The Use of Metronidazole to Isolate Nif⁻ Mutants of *Rhodopseudomonas capsulata*, and the Identification of a Mutant with Altered Regulatory Properties of Nitrogenase

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Metronidazole (2-methyl-5-nitroimidazole-1-ethanol) was used to select nitrogen fixation (Nif⁻) mutants of *Rhodopseudomonas capsulata*, either by enrichment with metronidazole, or by direct selection on agar medium containing the drug. By the latter method, approximately 50% of the isolates obtained were Nif⁻. Several Nif⁻ mutants showed the phenotype expected for mutants defective in electron transport to nitrogenase: their nitrogenase activity (determined as rate of acetylene reduction) relative to that of the wild-type strain was higher when assayed in toluene-treated cells in the presence of dithionite, than when assayed in resting cell suspensions with DL-malate as source of electrons. Glutamate-grown cells of one such mutant (RC5) resembled N-starved cells of the wild-type, and differed from glutamate-grown cells of the wild type in that nitrogenase activity in toluene-treated cells was unaffected by the addition of Mn²⁺ and the rate of H₂ production by resting cell suspensions was significantly higher than the rate of acetylene reduction. These observations implied that glutamate-grown cells of the mutant contained predominantly nitrogenase A, the form of nitrogenase which is independent of the Mn²⁺-dependent membrane-bound activating factor, suggesting that the mutant may be affected in the interconversion of nitrogenase between the regulatory forms A and R.

INTRODUCTION

The photosynthetic bacterium *Rhodopseudomonas capsulata* is able to grow with N₂ as sole N source, due to the presence of the enzyme nitrogenase. Also, when derepressed for nitrogenase synthesis in the absence of N₂, cultures of this organism exhibit vigorous, light-dependent H₂ evolution (Hillmer & Gest, 1977*a*). The reduction of N₂ to NH₄⁺ and of H⁺ to H₂ is catalysed by nitrogenase, which requires ATP and electrons from a low-potential donor ($E_h < -400$ mV) for activity (Yoch, 1978). In *R. capsulata*, these requirements can be met under a variety of conditions: anaerobic conditions, both in the light (Hillmer & Gest, 1977*a*) and in the dark (Madigan *et al.*, 1979); partially aerobic conditions in the light (Meyer *et al.*, 1978*a*); and microaerobic conditions in the dark (Siefert & Pfennig, 1980). Protection of nitrogenase against inactivation by O₂ may be afforded by respiration (Meyer *et al.*, 1978*b*).

The physiological electron donor to nitrogenase in *R. capsulata* is unknown, as is the mechanism by which the reducing power for nitrogen fixation is generated (Yoch, 1978). A ferredoxin may be involved in electron transport to nitrogenase, since such a compound has been isolated from *R. capsulata* and shown to donate electrons to nitrogenase *in vitro* (Jouanneau, 1982).

The drug metronidazole (2-methyl-5-nitroimidazole-1-ethanol) only appears to be toxic to anaerobic micro-organisms, including photosynthetic micro-organisms, following its reduction, which is dependent on low-potential electron carriers such as ferredoxin (Edwards & Mathison, 1970; O'Brien & Morris, 1972; Edwards *et al.*, 1973; Schmidt *et al.*, 1977). Consequently, metronidazole has been used for the selective enrichment of photosynthetic electron transfer mutants of green algae and cyanobacteria (Schmidt *et al.*, 1977; Guikema & Sherman, 1980).

More recently, it has been shown that, at low concentrations, metronidazole is a selective inhibitor of nitrogen fixation, both in cyanobacteria (Tetley & Bishop, 1979) and in *R. capsulata* (Hallenbeck & Vignais, 1981; Kelley & Nicholas, 1981). These observations suggested that metronidazole might be useful in the isolation of nitrogen fixation (Nif^-) mutants, particularly those defective in electron transport to nitrogenase.

We describe here the isolation of Nif^- mutants of *R. capsulata* using metronidazole, the screening of these mutants for strains defective in electron transport between physiological substrates and nitrogenase, and the altered regulatory properties of nitrogenase from one such mutant.

