encoding the 72-kDa dehydrogenase, *adhB*, encoding the 44-kDa cytochrome *c*-553 (a CO-binding cytochrome *c*), and *adhS*, encoding a 15-kDa protein, were cloned and the amino acid sequences of their products were deduced from the nucleotide sequences. The dehydrogenase and cytochrome genes were clustered with the same transcription polarity, as is the case in species of *Acetobacter*, another genus of acetic acid bacteria. These AdhA and AdhB subunits showed similarity in amino acid sequence to those from *Acetobacter* spp., whereas AdhS showed no similarity to the corresponding subunit of the ADH complex of *Acetobacter pasteurianus*. Consistent with this, *adhS* of *G. suboxydans* could not complement a defect in the corresponding subunit of *A. pasteurianus*. When the *adhA-adhB* gene cluster of *G. suboxydans* was expressed in an ADH-deficient mutant of *A. pasteurianus*, the transformant showed distinct ADH activity. The ADH complex was purified to near homogeneity and consisted of two subunits, the dehydrogenase and the cytochrome *c* subunits derived from *G. suboxydans*, without any other subunit. These data suggested that AdhS, the smallest subunit of ADH, from *G. suboxydans* is not essential for ADH activity in *A. pasteurianus*, in contrast to the essential role of *A. pasteurianus* AdhS, which is required for correct assembly of the dehydrogenase and cytochrome *c* subunits on the membrane.

The oxidation of ethanol to acetic acid by acetic acid bacteria is the most characteristic process in vinegar production (14). This ethanol oxidation is catalyzed by two membrane-bound enzymes, alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (1–3, 5–7, 16, 39). The ADHs (a-ADH) of *Acetobacter*, a genus of acetic acid bacteria widely used for industrial vinegar production, are classified into two groups: the two-component (a2-ADH) type and the three-component (a3-ADH) type. The former, which occurs 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) (39), while the latter, which occurs in *Acetobacter aceti* and *Acetobacter pasteurianus*, contains an additional 20-kDa subunit (subunit III) (25, 37). We have purified and characterized both types of ADHs (37, 39) and cloned all the genes encoding their subunits (21, 37, 38). These studies of *Acetobacter* enzymes have shown that all subunits in both a2-ADH and a3-ADH are essential for enzyme activity. It is thought that the two larger subunits are functionally required for electron transport (25, 39) and that the smallest subunit, present only in the a3-ADH from *A. aceti* and *A. pasteurianus*, contributes to the precise association of the two functional subunits on the membrane (21).

On the other hand, ADH (g3-ADH) of *Gluconobacter suboxydans*, a member of another genus of acetic acid bacteria, has not been adequately characterized. g3-ADH has been purified for elucidation of electron transport (3, 25), since *G. suboxydans* supposedly contains an efficient respiratory chain because of its ability to oxidize several sugars and sugar alcohols besides ethanol (14). Like a3-ADH, g3-ADH consists of three subunits (25, 26): a 72-kDa dehydrogenase subunit (subunit I), a 44-kDa cytochrome *c* subunit (subunit II), and a 15-kDa subunit (subunit III). However, neither the gene structures and amino acid sequences of g3-ADH nor the role of the smallest subunit has yet been elucidated.

The first objective of this study was to deduce the amino acid sequences of the three subunits of g3-ADH by cloning and sequencing the genes. The second objective was to determine whether the smallest subunit in g3-ADH plays a role essential for enzyme activity. Expression of the cloned genes in *A. pasteurianus* mutants deficient in ADH demonstrated that the smallest subunit played no obligatory role for ADH activity, in contrast to its counterpart in a3-ADH.

MATERIALS AND METHODS

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

Media and culture conditions. Sugar-rich medium (pH 6.5) used for *Gluconobacter* spp. consisted of 20 g of sodium gluconate, 5 g of glucose, 3 g of glycerol, 3 g of yeast extract (Wako Pure Chemicals), and 2 g of polypeptone (Wako Pure Chemicals) in 1 liter of water. YPG medium (pH 6.5) used for *Acetobacter* spp. consisted of 5 g of yeast extract (Wako Pure Chemicals), 3 g of polypeptone (Wako Pure Chemicals), and 30 g of glucose in 1 liter of water. Solid medium was made by adding 2% agar. Each strain of acetic acid bacteria was first cultured with shaking in a 50-ml test tube containing 5 ml of medium for 24 to 40 h at 30°C. A portion (1 to 5 ml) of the broth was inoculated into 100 ml of medium in a 500-ml shaking flask and further cultured with shaking at 30°C. Sugar-rich medium supplemented with allyl alcohol (0.002%) was used for positive selection of ADH-deficient mutants (21, 24). When necessary, ethanol at a final concentration of 3% was added to the medium at the start of the culture. *Escherichia coli* strains were routinely cultured in Luria broth (31). Ampicillin was added to the medium at a final concentration of 50 μg per ml when necessary.

DNA preparation and manipulation. Total DNA of acetic acid bacteria was prepared as described by Okumura et al. (29). DNA-DNA hybridization was

* Corresponding author. Phone: 81 (03) 3812-2111, ext. 5123. Fax: 81 (3) 5802-2931.

† Present address: Nakano Central Research Institute, Nakano Vinegar Co., Ltd., Nakamura-cho 2-6, Handa-shi, Aichi 475, Japan.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
G. suboxydans IFO12528	Wild type	IFO[b]
A. pasteurianus		
m00-09	Sm^r, *met*, reduced ADH activity (subunit III deficient); spontaneous mutant of NCI1452	21
m00-21	Sm^r, *met*, reduced ADH activity (subunit I and subunit II deficient); spontaneous mutant of NCI1452	21
m36-05	Sm^r, *met*, reduced ADH activity (subunit I deficient); spontaneous mutant of NCI1452	21
E. coli JM109	*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1*Δ(*lac-proAB*)/F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*	42
Plasmids		
pUC19	Ap^r, *lacZ′*	42
pMV24	Ap^r, *lacZ′*; *Acetobacter-E. coli* shuttle vector	17
pGADL192[c]	Ap^r; 4-kb *Pst*I fragment containing g3-*adhA* and an NH_2-terminal portion of g3-*adhB* on pUC19	This work
pGADL198[d]	Ap^r; 1.5-kb *Eco*RI fragment containing a COOH-terminal portion of g3-*adhB* on pUC19	This work
pGADL1911[e]	Ap^r; 5.6-kb *Pst*I-*Eco*RI fragment containing g3-*adhAB* reconstructed from both pGADL192 and pGADL198 on pUC19	This work
pGADS1922[f]	Ap^r; 3.6-kb *Sac*I fragment containing g3-*adhS* and g-*ppa* on pUC19	This work
pGADL2411[g]	Ap^r; 4.2-kb PCR fragment containing g3-*adhAB* from pGADL1911 subcloned onto pMV24	This work
pGADS2422[h]	Ap^r; 0.6-kb PCR fragment containing g3-*adhS* from pGADS1922 subcloned onto pMV24	This work

