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Regulation of Nitrogen Fixation in *Rhizobium* sp.

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Regulation of nitrogen fixation by ammonium and glutamate was examined in *Rhizobium* sp. 32H1 growing in defined liquid media. Whereas nitrogenase synthesis in *Klebsiella pneumoniae* is normally completely repressed during growth on NH_4^+, nitrogenase activity was detected in cultures of *Rhizobium* sp. grown with excess NH_4^+. However, an "ammonium effect" on activity was invariably observed in cultures grown on NH_4^+ as sole nitrogen source; the nitrogenase activity was, depending on conditions, 14 to 36% of that of comparable glutamate-grown cultures. Glutamate inhibited utilization of exogenous NH_4^+ and, in one of two procedures described, glutamate partially alleviated the ammonium effect on nitrogenase activity. NH_4^+, apparently produced from N_2, was excreted into the culture medium when growth was initiated on glutamate, but not when NH_4^+ was the sole source of fixed nitrogen for growth. These findings are discussed in relation to nitrogen fixation by *Rhizobium* bacteroids.

Until recently, rhizobia were thought to reduce dinitrogen to ammonium only when symbiotically associated with a suitable legume host. However, several reports (9, 11, 13, 14, 21) have now demonstrated nitrogenase activity (acetylene reduction) and nitrogen fixation (14) in certain strains of *Rhizobium* growing in complete absence of host plant material. These findings provide the means to study directly how nitrogen fixation is regulated in these agronomically important organisms.

Considerable progress toward understanding the processes regulating synthesis of nitrogenase has been made in the asymbiotic, anaerobically N_2-fixing bacterium, *Klebsiella pneumoniae*. In a wild-type strain, synthesis of nitrogenase is completely repressed by excess free ammonium ion (23), and this is true for all asymbiotic nitrogen-fixing bacteria studied so far. However, *Klebsiella* strains that are derepressed for nitrogenase synthesis in the presence of NH_4^+ have been constructed genetically (16, 20, 22): such mutants have altered regulatory and/or catalytic properties of their ammonium-assimilating (glutamate-forming) enzymes. In the presence of NH_4^+, GlnC$^-$ strains have derepressed levels of glutamine synthetase (EC 6.3.1.2) and simultaneously lack glutamate dehydrogenase activity (EC 1.4.1.4): these mutants retain up to 30% of their derepressed level of nitrogenase activity (20, 22). However, mutants isolated as glutamate- or glutamine-requiring auxotrophs from Asm$^-$ strains, which already lack glutamate synthase (EC 1.4.1.__) activity, retain up to 100% of their nitrogenase activity in the presence of NH_4^+ (16). Such fully derepressed strains are biochemically blocked in both the high-affinity (glutamine synthetase/glutamate synthase) and the low-affinity (glutamate dehydrogenase) pathways of ammonium assimilation; consequently, the NH_4^+ produced by N_2 fixation is excreted into the culture medium (18). Some excretion of fixed nitrogen as NH_4^+ is observed also in Asm$^-$ *Klebsiella* strains (17) and in *Azotobacter* (7) or blue-green algae (19) when glutamine synthetase and glutamate synthase are inhibited in vivo with the analogue methionine sulfoximine.

Ammonium- or nitrate-limited cultures of *Rhizobium*, in common with *Klebsiella*, assimilate NH_4^+ via the glutamine synthetase/glutamate synthase pathway (3), but present evidence argues against operation of this pathway in *Rhizobium* bacteroids. Although glutamine synthetase is detected in bacteroid extracts by the transferase assay (6, 10), biosynthetic assays (3, 15; or transferase assays aimed at measuring active unadenylylated enzyme only [10]) have recorded very low glutamine synthetase activity throughout nodule development (3, 10, 15). However, high glutamine synthetase activity is found in the supernatant (or plant) fraction prepared from legume nodules (3, 6, 15), and a 10-fold increase in glutamate synthase activity in this fraction has been dem-

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onstrated during nodule development in the lupin (15). Such observations have suggested that the bacteroid is not the site of ammonium assimilation; this view is consistent with an earlier observation that isolated soybean bacteroids excrete $^{15}NH_4^+$ when exposed to $^{15}N_2$ (1).

Since excretion of NH_4^+ under N_2-fixing conditions in *Klebsiella* is correlated with derepressed synthesis of nitrogenase, the present study was undertaken to investigate (i) whether nitrogenase synthesis was derepressed in ammonium-grown *Rhizobium* and (ii) whether any excretion of NH_4^+ could be detected under asymbiotic nitrogen-fixing conditions in this organism.