METHODS

Source, growth and maintenance of bacterial strains. Wild-type *R. capsulata* strain B10, and the Nif^- mutant, W15 (Wall *et al.*, 1975) were kindly supplied by the Photosynthetic Bacteria Group, Bloomington, Ind., U.S.A.

Bacteria were routinely cultivated in a mineral salts (RCV) medium (Weaver *et al.*, 1975; Hillmer & Gest, 1977a) supplemented with either DL-malate (40 mM) or DL-lactate (30 mM) as C source, and with $(\text{NH}_4)_2\text{SO}_4$ (7 mM) as N source. Complex (YPS) medium was also used (Weaver *et al.*, 1975). Solidified medium for agar plates contained 1.2% (w/v) purified agar. For growth on N_2 , RCV medium was modified by the omission of $(\text{NH}_4)_2\text{SO}_4$. Anaerobic, photosynthetic growth was achieved in 17 ml stoppered tubes, incubated in a glass-sided water bath maintained at 34 °C, and illuminated laterally by three 100 W incandescent lamps, placed at a distance of 15 cm. Anaerobic, photosynthetic growth on agar plates was achieved in GasPak anaerobic jars (BBL, Cockeysville, MD 21030, U.S.A.), under an atmosphere of $\text{H}_2/\text{N}_2/\text{CO}_2$. The jars were incubated at an ambient temperature of 28 °C between two banks of 100 W incandescent lamps placed 150 cm apart. Under these conditions, the temperature inside the jars was 32–34 °C. Aerobic cultures were grown in the dark, in Erlenmeyer flasks agitated at 300 r.p.m. at 30 °C in an environmental incubator shaker (New Brunswick Scientific Co.). Aerobic agar plates were incubated in the dark at 28–30 °C. Bacterial growth was monitored as the A_{660} of cultures in a Zeiss PMQII spectrophotometer. An A_{660} of 1.0 was taken to be equivalent to 0.5 mg cell dry wt ml^{-1} (Meyer *et al.*, 1978c) and corresponded to approximately 3×10^8 c.f.u. ml^{-1} .

Stock cultures were grown aerobically in YPS medium, and maintained in 40% (v/v) glycerol, at –20 °C.

Mutagenesis. An exponential phase photosynthetic culture of strain B10 was washed twice in TM buffer (50 mM Tris/maleate, pH 6.0) and resuspended to a density of approximately 10^9 c.f.u. ml^{-1} in TM buffer containing *N*-methyl-*N'*-nitrosoguanidine (100 $\mu\text{g ml}^{-1}$). After incubation at 30 °C for 30 min in the dark, during which time the number of viable organisms decreased by 95%, the culture was washed twice more in TM buffer to remove the mutagen. The mutagenized culture was then inoculated (1%, v/v) into several tubes of RCV medium, and the cultures grown photosynthetically to allow segregation of mutant genomes. Only one mutant was taken from each such culture to ensure that independent mutants were studied.

Isolation of Nif^- mutants with metronidazole. Mutants were isolated either by enrichment of mutagenized cultures with metronidazole, or by direct selection on agar medium containing metronidazole. Enrichment for Nif^- mutants was carried out by inoculating a mutagenized culture into NH_4^+ -free RCV medium containing metronidazole (1 mM; filter-sterilized) to a final density of 10^7 c.f.u. ml^{-1} . The suspension (4 ml) was then placed in a 12 ml glass vial fitted with a rubber serum stopper and illuminated at 30 °C for 6 h in a Warburg water bath, as described by Meyer *et al.* (1978c). After incubation with metronidazole, the suspension was centrifuged, and the pellet resuspended in RCV medium to a density of about 2×10^3 c.f.u. ml^{-1} . Samples (0.1 ml) were then spread on to YPS agar plates. When small colonies (<1 mm diam.) had appeared, usually after 2 d aerobic incubation, they were replica-plated on to RCV agar medium with or without NH_4^+ , and the replica-plates incubated photosynthetically for a further 2 d. Isolates which grew in the presence, but not in the absence of NH_4^+ were then purified by serial streaking on YPS agar medium, and individual colonies tested for their ability to grow on N_2 . The direct selection of Nif^- mutants was achieved by spreading samples (0.1 ml) of a mutagenized culture on to RCV agar medium supplemented with 200 μM -metronidazole and containing glutamate (7 mM) as sole added N source. Under these conditions, about 50% of the colonies which appeared after 5 to 6 d anaerobic incubation in the light were Nif^- .

