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Derepression of Nitrogenase Activity in Glutamine Auxotrophs of *Rhodopseudomonas capsulata*

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In contrast to wild-type cells, glutamine auxotrophs of the photosynthetic bacterium *Rhodopseudomonas capsulata* synthesize nitrogenase, produce H_2 (catalyzed by nitrogenase), and continue to reduce dinitrogen to ammonia in the presence of exogenous NH_4^+. The glutamine synthetase activity of such mutants is less than 2% of that observed in the wild type. It appears that glutamine synthetase plays a significant role in regulation of nitrogenase synthesis in *R. capsulata*.

Discovery of the N_2 fixation capacities of photosynthetic bacteria resulted from observations of the phenomenon of light-dependent production of H_2 (4). The latter process is a major metabolic activity in cultures of *Rhodospirillaceae* growing with certain amino acids as sources of N (8); exogenous ammonia prevents H_2 production, and it has been established that H_2 synthesis is catalyzed by nitrogenase (24). Control of N_2 fixation in vivo occurs through ammonia-mediated effects on nitrogenase, that is, repression of biosynthesis (5) and inhibition of its activity (5, 9). The molecular mechanisms of these controls remain unelucidated. From studies with non-photosynthetic diazotrophs, glutamine synthetase (L-glutamate: ammonia ligase [ADP]; EC 6.3.1.2; GS) has been implicated in the genetic regulation of nitrogenase synthesis (3, 20, 21). Studies on a representative of the *Rhodospirillaceae, Rhodopseudomonas capsulata,* have shown that GS is a key enzyme in the assimilation of N_2 and ammonia by the organism (10) and that glutamate dehydrogenase activity is undetectable (10). In addition GS activity is considerably elevated in cells grown on dinitrogen as sole N source (10), and evidence has been obtained (11) for regulation of GS activity in *R. capsulata* by adenylylation/deadenylylation, as demonstrated earlier with the enzyme from *Escherichia coli* (6, 26). Thus, GS is likely to be an important regulatory element in the assimilation of nitrogen by *R. capsulata*. To explore this possibility further, mutants of *R. capsulata* requiring glutamine for growth were isolated and examined for relevant metabolic properties.

Eight different glutamine auxotrophs (Gln^-) of wild-type *R. capsulata* strain B10 (13) were isolated after ethyl methane sulfonate mutagenesis (15). Three rounds of penicillin selection were carried out under photosynthetic growth conditions in a minimal malate plus NH_4^+ medium (RCVB medium described in reference 22) with growth between selections in RCVB supplemented with 6 mM L-glutamine (always freshly prepared and filter sterilized). The mutants showed wild-type pigmentation and cellular morphology and the ability to exchange genetic information via the "gene transfer agent" system unique to this species (23). The requirement for glutamine could not be satisfied by glutamate, aspartate, asparagine, alanine, or autoclaved yeast extract. All mutants reverted at frequencies expected for single-site alterations, 10^{-8} to 5×10^{-8}, with the majority of revertants having all mutant phenotypes restored to those of the wild-type strain. Mutant strains were routinely cultivated under photosynthetic (anaerobic) conditions in RCVB medium modified by omission of ammonia and supplementation with 12 mM glutamine.

The mutants were assayed for GS activity (γ-glutamyl transferase assay) by using a whole cell assay adapted from other procedures (2, 19). Cells in early stationary phase were treated with cetyltrimethylammonium bromide (0.1 mg/ml) and 0.1 mM $MnCl_2$ for 1 to 2 min before harvest by centrifugation (20,000 × *g* for 15 min). The cell pellet was washed once and resuspended in 15 mM mixed imidazole-Cl buffer (pH 7.5; 5 mM each of imidazole, 2-methylimidazole, and 2,4-dimethylimidazole) plus 1 mM $MnCl_2$. A 0.05-ml sample of the suspension was added to 0.45 ml of the assay mixture specified by Stadtman et al. (19), modified to contain 15 mM mixed imidazole buffer and 0.4 mg of cetyltrimethylammonium bromide per ml. After incubation for 15 min at 30°C, the reaction was terminated by

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adding 1.0 ml of a solution containing 0.5 M HCl, 2% trichloroacetic acid, and 0.83% $FeCl_3$ (12); the suspension was centrifuged, and the concentration of ferric γ-glutamyl hydroxamate in the supernatant fluid was determined by measuring absorbancy at 540 nm. GS activities observed in extracts of the mutants were ≤2% of those found with the parental B10 strain.

