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Characterization of a Cytochrome a_1 That Functions as a Ubiquinol Oxidase in *Acetobacter aceti*

MASAHIRO FUKAYA,[1]* KENJI TAYAMA,[1] TOSHIMI TAMAKI,[1] HIROAKI EBISUYA,[1] HAJIME OKUMURA,[1] YOSHIYA KAWAMURA,[1] SUEHARU HORINOUCHI,[2] AND TERUHIKO BEPPU[2]

Nakano Central Research Institute, Nakano Vinegar Co., Ltd., 2-6, Handa, Aichi 475,[1] and Department of Agricultural Chemistry, Faculty of Agriculture, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113,[2] Japan

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The terminal oxidase for ethanol oxidation in *Acetobacter aceti* was purified as a complex consisting of four subunits (subunits I, II, III, and IV) with molecular masses of 72, 34, 21, and 13 kDa, respectively. Spectrophotometric analysis and catalytic properties determined with the purified enzyme showed that it belonged to a family of cytochrome a_1 (*ba*)-type ubiquinol oxidases. A polymerase chain reaction with two oligonucleotides designed for amino acid sequences that are conserved in subunit I of the aa_3-type cytochrome *c* oxidases from various origins and of an *Escherichia coli o* (*bo*)-type ubiquinol oxidase was used for cloning the cytochrome a_1 gene. A 0.5-kb fragment thus amplified was used as the probe to clone a 4.5-kb *Kpn*I fragment that contained a putative open reading frame for the whole subunit I gene. The molecular weight and amino acid composition of the product of this open reading frame (*cyaA*) were the same as those of the purified protein from *A. aceti*. The amino acid sequence of CyaA was homologous to that of subunit I of the *E. coli o*-type ubiquinol oxidase. Nucleotide sequence analysis of the region neighboring the *cyaA* gene revealed that the genes (*cyaB*, *cyaC*, and *cyaD*) encoding the other three subunits (subunits II, III, and IV) were clustered upstream and downstream of the *cyaA* gene in the order *cyaB*, *cyaA*, *cyaC*, and *cyaD* and with the same transcription polarity, forming an operon. As expected from the enzymatic properties, CyaB, CyaC, and CyaD showed great similarity in amino acid sequence to the corresponding subunits of the *E. coli o*-type ubiquinol oxidase and aa_3-type cytochrome *c* oxidases.

The characteristic activity of the genus *Acetobacter* in vinegar production is the oxidation of ethanol, which is catalyzed by the two membrane-associated dehydrogenases, alcohol dehydrogenase and aldehyde dehydrogenase. The enzymatic properties of these dehydrogenases have been elucidated (1, 2, 9, 34), and the genes encoding these enzymes have been cloned and sequenced (10, 32, 33). Electrons generated in ethanol oxidation are thought to be directly transferred through the respiratory chain to the terminal oxidase via ubiquinone (20). *Acetobacter* strains were divided into two groups on the basis of the terminal oxidases; one contains cytochrome a_1, and the other contains cytochrome *d* (3). Williams and Poole (36, 37) observed that the amount of cytochrome *o*, as the terminal oxidase of *Acetobacter pasteurianus* NCIB 6428, increased under aerobic culture conditions. Recently, Matsushita et al. (17, 19) reported that cytochrome a_1 of *A. aceti*, which is composed of four subunits (55, 35, 22, and 18 kDa) containing heme *b*, heme *a*, and one copper ion, functions as a ubiquinol oxidase. They also examined the effect of aeration on the terminal ubiquinol oxidase and observed that a change in culture conditions caused a change in the composition of the terminal ubiquinol oxidase (17, 18).

To elucidate the genetic background of the terminal oxidase in ethanol oxidation, we first purified and characterized the terminal oxidase from a thermotolerant strain, *A. aceti* 1023 (24), a suitable strain for submerged acetic acid fermentation. We then cloned and characterized the gene cluster encoding all the subunits of the terminal oxidase. In this paper, we describe the enzymatic properties of the ubiquinol oxidase from *A. aceti* and the cloning and sequencing of the gene cluster. In addition, we compared the amino acid sequence of each subunit with those of other terminal oxidases, especially the *Escherichia coli o*-type ubiquinol oxidase, which contains heme *b*, heme *o*, and one copper ion (5, 22, 25).

To our knowledge, this is the first report on a gene encoding an a_1-type cytochrome functioning as a ubiquinol oxidase.

MATERIALS AND METHODS

Bacterial strains and plasmids. *A. aceti* 1023 (24) is an isolate from a vinegar factory. *E. coli* JM109, used as the host strain for cloning, and pUC9 and pUC18, used as the vector plasmids, were described by Yanisch-Perron et al. (38). pHC79 (12) is a cosmid vector for *E. coli*.

Media and culture conditions. *A. aceti* 1023 was grown in YPG medium (24) supplemented with 3% (vol/vol) ethanol under aerobic conditions with shaking as described previously (24). *E. coli* was routinely grown in Luria broth (6).

Materials. Q_1 was a product of Nisshin Flour Milling Co., Ltd. (Tokyo, Japan), and Q_1H_2 was prepared by the method of Rieske (27). DEAE-Toyopearl was from Toyo Soda (Tokyo, Japan). Cytochrome *c*-551 from *Pseudomonas aeruginosa* was obtained from Sigma Chemical Co. Ltd. All other reagents and materials were obtained from commercial sources.

Enzyme assay. Ubiquinol oxidase activity was measured

* Corresponding author.