[a] Ap^r, ampicillin resistance; Sm^r, streptomycin resistance.
[b] IFO, Institute for Fermentation, Osaka, Japan.
[c] A 4-kb *Pst*I fragment that hybridized with a region encoding an NH_2-terminal part of the *c*-553 gene was cloned in the *Pst*I site of pUC19 (Fig. 2).
[d] A 1.5-kb *Eco*RI fragment that hybridized with a region encoding an NH_2-terminal part of the *c*-553 gene was cloned in the *Eco*RI site of pUC19 (Fig. 2).
[e] The two fragments in pGADL192 and pGADL198 were combined to generate a 5.6-kb *Pst*I-*Eco*RI fragment in pUC19 by the standard recombinant DNA techniques (Fig. 2).
[f] A 3.6-kb *Sac*I fragment that hybridized with an oligonucleotide designed on the basis of the NH_2-terminal amino acid sequence of AdhS was cloned in the *Sac*I site of pUC19.
[g] A 4.2-kb fragment containing g3-*adhAB* was amplified by PCR, as described in Materials and Methods, and cloned into pMV24.
[h] A 0.6-kb fragment containing g3-*adhS* was amplified by PCR, as described in Materials and Methods, and cloned into pMV24 (Fig. 2).

performed by the standard method (31, 34) with a nylon membrane (Hybond-N$^+$; Amersham International plc.). [α-^{32}P]dCTP at 3 kCi/mmol for the Amersham multiprime DNA-labeling system was purchased from Amersham. Restriction endonucleases, Klenow fragment, and T4 DNA ligase were purchased from Takara Shuzo Co. (Kyoto, Japan). High-fidelity thermostable DNA polymerase (Expand) used for the PCR was purchased from Boehringer Mannheim GmbH. *E. coli* was transformed as described by Hanahan (18). *Acetobacter* strains were transformed by the electroporation method described by Wong et al. (41). DNA was manipulated by cloning into pUC19 (42). Nucleotide sequences of DNA fragments cloned in pUC vectors (42) were determined by the dideoxy chain termination method (32) on DSQ-1000 (Shimadzu) and Li-Cor model 4000L DNA sequencers. General recombinant DNA techniques were as described by Sambrook et al. (31).

Synthesis of oligomeric DNAs. Oligomer DNAs were synthesized on an Expedite nucleic acid synthesis system DNA synthesizer (PerSeptive Biosystems). Two primers were used for amplification by PCR of the NH_2-terminal region of the *c*-553 gene (35): 553-N (5′-GGGGATCCATTAACTCGGGACAG-3′, which extends from nucleotides [nt] 2787 to 2802 of the *c*-553 gene [see Fig. 3], with seven bases added to the 5′ end of the oligonucleotide to generate a *Bam*HI site) and 553-C (5′-AAAAGCTTCATTCGCCGCAATGGC-3′, complementary to the region from nt 3396 to 3381, with eight bases added to the 5′ end of the oligonucleotide to generate a *Hin*dIII site). Two probes for cloning of the subunit III gene of g3-ADH were designed on the basis of its NH_2-terminal amino acid sequence (see Results). These probes were III-mix (5′-CA[A/G]GA[A/G]CA[A/G]TCCCCNCCNCCNCCNCCNGCNCTNCA[A/G]GG-3′) and III-inosine (5′-GGNGTNCCITGIACIGCIGGIGGIGGIGGIGGGGAITGITC[C/T]TG-3′). Two pairs of PCR primers were used for subcloning of the g3-ADH genes: the pair g3-*adhS*-N (5′-GCGTCGACCTAAGGATACCGTGATGTTTCGTCG-3′, which extends from nt 333 to 357 [see Fig. 5], with eight bases added to the 5′ end of the oligonucleotide to generate a *Sal*I site) and g3-*adhS*-C (5′-GCTCTAGAGGCGGCGATACTACCGGCGGCCGG-3′, complementary to the region from nt 899 to 873, with five bases added to the 5′ end of the oligonucleotide to generate an *Xba*I site) and the pair g3-*adhAB*-N (5′-AAAGTCGACATGAACGACACCGTGCCCGAGC-3′, which extends from nt 9 to 30 [see Fig. 3], with eight bases added to the 5′ end of the oligonucleotide to generate a *Sal*I site) and g3-*adhAB*-C (5′-TCTCTAGACAGGGGTGGGGACGCTTATTGTGCG-3′, complementary to the region from nt 4228 to 4204, with eight bases added to the 5′ end of the oligonucleotide to generate an *Xba*I site). For amplification of the *Gluconobacter adhS* and *adhAB* regions, cloned fragments on pGADS1922 and pGADL1911, respectively, were used.

Enzyme assays. Cells were grown until mid- or late logarithmic phase and harvested by centrifugation. The cells were suspended in 10 mM potassium phosphate buffer (pH 6.0) and disrupted with an ultrasonifier (Branson Sonifier cell disrupter 250). The activity of ADH was measured by the ferricyanide method of Ameyama and Adachi (5).

Purification and amino acid sequencing of ADHs. Native ADH from *G. suboxydans* was purified from the membrane fraction of cells grown in sugar-rich medium supplemented with 2% ethanol as described by Tayama et al. (39). The g3-ADH AdhA-AdhB (herein referred to as g3-AdhA–g3-AdhB) complex from an *A. pasteurianus* transformant was purified similarly. The amino acid sequence of the NH_2-terminal region of each subunit was determined by Edman degradation (15) on an Applied Biosystems model 477A protein sequencer. Because the NH_2 termini of all the subunits were modified, their NH_2-terminal amino acid sequences were determined after treatment with pyroglutamate aminopeptidase (Takara Shuzo).

Immunoblot hybridization. Antisera raised against each of the a3-ADH subunits (37) were used for immunoblot detection of electrophoresed proteins by the method of Burnette (11). A polyvinylidene difluoride (PVDF) membrane was used for blotting. The blotted membrane was cut into three pieces, each corresponding to the mobility of one subunit, and pieces were then mixed with the appropriate antibodies (21).

Computer-aided analysis of nucleotide and amino acid sequences. The DNA sequence was analyzed by using the GENETYX sequence analysis program (Software Development). DNA and amino acid homology searches were performed at the National Center for Biotechnology Information, Bethesda, Md., by using the BLAST network service (4).

