Determination of C/N Ratios Required for De-Repression of Nitrogenase in *Rhodobacter capsulatus*

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Phototrophic continuous and batch cultures of *Rhodobacter capsulatus* were employed to identify the C/N ratio above which nitrogenase is de-repressed. The cultures were grown with limiting amounts of ammonium as source of bound nitrogen and with L-lactate or L-malate as sources of carbon and reducing equivalents. De-repression of nitrogenase was determined on the basis of the occurrence of dinitrogen fixation, acetylene reduction and *nifH* promoter activities as well as on the basis of hydrogen evolution and nitrogenase polypeptides. In continuous culture, cells started to fix dinitrogen, to reduce acetylene, to activate the *nifH* promoter and to form nitrogenase polypeptides, when consuming lactate per ammonium at a C/N ratio of about 6 (this ratio represents the number of C and N atoms consumed). With malate as carbon source all of the activities became detectable above a C/N ratio of about 8. Essentially the same C/N ratios were determined with batch cultures for the occurrence of N-limitation of growth and hydrogen evolution. The experimentally determined C/N ratios for nitrogenase de-repression essentially agreed with C/N ratio of 5.8 and 7.8 calculated for the assimilation of ammonium and either lactate or malate, into biomass of an elemental composition of $CH_{1.83}N_{0.183}O_{0.5}$. This means that the occurrence of N-limitation and nitrogenase de-repression is defined by a threshold C/N ratio required for biomass production. As experimentally and theoretically shown, this ratio depends on the reduction state of the carbon source. It is concluded that the C/N ratio of nutrient consumption represents an intracellular signal which is directly translated into nitrogenase de-repression.

Introduction

Biological fixation of dinitrogen is catalyzed by the nitrogenase enzyme complex, which is composed of two functional units, dinitrogenase and dinitrogenase reductase. Dinitrogen fixation is a highly energy-demanding and oxygen-sensitive process. Therefore, the enzyme complex is controlled tightly by fixed nitrogen, the energy status of the cells, and oxygen (Hill, 1992). Control of nitrogenase may take place at the level of both the activity and formation of the enzyme.

When compound nitrogen such as ammonium is added to diazotrophic cultures, several free-living organisms reversibly switch off their nitrogenase activity (Hill, 1992). In the phototrophic bacteria such as *Rhodospirillum rubrum* or *Rhodobacter capsulatus* and in some species of the chemotrophic *Azospirillum,* this switch-off is accompanied by reversible ADP-ribosylation of dinitrogenase reductase (Ludden and Roberts, 1996; Zhang *et al.*, 1997).

Expression of genes required for the synthesis and function of the classical nitrogenase (*nif* genes) is generally assumed to be regulated by the ratio of glutamine to 2-oxoglutarate representing the N status of the cells (Merrick, 1992). In *Klebsiella pneumoniae,* two different gene products have been described as possible sensors of the intracellular concentrations of glutamine and 2-oxoglutarate (Merrick, 1992; Kamberov *et al.,* 1995). As yet, however, the ratios of glutamine to 2-oxoglutarate are not known which respectively repress and de-repress nitrogenase. Since nitrogenase is de-repressed and dinitrogen fixation occurs when growth of the organisms becomes limited by fixed nitrogen, it is assumed that the supply of fixed nitrogen controls the N status of cells.

Results particularly obtained with batch cultures suggested that nitrogenase remains repressed as long as the cultures are growing at the expense of fixed nitrogen like ammonium or selected amino

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acids (Postgate, 1982; Hill, 1992). Yet, in the phototrophic bacteria *R. capsulatus* and *R. rubrum,* repression of nitrogenase disappears upon addition of an organic carbon source such as malate or pyruvate to cultures growing with amino acids (Hillmer and Gest, 1977, Hoover and Ludden, 1984; Moreno-Vivian *et al.*, 1989). Moreover, chemostat cultures supplied with ammonium expressed nitrogenase under steady state conditions, if simultaneously fed with sufficiently high amounts of an organic carbon source (Munson and Burris, 1969; Postgate, 1982; Hallenbeck, 1987; Bühler *et al.*, 1987). These results gave rise to the assumption that the C/N ratio rather than fixed nitrogen *per se* controls the intracellular N status. So far, however, the ratio at which carbon and nitrogen sources are supplied has not been distinguished from the ratio at which both are consumed. It is likely, however, that only the latter ratio influences the intracellular N status.

