Expression and regulation of Bradyrhizobium japonicum and Xanthobacter flavus CO2 fixation genes in a photosynthetic bacterial host.

D L Falcone and F R Tabita


Updated information and services can be found at:
http://jb.asm.org/content/175/3/866

CONTENT ALERTS

These include:

Receive: RSS Feeds, eTOCs, free email alerts (when new articles cite this article), more»

Information about commercial reprint orders: http://journals.asm.org/site/misc/reprints.xhtml
To subscribe to another ASM Journal go to: http://journals.asm.org/site/subscriptions/
Expression and Regulation of *Bradyrhizobium japonicum* and *Xanthobacter flavus* CO₂ Fixation Genes in a Photosynthetic Bacterial Host

DEANE L. FALCONE AND F. ROBERT TABITA*

Department of Microbiology and The Biotechnology Center, The Ohio State University, 484 West 12th Avenue, Columbus, Ohio 43210

Received 5 August 1992/Accepted 18 November 1992

Calvin cycle carbon dioxide fixation genes encoded on DNA fragments from two nonphotosynthetic, chemolithoautotrophic bacteria, *Bradyrhizobium japonicum* and *Xanthobacter flavus*, were found to complement and support photosynthetic growth of a ribulose 1,5-bisphosphate carboxylase-oxygenase (RubisCO) deletion mutant of the purple nonsulfur bacterium *Rhodobacter sphaeroides*. The regulation of RubisCO expression was analyzed in the complemented *R. sphaeroides* RubisCO deletion mutant. Distinct differences in the regulation of RubisCO synthesis were revealed when the complemented *R. sphaeroides* strains were cultured under photolithoautotrophic and photolithoautotrophic growth conditions, e.g., a reversal in the normal pattern of RubisCO gene expression. These studies suggest that sequences and molecular signals which regulate the expression of diverse RubisCO genes may be probed by using the *R. sphaeroides* complementation system.

The reductive pentose phosphate pathway, or Calvin cycle, is widely present in organisms capable of chemolithotrophic or phototrophic growth (17). Bacteria that are able to grow chemolithoautotrophically, such as *Alcaligenes eutrophus* (2), *Xanthobacter flavus* (14), and the soybean symbiont *Bradyrhizobium japonicum* (16), employ the Calvin reductive pentose phosphate pathway when CO₂ is the sole carbon source. When these organisms are grown on formate, CO₂ is released from formate via the activity of formate dehydrogenase and then assimilated by the Calvin cycle (2, 10, 19). The enzymes unique to the Calvin cycle include ribulose 1,5-bisphosphate carboxylase-oxygenase (RubisCO) and phosphoribulokinase (PRK). RubisCO catalyzes the primary carboxylation step of CO₂ fixation, and PRK catalyzes the formation of the CO₂ acceptor molecule ribulose 1,5-bisphosphate. Both enzyme activities have been detected in the organisms mentioned above (14, 17), and the structural genes have been isolated (1, 6, 8, 12). In these organisms, the two enzymes have been found to be coregulated (6, 8, 12).

The versatile purple nonsulfur photosynthetic bacteria fix CO₂ during photolithoautotrophic and photolithoautotrophic growth, and many species, such as *Rhodobacter capsulatus*, grow well chemolithoautotrophically under an H₂-CO₂-O₂ atmosphere, much like aerobic hydrogen-oxidizing bacteria. The regulation of expression of the Calvin cycle may be assessed, at least in part, by the level of RubisCO activity present in cell extracts. Since RubisCO catalyzes the primary step in carbon assimilation under autotrophic conditions, the levels of RubisCO protein and activity are tightly regulated, and the extent of derepression varies over a wide range of growth conditions (17).

