Expression and Regulation of Ribulose 1,5-Bisphosphate Carboxylase/Oxygenase Genes in *Mycobacterium* sp. Strain JC1 DSM 3803

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Ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) is the key enzyme of the Calvin reductive pentose phosphate cycle. Two sets of structural genes (*cbbLS-1* and *-2*) for form I RubisCO have been previously identified in the *Mycobacterium* sp. strain JC1, which is able to grow on carbon monoxide (CO) or methanol as sole sources of carbon and energy. Northern blot and reverse transcriptase PCR showed that the *cbbLS-1* and *-2* genes are expressed in cells grown on either carbon monoxide (CO) or methanol, but not in cells grown in nutrient broth. A promoter assay revealed that the *cbbLS-2* promoter has a higher activity than the *cbbLS-1* promoter in both CO- and methanol-grown cells, and that the activities of both promoters were higher in CO-grown cells than in methanol-grown cells. A gel mobility shift assay and footprinting assays showed that CbbR expressed in *Escherichia coli* from a *cbbR* gene, which is located downstream of *cbbLS-1* and transcribed in the same orientation as that of the *cbbLS* genes, specifically bound to the promoter regions of the *cbbLS-1* and *-2* genes containing inverted repeat sequence. A DNase I footprinting assay revealed that CbbR protected positions -59 to -3 and -119 to -78 of the *cbbLS-1* and *-2* promoters, respectively. Overexpression of CbbR induced the transcription of RubisCO genes in *Mycobacterium* sp. strain JC1 grown in nutrient broth. Our results suggest that the CbbR product from a single *cbbR* gene may positively regulate two *cbbLS* operons in the *Mycobacterium* sp. strain JC1 as is the case for *Rhodobacter sphaeroides* and *Cupriavidus necator*.

***Keywords*: RubisCO genes, two copies of *cbbLS* genes, gene expression and regulation, mycobacteria, carboxydobacteria, methylotrophic bacteria**

Ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO), the most abundant enzyme in nature, catalyzes the fixation of CO_2 to yield organic carbon (Ellis, 1979). RubisCOs are present in many archaea, bacteria, and eukarya (Delwiche and Palmer, 1996; Watson and Tabita, 1997; Ashida *et al.*, 2003; Finn and Tabita, 2003; Li *et al.*, 2005; Tabita *et al.*, 2007) and are divided into four groups (form I, II, III, and IV) based on molecular structure, sequence identity, presence and conservation of essential residues for CO_2 fixation, and ability to fix CO_2 (Tabita *et al.*, 2007).

Some bacteria possess multiple RubisCO genes. *Allochromatium vinosum* (previously *Chromatium vinosum*) has two sets of divergent green-like form I RubisCO genes (Viale *et al.*, 1989). *Cupriavidus necator* H16 (previously *Alcaligenes eutrophus* H16) and *Mycobacterium* sp. strain JC1 (previously *Acinetobacter* sp. strain JC1) possess two sets of nearly identical red-like form I RubisCO genes (Kusian *et al.*, 1995; Park *et al.*, 2009). *Rhodobacter azotoformans* has a set each of green- and red-like form I RubisCO genes (Uchino and

Yokota, 2003). *Thiobacillus denitrificans*, *Rhodobacter sphaeroides*, *Rhodobacter capsulatus*, *Halothiobacillus neapolitanus* (previously *Thiobacillus neapolitanus*), *Thiomonas intermedia* (previously *Thiobacillus intermedius*), and *Thiomicrospira pelophila* have a set each of form I and form II RubisCO genes (Gibson and Tabita, 1977a, 1977b; Shively *et al.*, 1986; English *et al.*, 1992; Stoner and Shively, 1993; Paoli *et al.*, 1995; Tourova *et al.*, 2006). *Acidithiobacillus ferrooxidans* (previously *Thiobacillus ferrooxidans*), *Thiomicrospira crunogena*, *Thiomicrospira kuenenii*, and *Hydrogenovibrio marinus* possess two sets of green-like form I and a set of form II RubisCO genes (Kusano *et al.*, 1991; Yaguchi *et al.*, 1994; Heinhorst *et al.*, 2002; Tourova *et al.*, 2006).

CbbR, a member of LysR type transcription regulators (LTTRs), positively regulates the transcription of RubisCO genes (Schell, 1993). LTTRs, including CbbR, typically activate transcription by binding to a region located 1 to 80 bp upstream of the transcriptional start site of RubisCO genes (Wang *et al.*, 1992; Parsek *et al.*, 1994; McFall *et al.*, 1997; Van Keulen *et al.*, 1998; Dubbs *et al.*, 2000; Frias *et al.*, 2000; Vichivanives *et al.*, 2000; Dubbs and Tabita, 2003; Dangel *et al.*, 2005). LTTRs usually bind to the consensus DNA binding motif, $T-N_{11}-A$, as a dimer or tetramer (Schell, 1993; Van Keulen *et al.*, 2003; Dubbs *et al.*, 2004).

