Expression and Regulation of *Bradyrhizobium japonicum* and *Xanthobacter flavus* CO_2 Fixation Genes in a Photosynthetic Bacterial Host

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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 photoheterotrophic 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 CO_2 is the sole carbon source. When these organisms are grown on formate, CO_2 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 CO_2 fixation, and PRK catalyzes the formation of the CO_2 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 CO_2 during photoheterotrophic and photolithoautotrophic growth, and many species, such as *Rhodobacter capsulatus*, grow well chemolithoautotrophically under an H_2-CO_2-O_2 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 CO_2 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 photoheterotrophic and photolithoautotrophic growth conditions.

MATERIALS AND METHODS

Bacterial strains and plasmids. *R. sphaeroides* HR (Sm^r) was the wild-type strain used in these studies. The RubisCO deletion derivative strain 16 (Sm^r Km^r Tp^r) (3) was used as the host strain for plasmids containing foreign CO_2 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 CO_2 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 CO_2 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 CO_2 fixation gene cluster similar to that found in *A. eutrophus* (6), including genes encoding PRK (*prk*) and RubisCO (*rbcL rbcS*), fructose 1,6-bisphosphatase (*fbp*), and a divergently transcribed gene located upstream from the cluster, *cfxR*.

Media and growth conditions. *R. sphaeroides* cells were grown under photoheterotrophic 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% CO_2-98.5% H_2. Aerobic growth was done in 250-ml baffle flasks containing 50 ml of medium in a 30°C incubator with

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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 photoheterotrophic 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 deletion 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 photoheterotrophic 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}

TABLE 1. Growth rate and RubisCO activity of *R. sphaeroides* 16 complemented with CO$_2$ fixation genes from *B. japonicum* (pCRM6) and *X. flavus* (pCD102)

Growth conditions[a] and R. sphaeroides strain	Generation time (h)	RubisCO activity (U/mg)[b]
Photoheterotrophic		
HR (wild type)	8.0	0.046
16(pCRM6)	7.2	0.503
16(pCD102)	7.2	0.294
Photolithoautotrophic		
HR (wild type)	24.0	0.357
16(pCRM6)	32.0	0.100
16(pCD102)	82.0	0.108

[a] Strains were grown photoheterotrophically with malate as the electron donor or photolithoautotrophically in an atmosphere of 1.5% CO$_2$–98.5% H$_2$.
[b] Micromoles of CO$_2$ fixed per minute per milligram of total soluble protein.

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 photoheterotrophically (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 photoheterotrophically. The high levels of RubisCO activity observed in photoheterotrophically 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 photoheterotrophic 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 photoheterotrophic 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 photoheterotrophic 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 photoheterotrophic conditions. This analysis revealed a ma-

TABLE 2. Effect of malate addition to photolithoautotrophically grown cultures of complemented and wild-type R. sphaeroides strains[a]

Strain	RubisCO activity (U/mg) at time (h)			
	0	6.5	17	25
HR (wild type)	0.265	0.056	0.064	0.046
16(pCRM6)	0.200	0.350	0.442	0.372
16(pCD102)	0.141	0.244	0.217	0.282

[a] Strains were grown photolithoautotrophically on 1.5% CO_2–98.5% H_2. After the initial sample was taken, malate was added. For definition of RubisCO activity units, see Table 1, footnote b.

TABLE 3. RubisCO activities of R. sphaeroides strains grown aerobically on minimal medium containing formic or malic acid as the carbon source

R. sphaeroides strain	RubisCO activity (mU/mg)[a]	
	Formate	Malate
HR (wild type)	3.0	ND[b]
16(pCRM6)	2.5	ND
16(pCD102)	7.9	0.56

[a] See Table 1, footnote b.
[b] ND, not detectable.

