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Nitrogen Metabolism in the Phototrophic Bacteria *Rhodocyclus purpureus* and *Rhodospirillum tenue*

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Studies of the nitrogen nutrition and pathways of ammonia assimilation in *Rhodocyclus purpureus* and *Rhodospirillum tenue* have shown that these two seemingly related bacteria differ considerably in aspects of their nitrogen metabolism. When grown photoheterotrophically with malate as carbon source, *R. purpureus* utilized only NH_4^+ or glutamine as sole nitrogen sources and was unable to fix N_2. By contrast, *R. tenue* was found to utilize a variety of amino acids as nitrogen sources and was a good N_2 fixer. No nitrogenase activity was detected in cells of *R. purpureus* grown on limiting ammonia, whereas cells of *R. tenue* grown under identical conditions reduced acetylene to ethylene at high rates. Regardless of the nitrogen source supporting growth, extracts of cells of *R. purpureus* contained high levels of glutamate dehydrogenase, whereas *R. tenue* contained only trace levels of this enzyme. Alanine dehydrogenase activity was absent from both species. We conclude that *R. purpureus* is incapable of fixing molecular nitrogen and employs the glutamate dehydrogenase pathway as the primary means of assimilating NH_4^+ under all growth conditions. *R. tenue*, on the other hand, employs the glutamine synthetase/glutamate synthase pathway for the incorporation of NH_4^+ supplied exogenously or as the product of N_2 fixation.

Recent molecular taxonomic studies employing analysis of 16S rRNA sequences have separated the *Rhodospirillaceae* into several taxonomic subgroups, each of which contains several morphologically distinct representatives which have traditionally appeared as separate species or genera (7). One such subgroup contains species from three genera, namely, *Rhodopseudomonas gelatinosa, Rhodospirillum tenue*, and *Rhodocyclus purpureus* (6, 7, 26). These organisms share in common a tubular intracytoplasmic membrane arrangement (5, 18, 19), quite distinct from the lamellar or vesicular membrane systems of the remaining members of *Rhodospirillaceae* and the *Chromatiaceae*. Biochemical analyses of the lipopolysaccharide of the *Rhodocyclus* species supports a strong affinity with *R. tenue*, since both contain the same fatty acids and sugars in their outer membrane (25). In addition, purple violet strains of *R. tenue* and the sole strain of *R. purpureus* have virtually identical carotenoid compositions (20), and their DNA base ratios are within 1% (23).

Despite the outward appearance of relatedness between *R. purpureus* and *R. tenue*, the carbon nutrition of these two organisms differs markedly (19). *R. purpureus* is very restricted in its carbon metabolism, using only a few organic acids and related compounds as sole carbon sources for photoheterotrophic growth (19). *R. tenue*, on the other hand, is typical of the majority of *Rhodospirillaceae*, most of which use a variety of organic acids, fatty acids, alcohols, and many sugars as carbon sources photosynthetically, or as carbon and energy sources chemotrophically in darkness (23). In addition, *R. purpureus* is the only nonmotile member of the *Rhodospirillaceae* and was the first species of this family found to require vitamin B_{12} (19). A few strains of *R. tenue* also require vitamin B_{12} (21).

With the objective of better defining the nitrogen nutrition of *Rhodospirillaceae*, we have examined the utilization of nitrogenous compounds and the route of ammonia assimilation in *R. tenue* and *R. purpureus*. We report herein that these two species differ considerably in their abilities to use organic nitrogen sources or N_2 in place of ammonia and in their primary enzymatic routes by which ammonia is incorporated.

MATERIALS AND METHODS

Bacterial strains. *R. purpureus* 6770 (DSM 168) and *R. tenue* 2761 (DSM 109, ATCC 25093) were obtained from N. Pfennig and J. Oelze, respectively. *Rhodopseudomonas capsulata*, B10 has been described previously (13).

