CbbR, a LysR-type transcriptional regulator from *Hydrogenophilus thermoluteolus*, binds two cbb promoter regions

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Abstract

The cbbR encoding the LysR-type transcriptional regulator is located downstream of cbbLSQOYA and this gene is located upstream of cbbFPT in divergent transcription. The two promoter regions with LysR-binding sites are located in the cbbL upstream region and in the cbbR–cbbF intergenic region. Electrophoretic mobility shift assays using a cell extract of *Escherichia coli* harboring a plasmid containing cbbR and the DNA fragments of promoter regions indicated that CbbR binds in both regions. NADPH caused differences in the complex of CbbR and DNA. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Microbiological Societies.

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1. Introduction

*Pseudomonas hydrogenothermophila* strain TH-1, an aerobic, thermo tolerant hydrogen-oxidizing bacterium, is a facultative chemolithotroph able to grow not only autotrophically using hydrogen, oxygen, and carbon dioxide, but also heterotrophically in organic media [1,2]. Our recent 16S rDNA sequence study indicated that this strain has a unique sequence and that it belongs to a new genus of the β subclass of Proteobacteria. We propose the name *Hydrogenophilus thermoluteolus* gen. nov., sp. nov., for this strain [3].

The strain is known to assimilate CO2 via the Calvin–Benson–Bassham (CBB) cycle. The key CBB cycle enzyme is ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO; EC 4.1.1.39), which fixes CO2. RubisCO, found in plants, algae, cyanobacteria, and most photo- and chemolithotrophic bacteria, is composed of eight large and eight small subunits (L8S8). The large subunits, which contain the active site, are encoded by the cbbL gene and the small subunits are encoded by the cbbS gene. The other form II RubisCO (Lx) is composed only of large subunits, encoded by the cbbM gene. The cbbLS genes were previously cloned from *H. thermoluteolus* genomic DNA and sequenced [4]. We found some open reading frames (ORF) downstream of cbbLS (Fig. 1) [4–6]. CbbQ and CbbO are thought to play an important role in the post-translational regulation of RubisCO, because co-expression of cbbQ with cbbLS in *Escherichia coli* affects the conformational state and the activity of the RubisCO of *H. thermoluteolus* [5,7,8]. On the other hand, cbbRFPT genes are found as a different operon in *H. thermoluteolus* (Fig. 1).

The cbbR genes are located upstream of cbbL in a divergent orientation in *Ralstonia eutropha* [9], *Xanthobacter flavus* [10], *Rhodobacter capsulatus* [11], *Nitrobrocher vulgaris* [12], *Chromatium vinosum* [13], *Thiobacillus ferrooxidans* [14], *Thiobacillus denitrificans* [15], and *Hydrogenophaga pseudoalva* (accession number U55038), and the cbbR genes are located upstream of cbbM in divergent orientation in *Thiobacillus denitrificans* [15], *Thiobacillus neapolitanus* [16], *Thiobacillus intermedium* [16], and a symbiont of the tubeworm *Riftiia pachyptila* [17]. On the other hand, the cbbRFPT genes are found as a different operon in *H. thermoluteolus* (Fig. 1).

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Promoter regions including LysR-binding sites are located between cbbL (cbbM or cbbF) genes and cbbR [9–21]. The CbbR binds to the promoter region upstream of cbbR in R. eutropha [9,22], Rh. sphaeroides [23], X. fumaricus [10,24], and T. ferrooxidans [14]. The CbbR binds to two LysR-binding sites (T-N11-A) [25] in the promoter regions. Two LysR-binding sites is defined as IR1 and IR2; IR1 located far from cbbL contained an approximately perfect inverted repeat, and IR2 located near to cbbL contained an imperfect inverted repeat in X. fumaricus [24]. The cbbR gene is not located upstream of cbbL in H. thermoluteolus, but only two LysR-binding sites are located in the promoter region upstream of cbbL [6], whereas the cbbR gene is located upstream of cbbF in this bacterium, and two LysR-binding sites are located in the promoter region upstream of cbbF (accession number AB042620).

In this study, we compared the amino acid sequence of CbbR from H. thermoluteolus to other CbbR sequences from autotrophic bacteria. We determined whether CbbR could bind to the cbb promoter region, and whether NADPH was an enhancer of CbbR binding to the DNA.

