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Identification of an Alternative Nitrogenase System in *Rhodospirillum rubrum*

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A second nitrogenase activity has been demonstrated in *Rhodospirillum rubrum*. This nitrogenase is expressed whenever a strain lacks an active Mo nitrogenase because of physiological or genetic inactivation. The alternative nitrogenase is able to support growth on N_2 in the absence of fixed N. V does not stimulate, nor does Mo or W inhibit, growth or activity under the conditions tested. The proteins responsible for this activity were identified by electrophoretic and immunological properties. The synthesis of these proteins was repressed by NH_4^+. The alternative nitrogenase reductase is ADP ribosylated in response to darkness by the system that regulates the activity of the Mo nitrogenase. The genes for the alternative nitrogenase have been cloned, and the alternative nitrogenase reductase has been expressed in an in vitro transcription-translation system.

The nitrogen-fixing bacterium *Azotobacter vinelandii* has been shown to have three different nitrogenases that are expressed under different growth conditions. The first and best-characterized nitrogenase is the classical Mo-containing nitrogenase encoded by the *nifHDK* genes. The second, a V-containing enzyme encoded by the *vnfHDGK* genes (12), is detected in wild-type *A. vinelandii* in the presence of V and the absence of Mo (8). This enzyme is also found in *Azotobacter chroococcum* (28). When both Mo and V are absent from the medium, a third system, encoded by the *anfHDGK* genes (11) and believed to contain only Fe in its cofactor (4), is derepressed. This enzyme has not been found in *A. chroococcum*.

The rationale for the presence of more than one nitrogenase in an organism is not clear. It is possible that metal availability plays a role here. Having a non-Mo-containing nitrogenase in environments where Mo is biologically limiting, such as soils in the tropics and southeastern United States (1), would allow an organism to still obtain fixed nitrogen from N_2. Support for this hypothesis is provided by the metal regulation of the three systems in *A. vinelandii*. A clear preference is demonstrated: Mo represses the expression of the second and third systems, and V represses the expression of the third system (10).

In *Rhodospirillum rubrum*, regulation of nitrogenase occurs at a level not seen in *A. vinelandii* or *A. chroococcum*. This regulation, a reversible posttranslational ADP ribosylation of dinitrogenase reductase (Rr2, for *R. rubrum* component II) (13, 19, 25), is carried out by two enzymes, dinitrogenase reductase ADP-ribosyl transferase (DRAT) (17) and dinitrogenase reductase-activating glycohydrolase (DRAG) (29), that respond to the levels of light and fixed N sensed by the cell (13). DRAT modifies Rr2 in response to darkness or fixed N and thereby inactivates the enzyme (21). When the ammonia is exhausted or light is again present, DRAG removes the ADP-ribose group and the enzyme is activated (13).

R. rubrum was not thought to have a second nitrogenase, but in the course of characterizing several mutations in *nifH* a second nitrogenase activity was noted. The conditions for the expression of this activity and the proteins responsible for it were investigated. The regulation of this system through posttranslational modification was characterized, and the gene for the second nitrogenase reductase was cloned and expressed in an in vitro system.

MATERIALS AND METHODS

Strains and growth. The strains used in this study are listed in Table 1. *R. rubrum* strains were grown in minimal medium anaerobically and photosynthetically before derepression. Minimal medium contains the following (per liter): 10.5 g of MOPS (morpholinopropanesulfonic acid), 4 g of malic acid, 1 g of NH_4Cl, 2.8 mg of H_3BO_4, 20 mg of disodium EDTA, 4 mg of ferric citrate, 1 mg of Na_2MoO_4, 600 mg of KH_2PO_4, 900 mg of K_2HPO_4, 250 mg of $MgSO_4$, 100 mg of $CaCl_2$, and 1 μg of biotin (pH 7.0). Nitrogenase derepression medium is minimal medium with 4 g of glutamic acid substituted for NH_4Cl and 0.75 g instead of 10.5 g of MOPS per liter. Cultures were derepressed by diluting minimal medium-grown cells 1:50 into derepression medium in anaerobe tubes with black butyl rubber stoppers and aluminum crimps (Bellco, Vineland, N.J.), flushing the headspace with Ar, and incubating the cultures anaerobically with illumination of 25 W/m^2 at 28°C until the cultures reached an optical density at 680 nm of 1 to 1.2 as measured on a Spectronic 21 spectrophotometer (Bausch & Lomb, Rochester, N.Y.). To determine growth under nitrogen-free conditions, cells were grown in minimal medium and washed in the nitrogen-free medium (minimal medium without NH_4Cl or MOPS), inoculated into 15 ml of nitrogen-free medium in a stoppered and crimped 110-ml vial, flushed with N_2 under sterile conditions and incubated in the light as described above. ADP ribosylation of derepressed cultures was initiated by the removal of the culture to darkness for 1 h. Derepression of nitrogen fixation was assayed in vivo by monitoring whole-cell acetylene reduction (13). Antibiotic levels for *R. rubrum* were 100 μg of streptomycin and 10 μg of kanamycin per ml in minimal medium. When used, $MnCl_2$, $NiCl_2$, $CuCl_2$, $ZnSO_4$, $CoCl_2$, K_2CrO_4, and VSO_4 were added at 10 μM; 1 mM Na_2WO_4 was used to inhibit nitrogenase.

