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Presence of a Vanadium Nitrogenase in *Azotobacter paspali*

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There have been no previous studies on the genetics of *Azotobacter paspali*, an aerobic bacterium which forms a highly specific diazotrophic association with Bahia grass (*Paspalum notatum*). We constructed *A. paspali* strains defective in the molybdenum nitrogenase so that alternative N_2ases could be studied. The cosmid vector pTBE and genomic DNA fragments (~50 kb) of *A. paspali* ATCC 23367 were used to construct a gene library in *Escherichia coli*. Recombinant cosmids containing sequences homologous to molybdenum nitrogenase *nifDK* structural genes were identified by hybridization. A 2.9-kb fragment bearing the putative *nifDK* genes of *A. paspali* was subcloned and mutagenized in vitro by the insertion of a kanamycin resistance gene cassette. The mutation was recombined into the chromosome of *A. paspali* with the suicide vector pCU101. One resultant mutant strain, AP2, was incapable of diazotrophic growth in a molybdenum-containing medium (Nif^-) without vanadium but grew well in a molybdenum-deficient medium with vanadium. The nitrogenase system in AP2 reduced acetylene to ethylene and produced ethane as 2.4% of the total products. Molybdenum levels as low as 10 nM prevented the diazotrophic growth of AP2, even in the presence of vanadium at levels up to 10 μM. These results are consistent with the existence of a vanadium nitrogenase system in *A. paspali*.

The aerobic dinitrogen (N_2)-fixing bacterium *Azotobacter paspali* forms a highly specific association with Bahia grass (*Paspalum notatum* Flügge). Nitrogen fixed by this bacterium may be transferred to Bahia grass under both field and greenhouse conditions (5). Our earlier study suggested that *A. paspali* possesses three genetically distinct N_2 fixation systems similar to those in other *Azotobacter* species (6). Usually, N_2 reduction is catalyzed by a molybdenum-containing nitrogenase (MoFe-N_2ase), but in some diazotrophs, molybdenum is not required. In *Azotobacter chroococcum* (25) and *Azotobacter vinelandii* (9), vanadium apparently substitutes for molybdenum in the vanadium nitrogenase (VFe-N_2ase). A third nitrogenase (Fe-N_2ase), which requires only iron, exists in *A. vinelandii* (2, 20). Each enzyme is encoded by a different set of structural genes: *nifHDK* for the MoFe-N_2ase, *vnfHDGK* for the VFe-N_2ase, and *anfHDGK* for the Fe-N_2ase (11, 23). Expression of each N_2ase system is controlled by the availability of metals. When molybdenum is present, only the MoFe-N_2ase is expressed. When molybdenum is absent and vanadium is present, only the VFe-N_2ase is expressed. When both molybdenum and vanadium are absent, only the Fe-N_2ase is expressed. The alternative nitrogenases can be detected by the formation of ethane (C_2H_6) in addition to ethylene (C_2H_4) in the standard acetylene reduction test for N_2 fixation (4, 20).

The potential importance of the VFe-N_2ase and Fe-N_2ase systems in natural environments is poorly understood. We initiated molecular genetic studies with *A. paspali* to construct strains defective in the MoFe-N_2ase so that its alternative N_2ases could be studied. Assuming that nitrogen fixed by *A. paspali* is transferred to Bahia grass, a genetic approach provides a means of addressing the potential importance of VFe-N_2ase and Fe-N_2ase systems.

