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Isolation and Partial Characterization of *Rhodopseudomonas sphaeroides* Mutants Defective in the Regulation of Ribulose Bisphosphate Carboxylase/Oxygenase

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Several mutants of *Rhodopseudomonas sphaeroides* defective in the derepression of the enzyme ribulose 1,5-bisphosphate carboxylase have been isolated by using the unstable Tn*5* vectors pJB4JI and pRK340. Transpositional insertion mutants obtained with pJB4JI were demonstrated to be incapable of increasing ribulose 1,5-bisphosphate carboxylase/oxygenase levels when grown on butyrate-bicarbonate medium or under conditions of carbon starvation, whereas the wild-type strain increased activity four- to eightfold. When the wild-type strain was starved for carbon in the presence of chloramphenicol, no derepression was observed. Crude extracts from mutant and wild-type strains had distinct and consistent differences in protein content as observed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Chromatographic evidence indicated that mutants were defective in the regulation of only one of the two forms of ribulose 1,5-bisphosphate carboxylase/oxygenase synthesized by *R. sphaeroides*.

Ribulose 1,5-bisphosphate (RuBP) carboxylase/oxygenase, the first and key enzyme of the Calvin cycle, is the primary catalyst of carbon fixation in plants and most photosynthetic bacteria. The enzyme catalyzes the fixation of carbon dioxide onto the enediol of RuBP to yield two molecules of D-3-phosphoglycerate. It is also the key enzyme in the competing, less efficient, photorespiratory pathway, catalyzing the oxygenolysis of RuBP to yield one molecule each of 3-phosphoglycerate and 2-phosphoglycolate.

Despite the obvious agricultural significance of this enzyme, little or nothing is known about its regulation at the molecular level. For this purpose *Rhodopseudomonas sphaeroides*, a member of the *Rhodospirillaceae*, provides a useful model system. Because of its versatility, mutants in the regulation of RuBP carboxylase/oxygenase can be obtained. When grown anaerobically under photoautotrophic conditions in an H_2–CO_2 atmosphere or photoheterotrophically with a reduced fatty acid, such as butyrate, as an electron donor, this organism requires high levels of RuBP carboxylase activity to grow (3, 15). However, when cultured photoheterotrophically with oxidized organic acids, such as malate, as the electron donor, only low levels of this enzyme are required (15). Therefore, mutant strains incapable of producing high levels of RuBP carboxylase activity may be obtained by selecting strains capable of photosynthetic growth on malate, but incapable of growth in the butyrate-bicarbonate medium or in an H_2–CO_2 atmosphere. In addition it has been shown that this organism produces two distinct structural forms of RuBP carboxylase/oxygenase, form I and form II (3). Form I RuBP carboxylase/oxygenase is similar to the enzyme produced by eucaryotic phototrophic organisms (10, 11) and is composed of an aggregate of eight large subunits that are responsible for the catalytic activity of the enzyme (11, 12) and eight small subunits that may play a regulatory role in catalysis (5). Form II RuBP carboxylase/oxygenase is a hexamer of six large subunits, lacking small subunits, and is thus similar to the dimer observed in *Rhodospirillum rubrum* (18). Immunological (3, 4) and peptide mapping studies (15) indicate that the large subunits from the form I and form II enzymes might be distinct proteins (J. L. Gibson, F. E. Waddill, and F. R. Tabita, manuscript in preparation). The significance of this duplicity of enzyme production and the regulation of these enzymes under various growth conditions is unknown.

In this study, we describe the characteristics of mutants obtained by transposon mutagenesis with plasmids pJB4JI, a "suicide" plasmid carrying Tn*5* (1), and pRK340 (R. Meyer, unpublished data), a heat-labile RK2 derivative also carrying Tn*5*. These mutants were isolated after ampicillin selection (9) under photolithotrophic growth conditions in an H_2–CO_2 atmosphere and have been shown to possess the growth charac-

teristics suggestive of regulatory mutants as described above. In addition, these mutants have been shown to be incapable of chemolithotrophic growth and incapable of increasing enzyme activity to wild-type levels in butyrate-bicarbonate medium or under carbon starvation conditions. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) protein profiles of wild-type and mutant cell-free extracts were compared, and interesting differences between the mutants and the parental strain were noted. Separation of form I and form II RuBP carboxylase/oxygenase revealed that, although mutants were capable of producing normal levels of form II activity under carbon starvation conditions, form I activity in mutant extracts was one-third that observed in wild-type extracts.

MATERIALS AND METHODS

Reagents. All biochemicals were purchased from the Sigma Chemical Co., St. Louis, Mo. All common compounds were of reagent-grade quality; $NaH[^{14}C]O_3$ (20 mCi/mmol) was purchased from Amersham Corp., Arlington Heights, Ill.

Bacterial strains and plasmids. The *R. sphaeroides* parental strain used in this study was a streptomycin-resistant derivative of strain ATCC 17023, capable of growth at 42°C (designated HR).

Plasmid pJB4JI is a suicide plasmid that encodes gentamycin resistance and carries transposon Tn*5*, which carries kanamycin and neomycin resistance inserted into a Mu phage genome (1). The pJB4JI donor strain was *Escherichia coli* strain 1830, a *pro*$^-$ *met*$^-$ auxotroph, and was kindly provided by Sharon Long (Harvard University).

Plasmid pRK340 is a heat-labile, carbenicillin-sensitive derivative of RK2 which carries Tn*5* in place of its native kanamycin resistance. The host strain is a Mu and naladixic-acid resistant *E. coli* C600 strain and was provided by Richard Meyer (The University of Texas at Austin).