The genetic linkage of the *gln* mutations was determined by using the gene transfer agent system (13). Genetic recombination in this system is mediated by a small phage-like nucleoprotein particle carrying randomly packaged double-stranded DNA pieces of 3×10^6 daltons (18). The procedures used were those of Yen and Marrs (27) which specify the use of peptone-yeast extract medium for production of the agent and growth of recipient cells. Mutant strains, as originally isolated, were unable to grow in this medium even when supplemented with 12 mM glutamine. This inhibition apparently resulted from increased sensitivity of the mutants to an unknown yeast extract component(s) which at higher concentrations (0.6 versus 0.3%) also inhibited growth of the wild-type strain. Secondary mutations which would allow growth arose spontaneously at a frequency of 10^{-5} to 10^{-6}, and one such derivative was isolated from each of the eight Gln^- strains for the genetic experiments.

To obtain an estimate of the distance between the *gln* mutations, the ratio test (7, 27) was applied to the results of crosses with one of the Gln^- mutants as gene transfer agent donor and the other seven as recipients. In this test, the numbers of glutamine-independent recombinants obtained from these crosses were compared to those from the corresponding crosses using gene transfer agent from a wild-type donor. To standardize the results, a rifampicin resistance marker (*rif-1*) was introduced into the donors, and the frequencies of Rif^r recombinants produced in separate selections were determined for each recipient. If the *gln* mutations were closely linked and cotransferred, very few prototrophic recombinants would be produced in mutant × mutant crosses. By comparison, crosses between mutations widely separated on the chromosome would give recombinants at a frequency comparable to that obtained with the wild-type donor. This analysis revealed cotransfer frequencies of 0.59 to 0.75 for the eight *gln* mutations which correspond to map distances of 0.13 to 0.23, calculated by the method of Yen and Marrs (27). Because only a small quantity of DNA is transferred by the agent (18), such cotransfer frequencies and map distances suggest that the mutations are clustered on the chromosome, possibly representing one or a small number of closely linked genes.

One of the glutamine auxotrophs, designated G29, was selected for more detailed analysis. This mutant is unable to use either N_2 or NH_4^+ as a source of nitrogen for growth, and its dependence on exogenous glutamine is shown in Fig. 1. Addition of 7.5 mM ammonium sulfate to the growth medium had no sparing effect on the glutamine required (with 10 to 14 mM glutamine necessary for maximal growth), indicating that G29 cannot assimilate ammonia. A similar dependence on glutamine was seen when cultures of the mutant are supplemented with N_2 as the potential N source. We interpret the foregoing results as conclusive evidence that *R. capsulata* uses the GS/glutamate synthase (EC 2.6.1.53) sequence of reactions as the primary pathway for the assimilation of ammonia, produced from N_2 or added exogenously, as was suggested by Johansson and Gest (10).

A comparison of the specific activities of GS and glutamate synthase in extracts of G29 and the wild-type B10 strain is given in Table 1. The mutant had less than 1% of the GS activity of

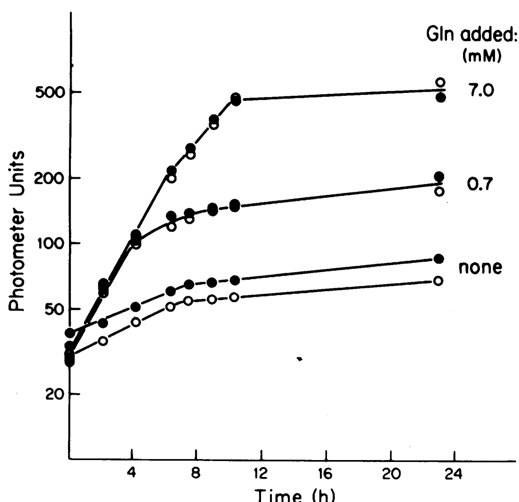

FIG. 1. *Growth of R. capsulata mutant G29 as a function of glutamine (Gln) concentration. The cultures were grown anaerobically with saturating illumination at 30 to 33°C in completely full screw-capped test tubes of 17-ml capacity. Inoculum consisted of cells grown photosynthetically in RCVB medium minus ammonium sulfate plus 12 mM Gln. Open symbols, RCVB medium modified by omission of ammonium sulfate; filled symbols, RCVB medium. Cultures were further supplemented with glutamine as indicated, and turbidity measurements were made intermittently using a Klett-Summerson photometer (filter 66).*

