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Regulation of CO$_2$ fixation in photosynthetic bacteria

Falcone, Deane Louis, Ph.D.

The Ohio State University, 1992
REGULATION OF CO₂ FIXATION IN PHOTOSYNTHETIC BACTERIA

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By
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The Ohio State University
1992

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FIELDS OF STUDY

Major Field: Microbiology
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INTRODUCTION

There are a number of biochemical pathways that enable autotrophic organisms to grow on carbon dioxide (CO₂) as the sole cellular carbon source (14). The most widespread route found in nature for the fixation of CO₂ is the Calvin reductive pentose phosphate pathway (13, 86). One reason the Calvin cycle predominates in the biosphere is due to the ability of these reactions to function in an aerobic environment; the basis of the pathways' presence in all eukaryotic photosynthetic organisms. The Calvin cycle is also found in an extensive range of prokaryotes, including aerobic and anaerobic chemolithotrophs and aerobic and anaerobic photosynthetic members. In facultatively autotrophic species, grown in the presence of heterotrophic matter, the precise functional role of CO₂ fixation is more subtle, where it appears to simultaneously increase the organisms' metabolic diversity, while supplementing the efficiency of existing heterotrophic growth modes.

The central enzyme of the Calvin reductive pentose phosphate pathway is ribulose 1,5-bisphosphate carboxylase-oxygenase (RubisCO), which catalyzes the primary reaction of CO₂ fixation. Because of the predominance of the Calvin cycle and the presence of RubisCO at extremely high levels within the cell, (62, 86), this enzyme has been described as the most abundant protein on earth (13). As described in the introductions to the following chapters, the RubisCO enzyme is remarkably conserved throughout evolution. This is based on comparisons of the
deduced primary structure of a large number of RubisCO proteins, including a RubisCO sequence obtained from an extinct plant species (33). The most common RubisCO structure (type I) is composed of eight large catalytic subunits and eight small subunits, of unknown function (2, 62). A second structural form, known only from certain prokaryotic sources, is composed of large subunits only (type II or form II). The form II RubisCO is structurally unique, although many residues at the active site are conserved in both type I and type II RubisCO large subunits (2, 62, 63, 91). However, as a result of physiological studies of the type I and type II RubisCO enzymes of the photosynthetic bacterium, *Rhodobacter sphaeroides*, several regulatory differences were observed.

RubisCO constitutes only one of two enzymes unique to the Calvin cycle, the other being phosphoribulokinase (PRK). PRK uses ATP to phosphorylate ribulose 5-phosphate (Ru5P) generating ribulose 1,5-bisphosphate (RuBP), the CO₂ acceptor molecule. Other reactions in the pathway serve to regenerate Ru5P and are catalyzed by enzymes common to other pathways of intermediary metabolism (86). A number of Calvin cycle enzymes are encoded within large transcriptional units in the bacteria *Rhodobacter sphaeroides*, *Alcaligenes eutrophus*, and *Xanthobacter flavus* (24, 25, 27, 34, 39, 59, 96, 97). Genes encoding the Calvin cycle enzymes, in addition to those mentioned above, are: fructose bisphosphate aldolase, (*fba*), fructose bisphosphatase (*fbp*), transketolase (*tkl*), pentose phosphate epimerase (*ppe*), and glyceraldehyde phosphate dehydrogenase (*gap*) (8, 24, 26, 28, 59, 96). All of these genes have been identified in *A. eutrophus*, and all but *ppe*, in *R. sphaeroides*. Genes encoding the large and small subunits of RubisCO (*rbcL rbcS*), as well as *fbp* and *prk*, have been identified in *X. flavus* (49, 59). As described in this dissertation, the
clustering of the various Calvin cycle enzymes permits coordinate control, since a single regulator locus, encoded by the \( cfxR \) gene, has been identified in the above organisms. In particular, for \( R. \) \textit{sphaeroides} and \( A. \) \textit{eutrophus}, a single \( cfxR \) transcriptional regulator gene has been shown to control two distinct CO\(_2\) fixation gene (\( cfx \)) gene clusters of the same organism (29, 97). The molecular regulation of Calvin cycle \( cfx \) genes thus must be largely determined by the interactions between \( cfx \) promoter regions and the \( cfxR \) gene product, which in \( A. \) \textit{eutrophus} has been shown to occur \textit{in vitro} (97).

The work presented in this dissertation addresses aspects of regulation of \( cfx \) genes in two photosynthetic bacteria, \textit{Rhodobacter sphaeroides} and \textit{Rhodospirillum rubrum}. Mutants of these strains were constructed in which the genes encoding RubisCO were deleted. The strains were then employed as hosts for complementation analyses, which was facilitated by the development of a RubisCO expression vector. The expression vector contains sequences upstream from the \( R. \) \textit{rubrum} \( rbpL \) gene; analysis of this upstream region of \( R. \) \textit{rubrum} DNA ultimately led to the identification of CO\(_2\) fixation genes within a uniquely organized \( cfx \) gene cluster. The RubisCO deficient mutant strains also facilitated an assessment of the Calvin cycle under diverse growth conditions. The behavior and capacity of diverse sources of RubisCO to support CO\(_2\)-dependent growth in \( R. \) \textit{sphaeroides} was also determined.
CHAPTER I

Expression of Endogenous and Foreign Rubisco Genes in a Rubisco Deletion Mutant of \textit{Rhodobacter sphaeroides}

INTRODUCTION

Ribulose 1,5-bisphosphate carboxylase-oxygenase (Rubisco) is a bifunctional enzyme that catalyzes the initial step in the carbon dioxide assimilatory pathway and photorespiratory pathway in virtually all photosynthetic organisms. Rubisco is thus positioned to regulate the flow of cell carbon through two competing metabolic pathways (2, 62, 85). The enzyme from photosynthetic organisms, including plants, algae, cyanobacteria and most photosynthetic bacteria is composed of eight large and eight small subunits (L$_g$S$_8$) (type I or form I). The large subunits ($M_r \sim$50,000), encoded by the \textit{rbcL} gene, contain the active site while the precise role of the small subunit ($M_r \sim$12-15,000), encoded by the \textit{rbcS} gene, is unclear, although it is required for full activity (2, 86). A much less common form of Rubisco, containing only large subunits (type II or form II), encoded by the \textit{rbpL} gene, is found in some purple nonsulfur photosynthetic bacteria. The enzyme from \textit{Rhodospirillum rubrum}, the first of this type isolated, is a homodimer of large subunits (84) that shows little overall homology to large subunits of L$_g$S$_8$ enzymes. Due to its comparatively simple structure and functional similarity to the plant enzyme, it is perhaps the most thoroughly studied Rubisco protein (2, 48, 86).
Rhodobacter sphaeroides, another purple nonsulfur photosynthetic bacterium, was also shown to synthesize form II RubisCO. In addition, this organism, like the related bacterium Rhodobacter capsulatus, synthesizes the more prevalent L_{8}S_{8} enzyme (form I) which resembles the RubisCO from plant, algal, and cyanobacterial sources (21, 22). Thus, R. sphaeroides and R. capsulatus synthesize two genetically and structurally distinct forms of RubisCO. In the case of R. sphaeroides, much is known about the arrangement and regulation of the RubisCO genes (15, 27, 23, 24, 25, 35, 36, 86), which are found in two unlinked genetic regions that encode many of the enzymes of the reductive pentose phosphate pathway (24, 25, 86). In both Rhodobacter sphaeroides and Rhodospirillum rubrum the regulation of RubisCO synthesis is sensitive to the CO_{2} concentration in the medium when cells are grown photolithoautotrophically. During photoheterotrophic growth, the levels of RubisCO depend on the nature and reduction level of the electron donor (41, 23, 86).

In this study we constructed an R. sphaeroides RubisCO deletion strain that served as a host for the expression of several different sources of RubisCO. Complementation of the RubisCO deletion strain was possible with DNA fragments containing either of two CO_{2} fixation gene clusters from R. sphaeroides or with a DNA fragment that contained the region upstream and including the rbpL gene from R. rubrum. The upstream region of the R. rubrum rbpL gene was also utilized as a promoter to express genes encoding RubisCO from different sources.
MATERIALS AND METHODS

Bacterial strains and plasmids. *Escherichia coli* JM109 (90) was used as the host strain for routine plasmid manipulations. *E. coli* HB101 (7) was used to maintain pVK101 and pVK102 plasmid derivatives. *E. coli* SM-10 (78) was used as the donor strain for *R. sphaeroides* strain constructions. *R. sphaeroides* HR (93) was derived from ATCC strain 17023 and was used as the wild-type strain in all growth experiments and as the parental strain for the RubisCO-deletion mutant. Plasmids and relevant characteristics used in this study are shown in Table 1.1.

Media and growth conditions. *R. sphaeroides* cells were grown under photoheterotrophic conditions in 9- or 22-ml screw-cap tubes or in bottles containing 400 ml malate-supplemented Ormerod's medium (66) bubbled with argon, as previously described (41), except that NaHCO₃ was omitted. Dimethyl sulfoxide (DMSO) was used, where noted, at a concentration of 40 mM. Photolithoautotrophic growth was attained by bubbling 400 ml cultures of minimal medium with 1.5% CO₂ in hydrogen (41). For photosynthetic growth on solid media, 1.8% Bacto-Agar (Difco Laboratories, Detroit, Mich.) was added to malate or minimal medium; plates were incubated in GasPak (Scientific Products Div., McGaw Park, Ill) anaerobic jars as previously described (93). Growth in large liquid cultures was monitored by measuring absorbance at 660 nm with a Beckman spectrophotometer; 9.0 ml culture tubes were directly inserted into a Bausch and Lomb Spectronic 20. All growth vessels were incubated in illuminated water baths at 32°C as previously described (93). Aerobic growth of *R. sphaeroides*, in complex peptone yeast extract (PYE) medium (93), was performed in Erlenmeyer flasks or agar plates at 30°C. Antibiotic
TABLE 1.1. Plasmids used in this study

<table>
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<th>Plasmid</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
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<tr>
<td>pUC8</td>
<td>Ap⁺,</td>
<td>(90)</td>
</tr>
<tr>
<td>pUC1318</td>
<td>Ap⁺, pUC vector with hybrid cloning site</td>
<td>(44)</td>
</tr>
<tr>
<td>pUC1813</td>
<td>Ap⁺, pUC vector with hybrid cloning site</td>
<td>(44)</td>
</tr>
<tr>
<td>pVK101</td>
<td>Tc⁺, broad-host-range vector</td>
<td>(46)</td>
</tr>
<tr>
<td>pVK102</td>
<td>Tc⁺, Km⁺ broad-host-range vector</td>
<td>(46)</td>
</tr>
<tr>
<td>pRK404</td>
<td>Tc⁺, broad-host-range vector, LacZ</td>
<td>(12)</td>
</tr>
<tr>
<td>pSUP202</td>
<td>Cm⁺ Tc⁺ Ap⁺ Tra'Mob⁺, pBR325 replicon</td>
<td>(78)</td>
</tr>
<tr>
<td>pXLGD4</td>
<td>pRK290 derivative with a 9.4-kb insert encoding lacZY genes</td>
<td>(12)</td>
</tr>
<tr>
<td>pJG336</td>
<td>Tc⁺, pVK102 with 24-kb insert of R. sphaeroides DNA encoding CO₂-fixation gene cluster A</td>
<td>(23, 24)</td>
</tr>
<tr>
<td>pJG106</td>
<td>Tc⁺, pVK102 with 26-kb insert of R. sphaeroides DNA encoding CO₂-fixation gene cluster B</td>
<td>(23, 24)</td>
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<tr>
<td>pJG29</td>
<td>Ap⁺, pUC8 containing R. sphaeroides rbcL, rbcS genes in a 4.0-kb SmaI insert</td>
<td>(23)</td>
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<tr>
<td>pJGE25</td>
<td>Ap⁺, pUC8 containing 1.8-kb EcoRI fragment from pJG336</td>
<td>J. Gibson,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>This laboratory</td>
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<tr>
<td>pRQ2</td>
<td>Tc⁺ Ap⁺ pBR322 containing R. sphaeroides rbpL gene in a 3.0-kb EcoRI insert</td>
<td>(70)</td>
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<tr>
<td>pRQ53</td>
<td>Ap⁺ pUC8 containing the 3.0-kb EcoRI insert present in pRQ2</td>
<td>(70)</td>
</tr>
<tr>
<td>pQW32</td>
<td>Tc⁺ pVK101 containing the 3.0-kb EcoRI insert present in pRQ2</td>
<td>This study</td>
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<tr>
<td>pCS75</td>
<td>pUC9 containing A. nidulans RubisCO genes in a 2.3 -kb PstI insert</td>
<td>(85)</td>
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<th>Plasmid</th>
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<tr>
<td>pUC13-K</td>
<td>pUC1318 containing a 1.44-kb HindIII insert of Tn5 encoding Km&lt;sup&gt;r&lt;/sup&gt; (Km&lt;sup&gt;r&lt;/sup&gt; cartridge)</td>
<td>This study</td>
</tr>
<tr>
<td>pUC1318::E25</td>
<td>pUC1318 containing 1.8-kb EcoRI-insert of pJGE25</td>
<td>This study</td>
</tr>
<tr>
<td>pUC1318::E25d::Km</td>
<td>pUC1318::E25 with SalI d and Km&lt;sup&gt;r&lt;/sup&gt; gene cartridge insert</td>
<td>This study</td>
</tr>
<tr>
<td>pSUP::E25 d ::Km</td>
<td>pSUP202 containing the EcoRI insert of pUC1318::E25::Km</td>
<td>This study</td>
</tr>
<tr>
<td>pUT89</td>
<td>pACYC184 containing a 2.6-kb BamHI insert of plasmid R751 encoding Tp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>R. J. Meyer (60)</td>
</tr>
<tr>
<td>pUC18-89</td>
<td>pUT89 as a SalI insert (Tp&lt;sup&gt;r&lt;/sup&gt; cartridge)</td>
<td>This study</td>
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<tr>
<td>pQW32d::Tp</td>
<td>pQW32 with PstI d and Tp&lt;sup&gt;r&lt;/sup&gt; gene cartridge insert</td>
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<td>pSUP::FII::DI</td>
<td>pSUP202 containing the 5.3-kb insert of pQW32d::Tp</td>
<td>This study</td>
</tr>
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<td>pRR116</td>
<td>pBR325 containing R. rubrum RubisCO gene in a 6.6-kb EcoRI insert</td>
<td>(80)</td>
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<tr>
<td>pUC8::116-I</td>
<td>pUC8 containing 2.0-kb SalI insert from pRR116 with RubisCO transcription start site in orientation I</td>
<td>This study</td>
</tr>
<tr>
<td>pUC8::116-II</td>
<td>pUC8 containing 2.0-kb SalI insert from pRR116 in orientation II</td>
<td>This study</td>
</tr>
<tr>
<td>pUC1813-116</td>
<td>pUC1813 containing 6.6-kb EcoRI R. rubrum DNA from pRR116</td>
<td>This study</td>
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<tr>
<td>pRKRP-1</td>
<td>pRK404 containing 1.45-kb HindIII-SalI insert from pUC8::116-I</td>
<td>This study</td>
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<tr>
<td>pUC13-75</td>
<td>pUC1318 containing 2.3-kb PstI insert of pCS75</td>
<td>This study</td>
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Table 1.1. (continued)

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<th>Plasmid</th>
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<td>pRKRP1-M</td>
<td>pRKRP-1 lacking the restriction site, <em>HindIII</em></td>
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<tr>
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<td>pRK404 containing 5.3-kb <em>HindIII</em> insert from pUC1813-116</td>
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<td>pRPS-53</td>
<td>pRKRP-1 containing 2.8-kb <em>BamHI</em> insert encoding <em>rbpL</em> from pRQ53</td>
<td>This study</td>
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<td>pRPS-75-I</td>
<td>pRKRP-1 containing 2.3-kb <em>BamHI</em> insert encoding <em>rbcL</em> <em>rbcS</em> genes from pUC13-75 in orientation I</td>
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<td>pRKRP1-M with 62-bp multiple cloning site inserted down from <em>R. rubrum</em> RubisCO mRNA initiation site</td>
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<td>pRPS4-29-I</td>
<td>pRPS-1 containing 4.0-kb <em>HindIII</em> insert encoding <em>rbcL</em> <em>rbcS</em> genes from pUC18-29 in orientation I</td>
<td>This study</td>
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<tr>
<td>pUC18::9.4lac</td>
<td>pUC1813 containing 9.4-kb insert encoding <em>lacZY</em> genes as a <em>HindIII</em> or <em>PstI</em> cartridge</td>
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<td>pRPSL-26</td>
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<td>pRPSL-13</td>
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<td>This study</td>
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concentrations used for selection of resistance markers in *R. sphaeroides* were 50 μg/ml for streptomycin (Sm), 25 μg/ml for kanamycin (Km), and 200 μg/ml for trimethoprim (Tp). Tetracycline (Tc) was used at 5 μg/ml for routine plasmid maintenance and initial selection of exconjugants, and 0.5 μg/ml was used for screening of tetracycline-sensitive strains. *E. coli* was grown in Luria-Bertani (LB) medium (54) at 37°C. Antibiotic concentrations were the same as with *R. sphaeroides*, except for tetracycline, which was used at 12.5 μg/ml. Other antibiotics for *E. coli* were ampicillin (Amp) at 50 μg/ml and chloramphenicol at 34 μg/ml.

**DNA manipulations and conjugation techniques.** Plasmid and chromosomal DNA isolation, digestion of DNA with restriction endonucleases, ligation of DNA with T4 ligase, transformation of *E. coli*, agarose gel electrophoresis of DNA, and Southern hybridization analysis were performed as previously described (15), except that the alkaline sodium dodecyl sulfate (SDS) (4) procedure was used for plasmid isolation. Transformation of *E. coli* with plasmid DNA was also performed using the one-step transformation-bacterial storage procedure (TSS) of Chung *et al.* (9). Conjugations were performed on filter pads for diparental matings, using *E. coli* strain SM-10 (78) and pSUP202 derivatives for the construction of the RubisCO-deletion strain. Triparental matings employed the helper plasmid pRK2013 (18) for the introduction of plasmid constructs into *R. sphaeroides* as previously described (93).

*R. sphaeroides rbcL, rbcS, rbpL* deletion construction. The plasmid derivative pSUP::E25d::Km was constructed to mediate double-recombination at the *rbcL*, *rbcS*, and *rbpL* loci in the bacterial chromosome (Fig. 1.1A). To construct pSUP::E25d::Km, a 749bp *SalI* fragment, which contains sequences coding for the last 130 codons of the large RubisCO subunit and
the first 115 codons of the small RubisCO subunit was removed from plasmid pUC1318::E25, which contains a 1.8-kb EcoRI fragment of \textit{R. sphaeroides} DNA (Fig. 1.1). A 1.44-kb \textit{SalI} fragment encoding resistance to Km, derived from pUC13-K, was inserted into the single remaining \textit{SalI} site in pUC1813::E25, and the resulting deletion-insertion fragment was subcloned into the narrow host-range, mobilizable vector pSUP202. Selection for the \textit{R. sphaeroides rbcL} \textit{rbcS} deletion strain, designated FIdA, was made by Km\textsuperscript{r} after mating pSUP::E25d::Km via \textit{E. coli} SM-10 by procedures previously described (15).

The construction of an \textit{rbpL} deletion (encoding form II RubisCO) was performed in an analogous manner (Fig. 1.1B). A 3.0-kb EcoRI \textit{R. sphaeroides} DNA insert, containing the \textit{rbpL} gene, was subcloned into the cosmid-cloning vector pVK101 from plasmid pRQ2 (70) giving pQW32. A 906-bp \textit{PstI} fragment, located within the coding sequence of \textit{rbpL} (91), was then removed from plasmid pQW32. The single remaining \textit{PstI} site in the pQW32-deleted intermediate was then used to insert a 2.6-kb \textit{PstI} fragment encoding trimethoprim resistance, derived from pUC18-89. This entire \textit{rbpL} deletion-insertion construct was subcloned as an EcoRI fragment to the single EcoRI restriction site in pSUP202. The resulting plasmid from these manipulations, pSUP::FII::DI, was introduced into both \textit{R. sphaeroides} HR wild type and the FIdA strain via conjugation with \textit{E. coli} SM-10 to acquire strains which had undergone gene replacement with the deleted \textit{rbpL}. Exconjugants were obtained by selection for Tp\textsuperscript{r} and screening for Te\textsuperscript{s}.

