Noncoupled NADH:Ubiquinone Oxidoreductase of *Azotobacter vinelandii* Is Required for Diazotrophic Growth at High Oxygen Concentrations

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The gene encoding the noncoupled NADH:ubiquinone oxidoreductase (NDH II) from *Azotobacter vinelandii* was cloned, sequenced, and used to construct an NDH II-deficient mutant strain. Compared to the wild type, this strain showed a marked decrease in respiratory activity. It was unable to grow diazotrophically at high aeration, while it was fully capable of growth at low aeration or in the presence of NH₄⁺. This result suggests that the role of NDH II is as a vital component of the respiratory protection mechanism of the nitrogenase complex in *A. vinelandii*. It was also found that the oxidation of NADPH in the *A. vinelandii* respiratory chain is catalyzed solely by NDH II.

Molecular nitrogen reduction is mediated by the nitrogenase complex, an enzyme known to be highly vulnerable to oxygen damage. Thus, the majority of bacteria are capable of reducing N₂ only in anaerobic or microaerobic conditions. Contrary to this, *Azotobacter vinelandii* is an obligate aerobe capable of fixing molecular nitrogen even at very high ambient O₂ concentrations. The purified nitrogenase complex of this bacterium was shown to be as susceptible to oxygen damage as those in other microorganisms (26). Dalton and Postgate hypothesized that the concentration of O₂ in the cytoplasm of *Azotobacter* is effectively reduced below nitrogenase-damaging levels by the extremely active respiration characteristic of this bacterium (5, 6). The existence of such a mechanism, termed respiratory protection, was later substantiated in a large number of studies. In particular, it was found that the *A. vinelandii* bd-type quinol oxidase is essential for respiratory protection (15, 21). It was also shown that respiration mediated by the bd-type oxidase is dominant in cells growing diazotrophically or at high oxygen concentrations (18, 24). Mutant strains with disruptions in the gene encoding the bd-type oxidase failed to fix N₂ at high ambient oxygen concentrations (15).

It would be logical to assume that in order to match the above-mentioned highly rapid oxygen consumption at the bd terminus of the *A. vinelandii* respiratory chain, there must exist a mechanism at the initial segment that feeds electrons into the chain just as effectively. Moreover, this putative enzyme should utilize a major respiratory substrate as well as have no coupling of the respiratory chain just as effectively. Moreover, this putative enzyme should utilize a major respiratory substrate as well as have no coupling of the respiratory chain just as effectively. Moreover, this putative enzyme should utilize a major respiratory substrate as well as have no coupling of the respiratory chain just as effectively. Moreover, this putative enzyme should utilize a major respiratory substrate as well as have no coupling of the respiratory chain just as effectively. Moreover, this putative enzyme should utilize a major respiratory substrate as well as have no coupling of the respiratory chain just as effectively.

In our previous study (3), it was shown that oxidation in *A. vinelandii* is mediated by two distinct NADH: ubiquinone oxidoreductases; one is coupled to the generation of the transmembrane proton potential (NDH I), and the other is not (NDH II). We also showed that the expression of NDH II, in contrast to that of NDH I, is induced by high O₂ concentration and by switching of the bacterial culture to diazotrophic growth. Induction of NDH II activity was observed under the same conditions in which induction of the bd-type oxidase was shown to take place (3). It is known that the latter effect is mediated by a regulatory *A. vinelandii* protein, CydR (homologous to FNR in *Escherichia coli*) (24, 25). It was shown that the *A. vinelandii* strains lacking this protein overproduce the bd-type oxidase even during growth at lowered O₂ concentrations. Later it was found that the cydR mutation also leads to induction of NDH II and repression of NDH I (3). Thus, at all growth conditions *NADH* is oxidized in the respiratory chain of this mutant almost entirely via NDH II.