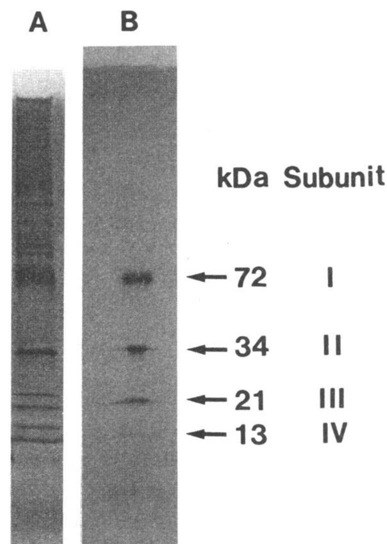

FIG. 1. SDS-polyacrylamide gel electrophoresis of cytochrome a_1 purified from *A. aceti* 1023. Lanes: A, preparation purified by DEAE-Toyopearl column chromatography; B, preparation in the final step of the purification. Each preparation (7.5 μg of protein) was stained with Coomassie blue.

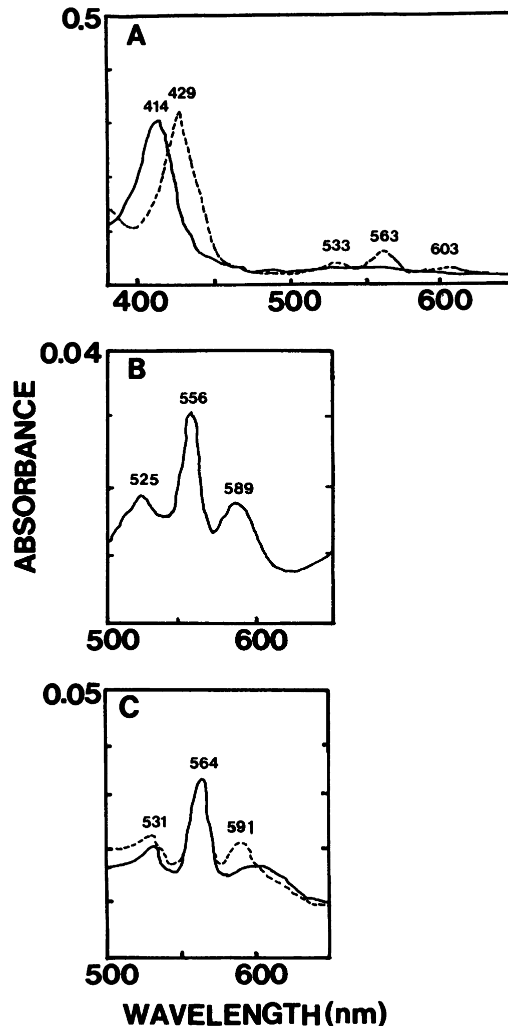

FIG. 2. Absolute absorption spectra, pyridine hemochromogen spectra, and reduced-minus-oxidized difference spectra of ubiquinol oxidase purified from *A. aceti* 1023. (A) Absolute absorption; spectra were determined at a protein concentration of 0.25 mg/ml. ——, oxidized; - - -, reduced. (B) Pyridine hemochromogen spectra; pyridine hemochromogen was prepared with purified ubiquinol oxidase (0.25 mg/ml). (C) Reduced-minus-oxidized difference spectra; spectra were determined at a protein concentration of 0.25 mg/ml in the absence (——) and presence (- - -) of 0.5 mM KCN.

spectrophotometrically at 30°C by monitoring the A_{275}. The reaction mixture in a total volume of 1 ml consisted of 50 mM potassium phosphate (pH 6.5), 200 μM Q_1H_2, 0.02% Tween 20, and an appropriate amount of enzyme. The activity was calculated by use of a millimolar extinction coefficient of 13.53.

Purification of ubiquinol oxidase from *A. aceti* 1023. Ubiquinol oxidase was purified from the membrane fraction of *A. aceti* 1023 essentially by the method of Matsushita et al. (19), except that the preparation obtained by DEAE-Toyopearl column chromatography was further purified by hydroxyapatite column chromatography. All the purification operations were done at 4°C.

Cells were suspended in 50 mM potassium phosphate buffer (KPB) (pH 6.5) and disrupted by passage through a French pressure cell at 20,000 lb/in^2. The disrupted cells obtained by centrifugation at 68,000 × *g* for 1 h were used as the membrane fraction (2.8 g). The membrane fraction was suspended in 28 ml of 50 mM KPB (pH 6.0), and Triton X-100 was added to the suspension to a final concentration of 1% (wt/vol). The mixture was stirred for 0.5 h and then centrifuged at 68,000 × *g* for 1 h. The resultant sediments (2.0 g) were suspended in 20 ml of 50 mM KPB (pH 6.5), and to this suspension *n*-octyl-β-D-glucopyranoside was added to a final concentration of 1.25% (wt/vol). The mixture was stirred for 0.5 h and then centrifuged at 68,000 × *g* for 1 h. Most of the ubiquinol oxidase activity was detected in the soluble fraction.

The supernatant was applied to a DEAE-Toyopearl column (bed volume, 30 ml) previously equilibrated with 50 mM KPB (pH 6.5) containing 1.0% (wt/vol) *n*-octyl-β-D-glucopyranoside and then eluted with a linear phosphate gradient (50 to 100 mM). The active fractions, which eluted at about 80 mM phosphate, were applied to a hydroxyapatite column (bed volume, 2 ml) previously equilibrated with 50 mM KPB (pH 6.5) containing 1.0% (wt/vol) *n*-octyl-β-D-glucopyranoside and then eluted with a linear phosphate gradient (50 to 1,000 mM). The active fractions, which eluted at about 700 mM phosphate, were collected and concentrated by ultrafiltration with a Diaflo YM10 membrane (Amicon Corp., Danvers, Mass.).

Analytical procedures. Absorption spectra were determined with a Hitachi U2000 spectrophotometer. The heme content was determined by measuring pyridine hemochromogen with millimolar extinction coefficients of 20.7 for heme *b* and 21.7 for heme *a*. The protein content was determined by a modification of the method of Lowry et al. (8). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (4 to 20% linear gradient for the acrylamide gel; pH 8.3) was performed as described previously (9).