\textbf{\textit{R. sphaeroides} expression vector construction.} Plasmid pRR116 (80, from C. R. Somerville) contains 6.6-kb of \textit{R. rubrum} DNA including the RubisCO gene. A 2.0-kb \textit{SalI} fragment from pRR116, which extends from
Figure 1.1. Maps of CO₂ fixation gene regions from *Rhodobacter sphaeroides*. (A), the form I cluster present on plasmid pJG336, and (B), the form II CO₂ cluster present on plasmid pJG106. The portions removed within the RubisCO structural genes and the deletion-insertion derivative plasmids used for the construction of the deletion mutants are indicated below each cluster. Plasmid diagrams below the maps are drawn to scale to indicate the approximate amount of homologous DNA on either end of the plasmid resistance cartridge insert (cross-hatched markings).
Fig. 1.1
approximately 1.90-kb upstream of the Rubisco gene to 196-bp into the Rubisco gene sequence (63), was subcloned into the SalI endonuclease site of pUC8 in both orientations. The resulting plasmids, pUC8::116-I and pUC8:116-II, were used to obtain smaller DNA fragments of *R. rubrum* DNA containing the mapped transcription initiation site for the Rubisco gene (51). A 1.45-kb *HindIII-BamHI* DNA fragment from pUC8::116-I, which extends approximately 1.0-kb upstream of the mRNA start site, was then ligated to the *HindIII-BamHI* sites in the broad-host-range vector, pRK404 (12). This derivative, pRKRPl, was then used as the promoter-vector plasmid for expressing the form II Rubisco gene (*rbpL*) from *R. sphaeroides* and the *rbcL rbcS* genes from the cyanobacterium *Anacystis nidulans* (*Synechococcus* sp. PCC6301). These constructs were made by insertion of a 2.8-kb *BamHI* fragment from plasmid pRQ53, containing the *R. sphaeroides* *rbpL* gene, giving plasmid pRPS-53, and insertion of a 2.3-kb *BamHI* fragment containing the *A. nidulans* Rubisco genes from plasmid pUC13-75, resulting in plasmid pRPS-75 (Fig. 1.2). The basic pRKRPl promoter construct was modified to allow the addition of a multiple cloning site sequence for the insertion of a wider range of DNA fragments downstream from the *R. rubrum* promoter. First, the *HindIII* site at the junction between vector DNA and *R. rubrum* DNA, opposite the end of the transcription start site, was removed by *HindIII* digestion, filled in with the Klenow fragment of DNA polymerase I, and religated with T4 DNA ligase. Then, into the single *BamHI* site of this intermediate, pRKRPl-M, a 62-bp *BamHI* polylinker of pUC1318 was inserted, giving the general expression vector construct, pRPS1. A 4.0-kb *HindIII* DNA fragment containing the *R. sphaeroides* form I Rubisco genes from pUC18-29 was then inserted into the *HindIII* site within the added
Figure 1.2. *R. sphaeroides* expression vector constructs. Cross-hatched marks indicate *R. rubrum* DNA. The cross-hatched arrow represents 1.45-kb of *R. rubrum* DNA extending 196-bp into the *R. rubrum rbpL* gene. Insertions into the promoter-vector construct, with DNA fragments encoding various RubisCO sequences, are made at the multiple cloning site junction, at the tip of the arrow. LS, approximate region coding for RubisCO large subunit; SS, approximate region coding for RubisCO small subunit. All constructs are in pRK404.
Type II RubisCO

R. rubrum RubisCO pRPS-116

R. sphaeroides RubisCO pRPS-53

Type I RubisCO

A. nidulans RubisCO pRPS-75

R. sphaeroides RubisCO pRPS4-29

Fig. 1.2
multiple cloning site in both orientations with respect to the *R. rubrum* promoter in pRPS1, resulting in pRPS4-29-I and pRPS4-29-II.

**Preparation of cell extracts.** Cells grown photosynthetically were withdrawn as 20- or 30-ml samples using a syringe-tubing withdrawal system so as not to disturb the anaerobicity of the growing culture. The cell samples were immediately washed in 0.1 M Tris HCl, pH 8.0, 1 mM EDTA, twice at 4.0°C; the cell pellets were stored at -70°C until needed. Frozen cells were thawed and resuspended in 1 ml of 50 mM Tris-HCl, pH 7.3, 5 mM EDTA, 5 mM DTT. Lysozyme was added to a final concentration 0.25 mg/ml, and after 15 min incubation at 37°C, the cells were sonicated at 4°C to complete cell lysis. Centrifugation in an Eppendorf microcentrifuge was performed at 4°C to pellet cell debris. Chromatophores were removed from the supernatant by treatment at 50°C for 10 min in the presence of 50 mM Mg²⁺, followed by an additional centrifugation for 10 min at 4°C (41). The resulting supernatant was used for all subsequent assays. RuBP carboxylase activity was measured by the assay previously described (95). Protein concentration was determined by a modification of the Lowry protein assay (55), using bovine serum albumin as the standard.

**Rocket immunoelectrophoresis.** The concentration of all forms of RubisCO in cell extracts was quantified by rocket immunoelectrophoresis, using antibodies specific for each of the four enzymes employed (41). Each of these antisera are specific for the respective antigens.
RESULTS

Construction of an *R. sphaeroides* RubisCO-deletion strain. *R. sphaeroides* strains lacking one of either form of RubisCO had previously been shown to be capable of both photoheterotrophic and photolithoautotrophic growth (15). Thus, strains obtained from the mating with pSUP::E25d::Km, possessing the proper antibiotic-resistance phenotype (Km\(^r\), Tc\(^s\)), were cultured photoheterotrophically in 22-ml tubes and cell extracts were examined for the presence of form I RubisCO by rocket immunoelectrophoresis. Three out of three Km\(^r\), Tc\(^s\) strains tested lacked any detectable form I RubisCO antigen (data not shown). Strains with the form I-negative phenotype were subsequently subjected to Southern hybridization analysis to confirm genetic replacement with the *rbcLrbcS* deletion construct. Figure 1.3, panel A, together with the restriction maps in Figure 1.1, verify the genetic replacement of the *rbcLrbcS* deletion construct. Most importantly, a hybridizing fragment at 1.8-kb is found in lane 1, Fig. 1.3A, corresponding to the intact fragment in the chromosome; this is replaced by a hybridizing fragment of 2.5-kb in the deletion strain (Fig. 1.3A), accounting for the removal of 750-bp of DNA and the insertion of the 1.44-kb fragment encoding Km\(^r\). The lack of any detectable sequences in the size range of the pSUP202 vector (~8kb) also supports a double-recombination event, as sequences homologous to the pSUP202 vector would have been indicative of a single cross-over with the insertion of the entire plasmid into the chromosome.

Southern blot analysis of the *rbpL* region is also shown (Fig. 1.3B). These results confirm the genetic replacement of the deleted-inserted fragment from pSUP::FII::DI for the intact *rbpL* allele in the chromosome.
Figure 1.3. Southern hybridization analysis of wild-type *R. sphaeroides* HR and *rbcL*/*rbcS*/*rblpL* deletion strain 16. (A). Autoradiograph of EcoRI-digested *R. sphaeroides* total DNA probed with pSUP::E25::Km. (B). Autoradiograph of EcoRI-digested *R. sphaeroides* total DNA probed with plasmid pSUP::FII::DI. Lane 1, *R. sphaeroides* HR DNA. Lane 2, *R. sphaeroides* strain 16 DNA.
The presence of the internal EcoRI site in the fragment encoding resistance to trimethoprim results in the generation of two hybridizing fragments, one at 3.8-kb and one at 1.1-kb (Fig. 1.1), thus providing further confirmation that a double-recombination had occurred in the chromosome. *R. sphaeroides* strain 16 therefore was genetically verified to contain chromosomal deletion-insertions at the regions encoding for the *rbcL rbcS* genes and the *rbpL* gene and was incapable of synthesizing form I or form II RubisCO as measured by immunological procedures (41).

**Analysis of the *R. sphaeroides* RubisCO-deletion strain 16.** Growth was possible for the dual RubisCO mutant strain under aerobic (chemoheterotrophic) conditions. Photoheterotrophic growth was possible only when the culture was supplemented with an alternate electron acceptor, such as DMSO to bypass CO₂ fixation as a means to maintain redox balance (57, 72) (Fig. 1.4). Strain 16 grew photoheterotrophically on malate in the presence of DMSO with a generation time of approximately 20 h compared to 8 h for wild-type strain HR. In the absence of DMSO as electron acceptor, strain 16 was not able to grow photoheterotrophically in malate, butyrate-bicarbonate, or complex (PYE) medium, nor did growth occur photolithoautotrophically (data not shown). Results were identical using standing- or argon-bubbled broth cultures or with agar-containing media.

**Complementation of *R. sphaeroides* RubisCO-deletion strain 16 with CO₂ fixation gene clusters from *R. sphaeroides*.** The two distinct RubisCO genes in this organism are encoded within two separate clusters of CO₂ fixation genes contained within the pVK102 cosmid derivatives pJG336 and pJG106 (24, 25, 27, 86) (Fig. 1.1). *R. sphaeroides* 16 derivatives, containing plasmid pJG336 or pJG106, were obtained by conjugation of *E. coli* HB101
(pJG336) or HB101 (pJG106) with strain 16 by selecting for all antibiotic resistance markers (Sm', Km', Tp' and Tc') under aerobic conditions. Several Tc' exconjugants were then assessed for photoheterotrophic and photolithoautotrophic growth on plates in anaerobic chambers. *R. sphaeroides* 16, containing plasmids pJG336 or pJG106, grew well both photoheterotrophically and photolithoautotrophically. Single colonies on PYE-Tc plates from each mating were inoculated to liquid malate media in 22-ml screw-cap tubes. Cells from these cultures were judged to be photosynthetically competent and were used to start 400 ml cultures bubbled with the appropriate gas mixtures. These particular growth conditions were used throughout the analysis of *R. sphaeroides* complemented strains, as photosynthetic growth of the wild type as well as RubisCO protein levels and activity profiles have been well characterized using this system (15, 41). Rocket immunoelectrophoresis of cell extracts derived from cells grown in this experiment indicated that only the enzyme encoded by the plasmid in question cross-reacted with antisera specific to that source of RubisCO (data not shown). Analysis of RubisCO activity and specific RubisCO synthesis in the complemented strains containing plasmids pJG336 and pJG106 grown under both photoheterotrophic and photolithoautotrophic conditions indicated that expression was regulated (Table 1.2). Specifically, both RubisCO specific activity and RubisCO protein levels increased in photolithoautotrophically-grown cells, similar to the pattern obtained with wild-type strain HR. Greater differences between the complemented strains were found in cells grown under photolithoautotrophic conditions, where the amount of RubisCO produced was 19% of the total soluble protein for form I, via plasmid pJG336, and 13.6% of the total soluble protein for form II, via plasmid pJG106. These
Figure 1.4. Growth curves of *R. sphaeroides* HR (solid line) and *R. sphaeroides* strain 16 (broken line) under photoheterotrophic conditions in the presence (•) and absence (○) of DMSO. Growth took place in screw-cap test tubes containing 0.4% malate.
Fig. 1.4
Table 1.2. Photoheterotrophic and photolithoautotrophic growth and RubisCO levels of *R. sphaeroides* HR and strain 16 complemented with plasmids pJG336 and pJG106.

<table>
<thead>
<tr>
<th><em>Rhodobacter sphaeroides</em> Strain</th>
<th>Gen. Time (h)</th>
<th>RubisCO Actyc (U/mg)</th>
<th>Protein (% Total)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Het&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Aut&lt;sup&gt;b&lt;/sup&gt;</td>
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</tr>
<tr>
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<td>32</td>
<td>0.03</td>
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<td>18</td>
<td>24</td>
<td>0.13</td>
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<sup>a</sup>Photoheterotrophic growth using malate as the electron donor.
<sup>b</sup>Photolithoautotrophic growth in an atmosphere of 1.5% CO<sub>2</sub> in H<sub>2</sub>.
<sup>c</sup>Expressed as units/mg protein.
<sup>d</sup>Expressed in terms of the RubisCO protein percentage of the total soluble protein as determined by rocket immunoelectrophoresis (41). I: form I RubisCO; II, form II RubisCO.
<sup>e</sup>Plasmid pJG336 contains 24 kb of *R. sphaeroides* DNA encoding the form I CO<sub>2</sub> fixation genes (24).
<sup>f</sup>pJG106 contains 26 kb of *R. sphaeroides* DNA encoding the form II CO<sub>2</sub> fixation genes (24).
levels, while amplified over the amounts found in the wild type, can most simply be attributed to a gene dosage effect since the complementing RubisCO-encoding genes are located on low copy plasmids and the increases in protein level average 2 to 3 fold over the amounts found in the wild-type strain, corresponding to a plasmid copy number of 2 to 3. Longer generation times under photoheterotrophic conditions for both *R. sphaeroides* 16(pJG336) and *R. sphaeroides* 16(pJG106) may also be a plasmid effect, since maintenance of the 45 to 47-kb plasmids may place additional burdens on the cell. The increased generation time under photolithoautotrophic conditions for *R. sphaeroides* 16(pJG336) may be a combination of this plasmid effect, and to cells bearing a higher concentration of form I RubisCO than that seen in the wild type, thereby taxing the growth of this strain even further. To summarize these results, complementation of the RubisCO-deletion strain 16 is possible by the introduction of plasmids pJG336 and pJG106, containing either of the two CO₂-fixation gene clusters, and expression from these gene clusters is regulated such that there is derepression of RubisCO synthesis in cells grown photolithoautotrophically.

**Construction of an *R. sphaeroides* expression vector.** Successful complementation of the *R. sphaeroides* RubisCO-deletion strain 16 with native CO₂ fixation gene clusters, using plasmids pJG336 and pJG106, demonstrated that strain 16 could be complemented by introduced DNA encoding the respective RubisCO genes. To study the physiological consequences of *R. sphaeroides* growing photosynthetically with different RubisCOs, an expression system was sought that could direct the synthesis of RubisCO in amounts that would allow complementation to photolithoautotrophic growth. A screen of various promoter expression
systems, including *R. sphaeroides* DNA fragments encoding Rubisco genes from plasmids pJG29 and pRQ2, allowed no real (promoter-driven) complementation. The slight growth that was seen in strains containing these plasmids or other plasmids containing *R. sphaeroides* DNA was later shown to be due to double-recombination back into the chromosome. This conclusion was based mainly on the presence of isolated colonies on plates incubated photosynthetically which had been streaked with single colonies (exconjugants) directly from the initial mating selection (Tc) plates. Constructs containing Rubisco gene fragments inserted downstream from the *Rhizobium meliloti* δ-ALAS promoter (50), and the *nifHDK* promoter from *Rhodobacter capsulatus* (68) also did not effectively complement (data not shown). Only a few isolated colonies appeared on plates streaked with a heavy inoculum in attempts to complement the *rbcLrbcS, rbpL* deletion strain to photosynthetic growth with plasmids containing the form I or form II Rubisco genes and DNA immediately upstream from these genes. In contrast, no growth whatsoever occurred with these plasmid promoter constructs when Rubisco genes from an organism other than *R. sphaeroides* were used, i.e., the Rubisco genes from *A. nidulans*. However, when the *R. sphaeroides rbcLrbcS, rbpL* mutant, harboring plasmid pRPS-116 was incubated on plates under photosynthetic conditions, confluent growth (no isolated colonies) resulted. Plasmid pRPS-116 contains the *R. rubrum rbpL* gene within a 5.3-kb insert of *R. rubrum* DNA, which includes DNA that extends approximately 1.40-kb upstream of the *rbpL* gene (63, 80). When this *R. rubrum* DNA fragment was manipulated, as described in Materials and Methods, and used to drive expression of other Rubisco genes, including those from *R. sphaeroides*, it was clear that expression was directed by the promoter on this fragment. Most convincing was the finding
that expression was orientation-dependent from the *R. rubrum* DNA insert. In the case of the *rbcLrbcS* genes from *A. nidulans*, *R. sphaeroides* strain 16, containing plasmid pRPS-75-I, showed confluent growth under photosynthetic conditions, while absolutely no growth occurred with pRPS-75-II, which contains the *A. nidulans* genes in the opposite orientation from the *R. rubrum* RubisCO promoter. The finding that expression of the various genes encoding RubisCO proteins is dependent on promoter activity from the *R. rubrum* DNA insert is supported by previous experiments involving pRK404 containing only the *A. nidulans* *rbcLrbcS* genes. In these cases, no complementation was detected when pRK404 containing the *A. nidulans* *rbcLrbcS* genes was introduced into *R. sphaeroides* 16. Negative complementation was obtained using *A. nidulans* genes inserted in either orientation in pRK404. Thus, the possibility of transcription initiating from either the plasmid-based *lacZ* gene, the gene encoding tetracycline resistance, or from some other cryptic promoter present on the parental vector molecule can be excluded. Expression of *R. sphaeroides* form I and form II RubisCO via the *R. rubrum* promoter was also dependent on orientation. Confluent growth resulted when *R. sphaeroides* genes encoding form I and form II RubisCO were in the proper orientation for transcription. A few isolated colonies were obtained when strain 16 cells harbored plasmids with RubisCO genes inserted in the opposite orientation with respect to the *R. rubrum* fragment, e.g. strain 16(pRPS4-29-II). These were found to be the result of recombination back into the chromosome. Taken together, these results suggest that no functional promoter resides on the inserted *R. sphaeroides* *rbcLrbcS* and *rbpL* fragments tested; expression is obligatorily directed by the *R. rubrum* promoter.
Analysis of *R. sphaeroides* 16 complemented with different RubisCO genes. Since the *R. rubrum* fragment encoding the presumptive promoter was shown to facilitate expression of various RubisCO genes and complement the *R. sphaeroides* RubisCO-deletion strain to photosynthetic growth, physiological analysis of these cells was undertaken. Cultures of *R. sphaeroides* 16 complemented with RPS-plasmids containing each of the four DNA inserts (encoding different RubisCO genes) were grown photoheterotrophically and photolithoautotrophically. All cultures, expressing each of the RubisCO enzymes under study, had doubling times of 12-13 h under photoheterotrophic growth conditions. This is about 5-6 h longer than wild-type strain HR (Table 1.3). Generation times of complemented strains under photolithoautotrophic conditions, ranged from 8 to 18 h longer than the wild type. Strain 16 cells harboring plasmids pRPS4-29 and pRPS-53, encoding the indigenous *R. sphaeroides* rbcL*rbcS* and *rbpL* genes, respectively, had the fastest photolithoautotrophic generation times of the complemented strains. Extended but variable lag times of up to 72 h following inoculation of photolithoautotrophic cultures often resulted for complemented strains, especially when the initial $A_{660}$ was below 0.1 (data not shown). Inoculating photolithoautotrophic cultures at an $A_{660}$ higher than 0.1 generally eliminated this lag but did not consistently lead to an increased overall growth rate.

The levels of RubisCO activity and immunologically quantitated RubisCO protein in strain 16 complemented by each of the *R. rubrum* promoter-structural gene constructs was determined (Table 1.3). Photoheterotrophic growth on malate of *R. sphaeroides* 16(pRPS4-29), bearing the *R. sphaeroides* rbcL*rbcS* genes, and *R. sphaeroides* 16(pRPS-116), which possesses the *rbpL* gene from *R. rubrum*, indicated that the RubisCO
Table 1.3. Photoheterotrophic and photolithoautotrophic growth and RubisCO levels of *R. sphaeroides* HR and strain 16 cells complemented with *R. rubrum* promoter-constructs containing different RubisCO genes.

<table>
<thead>
<tr>
<th>Rhodobacter sphaeroides Strain</th>
<th>Gen. Time (h)</th>
<th>RubisCO Levels</th>
<th>Proteind (% Total)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Het</td>
<td>Aut</td>
<td>Acty(^c)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HR</td>
<td>7</td>
<td>24</td>
<td>0.05</td>
</tr>
<tr>
<td>16(pRPS4-29)(^e)</td>
<td>12</td>
<td>33</td>
<td>0.05</td>
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<tr>
<td>16(pRPS-75)(^f)</td>
<td>13</td>
<td>42</td>
<td>0.12</td>
</tr>
<tr>
<td>16(pRPS-53)(^g)</td>
<td>12</td>
<td>32</td>
<td>0.12</td>
</tr>
<tr>
<td>16(pRPS-116)(^h)</td>
<td>13</td>
<td>39</td>
<td>0.07</td>
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</table>

\(^a\) Photoheterotrophic growth using malate as the electron donor.
\(^b\) Photolithoautotrophic growth in an atmosphere of 1.5% CO\(_2\) in H\(_2\).
\(^c\) Expressed as units/mg protein.
\(^d\) Expressed in terms of the RubisCO protein percentage of the total soluble protein as determined by rocket immunoelectrophoresis (41); I, form I RubisCO; II, form II RubisCO.

\(^e\) *R. rubrum* promoter - *R. sphaeroides* rbcLrbcS genes (form I).

\(^f\) *R. rubrum* promoter - *A. nidulans* rbcLrbcS genes.

\(^g\) *R. rubrum* promoter - *R. sphaeroides* rbpL gene (form II).

