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# THE PHOTOPRODUCTION OF $H_2$ AND $NH_4^+$ FIXED FROM $N_2$ BY A DEREPRESSED MUTANT OF *RHODOSPIRILLUM RUBRUM*

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## Summary

A mutant of *Rhodospirillum rubrum* has been isolated, after mutagenesis with nitrosoguanidine, which is characterized by its inability to grow in the light on malate-minimal media with exogenous ammonia or alanine, poor growth on glutamine and vigorous growth on glutamate. This mutant produces low levels of a key $NH_4^+$ assimilation enzyme, glutamate synthase (NADPH-dependent). It also exhibits significant derepression of nitrogenase biosynthesis in the presence of ammonia or alanine, being 15% derepressed for the former and about 70% derepressed for the latter.

Some of this mutant's fixed $N_2$ is excreted into the medium as $NH_4^+$ (1 $\mu$mol $NH_4^+$ per mg cell protein in 50 h). Nitrogenase-mediated $H_2$ production by this strain is considerable (42 $\mu$mol $H_2$ per mg cell protein in 50 h), approximately twice that of the wild type assayed under similar conditions.

These results demonstrate that genetic alteration of the photosynthetic $N_2$-fixer's $NH_4^+$ assimilation system disrupts the tight coupling of $N_2$ fixation and $NH_4^+$ assimilation normally observed in these organisms, enabling photochemical conversion steps to be utilized for the photoproduction of $NH_4^+$ and $H_2$.

## Introduction

Photosynthetic $N_2$-fixers have the potential of utilizing photochemical conversion steps for the production of $NH_4^+$ and/or $H_2$ in reactions catalyzed by nitrogenase. However, in most free-living $N_2$-fixers the synthesis of nitrogenase is repressed by relatively low amounts of $NH_4^+$ in the medium [1,2]. Moreover, when these organisms are fixing $N_2$ they normally do not excrete $NH_4^+$ or other products of $N_2$ fixation. This tight coupling of $N_2$ fixation and $NH_4^+$ assimilation is a major hindrance to the use of $N_2$-fixing cultures for the generation of $NH_4^+$ (and $H_2$).

The fine balance operating between the production and utilization of $NH_4^+$ fixed from $N_2$ has been disrupted chemically [3] and genetically [4,5] in heterotrophic bacteria. But to date derepressed $N_2$ fixation and the concomitant excretion of $NH_4^+$ have been achieved only chemically in free-living photosynthetic $N_2$-fixers. Stewart and Rowell [6] reported that the glutamate analog, methionine sulfoximine, alleviated the inhibitory effect of exogenous $NH_4^+$ on nitrogenase biosynthesis in the blue-green alga, *Anabaena cylindrica*, and that $N_2$-fixing cultures of this organism excreted $NH_4^+$. (see also ref. 7 for the effect of methionine sulfoximine on *A. cylindrica*.) In a recent paper [8] it was shown that $N_2$ fixation could also be derepressed by methionine sulfoximine in the purple, non-sulfur bacterium, *Rhodospirillum rubrum*, and that large amounts of extracellular $NH_4^+$ could be photoproduced from fixed $N_2$ in these cultures derepressed by the glutamate analog.

In this paper I describe a derepressed $N_2$ fixation mutant of *R. rubrum* and compare its photoproduction of $NH_4^+$ fixed from $N_2$ as well as its $H_2$ production to that of wild type *R. rubrum* under various culture conditions. Evidence is also presented which suggests that alteration of important $NH_4^+$ assimilation enzymes and pathways in this mutant may result in the partial derepression of its nitrogenase biosynthesis and excretion of some of its fixed $N_2$ as $NH_4^+$.