DNA preparation and manipulation. Total DNA and plasmid DNA from *A. aceti* and *E. coli* were prepared by previously described methods (23). Oligonucleotides were synthesized on a DNA synthesizer (model 380B; Applied

TABLE 1. Purification of cytochrome a_1 from *A. aceti*

Step	Protein (mg)	Ubiquinol oxidase activity	
		U	U/mg of protein
Membrane	365.4	1,247	3.4
Washed membrane	245.7	849	3.5
Octylglucoside extract	25.6	810	31.6
DEAE-Toyopearl	6.9	380	55.1
Hydroxyapatite	3.2	220	68.6

Biosystems, Foster City, Calif.). The conditions for the polymerase chain reaction were those described by Gould et al. (11), except that annealing was performed at 37°C. Southern blot hybridization was performed by the standard method (16). A cosmid bank of *Bam*HI-digested chromosomal fragments from *A. aceti* 1023 was constructed by use of pHC79. Restriction endonucleases, T4 DNA ligase, and *Taq* DNA polymerase were purchased from Takara Shuzo Co., Ltd. (Kyoto, Japan), and Perkin-Elmer Cetus and used in accordance with the instructions of the manufacturers.

Protein sequencing and determination of amino acid composition. Separation of each subunit of the ubiquinol oxidase was done by SDS-polyacrylamide gel electrophoresis (32). The NH_2-terminal amino acid sequence of each subunit was determined by an automated Edman degradation procedure. The amino acid composition of each subunit was determined by use of an amino acid analyzer (Hitachi L-8500) after acid hydrolysis.

DNA sequencing and sequence analysis. The nucleotide sequence was determined by the M13 dideoxy nucleotide method of Sanger et al. (28). The DNA sequence was analyzed by use of the GENETYX sequence program (Software Development Co., Ltd., Tokyo, Japan). The Kyte-Doolittle algorithm (15) was used to analyze hydropathy profiles.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper appear in the DDBJ, EMBL, and GenBank nucleotide sequence data bases under accession number D13815.

TABLE 2. Kinetic properties of cytochrome a_1 from two *A. aceti* strains

A. aceti strain	Ubiquinol oxidation		KCN K_i (μM)	O_2 K_m (μM)
	K_m (μM)	Turnover (no. of electrons/s/mol)		
1023	93.5	320	25	2.1
IFO 3284[a]	18.8	672	74	4.4

[a] Data were taken from Matsushita et al. (17).

RESULTS

Purification and catalytic properties of ubiquinol oxidase from *A. aceti* 1023. Because *A. aceti* 1023 did not grow with static cultivation, we used cells grown with shaking. Ubiquinol oxidase was solubilized by use of octylglucoside from the membrane prewashed with Triton X-100 to remove alcohol dehydrogenase and aldehyde dehydrogenase and was purified by DEAE-Toyopearl column chromatography. This preparation was subjected to hydroxyapatite column chromatography to remove some impurities that were still detected by SDS-polyacrylamide gel electrophoresis (Fig. 1). The summary of the purification is shown in Table 1. The

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Fragment I     Phe Gly His Pro Glu Val        Fragment II     Ala His Phe His Tyr Val

                    T       T       A                          A    A    A    A
Primer I       5'-TT  GGN CA  CCN GA  GT-3'   Primer II    5'-AC  TA  TG  AA  TG NGC-3'
                    C       C       G                          G    G    G    G
```

K A E Sp E B P Sm C E K 1 kb

C E K B K C

cyaB cyaA cyaC cyaD

FIG. 3. Restriction maps of the *Kpn*I fragment (upper bar) and the *Cla*I fragment (lower bar) and the amino acid sequences of the two fragments used for the synthesis of oligonucleotide primers. Amino acid sequences I and II were derived from two regions between P-246 and V-251 and between A-382 and V-387 of subunit I of the cytochrome *c* oxidase complex from thermophilic bacterium PS3 (14). A, *Acc*I; B, *Bgl*II; C, *Cla*I; E, *Eco*RI; K, *Kpn*I; P, *Pst*I; Sm, *Sma*I; Sp, *Sph*I. The broken line indicates the location of the 0.5-kb fragment amplified by the polymerase chain reaction. The sequencing strategy is indicated by solid arrows. Open arrows indicate the locations and directions of *cyaA*, *cyaB*, *cyaC*, and *cyaD*.

```
CTACAAGGTGTTTTCGCACTTCATTCATTCTGTCATCAGCATGATGTGCCGTGTTCCTTATGTTTTTAAAGGATAATGGCGCTGTGTCATGTGTCTATGG    100

CGGGTGGGGAATGTTGAAACGCGGTTTTTCTTGCTGTTGTCAGGCTTCTATGGCAGCAGAACAAAGAGTCTTAGTGAGGCTAATTTGATGTCAGAACCAA    200

GAGGCGTGCGATGAAAAACAAGTTACTGGCGAGAGTGGCGCGATTGGGCGGCCTGTCGTCAGCATTGCTGCTTGCCGGGTGTGAACTTGATGTTCTCGAC    300
       MetLysAsnLysLeuLeuAlaArgValAlaArgLeuGlyGlyLeuSerSerAlaLeuLeuLeuAlaGlyCysGluLeuAspValLeuAsp

CCGAAAGGCCCGGTTGGTGAGGGGGTTAAAACCCTAATTGCCACCTCCACAGTCGCAATGCTGATTGTTGTGATTCCGACCATCCTCGAGACGCTTCTGT    400
ProLysGlyProValGlyGluGlyValLysThrLeuIleAlaThrSerThrValAlaMetLeuIleValValIleProThrIleLeuGluThrLeuLeuPhe

TTGCCTGGCAGTATCGCCAGTCTAACACGAGCGCCGAGTATCTGCCGAAGTGGTGCCACTCCAACAAAATTGAAGTGACTATCTGGGGCGTGCCTTCCCT    500
 AlaTrpGlnTyrArgGlnSerAsnThrSerAlaGluTyrLeuProLysTrpCysHisSerAsnLysIleGluValThrIleTrpGlyValProSerLeu