The properties of the noncoupled NADH dehydrogenase (NDH II) and the conditions of its induction in *A. vinelandii* suggest the possible role of this enzyme in respiratory protection. To prove this assumption, in the current work we constructed an NDH II-deficient strain of *A. vinelandii* and tested its nitrogen-fixing capabilities at high ambient oxygen concentrations.

**MATERIALS AND METHODS**

Bacterial strains, plasmids, growth, and medium compositions. The *A. vinelandii* and *E. coli* strains used in this study are listed in Table 1. *A. vinelandii* cells were grown in modified Burke's media BS and BSN (BS with ammonia salts added) (7). The cells were grown in a rotary shaker at 250 rpm and 30°C. Antibiotics (where used) were added to the following final concentrations: rifampicin, 10 μg/ml; kanamycin, 1 μg/ml; ampicillin, 50 μg/ml; tetracycline, 10 μg/ml. *E. coli* cells were routinely grown in Luria-Bertani (LB) medium.

**Kₐₑ** The oxygen transfer coefficient (*K_aₑ*) was determined by the sulfite method as described previously (10) with some modifications: 100 g of KI/liter and 63.5 g of I₂/liter were used to prepare a 0.25 M iodine solution, and Na₉S₂O₃ was used instead of Na₂S₂O₃ for titration. To establish high aeration (measured *Kₐₑ* = 14 mmol of O₂ liter⁻¹ h⁻¹), 100 ml of growth medium was shaken in 1-liter flasks. Decreased aeration (*Kₐₑ* = 1.4 mmol of O₂ liter⁻¹ h⁻¹) was established by shaking 200 ml of medium in 350-ml flasks.

SBPs. Subcellular particles (SBPs) from *A. vinelandii* cells were prepared as described previously (2).

(i) **Respiration of *A. vinelandii* cells and SBPs.** Respiration of *A. vinelandii* cells and SBPs was monitored using a standard Clark-type electrode at 30°C. The following buffer was used as the respiration medium for SBPs: 60 mM KCl–2 mM...
MgSO₄·7H₂O, 20 mM HEPEs-KOH (pH 7.5). Respiration of whole *A. vinelandii* cells was measured in BSN medium. Logarithmic cells containing from 30 to 60 µg of overall protein were sampled from the growth medium and directly injected into a polarograph chamber.

(ii) Oxidation rates of NADH, NADPH, and reduced nicotinamide hypoxanthine dinucleotide (dNADH) by *A. vinelandii* SBPs. Oxidation rates were monitored by means of a Hitachi-557 spectrophotometer as described previously (3). All reduced pyridine dinucleotides were used at a final concentration of 150 µM.

Estimation of the Km values of A. vinelandii NDH I and NDH II for reduced pyridine nucleotide oxidation. Estimation of the Km values for NDH I and NDH II were carried out using SBPs from *A. vinelandii* strains DN165 and MK8, respectively. NADH and dNADH oxidation was monitored by registering the decrease in optical density at 340 nm (OD₃₄₀) (for both NADH and dNADH, mₚ= 6.22 mM⁻¹ cm⁻¹ was used). The rates of NADH and dNADH oxidation at different concentrations of these reduced pyridine dinucleotides were determined by analyzing the first derivative of the (d)NADH oxidation progress curves. Briefly, the (d)NADH concentration can be calculated from a direct OD₃₄₀-versus-time curve, while the values of the first derivative of the direct curve are proportional to the rates of the (d)NADH oxidation. The data obtained were fitted to the Michaelis-Menten equation using the nonlinear regression analysis method.

The Km value for NADPH was determined by recording initial rates of oxygen consumption by NADPH-oxidizing SBPs at different NADPH concentrations.

Construction of an NDH II-deficient *A. vinelandii* strain by site-directed mutagenesis. Amplification of the *A. vinelandii* ndh fragment was carried out using the PCR with degenerates primers ndhB1a [5'-CACCTG(C/G)TCCGAAAGCC(T/G)CAT and ndhB2a [5'-CCCGT(G/C)GGGGCC(A/C)GA(G/C)CCGC(AC) (17). The amplified ndh fragment was cloned into pGEM-T vector, resulting in plasmid pND7.

