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Derepression of Nitrogenase by Addition of Malate to Cultures of *Rhodospirillum rubrum* Grown with Glutamate as the Carbon and Nitrogen Source

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Rhodospirillum rubrum **grown in continuous culture with glutamate as the sole fixed C and N source produced no nitrogenase, and the cultures were characterized by high extracellular ammonium concentrations. Addition of organic acids derepressed nitrogenase. Glutamate dehydrogenase, glutamine synthetase, glutamate synthase, malate dehydrogenase, nitrogenase, and ammonium were assayed before and after malate addition.**

Nitrogenase is expressed in *Rhodospirillum rubrum* when it is cultured anaerobically with glutamate, limiting ammonium, or N_2 as the nitrogen source and organic acids as the carbon source (11, 18, 21, 23, 25). Nitrogenase reduces N_2 to ammonium which is utilized by the cell. When ammonium is in excess, nitrogenase synthesis is repressed. The effector of the repression of nitrogenase is not free ammonium but is probably an enzyme or intermediate involved in ammonium assimilation (13–15, 26).

The derepression of nitrogenase in the presence of ammonium has been achieved by altering pathways of ammonium assimilation either chemically or genetically. Derepression in *Klebsiella pneumoniae* and *Azotobacter vinelandii* occurs with the glutamine analogs methionine sulfone and methionine sulfoximine (13). Methionine sulfoximine has a similar effect in *R. rubrum* (31). The derepression of nitrogenase in *Rhodopseudomonas capsulata* was shown to be under the control of fixed N and oxygen (15).

We report a novel method for derepression of nitrogenase in *R. rubrum*. When *R. rubrum* is grown on glutamate as the sole N and C source, nitrogenase is repressed and ammonium levels in the medium are high (200 μM). Upon the addition of malate, β-hydroxybutyrate, acetate, or oxalacetate, the ammonium concentration decreases and nitrogenase is derepressed.

R. rubrum (ATCC 11170) was cultured in 0.5 liter of modified Ormerod medium (22) from which the malate was deleted (glutamate-only medium). Cultures were initiated with a 4% inoculum of 1- to 2-week-old stock cultures which had been maintained on the same medium. Cells for enzyme assays other than nitrogenase were collected and extracted according to the method of Nordlund and Ludden (22), except that the extraction was done aerobically with 50 mM morpholinepropanesulfonic acid–NaOH buffer, pH 7.6. Derepression of nitrogenase was initiated by the addition of 2 g of DL-malic acid in 10 ml of water (pH 6.7) to the culture. The requirement for CO_2 for growth on glutamate-only medium was tested in cultures grown in 100-ml bottles.

Whole-cell nitrogenase activity was assayed by acetylene reduction (19). Cell growth was measured as absorbance at 600 nm.

Glutamine synthetase (EC 6.3.1.2) (GS) was measured by the γ-glutamyl transferase reaction in the presence of 5 mM Mn^{2+} (27). Glutamate synthase (EC 1.4.1.13) was assayed according to the method of Dougall (7). Glutamate dehydrogenase (EC 1.4.1.3) (GDH) and malate dehydrogenase (EC 1.1.1.37) (MDH) were measured according to the methods described by Fahien and Cohen (8) and Anderson and Fuller (1). Malic enzyme (EC 1.1.1.40) was assayed according to the method of Sahl and Trüper (24). Protein was measured by biuret with bovine serum albumin as a standard. Ammonium was determined by the indophenol reagent (3) after microdiffusion (2). Cells were removed from the samples for ammonium assays by centrifugation for 2.5 min with a Beckman microfuge B. Rocket immunoelectrophoresis was performed as described by Frasch et al. (10).

Continuous culture experiments described here were carried out by a modification of the Munson and Burris procedure (21). A 500-ml water-jacketed culture vessel (8 by 10 cm) was used (produced by Jim Blaine, Department of Chemistry, University of California, Riverside). The culture vessel had a side port fitted with a serum stopper which allowed direct, rapid sampling of the culture. Flow of fresh medium (17 ml/h) into the vessel was regulated by a Gilson peristaltic pump, and the same pump was used to remove cells plus medium from the vessel. A continuous culture of *R. rubrum* was established on glutamate-only medium supplemented with 0.1% sodium bicarbonate. The culture was maintained under an atmosphere of helium at 30°C. Illumination was provided by a 150-W incandescent flood lamp directed at the vessel from a distance of 5 cm. The temperature was maintained at 30°C by flowing 28°C water through the water jacket of the vessel.