\(^h\) *R. rubrum* promoter - *R. rubrum* rbpL gene.
activities were similar to that found in wild-type strain HR. Strains 16(pRPS-75) and 16(pRPS-53), containing the \textit{A. nidulans} rbcL rbcS and the \textit{R. sphaeroides} rbpL genes, respectively, showed about two-fold higher RubisCO activity than the wild type. Compared to the wild-type strain, the amount of RubisCO protein was also higher in strains complemented by the various \textit{R. rubrum} promoter-RubisCO gene constructs under photoheterotrophic conditions. The higher RubisCO protein level found in the pRPS-complemented strains is similar to the complementation results obtained with the plasmids pJG336 and pJG106, which contain endogenous \textit{R. sphaeroides} promoter sequences (Table 1.2). Since pRK404-based plasmids have a copy number in \textit{R. sphaeroides} estimated at six per chromosome (11), a likely cause of the high levels of RubisCO may reflect high gene dosage using the pRPS expression vectors. The most dramatic effect of using the \textit{R. rubrum} RubisCO promoter to express the various RubisCO genes in \textit{R. sphaeroides} strain 16 was observed in cells grown photolithoautotrophically on 1.5\% CO$_2$ in H$_2$. Here, RubisCO protein levels and RubisCO activity increased well over the amounts found under photoheterotrophic conditions. In particular, strain 16 complemented with pRPS4-29, pRPS-53 and pRPS-116, containing the purple non-sulfur bacterial genes encoding \textit{R. sphaeroides} form I RubisCO, \textit{R. sphaeroides} form II RubisCO, and \textit{R. rubrum} RubisCO, respectively, produced a minimum of 34\% of the total soluble protein of the cell, with the \textit{R. rubrum} enzyme accumulating up to 45\% of the total soluble cell protein (Table 1.3). Somewhat less RubisCO protein was obtained in \textit{R. sphaeroides} 16(pRPS-75) cells grown photolithoautotrophically (encoding the \textit{A. nidulans} rbcLrbcS genes), about 15\% of the total cell protein. This was the lowest quantity of RubisCO produced in strains complemented with the \textit{R. rubrum}
promoter-constructs under this growth condition, though this level is still greater than the amount for *R. sphaeroides* form I and form II RubisCO combined in the wild type. RubisCO activity from photolithoautotrophically-grown cells showed the widest range among the strain 16 cultures complemented with different RubisCO genes. The highest RubisCO specific activity, calculated on the basis of the amount of specific RubisCO antigen synthesized, was obtained from extracts of cells containing the promoter-type II gene plasmid constructs. Strain 16 cells complemented by plasmid promoter-constructs containing the genes encoding form I enzymes from *R. sphaeroides* and *A. nidulans* exhibited RubisCO specific activities which were within the range of that found typically for purified enzymes obtained from the wild-type strain.

Analysis of photoheterotrophic growth of *R. sphaeroides* RubisCO deletion strain complemented by cyanobacterial RubisCO genes. The results discussed above clearly show that *R. sphaeroides* RubisCO deletion strain 16 is capable of both photoheterotrophic and photolithoautotrophic growth when foreign RubisCO molecules were expressed. As the data in Table 1.3 indicate, all RubisCO proteins were synthesized to appreciable levels with good enzymatic activity. An original rationale of these studies was to determine whether physiological problems arose due to the expression of foreign RubisCO genes. Results using the four sources of RubisCO to complement the *R. sphaeroides* deletion strain indicated no major physiological growth differences, with one possible exception, other than the previously mentioned extended lag which occurred under photolithoautotrophic growth conditions. Of particular interest was the response of photoheterotrophically-grown *R. sphaeroides* strain 16 cells expressing the *A. nidulans* *rbcLrbcS* genes. This strain, 16(pRPS-75),
showed a pronounced lag time when grown photoheterotrophically on 0.4% malate bubbled with argon (Fig. 1.5). Sealing the culture flask and stopping the argon bubbling eliminated the growth lag, with or without the addition of exogenous sodium bicarbonate. Thus, changing from a bubbled and agitated culture to a standing culture had an almost immediate effect in eliminating the prolonged lag in growth. Alternatively, exponential photoheterotrophic growth of argon bubbled cultures of \textit{R. sphaeroides} 16(pRPS-75) on malate ensued once the culture density reached an A$_{660}$ of about 0.5, which could take up to one week. Since these results occurred only with \textit{R. sphaeroides} 16 synthesizing the cyanobacterial enzyme, the effect was most likely a property of the \textit{A. nidulans} enzyme itself. No lag in growth was observed with strain 16 (pRPS-75) under photolithoautotrophic growth conditions, where CO$_2$ is continuously bubbled through the culture.

**Further characterization of \textit{R. rubrum} promoter expression.** In all instances where photosynthetic growth depended on the \textit{R. rubrum} promoter-RubisCO structural gene constructs, the amount of RubisCO protein and activity increased steadily as the cell culture density increased (data not shown). The RubisCO protein and activity values presented in Table 1.3 are data calculated from cell culture samples at stationary phase, and do not indicate this gradual increase; yet, this phenomenon is similar to the accumulation of RubisCO seen in the wild-type strain of both \textit{R. sphaeroides} and \textit{R. rubrum} (41, 73, 86). Thus, the \textit{R. rubrum} RubisCO gene promoter seems to function in \textit{R. sphaeroides} in the same manner as it does in \textit{R. rubrum}.

Additional analysis of expression from the \textit{R. rubrum} promoter was made with \textit{R. sphaeroides} cells grown aerobically. Since RubisCO is not
Figure 1.5. Photoheterotrophic growth on 0.4% malate of *R. sphaeroides* strain 16(pRPS-75), containing the *A. nidulans rbcLrbcS* genes. (O), standing culture containing 30 mM sodium bicarbonate with no argon bubbling; (•), culture maintained with argon bubbling and no added sodium bicarbonate.
Fig. 1.5
required for aerobic growth, both *R. sphaeroides* and *R. rubrum* wild-type cells show virtually no RubisCO expression when grown under highly aerated conditions. Thus, it was of interest to determine if the *R. rubrum* RubisCO promoter region was regulated similarly in the *R. sphaeroides* background. *R. sphaeroides* strain 16 (pRPS-53) (the *R. sphaeroides* *rbcL* gene construct) showed some activity (17.8 mU/mg) when grown aerobically on complex media in the dark, but no detectable activity was obtained in *R. sphaeroides* strain 16 (pRPS-75) (the *A. nidulans* *rbcL rbcS* gene construct). These results suggest that the *R. rubrum* RubisCO promoter is repressed under these conditions. Since these results were highly reproducible, the low levels of activity seen for pRPS-53 was most likely a property of this DNA insert, and not a result of readthrough occurring from the *R. rubrum* promoter under aerobic conditions. Overall, expression in *R. sphaeroides* of several different *rbcLrbcS, rbpL* genes, directed by the *R. rubrum* RubisCO promoter construct, displayed regulation that closely paralleled that seen in the native background. The regulation pattern seen with the *R. rubrum* promoter construct may be generalized: little or no expression under fully aerobic conditions, intermediate levels of expression under photoheterotrophic conditions and complete derepression (highest expression) under photolithoautotrophic conditions in an atmosphere of 1.5% CO₂ in H₂.

*R. rubrum rbpL* promoter activity was further tested by inserting a DNA fragment encoding the *lacZY* genes into the pRPS vector. Plasmid RPS-*lacZ* transcriptional fusions were then mobilized into *R. sphaeroides* HR, and the expression of β-galactosidase in the resulting *R. sphaeroides* strains determined. β-galactosidase activity was expressed in photoheterotrophically grown (malate) cells harboring plasmid pRPSL-26,
where the *lacZY* genes were in the correct orientation for transcription with respect to the *R. rubrum* *rbpL* promoter fragment (Table 1.4). The addition of DMSO to the growth media resulted in a reduction of β-galactosidase activity in the same *R. sphaeroides* cells grown photoheterotrophically (Table 1.4). Thus, the *R. rubrum* *rbpL* promoter in *R. sphaeroides* cells responds to the presence of an alternate electron acceptor by reducing the level of promoter activity. A qualitative assessment of *rbpL* promoter activity was made by plating *R. sphaeroides* cells containing the RPS-*lacZ* constructs to PYE plates containing X-gal. When incubated aerobically, colonies had normal pigmentation at the

Table 1.4. β-galactosidase expression via the *R. rubrum* *rbpL* promoter in *R. sphaeroides* HR wild-type cells grown photoheterotrophically on 0.4% malate.

<table>
<thead>
<tr>
<th><em>R. sphaeroides</em> Strain</th>
<th><em>lacZ</em> gene insert ORI&lt;sup&gt;a&lt;/sup&gt;</th>
<th>β-galactosidase (U/mg)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>-DMSO</th>
<th>+DMSO</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (wild type)</td>
<td>-</td>
<td></td>
<td>426</td>
<td>-</td>
</tr>
<tr>
<td>HR (pRPSL-26)</td>
<td>I</td>
<td></td>
<td>2628</td>
<td>1186</td>
</tr>
<tr>
<td>HR (pRPSL-13)</td>
<td>II</td>
<td></td>
<td>313</td>
<td>421</td>
</tr>
</tbody>
</table>

<sup>a</sup>ORI refers to orientation of the *lacZ* gene inserted into the plasmid promoter vector (pRPS-1). I: proper orientation with respect to the *rbpL* promoter; II: opposite orientation.

<sup>b</sup>β-galactosidase activity determined as described by Miller (61).
periphery, but turned violet-blue toward the center of the colony. This is most likely a response to the reduced oxygen tension at the center of the colony; an effect similar to the normal pigmentation pattern seen with colonies incubated aerobically on plates. Transferring the above PYE-X-gal agar plates with partially grown colonies to anaerobic conditions resulted in colonies that were deep violet-blue throughout.

**DISCUSSION**

This work describes the construction of a RubisCO-negative strain of *Rhodobacter sphaeroides* which lacks both form I and form II RubisCO. The construction was made by replacing wild-type alleles of each of the genes encoding the two RubisCO forms with deletion-insertion derivatives contained in suicide vectors. Genetic exchange was confirmed by Southern blot analysis. The *rbcLrbcS, rbpL*-deletion strain (strain 16) was unable to grow under photolithoautotrophic or photoheterotrophic conditions with CO$_2$ as the electron acceptor. However, if dimethyl sulfoxide was added as an alternative external electron acceptor, photoheterotrophic growth was possible for the RubisCO-deleted strain. This result was not surprising since the presence of DMSO can eliminate the otherwise absolute requirement for sodium bicarbonate for photoheterotrophic growth on butyrate (72). The finding of an alternate electron acceptor effectively substituting for CO$_2$ fixation is also consistent with recent studies involving insertions into the *cfx* genes of *R. sphaeroides* (35). The *cfx* genes, now known to encode fructose 1,6-bisphosphate aldolase, *fba*, are located just upstream of each of the two genes encoding RubisCO (24, 28, 34) (Fig. 1.1). Insertions into *cfxA* or *cfxB* essentially prevents expression of each of the
downstream RubisCO genes, thus endowing the strain with a RubisCO-negative phenotype (26, 28, 35, Chapter III).

This work extends earlier studies in which it was found that inactivation of either the \textit{rbcLrbcS} or \textit{rbpL} genes did not prevent photosynthetic growth of the resultant form I or form II negative strain. These studies (15) and more recent investigations (35) indicated that form I or form II negative strains may compensate for the lack of the other RubisCO enzyme. Here, we desired especially stable null mutations in genes encoding both of the two forms of RubisCO, so that attempts at complementation or selection of altered forms of RubisCO or detection of other means of CO$_2$ fixation would not be obscured or prevented. Previous attempts to construct the dual RubisCO mutant in \textit{R. sphaeroides} used a Tn5 insertion to inactivate one gene encoding RubisCO, and the insertion of a resistance cartridge to disrupt the other RubisCO-encoding gene. However, it was found that after prolonged incubation under photoheterotrophic conditions, the Tn5-inactivated RubisCO genes reverted. Presumably, precise excision of Tn5 resulted, leading to photosynthetic growth (data not shown). Corroborating these results is the high frequency of precise excision of Tn5 recently found in \textit{Pseudomonas aeruginosa} (31). Thus, the use of deletion derivatives of \textit{rbcLrbcS/rbpL} in this study permitted the construction of a strain which exhibited complete stability, in that no evidence of reversion has been observed even after prolonged photolithoautotrophic incubation.

Introduction of the \textit{R. sphaeroides} endogenous CO$_2$-fixation operons found in cosmid derivatives pJG106 and pJG336, into the RubisCO-deletion strain 16, resulted in complementation to photosynthetic growth. Regulation of RubisCO expression was normal when directed by
these plasmids since RubisCO synthesis increased in photolithoautotrophically-grown cells. The levels of RubisCO protein were uniformly greater in these complemented stains compared to the wild type, probably due to a gene dosage effect of using multiple-copy plasmids for complementation. Complementation of *R. sphaeroides* strain 16 was also possible with several foreign genes encoding RubisCO when the region upstream of the *R. rubrum rbpL* gene was used as the promoter. Again, expression was regulated similarly to the parent strain. Amplified levels of RubisCO expressed from the *R. rubrum* promoter construct were similar to the high amounts obtained with the cosmid derivatives pJG336 and pJG106. Here, however, both a plasmid copy effect and a more active promoter may account for the greatly increased levels. The latter possibility is reasonable since *R. rubrum* may produce levels of RubisCO that approach 50% of the total soluble protein (73).

Use of this expression vector construct, combined with the results of attempts at complementation using plasmids containing various lengths of sequence upstream from the form I or form II RubisCO encoding genes, indicated that the *R. sphaeroides rbcL/rbcS/rbpL* gene promoters were not immediately proximal to the RubisCO initiation codon. Indeed, positive complementation with the entire CO₂-fixation gene regions of plasmids pJG336 and pJG106, suggests that the RubisCO gene promoters are some distance upstream. This is in agreement with recent studies involving cartridge insertions into regions upstream of both RubisCO genes (26, 27, 28). Certainly, as shown in this study, double recombination back into the chromosome must be rigorously excluded as a potential trivial explanation for positive complementation. These results also suggest a fundamental difference in the promoter-RubisCO gene arrangement between *R.*
*sphaeroides* and *R. rubrum* in which the promoter for the *rbpL* gene is located just upstream of the gene, as shown here and by transcription mapping studies (51).

The ability of *R. sphaeroides* to grow photosynthetically using different sources of RubisCO is demonstrated. Under both photoheterotrophic and photolithoautotrophic conditions, ample quantities of all RubisCO's were produced using the *R. rubrum* promoter. The pattern of RubisCO specific activity in photosynthetically-grown strains, however, is curious since in those cells that produce form I RubisCO, including pJG336, the level of activity fails to attain that found in form II RubisCO-containing strains. Again, this is most evident in the pJG336 and pJG106 complementations, but also where the *R. rubrum* promoter is used for complementation. The specific activity of form I-type enzymes calculated from the levels of antigen found in crude extracts is similar to the specific activity of the enzyme found in extracts of the wild-type strain. However, the calculated specific activity found in extracts of form II RubisCO-complemented strains is consistently higher than the specific activity of the enzyme synthesized in the wild-type strain. One possibility for this is an alteration of enzymatic activity. *R. sphaeroides* cells modulate the activity of form I but not form II RubisCO in response to changes in the nature of the carbon source or CO₂ availability (42). Thus, it is conceivable that growth of *R. sphaeroides* under conditions where there is nearly three-fold higher RubisCO activity than normal (corresponding to the approximate increase in RubisCO protein), might disturb the metabolic balance of the cell such that there is a post-translational reduction of enzyme activity.

Use of the *R. rubrum* promoter constructs facilitated determination
of the physiological consequences of photosynthetic growth of \textit{R. sphaeroides} cells synthesizing foreign Rubisco. The most notable finding was the behavior of \textit{R. sphaeroides} strain 16 cells complemented with and expressing \textit{A. nidulans rbcL rbcS} genes. Growth of this strain differed significantly from either the wild-type strain HR or strain 16 complemented with other Rubisco genes, in that a pronounced lag occurred under photoheterotrophic conditions. This lag was eliminated by adding a sodium bicarbonate supplement and/or stopping the bubbling of argon through the culture, which is routinely used to ensure anaerobicity. Argon bubbling can quickly disperse CO\textsubscript{2} generated endogenously by the metabolism of malate by the cells. Thus, the lag in growth may be a manifestation of the removal of CO\textsubscript{2} from the culture. Normally, wild type, and other complemented strains, are not sensitive to the CO\textsubscript{2} concentration when growing photoheterotrophically on malate and thus do not require added NaHCO\textsubscript{3} (although they do require a low level of CO\textsubscript{2} fixation for photoheterotrophic growth as a means to dissipate excess electrons). The CO\textsubscript{2} sensitivity then, may reflect the dissimilar cellular environment in which the cyanobacterial enzyme is expressed. Cyanobacteria contain active CO\textsubscript{2} concentrating mechanisms which function under low CO\textsubscript{2} conditions (43, 64, 77). In addition, unlike \textit{R. sphaeroides}, cyanobacteria compartmentalize Rubisco into discrete structures, called carboxysomes, where CO\textsubscript{2} accumulation has been hypothesized to be due to the action of carbonic anhydrase (71) thereby obviating the need for Rubisco with a high affinity for CO\textsubscript{2}. The situation here, involving the expression of Rubisco from an aerobic cyanobacterium in an anaerobic photosynthetic bacterium, seems to be the converse of recent work with \textit{Synechocystis} sp. PCC6803 in which the wild-type \textit{rbcLrbcS} genes on the chromosome of the
cyanobacterium were replaced with the *R. rubrum* rbpL gene (67). The so-called "cyanorubrum" in that study took on properties dictated by the *R. rubrum* enzyme such as oxygen sensitivity and a high CO$_2$ requirement. The requirement of a high CO$_2$ concentration was presumably because the cyanorubrum failed to form carboxysomes, needed to concentrate CO$_2$ (67).

While not the major focus in undertaking this study, the development, use, and initial functional characterization of the *R. rubrum* RubisCO promoter is noteworthy because of its obvious applicability in expressing genes in *R. sphaeroides* and perhaps other related bacteria, especially purple non-sulfur bacteria. The availability of such a promoter for these organisms could be useful, mainly because of its suitability for different requirements. Significant features include strong activity, allowing high levels of expression, inducibility, and facile methods of control, including the ability to fully repress promoter activity under aerobic conditions and the ability to modulate promoter activity with DMSO. Additional applications include comparisons of post-translational regulatory mechanisms various bacteria use to modulate RubisCO activity. For example, *R. rubrum* has been shown to inactivate RubisCO in the presence of oxygen by endogenous factors, the system described here could demonstrate if this inactivation is strain dependent (10). The *R. sphaeroides* RubisCO-deletion strain and RubisCO promoter system will also be useful in characterizing mutagenized RubisCO proteins *in vivo* and in the selection for altered RubisCO molecules after random mutagenesis of RubisCO genes or gene regions. Further work in characterizing this promoter and studies using the strain promoter system to address genetic problems in *R. sphaeroides* are described in subsequent chapters (Chapters 3, 4).
CHAPTER II

Expression and Regulation of *Bradyrhizobium japonicum* and *Xanthobacter flavus* CO$_2$-Fixation Genes in a Photosynthetic Bacterial Host

INTRODUCTION

The presence of the reductive pentose phosphate pathway or Calvin cycle is widespread in organisms capable of chemolithotrophic or phototrophic growth (86). Bacteria able to grow chemolithoautotrophically, such as *Alcaligenes eutrophus* (5), *Xanthobacter flavus* (58), and the soybean symbiont *Bradyrhizobium japonicum* (79) 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 (Fdh) and then assimilated by the Calvin cycle (5, 53, 94). 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 (58, 86) and the structural genes have been isolated (1, 39, 46, 56). In these organisms, the activity for each enzyme has been found to be coregulated (1, 49, 56).

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 H₂, CO₂, O₂ atmosphere, much like aerobic hydrogen oxidizing bacteria. 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 (86).

In the present study, a RubisCO-deletion strain of the photosynthetic bacterium *Rhodobacter sphaeroides* (Chapter 1) served as a host, and was complemented to autotrophic growth by CO₂-fixation genes from two nonphotosynthetic bacteria, *Bradyrhizobium japonicum* and *Xanthobacter flavus*. The regulation of RubisCO expression showed some similarity to 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.** *Rhodobacter sphaeroides* strain HR (Sm') was the wild type strain used in these studies. The RubisCO-deletion derivative, strain 16 (Sm', Km', Tp') (16) (Chapter 1), was employed as the host strain for plasmids containing foreign CO₂-fixation gene clusters. *Escherichia coli* strains JM83 (pCD102) or JM109 (pCRM6) were used to maintain plasmids or were employed as donors in conjugation experiments. Plasmid pRK2013, used to effect mobilization of broad host-range
plasmids, was maintained in *E. coli* strain MM294 (18). Plasmids containing \( \text{CO}_2 \)-fixation genes from *Bradyrhizobium japonicum* (plasmid pCRM6) and *Xanthobacter flavus* (plasmid pCD102) were previously isolated from cosmid libraries (49, 56). 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 Dr. Todd Cotter. The \( \text{CO}_2 \)-fixation gene cluster from *Xanthobacter flavus* was obtained by complementation of autotrophic mutants of *X. flavus* (49) and was kindly provided by Dr. Wim Meijer. The 24-kb DNA insert in this cosmid clone was subsequently shown by DNA sequence analysis (59) to have a \( \text{CO}_2 \)-fixation gene cluster similar to that found in *A. eutrophus* (39), 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 l bottles containing 400 ml of Ormerod’s medium (66) plus 0.4% malate; cultures were bubbled with argon as previously described (41). Photolithoautotrophic growth was accomplished by bubbling 400 ml of Ormerod’s minimal medium with a gas mixture of 1.5% \( \text{CO}_2 /98.5\% \text{H}_2 \). Aerobic growth was achieved in 250-ml baffle flasks containing 50 ml 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. Antibiotic concentrations for selection of transconjugants and routine plasmid maintenance were as described previously (16). Photosynthetic growth on solid media was as described previously; 1.8% Bacto-Agar (Difco
Laboratories, Detroit, Mich.) was added to minimal medium and plates were incubated in anaerobic jars in a CO₂-H₂ atmosphere in the light (93).