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MATERIALS AND METHODS

Growth and maintenance of bacteria. Various bacteria and plasmids were used (Table 1). Strains of *A. paspali* were grown routinely under aerobic conditions in an orbital shaker (250 rpm) at 30°C in nephelometer flasks (250-ml capacity) containing 25 ml of a modified Burk's medium; this medium contained (in grams per liter of distilled water): sucrose (30), K_2HPO_4 (0.64), KH_2PO_4 (0.16), Na_2SO_4 (0.05), $MgCl_2 \cdot 6H_2O$ (0.2), $CaCl_2 \cdot 2_2O$ (0.07), and $FeSO_4 \cdot 7H_2O$ (0.015). This medium contained no added molybdenum, vanadium, or combined nitrogen source and was supplemented when required with molybdenum ($Na_2MoO_4 \cdot 2H_2O$; 10 μM), vanadium ($VOSO_4$; 10 μM), iron ($FeSO_4 \cdot 7H_2O$; 10 μM), tungsten ($NaWO_4 \cdot 2H_2O$; 10 μM), nickel ($NiCl_2 \cdot 6H_2O$; 10 μM), manganese ($MnSO_4 \cdot H_2O$; 10 μM), copper ($CuSO_4 \cdot 5H_2O$; 10 μM), ammonium acetate (10 mM), kanamycin (1 μg/ml), or neomycin (1 μg/ml). Growth was measured with a Klett-Summerson colorimeter equipped with a green filter. All doubling times were determined in triplicate. To prepare "scavenged" Burk's medium, *A. paspali* AP2 was incubated in modified Burk's medium containing ammonium acetate (1 mM) until a density of ~50 Klett units/ml was attained. The cells were removed by centrifugation at 10,000 × *g* for 10 min, and the culture supernatant was sterilized by filtration through a 0.22-μm-pore-size filter. This scavenged medium was used for growth studies in which the vanadium and molybdenum contents were controlled (26). Strains of *Escherichia coli* were grown aerobically at 37°C on Luria-Bertani medium (16) supplemented with (in micrograms per milliliter) kanamycin (25), neomycin (25), tetracycline (5), or ampicillin (100). When necessary, the media were solidified by the addition of agar (1.4%, wt/vol).

Molecular biology techniques. Chromosomal DNA of *A. paspali* was prepared as described by Robson et al. (24). Plasmid DNA was isolated by an alkaline lysis method (1), and DNA was electrophoresed in 0.8% (wt/vol) agarose in TAE buffer, as described by Maniatis et al. (16). Southern blots were prepared electrophoretically on GeneScreen membranes (New England Nuclear Corp., Boston, Mass.).

TABLE 1. Bacterial strains and plasmids used

Bacterium or plasmid	Genotype and/or phenotype	Reference or source
Bacteria		
A. paspali		
AP1	Wild type	ATCC 23367
AP2	Nif^- Km^r	This work
E. coli		
71/18	*thi supE* Δ(*lac-proAB*) [F′ *proAB lacI*^{q}ZΔM15]	18
LE392	F′ *hstR574* (r_K^-, m_K^-) *supE44 supF58 lacY1 galK2 galT22 metB1 trpR55*	19
HB101	F^- *thi-1 hsdS20* (r_B^-, m_B^-) *supE44 recA13 ara-14 leuB6 proA2 lacY1 rpsL20 xyl-5 mtl-1*	15
Plasmids		
pTZ19R	Ap^r, cloning vector	17
pTBE	Ap^r, *cos*	8
pUC4-KIXX	Ap^r, Km^r cassette	Pharmacia, Inc., Piscataway, N.J.
pAPN3	Ap^r, cosmid clone in pTBE *A. paspali nifDK* genes	This work
pAPNSa	Ap^r, 2.9-kb *A. paspali Sal*I fragment carrying *nifDK* genes in pTZ19R	This work
pAPNK7	Ap^r, Km^r, KIXX-Km^r cassette inserted into pAPNSa	This work
pAPNKM	Cm^r Km^r Tra (N type), p15A replicon, KIXX cassette in *A. paspali nifDK* gene locus	This work
pCU101	Cm^r Tra (N type), p15A replicon	27