Construction of the ndh::Tc strain of *A. vinelandii* was carried out as follows. A tetracycline resistance cassette from pHP45Tc was ligated into the Smal site of pND7, and a fl-fragment-containing plasmid (pND7Tc) bearing the ndh gene together with the unidirectionally transcribing tetracycline resistance cassette was selected. Competent *A. vinelandii* cells were transformed by the pND7Tc plasmid. This transformation resulted in a series of tetracycline-resistant clones. Screening of the clones with the unidirectionally transcribing tetracycline resistance cassette was performed by the insertion of the tetracycline resistance cassette into the 3' end of the *ndh* gene.

The 5' part of ndh was cloned by an inverse PCR technique (19). Two micrograms of *A. vinelandii* UW136 DNA was ligated with the PacI restriction. The rest of the restriction mixture was treated with T4 DNA ligase in a 300-µl volume. An individual 1 µl of the ligase mixture was used as the PCR template for the rev_ph (5'-GGTGGAGTCCGACCCGAC) and rev_mdh (5'-CACTACTTGGTGGCCCA) primers. A unique 2.8-kb PCR product was obtained and later used for sequencing of the 5' part of ndh.

Nucleotide sequence analysis was done by direct sequencing with both strands sequenced using synthesized oligonucleotide primers. This analysis was carried out by the sequencing group of the Engelhard Institute of Molecular Biology using an Applied Biosystems ABI 373A DNA sequencer.

**RESULTS**

Construction of an NDH II-deficient *A. vinelandii* strain. (i) Cloning of a fragment of the ndh gene from *A. vinelandii*. An ndh gene fragment from the *A. vinelandii* DNA was amplified by means of a PCR technique using degenerative primers (17). PCR with these primers and the *A. vinelandii* DNA as a template resulted in amplification of a variety of fragments of different lengths. The PCR products were separated using gel electrophoresis. In accordance with expected size of the ndh gene, the amplified product was searched for in the 400-bp band. This band was isolated, cloned into a pGEM-T vector (resulting in plasmid pND7), and sequenced. A BLASTX analysis of the acquired fragment (with the exclusion of primer sequences) showed that the putative peptide corresponding to this nucleotide fragment is homologous to NDH II from a wide variety of bacteria. The highest homologies were found with the sequence of NDH II from *Pseudomonas aeruginosa* (79%) and *Pseudomonas fluorescens* (77%). Thus, the ndh fragment of *A. vinelandii* DNA was cloned.

(ii) Construction of an ndh::TcT strain of *A. vinelandii*. The sequence data for the cloned ndh fragment revealed the presence of a unique Smal site located 263 bp away from the 5' end of the PCR product. Subsequently, a tetracycline resistance cassette from pHP45Tc was ligated into this Smal site of plasmid pND7, resulting in plasmid pND7Tc. In order to construct a strain of *A. vinelandii* with the insertion-inactivated

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**TABLE 1. Bacterial strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strains and plasmids</th>
<th>Characteristic(s)</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td>A. vinelandii UW136</td>
<td>Rif R</td>
<td>15</td>
</tr>
<tr>
<td>A. vinelandii MK8</td>
<td>UW136 cyd::Tra5 Rif Km'</td>
<td>15</td>
</tr>
<tr>
<td>A. vinelandii DN165</td>
<td>UW136 ndh::TcT Rif Te'</td>
<td>This work</td>
</tr>
<tr>
<td>A. vinelandii N24</td>
<td>UW136 ndh::PNDT6 Rif Te' AmpR</td>
<td>This work</td>
</tr>
<tr>
<td>E. coli TG1</td>
<td>F' traD36 lacPZAM15 proA BjugE (ΔlsdM-mcrB5) (rK mK msrB) thi (Δlac-proAB)</td>
<td>Promega</td>
</tr>
<tr>
<td>pGEM-T</td>
<td>PCR product cloning vector</td>
<td>Amp'</td>
</tr>
<tr>
<td>pHP45Tc</td>
<td>Tetracycline resistance</td>
<td>Amp'</td>
</tr>
<tr>
<td>pND7</td>
<td>pGEM-T with 400-bp fragment of A. vinelandii ndh; Amp'</td>
<td>This work</td>
</tr>
<tr>
<td>pND7Tc</td>
<td>pND7 with TcT inserted in Smal site of ndh fragment; Amp' Te'</td>
<td>This work</td>
</tr>
</tbody>
</table>

