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Cloning, sequencing, and mutagenesis of the cytochrome c_4 gene from *Azotobacter vinelandii*: characterization of the mutant strain and a proposed new branch in the respiratory chain

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Abstract

Azotobacter vinelandii is a free-living, nitrogen-fixing bacterium with a branched electron transport chain terminating with two terminal oxidases, cytochromes *d* and *o*. Cytochrome *o* is thought to receive its electrons from cytochromes *c*. The gene encoding cytochrome c_4 has been cloned and sequenced (termed the *cycA* locus). The deduced amino acid sequence contains a 20 residue signaling peptide sequence on the N-terminal end. Mutagenesis was performed by inserting a Km^r cassette into the structural gene. The subsequent mutant strains showed reduced amounts of cytochromes *c* (approximately 60% of wild-type levels) based on difference absorption spectra measurements. Heme staining confirmed the complete loss of cytochrome c_4 protein in the mutant strains. These mutants could grow and respire normally, like the wild type, under both diazotrophic or non-diazotrophic conditions. Surprisingly, the cytochrome *o* terminal oxidase was still turning over in membranes from the *cycA* mutants as evidenced by substrate-reduced CO difference spectra and inhibition experiments with the use of the cytochrome *o* inhibitor, chlorpromazine. Still, the levels of oxidation by ascorbate-TMPD were greatly reduced in the *cycA* mutants. Therefore, it is proposed that cytochrome c_4 does not exist in complex with cytochrome *o* as a multi-component terminal oxidase complex, yet still passes electrons to it in parallel like cytochrome c_5, as opposed to in an obligate sequential manner with cytochrome c_5. In this pathway the proposed new branch is at the ubiquinone to cytochromes *c* level.

Keywords: Cytochrome *c*; Electron transport; Nitrogen fixation; Respiratory protection; (*A. vinelandii*)

1. Introduction

Biological nitrogen fixation, the process by which atmospheric nitrogen is reduced to ammonia, is catalyzed by an enzyme system known as nitrogenase. This enzyme system, found only in a limited number of prokaryotes, is sensitive to oxygen and is extremely energy demanding (see [1] for a recent review). Because of these unusual demands on nitrogenase function, nitrogen-fixing aerobes have the unique problem of balancing the levels of intracellular oxygen to prevent inhibition of nitrogenase while carrying out oxygen-dependent respiration to allow for ATP synthesis. Thus the electron transport chains of these obligate aerobes have a dual role of consuming excess oxygen to protect nitrogenase and conserving energy via oxidative phosphorylation.

A commonly studied aerobic diazotroph is the free-living soil bacterium *Azotobacter vinelandii*. *A. vinelandii* has a branched electron transport chain which contains several flavin-dependent substrate dehydrogenases, ubiquinone Q-8, and at least six spectroscopically detectable cytochromes of which cytochromes *d* and *o* are terminal oxidases [2–5]. The branch site, which occurs at the quinone level, yields a cytochrome $b \rightarrow d$ and a $c \rightarrow o$ terminal pathway, as illustrated in Fig. 1. Cytochrome *b/d* has been cloned and sequenced [6], and has been shown to be up-regulated when *nif* genes are transcriptionally activated [7]. Mutants with a transposon inserted into the structural *b/d* gene could not fix nitrogen under air (20% oxygen) but could do so under low oxygen (1.5%), demonstrating that this pathway is involved in respiratory protection of nitrogenase [8].

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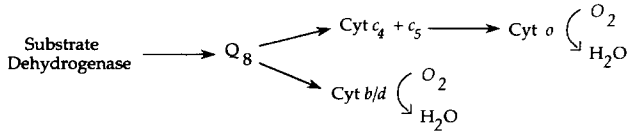

Fig. 1. The electron transport chain of *A. vinelandii*. Q_8, ubiquinone Q-8; Cyt, cytochrome. Adapted from Haddock and Jones [4].

In contrast, the cytochrome $c \rightarrow o$ pathway is thought to be involved in energy conservation. *A. vinelandii* has at least four cytochromes *c*, two major *c*-types, cytochromes c_4 and c_5, as well as two minor *c*-types, cytochromes *c*-551 and *c*-555 [9]. The function of the two minor cytochromes *c* is unknown, however the two major *c*-types ($c_4 + c_5$) are thought to transfer electrons in a sequential pathway from ubiquinone (or some unidentified intermediate) to the terminal oxidase cytochrome *o* [2–5]. There have been many bacterial and mitochrondrial cytochromes *c* studied and they mostly play roles in donating electrons to terminal oxidases [see [10,11] for a general review].

A cytochrome oxidase complex containing cytochromes c_4 and *o* has been purified from *A. vinelandii* membranes [12]. This complex was found to be able to oxidize ascorbate-*N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD) and was proposed to be a terminal oxidase complex. More recent work on cytochrome c_4 has concluded that it is not involved in ascorbate-TMPD oxidation, nor is it joined in a complex with cytochrome *o* as a terminal oxidase in membranes [13]. Thus there appear to be open questions on the nature of cytochrome c_4 in association with other cytochromes and therefore on the role of cytochrome c_4 in the electron transport chain. This work addresses the role of cytochrome c_4 by cloning and mutation of the gene encoding this protein and characterizing the subsequent mutant strains.

2. Materials and methods

2.1. Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this work are listed in Table 1. *A. vinelandii* strains were grown at 30° C in Burk's sucrose medium [14] with 2% (w/v) sucrose (BS), supplemented with 25 mM ammonium chloride (BSN) as a fixed nitrogen source, when needed. A rich modification (RMN) [15] of Burk's sucrose medium supplemented with ammonium was used in experiments in which the cytochrome *b*/*d* deletion was grown. This was done for ease of growth of the extremely respiratory deficient *b*/*d* mutant. Similar results were obtained when strains were grown in minimal Burk's sucrose medium as in RMN (unpublished data). Antibiotics were added at the following concentrations: kanamycin, 5 μg ml^{-1}; and spectinomycin, 5 μg ml^{-1} as needed. Batch cultures for isolation of membranes were grown in 2 l baffled flasks, containing 800 ml of medium and shaken vigorously at 220 rpm in a rotary shaker/incubator. For growth measurements, 20 ml cultures were shaken in 250 ml side-arm flasks at 220 rpm. Cell densities were measured at 540 nm with 0.1 optical absorbance unit equivalent to $2.2 \cdot 10^7$ cells ml^{-1} [16].

