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Cloning, Sequencing, Mutagenesis, and Functional Characterization of *draT* and *draG* Genes from *Azospirillum brasilense*

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The *Azospirillum brasilense draT* gene, encoding dinitrogenase reductase ADP-ribosyltransferase, and *draG* gene, encoding dinitrogenase reductase activating glycohydrolase, were cloned and sequenced. Two genes were contiguous on the *A. brasilense* chromosome and showed extensive similarity to the same genes from *Rhodospirillum rubrum*. Analysis of mutations introduced into the *dra* region on the *A. brasilense* chromosome showed that mutants affected in *draT* were incapable of regulating nitrogenase activity in response to ammonium. In contrast, a mutant with an insertion in *draG* was still capable of ADP-ribosylating dinitrogenase reductase in response to ammonium but was no longer able to recover activity after ammonium depletion. Plasmid-borne *draTG* genes from *A. brasilense* were introduced into *dra* mutants of *R. rubrum* and restored these mutants to an apparently wild-type phenotype. It is particularly interesting that *dra* mutants of *R. rubrum* containing *draTG* of *A. brasilense* can respond to darkness and light, since *A. brasilense* is a nonphotosynthetic bacterium and its *dra* system does not normally possess that regulatory response. The *nifH* gene of *A. brasilense*, encoding dinitrogenase reductase (the substrate of dinitrogenase reductase ADP-ribosyltransferase and dinitrogenase reductase-activating glycohydrolase), is located 1.9 kb from the start of *draT* and is divergently transcribed. Two insertion mutations in the region between *draT* and *nifH* showed no significant effect on nitrogenase activity or its regulation.

Nitrogen fixation, the reduction of molecular dinitrogen to ammonium, is catalyzed by the nitrogenase complex. This complex consists of dinitrogenase (MoFe protein), which contains the active site of dinitrogen reduction, and dinitrogenase reductase (Fe protein), which donates electrons to dinitrogenase (1). Nitrogen fixation is a very energy-demanding process and is therefore subject to elaborate regulatory control. Two kinds of regulation have been studied in detail. Transcriptional regulation exists in all studied nitrogen-fixed organisms. It is best characterized in *Klebsiella pneumoniae*, where the expression of the *nif* genes responds to fixed nitrogen and oxygen through both global and *nif*-specific systems (30). Posttranslational regulation, which has also been termed short-term inhibition (20) or switch-off (37), causes rapid control of nitrogenase activity. This regulatory approach has been found in diverse nitrogen-fixing bacteria (25).

Posttranslational regulation of nitrogenase activity has been best described in *Rhodospirillum rubrum*, where it involves the ADP-ribosylation of dinitrogenase reductase (25). Thus far, two enzymes have been shown to perform this regulation. Dinitrogenase reductase ADP-ribosyltransferase (DRAT; the gene product of *draT*) carries out the transfer of the ADP-ribose from NAD to the Arg-101 residue of one subunit of the dinitrogenase reductase dimer, resulting in inactivation of that enzyme. Dinitrogenase reductase-activating glycohydrolase (DRAG; the gene product of *draG*) removes the ADP-ribose from the covalently modified enzyme, restoring its activity.

Azospirillum spp. are microaerobic nitrogen-fixing bacteria associated with the roots of many important cereals and grasses (8). Regulation of nitrogenase activity in *Azospirillum* spp. has been examined (2). Whereas the addition of ammonium to *Azospirillum brasilense* and *A. lipoferum* results in modification of dinitrogenase reductase as in *Rhodospirillum rubrum*, the addition of ammonium to *Azospirillum amazonense* results in only partial inhibition without covalent modification of dinitrogenase reductase (17). *draTG*-hybridizing regions have been found in *A. brasilense* and *A. lipoferum* but not in *A. amazonense* (14). *draTG* genes have been cloned from *A. lipoferum* (13), and DRAG was recently purified from *A. brasilense* (24).