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.
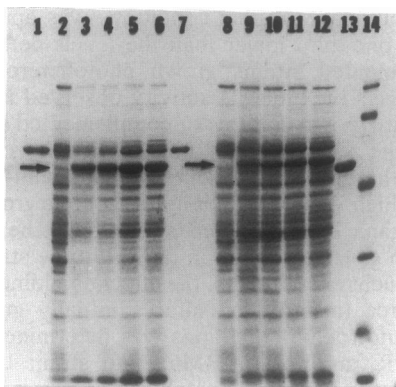
Since RubisCO is synthesized in both B. japonicum and X.

FIG. 1. SDS-PAGE (12% acrylamide) of extracts from photosynthetically grown R. sphaeroides 16 cells complemented with B. japonicum cfx genes (pCRM6) and X. flavus cfx genes (pCD102). Lanes: 1 and 7, purified cpn60 from R. sphaeroides; 2 and 8, extracts of R. sphaeroides HR (wild-type) cells grown photolithoautotrophically; 3 to 6, cell extracts of R. sphaeroides 16(pCRM6); 9 to 12, cell extracts of R. sphaeroides 16(pCD102); 3 and 9, extracts of photolithoautotrophically grown (1.5% CO_2, 98.5% H_2) cells prior to the addition of malate; 4 and 10, extracts of cells prepared 6.5 h after the addition of 0.4% malate; 5 and 11, extracts of cells prepared 17 h after the addition of malate; 6 and 12, extracts of cells prepared 25 h after the addition of malate; 13, large subunit of purified RubisCO from Anacystis nidulans (Synechococcus sp. strain pCC6301) (M_r 55,000); 14, commercially prepared (Bio-Rad) molecular weight markers: (from top) phosphorylase b, 97,400; bovine serum albumin, 66,200; ovalbumin, 45,000; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; and lysozyme, 14,400. The arrows indicate the band in extracts from the complemented strains corresponding to the large subunit of RubisCO.

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 CO_2 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 CO_2 fixation regulator genes, found to be associated with a variety of bacteria that possess the Calvin cycle, including *A. eutrophus*, *Chromatum vinosum*, *X. flavus*, and *R. sphaeroides* (5, 13, 18, 22). In each case, a regulator gene, designated *cfxR*, is situated upstream of the CO_2 fixation structural genes. Thus, it can be inferred that the two CO_2 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