Characterization of strains. The two-dimensional (2-D) gel electrophoresis protocol was that of Roberts et al. (26).

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TABLE 1. Strains and plasmids

Strain or plasmid	Relevant traits	Source or reference
R. rubrum		
UR2	Sm^r *nif*$^+$	14
UR201[a]	Sm^r *nifD1067*::*kan*	20
UR206[a]	Sm^r *nifH1068*::*kan* derivative of UR156 lacking pPH1JI	14
UR212	Sm^r *draT2*::*kan*	23
UR249[a]	Sm^r *nifH1071*	15
UR250[a]	Sm^r *nifH1072*	15
Plasmids		
pLWH3	pUC19 containing *A. vinelandii anfH*	11
pJWD3	pUC9 containing *A. vinelandii anfD*	11
pLAM3	pTZ19 containing *A. vinelandii vnfHDGK*	24
pLL101	pUC19 containing *R. rubrum nifH'DK'*	14
pTZ19	Ap^r	Pharmacia
pUC19	Ap^r	32
pBSKS(−)	Ap^r	Stratagene

[a] Lacks Mo nitrogenase activity.

TABLE 2. Protein accumulation

Strain	Medium[a]	Nitrogenase activity[b]	Accumulation of[c]:			
			Rr2	Rr1	Rr2*	Rr1*
UR2	MGC	1,010	+	+	−	−
UR249	MGC	51	+[d]	+	+	+
UR250	MGC	38	+[d,e]	+	+	+
UR206	MGC	98	−	−	+	+
UR201	MGC	ND[f]	+	−	+	+
UR2	MN$^-$	ND	+	+	−	−
UR2	MN$^-$ + WO_4^{2-}	ND	+	+	+	+
UR206	MN$^-$	ND	−	−	+	+
UR206	MN$^-$ + WO_4^{2-}	ND	−	−	+	+

[a] MGC, derepression medium; MN$^-$, nitrogen-free medium.
[b] Nanomoles of acetylene reduced per hour per milliliter of cells at an optical density at 680 nm of 1 (approximately equal to 0.15 mg of soluble protein).
[c] Based on appearance in 2-D gels.
[d] Inactive Rr2.
[e] Charge-changed Rr2.
[f] ND, not determined.

Proteins were labeled with 15 μCi of Tran^{35}S-label, a metabolic labeling reagent containing L-[^{35}S]methionine and L-[^{35}S]cysteine (ICN, Irvine, Calif.), per ml. Samples were prepared as described previously (3). The isoelectric points of the denatured proteins in a urea isoelectric focusing gel were determined by slicing a tube gel into 4-mm sections, soaking these sections in 0.5 ml of 6 M urea for 2 h at room temperature, and then measuring the pH. The protocol for Western immunoblots was that of Towbin et al. (30).

DNA methods. The standard DNA methods of Maniatis et al. (22) were employed. Probes were labeled by using the method of Feinberg and Vogelstein (6). Southern transfers of electrophoretically separated restriction digests were performed by using Genescreen II membranes (NEN, Boston, Mass.) and NaOH as the transfer medium (9). The lambda EMBL4 library of *R. rubrum* was previously described (7). Plasmids used for S-30 transcription-translation were purified by using Tip-20 columns (Qiagen, Studio City, Calif.). S-30 extracts from *Rhodobacter sphaeroides* were prepared by the protocol of Chory and Kaplan (5). The protein products of the in vitro transcription-translation reaction were labeled with [^{35}S]methionine (ICN).