E. coli strain DH5α (Gibco-BRL) used for plasmid growth and isolation was grown at 37° C in LB medium [17]. Antibiotics were added at the following concentrations: ampicillin, 200 μg ml^{-1}; kanamycin, 25 μg ml^{-1}; and spectinomycin, 25 μg ml^{-1} when required.

Table 1
Bacterial strains and plasmids

Strain/plasmid	Description	Origin/Reference
Strain		
Azotobacter vinelandii		
CA	Wild-type strain	[33]
CA Δ*cyd*::Ω3	*cyd* deletion, Ω cassette in the same orientation as the *cyd* locus	This work
CA *cycA*::Kan1	*cycA* with Km^r cassette inserted in opposite orientation to the *cycA* gene.	This work
CA *cycA*::Kan5	*cycA* with Km^r cassette inserted in the same orientation as the *cycA* gene.	This work
E. coli		
DH5α	Cloning host	Gibco-BRL
Plasmid		
pBluescript KSII(+)	Ampicillin^r	Stratagene
pUC4-KIXX	pUC4-K containing the kanamycin-resistance cassette from Tn5 and the bleomycin-resistance gene	[22], Pharmacia
pAL1	pBluescript KSII(+) with a 1.76 kb SalI fragment carrying the *A. vinelandii cycA* locus	This work
pTN1	pBluescript KSII(+) with an approximately 4.0 kb *Bgl*II fragment carrying the *A. vinelandii cycA* locus	This work
pTN1::Kan1	pTN1 with the 1.2 kb Km^r cassette from pUC4-KIXX inserted in the opposite orientation of the *cycA* gene within a unique *Hpa*I site in the *cycA* coding region	This work
pTN1::Kan5	Same as pTN1::Kan1 except the Km^r cassette is inserted in the same orientation as the *cycA* gene	This work

2.2. DNA manipulations

Genomic DNA from *A. vinelandii* was prepared by the CTAB/NaCl method [18]. All other DNA isolation and cloning techniques were performed using standard procedures [17]. The two degenerate primers used for PCR of *A. vinelandii* genomic DNA each contained a 'GGG' clamp sequence and an engineered *Eco*RI site on the 5′ end. The N-terminal primer, based on residues 41 through 49, sequence is 5′-TNAARCARATGCARGAYATH-AARGC-3′ (384-fold degenerate). The C-terminal primer, based on residues 160 though 167, sequence is 5′-CATDATCATRTTRTCNCCRTCRTT-3′ (192-fold degenerate). Standard reaction conditions for PCR were used [17] using 160 pmol of each degenerate primer and 10 ng of CA genomic DNA. 30 cycles were done at an annealing temperature of 45° C for 1 min and extension at 72° C for 95 sec. Southern hybridizations of *A. vinelandii* genomic DNA, phage clones, and plasmid subclones were performed using established protocols [17]. Probes for Southern blots and library screening were made from gel-purified restriction fragments using the Prime-it II kit (Stratagene) and [α-^{32}P]dATP or [α-^{32}P]dCTP (3000 Ci mmol^{-1}) (NEN Research Products).

The *A. vinelandii* genomic library constructed in lambda EMBL3 has been previously described [19]. The library was screened with the radiolabelled PCR subclone fragment using standard protocols [17]. After a secondary screen of positive plaques, single phage plaques were selected to produce high-titer stocks. Phage DNA was isolated from the high-titer stocks and further characterized. Plasmid pBluescript KSII(+) (Stratagene) was used for further subcloning. Plasmid pAL1 containing a 1.76 kb *Sal*I fragment carrying the *cycA* locus, constructed by cloning the gel-purified 1.76 kb *Sal*I fragment from phage DNA into the unique *Sal*I site in the polylinker of pBluescript KSII(+), was used for sequencing. Plasmid pTN1, which has an approximately 4.0 kb *Bgl*II fragment containing the *cycA* locus, was constructed by cloning the blunted gel-purified 4.0 kb *Bgl*II fragment from phage DNA into the unique *Eco*RV site in the polylinker of pBluescript KSII(+); thereby losing the *Bgl*II sites. This plasmid was used for mutant strain construction (as described below). The orientation of the insert in these two plasmids was determined by restriction mapping.

Plasmid pAL1 and various subclones of it were sequenced using the Sequenase Version 2.0 kit (U.S. Biochemicals) following the manufacturer's instructions for double stranded sequencing or at the DNA Analysis Facility of Johns Hopkins University School of Medicine on a Applied Biosystems 373a automated DNA sequencer (Applied Biosystems) using the fluorescent di-deoxy terminator method of cycle sequencing following ABI protocols [20,21]. DNA and amino acid sequences were analyzed using the PC/Gene software package (Intelligenetics). The DNA sequence was sequenced and confirmed in both strands and has been submitted to the EMBL/GenBank/DDBJ Nucleotide Sequence Data Libraries. The accession number is L37290.

2.3. Construction of A. vinelandii mutants

The in vitro mutagenesis of *cycA* was performed by cloning the gel-purified Km^r cassette (1.2 kb *Sma*I fragment) from plasmid pUC4-KIXX [[22], from Pharmacia] into the unique *Hpa*I site in plasmid pTN1. This yielded plasmids pTN1::Kan1 and pTN1::Kan5. The orientation of the Km^r cassette in pTN1::Kan1 and pTN1::Kan5 is in the opposite and same direction as the disrupted *cycA* gene, respectively, as determined by restriction mapping. Plasmids pTN1::Kan1 and pTN1::Kan5 were doubled-digested with *Apa*I and *Eco*RI (to release the insert from the pBluescript KSII(+) vector) and this linearized DNA was used to transform competent *A. vinelandii* CA cells as described below.

In vitro mutagenesis of the *A. vinelandii* cytochrome *b/d* gene was made as follows. Plasmid pAVP1, containing a 0.65 kb *Pst*I restriction fragment corresponding to the genomic DNA region directly upstream of the *cydAB* locus, which is cloned into the *Pst*I site within the polylinker of pBluescript SKII(+) [6] was doubled-digested with *Eco*RI and *Xho*I. The 1.3 kb *Eco*RI-*Xho*I restriction fragment located immediately downstream of the *cydAB* region [see Fig. 2 of [6]] was directionally subcloned into these sites yielding plasmid pΔ*cyd*. Plasmid pΔ*cyd* was linearized at the unique *Eco*RI site and blunted and the Ω cassette (spectinomycin^r) from plasmid pHP45Ω [23] was subcloned into it as a blunted 2.0 kb *Bam*HI fragment. This yielded plasmid pΔ*cyd*::Ω3 which contains the Ω cassette in the same orientation as the deleted *cydAB* genes. Correct structure and orientation was verified by restriction mapping. This plasmid was linearized at the unique *Xho*I site and the DNA was used to transform competent *A. vinelandii* wild-type cells as described below.

