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Nitrogen Fixation and Nitrogenase Activities in Members of the Family *Rhodospirillaceae*

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Strains of all 18 species of the family *Rhodospirillaceae* (nonsulfur photosynthetic bacteria) were studied for their comparative nitrogen-fixing abilities. All species, with the exception of *Rhodocyclus purpureus*, were capable of growth with N_2 as the sole nitrogen source under photosynthetic (anaerobic) conditions. Most rapid growth on N_2 was observed in strains of *Rhodopseudomonas capsulata*. Within the genus *Rhodopseudomonas*, the species *R. capsulata*, *R. sphaeroides*, *R. viridis*, *R. gelatinosa*, and *R. blastica* consistently showed the highest in vivo nitrogenase rates (with the acetylene reduction technique); nitrogenase rates in other species of *Rhodopseudomonas* and in most species of *Rhodospirillum* were notably lower. Chemotrophic (dark microaerobic) nitrogen fixation occurred in all species with the exception of one strain of *Rhodospirillum fulvum*; oxygen requirements for dark N_2 fixation varied considerably among species and even within strains of the same species. We conclude that the capacity to fix molecular nitrogen is virtually universal among members of the *Rhodospirillaceae* but that the efficacy of the process varies considerably among species.

The family *Rhodospirillaceae* (nonsulfur purple photosynthetic bacteria) currently contains 18 species placed in the four genera *Rhodopseudomonas*, *Rhodospirillum*, *Rhodocyclus*, and *Rhodomicrobium* (30). In 1949, Gest and Kamen demonstrated that *Rhodospirillum rubrum* was capable of producing molecular hydrogen (6) and fixing molecular nitrogen (12). Shortly thereafter, the remaining species of nonsulfur photosynthetic bacteria known at that time, as well as representatives of the purple and green sulfur bacteria, also were shown to be capable of fixing N_2 (14, 15).

Since the early 1950s, several new species of *Rhodospirillaceae* have been described, but despite a number of reviews devoted to nitrogen fixation and hydrogen metabolism in phototrophic and other free-living, nitrogen-fixing bacteria (7, 17, 21, 25, 29, 32), the capacity of many species of *Rhodospirillaceae* to fix N_2 remains undocumented. In this communication we present the results of comparative studies on the nitrogen-fixing abilities of all 18 species of *Rhodospirillaceae* (3, 4, 30) with sensitive assay techniques and a standardized testing protocol. Strains of *Rhodospirillaceae* obtained from various culture collections as well as newly isolated strains enriched from nature have been examined for their ability to grow with N_2 as the sole nitrogen source under both phototrophic and chemotrophic conditions and have been assayed for their in vivo nitrogenase contents. We report that the ability to fix nitrogen is a universal property of species of *Rhodospirillaceae* (with one exception) and that the capacity to fix N_2 is particularly well developed in the species *Rhodopseudomonas capsulata*.

MATERIALS AND METHODS

Bacterial strains. The sources of bacterial cultures used in this study are listed in Tables 1 and 2.

Growth media. Most strains were grown photosynthetically in a medium (RCVBNP) containing, per liter of deionized water: EDTA, 20 mg; $(NH_4)_2SO_4$, 1 g; DL-malate, 4 g; $MgSO_4 \cdot 7H_2O$, 200 mg; $CaCl_2 \cdot 2H_2O$, 75 mg; iron solution (31), 2 ml; trace elements solution [containing, per 250 ml of deionized water: $MnSO_4 \cdot H_2O$, 0.3975 g; H_3BO_3, 0.7 g; $Cu(NO_3)_2 \cdot 3H_2O$, 10 mg; $ZnSO_4 \cdot 7H_2O$, 60 mg; $NaMoO_4 \cdot 2H_2O$, 0.1875 g; $CoCl_2 \cdot 6H_2O$, 11.25 mg], 1 ml; thiamine hydrochloride, 1 mg; biotin, 15 μg; vitamin B_{12}, 20 μg; nicotinic acid, 1 mg; *para*-aminobenzoic acid, 0.5 mg; and phosphate solution (KH_2PO_4 [20 g] and K_2HPO_4 [30 g] in 500 ml of deionized water), 15 ml. The pH of the medium was adjusted to 6.8 and sterilized by autoclaving for 20 min. For growth on N_2, $(NH_4)_2SO_4$ was omitted from the medium.