Media. The defined malate-mineral salts medium (RCVM) used for photoheterotrophic growth of *R.*

purpureus and *R. tenue* contained (per liter of deionized water): disodium EDTA, 20 mg; $MgSO_4 \cdot 7H_2O$, 200 mg; $CaCl_2 \cdot 2H_2O$, 75 mg; KH_2PO_4, 0.6 g; K_2HPO_4, 0.9 g; $(NH_4)_2SO_4$, 1 g; DL malic acid, 4 g; thiamine hydrochloride, 1 mg; biotin, 15 μg; iron solution (containing, per 1,000 ml of deionized water, 800 mg of disodium EDTA and 1,000 mg of $FeNH_4(SO_4)_2 \cdot 12H_2O$), 2 ml; and trace element solution (containing, per 250 ml of deionized water, $CoCl_2 \cdot 6H_2O$, 11 mg; $MnSO_4 \cdot H_2O$, 398 mg; H_3BO_3, 700 mg; $Cu(NO_3)_2 \cdot 3H_2O$, 10 mg; $ZnSO_4 \cdot 7H_2O$, 60 mg; $NaMoO_4 \cdot 2H_2O$, 188 mg), 1 ml. All media were adjusted before autoclaving to pH 6.8. For growth of *R. purpureus*, medium RCVM was supplemented with 1 mg of vitamin B_{12} and 5 μg of *para*-aminobenzoic acid per liter, and the phosphate content of the medium was doubled. Tests for use of alternative nitrogen sources were carried out in RCVM medium minus $(NH_4)_2SO_4$, containing a single nitrogenous compound in the concentration specified in Table 1.

Growth conditions. Cells were grown photosynthetically in completely filled screw-capped tubes (17 ml) or glass bottles (250 ml). When N_2 served as nitrogen source, 500-ml Erlenmeyer flasks fitted with a side arm for measuring cell densities were used. Each flask was filled with 250 ml of RCVM medium minus NH_4^+ and contained a magnetic stirring bar. The contents of each flask were bubbled for 15 min with 1% CO_2 in N_2, inoculated with 1 to 5% inoculum, and sealed with a rubber stopper under a stream of 1% CO_2 in N_2.

For large-scale growth with N_2 as nitrogen source, flat-sided 1-liter bottles filled to the neck with medium and sealed with a rubber stopper were used. A gas mixture of 1% CO_2 in N_2 was continuously passed through the medium by using the sparging/sampling apparatus described by Madigan and Gest (13). Samples were removed anaerobically from the bottle with this same device (13). After inoculation, all vessels were routinely placed at 30°C in the dark for 1 to 2 h before being placed in the light. Photosynthetic growth was achieved by incubation at 30 to 33°C with continuous illumination of approximately 4,500 lx from 60-W Lumiline incandescent lamps (Sylvania). Cells grown with N_2 were stirred at approximately 150 rpm.

Measurement of bacterial growth. Cell numbers and cell mass were quantitated as follows. (i) Cell densities were measured turbidimetrically with a Klett-Summerson photometer fitted with a no. 66 (red) filter or with a Beckman DB spectrophotometer at 660 nm. (ii) Cell dry weights were determined by centrifugation of 20 ml of cell suspension and then washing the pellet once in deionized water and drying to constant weight at 90°C in preweighed aluminum foil dishes.

Preparation of cell-free extracts. Exponentially growing cells were harvested by centrifugation, washed once in 50 mM Tris-hydrochloride buffer (pH 7.6) and suspended in a small amount of the same buffer. β-Mercaptoethanol was added to *R. tenue* cell suspensions before disruption (10 mM final concentration). Crude extracts were obtained by passing cells (once) through a precooled French pressure cell operating at 20,000 lb/in^2 (4°C). For glutamate dehydrogenase or glutamate synthase assays, crude extracts were centrifuged at 106,000 × *g* for 90 min, and the supernatants were removed for enzyme assay. For glutamine synthetase assays, crude extracts were centrifuged at 32,000 × *g* for 30 min, and the supernatant (containing membranes) was removed for assay.