**Mobilization of plasmids to R. sphaeroides.** Broad host-range plasmids were introduced to *R. sphaeroides* strain 16 by triparental matings using the helper plasmid pRK2013 on filter pads as previously described (93). Selection for transconjugants was made 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 (Sm, Km, Tp) 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 (16). Ribulose 1,5-bisphosphate carboxylase activity was measured by the assay previously described (95). Protein concentration was determined with a modified Lowry protein assay (55), using bovine serum albumin as the standard. Denaturing polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was according to the method of Lugtenberg et al. (52).

**RESULTS**

In previous experiments, we demonstrated that plasmids which contained DNA fragments encoding either of the two *R. sphaeroides* CO₂-fixation gene clusters complemented a RubisCO-deletion strain of *Rhodobacter sphaeroides*. The levels of RubisCO expression were regulated under photoheterotrophic and photolithautotrophic conditions in the complemented strain, in the same manner as the wild-type strain (16) (Chapter 1).
After introduction of cosmids containing DNA that encoded CO₂-fixation genes from *Bradyrhizobium japonicum*, or *Xanthobacter 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, growth occurred for each single transconjugant tested. This demonstrated that 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 CO₂-fixation gene clusters, transconjugants were inoculated to 22-ml screw cap tubes containing Ormerod-malate minimal media (66). 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% CO₂ in 98.5% H₂, respectively. Growth rates under photoheterotrophic conditions were approximately equivalent for each of the two complemented strains compared to the wild-type strain (Table 2.1). Photolithoautotrophic growth rates, in contrast, were slower for strains complemented by either source of cfx genes, ranging from 8 to 58 h longer than the doubling time of wild-type strain HR (Table 2.1). Cultures of the complemented *R. sphaeroides* strains attained cell densities greater than 1.5 (A₆₆₀) 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 2.1). The amount of RubisCO activity detected
Table 2.1. Growth rate and RubisCO activity of *R. sphaeroides* strain 16 complemented with CO₂-fixation genes from *Bradyrhizobium japonicum* (pCRM6) and *Xanthobacter flavus* (pCD102).

<table>
<thead>
<tr>
<th><em>R. sphaeroides</em> Strain</th>
<th>Generation time (h)</th>
<th>RubisCO Activity (Units/mg)³</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (wild type)a</td>
<td>8.0</td>
<td>0.046</td>
</tr>
<tr>
<td>16 (pCRM6)a</td>
<td>7.2</td>
<td>0.503</td>
</tr>
<tr>
<td>16 (pCD102)a</td>
<td>7.2</td>
<td>0.294</td>
</tr>
<tr>
<td>HR (wild type)b</td>
<td>24.0</td>
<td>0.357</td>
</tr>
<tr>
<td>16 (pCRM6)b</td>
<td>32.0</td>
<td>0.100</td>
</tr>
<tr>
<td>16 (pCD102)b</td>
<td>82.0</td>
<td>0.108</td>
</tr>
</tbody>
</table>

a Strains were grown photoheterotrophically with malate as the electron donor.
b Strains were grown photolithoautotrophically in an atmosphere of 1.5%CO₂/98.5% H₂.
c µmol CO₂ fixed per min per mg total soluble protein.
in extracts from cells grown under photolithoautotrophic conditions was approximately 3 to 5 times 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, high RubisCO activity in cultures grown with an oxidized organic carbon source is essentially the opposite to what is 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% \( \text{CO}_2 \)/98.5% \( \text{H}_2 \), 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 2.2). 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 \textit{Bradyrhizobium} or \textit{Xanthobacter cfx} genes, 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 indicated time points in Table 2.2 were also examined by SDS-PAGE to establish whether there was a corresponding increase in RubisCO subunit polypeptide concentration after shifting from photolithoautotrophic to photoheterotrophic conditions. This analysis revealed a major band, just above the 55,000 molecular weight cyanobacterial large subunit standard,
Table 2.2. Addition of malate to photolithoautotrophically-grown cultures of complemented and wild-type *R. sphaeroides* strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Aut. growth</th>
<th>Time after malate addition (h)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>6.5</td>
<td>17</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>HR (wt)</td>
<td>0.265</td>
<td>0.056</td>
<td>0.064</td>
<td>0.046</td>
<td></td>
</tr>
<tr>
<td>16(pCRM6)</td>
<td>0.200</td>
<td>0.350</td>
<td>0.442</td>
<td>0.372</td>
<td></td>
</tr>
<tr>
<td>16(pCD102)</td>
<td>0.141</td>
<td>0.244</td>
<td>0.217</td>
<td>0.282</td>
<td></td>
</tr>
</tbody>
</table>

*a μmole CO₂ fixed per min per mg soluble protein
*b Photolithoautotrophic growth on 1.5% CO₂/98.5% H₂
Figure 2.1. SDS-PAGE (12% acrylamide) of extracts from photosynthetically grown *R. sphaeroides* strain 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; (3-6); cell extracts of *R. sphaeroides* 16 (pCRM6); (9-12), cell extracts of *R. sphaeroides* 16 (pCD102); (3 and 9), extracts of photolithoautotrophically grown (1.5% CO₂, 98.5% H₂) cells prior to the addition of malate; (4 and 10), extracts of cells prepared 6.5 h after the addition of malate (0.4%); (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 malate; (13), large subunit of purified RubisCO from *Anacystis nidulans* (*Synechococcus* sp. strain pCC6301) (M, 55,000); (14), commercially prepared (BioRad) 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; lysozyme, 14,400. The arrows indicate the band in extracts from the complemented strains corresponding to the large subunit of RubisCO.
that increased in intensity in extracts of the complemented strains as the
time after the addition of malate increased (Fig. 2.1). Moreover,
immunoblots, using antibodies to \emph{R. sphaeroides} form I RubisCO, showed
specific cross-reactivity with a 55,000 molecular weight band (data not
shown). The prominent approximate 60,000 molecular weight protein was
the \emph{R. sphaeroides} chaperonin 60 (cpn60) protein, as shown by immunoblots
employing antibodies to \emph{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 post-translational level.

Since RubisCO is synthesized in both \emph{B. japonicum} and \emph{X. flavus} after
aerobic growth on formate (53, 58), extracts of \emph{R. sphaeroides} strain 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 2.3). As a comparison,
complemented strains were also grown aerobically on malate, a growth
condition where it is known that RubisCO expression in wild-type \emph{R.
sphaeroides} is fully repressed (41). No substantial RubisCO activity was
detected in malate-grown cells except for a trace amount in strain 16
containing the \emph{X. flavus} plasmid pCD102 (Table 2.3). To test whether the
complementing cosmids could confer the ability of \emph{R. sphaeroides} to grow
anaerobically (photosynthetically) on formate, strain 16 containing each
of the plasmids was incubated in minimal medium plus formate in the light.
No growth of \emph{R. sphaeroides} 16 (pCD102) or \emph{R. sphaeroides} (pCRM6) was
observed.
Table 2.3. RubisCO activities of *R. sphaeroides* strain 16 complemented with CO\textsubscript{2} -fixation genes from *B. japonicum* (plasmid pCRM6) and *X. flavus* (plasmid pCD102) and grown aerobically on minimal media containing formic or malic acid as the carbon source.

<table>
<thead>
<tr>
<th>R. sphaeroides Strain</th>
<th>RubisCO Activity (mUnits/mg)<em>a</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>formate</td>
</tr>
<tr>
<td>HR (wt)</td>
<td>3.0</td>
</tr>
<tr>
<td>16(pCRM6)</td>
<td>2.5</td>
</tr>
<tr>
<td>16(pCD102)</td>
<td>7.9</td>
</tr>
</tbody>
</table>

*a* nmole CO\textsubscript{2} fixed per min per mg protein  
*b* N.D., not detectable

DISCUSSION

Complementation of a RubisCO deletion strain of *R. sphaeroides* by plasmids with DNA inserts encoding CO\textsubscript{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 manifest in a dissimilar background strain is not obvious. *R. sphaeroides* cells grown chemoheterotrophically with *cfx* genes from either *B. japonicum* or *X. flavus* showed a regulatory pattern of RubisCO synthesis more similar to the host bacterium rather than to the organisms from which the *cfx* genes were
derived. For example, aerobic repression of RubisCO in formate-containing media was apparent in *R. sphaeroides* cells grown with the plasmid-based *cfx* genes, even though this is opposite to what is normally observed for the aerobic bacteria *B. japonicum* or *X. flavus*. Thus, in this instance, the controlling environment of *R. sphaeroides* took precedence over the system that normally regulates RubisCO derepression of 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 amounts present in wild-type cells (Table 2.1). Moreover, *R. sphaeroides* cells growing with the heterologous *cfx* genes under photolithoautotrophic growth conditions displayed a reduction of 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 provide an indication of subtle differences in control of RubisCO expression among separate genera. Perhaps these differences may be the result of unique responses of regulatory sequences present in each gene cluster and to 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* (26, 59, 89, 97). 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 employed 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 \( Xanthobacter \) \textit{rbcLrbcS} 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 is entirely feasible using the \( R. sphaeroides \) expression system and the results provided in this investigation and in earlier studies (16) indicate that alterations of RubisCO function from both aerobic and anaerobic bacteria may be selected with this system.
CHAPTER III

Additional Studies with CO₂ Fixation Mutants of *Rhodobacter sphaeroides*

INTRODUCTION

The generation of a variety of CO₂-fixation structural gene mutants in *R. sphaeroides* enabled additional analyses to be performed. In this section, the physiological evaluation of strains described in Chapter one is continued. In particular, during the construction of the *R. sphaeroides* dual RubisCO mutant, strain 16, genes encoding either form I or form II RubisCO were, by necessity, inactivated independently, resulting in strains deficient in one RubisCO type or the other. This comparison is thus an extension of earlier work (15) conducted to determine how strains deficient in one form of RubisCO behave under various photosynthetic growth conditions. Earlier studies had shown that synthesis of form I RubisCO was predominant under low (1.5%) CO₂ concentrations in H₂ (41). Form II RubisCO, however, was more abundant under photoheterotrophic conditions with organic acids supplied as the electron donor and major carbon source. Work presented in this chapter describes the response of *R. sphaeroides* strains possessing only a single form of RubisCO under conditions of CO₂ limitation.

The second part of this chapter involves an examination of various cfx mutants of *R. sphaeroides*. These studies, performed in collaboration with Dr. Janet Gibson, extend previous analyses (27, 28) through
comparisons of recently acquired mutant strains impaired in the expression of part of the duplicated cfx gene clusters; in addition, mutants deficient in an entire cfx transcriptional unit (28) were examined in this study. From these studies, an important observation was made: that pronounced effects on growth resulted when only a portion of the complete cfx operon was expressed, as opposed to strains in which one of the two cfx operons were eliminated entirely.

Finally, a study was performed to help identify the physiological role for the cfx genes found immediately upstream from the rbcL and rbpL genes of each operon. At the time these experiments were performed, these genes (cfxA and cfxB) were unidentified, with uncertain function (25, 35). Recently, however, these genes were found to encode fructose bisphosphate aldolase (fba) (28); the current investigation still yields pertinent information relative to the essentiality of the fba gene products during photosynthetic growth. Finally, additional insights relative to the phenotype of various R. sphaeroides and Rhodospirillum rubrum cfx mutants are provided and the utility of the R. rubrum rbpL expression vector (Chapter 1) to address genetic problems in R. sphaeroides is documented.

MATERIALS AND METHODS

Media, strains and genetic techniques. All strains of Rhodobacter sphaeroides employed in the present study were derived from strain HR (93); various mutant derivatives were constructed using the methods described in Chapter 1. Insertion or deletion mutants in the fbpA, fbpB, and fbaA (cfxA) loci were made by Dr. Janet Gibson.

R. sphaeroides strains were cultured photolithoautotrophically using
variable mixtures of CO₂ and H₂ after adjusting gas rotameters (Matheson Scientific, Inc., Elk Grove Village, IL) to the desired CO₂ concentration. The final concentration of CO₂ (in H₂) was measured by gas chromatography using an Antek gas chromatograph. Both culture input and output CO₂ levels were determined to test whether CO₂ in the medium was saturating. Output CO₂ concentrations were measured from the culture outlet tube, reflecting the CO₂ concentration gas in the head space of the growth flask. For photolithoautotrophic growth at low levels of CO₂, cultures were initiated with a 2.0% inoculum of photoheterotrophically (malate)-grown cells. All other conditions for photoheterotrophic and photolithoautotrophic growth were performed using standard methods (16, 41).

Cell sampling procedures, extract preparations, and enzyme assays were performed as previously described (Chapter 1).

Construction of an R. sphaeroides mutant, strain 21, was accomplished by the inactivation of the specific alleles by site-directed mutagenesis using the same procedures as described for loci that encode RubisCO genes (Chapter 1) (16).

RESULTS AND DISCUSSION

Physiological analysis of Rhodobacter sphaeroides strains grown on limiting carbon dioxide levels. An earlier study had established that R. sphaeroides mutants lacking either form I or form II RubisCO were capable of photosynthetic growth, even under photolithoautotrophic growth conditions, with generation times that were not severely impaired (15). The duplication of RubisCO in R. sphaeroides was thus reasoned to be an
apparent functional redundancy. However, in cells grown photolithoautotrophically, the ratio of form I- to form II RubisCO protein is reversed compared to photoheterotrophically grown cells (41), implying that each RubisCO might have a discrete role under different environmental conditions. To further investigate this possibility, and to evaluate how strains of *R. sphaeroides* containing only one RubisCO type respond to conditions of CO₂ limitation, *R. sphaeroides* strains containing inactivated genes encoding either form I RubisCO (*rbcLrbcS*'), or form II RubisCO (*rbpL*'), were cultured under photolithoautotrophic conditions using reduced CO₂ concentrations. Throughout these studies, it is instructive to compare cultures grown with 1.5% CO₂/98.5% H₂, the standard gas mixture used for maximal derepression of RubisCO in *R. sphaeroides* (41). Typical results for RubisCO levels and growth rates at standard gas concentrations have been reported (Chapter 1, Table 1.2; Chapter 2, Table 2.2) (16, 41).

Previous studies established that wild-type *R. sphaeroides* grows with a generation time of about 24-25 h on 1.5% CO₂ in H₂ (16). The growth rates of the *rbpL*⁻ strain or *rbcLrbcS*⁻ strain were 1 to 6 h less on 0.7% CO₂/99.3% H₂ compared to the wild type grown on 1.5% CO₂ in H₂ (Fig. 3.1; Table 3.1). Thus, the overall growth rate was only slightly affected, although the cultures rarely achieved the final cell densities achieved when grown at 1.5% CO₂, which typically reaches an A₆₆₀ of 2.0. The specific activity for RubisCO was about 3-fold higher in extracts of the *rbcLrbcS*⁻ strain (containing form II RubisCO) compared to the *rbpL*⁻ strain (containing form I RubisCO (Table 3.1). However, the activity measurements did not coincide with the relative amounts of immunologically determined RubisCO protein for the two strains. The
strain expressed substantially higher form I protein levels, approximately 9.0% of the total soluble cell protein, compared to the \textit{rbcLrbcS} strain, which synthesized substantially lower amounts of form II RubisCO (Table 3.1). It is thus evident that there is a significant reduction form I RubisCO activity in cells cultured at 0.7% CO$_2$ in H$_2$. In previous work, it had been shown that \textit{R. sphaeroides} strains containing only the form I RubisCO synthesize similar amounts of RubisCO when grown in an atmosphere of 1.5% CO$_2$ in H$_2$ (15) as obtained here at 0.7% CO$_2$, however, the RubisCO specific activity of extracts from cells grown at 1.5% CO$_2$ was considerably higher, about 0.19 U/mg (15). The level of form II RubisCO synthesized in the \textit{rbcLrbcS} strain grown at 0.7% CO$_2$, represents 2.8% of the total soluble protein, well within the range found in cells grown at 1.5% CO$_2$ (16, 41), and the specific activity is not substantially changed. These results therefore indicate that differences in RubisCO specific activity might be due to some post-translational alteration of the form I RubisCO enzyme. To continue this analysis, cells grown at 0.7% CO$_2$ (Fig. 3.1) were used to inoculate cultures provided with a mixture of 0.5% CO$_2$ in H$_2$; perhaps there might be a further reduction of form I RubisCO activity, indicative of a trend as CO$_2$ becomes limiting for growth.

Since it is known that the levels of RubisCO expression and activity respond to the concentration of CO$_2$ bubbled through the culture (41, 73, 86), the input CO$_2$ level in each experiment at low CO$_2$ was measured over the duration of growth. In the first experiment, using an initial concentration of 0.7% CO$_2$, the average input CO$_2$ concentration was 0.68%. The output CO$_2$ concentration, sampled from the exhaust port of the culture apparatus, when cell densities reached an A$_{660}$ greater than 0.3,
Fig. 3.1. Growth curves for *R. sphaeroides rbcLrbcS' and *R. sphaeroides rbpL' strains grown photolithoautotrophically on 0.7% CO₂ in H₂. (•——•) rbcLrbcS'; (o——o) rbpL'.

Table 3.1. Growth rate and RubisCO activity of *R. sphaeroides* mutant strains grown in an atmosphere of 0.7% CO₂ in H₂ (as shown in Fig. 3.1).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Gen. Time (h)</th>
<th>Time Point</th>
<th>RubisCO S.A. (U/mg)</th>
<th>RubisCO Protein (% Total)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>form I</td>
<td>form II</td>
</tr>
<tr>
<td><em>rbcLrbcS'</em> (•——•)</td>
<td>31.0</td>
<td>1</td>
<td>0.130</td>
<td>0.0</td>
</tr>
<tr>
<td><em>rbcLrbcS'</em></td>
<td>26.0</td>
<td>1</td>
<td>0.049</td>
<td>9.0</td>
</tr>
<tr>
<td><em>rbcLrbcS'</em></td>
<td>2</td>
<td>0.034</td>
<td>8.5</td>
<td>0.0</td>
</tr>
</tbody>
</table>

*Time point as indicated on growth curves in Figure 3.1.*

*S.A., specific activity in µmol CO₂ fixed per min per mg total soluble protein. Protein expressed in terms of the percentage of the total soluble protein as determined by rocket immunoelectrophoresis (41).*
averaged 0.39% CO₂ in H₂. This finding indicated that the CO₂ concentration might be limiting for growth, perhaps explaining the inability of cultures to attain cell densities much higher than 1.0 at 660 nm. Similar measurements of input and output CO₂ gas concentrations were made for cultures maintained at 0.5% CO₂. In this instance, the input CO₂ concentration was stable with an average of 0.51%. The post-culture CO₂ concentration, however, maintained an average of 0.14%, at points measured when the cell culture densities (A₆₆₀) were between 0.40 and 0.88. To place these observations in the context of CO₂ released from cells growing photoheterotrophically on a fixed carbon source, samples of an R. sphaeroides culture growing on malate were measured in the same manner. In this case, cultures are bubbled with argon to maintain anaerobicity, so any CO₂ detected reflects CO₂ released from the cells as malate is metabolized. At mid-log phase, the concentration of CO₂ liberated was 0.17% and at late log phase, 0.12%. Thus, the concentration of CO₂ produced from a malate-grown culture is very close to the output levels obtained by photolithoautotrophic cultures growing at 0.5% CO₂ in H₂, suggesting that 0.12-0.17% CO₂ may be a basal level of CO₂ released from cultures as a result of normal cell growth and metabolism. These results also suggest that 0.5% CO₂ might be the lowest CO₂ concentration which will support photolithoautotrophic growth at reasonable rates.

