VnfY Is Required for Full Activity of the Vanadium-Containing Dinitrogenase in Azotobacter vinelandii

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A gene from Azotobacter vinelandii whose product exhibits primary sequence similarity to the NifY, NafY, NifX, and VnfX family of proteins, and which is required for effective V-dependent diazotrophic growth, was identified. Because this gene is located downstream from nifK in an arrangement similar to the relative organization of the nifK and nifY genes, it was designated vnfY. A mutant strain having an insertion mutation in vnfY has 10-fold less vnf dinitrogenase activity and exhibits a greatly diminished level of 49V label incorporation into the V-dependent dinitrogenase when compared to the wild type. These results indicate that VnfY has a role in the maturation of the V-dependent dinitrogenase, with a specific role in the formation of the V-containing cofactor and/or its insertion into apodinitrogenase.

Azotobacter vinelandii harbors three genetically distinct nitrogenase systems that are differentially expressed depending on the availability of metals in the medium: a nif-encoded Mo-containing nitrogenase, a vnf-encoded V-containing nitrogenase, and an naf-encoded iron-only nitrogenase (2). The V-containing nitrogenase contains an iron-vanadium cofactor (FeV-co) at its active site which is structurally and functionally analogous to the better-characterized FeMo-co of the Moadependent system. A functional V-containing nitrogenase requires the products of the structural genes for dinitrogenase (vnfDGK) and dinitrogenase reductase (vnfH) as well as several other nif and vnf gene products involved in the biosynthesis of FeV-co and the maturation of the nitrogenase component proteins. Much of what is known about the biosynthesis of FeV-co comes from analogous studies on FeMo-co biosynthesis. It is believed that the products of nifB, nifN, nifE, nifH, vnfX, and nifV are involved in the biosynthesis of FeV-co (see reference 12 for a review).

The involvement of VnfX in the biosynthesis of FeV-co is of particular interest with respect to the results described here. A vanadium-iron-sulfur cluster presumed to be similar to a precursor of FeMo-co biosynthesis accumulates on VnfX during FeV-co biosynthesis (16). Upon homocitrate addition, a newly formed homocitrate- and vanadium-containing cluster is transferred from VnfX to apodinitrogenase; the resulting dinitrogenase is able to reduce acetylene (15). VnfX is also able to bind structurally related metalloclusters as NifH-co or FeMo-co. The exact role of VnfX in FeV-co biosynthesis is not yet known.

To identify vnf genes whose products are involved in formation of a functional V-containing nitrogenase, we determined the nucleotide sequence of the genomic region located downstream from vnfY (see reference 12 for a review).

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To identify vnf genes whose products are involved in formation of a functional V-containing nitrogenase, we determined
formation of an active dinitrogenase under metal-sufficient growth conditions (8, 9, 14, 22).

To assess the involvement of VnfY in maturation of the V-containing nitrogenase, \( vnfY \) was disrupted by insertion mutagenesis using a kanamycin resistance gene cartridge. Procedures for \( A. \) \textit{vinelandii} transformation (11) and gene replacement (10) were performed as previously described, and strains used in this work are listed in Table 1. \( A. \) \textit{vinelandii} mutant strain DJ1254 is a \( nifDK \), tungsten-tolerant strain derived from the CA11.6 and DJ33 strains. The tungsten-tolerant strain DJ1254 is used in this study because the expression of its V-dependent nitrogenase is less sensitive to Mo contamination in the culture medium than it is in strain DJ33, most probably due to a defect in Mo/W uptake into the cell. The DJ1293 strain was generated by transformation of DJ1254 with plasmid FIG. 1. Structure of the \( A. \) \textit{vinelandii} chromosome 3' of the \( vnfDGK \) gene region. The locations of the kanamycin resistance cassette insertions within \( vnfY \) and ORF2 are indicated, together with the denomination (DJ) of the resulting mutant strain. S, SalI; X, XhoI.

![Diagram](image)

FIG. 2. Amino acid sequence alignment of VnfY, VnfX, NifX, NaFY, and NifY proteins from \( A. \) \textit{vinelandii}. The amino acid sequences are indicated by the single-letter code. Amino acid residues identical or similar in three or more of the aligned proteins are shaded in gray.