RCVBNP was modified for growing certain species of *Rhodospirillaceae*. *Rhodopseudomonas sulfidophila* was grown in RCVBNP supplemented with 0.05% sulfide (pH 7) and 3% (wt/vol) NaCl. *Rhodocyclus purpureus* and *Rhodopseudomonas globiformis* were grown in media described by Masters and Madigan (19) and Madigan and Cox (16), respectively. *Rhodopseudomonas sulfoviridis* was grown in RCVBNP with 0.02% (wt/vol) acetate, 0.05% (wt/vol) yeast extract, and 0.05% sulfide (pH 7). *Rhodopseudomonas acidophila* was grown in a medium containing, per liter of deionized water: NH_4Cl, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $CaCl_2 \cdot 2H_2O$, 0.05 g; NaCl, 0.4 g; SL-6 trace elements solution (22), 1 ml; iron solution (31), 1 ml; sodium succinate, 4 g; and KH_2PO_4 (made up in a separate solution [pH 5.5] and added after autoclaving), 1 g. The medium used for *Rhodospirillum salexigens* contained, per liter of deionized water: EDTA, 20 mg; $MgSO_4 \cdot 7H_2O$, 200 mg; $CaCl_2 \cdot 2H_2O$, 75 mg; trace elements solution (used in RCVBNP), 1 ml; iron solution (31), 1 ml; NaCl, 70 g; sodium glutamate, 4 mM; and acetate, 0.2% (wt/vol). After autoclaving, 15 ml of the phosphate solution in RCVBNP was added per liter.

Enrichment and isolation. Twenty-three new strains of *Rhodospirillaceae* were isolated from water, mud, and leaf litter according to the general enrichment protocol described by Madigan and Gest (17). The enrichment conditions were established to take advantage of the fact that *Rhodospirillaceae* typically fix N_2 under anaerobic photosynthetic conditions. The primary enrichment medium was similar to RCVBNP with the following exceptions: EDTA was omit-

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TABLE 1. Nitrogen fixation and nitrogenase activities in the genus *Rhodopseudomonas*

Species	Strain designation/origin[a]	Generation time (h)[b]	Chemotrophic (dark) N_2 fixation[c]	In vivo nitrogenase activity[d]
R. capsulata	B10 (PBG)	3.2	1.0	3,650
	St. Louis (PBG)	3.7	1.5	1,800
	6950 (DSM)	3.9	1.5	1,430
	KB1 (DSM)	4.0	2.0	3,700
	SP107 (PBG)	3.4	2.0	3,125
	EY3 (PBG)	3.0	2.0	4,900
	Ford	3.2	1.0	3,710
	DR	3.8	1.5	2,480
	4-6-4	3.6	2.0	2,340
	5-2-5	3.8	2.0	4,420
	29-21	4.1	1.5	2,510
	30-11	3.8	1.0	2,990
	31-9	3.5	1.5	3,010
R. sphaeroides	2.4.1 (vanNiel; PBG)	6.5	2.5	1,110
	2.4.3 (vanNiel; PBG)	5.0	2.0	1,875
	H6 (PBG)	3.5	2.0	1,940
	LC6 (PBG)	4.3	1.5	1,700
	KS1 (PBG)	3.9	2.5	1,260
	DG	3.2	1.0	2,260
	21-7	4.1	2.5	2,020
	Geller	5.5	2.0	2,755
R. gelatinosa	2.2.1 (vanNiel; PBG)	9.9	2.5	860
	Klemme (PBG)	8.6	2.0	1,060
	DSM 149 (Pfennig)	6.4	2.0	730
	31-92	9.6	3.0	1,600
	30-2	3.5	1.5	2,490
	Nelson	8.0	2.0	1,600
R. viridis	DSM 133 (= ATCC 19567) (DSM)	7	10.5	2,070
	UN	7	2.0	2,510
R. blastica	NCIB 11567 (Dow)	5.5	2.0	1,230
R. palustris	2.1.6 (vanNiel; PBG)	22	4.0	525
	GH (PBG)	9	3.0	540
	RP1 (PBG)	12	3.5	700
	Stone	7	2.0	540
	29-1	9	1.0	480
R. acidophila	DSM 145 (DSM)	5	3.0	750
	7050 (= ATCC 25092) (DSM)	8	1.5	880
	MO	12.5	5.0	500
R. sulfidophila	W4 (Hansen; PBG)	9	2.5	640
R. globiformis	7950 (Pfennig)	27	9.0	515
R. sulfoviridis[e]	DSM 729 (Trüper)	13	1.0	520