Photolithoautotrophic growth of the rbpL⁻ and rbcLrbcS⁻ strains at 0.5% CO₂/99.5% H₂ are presented in Figure 3.2. RubisCO activities of crude extracts obtained from each strain follow the same general pattern seen at 0.7% CO₂: the form II RubisCO specific activity in the rbcLrbcS⁻ strain is at least 3-fold greater than the specific activity of the form I RubisCO in the rbpL⁻ strain. Again, the level of form I RubisCO protein
in the \( rbpL^- \) strain is higher than the total RubisCO concentration of the form II enzyme in \( R. sphaeroides rbcLrbcS^- \), although both RubisCO activity and protein levels of each strain appear somewhat decreased from that obtained when cells were grown at 0.7% CO\(_2\) (Table 3.1). The lag seen in the \( rbcLrbcS^- \) \( R. sphaeroides \) strain is not necessarily a result of the decreased CO\(_2\) concentration since variable lags are common for \( R. sphaeroides \) when small inocula are used to start photolithoautotrophic cultures. The lag is thus probably a result of the initial low density of the culture (Fig. 3.2). Finally, an experiment was performed with 0.4% CO\(_2\), to confirm the observed trend relative to the reduction of RubisCO activity in the \( rbpL^- \) strain at 0.5% and 0.7% CO\(_2\); in addition, the response of the mutants was directly compared to the wild-type strain (Table 3.3). After photolithoautotrophic growth on 0.4% CO\(_2\) in H\(_2\), RubisCO activity in the \( rbcLrbcS^- \) strain was again observed to be higher than in the \( rbpL^- \) strain. Interestingly, the RubisCO specific activity in the \( rbcLrbcS^- \) strain is also higher than that found in the wild type, strain HR. Furthermore, the overall levels of both RubisCO activity and protein are slightly decreased over the amounts found in the \( rbcLrbcS^- \) and \( rbpL^- \) strains grown with 0.5% CO\(_2\) in H\(_2\) (Table 3.2).

The fact that the RubisCO activity is lower in the wild-type strain than the \( rbcLrbcS^- \) strain suggests that the overall level of RubisCO activity may be regulated in the wild type at both the level of enzyme synthesis and the level of enzyme activity. Perhaps in wild-type strain HR, the degree of derepression of the two RubisCO forms is adjusted, as well as specific modification of the form I RubisCO (41), in order to achieve a specific level of RubisCO activity. The \( rbpL^- \) strain, on the other hand, does not synthesize form I RubisCO, and the remaining form II enzyme is not
Fig. 3.2. Growth curves for *R. sphaeroides rbcLrbcS'* and *R. sphaeroides rbpL'* strains grown photolithoautotrophically on 0.5% CO₂ in H₂. (○—○) *rbcLrbcS'*(•—•) *rbpL'*. 

Table 3.2. Growth rate and RubisCO activity of *R. sphaeroides* mutant strains grown in an atmosphere of 0.5% CO₂ in H₂ (as shown in Fig. 3.2).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Gen. Time (h)</th>
<th>Time Point</th>
<th>RubisCO S.A. (U/mg)</th>
<th>RubisCO Protein (%) Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>rbcLrbcS'</em> (○—○)</td>
<td>42.0</td>
<td>1</td>
<td>0.114</td>
<td>0.0</td>
</tr>
<tr>
<td><em>rbcLrbcS'</em></td>
<td>2</td>
<td></td>
<td>0.103</td>
<td>0.0</td>
</tr>
<tr>
<td><em>rbpL'</em> (•—•)</td>
<td>27.0</td>
<td>1</td>
<td>0.028</td>
<td>5.3</td>
</tr>
<tr>
<td><em>rbpL'</em></td>
<td>2</td>
<td></td>
<td>0.035</td>
<td>5.3</td>
</tr>
</tbody>
</table>

"Time point as indicated on growth curves in Figure 3.2.

'S.A., specific activity in µmol CO₂ fixed per min per mg total soluble protein. 'RubisCO protein expressed in terms of the percentage of the total soluble protein as determined by rocket immunoelectrophoresis (41).
Table 3.3. RubisCO activities and protein levels of *R. sphaeroides* mutant and wild-type strains grown in an atmosphere of 0.4% CO₂ in H₂.

<table>
<thead>
<tr>
<th>Strain</th>
<th>(A_{660}^a)</th>
<th>RubisCO S.A. (^b) (U/mg)</th>
<th>RubisCO Protein (^c) (% Total)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>form I</td>
<td>form II</td>
</tr>
<tr>
<td>HR (wild type)</td>
<td>0.66</td>
<td>0.066</td>
<td>4.8</td>
</tr>
<tr>
<td>HR (wild type)</td>
<td>1.02</td>
<td>0.070</td>
<td>4.2</td>
</tr>
<tr>
<td>(rbcLrbcS')</td>
<td>0.77</td>
<td>0.111</td>
<td>0.0</td>
</tr>
<tr>
<td>(rbcLrbcS')</td>
<td>1.18</td>
<td>0.125</td>
<td>0.0</td>
</tr>
<tr>
<td>(rpbL')</td>
<td>0.78</td>
<td>0.017</td>
<td>3.1</td>
</tr>
<tr>
<td>(rpbL')</td>
<td>0.99</td>
<td>0.017</td>
<td>2.2</td>
</tr>
</tbody>
</table>

\(^a\)Density of culture when sampled. Second \(A_{660}\) is the final culture density.

\(^b\)S.A., specific activity in \(\mu\)mol CO₂ fixed per min per mg total soluble protein.

\(^c\)RubisCO protein, expressed in terms of the percentage of the total soluble protein as determined by rocket immunoelectrophoresis (41).
known to be subject to post-translational control. Thus on one level, the cell appears to respond to the requirement of higher RubisCO activity (due to the lack of form I RubisCO) by synthesizing greater amounts of form II RubisCO. The resulting total activity surpasses that found in the wild type, presumably because the cell no longer possesses the additional means of control that may be achieved by modulating the form I RubisCO enzyme. In this respect, the \( \text{rbcLrbcS}^{-} \) strain, which only synthesizes form II RubisCO, seems to have lost the sensitive level of regulation that resides with form I RubisCO. Consequently, in a strain that retains only form II RubisCO, RubisCO enzymatic activity appears to be essentially unregulated when compared to the activity in the wild type.

The presumptive post-translational modulation of form I RubisCO activity observed when cells are grown at low levels of \( \text{CO}_2 \) is in agreement with previous studies which demonstrated the reversible post-translational inactivation-reactivation of form I RubisCO in \( \text{R. sphaeroides} \) (42, 92). In the latter situation, form I RubisCO is specifically, but transiently inactivated when organic carbon is added to cells cultured under an atmosphere of 1.5% \( \text{CO}_2 \) in \( \text{H}_2 \). The reduction of RubisCO activity appears to be a response to an abrupt shift from a photolithoautotrophic to a photoheterotrophic environment, where the level of RubisCO necessary for growth is drastically reduced because of the abundance of a usable growth substrate. The condition described in the present study, however, is one of carbon limitation, and the need to decrease RubisCO activity is likely to be related to the ability of the cells to maintain viability during nutrient deprivation. It is not known if the process that leads to diminished form I RubisCO activity is the same in which form I inactivation has been observed.
Analysis of additional *cfx* mutants of *R. sphaeroides*. As mentioned previously, the *cfx* genes of *R. sphaeroides* are found within two distinct clusters or operons (24, 25, 27, 28, 81, 82) (Chapter 1; fig. 1.2). Previous work (Chapter 1), demonstrated that *R. sphaeroides* strains bearing mutations in both *rbcLrbcS* and *rbpL* were incapable of photolithoautotrophic growth, but could be fully complemented by expressing an introduced sequence that encoded only RubisCO. This finding, together with the determination of the nucleotide sequence of each *cfx* cluster (8, 27, 28), supports the notion that the 3' open reading frames within each transcriptional unit encode RubisCO. With such an arrangement, insertions into either *rbcLrbcS* or *rbpL*, result in mutant strains with a phenotype unobscured by secondary polar effects on known downstream Calvin cycle structural genes. The situation resulting from insertions into genes upstream of *rbcLrbcS* or *rbpL* however, is not so straightforward since these mutations lead to the elimination of all genes downstream from the original insertions. Thus, *R. sphaeroides* strains containing mutations in *fbpA*, *fbpB*, and *fbaA* possess a disruption in these genes plus all *cfx* genes encoded downstream. Mutant strains *fbpA*<sup>+</sup> and *fbpB*<sup>+</sup> can therefore be considered to lack, respectively, the entire *cfxA* or *cfxB* gene cluster entirely. Physiological analysis of these strains are compared with previously described RubisCO mutants (Table 3.4).

All *R. sphaeroides* mutant strains tested, *fbpA*<sup>+</sup>, *fbpB*<sup>+</sup>, *fbaA*<sup>+</sup>, *rbcLrbcS*<sup>+</sup> and *rbpL*<sup>+</sup> were capable of photolithoautotrophic growth. As compared to the wild-type strain HR, all mutant strains display the characteristic increase in RubisCO specific activity when extracts of CO<sub>2</sub>-grown cells were compared to extracts of malate-grown cells. However, the mutant strains exhibit altered levels of RubisCO activity and protein. For
example, in the \textit{f}b\textit{p}A' and \textit{f}b\textit{p}B' strains, RubisCO levels are higher than those measured in wild-type strain HR under both photolithoautotrophic and photoheterotrophic growth conditions. The observed increases in the \textit{f}b\textit{p}A' mutant are substantial, considering only the form II enzymes are present, which normally account for 33\% of total RubisCO in the wild type (41). The level of RubisCO antigen detected in CO$_2$-grown extracts of \textit{f}b\textit{p}A' (21\% of the soluble protein) is more than a 10-fold increase over the amount of form II RubisCO antigen in the wild type. A similar enhancement in the level of RubisCO is seen in extracts of CO$_2$-grown \textit{f}b\textit{p}B' strains, however the increase is not as dramatic. It should be noted that the specific activities of FBPase and phosphoribulokinase (PRK) were also determined for each of the mutant strains; these activities increased essentially in parallel with the rise in RubisCO activity, except for the \textit{r}bpL' strain (28). Thus, expression of genes upstream of \textit{r}bc\textit{L}r\textit{bc}S and \textit{r}bp\textit{L} within the operons are similarly augmented, which illustrates the probable coordinate regulation of upstream genes. The results from the analysis of the \textit{f}baA' strain more closely resemble the \textit{r}bc\textit{L}r\textit{bc}S' strain with respect to the overall induction level of RubisCO. This strain, however, similar to a previous analysis of \textit{prk} insertional mutants (35, 36), displays a pronounced lag time under photoheterotrophic conditions when the culture was bubbled with argon (28, 36). Since none of the other mutant strains analyzed in this study exhibited such a phenotype, the inability of the \textit{f}baA' strain to grow for several days is likely to be the result of the \textit{f}ba\textit{A} mutation, leading to a blockage of expression of the last half of the \textit{c}fc\textit{A} operon, while allowing the expression of the upstream half. The different growth phenotypes seen under photoheterotrophic conditions with malate-argon, may therefore be the effect of unequal levels of enzymes expressed from each of the two operons.
Table 3.4. RubisCO activity and protein levels of various cfx mutant strains and wild type *R. sphaeroides* grown photoheterotrophically and photolithoautotrophically in an atmosphere of 1.5% CO$_2$ in H$_2$.

<table>
<thead>
<tr>
<th><em>R. sphaeroides</em> strain</th>
<th>RubisCO Levels</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acty$^a$ (U/mg)</td>
<td>Protein$^b$ (%Total)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Het$^c$</td>
<td>Aut$^d$</td>
<td>Het</td>
</tr>
<tr>
<td>HR (wild type)</td>
<td>0.05</td>
<td>0.13</td>
<td>I: 1.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>II: 0.5</td>
</tr>
<tr>
<td><em>rbcLrbcS</em></td>
<td>0.04</td>
<td>0.17</td>
<td>II: 0.7</td>
</tr>
<tr>
<td><em>rbpL</em></td>
<td>0.04</td>
<td>0.09</td>
<td>I: 3.8</td>
</tr>
<tr>
<td><em>fbaA</em></td>
<td>0.05</td>
<td>0.09</td>
<td>II: 0.2</td>
</tr>
<tr>
<td><em>fbpA</em></td>
<td>0.08</td>
<td>0.36</td>
<td>II: 2.1</td>
</tr>
<tr>
<td><em>fbpB</em></td>
<td>0.06</td>
<td>0.17</td>
<td>I: 4.8</td>
</tr>
</tbody>
</table>

$^a$ Acty, specific activity in μmol CO$_2$ fixed per min per mg total soluble protein.

$^b$ RubisCO protein expressed in terms of the percentage of the total soluble protein as determined by rocket immunoelectrophoresis (41); I: Form I RubisCO; II: Form II RubisCO.

$^c$ Het, Photoheterotrophic growth on 0.4% malate bubbled with argon.

$^d$ Aut, Photolithoautotrophic growth on 1.5% CO$_2$, 98.5% H$_2$. 
In the case of \( fbpA' \), \( fbpB' \), \( rbcLrbcS' \), or \( rbpL' \) strains, growth rates under photoheterotrophic and photolithoautotrophic conditions are similar to the wild type. These mutations either inactivate an entire \( cfx \) cluster (\( fbp' \) mutants) or just the last gene in the cluster (RubisCO-encoding genes). Insertion into \( fbaA \) (or \( prkA \) or \( prkB \)) (28, 36), however, results in the inactivation of only a portion of the \( cfx \) operon, perhaps leading to disproportionate enzyme activity levels that normally are coordinately expressed. Such ordered expression may be necessary to maintain the proper flux of metabolites through the reductive pentose phosphate pathway so that the various intermediates required for the operation of the cycle, and for the regeneration of RuBP, are provided. The unequal expression of genes resulting from mutations in \( fbaA \), \( prkA \) and \( prkB \), may thus lead to the altered growth phenotypes observed in strains containing these mutations (28, 35, 36).

**Analysis of the role of two \( cfx \) loci in \( R. sphaeroides \).** Most of the genes of the two \( CO_2 \) fixation gene clusters of \( R. sphaeroides \) were identified by hybridization and sequence comparisons to genes of known function from other organisms (8, 24, 25, 27, 28). Two loci, however, initially known as \( cfxA \) and \( cfxB \), which are situated just upstream of \( rbcLrbcS \) and \( rbpL \), respectively, were among the last to be identified (28). Before these genes were identified, an experiment was devised to define the physiological role for the \( cfxA \) and \( cfxB \) loci. As indicated in the maps presented of the \( R. sphaeroides \) \( cfx \) operons, both of these loci have since been identified to encode fructose 1,6-bisphosphate aldolase (\( fba \)) genes, based on nucleotide sequence similarity to a recently identified class II aldolase gene from \( E. coli \) (28). However, the results described in this analysis are still informative, since they relate to the requirement of \( fbaA \) (\( cfxA \)) during
various photosynthetic growth modes. A specific \textit{cfxA'fbpB'} mutant strain of \textit{R. sphaeroides} was constructed; thus the importance of the \textit{fbaA} gene product could be assessed (Fig. 3.3). Because of polarity effects on downstream genes of the Calvin cycle structural gene operons, mutant analysis is hampered. The problem is also compounded by the duplication of the genes of interest within the two operons. Thus, we took advantage of the \textit{rbpL} promoter expression (pRPS) vector from \textit{R. rubrum} (Chapter 1) (16) to replace the desired gene products in the \textit{cfxA'fbpB'} background. The goal of the experiment was to attempt to identify conditions in which the \textit{fbaA} gene was required for growth, as well as establish the phenotype of a strain that lacked only the \textit{fbaA} gene within a single functioning operon. The strain design and plasmid promoter constructs used are represented in Figure 3.3. Insertions into \textit{fbaA} and \textit{fbpB} in the two \textit{cfxA} and \textit{B} operons, result in a strain unable to grow photosynthetically under all conditions tested. Based on previously described results, the insertion of a trimethoprim resistance gene cartridge results in a polar effect on the downstream \textit{rbcLrbcS} genes. Thus, the introduction of the plasmids shown in the lower half of Figure 3.3 to strain 21, result in strains in which both \textit{fbaA} (\textit{cfxA}) and \textit{rbcLrbcS} genes are restored with plasmid pRPS4-29, or simply \textit{rbcLrbcS}, in plasmid pRPS3-29. In this connection, it is important to note results presented in the last section, in which a strain bearing an insert in the \textit{fbpB} gene is deficient in the synthesis of the entire \textit{B} operon, yet retains the capacity for photolithoautotrophic growth. Thus, by expressing the \textit{fbaA} and \textit{rbcLrbcS} gene in \textit{trans}, the genotype should be functionally equivalent to the \textit{fbpB'} strain. Supplying \textit{fbaA} and \textit{rbcLrbcS} gene products in strain 21 via plasmid pRPS4-29 resulted in full complementation to all photosynthetic growth modes, including
Figure 3.3. Top. Schematic representation of the *R. sphaeroides* strain 21 genotype. A and B designate the distinct Calvin cycle gene (*cfx*) operons with antibiotic resistance cartridges inserted as indicated. Since insertions are polar on the expression of downstream genes, strain 21 entirely lacks the B *cfx* cluster and is only able to express *fbpA* and *prkA* from the A operon. Arrows indicate the extent of gene expression before transcription is blocked.

Bottom. Diagrams of inserts in pRPS plasmid constructs introduced to *R. sphaeroides* strain 21 to test for complementation to phototrophic growth. pRPS4-29 contains the *R. sphaeroides fbaA* (*cfxA*) gene encoding fructose 1,6-bisphosphate aldolase, and the *rbcLrbcS* genes encoding the large and small subunits of form I RubisCO. pRPS3-29 is the same as pRPS4-29, but lacks the *fbaA* gene sequence preceding *rbcLrbcS*. pRPS-53 possesses the *rbpL* gene encoding form II RubisCO. All constructs employ the *R. rubrum rbpL* promoter sequence (RPS) to drive expression of inserted genes. Insert ORI refers to the orientation of the DNA inserts downstream from the RPS promoter in correct (I) and opposite (II) orientation for expression. Photosynthetic growth refers to growth on plates on the indicated media in anaerobic chambers. (+), good growth; (-), no growth.
**R. sphaeroides strain 21**

![Diagram of genetic markers and plasmid constructs](image)

### Plasmid construct

<table>
<thead>
<tr>
<th>Plasmid construct</th>
<th>Insert Location</th>
<th>Photosynthetic Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ORI Malate Butyrate Minimal</td>
<td></td>
</tr>
<tr>
<td>pRPS4-29</td>
<td>(cfxA) rbcL rbcS</td>
<td>+ + + +</td>
</tr>
<tr>
<td>pRPS3-29</td>
<td>RPS rbcL rbcS</td>
<td>+ - - -</td>
</tr>
<tr>
<td>pRPS-53</td>
<td>RPS rbPL</td>
<td>+ - - -</td>
</tr>
</tbody>
</table>

**Fig. 3.3.**
photolithoautotrophic growth. However, when plasmid pRPS3-29 or pRPS-53 were introduced, encoding form I or form II Rubisco genes, respectively, growth was possible only under photoheterotrophic conditions using malate as the substrate. Growth did not occur photoheterotrophically in a butyrate-HCO$_3^-$ medium nor under photolithoautotrophic growth conditions with CO$_2$ in H$_2$ (Fig. 3.3, bottom). This demonstrates that the $fbaA$ gene product is necessary for photolithoautotrophic growth and is nonessential under photoheterotrophic conditions using oxidized substrates such as malate. The capacity for photoheterotrophic growth on oxidized carbon sources in a strain lacking the aldolase of the Calvin cycle indicates that under these physiological conditions, in which only relatively low levels of Calvin cycle genes are expressed, $R. sphaeroides$ must utilize a "nonphotosynthetic", i.e., heterotrophic aldolase. Since the photoheterotrophic growth described here is dependent on Rubisco, this study establishes a situation where the Calvin cycle must be functioning, but at levels able to be supported by supplemental ("non-Calvin cycle") enzyme activities. Limitations of the presumptive heterotrophic aldolase activity, however, are manifested by a lack of photoheterotrophic growth on the reduced substrate, butyrate (Fig. 3.3), and of course, under conditions where CO$_2$ is the sole source of carbon. Thus, growth conditions which require high levels of Calvin cycle enzymes obviously require levels of aldolase activity that are not provided by the "heterotrophic" enzyme. This situation differs from strains which contain insertions in $tklB$ or $gapB$, in which the "heterotrophic" transketolase or glyceraldehyde phosphate dehydrogenase provide the necessary levels of activity to support photolithoautotrophic growth (27).
CHAPTER IV

Identification and complementation analysis of CO₂ fixation genes from *Rhodospirillum rubrum*

INTRODUCTION

The development and use of the *R. rubrum* RubisCO gene (*rbpL*) expression vector (Chapter 1) in *Rhodobacter sphaeroides* led to an analysis of RubisCO regulation in *R. rubrum* itself. Since the nature and regulation of CO₂ fixation genes in *R. sphaeroides* has been more thoroughly investigated (8, 15, 27, 28, 29, 35, 36, 41, 42, 93), initial efforts at constructing a RubisCO deletion strain were made with that organism. However, *R. rubrum* is unique in that it is the only prokaryote thus far identified that possesses a single form of RubisCO composed exclusively of large subunits (84). The availability of the cloned *rbpL* gene from *R. rubrum* has allowed studies to be initiated on RubisCO gene expression. Previous physiological studies had established the degree of derepression of RubisCO synthesis in *R. rubrum* (73). Subsequently, Leustek et al. performed experiments to examine *rbpL* expression by *in vitro* and *in vivo* analysis of specific transcripts (51). This latter study verified that derepression occurred at the transcriptional level.