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<th>Strain</th>
<th>Genotype</th>
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<tbody>
<tr>
<td>DJ54</td>
<td>( \Delta nifH )</td>
<td>13</td>
</tr>
<tr>
<td>CA12</td>
<td>( \Delta nifHDK )</td>
<td>3</td>
</tr>
<tr>
<td>CA11.1</td>
<td>( \Delta nifHDKnfdDGK::spc )</td>
<td>21</td>
</tr>
<tr>
<td>DJ1254</td>
<td>( \Delta nifDK )</td>
<td>Dennis Dean, Virginia Tech</td>
</tr>
<tr>
<td>DJ1293</td>
<td>( \Delta nifDKnfdY::Kan )</td>
<td>This work</td>
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<tr>
<td>DJ1299</td>
<td>( \Delta nifDKORF2::Kan )</td>
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TABLE 1. \( A. \) \textit{vinelandii} strains used in this work

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When analyzed by anoxic native gel electrophoresis. The significance of this observation is not yet known. In control experiments using extracts prepared from CA12 and DJ1293 (developed with antibody to VnfD), sodium dodecyl sulfate-gel immunoblot analysis of vnf dinitrogenase revealed that the two strains accumulate similar levels of V-containing dinitrogenase (data not shown). Thus, insertional inactivation of vnfY prevents the effective incorporation of FeV-co into V-containing dinitrogenase without altering the accumulation of the complementary polypeptide.

It was previously shown that the $^{59}$V radiolabel accumulates on VnfX when the structural genes for the V-containing dinitrogenase are deleted (16). In the present study, the amount of $^{59}$V radiolabel associated with VnfX in extracts of a strain from which vnfDGK was deleted (CA11.1) was fivefold larger than that for a strain having an insertion mutation within vnfY (DJ1293) (see Fig. 3, lanes 2 and 3, respectively). Moreover, the amount of $^{59}$V label found associated with VnfX in extracts of DJ1293 was only slightly larger than the amounts exhibited by extracts of CA12.

The dinitrogenase and dinitrogenase reductase activities in crude extracts of $A$. vinelandii strain DJ1293 were also determined and compared to those in extracts of strains CA12 and DJ54 (Table 2). Dinitrogenase and dinitrogenase reductase activities in cell extracts were obtained after titration with an excess of the complementary component as described previously (17).
function of NiY/NafY in the Mo-dependent system—namely, to aid in the incorporation of finished cofactor into apodinitrogenase. If this were the only step impaired in the vnfY mutant strain, one would expect accumulation of $^{49}$V label on VnX at levels similar to those found in a strain from which vnfDGK was deleted. However, our results indicate that loss of VnY function results in a lower level of $^{49}$V incorporation into the V-containing dinitrogenase and also into VnX. Furthermore, no other protein(s) is $^{49}$V labeled in extracts of DJ1293. Thus, it is possible that VnY is involved in an early step in the biosynthesis of FeV-co, for example, in the incorporation of V into the pathway.

It is not surprising that VnY might have more than one role in the biosynthesis of FeV-co. As noted above, some members of this family of proteins show functional versatility and seem to be involved in more than the binding of FeMo-co or FeV-co precursors only. For example, NifX and NiY have been proposed to have a role in balancing $nif$ gene expression in Klebsiella pneumoniae (6, 20), and NafY acts like a chaperone that stabilizes the apo form of the $nif$ dinitrogenase in A. vinelandii (7, 14). However, VnY neither seems to regulate the expression of the $nif$ gene (since a vnfY mutant contains normal levels of VnDGK protein) nor seems to act as a chaperone because it is not found associated with purified apo-VnDGK (4).

Unlike the situation observed for the loss of NiY, NafY, NifX, or VnIX function, loss of VnY function has a clear effect on the maturation of V-containing dinitrogenase which is reflected in a lower level of incorporation of FeV-co into the V-containing dinitrogenase and, consequently, in lower dinitrogenase activity. The vnfY mutant strain is clearly and consistently defective in V-dependent diazotrophic growth when tested in solid medium (data not shown). These effects are not due to a polar effect of the insertion mutation within $nifY$ on the ORF2, because insertional inactivation of ORF2 does not affect V-dependent diazotrophic growth (data not shown). These observations should provide a physiological and biochemical basis for determining the specific role of VnY in V-containing dinitrogenase maturation, which should in turn provide insight into the possible roles of this family of proteins in the assembly and insertion of complex metalloclusters.

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