This study was performed to understand posttranslational regulation in *A. brasilense* and to provide biochemical and genetic analyses for comparison with the *R. rubrum* system.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and antibiotics. The strains used in this study are listed in Table 1. For chromosomal DNA isolation, *A. brasilense* was grown in LD medium (1% tryptone, 0.5% yeast extract, 0.25% NaCl [pH 6.8]). For derepression of nitrogenase, *A. brasilense* cells were inoculated into 50 ml of NfbHP-glutamate medium (29) in 250-ml flasks and grown with 100 rpm orbital shaking for 16 to 20 h at 30°C. Whole-cell nitrogenase activity (acetylene reduction) was monitored as follows: A 1-ml sample of cells was injected into a stoppered 9-ml vial containing 20% air, 10% acetylene, and 70% argon and incubated for 10 min at 160 excursions per min on a reciprocal shaker, and 0.5 ml of 4 N NaOH was injected to stop the activity in the assay. The ethylene produced was measured by gas chromatography. Derepression of *R. rubrum* and assay of whole-cell acetylene reduction were carried out by the methods of Kanemoto and Ludden (18) and Lehman and Roberts (21).

Antibiotics were used at the following concentrations: for *A. brasilense*, 25 mg of ampicillin, 12.5 mg of kanamycin, 3 mg of nalidixic acid, and 7.5 mg of chloramphenicol per liter; for *R. rubrum*, 1 mg of tetracycline and 100 mg of strepto-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype and description	Source or reference
A. brasilense		
Sp7	Wild type, Ap^r Nx^r	17
UB2	*draT1*::*kan* Ap^r Nx^r Km^r	This study
UB3	Δ*draT2*::*kan* Ap^r Nx^r Km^r	This study
UB4	*draG3*::*kan* Ap^r Nx^r Km^r	This study
UB5	*dnr-1*::*kan* in region between *dra* and *nif*, Ap^r Nx^r Km^r	This study
UB6	*dnr-2*::*kan* in region between *dra* and *nif*, Ap^r Nx^r Km^r	This study
R. rubrum		
UR2	Wild type, Sm^r	12
UR212	*draT2*::*kan*, polar, Sm^r Km^r	23
UR213	*draT3*, nonpolar with 15-bp insertion, Sm^r	23
UR214	*draG4*::*kan*, polar, Sm^r Km^r	23
UR215	*draG5*, nonpolar with 24-bp insertion, Sm^r	23
Plasmids		
pUC19	Ap^r, pMB1 replicon	36
pBSKS(−)	Ap^r, pMB1 replicon	Stratagene
pSUP202	Ap^r Tc^r Cm^r, pMB1 replicon	33
pRK404	Tc^r, RK2 replicon	7
pRK2013	Km^r, Tra^+, ColE1 replicon	11
pUC4K	Km^r gene from Tn*903* in pBR322, Ap^r Km^r	35
pYPZ103	*A. brasilense draTG* (6.7-kb *Sal*I fragment) in pUC19, Ap^r	This study
pYPZ106	*A. brasilense draTG* (5.9-kb *Sal*I-*Xho*I fragment) in pRK404, Tc^r	This study

mycin per liter; for *Escherichia coli*, 100 mg of ampicillin, 50 mg of kanamycin, 25 mg of chloramphenicol, and 12.5 mg of tetracycline per liter.

DNA hybridization and other DNA methods. Colony/plaque Screen hybridization membrane (NEN Research Products, Boston, Mass.) was used for colony hybridization according to manufacturer's specifications. For Southern hybridization, electrophoretically separated DNA was transferred to GeneScreen membranes (NEN) by alkaline transfer (31). Hybridization conditions (26), washing conditions (14), and probe labeling (10) were as described previously. For isolation of *A. brasilense* DNA, cells from a 2.5-ml culture grown overnight in LD medium with appropriate antibiotics were harvested by centrifugation. The pellet was washed with 100 mM Tris (pH 8.0)–1 mM EDTA–100 mM NaCl, resuspended in 500 μl of 50 mM Tris (pH 8.0)–20 mM EDTA–2 mg of lysozyme per ml, and incubated at 37°C for 1 h. Then 0.5% sodium dodecyl sulfate and proteinase K (250 μg/ml) were added and incubated at 50°C for 5 h. The lysate was extracted with phenol, and DNA was precipitated with ethanol as described previously for *R. rubrum* (12) and resuspended in 200 μl of glass-distilled water. Transformation was carried out by the method of Hanahan (16). A Geneclean Kit (Bio 101 Inc., La Jolla, Calif.) was used for purification of specific DNA fragments separated on agarose gel. Other DNA manipulations were performed by standard methods (26).