GATTATCCTCTTCCTGGCAGTGATTACCTATCAGACCTGTCATTCTCTGGACCCGTATAAGCCACTTGAAGCAGAAGCAAATACCAAGCCTCTGCACGTT    600
IleIleLeuPheLeuAlaValIleThrTyrGlnThrCysHisSerLeuAspProTyrLysProLeuGluAlaGluAlaAsnThrLysProLeuHisVal

GAAGTGGTGGCTCTGGACTGGAAATGGCTGTTCATCTACCCGGAACAGGGTATTGCAACGGTCAATCAGCTGGCCATTCCGGTCAACACCCCGATTGACT    700
GluValValAlaLeuAspTrpLysTrpLeuPheIleTyrProGluGlnGlyIleAlaThrValAsnGlnLeuAlaIleProValAsnThrProIleAspPhe
                        EcoRI                                                   SphI
TTAACATTACCTCTGATTCCGTGATGAATTCCTTCTTCATCCCGCGTCTTGGTTCCATGATTTACGCCATGGCAGGCATGCAGACCCAGCTTCATCTGCT    800
 AsnIleThrSerAspSerValMetAsnSerPhePheIleProArgLeuGlySerMetIleTyrAlaMetAlaGlyMetGlnThrGlnLeuHisLeuLeu
                                                                                                  EcoRI
GGCAAGCGAACCGGGCGACTATCTGGGTGAGTCTGCCAACTACAGTGGCCGTGGTTTTTCTGACATGAAATTCCATACGCTTGCTGTGAGCGGCGATGAA    900
AlaSerGluProGlyAspTyrLeuGlyGluSerAlaAsnTyrSerGlyArgGlyPheSerAspMetLysPheHisThrLeuAlaValSerGlyAspGlu

TTCAATGCCTGGGTTGAAAAGGTGAAGTCTTCTTCCGAGCAGCTGGATAGCCAGACCTATCCGAAACTTGCTGCCCCAAGCGAAAACCCCGTCGAATATT   1000
PheAsnAlaTrpValGluLysValLysSerSerSerGluGlnLeuAspSerGlnThrTyrProLysLeuAlaAlaProSerGluAsnProValGluTyrPhe

TCGCGCATGTTGAACCCGGCATGTTCAACACGATCGTTGCCAAGTACAACAACGGCATGGTCATGGACAAAAGCACTGGCAAAATGATCCAGGTGCAGCA   1100
 AlaHisValGluProGlyMetPheAsnThrIleValAlaLysTyrAsnAsnGlyMetValMetAspLysSerThrGlyLysMetIleGlnValGlnGln

GTCTGCGATGTCCGACATGAATATGAAGGAATAGGATCTATGCTAGGGAGACTATCGCTCTCGGCCATCCCCTTGGATGTGCCGATCCTGGTAGGGACGT   1200
SerAlaMetSerAspMetAsnMetLysGlu        MetLeuGlyArgLeuSerLeuSerAlaIleProLeuAspValProIleLeuValGlyThrPhe

TCATCGGCGTTGTCATTGTCGGTGTCGCGGTACTGGGACTTATTACGTATTACGGTAAGTGGGGCTACCTCTGGAAAGAGTGGTTCACTTCTGTCGATCA   1300
 IleGlyValValIleValGlyValAlaValLeuGlyLeuIleThrTyrTyrGlyLysTrpGlyTyrLeuTrpLysGluTrpPheThrSerValAspHis

CAAGCGTCTAGCCGCGATGTACATCATCTTGGCACTGGTCGCTCTTTTCCGTGGTTTTGCTGACGCTATCATGATGCGTACCCAGCTTGCGTTGGCGTAT   1400
LysArgLeuAlaAlaMetTyrIleIleLeuAlaLeuValAlaLeuPheArgGlyPheAlaAspAlaIleMetMetArgThrGlnLeuAlaLeuAlaTyr
                                       BglII
GCAGGTAACCCAGGCTATCTACCACCACACCATTACGATCAGATCTTCTCCGCTCACGGAACGATCATGATCTTCTTCCTGGCCATGGCGTTCATGACCG   1500
AlaGlyAsnProGlyTyrLeuProProHisHisTyrAspGlnIlePheSerAlaHisGlyThrIleMetIlePhePheLeuAlaMetAlaPheMetThrGly
                        PstI
GTCTGTTCAACTTCATCGTGCCTCTGCAGATTGGTGCGCGTGACGTTGCCTTCCCGTTCCTGAACAACCTGAGCTTCTGGATGACGGCTGTTGCGTTTAT   1600
 LeuPheAsnPheIleValProLeuGlnIleGlyAlaArgAspValAlaPheProPheLeuAsnAsnLeuSerPheTrpMetThrAlaValAlaPheIle

TCTGGTGAACGTTTCTCTGTTCATTGGTGAATTTTCGCAGTGCGGCTGGTTGGCATATCCGCCTCTGTCCGAAAATCAGTTCAGCCCTGGCGTTGGCGTA   1700
LeuValAsnValSerLeuPheIleGlyGluPheSerGlnCysGlyTrpLeuAlaTyrProProLeuSerGluAsnGlnPheSerProGlyValGlyVal

GATTACTATATCTGGGCCGTTCAGATTTCTGGTGTTGGCACGCTGCTGACCGGTGTGAACTTCTTTGTGACCATCGTGAAAATGCGCGCTCCGGGAATGA   1800
AspTyrTyrIleTrpAlaValGlnIleSerGlyValGlyThrLeuLeuThrGlyValAsnPhePheValThrIleValLysMetArgAlaProGlyMetThr

CCTGGAGGAAAATGCCGGTTTTCACCTGGACAGCTCTCTGTGCTTCCATCCTGATCATGGTGGCCTTCCCTGTTCTGACAGTTGCTGTTGGTCTGCTGGG   1900
 TrpArgLysMetProValPheThrTrpThrAlaLeuCysAlaSerIleLeuIleMetValAlaPheProValLeuThrValAlaValGlyLeuLeuGly