Nucleotide sequence accession number. The nucleotide sequences of the dehydrogenase and subunit III genes will appear in the DDBJ, EMBL, and Gen-

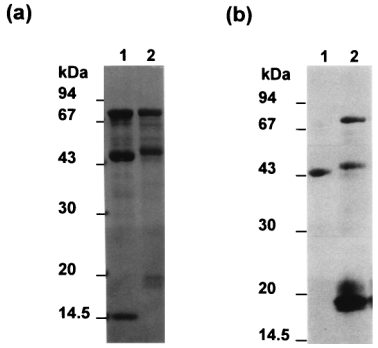

FIG. 1. Gel electrophoretic analysis (a) and immunoblot analysis (b) of g3-ADH and a3-ADH. (a) g3-ADH (10 μg of protein) purified from *G. suboxydans* IFO12528 (lane 1) and a3-ADH (10 μg of protein) purified from *A. pasteurianus* NCI1452 (lane 2) were electrophoresed in an SDS-polyacrylamide gel. The proteins were stained with Coomassie brilliant blue R-250. (b) The two ADHs were similarly electrophoresed and transferred to a PVDF membrane. The PVDF blot was then cut into three pieces, each of which contained one of the three subunits, and the pieces were reacted individually with antisera raised against each subunit of the a3-ADH complex. Among the three subunits of g3-ADH, only subunit II reacted with the antibody.

Bank nucleotide sequence databases with the accession numbers D86375 and D86440, respectively.

RESULTS

NH_2-terminal amino acid sequences of the three components of ADH in *G. suboxydans*. In order to clone the genes encoding the three components of the ADH of *G. suboxydans* IFO12528 by the DNA-probing method, we purified the enzyme complex and determined the NH_2-terminal amino acid sequence of each subunit by the Edman degradation procedure (15). Purified g3-ADH with its specific activity of 260 U/mg of protein consisted of a 72-kDa dehydrogenase subunit (subunit I), a 44-kDa cytochrome *c* subunit (subunit II), and a 15-kDa subunit (subunit III), as estimated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (Fig. 1a). A comparison of the electrophoretic patterns of g3-ADH from *G. suboxydans* and of a3-ADH from *A. pasteurianus* is shown in Fig. 1a. The mobilities of the two larger polypeptides were almost the same, whereas those of the smallest ones were quite different (15 kDa for g3-ADH and 20 kDa for a3-ADH) (Fig. 1a). Reactivity of each subunit of g3-ADH with the polyclonal antibodies raised against each subunit of a3-ADH (37) is shown in Fig. 1b. Only subunit II of g3-ADH cross-reacted with the antiserum raised against subunit II of a3-ADH, suggesting close similarity between the subunits II from the two species.

The NH_2-terminal amino acids of the three subunits of g3-ADH could not be determined by Edman degradation, probably due to a modification of the NH_2 terminus. We then treated the subunits with pyroglutamate aminopeptidase, because the NH_2 termini of the subunits of a3-ADH were pyroglutamate (17, 38). As expected, the aminopeptidase treatment gave sequences as follows: Glu-Asp-Thr-Gly-Ala-Ile-Ser-Ser-Asp-Asn-Gly-Gly-His for subunit I, Asp-Ala-Asp-Glu-Ala-Leu-Ile-Lys-Arg-Gly-Glu-Tyr-Val-Ala-Arg for subunit II, and Glu-Gln-Ser-Pro-Pro-Pro-Pro-Pro-Ala-Val-Gln-Gly-Thr-Pro-Gly for subunit III. The amino termini of the three subunits deduced from the nucleotide sequences were Gln, as anticipated (see below).

Cloning and nucleotide sequence of the gene cluster encoding subunit I and subunit II of g3-ADH. A computer-aided homology search predicted that subunit II of g3-ADH would be identical to the *c*-553 protein from *G. suboxydans* IFO12528

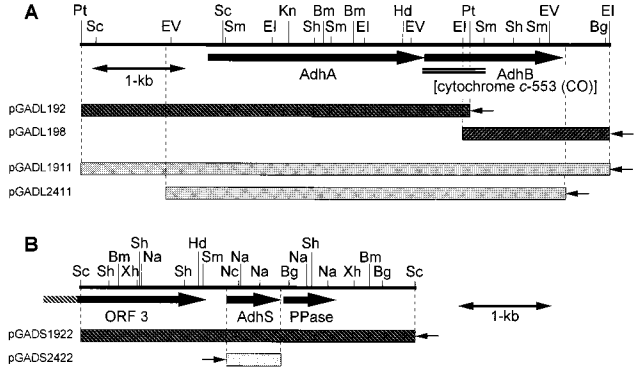

FIG. 2. Restriction maps of the regions encoding the 72-kDa dehydrogenase and 44-kDa cytochrome *c* subunits of g3-ADH (A) and the 3.6-kb *Sac*I fragment encoding g3-AdhS and neighboring genes (B). (A) The *Pst*I and *Eco*RI fragments on pGADL192 and pGADL198 were originally cloned in pUC19. pGADL1911 contains the indicated 5.6-kb *Pst*I-*Eco*RI fragment in pUC19. pGADL2411 contains the subcloned 4.2-kb fragment in pMV24. The thick arrows indicate the location and direction of the dehydrogenase and cytochrome *c* genes. The probe used for cloning this region is indicated by the double bars. The thin arrows indicate the direction of the *lac* promoter in the vectors. (B) The 3.6-kb *Sac*I fragment on pGADS1922 was originally cloned in pUC19. pGADS2422 contains the subcloned 0.6-kb fragment encoding only g3-AdhS in pMV24. The thick arrows indicate the location and direction of three ORFs. The thin arrows indicate the direction of the *lac* promoter in the vectors. Bg, *Bgl*II; Bm, *Bam*HI; EI, *Eco*RI; EV, *Eco*RV; Hd, *Hin*dIII; Kn, *Kpn*I; Na, *Nae*I; Nc, *Nco*I; Pt, *Pst*I; Sc, *Sac*I; Sh, *Sph*I; Sm, *Sma*I, Xh, *Xho*I; PPase, inorganic pyrophosphatase.

(35), since the NH_2-terminal amino acid sequence of subunit II of g3-ADH was identical to the NH_2-terminal portion of the *c*-553 gene. On the basis of the structure of the gene cluster encoding subunits I and II in *Acetobacter* spp. (20, 37, 38), we expected that subunit I of g3-ADH would be encoded by a region just upstream of the *c*-553 gene. Total DNA from *G. suboxydans* digested with several restriction endonucleases was hybridized with a ^{32}P-labeled DNA fragment covering the sequence from nt 2783 to 3400 of the *c*-553 gene (Fig. 2) as a probe. Approximately 4-kb *Pst*I fragments and 1.5-kb *Eco*RI fragments showing positive hybridization with the probe were recovered from gel slices and cloned into the corresponding sites of pUC19, resulting in pGADL192 and pGADL198, respectively. The restriction map of the cloned 5.6-kb region is shown in Fig. 2. This fragment was found to contain both subunits I and II, as shown below. The complete nucleotide sequence of the region is shown in Fig. 3. We named these genes g3-*adhA* and g3-*adhB*, respectively.