2. Materials and methods

2.1. Bacterial strains, vectors, and growth condition

E. coli strains JM109 [26], DH5α (Takara shuzo, Tokyo, Japan), and BL21(DE3) (Novagen, Madison, WI, USA) [27] were routinely cultivated at 37°C in Luria-Bertani (LB) medium [28] with 100 μg ml⁻¹ ampicillin. pBlue-script II KS⁺ (Stratagene, La Jolla, CA, USA) was used for the subcloning of a promoter region. pET11a (Nova-gen) was used for the expression of CbbR. pUC118 (Takara shuzo) was used in electrophoretic mobility shift assays.

2.2. Construction of the plasmid

Oligodeoxyribonucleotide primers were designed to prepare the cbbR gene. The sequences of the primers were 5′-CAGGGAGAACAATGAAACG-3′ (primer 1: 5′ terminal of the cbbR gene including a Ndel site) and 5′-TACCAGATCTTTTGTCAGGAAACTA-3′ (primer 2: 3′ terminal of the cbbR gene and a BamHI site). The DNA fragment between primer 1 and primer 2 was amplified from chromosomal DNA extracted from strain TH-1 by polymerase chain reaction (PCR). The DNA amplified by PCR was digested with Ndel and BamHI and inserted into the Ndel and BamHI sites of pET11a. This ligation mixture was used to transform E. coli DH5α, and the correct sequence was confirmed (pKT23; Fig. 1).

pYAH203 was constructed by ligating the EcoRI–BamHI region of pKY2 [4] containing the upstream region of cbbL into the EcoRI and BamHI sites of pBluescript II KS⁺.

Other recombinant DNA manipulations were carried out as described by Sambrook et al. [28].

The DNA sequence of pKT23 was determined according to the method described previously [4,6].

2.3. Expression of CbbR and preparation of cell extract

E. coli strain BL21(DE3) cells harboring pKT23 or pET11a were grown at 37°C in 20 ml LB medium with 100 μg ml⁻¹ ampicillin and 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells suspended in 5 ml Tris buuffer (25 mM Tris–HCl, 10 mM EDTA, 1 mM dithiothreitol; pH 7.8) were disrupted by an ultrasonic generator (Branson Sonifier model 250D, Branson). The sonicating conditions were 50 W for 5 min at 4°C. The cell extract was centrifuged at 50 000 rpm for 15 min at 4°C to remove cell debris.

Proteins in the cell extract were separated by electrophoresis in a 12.5% polyacrylamide gel (PAGE) in the presence of sodium dodecyl sulfate (SDS). Protein was visualized by Coomassie brilliant blue.

Protein concentration was measured by a Bio-Rad protein assay dye reagent concentrate kit (Bio-Rad Lab., Richmond, CA, USA) using bovine serum albumin (BSA) as a standard.

2.4. Preparation of DNA fragments used in binding studies and labeling of DNA fragments

The cbbL upstream region was amplified by PCR from pYAH203 by using oligonucleotides primer 3 (5′-ACATCGGAATGCCATTATT-3′) and primer 4 (5′-GGGCGCTGAGAAGACTATA-3′). The cbbR-cbbF intergenic region was amplified by PCR from pKT20 using oligonucleotides primer 5 (5′-ACGCATGTCAGCTGTTC-3′) and primer 6 (5′-GTCATGCTGCAAGGATAAA-3′) (Fig. 2).

To obtain DNA fragments for use in binding studies, PCR products were recovered from agarose gel and purified by phenol treatment. The DNA fragments were labeled with [γ⁻³²P]ATP by MEGALABEL (Takara shuzo).

2.5. Electrophoretic mobility shift assay

Cell extracts of E. coli harboring pKT23 or pET11a were incubated with the labeled DNA (5000–10 000 cpmp) in incubation buffer (10 mM Tris–HCl, 50 mM KCl, 1 mM dithiothreitol, 10 mM EDTA, 50 μg ml⁻¹ BSA, 5% glycerol, 2 μg calf thymus DNA) for 30 min at 20°C. After incubation, the mixtures were loaded on a 6% non-denaturing acrylamide gel. The gel was subsequently dried and the radiolabeled protein–DNA complexes were then detected by autoradiography using X-ray film.
3. Results

3.1. Comparison of CbbR amino acid sequence

The cbbR gene encoding the LysR-type transcriptional regulator is located upstream of cbbF in divergent transcription (Fig. 1). The highest sequence identity (43%) was found with CbbR from R. eutropha [9] and Rb. capsulatus (CbbRI) [11]. This protein shared approximately 40% identity with CbbR from Rb. sphaeroides [18], Rb. capsulatus (CbbRII) [19], X. flavus [10], C. vinosum [13], T. ferrooxidans [14], T. denitri¢cans (CbbRI and CbbRII) [15], T. intermedius [15], T. neapolitanus [15], S. meliloti (AF211846), Rs. rubrum [20], H. pseudo£ava (U55038), and cyanobacterium [29], and shared approximately 30% identity with CbbR encoded on red plastid [30] (AF041468; U30821).