R. rubrum grown with glutamate as the sole fixed N and C source required CO_2 for growth (4, 5, 12), whereas cells grown with glutamate plus malate or ammonium plus malate medium did not exhibit this requirement. Cells were grown in stoppered 100-ml serum vials while shaking the cultures in an illuminated respirometer water bath, and CO_2 was removed with a KOH trap. Cells grown with glutamate plus CO_2 had a doubling time of 16 h, whereas cells grown with glutamate in the absence of CO_2 (CO_2 trapped by a KOH trap) had a doubling time of 73 h or more. Addition of malate or succinate, but not acetate, to the medium eliminated the need for CO_2.

Figure 1 shows the time course of ammonium ion accumulation and derepression of nitrogenase during a continuous culture of *R. rubrum* cells on glutamate-only medium. The absorbance at 600 nm of the culture during this experiment was 2.0. Ammonium ion accumulated in the medium up to a

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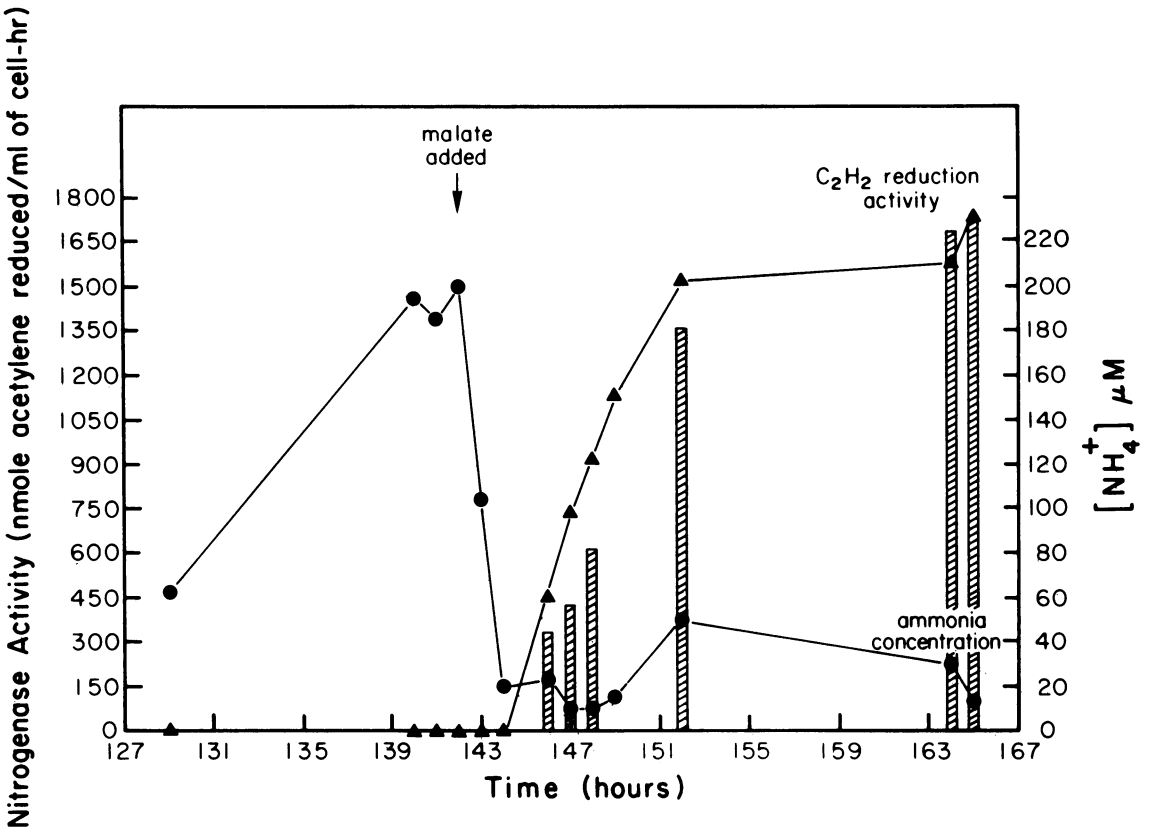

FIG. 1. Changes in $[NH_4^+]$, nitrogenase activity, and iron protein during derepression of nitrogenase by addition of malate to a glutamate-grown culture. *R. rubrum* was grown in continuous culture as described in the text. After 142 h of growth, malate was added to a final concentration of 25 mM (addition indicated by arrow in figure). The malate concentration was maintained at a high level by providing medium containing 25 mM malate through the feed line for the continuous culture. The concentration of NH_4^+ in the medium was followed before and after the addition of malate (●). Whole-cell nitrogenase activity was followed by acetylene reduction (▲). The level of iron protein in extracts was determined by rocket immunoelectrophoresis (hatched bars) (arbitrary units).

concentration of 200 μM, and nitrogenase was completely repressed. At 142 h, malate was added to the culture vessel to a final concentration of 25 mM, and the line feeding fresh medium was switched to a reservoir containing malate plus glutamate medium. Within 4 h of the addition of malate, the ammonium level decreased to 20 μM or less, and nitrogenase was detected by whole-cell acetylene reduction activity. The de novo synthesis of nitrogenase was shown by the increase in iron protein antigen in the extract as detected by rocket immunoelectrophoresis.