Isolation of Nif^- mutants with penicillin. The enrichment of cultures with penicillin was carried out as described above for enrichment with metronidazole, but with the following modifications: (a) bacteria were harvested from a mutagenized culture in the mid-exponential phase of growth, washed twice in NH_4^+ -free RCV medium, and resuspended in the same medium to a density of about 2×10^8 c.f.u. ml^{-1} ; (b) the suspension was gassed with N_2 , incubated in the light for 6 h to consume endogenous N sources, and then incubated for a further 24 h in the presence of penicillin (100 U ml^{-1} ; filter sterilized); (c) survivors were washed twice in NH_4^+ -free RCV medium to remove penicillin, before plating on to YPS agar medium.

Derepression of nitrogenase. Cultures were grown photosynthetically, either in medium containing glutamate (7 mM) as sole added N source (glutamate-grown cultures) or in medium containing a limiting concentration of NH₄⁺ (2 mM) (N-starved cultures), and harvested after 16–18 h and 40 h incubation, respectively.

Assay of nitrogenase activity in intact organisms. Resting bacterial suspensions were prepared as described by Meyer *et al.* (1978c) except that malate (40 mM) was given as substrate, and the concentration of potassium phosphate in the suspension medium was increased to 30 mM. Nitrogenase (acetylene reducing) activity was assayed by the gas-liquid chromatographic measurement of ethylene, as described by Meyer *et al.* (1978c). All solutions were saturated with O₂-free argon, and all transfers made anaerobically in syringes pre-flushed with argon.

Assay of nitrogenase activity in toluene-treated cells. Bacteria were harvested by centrifugation, and resuspended to a density of 0.6–1.4 mg dry wt ml⁻¹ in a medium containing Tris/HCl (0.125 M, pH 8.0), sodium dithionite (2 mM) and dithiothreitol (0.5 mM). A sample (0.6 ml) of the suspension was then injected into an argon-filled 12 ml vial fitted with a rubber serum stopper. Toluene (10 µl) was added and the suspension agitated at 200 r.p.m. for 3 min at room temperature in a rotary shaker. A solution (0.2 ml) containing Tris/HCl (0.1 M, pH 7.4) and sodium dithionite (25 mM) was then added, followed by 0.2 ml of an ATP-generating solution, which contained (ml⁻¹): 50 µmol MgCl₂, 15 µmol ATP, 125 µmol creatine phosphate, 5 mg bovine serum albumin, 0.25 mg creatine kinase (ATP:creatine *N*-phosphotransferase; EC 2.7.3.2.), 12 µmol NaOH, and 125 µmol MOPS pH 6.8. The suspension was shaken at 30 °C in the dark in a Warburg water bath, and the reaction started, after 5 min pre-incubation, by the injection of 1 ml acetylene into the gas phase. The reaction was terminated after 60 min by the addition of 0.2 ml 30% (w/v) trichloroacetic acid. The rate of acetylene reduction was constant up to at least 90 min after the addition of acetylene and was proportional to cell density in the range 0.2–1.4 mg dry wt ml⁻¹ at specific activities of 20–200 nmol h⁻¹ (mg dry wt)⁻¹.

H₂ production by resting cells. H₂ production was assayed under the same conditions as were used to measure acetylene reduction, but without the addition of acetylene to the gas phase. The H₂ produced was determined by gas-liquid chromatography, as described by Colbeau *et al.* (1980), and the chromatograph was calibrated by the injection of known quantities of H₂.

Chemicals. Metronidazole was a generous gift from SPECIA, Paris, France. Sodium dithionite was obtained from Fluka, and *N*-methyl-*N'*-nitrosoguanidine from Serva. Tris, MOPS, ATP, creatine phosphate, bovine serum albumin (Fraction V) and creatine kinase (Type I) were supplied by Sigma. Acetylene (>99.5%), ethylene (>99.9%) and ultrapure (<5 p.p.m. O₂) argon, N₂ and H₂ were from L'Air Liquide, Grenoble, France. All other chemicals were obtained from either Sigma or Prolabo.