MATERIALS AND METHODS

Bacterial strain and culture media. *Rhizobium* sp. 32H1, originally isolated by J. C. Burton of the Nitragin Co., Milwaukee, was obtained from J. Tjepkema, Oregon State University, Corvallis, Ore.; stocks were maintained on agar slants of glucose-yeast extract-peptone (GYP) medium (21). For preparation of inocula, *Rhizobium* sp. 32H1 was cultured in K1 medium (modified from Keister [9]) containing 1.0 g of KH_2PO_4, 1.0 g of yeast extract (Difco), 5.0 g of D-mannitol, 5.0 g of potassium gluconate, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of $CaCl_2 \cdot 2H_2O$, 0.01 g of $FeSO_4 \cdot 7H_2O$, and 0.01 g of $Na_2MoO_4 \cdot 2H_2O$ per liter, neutralized to pH 7.2 with NaOH. Screw-capped culture tubes (160 by 15 mm) containing 5 ml of inoculated medium were inclined at 40° from vertical, and incubated at 30°C on a rotary shaker. For growth under nitrogenase-inducing conditions, the medium was either (i) Tjepkema and Evans (TE) medium containing malate as carbon source (21), or (ii) K2 medium (the same as K1, except that KH_2PO_4 concentration was increased to 6.8 g/liter and $CaCl_2 \cdot 2H_2O$ was omitted). Both media were routinely prepared with nitrogen source omitted; monosodium glutamate and/or $(NH_4)_2SO_4$ were added subsequently as required.

Culture procedures for obtaining nitrogenase activity. Procedure i was based on that of Tjepkema and Evans (21). *Rhizobium* sp. 32H1 from GYP medium was suspended in 5 ml of TE (N-free) medium to give approximately 5×10^8 organisms/ml, as determined by microscopy counting using a Petroff-Hauser chamber. Portions (0.2 ml) of this suspension were added to 5 ml of TE medium containing the desired nitrogen source(s) in test tubes (14 by 125 mm). The cotton plug was pushed down to allow sealing with a sterile serum stopper that was pushed in completely. Tubes were evacuated and filled with argon four times; oxygen and acetylene were added to 0.01 and 0.08 atm, respectively. Tubes, in triplicate for each experimental treatment, were inclined at 60° from vertical and shaken on a rotary shaker at 25°C.

Procedure ii was a modification of the procedure of Keister (9). Isolated colonies obtained on GYP medium after 7 to 10 days were inoculated into K1 medium and grown for 3 days to a density of between 2×10^9 and 4×10^9 organisms/ml. This culture was washed once with K2 (nitrogen-free) medium, and 0.1 ml of the final suspension was added to 2 ml of K2 medium containing the desired nitrogen sources. The growth vessel was a prescription bottle (about 65-ml volume) sealed with a serum stopper; the hollow stopper was plugged with cotton to allow aseptic addition and removal of gases. Bottles were evacuated and filled with argon (or N_2) four times; when required, acetylene was subsequently added by syringe to 0.09 atm. Cultures, in triplicate, were incubated at 25°C with gentle rotary shaking.

Criteria of pure culture. All data presented here were obtained with cultures adjudged to be pure cultures of *Rhizobium* by (i) their failure to grow on tryptic soy agar (Difco), (ii) their appearance as isolated colonies on GYP agar only after 3 to 4 days and development of colonies to 2.5-mm diameter after a further 3 to 6 days, and (iii) their ability to form root nodules on *Phaseolus aureus* (mung bean). Checks i and ii were performed on all cultures at the end of experiments. For iii, single-colony isolates were made from the original stock culture and from typical nitrogenase-positive cultures at the end of experiments. Mung beans were surface sterilized, germinated, and inoculated with *Rhizobium* as described by Beringer (2). Inocula were prepared here from 3-day cultures grown on K1 medium. Nodules were easily visible 2 weeks after addition of bacteria; after 1 more week, inoculated plants were taller and had produced more leaves than uninoculated controls.

Assays and analyses. Growth of tube cultures (procedure i) was followed by measuring absorbance at 540 nm; tubes were inserted directly into a Bausch and Lomb Spectronic 20 colorimeter. Reduction of acetylene to ethylene was accepted as a measure of nitrogenase activity; gas samples (50 μl) were removed by syringe, and ethylene was determined by vapor-phase chromatography as previously described for this laboratory (17). Protein was determined by the procedure of Lowry et al. (12); washed culture samples were predigested for 1 h in 0.3 N NaOH at 60°C. Ammonium was determined in culture supernatants using the colorimetric method of Chaney and Marbach (4) as previously described (23).

