JOURNAL OF BACTERIOLOGY, Feb. 1977, p. 718-723

Vol. 129, No. 2
Printed in U.S.A.

Nitrogen Fixation in Nitrate Reductase-Deficient Mutants of Cultured Rhizobia

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Received for publication 11 August 1976

Forty-eight mutants unable to reduce nitrate were isolated from "cowpea" *Rhizobium* sp. strain 32Hl and examined for nitrogenase activity in culture. All but two of the mutants had nitrogenase activity comparable with the parental strain and two nitrogenase-defective strains showed alterations in their symbiotic properties. One strain was unable to nodulate either *Macroptilium atropurpureum* or *Vigna unguiculata* and, with the other, nodules appeared promptly, but effective nitrogen fixation was delayed. These results, and the relatively low proportion of nitrate reductase mutants with impaired nitrogenase activity, do not support the proposed commonality between nitrogenase and nitrate reductase in cowpea rhizobia. Inhibition studies of the effect of nitrate and its reduction products on the nitrogenase activity in cultured strains 32Hl and the nitrate reductase-deficient, Nif$^+$ strains, indicated that nitrogenase activity was sensitive to nitrite rather than to nitrate.

Until recently, attempted studies of the genetics and control mechanisms of N_2 fixation and assimilation by rhizobia necessarily entailed consideration of both legume and microsymbiont. However the discovery (7, 11, 13, 15, 24) and delineation (2, 5, 6, 8) of conditions sufficient to induce nitrogenase activity in some cultured *Rhizobium* sp. should greatly facilitate genetic studies. To date we have been unable to determine the conditions required for growth of the cowpea strain, 32Hl, solely on the products of N_2 fixation. Accordingly, we were forced to use less direct methods in attempts to isolate mutant strains deficient in nitrogenase activity in culture.

One possible approach lay through the postulated commonality of the Mo-Fe subunit of nitrate reductase and nitrogenase proteins (14). The isolation of mutant strains of *Rhizobium meliloti*, which were deficient in nitrate reductase activity, was reported (10, 23). However, only symbiotic nitrogen fixation could be examined in this species.

In this study, we examine the relationship between asymbiotic nitrogenase and nitrate reductase activities in chlorate-resistant mutants of cowpea rhizobia strain 32Hl. The inhibition of nitrogenase by nitrate and its reduction products was studied by using some of these mutants.

MATERIALS AND METHODS

All chemicals were of reagent grade and were not further purified. *N*-1-naphthylethylene-diamine dihydrochloride was obtained from Sigma Chemical Co.

Bacterial cultures and growth conditions. Cultures of the cowpea strain 32Hl (*Rhizobium* sp.) (21) and the derived mutants were grown from single colony isolates on yeast-mannitol agar (YMA) or yeast-arabinose agar, a medium derived from YMA by replacement of mannitol with arabinose. For tests of nitrate reductase activity, the cultures were grown in yeast arabinose broth or CS8 medium (CS7 medium of Pagan et al. [15] with potassium phosphate buffer increased to 30 mM, arabinose increased to 100 mM, and 25 mM succinate included), or Bergersen synthetic medium (1), modified by replacing sodium glutamate with 5 mM KNO_3 (BN), where stated in the text. Growth under conditions described as "anaerobic" was in a vacuum dessicator filled with N_2 or in bottles entirely filled with medium with the lids screwed on tightly. All cultures were incubated at 30°C.

Mutant isolation. Chlorate can function as an alternative substrate to nitrate, and any strains possessing nitrate reductase activity will catalyze the conversion of chlorate to the toxic product, chlorite (17). Strain 32Hl cultured on YMA was suspended in sterile distilled water, and approximately 10^6 cells per plate were spread on 20 CS7 (15) or 20 BN plates containing 16.3 mM of $KClO_3$. The plates were cultured under N_2 for 2 weeks. Single-colony isolates of 283 chlorate-resistant mutants were obtained and checked for nitrate reduction by a modification of the rapid screening method developed by Ruiz-Herrera et al. (19). The isolates were grown as small patches on CS7 plates, and a thick mass of bacterial growth was transferred to a 1-cm^2 piece of filter paper. The filter papers were put on glass petri dishes, and 2 drops of a buffer solution (0.05 M potassium phosphate, pH 8.0) containing 1 M sodium

formate and 100 mM KNO_3 was added, and then they were incubated at 30°C for 2 h. Excess liquid was removed by partially drying the papers at 60°C for 10 min. The production of nitrite was detected by adding a drop of the sulfanilamide-naphthylethylene diamine dihydrochloride reagent dissolved in 50% ethanol (19). Forty-eight of the presumptive nitrate reductase mutants were chosen at random, inoculated onto 10 CS7 plugs (15), and incubated at 30°C. At 4 and 6 days, five plugs of each isolate were examined for their ability to fix N_2 in culture as determined by the acetylene-dependent ethylene production technique as used by Gibson et al. (6).