RESULTS

Isolation of Nif⁻ mutants with metronidazole

The effect of metronidazole (1 mM) on photosynthetic cultures of *R. capsulata* is shown in Fig. 1. Under N₂-fixing conditions, incubation in the presence of metronidazole resulted in a 10³-fold decrease in the number of c.f.u. ml⁻¹ after 6 h, and little subsequent decrease in cell viability was observed. In contrast, treatment of cultures in the presence of NH₄⁺ had no effect on growth after 6 h incubation, although after 24 h incubation a 10⁴-fold decrease in the number of c.f.u. was observed. If the initial cell density exceeded approximately 5 × 10⁷ c.f.u. ml⁻¹, or if YPS medium was used, metronidazole had no significant effect on viability over a 24 h period. At 1 mM, metronidazole had no effect on dark, aerobic growth, but at concentrations higher than 5 mM, dark, aerobic growth was completely inhibited.

After treatment of mutagenized cultures with metronidazole under N₂-fixing conditions, the proportion of Nif⁻ mutants among the survivors was 0.1 to 1%, compared to a frequency of <0.01% after treatment of non-mutagenized cultures, or without treatment with the drug in mutagenized cultures. Treatment with metronidazole was therefore effective in enriching mutagenized cultures for Nif⁻ mutants. Metronidazole could also be used to enrich mutagenized cultures for photosynthetic (Pho⁻) mutants. After 24 h incubation with 1 mM-metronidazole in the presence of NH₄⁺, about 10% of the survivors were unable to grow photosynthetically.

The effect of metronidazole on the growth of *R. capsulata* strain B10 on RCV agar medium was also studied. Under photosynthetic conditions, with either N₂, glutamate or NH₄⁺ as N source, growth was completely inhibited by metronidazole at 10 µM, 50 µM or 100 µM, respectively. Dark, aerobic growth was completely inhibited by 1 mM-metronidazole. When a mutagenized culture was spread on to RCV agar medium containing glutamate as sole added N

Fig. 1. Effect of metronidazole on viability of photosynthetic cultures of *R. capsulata* strain B10. A culture in the early stationary phase of photosynthetic growth was inoculated into RCV medium (○), RCV medium plus 1 mM-metronidazole (●), NH_4^+ -free RCV medium (□), or NH_4^+ -free RCV medium plus 1 mM-metronidazole (■). The cultures were gassed with N_2 , and shaken at 30 °C, with illumination. Samples (0.1 ml) were withdrawn at intervals and suitable dilutions plated on to YPS agar medium to determine the number of c.f.u. ml^{-1} .

source and 200 μM -metronidazole, about 50% of the colonies which appeared after photosynthetic incubation were Nif^- . Colonies also appeared on RCV agar medium containing NH_4^+ and metronidazole, but 37 out of 37 colonies tested were Nif^+ .

Growth characteristics of Nif^- mutants

The Nif^- strains used in the present study are listed in Table 1. The Nif^- phenotype of these mutants was tested by replica-plating colonies, growing aerobically on YPS agar medium, on to NH_4^+ -free RCV agar medium. No growth on NH_4^+ -free medium was observed after 2 d anaerobic incubation in the light. Strains RC2, RC4 and RC5, were 'leaky' for growth on N_2 , and showed confluent growth when 0.1 ml of culture, containing approximately 10^9 c.f.u. ml^{-1} , was spread on to an agar plate containing NH_4^+ -free RCV medium. Non-leaky strains, where tested, reverted to the Nif^+ phenotype at a frequency of 10^{-7} – 10^{-9} c.f.u. $^{-1}$. All strains grew at similar rates to the wild-type in RCV medium containing either 2 mM- or 15 mM- NH_4^+ . When cultures were grown with limiting NH_4^+ , a constant absorbance was reached after 16–24 h in all cases, and the value of this absorbance was similar to or slightly higher than in cultures of strain B10.

