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CbbR, a LysR-Type Transcriptional Activator, Is Required for Expression of the Autotrophic CO_2 Fixation Enzymes of *Xanthobacter flavus*

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Xanthobacter flavus **is able to grow autotrophically with the enzymes of the Calvin cycle for the fixation of CO_2, which are specified by the *cbbLSXFP* gene cluster. Previously, the 5′ end of an open reading frame (*cbbR*), displaying a high sequence similarity to the LysR family of regulatory proteins and transcribed divergently from *cbbLSXFP*, was identified (W. G. Meijer, A. C. Arnberg, H. G. Enequist, P. Terpstra, M. E. Lidstrom, and L. Dijkhuizen, Mol. Gen. Genet. 225:320–330, 1991). This paper reports the complete nucleotide sequence of *cbbR* and a functional characterization of the gene. The *cbbR* gene of *X. flavus* specifies a 333-amino-acid polypeptide, with a molecular weight of 35,971. Downstream from *cbbR*, the 3′ end of an open reading frame displaying a high similarity to ORF60K from *Pseudomonas putida* and ORF261 from *Bacillus subtilis* was identified. ORF60K and ORF261 are located at the replication origin of the bacterial chromosome. Inactivation of *cbbR*, via the insertion of an antibiotic resistance gene, rendered *X. flavus* unable to grow autotrophically. This was caused not by an inability to oxidize autotrophic substrates (e.g., formate) but by a complete lack of expression of the *cbb* genes. The expression of the CbbR protein in *Escherichia coli* was achieved by placing *cbbR* behind a strong promoter and optimization of the translational signals of *cbbR*. CbbR binds specifically to two binding sites in the *cbbR-cbbL* intergenic region.**

Xanthobacter flavus is a gram-negative autotrophic bacterium, using methanol, formate, and molecular hydrogen as its energy source. During methylotrophic growth, methanol is oxidized via a PQQ-dependent methanol dehydrogenase to formaldehyde, and subsequently to formate and carbon dioxide by formaldehyde and formate dehydrogenases. While growing on methanol, formate, or molecular hydrogen, *X. flavus* employs the Calvin cycle to assimilate CO_2 (27). Ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisC/O) and phosphoribulokinase are characteristic for this CO_2 assimilation pathway. Other enzymes functioning in the Calvin cycle are also required during heterotrophic growth, e.g., fructose 1,6-bisphosphatase (41).

Several genes encoding Calvin cycle enzymes have been cloned from *X. flavus*, and these were referred to as *cfx* genes (26). Recently, a new nomenclature (*cbb*) was adopted for these genes, creating a uniform designation for the Calvin cycle genes in *Rhodobacter sphaeroides*, *Alcaligenes eutrophus*, and *X. flavus* (42). In addition to the RuBisC/O-encoding genes (*cbbL*, *cbbS*), several other Calvin cycle genes have been cloned from *R. sphaeroides*, *A. eutrophus*, and *X. flavus* and characterized (2, 22, 26, 28, 43). In *X. flavus*, the genes encoding RuBisC/O and phosphoribulokinase (*cbbP*) are most likely organized in an operon, together with the fructose 1,6-bisphosphatase-encoding gene (*cbbF*) and a gene (*cbbX*) encoding a protein with unknown function (22, 25, 26, 28). Detailed physiological studies made it clear that the levels of available carbon and energy sources determine whether, and to what extent, the *cbbLSXFP* operon is expressed. Maximal expression requires the absence of multicarbon substrates and the presence of methanol, formate, or hydrogen (7, 8, 26, 27).

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The molecular basis of the regulation of the *cbbLSXFP* operon in *X. flavus* is unclear. Upstream of the *cbbLSXFP* operon, a gene (*cbbR*) transcribed divergently from *cbbLSXFP* and belonging to the LysR class of regulatory proteins was identified (25, 26). Proteins belonging to this class generally are transcriptional activators, controlling a wide range of metabolic processes (14, 36). Most LysR-class activators bind to the DNA between the genes they control and the gene by which they are encoded. The binding sites of the LysR-type proteins have a common motif (T-N_{11}-A) as the core of an inverted repeat, designated the LysR motif (12). A 5-bp inverted repeat containing a LysR motif is present in the *cbbR-cbbL* intergenic region of *X. flavus* (12, 25, 26). In this paper, we describe the characterization of *cbbR* and its gene product. From the results, we conclude that *cbbR* is a transcriptional activator of the *cbbLSXFP* operon. CbbR binds specifically to two sites in the *cbbR-cbbL* intergenic region.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1.

Media and growth conditions. *X. flavus* was grown on minimal medium (23), supplemented with succinate (15 mM), gluconate (15 mM), methanol (0.5%), or formate (20 mM) as previously described (27). Autotrophic growth on an H_2-CO_2-air mixture was done as described previously (27). *X. flavus* was grown on formate in a batch fermentor, with a working volume of 3 liters. The pH was kept constant by automatic titration with formic acid (25% [vol/vol]). *Escherichia coli* strains were grown on Luria-Bertani (LB) medium at 37°C (32). When appropriate, the following supplements were added (concentrations given in micrograms per milliliter, except as otherwise noted): ampicillin, 100; 5-bromo-4-

TABLE 1. Bacteria and plasmids used in this study

Strain or plasmid	Genotype or characteristics	Source or reference
Strains		
E. coli		
JM101	Δ(*lac-proAB*) [F$^+$ *proAB lacI*^{q}ZΔM15]	49
S17-1	*thi pro res mod*$^+$ Sm^r Tp^r *recA* RP4-2 (Tc::Mu; Km::Tn*7*)	38
C600	*supE hsdR*	32
X. flavus		
H4-14		24
R22	*cbbR*::Km^r	This study
Plasmids		
pTZ18U	Ap^r *lacZ'*	Bio-Rad
pJRD184	Ap^r Tc^r	15
pUC4K	Ap^r Km^r	45
pSUP5011	Ap^r Km^r Cm^r Tn*5*::Mob	37
pPROK-1	Ap^r *tacp*	Clontech
pXA1	Tc^r incP1 *mob cbbR* ϕ(*cbbL'-lacZ*)	26
pXA2	Tc^r incP1 *mob 'cbbR* ϕ(*cbbL'-lacZ*)	This study
pSR1	Ap^r *cbbR cbbL'*	This study
pSR7	Ap^r *cbbR' cbbL'*	This study
pSR10	Ap^r *'cbbR*	This study
pKR1	Ap^r Km^r *cbbR*::Km^r	This study
pKR2	Ap^r Tc^r Km^r *cbbR*::Km^r	This study
pKR3	Ap^r Tc^r Km^r *mob cbbR*::Km^r	This study
pER93	Ap^r *cbbR'*, ATG initiation codon	This study
pER94	Ap^r ϕ(*tacp-cbbR*), ATG initiation codon	This study

chloro-3-indolyl-β-D-galactoside (X-Gal), 20; isopropyl-β-D-thiogalactoside (IPTG), 0.1 mM; kanamycin, 50 (*E. coli*) or 5 (*X. flavus*); and tetracycline, 12.5 (*E. coli*) or 5 (*X. flavus*). Agar was added for solid media (1.5%).