Cloning of the *draTG* gene region. Total DNA from *A. brasilense* was digested with *Sal*I and probed with the *draTG* gene of *A. lipoferum* (4.5-kb *Bam*HI-*Eco*RI fragment from pHAF102 [13]), revealing a 6.7-kb region of hybridization. This region of the gel was excised, and the fragments were ligated into pUC19 digested with the same enzyme. Transformants containing insert DNA (white colonies on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside plates containing ampicillin) were screened by colony hybridization with the probe described above. A plasmid from one such transformant, termed pYPZ103, was isolated; the restriction map of the insert is shown in Fig. 1.

Construction of a *draTG*$^+$ vector and its mobilization into *R. rubrum*. A *Sal*I-*Xho*I fragment containing *draTG* of *A. brasilense* from pYPZ103 (Fig. 1) was cloned into the broad-host-range plasmid pRK404, producing pYPZ106. pYPZ106 and pRK404 were mobilized from *E. coli* DH5α (GIBCO BRL, Gaithersburg, Md.) to *R. rubrum* by the methods of Liang et al. (23) as follows. Midlog-phase *E. coli* DH5α containing pRK404 or pYPZ106 and *E. coli* DH5α containing helper plasmid pRK2013 were mixed with fresh overnight *R. rubrum* cells in a microcentrifuge tube (100 μl of each). After centrifugation, the pellet was washed and resuspended in 50 μl of SMN (12) medium, spread on a sterile membrane filter (0.45-μm pore size, 25-mm diameter; Gelman Sciences Inc., Ann Arbor, Mich.) on an SMN plate and incubated at 30°C for 24 h. Cells were washed off with 1 ml of SMN medium, diluted, and plated on selective SMN plates containing streptomycin and tetracycline. Sm^r Tc^r *R. rubrum* colonies were purified for further characterization.

Mutagenesis of *draTG* region and reintroduction into *A. brasilense*. pYPZ103 was partially digested with *Hin*cII or *Nae*I and ligated with a 1.4-kb *Hin*cII fragment of the Km^r cassette gene from pUC4K. Km^r transformants from these ligations contained plasmids with the cassette inserted in one of three *Hin*cII sites and in one *Nae*I site (Fig. 1). Another had a deletion of a 1.2-kb *Hin*cII fragment, including part of *draT*, and the region was replaced with the cassette gene (Fig. 1). *Sal*I fragments containing these mutagenized regions were separately subcloned into pSUP202 at the *Sal*I site.

These plasmids were transformed into *E. coli* S17-1 (33) and conjugated into *A. brasilense* by the method described previously for *R. rubrum* (23), with some modifications. *E. coli* S17-1 donor strains carrying each of the five different plasmid constructs were grown in LB medium (26) containing ampicillin, kanamycin, and chloramphenicol at 37°C