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Thus, the ndh fragment of *A. vinelandii* DNA was cloned.
fragment replacing the wild-type sequence, competent \textit{A. vinelandii} cells were transformed using plasmid pNDT6 (this plasmid is unstable in \textit{A. vinelandii} because of its ColE1 replicon). An Amp$^\beta$ Tc$^c$ clone (DN165) characteristic of a double-cross-over introduced mutation was selected. Localization of this mutation in the \textit{ndh} gene of the acquired strain was verified by PCR analysis.

In order to clone the entire \textit{A. vinelandii} \textit{ndh} gene, the plasmid rescue technique was applied. This method is based on obtaining a strain with studied gene disruption by a single-cross-over plasmid insert. Briefly, DNA of such a mutant is cut by a variety of different endonucleases, and the fragments are ligated and used to transform \textit{E. coli} cells. Such a procedure may lead to obtaining a clone bearing the plasmid with the insert of the DNA which flanked the crossover site in the chromosome (8). This procedure may be successful provided there are two restriction sites in the flanking DNA regions that are near enough to the start and stop codons of the studied gene while there are no such sites inside the gene. In our case, the N24 single recombinant \textit{A. vinelandii} strain was used. A desired clone was obtained only by using the \textit{AatII} restriction enzyme (see Materials and Methods for details). As this restriction enzyme is located in the pNDT6 polylinker, we succeeded in cloning and sequencing only the DNA fragment flanking the pNDT6 plasmid insert from 3’ side of the \textit{ndh} gene.

In order to clone the 5’ side of \textit{ndh}, the inverse PCR technique was used. This involved cutting the wild-type DNA with an appropriate endonuclease, ligating the fragments, amplifying the fragment carrying the part of the known sequence of \textit{ndh} with inverse primers, and, finally, isolating and sequencing the amplified DNA. Again, this technique can be successful provided that one restriction site is in the known region of \textit{ndh} while the second site is in the 5’-flanking DNA close enough so that the resulting fragment can be ligated and amplified. Based upon the determined sequence of \textit{ndh}, a set of restriction enzymes was chosen. The choice of the \textit{PstI} restriction enzyme proved to be successful and led to cloning and sequencing of the 5’ part of \textit{ndh} (see Materials and Methods for details).

Thus, having applied the inverse PCR and the plasmid rescue techniques, we succeeded in sequencing the whole \textit{A. vinelandii ndh} gene. The resulting data were deposited into GenBank (accession number AF346487).

Properties of the \textit{NDH II}-deficient \textit{A. vinelandii} strain. (i) Respiration rates of whole \textit{A. vinelandii} cells and subbacterial particles. As experiments showed, the DN165 mutant cells have a much lower respiratory activity than the wild type. Generally, the wild-type cells grown in highly aerated BSN medium consumed 2.1 ± 0.6 μg-atom of oxygen per min per mg of protein, whereas the figure for DN165 was as low as 0.4 ± 0.1 (average from three independent experiments).