Comparison of CbbR and RbcR from red plastid primary structure indicated three conserved regions (Fig. 3). Region 1 was a helix-turn-helix motif that is a DNA-binding motif [25]. The region shared high similarity with that of another LysR-type transcriptional activator (data not shown). Region 2 is part of the coinducer recognition/response domain. Amino acid substitutions at this region eliminate transcription activation, but not DNA binding [25]. Region 3, called the conserved carboxy-terminal domain, is important for coinducer binding or DNA binding [25]. Regions 2 and 3 did not share high similarity with the other LysR-type transcriptional activators (data not shown).

3.2. CbbR binds the cbbL upstream region and the cbbR–cbbF intergenic region

The cbbR gene from H. thermoluteolus was placed under the control of the T7 promoter of pET11a for the expression of CbbR. This plasmid was then transformed into E. coli BL21(DE3). Overproduction of an approximately 35-kDa protein was detected in SDS–PAGE when CbbR was induced by IPTG (data not shown).

The cbbL upstream region and cbbR–cbbF intergenic region are thought to be promoter regions which include two LysR-binding sites (T-N11-A) [25]. Both IR1s located far from cbbL and cbbF contained an approximately perfect inverted repeat, and both IR2s located near to cbbL and cbbF contained an imperfect inverted repeat (Fig. 2). The binding of CbbR to IR1 and IR2 of R. eutropha [9,22], Rb. sphaeroides [23], X. flavus [10,24], and T. ferrooxidans [14] has been shown in vitro. The 98-bp fragment upstream of cbbL and the 97-bp fragment between cbbR and cbbF were amplified by PCR. Electrophoretic...
mobility shift assays were performed with cell extracts of *E. coli* BL21(DE3) harboring pKT23. Two retarded bands representing promoter-binding activity were found in a binding assay mixture containing CbbR and the cbbR-cbbF intergenic region (Fig. 4A, lane 3). No binding activity was detected in a binding assay mixture containing no protein or no CbbR (Fig. 4A, lanes 1 and 2). Addition of the unlabeled promoter regions at a 50-fold molar excess abolished binding of CbbR in this region (Fig. 4A, lane 3). No binding activity was detected in a binding assay mixture containing the cbbL upstream region (Fig. 4B, lanes 1–5). These results clearly indicated the specificity of CbbR binding to the *cbbL* upstream region. It is thought that complex 2 is formed by CbbR binding to only one LysR-binding site (perfect inverted repeat), and complex 1 is formed by the CbbR binding to both LysR-binding sites [24].

### 3.3. NADPH changes the complex of CbbR and promoter regions

LysR-type transcriptional regulator proteins activate transcription following the binding of ligands [25]. NADPH enhances the DNA binding of CbbR in *X. flavus* [24]. We examined whether NADPH enhances the DNA binding of CbbR from *H. thermoluteolus*. The *cbbL* upstream region was used, and compounds associated with energy metabolism (NAD, NADH, NADP, and NADPH) containing the *cbbL* upstream region (Fig. 4B, lanes 1–5). These results clearly indicated the specificity of CbbR binding to the *cbbL* upstream region. It is thought that complex 2 is formed by CbbR binding to only one LysR-binding site (perfect inverted repeat), and complex 1 is formed by the CbbR binding to both LysR-binding sites [24].