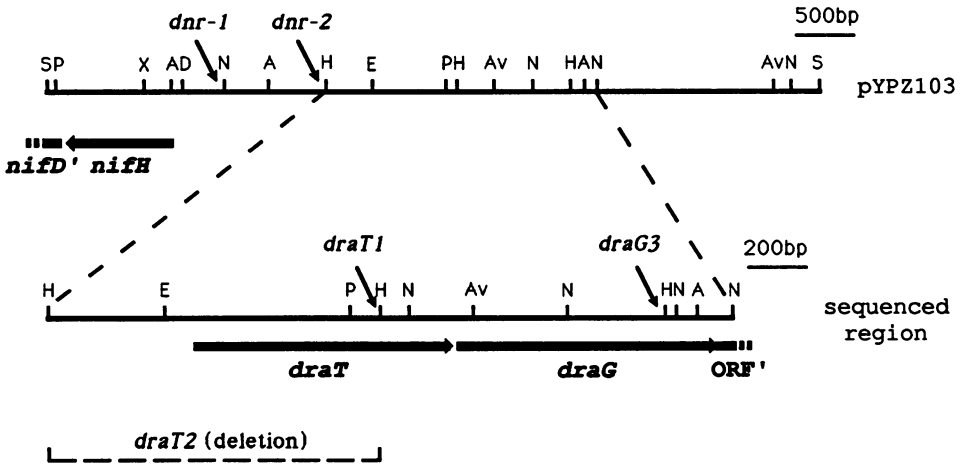

FIG. 1. Restriction map of the *draTG* region of *A. brasilense* Sp7. Restriction sites: A, *Acc*I; Av, *Ava*I; D, *Dra*I; E, *Eco*RI; H, *Hin*cII; N, *Nae*I (not all sites are shown); P, *Pst*I; S, *Sal*I; X, *Xho*I. The top line is a restriction map of the *Sal*I fragment cloned into pYPZ103, and the bottom line shows the sequenced region. *draT*, *draG*, and open reading frame (ORF) regions were identified through the DNA sequence (Fig. 2). The *nifH* and *nifD'* regions were identified by Southern hybridization and published DNA sequence of these genes (5, 9). The sites of introduced mutations are indicated by arrows except for the *draT2* deletion, which is shown by the dashed line at the bottom.

overnight. These cultures were diluted 1:5 into fresh LB without antibiotics and grown for 2 to 3 h. the *A. brasilense* recipient was grown overnight in LD medium containing ampicillin and nalidixic acid at 30°C; the cultures were then diluted 1:5 into fresh LD medium without antibiotics and grown for 2 to 3 h. Then 0.3 ml of *E. coli* and 1.5 ml of *A. brasilense* cells were separately collected in microcentrifuge tubes, and the pellets were washed with LD medium. Cells were mixed together in 50 μl of LD medium and spread on a 0.45-μm-pore-size membrane filter (see above) set on an LD plate. After 30 h of growth at 30°C, cells were washed from the filter with 1 ml of LD medium, diluted, and plated on LD plates containing nalidixic acid and kanamycin.

Nx^r Km^r *A. brasilense* colonies were picked and replica printed to identify Cm^s colonies resulting from a double-crossover recombination event. Such events reflect replacement of the wild-type region with the mutant one, rather than integration of the entire plasmid. Cm^s colonies were rather commonly found (typically 10 to 30% of Nx^r Km^r colonies). In contrast, resolution of merodiploid Nx^r Km^r Cm^r strains (the plasmid integrants from single-crossover events) to a Nx^r Km^r Cm^s phenotype was extremely rare ($<10^{-3}$), so this approach was not used for mutant construction.

DNA sequencing. A 2.4-kb *Hin*cII-*Nae*I fragment containing the *draTG* gene from pYPZ103 (Fig. 1) was cloned into pBSKS(−) for DNA sequencing. The Erase-a-Base system (Promega Corp., Madison, Wis.) for nested deletion with exonuclease III was used to generate sets of deletion mutants from both directions. The DNA sequences were determined for both strands by the dideoxy method (32) with a Sequenase version 2 sequencing kit (U.S. Biochemical Corp. Cleveland, Ohio). Because of the high G+C content of *A. brasilense* DNA (34), a highly denaturing formamide gel was used as recommended by U.S. Biochemical. Some serious compressions were resolved by the substitution of dITP for dGTP in the sequencing reaction. The DNA sequence was analyzed by using the software of the University of Wisconsin Genetics Computer Group (4).

Nucleotide sequence accession number. The nucleotide sequence reported in this paper is in the GenBank data base under accession number M87319.