Enzyme assays. Glutamate dehydrogenase (E.C. 1.4.1.3) and glutamine-oxoglutarate aminotransferase (glutamate synthase, E.C. 1.4.1.13) activities were assayed by measuring the rate of NAD(P)H oxidation as described by Meers et al. (16). Glutamine synthetase (E.C. 6.3.1.2) was assayed via the γ-glutamyl transferase and biosynthetic assays of Bender et al. (2). Tests for glutaminase activity were carried out in crude extracts by the method of Brown and Tata (4). Nitrogenase assays were performed as previously described (12, 15).

Protein determination. The protein content of cell extracts was estimated by the method of Lowry et al. (11), using bovine serum albumin (Sigma Chemical Co.) as standard.

Ammonia determination. Ammonia levels in growing cultures were determined colorimetrically (1) on the supernatant fluid remaining after centrifuging cell suspensions.

RESULTS

Nitrogen nutrition of *R. purpureus* and *R. tenue*. Table 1 lists the nitrogenous compounds tested as sole nitrogen sources for photoheterotrophic growth of *R. purpureus* and *R. tenue*. Other than NH_4^+, only glutamine was found to support the growth of *R. purpureus*, although slight growth could be obtained with yeast extract. Growth of *R. purpureus* on N_2 as sole nitrogen source could not be demonstrated. By contrast, *R. tenue* utilized a number of nitrogen sources besides NH_4^+, and particularly good growth was achieved when glutamate, aspartate, or N_2 served as nitrogen source (Table 1). Cultures of *R. tenue* grown on nitrogen sources other than N_2 or NH_4^+ produced a strong positive gas pressure which was assumed to be H_2 (14, 17).

Attempts to grow *R. purpureus* on N_2 and induce nitrogenase activity. After observing that N_2 would not support growth of *R. purpureus* (Table 1), attempts were made to induce nitrogenase activity by the addition of reducing agents and additional organic constituents. Flasks containing medium RCVM (minus NH_4^+) and vitamins B_{12} and *para*-aminobenzoic acid were supplemented with one of the following: 2, 1, or 0.5 mM NH_4^+ (all growth limiting); 0.05% (wt/vol) sodium ascorbate; 0.05% (wt/vol) cysteine; 10 mM glutamate; 10 mM pyruvate; 0.015% (wt/vol) hydrogen sulfide; fivefold increases in the iron and trace element concentrations; 10 mM 2-oxoglutarate; 10 mM 2-oxoglutarate plus 0.1% (wt/vol) oxaloacetate and 0.1% sodium ascorbate; 0.1% sodium bicarbonate; or 0.1% (wt/vol) yeast extract. Each flask was flushed with 1% CO_2 in N_2, inoculated with a culture of *R. purpureus* grown on limiting NH_4^+, and incubated photosynthetically. After a 72-h incubation period, no increase in culture turbidity was observed in any flask, except for sparse

TABLE 1. Utilization of nitrogenous compounds as sole nitrogen source by *R. purpureus* and *R. tenue*[a]

Nitrogen source[b]	Growth[c]	
	R. purpureus 6770	*R. tenue* 2761
Alanine	0	+
Arginine	0	+
Aspartate	0	++++
Glutamate	0	++++
Glutamine	++++	+++
Lysine	0	+
Methionine	0	+
Serine	0	+
Threonine	0	+
Ammonia	++++	++++
Casamino Acids	0	++
Peptone	0	++
Urea	0	+
Yeast extract	+	++
N_2	0	+++
Control (no added nitrogen source)	0	0

[a] Cells were grown in 17-ml screw-capped tubes containing medium RCVM minus NH_4^+ and the nitrogen source indicated at a final concentration of 10 mM (0.1% for complex substrates). Tubes were incubated photosynthetically (anaerobically) at 30°C and 4,500 lx for 72 h.

[b] Not utilized by either species: adenine, asparagine, cysteine, cytosine, glycine, histidine, isoleucine, leucine, phenylalanine, tryptophan, valine, and nitrate.