In vitro methods. Crude extracts of *R. rubrum* were prepared by sonication (31). To determine the modifiability of dinitrogenase reductase, crude extracts were labeled in vitro with ^{32}P-NAD (18). Published methods were used to assay extracts for DRAT activity (16). DRAG assays were performed as described by Saari et al. (29).

RESULTS

Derepression of a second nitrogenase activity. UR249, a Nif$^-$ strain that contains a nonpolar, 1-bp change (Gly-100 to Ala-100) in *nifH*, displayed acetylene reduction activity of 51 nmol · h^{-1} · ml^{-1} of whole cells when derepressed in derepression medium for 48 h. Acetylene reduction activity was also detected in the two other Nif$^-$ strains examined: UR206, a *nifH* insertion mutant, and UR250, a *nifH* nonpolar point mutant (Arg-101 to Val-101) (Table 2). This second nitrogenase activity was not detected when these strains were originally characterized (14, 15). It was observed only after a change in the derepression medium formulation (the concentration of MOPS was lowered).

This acetylene reduction activity was detected in medium containing 4 μM Mo, the normal amount used in derepressing the *nif* regulon but a level sufficient to block the synthesis of *vnf* and *anf* gene products in *A. vinelandii* (2, 27). Omitting Mo from the medium did not stimulate the activity. No significant stimulation or inhibition of activity in UR206 was seen upon the addition of salts of Mn, Ni, Cu, Zn, Co, W, Cr, or V. The lack of inhibition by WO_4^{2-} is in contrast to the inhibitory effect of WO_4^{2-} on the activity of the Mo-containing nitrogenase previously described in *R. rubrum* (20). Removing EDTA from derepression medium in the presence or absence of metals listed above also did not stimulate the acetylene reduction activity of UR206.

Growth in derepression medium does not require that a strain be Nif$^+$, so the ability of this second nitrogenase activity to support growth in a medium containing N_2 as the sole source of N was tested as follows. UR206, which lacks the Mo nitrogenase, was inoculated into nitrogen-free medium with a headspace of N_2 and was found to grow with a doubling time of 14.7 h. The culture reduced acetylene at the rate of 24 nmol · h^{-1} · ml^{-1} of whole cells. WO_4^{2-} (1 mM) did not inhibit growth or activity, nor did VSO_4 stimulate them. Since WO_4^{2-} does inhibit the known nitrogenase from *R. rubrum*, the growth of the wild type (UR2) was examined on nitrogen-free medium under N_2 with and without 1 mM Na_2WO_4. WO_4^{2-} slowed the doubling time of UR2 from 6 h to 8 h, and the maximal activity was approximately the same as that of UR206 under the same conditions. When these two strains were derepressed in derepression medium with and without WO_4^{2-}, the same effects on activity were noted.

Correlation of the new nitrogenase activity and the presumptive second nitrogenase proteins. When strains were derepressed for the second nitrogenase activity and subjected to 2-D gel analysis, three new major proteins were observed (spots a, b, and c in Fig. 1). These proteins were synthesized and accumulated only after derepression. When UR249 (Fig. 1), UR250, and UR2 (in WO_4^{2-}-containing medium) were derepressed, both the previously identified nitrogenase proteins and the newly observed proteins were