RESULTS

Effects of fixed nitrogen sources on nitrogenase activity. Procedure i. *Rhizobium* sp. 32H1 was grown under the conditions of procedure i for obtaining nitrogenase activity (see Materials and Methods); glutamate or NH_4^+, or a combination of both of these, was added as nitrogen source. Since the growth curve obtained was unaffected by the amount or the form of fixed nitrogen added (Fig. 1A), the nitrogen source did not limit growth in exponential phase nor was cessation of growth due to exhaustion of nitrogen source. The doubling time in exponential phase was 15 h.

Irrespective of nitrogen source, nitrogenase

activity was detected 35 h after inoculation (Fig. 1B); this time coincided with the end of the exponential phase of growth (Fig. 1A). Acetylene was reduced at a linear rate for a period of about 15 h, after which activity declined. With glutamate as sole nitrogen source, no significant difference in nitrogenase activity was observed between cultures grown with 8.3 μg of N per ml and those grown with 10 times as much (Fig. 1B; conditions ii and iii, Table 1). However, activities after growth on NH_4^+ (21.3 or 213 μg of N per ml), whether added as sole nitrogen source or in combination with glutamate, were, on average, 14% of the activities obtained with glutamate-grown cultures (Fig. 1B; conditions iv to ix, Table 1). Residual NH_4^+

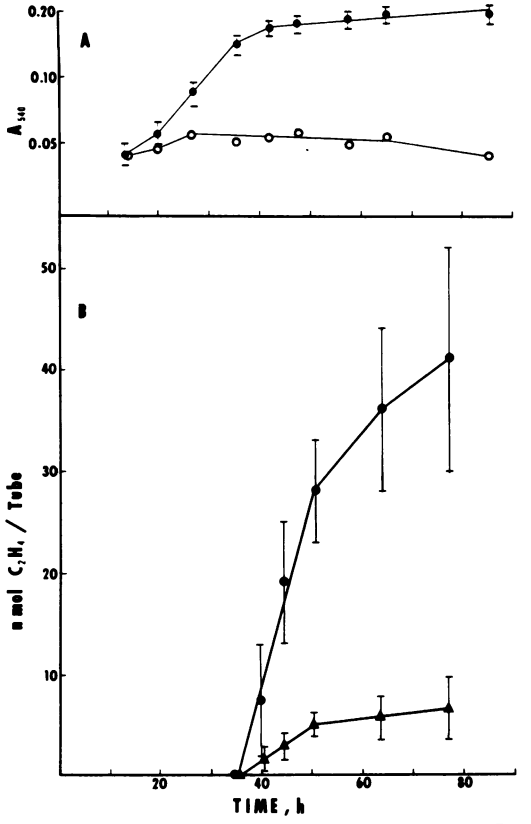

FIG. 1. *(A) Growth of Rhizobium sp. 32H1 in TE medium under an initial gas phase of 0.01 atm of O_2 and 0.08 atm of acetylene in argon. Symbols: (○) No added nitrogen source; (●) glutamate or NH_4^+, or both, as nitrogen source (i.e., conditions ii to ix, Table 1). (B) Nitrogenase activities in same experiments. Symbols: (●) glutamate as nitrogen source (conditions ii and iii, Table 1); (▲) NH_4^+, or glutamate plus NH_4^+, as nitrogen source (conditions iv to ix, Table 1). Each treatment was performed in triplicate; data points are means of pooled data (plus or minus standard errors).*

was detected in culture supernatant of all such ammonium-grown cultures at the end of the experiment. Both glutamate (8.3 or 83 μg of N per ml) and NH_4^+ (21.3 or 213 μg of N per ml) were effective nitrogen sources for growth, but utilization of 21.3 μg of NH_4^+-N per ml, as measured by its disappearance from the medium, was inhibited by glutamate (compare conditions vi and viii with condition iv in Table 1). However, glutamate did not relieve the ammonium effect on nitrogenase activity in experiments vi and viii. Here, presumably, the ammonium effect was independent of significant ammonium assimilation.

No nitrogenase activity was obtained in the absence of an added nitrogen source, but then such cultures showed very little growth (Table 1, condition i; Fig. 1A).

Effects of fixed-nitrogen sources on nitrogenase activity. Procedure ii. Preliminary experiments using the growth conditions of procedure ii produced considerably higher nitrogenase activities than those in Table 1. The effects of fixed-nitrogen sources on nitrogenase activity were reexamined, therefore, under this second set of conditions. Acetylene reduction was monitored from about 40 h after inoculation; ethylene was determined every 10 h until a linear rate of acetylene reduction was obtained. Bacterial protein and supernatant ammonium concentration of all cultures were measured after 140 h, by which time acetylene reduction rates, under all conditions used, had started to decline and further growth was negligible (not shown). The nitrogenase specific activities in Table 2 were calculated from the linear rate of acetylene reduction and the bacterial protein measured at 140 h after inoculation.

