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Cloning, Characterization, and Expression in *Escherichia coli* of the Genes Encoding the Cytochrome *d* Oxidase Complex from *Azotobacter vinelandii*†

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***Azotobacter vinelandii* is a free-living nitrogen-fixing bacterium that has one of the highest respiratory rates of all aerobic organisms. Based on various physiological studies, a *d*-type cytochrome has been postulated to be the terminal oxidase of a vigorously respiring but apparently uncoupled branch of the electron transport system in the membranes of this organism. We cloned and characterized the structural genes of the two subunits of this oxidase. The deduced amino acid sequences of both subunits of the *A. vinelandii* oxidase have extensive regions of homology with those of the two subunits of the *Escherichia coli* cytochrome *d* complex. Most notably, the histidine residues proposed to be the axial ligands for the b hemes of the *E. coli* oxidase and an 11-amino-acid stretch proposed to be part of the ubiquinone binding site are all conserved in subunit I of the *A. vinelandii* oxidase. The *A. vinelandii* cytochrome *d* was expressed in a spectrally and functionally active form in the membranes of *E. coli*, under the control of the *lac* or *tac* promoter. The spectral features of the *A. vinelandii* cytochrome *d* expressed in *E. coli* are very similar to those of the *E. coli* cytochrome *d*. The expressed oxidase was active as a quinol oxidase and could reconstitute an NADH to oxygen electron transport chain.**

Nitrogen-fixing bacteria have in common the ability to utilize atmospheric nitrogen gas as their sole source of nitrogen for metabolic biosynthesis. Otherwise, they represent species from rather different taxonomic groups, with very different physiologies and living in diverse ecological niches. Another common theme in bacterial nitrogen fixation is that these organisms must employ some mechanism to protect the nitrogenase components from oxygen. This is because all of the nitrogenases that have been purified and studied to date are sensitive to irreversible inactivation by oxygen, irrespective of whether they are isolated from anaerobic, facultatively aerobic, strictly aerobic, symbiotically associated, or even photosynthetic bacteria (29).

Among the nitrogen-fixing bacteria, those of the genus *Azotobacter* are interesting in that they carry out nitrogen fixation under fully aerobic growth conditions. Over 20 years ago, Postgate and coworkers proposed the operation of a "respiratory protection" mechanism in nitrogen-fixing *Azotobacter* species (for a review, see reference 30). Nitrogen-fixing *Azotobacter* cells are known to have one of the highest respiratory rates of all aerobic organisms (33). It has been proposed that this very high respiration helps maintain the intracellular oxygen concentration at a concentration low enough for proper nitrogenase function. The electron transport chain of *Azotobacter vinelandii* has been studied in some detail (16–18, 33) and is composed of a branched system with either cytochrome *o* or cytochrome *d* as the terminal oxidase. It has been proposed that the *d*-type cytochrome is the main component responsible for the high respiratory rates of nitrogen-fixing *A. vinelandii* cells, culminating a vigorously respiring but apparently uncoupled branch of the respiratory chain. Here, we report our initial cloning of the structural genes of the two subunits of the *A. vinelandii* cytochrome *d* complex. The deduced protein sequences of the genes reveal areas of high homology with the *Escherichia coli cyd* structural genes. Furthermore, the *A. vinelandii* genes have been spectrally and functionally expressed in *E. coli*. The results presented here demonstrate that the *bd*-oxidase of *A. vinelandii* is an active quinol oxidase when expressed in the heterologous *E. coli* host. The *A. vinelandii cyd* locus has been recently cloned independently by Kelly et al., and *A. vinelandii* strains with transposon insertions in this region have been generated (19).

(A preliminary report of this work was presented previously [26a].)

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. *A. vinelandii* CA (4) (kindly provided by Paul Bishop, North Carolina State University) was the wild-type strain used as the source of genomic DNA for this study. Growth of *A. vinelandii* was as described previously (33). *E. coli* HB101 and XL-1 Blue (Stratagene, Inc., La Jolla, Calif.) were used as the hosts in all of the recombinant DNA experiments. *E. coli* GR84N (13) (kindly provided by Robert Gennis, University of Illinois) was the host used for the expression of *A. vinelandii* cytochrome *d*. *E. coli* strains were routinely grown in LB medium (3) at 37°C with added antibiotics as described below.

Plasmids and bacteriophages. Plasmid pBR322 (5) was used for the initial cloning of the *A. vinelandii* cytochrome *d* region. Cloning into this plasmid disrupted the ampicillin resistance gene, and the clone was maintained by growth of the bacteria on medium with 12.5 μg of tetracycline per ml. Essentially all of the subsequent cloning and subcloning manipulations were done in pBluescript KSII(+) or SKII(+) (Stratagene). Cells harboring these plasmids were grown on media containing 100 μg of ampicillin per ml. For the

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purpose of cloning the *A. vinelandii* genomic region surrounding the *cyd* region, size-fractionated restriction digests of *A. vinelandii* genomic DNA were used for the construction of minilibraries in λ-ZAP (Stratagene) as recommended by the supplier. Three separate libraries were constructed that contained *Eco*RI fragments of approximately 8 to 11 kb, *Xho*I fragments of 5 to 7 kb, and *Xho*I fragments of 2.5 to 3.5 kb, respectively. The libraries were screened with the 2.8-kb *Pst*I-*Eco*RI insert from pTB11 (see below). Positive clones in λ-ZAP were subcloned by in vivo excision as clones in pSK(−), as recommended by Stratagene, and verified by restriction mapping. The expression vector pKK233.2 (1) (Pharmacia LKB Biotechnology, Inc., Piscataway, N.J.) was used for some of the expression experiments. Plasmid pBG1, containing the *lacI*^{q} gene cloned in plasmid pACYC177, was a kind gift of Robert Garcea, Dana Farber Cancer Research Center, Boston, Mass. Cells harboring this plasmid were selected with 20 μg of kanamycin per ml.

DNA isolation and recombinant DNA methods. High-molecular-weight *A. vinelandii* genomic DNA was isolated as described previously (20). The restriction endonucleases and other enzymes used for in vitro manipulations were from Boehringer-Mannheim, Inc. (Indianapolis, Ind.), and New England BioLabs, Inc. (Beverly, Mass.) and were used as indicated by the manufacturers. Plasmid transformation into frozen competent cells and plasmid minipreparations were done by standard protocols (3).

Southern hybridization. *A. vinelandii* genomic DNA and plasmid DNA from the different recombinant clones were digested with restriction enzymes and resolved by running aliquots on agarose gels by established methods. DNA was transferred from the gels onto nitrocellulose (Schleicher and Schuell Inc., Keene, N.H.) by capillary transfer, prehybridized, and then hybridized to ^{32}P-labeled probes by standard methods (3). Salmon sperm DNA was omitted from the prehybridization and hybridization reactions. Hybridization was carried out overnight in 50% formamide at 42°C. The blots were washed three times at room temperature in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) and two more times at 42°C in 0.5× SSC–0.1% SDS. Autoradiograms were developed from exposure of the blots to Kodak XAR-5 film at −70°C with an intensifying screen. The high-specific-activity ^{32}P-labeled probes used for the hybridization experiments were prepared by using the random-primer labeling method (9) with gel-purified restriction fragments and [^{32}P]dCTP (New England Nuclear Corp., Boston, Mass.).

DNA sequencing and analysis. Overlapping deletions of regions that were to be sequenced were constructed with the exonuclease III/mung bean deletion kit from Stratagene as recommended by the manufacturer. The deletions and specific subclones of specific restriction fragments were sequenced after single-strand rescue (with the protocol supplied by Stratagene for pBluescript) with the Sequenase II kit from U.S. Biochemical Corp., Cleveland, Ohio. G+C-rich regions were sequenced with dITP, in conjunction with dGTP, as recommended by the manufacturer for Sequenase. Some regions were further sequenced in the presence of the single-strand binding protein (U.S. Biochemical Corp.) as recommended by the manufacturer. Sequencing was done with ^{35}S-dATP (New England Nuclear). Oligonucleotide primers supplied by Stratagene were used for sequencing most of the templates. The junction between the *A. vinelandii* subunit I and the vector and the junction between the two subunits of the *A. vinelandii* cytochrome *d* were sequenced by using custom-made oligonucleotides synthesized on an Applied Biosystems DNA synthesizer (kindly provided by Douglas Fambrough and Jeremy Berg). Double-stranded sequencing of constructs in pKK233.2 was performed on plasmid mini-preparations after alkaline denaturation as recommended in the instructions for Sequenase.

The nucleotide sequence data were compiled and analyzed by using the PC/GENE software package (IntelliGenetics, Inc., Mountain View, Calif.). The nucleotide sequences were compared with the unannotated EMBL nucleotide sequence data bank (release 21), and the deduced amino acid sequences were compared with the Swiss-Prot protein sequence data bank (release 13).