In chemoheterotrophic bacteria, the organic carbon source is not only assimilated into biomass but also dissimilated in the course of energy regeneration. Therefore, in these organisms, the C/N ratio at which nitrogenase is de-repressed may be subject to considerable variation (Bühler *et al.*, 1987). Inevitably, this must obscure a direct relationship between the C/N ratio and the control of nitrogenase. This problem should not occur in photoheterotrophic bacteria, which are able to utilize light as their only source of energy. In the present study, we determined C/N ratios for the repression/de-repression of nitrogenase in *R. capsulatus* growing in batch and continuous culture systems. The results show that the occurrence of nitrogenase correlates directly with the stoichiometry of substrate assimilation into biomass.

Materials and Methods

Organisms and growth conditions

Rhodobacter capsulatus wild type strain B10 and a *nifH-lacZ* fusion mutant of strain B10 (Klein *et al.*, 1996) were grown photoheterotrophically in continuous chemostat and batch cultures in medium RCVBN (Weaver *et al.*, 1975) at 30 °C. The medium contained ammonium sulfate as only source of combined nitrogen and L-malate of L-lactate as electron donors and carbon sources. Chemostat cultures were grown at a constant pH of 7.0 with stirring (350 rpm) in a 1.5 l fermenter (Biostat S or M, B. Braun Biotech International). Cultures were illuminated with 30 klx of incandescent light as determined at the surface of the culture vessel. In order to maintain anaerobic conditions, continuous cultures were steadily gassed with N_2 (99.99%) and oxygen-free argon, respectively. Batch cultures inoculated at a density of about 0.06 mg cell protein per ml of medium were grown in bottles (50 ml) sealed with serum rubber stoppers.

Determination of nitrogenase activities

Cellular nitrogenase activities were determined *in situ* either by dinitrogen fixation or by hydrogen formation. Alternatively, cell samples removed from the culture vessels were used to determine nitrogenase activity by the acetylene reduction assay.

In situ-dinitrogen fixation by chemostat cultures was estimated on the basis of the nitrogen contents of cell samples and the dilution rate as described (Klein *et al.*, 1993). For this purpose, nitrogen contents of cells were determined by the micro-Kjeldahl method according to Beloserski and Proskurjakow (1956). Nitrogenase-mediated *in situ*-hydrogen formation was followed by collecting the gas with sterile syringes injected through serum stoppers into the hermetically sealed batch culture bottles. The amount of hydrogen in the gas phase was analyzed after separation on Porapak N (60–80 Mesh) with a Shimadzu GC 8A gas chromatograph equipped with a thermal conductivity detector. Acetylene reduction assays were performed under optimal test conditions as described (Klein *et al.*, 1993) with the exception that the electron donors (D,L-malate or L-lactate) were added to final concentrations of 20 mM.

Determination of nitrogenase polypeptides

Separation of nitrogenase polypeptides by sodium dodecyl sulfate (SDS) polyacrylamide electrophoresis (PAGE) and detection of the polypeptides by enzyme linked immunological assays were performed as previously (Klein *et al.*, 1996). To prevent artificial ADP-ribosylation of component 2 of nitrogenase (i. e. Rc2 of *R. capsulatus*), all steps required for the preparation of samples for PAGE were performed at a temperature of 4 °C.

Miscellaneous determinations

The *nifH* promoter activity of *R. capsulatus* was analyzed by the activity of β-galactosidase of the *nifH-lacZ* fusion mutant (Klein *et al.*, 1996). Protein was quantified according to Schacterle and Pollack (1973). Concentrations of L-malate and L-lactate were determined with test kits with detection limits of 0.15 and 0.33 mM, respectively (Boehringer Mannheim, Germany). Ammonium was measured with an ammonium-sensitive electrode featuring lower detection limit of about 5 μM (Ingold, Frankfurt, Germany).