Nitrate reductase activity in whole cells. Cells from 32H1 and eight randomly selected chlorate-resistant strains were grown in 30 ml of CS8 under N_2 to late log phase and then washed and suspended in a final volume of 0.5 to 2.0 ml in 50 mM potassium phosphate buffer (pH 6.8). Samples of the suspension containing 100 to 200 μg of protein were added to the assay mixture (100 mM KNO_3 and 1 M sodium formate in 50 mM phosphate buffer, final volume of 2.5 ml). After 5 to 30 min at 22°C under a stream of N_2, the reaction was terminated by the addition of 0.75 ml of the sulfanilamide-naphthylethylene diamine dihydrochloride reagent (22). Absorbance at 540 nm was determined after 30 min on a Varian Techtron spectrophotometer, and nitrate production was corrected for that observed in the assays with no added sodium formate. Enzyme activity was linear for up to 30 min at the protein concentrations used.

Nitrate reductase activity in cell extracts. Cells of the various strains were grown in yeast arabinose liquid medium until the late log phase; then they were aseptically washed and suspended in BN medium (100 ml), completely filling the containers with the lids screwed on tightly, and the cultures were incubated for an additional 2 days. The cells were then washed in 100 mM potassium phosphate buffer (pH 7.5), resuspended in approximately 5 ml of the buffer, and then sonically treated at 80 W for 3 min (six 30-s bursts) with a Branson B-12 sonicator. The nitrate reductase assay, with methyl viologen as electron donor, was as described by Kennedy et al. (9) except that the reaction was conducted in open tubes under a stream of N_2.

Symbiotic effectiveness tests. Five nitrate reductase-deficient mutant strains were examined for their symbiotic effectiveness with *Macroptilium atropurpureum* ("Sirato") grown on agar slopes (25). Each mutant strain was tested on 30 plants. In addition, strains 32H1 and NR293 were also used to inoculate *Vigna unguiculata* cv. Meringa grown in vermiculite (150-ml flasks) with an 18-h, 28°C/6-h, 25°C light/dark cycle (1,500 lm).

Serology. The immuno-diffusion analysis was conducted according to Dudman (4).

Inhibition studies. A culture transfer system was used (W. R. Scowcroft, A. H. Gibson, and J. D. Pagan, Biochem. Biophys. Res. Commun., in press) where bacteria were grown on Millipore filter disks. When actively fixing nitrogen, the bacteria were transferred to CS10 media (21a) containing various inhibitors (nitrate, nitrite, hydroxylamine, and ammonium). Acetylene reduction assays were done after 1 to 2 h of incubation.

Protein. Protein concentration was determined in whole cells (3) or in cell extracts (12).

RESULTS

Preliminary investigations indicated that nitrate reductase in strain 32H1 was constitutive and functioned only under anaerobic conditions. Futhermore, this parental strain was able to grow under anaerobic conditions in media containing nitrate as the sole source of combined nitrogen. Limited growth of 32H1 does occur on agar, or in liquid cultures, without the addition of combined nitrogen. This indicates that 32H1 can scavenge nitrogen from agar and/or NH_3 from the atmosphere. Growth of 32H1 was completely suppressed only when grown in nitrogen-free liquid, under an atmosphere of N_2, over 2 M H_2SO_4.

Mutant isolation and nitrate reductase activities. Nitrate reductase-deficient isolates were sought by selecting clones resistant to chlorate (17). Of the 283 chlorate-resistant mutants, 145 appeared to have little or no nitrate reductase activity as detected by the rapid screening procedure. Nitrate reductase activity in the whole cells and in crude cell sonic extracts was examined in eight strains chosen at random. Whole cells of the parental strain 32H1, with formate as the electron donor, converted nitrate to nitrite at a rate of 140 nmol/mg of whole cell protein per h. Comparatively little nitrate reductase activity was detected in the eight representative mutant strains under these conditions (Table 1).

The assays on cell extracts tested whether the inability of the mutant strains to convert nitrate to nitrite was due to defective nitrate reductase rather than to lesions in the nitrate uptake system. Again, comparatively little activity was detected in five mutant strains assayed (Table 1).

