CbbR, a LysR-Type Transcriptional Activator, Is Required for Expression of the Autotrophic CO2 Fixation Enzymes of Xanthobacter flavus

E. R. E. van den Bergh, L. Dijkstra, and W. G. Meijer*

Department of Microbiology, University of Groningen, Kerkaal 30, 9751 NN Haren, The Netherlands

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Xanthobacter flavus is able to grow autotrophically with the enzymes of the Calvin cycle for the fixation of CO2, 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. Dijkstra, 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.

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-N11-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 H2-CO2-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-

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* Corresponding author.
TABLE 1. Bacteria and plasmids used in this study

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Plasmids

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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 phosphate-dependent 14CO2 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 (Genecreen 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-32P]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 35S-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 SalI fragment from pUC4K, encoding kanamycin resistance, was inserted into the unique SalI site within cbbR. The resulting plasmid, pKR1, was digested with XbaI, treated with Klenow enzyme, and digested with KpnI. The DNA fragment containing cbbR::Km was subsequently ligated into HpaI- and KpnI-digested pJR184 (pKR2). The mob site from pSUP9011 was isolated as a BamHI 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 in X. flavus R22.

Expression of CbbR in E. coli. A 558-bp DraI-SalI fragment containing the 5' end of cbbR was mutagenized via the method described by Kunkel et al. (19), with a synthetic oligonucleotide (5'-TGAGGACTTACGGAAGAATTCGTTCCGCCCCACTGGAC-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 EcoRI restriction site (pER93). Sequencing of the DraI-SalI fragment confirmed that only the desired mutations had occurred. Subsequently, a SalI-HindIII fragment containing the 3' end of cbbR was ligated into pER93. The mutagenized cbbR was then cloned as an EcoRI-HindIII 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...
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 × 10^2 kPa/m²). 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 HindIII and EcoRV, liberating a 307-bp fragment containing the cbbR-cbbL intergenic region. The HindIII-EcoRV fragment was labelled with [α-32P]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 non-denaturing acrylamide gel (4%), and the HindIII-EcoRV fragment was isolated via electroelution (32).

**DNA binding assay.** Various amounts of cell extract were incubated with the labelled HindIII-EcoRV 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% non-denaturing 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 EcoRV-BamHI 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 BamHI-EcoRV fragment (pXAl) restored autotrophic growth of X. flavus R22, comparable to that of the wild type. However, pXAl2, containing a SalI-EcoRV fragment with a truncated cbbR gene (Fig. 1), was not able to complement X. flavus R22. 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, RuBiS/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 CO₂ by RuBiS/O, since RuBiS/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 (19). 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 of 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 HindIII-EcoRV fragment containing the cbbR-cbbL intergenic region (Fig.
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
flavus and sphaeroides, B. eutrophus was subsequently (CfxR), eutrophus was cbbR determined. 

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experiments. via determined

dashes instead of genes in similar sequence, amino terminus of Chromatium vinosum, A. ferrooxidans. It is 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 (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.

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).

 assay with extracts of E. coli(pER94), retardation of the labelled HindIII-EcoRV 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 RubisCO-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 O2 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.

A

GSVPVIGSLYLLGALFLVEVYTFVQGCQL--EPAPDPTQG1FNBGFIIPFMMLDIYFAAGLVLYAYK1VTLSVTQAV 77

60K

TDL5IEFDFLFL--PI053ATM1FQGRL--NPTPPDMQAXK1DNPFI1FPPFWFPAAGLVLWyVWWNC1LS1Q0WY 546

261

FDL5IEFDFLFL--PI053ATM1FQGRL--EAG1AANQFP1AM1LW1MP1H1IVFA1NPFAPALSSLYWVVGLHP1M1ATFPL 241

B

IMRRVGYK1LWNDINIGP1KXSAAG 106

60K

IFVR----IEAA-TEKAA 560

261

IKPD0----ITKFPQPORAGKXK 261

Time after formate addition (h) 0 -2 1 2 3 4 5 6 7

Time after formate addition (h) 0 -2 1 2 3 4 5 6 7

Formate respiration: RubisC/O protein

RubisC/O activity

Time after formate addition (h) 0 -2 1 2 3 4 5 6 7

Formate respiration: RubisC/O protein

Downloaded from j.asm.org on May 24, 2007
In this paper, we present evidence that X. flavus R22 was indistinguishable from the wild-type strain. It also shows that the dissimilation of formate to CO₂ 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 −40 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 σ⁵⁴ 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 (TTCCAG-N₅⁻₁-CTGAA [IR₁]), containing the LysR motif, is present in the cbbR-cbbL intergenic region of X. flavus (12, 26). IR₁ is centered at the −65 position with respect to the cbbLSXFP transcription start. A second imperfect inverted repeat (IR₂) is centered at position −43 and is similar to IR₁ (Fig. 2). The right half of IR₁ has only one mismatch compared with the right half of IR₂, 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 IR₁ and IR₂ 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 IR₁ and IR₂ 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.
ACKNOWLEDGMENTS

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REFERENCES