Media and growth conditions. PYE medium is a complex medium consisting of 0.3% peptone, 0.3% yeast extract, 10% Ormerod basal salts (13), and the following vitamin supplements: 15 μg of biotin and 1 mg each of niacin and thiamine per liter. Heterotrophic growth on defined medium was performed with Ormerod medium containing 0.6% malate or 0.2% butyrate with the addition of 0.1% sodium bicarbonate. Solid medium was made by adding 2% agar. Plates were incubated aerobically in the dark at 30°C. Anaerobic growth was accomplished by incubating plates in a GasPak (Scientific Products Div., McGaw Park, Ill.) anaerobic jar. Small liquid cultures were grown in screw-cap tubes filled to the top with media. Larger cultures were grown in 1-liter bottles continuously bubbled with argon. All growth experiments were performed at 35°C in temperature-controlled water baths illuminated by four 60-W soft white incandescent light bulbs on each side of the bath about 15 cm from the growth vessels. Growth was followed by measuring the absorption at 650 nm.

Minimal salts medium (MSM) consisted of Ormerod medium, without an added carbon source, containing 10 μM $NiCl_2$. Photoautotrophic cultures were grown by incubating solid MSM in a GasPak or by bubbling liquid MSM with a mixture of 5% CO_2–95% H_2. Chemolithotrophic growth was performed on solid MSM in jars initially flushed with H_2 and supplemented with 5% CO_2 and 5% O_2.

For the purpose of comparison of growth of wild-type, mutant, and revertant strains on the various media, strains were streaked from liquid PYE cultures onto the appropriate solid media. PYE and malate plates were incubated for 3 to 5 days, and MSM plates were incubated for 2 weeks under photoautotrophic conditions and for 2 months under chemolithotrophic conditions. At this time, the growth of strains were compared, making HR the standard (+++). No growth was defined as no visible colony development, and growth of the other strains was graded relative to these two extremes.

E. coli was grown on TYE broth containing 1% tryptone, 0.5% yeast extract, and 0.5% NaCl. Flasks were incubated with shaking at 30°C.

Conjugation. Equal volumes of a stationary-phase culture of recipient and an overnight donor culture were collected on a membrane filter (0.45-μm pore size; Millipore Corp., Bedford, Mass.), which was then placed on a PYE plate and incubated overnight at 30°C. Filters were then washed with 9 ml of sterile phosphate buffer. Tenfold dilutions were then plated on selective media. Transconjugants containing pJB4JI were selected on PYE plates supplemented with 100 μg of streptomycin per ml and either 50 μg of gentamycin per ml or 50 μg of neomycin sulfate per ml. After 3 days of growth, transconjugants were screened for resistance to the unselected antibiotic (neomycin or gentamycin, respectively). Transconjugants containing pRK340 were selected on plates containing 100 μg of streptomycin per ml and 50 μg of kanamycin per ml and screened for resistance to 10 μg of tetracycline per ml.

Selection of mutants. Transconjugants shown to carry both plasmid- and transposon-encoded drug resistances were selected and purified. Strains carrying pJB4JI were then cultured anaerobically on PYE to the stationary phase. Cells were harvested and washed three times in 10 ml of cold sterile MSM. Cell material was then suspended in 20 ml of MSM. Cultures were incubated photoautotrophically for 2 h to allow exhaustion of intracellular pools. Ampicillin was then added to a final concentration of 100 μg/ml. Incubation was continued for 18 h, at which point cells were harvested and washed before a second cycle of ampicillin selection was applied. After a third cycle of ampicillin selection, 0.1-ml samples of culture were plated on malate plates and incubated aerobically. A total of 200 randomly selected colonies were then gridded systematically on fresh malate plates. Colonies were then screened for growth when incubated anaerobically on malate, butyrate, and MSM. Mutants were selected which did not grow on either butyrate or MSM.

Since mutagenesis with pRK340 requires a heat induction step, transconjugants were grown aerobically in liquid PYE in a shaker flask at 30°C. After cultures reached early log phase they were transferred to 37°C. After heat induction, strains were prepared for, and subjected to, ampicillin selection as described above.

Derepression on butyrate-bicarbonate and carbon-free media. Cell material from 20 ml of a stationary-phase, anaerobically grown PYE culture was harvested and washed twice with sterile malate medium and subsequently transferred to 800 ml of malate medium. After anaerobic growth in the presence of argon, cell material was again harvested and either washed once with butyrate medium and suspended in 800 ml of butyrate medium or washed three times in MSM and suspended in 800 ml of MSM. Cultures to be transferred to butyrate medium were precultured on malate to the stationary phase, whereas log-phase cultures were used for carbon starvation experiments. In either case, 10-ml samples were immediately removed. After the absorbance at 650 nm was determined, cell samples were washed once in 5 ml of 50 mM Tris-hydrochloride–1 mM EDTA (pH 7.5) and then suspended in 0.5 ml of 25 mM Tris-hydrochloride buffer (pH 7.5) containing 1 mM EDTA and 5 mM 2-mercaptoethanol and frozen at −70°C. Cultures suspended in butyrate and carbon starvation media were then incubated with argon for 10 and 3 days, respectively; 10-ml samples were removed each day and treated as above. After the end of the experiment, samples were permeabilized with toluene and assayed for carboxylase activity by the whole cell method previously described (16).