Construction of the expression plasmids. Plasmid pTB11, containing most of the coding region for subunit I and all of the coding region for subunit II of the *A. vinelandii* cytochrome *d*, is described below. The region surrounding this area was restriction mapped from the λ-ZAP genomic clones containing that segment, and a 0.7-kb *Pst*I-*Pst*I fragment located immediately adjacent to the insert in pTB11 (and containing the N terminus and the 5′-upstream region for subunit I) was subcloned in the opposite orientation relative to the polylinkers in pBluescript KSII(+) and SKII(+), yielding plasmids pAVP9 and pAVP1, respectively. The sequence presented in Fig. 3 represents the complete sequence of the insert in pTB11, with the additional sequences up to a *Sac*I site from these two plasmids. Plasmids pKE2 and pMH3 were constructed by joining the insert from pTB11 with the *Kpn*I-*Pst*I fragment from pAVP1 [in pKSII(+)] and the *Sac*I-*Pst*I fragment from pAVP9 [in pSKII(+)], respectively. The junctions created in these subclonings were sequenced and were found to be as predicted. Plasmid pOEC3 was constructed in two parts. First, a 38-bp *Ple*I (blunted)-*Pst*I fragment of pAVP1 was subcloned into the *Nco*I (blunted)-*Pst*I sites of the expression vector pKK233.2 (1), yielding plasmid pFM6. The complete construct was then made by joining the insert from pTB11, as a *Pst*I-*Hin*dIII fragment (containing, in addition to the *A. vinelandii* insert, 38 bp from between the *Eco*RI and *Hin*dIII sites of pBR322), to *Pst*I-*Hin*dIII-digested pFM6 to yield pOEC3. The created junction, including the vector-derived *tac* promoter region, was directly sequenced by double-stranded sequencing. Plasmid pECcyd2 was constructed by subcloning a 3.8-kb *Nru*I fragment of plasmid pNG2 (13) into the *Eco*RV site of pSKII(+). This *Nru*I fragment contains the entire coding region for both subunits of the *E. coli* cytochrome *d* gene, with approximately 1 kb of upstream sequences (the *cyd* promoter [10]). The orientation of this fragment aligns the *cyd* promoter in the same direction with the vector-derived *lac* promoter. Both this plasmid and plasmid pECcyd5 (with the insert in the opposite orientation) directed the overexpression of the *E. coli* cytochrome *d* in transconjugants bearing them.

Expression experiments. Plasmids pKE2, pMH3, pOEC3, pSKII(+), pKK233.2, and pECcyd2 were cotransformed with plasmid pBG1 into *E. coli* GR84N cells. Transconjugants were routinely grown on LB medium supplemented with 100 μg of ampicillin per ml, 20 μg of kanamycin per ml, and 0.3% glucose. For the expression experiments, 100-ml cultures started from single colonies were grown to full density (12 to 14 h at 37°C with shaking at 250 rpm). The whole 100 ml of culture was then used to inoculate 300 ml of prewarmed fresh medium with 200 μg of ampicillin per ml (without kanamycin and glucose), with or without isopropyl-β-D-galactopyranoside (1 mM, final concentration). These cultures were grown with rapid shaking (250 rpm) for 8 h.

The cultures were chilled on ice, and the cells were harvested by centrifugation and washed with 50 mM sodium phosphate buffer with 2.5 mM magnesium chloride (pH 7.0). The subsequent preparation of membranes were carried out at 4°C. The washed cells were broken by two passages through a French pressure cell (SLM-Aminco Instruments, Inc., Urbana, Ill.) at 10,000 lbs/in^2 in the presence of 1 mM phenylmethylsulfonyl fluoride and a small amount of pancreatic DNase. The lysates were centrifuged at 12,000 × *g* for 15 min. The supernatant (crude extract) was further centrifuged at 138,000 × *g* for 2 h to yield the membrane fraction (pellet) and the cytosolic fraction (supernatant). The membranes were washed by resuspending them in the same buffer and recovered by centrifugation at 138,000 × *g* for 1 h. The washed membranes were resuspended in the same buffer and kept on ice until used. *A. vinelandii* membranes were prepared as described previously (33).

SDS-PAGE and immunoblotting. SDS-polyacrylamide gel electrophoresis (PAGE) was carried out as described previously (23), with the modification that the samples were not boiled in the SDS sample buffer, which causes aggregation of the *E. coli* cytochrome *d* subunits (26). For immunoblotting, the proteins resolved on polyacrylamide gels were electrophoretically transferred onto nitrocellulose by established methods (32) with a Transphor apparatus (Hoeffer Scientific Instruments, San Francisco, Calif.). Blots were then blocked and incubated with antibodies in BLOTTO (14). Monoclonal antibody A14-5 (7) and strip-purified polyclonal antibodies prepared against subunit II of *E. coli* cytochrome *d* (both kindly supplied by Kris Oden, University of Illinois) were used at dilutions of 1:2,000 and 1:25, respectively. Immunoreactive bands were further reacted with alkaline phosphatase-conjugated anti-mouse (for A14-5) or anti-rabbit (for the polyclonal antibodies) immunoglobulin G antibodies (Boehringer-Mannheim) and visualized with 5-bromo-4-chloro-3-indolyl phosphate and *p*-nitro blue tetrazolium chloride as recommended by Boehringer-Mannheim.

Oxidase assays. All of the assays were performed at room temperature (23°C) in 100 mM sodium phosphate buffer (pH 7.0). The oxygen consumption rates were measured in a magnetically stirred 5-ml chamber with an amperometric oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio). The concentration of oxygen in air-saturated buffer at 23°C was taken to be 250 μM. Assays were initiated with the addition of the samples, followed by the substrates. The substrates used were 200 μM ubiquinone-1 (kind gift of Peter Sorter, Hoffman-La Roche, Inc., Nutley, N.J.) with 2 mM dithioerythritol, 2 mM *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD)–5 mM sodium ascorbate, 5 mM NADH, 20 mM sodium D,L-lactate, or 20 mM sodium succinate. All of the substrate concentrations used were sufficient for maximal activity. TMPD oxidase rates were corrected for the small but significant auto-oxidation rate of this substrate.

Spectroscopic analysis. All spectra were measured with a Perkin-Elmer model 557 dual-beam spectrophotometer. The reduced-minus-oxidized spectra were measured at room temperature. Membrane samples were reduced or oxidized with a few grains of sodium dithionite or potassium ferricyanide, respectively. The total cytochrome *b* and cytochrome *d* contents of GR84N membranes were calculated by using the extinction coefficients 17.5 mM^{-1} cm^{-1} (560 to 580 nm) (12) and 7.4 mM^{-1} cm^{-1} (628 to 607 nm) (24), respectively.

Protein determination. Protein assays were performed with the BCA protein assay kit from Pierce, Inc., Rockford, Ill. with bovine serum albumin as the standard. Samples were initially mixed with an equal volume of SDS sample buffer (23) without mercaptoethanol.

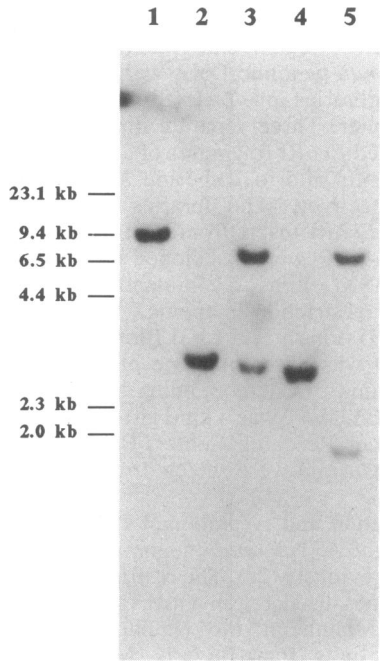

FIG. 1. Hybridization of the *E. coli* cytochrome *d* structural genes to restriction digests of *A. vinelandii* genomic DNA. Each lane contains 5 μg of genomic DNA from *A. vinelandii* CA digested with different restriction enzymes. The digested DNA was size fractionated on a 0.8% agarose gel, transferred onto nitrocellulose, and probed with the 3.8-kb *Nru*I fragment of pNG2 as described in Materials and Methods. Lanes: 1, *Eco*RI digest; 2, *Pst*I digest; 3, *Xho*I digest; 4, *Eco*RI and *Pst*I digest; 5, *Eco*RI and *Xho*I digest. Molecular size markers are phage lambda DNA digested with *Hin*dIII.