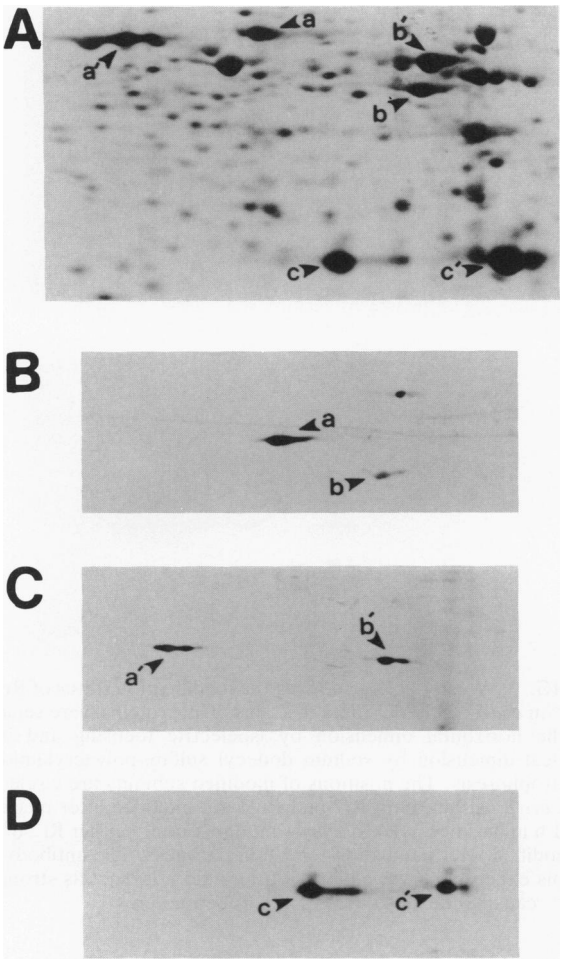

FIG. 1. 2-D gel and Western blot analyses of nitrogenase proteins. Panel A is an autoradiogram of derepressed UR249. The letter designations are as follows: a′ and b′, subunits of Rr1; c′, Rr2; a and b, subunits of Rr1*; and c, Rr2*. Panels B (UR206), C (UR2), and D (UR2 and UR206) are Western blots of 2-D gels. The same letter codes are used. Due to reduced recognition of Rr1* by the antibody against Rr1, more protein was used for the blot in panel B, and the background is correspondingly darker. Antibodies used in panels B and C were raised against Rr1; those used in panel D were against Rr2.

present, arguing that the two sets of proteins are different (Table 2). Also, the dinitrogenase reductase of UR250 is charge changed due to a point mutation, but none of the three new proteins is charge changed, implying that these new proteins are not altered *nif* gene products. The three new proteins are also present in UR206, which does not synthesize or accumulate the Mo nitrogenase components.

When Western blots were done with samples derepressed for the second nitrogenase activity, proteins a and b cross-reacted with a polyclonal antibody to dinitrogenase (Rr1, for *R. rubrum* component I) and protein c cross-reacted with an antibody to dinitrogenase reductase (Rr2) (Fig. 1). The degree to which the proteins cross-reacted varied. Protein a was much more cross-reactive than protein b with the Rr1 antibody. This may be due to greater conservation of protein structure in protein a than in protein b, or it may be due to the composition of the polyclonal antibody, reflecting a smaller number of denaturation-resistant epitopes that are shared. The proteins that are homologous to Rr1 are referred to as Rr1*, and the protein that is homologous to Rr2 is referred to as Rr2*.

Rr2* and the two components of Rr1* are similar in molecular weight to Rr2 and Rr1, respectively. Rr2* has the same molecular weight as Rr2 but is shifted approximately 0.3 pH unit (6.6 to 6.9). The two denatured subunits of Rr1* have pIs of 7.0 and 6.8; the pIs of the two subunits of Rr1 are 7.3 and 6.8.

Regulation of synthesis of the presumptive second nitrogenase proteins. As noted in Table 2, Rr1* and Rr2* were synthesized in a variety of Nif^- mutants. The proteins were synthesized when Rr1 was not made (UR201), when inactive Rr2 was accumulated (UR249 and UR250), and when neither Rr1 nor Rr2 was made (UR206). The proteins of the second system were also synthesized when Rr1 activity was blocked by the addition of WO_4^{2-}. There thus appears to be no absolute requirement for the presence or the absence of either Rr1 or Rr2 in the derepression of the second system. The characteristic of all strains displaying the second system is a lack of an active Mo nitrogenase system because of physiological or genetic inactivation.

The synthesis of the second system reacted like the first system to several environmental stimuli. When UR249 was exposed to 2 mM NH_4^+ for 1 h, synthesis of new Rr1* and Rr2* ceased (Fig. 2). When the same strain was subjected to 1 h of darkness, overall protein synthesis was greatly decreased, but Rr1* and Rr2* were still the major newly synthesized proteins (Fig. 2). The identical results were obtained with UR206 (data not shown). The synthesis of Rr1 and Rr2 was similar to that of Rr1* and Rr2* in UR249 (Fig. 2) and in UR2 (data not shown).

Curiously, when the time course of derepression of UR206 in derepression medium was studied, a significant lag was noted between enzyme accumulation and whole-cell activity. Synthesis of Rr1* and Rr2* was seen by 16 h; significant accumulation of these proteins was detectable, as judged by Coomassie staining, by 25 h. However, significant acetylene reduction activity was not detected until 48 h. The reason for this delay is unclear.