RESULTS AND DISCUSSION

Location of the *dra* and *nif* genes in the cloned region. The *dra*-hybridizing region was cloned by hybridization with an *A. lipoferum* probe (Fig. 1). The 2.8-kb *Sal*I-*Eco*RI fragment of pYPZ103 contains the functional *nifH* gene, since the restriction map of this region is identical to that published for the *nifHDK* region from the same strain (5, 9), it hybridizes to a *nifH* probe, and a cassette insertion renders the strain Nif^- (data not shown). The *nifH* gene is also found near *draTG* in both *R. rubrum* and *A. lipoferum* (12, 13).

Whereas some hybridization with *K. pneumoniae nifJ* was found in a region between *draT* and *nifH* in *A. lipoferum* (13), none was detected in the cloned *A. brasilense* region with a *K. pneumoniae nifJ* probe (3.7-kb *Sal*I fragment from pJC372 [3]) (data not shown), consistent with another report (15).

DNA sequence of *dra* region. The 2.4-kb *Hin*cII-*Nae*I fragment was completely sequenced on both strands. Analysis of the sequenced region revealed open reading frames appropriate for *draT* and *draG* and a potential open reading frame next to *draG* (Fig. 1; data not shown). A codon preference plot, reflecting codon usage in the *A. brasilense* *nifH* (5) and *glnB* (6) genes, showed good codon choice for these three open reading frames (data not shown) and reasonable G+C preference of 91% in the third position.

The 888-bp *draT* gene would encode a 33.7-kDa protein. In comparison with the same gene of *R. rubrum*, *draT* shows 52% identity and 70% similarity at the amino acid level and 65% identity at the nucleic acid level. Within *draT*, there seem to be several regions of extreme conservation, probably reflecting the involvement of those regions of the gene product in its catalytic function or its regulation. In *R. rubrum*, *draT* uses the unusual initiation codon of TTG (based on N-terminus determination [12]), but *A. brasilense* *draT* does not have a TTG at the same position.

The 894-bp *draG* gene would encode a protein of 32.4 kDa, consistent with the published 33.5-kDa size (24). It shows 62% identity and 77% similarity at the amino acid level and 70% identity at the nucleic acid level to *draG* of *R. rubrum*. The comparison of the *draTG* sequence with GenBank

sequences showed significant similarity only to the *draTG* genes of *R. rubrum*; they were not similar to the ADP-ribosyltransferases of bacterial toxins in the data banks.

Only a short region downstream of the 3'end of *draG* has been sequenced thus far; it contains the first 14 codons of an open reading frame that shows extensive similarity (79% identity and 86% similarity) to the appropriate portion of the predicted 15-kDa product encoded in the same position in *R. rubrum*. In *R. rubrum*, the product of this open reading frame appears to have a secondary role in the regulation of the DRAG-DRAT system (23).

There are three different open reading frames in the region immediately upstream of *draT*, but all have a high level of unusual codons, suggesting that they are not coding regions.

Generation and physiological characterization of constructed *A. brasilense* mutants. To identify the functionality of this region of the *A. brasilense* chromosome, the *draTG* region and that between *draT* and *nifH* were mutagenized by Km^r cassette insertions or by a specific deletion as described in Materials and Methods. All mutants were verified by Southern analysis.

In *R. rubrum* the physiological characterization of *dra* mutants has shown the in vivo functionality of the gene products and the fact that DRAT is itself subject to post-translational regulation (23). To answer similar questions about *A. brasilense*, the wild type and five constructed mutants were derepressed for nitrogenase in a NfbHP-glutamate medium, treated with different concentrations of ammonium, and monitored for nitrogenase activity. Wild-type *A. brasilense* showed rapid and complete loss of whole-cell nitrogenase activity, which was recovered when the ammonium was metabolized (Fig. 2A). The recovery time depended on the concentration of ammonium used. These results are similar to the response of *R. rubrum* to light-dark shifts, except *R. rubrum* always retains approximately 15% of the nitrogenase activity under all conditions and seems to display a slower response to modification signals (23).