Radioactive probes for DNA hybridization were labelled by nick translation with [^{32}P]dCTP (3,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) (21). Hybridizations were performed in roller bottles for 16 h at 42°C in formamide (45%), 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), dextran sulfate (10%, wt/vol), and Carnation nonfat dried milk (0.5%, wt/vol) (12). Blots were washed twice at room temperature in 100-ml amounts of 1× SSC–0.1% (wt/vol) sodium dodecyl sulfate (SDS) and twice at 65°C in 100-ml amounts of 0.5× SSC–0.1% (wt/vol) SDS. Autoradiography was carried out with Kodak X-OMAT AR film. Radioactive DNA molecular weight markers were prepared by end labelling *Hin*dIII restriction digests of phage λ DNA with DNA polymerase (Klenow fragment) and ^{35}S-dATP. Previously we showed that an *nifDK* gene probe from *A. chroococcum* hybridized to genomic DNA from *A. paspali* (6). Therefore, we used the same probe to identify and clone homologous sequences from the pTBE cosmid (8) library. The *nifDK* probe was prepared with the 3-kb *Sal*I-*Sac*I fragment from pER4 containing *nifD* and *nifK* from *A. chroococcum* (13, 14).

Genomic library construction. The cosmid vector pTBE and genomic DNA fragments (~50 kb) of *A. paspali* AP1 were used to construct a gene library in *E. coli* LE392. The pTBE "arms" were made by digesting batches of the cosmid with either *Hpa*I or *Cla*I. The arms were treated with calf alkaline phosphatase and digested with *Bam*HI. The DNA of *A. paspali* was partially digested with *Sau*3AI and size fractionated on a NaCl gradient. The DNA fragments were ligated to the cosmid arms, packaged into λ particles in vitro, and transfected into *E. coli* LE392. Transfectants were selected on Luria-Bertani agar containing ampicillin.

Other genetic techniques. The conjugative vector pCU101, which contains the N-type *tra* genes, allows marker exchange mutagenesis in *A. chroococcum* (22). For conjugations between *A. paspali* and *E. coli* containing derivatives of pCU101, recipients were grown for 3 days on Burk's agar plates amended with ammonium acetate (5 mM). Donors were grown on Luria-Bertani agar plates with the appropriate antibiotics for 2 days at 37°C prior to mating. Donors and recipients were resuspended in 1 ml of 1× PEM buffer (24). A 1:10 mixture of donors and recipients was spotted onto Burk's agar amended with nutrient broth (0.2 g/liter) and ammonium acetate (5 mM) and incubated for 3 days at 30°C. Exconjugants were selected and purified twice on Burk's agar containing the appropriate antibiotics and ammonium acetate (1 mM). Transformations of *E. coli* were performed by the method of Dagert and Ehrlich (3).

Assay of nitrogenase in whole cells. Strains of *A. paspali* were grown for 18 h in Burk's medium amended with ammonium acetate (10 mM). To follow derepression of nitrogenase activity, 0.5-ml samples of cells were inoculated into duplicate 250-ml nephelometer flasks containing 25 ml of Burk's medium amended with various metals and limiting levels of ammonium acetate (1 mM). Cultures were capped with Subba seals, and 25 ml of C_2H_2 was injected. Cultures were incubated on a rotary shaker at 200 rpm at 30°C. Periodically, a 0.2-ml sample of the gas headspace was analyzed for C_2H_4 and C_2H_6 with a Varian 3400 gas chromatograph equipped with a Porasil C column and a flame ionization detector.

RESULTS

Cloning of the MoFe-N_2ase structural genes of *A. paspali*. Twenty-five clones of the *A. paspali* gene library hybridized to the *nifDK* probe, and five of these were randomly selected for further study. All five contained plasmids, which when digested had a number of restriction fragments in common, consistent with their being derived from overlapping portions of the *A. paspali* genome. The cloning of *nifDK*-like sequences in these plasmids was confirmed by digesting the