In the present study, a RubisCO deletion strain of the photosynthetic bacterium *Rhodobacter sphaeroides* served as the host and was complemented for autotrophic growth by CO₂ fixation genes from two nonphotosynthetic bacteria, *B. japonicum* and *X. flavus*. The regulation of RubisCO expression showed some similarity to that in the foreign host strain, such as aerobic repression, but important differences were noted under photolithoautotrophic and photolithoautotrophic growth conditions.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** *R. sphaeroides* HR (Sm') was the wild-type strain used in these studies. The RubisCO deletion derivative strain 16 (Sm' Km' Tp') (3) was used as the host strain for plasmids containing foreign CO₂ fixation gene clusters. *Escherichia coli* JM83 (pCD102) and JM109 (pCRM6) were used to maintain plasmids or as donors in conjugation experiments. Plasmid pRK2013, used to effect mobilization of broad-host-range plasmids, was maintained in *E. coli* MM294 (4). Plasmids containing CO₂ fixation genes from *B. japonicum* (plasmid pCRM6) and *X. flavus* (plasmid pCD102) were previously isolated from cosmid libraries (8, 12). The cosmid from *B. japonicum* contains a 20-kb DNA insert and was shown to possess formate dehydrogenase-, RubisCO-, and PRK-encoding genes and was generously provided by Todd Cotter. The CO₂ fixation gene cluster from *X. flavus* was obtained by complementation of autotrophic mutants of *X. flavus* (8) and was kindly provided by Wim Meijer. The 24-kb DNA insert in this cosmid clone was subsequently shown by DNA sequence analysis (14) to have a CO₂ fixation gene cluster similar to that found in *A. eutrophus* (6), including genes encoding PRK (prk) and RubisCO (*rbcL rbcS*), fructose 1,6-bisphosphatase (fhp), and a divergently transcribed gene located upstream from the cluster, *cfr*.

**Media and growth conditions.** *R. sphaeroides* cells were grown under photolithoautotrophic conditions in 22-ml screw-cap tubes or in 0.5-liter bottles containing 400 ml of Ormerod's medium (15) plus 0.4% malate; cultures were bubbled with argon as described previously (7). Photolithoautotrophic growth was accomplished by bubbling 400 ml of Ormerod's minimal medium with a gas mixture of 1.5% CO₂-98.5% H₂. Aerobic growth was done in 250-ml baffle flasks containing 50 ml of medium in a 30°C incubator with
shaking at 280 rpm. Formate was used as the organic carbon source by adding filter-sterilized formic acid to minimal Ormerod's medium to a final concentration of 22 mM. The antibiotic concentrations used for selection of transconjugants and routine plasmid maintenance have been described previously (3). Photosynthetic growth on solid medium was achieved as described previously; 1.8% Bacto-Agar (Difco Laboratories, Detroit, Mich.) was added to minimal medium, and the plates were incubated in anaerobic jars in a CO$_2$-H$_2$ atmosphere in the light (20).

**Mobilization of plasmids to *R. sphaeroides***. Broad-host-range plasmids were introduced into *R. sphaeroides* 16 by triparental matings with the helper plasmid pRK2013 on filter pads as described previously (20). Transconjugants were selected by plating the mixture of cells from the filter pads onto peptone yeast extract (PYE) agar plates that included all antibiotics for selection of the recipient strain (spectinomycin, kanamycin, and trimethoprim) plus tetracycline (5.0 μg/ml). Plates were incubated aerobically in the dark at 30°C.

**Rubisco assays**. Cell extracts were prepared by a lysozyme sonication method as described previously (3). Rubisco activity was measured by the assay described previously (21). Protein concentration was determined by a modified Lowry protein assay (11), with bovine serum albumin as the standard. Denaturing polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) was done by the method of Lugtenberg et al. (9).

**RESULTS**

In previous experiments, we demonstrated that plasmids which contained DNA fragments encoding either of the two *R. sphaeroides* CO$_2$ fixation gene clusters complemented a Rubisco deletion strain of *R. sphaeroides*. The levels of Rubisco expression were regulated under phototrophic and photolithoautotrophic conditions in the complemented strain in the same manner as in the wild-type strain (3).

After introduction of cosmids containing DNA that encoded CO$_2$ fixation genes from *B. japonicum* or *X. flavus* (plasmids pCRM6 and pCD102, respectively), transconjugants from the tetracycline-containing PYE plates were streaked to minimal and minimal-malate agar plates. After incubation under photosynthetic conditions, each single transconjugant tested grew. This demonstrated that the Rubisco encoded by each of the two foreign cfx gene cosmids was expressed to a level adequate to support photosynthetic growth of the *R. sphaeroides* Rubisco-growing host strain.