[a] Strain origins: PBG, Photosynthetic Bacteria Group, H. Gest, Indiana University; DSM, Deutsch Sammlung von Mikroorganismen, Göttingen, Federal Republic of Germany; Pfennig, N. Pfennig, Universität Konstanz, Konstanz, Federal Republic of Germany; Trüper, H. G. Trüper, Rheinische Friederich-Wilhelms-Universität, Bonn, Federal Republic of Germany; Dow, C. Dow, University of Warwick, Coventry, United Kingdom; Hansen, T. Hansen, University of Groningen, Groningen, Holland. Strains with no source listed in parentheses were isolated in the present work.

[b] Photosynthetic growth with N_2 as the sole nitrogen source; cultures were stirred at ca. 150 rpm.

[c] Distance from the agar surface to pigmented growth band in millimeters.

[d] Nanomoles of ethylene produced per hour per milligram of cells (dry weight).

[e] Medium contained 0.05% (wt/vol) yeast extract.

ted, DL-malate was replaced with 0.4% (wt/vol) sodium succinate, 1 mM NH_4Cl was added in place of 1 g of $(NH_4)_2SO_4$ per liter, and 0.1 g of yeast extract was added per liter. The pH was adjusted before autoclaving to 6.8. Enrichments were established with 2 to 4 g of mud or leaf litter or 0.1 to 20 ml of pond, lake, or ditch water sample and were incubated under N_2-CO_2 (99:1) at 30 to 32°C. Positive enrichments were red, brown, or purple-red after ca. 1 week. Pure cultures were obtained by streaking enrichments on plates of the same medium (solidified with 1.5% agar) and incubating them anaerobically in an atmosphere of N_2-H_2-CO_2 (GasPak jars, BBL Microbiology Systems). Colonies

TABLE 2. Nitrogen fixation and nitrogenase activities in *Rhodospirillum*, *Rhodomicrobium*, and *Rhodocyclus* species

Species	Strain designation/origin[a]	Generation time (h)	Chemotrophic (dark) N_2 fixation	In vivo nitrogenase activity
Rhodospirillum rubrum	1.1.1. (vanNiel; PBG)	8.5	2.0	750
	Utah (PBG)	9.0	3.0	1,175
	21-3	7.6	5.0	1,090
	BC	9.8	2.0	715
R. tenue	DSM 110 (DSM)	11.0	5.0	210
	2761 (= ATCC 25093) (Oelze)	8.8	3.0	1,400
	LA (PBG)	10.0	2.5	890
R. fulvum	1360 (DSM)	7.0	NG[b]	390
	2860 (Biebl)	6.4	6.0	930
	4	8.2	4.5	720
R. molischianum	14031 (ATCC)	8.8	12.1	1,030
R. photometricum	DSM 122 (Biebl)	11.0	6.0	153
	27871 (ATCC)	4.7	5.0	755
	SP113 (PBG)	7.5	11.0	730
	3-4-8	5.3	3.5	760
R. salexigens[c]	DSM 2132 (Drews)	8.8	At surface	630
Rhodomicrobium vannielii	DSM 162 (= ATCC 17100) (Pfennig)	4.3	1.5	660
	DSM 163 (DSM)	9.8	2.0	510
	EY33 (PBG)	5.7	2.5	820
	SI	8.0	5.0	1,350
	Kelly	6.5	3.5	1,570
Rhodocyclus purpureus	6770 (Pfennig)	NG	NG	<0.001

[a] Strain origins as listed in Table 1, footnote *a*, plus the following: Oelze, J. Oelze, Universität Freiburg, Freiburg, Federal Republic of Germany; ATCC, American Type Culture Collection, Rockville, Md.; Drews, G. Drews, Albert-Ludwigs-Universität, Freiburg, Federal Republic of Germany; Biebl, H. Biebl, Gesellschaft Für Biotechnologische, Braunschweig, Federal Republic of Germany. For explanatory material for other columns in this table, see Table 1.

[b] NG, No growth.

[c] Medium contained 4 mM sodium glutamate.

were picked and streaked a minimum of three times to ensure that the cultures were pure. Isolates were identified as to species based on microscopy and pigmentation and were then grown to the mid-log phase and frozen at −80°C until needed.