REFERENCES

1. **Andersen, K., and M. Wilke-Douglas.** 1987. Genetic and physical mapping and expression in *Pseudomonas aeruginosa* of the chromosomally encoded ribulose bisphosphate carboxylase genes of *Alcaligenes eutrophus*. J. Bacteriol. **169:**1997–2004.
2. **Bowein, B., and H. G. Schlegel.** 1981. Physiology and biochemistry of aerobic hydrogen oxidizing bacteria. Annu. Rev. Microbiol. **35:**405–452.
3. **Falcone, D. L., and F. R. Tabita.** 1991. Expression of endogenous and foreign ribulose 1,5-bisphosphate carboxylase-oxygenase (RubisCO) genes in a RubisCO deletion mutant of *Rhodobacter sphaeroides*. J. Bacteriol. **173:**2099–2108.
4. **Figurski, D., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of the plasmid RK2 dependent on a plasmid function provided *in trans*. Proc. Natl. Acad. Sci. USA **76:**1648–1652.
5. **Gibson, J. L., and F. R. Tabita.** Submitted for publication.
6. **Huseman, M., R. Klintworth, V. Büttcher, J. Salnikow, C. Weissenborn, and B. Bowein.** 1988. Chromosomally and plasmid encoded gene clusters for CO_2 fixation (*cfx*) genes in *Alcaligenes eutrophus*. Mol. Gen. Genet. **214:**112–120.
7. **Jouanneau, Y., and F. R. Tabita.** 1986. Independent regulation of form I and form II ribulose bisphosphate carboxylase-oxygenase in *Rhodopseudomonas sphaeroides*. J. Bacteriol. **165:**620–624.
8. **Lehmicke, L. G., and M. E. Lidstrom.** 1985. Organization of genes necessary for growth of the hydrogen-methanol autotroph *Xanthobacter* sp. strain H4-14 on hydrogen and carbon dioxide. J. Bacteriol. **162:**1244–1249.
9. **Lugtenberg, B., J. Mujers, R. Peters, P. Van der Hock, and L. Van Alpen.** 1975. Electrophoretic resolution of the major outer membrane protein of *E. coli* into bands. FEBS Lett. **58:**254–258.
10. **Manian, S. S., and F. O'Gara.** 1982. Induction and regulation of ribulose bisphosphate carboxylase activity in *Rhizobium japonicum* during formate-dependent growth. Arch. Microbiol. **131:** 51–54.
11. **Markwell, M. A., S. M. Hass, L. Buber, and N. E. Tolbert.** 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal. Biochem. **87:**206–210.
12. **McClung, C. R., and B. K. Chelm.** 1987. A genetic locus essential for formate-dependent growth of *Bradyrhizobium japonicum*. J. Bacteriol. **169:**3260–3267.
13. **Meijer, W. G., A. C. Arnberg, H. G. Enquist, P. Tupestra, M. E. Lidstrom, and L. Dijkhuizen.** 1991. Identification and organization of carbon dioxide fixation genes in *Xanthobacter* H4-14. Mol. Gen. Genet. **225:**320–330.
14. **Meijer, W. G., L. M. Croes, B. Jenni, L. G. Lehmicke, M. E. Lidstrom, and L. Dijkhuizen.** 1990. Characterization of *Xanthobacter* strains H4-14 and 25A and enzyme profiles after growth under autotrophic and heterotrophic conditions. Arch. Microbiol. **153:**360–367.
15. **Ormerod, J. G., K. D. Ormerod, and H. Gest.** 1961. Light dependent utilization of organic compounds and photoproduction of hydrogen by photosynthetic bacteria: relationship with nitrogen metabolism. Arch. Biochem. Biophys. **94:**449–463.
16. **Simpson, F. B., R. J. Maier, and H. J. Evans.** 1979. Hydrogen-stimulated CO_2 fixation and co-ordinate induction of hydrogenase and ribulose bisphosphate carboxylase in a H_2-uptake positive strain of *Rhizobium japonicum*. Arch. Microbiol. **123:** 1–8.
17. **Tabita, F. R.** 1988. Molecular and cellular regulation of autotrophic carbon dioxide fixation in microorganisms. Microbiol. Rev. **52:**155–189.
18. **Viale, A. M., H. Kobayashi, T. Akayawa, and S. Henikoff.** 1991. *rcbR*, a gene coding for a member of the LysR family of transcriptional regulators, is located upstream of the expressed set of ribulose 1,5-bisphosphate carboxylase/oxygenase genes in the photosynthetic bacterium *Chromatium vinosum*. J. Bacteriol. **172:**5224–5229.
19. **Weaver, C. A., and M. E. Lidstrom.** 1985. Methanol dissimilation in *Xanthobacter* H4-14: activities, induction, and comparison to *Pseudomonas* AM1 and *Paracoccus denitrificans*. J. Gen. Microbiol. **131:**2183–2197.
20. **Weaver, K. E., and F. R. Tabita.** 1983. Isolation and partial characterization of *Rhodopseudomonas sphaeroides* mutants defective in the regulation of ribulose bisphosphate carboxylase/oxygenase. J. Bacteriol. **156:**507–515.
21. **Whitman, W., and F. R. Tabita.** 1976. Inhibition of ribulose 1,5-bisphosphate carboxylase by pyridoxal 5-phosphate. Biochem. Biophys. Res. Commun. **71:**1034–1039.
22. **Windhövel, U., and B. Bowein.** 1991. Identification of *cfxR*, an activator gene of autotrophic CO_2 fixation in *Alcaligenes eutrophus*. Mol. Microbiol. **5:**2695–2705.