RESULTS AND DISCUSSION

Cloning and nucleotide sequence of *A. vinelandii* DNA homologous to the structural genes of *E. coli* cytochrome *d*. Previous work utilizing antibodies raised to each of the subunits of the *E. coli* cytochrome *d* complex had shown immunoreactive material to be present in a number of gram-negative bacteria including *A. vinelandii*, known to have cytochrome *d* (21). Using a 3.8-kb *Nru*I restriction fragment from plasmid pNG2, which contains the structural genes for both subunits of the *E. coli* cytochrome *d* (the *cydA* and *cydB* loci) (11, 13), as a hybridization probe, we found homologous DNA sequences in genomic Southern blots of *A. vinelandii* DNA (Fig. 1).

Although the hybridization of this probe to *A. vinelandii* genomic DNA was evident under high-stringency hybridization conditions, our initial attempts to clone the homologous region by colony hybridization techniques were unsuccessful. This was presumably due to the presence of a perfect copy of the gene in the chromosome of the host *E. coli*, giving an unacceptably high background signal. For this reason, *A. vinelandii* genomic DNA was double digested with *Eco*RI and *Pst*I and size fractionated, and a minilibrary of restriction fragments of approximately 2.8 to 3.2 kb was constructed in pBR322. The screening of isolated plasmid DNAs from approximately 80 individual colonies yielded

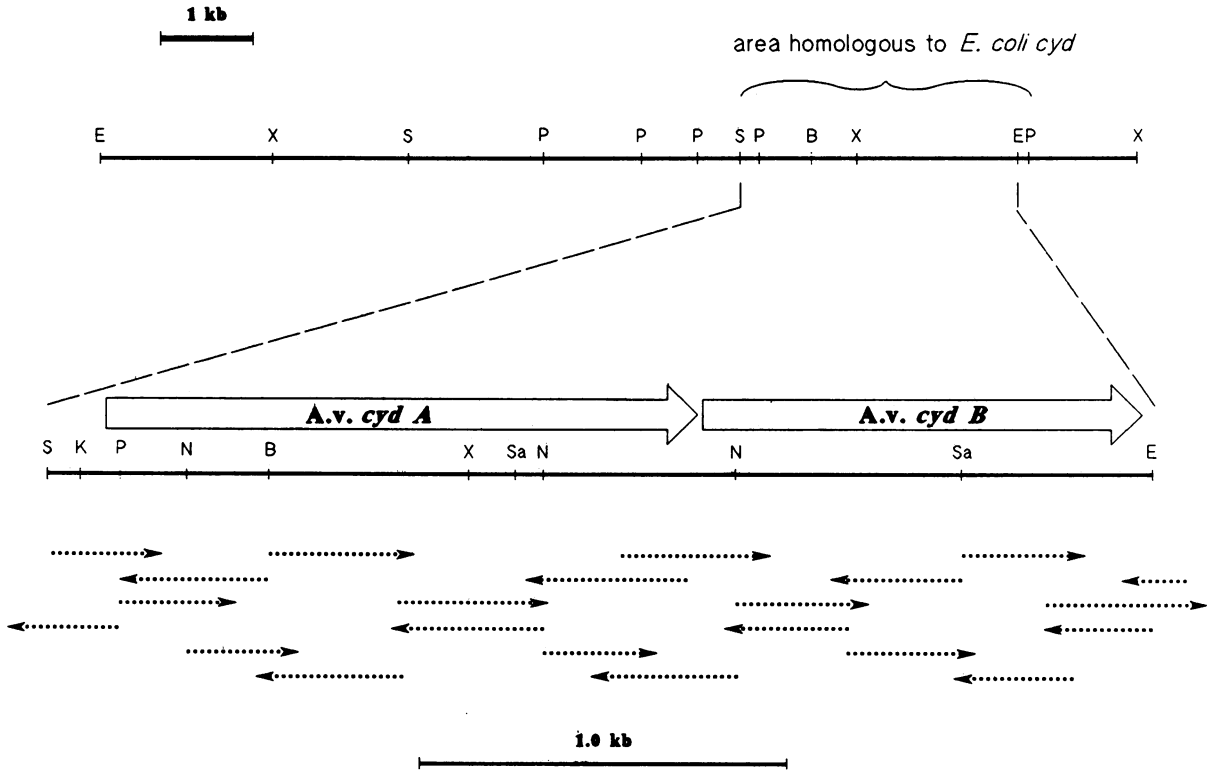

FIG. 2. Restriction map of the cloned *A. vinelandii* region, including and surrounding the area of hybridization to the *E. coli cyd* genes. The expanded region is the area containing the complete coding regions for both of the subunits of the *A. vinelandii* cytochrome *d* complex, including representative subclones and nested deletions used for sequencing the region. The insert from plasmid pTB11 corresponds to the *Pst*I-*Eco*RI region in the expanded diagram. Restriction sites: B, *Bam*HI; E, *Eco*RI; K, *Kpn*I; N, *Nco*I; P, *Pst*I; S, *Sac*I; Sa, *Sal*I; X, *Xho*I.

one positive clone, pTB11. Although the radiolabeled *E. coli cyd* probe hybridized strongly to various restriction digests of pTB11, on an equimolar basis the hybridization signal to itself (i.e., pNG2) was about 30- to 50-fold stronger (data not shown), possibly explaining our earlier problems with the colony hybridization approach.

By using the insert from plasmid pTB11 as a hybridization probe and separately screening size-selected *Eco*RI and *Xho*I minilibraries constructed in λ-ZAP, we were able to further clone approximately 12 kb of contiguous *A. vinelandii* genomic DNA. In addition to the 2.8-kb region corresponding to pTB11, the cloned region contains approximately 7 kb of DNA leftward of the *Pst*I site and approximately 1.3 kb rightward of the *Eco*RI site. A restriction map of the cloned region is shown in Fig. 2.

The fact that the area homologous to the *E. coli cyd* structural genes is, in fact, the *A. vinelandii cyd* locus was demonstrated by sequencing this region. The sequencing strategy and the nucleotide sequence of a 2,966-bp *Sac*I-*Eco*RI fragment, containing the entire region corresponding to the pTB11 insert and approximately 200 bp 5′ of the *Pst*I site, are shown in Fig. 2 and 3, respectively. The sequenced region contains two open reading frames (ORFs), each preceded by a typical Shine-Dalgarno sequence (31). ORF1 encodes a protein of 537 residues, with a predicted molecular weight of 59,719. ORF2, starting at 10 bp downstream of ORF1, encodes a protein of 379 residues corresponding to a molecular weight of 42,060. Sequences up to 200 bp upstream from ORF1 did not reveal the presence of any recognizable prokaryotic promoter elements. Northern blot analysis in conjunction with nuclease protection experiments on RNA isolated from *A. vinelandii* showed ORF1 and ORF2 to be cotranscribed. We have determined the transcription start site for the *cyd* mRNA to be approximately 280 bp upstream of the translation start site of ORF1 (26b).

A scan of the EMBL nucleotide sequence data bank and the Swiss-Prot protein sequence data bank revealed that the *E. coli cyd* locus and the deduced amino acid sequences of the two subunits of *E. coli* cytochrome *d* encoded by the *cydA* and *cydB* structural genes (11) are the only entries with a significant score of homology with the sequenced area of *A. vinelandii* DNA. The alignment of the deduced amino acid sequence of ORF1 with that of *cydA* and the alignment of ORF2 with *cydB* are shown in Fig. 4 and 5, respectively. The striking degree of homology between the *A. vinelandii* ORFs and the two subunits of the *E. coli* oxidase and the results of the expression of the *Azotobacter* oxidase in *E. coli* (see below) unambiguously show the cloned region to be the structural genes of the *A. vinelandii* cytochrome *d*.