Enzyme assays. Cell extracts were prepared as described previously (26). RuBisC/O activity was determined by measuring the ribulose bisphosphate-dependent $^{14}CO_2$ fixation in cell extracts (11). The maximum capacity of washed cells to oxidize formate was determined according to Dijkhuizen and Harder (9). Protein was determined according to Bradford (3).

Immunological techniques. The amount of RuBisC/O protein was determined by a modified rocket immunoelectrophoresis protocol (17, 21), with antibodies raised against purified *X. flavus* RuBisC/O (26).

DNA manipulations. Plasmid DNA was isolated via the alkaline lysis method of Birnboim and Doly (1). Chromosomal DNA was isolated following cell lysis with sodium dodecyl sulfate (SDS) as described by Lehmicke and Lidstrom (22). DNA-modifying enzymes were obtained from Boehringer (Mannheim, Germany) and were used according to the manufacturer's instructions. DNA fragments were isolated from agarose gels by adsorption to glass (Geneclean kit; Bio 101, La Jolla, Calif.). Other DNA manipulations were done according to standard protocols (32).

Southern hybridizations. DNA was transferred to nylon membranes (GeneScreen Plus, DuPont) via capillary transfer as specified by the manufacturer. Prehybridization, hybridization, and washing conditions were done as suggested by the manufacturer at 65°C. DNA fragments used as probes were labelled with [α-^{32}P]dCTP with the random primed labelling kit supplied by Boehringer.

Nucleotide sequencing. A DNA fragment containing the *cbbR* gene was cloned in two orientations in pTZ18U, generating pSR1 and pSR10. A nested set of unidirectional deletions of pSR1 and pSR10 was created by digestion with exonuclease III and mung bean nuclease essentially as described by Henikoff (13). Infection of *E. coli* JM101 containing pSR1, pSR10, and their derivatives with the helper phage M13K07 (46) and purification of single-stranded DNA were done as described previously (32). Dideoxy sequencing reactions were performed with modified T7 DNA polymerase (Sequenase; U.S. Biochemical Corporation, Cleveland, Ohio) and ^{35}S-dATP as recommended by the manufacturer. In addition to the sequencing reactions employing dGTP, at least one strand was also sequenced with dITP to eliminate compressions. The nucleotide sequence data were compiled and analyzed with the programs supplied in the PC/GENE software package (IntelliGenetics, Mountain View, Calif.).

Mobilization of plasmids. Mobilization of plasmids with *E. coli* S17-1 containing the appropriate plasmids as the donor was performed essentially as described by Simon et al. (38).

Construction of an *X. flavus cbbR* mutant. A 1.3-kb *Sal*I fragment from pUC4K, encoding kanamycin resistance, was inserted into the unique *Sal*I site within *cbbR*. The resulting plasmid, pKR1, was digested with *Xba*I, treated with Klenow enzyme, and digested with *Kpn*I. The DNA fragment containing *cbbR*::Km was subsequently ligated into *Hpa*I- and *Kpn*I-digested pJRD184 (pKR2). The *mob* site from pSUP5011 was isolated as a *Bam*HI fragment and ligated into pKR2, yielding pKR3. *E. coli* S17-1 was transformed with pKR3, and the plasmid was subsequently mobilized to *X. flavus*. Exconjugants were plated on minimal medium containing succinate and kanamycin. Since pKR3 is unable to replicate in *X. flavus*, kanamycin resistance can be acquired only if the mutated *cbbR* gene is integrated into the chromosome. Kanamycin-resistant colonies appeared with a frequency of 10^{-7} and were subsequently screened for tetracycline susceptibility, indicating the loss of vector sequences. Southern hybridization experiments confirmed that a double recombination event had taken place, replacing the *cbbR* gene with *cbbR*::Km^r in *X. flavus* R22.

Expression of CbbR in *E. coli*. A 558-bp *Dra*I-*Sal*I fragment containing the 5′ end of *cbbR* was mutagenized via the method described by Kunkel et al. (19), with a synthetic oligonucleotide (5′-GTAGGCATTCAGGAAAGAATTCAT GGCGCCCCACTGGAC-3′) synthesized by an Applied Biosystems 381A DNA synthesizer. In this way, the GTG initiation codon of *cbbR* was changed into an ATG codon preceded by an *Eco*RI restriction site (pER93). Sequencing of the *Dra*I-*Sal*I fragment confirmed that only the desired mutations had occurred. Subsequently, a *Sal*I-*Hin*dIII fragment containing the 3′ end of *cbbR* was ligated into pER93. The mutagenized *cbbR* was then cloned as an *Eco*RI-*Hin*dIII fragment into the expression vector pPROK-1 (Clontech Laboratories, Palo Alto, Calif.), yielding pER94. In pER94, the expression of *cbbR* is under the control of the *tac* promoter.

E. coli C600 transformed with pER94 or pPROK-1 was grown on LB medium, diluted into fresh LB medium, and grown until an optical density at 663 nm of 0.5 was reached. IPTG was added to a final concentration of 1 mM, and growth was allowed to proceed for an additional 4 h. Cells were harvested via centrifugation, washed once in ice-cold binding buffer (25 mM Tris-HCl, 1 mM EDTA, 0.1 mM

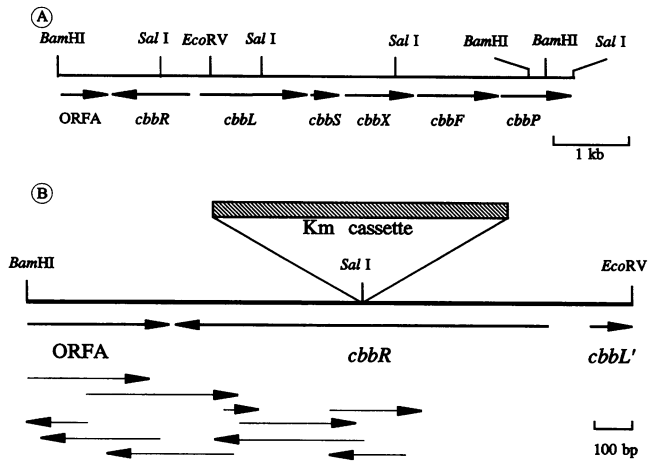

FIG. 1. Restriction map of the *cbb* operon (A) and the *cbbR* gene and flanking regions from *X. flavus* (B). The positions and directions of transcription of the genes and ORFA are indicated by arrows below the restriction map. The insertion of the kanamycin resistance cassette in *cbbR* is indicated with a striped bar. The sequence strategy is shown beneath the restriction map.

dithiothreitol, 15% [vol/vol] glycerol), and resuspended in the same buffer. Extracts were prepared freshly by passing the cell suspension twice through a French pressure cell (1.4×10^5 kN/m^2). Cell debris was removed by centrifugation, and the resulting cell extract was used in the DNA binding assay.