Highly significant nitrogenase activities were observed in ammonium-grown cultures, although for "low" and "high" ammonium conditions, respectively, specific activities were only 35 and 23% of the glutamate-grown cultures (compare conditions iv and v with conditions ii and iii in Table 2). Once again, glutamate inhibited ammonium uptake, where this was easily detectable with the low concentration of NH_4^+ (compare conditions vii and vi with condition iv, Table 2). However, in contrast to the results obtained using procedure i, nitrogenase activities of cultures grown on glutamate plus NH_4^+ were higher than those of cultures grown on NH_4^+ alone: the ammonium effect on nitrogenase activity was relieved, at least partially, by glutamate and, in this respect, the higher concentration of glutamate was more effective (Table 2).

Fate of N_2 fixed by *Rhizobium* sp. 32H1. The total acetylene reduced in a culture grown un-

TABLE 1. *Growth, NH_4^+ usage, and nitrogenase activities of Rhizobium sp. 32H1 in TE medium (procedure i) with glutamate and/or NH_4^+ as nitrogen source(s)*

Expt	Nitrogen source (μg of N/ml)		Growth yield[a] (mg of bacterial protein/ml)	Net NH_4^+ used (−) or produced (+) (μg of NH_4^+-N/ml)	Nitrogenase act[b]
	Glutamate	NH_4^+			
i	0	0	0.002 ± 0.000	0	0.0
ii	8.3	0	0.020 ± 0.001	0	18.7 ± 3.3
iii	83	0	0.019 ± 0.001	0	
iv	0	21.3	0.029 ± 0.002	−6 ± 2	2.7 ± 0.6
v	0	213	0.024 ± 0.001	ND[c]	
vi	8.3	21.3	0.026 ± 0.005	+1 ± 1	
vii	8.3	213	0.025 ± 0.002	ND	
viii	83	21.3	0.024 ± 0.001	−1 ± 1	
ix	83	213	0.024 ± 0.001	ND	

[a] Measured at 100 h after inoculation.
[b] Nanomoles of C_2H_4 produced per hour per milligram of bacterial protein; based on initial rate of acetylene reduction and protein content of culture at 100 h.
[c] ND, Not determined.

TABLE 2. *Growth, NH_4^+ usage, and nitrogenase activities of Rhizobium sp. 32H1 in K2 medium (procedure ii) with glutamate and/or NH_4^+ as nitrogen source(s)*

Expt	Nitrogen source (μg of N/ml)		Growth yield[a] (mg of bacterial protein/ml)	Net NH_4^+ used (−) or produced (+) (μg of NH_4^+-N/ml)	Nitrogenase act[b]
	Glutamate	NH_4^+			
i	0	0	0.006 ± .001	0.0	0
ii	8.3	0	0.029 ± .004	+0.3 ± 0.2	359 ± 27
iii	83	0	0.033 ± .004	+0.6 ± 0.2	363 ± 41
iv	0	21.3	0.051 ± .014	−14 ± 4	128 ± 33
v	0	213	0.045 ± .010	ND[c]	82 ± 23
vi	8.3	21.3	0.039 ± .002	0 ± 4	184 ± 38
vii	8.3	213	0.041 ± .003	ND	118 ± 6
viii	83	21.3	0.041 ± .011	+1 ± 1	237 ± 17
ix	83	213	0.055 ± .014	ND	220 ± 30

[a] Measured at 140 h after inoculation.
[b] Nanomoles of C_2H_4 produced per hour per milligram of bacterial protein.
[c] ND, Not determined.

der the conditions of procedure ii was high enough to represent a significant gain of fixed N to the culture if argon and acetylene were to be replaced by N_2. For example, 1,316 ± 284 nmol of C_2H_4 was produced in a 150-h period with glutamate as nitrogen source (8.3 μg of N per ml), and 654 ± 28 nmol of C_2H_4 was produced with an equivalent concentration of NH_4^+. Assuming the theoretical ratio of 3 mol of C_2H_4 reduced for 1 mol of N_2, the potential for fixation was 12.3 ± 2.6 μg of N for glutamate-grown cultures and 6.1 ± 0.3 for NH_4^+-grown cultures. Whether N_2 fixed by *Rhizobium* sp. 32H1 under these conditions contributed to growth of the organism or whether any fixed N was excreted in the form of NH_4^+ were important questions.