To analyze the regulation of the CO₂ fixation genes of *R. rubrum*, we first inactivated the *rbpL* gene. Unlike results obtained with *R. sphaeroides*, the *rbpL* deletion strain of *R. rubrum* was capable of
photoheterotrophic growth using CO₂ as an electron acceptor. In addition, the ability of the *R. sphaeroides* and *R. rubrum* RubisCO deletion strains to be complemented to photolithoautotrophic growth differed significantly.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** *Escherichia coli* JM109 (98) was used for all routine plasmid manipulations and as the donor in conjugation experiments with *R. sphaeroides* and *R. rubrum*. *E. coli* HB101 (7) was used to maintain pVK102 plasmid derivatives. *E. coli* SM-10 (78) was used as the donor in *R. rubrum* strain constructions. *R. rubrum* Str-2, a spontaneous streptomycin-resistant derivative of strain S1 (ATCC 11170), was used as the wild type in all growth experiments and as the parental strain for the *rbpL* deletion mutant construction. Plasmid vectors and constructs used in this study with relevant characteristics are listed in Table 4.1.

**Media and growth conditions.** Both *R. rubrum* and *R. sphaeroides* cells were grown under photoheterotrophic conditions in Ormerod's medium (66) containing malate as previously described (41). Photolithoautotrophic growth was attained by bubbling minimal Ormerod's medium with a gas mixture of 1.5% CO₂ / 98.5% H₂ (41). Photosynthetic growth on agar-containing media was achieved by incubating plates containing minimal or minimal-malate media in Gas Pak anaerobic jars (Scientific Products Div., McGaw Park, IL) as previously described (93). Growth was monitored by measuring the A₆₆₀ of samples with a Beckman spectrophotometer. Cultures were incubated at 28 - 32 °C behind banks of incandescent lights as previously described (41). Aerobic growth of *R. rubrum* was performed
### TABLE 4.1. Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pK18, pK19</td>
<td>Km&lt;sup&gt;r&lt;/sup&gt;, pUC derivatives</td>
<td>(69)</td>
</tr>
<tr>
<td>pUC1813</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt;, pUC vector with hybrid cloning site</td>
<td>(44)</td>
</tr>
<tr>
<td>pRK404</td>
<td>Tc&lt;sup&gt;r&lt;/sup&gt;, broad-host-range vector, LacZ</td>
<td>(12)</td>
</tr>
<tr>
<td>pRK415</td>
<td>Tc&lt;sup&gt;r&lt;/sup&gt;, pRK404 with modified multiple cloning site</td>
<td>(45)</td>
</tr>
<tr>
<td>pSUP202</td>
<td>Cm&lt;sup&gt;r&lt;/sup&gt; Tc&lt;sup&gt;r&lt;/sup&gt; Ap&lt;sup&gt;r&lt;/sup&gt; Tra&lt;sup&gt;-&lt;/sup&gt;Mob&lt;sup&gt;+&lt;/sup&gt;, pBR325 replicon</td>
<td>(78)</td>
</tr>
<tr>
<td>pUC4K</td>
<td>Km&lt;sup&gt;r&lt;/sup&gt; gene cartridge in pUC vector</td>
<td>(87)</td>
</tr>
<tr>
<td>pJG336</td>
<td>Tc&lt;sup&gt;r&lt;/sup&gt;, pVK102 with 24-kb insert of <em>R. sphaeroides</em> DNA encoding CO&lt;sub&gt;2&lt;/sub&gt;-fixation gene cluster A</td>
<td>(23, 24)</td>
</tr>
<tr>
<td>pJG106</td>
<td>Tc&lt;sup&gt;r&lt;/sup&gt;, pVK102 with 26-kb insert of <em>R. sphaeroides</em> DNA encoding CO&lt;sub&gt;2&lt;/sub&gt;-fixation gene cluster B</td>
<td>(23, 24)</td>
</tr>
<tr>
<td>pRR116</td>
<td>pBR325 containing <em>R. rubrum</em> RubisCO gene in a 6.6-kb <em>EcoRI</em> insert</td>
<td>(80)</td>
</tr>
<tr>
<td>pRR2119</td>
<td>pBR322 derivative containing <em>R. rubrum rbpL</em> gene in a 2.4-kb <em>Sau3A</em> insert from pRR116</td>
<td>(80)</td>
</tr>
<tr>
<td>pSUPDI5</td>
<td>pSUP202 containing 3.15-kb <em>EcoRI</em> fragment encoding the <em>R. rubrum rbpL</em> deletion-insertion construct</td>
<td>This study</td>
</tr>
<tr>
<td>pRPS116</td>
<td>pRK404 containing 5.3-kb <em>HindIII</em> fragment derived from pRR116</td>
<td>This study</td>
</tr>
<tr>
<td>pRPSE66</td>
<td>pRK415 containing the <em>R. rubrum</em> 6.6-kb <em>EcoRI</em> fragment from pRR116</td>
<td>This study</td>
</tr>
<tr>
<td>pRPS101</td>
<td>pRK404 containing a 20-kb <em>BamHI</em> fragment of <em>R. rubrum</em> DNA from a λ library insert in λRR201 (80)</td>
<td>This study</td>
</tr>
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</table>
TABLE 4.1. Plasmids used in this study (cont.)

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRPS523</td>
<td>pRK404 containing a 523-bp region upstream of <em>R. rubrum</em> <em>rbpL</em> as a <em>BamH1-AluI</em> insert from pRR116</td>
<td>This study</td>
</tr>
<tr>
<td>pRKRX8</td>
<td>pRK415 containing 8.0-kb <em>XhoI</em> fragment encoding <em>rbpL</em> isolated from <em>R. rubrum</em> chromosomal DNA</td>
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</tr>
<tr>
<td>pRPS5C</td>
<td>pRK415 containing deletion derivative of pRKRX8 with 1.48-kb DNA upstream of <em>rbpL</em></td>
<td>This study</td>
</tr>
<tr>
<td>pRPS4A</td>
<td>pRK415 containing deletion derivative of pRKRX8 with 1.82-kb DNA upstream of <em>rbpL</em></td>
<td>This study</td>
</tr>
<tr>
<td>pRPSRV</td>
<td>pRK415 containing 4.5-kb <em>EcoRV</em> insert of pRKRX8 with 2.03-kb DNA upstream of <em>rbpL</em></td>
<td>This study</td>
</tr>
<tr>
<td>pRS115</td>
<td>pRK415 containing a 9.4-kb <em>HindIII-EcoRI</em> subclone of pJG336 encoding <em>R. sphaeroides</em> form I <em>cfx</em> genes</td>
<td>This study</td>
</tr>
<tr>
<td>pRPSRVFI</td>
<td>pRK415 containing a 2.03-kb upstream fragment from pRPSRV with the <em>R. sphaeroides fba, rbcLrbcS</em> genes inserted downstream</td>
<td>This study</td>
</tr>
</tbody>
</table>
in Erlenmeyer flasks containing complex peptone yeast extract (PYE) medium (93) or minimal-malate medium. *R. rubrum* strains carrying broad host-range plasmids to be used to determine *rbpL* expression and for other physiological analyses were first grown aerobically in minimal-malate medium with full antibiotic selection (Sm, Km and Tc). Cultures were then harvested, washed and resuspended to a cell density corresponding to an *A*$_{660}$ of approximately 0.5 in minimal-malate medium lacking antibiotics. Such cultures were then incubated anaerobically for phototrophic growth. This protocol was followed to minimize effects of plasmid loss after transferring cultures to photosynthetic growth conditions where the light-sensitive antibiotic, tetracycline, was not used. PYE medium containing 1.8% agar was employed for routine aerobic growth and genetic manipulations. Antibiotic concentrations used for selection of resistance markers in *R. rubrum* were (in µgrams per milliliter) streptomycin (Sm), 50; kanamycin (Km), 25; and tetracycline (Tc), 5. These levels were used for routine plasmid maintenance and selection of exconjugants. *E. coli* was grown in Luria-Bertani (LB) medium (54) with antibiotic concentrations previously described (16, Chapter 1). When necessary, LB agar plates contained 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) and isopropyl-β-D-thiogalactoside (IPTG) at concentrations of 40 µg/ml and 0.1 mM, respectively.

**Deletion mutagenesis of *rbpL***. Digestion of pRR2119 (80) with *SalI* removes two internal fragments from within the *rbpL* gene (63). Religation of the *SalI*-digested plasmid generated the deletion derivative, pRR2119d. A 1.4 kb-*SalI* cassette from pUC4K (87, 90) was then inserted into the remaining *SalI* site of pRR2119d. The 3.15-kb *EcoRI* fragment containing the deletion - insertion construct was ligated into pSUP202 (78) generating
pSUPDI5. Selection for the *R. rubrum rbpL* deletion strain was made by mating pSUPDI5, via *E. coli* SM-10, to *R. rubrum* Str-2 on nonselective PYE plates, and then plating cell mixtures from overnight incubations to PYE agar plates containing Sm and Km. Double recombinants were scored as those Sm<sup>r</sup>, Km<sup>r</sup> transconjugants that were Tc<sup>s</sup>, indicating the loss of plasmid vector sequences (78).

**Cloning.** A chromosomal *XhoI* fragment that hybridized to a 1.2-kb probe derived from the region upstream of the *rbpL* gene from pRR116 (80), was isolated from *R. rubrum* total DNA. *XhoI*-digested chromosomal DNA was separated by agarose gel electrophoresis and the region of the gel corresponding to the size of the *XhoI* hybridizing fragment was eluted using a Schleicher and Schull, Inc. (Keene, NH) "elutrap" apparatus. After phenol extraction and precipitation, the fragments were ligated into *SalI*-digested pUC1318 (44) and transformed into competent JM109 *E. coli* cells. White transformants on X-gal/ IPTG LB plates from the ligation were picked to fresh plates. Colonies were transferred to nitrocellulose membrane filters for colony hybridizations, which were performed by standard techniques (3) using the 1.2-kb DNA fragment upstream from *rbpL* as a probe. The overnight hybridization, performed in a Hybaid rotisserie hybridization oven (National Labnet Co., Woodbridge, NJ), was followed by two stringent washes (65°C, 1 X SSC, 0.1% SDS). The membranes were then exposed to X-OMAT AR film (Eastman Kodak, Rochester, NY). Plasmids from colonies giving positive signals were digested with various restriction enzymes known to cut within the sequenced *rbpL* gene (63) and compared to the restriction digest pattern of pRR116 to verify cloning of the desired *rbpL* containing fragment.

**DNA Sequencing.** Sets of nested deletions were made by
exonuclease III digestion by established protocols (3, 37) on subclones of the 8.0-kb XhoI DNA fragment isolated from R. rubrum total DNA. Sequencing of the various deletions was performed on double-stranded DNA templates in plasmids pK18 or pK19 (69) by the dideoxy chain termination method (74) using the Sequenase kit (version 2.0) from United States Biochemical (U.S.B.) (Cleveland, OH). 35S-dATP was obtained from DuPont New England Nuclear. Universal and reverse commercial primers were purchased from U.S.B. or New England Biolabs (Beverly, MA). Occasionally, dITP was used in place of dGTP to help resolve G+C compressions. Sequence analyses were performed using the International Biotechnologies software prepared by J. Pustell and the University of Wisconsin Genetics Computer Group package (Madison, WI).

Enzyme assays. All extracts for enzyme and immunological assays were prepared as described previously (16, Chapter 1). Ribulose 1, 5-bisphosphate carboxylase activity was measured by the assay described previously (95). Total protein concentration was determined by a modified Lowry protocol described by Markwell (55).

Immunological methods. Rocket immunoelectrophoresis was performed according to the procedure described previously (41). Antibodies specific to each RubisCO enzyme allowed quantitation of each protein in crude extracts by comparison to standards of the respective purified RubisCO protein.

DNA manipulations and conjugation techniques. Chromosomal DNA isolation from R. rubrum was performed essentially as described (17) using the sodium sarkosyl lysate procedure, scaled down to a starting cell culture volume of 60-ml. Conjugations were performed on filter pads for all matings (93). The helper plasmid pRK2013 (18) was used in triparental
matings for the routine mobilization of plasmids into *R. rubrum*. Diparental matings (for the construction of the *R. rubrum* *rbpL* deletion strain) employed *E. coli* strain SM-10 (78) as previously described for *R. sphaeroides* (16, Chapter 1).

All routine DNA manipulations including restriction endonuclease digestions, ligations and agarose gel electrophoresis were performed according to standard methods (3). Labelling of DNA fragments with $^{32}$P for use in Southern and colony hybridizations was by the random primed procedure using a kit supplied by Boehringer Mannheim (Mannheim, W. Germany).

**RESULTS**

**Generation and characterization of an *R. rubrum* RubisCO deletion strain.** The genetic techniques used to construct a dual RubisCO deletion strain of *Rhodobacter sphaeroides* (16, Chapter 1) were applied to delete the single RubisCO-encoding gene, *rbpL*, in *Rhodospirillum rubrum*. Transconjugants from matings with *R. rubrum* selected on Sm, Km media that displayed Tc-sensitivity arose at a frequency of 0.5%. Southern hybridization analysis of *EcoRI*-digested chromosomal DNA isolated from *R. rubrum* wild-type and presumptive *rbpL* strains, probed with an *rbpL*-containing DNA fragment, indicated the expected increase in size of the hybridizing fragment from the *rbpL* mutant. These results confirmed that the expected genetic replacement in the chromosome had occurred in the deletion construct.

When cultured under photosynthetic (anaerobic) conditions, the *rbpL* strain was incapable of photolithoautotrophic growth but was able to
grow under photoheterotrophic conditions in a defined malate-containing media or on complex (PYE) media. Such a phenotype sharply contrasted to results with RubisCO deletion strains of *Rhodobacter sphaeroides*, which were unable to grow under photoheterotrophic conditions with CO₂ as the electron acceptor. Cell extracts of *R. rubrum rbpL* strains that were cultured under photoheterotrophic conditions on malate displayed no RubisCO enzymatic activity nor RubisCO protein as determined immunologically (data not shown). Thus, *R. rubrum* has the capacity for photoheterotrophic growth in the absence of CO₂ reduction through the Calvin cycle.

Upon photoheterotrophic growth on malate, *R. rubrum* wild-type strain Str-2 had a generation time of 6 h compared with 13 h for *rbpL* strain I-19 and 9 h for strain I-32 (Fig. 4.1). *R. rubrum* strain I-32 was used as a positive control in growth experiments since it maintains plasmid pSUPDI5 integrated into the chromosome, and thus continues to synthesize RubisCO and grow photolithoautotrophically (data not shown). Since strain I-32 possesses additional plasmid sequences, including the kanamycin-resistance gene, that are also present in I-19, the overall growth rate attained by the *rbpL* strain is probably best reflected by comparison to strain I-32 (Fig. 4.1). Growth rates for cultures under nonselective, aerobic conditions for the *rbpL* and wild-type strains were similar (data not shown). While the *R. rubrum rbpL* deletion strain grew in 0.4% malate media under photoheterotrophic conditions, no growth was obtained using the reduced fatty acid, butyrate. It therefore appears that RubisCO activity is essential for photoheterotrophic growth on reduced substrates such as butyrate, a substrate which is readily utilized by wild-type *R. rubrum*. In addition, unlike the situation with RubisCO deficient strains of *R. sphaeroides*, where
Fig. 4.1. Growth curves of *R. rubrum* wild-type Str-2 (•—•), *R. rubrum rbpL* deletion strain I-19 (○—○) and *R. rubrum* I-32 (•--•) under photoheterotrophic conditions using 0.4% malate as carbon source and electron donor. Growth was measured in 800-ml cultures in 1-L flasks bubbled with argon.
reduction of an alternate electron acceptor such as dimethyl sulfoxide (DMSO) bypasses the requirement for RubisCO under photoheterotrophic conditions (16, Chapter 1). *R. rubrum rbpL* was unable to grow on butyrate in the presence of DMSO (data not shown). The ability of DMSO to substitute for CO₂ fixation during photoheterotrophic growth on reduced compounds thus represents another distinction between these two genera of photosynthetic bacteria.

**Complementation of *R. rubrum rbpL* strain I-19 with CO₂ fixation gene clusters from *Rhodobacter sphaeroides***. The two Calvin cycle gene clusters from *R. sphaeroides* exist as distinct transcriptional units, resulting in the duplication of many genes of the reductive pentose phosphate pathway, including the genes encoding enzymes unique to the cycle, i.e., RubisCO and phosphoribulokinase (24, 25, 34). Each of the two *R. sphaeroides cfx* clusters have been shown to be present on cosmid clones pJG336 (*cfx A* operon) and pJG106 (*cfx B* operon) (8, 24, 27, 28). *R. rubrum rbpL* derivatives containing plasmids pJG336 or pJG106 were obtained by triparental matings with *R. rubrum rbpL* and the appropriate *E. coli* host strains. Single exconjugants were initially screened for photosynthetic growth on plates in anaerobic chambers. The *R. rubrum rbpL* strain, harboring pJG336 or pJG106, grew well both photoheterotrophically and photolithoautotrophically. Complemented strains were then grown in liquid cultures under photolithoautotrophic conditions for physiological analysis (Table 4.2). Both *cfx* gene clusters from *R. sphaeroides* supported photolithoautotrophic growth at rates 6- to 9-h longer than the wild-type Str-2; both complemented I-19 strains achieved final cell densities (*A*₆₆₀) greater than 1.5. The presence of RubisCO activity and specific RubisCO synthesis confirmed expression of
the distinct genes encoding RubisCO from plasmids pJG336 and pJG106. The absence of the native \textit{R. rubrum} enzyme in both complemented strains was verified by rocket immunoelectrophoresis using antisera directed against \textit{R. rubrum} RubisCO, which further established that photolithoautotrophic growth was due solely to the activity of the \textit{R. sphaeroides} enzymes (data not shown). \textit{R. sphaeroides} RubisCO synthesized in the \textit{R. rubrum rbpL}+ complemented strains did not accumulate to the same levels as \textit{R. rubrum} RubisCO produced in the wild-type strain (Table 4.2). RubisCO activity in the complemented strains was 10- to 20-fold lower than that found in extracts from wild-type strain Str-2, correlating with the low level of RubisCO protein detected in the complemented strains. Both the A and B \textit{cfx} operons from \textit{R. sphaeroides} were therefore capable of \textit{trans} complementation of the \textit{rbpL}+ \textit{R. rubrum} mutant to photolithoautotrophic growth. These results also demonstrate that photolithoautotrophic growth is possible in \textit{rbpL}+ complemented strains containing foreign \textit{cfx} gene clusters, resulting in RubisCO specific activities that are less than 10-fold that of the wild type.

\textbf{Analysis of expression and complementation attempts with \textit{rbpL}+ containing DNA fragments from \textit{R. rubrum}.} An \textit{R. rubrum} DNA fragment encoding the \textit{rbpL} gene (pRPS116) was previously employed to complement an \textit{R. sphaeroides} RubisCO deletion strain to photosynthetic growth (16, Chapter 1). Expression of \textit{rbpL} directed by the \textit{R. rubrum} DNA fragment was found to be regulated; consequently, the 1.45-kb region upstream of the \textit{rbpL} gene was subcloned and employed as a promoter sequence in an expression vector for \textit{R. sphaeroides} (16, Chapter 1). Curiously, this \textit{R. rubrum rbpL} promoter fragment did not function to express \textit{rbpL} (or other inserted genes) in the \textit{R. rubrum} RubisCO deletion strain I-19; this was
Table 4.2. Photolithoautotrophic growth and RubisCO levels of *Rhodospirillum rubrum* wild type (Str-2) and I-19 *rbpL-* strains complemented with *Rhodospirillum sphaeroides cfx* gene clusters in plasmids pJG336 (*cfxA* operon) or pJG106 (*cfxB* operon).

<table>
<thead>
<tr>
<th><em>Rhodospirillum rubrum</em> Strain</th>
<th>Gen. Time (h)</th>
<th>RubisCO S. A. (U/mg)a</th>
<th>Protein (% Total)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Str-2</td>
<td>18</td>
<td>0.128</td>
<td>10.0</td>
</tr>
<tr>
<td>I-19(pJG336)</td>
<td>24</td>
<td>0.010</td>
<td>1.2</td>
</tr>
<tr>
<td>I-19(pJG106)</td>
<td>27</td>
<td>0.005</td>
<td>0.9</td>
</tr>
</tbody>
</table>

*a*RubisCO specific activity (S.A.) in μmol CO₂ fixed per min per mg total soluble protein.