[c] Growth measurements were made in a Klett-Summerson photometer. Units: 0, no growth; +, 25 to 100 photometer units (PU); ++, 100 to 200 PU; +++, 200 to 300 PU; ++++, >300 PU. For *R. purpureus*, 100 PU = 0.25 mg cell dry weight per ml $\simeq 1.5 \times 10^9$ cells per ml/ for *R. tenue*, 100 PU = 0.25 mg cell dry weight per ml $\simeq 1.3 \times 10^9$ cells per ml.

growth in the flasks containing yeast extract (see Table 1) and variable cell densities in the flasks supplemented with growth-limiting levels of NH_4^+.

To ensure that the supply of N_2 was adequate for N_2 fixation in the above experiments, cultures of *R. purpureus* and *R. capsulata* (chosen as a positive N_2-fixing control) were incubated photosynthetically in bottles of RCVM containing only 1 mM NH_4^+ and were constantly sparged with 1% CO_2 in N_2. Samples were removed periodically, and growth and residual NH_4^+ levels were determined. The results are shown in Fig. 1. *R. purpureus* actively consumed NH_4^+ and grew at normal rates (doubling time, 3.5 h) until NH_4^+ levels were virtually undetectable. At this point (7.75 h postinoculation), growth ceased. During the next 5 h, the turbidity of the culture did not increase, even though saturating levels of N_2 were available. At 12.25 h postinoculation, additional NH_4^+ was added, and the culture immediately began to grow until NH_4^+ once again became growth limiting. During the next 14 h, the density of the *R. purpureus* culture remained relatively constant, but shortly thereafter cell lysis began (data not shown). Figure 1 also shows the result of a control experiment in which *R. capsulata* was inoculated in precisely the same manner as *R. purpureus*. In this case, growth continued after NH_4^+ exhaustion but at a slightly slower rate, typical of N_2-grown *R. capsulata* (24). When NH_4^+ was added, however, the culture immediately began growing at the original rate (doubling time, 2.5 h). These results demonstrate that under nutritional conditions which support growth and N_2 fixation in *R. capsulata*, *R. purpureus* is unable to grow, apparently from an inability to fix N_2.

Table 2 shows the results of nitrogenase assays performed with intact cells of *R. purpureus* and *R. tenue* grown under various nitrogen

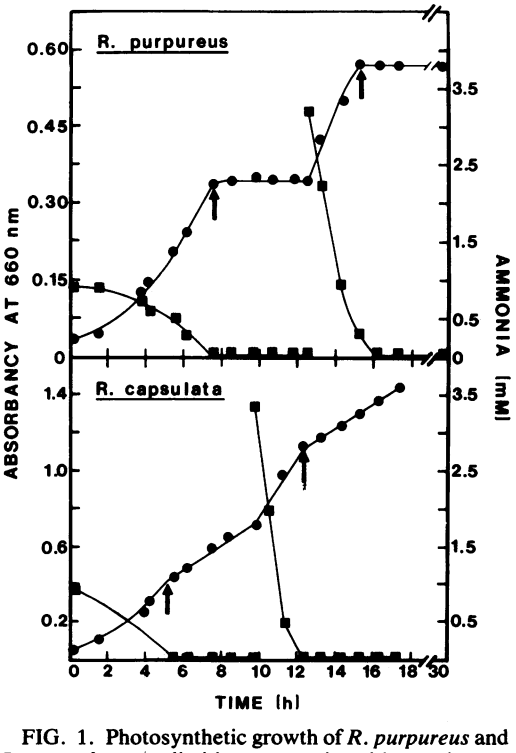

FIG. 1. Photosynthetic growth of *R. purpureus* and *R. capsulata* on limiting ammonia with continuous sparging with N_2. Cells of both species were grown in 1-liter bottles as described in the text. Arrows indicate the point at which ammonia was no longer detectable in culture fluids. Additional ammonia (3.5 mM) was added at 12.25 h (*R. purpureus*) or 10 h (*R. capsulata*). Symbols: ●, growth (absorbance at 600 nm); ■, ammonia concentration (millimolar). Both organisms were grown at 30°C and 4,500 lx.