Growth conditions. Cells were grown photosynthetically in completely filled 17-ml screw-capped tubes. When N_2 served as the sole nitrogen source, 250-ml Ehrlenmeyer flasks filled with 100 ml of the appropriate medium minus NH_4^+ and containing a magnetic stirring bar were used. Media were bubbled with 1% CO_2 in N_2 for 10 min and then inoculated. Flasks were sealed with a rubber stopper under a stream of N_2-CO_2. All cultures were incubated at 32°C in a light intensity from 3,450 to 5,380 lx (incandescent 40-W lamps). The ability to fix N_2 in the dark under microaerobic conditions was determined in growth tests similar to those described by Siefert and Pfennig (27). In these tests, 17-ml tubes were filled with 6 to 10 ml of medium (minus NH_4^+) containing 1.5% agar. Molten agar tubes were inoculated with 0.1 ml of culture, inverted several times to distribute the inoculum, and immediately placed in an ice bath to quickly solidify the agar. Culture tubes were loosely capped, and the tubes were placed in the dark at 32°C.

Measurements of growth. Growth rates were calculated by taking intermittent turbidity readings with a Klett-Summerson photometer fitted with a no. 66 (red) filter. Dry weights were determined as previously described (19).

Nitrogenase assay. In vivo nitrogenase (acetylene reduction) activity was determined by removing an 11.2-ml sample of the mid-log-phase (100 to 150 photometer units in most cases) culture under a continuous stream of argon and injecting it directly into a preflushed (argon) 70-ml serum vial. The vials were again flushed with argon, vented to atmospheric pressure, and placed at 32°C in the light (≈8,000 lx). After 15 min of preincubation, 7 ml of acetylene (produced by hydrating calcium carbide crystals) was injected into each vial. The vials were periodically shaken, and gas samples removed at intervals were assayed for their ethylene content in a Varian model 2440 gas chromatograph fitted with a flame ionization detector and a Porapak R column operated at 70°C.

RESULTS

Isolation of *Rhodospirillaceae* with N_2 as the sole nitrogen source. More than one-third of the strains of *Rhodospirillaceae* tested for comparative nitrogen-fixing ability (Tables 1 and 2) were isolated in the present work with an enrichment technique in which N_2 served as the sole nitrogen source. Enrichments of this type invariably select for *Rhodospirilla-*

ceae, due to the nature of the culture conditions employed: anaerobiosis, lack of alternative electron acceptors, respiratory substrates as main carbon sources, N_2 as the sole nitrogen source, and light as the chief energy source (17). From primary liquid enrichments, pure cultures were quickly obtained by streaking agar plates of the same medium and incubating them anaerobically in an N_2-containing atmosphere. All isolates obtained herein were identified as to species via a combination of cultural characteristics, primarily cellular morphology, pigmentation, and comparison with reference strains.

The majority of the enrichment cultures established with pond or lake water, decaying leaf litter, mud, or soil as the inoculum resulted in the isolation of *Rhodopseudomonas* species, in particular *R. capsulata*, *R. sphaeroides*, *R. gelatinosa*, and *R. palustris* (Table 1). By comparison, primary enrichments containing *Rhodospirillum* species were much less common, and the most frequently observed phototrophic spirilla were members of the so-called brown rhodospirilla: *R. fulvum*, *R. molischianum*, and *R. photometricum* (Table 2). Enrichments containing large numbers of *Rhodospirillum rubrum* or *Rhodomicrobium vannielii* were rare.

Nitrogen fixation in species of *Rhodopseudomonas*. The nitrogen-fixing properties of all 10 recognized species of the genus *Rhodopseudomonas* are listed in Table 1. Growth on N_2 as the sole nitrogen source under photosynthetic (anaerobic) conditions was demonstrated for every strain of each of the species examined (Table 1). Shortest generation times were consistently observed in strains of *R. capsulata* (mean generation time, 3.6 h) and *R. sphaeroides* (mean generation time, 4.5 h), with relatively little intraspecies strain variation. Considerably longer generation times were noted for the remaining *Rhodopseudomonas* species, and intraspecies variation was particularly noticeable among strains of the species *R. gelatinosa* and *R. palustris* (Table 1).