## Materials and Methods

*Mutagenesis and selection.* Strain IIC No. 31 of *R. rubrum* was isolated as a glutamate auxotroph in the presence of $NH_4^+$ after mutagenesis with nitrosoguanidine of wild type strain S-1 (obtained from Professor M.D. Kamen). The experimental procedure employed follows: (1) Treatment of an early stationary phase wild type culture with 100 $\mu$g nitrosoguanidine per ml for 30 min; (2) Anaerobic incubation in enriched malate-minimal medium [9] supplemented with 10 mM L-glutamate in the light (after a brief dark preincubation) for several generations growth to permit segregation of the genome; (3) Resuspension of cells in minimal medium supplemented with 10 mM $NH_4Cl$ in the presence of 1000 units penicillin per ml to select for cells unable to utilize exogenous $NH_4^+$ for growth; (4) Selection of colonies able to grow anaerobically in the light on enriched (minimal medium supplemented with 0.1% yeast extract and L-glutamate) plates but unable to grow on $NH_4^+$ supplemented plates; (5) Replicate plating to determine the spectrum of nitrogeneous compounds which will support growth of the selected colonies.

*Cultivation of photosynthetic bacteria.* Growth of the mutant and wild type cultures on minimal media with limiting nitrogen source supplements and determination of absorbances were as described previously [8].

*$C_2H_2$ reduction and $H_2$ evolution assays.* Preparation and incubation of culture samples for $C_2H_2$ reduction assays as well as the determination of the amounts of $C_2H_2$ and $C_2H_4$ in a sample's gas phase by gas chromatography were also as described earlier [8] (see also caption Fig. 2). $H_2$ production assays were similar to $C_2H_2$ reduction assays except that samples were under argon rather than $N_2$, $C_2H_2$ was not added to the samples, and the amount of $H_2$ in a sample's gas phase was determined by means of a Varian thermal conductivity gas chromatograph. Argon and dark controls for nitrogenase activity and $NH_4^+$

production assays have also been described [8].

*Chemical and biochemical analyses.* The amount of glutamate remaining in the medium during growth of the *R. rubrum* cultures on minimal media supplemented with L-glutamate was determined with a Beckman automatic amino acid analyzer. All other chemical and biochemical analyses (extracellular $NH_4^+$, whole cell protein, protein, and the specific activities of glutamate dehydrogenase, glutamine synthetase and glutamate synthase) were as previously described [8].

## Results

*Nitrogen sources utilized for growth*

*R. rubrum* mutant strain IIC No. 31 exhibits glutamate auxotrophy in the presence of $NH_4^+$. Its vigorous photosynthetic growth on L-glutamate as a nitrogen source contrasts sharply with its poor growth (>50 h lag time) on L-glutamine and its inability to grow on $NH_4^+$ or L-alanine (Fig. 1B). A reversion to wild type occurs in those cultures supplemented with $NH_4^+$ or L-alanine after about 120 h, which is indicative of a point mutation in strain IIC No. 31. In comparison, the growth patterns of the wild type on all four nitrogenous compounds (Fig. 1A) are similar to that of the mutant on L-glutamate. Growth characteristics of wild type and mutant in media with 6 mM of nitrogenous supplements (not shown) resemble those shown in Fig. 1.

