The criteria necessary in the design of this particular O_2 storage system probably include: (i) a high-affinity hemoglobin that slowly dissociates and rapidly reassociates O_2 to reduce the amount of free O_2 diffusing out of the nerve and that, combined with high cooperativity, restricts O_2 unloading to a very low and narrow PO_2 range so that the diffusion gradient is always small; (ii) diffusion distances, which are smaller from glial cells to axons than from glial cells across the collagen sheath to the outside, that favor O_2 consumption by axon bundles; and (iii) O_2 consumption rates of active nerves that are equivalent to the O_2 unloading rates. A measure of how much of the unloaded O_2 is actually consumed by the nerve as opposed to how much is lost could indicate how efficiently the hemoglobin functions in storage. Preliminary calculations based on O_2 unloading rates, O_2 consumption rates, heme concentration, and the anatomy of the nerve suggest that more than 80 percent of the unloading O_2 could be consumed by the nerve during high activity, demonstrating an appropriate match between hemoglobin O_2 supply and neural O_2 demand. The molecular phenomena influencing the long unloading time and a model relating the important variables to long-term O_2 supply by hemoglobin will be discussed elsewhere (*18*).

Tellina alternata, unlike the filter-feeding *T. plebeius*, does not inhabit a permanent burrow, because its mode of deposit feeding quickly depletes the surrounding region of food and it must continually burrow through anoxic sediment to new locations. When laterally burrowing several centimeters below the sediment surface, it does not maintain siphonal contact with the surface and therefore does not seem to have access to a source of O_2. It seems reasonable that its slowly unloading neural O_2 stores may enable continued nervous function and burrowing activity even in anoxic sediments for an extended period of time, allowing it to reach fresh deposits.

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9. In the compound c-v nerve, a supramaximal stimulus was used to recruit a maximum number of axons for action potential propagation. A decline in action potential amplitude during oxygen deprivation was assumed to be due to a decrease in the total number of axons that could maintain excitability as a result of the declining PO_2.
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11. Data from electron micrographs of c-v nerve cross sections revealed similar numbers and sizes of axons in both bivalves.
12. The red pigment is referred to as hemoglobin because it displays a typical oxyhemoglobin spectrum (peaks: a = 575 nm; b = 540 nm) and carboxyhemoglobin spectrum. The deoxy spectrum is unusual in having two peaks (560 nm and 530 nm), which have been ascribed to a nitrogenous or sulfurous ligand binding to the ferrous heme iron when it is deoxygenated [J. B. Wittenberg and B. A. Wittenberg, *Comp. Biochem. Physiol.* **51A**, 425 (1975)].
13. The affinity characteristics of the hemoglobin do not seem to be *p*H-sensitive from 6.6 to 7.8. However, because this is an in situ preparation, it is difficult to be certain that the intracellular *p*H is in equilibrium with the *p*H of the solution that bathes the intact nerve. Although this hemoglobin has been isolated as a monomer having a molecular weight of 20,000 in its oxygenated state, it displays several characteristics in addition to high cooperativity that suggest that it forms large aggregational polymers upon deoxygenation (D. W. Kraus and J. M. Colacino, in preparation). The existence of this property in tissue hemoglobins that deliver O_2 in a precise manner has been reported [R. M. G. Wells *et al.*, *J. Comp. Physiol.* **142**, 515 (1981); L. T. Tam and A. F. Riggs, *J. Biol. Chem.* **259**, 2610 (1984)].
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16. To ensure that the long unloading time was a property of the hemoglobin and not due to the gas delivery system, we calculated that with a gas slide chamber volume of 0.3 ml, a gas flow rate of 70 ml/min, and a maximum diffusion distance of 80 μm, the time required for the chamber and sample to reach 99.999 percent of the flushing gas was 1.0 minute or less (*7*).
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Nitrogen Fixation by *Azotobacter vinelandii* Strains Having Deletions in Structural Genes for Nitrogenase