Preparation of extracts. Endpoint cultures of carbon starvation experiments and stationary-phase malate cultures were harvested and suspended to 1/100 volume in 25 mM Tris-hydrochloride buffer (pH 7.5) containing 1 mM EDTA and 5 mM 2-mercaptoethanol. Cells were then lysed in a French pressure cell at 20,000 lb/in^2. Cell debris was removed by centrifugation at 37,000 × *g* for 10 min or 120,000 × *g* for 60 min. The protein content of the resulting supernatants was determined by the absorbance at 280 and 260 nm (6) and by the method of Bradford (2).

Electrophoresis. After samples were adjusted to equal protein concentrations, they were subjected to SDS-PAGE in 0.75-mm slab gels by the procedure of Lugtenburg et al. (7). Slab gels were stained with 0.1% Coomassie blue R in 25% isopropanol–10% acetic acid and destained with 10% acetic acid–10% isopropanol.

Column chromatography. Form I and form II RuBP carboxylase/oxygenase were partially purified from cell-free extracts of carbon-starved cultures by diethylaminoethyl cellulose column chromatography as previously described (4). Samples (25 μl) of each fraction were assayed for RuBP carboxylase activity. Active fractions from both peaks were pooled and concentrated by ultrafiltration in an Amicon model 12 ultrafiltration cell. Protein content of the concentrate was determined as before, and 10-μl samples were assayed for activity.

RESULTS

Plasmid transfer. To determine the initial stability of plasmid pJB4JI in *R. sphaeroides*, two selection systems were employed. Transconjugants selected on plates containing streptomycin and gentamycin would be expected to carry both plasmid and transposon. Transconjugants selected on plates containing streptomycin and neomycin would include those strains selected by the above system and strains in which the plasmid had segregated out, leaving a copy of the transposon, Tn*5*, behind. Since transfer frequencies were not significantly different between the two systems (both were around 10^{-5} per donor cell), the plasmid appears to be fairly stable in this organism. In addition, when transconjugants were tested for resistance to the unselected antibiotic, greater than 90% displayed such resistance, and the frequency of gentamycin- and neomycin-resistant clones was no greater in strains initially selected for gentamycin resistance than in strains selected for neomycin resistance.

Transconjugants of *R. sphaeroides* carrying pRK340 were selected on plates containing streptomycin and kanamycin. Because of contamination of the recipient strain with a spontaneous kanamycin-resistant mutant, transconjugants had to be screened for tetracycline resistance. Thus, precise transfer frequencies were not obtained, but were estimated at about 10^{-4} per donor cell. Strains carrying both tetracycline and kanamycin resistances were selected for further study.

Isolation of Aut$^-$ mutants. After ampicillin selection under autotrophic conditions, survivors were screened for growth on minimal salts media. Of 200 pJB4JI containing clones screened, 7 were found to be incapable of growth on MSM while growing normally on malate medium under anaerobic conditions in the light. Three of these mutants, KW44/8, KW45/143, and KW25/11 were also found to be incapable of anaerobic photosynthetic growth in a butyrate-bicarbonate media. These mutants were selected for further study.

After isolation, mutants were screened for maintenance of plasmid- and Tn*5*-encoded drug resistances. Immediately after selection, mutants were found to express both Tn*5*-encoded neomycin and kanamycin resistance and the plasmid-encoded gentamycin resistance. After several passages on nonselective medium, gentamycin-sensitive strains were isolated. These strains were of two general types: (i) strains that retained both kanamycin resistance and the mutant phenotype and (ii) strains that had lost the kanamycin resistance and regained some ability to grow photoautotrophically. The latter strains were designated as revertants, whereas the former were perpetuated on kanamycin-containing media for further characterization. All further experiments were performed with strains that had lost the plasmid-encoded gentamycin resistance.

Four of 200 clones screened after ampicillin selection of pRK340-containing strains were found to be incapable of normal growth on butyrate-bicarbonate medium or in an H_2–CO_2

atmosphere on minimal salts medium. These strains were also found to carry both plasmid- and Tn*5*-encoded resistances, so it is at present impossible to tell whether the mutant characteristics are due to transpositional insertion. Attempts to isolate tetracycline-sensitive, kanamycin-resistant strains from these pRK340-containing strains have failed. pRK340-induced mutants were extremely unstable even under selection for the transposon-encoded kanamycin resistance.

The growth characteristics of mutants and revertants are summarized in Table 1. All subsequent work has been done with pJB4JI-induced mutants since the nature of the lesion is more certain and more stable.

Chemolithotrophic growth. It has recently been reported that a close relative of *R. sphaeroides*, *Rhodopseudomonas capsulata*, is capable of chemolithotrophic growth (8). To test the hypothesis that the *R. sphaeroides* mutants are defective in some aspect of carbon dioxide assimilation, the wild type and two mutants, KW45/143 and KW44/8, were streaked for growth on MSM medium and incubated in a gas atmosphere of 90% H_2–5% CO_2–5% O_2 under the conditions described above. Although growth was extremely slow, good colony development was observed on plates inoculated with the parental strain. In contrast, only two colonies were seen on plates inoculated with strain KW44/8, and none were seen on plates inoculated with strain KW45/143. Taken together with the conditions of selection, i.e., no light-dependent growth on butyrate-bicarbonate medium or in an H_2–CO_2 atmosphere in the absence of any fixed carbon source, it is apparent that these mutants are defective in some aspect of their CO_2 fixation capacity.