Posttranslational regulation. When derepressed *R. rubrum* cells are exposed to darkness, Rr2 is inactivated by the covalent addition of ADP-ribose (25). UR206 was derepressed and then placed in the dark for 1 h. The analysis of samples before and after exposure to darkness by 2-D gel electrophoresis and Western blotting showed the appearance of a second anti-Rr2-cross-reactive protein that was slightly larger and more acidic than Rr2* (Fig. 3). This protein showed an increase in molecular weight with respect to Rr2* similar to that seen when Rr2 is ADP ribosylated (Fig. 3). To test for ADP ribosylation of Rr2*, an extract of derepressed UR206 was incubated with ADP, Mg^{2+}, and ^{32}P-NAD. After 2-D gel electrophoresis and autoradiography, one spot was detected that corresponded to the position of the darkness-induced protein visualized by Western blotting (data not shown). The characteristics of this dark-induced protein, namely, its increased molecular weight, its cross-reaction with anti-Rr2 antibodies, and its incorporation of ^{32}P derived from ^{32}P-NAD, argue that Rr2* is ADP ribosylated in response to darkness, as is Rr2.

To determine the identity of the enzyme involved in the ADP ribosylation of Rr2*, a mutant (UR212) lacking DRAT (because of an insertion in *draT*, the structural gene for DRAT) was derepressed in the presence of WO_4^{2-} and then incubated in the dark for 1 h. Western blot analysis demon-

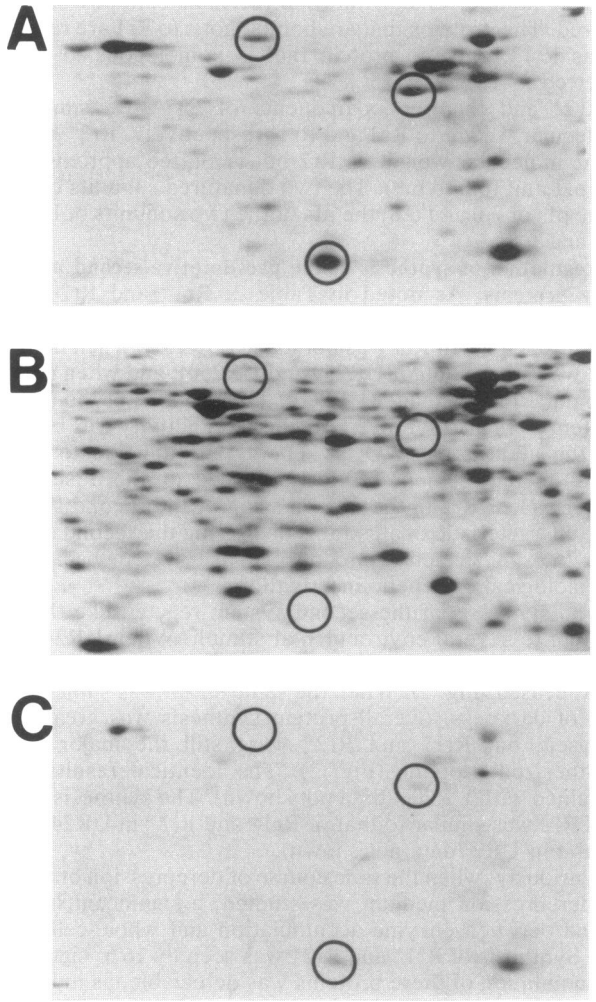

FIG. 2. Autoradiograms of 2-D gels showing the regulation of the synthesis of the alternative nitrogenase. The circles mark the positions of Rr1* (upper two spots in each panel) and Rr2*. Rr1 and Rr2 are also present but are not circled. (A) Synthesis in derepressed UR249. Rr1* and Rr2* are present. (B) Synthesis in derepressed UR249 1 h after the addition of 2 mM NH_4^+. Rr1* and Rr2* are no longer synthesized. (C) Synthesis in derepressed UR249 1 h after a shift to darkness. Overall protein synthesis is lowered, but Rr1* and Rr2* are still synthesized. For unknown reasons, the left subunit of Rr1* is often difficult to visualize.