The net yield of bacterial protein and the NH_4^+ produced, or remaining, in the culture supernatant were determined, therefore, in cultures grown for 150 h with and without a low concentration of fixed N_2 (glutamate or NH_4^+). The contribution of N_3 was assessed by comparing results from cultures grown in the presence and in the absence of N_2 (Table 3); argon replaced N_2 in its absence, and acetylene was omitted. In all cases, cultures grown with N_2 yielded more bacterial protein than cultures grown without N_2, although when no fixed-nitrogen source was added, very little growth was obtained, and an increase in growth over the level found in the blank was only just detectable. In ammonium-grown cultures, the presence of N_2 increased the protein yield by 32%. Virtually all the NH_4^+ disappeared from the medium both in the presence and absence of N_2, so no export of NH_4^+ produced from N_2 was detected. In glutamate-grown cultures, however, N_2 not only increased the protein yield by 75%, but also increased the production of NH_4^+ in the culture supernatant by 7 μg of N. Presumably, NH_4^+ produced by nitrogen fixation was completely assimilated when NH_4^+ was the only nitrogen source for growth but, in the

TABLE 3. *Contribution of N_2 to bacterial protein and ammonium production in Rhizobium sp. 32H1*

Nitrogen source[a]	Net yield of bacterial protein[b] (mg [mean])	Total NH_4^+-N in supernatant (μg of N [mean])
Glutamate + N_2	0.107	9.4
Glutamate	0.061	2.3
NH_4^+ + N_2	0.099	0.4
NH_4^+	0.075	0.5
N_2 only	0.003	0.0

[a] Glutamate or NH_4^+ was added as 16.6 μg of N per 2 ml of culture; argon was used for gas phase in absence of N_2.

[b] Value from protein determinations on controls with no nitrogen source has been subtracted.

presence of glutamate, a significant proportion of the NH_4^+ was excreted.

DISCUSSION

Two culture methods were used here to examine the effect of fixed-nitrogen sources on nitrogenase activity in *Rhizobium* sp. In both cases, significant activity was detected in cultures grown with NH_4^+ in excess of growth-limiting concentrations. From these data, nitrogenase synthesis by strain 32H1 must be considered derepressed, or at least partially so, in the presence of NH_4^+. This is unique among wild-type nitrogen-fixing organisms studied so far; further work will be needed to establish whether or not derepressed synthesis of nitrogenase is a feature of all *Rhizobium* strains.

Although nitrogenase activity was found in ammonium-grown cultures, such activity was invariably lower than that of glutamate-grown cultures. This "ammonium effect" may represent partial repression of nitrogenase synthesis or an inhibitory effect on nitrogenase activity of whole organisms such as that observed with the aerobic nitrogen-fixer *Azotobacter* (5, 8). In both procedures, the presence of glutamate in the medium inhibited uptake of NH_4^+ when this could be accurately measured, but only with procedure ii did inhibition of ammonium uptake result in relief of the ammonium effect on nitrogenase activity. The ammonium effect observed using procedure ii, therefore, depended on ammonium utilization, as would be expected for ammonium repression (7); the ammonium effect with procedure i was independent of significant ammonium utilization and may have represented an inhibitory effect on acetylene reduction by intact organisms. Which of the two procedures used here is the more relevant to nitrogen fixation by *Rhizobium* bacteroids within a legume root nodule is open to debate, but obviously procedure ii is much more favorable for the development of nitrogenase activity.

When cultures of *Rhizobium* sp. were provided with NH_4^+ and N_2, both contributed to growth: *Rhizobium* sp. had the capacity to assimilate both exogenously supplied NH_4^+ and that produced by nitrogen fixation. However, N_2 fixed in the presence of glutamate contributed both to growth and to excreted NH_4^+. Glutamate, therefore, inhibited not only utilization of exogenous NH_4^+ (see above) but also utilization of NH_4^+ produced intracellularly by N_2 fixation. Presumably, glutamate either inhibited activities or repressed synthesis of ammonium-assimilating enzymes under these conditions. Scaling up the present procedures for obtaining symbiotic nitrogen fixation in *Rhizobium* will be necessary to obtain sufficient material on which to perform the pertinent enzyme assays.

From the previous work of several laboratories, it would seem likely that *Rhizobium* bacteroids excrete NH_4^+, which is assimilated subsequently by plant enzymes. If so, glutamate (or a related amino acid or analogue) may play a key role in promoting ammonium excretion and thereby establishing a symbiotic rather than a parasitic infection.

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