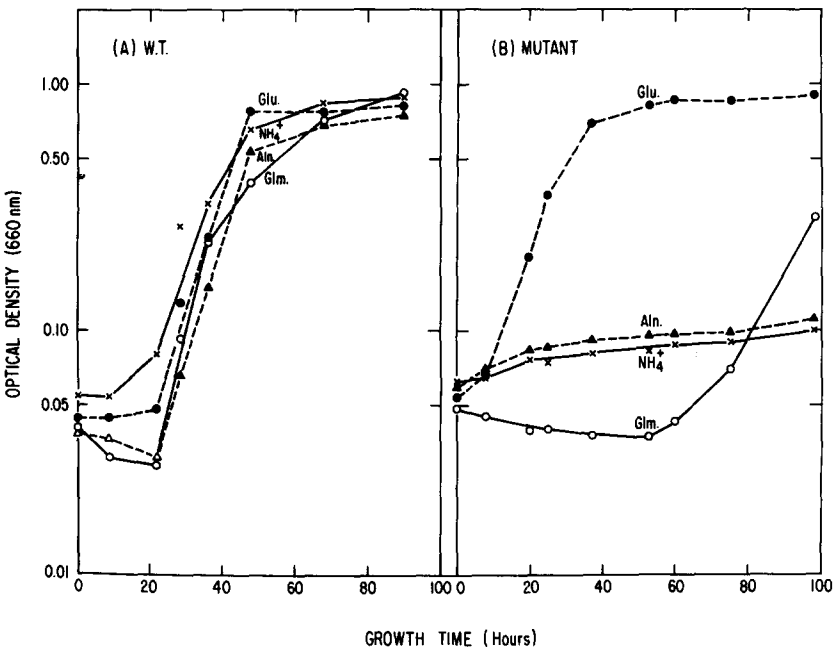


**Fig. 1. Photosynthetic growth of wild type (W.T.) and mutant IIC No. 31 cultures of *R. rubrum* with various nitrogen sources. Filled screw-capped tubes containing malate-minimal media supplemented (0.125%) with the indicated nitrogen sources: L-glutamate (●------●), L-glutamine (○———○), L-alanine (▲- - - -▲), and $NH_4Cl$ (X———X) were inoculated with washed cells from malate-minimal, limiting glutamate-grown *R. rubrum* wild type or mutant cultures. Growth of the cultures (incubated at $T = 30°C$ and $I = 1 \cdot 10^4$ ergs/cm$^2$ per s) was then followed by turbidity measurements (absorbance at 660 nm).**

*Derepression of nitrogenase for various nitrogenous compounds*

As measured by incubation of $C_2H_2$ reduction activity, mutant IIC No. 31 exhibits significant derepression of nitrogenase biosynthesis in the presence of 10 mM exogenous $NH_4^+$ or alanine: 15% and nearly 70%, respectively (Table I). It also displays low (5%) derepression for glutamine (Table I). The induction of $C_2H_2$ reduction activity in the wild type is over 98% repressed by similar concentrations of any of these nitrogenous compounds (Table I). 10 mM glutamate has no repressive effect on nitrogenase biosynthesis in either the mutant or the wild type (Table I). $H_2$ evolution catalyzed by nitrogenase, that which is light dependent, insensitive to 3% CO and inhibited by $N_2$ but not by $N_2$ + 3% CO (data not shown) [10], also points to nitrogenase biosynthesis being derepressed in the mutant for $NH_4^+$, alanine and glutamine but being severely repressed in the wild type for the same compounds (Table I).

*Photoproduction of ammonia from molecular nitrogen*

Grown photosynthetically on minimal medi with limiting amounts of glutamate as a nitrogen source both the mutant and the wild type initiate nitrogenase biosynthesis as the glutamate in the medium become exhausted. When fixing $N_2$ (assayed as $C_2H_2$ reduction activity under $N_2$) the mutant excretes significantly higher amounts of $NH_4^+$ into the medium than does the wild type. After a 60 h incubation period in the light under $N_2$ the net $NH_4^+$ production of the mutant is over 13 times greater than the low background net $NH_4^+$ production of the wild type (Fig. 2). When grown on limiting $NH_4Cl$ rather than limiting glutamate no net increase in $NH_4^+$ is detected in the wild type culture medium [8]. The mutant's nitrogenase activity corresponds, on an electron transfer basis, to levels of fixed $N_2$ greater then those detected in the medium. Since the mutant is capable of slow growth under $N_2$-fixing conditions the remainder of its fixed $N_2$ must be utilized for cellular repair and growth.