Strain RC34 differed from strain B10, and from other Nif^- mutants tested, in being unable to grow with glutamate as sole added N source. This strain also grew more slowly than strain B10 on YPS agar medium. Revertants of strain RC34 which were able to grow on glutamate, were Nif^+ , and grew at the same rate as the wild-type strain on YPS agar medium, suggesting that the pleiotropic characteristics of this strain were the result of a single mutation.

Nitrogenase activities of Nif^- mutants

The nitrogenase activities of N-starved cells were measured both *in vivo*, i.e. in intact cells, with malate as source of reducing equivalents for nitrogenase, and *in situ*, i.e. in toluene-treated cells, with sodium dithionite as electron donor (Table 1). Treatment with toluene renders cells permeable to small molecules. This method has been used previously in *R. capsulata* for the whole-cell assay of ribulosebisphosphate carboxylase (Tabita *et al.*, 1978) and has been used for the *in situ* assay of nitrogenase in *A. vinelandii* (Haaker & Veeger, 1977).

In strain B10, the rate of acetylene reduction *in situ* varied from 6.9 to 11% of the rate measured *in vivo*. Duplicate measurements of activity on the same culture varied by less than

Table 1. *In situ* and *in vivo* nitrogenase activities of Nif⁻ mutants of *R. capsulata*

Strains were grown photosynthetically in RCV medium containing growth-limiting NH₄⁺ (2 mM) and harvested after 40 h. *In vivo* nitrogenase activity was measured in resting bacterial suspensions provided with DL-malate as source of electrons. *In situ* nitrogenase activity was determined in toluene-treated cells of the same culture, with dithionite as electron donor. The values shown for the wild-type represent the range of activities measured in cells from a large number of different cultures. The values shown for the mutant strains are the average of at least two determinations on each strain. The ratio of *in situ* to *in vivo* activity did not vary by more than two-fold between different cultures of the same strain. Mutant types (I–IV) are defined in the text.

Strain	Phenotype	Specific nitrogenase activity [nmol C ₂ H ₂ reduced h ⁻¹ (mg dry wt) ⁻¹]		Mutant type
		<i>in vivo</i>	<i>in situ</i>	
B10	Wild-type	700–1410	70–170	—
W15	Nif ⁻	<0.5	<0.5	I
(1) Mutants isolated after metronidazole-enrichment of cultures				
RC1	Nif ⁻	<0.5	<0.5	I
RC2	Pho ⁻	380	31.0	II
RC3	Nif ⁻	<0.5	<0.5	I
RC4	Nif ⁻	53	6.0	II
RC5	Nif ⁻	42	29	III
RC6	Nif ⁻	11.2	5.3	III
RC7	Nif ⁻	33.5	16.7	III
RC8	Nif ⁻	<0.5	<0.5	I
RC9	Nif ⁻	106	5.2	II
RC10	Nif ⁻	60.5	14.6	III
RC11	Nif ⁻	1490	143	II
RC12	Nif ⁻	1300	79.9	II
RC13	Nif ⁻	194	49	III
RC14	Nif ⁻	1640	76	II
RC15	Nif ⁻	<0.5	11.7	IV
(2) Mutants isolated after direct selection on agar medium containing metronidazole				
RC16	Nif ⁻	6.7	0.5	II
RC17	Nif ⁻	1.7	7.8	IV
RC18	Nif ⁻	<0.5	<0.5	I
RC19	Nif ⁻	<0.5	<0.5	I
RC20	Nif ⁻	<0.5	<0.5	I
RC21	Nif ⁻	<0.5	<0.5	I
RC22	Nif ⁻	<0.5	<0.5	I
RC23	Nif ⁻	<0.5	<0.5	I
RC24	Nif ⁻	<0.5	<0.5	I
RC25	Nif ⁻	255	8.9	II
(3) Mutants isolated after penicillin-enrichment of cultures				
RC26	Nif ⁻	56	22.5	III
RC27	Nif ⁻	<0.5	7.1	IV
RC28	Nif ⁻	<0.5	17.4	IV
RC29	Nif ⁻	2.3	6.4	IV
RC30	Nif ⁻	1220	93.3	II
RC31	Nif ⁻	493	53.2	II
RC32	Nif ⁻	<0.5	<0.5	I
RC33	Nif ⁻	<0.5	<0.5	I
RC34*	Nif ⁻	12.7	20.0	III
RC35	Nif ⁻	1117	44.1	II

* Also unable to utilize glutamate as sole N source for growth.