A. brasilense UB2 (*draT1*) and UB3 (*draT2*) showed no loss of nitrogenase activity when treated with 1 and 2 mM NH_4Cl (Fig. 2B; data not shown), consistent with the loss of DRAT function and its requirement for the regulatory response. The slight initial decrease in nitrogenase activity was probably caused by a perturbation of the dissolved oxygen concentration during ammonium addition, since a control with added water behaved similarly.

When treated with lower concentrations of ammonium (0.25 and 0.5 mM NH_4Cl), *A. brasilense* UB4 (*draG3*) completely lost nitrogenase activity and no recovery was detected over 90 min, consistent with a nonfunctional DRAG and the resultant inability to activate modified dinitrogenase reductase (Fig. 2B; data not shown). As in the case of *R. rubrum* mutants affected in *draG*, strain UB4 displays active dinitrogenase reductase under derepression conditions, suggesting that DRAT is not active under these conditions.

For identification of possible functions encoded between *draT* and *nifH*, two mutants, *A. brasilense* UB5 (*dnr-1*) and UB6 (*dnr-2*), with mutations in this region were generated (Fig. 1). There was no significant difference between these mutants and wild type in nitrogenase activity or in response to ammonium (Fig. 2B). Any products encoded by this region are therefore not essential for nitrogenase activity and its regulation, although more subtle involvement is possible.

Expression of *A. brasilense draTG* in *R. rubrum*. To verify further the functionality of the cloned genes, pYPZ106 (containing the *draTG* genes of *A. brasilense*) and pRK404