The open reading frame (ORF) corresponding to g3-*adhA* starts with ATG (nt 474 to 476) and terminates with TGA (nt 2745 to 2747) and encodes a polypeptide composed of 757 amino acids. A possible ribosome-binding sequence (33), AAA GGA, is present 10 nt upstream of the ATG start codon. The NH_2-terminal amino acid sequence determined with the purified g3-AdhA is present in the deduced amino acid sequence at positions 36 to 50. Gln-35 appears to be modified in vivo into pyroglutamate, because the NH_2-terminal sequence was obtained only after treatment with pyroglutamate aminopeptidase. The additional 34 amino acids at the NH_2 terminus show features typical of a signal peptide (40), i.e., positively charged residues at the NH_2 terminus followed by a stretch of hydrophobic residues. g3-AdhA shows significant similarity (approximately 70%) in amino acid sequence to the corresponding subunits of a2-ADH (38) and a3-ADH (19, 20, 37) (Fig. 4) despite little or no detectable cross-reactivity between g3-AdhA and the anti-a3-AdhA antibody (Fig. 1b). Because of the

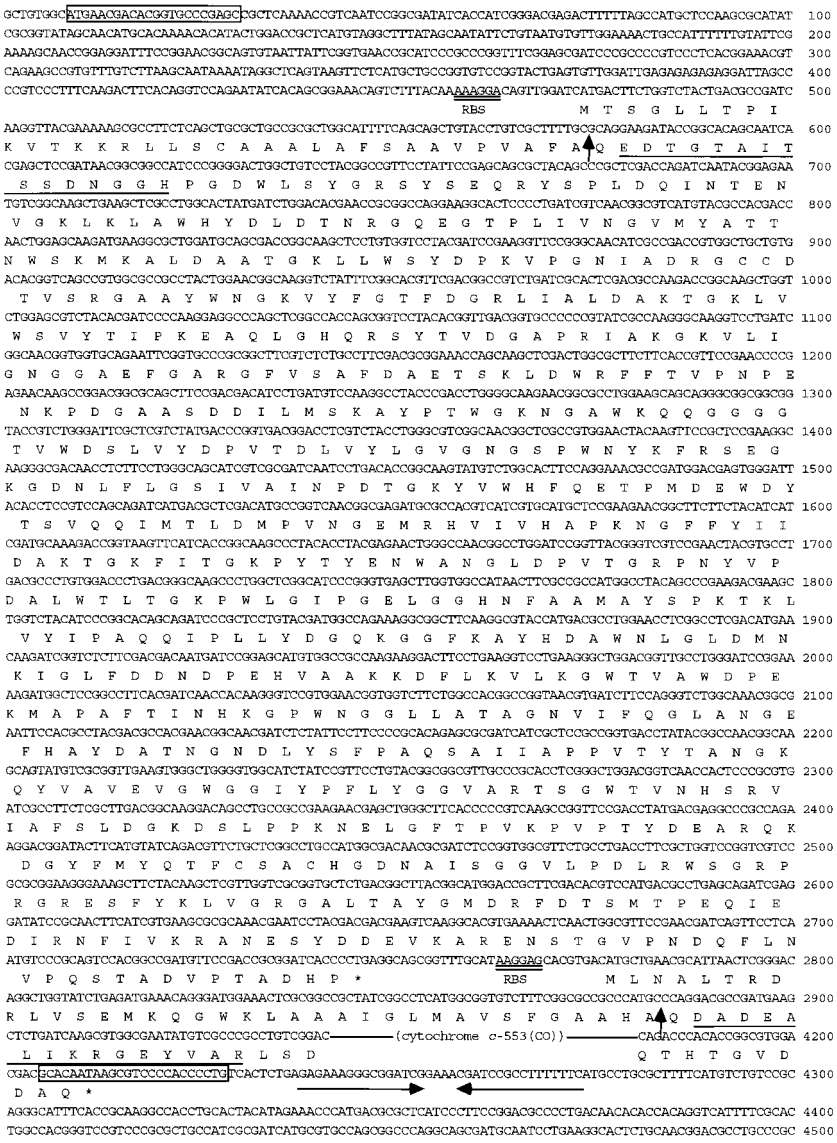

FIG. 3. Nucleotide and deduced amino acid sequences of the 5.6-kb region encoding dehydrogenase and cytochrome *c* subunits of g3-ADH. The NH_2-terminal amino acid sequences determined for the purified proteins by the Edman degradation procedure are underlined. RBS, potential ribosome-binding sequence. The signal peptide cleavage sites are marked by vertical arrows. An inverted repeat, which possibly serves as a rho-independent transcriptional terminator, is indicated by facing arrows. The primer sequences used for subcloning of the g3-*adhAB* gene cluster are boxed.

similarity (approximately 60%) in G+C content between the DNAs of *Gluconobacter* and *Acetobacter*, the nucleotide sequences encoding g3-ADH and a3-ADH also have about 70% identity. In the amino acid sequence of g3-AdhA, two putative pyrroquinoline quinone (PQQ)-binding motifs (nt 629 to 716 and 1308 to 1376) (10) and one heme-binding motif (nt 2430 to 2444) (24) were present. One molecule of PQQ is thought to be bound by two PQQ-binding motifs. PQQ-binding motifs are also present in other quinoprotein dehydrogenases, such as glucose dehydrogenases (12, 13) and methanol dehydrogenases (9). The presence of these motifs supports the idea that subunit I of ADHs in acetic acid bacteria is a quinohemoprotein dehydrogenase (25).

The ORF corresponding to g3-*adhB* is located 29 bp downstream of the termination codon of g3-*adhA*. This ORF, which starts at ATG (nt 2777 to 2779) and terminates at TAA (nt 4211 to 4213), encodes a 478-amino-acid protein that is similar to *c*-type cytochromes (28) and identical to the protein encoded by the membrane-bound cytochrome *c*-553 gene (a CO-binding type) (35, 36). A possible ribosome-binding sequence (33), AAGGAG, is present 8 nt upstream of the ATG codon. The NH_2-terminal amino acid sequence determined with the purified g3-AdhB is present in the deduced amino acid sequence at positions 38 to 52. Gln-37 appears to be modified into pyroglutamate. The extra 36 amino acids at the NH_2 terminus also appear to be a signal peptide (40), suggesting that g3-AdhA and g3-AdhB translocate through the cytoplasmic membrane to its outer surface or into the periplasmic space. An inverted repeat sequence (nt 4238 to 4273) present 24 bp downstream of g3-*adhB* may serve as a rho-independent transcriptional terminator (30). g3-AdhB also shows significant similarity (approximately 70%) in amino acid