To determine the physiological response of *R. sphaeroides* growing photosynthetically with foreign CO$_2$ fixation gene clusters, transconjugants were inoculated into 22-ml screw-cap tubes containing Ormerod's minimal medium with malate (15). These cells were then inoculated to 400-ml cultures containing malate or minimal medium, which were bubbled with argon gas or a mixture of 1.5% CO$_2$ in 98.5% H$_2$, respectively. Growth rates under phototrophic conditions were approximately equivalent for each of the two complemented strains compared with that of the wild-type strain (Table 1). Photolithoautotrophic growth rates, in contrast, were slower for strains complemented by either source of the cfx genes, ranging from 8 to 58 h longer than the doubling time of wild-type strain HR (Table 1). Cultures of the complemented *R. sphaeroides* strains attained an A$_{660}$ of greater than 1.5 under photolithoautotrophic conditions, indicating that there was nothing limiting growth.

To determine the level of Rubisco in these cells, extracts were prepared and assayed for Rubisco activity. For malate-grown cultures of *R. sphaeroides* strains harboring the foreign cfx genes, Rubisco levels were increased 6- to 10-fold over the activity found in extracts from the wild-type strain (Table 1). These high levels of Rubisco are probably not solely due to the introduction of multiple copies of the genes, since similar experiments with plasmids containing the form I and form II Rubisco genes of *R. sphaeroides* yielded the usual repressed levels of Rubisco when the cells were cultured phototrophically (3). The amount of Rubisco activity detected in extracts from cells grown under photolithoautotrophic conditions was approximately one-fifth to one-third lower than the levels determined for the complemented strains grown photolithoautotrophically. The high levels of Rubisco activity observed in photolithoautotrophically grown cultures of complemented strains indicated that the regulation of Rubisco activity or synthesis was unlike that normally observed for the wild-type strain. In fact, the high Rubisco activity in cultures grown with an oxidized organic carbon source is essentially the opposite of the situation generally found in the wild-type strain.

In subsequent experiments, the effect of adding an organic carbon source (malate) to cultures initially grown under photolithoautotrophic conditions was determined. Cultures of strain HR, strain 16(pCRM6), and strain 16(pCD102), grown to an A$_{660}$ of approximately 0.5 under an atmosphere of 1.5% CO$_2$-98.5% H$_2$, were shifted to phototrophic conditions by adding malate to a final concentration of 0.4%. Rubisco activities were then assessed at various times after the addition of malate to each culture (Table 2). The observed effect was as expected for the wild-type strain; there was a sharp drop in Rubisco activity at the first time point (6.5 h) after the shift to phototrophic conditions. Rubisco activity in strain 16, expressing the *Bradyrhizobium* or *Xanthobacter* cfx gene, increased over the level present before the addition of malate, in agreement with the pattern of activity found when strains were continuously cultured under phototrophic conditions. Cell extracts taken from the time points indicated in Table 2 were also examined by SDS-PAGE to establish whether there was a corresponding increase in Rubisco subunit polypeptide concentration after the shift from photolithoautotrophic to phototrophic conditions. This analysis revealed a ma-
jor band, just above the 55,000-molecular-weight cyanobacterial large-subunit standard, that increased in intensity in extracts of the complemented strains as the time after the addition of malate increased (Fig. 1). Moreover, immunoblots made with antibodies to *R. sphaeroides* form I RubisCO specifically cross-reacted with the 55,000-molecular-weight band (data not shown). The prominent approximately 60,000-molecular-weight protein was the *R. sphaeroides* chaperonin 60 (cpn60) protein, as shown by immunoblots with antibodies to *R. sphaeroides* cpn60 (data not shown). The results from the SDS-PAGE experiments have thus established that a protein with the expected molecular weight of the large subunit coincided with the observed increase in RubisCO activity, suggesting a regulatory response at the level of protein expression and not at the posttranslational level.

Since RubisCO is synthesized in both *B. japonicum* and *X. flavus* after aerobic growth on formate (10, 14), extracts of *R. sphaeroides* 16 harboring either pCRM6 or pCD102 were examined for the presence of RubisCO activity after aerobic growth on formate. Low but measurable levels of activity were obtained in all cases (Table 3). As a comparison, complemented strains were also grown aerobically on malate, a growth condition that is known to fully repress RubisCO expression in wild-type *R. sphaeroides* (7). No substantial RubisCO activity was detected in malate-grown cells except for a trace amount in strain 16 containing the *X. flavus* plasmid pCD102 (Table 3). To test whether the complementing cosmids could confer the ability to grow anaerobically (photosynthetically) on formate on *R. sphaeroides*, strain 16 containing each of the plasmids was incubated in minimal medium plus formate in the light. No growth of *R. sphaeroides* 16(pCD102) or *R. sphaeroides* 16(pCRM6) was observed.