Analysis of the deduced amino acid sequence of the *A. vinelandii cyd* locus. The deduced amino acid sequence of ORF1 (hereafter referred to as *A. vinelandii cydA*) is 69% identical to that of *E. coli cydA* throughout its length; if the two sequences are further adjusted for conserved changes, they are 83% similar. The hydropathy plot of the deduced amino acid sequence of the *A. vinelandii* gene is shown in Fig. 6A. The results from the sequence alignments, combined with a comparison of the hydropathy plots of the two *E. coli* protein subunits (11), make the two sequences almost

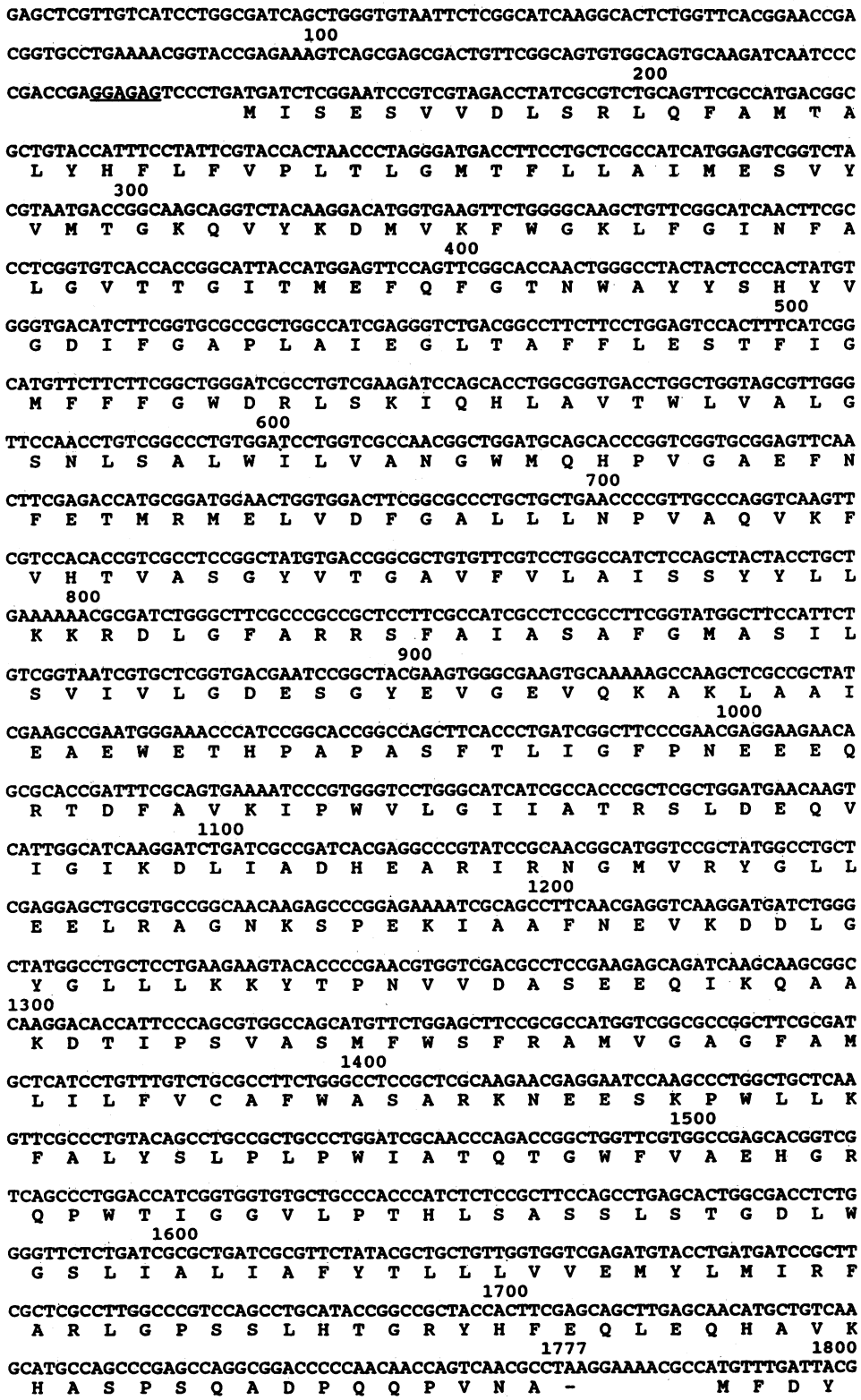

FIG. 3. Nucleotide sequence and deduced amino acid sequence of the *A. vinelandii cydA* and *cydB* structural genes. Potential ribosome binding sites are underlined.

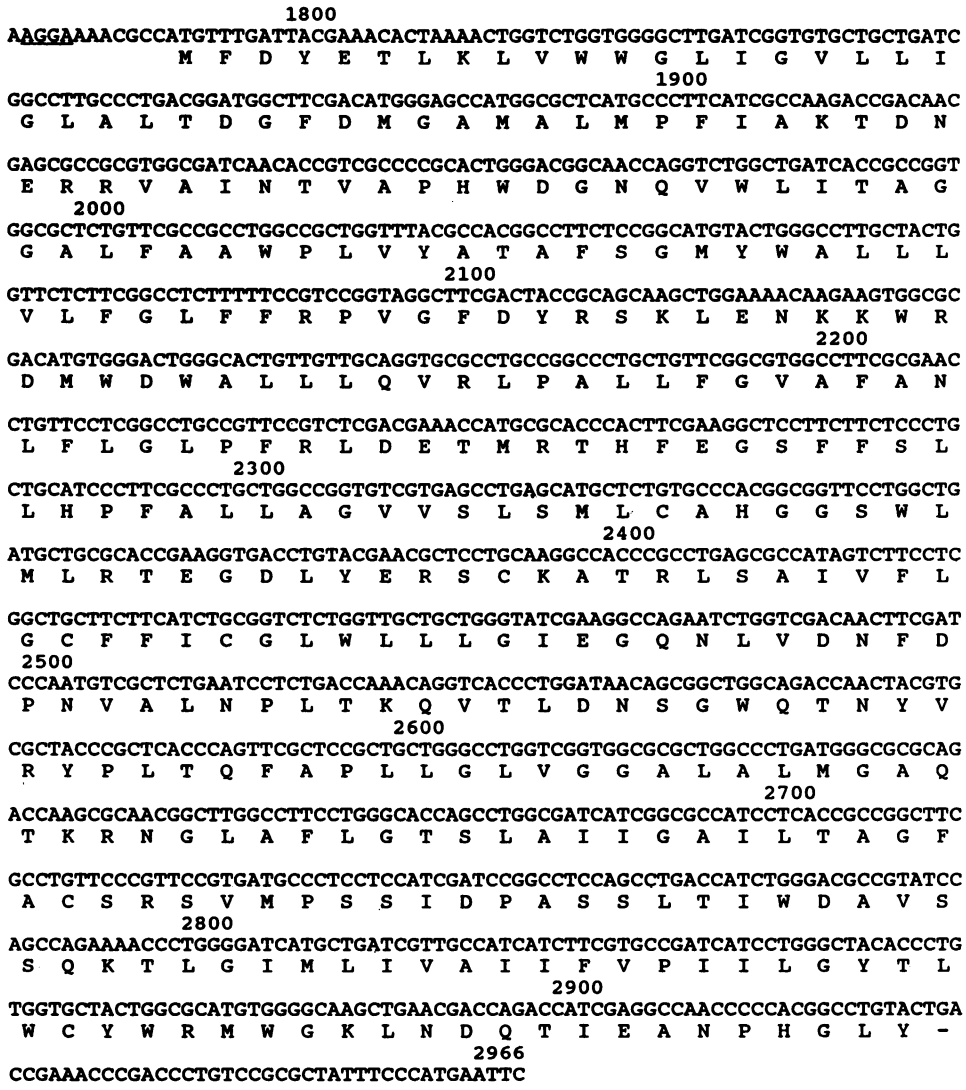

FIG. 3—*Continued.*

indistinguishable and practically superimposable. Two essential histidine residues, His-19 and His-186, which have been proposed to be heme axial ligands in the *E. coli* enzyme based on site-directed mutagenesis experiments (8), are both present (corresponding to His-21 and His-188 in the *A. vinelandii* sequence), and the residues surrounding them are essentially identical. All five histidine residues present in subunit I of the *E. coli* oxidase have been conserved in *A. vinelandii*, although the latter has seven additional histidines. Additionally, work from the Gennis group has characterized two different monoclonal antibodies that bind to the *E. coli* cytochrome *d* complex and specifically inhibit the quinol oxidase activity of the enzyme. The epitope for both of these antibodies has been mapped to a stretch of 11 amino acids in subunit I (7). This same stretch is also present in the deduced sequence of subunit I of the *A. vinelandii* oxidase. The conservation of this site and of His-186, which has been shown to be the axial ligand for the *E. coli* cytochrome b_{558} component (8), strongly suggests that the *A. vinelandii* oxidase is a quinol oxidase.

The overall similarity between the deduced sequence of ORF2 (hereafter called *A. vinelandii cydB*) and *E. coli cydB* is also high (68%). The hydropathy profile of this subunit (Fig. 6B) is also quite similar to that of *E. coli cydB*. The two sequences (both 379 residues in length) can in fact be aligned without inserting any gaps in either sequence. The highest level of homology in the two sequences is in the N-terminal third of both subunits. The results from the site-directed mutagenesis of the four histidine residues in *E. coli cydB* showed all of those residues to be dispensable for both the activity and the visible spectroscopic signatures of the enzyme (8). Two of the four histidines from the *E. coli* subunit II are conserved in *A. vinelandii*, although the latter has two additional histidines.