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The mechanism of regulation of the *cbb* operon by CbbR varies between species. CbbR regulates the *cbb*₁ operon by interacting with RegA in *R. sphaeroides* (Dubbs *et al.*, 2000). In *Xanthobacter flavus*, CbbR binds to the inverted repeats of the *cbb* operon differently in the presence and absence of NADPH and controls the expression of the operon by bending the DNA (Van Keulen *et al.*, 2003). In the regulation of multiple RubisCO genes, either one or two CbbRs regulate RubisCO genes. For example, a single CbbR positively regulates two *cbb* operons in *R. sphaeroides* and *C. necator* (Gibson and Tabita, 1993; Kusian *et al.*, 1995). Two CbbR proteins encoded by two independent *cbbR* genes located upstream of *cbbLS-1* and *cbbLS-2* regulate *cbbLS-1* and *cbbLS-2*, respectively, in *R. capsulatus* (Dubbs *et al.*, 2004). In *H. marinus*, two CbbR proteins from different *cbbR* genes located upstream of *cbbLS-1* and *cbbM* are involved in the regulation of *cbbLS-1* and cbbM, respectively, but neither CbbR has been found to regulate the *cbbLS-2* genes (Toyoda *et al.*, 2005). There has been no report on the regulation of RubisCO genes in mycobacterium.

Mycobacterium sp. strain JC1 is a carboxydobacterium that is able to grow on carbon monoxide (CO) or methanol as a sole carbon and energy source (Cho *et al.*, 1985; Ro *et al.*, 1997; Song *et al.*, 2002). When this strain is grown on either of these substrates it exhibits RubisCO activity, indicating that it employs the Calvin cycle for C_1 assimilation (Ro *et al.*, 1997; Park *et al.*, 2003). Two copies of the RubisCO genes, *cbbLS-1* and *cbbLS-2*, encoding a novel form I RubisCO (form IE), and a putative *cbbR* gene (GenBank accession no. FJ042653) located downstream of *cbbLS-1*, were recently identified in *Mycobacterium* sp. strain JC1 (Park *et al.*, 2009). In this work, we provide the first report on the basic mechanism of expression and regulation of the RubisCO genes in mycobacteria using *Mycobacterium* sp. strain JC1.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Reference or source
Bacterial strains		
Mycobacterium sp. strain JC1	Wild type (DSM 3803)	Cho *et al.* (1985)
E. coli		
DH5α	*supE44lac169(φ80lacZΔM15)hsdR17recA1endA1gyrA96 thi-1relA1*	GIBCOBRL
BL21(DE3)	F⁻ *ompT hsdS* B(r_B⁻ m_B⁻) *gal dcm*	Promega
Plasmids		
pBluescriptII KS+	2,961 bp plasmid derived from pUC19, Amp^r	Stratagene
pGEM-7Zf(-)	3,000 bp cloning vector, Amp^r	Promega
pGEM-T Easy	3,018 bp linear plasmid vector for direct subcloning of PCR product, Amp^r	Promega
pGEX-4T1	4,950 bp plasmid for high-level intracellular expression of genes, Amp^r	Pharmacia
pGL3-Basic	4,818 bp promoterless, luciferase reporter vector, *luc*⁺, Amp^r	Promega
pMV306AC	pMV306 with acetamide promoter, Kan^r	Kang *et al.* (2005)
pNBV1	5.8 kb plasmid derived from p16R1, Hyg^r	Howard *et al.* (1995)
pKO	8,366 bp vector containing *sacB* gene for sucrose counter-selection, Hyg^r and Kan^r.	Sherman *et al.* (2001)
pUC18	2,686 bp cloning vector, Amp^r	Fermentas
pHJ1	pBluescript II KS+ containing 7.2 kb *Pst*I fragment from lambda clone 1	Park *et al.* (2009)
pDO12	pGEM-7Zf(-) containing 2,027 bp *Kpn*I/*Xba*I fragment from pEHP12	This study
pDO13	pNBV1 containing 2,082 bp *Bst*XI/*Xba*I fragment from pDO12	This study
pDO15	pGEM-7Zf(-) containing 2,135 bp *Kpn*I/*Xba*I fragment from pEHP22	This study
pDO16	pNBV1 containing 2,190 bp *Bst*XI/*Xba*I fragment from pDO15	This study
pDO17	pGEM-7Zf(-) containing 1.6 kb *Kpn*I/*Xba*I fragment from pGL3-Basic	This study
pDO18	pNBV1 containing 1.6 kb *Bst*XI/*Xba*I fragment from pDO17	This study
pEHP09	pGEX-4T-1 containing 927 bp PCR product for CbbR over-expression	This study
pEHP1	pGEM-T Easy containing PCR product of a 247 bp promoter region of *cbbL-1* gene of *Mycobacterium* sp. strain JC1	This study
pEHP11	pUC18 containing 311 bp *Sal*I/*Sph*I fragment from pEHP1	This study
pEHP12	pGL3-Basic containing 340 bp *Hin*dIII/*Sac*I fragment from pEHP11	This study
pEHP2	pGEM-T Easy containing PCR product of a 343 bp promoter region of *cbbL-2* gene of *Mycobacterium* sp. strain JC1	This study
pEHP21	pUC18 containing 407 bp *Sal*I/*Sph*I fragment from pEHP2	This study
pEHP22	pGL3-Basic containing 436 bp *Hin*dIII/*Sac*I fragment from pEHP21	This study
pJL1	pGEM-T Easy containing PCR product of a *cbbR* gene with His-tag	This study
pJL2	pMV306AC containing a hygromycin resistant gene from pKO	This study
pJL3	pJL2 containing 931 bp fragment from pJL1 digested with *Nde*I and *Pvu*II	This study