*b*Expressed in terms of the RubisCO protein, percentage of the total soluble protein, as determined by rocket immunoelectrophoresis (41).
tested by determining the RubisCO activity and antigen levels in extracts. To ascertain whether the deletion mutation in the chromosomal \textit{rbpL} gene affected the plasmid-based \textit{rbpL} promoter activity in trans, plasmid constructs containing DNA inserts encoding RubisCO molecules that were antigenically distinct from the \textit{R. rubrum} enzyme, were conjugated into wild-type \textit{R. rubrum}. No trace of the plasmid-encoded RubisCO antigen was detected, indicating that the deletion-insertion mutation in the genome was not the cause of the inability to express \textit{rbpL} from the inserted plasmids.

DNA fragments possessing additional sequence upstream of \textit{rbpL} were then isolated to ascertain if \textit{rbpL} expression in the RubisCO deletion strain required such DNA in cis. Two fragments (from C. R. Somerville) (80), a 6.6-kb \textit{EcoRI} \textit{R. rubrum} DNA insert, and a 20-kb \textit{BamHI} insert, were cloned into plasmids pRK415 and pRK404, respectively, and transferred to \textit{R. rubrum} \textit{rbpL}' by conjugation. No complementation by the introduced DNA fragments was observed. However, RubisCO activity and antigen were present in extracts from photoheterotrophically grown cells (Fig. 4.2), demonstrating that sequences upstream of \textit{rbpL} are essential for \textit{rbpL} expression.

Because the \textit{R. rubrum} DNA fragments thus far tested did not result in complementation of the \textit{R. rubrum} \textit{rbpL}' strain, it was expected that additional DNA upstream of \textit{rbpL} might be necessary for full complementation to photolithoautotrophic growth. Such a requirement appeared likely, considering the positive complementation results obtained with the entire \textit{cfx} gene clusters from \textit{R. sphaeroides} (Table. 4.2). Thus, a fragment with additional DNA sequence upstream of \textit{rbpL} was sought. Southern blot analysis of \textit{R. rubrum} chromosomal DNA digested with a
Fig. 4.2. Complementation and \textit{rbpL} expression analysis of \textit{R. rubrum} DNA fragments containing the \textit{rbpL} gene. Complementation refers to restoration of photolithoautotrophic growth of RubisCO deletion strains of \textit{R. rubrum} and \textit{R. sphaeroides}. Levels of RubisCO expression in photoheterotrophically-grown \textit{R. rubrum} strain I-19 harboring the indicated plasmid. RubisCO specific activity, expressed as \( \mu \text{mol (U) CO}_2 \) fixed per min per milligram total soluble protein. RubisCO protein, expressed in terms of the total soluble protein, as determined by rocket immunoelectrophoresis (41).
### R. rubrum DNA fragment

<table>
<thead>
<tr>
<th>DNA Fragment</th>
<th>Complementation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>pRPS116</strong></td>
<td>- + 0.0 0.0</td>
</tr>
<tr>
<td>5.3 kb</td>
<td></td>
</tr>
<tr>
<td>1.45 kb</td>
<td></td>
</tr>
<tr>
<td><strong>pRPSE66</strong></td>
<td>- + 0.03 1.7</td>
</tr>
<tr>
<td>6.6 kb</td>
<td></td>
</tr>
<tr>
<td>2.75 kb</td>
<td></td>
</tr>
<tr>
<td><strong>pRPS101</strong></td>
<td>- + 0.05 2.7</td>
</tr>
<tr>
<td>20 kb</td>
<td></td>
</tr>
<tr>
<td>3.10 kb</td>
<td></td>
</tr>
</tbody>
</table>

### Rubisco

<table>
<thead>
<tr>
<th>Strain</th>
<th>S.A. (U/mg)</th>
<th>Protein (% Total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R. rub.</td>
<td>R. sph.</td>
<td></td>
</tr>
<tr>
<td>Str-2 (WT):</td>
<td>0.06</td>
<td>2.6</td>
</tr>
</tbody>
</table>

Fig. 4.2
variety of restriction endonucleases and probed with a 1.2-kb EcoRI-HindIII DNA fragment upstream of *rbpL* revealed an 8.0-kb *XhoI* hybridizing fragment. Since a *XhoI* site is present about 1.0-kb downstream of the *rbpL* gene, the 8.0-kb fragment should contain more sequence upstream of *rbpL* than the clones already acquired. The 8.0-kb *XhoI* fragment was isolated from *R. rubrum* Str-2 DNA as described in Materials and Methods and was shown by restriction endonuclease analysis to possess 5.0-kb upstream of *rbpL* (Fig. 4.3). When a plasmid (pRKRX8) containing this 8.0-kb insert was mated into strain I-19, *rbpL* was expressed as shown by immunologically quantitating the RubisCO protein (Fig. 4.3), in agreement with *R. rubrum* DNA fragments tested previously (Fig. 4.3). The 8.0-kb clone was also able to complement *R. sphaeroides* RubisCO deletion strains to all photosynthetic growth modes.

Several conclusions regarding complementation and RubisCO expression in *R. rubrum* and *R. sphaeroides* can be summarized. First, requirements for expression of the *R. rubrum* *rbpL* gene differ in the two strains. An insert encoding *rbpL* and 1.45-kb of upstream sequence promotes *rbpL* expression in *R. sphaeroides* but not in *R. rubrum* unless additional sequence preceding *rbpL* is present (Fig. 4.2). Second, although we have demonstrated that various *R. rubrum* DNA fragments result in *rbpL* expression in a *R. rubrum* *rbpL*<sup>-</sup> host, these fragments are unable to complement the *rbpL*<sup>-</sup> strain to photolithoautotrophic growth. Since the only sequences able to phenotypically complement *R. rubrum* *rbpL*<sup>-</sup> are the entire *cfx* gene clusters from *R. sphaeroides*, the genetic requirements for complementation of each RubisCO deletion strain differ.

**Nucleotide sequence analysis.** To examine sequences involved in the regulation of *rbpL*, and to ascertain the different requirements for
Fig. 4.3. Physical and genetic map of the *R. rubrum* CO₂ fixation gene cluster and various fragments used in expression analysis.

A. Restriction map of the 8.0-kb *XhoI* fragment isolated from *R. rubrum* Str-2 DNA. Arrows beneath indicate regions sequenced from subclones derived from exonuclease III digestions.

B. Diagram showing orientation and identity of genes upstream of *rbpL*. Schematic below represents plasmid inserts used to delimit lengths of DNA upstream of *rbpL* (in kilobase pairs) required for *rbpL* expression in *R. rubrum* and *R. sphaeroides* RubisCO deletion strains. Complementation refers to restoration of photolithoautotrophic growth of RubisCO deletion strains as indicated by, (+); and, (-) for no complementation to photolithoautotrophic growth. RubisCO expression, as determined by rocket immunoelectrophoresis of extracts of photoheterotrophically grown *R. rubrum* strain I-19 cells containing the respective plasmids.

N.D., not determined.
A

Xhol  BarnHI  HindIII  Xhol

B

tkl  prk  fbp  ps  SXR  rbpl

<table>
<thead>
<tr>
<th>Complementation</th>
<th>RubisCO Expression (%) Total Ptn</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R. rub.</td>
</tr>
<tr>
<td>pRR523</td>
<td>ND</td>
</tr>
<tr>
<td>pRPS116</td>
<td>-</td>
</tr>
<tr>
<td>pRPS5C</td>
<td>-</td>
</tr>
<tr>
<td>pRPS4A</td>
<td>-</td>
</tr>
<tr>
<td>pRPSRV</td>
<td>-</td>
</tr>
<tr>
<td>pRKRX8</td>
<td>-</td>
</tr>
</tbody>
</table>

Fig. 4.3
\textit{rbpL} expression in \textit{R. rubrum} and \textit{R. sphaeroides}, the nucleotide sequence of the region upstream from the \textit{R. rubrum} \textit{rbpL} was determined. Approximately 2.0-kb 5' of \textit{rbpL} was sequenced on both strands as indicated (Fig. 4.3A). A protein data base search of the translated \textit{R. rubrum} DNA sequence was performed for all six reading frames. Two divergently oriented open reading frames (ORF's) were tentatively identified. The first ORF revealed a gene situated upstream and oriented in the same direction of transcription as \textit{rbpL} that shows high identity to \textit{cfxR} genes, which encode LysR-type transcriptional activator proteins (38) recently identified in a number of autotrophic bacteria that utilize the Calvin cycle (29, 59, 89, 97). In particular, the deduced amino acid sequence is 48\% identical to the deduced \textit{cfxR} polypeptide sequence from \textit{Alcaligenes eutrophus} (97) and 69\% similar when conservative amino acid substitutions are considered. The \textit{cfxR} gene from the purple nonsulfur bacterium \textit{Chromatium vinosum} (89) shows 44\% amino acid residues identical with 64\% similarity (Fig. 4.4). The predicted molecular weight of the \textit{R. rubrum} \textit{cfxR} deduced product is 32,567 with an estimated pI of 9.66.

Adjacent to the \textit{cfxR} gene is an open reading frame (ORF) oriented divergently from \textit{cfxR}; from comparison to a recently determined nucleotide sequence of a \textit{cfx} gene from \textit{A. eutrophus} (96, unpublished), this ORF was identified as the pentose phosphate epimerase gene (\textit{ppe}). The translated sequence of the ORF is 65\% identical to \textit{ppe} from \textit{A. eutrophus} and 69\% similar. The placement of \textit{cfxR} and \textit{ppe} genes within the 1.9-kb region upstream of \textit{rbpL} permits analysis of the intergenic spaces, regions which might be important for control of both \textit{rbpL} and \textit{ppe} expression. The intergenic region between \textit{ppe} and \textit{cfxR} is 147 base pairs, while there are 178 base pairs between the \textit{cfxR} and \textit{rbpL} genes, which are oriented in the
Figure 4.4. Sequence comparisons of the \textit{cfxR} genes of various bacteria. Deduced amino acid sequences of \textit{cfxR} of \textit{R. rubrum} (Rr) compared with sequences from \textit{A. eutrophus} (Ae) (97) and \textit{C. vinosum} (Cv) (89). Identical residues to \textit{A. eutrophus} are marked with asterisks. Residues identical in all three \textit{cfxR} genes are shaded.
Fig. 4.4.
same direction. Three interrupted palindromes fitting the consensus, 5'T-N₁₁-A-3', which is the proposed control region for the LysR family of regulators (20, 30), are present as indicated (Fig. 4.5). Their locations, in front of the genes encoding rbpL and ppe, implicate the intergenic regions as sites for cfxR to elicit transcriptional control.

In addition, partial nucleotide sequence analysis, and Southern hybridization studies using R. sphaeroides gene probes, resulted in the identification of additional cfx genes. The identity of the partially sequenced genes was readily apparent due to the high degree of sequence similarity to cfx genes from R. sphaeroides (8, 27, 28). When translated and compared to the amino acid sequences of cfx polypeptides from R. sphaeroides, at least 40% identity was obtained for the sequenced portions of the DNA from R. rubrum. The ORF downstream and oriented in the same direction as the ppe gene is fbp, coding for fructose 1,6-bisphosphatase. Following fbp is the gene encoding phosphoribulokinase (prk) and finally transketolase (tkl) occupies the remaining sequence upstream of rbpL on the 8.0-kb XhoI fragment. Based on the size of the R. sphaeroides tkl gene, the tkl sequence extends approximately 240 base pairs beyond the XhoI site at the end of the 8.0-kb insert, which would likely prevent the synthesis of a functional transketolase enzyme from the XhoI fragment. Thus, the cfx gene region from R. rubrum encodes many of the genes found as clusters in different autotrophic bacteria and a conserved gene order is maintained; e.g., ppe, fbp, prk and tkl (59, 96, 97). However, R. rubrum represents a distinct cfx gene organization in the placement and orientation of both cfxR and rbpL within the cluster, which could imply a mechanism of regulation dissimilar to cfx genes from other organisms.
Figure 4.5. Nucleotide sequence and deduced amino acid sequences for the \textit{R. rubrum cfxR} gene and the intergenic spaces between \texttt{ppe} and \textit{cfxR} and \texttt{rbpL} and \textit{cfxR}. Arrows over the initiation site of each gene indicate direction of transcription. Regions underlined indicate potential LysR-type activator binding sites with the T and A in the putative consensus, T-N$_{11}$-A (30), indicated by $\|$ , upstream of both \texttt{ppe} and \texttt{rbpL} genes.
Further analysis of *rbpL* expression in *R. rubrum* and *R. sphaeroides.*

With the nucleotide sequence upstream of *rbpL* determined, the different lengths of sequence required for *rbpL* expression in the *R. sphaeroides* and *R. rubrum* background was evaluated. An EcoRV DNA fragment encoding *rbpL* and retaining 2.0-kb upstream of *rbpL* was subcloned from pRPSRV (Fig. 4.3B). This 4.6-kb insert was found to contain the shortest DNA region 5′ of *rbpL* that promoted *rbpL* expression in the *R. rubrum* RubisCO deletion strain (Fig. 4.3B). By subcloning truncated inserts derived from exonuclease III treated plasmid constructs, the delimited ends upstream of *rbpL* could be tested for their ability to direct *rbpL* expression *in vivo.* Extracts of *R. rubrum* I-19 cells grown photoheterotrophically, containing the deletion constructs pRPSSC and pRPS4A, displayed no RubisCO protein or activity (Fig. 4.3B). All *R. rubrum* DNA insert-constructs were also introduced to the *R. sphaeroides* RubisCO deletion strain to check for *rbpL* expression and therefore confirm the functional integrity of each plasmid construct. For constructs pRPS5C and pRPS4A, expression was demonstrated in *R. sphaeroides* RubisCO deletion strain 16. The nucleotide sequence critical for expression of *rbpL* in *R. rubrum* thus lies within 208 base pairs on the 5′ end of pRPSRV (Fig. 4.3B). *R. rubrum rbpL* promoter activity was thus evident for all plasmids introduced in *R. sphaeroides*, except pRPS523, which includes 327 base pairs in front of the *rbpL* translational start codon and within which an *rbpL* transcriptional initiation site has been mapped (51). The identity of the upstream gene proximal to *rbpL* as *cfxR* thus implicates CfxR as a requirement for *rbpL* expression in the *R. sphaeroides* host strain (Fig. 4.3B).

*R. rubrum* I-19 cells containing the 6.6-kb, 8.0-kb and 20-kb *R. rubrum*
DNA inserts, in plasmids pRPSE66, pRKRX8 and pRPS101, respectively, show a pattern of RubisCO synthesis that ranges from 1.7-5.8%, of the total soluble protein (Figs. 4.2 and 4.3B). However, in *R. rubrum rbpL* carrying pRPSRV, a plasmid which contains a 4.6-kb DNA insert, the extent of RubisCO expression is consistently 4- to 10-fold higher than that obtained with the other *rbpL*-encoding inserts.

**Additional characterization of *R. rubrum rbpL* complementation.**

The results demonstrated the inability of any of the *R. rubrum* DNA fragments thus far obtained to complement *R. rubrum* strain I-19 to photolithoautotrophic growth (Figs. 4.2 and 4.3). Positive complementation has only been observed with the entire *cfx* gene clusters from *R. sphaeroides* (Table 4.2). Because the *R. sphaeroides cfx* gene clusters are present in cosmids within 26- to 30-kb DNA inserts (24, 25), a 9.7-kb fragment, which primarily contains only the characterized *cfx* genes of the A cluster was isolated after subcloning EcoRI partial digests of pJG336 into pRK415. This subclone, pRS115, excludes at least 14-kb of downstream DNA present in the parental cosmid. After introduction of the subcloned derivative to the *R. rubrum rbpL* strain, complementation was still observed and RubisCO specific activities were in the range of those determined previously for cells harboring the entire cosmid (plasmid pJG336) (Table 4.2). Thus, *R. sphaeroides* DNA sequences, in addition to the known *cfx* genes, do not likely play a role in restoring the *R. rubrum rbpL* strain to photolithoautotrophic growth.

Finally, an experiment was designed in order to conclusively establish that the inability to complement *rbpL* strain I-19 with *R. rubrum* DNA was not due to some property inherent in the *R. rubrum* RubisCO enzyme itself; this experiment might also provide further evidence that successful
complementation of strain I-19 with the *R. sphaeroides* *cfx* gene clusters required the expression of an entire group of *cfx* genes. For these studies, an expression vector was constructed using the same *rbpL* upstream sequence which had previously been found to yield highly overexpressed *rbpL* in *R. rubrum*. The 2.03-kb upstream portion of pRPSRV (Fig. 4.3B) was subcloned as an *EcoRV-XbaI* fragment to vector pRK415, to which the *R. sphaeroides* genes encoding fructose 1,6-bisphosphate aldolase (*fba*) and the large and small subunit genes of RubisCO (*rbcLrbcS*) were inserted downstream. The construct, pRPSRVFI, introduced to *R. rubrum rbpL* by conjugation, resulted in no complementation to photolithoautotrophic growth. *R. sphaeroides* form I RubisCO was detected in extracts from photoheterotrophically grown cells, however, with a specific activity of 0.553 U/mg and a protein concentration that represented 10.7% of the total soluble protein. The pRPSRVFI construct did support photolithoautotrophic growth of the *R. sphaeroides* RubisCO deletion strain, where the level of RubisCO protein was over 26% of the total soluble protein, with a specific activity of 0.17 U/mg. Taken together, with the lack of complementation seen with the 8.0-kb *R. rubrum* DNA insert in pRKRX8, these results suggest that positive complementation depends on the coordinated synthesis of a complete set of CO₂ fixation enzymes.

**DISCUSSION**

The isolation of an *R. rubrum* strain that lacks RubisCO represents the second such mutant of a purple, nonsulfur photosynthetic bacterium. The *R. rubrum rbpL* deletion strain, I-19, like that of the RubisCO double
deletion strain of *R. sphaeroides*, is unable to grow photolithoautotrophically on a gas mixture of CO₂ and H₂. However, unlike the phenotype of the *R. sphaeroides* RubisCO deletion strain, which is incapable of photoheterotrophic growth on substrates such as malate and succinate without an external electron acceptor such as dimethyl sulfoxide, the *R. rubrum rbpL* deletion strain is able to grow photosynthetically on these substrates.

No traces of either RubisCO activity or antigen were detected in *R. rubrum rbpL* cell extracts from photoheterotrophically grown cultures, establishing that RubisCO is not essential under these photosynthetic conditions. This result tends to support the findings of Buchanan et al. (6), who demonstrated the existence of an alternate CO₂ reduction pathway in *R. rubrum*, specifically the reverse TCA cycle, in which pyruvate synthase and α-ketoglutarate synthase are key CO₂ fixation enzymes. In addition, whole-cell CO₂ incorporation studies suggest that some cellular carbon is provided by CO₂ (X. Wang and F. R. Tabita, unpublished). It must be emphasized, however, that the RubisCO deficient *R. rubrum* strain I-19 grows only photoheterotrophically (e.g., on malate with CO₂ as electron acceptor) and not photolithoautotrophically with CO₂ as the sole source of carbon like the green sulfur bacteria, which seem to use the reverse TCA cycle as the primary CO₂ assimilation pathway (14). Therefore, in *R. rubrum* the reverse TCA cycle, or some other CO₂ reduction pathway, does not appear to function solely to provide cellular carbon, but probably also helps to maintain a redox balance within the cell. While maintenance of a redox balance through the use of CO₂ as an electron acceptor, appears to contribute to the ability of *R. rubrum* I-19 to grow on malate, strain I-19 is incapable of photoheterotrophic growth on the more reduced
substrate butyrate. Conversely, in the *R. sphaeroides* RubisCO deletion strain, the presence of DMSO allows for photoheterotrophic growth on various carbon substrates, including butyrate (16, Chapter 1). This illustrates another distinction between the *R. rubrum* and *R. sphaeroides* RubisCO deletion strains, as DMSO does not support photoheterotrophic growth of *R. rubrum* I-19 on butyrate despite the apparent ability of *R. rubrum* to reduce DMSO (76). If the reverse TCA cycle or some other pathway operates to maintain redox balance, the above differences may indicate the limitation of this form of CO₂ fixation, since growth appears to be sustained only on oxidized substrates, such as malate or succinate.

Successful restoration of *R. rubrum rbpL* to photolithoautotrophic growth with either of the two *cfx* gene clusters from *R. sphaeroides* demonstrated that strain I-19 could be complemented by introduced, foreign DNA. Low levels of expression of the *R. sphaeroides* RubisCO-encoding genes in *R. rubrum* strain I-19 was evident, since ten-fold less RubisCO specific activity and RubisCO protein were obtained. This is in contrast to earlier results where *R. sphaeroides* RubisCO deletion strains carrying cosmids encoding *cfx* gene clusters led to elevated levels of RubisCO protein and activity, presumably because of gene dosage (16, Chapter 1). Depressed RubisCO activity and protein was also exhibited by the *R. rubrum rbpL* strain complemented by an *R. sphaeroides* DNA fragment which encodes the *cfxA* gene cluster almost exclusively. The reason why there are diminished levels of RubisCO in *R. rubrum* strain I-19 when the *R. sphaeroides* genes are expressed in this strain is not understood at present. However, these results certainly appear to accentuate differences between the two RubisCO deficient strains, particularly in the capacity to express introduced *cfx* genes in trans to wild-type levels.
Attempts to complement *R. rubrum rbpL* strain I-19 with *R. rubrum* DNA fragments were totally unsuccessful. On the other hand, every *R. rubrum rbpL* containing DNA fragment that was tested was found to complement the *R. sphaeroides* RubisCO negative strain to all photosynthetic growth modes; the only exception was plasmid pRR523, which contained an insert with only 327 base pairs upstream of *rbpL* (Fig. 4.3B). These findings further illustrate the basic distinctions between *R. rubrum* and *R. sphaeroides*.