Preparation of the DNA fragment used in the binding study. Plasmid pSR7 (5 μg) was digested with *Hin*dIII and *Eco*RV, liberating a 307-bp fragment containing the *cbbR-cbbL* intergenic region. The *Hin*dIII-*Eco*RV fragment was labelled with [α-^{32}P]dCTP (3,000 Ci/mmol, 1 mCi/ml) by filling in the recessive ends with the Klenow fragment of DNA polymerase (32). The labelling mixture was subsequently applied to a nondenaturing acrylamide gel (4%), and the *Hin*dIII-*Eco*RV fragment was isolated via electroelution (32).

DNA binding assay. Various amounts of cell extract were incubated with the labelled *Hin*dIII-*Eco*RV fragment of pSR7 (9,000 cpm) in binding buffer with 50 μg of bovine serum albumin per ml and 100 μg of salmon sperm DNA per ml at 30°C. After 30 min, the samples were loaded on a 6% nondenaturing acrylamide gel in Tris-borate buffer and run at 4°C at 10 V/cm (32). The gels were subsequently dried and autoradiographed with intensifying screens at −80°C.

Nucleotide sequence accession number. The nucleotide sequence presented in this paper was entered into the EMBL nucleotide sequence data base under accession number Z22705.

RESULTS

Nucleotide sequence of *cbbR* and downstream DNA. We previously reported the nucleotide sequence of the 5′ end of the *cbbR* gene (25, 26). To further characterize *cbbR*, its complete nucleotide sequence was determined according to the strategy depicted in Fig. 1. The nucleotide sequence of the *Eco*RV-*Bam*HI fragment containing ORFA and *cbbR* is shown in Fig. 2. The *cbbR* gene specifies a 333-amino-acid protein with a molecular weight of 35,971. In the nucleotide sequence downstream from *cbbR*, the 3′ end of an open reading frame (ORFA) was detected. The deduced amino acid sequence of ORFA was compared with sequences in the PIR protein data base (release 34.0) with the program FASTA. This revealed a high degree of similarity with the carboxyl-terminal part of ORF60K from *Pseudomonas putida* and ORF261 from *Bacillus subtilis* (29). A comparison of ORFA with the ORF60K and ORF261 proteins is shown in Fig. 3.

Construction of a *cbbR* mutant. The functionality of *cbbR* was tested by inserting a kanamycin resistance gene into *cbbR* via a double recombination event, as outlined in Materials and Methods. The resulting mutant, *X. flavus* R22, was unable to grow autotrophically with methanol, formate, or molecular hydrogen as an electron donor. Heterotrophic growth on media containing succinate or gluconate as carbon source was indistinguishable from that of the wild-type strain. Introduction of an intact *cbbR* gene on a *Bam*HI-*Eco*RV fragment (pXA1) restored autotrophic growth of *X. flavus* R22, comparable to that of the wild type. However, pXA2, containing a *Sal*I-*Eco*RV fragment with a truncated *cbbR* gene (Fig. 1), was not able to do so. It is therefore concluded that the inability of *X. flavus* R22 to grow autotrophically is not caused by a second site mutation but is due to the disruption of *cbbR*.

Characterization of *X. flavus* R22. The disruption of *cbbR* prevents autotrophic growth of *X. flavus* R22. This can be explained by assuming that CbbR activates the transcription of the *cbb* genes or, alternatively, that CbbR is required for the oxidation of methanol, formate, and molecular hydrogen. To distinguish between these possibilities, the *cbbLSXFP* genes were induced by adding formate (20 mM) to cultures of *X. flavus* and the *cbbR* mutant strain R22, growing exponentially on gluconate (5 mM) in a batch fermentor.

Immediately after the addition of formate, the capacity to oxidize formate increased equally in both wild-type and mutant strains. Two hours after the addition of formate, RuBisC/O protein and activity were observed in the cell extracts of *X. flavus*, but remained undetectable in *X. flavus* R22 (Fig. 4). These results clearly show that an intact *cbbR* gene is not required for the oxidation of formate. A functional *cbbR* gene is required for the fixation of CO_2 by RuBisC/O, since RuBisC/O protein and activity are absent in *X. flavus* R22.

Expression of CbbR in *E. coli*. Genes initiating with a GTG codon, such as *cbbR*, are translated less efficiently than genes starting with an ATG codon (18). Furthermore, regulatory proteins belonging to the LysR class are frequently subject to autoregulation, which would preclude a high expression of CbbR (12, 14). Because of this, the GTG initiation codon of *cbbR* was replaced by an ATG codon, and the region upstream of *cbbR* was replaced with a *tac* promoter and a strong *E. coli* ribosome binding site (pER94). *E. coli* C600 was transformed with pER94 and pPROK-1, and expression from the *tac* promoter was subsequently induced with IPTG, as outlined in Materials and Methods. Analysis of the cell extracts on a denaturing acrylamide gel (Fig. 5) showed that a protein with an apparent molecular weight of 36,000 was expressed in *E. coli*(pER94) but not in *E. coli*(pPROK-1). The observed molecular weight of this protein is in good agreement with the molecular weight of CbbR predicted from the deduced amino acid sequence.

CbbR binds to the *cbbR-cbbL* intergenic region. The ability of CbbR to specifically bind to the *cbbR-cbbL* intergenic region was determined via a band-shift assay. Increasing amounts of cell extract of *E. coli*(pPROK-1) or *E. coli*-(pER94) were incubated with a labelled *Hin*dIII-*Eco*RV fragment containing the *cbbR-cbbL* intergenic region (Fig.