Results

De-repression of nitrogenase in continuous cultures

In order to define the nutritional conditions for de-repression of nitrogenase, *R. capsulatus* was grown phototrophically in continuous chemostat cultures. The feed medium supplied at constant dilution rates (D) contained 5 mM of ammonium and different concentrations of either L-lactate or L-malate. As soon as steady states were reached after at least five exchanges of the culture volume, samples were removed from the culture vessel and analyzed for cell-bound nitrogen as well as for residual amounts of ammonium, lactate or malate in the spent medium. Under all of the conditions tested, both carbon sources were essentially consumed by the organisms. But, at the lower C/N ratios, residual concentrations of ammonium remained detectable. (In the present communication, the C/N ratio defines the ratio of carbon atoms of the substrate per atoms of the fixed-nitrogen source ammonium). The results depicted in Fig. 1 show that the steady state concentration of ammonium decreased with increasing the C/N ratio in the feed medium. At C/N ratios of about 6 and 8, respectively, all of the ammonium was utilized by cultures growing on lactate (Fig. 1a) and malate (Fig. 1b). At the higher C/N ratios, the carbon sources as well as ammonium were essentially consumed, which means that the C/N ratio of nutrients supplied corresponded to the C/N ratio of nutrients utilized.

In spite of the depletion of ammonium, the steady state level of N contained in biomass steadily increased with increasing the C/N ratio. Consequently, biomass production by the cultures was carbon-limited over the entire range of C/N ratios shown in Fig. 1. Lasting carbon limitation, however, was only possible because ammonium limitation was compensated for by dinitrogen fixation above C/N ratios of 6 (lactate) and 8 (malate). The occurrence of dinitrogen fixation at defined C/N ratios leads to the question if, at these C/N ratios, nitrogenase was expressed and/or activated.

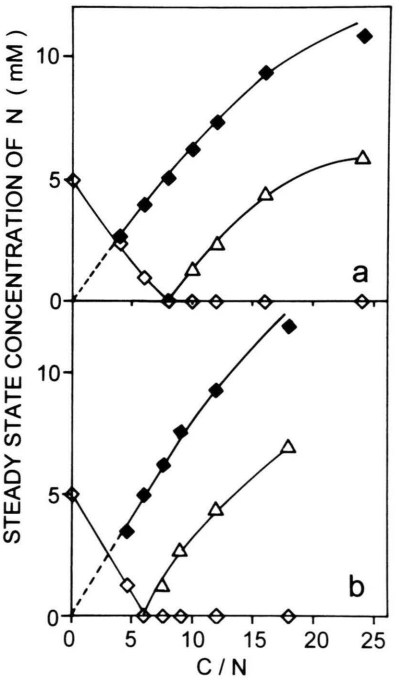

Fig. 1. Dependence of ammonium consumption and nitrogen fixation on the C/N ratio in *Rhodobacter capsulatus*. The organisms were grown phototrophically in continuous culture with 5 mM of ammonium and concentrations of L-malate (a) and L-lactate (b) as indicated. The dilution rates were $D = 0.075\ h^{-1}$ and $D = 0.06\ h^{-1}$ with malate and lactate, respectively. The C/N ratio represents the number of atoms of the carbon sources consumed per atom of N of ammonium. Constant gassing of the cultures with nitrogen provided anaerobiosis as well as the substrate for diazotrophic growth. In order to confirm steady states, continuous cultures were grown for at least five changes of the culture volume. Each steady state was maintained for three to five days, in the course of which five to fifteen culture samples were analyzed. The figure represents mean values of the resulting data. Results were confirmed by at least two independent continuous culture experiments. Steady state concentrations of N per liter: ammonium in the spent medium (◇); biomass (◆); biomass minus 5 mmol of ammonium added per liter = N fixed by nitrogenase per liter (△).

Expression of nitrogenase polypeptides was studied at the level of the *nifH* promoter activity as well as at the level of the relative cellular contents of the three polypeptides of nitrogenase, *i.e.* the α and β polypeptides of component 1 (Rc1) and the polypeptide of component 2 (Rc2). The results compiled in Table I reveal that, in cultures grown with malate, β-galactosidase activity could be detected when the sources of carbon and fixed nitrogen were consumed at a C/N ratio of 7.5, while in cultures grown with lactate the activity became measurable at a C/N ratio of 6.25. Expression of nitrogenase polypeptides was studied by SDS-PAGE and Western-blot analyses of samples from cultures adapted to different C/N ratios where lactate was the carbon source. The results showed that cells grown with lactate and ammonium at a C/N ratio of 6 exhibited faint but significant bands of the polypeptides of nitrogenase (Fig. 2). At a lower C/N ratio of 4.5 neither one of these polypeptides could be detected, while at higher C/N ratios of 7.5 and 15 the polypeptides exhibited rather high cellular levels. This suggests that cells were induced to form active nitrogenase polypeptides when consuming lactate and ammonium at a C/N ratio of about 6.