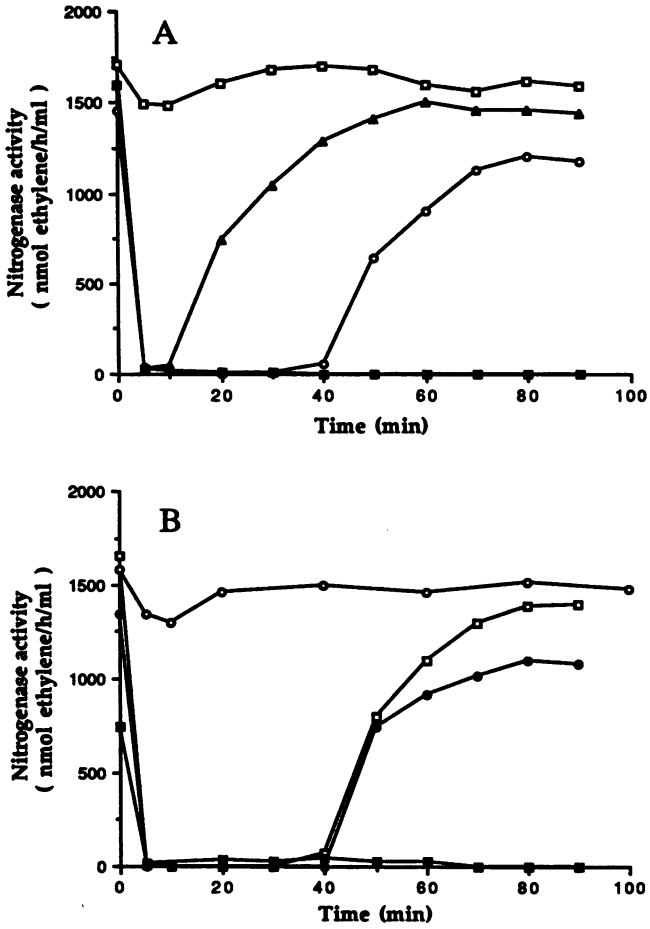

FIG. 2. (A) Regulation of nitrogenase activity by ammonium addition in the *A. brasilense* Sp7 wild type. Cells were derepressed for nitrogenase for 16 to 20 h before time zero, when ammonium chloride was added. Samples of 1 ml of culture were withdrawn and assayed for nitrogenase activity at the time points indicated. Ammonium chloride was added to a final concentration of 0.5 mM (▲), 1 mM (○), or 2 mM (■). An equivalent volume of water was added to the control (□). Nitrogenase activity is expressed as nanomoles of ethylene produced per hour per milliliter of cells at an optical density at 600 nm of 5 (approximately equal to 1.5 mg of protein); for these assays, the optical densities of all cultures were between 5.0 and 6.0. (B) Regulation of nitrogenase activity by ammonium in *A. brasilense* mutants. Ammonium chloride was added at time zero to the following concentrations: 2 mM for UB2 (*draT1*) (○), 0.25 mM for UB4 (*draG3*) (■), 1.0 mM for UB5 (*dnr-1*) (□), and 1.0 mM for UB6 (*dnr-2*) (●).

were transferred into *R. rubrum dra* mutants UR212, UR213, UR214, and UR215 (23). Regulation of nitrogenase activity by darkness and light in these transconjugants is shown in Table 2. UR212 and UR213 (*draT* mutants) carrying pRK404 (negative control) showed no loss of whole-cell nitrogenase activity after exposure to darkness, as reported for the same strains without the plasmid (23). In contrast, the presence of pYPZ106 (*dra*$^+$ from *A. brasilense*) restored the ability to regulate nitrogenase activity in response to darkness. UR214 and UR215 (mutants affected in *draG*) containing pRK404 showed no recovery of nitrogenase activity after reillumination, again consistent with the published report, but pYPZ106 (*dra*$^+$) also restored this regulatory response.

Since *A. brasilense* is a nonphotosynthetic bacterium, it is

TABLE 2. Effects of darkness and light on nitrogenase activity in *R. rubrum* transconjugants

Strain	Plasmid	Nitrogenase activity[a] (% of original activity)		
		Initial[b]	Darkness[c]	Reillumination[d]
UR2 (wild type)	pRK404 (*dra*$^-$)	1,570	240 (15)	1,760 (112)
UR212 (*draT2*, polar)	pRK404 (*dra*$^-$)	950	880 (93)	1,020 (107)
UR213 (*draT3*, nonpolar)	pRK404 (*dra*$^-$)	1,050	1,210 (115)	1,180 (112)
UR214 (*draG4*, polar)	pRK404 (*dra*$^-$)	720	80 (11)	90 (13)
UR215 (*draG5*, nonpolar)	pRK404 (*dra*$^-$)	750	150 (20)	180 (24)
UR212	pYPZ106 (*dra*$^+$)	840	280 (33)	870 (104)
UR213	pYPZ106 (*dra*$^+$)	800	230 (29)	800 (100)
UR214	pYPZ106 (*dra*$^+$)	910	210 (23)	980 (108)
UR215	pYPZ106 (*dra*$^+$)	670	190 (28)	800 (119)

[a] Units of nitrogenase activity: nanomoles of ethylene produced per hour per milliliter of cells at an optical density at 680 nm of 1 (approximately equal to 0.15 mg of soluble protein).
[b] Nitrogenase activity before treatment with darkness.
[c] Nitrogenase activity after 40 min of darkness.
[d] Nitrogenase activity after 20 min of reillumination.

interesting that its *draTG* gene products also can respond to darkness and light in this heterologous background. Although there have been several studies of signal transduction to this system, including the effects of energy charge, pyridine nucleotide pools, and amino acid pools (18, 19, 22, 27, 28), the small molecules and protein factors involved are unknown. The most striking difference between the response of these transconjugants and that of the *R. rubrum* wild type was that the transconjugants possessed a slightly higher percentage of residual nitrogenase activity. They also showed a slower response to darkness and a faster response to ammonium than did wild-type *R. rubrum* (data not shown).

Conclusion. Our mutational analysis of the *draTG* region of *A. brasilense* confirms the function of these genes in the regulation of nitrogenase activity. It also demonstrates that the *draTG* genes are not essential for growth, nor are there duplicated genes or redundant functions in this organism. Although the general behavior of the DRAG-DRAT system in *A. brasilense* is similar to that seen in *R. rubrum*, there are a number of interesting differences in the timing and extent of the regulatory response that make the comparative analysis of the two systems important.

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