Chemotrophic (dark microaerobic) N_2 fixation was demonstrated with the auxanographic (agar-deep) technique (27) in strains of all species of *Rhodopseudomonas* (Table 1). A fully pigmented growth zone was visible within 3 to 4 days with the rapid-growing species of *Rhodopseudomonas*. Bands in *R. palustris* and *R. globiformis*, on the other hand, were observed only after 1 to 2 weeks of incubation. The location of the growth zone under these conditions gives a relative indication of oxygen sensitivity during dinitrogen fixation as reflected by the inherent respiratory potential of the species (27). Of interest in this connection was the finding that *R. capsulata*, *R. sphaeroides*, and *R. blastica* were, on a whole, more tolerant of O_2 while fixing N_2 chemotrophically than were the remaining species of *Rhodopseudomonas*. It is also of interest that significant intraspecies strain variation in oxygen tolerance occurs, since certain species (e.g., *R. palustris*) were observed to contain both highly oxygen-tolerant and -intolerant strains (Table 1). One strain of *R. viridis* and *R. globiformis* required very low oxygen tensions for dark N_2 fixation; the location of the growth band indicated that these organisms were much more O_2 sensitive than were any of the remaining *Rhodopseudomonas* species.

In vivo nitrogenase activities measured in logarithmic-phase cells via the acetylene reduction technique varied markedly from species to species within the genus *Rhodopseudomonas* (Table 1). The highest average specific nitrogenase activities were found in strains of *R. capsulata* ($\simeq$3 μmol of ethylene per h per mg [dry weight]). Nitrogenase activities in strains of *R. sphaeroides* were significantly lower than those in strains of *R. capsulata*, but were, on the average, significantly higher than those in other *Rhodopseudomonas* species. With the exception of newly isolated strains of *R. gelatinosa*, no significant differences in enzyme levels were found between strains of *Rhodospirillaceae* isolated in the present study (i.e., with N_2 as the sole nitrogen source) and reference strains presumably isolated with ammonia as the major or sole nitrogen source. Finally, despite their frequent appearance in enrichment cultures, pure cultures of *R. palustris* consistently expressed significantly lower nitrogenase activities than did the majority of the remaining *Rhodopseudomonas* species.

Nitrogen fixation in species of *Rhodospirillum*, *Rhodomicrobium*, and *Rhodocyclus*. Table 2 lists the nitrogen-fixing properties of members of the *Rhodospirillum-Rhodomicrobium-Rhodocyclus* group. *Rhodocyclus purpureus* was the only member of the family *Rhodospirillaceae* found to be incapable of fixing molecular nitrogen. In general, photosynthetic growth rates on N_2 and nitrogenase levels in species of *Rhodospirillum* and in the strains of *Rhodomicrobium vannielii* tested were significantly lower than those of the *Rhodopseudomonas capsulata-Rhodopseudomonas sphaeroides* group. Wide intraspecies strain variation in nitrogenase activity was observed in the three strains of *Rhodospirillum tenue* examined. Strain DSM110, although capable of reasonable growth rates on N_2, consistently showed very low in vivo nitrogenase activities when compared with the type strain of the species, *R. tenue* ATCC 25093 (Table 2).

Rhodospirillum salexigens, a newly described member of the family *Rhodospirillaceae* (3), was incapable of growing photosynthetically on either NH_4^+ or N_2 as the sole nitrogen source, and in accordance with previous findings (3), the organism grew only when substrate levels of glutamate were added to the growth medium. Cultures of *R. salexigens* grown with 4 mM glutamate and N_2 as nitrogen sources, however, produced readily detectable levels of nitrogenase (Table 2). Bearing in mind the glutamate requirement, however, we concluded that the contribution of nitrogen fixation to the overall nitrogen nutrition of *R. salexigens* remains unclear at this time.

Chemotrophic N_2 fixation occurred in all species of *Rhodospirillum*, but growth under these conditions was extremely slow. The average time required for a pigmented growth band to occur in these species was 1 to 2 wks; in some cases, prolonged incubation for up to 1 mo was needed. *R. fulvum* 1360 did not grow on N_2 chemotrophically in darkness. Most of the *Rhodospirillum* species grew microaerobically in darkness on N_2 only at reduced oxygen tensions as evidenced by the depth of the growth band in agar tubes. The type strain of *R. molischianum* (ATCC 14031) was most oxygen sensitive and grew very slowly in the dark in a band more than 1 cm from the agar surface. *R. salexigens* grew in darkness only when glutamate was present. In this case, the growth band was located at the surface of the agar, suggesting that N_2 fixation was not occurring in this organism under these growth conditions.