Based on a systematic compilation and analysis of sequences of membrane-bound hemoproteins known to interact with quinones, Degli Esposti devised a procedure to predict whether particular histidine residues in a membrane-bound hemoprotein are heme ligands (6). Briefly, three separate parameters, a histidine index, an SER parameter (dealing with the number of specifically enriched residues surrounding the particular histidine), and a score of local

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                                        *
AVCYDSUB1 - MISESVVDLSRLQFAMTALYHFLFVPLTLGMTFLLAIMESVYVMTGKQVY -50
            :.   .:.:::::::.::.:::::::::::::.:::::::.::::..:::.:
ECCYDSUB1 - ML--DIVELSRLQFALTAMYHFLFVPLTLGMAFLLAIMETVYVLSGKQIY -48

AVCYDSUB1 - KDMVKFWGKLFGINFALGVTTGITMEFQFGTNWAYYSHYVGDIFGAPLAI -100
            ::: ::::::::::::::::.::.:::::::::::.:::::::::::::::::
ECCYDSUB1 - KDMTKFWGKLFGINFALGVATGLTMEFQFGTNWSYYSHYVGDIFGAPLAI -98

AVCYDSUB1 - EGLTAFFLESTFIGMFFFGWDRLSKIQHLAVTWLVALGSNLSALWILVAN -150
            ::: :::::::::.:.:::::::: :.::. ::::::::::::::::::::
ECCYDSUB1 - EGLMAFFLESTFVGLFFFGWDRLGKVQHMCVTWLVALGSNLSALWILVAN -148
                                                *
AVCYDSUB1 - GWMQHPVGAEFNFETMRMELVDFGALLLNPVAQVKFVHTVASGYVTGAVF -200
            :::: :. ..:::::::::.: :  :.:::::::::::::::::::::.:
ECCYDSUB1 - GWMQNPIASDFNFETMRMEMVSFSELVLNPVAQVKFVHTVASGYVTGAMF -198

AVCYDSUB1 - VLAISSYYLLKKRDLGFARRSFAIASAFGMASILSVIVLGDESGYEVGEV -250
            .: ::..:.:: ::: ::.:::::::..::::..:::::::::::::.:.:
ECCYDSUB1 - ILGISAWYMLKGRDLAFAKRSFAIAASFGMAAVLSVIVLGDESGYEMGDV -248

AVCYDSUB1 - QKAKLAAIEAEWETHPAPASFTLIGFPNEEEQRTDFAVKIPWVLGIIATR -300
            ::.:::::::::::: ::::.::: : :  ::    ::. ::. :::::::
ECCYDSUB1 - QKTKLAAIEAEWETQPAPAAFTLFGIPDQEEETNKFAIQIPYALGIIATR -298

AVCYDSUB1 - SLDEQVIGIKDLIADHEARIRNGMVRYGLLEELRAGNKSPEKIAAFNEVK -350
            :.:  :::.:.:.  :: ::::::  : ::: ::.:          :: .:
ECCYDSUB1 - SVDTPVIGLKELMVQHEERIRNGMKAYSLLEQLRSGSTDQAVRDQFNSMK -348

AVCYDSUB1 - DDLGYGLLLKKYTPNVVDASEEQIKQAAKDTIPSVASMFWSFRAMVGAGF -400
             ::::::::::.::::: ::.: :: ::.::.:: :: ....:: ::  ::
ECCYDSUB1 - KDLGYGLLLKRYTPNVADATEAQIQQATKDSIPRVAPLYFAFRIMVACGF -398

AVCYDSUB1 - AMLILFVCAFWASARKNEESKPWLLKFALYSLPLPWIATQTGWFVAEHGR -450
            .: .   .::.  :     : :::. ::: .::::::  .:::::: ::
ECCYDSUB1 - LLLAIIALSFWSVIRNRIGEKKWLLRAALYGIPLPWIAVEAGWFVAEYGR -448

AVCYDSUB1 - QPWTIGGVLPTHLSASSLSTGDLWGSLIALIAFYTLLLVVEMYLMIRFAR -500
            :::.:: :::: .. :::..:::  :.. .   ::: :: :..:: .:::
ECCYDSUB1 - QPWAIGEVLPTAVANSSLTAGDLIFSMVLICGLYTLFLVAELFLMFKFAR -498

AVCYDSUB1 - LGPSSLHTGRYHFEQLEQHAVKHASPSQADPQQPVNA -537
            :::::: ::::::::         .. .:
ECCYDSUB1 - LGPSSLKTGRYHFEQ-------SSTTTQPAR       -522
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FIG. 4. Alignment of the amino acid sequence of the *A. vinelandii cydA* with the *E. coli cydA*. The amino acid sequences were aligned by using the PC/GENE software package (IntelliGenetics, Inc.). Colons indicate identical residues, and periods indicate conserved substitutions. His-19 and His-186 in the *E. coli* sequence (corresponding to His-21 and His-188 in the *A. vinelandii* sequence) are marked with asterisks. The amino acid stretch that is known to be the epitope of the monoclonal antibody A14-5 against the *E. coli* subunit I (9) is set in boldface type and underlined.

sequence similarity to a compiled data base of sequences surrounding histidine residues that are known to be heme ligands (the QB data base [6]), are calculated. Based on the overall scores from the three basic parameters, a prediction can be made on the qualification of a particular histidine residue to act as a heme ligand. This analysis successfully predicted His-19 and His-186 of the *E. coli cydA* to be good candidates as heme ligands (6). When this analysis is applied to the histidine residues in the *A. vinelandii cydA*, both His-21 and His-188 (and no others) stand out as good candidates for heme ligands. This result is not surprising, and, in light of the high degree of homology of the sequences surrounding both of these residues, His-21 and His-188 almost certainly must have the same function in the *A. vinelandii* oxidase.

Interestingly, however, His-166 in the *A. vinelandii cydB* (whose homolog in the *E. coli* gene is Asn-166), if analyzed by this method, has a histidine index of 0.898, an SER of 5, and, when compared with the QB data base, an alignment score of +6 or more with 10 separate sequences (the highest score is +11). All three scores, by the criteria of Degli Esposti (6), would strongly suggest this residue to be a heme ligand.

Expression of *A. vinelandii* cytochrome *d* in *E. coli*. Three different plasmids that were able to express the *A. vinelandii* cytochrome *d* under the control of a regulatable *E. coli* promoter were constructed in vitro by combining the entire *Pst*I-*Eco*RI insert from pTB11 with additional sequences located 5′ upstream of the *Pst*I site to form the entire coding sequence for subunits I and II. Plasmids pMH3 and pKE2 contained sequences up to the *Sac*I site (located 164 bp upstream of the initiator ATG) and the *Kpn*I site (located 74 bp upstream of the initiator ATG) (Fig. 2), respectively. The parent plasmids for pMH3 and pKE2 were pBluescript SKII(+) and pKSII(+), respectively. Both constructs were designed to align the *lac* promoter from the vector in the correct orientation relative to the coding region. The translation of both of the subunits in these constructs would be expected to be promoted by the *A. vinelandii* Shine-Dalgarno sequences, which seem to closely resemble those of *E. coli* (Fig. 3). In addition, a more streamlined construct (pOEC3) was also made in the expression vector pKK233.2 (detailed in Materials and Methods). In this construct, the presumptive initiator ATG of subunit I was fused directly to a vector-derived ATG and the *lac* ribosome-binding site, placing the entire coding region of both subunits under the

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AVCYDSUB2 - MFDYETLKLVWWGLIGVLLIGLALTDGFDMGAMALMPFIAKTDNERRVAI -50
            : ::: :. .:: :.:::::: :.:::::::   :  :. . : :::. :
ECCYDSUB2 - MIDYEVLRFIWWLLVGVLLIGFAVTDGFDMGVGMLTRFLGRNDTERRIMI -50

AVCYDSUB2 - NTVAPHWDGNQVWLITAGGALFAAWPLVYATAFSGMYWALLLVLFGLFFR -100
            :..:::::::::::::::::::::::.:::.:::: : :..:::  ::::
ECCYDSUB2 - NSIAPHWDGNQVWLITAGGALFAAWPMVYAAAFSGFYVAMILVLASLFFR -100

AVCYDSUB2 - PVGFDYRSKLENKKWRDMWDWALLLQVRLPALLFGVAFANLFLGLPFRLD -150
            ::::::::::.:  .:: :::: . .   .: :. :::: ::  :.:: .:
ECCYDSUB2 - PVGFDYRSKIEETRWRNMWDWGIFIGSFVPPLVIGVAFGNLLQGVPFNVD -150

AVCYDSUB2 - ETMRTHFEGSFFSLLHPFALLAGVVSLSMLCAHGGSWLMLRTEGDLYERS -200
            : .:  . : :: :: :: :::::::. :. . : ..: .:: :.:  :.
ECCYDSUB2 - EYLRLYYTGNFFQLLNPFGLLAGVVSVGMIITQGATYLQMRTVGELHLRT -200