Batch cultures of *R. rubrum* grown on glutamate-only medium typically showed three phases of growth as characterized by changes in the growth rate. Before malate was added to the culture, doubling times were between 16 and 30 h. Upon the addition of malate, the doubling times decreased to between 7 and 10 h. After a few hours, nitrogenase activity appeared, and the doubling times increased to between 18 and 39 h.

Enzymes which metabolize malate and glutamate were assayed to test whether they might play some role in nitrogenase derepression under these conditions. The steady-state level of MDH specific activity did not change significantly in continuous culture upon the addition of malate. However, in the experiment shown, fluctuation of enzyme activity was seen at the time of malate addition (Fig. 2). This fluctuation in MDH activity was not seen in all experiments. Similarly, in continuous culture, the steady-state specific activities of GDH did not vary after the addition of malate. An increase in GDH specific activity in extracts from 4.0 to 6.0 nmol of NADH per min per mg was observed 2 h after malate addition. The specific activities of malic enzyme were low and increased ca. twofold over the course of the experiment. GS activities varied inversely with the concentration of ammonium in the medium (Fig. 2). Activity of GS increased ca. 20-fold, whereas GS antigen in the extract increased only twofold, indicating that the GS present was activated. Data shown in Fig. 1 and 2 were collected during the same continuous culture experiment. Note that the transferase assay for GS was performed in the absence of high magnesium in these experiments. In batch cultures both MDH and GDH activities increased after malate addition, although not to the extent that GS and nitrogenase activities were observed to increase (data not shown).

Nitrogenase was derepressed in glutamate-only cultures of *R. rubrum* by a number of other carbon sources in addition to malate (Table 1). α-Ketoglutarate, the product of glutamate oxidation by GDH, was not capable of derepressing nitrogenase in these cells.

In approaching the question of how glutamate derepresses nitrogenase in *R. rubrum*, a method for the rapid and apparently specific derepression of nitrogenase in continuous culture of *R. rubrum* was found. This method involves the addition of malate or other suitable carbon sources to cells growing with glutamate as the sole carbon and nitrogen source. Hillmer and Gest (16) had previously noted that the