A. vinelandii wild-type strain CA was made competent by a simplified modified protocol [24]. After transformation and growth on non-selective CM agar medium, cells were plated onto either BSN or RMN plates containing the appropriate antibiotic. Antibiotic resistant colonies were subcultured four times on the appropriate antibiotic plates. This yielded the mutant strains: CA *cycA*::Kan1, CA *cycA*::Kan5, and CA Δ*cyd*::Ω3. Mutant strains CA-*cycA*::Kan1 and CA *cycA*::Kan5 have the Km^r cassette in the opposite and same direction as the *cycA* gene respectively. Mutant strain CA Δ*cyd*::Ω3 has the Ω cassette in the same direction as the deleted *cydAB* locus. This cytochrome *b/d* deletion mutant has the same physiological characteristics as the previously described cytochrome *d* transposon mutant [8]. The genomic organization of the desired deletion and/or insertion in the mutant strains were confirmed by genomic Southern blots.

2.4. Preparation of membranes

Cells were harvested at late log phase (optical absorbance at 540 nm of 0.7-0.9). Whole cells were resuspended in a 50 mM phosphate buffer (pH 7.4) with 2.5 mM $MgCl_2$ and stirred overnight at 4° C to remove endogenous substrates. Membranes were isolated from the cell suspension as previously described [25].

2.5. Protein determination

Protein concentrations were determined using the BCA protein assay kit (Pierce) with bovine serum albumin as the standard.

2.6. Gel electrophoresis, protein and heme staining

SDS-polyacrylamide gel electrophoresis (PAGE) and Coomassie brilliant blue staining was performed using standard methods [17]. Prior to SDS-PAGE, membranes were extracted with n-butanol as previously described [26]. The single-step partially purified membranes were heated with SDS sample buffer that did not contain β-mercaptoethanol at 90° C for 5 min immediately before electrophoresis on 12.5% gels. Gels were stained for heme *c* by peroxidase action on 3,3′-dimethoxybenzidine as previously described [27].

2.7. Whole-cell and membrane respiratory rate measurements

For whole-cell respiratory rate measurements, aliquots of cultures diluted with medium were transferred into a sealed gas-tight, magnetically stirred 8-ml chamber. Respiratory rates were measured at room temperature with a YSI 5331 amperometric oxygen electrode (Yellow Springs Instruments).

Membrane respiratory rates were done at room temperature in a 50 mM phosphate buffer (pH 7.4) with 2.5 mM $MgCl_2$, in the same chamber as described above. The reaction was initiated by the addition of the membrane samples followed by the substrate. The substrates used were 5 mM NADH, 10 mM malate, 20 mM succinate, or 4 mM ascorbate–2 mM TMPD. The ascorbate-TMPD respiratory rates were corrected for the small but significant auto-oxidation rate of this substrate. For the chlorpromazine inhibition, the same substrates were used, except that the membrane samples were preincubated with either 100 μM or 500 μM chlorpromazine for 5 min before addition of substrate. The concentration of oxygen in air-saturated medium or buffer was taken to be 250 μM at room temperature.

2.8. Spectroscopic analysis

Difference spectra were performed at room temperature with a Beckman DU-70 spectrophotometer (Beckman Instruments). 1.0 ml of membrane samples were placed in a semi-micro quartz cuvette with a 10-mm pathlength (Savant Instruments) and tightly stoppered with a sleeve type rubber stopper to keep the samples anaerobic when necessary. Membranes were reduced or oxidized with a few grains of sodium dithionite or ammonium persulfate, respectively. To reduce membranes with NADH, the stoppered cuvette containing the membrane samples was flushed with argon gas for 2 min to remove oxygen. 5 mM NADH was then added and the sample was equilibrated at room temperature for 15–20 min with substrate before recording spectra. For CO difference spectra, reduced membrane samples were flushed with CO for 2 min before recording spectra. Two spectral control experiments were performed. An inhibitor to the cytochrome *o* pathway, chlorpromazine (at 100 μM) [39] was used along with reductant (5 mM NADH) to document the significance of the trough at 558 nm. The cytochrome *o* trough at 558 nm was diminished by chlorpromazine for all samples (wild type and all mutants). This result is like that previously described for *A. vinelandii* membranes [39]. To ensure that the samples remained reduced after CO bubbling, CO difference spectra were recorded at intervals up to 30 min; the spectra were unchanged during this period. The concentrations of cytochromes were calculated from dithionite-reduced-minus-persulfate-oxidized difference spectra using the following extinction coefficients: total cytochrome *b*, 17.5 mM^{-1} cm^{-1} (560 –580 nm) [28]; total cytochrome *c*, 17.3 mM^{-1} cm^{-1} (551–538 nm) [29]; cytochrome *d*, 7.4 mM^{-1} cm^{-1} (628–607 nm) [30]; and cytochrome *o*, 42.2 mM^{-1} cm^{-1} (562–575 nm) [31].

3. Results

3.1. Cloning, sequencing, and analysis of the cytochrome c_4 gene

Based on the published amino acid sequence of the purified *A. vinelandii* cytochrome c_4 protein [32], two degenerate oligonucleotide primers were synthesized corresponding to areas near the N-terminal and the C-terminal ends of the sequence. The N-terminal primer was deduced from amino acid residues 41 through 49 and the C-terminal primer was deduced from residues 160 through 167 (see Fig. 3 below). *A. vinelandii* wild-type strain CA [33] genomic DNA was amplified using these two primers and the reaction yielded a product of the expected size (0.38 kb). This PCR fragment was subcloned and partially sequenced. The translated PCR DNA sequence corresponded exactly with the previously published cytochrome c_4 protein sequence. This PCR fragment was radiolabelled and used to probe various restriction digests on genomic Southern blots and a lambda EMBL3 gene bank (data not shown). Three different lambda EMBL3 clones were selected as positive during the library screening and they all