TATGGATCGTTACTTCGGGATGCACTTCTTCACCAATGATGGTGGCGGCAACCAGATGATGTACCTGAACCTGATCTGGGCTTGGGGTCATCCGGAAGTT   2000
MetAspArgTyrPheGlyMetHisPhePheThrAsnAspGlyGlyGlyAsnGlnMetMetTyrLeuAsnLeuIleTrpAlaTrpGlyHisProGluVal

TACATTCTCGTTATTCCTGCCTTCGGTGTGTTTTCGGAAGTTGTTCCTGCATTTTCCGGTAAGCCGCTGTTTGGTTACAGCACCATGGTTTACGCAACCT   2100
TyrIleLeuValIleProAlaPheGlyValPheSerGluValValProAlaPheSerGlyLysProLeuPheGlyTyrSerThrMetValTyrAlaThrCys

GCTCCATCATGGTTCTGTCCTTCCTTGTGTGGGTTCACCACTTCTTCACAATGGGCGCTGGTCCGGACGTGAATGCCTTCTTTGGTATTGCGACCATGAT   2200
 SerIleMetValLeuSerPheLeuValTrpValHisHisPhePheThrMetGlyAlaGlyProAspValAsnAlaPhePheGlyIleAlaThrMetIle

CATCTCCATTCCTACCGGTATTAAGCTGTTTAACTGGCTGTTCACCATGTATAAGGGCCGCATTCAGTTCCATGCCTGCATGTACTGGGCTGTTGGCTTC   2300
IleSerIleProThrGlyIleLysLeuPheAsnTrpLeuPheThrMetTyrLysGlyArgIleGlnPheHisAlaCysMetTyrTrpAlaValGlyPhe
                                                    SmaI
ATGATCACCTTCACCATCGGTGGTATGACCGGCGTTATGCTGGCTATCCCGGGTGCTGACTTTGTTCTGCATAACTCCCTGTTCCTGATTGCCCACTTCC   2400
MelIleThrPheThrIleGlyGlyMetThrGlyValMetLeuAlaIleProGlyAlaAspPheValLeuHisAsnSerLeuPheLeuIleAlaHisPheHis

ATAATACCATTATCGGTGGTGTGTATTTCGGTTACATCTGCGGCATGAACTTCTGGTTCCCGAAGGTGATGGGCTTCAAGCTGGATGAAACCTGGGGCAA   2500
 AsnThrIleIleGlyGlyValTyrPheGlyTyrIleCysGlyMetAsnPheTrpPheProLysValMetGlyPheLysLeuAspGluThrTrpGlyLys

GCGCGCTTTCTGGTTCTGGTTTGTTGGCTTCTATTGCGCATTCGTACCGCTCTACATCGTCGGTTTCGAAGGCATGACCCGTCGTCTGAACCACTACGAC   2600
ArgAlaPheTrpPheTrpPheValGlyPheTyrCysAlaPheValProLeuTyrIleValGlyPheGluGlyMetThrArgArgLeuAsnHisTyrAsp

AATCCAGCTTGGCACCCGTGGCTGCTGGTTGCTGAAGTTGGTGCAGTTCTGGTTATGCTTGGTATCGCTTGCCAGCTTACTCAGCTGTATGTTTCCATCC   2700
AsnProAlaTrpHisProTrpLeuLeuValAlaGluValGlyAlaValLeuValMetLeuGlyIleAlaCysGlnLeuThrGlnLeuTyrValSerIleArg
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FIG. 4. Nucleotide and deduced amino acid sequences of the region encoding the a_1-type ubiquinol oxidase of *A. aceti*. The deduced amino acid sequences of the four subunits are shown below the nucleotide sequence. The amino acid sequences that were determined by direct amino acid sequencing are doubly underlined. The putative ribosome-binding sequences are underlined. The broken line indicates a sequence similar to the proposed sequence responsible for regulation by oxygen of the *E. coli cyo* operon (21).

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GTGACCGCAACCTGCCGCAGAACCGCGACGTGACCGGTGATCCATGGAATGGCCGTACGCTGGAATGGTCCACTTCTTCTCCGCCGCCGGTTTACAACTT   2800
  AspArgAsnLeuProGlnAsnArgAspValThrGlyAspProTrpAsnGlyArgThrLeuGluTrpSerThrSerSerProProProValTyrAsnPhe
                                                                   ClaI
CGCTATCGTTCCTCACGTGCACGAACTTGATACGTTCATGCTTGATAAGGAAAATGGTATCGATACCCGTCAGGCTGGTGCTCAGTACGAAGCAATCCAC   2900
 AlaIleValProHisValHisGluLeuAspThrPheMetLeuAspLysGluAsnGlyIleAspThrArgGlnAlaGlyAlaGlnTyrGluAlaIleHis

ATGCCCAAGAACACCTCCTTTGGGTCTGGCTTGTGCAAGTGTTCCGCTCTGATCTTCGGTTTTGCTGCGGTTTGGTACATCTGGTGGCTGGCTGCTGTTG   3000
MetProLysAsnThrSerPheGlySerGlyLeuCysLysCysSerAlaLeuIlePheGlyPheAlaAlaValTrpTyrIleTrpTrpLeuAlaAlaValGly

GTCTTGTTGGCGTTATCGGTACGGTAATCGCTCGCAGCGCCGATAAGGATATTGATTACTATATCCCTGCCGAAGAGGTTGCCCGGATTGAAAACGAGCA   3100
  LeuValGlyValIleGlyThrValIleAlaArgSerAlaAspLysAspIleAspTyrTyrIleProAlaGluGluValAlaArgIleGluAsnGluHis

CACCCGTAAACTGATGGCACAGGCAGCTGAATAAAATGGCACAGAACACAACTGTTCAGACCGCAGGCCATGACGAACATCACCACGAATCTCCGGTGGT   3200
 ThrArgLysLeuMetAlaGlnAlaAlaGlu    MetAlaGlnAsnThrThrValGlnThrAlaGlyHisAspGluHisHisHisGluSerProValVal