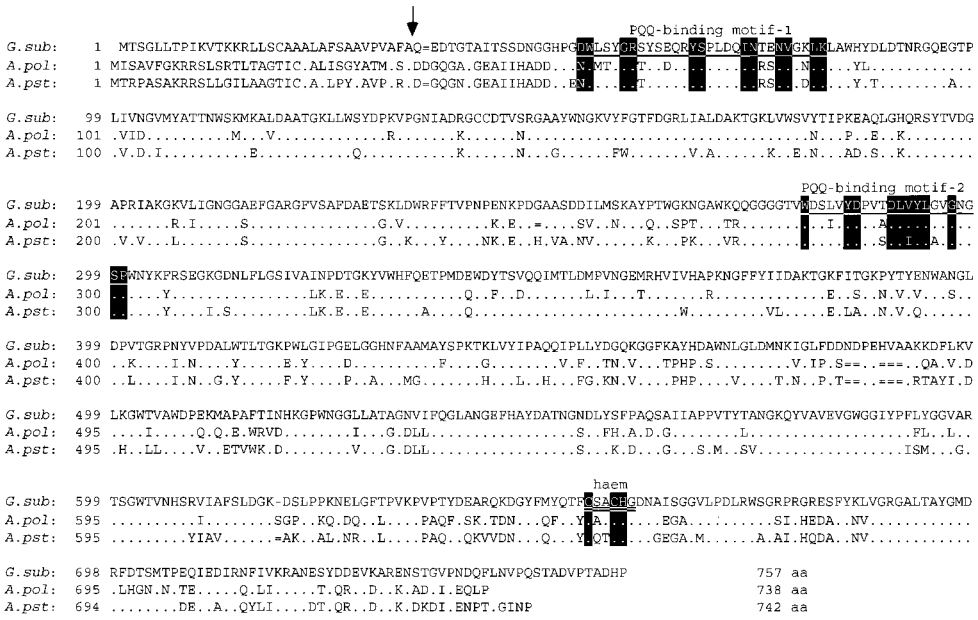

FIG. 4. Alignment of amino acid sequences of the dehydrogenase subunits from *G. suboxydans* IFO12528 (*G.sub*), *A. polyoxogenes* NCI1028 (*A.pol*), and *A. pasteurianus* NCI1452 (*A.pst*). Dots represent amino acids identical to those of g3-AdhA. Black boxes in the PQQ-binding and heme-binding motifs are highly conserved amino acids in the respective motifs. Gaps (=) were introduced to obtain maximal matching. Two PQQ-binding motifs are indicated by underlines, and a heme-binding site is marked by a double underline. The signal peptide cleavage site is indicated by a vertical arrow. aa, amino acids.

sequence to the cytochrome *c* subunits of a-ADHs composed of two components (38) and three components (20, 37) (data not shown).

Cloning and nucleotide sequence of the gene encoding subunit III of g3-ADH. In order to clone the gene encoding subunit III of g3-ADH (g3-*adhS*), we synthesized two oligonucleotides to be used as hybridization probes on the basis of the NH_2-terminal amino acid sequence of AdhS. An approximately 3.6-kb *Sac*I fragment in the total DNA of *G. suboxydans* showed positive hybridization with the two probes. It was recovered from gel slices and inserted into the *Sac*I site of pUC19. One colony among about 1,000 ampicillin-resistant transformants gave a positive signal by colony hybridization with the two probes, and the recombinant plasmid, named pGADS1922, was recovered from the transformant. The restriction map of the cloned 3.6-kb *Sac*I fragment is shown in

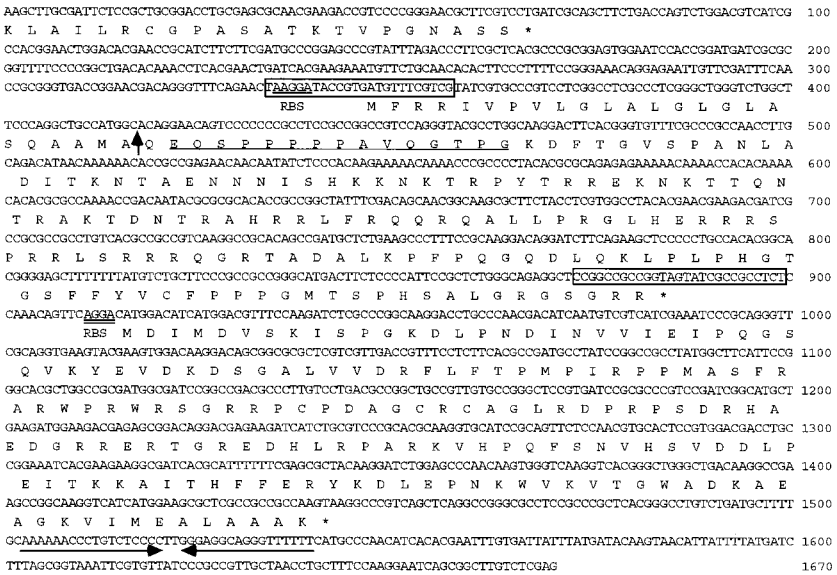

FIG. 5. Nucleotide and deduced amino acid sequences of the 3.6-kb *Sac*I fragment encoding the 15-kDa small subunit of g3-ADH and two additional ORFs. The NH_2-terminal amino acid sequence of subunit III of g3-ADH determined for the purified protein by the Edman degradation procedure is underlined. RBS, potential ribosome-binding sequence. The signal peptide cleavage site is marked by a vertical arrow. An inverted repeat, which possibly serves as a rho-independent transcriptional terminator, is shown by facing arrows. The primer sequences used for subcloning of the g3-*adhS* gene are boxed.

```
G. sub:   1 MDIMDVSKISPGKDLPNDINVVIEIPQGSQ-VKYEVDKDSGALVVDRFLFTPMPIRPPMASFRARWPRWRSGR------RPCP---DAGCRCAGLRDPRP
              * . ....*****.** ******  .. .***.**.**** ****. *.*   *   ..  .     ..         * *   ..  **  .   .
E. col:   1     MSLLNVPAGKDLPEDIYVVIEIPANADPIKYEIDKESGALFVDRFMSTAM-FYPCNYGYINHTLSLDGDPVDVLVPTPYPLQPGSVIRCRPVGVLKM

G. sub:  91 SDRHAEDGRRERTGREDHLRPARKVHPQPSNVHSVDDLPEITKKAITHFFERYKDLEPNKWVKVTGWADKAEAGKVIMEAL--AAAK
             .*  .**..          *  *.  .. ..  *.****. *  *.****.*****  ***** ** .   *   *. ..  *  *
E. col:  97 TDEAGEDAKLVAV-------PHSKLSKEYDHIKDVNDLPELLKAQIAHFFEHYKDLEKGKWVKVEGWENAEAAKAEIVASFERAKNK
```

FIG. 6. Alignment of amino acid sequences of the ORF downstream of g3-AdhS (*G. sub*) and the inorganic pyrophosphatase of *E. coli* (*E. col*). Identical (∗) and conserved (·) residues are indicated. Gaps (−) are introduced to obtain maximal matching.

Fig. 2, and the complete nucleotide sequence of the fragment is shown in Fig. 5. There are two perfect ORFs and one 5′-truncated imperfect ORF in the region.