![Diagram of CbbR binding to promoter regions](image)

Fig. 4. Gel mobility shift assay for binding of CbbR to the promoter regions. Arrows indicate the positions of the unbound DNA and the protein–DNA complex of high (complex 1) and low (complex 2) electrophoretic mobility. A: Retardation of the 98-bp *cbbR-cbbF* intergenic region. DNA fragment on a 6% non-denaturing polyacrylamide gel, after incubation with cell extracts of *E. coli* (10 μg) transformed with pKT23 and pET11a. Lane 1, no protein added (labeled DNA only); lane 2, extract of *E. coli* (pET11a); lane 3, extract of *E. coli* (pKT23); lane 4, extract of *E. coli* (pKT23) with a 50-fold molar excess of unlabeled 97-bp *cbbR-cbbF* intergenic region; lane 5, extract of *E. coli* (pKT23) with a 50-fold molar excess of unlabeled 123-bp pUC118 multi-cloning site DNA fragment. B: Retardation of the 98-bp *cbbL* upstream region. Lane 1, no protein added (labeled DNA only); lane 2, extract of *E. coli* (pET11a); lane 3, extract of *E. coli* (pKT23); lane 4, extract of *E. coli* (pKT23) with a 50-fold molar excess of unlabeled 100-bp *cbbL* upstream region; lane 5, extract of *E. coli* (pKT23) with a 50-fold molar excess of unlabeled 123-bp pUC118 multi-cloning site DNA fragment.

![Diagram of CbbR binding to promoter regions](image)

Fig. 5. Effect of pyridine dinucleotides (20 mM) on the DNA-binding characteristics of CbbR analyzed by gel mobility shift assay. Retardation of the 98-bp *cbbL* upstream region DNA fragment on a 6% non-denaturing polyacrylamide gel, after incubation with cell extracts of *E. coli* (10 μg) transformed with pKT23 and pET11a. Lane 1, no protein added (labeled DNA only); lane 2, extract of *E. coli* (pET11a); lane 3, extract of *E. coli* (pKT23); lane 4, extract of *E. coli* (pKT23) plus NAD; lane 5, extract of *E. coli* (pKT23) plus NADH; lane 6, extract of *E. coli* (pKT23) plus NADPH; lane 7, extract of *E. coli* (pKT23) plus NADP.
were tested as enhancers of binding. None of these nucleotides enhanced DNA-binding activity. However, NADPH caused a greater shift of complex 1, and the disappearance of complex 2 (Fig. 5). It was thought that the binding of CbbR to the promoter region was influenced by NADPH.

4. Discussion

There is no cbbR in the cbbL upstream region of H. thermoluteolus (Fig. 1), whereas the binding of CbbR to the cbbL upstream region of H. thermoluteolus was shown by electrophoretic mobility shift assay (Fig. 4). This is the first report to show the binding of CbbR to a promoter region not located upstream of cbbR. It was thought that the CbbR controlled two operons (cbbLSQOYA and cbbFPT). It is indicated that the expression of cbb genes is controlled by CbbR encoded by the gene that is located in another gene cluster and CbbR controlled two cbb operons in R. eutropha (duplicate cbb clusters are located in chromosome and plasmid) [9]. Rh. sphaeroides (cbbFAPALSXY and cbbFPTGAM) [18,23,31], Rh. capsulatus (cbbLSQO and cbbFPTGAM) [32], and X. flavus (cbbLSXFPTAZE and gap-pgk) [33].

NADPH enhances the DNA binding of CbbR in X. flavus [24]. NADPH appears to influence the binding of CbbR to the cbbL upstream region in H. thermoluteolus (Fig. 5). Region 2, which is a coinducer recognition/response domain, shared significant identity among the CbbR from autotrophic bacteria (Fig. 3). It is thought that NADPH enhances the DNA binding of CbbR from autotrophic bacteria. NADPH causes a greater shift and a large amount of complex 1 and disappearance of complex 2 in X. flavus [24]. On the other hand, NADPH did not increase the amount of complex 1 significantly in H. thermoluteolus (Fig. 5). It is indicated that addition of NADPH relaxes DNA bending by CbbR, and it is thought that DNA bending increases the promoter activity of cbb genes. It was thought that the relaxation of DNA bending by NADPH caused a greater shift of complex 1 in H. thermoluteolus.

Two CbbR–DNA complexes are observed in electrophoretic mobility shift assays using CbbR and promoter region and NADPH caused a greater shift of complex 1 in H. thermoluteolus (Figs. 4 and 5) and X. flavus [10,24]. The cbb genes from H. thermoluteolus were regulated by the same mechanism as in X. flavus. On the other hand, only one complex is indicated in that from Rh. sphaeroides [23] and T. ferrooxidans [14], and electrophoretic mobility shift assays using higher concentrations of CbbR protein and promoter region from R. eutropha indicate four CbbR–DNA complexes [9]. Thus, the CbbR–DNA complex is quite different among several autotrophic bacteria in spite of the absence of significant differences of amino acid sequences among all CbbR.

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References