TABLE 2. Tests for nitrogenase activity (acetylene reduction) in cells of *R. purpureus* 6770 and *R. tenue* 2761 grown on various nitrogen sources

Organism	Nitrogen source for growth	Nitrogenase activity[a] (nmol of C_2H_4 produced per h per mg dry weight of cells)
R. purpureus	15 mM ammonia	<0.01
	1 mM ammonia	<0.01
	30 mM glutamine	<0.01
	0.1% yeast extract	<0.01
R. tenue	15 mM ammonia	<0.01
	1 mM ammonia	1,400
	30 mM glutamine	187
	10 mM glutamate	1,103
	N_2	1,393

[a] Cells of *R. purpureus* and *R. tenue* were grown to mid-log phase on the nitrogen source listed (at 30°C and 4,500 lx) and were assayed as described in the text.

nutritional conditions. When cultures of *R. purpureus* or *R. tenue* were grown on excess NH_4^+, no nitrogenase activity, as expected, was detected. When *R. tenue* was grown on limiting NH_4^+, various amino acids, or N_2, nitrogenase activity was readily detected (Table 2). Cells of *R. purpureus* grown on limiting NH_4^+, glutamine, or yeast extract, however, did not reduce acetylene and were not observed to produce H_2 in completely filled bottles or tubes.

Enzymes of NH_4^+ assimilation in *R. purpureus* and *R. tenue*. The results of tests for glutamate dehydrogenase, glutamate synthase, and glutamine synthetase from extracts of *R. purpureus* and *R. tenue* grown on various nitrogen sources are shown in Table 3. Cells of *R. purpureus* contained high levels of an NADPH-linked glutamate dehydrogenase whether grown on excess or limiting NH_4^+ or glutamine, and the levels of this enzyme appeared to bear some correlation to the initial NH_4^+ content of the medium. In this connection, levels were highest in cells of *R. purpureus* grown on excess NH_4^+ and lowest in cells grown on glutamine. Tests for alanine dehydrogenase in *R. purpureus* were made by substituting pyruvate for 2-oxoglutarate in the glutamate dehydrogenase assay system; no alanine dehydrogenase activity could be demonstrated. Glutamate synthase (NADH linked) and glutamine synthetase activities in *R. purpureus* were inversely correlated with the initial concentration of NH_4^+ in the medium. Maximal activities of these enzymes were observed in glutamine-grown cells, which probably reflects a low NH_4^+ pool under these nutritional conditions. In addition, no glutaminase activity was detected in crude extracts of glutamine-grown *R. purpureus*; it is therefore likely that glutamate originates from the activity of glutamate synthase when glutamine serves as sole nitrogen source for this organism.

Unlike *R. purpureus*, cells of *R. tenue* contained barely detectable levels of an NADPH-linked glutamate dehydrogenase, regardless of the nitrogen source used to support growth. Tests for alanine dehydrogenase in *R. tenue* also were negative. *R. tenue* had fairly constant levels of an NADH-linked glutamate synthase under all nitrogen growth conditions tested. Glutamine synthetase levels in *R. tenue* were greatly elevated (10- to 20-fold) in cells grown on glutamate or N_2 as compared with cells grown with excess NH_4^+.

DISCUSSION

The results presented here show that *R. purpureus* is severely restricted in its ability to utilize alternative sources of nitrogen for biosyn-

TABLE 3. Assays of ammonia assimilation enzymes in extracts of *R. purpureus* 6770 and *R. tenue* 2761 grown on various nitrogen sources

Organism	Nitrogen source for growth	Enzyme activity (nmol/min per mg of protein)[a]			
		Glutamate dehydrogenase[b]	Glutamate synthase[c]	Glutamine synthetase[d]	
				Transferase	Biosynthetic
R. purpureus	15 mM ammonia	1,750	63	156	21
	2 mM ammonia	903	105	120	54
	0.5 mM ammonia	431	136	202	126
	30 mM glutamine	500	174	256	419
R. tenue	15 mM ammonia	4	78	133	27
	30 mM glutamine	0	65	332	140
	10 mM glutamate	5	68	1,051	164
	N_2	4	79	1,123	552