Since the results reported so far were obtained with cultures grown at a constant D, it was of interest to know, if D influenced the formation and function of nitrogenase. De-repression of nitrogenase was not influenced by the dilution rate, because nitrogenase could not be detected in cultures consuming lactate and ammonium at C/N ratios below 6 and dilution rates of $D = 0.06\ h^{-1}$ and $D = 0.12\ h^{-1}$ (data not shown). But at higher C/N ratios, the activity of *in situ*-dinitrogen fixation almost doubled if the dilution rate was increased from $D = 0.06\ h^{-1}$ to $D = 0.12\ h^{-1}$ (Table II). Moreover, the results suggest that the activity of *in situ*-dinitrogen fixation depended either on the C/N ratio or, at a given C/N ratio, on D rather than on changes in the concentrations of either lactate or ammonium. In addition to *in situ*-dinitrogen fixation, nitrogenase activities of culture samples were determined by acetylene reduction assayed under optimal test conditions. This activity increased, as well, with increasing either D or the C/N ratio (Table II). The respective increments, however, were less pronounced than in the case of *in situ*-dinitrogen fixation.

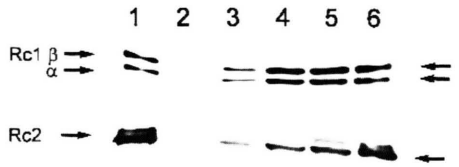

Fig. 2. De-repression of the nitrogenase enzyme complex of *Rhodobacter capsulatus* grown in continuous culture at different C/N-ratios. The cultures were supplied with 5 mM of ammonium and different concentrations of L-lactate as described in Fig. 1. Identical amounts (30 μg) of cell samples from steady states were subjected to SDS-PAGE followed by Western blotting and the enzyme-linked immunosorbent assay of the nitrogenase polypeptides Rc1 α and β as well as Rc2. Lanes 1 and 6: purified nitrogenase polypeptides; lanes 2–5 cells grown at C/N-ratios of 4.5, 6, 7.5 and 15, respectively.

Table I. The dependence of the *nifH* promoter activity in a *nifH-lacZ* double cross-over mutant of *Rhodobacter capsulatus* on the ratio of either lactate or malate consumption per ammonium (C/N represents the ratio of atoms consumed). The organisms were grown phototrophically in continuous culture with 5 mM ammonium and different concentrations of either malate or lactate at a dilution rate of $D = 0.1\ [h^{-1}]$.

Carbon Source	C/N	*nifH* Promoter Activity*
L-Malate	7.1	n.d.**
	7.5	1.0
	8.0	7.9
L-Lactate	5.3	n.d.**
	6.0	n.d.**
	6.25	0.4
	6.5	1.4
	7.0	2.9

* Specific activity of β-galactosidase [nmol min^{-1} mg^{-1}].
** Not detectable.

De-repression of nitrogenase in batch culture

Studies performed with batch cultures on the control of nitrogenase by the C/N ratio are hampered by the fact that the concentrations of nutrients as well as the culture density are steadily changing. If, however, batch cultures are allowed to utilize the sources of carbon and fixed nitrogen as complete as possible, it should be possible to determine the C/N ratio above which nitrogenase is expressed and/or activated. In the present study, phototrophic batch cultures of *R. capsulatus* were supplied with RCVBN medium containing initial concentrations of 5 mM ammonium and different

Table II. Dependence of cellular nitrogenase activities (*in situ*-N_2 fixation and acetylene reduction assayed with samples taken from the culture) of *Rhodobacter capsulatus* on the dilution rate (*D*) and the ratio of lactate and ammonium consumption (C/N represents the ratio of atoms consumed). The organisms were grown phototrophically in a chemostat system gassed with nitrogen (N_2).