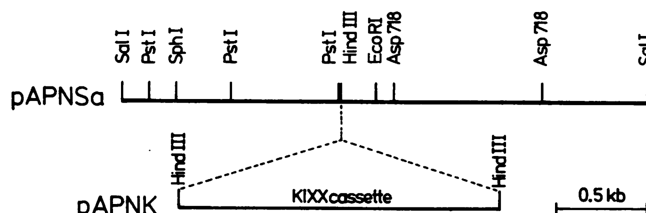

FIG. 1. Physical map of the putative *nifDK* locus from *A. paspali* inserted in pAPNSa and construction of pAPNK. Plasmid pAPNSa contains the 2.9-kb *Sal*I genomic DNA fragment of *A. paspali*, which hybridizes to the *A. chroococcum nifDK* probe and which was isolated from cosmid pAPN3 and cloned into pTZ19R. Plasmid pAPNK carries the kanamycin resistance gene KIXX cassette in the unique *Hin*dIII site of the insert in pAPNSa.

plasmids with restriction enzymes and by blotting and exposing the resultant fragments to the *nifDK* probe. Each cosmid contained a 2.9-kb *Sal*I fragment which hybridized to the probe. One cosmid (pAPN3) was randomly selected and digested with *Sal*I. The 2.9-kb fragment was purified and cloned into pTZ19R to create plasmid pAPNSa, which was then physically mapped (Fig. 1).

Mutagenesis of putative *nifDK* genes in *A. paspali*. To establish that the 2.9-kb *Sal*I fragment cloned in pAPNSa contained the functional homologs of the *nifDK* genes, the 1.4-kb kanamycin resistance gene from pUC4-KIXX was isolated as a *Hin*dIII fragment and cloned into the *Hin*dIII site within the 2.9-kb insert in pAPNSa. This created plasmid pAPNK (Fig. 1). The mutation was then recombined into the chromosome.

Conjugation was used to introduce DNA into *A. paspali*. Plasmid pAPNK was linearized with *Bam*HI and cloned in its entirety into the *Bam*HI site in pCU101 (to create plasmid pAPNKM). When *E. coli* HB101 (pAPNKM) was mated with *A. paspali*, Km^r exconjugants were obtained at $\sim 10^{-6}$ per recipient on Burk's medium amended with kanamycin. Several Km^r isolates were found to be Nif^- (i.e., incapable of growth on N_2 in Mo-containing Burk's medium). Several of these Nif^- isolates were probed with the *nifDK* probe from *A. chroococcum*; in *A. paspali* AP2, the 2.9-kb *Sal*I *nifDK*-hybridizing genomic fragment was absent but was replaced by two new hybridizing fragments consistent in size with a restriction fragment length polymorphism arising from an insertion of the kanamycin resistance gene into the correct locus of the chromosome. We concluded that strain AP2 carries a gene replacement in the *nifDK* locus.

N_2 fixation in AP2. Unlike the wild-type strain, AP2 was incapable of growth in N-free medium when Mo was added, but it grew well (doubling time, 5.5 h) when molybdenum was omitted and vanadium was added (Fig. 2). When fixing N_2 in a vanadium-containing medium, AP2 grew more slowly than AP1 (doubling time, 3.4 h). When NH_4^+ was supplied as a nitrogen source, the strains grew at comparable rates (doubling times, 3.1 h). Logarithmically growing cultures of AP2 exhibited an average nitrogenase-specific activity of 34 nmol of C_2H_2 reduced per min per mg of protein. C_2H_4 was the major product, with up to 2.4% of the total as C_2H_6. Molybdenum prevented the growth and expression of V-dependent N_2ase activity in AP2. In cultures initially provided with 10 μM $VOSO_4$, addition of molybdenum at levels as low as 10 nM caused >97% inhibition of N_2ase activity. AP2 failed to grow in a medium without added molybdenum and vanadium (Fig. 2), but it did exhibit low levels of C_2H_4 formation (<4% of the amount of C_2H_4