AVCYDSUB2 - CKATRLSAIVFLGCFFICGLWLLLGIEGQNLVDNFDPNVALNPLTKQVTL -250
              .. ..:.: : :: . :.:.. ::.:  .    :   : ::: : :
ECCYDSUB2 - RATAQVAALVTLVCFALAGVWVMYGIDGYVVKSTMDHYAASNPLNKEVVR -250

AVCYDSUB2 - DNSGWQTNYVRYPLTQFAPLLGLVGGALALMGAQTKRNGLAFLGTSLAII -300
            .   :  :.   :.    : ::.:   :... :   .  .::. .::..
ECCYDSUB2 - EAGAWLVNFNNTPILWAIPALGVVLPLLTILTARMDKAAWAFVFSSLTLA -300

AVCYDSUB2 - GAILTAGFACSRSVMPSSIDPASSLTIWDAVSSQKTLGIMLIVAIIFVPI -350
              ::::: :    :::::    .:::.::: ::: :: .:  ::.. :::
ECCYDSUB2 - CIILTAGIAMFPFVMPSSTMMNASLTMWDATSSQLTLNVMTWVAVVLVPI -350

AVCYDSUB2 - ILGYTLWCYWRMWGKLNDQTIEANPHGLY -379
            :: :: ::::.:.:..    :: : : ::
ECCYDSUB2 - ILLYTAWCYWKMFGRITKEDIERNTHSLY -379
```

FIG. 5. Alignment of the amino acid sequence of the *A. vinelandii cydB* with the *E. coli cydB*. The symbols are defined in the legend to Fig. 4.

control of the strong hybrid *tac* promoter. By its design, this construct fuses an additional methionine residue to the N terminus of subunit I. Finally, a 3.8-kb *Nru*I fragment of plasmid pNG2, subcloned in pSKII(+), was used for the expression of *E. coli* cytochrome *d*, under the control of its own promoter (pEccyd2).

E. coli strain GR84N, a *cyd* mutant that makes no immunologically or spectroscopically detectable cytochrome *d* (13), was used as the host for the expression of the *A. vinelandii* cytochrome. To aid in repressing unwanted expression from the *lac* or *tac* promoters on the high-copy-number plasmids, GR84N cells were cotransformed with plasmid pBG1, a plasmid compatible with ColE1-derived plasmids, which contains the *lacI*^{q} gene. Monoclonal antibody A14-5 against the *E. coli* subunit I, which also inhibits the quinol oxidase activity of the *E. coli* oxidase (7) (see above), and affinity purified polyclonal antibodies to *E. coli* subunit II were used for the immunodetection of proteins expressed in the various transconjugants.

All three of the designed constructs, when transformed into GR84N cells, allowed for the expression of the *A. vinelandii* oxidase, which was detectable immunologically and spectroscopically and by its activity. In none of the constructs could the oxidase be fully repressed. In fact, the level of expression was not significantly higher upon the addition of 1 mM isopropyl-β-D-galactopyranoside to fully activate the *lac* or *tac* promoters (data not shown). All of the results presented here are for cells grown without isopropyl-β-D-galactopyranoside and were essentially identical to all three of the *A. vinelandii* cytochrome *d* expression plasmids; only the actual data for one of these (pOEC3) are shown. The expression of the cloned *E. coli* cytochrome *d* from its own promoter is maximal in the late log phase of growth (10) and does not require any inducers.

Plasmid pOEC3 in *E. coli* GR84N directed the expression of a protein that cross-reacted immunologically with monoclonal antibody A14-5 on SDS-PAGE immunoblots (Fig. 7). In addition, this antibody cross-reacted with a single band with an apparent molecular mass of 55 kDa in cell extracts of *A. vinelandii*. This protein is presumably subunit I of the *A. vinelandii* cytochrome *d*. The slightly larger size of this protein, compared with that of *E. coli* subunit I, is in good agreement with the larger size predicted from the sequence (59.7 kDa for *A. vinelandii* subunit I versus 58.2 kDa for *E. coli* subunit I). In addition, cell fractionation experiments showed that the *A. vinelandii* protein expressed in GR84N was correctly localized to the membrane fraction, as is the *E. coli* oxidase.

Previously, affinity polyclonal antibodies made against subunit II of the *E. coli* cytochrome *d* were shown to cross-react with a protein in *A. vinelandii* on dot blots (21). On immunoblots of SDS-PAGE-resolved *A. vinelandii* extracts, we found that this antibody cross-reacts with a protein of the same mobility as that of subunit I (apparent molecular mass of 55 kDa). The antibody, however, does not detect any cross-reacting proteins in GR84N transconjugants harboring any of the three expression constructs. Additionally, after two to three rounds of reuse, this polyclonal antibody preparation lost almost all of its cross-reactivity with any protein in the *A. vinelandii* extracts, but the loss of cross-reactivity to *E. coli* subunit II was negligible (data not shown). Our results indicate that this antibody does not cross-react with *A. vinelandii* subunit II and is thus not useful for monitoring the expression of this subunit in *E. coli*. Although our results have not allowed for the positive identification of the *A. vinelandii cydB* gene product expressed in *E. coli*, the sequences corresponding to this subunit are absolutely essential for the expression of the *A. vinelandii* oxidase in a spectroscopically detectable and enzymatically active form in *E. coli*, as described below.

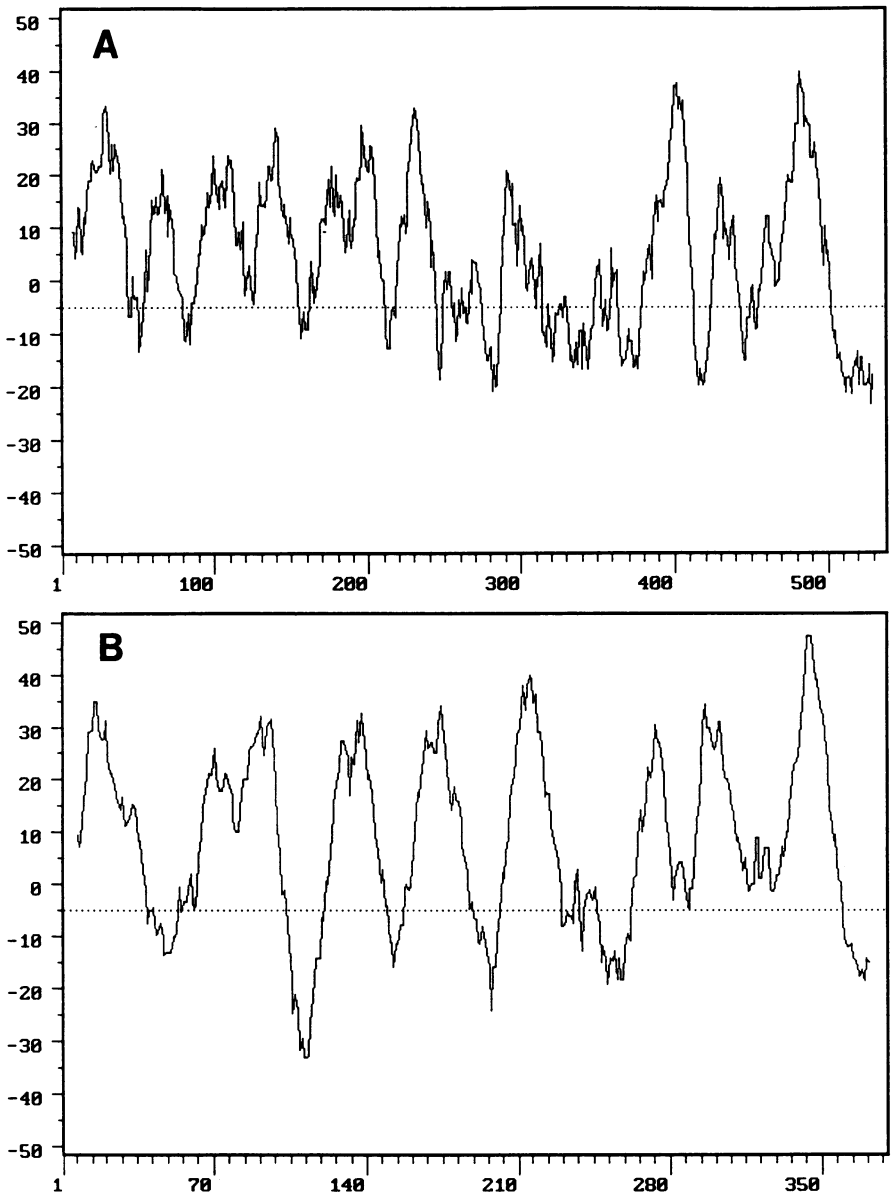

FIG. 6. Hydropathy plots of the deduced amino acid sequences of the *A. vinelandii cydA* (subunit I) (A) and *cydB* (subunit II) (B). (A) Hydropathic index of AVCYDA from amino acids 1 to 537 (GRAVY = 3.21). (B) Hydropathic index of AVCYDA from amino acids 1 to 379 (GRAVY = 5.55). The plots were generated with the program SOAP from the PC/GENE software package, which uses the Kyte and Doolittle algorithm (24) and a window size of 15 residues.

In addition to immunoreactive material, pelleted cells of *E. coli* GR84N transconjugants harboring pOEC3 had a faint green color, which is characteristic of cells that overexpress the *E. coli* cytochrome *d* (13). Reduced-minus-oxidized spectra of membranes of GR84N cells with pOEC3 are shown in Fig. 8. The *A. vinelandii* cytochrome *d* has not been previously purified, and its spectral features have been only characterized in *A. vinelandii* membranes in the presence of the other membrane-bound hemoproteins of this organism (mainly cytochrome *o* and cytochromes c_4 and c_5 [16–18]). The spectral features of the *A. vinelandii* oxidase expressed in a *cyd E. coli* background are essentially identical to those of the *E. coli* cytochrome *d* (Fig. 8B and C). In addition to the characteristic peak at 627 nm, the *A. vinelandii* oxidase has absorption peaks at 595 and 560 nm. The peak at 560 nm in the *A. vinelandii* spectrum is presumably due to cytochrome b_{560}, the homolog of the cytochrome b_{558} component of the *E. coli* cytochrome *d* complex. The absorption peak at 595 nm in both *E. coli* and *A. vinelandii* was previously referred to as cytochrome a_1 (18, 24), but work with the purified cytochrome *d* of *E. coli* has conclusively identified this component as being a part of the cytochrome *d* complex (cytochrome b_{595}) (24, 28). The presence of the peak at 595 nm in *E. coli* expressing the *A. vinelandii* cytochrome *d* further demonstrates the identity of the *A. vinelandii* cytochrome a_1 as the cytochrome b_{595} component of this organism. Expression plasmids with deletions in the region corresponding to the *A. vinelandii cydB*