Derepression of RuBP carboxylase/oxygenase. It has been previously shown that members of the *Rhodospirillaceae* derepress RuBP carboxylase/oxygenase synthesis to high levels during the stationary phase when grown with butyrate as the electron donor (15, 17). This appears to be due, at least in part, to the depletion of carbon dioxide from the medium (14). In addition, when wild-type *R. sphaeroides* was grown on malate and transferred, after extensive washing, to a medium containing no exogenous carbon, RuBP carboxylase/oxygenase levels were increased substantially. Thus, experiments were designed to determine the capacity of mutant strains to derepress the synthesis of RuBP carboxylase/oxygenase in butyrate-bicarbonate media and under carbon starvation conditions.

In experiments with wild-type and mutant strains transferred to the butyrate-bicarbonate media or subjected to starvation in a medium devoid of exogenous carbon, only the wild-type strain was capable of increasing RuBP carboxylase/oxygenase levels significantly. Moreover, protein synthesis was shown to be required by wild-type strain HR for the observed increase in activity under carbon starvation conditions, since 25 μg of chloramphenicol per ml virtually blocked any increase in RuBP carboxylase activity during the starvation stage (Fig. 1). As expected, mutant strain KW25/11 failed to attain high levels of RuBP carboxylase activity in the presence or absence of chloramphenicol.

Revertants tested for RuBP carboxylase activity under carbon starvation conditions were observed to have elevated levels of activity initially

TABLE 1. Growth characteristics of mutant strains of *R. sphaeroides*[a]

Strain[b]	Growth on the following media:					Reversion frequency[c]
	PYE	Malate	Butyrate	H_2–CO_2	H_2–O_2–CO_2	
HR	+++	+++	+++	+++	+++	
KW44/8	+++	+++	−	−	−	$<10^{-9}$
KW25/11	+++	+++	−	−	ND[d]	$<10^{-9}$
KW45/143	+++	+++	−	−	−	$\sim10^{-6}$
KW44/8R	+++	+++	++	++	ND	
KW25/11R	+++	+++	+	+	ND	
KW45/143R	+++	+++	++	+	ND	
RK404	+++	++	+	−	ND	$\sim10^{-1}$
RK408	+++	++	+	+	ND	$\sim10^{-1}$
RK416	+++	+++	+	−	ND	$\sim10^{-1}$
RK417	+++	++	+	−	ND	$\sim10^{-1}$

[a] All strains were tested for anaerobic growth in the indicated solid media; excellent growth (+++); good growth (++); poor growth (+); no growth (−).

[b] All strains were derived from strain HR, which is streptomycin resistant and capable of growth at 42°C.

[c] Reversion frequencies were determined by growing strains to the stationary phase on PYE and then plating on MSM.

[d] ND, Not determined.

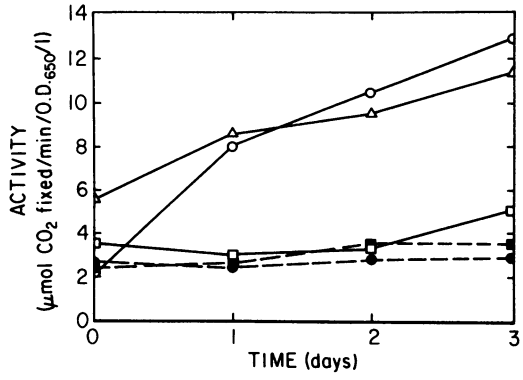

FIG. 1. Increase in RuBP carboxylase activity by *R. sphaeroides* wild-type, mutant, and revertant strains under conditions of carbon starvation in the presence and absence of chloramphenicol. The absorbancy at 650 nm of the cultures was between 1.1 and 1.3. Chloramphenicol was added upon initiation of carbon starvation to a final concentration of 25 μg/ml. Activity was determined by whole cell assay (16). Methods are described in the text. Symbols: ○, HR strain; ●, HR strain starved in the presence of chloramphenicol; □, strain KW25/11; ■, strain KW25/11 starved in the presence of chloramphenicol; △, strain KW25/11R.

upon transfer from malate to carbon starvation medium. This elevation was particularly apparent in experiments done with the revertant of strain KW25/11 (Fig. 1). Although the increase in RuBP carboxylase/oxygenase activity was only twofold in this case, because the initial activity was so high, this increase resulted in a final level of RuBP carboxylase 5.5-fold higher than the initial level of activity found in the wild-type malate-grown HR strain. This compared favorably with the 6.8-fold increase observed in experiments with strain HR. This initial elevation of RuBP carboxylase activity was less pronounced in experiments with revertants of the other two mutant strains, KW44/8 and KW45/143. This was particularly interesting in view of the fact that KW25/11R grew the poorest of the three revertants on minimal salts media under an H_2–CO_2 atmosphere.

SDS-PAGE profiles. To visualize any protein differences between the parental and mutant strains that might correlate with observed phenotypic differences, samples of the soluble portion of extracts obtained from endpoint cultures of carbon starvation experiments were run on SDS-polyacrylamide gels (Fig. 2 and 3).