TABLE I

REPRESSION OF NITROGENASE ACTIVITY ($C_2H_2$ REDUCTION AND $H_2$ EVOLUTION) BY VARIOUS NITROGENOUS COMPOUNDS

The indicated nitrogenous compounds (10 mM) were added to samples grown on limiting glutamate just before induction of nitrogenase activity. Nitrogenase activity was then assayed after 16 h incubation of the wild type and 7 h incubation of the mutant (both were incubated in the light, $I = 6 \cdot 10^4$ ergs/cm$^2$ per s, at $T = 30°C$ under an argon atmosphere). Control $C_2H_2$ reduction rates (100%) correspond to 574.7 nmol $C_2H_4$/mg cell protein per h in the wild type and to 836.5 nmol $C_2H_4$/mg cell protein per h in the mutant. Control $H_2$ production (100%) corresponds to 14.5 μmol $H_2$/mg cell protein in the wild type and to 2.2 μmol $H_2$/mg cell protein in the mutant.

| N source | Nitrogenase activity (percent of control specific activity) | | | |
|---|---|---|---|---|
| | $C_2H_2$ reduction | | $H_2$ evolution | |
| | Wild type | Mutant | Wild type | Mutant |
| — (control) | 100 | 100 | 100 | 100 |
| Glutamine | 0.16 | 4.96 | 0.09 | 16.88 |
| $NH_4Cl$ | 0.16 | 15.50 | 0.07 | 6.16 |
| Alanine | 1.08 | 68.58 | 29.34 | 55.04 |
| Glutamate | 86.25 | 90.92 | 78.79 | 94.90 |