[a] All assays performed at 25°C.
[b] α-Ketoglutarate + NH_3 + NADPH → glutamate.
[c] Glutamine + α-ketoglutarate + NAD(P)H → 2 glutamate.
[d] Transferase, γ-glutamyl transferase (reverse) reaction; biosynthetic, forward reaction.

thetic purposes. *R. tenue*, on the other hand, is considerably more versatile in this connection, as are most other *Rhodospirillaceae* (10, 14). *R. purpureus* resembles *Rhodopseudomonas acidophila* in its nitrogen metabolism, because neither of these organisms is able to utilize glutamate or aspartate as sole nitrogen sources, yet both species utilize glutamine (8). *R. acidophila* differs from *R. purpureus*, however, in its ability to utilize alanine, asparagine, nitrate, and N_2, in addition to glutamine (8).

It is clear that the major representatives of the family *Rhodospirillaceae* do fix N_2 (10, 14, 17). The *Rhodocyclus* species, on the other hand, is apparently unable to fix N_2, and this represents the first documented case of a species of *Rhodospirillaceae* being unable to fix N_2. Lack of an active nitrogenase in *R. purpureus* was confirmed by the inability of cells to reduce acetylene when grown to NH_4^+ exhaustion or when grown on glutamine or yeast extract. We cannot rule out the possibility that *R. purpureus* requires an unusual compound or compounds for nitrogenase synthesis or activity, but if *R. purpureus* is capable of N_2 fixation, the necessary prerequisites are not those of typical *Rhodospirillaceae*.

Levels of glutamate dehydrogenase measured in *R. purpureus* were considerably higher than those reported from other *Rhodospirillaceae* (3). Glutamine synthetase and glutamate synthase levels were, however, within the range of those found in several species of *Rhodospirillaceae* (3). These findings lead to the conclusion that glutamate dehydrogenase, a constitutive enzyme in *R. purpureus*, probably represents the major route of NH_4^+ incorporation in this organism, regardless of growth conditions. Constitutive glutamate dehydrogenases have also been reported from certain *Rhodospirillaceae* (3) and from *Escherichia coli* (22). In the latter case, some glutamate dehydrogenase is made even when NH_4^+ levels fall far below the K_m of the enzyme (22). By contrast, in organisms such as *Aerobacter aerogenes* and *Pseudomonas fluorescens*, glutamate dehydrogenase levels are almost totally repressed when cells of these species are grown under limited NH_4^+ as compared to NH_4^+ excess conditions (16).

The major route of NH_4^+ assimilation in *R. tenue* clearly involves the coupled glutamine synthetase/glutamate synthase reaction sequence. *R. tenue* synthesizes only traces of glutamate dehydrogenase, and it is doubtful whether this enzyme has any physiological significance. (Noteworthy in this connection is the finding that growth of *R. tenue* is significantly inhibited by the glutamate analog and glutamine synthetase inhibitor, methionine sulfoximine; the latter has no effect on growth of *R. purpureus* [data not shown].) The glutamate dehydrogenase pathway is not universally present in microorganisms and is absent from certain phototrophic bacteria such as *R. capsulata* (9) and *Rhodopseudomonas globiformis* (12). In these organisms, the glutamine synthetase/glutamate synthase pathway is active whether N_2 or NH_4^+ serves as nitrogen source, but under nitrogen-fixing conditions, glutamine synthetase levels are typically elevated (9, 12). This phenomenon was also observed in *R. tenue*.

In conclusion, even though *R. purpureus* and *R. tenue* show strong similarities in their photosynthetic pigments and membrane arrangement, DNA base ratios, and 16S rRNA class, they differ distinctly in the basic physiology of their nitrogen metabolism. The inability of *R. purpureus* to fix N_2 and the utilization of the glutamate dehydrogenase pathway as the primary means of NH_4^+ assimilation stand in marked contrast to the active N_2-fixing characteristics and dependence on the glutamine synthetase/glutamate synthase pathway for NH_4^+ assimilation of *R. tenue*. Our findings support the carbon nutritional studies of Pfennig (19), which also point to a physiological dichotomy between *R. purpureus* and *R. tenue*.

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