In Fig. 2A, cell-free extracts of wild-type and mutant KW25/11 strains obtained from cultures grown anaerobically on malate and under carbon starvation conditions in the presence and absence of chloramphenicol are compared. Several distinct and consistent differences occur, particularly among the proteins of low molecular weight. One very significant protein band is present at the position marked L1. Protein L1 ($M_r \cong 18{,}000$) is synthesized by wild-type cells after the initiation of carbon starvation since it is absent in extracts from both malate-grown and chloramphenicol-treated carbon-starved cells. Moreover, protein L1 is absent from cell-free extracts of carbon-starved cultures of strains

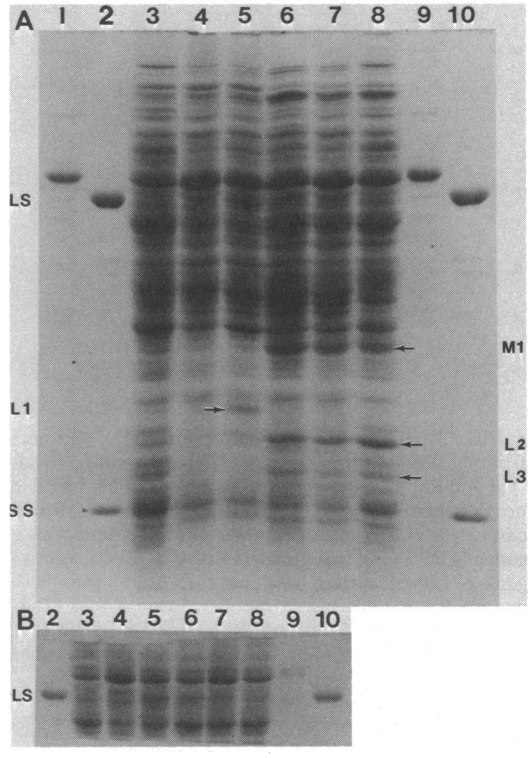

FIG. 2. (A) SDS-slab gel electrophoresis of extracts obtained from *R. sphaeroides* mutant and wild-type strains grown under various conditions. Each lane received 25 μl of a solution of 5 mg of protein per ml as follows: 1 and 9, homogeneous *R. sphaeroides* form II RuBP carboxylase/oxygenase; 2 and 10, homogeneous *R. sphaeroides* form I RuBP carboxylase/oxygenase; 3, extract from malate-grown wild-type strain HR; 4, extract from strain HR starved for carbon in the presence of chloramphenicol; 5, extract from carbon-starved strain HR; 6, extract from carbon-starved strain KW25/11; 7, extract from strain KW25/11 starved for carbon in the presence of chloramphenicol; 8, extract from malate-grown strain KW25/11. Carbon-starved extracts were obtained from endpoint cultures of experiments represented in Fig. 1. Bands labeled M1, L2, and L3 are novel proteins produced only by mutant cells. L1 is produced only by carbon-starved wild-type cells. LS and SS are the large and small subunits of form I, respectively. (B) SDS-slab gel of the same extracts showing more clearly the differences in protein bands which comigrate with purified form I and form II RuBP carboxylase/oxygenase.

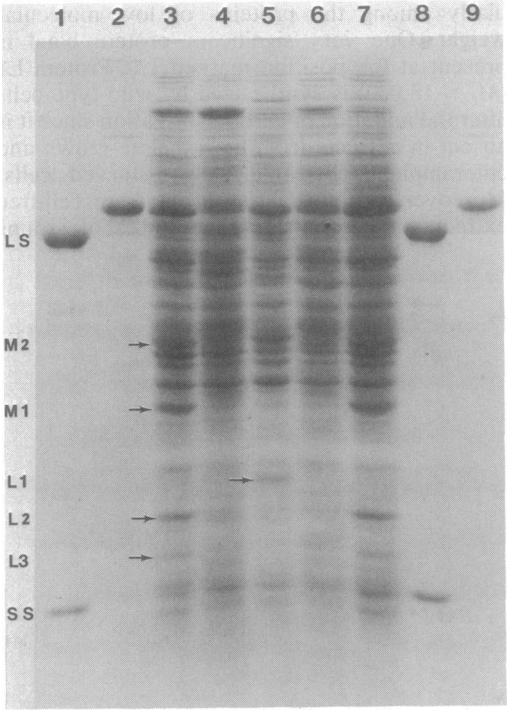

FIG. 3. SDS-slab gel electrophoresis of extracts from carbon-starved cultures of *R. sphaeroides* wild-type, mutant, and revertant strains. Lanes 1 and 8 contain homogeneous *R. sphaeroides* form I RuBP carboxylase/oxygenase. Lanes 2 and 9 contain homogeneous *R. sphaeroides* form II RuBP carboxylase/oxygenase. Lanes 3 through 7 contain 15 μl of a sample (6 mg/ml) of the following extracts: 3, strain KW25/11; 4, strain KW25/11R; 5, strain HR; 6, strain KW44/8R; 7, strain KW44/8. Extracts of these cultures were obtained from the experiments represented in Fig. 1.

KW25/11 and KW44/8 (Fig. 3). These two facts suggest an important role for this protein in RuBP carboxylase/oxygenase derepression under carbon starvation conditions.

Proteins L2 ($M_r \cong 16{,}000$) and M1 ($M_r \cong 25{,}000$) are present in both mutants and absent in the wild-type strain regardless of the presence or absence of chloramphenicol (Fig. 3). They are also present in extracts of malate-grown mutant cells, but not in the wild type (Fig. 2A). Protein L3 ($M_r \cong 14{,}000$), on the other hand, is decreased under chloramphenicol treatment of KW25/11 and is present in low amounts in malate extracts (Fig. 2A). It is produced by both mutants (Fig. 3), and it is not produced to proportions visible on SDS-PAGE by the wild type under any conditions (Fig. 2A).