The ORF corresponding to subunit III of g3-ADH starts with ATG (nt 347 to 349) and terminates with TAG (nt 1252 to 1254), encoding a polypeptide composed of 180 amino acids. A possible ribosome-binding sequence (33), AAGGA, is present 7 nt upstream of the ATG codon. The NH_2-terminal amino acid sequence determined with purified g3-AdhS is present in the deduced amino acid sequence at positions 26 to 40. Gln-25 appears to be modified into pyroglutamate in vivo, as in the two larger subunits. The extra 24 amino acids at the NH_2 terminus also appear to be a signal peptide (40). g3-AdhS shows no significant similarity in amino acid sequence or nucleotide sequence to a3-AdhS (21), in contrast to AdhA and AdhB, each of which has high homology between *Acetobacter* spp. and *Gluconobacter* spp. No proteins homologous with g3-AdhS have been registered in any protein data bank.

The second ORF is present 29 or 38 bp downstream of the termination codon of the g3-*adhS* gene. This ORF, which starts at ATG (nt 916 to 918 or 1441 to 1443) and terminates at TAA (nt 2709 to 2711), is a protein composed of 175 or 172 amino acids and shows similarity in amino acid sequence to the inorganic pyrophosphatase (encoded by *ppa*) in *E. coli* (23) (Fig. 6). An inverted repeat sequence present 59 bp downstream of this ORF (nt 2771 to 2807) may serve as a rho-independent transcriptional terminator (30). The absence of a sequence similar to signal peptides suggests that this protein is present in the cytoplasm. The details about this gene, including its expression and the properties of the enzyme it encodes, will be reported elsewhere. An additional truncated ORF is present 274 bp upstream of the ATG codon of the g3-*adhS* gene. The deduced amino acid sequence of the product of this ORF shows no similarity to the proteins registered in the databases. We have not characterized this ORF further.

Expression of the g3-*adh* genes in *A. pasteurianus*. We first expressed the g3-*adhS* gene in an *A. pasteurianus* mutant strain, m00-09 (21), which shows reduced ADH activity because of a defect in the a3-*adhS* gene. We placed the region from nt 333 to 899 (Fig. 5), containing the whole g3-*adhS* gene, on an *E. coli-Acetobacter* shuttle vector plasmid, pMV24 (17), and introduced the resulting plasmid, pGADS2422 (Fig. 2), into mutant m00-09 by the electroporation method (41). We randomly picked five ampicillin-resistant transformants and measured their ADH activities in the cell lysates. None of the transformants showed any ADH activity, although SDS-polyacrylamide gel electrophoretic analysis of the cell lysate of mutant m00-09 (pGADS2422) revealed the presence of a distinct 15-kDa protein band which was not present in the lysate similarly prepared from this mutant without the plasmid. Consistent with this, all of the transformants were resistant to allyl alcohol, showing that, due to the lack of ADH activity in the transformants, allyl alcohol was not converted to the corresponding aldehyde, which is toxic to the cell in a very small amount. It was thus apparent that the exogenous g3-*adhS* gene was not able to complement the defect in a3-*adhS* in mutant m00-09.

We next expressed the g3-*adhAB* genes in an *A. pasteurianus* mutant strain, m00-21 (21), which shows reduced ADH activity because of a defect in both a3-*adhA* and a3-*adhB*. We placed the region from nt 9 to 4228 (Fig. 3), containing the whole g3-*adhAB* region, in pMV24, and the resulting plasmid, pGADL2411 (Fig. 2), was introduced into mutant m00-21. We randomly picked five transformants and measured their ADH activities. All of the transformants showed distinct ADH activity, but one order of magnitude lower than that expressed by the parental strain, *A. pasteurianus* NCI1452 (21, 37). In order to determine whether the ADH activity detected in the transformants resulted from a large amount of a low-activity enzyme complex or a small amount of a high-activity enzyme complex, we purified the enzyme showing ADH activity from one of the m00-21 transformants containing pGADL2411. Since the ADH activity was detected mainly in the membrane fraction, we solubilized the activity from the membrane with 1.0% Triton X-100 and then purified it by two steps of column chromatography on DEAE-Toyopearl and hydroxyapatite columns. The result of purification is summarized in Table 2. The purified ADH was composed of two larger subunits (Fig. 7a), while the smallest subunit polypeptide corresponding to a3-AdhS derived from the host strain was not detected by immunoblot analysis (Fig. 7b). The two larger subunits had NH_2-terminal amino acid sequences identical to those of the corresponding subunits of g3-ADH when analyzed by the Edman degradation procedure, suggesting that the ADH preparation showing distinct ADH activity which was purified from the *A. pasteurianus* transformant consisted of the two subunits derived from the exogenous g3-*adhAB* genes from *G. suboxydans*. The specific activity of the ADH preparation was 49.6 U/mg of protein (Table 2), approximately one-fifth of that of the native g3-ADH.

DISCUSSION

We have deduced the primary structures of the three subunits of ADH of *G. suboxydans* from the nucleotide sequences of the cloned genes. Comparison of the primary translation products predicted from the nucleotide sequences and the NH_2-terminal amino acid sequences of the purified proteins shows the presence of a typical signal sequence at their NH_2

TABLE 2. Summary of purification of the ADH complex from *A. pasteurianus* m00-21(pGADL2411)

Purification step	Total activity (U)	Total protein (mg)	Sp act (U/mg)	Yield (%)
Membrane fraction	120.5	140	0.86	100
Triton X-100 supernatant	76.3	36	2.12	63.3
DEAE-Toyopearl	44.8	3.16	14.2	37.2
Hydroxyapatite	37.8	0.76	49.6	31.4

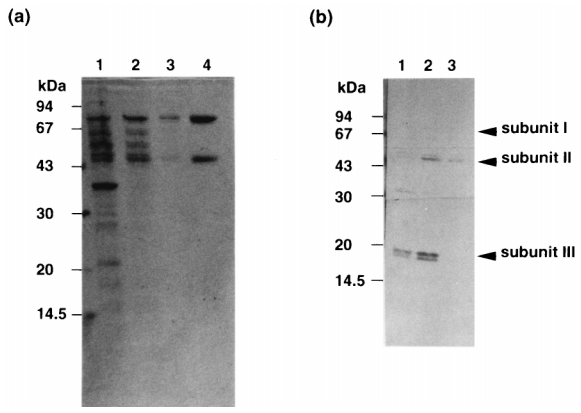

FIG. 7. SDS-polyacrylamide gel electrophoresis (a) and immunoblot analysis (b) of the ADH complex purified from the *A. pasteurianus* m00-21 transformant harboring pGADL2411. (a) Each preparation was stained with Coomassie brilliant blue R-250. Lane 1, preparation from the membrane fraction (30 μg of protein); lane 2, Triton X-100-solubilized fraction (30 μg of protein); lane 3, preparation purified by DEAE-Toyopearl column chromatography (5 μg of protein); lane 4, preparation (in the final step) purified by hydroxyapatite column chromatography (5 μg of protein). (b) Antisera raised against each of the a3-ADH subunits were used. The PVDF blot of the gel was cut into three pieces, each corresponding to the mobility of one subunit, and pieces were reacted with the appropriate antibodies individually. The presence of two bands representing subunit III is due to a rigid structure of this subunit probably caused by many S-S bonds (21). Lanes 1, 2, and 3 correspond to lanes 1, 2, and 4 in panel a, respectively.