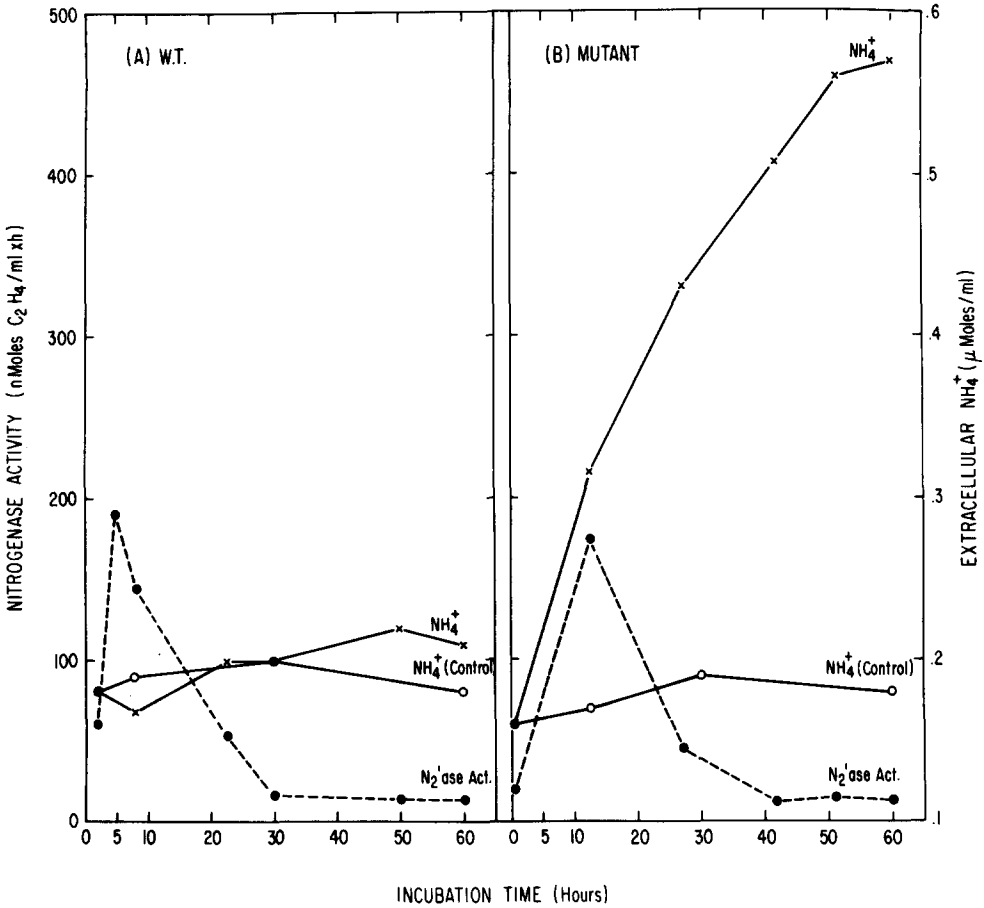


Fig. 2. $C_2H_2$ reduction and $NH_4^+$ production by wild type (W.T.) and mutant IIC No. 31 cultures of *R. rubrum*. Cultures were grown photosynthetically ($I = 2 \cdot 10^4$ ergs/cm$^2$ per s; at room temperature) on minimal media with limiting glutamate (5 mM) until the late exponential growth phase. After the cultures were flushed for 10 min with argon/3%$CO_2$, 2-ml samples [8] were injected into 6 ml serum-stoppered microfernbach flasks which had been preflushed either with $N_2$ ($C_2H_2$ reduction, •- - - - -•, and $NH_4^+$ production, X———X, assays) or argon (control $NH_4^+$ production, ○———○, assays). The flasks were vented and placed in a constant temperature shaking bath adapted for illumination ($I = 6 \cdot 10^4$ ergs/cm$^2$ per s; $T = 30°C$) at incubation time = 0. At the specified times the samples were vented again and 0.5 ml $C_2H_2$ injected to assay $C_2H_2$ reduction activity. At the end of approx. 60 min the samples were removed and placed in the dark. The amount of $C_2H_2$ and $C_2H_4$ in a sample's gas phase was determined immediately using gas chromatography. The absorbance, determined at 680 nm and at 1200 nm, the cellular protein content, and the extracellular $NH_4^+$ content of the samples were then determined as previously described [8].

On a cell protein basis, the maximum amount of $NH_4^+$ photoproduced from $N_2$ in a 50 h experiment is approx. 1 $\mu$mol per mg cell protein by the mutant and 0.14 $\mu$mol per mg cell protein by the wild type (Table II). In duplicate samples (not shown) incubated in the light under argon less than 0.1 $\mu$mol $NH_4^+$ per mg cell protein is detected in both wild type and mutant cultures (see also Fig. 2). $NH_4^+$ production in additional control samples of wild type and mutant which were incubated under $N_2$ in the dark is also negligible. The addition of glutamate just prior to the incubation of nitrogenase does not enhance the photoproduction of $NH_4^+$ in either the mutant or wild type although it does

considerably promote cell growth. These control experiments strongly suggest that the elevated level of extracellular $NH_4^+$ found in the mutant cultures is photoproduced from fixed $N_2$.

*Photoproduction of hydrogen*

Hydrogen production, assayed under an argon atmosphere (see Materials and Methods), by mutant IIC No. 31 is considerable (approximately twice that of the wild type; Table II). This nitrogenase-mediated activity (see above) also shows no enhancement with the addition of increasing amounts of glutamate.

*Ammonia assimilation enzymes*

The derepression of nitrogenase biosynthesis and excretion of $NH_4^+$ fixed from $N_2$ by strain IIC No. 31 are consistent with the activity levels of important $NH_4^+$ assimilation enzymes. The specific activities of glutamine synthetase, glutamate synthase and glutamate dehydrogenase determined in mutant cultures which were assayed both in the presence and absence of extracellular $NH_4^+$ are given in Table III. These biochemical analyses may be compared with data obtained under similar conditions in wild type *R. rubrum* cultures (see Table III and ref. 8). NADPH-dependent glutamate dehydrogenase activity is negligible in the mutant and wild type [8] both in the absence and presence of exogenous $NH_4^+$ (Table III). Levels of NADPH-dependent glutamate synthase activity are consistently low in the mutant (less than 50% of the values observed in the wild type) and do not vary with the source of nitrogen available or correlate directly with nitrogenase activity as observed in the wild type [8]. Mutant cultures assayed with $N_2$ as the sole source of nitrogen exhibit high glutamine synthetase activities which are comparable with those found in wild type cultures under similar conditions (Table III and ref. 8). In the presence of