In addition to the differences observed relative to the production of low-molecular-weight proteins, some interesting observations can be made concerning the two enzyme forms from wild-type and mutant cells. The two RuBP carboxylase/oxygenase forms can be separated on SDS-PAGE gels and identified by their comigration with homogeneous enzyme (Fig. 2A). It is interesting to note that only a slight difference exists in the intensity of bands comigrating with the form II enzyme from extracts of carbon-starved wild-type and mutant cells, about 12% more in the wild-type, according to densitometric tracings of the gel shown in Fig. 2B. Differences between soluble extracts of wild-type malate and carbon-starved cultures were also small, showing about a 30% increase in carbon-starved cells.

Observable intensity differences between protein bands of mutant and wild-type extracts of carbon-starved cells which comigrate with form I large subunit are variable and slight. However, a clear difference is seen with extracts obtained from wild-type cells incubated in the presence and absence of chloramphenicol (Fig. 2B). In extracts of carbon-starved cultures incubated with chloramphenicol, this band is absent, and a second protein of higher molecular weight than the form I large subunit is observed. This band is also present in extracts of malate cultures and may represent a precursor of the form I large subunit or may be related to the microheterogeneity of large subunits that is often found associated with this enzyme. In extracts of wild-type strains not treated with chloramphenicol, the band comigrating with the large subunit is noticeably enhanced upon carbon starvation. Such clear differences are not observed in extracts of carbon-starved mutant cultures.

In Fig. 3, extracts of two revertants grown under carbon starvation conditions are compared with their mutant parents and the wild type. Revertants produce neither the L2, L3, or M1 proteins present in mutants nor the L1 protein produced by the wild type. In addition, a larger protein ($M_r \cong 34{,}000$), M2, present in both wild-type and mutant strains, is absent in the revertant of KW25/11 and decreased in the revertant of KW44/8. Other than a slight increase in form I RuBP carboxylase/oxygenase in KW25/11R over its mutant parent, no significant differences in bands comigrating with pure RuBP carboxylase/oxygenase preparations are observed.

Column chromatography. The activity of both RuBP carboxylase/oxygenase enzymes was determined after separating the two enzyme forms by diethylaminoethyl cellulose column chromatography. Very little activity of either enzyme form was seen in extracts of wild-type cells starved for carbon in the presence of chloramphenicol (Fig. 4A). Extracts of wild-type cultures starved for carbon in the absence of chloramphenicol were found to have increased levels

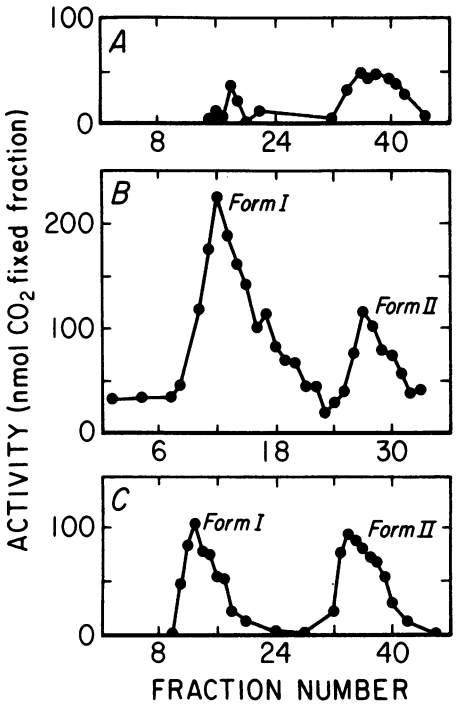

FIG. 4. Partial purification of form I and form II RuBP carboxylase/oxygenase from cell-free extracts of (A) wild-type HR starved for carbon in the presence of chloramphenicol, (B) carbon-starved HR strain, and (C) carbon-starved strain KW25/11. Extracts were obtained from endpoint cultures of experiments represented in Fig. 1. Protein was eluted from a diethylaminoethyl cellulose column by application of a linear 0.1 to 0.3 M NaCl gradient (3).

of form I and form II activity, with form I predominating almost 3:1 (Fig. 4B). Specific activities determined from pooled, concentrated fractions from the form I and form II activity peaks were 0.33 and 0.085 μmol of CO_2 fixed per min per mg of protein, respectively. Extracts of carbon-starved mutant cultures were found to possess form II activity levels comparable to those of wild-type cells, but form I activity levels were much lower than in the wild type (Fig. 4C). Specific activities of form I and form II enzymes from mutant strain KW25/11 were 0.11 and 0.14 μmol of CO_2 fixed per min per mg of protein. These results suggest that the mutant is defective only in the regulation of form I RuBP carboxylase/oxygenase.

DISCUSSION

In this study we have demonstrated the usefulness of plasmid pJB4JI as a vector for transpositional mutagenesis of *R. sphaeroides*. The plasmid is transferred from *E. coli* donors at relatively high frequencies, and both the Tn*5*-encoded kanamycin and plasmid-encoded gentamycin resistances are selectable. Although the plasmid is somewhat more stably maintained than expected, strains that have lost the plasmid can be isolated after a reasonable amount of time after several passages on nonselective media. Mutants obtained with this plasmid are extremely stable when maintained under conditions requiring Tn*5* maintenance. A second plasmid, pRK340, was also used as a Tn*5* vector. This plasmid was maintained over long periods of time and may require higher temperatures to facilitate its loss. Mutants obtained with this plasmid were unstable even under Tn*5* selection conditions, as expected, since one plasmid copy of Tn*5* would still be available to the cell if the chromosomal copy was lost.