The growth rate of five mutant strains (NR34, NR165, NR200, NR241, and NR293) in Bergersen medium (1), with mannitol and Na_2HPO_4 replaced by arabinose and K_2HPO_4, was equal to, or greater than, that of 32H1. No growth of the mutant strains occurred when sodium glutamate was replaced with 6 mM $NaNO_3$, except for slight growth of NR293.

Nitrogenase activities. Forty-eight of the nitrate reductase-deficient isolates were taken at random and examined for nitrogenase activity in culture. They were assayed 4 and 6 days after inoculation on CS8, and the rates of ethylene production were compared with that of the parent strain 32H1. With two exceptions, all the mutants had functional nitrogenase, and

the rates of acetylene reduction were similar to that of strain 32H1. The two strains, NR293 and NR165 had much lower nitrogenase activity and, in repeated experiments, NR293 had less than 2% (i.e., less than 0.3 nmol of C_2H_4 per sample per h) of the activity in the parental strain, whereas NR165 had about 20%. The mutant strains grew at least as well as 32H1 under the conditions used.

Strain purity checks. Isolates NR165, NR293, three randomly chosen mutants, and strain 32H1 were examined serologically as a purity check. Antisera prepared against 32H1 was used (4), and all five mutants had a similar reaction with the antisera as did the parental strain (Fig. 1).

TABLE 1. *Nitrate reductase activities of strain 32H1 and derived nitrate reductase mutants in whole cells and in cell extracts*

Strain	Sp act[a]
Whole cells	
32H1	140
NR241	0.4
NR200	2.0
NR315	1.5
NR158	1.3
NR34	1.1
NR293	0.8
NR165	0.4
NR292	0.4
Cell extracts	
32H1	540
NR241	22
NR200	10
NR34	8
NR293	<1
NR165	<1

[a] Specific activities are nmol of nitrite produced per hour per milligram of protein.

Symbiotic effectiveness tests. The ability of strains 32H1, NR34, NR200, NR241, NR165, and NR293 to nodulate Sirato was investigated. All except NR293 were able to nodulate Sirato within 6 to 8 days of inoculation, and subsequent plant growth was comparable to that achieved by plants inoculated with strain 32H1. Strain NR165 was comparatively slow in establishing an effective symbiosis, since plants remained comparatively yellow and stunted for 5 to 7 days after the plants inoculated with 32H1 had become green. However, by 21 days, the NR165-nodulated plants were green, and acetylene reduction rates per milligram of nodule tissue were comparable to those found with 32H1-nodulated plants. Strain NR293 failed to nodulate Sirato or *Vigna unguiculata*, even after 4 weeks.

Isolates were made from large, pink nodules, and the nitrate reductase activities were assayed (Table 2). Nitrate reductase activity was low or undetectable in these isolates. Thus, the nitrogenase activity observed in the nodules of strain NR165 was not due to a reversion of the nitrate reductase mutation.

Inhibition of nitrogenase activity. Nitrogenase activity in many N_2-fixing organisms is sensitive to nitrate. Nitrate and its reduction products were examined for their effects on the

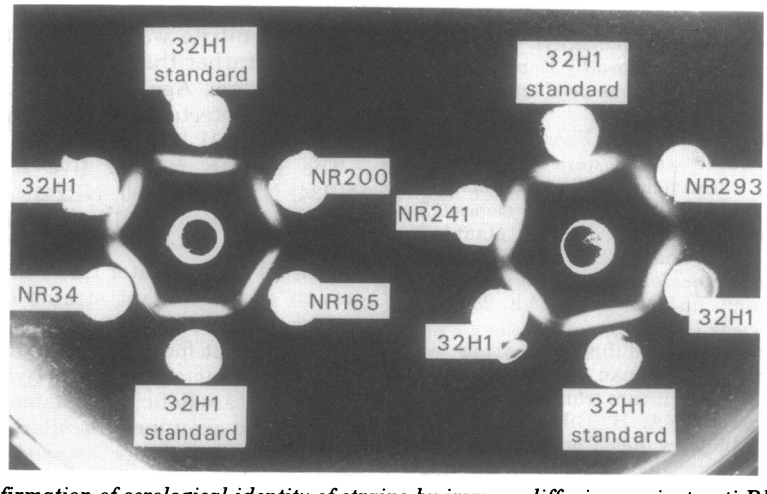

FIG. 1. *Confirmation of serological identity of strains by immunodiffusion against anti-Rhizobium 32H1 antiserum (in the central wells). Cultures grown on yeast-mannitol agar medium were applied as dense suspensions to the outer wells as shown. All the strains examined gave reactions of identity with the 32Hl standard.*