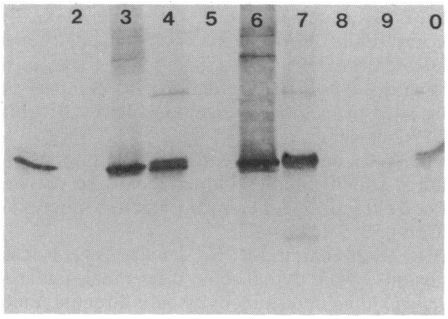

FIG. 7. Immunological detection of the *A. vinelandii* cytochrome *d* subunit I in *A. vinelandii* membranes and expressed in *E. coli* GR84N. Proteins were resolved by SDS-PAGE on 12.5% polyacrylamide gels, transferred onto nitrocellulose, and probed with monoclonal antibody A14-5, as described in Materials and Methods. All lanes contain 20 μg of total protein. Lanes: 1, *A. vinelandii* CA membranes; 2, GR84N/pKK233.2 crude extract; 3, GR84N/pECcyd2 crude extract; 4, GR84N/pOEC3 crude extract; 5, GR84N/pKK233.2 membranes; 6, GR84N/pECcyd2 membranes; 7, GR84N/pOEC3 membranes; 8, GR84N/pKK233.2, cytosolic fraction; 9, GR84N/pECcyd2, cytosolic fraction; 10, GR84N/pOEC3, cytosolic fraction.

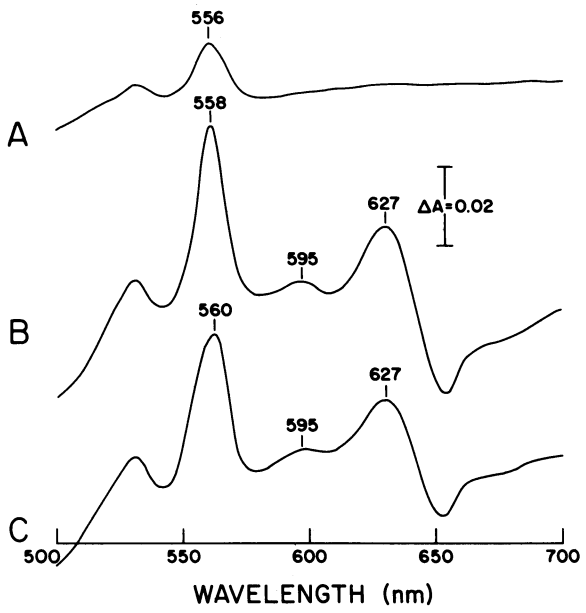

FIG. 8. Reduced-minus-oxidized absorption spectra of membranes isolated from GR84N transconjugants: A, GR84N/pKK233.2 (protein concentration, 3.6 mg/ml); B, GR84N/pECcyd2 (protein concentration, 0.97 mg/ml); C, GR84N/pOEC3 (protein concentration, 2.7 mg/ml).

do not express either the absorption peak at 595 nm or that at 627 nm in reduced-minus-oxidized absorption spectra, showing that this region is necessary for the expression and assembly of a functional oxidase (data not shown).

Finally, membrane preparations from *E. coli* GR84N transconjugants expressing the *A. vinelandii* cytochrome *d* were found to have ubiquinol-1-dependent oxidase activity that was about fourfold higher than the oxidase activity of cells transformed with the parent nonrecombinant plasmid [pKK233.2 or pSKII(+)] and about 40% of the oxidase activity of transconjugants overexpressing the *E. coli* cytochrome *d* (pEccyd2) (Table 1). The background quinol oxidase activity of GR84N cells is due to the endogenous cytochrome *o* present in these cells (12). That the fourfold increase in the quinol oxidase activity of the transconjugants expressing the *A. vinelandii* oxidase is, in fact, due to the activity of this component is demonstrated by the insensitivity of this activity to inhibition by 100 μM cyanide (Table 1). Purified *E. coli* cytochrome *o* is inhibited 85% by this concentration of cyanide (25), and we also found the background quinol oxidase activity of GR84N transconjugants bearing nonrecombinant plasmids to be inhibited similarly (82%) by 100 μM cyanide. Both the *E. coli* and *A. vinelandii* cytochrome *d*s are relatively insensitive to the concentrations of cyanide that inhibit cytochrome *o* (15, 26).

The immediate electron donor to the *A. vinelandii* cytochrome *d* was not previously known. The conserved features between the *A. vinelandii* and *E. coli* subunit I and the direct demonstration of the quinol oxidase activity of the *A. vinelandii* cytochrome *d* expressed in *E. coli* clearly show this oxidase to be a quinol oxidase. By the classification of Anraku (2), the *A. vinelandii* cytochrome *d* is thus a type IIB oxidase (a quinol oxidase with hemes b and d).

Both the cytochrome *d* and cytochrome *o* of *E. coli* can also oxidize the reduced form of the artificial electron donor TMPD. In *A. vinelandii* membranes, the cytochrome *d* branch of the respiratory chain cannot efficiently oxidize TMPD (15). Interestingly, when expressed in *E. coli*, the *A. vinelandii* cytochrome *d* has significant levels of cyanide-insensitive TMPD oxidase activity (Table 1). Perhaps the membrane environment of the foreign host (*E. coli*) affects TMPD accessibility to the *A. vinelandii* enzyme.

The ability of the *A. vinelandii* cytochrome *d* expressed in *E. coli* to reconstitute a complete electron transport chain from a primary dehydrogenase to oxygen was tested by measuring NADH-, lactate-, and succinate-dependent respiration and its inhibition by cyanide in membranes. The *A.*

TABLE 1. Heme content and oxidase specific activity of membranes from *E. coli* GR84N transconjungants harboring the various expression plasmids

	Heme content (nmol/mg of protein)		Oxygen uptake, nmol/min/mg of protein[a] (% inhibition with 100 μM cyanide)[b]			
	Cytochrome *b*	Cytochrome *d*	UQ-1[c]	TMPD	NADH	D,L-Lactate
pKK233.2 and pBG1	0.21	0	330 (81)	21 (80)	45 (85)	53 (80)
pECcyd2 and pBG1	2.48	2.23	2,890 (8)	185 (15)	370 (10)	350 (12)
pOEC3 and pBG1	0.53	0.62	1,165 (14)	47 (15)	124 (15)	10 (50)

[a] Averages of three separate determinations with each of the four substrates.
[b] Samples were preincubated with 100 μM cyanide for 5 min at room temperature (without a substrate), and the assay was initiated with the addition of a substrate.
[c] UQ-1, ubiquinone-1.

vinelandii oxidase could reconstitute a cyanide-insensitive respiratory chain from NADH to oxygen (Table 1). This heterologous oxidase, however, when expressed in *E. coli*, could not support significant levels of lactate- or succinate-dependent respiration (Table 1; data for succinate not shown). This is surprising in view of the fact that the dehydrogenases for all of these substrates are primary dehydrogenases, which feed the electrons from the oxidation of their respective substrates directly into the ubiquinone pool (27). It is possible that the expression of the *A. vinelandii* oxidase in these cells differentially affects the level or activity of the different dehydrogenases. Previous work from this laboratory has shown that the electron transport from a membrane-bound hydrogenase in *A. vinelandii* terminates almost exclusively with cytochrome *d* (34). A possible explanation for the results in *E. coli* may lie in some type of structural organization (i.e., compartmentalization) of particular dehydrogenases with a specific terminal oxidase. These observations are currently under further study.

Recently, Kelly et al. have also reported on the cloning of the genomic area of *A. vinelandii* homologous to the *E. coli cyd* locus (19). The direction of transcription of the genes was determined by using *lacZ* fusions and is in agreement with our results. Targeted transposon Tn*5* mutagenesis was then used to create an insertion mutant in the *cydB* region of *A. vinelandii*. The resulting mutation produced a spectroscopically Cyd$^-$ phenotype, abolishing the b_{595} and *d* peaks in reduced-minus-oxidized spectra. Interestingly, these mutants lost their ability to fix nitrogen under normal atmospheric levels of oxygen but could still fix nitrogen under microaerophilic growth conditions. Their results are in good agreement with the proposed function of cytochrome *d* in the respiratory protection mechanism of *A. vinelandii*.