We have used the pJB4JI mutagenesis system, combined with ampicillin selection, to isolate mutants defective in the regulation of RuBP carboxylase/oxygenase activity and synthesis. In the initial experiment, seven mutants incapable of growth on H_2 and CO_2 were isolated. Of these, three, KW25/11, KW44/8, and KW45/143, were found to be incapable of photoheterotrophic growth in a butyrate-bicarbonate medium or photolithotrophic growth in an H_2–CO_2 atmosphere. Since CO_2 fixation is the only common growth requirement, the metabolic defect is apparently at some step involved in CO_2 fixation. To eliminate the possibility that the defect might be due to some defect in the photosynthetic apparatus, two strains, KW44/8 and KW45/143, were tested for chemolithotrophic growth in a CO_2–H_2–O_2 atmosphere. Neither strain could grow under these conditions, whereas the wild-type strain grew normally. In addition, all mutant strains grew normally when cultured photoheterotrophically in a malate medium, indicating that the photosynthetic apparatus remains intact.

That the genetic lesions of the mutants are caused by transpositional insertion of the Tn*5* is indicated by two lines of evidence: (i) all mutant strains have lost the plasmid-encoded gentamycin resistance, but retain the transposon-encoded kanamycin resistance; and (ii) revertants of all three mutants have been isolated which have lost kanamycin resistance concomitantly with regaining some wild-type growth characteristics. In the absence of direct physical visualization, this is the strongest evidence possible to indicate that these are indeed transposon-mediated mutants.

Although the growth characteristics suggest that these mutants are defective in some aspect of CO_2 assimilation, it was necessary to observe directly the increase, or lack thereof, of enzyme activity under conditions that normally result in a high level of the key enzymes of CO_2 assimila-

tion. Since the mutants cannot grow under these conditions, it was necessary to devise experiments such that the wild-type organism was also incapable of growth to make conditions as equivalent as possible. For this purpose two systems were investigated: (i) derepression of enzyme synthesis in stationary-phase cultures in a butyrate-bicarbonate medium (15) and (ii) derepression of log-phase cultures under conditions of carbon starvation.

In both experiments the mutants, although capable of increasing enzyme activity somewhat, could not attain levels of RuBP carboxylase activity achieved by the wild type. RuBP carboxylase was the enzyme assayed since it is known to be most sensitive to such manipulations of the cultural conditions. The level of phosphoribulokinase, the other enzyme unique to the Calvin cycle, does not change as dramatically as RuBP carboxylase/oxygenase (unpublished observations). It seems fairly obvious from these results that these mutants are indeed defective in some aspect of RuBP carboxylase derepression.

Carbon starvation experiments with the wild-type strain HR in the presence of chloramphenicol indicated to us that de novo synthesis of protein was required at the initiation of starvation for the observed rise in RuBP carboxylase activity to occur. Therefore, the protein content of extracts of wild-type and mutant strains grown under various conditions was compared by SDS-PAGE to determine whether the mutant strains lacked proteins synthesized by the wild type after the initiation of carbon starvation.

Although small differences existed in the amounts of RuBP carboxylase/oxygenase enzymes, the most striking differences between mutant and wild-type extracts occur among several proteins of low molecular weight. The most significant of these is L1. The fact that this protein is only present in wild-type cells after the initiation of carbon starvation (Fig. 2) and is absent from two independently isolated mutants (Fig. 3) makes it a prime target for further investigation of its role as a potential positive effector molecule in the regulation of the RuBP carboxylase/oxygenase enzyme. In addition, three proteins, L2, L3, and M1, which are either absent or produced in too small a quantity in the wild type to be prominent on SDS-slab gels (Fig. 2), appear in mutants. It is possible that they are transposon-induced anomalies caused by premature termination of transcription due to transposon insertion or to some kind of polar effect. On the other hand, the possibility that one or more of these proteins is an overproduced negative effector molecule must be considered.

When revertants of these mutant strains are starved for carbon, neither L1 nor the novel mutant proteins is produced (Fig. 3). Since these revertants still do not regulate the RuBP carboxylase/oxygenase enzyme in a normal manner (Fig. 1), the possibility remains that L1 is an important positive regulatory protein. However, the fact that all revertants isolated thus far are kanamycin sensitive indicates that some defect caused by the insertion of the transposon, other than the loss of L1, must be relieved before the cell can grow autotrophically. What this defect is is unknown. It should also be noted that another protein, M2, disappears in both revertants investigated (Fig. 3). Whether this protein has a role in relieving the mutant phenotype has yet to be determined.

Finally, some differences in the bands which comigrate with homogeneous preparations of the two forms of RuBP carboxylase/oxygenase are worthy of note. Although very little difference exists between the amount of form II enzyme present in carbon-starved mutant and wild-type strains, some interesting differences exist between the amounts and migration properties of the form I enzyme when the two strains are grown under various conditions (Fig. 2A and B). Slightly more form I enzyme is seen in extracts of carbon-starved wild-type cells than in the mutants. In addition, when the wild-type strain is starved for carbon in the presence of chloramphenicol, a band of slightly higher molecular weight than form I is observed. This band disappears when the wild type is starved for carbon in the absence of chloramphenicol, and the band which comigrates with pure form I RuBP carboxylase/oxygenase intensifies. No such difference is observed between extracts of mutant cells starved for carbon in the presence or absence of chloramphenicol. The possibility that this higher-molecular-weight band could be a precursor of form I RuBP carboxylase/oxygenase is under investigation.