termini which is consistent with the localization of the ADH complex. g3-AdhA shows significant similarity in amino acid sequence to the dehydrogenase subunits of both two-component- and three-component-type ADHs from several *Acetobacter* spp. (19, 37, 38). In addition, the presence of two PQQ-binding consensus motifs in g3-AdhA, which supposedly bind one PQQ molecule, is in agreement with the data obtained from enzymatic characterization (27). g3-AdhB, which has been found to be identical to cytochrome *c*-553, shows considerable similarity in amino acid sequence to the cytochrome subunits (subunit II) of the ADH complexes in acetic acid bacteria, as was expected from its cross-reactivity with the antibody raised against subunit II of a3-ADH. The genes encoding the dehydrogenase and cytochrome *c* subunits appear to form a polycistron, like the ones in *A. polyoxogenes* and *A. pasteurianus*. All of these data show that g3-AdhA serving as the primary dehydrogenase subunit and g3-AdhB serving as the cytochrome for ubiquinone reduction comprise a membrane-bound quinohemoprotein-cytochrome *c* system very similar in electron transfer and ubiquinone reduction to the a3-AdhA-AdhB and a2-AdhA-AdhB complexes of *Acetobacter* spp.

g3-AdhS, the smallest subunit, shows no similarity in amino acid sequence or size to a3-AdhS, which was expected from its lack of reactivity with the antibody raised against subunit III of a3-ADH. No proteins homologous with g3-AdhS were found in any protein data bank. As expected from the difference between g3-AdhS and a3-AdhS, g3-*adhS* could not restore the ADH activity of the *A. pasteurianus* mutant deficient in a3-AdhS. Furthermore, the ADH complex purified from the a3-AdhAB-deficient *A. pasteurianus* mutant harboring a g3-*adhAB* gene cluster was free of a3-AdhS. It is therefore likely that g3-AdhS is dispensable for g3-AdhA and g3-AdhB to assemble correctly on the membrane to form a complex able to express ADH activity in *A. pasteurianus*, although the present study does not rule out the possibility that a3-AdhS from the host might contribute to the assembly of the two subunits to some extent and be dissociated from the complex. This will be made clear when an *A. pasteurianus* mutant deficient in all three Adh subunits is obtained. These features of g3-AdhS make a vivid contrast to those of a3-AdhS of *A. pasteurianus*, which is required as a molecular coupler of a3-AdhA and a3-AdhB on the cytoplasmic membrane (21). Further study is required for elucidation of the role of g3-AdhS during the assembly of the three subunits.

The ADH complex consisting of g3-AdhA and g3-AdhB still showed ADH activity, but one order of magnitude lower than that of the native g3-ADH complex. Matsushita et al. (27) reported that the g3-AdhA–g3-AdhS complex showed weak activity (approximately 100 U/mg of protein at pH 5.0, about two times higher than the activity of the g3-AdhA–g3-AdhB complex). These data obviously indicate that g3-AdhS is nonessential for the activity of g3-ADH but that it might contribute to the correct interaction between g3-AdhA and g3-AdhB as the electron transport pathway or to the stability of the g3-ADH complex. In order to examine the role of g3-AdhS of g3-ADH, we have tried to construct hybrid ADH complexes, such as g3-AdhA–a3-AdhB–a3-AdhS and g3-AdhA–a3-AdhB–g3-AdhS, in combinations of the cloned genes and our collection of *A. pasteurianus* mutants. The *A. pasteurianus* transformants with these genetic backgrounds showed weak and variable ADH activities, probably because a complex between g3-AdhA and a3-AdhB was not efficiently formed, thus resulting in proteolysis of the subunits. g3-AdhA without g3-AdhB or a3-AdhB is presumably sensitive to proteases, since a3-AdhA rapidly disappears in the absence of a3-AdhB (21). The instability of these hybrid ADH complexes has hampered the purification and characterization of the hybrids.

We have so far collected a set of genes encoding all the components of g3-ADH, a3-ADH, and a2-ADH in acetic acid bacteria (21, 37, 38). This set of genes will make it possible to construct in vitro any kind of hybrid ADH among g3-ADH, a3-ADH, and a2-ADH by using each subunit produced individually from *A. pasteurianus* or *E. coli* and to characterize them enzymatically. These genes and nucleotide sequences will also facilitate protein engineering work by means of site-directed mutagenesis (22) for detailed analysis of each subunit and for generation of improved ADHs for industrial vinegar production.

ACKNOWLEDGMENT

We are grateful to Kazuko Okamoto (Department of Biotechnology, University of Tokyo) for her help in determining the amino acid sequences.

REFERENCES

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.** 1978. Purification and characterization of particulate alcohol dehydrogenase from *Gluconobacter suboxydans*. Agric. Biol. Chem. **42:**2045–2056.
3. **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.
4. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
5. **Ameyama, M., and O. Adachi.** 1982. Alcohol dehydrogenase from acetic acid bacteria, membrane-bound. Methods Enzymol. **89:**450–457.
6. **Ameyama, M., and O. Adachi.** 1982. Aldehyde dehydrogenase from acetic acid bacteria, membrane-bound. Methods Enzymol. **89:**491–497.
7. **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.
8. **Ameyama, M., K. Matsushita, E. Shinagawa, and O. Adachi.** 1987. Sugar-