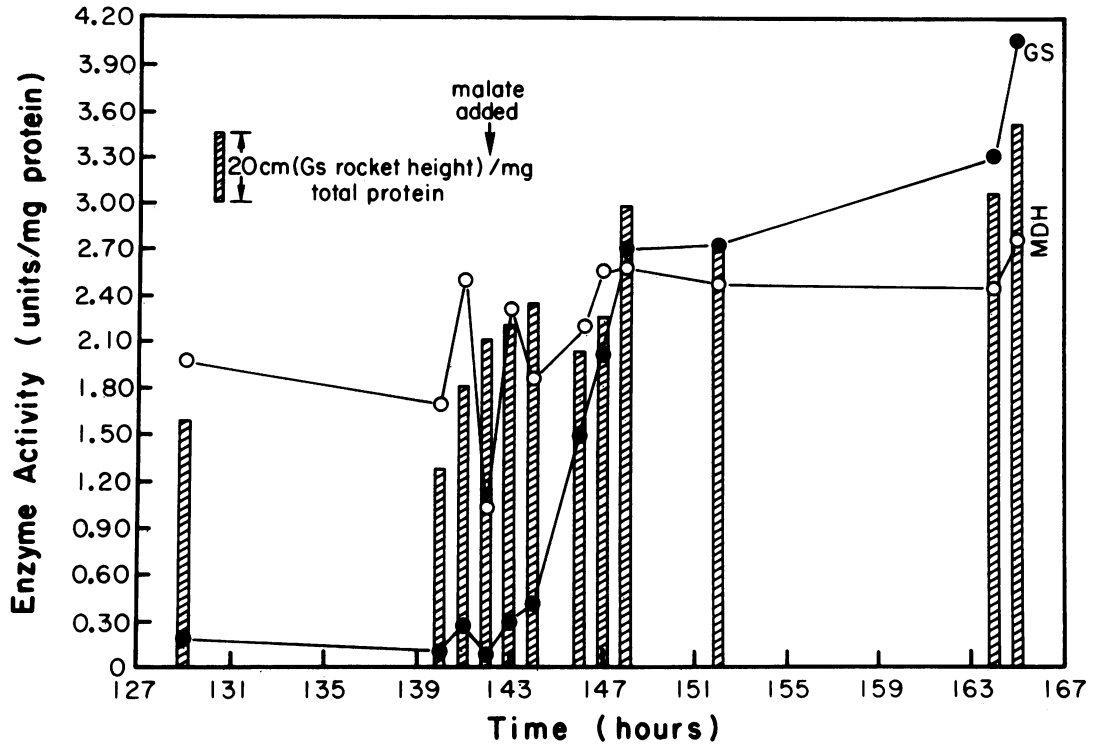

FIG. 2. MDH and GS activities in extracts of *R. rubrum* during derepression of nitrogenase by addition of malate to cells grown in continuous culture on glutamate-only medium. *R. rubrum* was grown in continuous culture as described in the text. After 142 h of growth, malate was added to a final concentration of 25 mM and maintained at that level. The culture was sampled and extracts were prepared as described in the text. MDH (○) and GS (●) activities (unit = 1 μmol/min) were determined as described in the text, and the amount of GS protein present in each extract was determined by rocket immunoelectrophoresis (hatched bars) (arbitrary units).

carbon-to-nitrogen ratio supplied to *R. capsulata* cells affected the rate of nitrogenase-dependent hydrogen evolution. We have found that whole cells grown with glutamate as the carbon and nitrogen source produce sufficient ammonium to repress nitrogenase entirely. Addition of amino acceptors resulted in decrease of free ammonium in the medium, presumably by assimilation to amino acids, and synthesis of nitrogenase. The advantage of this method of nitrogenase derepression is that it allows rapid nitrogenase derepression by the addition of a growth medium constituent to the cells; the cells do not need to be centrifuged or exposed to air or darkness to achieve derepression.

The effect is not specific to malate, as other carbon sources also result in derepression of nitrogenase. The carbon sources which did not derepress (α-ketoglutarate and citrate) are not good growth substrates for *R. rubrum*, and they appear to inhibit growth on glutamate-only medium. Furthermore, α-ketoglutarate is the product of glutamate oxidation by GDH.

The rapid increase in GS activity that is detected within an hour of the addition of malate is expected as GS activity in most enteric bacteria has been shown to respond to ammonium levels in the medium (14, 17, 30). The level of GS antigen increases only twofold during the derepression of nitrogenase, a change similar to that seen for GOGAT and malic enzyme activities. A 20-fold increase in GS transferase activity is seen during the derepression. Note that the GS transferase assay is performed in the presence of manganese with no added magnesium in these experiments. In *E. coli*, such an assay measures total GS present (both adenylylated and nonadenylylated forms) (27). Thus, there may be a difference in the regulation of GS in *R. rubrum*, as suggested by other workers (6, 9, 32).

Both in vivo acetylene reduction activity and iron protein antigen concentration in extracts extrapolate to zero at a point 2 h after malate addition. The ammonium concentration in the medium at this point is 20 μM, indicating that ammonium repression of *nif* in *R. rubrum* operates at about this concentration in the glutamate plus malate medium. The ammonium concentration in the medium remains at about this level for the next 12 h as nitrogenase activity is fully expressed, although one measurement of ammonium indicated the presence of 50 μM ammonium in the medium. The

TABLE 1. Derepression of nitrogenase in glutamate-only cultures by various carbon sources in batch cultures[a]

Added C source	C_2H_2 reduction activity (nmol/ml per h)	Initial A_{600}	Final A_{600}
None	62	1.262	2.160
Malate	1,737	1.270	3.295
Succinate	2,214	1.208	3.150
α-Ketoglutarate	0	1.170	1.500
Oxalacetate	754	1.220	3.200
Citrate	0	1.114	1.520
Acetate	2,864	1.228	3.980
β-Hydroxybutyrate	2,520	1.204	4.455
Malonate	0	1.270	1.540

[a] Acetylene reduction activities were taken immediately after adding the carbon source, and 24 h later. All carbon sources were added anaerobically in 50 mM morpholinepropanesulfonic acid–NaOH buffer, pH 6.7. No acetylene reduction activity was observed immediately after adding the carbon source. A_{600}, Absorbance at 600 nm.

levels of nitrogenase derepressed are similar to those observed for cells grown on N_2, glutamate plus malate, or limiting-ammonium media. Furthermore, analysis of subunits indicates that the iron protein that is present is fully active (20) (data not shown).

The lag seen for derepression of nitrogenase activity in *R. rubrum* is similar to that observed in *A. vinelandii* (28) and *K. pneumoniae* (29). The activation of GS after ammonium depletion does not occur immediately, but occurs over a 4-h period.

It is hoped that this method will allow the study of nitrogenase derepression in *R. rubrum* and provide some insight as to why glutamate serves to derepress nitrogenase in these bacteria.

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