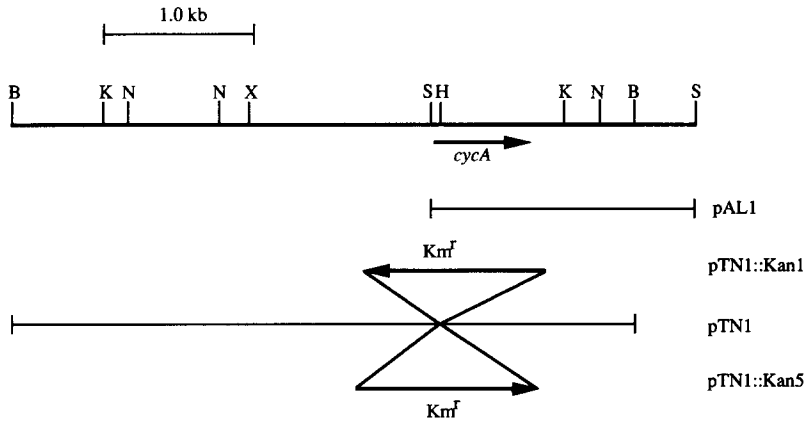

Fig. 2. Restriction map of the genomic region containing the *A. vinelandii cycA* locus. The position and direction of the *cycA* gene is indicated by an arrow below the restriction map. Plasmid pAL1, used for sequencing, contains a 1.76 kb *Sal*I fragment. Plasmid pTN1, used for mutagenesis, contains an approximately 4.0 kb *Bgl*II fragment. Plasmids pTN1::Kan1 and pTN1::Kan5 were used for mutant strain construction, as described in Materials and methods, and contain a Km^r cassette with the orientation of the cassette indicated by the direction of the arrow representing the Km^r cassette. Restriction endonuclease sites are: B, *Bgl*II; H, *Hpa*I; K, *Kpn*I; N, *Nco*I; S, *Sal*I; X, *Xho*I.

contained a common 1.76 kb *Sal*I restriction fragment, which also hybridized in the genomic Southern blots. After further restriction mapping, two fragments were subcloned from the lambda EMBL3 clones into a plasmid cloning vector. These were the 1.76 kb *Sal*I fragment, in plasmid pAL1, which was completely sequenced on both strands,

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                                        "RBS"
GTCGACAACAACACTTTTCTTGAGCCGTGATTTGGATTAGCTGATGAACAAAGCACTCGTG   61
                                                M  N  K  A  L  V  -15

ACTCTGCTGTTAACCCTGGGGATCACCGGCCTTGCCCACGCCGCGGGAGATGCCGCCGCCG  122
 T  L  L  L  T  L  G  I  T  G  L  A  H  A  A  G  D  A  A  A     6
                                          ▲
GCCAAGGCAAGGCGGCCGTCTGCGGCGCCTGCCACGGCCCGGACGGCAACAGCGCGGCCCC  183
G  Q  G  K  A  A  V  C  G  A  C  H  G  P  D  G  N  S  A  A  P  27

CAACTTCCCGAAACTGGCCGGCCAGGGCGAGCGCTATCTGCTCAAGCAGATGCAGGACATC  244
  N  F  P  K  L  A  G  Q  G  E  R  Y  L  K  Q  M  Q  D  I     47

AAGGCCGGCACCAAGCCGGGCGCTCCCGAAGGCAGCGGTCGCAAGGTGCTGGAAATGACCG  305
 K  A  G  T  K  P  G  A  P  E  G  S  G  R  K  V  L  E  M  T    67

GCATGCTCGACAACTTCAGCGACCAGGACCTGGCCGACCTCGCCGCCTACTTCACCTCGCA  366
G  M  L  D  N  F  S  D  Q  D  L  A  D  L  A  A  Y  F  T  S  Q  88

GAAGCCGACCGTCGGCGCAGCCGATCCGCAATTGGTGGAAGCCGGCGAGACGCTCTACCGC  427
  K  P  T  V  G  A  A  D  P  Q  L  V  E  A  G  E  T  L  Y  R  108

GGCGGCAAGCTGGCCGACGGCATGCCGGCCTGTACCGGTTGCCATTCGCCGAACGGCGAGG  488
 G  G  K  L  A  D  G  M  P  A  C  T  G  C  H  S  P  N  G  E   128

GCAACACCCCGGCCGCCTATCCGCGCCTGAGCGGCCAGCATGCCCAGTACGTTGCCAAGCA  549
G  N  T  P  A  A  Y  P  R  L  S  G  Q  H  A  Q  Y  V  A  K  Q  149

GCTCACCGACTTCCGCGAAGGCGCGCGCACCAACGATGGCGACAACATGATCATGCGCTCC  610
  L  T  D  F  R  E  G  A  R  T  N  D  G  D  N  M  I  M  R  S  169

ATCGCCGCCAAGTTGAGCAACAAGGACATCGCTGCGATCTCCAGCTACATCCAGGGCCTGC  671
 I  A  A  K  L  S  N  K  D  I  A  A  I  S  S  Y  I  Q  G  L   189

ACTGAGTCCGGCTCGTCGGCGTTGCGAAAAGGGGGGGGGATAGCCGCCCTTTTTCATTGGC  732
H  -                    ————————>       <————————              190
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Fig. 3. Nucleotide sequence and deduced amino acid sequence of the *A. vinelandii cycA* structural gene. A possible ribosome-binding site ('RBS') is underlined. A predicted stem loop structure located downstream of the stop codon is indicated by converging arrows. The numbering system for the protein has been kept the same as per the previously published sequence with the initial methionine at position −20 and position number 1 at alanine. Amino acid residues in italics represent the signaling peptide sequence with the site of cleavage denoted by ▲. The residues underlined represent either the heme *c* binding motif of C-X-Y-C-H or a methionine predicted to be a sixth ligand. The two sets of residues in bold were used to make the degenerate primers used for PCR (see Materials and methods). This sequence data appears in the EMBL/GenBank/DDBJ Nucleotide Sequence Data Libraries under the accession number L37290.

and an approximately 4.0 kb *Bgl*II fragment in plasmid pTN1 (Fig. 2).