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REFERENCES

1. **Amann, E., and J. Brosius.** 1985. 'ATG vectors' for regulated high-level expression of cloned genes in *Escherichia coli*. Gene **40**:183–190.
2. **Anraku, Y.** 1988. Bacterial electron transport chains. Annu. Rev. Biochem. **57**:101–132.
3. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1987. Current protocols in molecular biology. Wiley Interscience, New York.
4. **Bishop, P. E., D. M. L. Jarlenski, and D. R. Hetherington.** 1980. Evidence for an alternative nitrogen fixation system in *Azotobacter vinelandii*. Proc. Natl. Acad. Sci. USA **77**:7342–7346.
5. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heynecker, and H. W. Boyer.** 1977. Construction of useful cloning vectors. Gene **2**:95–113.
6. **Degli Esposti, M.** 1989. Prediction and comparison of haeme-binding sites in membrane haemoproteins. Biochim. Biophys. Acta **977**:249–265.
7. **Dueweke, T. J., and R. B. Gennis.** 1990. Epitopes of monoclonal antibodies which inhibit ubiquinol oxidase activity of *Escherichia coli* cytochrome d complex localize functional domain. J. Biol. Chem. **265**:4273–4277.
8. **Fang, H., R.-J. Lin, and R. B. Gennis.** 1989. Location of heme axial ligands in the cytochrome d terminal oxidase complex of *Escherichia coli* determined by site-directed mutagenesis. J. Biol. Chem. **264**:8026–8032.
9. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction fragments to high specific activity. Anal. Biochem. **132**:6–13.
10. **Georgiou, C. D., T. J. Dueweke, and R. B. Gennis.** 1988. Regulation of expression of the cytochrome *d* terminal oxidase in *Escherichia coli* is transcriptional. J. Bacteriol. **170**:961–966.
11. **Green, G. N., H. Fang, R.-J. Lin, G. Newton, M. Mather, C. D. Georgiou, and R. B. Gennis.** 1988. The nucleotide sequence of the cyd locus encoding two subunits of the cytochrome d terminal oxidase complex of *Escherichia coli*. J. Biol. Chem. **263**:13138–13143.
12. **Green, G. N., and R. B. Gennis.** 1983. Isolation and characterization of an *Escherichia coli* mutant lacking cytochrome *d* terminal oxidase. J. Bacteriol. **154**:1269–1274.
13. **Green, G. N., J. E. Kranz, and R. B. Gennis.** 1984. Cloning of the cyd gene locus coding for the cytochrome d complex of *Escherichia coli*. Gene **32**:99–116.
14. **Johnson, D. A., J. W. Gautsch, J. R. Sportman, and J. H. Elder.** 1984. Improved technique utilizing nonfat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. Gene Anal. Tech. **1**:3–8.
15. **Jones, C. W.** 1973. The inhibition of *Azotobacter vinelandii* terminal oxidases by cyanide. FEBS Lett. **36**:347–350.
16. **Jones, C. W., and E. R. Redfearn.** 1966. Electron transport in *Azotobacter vinelandii*. Biochim. Biophys. Acta **113**:467–481.
17. **Jones, C. W., and E. R. Redfearn.** 1967. Preparation of red and green electron transport particles from *Azotobacter vinelandii*. Biochim. Biophys. Acta **143**:354–362.
18. **Jones, C. W., and E. R. Redfearn.** 1967. The cytochrome system of *Azotobacter vinelandii*. Biochim. Biophys. Acta **143**:340–353.
19. **Kelly, M. J. S., R. K. Poole, M. G. Yates, and C. Kennedy.** 1990. Cloning and mutagenesis of genes encoding the cytochrome bd terminal oxidase complex of *Azotobacter vinelandii*: mutants deficient in the cytochrome *d* complex are unable to fix nitrogen in air. J. Bacteriol. **172**:6010–6019.
20. **Konderosi, A., E. Konderosi, C. E. Pankhurst, W. J. Broughton, and Z. Banfalvi.** 1982. Mobilization of a *Rhizobium meliloti* megaplasmid carrying nodulation and nitrogen fixation genes into other rhizobia and *Agrobacterium*. Mol. Gen. Genet. **188**:433–439.
21. **Kranz, R. G., and R. B. Gennis.** 1985. Immunological investigation of the distribution of cytochromes related to the two terminal oxidases of *Escherichia coli* in other gram-negative bacteria. J. Bacteriol. **161**:709–713.
22. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157**:105–132.
23. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
24. **Lorence, R. M., J. G. Koland, and R. B. Gennis.** 1986. Coulometric and spectroscopic analysis of the purified cytochrome d complex of *Escherichia coli*: evidence for the identification of "cytochrome a_1" as cytochrome b_{595}. Biochemistry **25**:2314–2321.
25. **Matsushita, K., L. Patel, and H. R. Kaback.** 1984. Cytochrome o type oxidase from *Escherichia coli*. Characterization of the enzyme and mechanism of electrochemical proton gradient generation. Biochemistry **23**:4703–4714.

26. **Miller, M. J., and R. B. Gennis.** 1983. The purification and characterization of the cytochrome d terminal oxidase complex of the *Escherichia coli* aerobic respiratory chain. J. Biol. Chem. **258:**9159–9165.

26a. **Moshiri, F., A. Chawla, and R. J. Maier.** 1990. Abstr. 90th Annu. Meet. Am. Soc. Microbiol. 1990.

26b. **Moshiri, F., E. G. Smith, J. P. Taormino, and R. J. Maier.** Transcriptional regulation of cytochrome d in nitrogen-fixing Azotobacter vinelandii. Evidence that up-regulation during N_2-fixation is dependent on *ntrA*. J. Biol. Chem., in press.

27. **Poole, R. K., and W. J. Ingledew.** 1987. Pathways of electrons to oxygen, p. 170–200. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.

28. **Poole, R. K., and H. D. Williams.** 1987. Proposal that the function of the membrane-bound cytochrome a_1-like haemoprotein (cytochrome b-595) in *Escherichia coli* is a direct electron donation to cytochrome d. FEBS. Lett. **217:**49–52.

29. **Postgate, J. R.** 1982. Fundamentals of nitrogen fixation. Cambridge University Press, Cambridge.

30. **Robson, R. L., and J. R. Postgate.** 1980. Oxygen and hydrogen in biological nitrogen fixation. Annu. Rev. Microbiol. **34:**183–207.

31. **Shine, J., and L. Dalgarno.** 1974. The 3′-terminal sequence of *Escherichia coli* 16S ribosomal RNA: complementarity to nonsense triplets and ribosome binding sites. Proc. Natl. Acad. Sci. USA **74:**1342–1346.

32. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.

33. **Williams, A. M., and P. W. Wilson.** 1954. Adaptation of *Azotobacter* cells to tricarboxylic acid substrates. J. Bacteriol. **67:** 353–360.

34. **Wong, T.-Y., and R. J. Maier.** 1984. Hydrogen-oxidizing electron transport components in nitrogen-fixing *Azotobacter vinelandii*. J. Bacteriol. **159:**348–352.