GTTCGGGTTCTGGGTCTATCTGATGACGGACTGCATTATCTTTGGCACGCTTTTTGCCGCGTTTGCAGTTCTCCATAACCAGTTCAACGGTGGTCCAACG   3300
 PheGlyPheTrpValTyrLeuMetThrAspCysIleIlePheGlyThrLeuPheAlaAlaPheAlaValLeuHisAsnGlnPheAsnGlyGlyProThr
                EcoRI
GGCCACGAACTGTTCGAATTCGGTGGGCTTGGGCTGGAAACAGCCCTCCTGCTGGTTTCGTCCATCACTTATGGGTTTGGCATGATTGCCGCCCATAAAA   3400
GlyHisGluLeuPheGluPheGlyGlyLeuGlyLeuGluThrAlaLeuLeuLeuValSerSerIleThrTyrGlyPheGlyMetIleAlaAlaHisLysSer

GCCAGGTTTCCAAGGTTATCCTTTGGCTTGGCCTTACCTTCCTGCTGGGCCTTGGCTTTGTGGGGCTGGAACTGCGTGAATTTGCGCACATGATCGCAGA   3500
  GlnValSerLysValIleLeuTrpLeuGlyLeuThrPheLeuLeuGlyLeuGlyPheValGlyLeuGluLeuArgGluPheAlaHisMetIleAlaGlu

AGGCGCCGGTCCGGATCGCAGTGCATTCCTGTCTGCGTTCTTTACGCTGGTGTCTACTCACGGTCTGCATGTCACGTGTGGTCTGATCTGGATTGTTACC   3600
 GlyAlaGlyProAspArgSerAlaPheLeuSerAlaPhePheThrLeuValSerThrHisGlyLeuHisValThrCysGlyLeuIleTrpIleValThr

CTGATCGTTCAGCTGATGGGTACGACTGAAATCCCGGAACGTATGATGAATAAGCTCACCTGCCTGAGCCTGTTCTGGCACTTTCTGGATATCGTCTGGA   3700
LeuIleValGlnLeuMetGlyThrThrGluIleProGluArgMetMetAsnLysLeuThrCysLeuSerLeuPheTrpHisPheLeuAspIleValTrpIle

TCTGCGTTTTCACCTATGTCTATCTGGCGAGCATGATCTGATGAGCAATCCGCATACATCCTCCTCAGGCGAGAGCCACGGTAGCGTATCTTCTTACATT   3800
  CysValPheThrTyrValTyrLeuAlaSerMetIle  MetSerAsnProHisThrSerSerSerGlyGluSerHisGlySerValSerSerTyrIle

ATCGGGTTTGTTCTTGCCGTTGTCCTGACGGTGCTGTCGTTTGGCGTGGTGATGACCCCACAGCCTTCTCCAGCAGGCACTCTGGCTGCTATTTCAGCTC   3900
IleGlyPheValLeuAlaValValLeuThrValLeuSerPheGlyValValMetThrProGlnProSerProAlaGlyThrLeuAlaAlaIleSerAlaLeu

TCGCTCTGGTTCAGGTTCTGGTGCATCTGCACTACTTCCTGCACATGGGCGGTAGCTCCGAACAGCGCTGGAACAATATGTGCTTTGTTTTCACCGTTGC   4000
  AlaLeuValGlnValLeuValHisLeuHisTyrPheLeuHisMetGlyGlySerSerGluGlnArgTrpAsnAsnMetCysPheValPheThrValAla
                         KpnI
GTTTGTGGCCATCCTGATTGTTGGTACCGTGTTCATCATGAACAACACCGAACATATGATGTCCCGCTAATATGGTTTGCCTTTCACCGCTGCCTTAAGT   4100
 PheValAlaIleLeuIleValGlyThrValPheIleMetAsnAsnThrGluHisMetMetSerArg

GGCGGTGA                                                                                             4108
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FIG. 4—*Continued.*

purified enzyme yield was 3.2 mg, and the specific activity was 68.6 U/mg of protein. The overall process resulted in about a 24-fold purification and a yield of about 20%. SDS-polyacrylamide gel electrophoresis revealed the presence of four subunits with molecular masses of 72 kDa (subunit I), 34 kDa (subunit II), 21 kDa (subunit III), and 13 kDa (subunit IV) (Fig. 1). The absolute absorption and pyridine hemochromogen spectra of the enzyme are shown in Fig. 2. The ubiquinol oxidase exhibited an absolute absorption spectrum having α and γ bands at 563 and 429 nm, respectively, in the reduced form and a γ band at 414 nm in the oxidized form. The pyridine hemochromogen exhibited two α bands, at 556 and 589 nm. The maximum absorption of the purified enzyme reduced with sodium dithionite in the absence of cyanide was observed at 564 nm, and the addition of cyanide brought forth a band at 591 nm (Fig. 2). These results indicated that this oxidase contained heme *b* and heme *a*, and the contents were estimated to be 4.1 nmol/mg of protein for heme *b* and 3.9 nmol/mg of protein for heme *a*.

The kinetic properties of the enzyme are summarized in Table 2. The enzyme oxidized ubiquinol but exhibited no activity for reduced horse heart cytochrome *c*, yeast cytochrome *c*, or cytochrome *c*-551 from *P. aeruginosa* and exhibited low activity for *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (1.5 U/mg of protein). The optimum pH was between 4.0 and 6.5. Tween 20 and Triton X-100 (0.01 to 0.02% [wt/vol]) activated the ubiquinol oxidase activity about fivefold. These results suggest that this oxidase is a cytochrome a_1 (*ba*) that functions as a ubiquinol oxidase and that it exhibits properties similar to those of a cytochrome a_1 detected in another strain of *A. aceti* (19).