The relevant portion of the nucleotide sequence as well as the deduced amino acid sequence is shown in Fig. 3. The structural gene (now referred to as *cycA*) was readily identified as an open reading frame of 210 amino acids by comparison to the previously published amino acid sequence. The deduced amino acid sequence has one distinct difference, an additional 20 amino acid residues at the N-terminal end. These additional residues form a typical signal sequence found in proteins which are transported through the inner, cytoplasmic membrane to the periplasmic side [34]. A positively charged residue (lysine) is followed by a central hydrophobic region and finally a polar cleavage region. Even with the addition of 20 residues, the numbering system for the protein has been kept the same as per the previously published protein sequence, with the initial methionine at position −20 and position number 1 at alanine (Fig. 3). The precursor protein has a calculated molecular mass of 21 687 Da and the mature protein a calculated molecular mass of 19 655 Da. Beyond the signal sequence, the deduced amino acid sequence is in exact agreement with the previously described sequence including two heme *c* binding motifs of C-X-Y-C-H and two possible sixth heme ligand methionines at positions 66 and 167 [35]. The nucleotide sequence also contains a possible ribosome-binding site preceding the initiation codon and a 10 bp inverted repeat following the termination codon (Fig. 3). This inverted repeat may form a stem-loop structure in the mRNA containing a stem of 10 bp with a calculated free energy value of −19.2 kcal and may act as a rho-independent terminator.

Further sequence analysis of areas immediately upstream and downstream of the *cycA* locus did not reveal any other possible open reading frames, including ones encoding other cytochromes *c*. Comparisons of the amino acid sequence of *A. vinelandii* cytochrome c_4 with other bacterial cytochromes *c*, including c_4 and *c*-551, have been previously reported [35–37]. Recently, a nucleotide sequence for a cytochrome c_4 has been published from *Pseudomonas stutzeri* [38]. There is 79% identity between the mature protein sequences of *A. vinelandii* cytochrome c_4 and *P. stutzeri* cytochrome c_4, and 85% identity between their signaling peptide sequences.

3.2. Construction of a *cycA* Km^r insertional mutant in *A. vinelandii*

Plasmid pTN1 contains the complete structural gene of cytochrome c_4 as well as enough flanking DNA to allow for recombination events on both sides of the gene. The *cycA* gene has an unique *Hpa*I site within the sequence. A kanamycin-resistance cassette (Km^r) was inserted into the *Hpa*I site in both orientations (i.e. in the same direction as the *cycA* gene and in the opposite direction) in plasmid pTN1. These plasmids are designated as pTN1::Kan1 and pTN1::Kan5 (Fig. 2). Linearized gel-purified restriction fragments from these two plasmids containing the insertion were transformed into competent *A. vinelandii* wild-type strain CA. After an incubation period to allow for homolo-

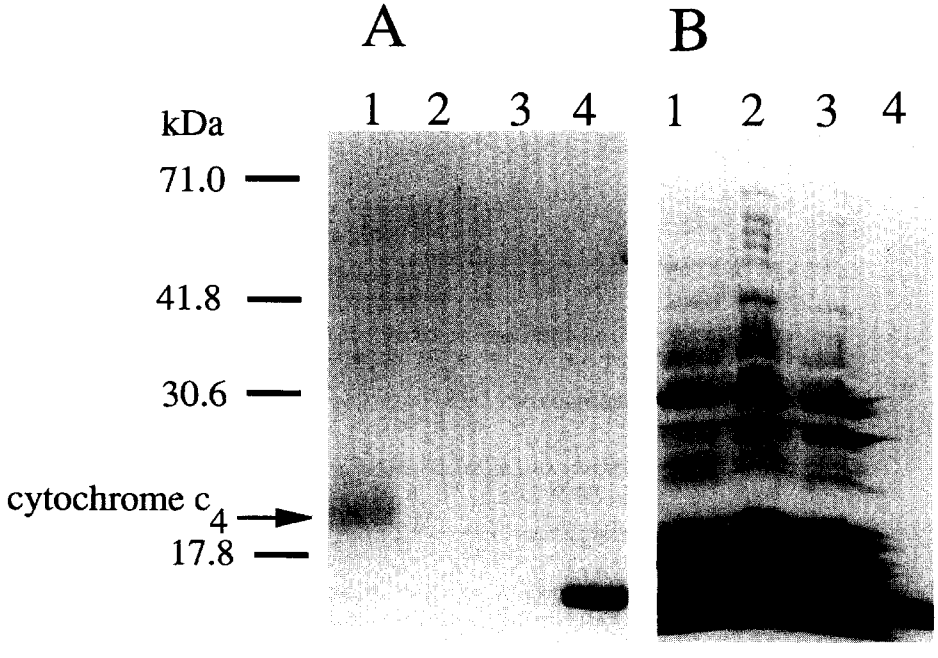

Fig. 4. A. SDS-PAGE of *n*-butanol extracted, partially purified membranes from strains of *A. vinelandii* on 12.5% polyacrylamide gels. Approximately 85 μg of membrane proteins were loaded in each lane after being heated with SDS sample buffer (w/o β-mercaptoethanol) at 90° C for 5 min. 5 μg of horse heart cytochrome *c* was used as a control. The gel was stained for heme with 3,3'-dimethoxybenzidine. Lanes: 1, CA; 2, CA*cycA*::Kan1; 3, CA*cycA*::Kan5; 4, horse heart cytochrome *c*. B. An identically loaded gel as in panel A stained for total protein using Coomassie brilliant blue.

gous recombination, transformants were selected for kanamycin resistance, and subcultured several times on kanamycin- and ammonium-supplemented agar plates to allow for selection of pure colonies. This yielded the mutant strains CA *cycA*::Kan1 and CA *cycA*::Kan5 with the Km^r cassette inserted in the opposite orientation and same orientation as the *cycA* gene, respectively. The genomic DNA from these mutants as well as from wild-type cells were probed with both the Km^r cassette and a fragment of the *cycA* structural gene in Southern blots, demonstrating that indeed the Km^r cassette was present in the mutants and not in the wild type and the *cycA* gene was disrupted in the mutants and not in the wild type (data not shown).

3.3. Heme staining of the cycA mutants

To investigate whether the Km^r insertional mutagenesis had resulted in a null mutation of the *cycA* gene, SDS-polyacrylamide gel electrophoresis and heme staining were performed. Membranes, isolated from both the wild-type and mutant strains, were treated with n-butanol as described in Materials and methods; this results in a partial purification of *c*-type cytochromes [26]. As seen in Fig. 4A (heme stain) only the wild-type lane contained cytochrome c_4 while both of the mutants CA *cycA*::Kan1 and CA *cycA*::Kan5 had no traces of the protein. Coomassie brilliant blue staining of an identically loaded gel confirms that approximately the same amount of protein was loaded in each lane (Fig. 4B). Additionally, the apparent molecular weight of the heme stained product corresponds very well with the calculated molecular mass of the mature protein (19 655 Da) and with those reported previously on purified *A. vinelandii* cytochrome c_4 [26].