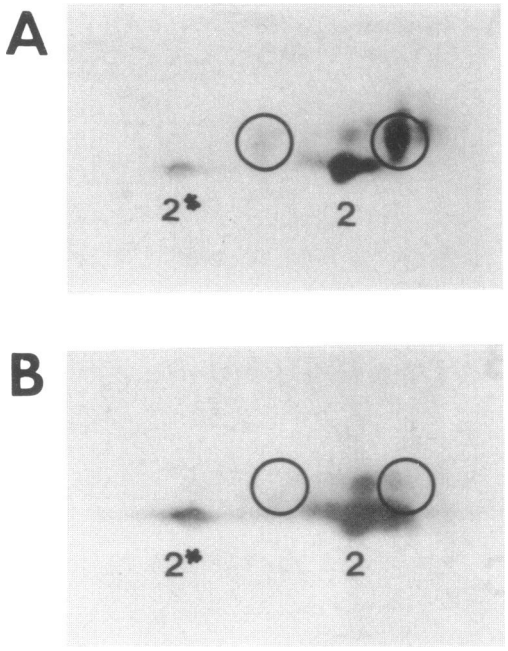

FIG. 3. Western blots showing the modification status of Rr2 and Rr2* in *draT*$^+$ and *draT* mutant strains. The proteins were separated in the horizontal dimension by isoelectric focusing and in the vertical dimension by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The positions of modified subunits are circled. (A) In a *draT*$^+$ strain, both Rr2 and Rr2* are modified after incubation for 1 h in darkness. (B) In a *draT* mutant strain, neither Rr2 or Rr2* is modified after incubation for 1 h in darkness. The antibody used in this experiment was against Rr2 and did not react as strongly to Rr2*, causing the signal from Rr2* to be fainter.

strated that Rr2* was present but was not modified in response to darkness (Fig. 3). The same lack of modification was noted for the Rr2 present in this strain. This lack of modification was probably not due to the inhibition of DRAT by WO_4^{2-}, because both dinitrogenase reductases were modified in response to darkness in the presence of WO_4^{2-}. These results strongly suggest that DRAT is essential in the posttranslational modification of Rr2*.

In vitro nitrogenase activity. When crude extracts of derepressed UR206 were assayed for acetylene reduction under the conditions used for Mo nitrogenase, a low level of activity was seen (0.11 nmol · min^{-1} · mg of $protein^{-1}$). This activity was not increased when the extract was incubated with DRAG, suggesting that the low activity was not due to modification. Low acetylene reduction activity by alternative nitrogenases has been observed repeatedly (8, 24, 28).

Isolation of the gene encoding Rr2*. Two alternative dinitrogenase reductases have been cloned from *A. vinelandii*: *vnfH* and *anfH*. These two genes were used to probe chromosomal digests of *R. rubrum* UR2 and UR206 (Fig. 4). The *anfH* probe hybridized strongly to one or more fragments in each lane; it was detectable even after a 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) wash at 65°C. These fragment sizes were as follows: for *Eco*RI, 1.9 and 9 kb; for *Hin*dIII, 4.0 kb; and for *Bam*HI, 8 kb. These fragments did not coincide with those that hybridized to the *R. rubrum nifH* probe, including the cassette-mutagenized fragment of UR206. In the latter strain the *nifH*-hybridizing band increased in molecular weight, but the *anfH*-hybridizing band did not. When the *vnfH* probe was employed, the resulting fainter hybridizing bands corresponded to those seen with the *anfH* and *nifH* probes. When the *anfD* gene was used to probe these digests, the 9-kb *Eco*RI fragment, the 4.0-kb *Hin*dIII fragment, and the 8-kb *Bam*HI fragment were again observed. Additionally, two more *Hin*dIII bands (1.0 and 0.6 kb) and a *Bam*HI band (0.8 kb) were detected.

The *A. vinelandii anfH* gene was used to probe a lambda EMBL4 library of *R. rubrum*, and several phages were isolated that carried an *anfH*-hybridizing region. Further characterization of the phages by Southern hybridizational analysis demonstrated that the inserts corresponded to the chromosomal regions detected above. The 4.0-kb *Hin*dIII fragment was subcloned into pBSKS(−), creating pLL133. This plasmid contained a region of *R. rubrum* DNA hybridizing to the *anfHD'* sequence of *A. vinelandii*.