TABLE II

NET $NH_4^+$ AND $H_2$ PRODUCTION AS A FUNCTION OF INCUBATION TIME AFTER GROWTH ON LIMITING GLUTAMATE

Cultures of wild type and mutant *R. rubrum* were grown photosynthetically on limiting amounts of glutamate (5 mM). Just prior to the induction of nitrogenase activity samples (2 ml) were removed and incubated (incubation time = 0) in the light ($I = 6 \cdot 10^4$ ergs/cm$^2$ per s) at $T = 30°C$ under argon for the $H_2$ assays and under $N_2$ for the $NH_4^+$ assays (see also caption Fig. 2). Results are expressed in μmol/mg cell protein.

| Incubation time (h) | Net $NH_4^+$ production | Net $H_2$ production |
|---|---|---|
| Wild type | | |
| 0.42 | 0.187 | 0 |
| 8.58 | 0.140 | 15.28 |
| 22.75 | 0.120 | 19.05 |
| 30.08 | 0.129 | 22.54 |
| 50.66 | 0.139 | 23.92 |
| Mutant (IIC No. 31) | | |
| 1.0 | 0.390 | 0.30 |
| 12.75 | 0.602 | 6.93 |
| 26.75 | 0.787 | 26.00 |
| 41.69 | 0.861 | 36.86 |
| 50.61 | 0.972 | 42.33 |

TABLE III

THE SPECIFIC ACTIVITIES OF AMMONIA ASSIMILATORY ENZYMES IN MUTANT CULTURES INCUBATED WITH AND WITHOUT $NH_4^+$

*R. rubrum* mutant IIC No. 31 was grown with magnetic stirring and $N_2/CO_2$ (2.5%) flushing in 3 l of malate-minimal medium supplemented with 5 mM L-glutamate in the light at 30°C. After 16 h of growth absorbance at 680 nm = 0.80; nitrogenase activity = 0), the culture was divided into two 1.5-l samples. To one sample was added 10 mM $NH_4Cl$; no additions were made to the other sample. Both samples were incubated, as described above, for 7 h and then nitrogenase activities and enzyme activities were determined. Results are expressed in nmol/min per mg protein.

| Incubation conditions | Glutamine synthetase (plus $Mn^{2+}$) | Glutamate synthase (plus NADPH) | Glutamate dehydrogenase (plus NADPH) |
|---|---|---|---|
| Glutamate, — | 1875 | 8.1 | <1 |
| Glutamate, + $NH_4Cl$ | 1120 | 6.3 | <1 |
| (included from ref. 8 for comparison:) | | | |
| Glutamate, — (wild type) | 2011 | 16.6 | <1 |

excessive $NH_4^+$, glutamine synthetase activity in the mutant is much higher than in the wild type however. Also, as shown above (Table I), the mutant strain continues to synthesize nitrogenase in the presence of 10 mM $NH_4^+$ (up to 15% the level produced in the absence of $NH_4^+$), whereas synthesis of nitrogenase in the wild type is completely repressed by 10 mM $NH_4^+$ (see also ref. 8). In the absence of $NH_4^+$ the nitrogenase activity of the mutant is similar to that of the wild type (Fig. 2).