10%. No acetylene reduction was detected in the dark in the absence of toluene, and the rate of acetylene reduction decreased at toluene concentrations higher than 1% (v/v). A small and variable increase in activity (approximately 20%) could be obtained by treating 1 ml cell suspension with 0.5 ml toluene, allowing the suspension to stand at 0 °C for 10 min, and then withdrawing samples for assay from beneath the toluene layer (cf. Tabita *et al.*, 1978).

The Nif^- mutants isolated either with metronidazole or with penicillin could be classified into four types, depending on their relative nitrogenase activities *in situ* and *in vivo* (Table 1). Type I mutants lacked detectable nitrogenase activity in either assay. Type II mutants were those in which the specific nitrogenase activity *in situ* relative to that measured *in vivo* (3.5–12%) was similar to, or slightly lower, than in strain B10. Type III mutants showed a rate of acetylene reduction *in situ* which was 20–100% of the rate measured *in vivo*. Finally, in Type IV mutants, nitrogenase activity could be measured *in situ* (6.4–17.4 nmol C_2H_2 reduced h^{-1} (mg dry wt) $^{-1}$), but nitrogenase activity *in vivo* was either very low [1–2 nmol C_2H_2 reduced h^{-1} (mg dry wt) $^{-1}$] or undetectable. The rate of reversion to Nif^+ was determined by replica-plating, and was $< 1 \times 10^{-3}$ c.f.u. $^{-1}$ in all cultures assayed.

Regulation of nitrogenase activity in strain B10 and the Nif^- mutant, RC5

In toluene-treated, N-starved cells of strain B10, acetylene reduction showed no lag period, was linear with time up to at least 90 min after the addition of acetylene, and was unaffected by the addition of Mn^{2+} (Fig. 2). In contrast, in glutamate-grown cells, a lag period of 30–50 min was observed before a linear rate of acetylene reduction was achieved and the duration of this lag period was reduced to 10–20 min in the presence of 0.5 mM- MnCl_2 (Fig. 2). In toluene-treated, glutamate-grown cells of strain RC5, a shorter lag period (10–20 min) was observed, which was unaffected by the presence of Mn^{2+} (Fig. 3).

The rates of H_2 production and acetylene reduction by resting cell suspensions of strains B10 and RC5 are compared in Table 2. In glutamate-grown cells of strain B10 the two rates were similar, while in N-starved cells the rate of H_2 production was 50–80% higher than the rate of acetylene reduction. In strain RC5, however, the rate of H_2 production was higher than the rate of acetylene reduction in both N-starved and glutamate-grown cells. H_2 production by resting cell suspensions was completely inhibited (> 99%) by the addition of acetylene (8.6%, v/v) to the gas phase, and no uptake of H_2 could be detected in the presence of acetylene.

Fig. 2

Fig. 3

Fig. 2. Kinetics of acetylene reduction by toluene-treated cells of strain B10. N-starved ($\circ$, $\bullet$) or glutamate-grown ($\square$, $\blacksquare$) cells (1.4 mg dry wt) were permeabilized with toluene, and assayed for dithionite-dependent acetylene reduction as described in Methods. Assays were performed either in the presence ($\bullet$, $\blacksquare$) or in the absence ($\circ$, $\square$) of 0.5 mM- MnCl_2 . The arrows indicate the time of addition of acetylene to the gas phase.

Fig. 3. Kinetics of acetylene reduction by toluene-treated cells of strain RC5. Cells were either N-starved ($\circ$, $\bullet$) or glutamate-grown ($\square$, $\blacksquare$) and were assayed in the absence ($\circ$, $\square$) or in the presence ($\bullet$, $\blacksquare$) of 0.5 mM- MnCl_2 . Conditions were as described in the legend to Fig. 2.