3.4. Growth and nitrogen fixing ability of the cycA mutants

Growth of the *cycA* mutants was not affected whether the cells were starved of oxygen or of fixed nitrogen. In fact, wild-type and *cycA* mutants had similar doubling times in either Burk's sucrose medium supplemented with ammonium or in ammonium-free medium. During diazotrophic growth conditions, the wild type (CA) doubled every 4.2 h while CA *cycA*::Kan1 doubled every 4.3 h and CA *cycA*::Kan5 doubled every 3.8 h. Similar results were found during non-diazotrophic growth conditions with CA doubling every 5.0 h, CA *cycA*::Kan1 doubling every 5.4 h, and CA *cycA*::Kan5 doubling every 5.4 h. To further test the similarities in growth of the *cycA* mutants with the wild type, whole cell respiration assays were performed using standard Burk's medium with sucrose as the sole carbon source. When grown in ammonium-free medium, the oxygen consumption rates (in nmol O_2 min^{-1} 10^8 $cells^{-1}$ ± standard deviation) were: CA, 212 ± 21; CA-*cycA*::Kan1, 219 ± 14; and CA *cycA*::Kan5, 220 ± 14. In ammonium-supplemented medium, respiratory rates for all the strains was less than in ammonium-free medium; strain CA had a rate of 157 ± 9, CA *cycA*::Kan1 and CA *cycA*::Kan5 had rates of 146 ± 8 and 146 ± 18, respectively. As a control for the effect of the lack of the *b / d* oxidase pathway a deletion strain CA *Δcyd*::*Ω*3 (described in Materials and methods) was also used. This mutant had the same phenotype as a previously described cytochrome *d* mutant [8]. It had an oxygen consumption rate of 24 ± 2.

3.5. Spectral analysis of mutants

Room-temperature dithionite-reduced-minus-persulfate-oxidized difference spectra of membranes from wild-type CA cells demonstrated total cytochrome *d* (peak at

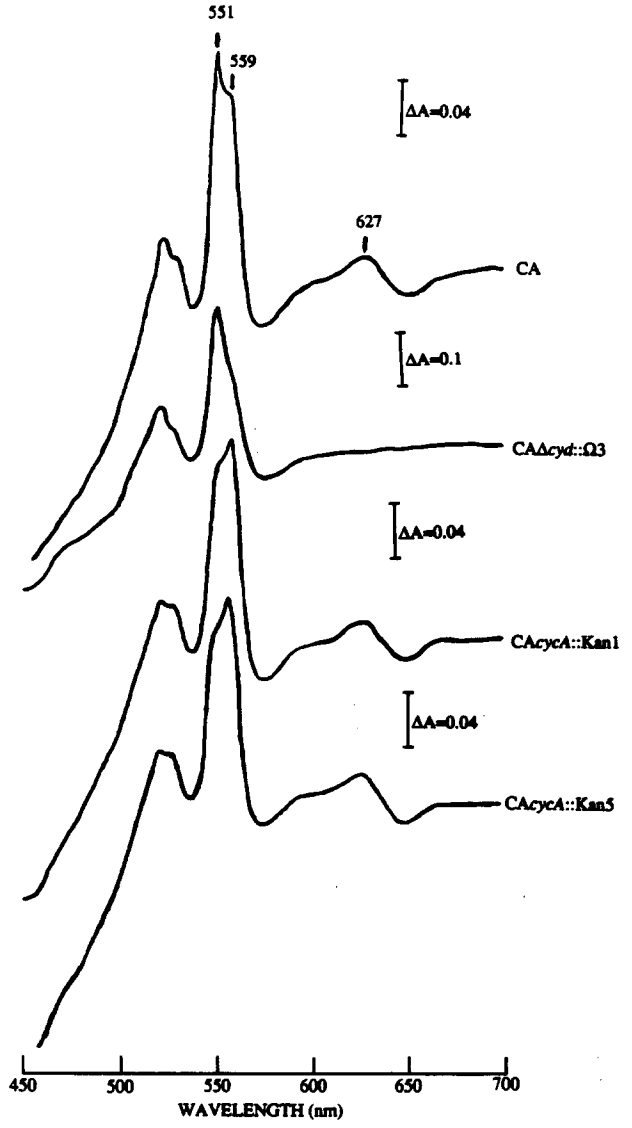

Fig. 5. Room-temperature dithionite-reduced-minus-persulfate-oxidized difference spectra of membranes isolated from strains of *A. vinelandii*. The membrane protein concentrations were as follows: CA, 10.44 mg ml^{-1}; CA *Δcyd*::*Ω*3, 13.00 mg ml^{-1}; CA *cycA*::Kan1, 13.05 mg ml^{-1}; CA *cycA*::Kan5, 11.22 mg ml^{-1}. The position of the absorption maximum of different peaks are indicated in nm. The absorption scales are shown as optical density units (*ΔA*).

627 nm), *b* (shoulder at 559 nm), and *c* (peak at 551 nm) as described previously [25] (Fig. 5). As expected, the cytochrome *b / d* deletion lacks a peak at 627 nm and the shoulder at 559 nm has been reduced, showing the loss of heme *d* and reduced amount of heme *b*. The *cycA* mutants CA*cycA*::Kan1 and CA*cycA*::Kan5 had identical spectra, with the peak at 551 nm reduced to a shoulder in comparison to the wild type, and a peak at 559 nm, where the wild type exhibits only a shoulder (Fig. 5). These spectra clearly demonstrate a significant reduction in the total amount of *c*-type cytochromes in the *cycA* mutants.

Cytochrome concentrations were calculated based on the reduced-minus-oxidized difference spectra as shown in Table 2. Cytochrome content was found to be very similar between the wild-type and the *cycA* mutants except for the amounts of cytochromes *c*. There is an approximate 60% loss of cytochromes *c* in the *cycA* mutant strains. Once again, as expected there was no detectable amounts of cytochrome *d* in the cytochrome *b / d* deletion mutant.