From the results obtained thus far, it is apparent that the inability to complement *R. rubrum* I-19 may be resolved into two separate issues, each of which relate to fundamental differences between *R. rubrum* and *R. sphaeroides*. First of all, there are *R. rubrum* DNA inserts which promote *rbpL* expression in *R. sphaeroides* that are not expressed in *R. rubrum* itself. The second, distinct problem is with the *R. rubrum* DNA fragments that can direct *rbpL* expression in *R. rubrum*. These DNA fragments remain unable to complement *R. rubrum* I-19 to photolithoautotrophic growth even if *rbpL* expression is directed by a 20-kb *R. rubrum* DNA insert (Fig. 4.2). This finding lessens the possibility that chromosomal sequences downstream of *rbpL* may be required for complementation of *R. rubrum* I-19; i.e., those sequences that may be transcriptionally blocked by the Km<sup>f</sup> cartridge inserted within in *rbpL* do not appear to be important. Judging from the fact that the *R. rubrum rbpL* strain may be complemented to photolithoautotrophic growth by the *R. sphaeroides cfx* gene clusters at relatively low levels of RubisCO activity and protein (Table 4.2), the level of RubisCO synthesis in *R. rubrum* should have been adequate to support photolithoautotrophic growth in this organism (Fig. 4.2). Thus, the notion that additional DNA upstream of *rbpL* might be required for
complementation of *R. rubrum* provided the incentive to isolate an *R. rubrum* DNA fragment that contained additional sequences.

An 8.0-kb *R. rubrum* DNA insert encoding *rbpL* was acquired by direct isolation of *XhoI*-digested chromosomal fragments from agarose gels. The insert contained 5.0-kb of DNA sequence upstream of *rbpL*, but did not complement *R. rubrum* I-19 after the fragment was transferred to strain I-19 by conjugation with a broad host-range vector. This 8.0-kb clone was chosen as the source of DNA for nucleotide sequence analysis because it directed the synthesis of *rbpL* in *R. rubrum* and complemented the *R. sphaeroides* RubisCO deletion strain. In addition, this DNA fragment was derived from the parental Str-2 (wild-type) strain from which the *rbpL* strain I-19 was isolated.

A total of five open reading frames were identified in the region upstream of *rbpL*, four of which were identified as genes that encode enzymes of the Calvin reductive pentose phosphate pathway. Most of the genes were readily recognized by sequence comparisons to the corresponding genes from *R. sphaeroides* (8, 27, 28). The high degree of sequence similarity of the translated products permitted identification even in regions where only portions of the genes were sequenced, i.e., *fbp*, *prk* and *tkl* (Fig. 4.3A). The remaining *cfx* genes, *ppe* and *cfxR* were sequenced entirely, on both strands, and like the preceding three genes, *cfxR* was promptly identified by the high degree of sequence similarity to *cfxR* genes from *Chromatium vinosum* (89), *Alcaligenes eutrophus* (97) and *R. sphaeroides* (Gibson and Tabita, submitted). The *ppe* gene, on the other hand, coding for pentose phosphate epimerase, was not identified until a similar sequence in *A. eutrophus* was identified by Bowein *et al.*, (unpublished). Thus all the sequence upstream of *rbpL* on the *XhoI*
fragment was assigned a coding function. The gene identified as tkl, distal from rbpL on the \textit{XhoI} fragment, is not expected to encode a complete polypeptide. Based on sequence comparisons and the length of the \textit{R. sphaeroides} tkl, the coding region extends beyond the \textit{XhoI} site at the 5' end of the \textit{R. rubrum} fragment.

As previously noted, the sequences of the individual \textit{R. rubrum} cfx genes show high similarity to cfx genes from other autotrophic bacteria. Also apparent is the conserved organization of the cfx genes within the cluster. For example, the tandem arrangement, \textit{fbp}, \textit{prk} and \textit{tkl} is the same order found in the operons from \textit{R. sphaeroides} (8), \textit{A. eutrophus} (96), \textit{Rhodobacter capsulatus} (J. Shively, unpublished); \textit{fbp}, and \textit{prk} in \textit{Xanthobacter flavus} (59) are also arranged similarly. However, the \textit{R. rubrum} cfx gene organization is unique in that the transcriptional regulator gene, \textit{cfxR}, is situated within the cluster and in the same transcriptional orientation as \textit{rbpL}. This arrangement of structural genes and regulator gene is similar to the situation in \textit{Rhizobia} and related species (20, 75) where \textit{nodD} is analogous to \textit{cfxR} (75). Moreover, the product of the \textit{nodD} gene was shown to regulate transcription by interacting at sites known as \textit{nod} boxes upstream of the genes to be regulated (19, 20). Homologous sites upstream of the cfx A and B clusters in \textit{R. sphaeroides} (29), and upstream of \textit{ppe} and perhaps \textit{rbpL} in \textit{R. rubrum} (Fig. 4.5), warrant further analysis in order to ascertain the precise role that CfxR plays in controlling transcription.

The dissimilar requirements for \textit{rbpL} expression in \textit{R. rubrum} and \textit{R. sphaeroides} remains largely unresolved. The high levels of regulated expression by the upstream \textit{rbpL} sequence in \textit{R. sphaeroides}, as well as mRNA initiation mapping data (51), substantiates that this region contains
a genuine promoter. In addition, the lack of promoter activity exhibited by plasmid pRR523 (Fig. 4.3B) in *R. sphaeroides* substantiates the requirement for the intact *cfxR* gene, since this construct contains the entire intergenic space between *cfxR* and *rbpL*. Additional sequences upstream of *cfxR* necessary for expression in *R. rubrum* may be important to initiate transcription. The presence of the upstream *ppe* gene sequence may help stabilize the initiation complex between CfxR and the region between *ppe* and *cfxR* since the entire intergenic region is included within the nonfunctional pRPS116 construct.

The enhanced level of *rbpL* expression with constructs containing 2.0-kb of *R. rubrum* DNA upstream of *rbpL* (plasmid pRPSRV) is distinctive, since the elevated RubisCO levels observed have been obtained with only this insert, yet all the other fragments tested are maintained within the same replicon (Fig. 4.3B). The pronounced synthesis is likely to be an increase in *rbpL* promoter activity in plasmid pRPSRV, since similar levels of expression were observed with foreign genes inserted downstream of the 2.0-kb sequence, i.e. the *R. sphaeroides fba* and *rbcLrbcS* genes. The molecular basis for the different levels of expression obtained with the constructs depicted in Fig. 4.3B is not known at present, but the presence of adjacent *cfx* genes may have an important influence. For example, depending on the additional *cfx* genes expressed, an imbalance of pentose phosphate pathway intermediates may result, thereby signalling an effect on *rbpL* expression. Such an interpretation was alluded to with *R. sphaeroides* mutants interrupted in one of the two *cfx* gene clusters (28, Chapter 3). Disruption of one of the *R. sphaeroides cfx* operons effectively results in the expression of genes from only the intact operon. Strains bearing such mutations displayed altered RubisCO protein levels, implying
that a perturbed balance of active cfx enzymes affected the expression of other cfx genes (28, Chapter 3).

An unequivocal explanation for the inability thus far to complement the R. rubrum rbpL' strain (I-19) to photolithoautotrophic growth with R. rubrum DNA has not been realized as yet. Nevertheless, several lines of evidence support the premise that a complete set of cfx enzymes must be coordinately expressed. One result favoring this idea was the positive complementation of R. rubrum strain I-19 with cfx gene clusters from R. sphaeroides. Genes identified on the 8.0-kb R. rubrum DNA fragment do not represent an entire set of cfx genes, thus when a plasmid bearing an insert encoding various cfx genes is introduced into R. rubrum I-19, it is entirely conceivable that unequal levels of the respective gene products may be produced relative to the amounts expressed from chromosomal gene copies. Certainly, the levels of expression of plasmid-borne genes are usually increased due to the multicopy vectors on which they are transferred. However, these interpretations are further complicated by the recent finding that a single regulator protein, CfxR, appears to be responsible for controlling not only the level of RubisCO synthesis, but also seems to interact at numerous potential sites, thus influencing and controlling an assorted group of genes. It is thus apparent that the overall regulation of the CO₂ fixation genes in R. sphaeroides and R. rubrum may be quite complex and subject to subtle alterations. This will require additional analysis to further clarify the role of other components important for control, which hopefully might eventually lead to the identification of molecules or metabolites that play a role in transducing or signalling the regulator genes.
CHAPTER V

GENERAL DISCUSSION

When growing photolithoautotrophically, purple nonsulfur bacteria rely on the Calvin reductive pentose phosphate pathway for the assimilation of most of the carbon required by the cell. Under photoheterotrophic growth conditions, as the organic substrate is oxidized, the reduction of CO₂ serves primarily to maintain an overall balance of the redox state of the cell. The latter function was suggested many years ago by Stanier et al (83) and is supported by the diminished Calvin cycle enzyme levels obtained in extracts of cells grown in the presence of alternate electron acceptors such as dimethyl sulfoxide (35). Exogenous electron acceptors were also shown to substitute for the otherwise essential bicarbonate requirement for photoheterotrophic growth on reduced substrates such as butyrate. In this work, the ability of alternate electron acceptors to completely bypass the requirement of Calvin cycle-dependent CO₂ fixation was tested directly. The ability of an R. sphaeroides RubisCO deficient strain to grow on reduced substrates in the presence of a suitable electron acceptor fully substantiates the original suggestion that these organisms fix CO₂ via the Calvin cycle to maintain the cellular redox balance when growing photoheterotrophically (Chapter 1).

Since this work involved the construction and analysis of RubisCO deletion strains of two genera of purple nonsulfur photosynthetic bacteria, their growth capabilities can be compared. As shown (Fig. 5.1), R.
*sphaeroides* strain 16, lacking both RubisCO enzyme forms encoded by this organism, is unable to grow photosynthetically on the carbon and electron sources indicated, including malate under photoheterotrophic growth conditions. The *R. rubrum* RubisCO deficient strain, I-19, displays the same growth characteristics as *R. sphaeroides* 16, except that I-19 has the capacity for photoheterotrophic growth on malate with CO₂ as electron acceptor. In addition, the alternate electron acceptor DMSO does not substitute for CO₂ in *R. rubrum* strain I-19. Thus, as noted in Chapter 4, *R. rubrum* strain I-19 has some way of disposing of excess reducing equivalents to CO₂ when cultured photoheterotrophically on malate, presumably using alternative CO₂ fixation reactions, such as enzymes of the reductive tricarboxylic acid cycle (TCA), known to be present in *R. rubrum* (6).

The distinction between the photosynthetic growth capabilities of the two RubisCO deficient strains, however, is not as fundamental as initially assumed, as a derivative of *R. sphaeroides* 16 was isolated that acquired the ability to grow photoheterotrophically using malate as electron donor. This strain was initially isolated when *R. sphaeroides* strain 16, previously grown aerobically on complex (PYE) medium, was inoculated into malate minimal medium and allowed to incubate in the light for approximately two months. Upon examination of extracts from the malate grown culture, the strain was found to have no RubisCO activity nor traces of form I or form II RubisCO antigen. This isolate, designated *R. sphaeroides* strain 16-PHC (for *photoheterotrophic* competent), retained the photoheterotrophic growth phenotype after the strain was subcultured under nonphotosynthetic aerobic conditions on complex media. Strain 16-PHC thus appears to be a true genetic variant since photoheterotrophic growth on malate occurs
Fig. 5.1 Summary of growth capabilities for *R. sphaeroides* and *R. rubrum* RubisCO deletion strains.
with lag times that are typical for photosynthetic bacterial strains precultured aerobically and then transferred to photoheterotrophic conditions. Reisolation of a photoheterotrophic-competent variant from the parental *R. sphaeroides* 16 was made by incubating *R. sphaeroides* strain 16 under the same photosynthetic growth conditions on minimal malate liquid media. Cultures started with single colonies from PYE complex medium plates resulted in the isolation of a strain of *R. sphaeroides* 16 for each colony tested. Growth was observed in the sealed test tubes after a incubation period of about six weeks. During the incubation period, cultures were monitored for the first sign of turbidity to determine a minimum time in which the photoheterotrophic "competent" phenotype would develop. The culture medium remained virtually clear for most of the incubation period except for cell clumps from the initial inoculum. After a 5 to 6 week period, a sudden onset of growth occurred with a doubling time of 7.7 h. After streak purification, photoheterotrophic isolates were again cultured to confirm the original RubisCO-negative phenotype. All strain 16-PHC isolates lacked RubisCO entirely and remained incapable of photolithoautotrophic and photoheterotrophic growth on butyrate. The *R. sphaeroides* 16-PHC phenotype thus appears very similar to *R. rubrum* 1-19 in that both strains possess the ability to grow photoheterotrophically on malate with CO₂ as electron acceptor (Fig. 5.1). *R. sphaeroides* 16-PHC was able to attain densities (660 nm) of over 2.0 when grown on malate. The acquisition of this photoheterotrophic growth phenotype is presumably due to some form of genetic alteration because of the extended time required to select for the strain; when the strain is transferred to the malate minimal medium from nonphotosynthetic growth conditions, the phenotypic characteristics remain stable. The capacity of
RubisCO deficient strains of *R. sphaeroides* to develop the photoheterotrophic-competent phenotype exemplifies the metabolic diversity already recognized for this group of organisms. In this respect, the possibility exists that the photoheterotrophic growth capability is due to a latent biochemical pathway, such as a remnant of a more primitive growth mode that has resurfaced in the RubisCO deletion strains. Since the phenotype is comparable to *R. rubrum* I-19, the attempt to detect enzyme activities critical for the operation of the reverse tricarboxylic acid cycle was made. No enzymatic reactions characteristic of the reverse TCA cycle were detected, i.e., the ferredoxin-dependent pyruvate synthase and α-ketoglutarate synthase (X. Wang, F.R. Tabita, unpublished) and pyruvate carboxylase and phosphoenol pyruvate carboxylase apparently do not play a role as well. Thus, the mechanism by which excess electrons derived from substrate (malate) oxidation are dissipated in the RubisCO deletion strains remains to be established. Recently, the observation was made that both *R. sphaeroides* strain 16 and *R. rubrum* I-19 can grow photoheterotrophically an acetate, thus further extending the range of carbon sources capable of being utilized by the RubisCO mutant strains (Y. Qian, F. R. Tabita, unpublished). This finding substantiates the early proposal (83), that little reduction of CO₂ should take place when cells are grown on organic substrates such as acetate, which are more oxidized than cell carbon. The ability of *R. sphaeroides* 16 to grow photoheterotrophically on acetate, and not on malate, demonstrates the significance of the substrate reduction level for photoheterotrophic growth. Presumably, the minor amount of CO₂ reduction that is necessary for malate assimilation may be accounted for by existing CO₂ uptake reactions.

Physiological manifestations of the distinct regulatory properties of
the two forms of RubisCO of *R. sphaeroides* were repeatedly noted in this study. One recurring observation was the apparent inactivation of form I RubisCO when *R. sphaeroides* cells were cultured under non-ideal conditions. When the plasmid-based *R. rubrum* promoter (pRPS, Chapter 1) was used to overexpress the form I RubisCO in *R. sphaeroides*, the levels of activity never approached the activity expected for the amount of form I protein synthesized (Tables 1.2, 1.3). On the other hand, when form II RubisCO was expressed to artificially high levels in *R. sphaeroides* strain 16, enzymatic activity was up to 10 times higher than that of the form I type enzymes (Table 1.3). The other situation that resulted in diminished form I RubisCO activity occurred when *R. sphaeroides* strains possessing only a single RubisCO type were incubated under limiting CO₂ conditions (Chapter 3). Thus, the enzyme affected under circumstances that necessitate modulation of RubisCO activity appears to be form I RubisCO and not the form II enzyme.

The differences in the genetics of CO₂ fixation between *R. sphaeroides* and *R. rubrum* were presented in Chapter 4. The major distinction, that only DNA fragments introduced to *R. rubrum* I-19 encoding "complete" CO₂ fixation gene operons can complement it to photolithoautotrophic growth, was suggested by the lack of complementation with an *R. rubrum* DNA fragment which expressed RubisCO and encoded a number of CO₂ fixation genes. That complementation of *R. rubrum* I-19 requires the expression of an entire group of *cfx* genes was also supported by results from the analysis of various *R. sphaeroides* *cfx* gene mutants described in Chapter 3. In one case (the *fbaA*⁻*fbpB*⁻ strain), the genotype of *R. sphaeroides* was modified such that only a specific set of *cfx* genes were expressed. The resultant phenotype,
the ability to grow photoheterotrophically on malate, resembled the non-complemented *R. rubrum* strain or *R. sphaeroides* strain 16-PHC. This experiment illustrated that a strain deficient in a single *cfx* gene (*fba*) can confer the specific phenotype of photoheterotrophic growth on certain oxidized substrates, such as malate, but not reduced substrates such as butyrate. Mutant strains of *R. sphaeroides* that express only part of a *cfx* gene cluster exhibited pronounced lag times during photoheterotrophic growth. As described (Chapter 3), *R. sphaeroides* strains with mutations leading to the expression of an entire *cfx* operon but only incomplete expression of the other *cfx* cluster, presumably resulted in an imbalance in the level of enzymes duplicated within both clusters. Specifically, in the strain characterized in Chapter 3, the inactivation of the *fbaA* gene, encoding fructose 1,6-bisphosphate aldolase (*Fba*), results in a strain that synthesizes two genomic copies of *fbp* and *prk*, encoding fructose bisphosphatase (*FBPase*) and phosphoribulokinase (*PRK*), respectively, but one copy of *Fba*, encoded by *fbaB*. The outcome of such a situation, as pointed out by Dr. Janet Gibson (28), is a probable imbalance in the proper levels of fructose 1,6-bisphosphate since FBPase and Fba catalyze the breakdown and synthesis of fructose 1,6-bisphosphate, respectively. If fructose 1,6-bisphosphate is a signal metabolite or a precursor to one, the above scenario might lead to aberrant regulation of the *cfx* genes as a whole.

The identification of a cluster of Calvin cycle CO₂ fixation genes from *R. rubrum* permits a comparison of the genetic organization of *cfx* genes from other bacteria (Fig. 5.2). Clustering of the genes from *R. rubrum*: *ppe, fbp, prk*, and *tkl*, are found in other organisms, including
Fig. 5.2. Comparison of cfx gene organization from 1, *Rhodospirillum rubrum*; 2, *Rhodobacter sphaeroides* (8, 27, 28); 3, *Rhodobacter capulatus* (Shively, unpublished); 4, *Chromatium vinosum* (89); 5, *Alcaligenes eutrophus* (97). Duplications of cfx genes occur as indicated. The duplicated cfx cluster in *A. eutrophus* is due to one copy encoded on an endogenous plasmid. The curved arrows indicate those cases in which the cfxR gene product has been shown to effect expression and/or shown to directly interact in DNA binding experiments (29, 89, 97).
Fig. 5.2
nonphotosynthetic bacteria. The most recurrent organization is the
tandemly arranged, *fbp*, *prk*, and *tkl* genes. Other structural genes
present, including *rbcLrbcS* are in somewhat different configurations, the
most common being those *cjx* clusters in which the first genes are *rbcLrbcS*,
as exemplified by *Alcaligenes eutrophus* (97) and *Xanthobacter flavus* (59).

It is expected that a number of the *cjx* genes identified in *R. rubrum*
will be cotranscribed. This is based on the close spacing between the genes
and the overall similarity to the *cjx* genes from *R. sphaeroides*, in which
cotranscription of each gene cluster is supported by a considerable amount
of experimental evidence (8, 24, 25, 27, 28, 34, 35). The reason for such a
grouping, other than to facilitate coordinate control, may also be to allow
the formation of multienzyme complexes. Early work reported with *R. rubrum*
demonstrated the copurification of two Calvin cycle enzymes (40).
In that study, FBPase and PRK were isolated from *R. rubrum* as a complex.
The possibility for even larger complexes composed of several Calvin cycle
enzymes also exists. In spinach, a five-enzyme complex was isolated (32).
While the enzyme activities identified were not all those represented within
the *cjx* gene clusters indicated in Fig. 5.2, the possibility exists that such
complexes might be found in autotrophic bacteria. The functional
advantage of such a complex would be the channeling of Calvin cycle
intermediates from one active site to another, reducing inefficiency due to
potential diffusion (32).
LIST OF REFERENCES