Table 2. Effect of N source on the relative rates of H₂ production and acetylene reduction by resting bacterial suspensions

Assays were performed as described in Methods.

Strain	Nitrogenase activity [$\mu\text{mol h}^{-1} (\text{mg dry wt})^{-1}$]			
	N-starved cells		Glutamate-grown cells	
	Acetylene reduction	H ₂ production	Acetylene reduction	H ₂ production
B10 (Expt 1)	0.76	1.35	4.71	5.60
B10 (Expt 2)	1.39	2.16	1.33	1.27
RC5	0.021	0.048	1.05	2.06

DISCUSSION

Nif⁻ mutants of *R. capsulata* have previously been isolated by penicillin enrichment (Wall *et al.*, 1975). Here, we have shown that metronidazole can also be used to enrich for Nif⁻ mutants of this organism.

The basis of this enrichment procedure was the selective toxicity of metronidazole towards N₂-fixing cells. Previous observations of the selective inhibition of N₂ fixation and of diazotrophic growth by metronidazole in *R. capsulata* have been adduced as evidence that a low-potential electron carrier, such as ferredoxin, functions in electron transport to nitrogenase in this organism (Hallenbeck & Vignais, 1981; Kelley & Nicholas, 1981). Studies in the obligate anaerobes *Trichomonas vaginalis* (Edwards & Mathison, 1970) and *Clostridium acetobutylicum* (O'Brien & Morris, 1972) have implicated the reduction of metronidazole by ferredoxin in the toxic action of this drug. However, in diazotrophic organisms, such as *R. capsulata*, the possibility that metronidazole is reduced by the low potential Fe protein of nitrogenase has not been ruled out (cf. Hallenbeck & Vignais, 1981). Moreover, the observation that both photosynthetic and dark, aerobic growth in the presence of NH₄ are inhibited by metronidazole, indicates that metronidazole is reduced under these conditions also. The nature of the low-potential reductant for metronidazole, under either N₂-fixing or nitrogenase-repressed conditions, is unknown.

Nif⁻ mutants possibly defective in electron transfer to nitrogenase were identified by comparing nitrogenase activity with sodium dithionite as electron donor to that with the physiological substrate DL-malate as source of electrons. In the present study, dithionite-dependent nitrogenase activity was measured *in situ* in toluene-treated cells, rather than *in vitro* in crude extracts, since smaller culture volumes were required and the risk of O₂-damage to nitrogenase was minimized. Nif⁻ mutants were classified into four types, depending on their relative nitrogenase activity with dithionite or with malate as source of electrons (Table 1). Type I mutants lacked detectable nitrogenase activity with either substrate and were obtained by all three isolation procedures used, but direct selection on agar medium containing metronidazole appeared to favour the isolation of this type of mutant. Type II mutants generally showed low nitrogenase activities compared to strain B10, but in some mutants (e.g. RC11, RC12) the rate of acetylene reduction was similar to that measured in the wild-type strain, both in intact and in toluene-treated cells. The latter mutants may be analogous to the *nif V* mutants of *Klebsiella pneumoniae*, which synthesize a nitrogenase capable of reducing acetylene but not N₂ (McLean & Dixon, 1981). In view of the growth characteristics of these strains, it seems unlikely that they are defective in ammonia assimilation.

In Type III and Type IV mutants, the rate of acetylene reduction relative to that shown by strain B10 was higher when measured *in situ* than when measured *in vivo*. Type IV mutants showed a much lower nitrogenase activity *in vivo* than did Type III mutants.

These strains cannot yet be identified conclusively as electron transport mutants, since, in the *in situ* assay for nitrogenase, cells were provided with ATP as well as an artificial electron donor. It is possible that the primary lesion in these mutants was in ATP synthesis rather than in electron transport to nitrogenase. Moreover, Type III and Type IV mutants accounted for a

similar proportion of the mutants isolated with either penicillin or metronidazole, so metronidazole did not enrich selectively for this type of mutant. One 'Type III' mutant isolated with penicillin (RC34) appeared to be a pleiotropic mutant defective in N metabolism. This strain differed from the pleiotropic mutant, W11, described by Wall *et al.* (1977), since after derepression of nitrogenase synthesis, the nitrogenase activity of this strain was much lower than in the wild-type strain. This strain may represent a new class of regulatory mutant.