We also determined the NH_2-terminal amino acid sequence of each subunit by automated Edman degradation after separation of the subunits by SDS-polyacrylamide gel electrophoresis, as follows: subunit II, Cys-Glu-Leu-Asp-Val-Leu-Asp-Pro-Lys; subunit III, Ala-Gln-Asn-Thr-Thr-Val-Gln-Thr-Ala-Gly-His; and subunit IV, Ser-Asn-Pro-His-Thr-Ser-Ser-Ser-Gly-Glu. The NH_2-terminal sequence of subunit I could not be determined, probably because of some modification at the NH_2 terminus. The sequence of subunit II coincided well with that (Gly-Glu-Leu-Asp-Val-Leu-Asp-Pro-Gly) of subunit II from *A. aceti* IFO 3284 (17).

Cloning of the gene encoding subunit I of cytochrome a_1 from *A. aceti*. Cytochrome a_1 of *A. aceti* seemed to be closely related to cytochrome *o* of *E. coli* because of the similar molecular weights of the four subunits, the same prosthetic group, heme *b*, and similar kinetics for quinol, although cytochrome *o* contains a different heme moiety, heme *o* (17). Puustinen and Wikstrom (25) found that heme *o* of the *bo*-type terminal oxidase of *E. coli* is a heme A-like molecule. Cloning and characterization of the gene encoding cytochrome a_1 were expected to yield information at the molecular level to elucidate the structural and functional similarities and differences between these two cytochromes.

There are several blocks of conserved amino acid sequences among aa_3-type bacterial cytochrome *c* oxidases (7, 14, 26, 29–31). Several of these blocks are also conserved in the *E. coli o*-type ubiquinol oxidase (4), especially in subunit I. Therefore, we synthesized oligonucleotide primers I and II

(Fig. 3) on the assumption that the conserved sequences are also present in the corresponding subunit of the *A. aceti* a_1-type ubiquinol oxidase. These two oligomers were used as the opposing primers in the polymerase chain reaction, and total DNA of *A. aceti* 1023 was used as the template for the reaction. Agarose gel electrophoretic analysis of the reaction mixture revealed the presence of an amplified 0.5-kb fragment, and this fragment was cloned into the *Sma*I site of pUC9 for nucleotide sequencing. The nucleotide sequence of the 0.5-kb fragment revealed an open reading frame (ORF) that can encode an amino acid sequence similar to those of subunit I of cytochrome *c* oxidases and the *E. coli* *o*-type ubiquinol oxidase. These findings suggested that the 0.5-kb fragment encoded an internal part of subunit I of the *A. aceti* a_1-type ubiquinol oxidase.

We used this 0.5-kb fragment as a hybridization probe for cloning of the entire structural gene encoding subunit I. We first selected one of the positive clones (pMCOX8) from an *A. aceti* 1023 cosmid bank consisting of 1,000 independent clones. The positive clone contained a 30-kb insert on pHC79. Southern hybridization experiments with the 0.5-kb fragment as the probe and pMCOX8 as the target showed that the probe hybridized with a 4.3-kb *Kpn*I fragment. We then cloned the 4.3-kb *Kpn*I fragment and constructed its restriction map (Fig. 3). We determined the nucleotide sequence of the region between the *Acc*I and *Kpn*I sites by using the strategy shown in Fig. 3. Figure 4 shows the complete nucleotide sequence (nucleotides [nt] 1 to 4028). We found an ORF (nt 1140 to 3134) encompassing the 0.5-kb fragment (nt 1986 to 2402). A putative ribosome-binding sequence was present 8 nt upstream of the start codon. The aforementioned ORF, starting at ATG (nt 1140 to 1142) and terminating at TAA (nt 3132 to 3134), encoded a protein of 664 amino acid residues with a molecular mass of 75,100 Da, consistent with that (72 kDa) of the purified enzyme, as determined by SDS-polyacrylamide gel electrophoresis. The deduced amino acid sequence showed end-to-end similarity to that of the *cyoB* gene encoding subunit I of the *E. coli* *o*-type ubiquinol oxidase (4), with 67% identity, as well as to those of thermophilic bacterium PS3 (14) and *Paracoccus denitrificans* PD COI (26) (with 44 and 36% identities, respectively). The 7 histidine residues that are putative ligands for heme and Cu_B in the aa_3-type cytochromes (5, 13) and in *E. coli* CyoB (4) were conserved. Although the NH_2-terminal amino acid sequence of purified subunit I could not be determined, probably because of a modification, the amino acid composition calculated from the nucleotide sequence matched that of the purified protein (data not shown), indicating the absence of a cleaved signal sequence, as in the case of *E. coli* CyoB (22). We concluded from these data that this ORF (*cyaA*) encoded subunit I of the *A. aceti* a_1-type ubiquinol oxidase.

Nucleotide sequencing of the region neighboring the *cyaA* gene. Since the genes encoding the subunits of aa_3-type cytochrome *c* oxidases from other microorganisms and the *E. coli* ubiquinol oxidase are clustered in the order of subunit II, subunit I, subunit III, and subunit IV (4, 14, 31), the genes encoding the other subunits of the ubiquinol oxidase of *A. aceti* were also expected to be clustered near the *cyaA* gene. As expected, there was an ORF (nt 211 to 1131 in Fig. 4) upstream of the *cyaA* gene. This ORF (*cyaB*), separated by 8 nt from the start codon of *cyaA*, encoded a protein of 307 amino acid residues with a molecular mass of 34,900 Da. The NH_2-terminal amino acid sequence of purified subunit II was found to be encoded by this ORF (nt 280 to 306), indicating that the NH_2-terminal 23 amino acids, with the defined structural features of leader peptides for secretion (35), are processed from the primary translation product. The molecular mass of the mature protein, 31,586 Da, predicted from the nucleotide sequence, was consistent with that (34 kDa) of purified subunit II, as determined by SDS-polyacrylamide gel electrophoresis, and the predicted amino acid composition also matched that of the purified protein (data not shown). The deduced amino acid sequence of CyaB was similar to that of the product of the *cyoA* gene, encoding subunit II of the *E. coli* *o*-type ubiquinol oxidase, with 52% identity, and also to those of the products of the corresponding genes of PS3 (*caaA*) and PD COII, with about 27% identity. These findings demonstrated that this ORF (*cyaB*) encoded subunit II of the *A. aceti* ubiquinol oxidase.