TABLE 2. *Nitrate reductase activities of strains isolated after nodule passage*

Strain	No. of nodule isolates[a]	Sp Act (nmol/h per mg of protein)
32H1	1	105
NR200	1	0.1
NR200	1	<0.1
NR165	4	<0.1
NR34	1	1.0
NR34	2	<0.1

[a] Single colony isolates were made after passage through nodules, and the strains were grown anaerobically in CS8 medium before whole cell nitrate reductase activities were determined.

nitrogenase activity in cultures of strain 32H1. When the cultures reached activity levels of approximately 20 nmol of C_2H_2 reduced per sample per h, the cultures on the Millipore disks were transferred to CS10 agar with either no additions, nitrate, nitrite, hydroxylamine, or ammonia (all at 0.3 mM) (Fig. 2). Nitrogenase activity was about 85% inhibited by either 0.3 mM nitrate or its first reduction product, nitrite (Fig. 2 and 3), whereas the same concentration of either hydroxylamine or ammonia did not appear to affect the activity (Fig. 2). Both nitrate and nitrite caused approximately 50% inhibition at 0.1 mM nitrogen (Fig. 3).

By using the nitrate reductase-deficient strains, the question as to whether nitrate, or the nitrite produced from it, was the inhibitor of nitrogenase activity, was investigated. NR158 and NR292 were partially inhibited by 0.1 mM nitrate, whereas NR200, NR34, and NR241 were insensitive to this concentration (Table 3). Little inhibition of acetylene reduction by NR34 and NR241 was observed, even when up to 10 mM KNO_3 was included in the medium. In contrast, the strains remained largely sensitive to 0.3 mM nitrite.

DISCUSSION

Selection of chlorate-resistant derivatives of strain 32H1 was used as a possible means for obtaining nitrogenase-defective strains. Recent reports delineated the conditions for the induction and maintenance of nitrogenase activity (2, 5, 6, 8) in cowpea rhizobia, strain 32H1, in culture. Thus, we were able to examine the effects of lesions in nitrate reductase on nitrogenase activity independently of the plant host. Some of these strains, together with the parental strain, provided a means of determining whether nitrate or its reduction products were inhibitory to nitrogenase activity in cultured rhizobia.

Chlorate-resistant mutants are usually deficient in nitrate reductase A but, at least in

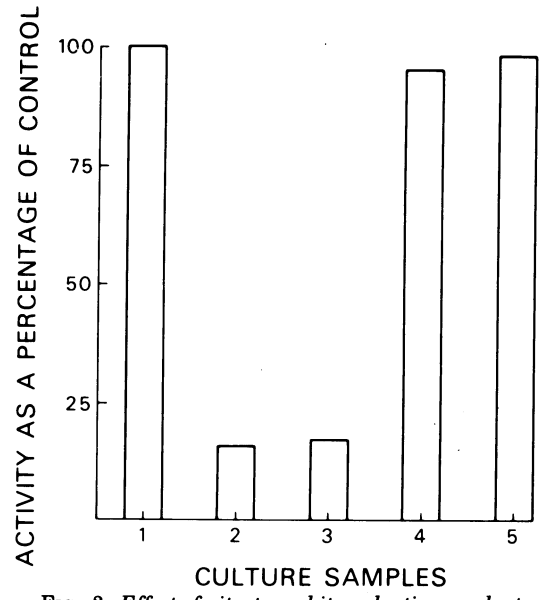

FIG. 2. *Effect of nitrate and its reduction products on nitrogenase activity in 32H1. The medium to which actively fixing cultures were transferred contained KNO_3 (2), KNO_2 (3), hydroxylamine (4) or NH_4^+ (5), each at 0.3 mM of nitrogen, or none of these (1). Activities are expressed as a percentage of the control samples (1).*

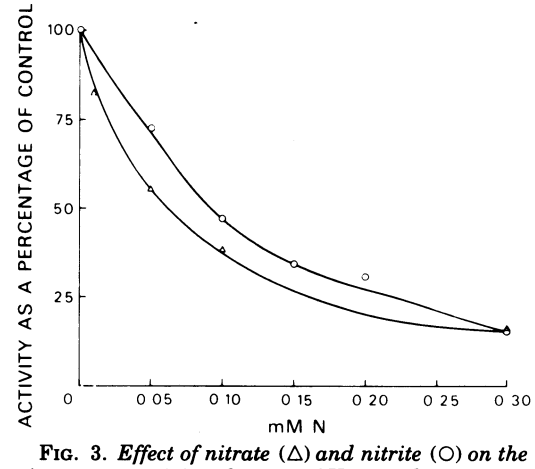

FIG. 3. *Effect of nitrate (△) and nitrite (○) on the nitrogenase activity of strain 32H1 in culture.*

enteric bacteria, they may also be defective in nitrate reductase B and many of the associated enzymes (16). By examining the nitrate reductase in cell extracts, with methyl viologen as electron donor, it was ascertained that the mutants were defective in nitrate reductase rather than in the loss of activity resulting from lesions in formate dehydrogenase (16), or had an altered permeability to nitrate.