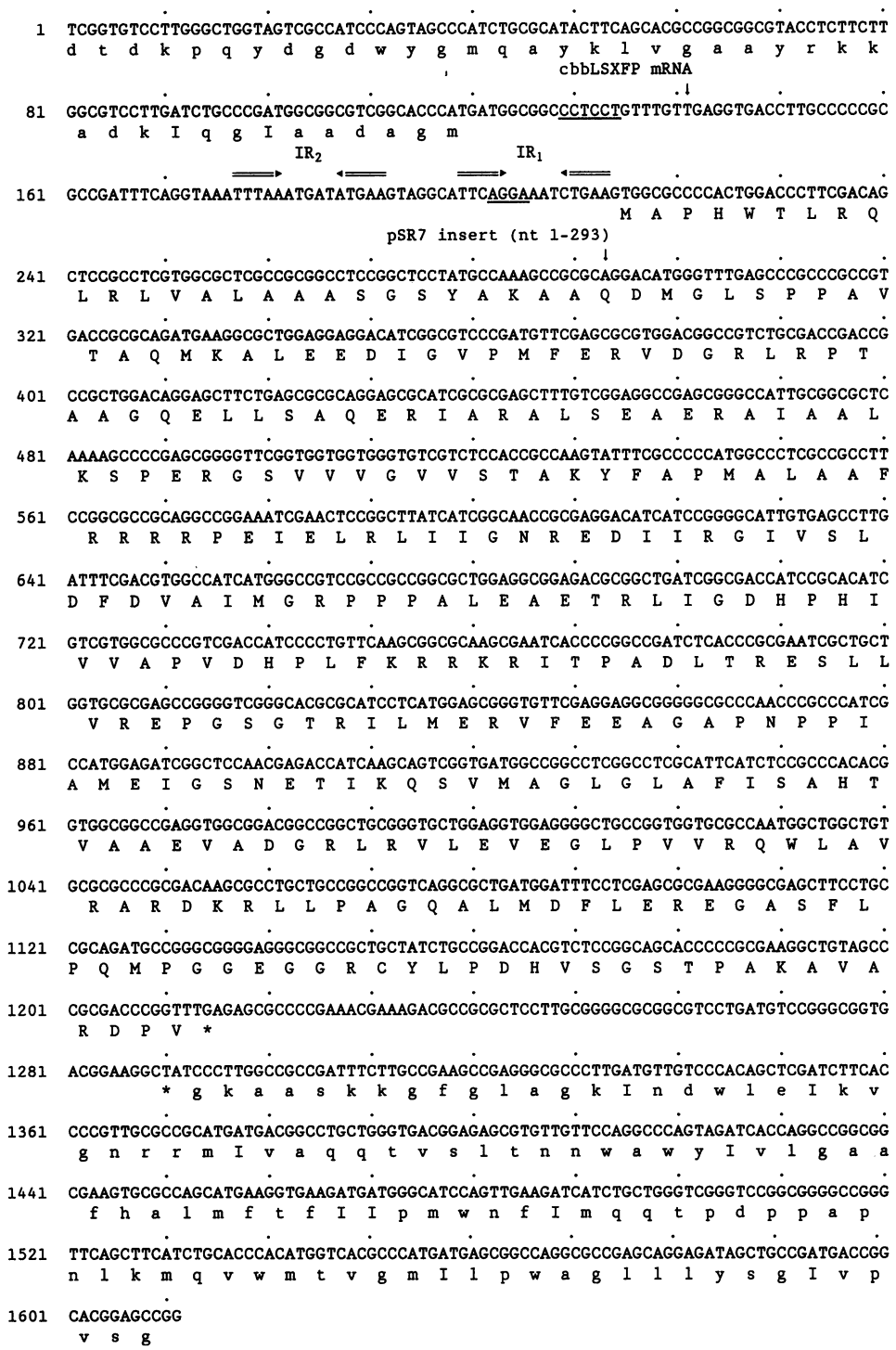

FIG. 2. Nucleotide sequence of the *Eco*RV-*Bam*HI DNA fragment encoding the 5′ end of *cbbL*, *cbbR*, and ORFA. The translations of ORFA and *cbbL′* are from the reverse complement (lowercase letters). Amino acids are represented by the single-letter code. A stop codon is indicated by an asterisk. Putative ribosome binding sites are underlined. The transcription start site of *cbbL* (26) is indicated by a vertical arrow. Arrows indicate the positions of IR_1 and IR_2.

6a). When cell extract of *E. coli*(pER94) was used in the binding assay, two retarded bands were observed. The intensity of the upper, more retarded band increased when higher concentrations of cell extract were used. In contrast, retardation was not observed when extracts of *E. coli* (pPROK-1) were used at identical concentrations.

When a 100-fold molar excess (with regard to the labelled fragment) of unlabelled pSR7 was included in the binding

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          . .. .●  ●.. ● . ..● .●    .  ●  . .●●●.      ●.●.● .●●  ●  ..●
A     GSVPVIGSYLLLGAWPLIMGVTMWVQMKL---NPAPPDPTQQMIFNWMPIIFTFMLAHFAAGLVIYWAWNNTLSVTQQAV   77
60K   TDLSIKDPFFIL---PIIMGATMFIQQRL---NPTPPDPMQAKVMKMMPIIFTFFFLWFPAGLVLYWVVNNCLSISQQWY  546
261   FDLGEKDPYYIL---PIVAGVATFVQQKLMMAGNAQQNPQMAMMLWIMPIMIIVFAINFPAALSLYWVVGNLFMIAQTFL  241

          ●          ●..
A     IMRRNGVKIELWDNIKGALGFGKKSAAKG                                              106
60K   ITRR----IEAA-TKKAAA                                                        560
261   IKGPD--IKKNPEPQKAGGKKK                                                     261
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FIG. 3. Comparison of the deduced ORFA (A) amino acid sequence with those of ORF60K (60K) from *P. putida* and ORF261 (261) from *B. subtilis* (29). Solid circle, identical residue; dot, conservative substitution according to the scheme PAGST, QNED, ILVM, HKR, FWY, C; dashes denote gaps introduced to maximize identities.

assay with extracts of *E. coli*(pER94), retardation of the labelled *Hin*dIII-*Eco*RV fragment was abolished. However, addition of a 100-fold molar excess of vector (pTZ18U) instead of pSR7 did not affect retardation (Fig. 6b). It is therefore concluded that CbbR specifically binds to the *cbbR-cbbL* intergenic region.