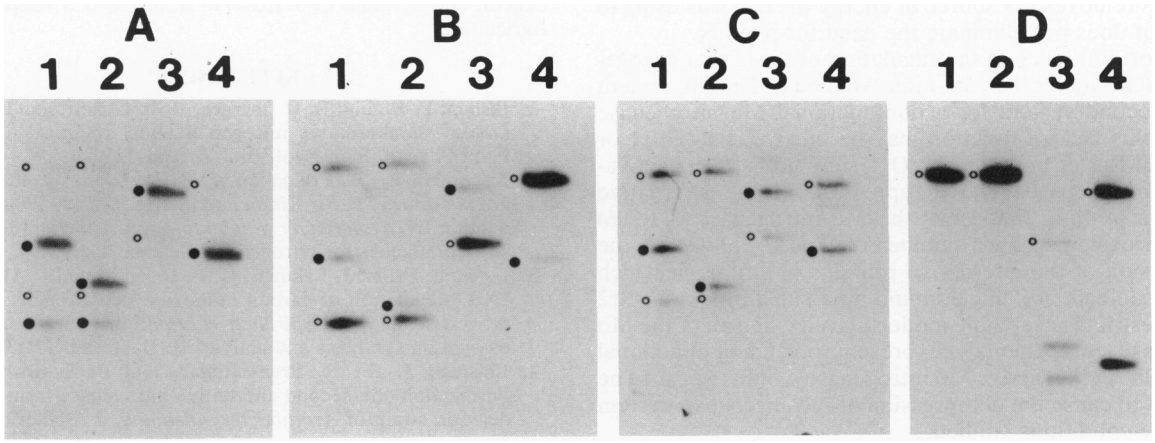

FIG. 4. Hybridization of *nifH*, *anfH*, *vnfH*, and *anfD* probes to the *R. rubrum* chromosome. The numbered lanes are the same in each panel. Lanes: 1, UR206 digested with *Eco*RI; 2, UR2 digested with *Eco*RI; 3, UR2 digested with *Hin*dIII; and 4, UR2 digested with *Bam*HI. (A) Hybridization pattern with *nifH* (pLL101) as a probe. The most intense bands detected with this probe are marked with closed circles on this and other panels for reference. (B) Pattern with *anfH* (pLWH3) as a probe. The most intense bands detected with this probe are marked with open circles on this and other panels for reference. There is a faint signal from the *nifH* bands (closed circles). (C) Pattern with *vnfH* (pLAM3) as a probe. This probe hybridized to the same fragments as those detected by *nifH* and *anfH*. (D) Pattern with *anfD* (pJWD3) as a probe. Some of the bands that hybridized with *anfH* were also detected here (open circles). In lanes 3 and 4, additional bands were detected, suggesting that the *anfH*- and *anfD*-homologous regions of *R. rubrum* are contiguous.

To determine whether pLL133 does carry the gene encoding Rr2*, an in vitro transcription-translation reaction was performed with an *R. sphaeroides* S-30 extract; the labeled products, spiked with unlabeled Rr2*-containing extracts, were separated on 2-D gels and transferred for Western blot analysis. The developed Western blot was then exposed to film. The major labeled product of the pLL133-containing reaction was one that coelectrophoresed with the Rr2* signal from the Western blot (Fig. 5). This product was not present in the vector or in DNA controls (data not shown).

DISCUSSION

R. rubrum has a second nitrogenase activity that was detected by acetylene reduction when the primary nitrogenase was nonfunctional. This in vivo acetylene reduction activity was very low compared with that in the wild type. For comparison, *K. pneumoniae* mutants possessing this percentage of wild-type acetylene reduction activity are phenotypically Nif$^-$ (26). However, the second nitrogenase system of *R. rubrum* is able to support growth on N_2 in the absence of fixed N, although the doubling time is longer. This disparity between acetylene reduction and nitrogen fixation is not an unusual situation, since all non-Mo nitrogenases characterized to date have been shown to be poor reducers of acetylene (2, 28). This poor acetylene reduction also is seen when the activity is measured in crude extracts. A contributing factor to this low acetylene reduction activity may be nonoptimal derepression conditions. We have only derepressed the alternative system in *nif* mutants and the WO_4^{2-}-inhibited wild type; it is unclear whether there are natural conditions for more appropriate derepression of this system. Fe was always present in our media and might be the only metal in the cofactor of this second system, as is currently posited for *A. vinelandii* nitrogenase 3, the product of *anfHDGK* (11).