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Phenotypic reversal of Nif$^-$ mutant strains to Nif$^+$ under molybdenum-deficient conditions has been cited as evidence that *Azotobacter vinelandii* possesses two nitrogen fixation systems: the conventional molybdenum-enzyme system and an alternative nitrogen-fixation system. Since explanations other than the existence of an alternative system were possible, deletion strains of *A. vinelandii* lacking the structural genes for conventional nitrogenase (nifHDK) were constructed. These strains were found to grow in molybdenum-deficient nitrogen-free media, reduce acetylene (at low rates), and incorporate molecular nitrogen labeled with nitrogen-15. Thus it can be concluded that the phenotypic reversal phenomenon cannot be due to altered phenotypic expression of *nif* mutations under molybdenum-deficient conditions, but is due to the existence of an alternative nitrogen-fixation system in *A. vinelandii* as originally proposed.

SEVERAL YEARS AGO WE REPORTED that Nif$^-$ mutant strains of *Azotobacter vinelandii* containing mutational lesions in the structural genes for nitrogenase underwent phenotypic reversal to Nif$^+$ when cultured in molybdenum-deficient nitrogen-free media (*1, 2*). To account for this phenotypic reversal, we proposed the existence of an alternative N_2 fixation system expressed under conditions of molybdenum starvation and repressed in the presence of molybdenum (*1*). It can be argued, however, that phenotypic reversal of Nif$^-$ mutants under conditions of molybdenum starvation is due to increased leakiness of the mutant phenotypes and not to derepression of an alternative N_2-fixation system. Mutant strains containing deletions in the structural genes for nitrogenase (*nifHDK*) should distinguish between these two explanations since mutational alterations caused by deletions should preclude leaky mutant phenotypes. Here we report N_2 fixation by Nif$^-$ mutant strains containing deletions in *nifHDK*.

Deletions in *nifHDK* were constructed by removing internal restriction fragments from cloned DNA's containing these genes.

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Table 1. Activities of dinitrogenase and dinitrogenase reductase in cell-free extracts of *nif* deletion strains. Growth of cells in molybdenum-deficient medium containing ammonium acetate, derepression for nitrogenase in the presence or absence of molybdenum, preparation of cell-free extracts, and the acetylene reduction assay have been described (*1*). The dinitrogenase and dinitrogenase reductase activities of cell-free extracts were determined by titration with an excess of the complementary component. Specific activities are given in nanomoles of C_2H_4 per minute per milligram of protein. The dinitrogenase and dinitrogenase reductase used in the complementation assay were purified from *A. vinelandii* by the method of Shah and Brill (*13*) and had specific activities of 2000 and 1360 nmol of C_2H_4 per minute per milligram of protein, respectively. The protein concentrations of the cell-free extracts ranged from 12 to 15 mg/ml. The values given are means of duplicate determinations. The error range is ±5% of the means.

Strain	Molybdenum	Specific activities		
		Extract	Dinitrogenase	Dinitrogenase reductase
CA	Present	35.09		
CA11	Present	0.00	0.01	0.01
CA11	Absent	0.13	0.11	11.40
CA12	Present	0.00	0.01	0.01
CA12	Absent	0.15	0.14	11.00
CA13	Present	0.01	0.01	73.00
CA13	Absent	0.17	0.15	18.80

The deletion plasmid, pDB11, was generated by removing a 5.3-kilobase-pair (kbp) Bgl II fragment from a 12.7-kbp Xho I insert carried by pDB3, which contains the entire *nifHDK* cluster from *A. vinelandii* (*3*). This deletion removed both *nifD* and *nifK* plus approximately two-thirds of *nifH* (Fig. 1). A second plasmid, pDB12, was constructed by removing three Kpn I fragments from pDB3. This 3.3-kbp deletion extended through about two-thirds of *nifH* to the middle of *nifK* (Fig. 1). *A. vinelandii* strains carrying the deletions contained by pDB11 and pDB12 were constructed by congression (coincident transformation of a competent cell by genetic markers on separate DNA molecules) (*4, 5*). The Nif^- isolates resulting from the congression crosses with pDB11 (strain CA11) and pDB12 (strain CA12) were sensitive to streptomycin [the vector for pDB11 and pDB12 was pKT230 (*6*)], suggesting that these plasmids no longer resided in these strains.