TABLE 1. *Ammonia assimilatory enzymes in R. capsulata B10 (wild type) and mutant G29*

Strain[a]	GS[b] (nmol/min per mg of protein)	GOGAT[c] (nmol/min per mg of protein)
B10	1970	8.2
G29	≤5	7.4

[a] Cells were grown photosynthetically to early stationary phase in completely filled 165-ml square bottles at 30 to 33°C in saturating light (about 6,500 lux) provided by a bank of three 60-W Lumiline lamps. Medium was RCVB modified by omission of $(NH_4)_2SO_4$ and addition of 10 mM glutamine.

[b] For GS γ-glutamyl transferase assays, crude extracts were prepared as described by Johansson and Gest (11) except that the cell lysate was centrifuged at 140,000 × *g* for 90 min and the supernatant fluid and "fluffy" layer (see description in reference 11) were removed for assay. Activities were determined by the procedure of Stadtman et al. (19) except that the buffer was 15 mM mixed imidazole-Cl (pH 7.5).

[c] For glutamate synthase (GOGAT) assays, crude extracts were prepared as detailed by Johansson and Gest (10), and assays were performed by the method of Meers et al. (14) except that the assay mixtures contained 125 μM NADPH and the pH was 7.5.

TABLE 2. *Nitrogenase activities of R. capsulata B10 (wild type) and the Gln⁻ mutant G29*

Strain	Nitrogen[a] source	Acetylene[b] reduction	Hydrogen[c] production
B10	Gln	2.06	44
B10	Gln + NH_4^+	<0.01	<1
G29	Gln	2.17	40
G29	Gln + NH_4^+	3.00	54

[a] The medium was RCVB lacking ammonium salts supplemented with 7.5 mM $(NH_4)_2SO_4$ and/or 5 mM glutamine (Gln) for the acetylene reduction assays. The medium for hydrogen production was the same except that the glutamine concentration was 4 mM.

[b] Acetylene reduction (16) is expressed as micromoles of ethylene per hour·milligram (dry weight). Cells were grown photosynthetically to the stationary phase in glass syringes. A 1-ml portion of this culture was directly injected into a 12-ml stoppered vial which had previously been flushed with argon. After injection of 0.5 ml of acetylene, the vials were incubated at 33°C for 1 h in saturating light (about 6,500 lux) with occasional shaking. Samples were then placed in darkness until estimation of ethylene by gas chromatography (Hewlett-Packard model 402 analyzer fitted with a Porapak R column operating at 75°C).

[c] Hydrogen production (8) is expressed as microliters of H_2 per hour·milligram (dry weight). Cultures (10 ml) were grown at saturating light intensity, about 6,500 lux, in ordinary 20-ml syringes at 33°C. As gas (determined to be primarily H_2, see reference 8) accumulated, the plunger was displaced upward, allowing a direct quantitation of gas production.

the parental strain. The possible presence of an inhibitor of GS activity in G29 extracts was ruled out by experiments in which extracts of G29 and B10 were mixed. Although glutamate synthase levels were relatively low in cells grown as described, there was no appreciable difference between the two strains.