NDH- and dNDH-oxidizing activities of the \textit{A. vinelandii} DN165 SBPs were also tested (Table 2). It is well known that NDH I can oxidize either NADH or its analog dNADH, while NDH II utilizes only NADH and not dNADH (3). SBPs from wild-type \textit{A. vinelandii} oxidized NADH at much higher rates than dNADH due to operation of both NDH dehydrogenases (Table 2). The difference between NDH and dNDH oxidase rates is much more profound in the MK8 (ΔcydR) strain, which contains very low levels of NDH I (3). On the other hand, in the constructed NDH II-deficient mutant strain DN165, NDH and dNDH oxidation rates were equal and low (Table 2). This confirms that the DN165 mutant does not possess significant NDH II activity.

(ii) Participation of the \textit{A. vinelandii} NDH II in respiratory protection. Growth of three \textit{A. vinelandii} strains was tested at different aeration levels in media with (BSN) and without (BS) ammonium acetate. As seen from Fig. 1, at high aeration ($K_{L,a} = 14$ mmol of O$_2$ liter$^{-1}$ h$^{-1}$) the DN165 strain lacking NDH II is capable of growing in the presence of NH$_4^+$ at a rate comparable to that of the wild-type strain (doubling time, 150 and 140 min, respectively), but contrary to the wild type, it failed to grow at a high oxygen concentration if N$_2$ fixation was necessary. For comparison, Fig. 1 also includes growth curves for \textit{A. vinelandii} MK8, which was shown to most preferably oxidize NADH via NDH II (3). As seen from Fig. 1C, the absence of NDH I activity (in contrast to NDH II) does not lead to any increase in O$_2$ sensitivity of the N$_2$-fixing bacterial culture.

The data presented in Fig. 2 shows that growth rates of the wild type and the mutant strain DN165 at low aeration ($K_{L,a} = 1.4$ mmol of O$_2$ liter$^{-1}$ h$^{-1}$) were similar both in the presence and in the absence of NH$_4^+$. An assumption can be drawn that NDH II is vital for the protection of the nitrogenase complex against oxygen insult, i.e., during diazotrophic growth at high ambient oxygen concentration.

(iii) Role of NDH II in the NADPH oxidation activity of \textit{A. vinelandii}. Earlier studies (13) have shown that \textit{A. vinelandii} membranes are capable of oxidizing NADPH. Ackrell and coworkers (1) have postulated the presence of a specific enzyme mediating this reaction. In the present study, we tested the effect of \textit{ndh} mutation on the NADPH-oxidizing activity of \textit{A. vinelandii}. Our data show (Table 2) that SBPs from \textit{A. vinelandii} strain DN165 completely lack the ability to oxidize NADPH. This provides proof that NDH II is solely responsible for the NADPH dehydrogenase activity in the respiratory chain of \textit{A. vinelandii}. It is noteworthy that a similar conclusion has been recently drawn for NDH II of \textit{Corynebacterium glutamicum} (17).

(iv) Estimation of $K_m$ values of \textit{A. vinelandii} NDH I and NDH II for reduced pyridine nucleotide oxidation. Using \textit{A. vinelandii} strains that respire almost solely via NDH I (MK8 [3]) or entirely via NDH II (DN165), we measured the $K_m$ values of these enzymes for various respiratory substrates (Table 3). Tables 2 and 3 show that NDH I is capable of oxidizing both NADH and dNADH, but not NADPH, while NDH II oxidizes NADH and NADPH but cannot utilize dNADH. The affinity of NDH II for NADPH is very poor, which indicates

<table>
<thead>
<tr>
<th>Strain</th>
<th>Activitya</th>
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<tr>
<td></td>
<td>NADH</td>
</tr>
<tr>
<td>UW136 (wild type)</td>
<td>8.2</td>
</tr>
<tr>
<td>MK8 (ΔcydR)</td>
<td>10.4</td>
</tr>
<tr>
<td>DN165 (Δndh)</td>
<td>1.6</td>
</tr>
</tbody>
</table>

a The concentration of all dinucleotides was 150 μM. Activities are given in micromoles of reduced pyridine dinucleotides oxidized per minute per milligram of protein.
that it is not a major substrate under physiological conditions. When comparing \( K_m \) values for NADH, we expected to see higher values for NDH II than for NDH I, as in theory this would render NDH II efficient only when NDH I is either substrate saturated or limited by respiratory control. Test results showed that the \( K_m \) value of NDH II for NADH turned out to be twice that of NDH I (Table 3). It is possible, however, that the electron flow through the two different NADH dehydrogenases is downregulated at the level of quinone, i.e., via their different affinities for ubiquinone-8. Investigation of this speculation, however, is left for future research.