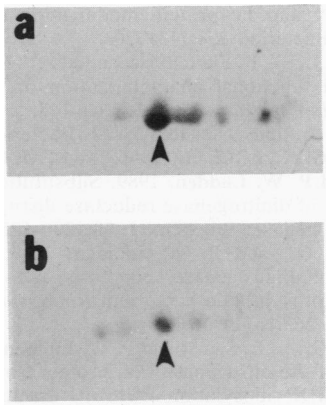

FIG. 5. Products of the in vitro transcription-translation reaction with pLL133. (a) 2-D Western blot of the reaction products spiked with unlabeled Rr2*-containing extract. The arrow identifies the major Rr2* product. The minor spots to either side are most likely Rr2*s that are charge changed because of misincorporation during the in vitro reaction. The rightmost spot is a small amount of Rr2. (b) Autoradiogram of the Western blot in panel a. Only Rr2* and the minor spots noted above are present.

Candidates for the proteins responsible for this new activity were detected in cell extracts of UR206 and several other strains. These proteins were coinduced with the activity, and their molecular weights were similar to those of known nitrogenases. Western analysis demonstrated that these proteins were cross-reactive with antibodies to Rr1 and Rr2. That these new proteins were not Rr1 and Rr2 was shown by their presence in a NifHDK$^-$ strain (UR206) and by their electrophoretic properties, which were strikingly different from those of Rr1 and Rr2 in UR249.

The regulation of synthesis of this second nitrogenase is similar to that of the Mo nitrogenase. It is synthesized in response to a deficiency of fixed N. Darkness does not stop this synthesis, but the addition of NH_4^+ does. These responses are not unexpected. Cessation of nitrogenase synthesis in response to fixed N is seen in all nitrogen fixers, because the cell recognizes that it no longer needs to fix N_2.

Darkness removes the source of energy the cell was using to fix N_2 but does not eliminate the need for fixed N.

A major difference in the regulation of these two nitrogenase systems under the conditions studied is the requirement by the second system for a nonfunctional Mo nitrogenase system; this lack of function may be due to lack of Rr1 or functional Rr2 or both or to WO_4^{2-} inhibition of the activation of nitrogenase. The derepression of the alternative system in a strain that lacks the Mo nitrogenase (UR206) might be explained by a number of models; however, the derepression of the alternative proteins in strains in which the only defects are single amino acid substitutions in Rr2 greatly restricts acceptable models. Even when all of the Mo nitrogenase components are present, including functional Rr1, the lack of an active Mo nitrogenase would appear to be sufficient to cause the derepression of the alternative system under the conditions studied.

The lag between protein accumulation of the alternative nitrogenase and whole-cell acetylene reduction activity during derepression is marked. Although part of the lag may be due to poor reduction of acetylene by the alternative nitrogenase, other factors would seem to be at play here, since the assay is sensitive enough to detect low activity. One possibility is the deficiency of a crucial medium component. This component is not V, since supplementation of the medium did not alleviate this lag (data not shown). It is also possible that the methods used to derepress the alternative nitrogenase are sufficiently nonphysiological that they result in poor derepression of auxiliary functions that are necessary for activity.

Posttranslational regulation of Rr2* also occurs in a manner analogous to that of the posttranslational regulation of Rr2. In response to darkness, Rr2* is modified in whole cells. In a mutant lacking DRAT, Rr2* is not modified in response to darkness. The incorporation of the label from ^{32}P-NAD into Rr2* in vitro also correlates with the result seen for Rr2 under the same conditions (13). These results argue that Rr2* is ADP ribosylated by DRAT in response to darkness, just as Rr2 is. Preliminary sequence data confirm that the site of ADP ribosylation in other dinitrogenase reductases, an arginine, and the surrounding residues are conserved in Rr2* (data not shown).

The lack of vanadium stimulation suggests that this enzyme is not a V-containing enzyme like that encoded by the *A. vinelandii vnfHDK* genes but is possibly more like the enzyme encoded by *A. vinelandii anfHDGK*. The results of Southern hybridizations to the *R. rubrum* chromosome agree with this supposition, demonstrating stronger hybridization under more stringent conditions. When an *anfH*-homologous *R. rubrum* fragment was transcribed and translated in an in vitro system, the major product of the reaction was Rr2*, whose identity was confirmed by coelectrophoresis. This suggests that the alternative nitrogenase from *R. rubrum* is most closely related to nitrogenase 3 from *A. vinelandii*. The exact degree of relatedness will not be known until the *R. rubrum* gene is sequenced and the identity of metal center of the purified Rr1* is determined.

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