DISCUSSION

We previously identified the 5′ end of an open reading frame (*cbbR*), transcribed divergently from *cbbLSXFP*, as a putative LysR-type regulatory protein (25, 26). Similar genes were subsequently detected upstream from the RuBisC/O-encoding genes in *Chromatium vinosum*, *A. eutrophus*, *R. sphaeroides*, and *Thiobacillus ferrooxidans*. It was shown that they are required for the expression of the Calvin cycle genes (20, 43, 44, 48). To characterize the *cbbR* gene from *X. flavus* in more detail, the complete nucleotide sequence of *cbbR* was determined. Comparison of the deduced amino acid sequence of CbbR with those of proteins from *A. eutrophus* (CfxR), *C. vinosum* (RbcR), and *T. ferrooxidans* (RbcR) revealed a high degree of similarity throughout the sequence, reflecting their common function (Fig. 7). In contrast, only the amino termini of the CbbR proteins were similar to those of other LysR-class proteins (Fig. 7). In the amino terminus of LysR-type proteins, a helix-turn-helix motif is present, suggesting that this part of the protein interacts with DNA (14). This is supported by an analysis of mutant NahR and NodD proteins (4, 16, 33, 40). From these mutant studies, it was shown that the carboxyl-terminal part of the LysR-class protein is also required for DNA binding, although it remains unclear how it participates in this (4, 33, 40). Two residues that are important for transcription activation by NahR, Pro-35 and Gly-203, are conserved in CbbR (16, 33) (Fig. 7).

Downstream from *cbbR*, the 3′ end of a gene displaying a high similarity to ORF60K from *P. putida* and ORF261 from *B. subtilis* was found. ORF60K and ORF261 are located at the replication origin of the chromosome of these bacteria. The function of these open reading frames is unknown. The replication origin covers about 20 kb and represents a highly conserved region in eubacteria (29, 39, 50). This could indicate that the *cbb* gene cluster is adjacent to the *X. flavus* chromosomal replication origin. Sequence analysis of the *cbbR* downstream region will show whether this is indeed the case.

The function of *cbbR* was assessed via gene disruption. The resulting *cbbR* mutant, *X. flavus* R22, failed to grow autotrophically because of its inability to induce *cbbLSXFP*. The failure to induce *cbbLSXFP* could be caused by an impairment of the capability to oxidize autotrophic substrates, e.g., formate. This is, however, not the case, since in this respect

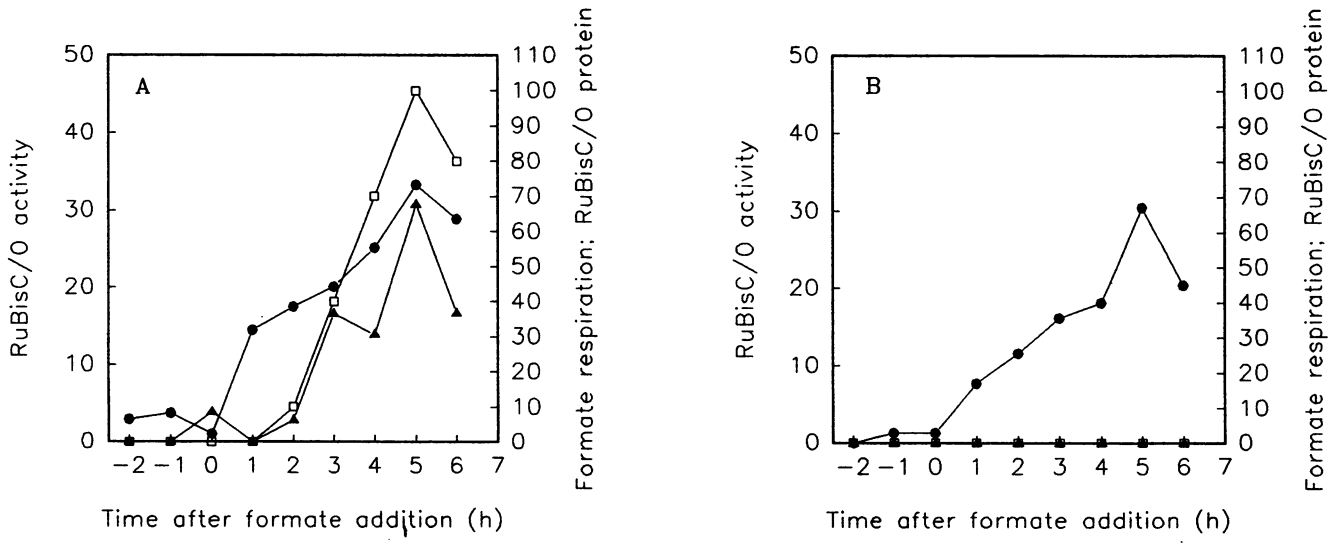

FIG. 4. Enzyme profiles of *X. flavus* (A) and *X. flavus* R22 (B) growing on 5 mM gluconate. Shown are results of addition of 20 mM formate and automatic titration with formic acid (25% [vol/vol]) at $t = 0$ h. ●, formate respiration, in nmoles of O_2 per minute per milligram of protein; ▲, RuBisC/O activity, in nmoles per minute per milligram of protein; □, RuBisC/O protein. The RuBisC/O protein concentration was determined via immunodetection, as described in Materials and Methods and is expressed as a percentage of the highest concentration in the experiments.

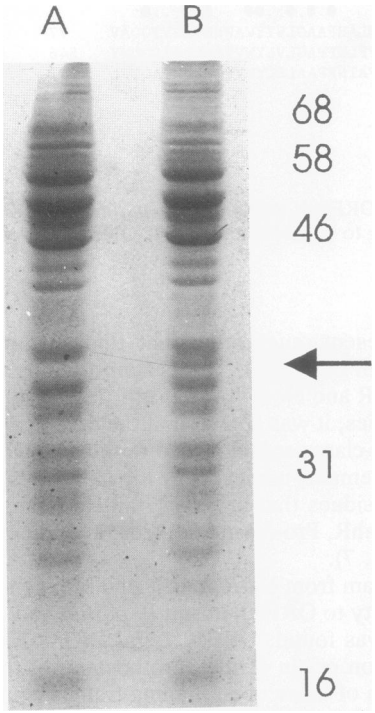

FIG. 5. Expression of CbbR in *E. coli*. *E. coli*(pER94) and *E. coli*(pPROK-1) were grown on LB medium and induced with IPTG. Cell extracts were analyzed on a 12.5% denaturing SDS-polyacrylamide gel. Lane A, *E. coli*(pPROK-1); lane B, *E. coli*(pER94). The arrow indicates the position of the additional protein in the extract of *E. coli*(pER94). The numbers refer to the molecular mass standards in kilodaltons.