A further complexity was introduced by the observation of altered regulatory properties of nitrogenase in the Type III mutant, RC5. In the purple non-sulphur bacterium *Rhodospirillum rubrum*, it has been proposed that nitrogenase exists in two regulatory forms, nitrogenase A and nitrogenase R (Carithers *et al.*, 1979). According to this model, nitrogenase is found predominantly in the A form in N-starved cells, and is converted to the R form by the addition of NH_4^+ , which also causes a rapid inhibition of nitrogenase activity (Carithers *et al.*, 1979; but cf. Sweet & Burris, 1981; Alef *et al.*, 1981). Furthermore, it has been proposed that the R form exists in two states, active and inactive; the former is found in cells grown either on glutamate or on N_2 and appears to be converted spontaneously to the inactive form in cell-free extracts (Yoch & Cantu, 1980). Nitrogenase purified from either glutamate-grown or N_2 -grown cells of *R. rubrum* is inactive, and requires activation by a Mn^{2+} -, Mg^{2+} - and ATP-dependent membrane-bound activating factor (AF) (Ludden & Burris, 1976; Nordlund *et al.*, 1977). During activation by AF, an 'adenine-like' molecule is removed from the Fe protein, which also contains covalently bound pentose and phosphate groups (Ludden & Burris, 1978, 1979). Similar properties of nitrogenase have been observed in *R. capsulata* (Yoch, 1979; Hallenbeck *et al.*, 1982) and in *Rhodospseudomonas palustris* (Zumft & Castillo, 1978), and this regulatory mechanism appears to be unique to the purple non-sulphur bacteria (Ludden & Burris, 1979; Yoch, 1979).

The results presented in Fig. 2 indicate that inactivation of nitrogenase in extracts of glutamate-grown cells also occurs when the cells are permeabilized with toluene. Similarly, nitrogenase activity in toluene-treated, glutamate-grown cells showed a pronounced lag period (cf. Ludden & Burris, 1976; Zumft & Castillo, 1978; Yoch, 1979). This lag period was greatly diminished by the addition of Mn^{2+} at a concentration (0.5 mM) found to be optimal for the activation of nitrogenase by AF (Ludden & Burris, 1976; Yoch, 1979). The final rate of acetylene reduction was the same in the presence or absence of Mn^{2+} , presumably because the toluene-treated cells were not depleted of Mn^{2+} (cf. Yoch, 1979).

In strain RC5, the lag period in acetylene reduction by toluene-treated, glutamate-grown cells was shorter than in strain B10, and was unaffected by the addition of Mn^{2+} (Fig. 3). This suggested either that the mutant was affected in the interconversion between the active and inactive forms of nitrogenase R, or that nitrogenase in glutamate-grown cells of the mutant was predominantly in the A form. Evidence in favour of the latter hypothesis was obtained from measurements of the rate of H_2 production by resting bacterial suspensions (Table 2). Glutamate-grown cells of strain B10, containing nitrogenase R, showed similar rates of H_2 production and acetylene reduction, while N-starved cells, containing nitrogenase A, produced H_2 at approximately twice the rate at which they reduced acetylene. On the other hand, both N-starved and glutamate-grown cells of strain RC5 showed higher rates of H_2 production than of acetylene reduction, suggesting that nitrogenase A was present in both types of cell. The rate of H_2 uptake via hydrogenase was probably negligible under the assay conditions used, since, firstly, high concentrations of organic substrate were present, which are known to inhibit the photo-reduction of CO_2 (Hillmer & Gest, 1977*b*; Kelley *et al.*, 1977; although see Khanna *et al.*, 1980) and, secondly, no H_2 uptake was detectable in the presence of acetylene, which completely inhibited H_2 evolution.

The further characterization of strain RC5, and other Nif^- mutants isolated in this study, should provide insights into the mechanism of the short-term regulation of nitrogenase in *R. capsulata*. Further studies are also needed to determine whether Type III and Type IV mutants carry lesions in electron transport to nitrogenase.

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