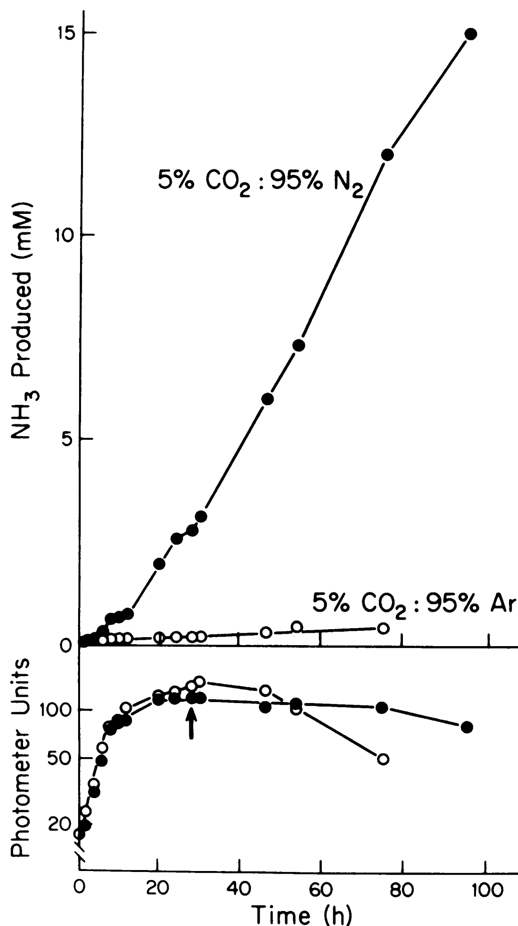

FIG. 2. *Accumulation of NH_4^+ by R. capsulata Gln⁻ mutant G29. Two Roux bottle cultures were set up, containing RCVB medium modified by omission of ammonium sulfate and addition of 0.7 mM glutamine; 15 ml of inoculum (grown photosynthetically in RCVB medium minus ammonium sulfate plus 5 mM glutamine) was added per 800 ml of medium. The cultures, at 29°C, were sparged (about 200 ml/min) with either 5% CO_2 in N_2 (●) or 5% CO_2 in Ar (○) for 15 min before initiation of illumination (7,500 lux) and throughout the course of the experiment. Samples were periodically removed for ammonia determination (upper panel; see reference 8 for details) and turbidity measurements (lower panel; as in Fig. 1). The arrow indicates time of addition of penicillin (10 U/ml) to prevent growth of revertants.*

Constitutive synthesis of the nitrogenase enzyme complex is a phenotypic characteristic of the eight Gln$^-$ mutants of *R. capsulata* described here and was evidenced visually by the production of H_2 in the presence of up to 30 mM ammonium salts. In Table 2, it can be seen that acetylene reduction assays of nitrogenase in the mutant G29 correlated well with the rates of H_2 production, clearly confirming the derepression of the enzyme complex in the presence of NH_4^+.

Genetic disruption of the normally close coupling between production and biosynthetic utilization of ammonia, resulting in derepression of nitrogenase, has been demonstrated with *Klebsiella pneumoniae* (1, 17) and *Rhodospirillum rubrum* (25); thus, certain mutants show a continuous conversion of N_2 to NH_4^+, which accumulates in the medium. *R. capsulata* mutant G29 shows a comparable excretion of NH_4^+ produced from dinitrogen (Fig. 2). The culture was grown photosynthetically on limiting glutamine as N source and gassed throughout the experiment with 5% CO_2 in N_2. Production of NH_4^+ was exponential during the first 8 h and continued at a linear rate for 3 days after growth had ceased. The maximum rate of NH_4^+ production in this trial was 0.6 μM/h·mg (dry weight), which is roughly the same as reported with derepressed *K. pneumoniae* mutants (1). Note (Fig. 2) that in the control culture gassed with 5% CO_2 in argon, very little NH_4^+ was excreted before cell lysis began at approximately 45 h. It is of interest that H_2, as sole reductant, was found to support a low rate of conversion of N_2 to NH_4^+ by resting cells of G29, although other experiments showed that H_2 inhibited reduction of N_2 when malate was the electron source (more than 50% inhibition under an atmosphere of 45% H_2–45% N_2–10% CO_2).

The isolation of a Gln$^-$ mutant of *R. capsulata* that is derepressed for nitrogenase activity in the presence of excess ammonia supports the hypothesis (20) that glutamine synthetase is involved in the regulation of N_2 fixation. Because this phenotype appears to derive from a single-site mutation, our results indicate that ammonia repression and inhibition of nitrogenase activity in *R. capsulata* may be responses to the same molecular signal.

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