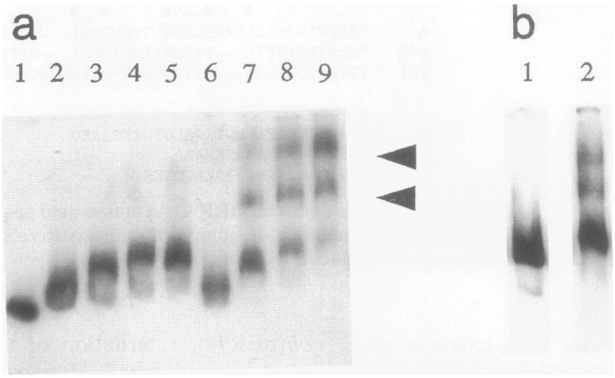

FIG. 6. (a) Retardation of the *Hin*dIII-*Eco*RV DNA fragment on a 6% nondenaturing polyacrylamide gel, after incubation with cell extracts from *E. coli* transformed with pPROK-1 and pER94. Lanes 2 to 5, cell extract of *E. coli*(pPROK-1); lanes 6 to 9, cell extract of *E. coli*(pER94). Protein concentrations in the binding assay (in micrograms): lane 1, 0; lanes 2 and 6, 10; lanes 3 and 7, 22; lanes 4 and 8, 33; lanes 5 and 9, 44. Arrowheads indicate the positions of the retarded bands. (b) Retardation of the *Hin*dIII-*Eco*RV DNA fragment on a 6% nondenaturing polyacrylamide gel, after incubation with cell extracts from *E. coli*(pER94) and a 100-fold molar excess of pSR7 (lane 1) or pTZ18U (lane 2).

X. flavus R22 was indistinguishable from the wild-type strain. It also shows that the dissimilation of formate to CO_2 and assimilation via the Calvin cycle are not regulated coordinately. This agrees with the results of previous experiments, in which the ratio of acetate and formate in the feed of a carbon-limited chemostat was varied (7, 8).

The requirement of a functional *cbbR* gene for the expression of *cbbLSXFP* strongly suggests that CbbR is a regulatory protein that binds to the *cbbLSXFP* promoter and subsequently activates transcription. Specific binding of CbbR to a DNA fragment containing the *cbbR-cbbL* intergenic region was demonstrated via a band-shift assay. Two retarded bands were observed. The intensity of the second, more retarded band increased with the concentration of CbbR in the binding assay. This is interpreted as the consecutive binding of CbbR to a high- and a low-affinity sites on the DNA fragment.

The presence of two binding sites (sites I and II) has been shown for several LysR-type proteins, such as TrpI, NahR, CatR, and IlvY (5, 16, 30, 47). The presence of an inducer is not required for binding to site I, although binding may be enhanced by the effector molecule. However, the affinity of the LysR-type regulatory protein for the second site is generally lower than that for site I. In several instances, binding to site II is observed only in the presence of the inducer (5, 10, 16, 30, 31, 34, 47). In all these examples, binding site I is generally located at position −60 at the respective promoter. Occupation of site I at the −60 position is associated with repression of the gene encoding the regulator, creating an autoregulatory circuit. The second binding site is at the −40 position, and occupation of this site is required for activation of transcription. Transcriptional activators interacting with σ^{70} RNA polymerase generally bind at −40, next to the binding site of RNA polymerase. It would thus allow the regulatory protein to contact the RNA polymerase, which is believed to be required for transcription activation (6).

It has been noted that a 5-bp inverted repeat (TTCAG-N_5-CTGAA [IR_1]), containing the LysR motif, is present in the *cbbR-cbbL* intergenic region of *X. flavus* (12, 26). IR_1 is centered at the −65 position with respect to the *cbbLSXFP* transcription start. A second imperfect inverted repeat (IR_2) is centered at position −43 and is similar to IR_1 (Fig. 2). The right half of IR_2 has only one mismatch compared with the right half of IR_1, whereas the left half of the inverted repeat is more degenerate. In a footprinting experiment using RbcR (CbbR) from *T. ferrooxidans*, it was shown that RbcR protected a region in the *rbcL* promoter from −14 to −75 from DNase activity. In the protected area, two inverted repeats are present, centered at −65 and −43, at positions identical to those of IR_1 and IR_2 in the *cbbLSXFP* promoter from *X. flavus*. The presence and localization of these two LysR motif-containing inverted repeats in the *cbbLSXFP* promoter of *X. flavus* are also strikingly similar to those of binding sites I and II of TrpI, CatR, IlvY, and NahR, discussed above. By analogy, we therefore propose that IR_1 and IR_2 in the *cbbLSXFP* promoter represent high- and low-affinity binding sites of CbbR.

Physiological studies have shown that the energy and carbon status of the cell control the expression of the Calvin cycle genes in *X. flavus* (7, 8, 27). It is clear from the results presented in this paper that CbbR plays an important role in transducing these signals to the *cbbLSXFP* promoter. We do not yet understand how these signals are transduced and what metabolites, if any, interact with CbbR. Current research aims to answer these questions.