The SDS-PAGE results indicate that the major difference between the mutant and wild-type strains is in the regulation of form I RuBP carboxylase/oxygenase. Separation of the two forms of the enzyme from extracts of carbon-starved mutant and wild-type cells by diethylaminoethyl cellulose chromatography confirmed this observation (Fig. 4). Mutants appear to be capable of attaining wild-type levels of form II enzyme under these conditions. In fact, the specific activity of the form II enzyme partially purified from the mutant was 50% higher than in the wild type. However, the specific activity of the form I enzyme isolated from wild-type cells is threefold higher than that isolated from the mutant. Therefore, it appears that the primary defect in these mutants is in the regulation of form I RuBP carboxylase/oxygenase, whereas the form II enzyme is regulated normally, at

least under conditions of carbon starvation. These results imply that some aspects of the regulation of the two forms of RuBP carboxylase/oxygenase present in *R. sphaeroides* are separate. However, although the results in Fig. 4B indicate that form I activity predominates under conditions of carbon starvation, this is not the case when cells are grown photoheterotrophically on a butyrate-bicarbonate medium or photoautotrophically (5; F. R. Tabita, submitted for publication), where form II is more highly active. Therefore, if these mutants were only blocked in form I derepression they might be expected to grow normally under these conditions. Since they do not, it is likely that form I and form II RuBP carboxylase/oxygenase derepression, although separate in some aspects, is still linked in some way.

By continuing to physiologically characterize these mutants, and by isolating the genes responsible for the observed defects we hope to further clarify the regulation of RuBP carboxylase/oxygenase in *R. sphaeroides*.

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LITERATURE CITED

1. **Beringer, J. E., J. L. Beynon, A. V. Buchanon-Wallaston, and A. W. B. Johnston.** 1978. Transfer of the drug resistance transposon Tn5 to *Rhizobium*. Nature (London) **276**:633–634.
2. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
3. **Gibson, J. L. and F. R. Tabita.** 1977. Different molecular forms of D-ribulose 1,5-bisphosphate carboxylase from *Rhodopseudomonas sphaeroides*. J. Biol. Chem. **252**:943–949.
4. **Gibson, J. L., and F. R. Tabita.** 1977. Characterization of antiserum directed against form II ribulose 1,5-bisphosphate carboxylase from *Rhodopseudomonas sphaeroides*. J. Bacteriol. **131**:1020–1022.
5. **Gibson, J. L., and F. R. Tabita.** 1979. Activation of ribulose 1,5-bisphosphate carboxylase from *Rhodopseudomonas sphaeroides*: probable role of small subunit. J. Bacteriol. **140**:1023–1027.
6. **Klackar, H.** 1947. Differential spectrophotometry of purine compounds by means of specific enzymes. J. Biol. Chem. **167**:461–475.
7. **Lugtenberg, B., J. Meijers, R. Peters, P. van der Hoak, and L. van Alpen.** 1975. Electrophoretic resolution of the "major outer membrane protein" of *E. coli* into bands. FEBS Lett. **58**:254–258.
8. **Madigan, M. T., and H. Gest.** 1979. Growth of the photosynthetic bacterium *Rhodopseudomonas capsulata* chemoautotrophically in darkness with H_2 as the energy source. J. Bacteriol. **127**:542–530.
9. **Marrs, B., S. Kaplan, and W. Shepherd.** 1980. Isolation of mutants of photosynthetic bacteria. Methods Enzymol. **69**:29–37.
10. **McFadden, B. A.** 1973. Autotrophic CO_2 assimilation and the evolution of ribulose diphosphate carboxylase. Bacteriol. Rev. **37**:289–319.
11. **McFadden, B. A., and F. R. Tabita.** 1974. D-ribulose 1,5-diphosphate carboxylase and the evolution of autotrophy. Biosystems **6**:93–112.
12. **Norton, I. L., M. H. Welch, and F. C. Hartman.** 1975. Evidence for essential lysyl residues in ribulose bisphosphate carboxylase by use of the affinity label 3-bromo-1,4dihydroxy-2-butanone 1,4-bisphosphate. J. Biol. Chem. **250**:8062–8068.
13. **Ormerod, J. G., K. O. Ormerod, and H. Gest.** 1961. Light dependent utilization of organic compounds and photoproduction of hydrogen by photosynthetic bacteria; relationships with nitrogen metabolism. Arch. Biochem. Biophys. **94**:449–463.
14. **Sarles, L. S., and F. R. Tabita.** 1983. Derepression of the synthesis of D-ribulose 1,5-bisphosphate carboxylase/oxygenase from *Rhodospirillum rubrum*. J. Bacteriol. **153**:458–464.
15. **Tabita, F. R.** 1981. Molecular regulation of carbon dioxide assimilation in autotrophic microorganisms, p. 70–81 *In* H. Dalton (ed.), Microbial growth on C_1 compounds. Heydon and Sons, London.
16. **Tabita, F. R., P. Caruso, and W. Whitman.** 1978. Facile assay of enzymes unique to the Calvin cycle, with special reference to ribulose 1,5-bisphosphate carboxylase. Anal. Biochem. **84**:462–472.
17. **Tabita, F. R., and B. A. McFadden.** 1974. D-ribulose 1,5-bisphosphate carboxylase from *Rhodospirillum rubrum*. I. Levels, purification, and effects of metallic ions. J. Biol. Chem. **249**:3453–3458.
18. **Tabita, F. R., and B. A. McFadden.** 1974. D-ribulose 1,5-bisphosphate carboxylase from *Rhodospirillum rubrum*. II. Quarternary structure, composition, catalytic and immunological properties. J. Biol. Chem. **249**:3459–3464.