Of the 48 nitrate reductase mutant strains

TABLE 3. *The effect of nitrate and nitrite on the nitrogenase activities of cultures actively reducing acetylene*

Bacterial strain	Nitrogenase activities with inhibitors (%)[a]		
	None	0.1 mM KNO_3	0.3 mM KNO_2
32H1	100	34	11
NR34	100	100	34
NR241	100	100	48
NR200	100	100	
NR158	100	63	
NR292	100	68	

[a] The activities were calculated for each strain as a percentage of that obtained in the absence of any added KNO_3 or KNO_2. Mean values based on 4 determinations. Activity of 32H1 in the absence of inhibitors was 31 ± 3.8 nmol of C_2H_2 reduced per disk per hour.

examined in this study, 46 did not have impaired nitrogenase activity in culture. This contrasts with the results of Kondorosi et al. (10) and Sik et al. (23) with *Rhizobium meliloti*, where approximately one-third of the nitrate reductase-deficient mutants lacked nitrogenase activity in symbiosis with *Medicago sativa*, and where one-half showed impaired nitrogenase activity. They concluded that the pleiotropic effect of the nitrate reductase mutations on nitrogen fixation was due to alterations in a Mo-Fe protein subunit common to both enzymes. We cannot draw the same conclusion for strain 32H1.

Moreover, Kennedy et al. (9) found that a possible similarity between a bacteroid constitutive subunit of nitrate reductase and the molybdoprotein component of nitrogenase in *R. japonicum*, another slow growing species, was unlikely, "because of the great difference observed in their respective [molecular] weights." This is consistent with our results, i.e., 46 of 48 nitrate reductase-deficient isolates had normal nitrogenase activities in culture. The pleiotropic effect of the mutation in the cowpea strains NR293 and NR165 may have been due to a deficiency in some enzyme(s) required for the function of both nitrate reductase and nitrogenase, for example, Mo uptake and utilization.

When tested for nodulation and symbiotic nitrogen fixation, one of the mutants, NR165, apparently had low nitrogenase activities in the early stages of plant growth. However, at final harvest the C_2H_2-reducing activity per gram of nodule weight was comparable to that in nodules formed by strain 32H1. This could not be attributed to reversion of the mutation(s), since single-colony isolates from NR165 nodules were defective in nitrate reductase activity and had comparatively poor nitrogenase activity in culture. The mutation(s) in strain NR293 also affected nitrate reductase and caused both loss of nitrogenase activity in culture and ability to form nodules on either Sirato or *Vigna unguiculata*. It has been suggested that the genes for nitrogen fixation and possibly some genes controlling symbiosis are located on a plasmid in some rhizobia (20). The parental strain 32H1 contained a plasmid, but this was also found in the non-nodulating strain NR293 (J. B. Langridge, personal communication). Because so little is known about the genes in rhizobia involved in the establishment of effective symbiosis, it is difficult to speculate on the nature of a possible mutation affecting the three functions: nitrate reduction, nitrogen fixation, and nodule initiation and development.

In this study it was found that nitrogenase activity in cultured cells of *Rhizobium* cowpea was sensitive to both nitrate and nitrite, but not to the further reduction products, hydroxylamine and ammonia, at low concentrations. From the results with the mutant strains, it was apparent that nitrogenase activity was markedly less sensitive to nitrate. This is consistent with the finding that nitrite inhibited nitrogenase activity in soybean bacteroids, and that the major inhibitory effect of nitrate was a consequence of its reduction to nitrite (9, 18). However, this study does not eliminate the possibility that in cultured rhizobia the reduction of nitrate or nitrite competes with nitrogenase for electrons, thereby lowering the activity of the latter. Additional studies (A. H. Gibson and J. D. Pagan, Planta, in press) examined whether N_2 fixation in such nitrate reductase-deficient strains remains comparatively insensitive to nitrate in the symbiotic situation.

ACKNOWLEDGMENTS

We wish to gratefully acknowledge the devoted technical assistance of M. Ashby, Y. Hort, and L. McCurley.

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