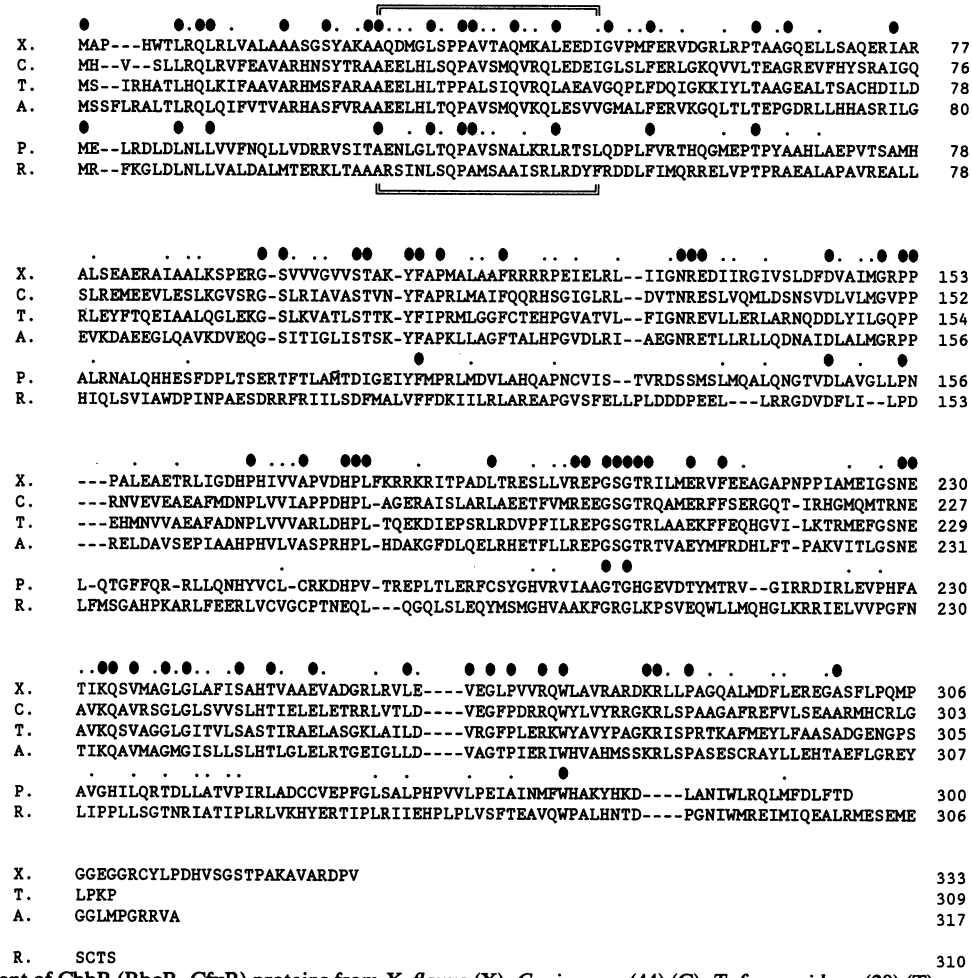

FIG. 7. Alignment of CbbR (RbcR, CfxR) proteins from *X. flavus* (X), *C. vinosum* (44) (C), *T. ferrooxidans* (20) (T), and *A. eutrophus* (48) (A) and comparison with NahR from *P. putida* (35) (P) and NodD from *Rhizobium leguminosarum* (4) (R). Solid circle, identical residue; dot, conservative substitution according to Fig. 3. The solid circles and dots above the CbbR alignment indicate similarities in the CbbR alignment, whereas the solid circles and dots below the CbbR alignment identify similarities between the CbbR proteins and NahR and NodD. The helix-turn-helix motif is indicated by lines above and below the sequence. Dashes denote gaps introduced to maximize identities.

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REFERENCES

1. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
2. **Bowien, B., R. Bednardski, B. Kusian, U. Windhövel, A. Freter, J. Schäferjohann, and J.-G. Yoo.** 1993. Genetic regulation of CO_2 assimilation in chemotrophs, p. 481–491. *In* J. C. Murrell and D. P. Kelly (ed.), Microbial growth on C_1 compounds. Intercept, Andover, England.
3. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
4. **Burn, J. E., W. D. Hamilton, J. C. Wootton, and A. W. B. Johnston.** 1989. Single and multiple mutations affecting properties of the regulatory gene *nodD* of *Rhizobium*. Mol. Microbiol. **3**:1567–1577.
5. **Chang, M., and I. P. Crawford.** 1990. The roles of indoleglycerol phosphate and the TrpI protein in the expression of *trpBA* from *Pseudomonas aeruginosa*. Nucleic Acids Res. **18**:979–988.
6. **Collado-Vides, J., B. Magasanik, and J. D. Gralla.** 1991. Control site location and transcriptional regulation in *Escherichia coli*. Microbiol. Rev. **55**:371–394.
7. **Croes, L. M., W. G. Meijer, and L. Dijkhuizen.** 1991. Regulation of methanol oxidation and carbon dioxide fixation in *Xanthobacter* strain 25a grown in continuous culture. Arch. Microbiol. **155**:159–163.
8. **Dijkhuizen, L., L. M. Croes, and W. G. Meijer.** 1989. Mixotrophic growth, p. 21–32. *In* G. Hamer, T. Egli, and M. Snozzi (ed.), Mixed and multiple substrates and feedstocks. Hartung-Gorre Verlag, Konstanz, Germany.
9. **Dijkhuizen, L., and W. Harder.** 1975. Substrate inhibition in *Pseudomonas oxalaticus* OX1: a kinetic study of growth inhibition by oxalate and formate using extended cultures. Antonie Leeuwenhoek **41**:135–146.
10. **Gao, J., and G. N. Gussin.** 1991. Mutations in TrpI binding site II that differentially affect activation of the *trpBA* promoter of *Pseudomonas aeruginosa*. EMBO J. **10**:4137–4144.
11. **Gibson, J. L., and F. R. Tabita.** 1977. Different molecular forms of D-ribulose-1,5-bisphosphate carboxylase from *Rhodopseudomonas sphaeroides*. J. Biol. Chem. **252**:943–949.
12. **Goethals, K., M. van Montagu, and M. Holsters.** 1992. Conserved motifs in a divergent *nod* box of *Azorhizobium caulinodans* ORS571 reveal a common structure in promoters regulated by LysR-type proteins. Proc. Natl. Acad. Sci. USA **89**:1646–1650.