Figure 1 summarizes the in vivo nitrogenase and generation time data for all species of the family *Rhodospirillaceae*. Not surprisingly, the generation time of a given species was roughly inversely proportional to its nitrogenase content. The data of Fig. 1 also emphasize the superior nitrogen-fixing properties of *Rhodopseudomonas capsulata*, *Rhodopseudomonas sphaeroides*, and *Rhodopseudomonas viridis* as compared with the remaining species of the family *Rhodospirillaceae* when nitrogenase levels were measured in mid-log-phase cells growing on dinitrogen.

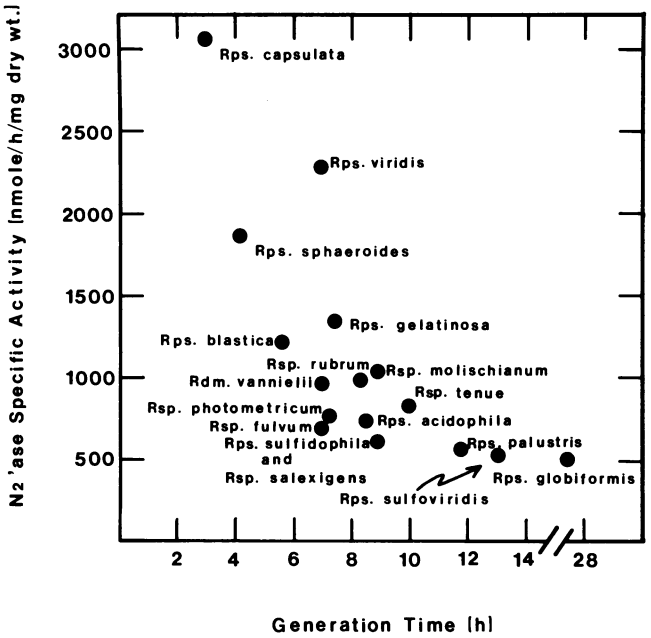

FIG. 1. Mean in vivo nitrogenase (N_2'ase) contents and growth rates of N_2-grown *Rhodospirillaceae*. The data represent the arithmetic mean of the nitrogenase values and growth rates listed for each species in Tables 1 and 2. Rps., *Rhodopseudomonas*; Rsp., *Rhodospirillum*; Rdm., *Rhodomicrobium*.

DISCUSSION

The results of this study have demonstrated that the ability to fix dinitrogen is a solid taxonomic marker in species of the family *Rhodospirillaceae*, with the exception of *Rhodocyclus purpureus*. The relative efficacy of nitrogen fixation, however, varies considerably among species of this family. Although it should be cautioned that absolute levels of nitrogenase measured in vivo in *Rhodospirillaceae* can vary dramatically, depending on whether cells are grown on N_2 or under nitrogen-limiting conditions (1), our studies used N_2-grown cells exclusively (with the exception of *Rhodospirillum salexigens* and *Rhodopseudomonas sulfoviridis* in which glutamate and yeast extract, respectively, were required for growth) to ensure the validity of interspecies comparisons of nitrogen fixation. Quantitative analysis with uniform assay procedures to test each species has clearly shown that strains of *Rhodopseudomonas capsulata* are far superior to the remaining *Rhodospirillaceae* in terms of nitrogenase expression and rapid growth on dinitrogen. To some extent it appears that *Rhodopseudomonas viridis* and *Rhodopseudomonas sphaeroides* also possess superior N_2-fixing properties; however, it should be cautioned that this conclusion in the case of *R. viridis* is based on the examination of only two strains. A recent study of nitrogen fixation in a third strain of *R. viridis*, however, supports our conclusions concerning its nitrogen-fixing abilities (10). The remaining members of the genus *Rhodopseudomonas* and the species in the *Rhodospirillum-Rhodomicrobium* group appear to be comparatively less adept N_2 fixers, yet most species remain capable of growing on N_2 at reasonable rates.