Room-temperature (CO + reduced)-minus-reduced difference spectra, using either dithionite or the physiological substrate NADH as reductant, was also performed (Fig. 6). Dithionite and NADH each reduced cytochromes *o* and *d* as seen by troughs at 558 nm and 624 nm, respectively in wild-type membranes, as seen previously [25]. The cy-

Table 2
Cytochrome concentration in membranes isolated from different strains of *A. vinelandii*

Strain [a]	Concentration (nmol mg protein^{-1}) [b] of cytochrome			
	Total *b*	Total *c*	*d*	*o*
CA	0.860	1.04	0.181	0.291
CA *Δcyd*::Ω3	0.782	1.29	ND [c]	0.248
CA *cycA*::Kan1	0.719	0.601	0.138	0.246
CA *cycA*::Kan5	0.807	0.626	0.141	0.281

[a] Cultures of *A. vinelandii* were grown in RMN with added antibiotic where appropriate and membranes were isolated as described in Materials and methods.
[b] Cytochrome concentrations were determined by reduced-minus-oxidized difference spectroscopy using extinction coefficients as described in Materials and methods.
[c] ND, not detected.

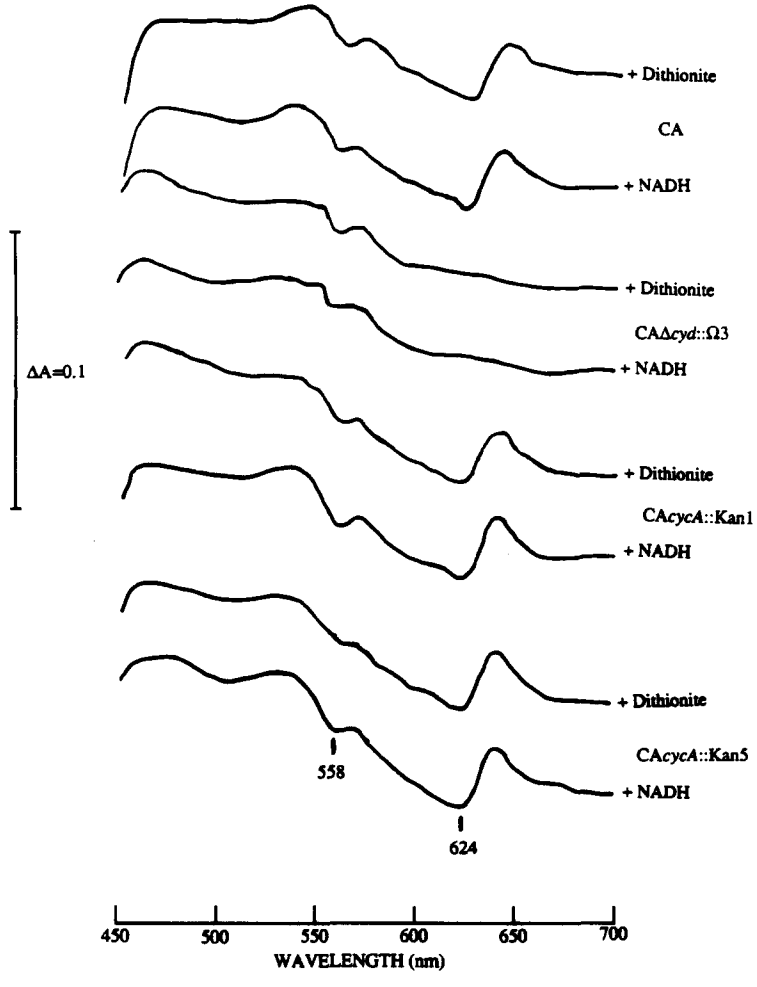

Fig. 6. Room-temperature CO difference spectra of dithionite- and NADH-reduced membranes isolated from strains of *A. vinelandii*. The membrane protein concentrations are the same as in Fig. 4. The position of the absorption minimum of different troughs are indicated in nm, and the absorption scale is shown as optical density units (ΔA).

Table 3
Respiration rates from membranes isolated from different strains of *A. vinelandii*

Substrate [a]	Respiration rates (nmol min^{-1} mg $protein^{-1}$) [b] of *A. vinelandii* strains [c] (% inhibition with chlorpromazine) [d]			
	CA	CA Δ*cyd*::Ω3	CA *cycA*::Kan1	CA *cycA*::Kan5
NADH	444 ± 36 (68)	123 ± 12	538 ± 17 (71)	540 ± 34 (73)
Malate	597 ± 62 (74)	159 ± 12	360 ± 18 (67)	360 ± 15 (69)
Succinate	199 ± 22 (71)	42 ± 5	201 ± 12 (60)	210 ± 8 (70)
Ascorbate-TMPD	1895 ± 149 (64)	1878 ± 143	301 ± 20 (60)	438 ± 17 (60)

[a] The substrates were 5 mM NADH, 10 mM malate, 20 mM succinate, or 4 mM ascorbate-2 mM TMPD.
[b] The respiratory rates shown were determined amperometrically using an oxygen electrode. Each value is a mean ± standard deviation of the values obtained from four separate determinations.
[c] Cultures of *A. vinelandii* were grown and membranes were isolated as described in Table 2.
[d] Membranes were preincubated with chlorpromazine for 5 min (w/o substrate) and then the assay was initiated with the addition of substrate. 100 μM chlorpromazine was used with all substrates except for ascorbate-TMPD when 500 μM was added.

tochrome *d* mutant lacked the trough at 624 nm but still had the trough at 558 nm using either substrate as a reductant, demonstrating the lack of the cytochrome *b / d* terminal oxidase (Fig. 6). Surprisingly, the two *cycA* mutants exhibited spectra identical to the wild type. Both cytochromes *o* and *d* were still reduced in the mutants, as evidenced by the troughs at 558 nm and 624 nm, when using either dithionite or NADH as a reductant (Fig. 6). Notably, the amount of cytochrome *o* reduced by NADH in the mutants was essentially indistinguishable from that in the wild type. Therefore, cytochrome *o* is still active as a terminal oxidase in the *cycA* mutants.