13. **Henikoff, S.** 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene **28:**351–359.
14. **Henikoff, S., G. W. Haughn, J. M. Calvo, and J. C. Wallace.** 1988. A large family of bacterial activators. Proc. Natl. Acad. Sci. USA **85:**6602–6606.
15. **Heusterspreute, M., V. H. Thi, S. Emery, S. Tournis-Gamble, N. Kennedy, and J. Davison.** 1985. Vectors with restriction site banks. IV. pJRD184, a 3793-bp plasmid vector having 43 unique restriction sites. Gene **39:**299–304.
16. **Huang, J., and M. A. Schell.** 1991. *in vivo* interactions of the NahR transcriptional activator with its target sequences. J. Biol. Chem. **266:**10830–10838.
17. **Jouanneau, Y., and F. R. Tabita.** 1986. Independent regulation of synthesis of form I and form II ribulose bisphosphate carboxylase-oxygenase in *Rhodobacter sphaeroides*. J. Bacteriol. **165:**620–624.
18. **Khudyakof, Y. E., V. S. Neplyueva, T. A. Kalinina, and V. D. Smirnov.** 1988. Effect of structure of the initiator codon on translation in *E. coli*. FEBS Lett. **232:**369–371.
19. **Kunkel, T. A., J. D. Roberts, and R. A. Zakour.** 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. **154:**367–382.
20. **Kusano, T., and K. Sugawara.** 1993. Specific binding of *Thiobacillus ferrooxidans* RbcR to the intergenic sequence between the *rbc* operon and the *rbcR* gene. J. Bacteriol. **175:**1019–1025.
21. **Laurell, C. B.** 1966. Quantitative estimation of proteins by electrophoresis in agarose gels containing antibodies. Anal. Biochem. **15:**45–52.
22. **Lehmicke, L. G., and M. E. Lidstrom.** 1985. Organization of genes necessary for growth of the hydrogen-methanol autotroph *Xanthobacter* sp. strain H4-14 on hydrogen and carbon dioxide. J. Bacteriol. **162:**1244–1249.
23. **Levering, P. R., J. P. van Dijken, M. Veenhuis, and W. Harder.** 1981. *Arthrobacter* P1, a fast growing versatile methylotroph with amine oxidase as a key enzyme in the metabolism of methylated amines. Arch. Microbiol. **129:**72–80.
24. **Lidstrom-O'Conner, M. E., G. L. Fulton, and A. E. Wopat.** 1983. '*Methylobacterium methanolicum*': a syntrophic association of two methylotrophic bacteria. J. Gen. Microbiol. **129:** 3139–3148.
25. **Meijer, W. G.** 1990. Regulation of carbon dioxide fixation in facultatively autotrophic bacteria. Ph.D. thesis. University of Groningen, Groningen, The Netherlands.
26. **Meijer, W. G., A. C. Arnberg, H. G. Enequist, P. Terpstra, M. E. Lidstrom, and L. Dijkhuizen.** 1991. Identification and organization of carbon dioxide fixation genes in *Xanthobacter flavus* H4-14. Mol. Gen. Genet. **225:**320–330.
27. **Meijer, W. G., L. M. Croes, B. Jenni, L. G. Lehmicke, M. E. Lidstrom, and L. Dijkhuizen.** 1990. Characterization of *Xanthobacter* strains H4-14 and 25a and enzyme profiles after growth under autotrophic and heterotrophic growth conditions. Arch. Microbiol. **153:**360–367.
28. **Meijer, W. G., H. G. Enequist, P. Terpstra, and L. Dijkhuizen.** 1990. Nucleotide sequences of the genes encoding fructosebisphosphatase and phosphoribulokinase from *Xanthobacter flavus* H4-14. J. Gen. Microbiol. **136:**2225–2230.
29. **Ogasawara, N., and H. Yoshikawa.** 1992. Genes and their organization in the replication origin region of the bacterial chromosome. Mol. Microbiol. **6:**629–634.
30. **Parsek, M. R., D. L. Shinabarger, R. K. Rothmel, and A. M. Chakrabarty.** 1992. Roles of CatR and *cis-cis*-muconate in activation of the *catBC* operon, which is involved in benzoate degradation in *Pseudomonas putida*. J. Bacteriol. **174:**7798–7806.
31. **Rothmel, R. K., D. L. Shinabarger, M. R. Parsek, T. L. Aldrich, and A. M. Chakrabarty.** 1991. Functional analysis of the *Pseudomonas putida* regulatory protein CatR: transcriptional studies and determination of the CatR DNA-binding site by hydroxyl-radical footprinting. J. Bacteriol. **173:**4717–4724.
32. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
33. **Schell, M. A., P. H. Brown, and S. Raju.** 1990. Use of saturation mutagenesis to localize probable functional domains in the NahR protein, a LysR-type transcription activator. J. Biol. Chem. **265:**3844–3850.
34. **Schell, M. A., and E. F. Poser.** 1989. Demonstration, characterization, and mutational analysis of NahR protein binding to *nah* and *sal* promoters. J. Bacteriol. **171:**837–846.
35. **Schell, M. A., and M. Sukordhaman.** 1989. Evidence that the transcription activator encoded by the *Pseudomonas putida nahR* gene is evolutionarily related to the transcription activators encoded by the *Rhizobium nodD* genes. J. Bacteriol. **171:**1952–1959.
36. **Schlaman, H. R. M., R. J. H. Okker, and B. J. J. Lugtenberg.** 1992. Regulation of nodulation gene expression by NodD in rhizobia. J. Bacteriol. **174:**5177–5182.
37. **Simon, R.** 1984. High frequency mobilization of Gram-negative bacterial replicons by the *in vitro* constructed Tn5-Mob transposon. Mol. Gen. Genet. **196:**413–420.
38. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for in vitro engineering: transposon mutagenesis in Gram-negative bacteria. Bio/Technology **1:**784–790.
39. **Smith, D. W., T. W. Yee, C. Baird, and V. Krishnapillai.** 1991. Pseudomonad replication origins: a paradigm for bacterial origins? Mol. Microbiol. **5:**2581–2587.
40. **Spaink, H. P., C. A. Wijffelman, R. J. H. Okker, and B. E. J. Lugtenberg.** 1989. Localization of functional regions of the *Rhizobium nodD* product using hybrid *nodD* genes. Plant Mol. Biol. **12:**59–73.
41. **Tabita, F. R.** 1988. Molecular and cellular regulation of autotrophic carbon dioxide fixation in microorganisms. Microbiol. Rev. **52:**155–189.
42. **Tabita, F. R., J. L. Gibson, B. Bowien, L. Dijkhuizen, and W. G. Meijer.** 1992. Uniform designation for the genes of the Calvin-Benson-Bassham reductive pentose phosphate pathway of bacteria. FEMS Microbiol. Lett. **99:**107–110.
43. **Tabita, F. R., J. L. Gibson, D. L. Falcone, X. Wang, L.-A. Li, B. A. Read, K. C. Terlesky, and G. C. Paoli.** 1993. Current studies on the molecular biology and biochemistry of CO_2 fixation in phototrophic bacteria, p. 469–479. *In* J. C. Murrell and D. P. Kelly (ed.), Microbial growth on C_1 compounds. Intercept, Andover, England.
44. **Viale, A. M., H. Kobayashi, T. Akazawa, and S. Henikoff.** 1991. *rcbR*, a gene coding for a member of the LysR family of transcriptional regulators, is located upstream of the expressed set of ribulose 1,5-bisphosphate carboxylase/oxygenase genes in the photosynthetic bacterium *Chromatium vinosum*. J. Bacteriol. **173:**5224–5229.
45. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19:**259–268.
46. **Vieira, J., and J. Messing.** 1987. Production of single-stranded plasmid DNA. Methods Enzymol. **153:**3–11.
47. **Wek, R. C., and G. W. Hatfield.** 1988. Transcriptional activation at adjacent operators in the divergent-overlapping *ilvY* and *ilvC* promoters of *Escherichia coli*. J. Mol. Biol. **203:**643–663.
48. **Windhövel, U., and B. Bowien.** 1991. Identification of cfxR, an activator gene of autotrophic CO_2 fixation in *Alcaligenes eutrophus*. Mol. Microbiol. **5:**2695–2705.
49. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.
50. **Yoshikawa, H., and N. Ogasawara.** 1991. Structure and function of DnaA and the DnaA-box in eubacteria: evolutionary relationships of bacterial replication origins. Mol. Microbiol. **5:**2589–2597.