Several approaches were used to verify the presence and extent of the deletions in strains CA11 and CA12. Neither dinitrogenase nor dinitrogenase reductase proteins were detected by two-dimensional gel electrophoresis in cell extracts of these strains derepressed for nitrogenase in the presence of 1 μM Na_2MoO_4. In vitro complementation assays involving the addition of purified dinitrogenase or dinitrogenase reductase to extracts from derepressed cells of strain CA11 and CA12 showed that these strains lacked detectable activity for both dinitrogenase and conventional dinitrogenase reductase (Table 1). In strain CA13, which contains a 470-bp deletion in *nifK* (*5*), only dinitrogenase reductase activity could be detected (Table 1). Strains CA11 and CA12, however, showed a dinitrogenase reductase–like activity when extracts from molybdenum-deficient derepressed cells were used (Table 1). These results are in agreement with previous results with Nif^- mutants that lack dinitrogenase reductase activity in the presence of molybdenum (*7*). Low, but detectable, activity was present in cell extracts of the deletion strains derepressed under molybdenum-deficient conditions (Table 1).

Marker rescue experiments were conducted with the plasmids pLB1 (*5*), pLB3A (*5*), and pMJH1 (consisting of a 1.35-kbp Sma I–Eco RI fragment containing *nifH* cloned into pUC9) that carry inserts having end points that fall within the regions covered by the putative deletions in strains CA11 and CA12. Results of these transformation crosses were negative; that is, none of the inserts carried by these plasmids were capable of correcting the *nif* mutations carried by strains CA11 and CA12. As expected, these mutations were corrected by pDB3 (1.1×10^{-3} and 8.3×10^{-4} Nif^+ transformants per cell plated, respectively, for strains CA11 and CA12).

Final verification that strains CA11 and CA12 contain *nifHDK* deletions was provided by Southern blot hybridization analysis. Figure 2 shows the results of experiments in which genomic DNA's from strains CA, CA11, and CA12 were digested with Eco RI, Xho I, and Sma I. These genomic digests were probed with a 6.2-kbp Sma I fragment containing *nifHDK*, which spans the deletions in strains CA11 and CA12 (Fig. 1). In Eco RI digests (Fig. 2A) the 2.6- and 1.4-kbp fragments were missing from both strains. The 2.6-kbp fragment contains *nifK* whereas the 1.4-kbp fragment contains *nifD* (*5*). The 14- and 4.1-kbp fragments represent *nifH*-like sequences located outside of the *nifHDK* cluster. The

Table 2. Acetylene reduction and $^{15}N_2$ incorporation by *nif* deletion strains. Acetylene reduction and $^{15}N_2$ incorporation assays on cell suspensions were conducted essentially as described (*1*). Portions (10 ml) of cell suspension in 25-ml serum bottles fitted with rubber stoppers were used for in situ acetylene reduction and $^{15}N_2$-incorporation assays. The bottles were briefly evacuated and flushed with a gas mixture of 80 percent argon and 20 percent oxygen. The final composition of the gas phase was 69.3 percent argon, 17.3 percent oxygen, and 13.3 percent acetylene or $^{15}N_2$ (containing 92.41 percent ^{15}N). Samples were incubated for 15 minutes with vigorous shaking at 30°C. In cultures grown to low density (10 to 12 Klett; 1 Klett unit = 3×10^6 cells per milliliter), cell suspensions were centrifuged at 6000*g* for 5 minutes at room temperature. The cell pellet was resuspended in the supernatant to a density of approximately 50 Klett units, and 10-ml portions of this suspension were used in the assays. Protein concentrations in cell suspensions were determined by the method of Lowry *et al.* (*15*), after solubilizing the cells by heating in 0.5*N* NaOH for 30 minutes in a boiling water bath. Bovine serum albumin, treated in the same manner, was used as standard. Protein concentrations ranged from 2.2 to 3.8 mg/ml in the cell suspensions. The values given are the means of triplicate samples. The error range is ±10 percent of the means. Specific activity is given in nanomoles of C_2H_4 per minute per milligram of protein. Incorporation of ^{15}N is given in micrograms of nitrogen per hour per milligram of cellular nitrogen.