**DISCUSSION**

Complementation of a RubisCO deletion strain of *R. sphaeroides* by plasmids with DNA inserts encoding CO₂ fixation genes from two nonphotosynthetic microorganisms demonstrated the functional relatedness among these three genera. While the recognition of foreign promoters was not surprising in view of the similar G+C content of these bacteria, the means by which regulation of RubisCO expression might be manifested in a dissimilar background strain is not obvious. *R. sphaeroides* cells grown chemoheterotrophically with a *cfx* gene from either *B. japonicum* or *X. flavus* showed a regulatory pattern of RubisCO synthesis more similar to that of the host bacterium than to that of the organism from which the *cfx* gene was derived. For example, aerobic repression of RubisCO in formate-containing medium was apparent in *R. sphaeroides* cells grown with the plasmid-borne *cfx* genes, even though this is opposite to what is normally observed for the aerobic bacteria *B. japonicum* and *X. flavus*. Thus, in this instance, the controlling environment of *R. sphaeroides* took precedence over the system that normally regulates RubisCO derepression in the authentic host. However, under photosynthetic growth conditions, the control of RubisCO expression differed substantially from what is usually observed in *R. sphaeroides*. The levels of RubisCO activity and expression in photoheterotrophically grown cells of the complemented *R. sphaeroides* strains were essentially reversed from the levels in wild-type cells (Table 1). Moreover, *R. sphaeroides* cells growing with the heterologous *cfx* genes under photolithoautotrophic growth conditions displayed a reduction in RubisCO activity, which was reflective of the decreased amount of RubisCO large subunits observed on SDS-PAGE gels, again
Contrary to the response of the wild-type strain under the same conditions.

This basic distinction in regulatory characteristics may indicate subtle differences in the control of RubisCO expression among separate genera. Perhaps these differences are the result of unique responses of regulatory sequences present in each gene cluster and of intracellular signals elicited under photolithoautotrophic and photoheterotrophic growth conditions. The means by which intracellular signals are processed may be considered in reference to recent findings of CO2 fixation regulator genes, found to be associated with a variety of bacteria that possess the Calvin cycle, including _A. eutrophus_, _Chromatium vinosum_, _X. flavus_, and _R. sphaeroides_ (5, 13, 18, 22). In each case, a regulator gene, designated _cfxR_, is situated upstream of the CO2 fixation structural genes. Thus, it can be inferred that the two CO2 fixation gene clusters used in this study encode presumptive _cfxR_ genes and that these are expressed. If this is in fact the case, then _R. sphaeroides_ cells harboring the foreign Calvin cycle genes express at least two regulator genes, one from the host and one from the introduced plasmid. Such a situation could have complex effects on the outcome of RubisCO expression. For example, the physiological state which leads to increased synthesis of RubisCO in the native _X. flavus_ cell may repress synthesis of RubisCO-encoding genes in a foreign host.

It is anticipated that the findings presented in this study will assist in defining potential signals that have a role in governing RubisCO expression. In addition, high-level expression of the _Bradyrhizobium_ and _Xanthobacter_ _rbcL_ and _rbcS_ genes in _R. sphaeroides_ will provide a convenient and readily available source of large amounts of RubisCO for subsequent purification and enzymological characterization. Certainly, biological selection of mutant _Bradyrhizobium_ and _Xanthobacter_ RubisCO enzymes with the _R. sphaeroides_ expression system is entirely feasible, and the results provided in this investigation and in earlier studies (3) indicate that alterations of RubisCO function in both aerobic and anaerobic bacteria may be studied with this system.

**ACKNOWLEDGMENTS**

We thank Todd Cotter from the laboratory of B. K. Chelm for the gift of plasmid pCRM6 and Wim Meijer for plasmid pCD102. We thank Betsy Read and Katherine Terlesky for providing, respectively, _Synechococcus_ sp. strain 6301 recombinant RubisCO and _R. sphaeroides_ cpn60.

This investigation was supported by NRICGP grant 91-37306-6325 from the U.S. Department of Agriculture.

**REFERENCES**

5. Gibson, J. L., and F. R. Tabita. Submitted for publication.