A more detailed study of the nitrogen metabolism of *Rhodocyclus purpureus* has confirmed the inability of this organism to fix N_2 (19). The possibility that *R. purpureus* can fix dinitrogen under some unusual nutritional conditions, however, cannot be ruled out. *R. purpureus* grows well with NH_4^+ or glutamine as the sole nitrogen source in a mineral salts-vitamin medium; no growth with N_2 as the sole nitrogen source occurs under these conditions, even if the medium is supplemented with various additional organic components (19). Perhaps the unusual habitat of *R. purpureus* (a swine waste lagoon; 23) has selected for the Nif$^-$ phenotype in this organism, since it would be expected that such a habitat would be rich in amines and ammonia.

The finding that most species of *Rhodospirillaceae* can fix N_2 chemotrophically in darkness is in agreement with previous determinations of this capacity in selected *Rhodospirillaceae* (16, 18, 27). Members of the brown *Rhodospirillum* species and *Rhodopseudomonas globiformis* appeared to be the most oxygen sensitive when growing chemotrophically in darkness on N_2; strains of these species either did not grow in the dark or grew only at extremely low O_2 tensions. It is likely that the depth of the growth zone in N_2-fixing, agar-deep cultures of *Rhodospirillaceae* reflects the relative ability of a particular species to protect its nitrogenase by removing oxygen via respiration; "respiratory protection" is thought to account for protection of nitrogenase in aerobic N_2 fixers such as *Azotobacter* sp. (26).

Although the actual O_2 concentration at the point of the growth band was not quantitated in the present work, the microaerophilic conditions required for chemotrophic N_2 fixation by even the more oxygen tolerant of the *Rhodospirillaceae* may be quite demanding. Hochman and Burris (9) demonstrated that dissolved O_2 concentrations of less than 1 μM severely inhibited nitrogenase activity in *Rhodopseudomonas capsulata* and *Rhodospirillum rubrum*. Others investigating this same problem but using different techniques have reported nitrogenase inhibition in *R. capsulata* only at substantially higher O_2 concentrations, in the range of 5 to 160 μM (20, 27). In the latter reports, it was shown that oxygen inhibition was markedly greater in cells of *R. capsulata* fixing N_2 photosynthetically than for cells fixing N_2 in the dark. Since light is known to sharply inhibit respiratory processes in purple bacteria (2), it seems logical to assume that oxygen tolerance during dark N_2 fixation by *Rhodospirillaceae* is a direct reflection of the inherent respiratory potential of each species. Similar conclusions have been reached in studies of chemotrophic N_2 fixation by the purple sulfur bacterium *Thiocapsa* sp. (11).

The ecological significance of nitrogen fixation by phototrophic bacteria in nature has not been extensively addressed. However, in situ experiments have shown that green photosynthetic bacteria of the genus *Pelodictyon*, perhaps in conjunction with *Rhodospirillum* and *Rhodomicrobium* species, fix considerable amounts of dinitrogen in the anaerobic zones of certain deep Norwegian lakes (28). In anaerobic environments, *Rhodospirillaceae* must compete with chemotrophic organisms for organic compounds which are required by the phototrophs as a source of carbon and as a source of electrons for the reduction of N_2 to ammonia. *Rhodospirillaceae* possess a physiological advantage over many chemotrophs, however, in that phototrophs can utilize a variety of highly reduced organic compounds which cannot be used by chemotrophic organisms in the absence of an electron acceptor (24). A relevant example of these interactions is the rice paddy ecosystem in which *Rhodospirillaceae*, and possibly members of the family *Chromatiaceae* as well, contribute significant amounts of fixed nitrogen to rice plants (8, 13). Epiphytic *Rhodospirillaceae*, growing attached to freshwater macrophytes such as *Myriophyllum* sp., also have been shown to be active nitrogen fixers (5).

In conclusion, it is clear that *Rhodospirillaceae* are an

active group of dinitrogen fixers and are undoubtedly of some ecological importance. Since *Rhodospirillaceae* fix N_2 in darkness as well as photosynthetically, it is likely that a portion of the dinitrogen fixed and released in a variety of habitats by free-living microorganisms is contributed by facultative phototrophic bacteria. Our results would suggest that the prime candidates for consideration in this connection are members of the genus *Rhodopseudomonas*, in particular *R. capsulata* and *R. sphaeroides*. The ability of these species to produce high levels of nitrogenase and to grow rapidly on N_2 also implies that *R. capsulata* and *R. sphaeroides* would be the predominant phototrophic species in nitrogen-limited environments.

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