Strain	Genotype	Culture density (Klett units)	Molybdenum	Specific activity	Excess ^{15}N (atom %)	^{15}N incorporation
CA	Wild type	46	Present	18.70	1.4228	30.91
CA	Wild type	12	Absent	5.72	0.4495	9.77
CA	Wild type	45	Absent	1.96	0.1667	3.62
CA11	Δ *(nifHDK) 11*	10	Absent	3.30	0.1902	4.13
CA11	Δ *(nifHDK) 11*	57	Absent	0.74	0.0856	1.86
CA12	Δ *(nifHDK) 12*	44	Absent	1.14	0.1221	2.65
CA13	Δ*nifK13*	48	Absent	0.84	0.1224	2.66

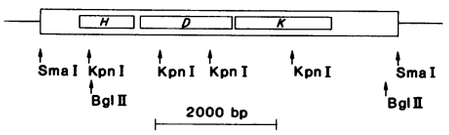

Fig. 1. Physical map showing restriction endonuclease sites in the *nif* structural gene region of the *A. vinelandii* genome that are relevant to construction of the deletion strains CA11 and CA12.

4.1-kbp fragment is not the previously reported 4.1-kbp partial digestion product (which hybridized to *nifD* and *nifK*) found in Eco RI digests of DNA from strain CA (*5*). On the basis of the Eco RI fragment sizes, the deletions can be estimated as 4.4 kbp for strain CA11 and 3.7 kbp for strain CA12. The 6.2-kbp *nifHDK* probe hybridized to a 12.7-kbp fragment and a 8.0-kbp fragment in Xho I digests of DNA from strain CA (Fig. 2B, lane 1). The 12.7-kbp fragment contains the *nifHDK* cluster (*3*), whereas the 8.0-kbp fragment is homologous to *nifH* but not to *nifD* and *nifK*. In Xho I digested DNA from strain CA11, the 12.7-kbp fragment seems to have been shortened to about 8.0-kbp (Fig. 2B, lane 2) and it comigrates with the 8.0-kbp fragment. The difference in size between the 12.7-kbp Xho I fragment and the shortened 8.0-kbp fragment suggests that the extent of the deletion in strain CA11 is approximately 4.7 kbp. Similarly the deletion carried by strain CA12 seems to be about 3.5 kbp (Fig. 2B, lane 3). The 6.2-kbp Sma I fragment present in Sma I digests of DNA from strain CA (Fig. 2C, lane 1) was shortened by 5.25 kbp in Sma I digests of DNA from strain CA11 and by 3.8 kbp in Sma I digests of DNA from strain CA12. In addition, a 9.0-kbp fragment and a fragment of almost identical size to the 6.2-kbp Sma I fragment were present in Sma I digests of genomic DNA's from strains CA, CA11, and CA12 (Fig. 2C). These fragments show sequence homology to *nifH*, but not to *nifD* or *nifK* (*7a*). Thus these fragments along with the two Eco RI fragments (14 and 4.1 kbp) and 8.0-kbp Xho I fragment may indicate the presence of the *nifH*-like genes which in turn could be involved with the proposed alternative N_2 fixation system.

The data from the Eco RI, Xho I, and Sma I digests indicate that the size of the deletion carried by strain CA11 is 4.4 to 5.25 kbp and the deletion carried by strain CA12 is 3.5 to 3.7 kbp. The variation in deletion size calculated from different restriction enzyme digests is probably due to the difficulty in estimating precise fragment sizes from autoradiograms. Nevertheless, these values are in reasonable agreement with sizes of the deletions generated in vitro in pDB11 and pDB12.