D	Lactate	Ammonium	C/N	Nitrogenase activity	
[h^{-1}]	[mM]	[mM]		N_2-Fixation*	Acetylene reduction*
0.12	10	4	7.5	2.4	19 ± 2
	5	2	7.5	3.0	20 ± 3
	10.5	3	10.5	5.7	24 ± 4
	10	2	15	7.7	34 ± 2.5
	5	1	15	8.0	31 ± 3.3
	11	2	16.5	9.0	33
	5	0.5	30	10.5	48 ± 10
0.06	10	4	7.5	1.2	15 ± 1.3
	5	2	7.5	1.5	19 ± 3.3
	10.5	3	10.5	3.0	23 ± 7
	10	2	15	3.9	22 ± 1
	5	1	15	3.8	27 ± 3
	5	0.5	30	6.5	31 ± 8

* nmol min^{-1} mg^{-1}.

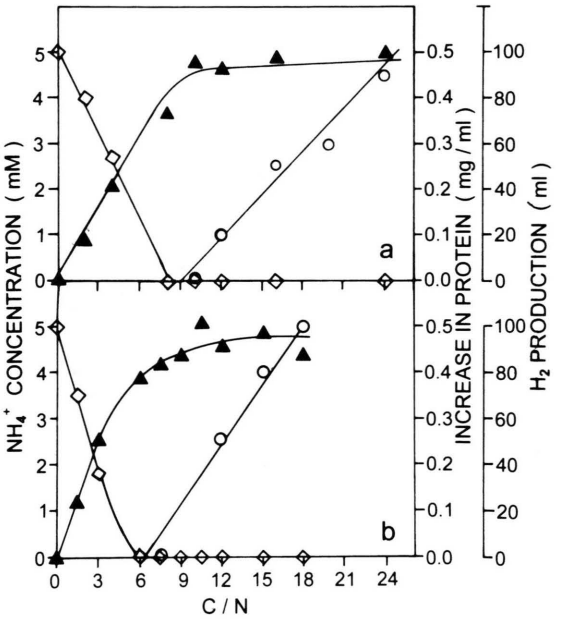

Fig. 3. Biomass formation and hydrogen evolution by phototrophic batch cultures of *Rhodobacter capsulatus* supplied with 5 mM of ammonium and different concentrations of L-malate (a) or L-lactate (b). The C/N ratio represents the number of atoms of the carbon sources consumed per atom of N of ammonium. The data depicted represent mean values obtained with three to seven independent bottles per batch culture experiment. Each experiment was repeated a least once. Hydrogen evolution (○) is presented as the total volume of gas (ml) accumulated by 50 ml of culture in the course of 90 h of incubation in the light. The increase in cell protein concentration (▲) was calculated on the amount of protein determined after 90 h of cultivation less the amount of protein of the inoculum. Ammonium concentration in the spent medium (◇) determined after 90 h of cultivation.

concentrations of either L-malate or L-lactate as detailed below. Since the batch cultures were kept in hermetically sealed bottles without addition of dinitrogen gas (except for dinitrogen initially dissolved in the fresh culture medium), the occurrence of nitrogenase was determined on the basis of hydrogen production. The cultures were incubated for 90 h to warrant optimal consumption of the carbon substrate as source of reducing equivalents. In fact, determinations of malate and lactate, respectively, in the spent media showed that both of the substrates were completely consumed by the organisms after 90 h. The uptake of ammonium, however, depended on the initial C/N ratio as well as on the carbon source. With malate, ammonium became undetectable at a C/N ratio of about 8, and with lactate this ratio was about 6 (Fig. 3). Up to these ratios, cell protein formation increased with increasing the C/N ratio. This means that biomass production was carbon-limited. Upon ammonium limitation, biomass production approached a constant level. Concomitantly, cultures started to evolve hydrogen. The total volume of hydrogen accumulated per culture increased with increasing the initial C/N ratio.

Interestingly, under conditions of carbon-limitation, the sources of carbon and nitrogen were consumed at constant ratios, which essentially agreed with the respective ratios at about which cells started to exhibit nitrogenase activity (Fig. 4). At higher C/N ratios, carbon and fixed nitrogen were completely consumed, i.e. the ratios at which C and N were consumed and supplied, respectively, became identical.