**DISCUSSION**

The current work clearly indicates that NDH II in *A. vinelandii* is vital for diazotrophic growth at high ambient oxygen concentrations. Thus, the result may suggest that the role of NDH II is as a key component of the oxygen protection mechanism in this bacterium. In earlier work (15, 21), a similar function has been assigned to the *bd*-type terminal oxidase from the same organism. Studies by our group (3) found NDH II induction by increased ambient oxygen concentration or by the absence of NH\(_4^+\) in the medium, i.e., under the same conditions that induce cytochrome *bd* (18, 24). Such coordinated expression of both of these enzymes is achieved through their joint induction by the CydR regulator protein (3, 24, 25).

Taking into account the results of previous work (2, 3, 15, 16, 21), the role of NDH II is as a key component of the oxygen protection mechanism in this bacterium.
The A. vinelandii respiratory chain may be described by the scheme shown in Fig. 3. It is assumed that A. vinelandii cells possess at least two respiratory chains differing in enzyme composition and physiological function. One of them is completely coupled (thin arrows); it includes NDH I (3), the bc₁-complex (2), cytochromes c₄ and c₅ (22), and the o-type oxidase (16). The other chain (thick arrows) is much simpler and includes the uncoupled NDH II (3) and the bd-type quinol oxidase (15). The energy-conserving efficiency of this chain \( H^+/e^- = 1 \) \([H^+/e^-] \) is the number of \( H^+ \) ions pumped across the membrane for every one electron passed down the respiratory chain \) is much lower than that of the coupled one \( (H^+/e^- = 5) \) \( (2, 3) \). This allows avoidance of limitations of oxygen consumption rates by systems utilizing transmembrane electrochemical proton potential \( (\Delta \mu H^+) \).

It is also worth noting that both NDH II and the bd oxidase possess unusually high specific activities (more so for NDH II), i.e., 560 \( \mu \)g-atom of oxygen min\(^{-1}\) mg of protein\(^{-1}\) \( (14) \) and 106 mmol of NADH min\(^{-1}\) mg of protein\(^{-1}\) \( (4) \) for the bd-type quinol oxidase and NDH II, respectively. Such high rates may be due to the absence of the proton-translocating function for these enzymes (the function of the bd-type oxidase does in fact generate \( \Delta \mu H^+ \) but solely due to scalar proton effects \( [2, 12] \)). Exceptionally high turnover of NDH II should be favorable for optimally efficient utilization of the membrane-occupied space which can limit overall rates of oxygen consumption in a cell. Such a high turnover rate of the respiratory protection chain is what makes it possible to decrease the intracellular oxygen level required for the operation of the nitrogenase.

While the respiratory chain in the inner membrane of animal mitochondria possesses a single NADH-oxidizing enzyme (complex I, analogous to bacterial NDH I) \( (23) \), most bacteria also harbor a second enzyme (i.e., NDH II) the physiological role of which has not yet been revealed. The analysis of the A. vinelandii NDH II sequence indicates that this protein is very similar to the NDH II of microorganisms from the gamma subdivision of proteobacteria. This suggests that NDH II from A. vinelandii is a typical representative of this class of enzymes and that in other organisms harboring NDH II the enzyme may also be employed similarly, i.e., for very fast quinone reduction (or very fast NADH oxidation). The possible physiological role(s) of NDH II in bacteria other than Azotobacter is left for future research.

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REFERENCES