Preliminary tests indicated that the deletion strains would grow on solid Burk nitrogen-free medium provided that precautions were taken to exclude molybdenum from the medium (*1*). In molybdenum-deficient nitrogen-free liquid medium, doubling times ranged between 3.5 and 4.0 hours. Definitive proof, however, that these strains are capable of N_2 fixation under molybdenum-deficient conditions was provided by acetylene-reduction and $^{15}N_2$-incorporation experiments with whole cells. The three deletion strains reduced acetylene and incorporated $^{15}N_2$ with rates being the highest under conditions of low cell density (~10 Klett units) (Table 2). Experimental conditions, however, were not optimized for maximum rates. Compared with the deletion strains, strain CA showed greater rates of acetylene reduction and $^{15}N_2$ incorporation under molybdenum-deficient conditions. This might be due to a small contribution by the conventional N_2 fixation system. The deletion strains did not reduce acetylene or incorporate $^{15}N_2$ when derepressed for 6 hours in the presence of 1 μM Na_2MoO_4.

The phenotypic reversal phenomenon cannot be due to increased leakiness of *nif* mutations under molybdenum-deficient conditions, but instead is due to the existence of an alternative N_2 fixation system in *A. vinelandii*. Although the exact biochemical nature of this system remains unknown, the alternative N_2 fixation system is expressed under molybdenum-deficient conditions and does not depend on conventional *nifHDK* genes. Recently, Eady and Robson (*8*) have characterized N_2 fixation by wild-type *A. vinelandii* in batch and chemostat cultures under conditions in which molybdenum was limiting and found a novel substrate reduction pattern: acetylene was a relatively poor substrate compared with N_2 and H^+. Also, under the conditions used, molybdenum was essential for diazotrophy. However, we have no evidence that molybdenum is essential for diazotrophic growth of strain CA11 either in batch or continuous culture. It is tempting to speculate that the finding of multiple copies of *nifH*-like sequences in other diazotrophs (*9–12*) may indicate the presence of alternative N_2 fixation systems adapted to function under less than optimum conditions, such as molybdenum deficiency.

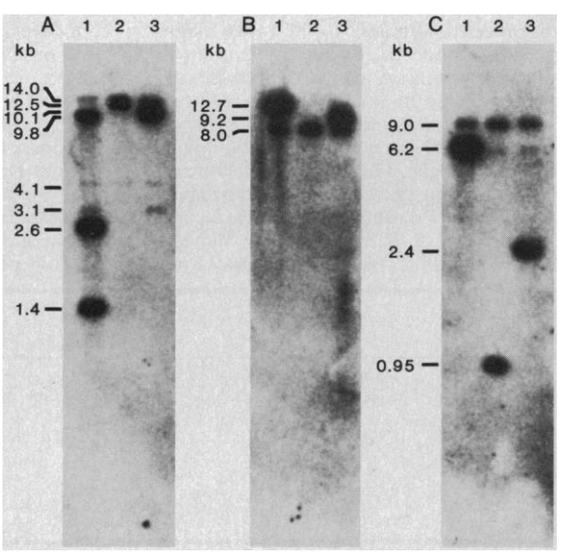

Fig. 2. Autoradiogram showing hybridization of a ^{32}P-labeled *nifHDK* probe to Eco RI, Xho I, and Sma I digests of genomic DNA's from the *A. vinelandii* deletion strains. (A) DNA's digested with Eco RI; (B) DNA's digested with Xho I; (C) DNA's digested with Sma I. Lane 1, strain CA DNA; lane 2, strain CA11 DNA; and lane 3, strain CA12 DNA. Preparation of DNA, restriction endonuclease reactions, agarose gel electrophoresis, and nick translation were conducted as described (*5*). Each lane contained approximately 10 μg of DNA. Transfer of DNA from agarose gels to Gene Screen (New England Nuclear) and DNA hybridization were carried out as described by the suppliers.

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