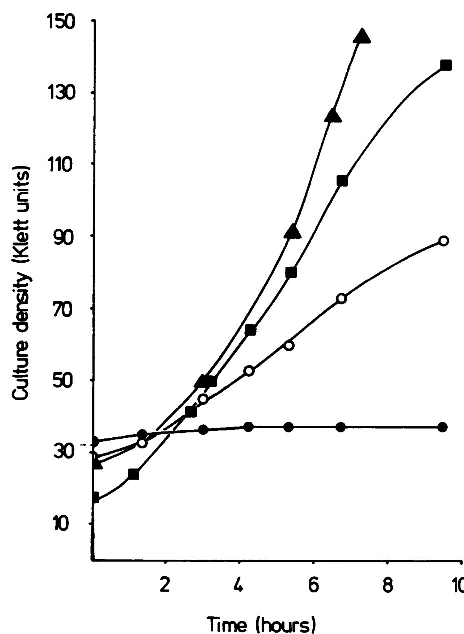

FIG. 2. Growth characteristics of *A. paspali* AP1 and AP2. *A. paspali* AP1 (wild type) was grown in Burk's medium amended with molybdenum (squares). *A. paspali* AP2 (presumptive Nif^-) was grown in Burk's medium with ammonium acetate and no added metals (triangles), with no ammonium acetate and with vanadium added (open circles), and with no ammonium acetate or any metals added (closed circles). Values are averages for three replicate cultures.

formed when vanadium was supplied). This scant activity could be due either to low levels of the VFe-N_2ase using traces of vanadium or to a third system partly repressed by traces of molybdenum or vanadium in the medium. To distinguish between these two possibilities, AP2 was grown in scavenged medium. When ammonium was added as a nitrogen source, AP2 grew well; when N_2 was the nitrogen source, AP2 failed to grow or exhibit significant nitrogenase activities unless vanadium was added. Supplementation of this scavenged medium with iron, tungsten, copper, manganese, or nickel did not stimulate N_2 fixation in AP2. Therefore, diazotrophic growth of *A. paspali* AP2 appeared to be dependent on vanadium.

DISCUSSION

In this work, we cloned the presumptive *nifDK* genes from *A. paspali* and used this DNA to construct a site-directed mutation in the corresponding chromosomal locus. The resultant strain, AP2, (i) was incapable of fixing N_2 in a medium containing Mo (10 nM molybdenum lowered N_2ase activity by 97%), (ii) was capable of fixing N_2 when molybdenum was omitted and vanadium was added, and (iii) reduced C_2H_2 to C_2H_4 with up to 2.4% of the total product as C_2H_6. The requirement of vanadium for diazotrophic growth and the formation of ethane suggest that *A. paspali* contains a VFe-N_2ase system similar to those described for *A. chroococcum* and *A. vinelandii*. These findings complement our earlier study in which *vnfDGK* structural gene probes hybridized to genomic DNA of *A. paspali* (5). The sensitivity of the VFe-N_2ase to molybdenum closely matches that described for *A. vinelandii* (10).

Hybridization studies with *nif*, *vnf*, and *anf* gene probes suggest that *A. paspali* contains not only MoFe- and VFe-N_2ases but also the Fe-N_2ase (5). In *A. vinelandii*, the Fe-N_2ase is expressed in the absence of molybdenum and vanadium (2, 19). We found no evidence for the expression of a similar system in *A. paspali*, even when cultured in scavenged Burk's medium. It is still possible that a mutation in *nifD* or *nifK* blocks expression of a third system, even though mutations in comparable genes in *A. vinelandii* do not prevent expression of the Fe-N_2ase (11).

DNA was introduced by conjugation into *A. paspali* with plasmids containing the N-type *tra* genes. This establishes *A. paspali* as a genetically amenable organism. An N-type *tra* plasmid was used for the creation of a site-directed mutation in the *A. paspali* genome. We have also introduced transposon Tn*5* at low frequency into *A. paspali* (7), which shows that this system should also prove useful for isolating random mutants. The *A. paspali*-Bahia grass association is an interesting and little-explored example of a specific plant-bacterium association. The ability to create both random and site-directed mutants should open the way to explore the genetic basis of this interaction.

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