Discussion

The results obtained with both continuous and batch cultures showed that nitrogenase was de-repressed when the cells consumed lactate and malate and the fixed-nitrogen sources at ratios of about 6 and 8, respectively. Explanation of these values, which were independent of light intensity

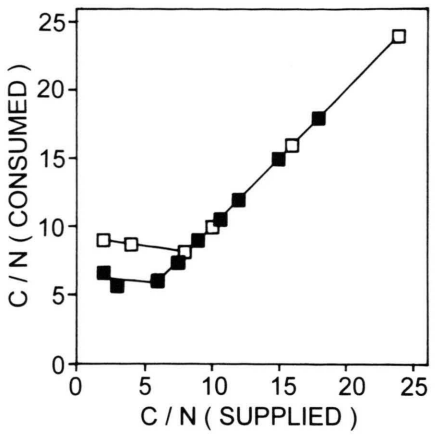

Fig. 4. The ratio of C and N atoms at which the carbon sources L-lactate (■) and L-malate (□) were supplied and consumed, respectively, per unit of ammonium by phototrophic batch cultures of *Rhodobacter capsulatus* grown as in Fig. 3.

and D, is possible after calculation of the stoichiometry of substrate assimilation into biomass. Previous calculations of biomass formation in *R. capsulatus* were based on an elemental composition of $CH_{1.83}N_{0.183}O_{0.5}$ (Klein *et al.*, 1991). Accordingly, the following equation describes the requirements of the assimilation of ammonium in combination with one mol of lactate into biomass:

$$C_3H_6O_3 + 0.55\ NH_3 + 0.426\ NADH_2 \rightarrow$$
$$C_3H_{5.5}N_{0.55}O_{1.5} + 1.5\ H_2O + 0.426\ NAD$$

0.426 moles of $NADH_2$, which are required because lactate is more oxidized than biomass, must be provided by dissimilation of 0.071 mol of lactate according to:

$$C_3H_6O_3 + 3\ H_2O + 6\ NAD \rightarrow 3\ CO_2 + 6\ NADH_2$$

Thus, biomass formation from lactate and ammonium is defined by the following equation:

$$1.071\ C_3H_6O_3 + 0.55\ NH_3 \rightarrow$$
$$C_3H_{5.5}N_{0.55}O_{1.5} + 1.3\ H_2O + 0.213\ CO_2$$

The corresponding C/N ratio amounts to 5.8.

From the above calculations it follows that the C/N ratio should increase when the substrate is more oxidized than lactate. Accordingly, the calculated C/N ratio for biomass formation from malate and ammonium is 7.8.

Both of the calculated C/N ratios predict that at higher ratios the cultures will be growing N-limited and N-sufficient at lower ratios. However, N-limitation can be prevented and constancy of the C/N ratio required for biomass production is warranted, if diazotrophic organisms fix dinitrogen. Under conditions of N-sufficiency, the cultures may be expected to consume only the amount of fixed nitrogen needed for biomass formation. The data of the present study support the above predictions.

The occurrence of dinitrogen fixation under conditions of N-limitation does not necessarily mean that at the same C/N ratios nitrogenase polypeptides are formed or the *nifH* promoter is activated. In the present case, however, essentially identical C/N ratios were determined for the regulation of nitrogenase at all of the three levels. This suggests that the signal provided by the ratio at which malate or lactate and fixed nitrogen are consumed is directly transduced into a physiological response, i.e. into de-repression of *nif* gene transcription. Since ammonium is assimilated in *R. capsulatus* directly into glutamine *via* the glutamine synthetase/glutamate synthase pathway (Borghese and Wall, 1992), the present results suggest that the C/N ratio represents the ratio of 2-oxoglutarate to glutamine. As compared with the 2-oxoglutarate/glutamine ratio, this means that the C/N ratio of nutrients consumption provides an experimentally more accessible parameter of defining the N status of the cells.

C/N ratios of the assimilation of most of the substrates utilized by *R. capsulatus* in combination with ammonium are in the range between about 6 and 8. Control experiments showed that, with different carbon sources and ammonium, nitrogenase is de-repressed if the C/N ratio increases above the respective calculated value. Yet, prediction of C/N ratios for nitrogenase de-repression was less successful if cultures were grown with an amino acid as nitrogen and carbon source in combination with another carbon source like malate or lactate. This is hardly surprising because formation of glutamine from other amino acids is less direct than from ammonium.

Finally, it should be noted that C/N ratios for nitrogenase de-repression determined above apply not only to *R. capsulatus* but also to other phototrophic bacteria like *Rhodospirillum rubrum* (Zeiger and Oelze, unpublished).

Acknowledgement

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