## Discussion

$NH_4^+$ fixed by nitrogenase from $N_2$ is assimilated into amino acids by the concerted action of reactions 1 and 2, catalyzed by the enzymes: glutamine synthetase and glutamate synthase (see refs. 11 and 12).

$$\text{glutamate} + NH_4^+ + \text{ATP} \xrightarrow{\text{glutamine synthetase}} \text{glutamine} + \text{ADP} + P_i \quad (1)$$

$$\alpha\text{-ketoglutarate} + \text{glutamine} + \text{NAD(P)H} \xrightarrow{\text{glutamate synthase}} 2\ \text{glutamate} + \text{NAD(P)}^+ + H^+ \quad (2)$$

Since glutamate dehydrogenase activity is negligible in *R. rubrum* cultures (Table III, ref. 8) the above pathway appears to be also the major route of $NH_4^+$ assimilation when $NH_4^+$ is supplied exogenously in high or low amounts.

$N_2$-fixing wild type *R. rubrum* normally releases little (glutamate-grown cultures) or no ($NH_4Cl$-grown cultures) $NH_4^+$ fixed from $N_2$ into the culture medium (Table II, Fig. 2, ref. 8). However, it has been possible to chemically disrupt the control mechanism(s) governing $N_2$ fixation and $NH_4^+$ assimilation in *R. rubrum* causing cultures to excrete considerable quantities of fixed $N_2$ as $NH_4^+$ [8]. Evidence presented in this paper indicates that genetic alteration of $NH_4^+$ assimilation also results in derepression of nitrogenase biosynthesis and the photoproduction of extracellular $NH_4^+$ from $N_2$ (Fig. 2, Tables I and II).

Growth data (Fig. 1) of the *R. rubrum* mutant (strain IIC No. 31)

derepressed for nitrogenase biosynthesis, which is described in Results, show a block in reactions converting $NH_4^+$ to glutamate. The cell's requirement for glutamate, an essential metabolite, can be satisfied by glutamine but only after a long lag time (Fig. 1). Strain IIC No. 31 resembles the glutamate-requiring strains of *Klebsiella pneumoniae* (e.g. $Glu^-$ strain SK-24 [4,5]) which lack both glutamate synthase and glutamate dehydrogenase (see Table III). These $Glu^-$ strains of *K. pneumoniae*, a $N_2$-fixing heterotroph, also excrete $NH_4^+$ fixed from $N_2$ [4,5]. The slow growth of mutant IIC No. 31 under $N_2$-fixing conditions indicates that some of its fixed $N_2$ is assimilated for cellular growth and repair although no growth could be detected with exogenously supplied $NH_4^+$ at a concentration as low as 1.5 mmolar. The mutant's low level assimilation of fixed $N_2$, which is consistent with its low glutamate synthase activity under $N_2$-fixing conditions (Table III), could account for its decreased production of extracellular $NH_4^+$ from $N_2$ and its lower degree of nitrogenase biosynthesis derepression relative to the non-growing, methionine sulfoximine-derepressed, wild type (Tables I and II, Fig. 2, ref. 8).

Genetic blocks in $NH_4^+$ assimilation which prevent glutamate formation in sufficient amounts to yield repressive levels of glutamine and glutamate have been suggested as an explanation for the derepressed biosynthesis of nitrogenase and glutamine synthetase in *K. pneumoniae* [4,13]. Genetic alteration of the *R. rubrum* mutant's $NH_4^+$ assimilation system leading to glutamate auxotrophy in the presence of $NH_4^+$ may also result in the observed derepression of both nitrogenase and glutamine synthetase biosynthesis (Tables I and III) and excretion of fixed $N_2$ as $NH_4^+$ into the medium.

Strain IIC No. 31 when growing photosynthetically on limiting glutamate minimal medium photoproduces approx. 13 times the amount of $NH_4^+$ and evolves about twice the amount of $H_2$ as does the wild type under similar experimental conditions (Fig. 2, Table II). These results imply a potential of similarly derepressed mutant cultures of photosynthetic bacteria which utilize inexpensive carbon sources and of blue-green algae, which utilize $CO_2$, as solar energy conversion systems for the production of $NH_4^+$ and/or $H_2$.

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