oxidizing respiratory chain of *Gluconobacter suboxydans*. Evidence for a branched respiratory chain and characterization of respiratory chain-linked cytochromes. Agric. Biol. Chem. **51:**2943–2950.
9. **Anderson, D. J., C. J. Morris, D. N. Nunn, C. Anthony, and M. E. Lidstrom.** 1990. Nucleotide sequence of the *Methylobacterium extorquens* AM1 *moxF* and *moxJ* genes involved in methanol oxidation. Gene **90:**173–176.
10. **Bairoch, A.** 1992. PROSITE: a dictionary of sites and patterns in proteins. Nucleic Acids Res. **20:**2013–2018.
11. **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.
12. **Cleton-Jansen, A.-M., N. Goosen, G. Odle, and P. van de Putte.** 1988. Nucleotide sequence of the gene coding for quinoprotein glucose dehydrogenase from *Acinetobacter calcoaceticus*. Nucleic Acids Res. **16:**6228.
13. **Cleton-Jansen, A.-M., N. Goosen, O. Fayet, and P. van de Putte.** 1990. Cloning, mapping, and sequencing of the gene encoding *Escherichia coli* quinoprotein glucose dehydrogenase. J. Bacteriol. **172:**6308–6315.
14. **De Ley, J., J. Swings, and F. Gosselé.** 1984. Key to the genera of the family *Acetobacteraceae*, p. 268–278. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore, Md.
15. **Edman, P., and G. Begg.** 1967. A protein sequenator. Eur. J. Biochem. **1:**80–91.
16. **Fukaya, M., K. Tayama, H. Okumura, Y. Kawamura, and T. Beppu.** 1989. Purification and characterization of membrane-bound aldehyde dehydrogenase from *Acetobacter polyoxogenes* sp. nov. Appl. Microbiol. Biotechnol. **32:**176–180.
17. **Fukaya, M., K. Tayama, T. Tamaki, H. Tagami, H. Okumura, Y. Kawamura, and T. Beppu.** 1989. Cloning of the membrane-bound aldehyde dehydrogenase gene of *Acetobacter polyoxogenes* and improvement of acetic acid production by use of the cloned gene. Appl. Environ. Microbiol. **55:**171–176.
18. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.
19. **Inoue, T., M. Sunagawa, A. Mori, C. Imai, M. Fukuda, M. Takagi, and K. Yano.** 1989. Cloning and sequencing of the gene encoding the 72-kilodalton dehydrogenase subunit of alcohol dehydrogenase from *Acetobacter aceti*. J. Bacteriol. **171:**3115–3122.
20. **Inoue, T., M. Sunagawa, A. Mori, C. Imai, M. Fukuda, M. Takagi, and K. Yano.** 1992. Nucleotide sequence of the gene encoding the 45-kilodalton subunit of alcohol dehydrogenase from *Acetobacter aceti*. J. Ferment. Bioeng. **73:**419–424.
21. **Kondo, K., T. Beppu, and S. Horinouchi.** 1995. Cloning, sequencing, and characterization of the gene encoding the smallest subunit of the three-component membrane-bound alcohol dehydrogenase from *Acetobacter pasteurianus*. J. Bacteriol. **177:**5048–5055.
22. **Kunkel, T. A.** 1985. Rapid and efficient site-directed mutagenesis without phenotype selection. Proc. Natl. Acad. Sci. USA **82:**488–492.
23. **Lahti, R., T. Pitkäranta, E. Valve, I. Ilta, E. Kukko-Kalske, and J. Heinonen.** 1988. Cloning and characterization of the gene encoding inorganic pyrophosphatase of *Escherichia coli* K-12. J. Bacteriol. **170:**5901–5907.
24. **Magnet, R.** 1967. Mutants partially deficient in alcohol dehydrogenase in *Schizosaccharomyces pombe*. Arch. Biochem. Biophys. **121:**194–201.
25. **Matsushita, K., Y. Takaki, E. Shinagawa, M. Ameyama, and O. Adachi.** 1992. Ethanol oxidase respiratory chain of acetic acid bacteria: reactivity with ubiquinone of pyrroloquinoline quinone-dependent alcohol dehydrogenases purified from *Acetobacter aceti* and *Gluconobacter suboxydans*. Biosci. Biotechnol. Biochem. **56:**304–310.
26. **Matsushita, K., T. Yakushi, Y. Takaki, H. Toyama, and O. Adachi.** 1995. Generation mechanism and purification of an inactive form convertible in vivo to the active form of quinoprotein alcohol dehydrogenase in *Gluconobacter suboxydans*. J. Bacteriol. **177:**6552–6559.
27. **Matsushita, K., T. Yakushi, H. Toyama, E. Shinagawa, and O. Adachi.** 1996. Function of multiple heme *c* moieties in intramolecular electron transport and ubiquinone reduction in the quinohemoprotein alcohol dehydrogenase-cytochrome *c* complex of *Gluconobacter suboxydans*. J. Biol. Chem. **271:** 4850–4857.
28. **Meyer, T. E., and M. D. Kamen.** 1982. New perspectives on *c*-type cytochromes. Adv. Protein Chem. **35:**105–212.
29. **Okumura, H., T. Uozumi, and T. Beppu.** 1985. Construction of plasmid vector and genetic transformation system for *Acetobacter aceti*. Agric. Biol. Chem. **490:**1011–1017.
30. **Rosenberg, M., and D. Court.** 1979. Regulatory sequences involved in the promotion and termination of RNA transcription. Annu. Rev. Genet. **13:** 319–353.
31. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
32. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
33. **Shine, J., and L. Dalgarno.** 1974. The 3′-terminal sequence of *Escherichia coli* 16S ribosomal RNA: complementarity to nonsense triplets and ribosomal binding sites. Proc. Natl. Acad. Sci. USA **71:**1342–1346.
34. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.
35. **Takeda, Y., and T. Shimizu.** 1991. Cloning and sequencing of the gene encoding cytochrome *c*-553 (CO) from *Gluconobacter suboxydans*. J. Ferment. Bioeng. **72:**1–6.
36. **Takeda, Y., and T. Shimizu.** 1992. Expression of cytochrome *c*-553 (CO) gene that complements the second subunit deficiency of membrane-bound alcohol dehydrogenase in *Gluconobacter suboxydans* subsp. α. J. Ferment. Bioeng. **73:**89–93.
37. **Takemura, H., K. Kondo, S. Horinouchi, and T. Beppu.** 1993. Induction by ethanol of alcohol dehydrogenase activity in *Acetobacter pasteurianus*. J. Bacteriol. **175:**6857–6866.
38. **Tamaki, T., M. Fukaya, H. Takemura, K. Tayama, H. Okumura, Y. Kawamura, M. Nishiyama, S. Horinouchi, and T. Beppu.** 1991. Cloning and sequencing of the gene cluster encoding two subunits of membrane-bound alcohol dehydrogenase from *Acetobacter polyoxogenes*. Biochim. Biophys. Acta **1088:**292–300.
39. **Tayama, K., M. Fukaya, H. Okumura, Y. Kawamura, and T. Beppu.** 1989. Purification and characterization of membrane-bound alcohol dehydrogenase from *Acetobacter polyoxogenes* sp. nov. Appl. Microbiol. Biotechnol. **32:**181–185.
40. **von Heijne, G.** 1985. Signal sequence, the limits of variation. J. Mol. Biol. **184:**99–105.
41. **Wong, H. C., A. L. Fear, R. D. Calhoon, G. H. Eichinger, R. Mayer, D. Amikam, M. Benziman, D. H. Gelfand, J. H. Meade, A. W. Emerick, R. Burner, A. Ben-Bassat, and R. Tal.** 1990. Genetic organization of cellulose operon in *Acetobacter xylinum*. Proc. Natl. Acad. Sci. USA **87:**8130–8134.
42. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.