Subunit II of the cytochrome *c* oxidase is assumed to act as a Cu_A-binding component, and the probable binding sites, two histidines and two cysteines, have been proposed (5, 13). These residues are not conserved in subunit II of the oxidase of *A. aceti* as they are not in *E. coli* *cyo*-encoded oxidase subunit II. This fact is consistent with the fact that cytochrome a_1 contains one copper per molecule (17).

There was an additional ORF (nt 3136 to 3738) in the region downstream of *cyaA* and separated from it by 4 nt; this ORF started at ATG (nt 3136 to 3138) and terminated at TAG (nt 3739 to 3741). It encoded a protein of 201 amino acid residues with a molecular mass of 27,000 Da, consistent with that of subunit III, as determined by SDS-polyacrylamide gel electrophoresis. The NH_2-terminal amino acid sequence of purified subunit III was found to be encoded by this ORF (nt 3139 to 3171). The amino acid composition calculated from the nucleotide sequence also matched that of purified subunit III (data not shown). There was a 54% match of the amino acid sequence with that of subunit III of the *E. coli* *o*-type ubiquinol oxidase (*cyoC*), a 38% match with that encoded by PS3 *caaC*, and a 25% match with that encoded by PD COIII. These findings indicated that this ORF (*cyaC*) encoded subunit III of the *A. aceti* ubiquinol oxidase.

Glu-38 in subunit III of the cytochrome *c* oxidase (14), which is presumably involved in binding an inhibitor, dicyclohexylcarbiimide (DCCD), corresponds to Asp-36 of *E. coli* CyoC (4). Its counterpart in *A. aceti* CyaB is Asp-32.

We found an additional ORF starting 2 nt downstream of the coding region of the *cyaC* gene (nt 3741 to 4067). Since there was no termination codon within the 4.5-kb *Kpn*I fragment (Fig. 4), we cloned the 5.0-kb *Cla*I fragment that shared the 1.2-kb *Cla*I-*Kpn*I region with the 4.3-kb *Kpn*I fragment, as shown in Fig. 3, and sequenced the region adjacent to the *Kpn*I site (nt 4029 to 4108), in which termination codon TAA (nt 4068 to 4070) was present. This ORF (*cyaD*) encoded a protein of 109 amino acid residues with a molecular mass of 12,300 Da, in agreement with that (13 kDa) of purified subunit IV. The NH_2-terminal amino acid sequence of purified subunit IV was found to be encoded by this ORF (nt 3744 to 3773), and the amino acid composition of the mature protein predicted from the nucleotide sequence matched well that of purified subunit IV (data not shown). There was a 53% match of the amino acid sequence with that of subunit IV of the *E. coli* ubiquinol oxidase and a 33% match with that encoded by PS3 *caaD*. These findings clearly indicated that *cyaD* encoded subunit IV of the *A. aceti* ubiquinol oxidase.

We found a sequence (nt 56 to 79; CCTTATGTTTTTA AAGGATAATGG) similar to the putative sequence (ATAA TTATTTGTTAAATAATTGT) responsible for the regulation by oxygen of the *E. coli* *cyo* operon (21), which is

located 130 bp upstream of the coding region of the *cya* operon, although it is unclear whether this sequence serves as a regulatory element in this bacterium.

The Kyte-Doolittle hydropathy profiles of the four gene products resembled those of the corresponding subunits of the *E. coli o*-type ubiquinol oxidase (4) (data not shown) and suggested that these gene products have transmembrane segments similar to those found in the *E. coli cyo* gene product.

From all the results described above, we concluded that the entire gene cluster encoding all four subunits of the a_1-type ubiquinol oxidase of *A. aceti* 1023 was cloned and that these genes form an operon with the same transcriptional polarity as the *E. coli o*-type ubiquinol oxidase gene and aa_3-type cytochrome *c* oxidase genes.

DISCUSSION

The *A. aceti* a_1 (*ba*)-type ubiquinol oxidase is considered to be closely related in structure and function to the *E. coli o* (*bo*)-type ubiquinol oxidase rather than to the aa_3-type cytochrome *c* oxidases because of the substrate, the heme moiety, and the number of copper molecules. Nucleotide sequencing of the cloned DNA fragment revealed similarities in the amino acid sequences of the four subunits between the *A. aceti* a_1-type and the *E. coli o*-type ubiquinol oxidases, as expected. The *A. aceti* a_1-type oxidase also shares several regions with the aa_3-type cytochrome *c* oxidases. The genes encoding the *A. aceti* a_1-type ubiquinol oxidase complex are clustered, as in the case of the genes encoding the *E. coli o*-type ubiquinol oxidase complex and the aa_3-type cytochrome *c* oxidases. In addition, they are aligned in the same order (subunits II, I, III, and IV) (4, 14, 26). These findings indicate that the a_1-type ubiquinol oxidase of *A. aceti* is also a member of the superfamily of heme-copper oxidases (4), including aa_3-type cytochrome *c* oxidases and the *E. coli o*-type ubiquinol oxidase, despite the fact that the substrate (cytochrome *c* or ubiquinol), the heme (aa_3, *bo*, or *ba*), and the number of copper molecules (Cu_A and Cu_B or Cu_B) are different. These findings also suggest that a comparison of the *cya* genes of *A. aceti* with the *cyo* genes of *E. coli* will help identify the catalytic domain and mechanism of electron transfer in these oxidases and also provide a clue for defining the essential amino acid residues responsible for the catalytic function in various types of terminal oxidases.

We have also purified a ubiquinol oxidase with similar properties from *A. polyoxogenes* and cloned a gene homologous to the *cya* operon by using a part of the cloned *cya* gene as a probe (unpublished results). The results imply that *Acetobacter* strains generally contain an a_1-type ubiquinol oxidase as a terminal oxidase for ethanol oxidation in acetic acid fermentation.

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