3.6. Oxygen uptake rates and inhibition by chlorpromazine

Respiratory rates were determined for membranes from CA, CA Δ*cyd*::Ω3, CA *cycA*::Kan1, and CA *cycA*::Kan5 using various physiological substrates and the artificial electron donor ascorbate-TMPD (Table 3). Oxygen uptake rates with the different physiological substrates were similar for the wild-type and both *cycA* mutants. However, the respiratory rate with ascorbate-TMPD was significantly lower in the *cycA* mutants as compared to the wild type. Chlorpromazine has been previously shown to inhibit the cytochrome $c \rightarrow o$ pathway of *A. vinelandii* membranes [39]. The percent inhibition by chlorpromazine of the *cycA* mutants was similar to that of the wild type (ranging from 60% to 74%), suggesting that cytochrome *o* is still functioning as a terminal oxidase in the cytochrome c_4 mutants. As seen previously, the cytochrome *d* deletion strain had much lower respiratory rates with the physiological substrates compared to the wild type; however, it had oxygen uptake rates with ascorbate-TMPD like the wild type, indicating that the cytochrome $c \rightarrow o$ pathway was functioning normally.

4. Discussion

Many components in the electron transport chain of *A. vinelandii* have been identified and studied previously, leading to a proposed complex branched scheme of electron transport [4,5]. However, the genes encoding most of these components have not been cloned, thus well defined structural gene mutants have not been constructed to unambiguously determine the function of these components. The exception is the cytochrome *b / d* terminal oxidase complex, which has been well characterized [6–8]. Mutants in the cytochrome $c \rightarrow o$ pathway have been previously reported [40–42]. However, these mutants were made with the powerful mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and thus the precise location of the mutation could not be localized. Furthermore, the reported mutants contained multiple defects. The report here describes the results of cloning the *cycA* gene and creation of a well defined structural gene mutant in cytochrome c_4.

Not surprisingly, the deduced amino acid sequence based on the DNA sequence matched exactly with the previously published sequence based on amino acid sequencing of the purified protein [32]. As expected, a 20 amino acid residue signaling peptide sequence was discovered, since it had been previously shown that cytochrome c_4 is membrane bound but faces the periplasmic side of the membrane [13,26]; thus this provides the mechanism by which to transport the protein through the hydrophobic inner, cytoplasmic membrane. The residues of the signal sequence meet all the requirements of a typical signaling peptide sequence [34]. Based on the determined nucleotide sequence, it appears that the cytochrome c_4 gene is contained in a single operon without any other co-transcribed genes; no other apparent open reading frames were identified nearby, and a termination stem-loop structure followed the stop codon.

Spectrally, it appears that the amount of cytochromes *c* in the *cycA* mutants is reduced approximately 60% as compared to the wild type. Heme staining confirmed the loss of cytochrome c_4 in the mutant strains. Interestingly, the mutants were still able to reduce cytochrome *o* with NADH as shown by CO difference spectra using that physiological substrate as the reductant. Therefore, it is concluded that cytochrome c_4 is probably not situated in complex with cytochrome *o* as a multi-component termi-

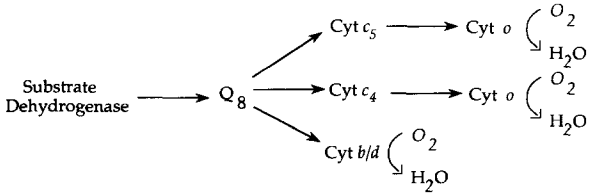

Fig. 7. Revised diagram of the electron transport chain of *A. vinelandii* incorporating the proposed additional branch at Q_8. Q_8, ubiquinone Q-8; Cyt, cytochrome.

nal oxidase complex. However, it is still independently involved in transferring electrons to cytochrome *o*. Mutants lacking cytochrome c_4 have normal respiratory ability with physiological substrates but have greatly reduced levels of ascorbate-TMPD oxidation rates. The oxidation of the artificial electron donor ascorbate-TMPD has been shown to proceed through *c*-type cytochromes to the cytochrome *o* terminal oxidase [43,44]. The percent inhibition of oxygen uptake by chlorpromazine was similar in the *cycA* mutants as the wild type, demonstrating that cytochrome *o* is active in the mutants. It was shown previously that chlorpromazine inhibits only the cytochrome $c \rightarrow o$ pathway [39].

Based on the evidence that cytochrome *o* is still turning over in a cytochrome c_4 deleted mutant, a new path of electron flow is proposed whereby electrons flow in parallel from ubiquinone to cytochrome c_4 to cytochrome *o* and separately from ubiquinone to cytochrome c_5 to cytochrome *o* (Fig. 7) but not in an obligate sequence of ubiquinone to cytochromes c_4c_5 to cytochrome *o* as previously thought [2–5]. A similar parallel scheme has been previously proposed [45], however their conclusion was based on the assumption that exogenous *A. vinelandii* cytochromes c_4 and c_5 donate electrons to endogenous c_4 and c_5, respectively. The current work gives further evidence for this proposed new branch with genetic and in vivo physiological data.

The proposed new separate but parallel *c*-type electron flow pathway may explain the lack of growth differences between the *cycA* mutants and the wild type. It has been proposed that there are three sites of proton translocation in the electron transport chain of *A. vinelandii*, at the NADH dehydrogenase site, at the ubiquinone site, and at the cytochrome *o* terminal oxidase site [4,5]. However, there is evidence that of the terminal pathways, only the cytochrome c_5 to *o* pathway is coupled to ATP synthesis [45]. Therefore the cytochrome c_4 to *o* pathway may be another fast-respiring but non-phosphorylating pathway, consuming excess oxygen to protect nitrogenase, like the cytochrome *b/d* branch [8]. Additionally, cytochromes *c* mutants may be lacking only one of the three proposed proton translocation sites while the other two function normally; then we could expect a cytochromes *c* mutant to have growth similarities to the wild type.

Recently, an *A. vinelandii* mutant has been constructed in the terminal oxidase cytochrome *o* [46]. This mutant was identical to the *cycA* mutants in its ability to fix nitrogen in air and its lack of any apparent growth differences from the wild type. However, this work was based on mutagenesis of a PCR fragment amplified from *A. vinelandii* genomic DNA. Presumably, work is underway to clone the genomic region corresponding to cytochrome *o* and the performance of physiological analyses on subsequent mutant strains.

Thus far a portion of the sequential cytochrome $c \rightarrow o$ pathway in *A. vinelandii* has been elucidated, but much work needs to be done to completely determine the nature and function of this pathway. It would be of great interest to determine the role of cytochrome c_5 by similar methods. Further investigations are needed to study the role of respiration and its relationship with nitrogen fixation.

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