Materials and Methods

Bacterial strains, phages, plasmids, and cultivation conditions

The bacterial strains and plasmids used in this work are described in Table 1. *Mycobacterium* sp. strain JC1 DSM 3803 was grown at 37°C in standard mineral base (SMB) medium (Kim and Hegeman, 1981) supplemented with 30% (v/v) CO (SMB-CO) or 1% (v/v) methanol (SMB-MeOH), in Luria-Bertani medium (LB), or in Nutrient broth (NB). *Escherichia coli* strains were cultivated at 37°C in LB.

Reverse transcriptase PCR (RT-PCR)

To detect the transcription of the *cbbL-1* and *-2* genes in cells grown in SMB-CO, SMB-MeOH, or NB by RT-PCR, three synthetic primers, CbbL12-F, CbbL1-R, and CbbL2-R, were synthesized. The CbbL12-F primer is a 20-mer; 5'-CAG ATAGATGGAACGCGGGA-3', and corresponds to nucleotide positions 2 to 21 bp downstream of the *cbbL-1* and *-2* start codons. The CbbL1-R primer; 5'-CAGGCCCAACTT GGGCTTGG-3' and CbbL2-R primer; 5'-TGGCCTGGTAG TGCTCGAAG-3' are 20-mers that are complementary to nucleotides 497 to 516 bp and 204 to 223 bp downstream of the *cbbL-1* and *-2* start codons, respectively.

Reverse transcription was performed with SuperScript III First-Strand Synthesis System (Invitrogen, USA) according to the manufacturer's instruction. The PCR mixture contained 2 μl of 2.5 mM each dNTP, 20 pmol each of appropriate primers, 1 μl of synthesized cDNA, and 0.5 U *Taq* polymerase in 50 μl reaction buffer [PCR reaction buffer: 50 mM Tris-HCl; pH 8.3, 250 μg BSA per ml, 1% (w/v) Ficoll, and 1.5 mM MgCl$_2$].

RNA isolation and Northern blot analysis

Total RNA was isolated from cells harvested at the mid-exponential growth phase using TRIzol reagent (Invitrogen), according to the method previously described by Seo *et al.* (2007). Northern blots were performed by the method described in Sambrook *et al.* (1989). The 515 bp *cbbL-1* DNA fragment obtained by PCR with CbbL12-F and CbbL1-R was used as a probe in the northern blots.

Primer extension

A primer-extension assay was performed using the avian myoblastosis virus reverse transcriptase primer extension system (Promega, USA) and a 21-mer oligonucleotide primer; 5'-GCCGGCAGGAGTCAGCCGCAC-3', which is complementary to nucleotide positions 160 to 180 downstream of the translational start codon of the *Mycobacterium* sp. strain JC1 *cbbR* gene (GenBank accession no. FJ042653), following the method of Sambrook *et al.* (1989). Total RNA was prepared from cells in early stationary phase in SMB-CO. Primer extension was performed with 5.6 μg RNA at 60°C annealing temperature.

Construction of reporter plasmids

To amplify the putative *cbbLS-1* and *-2* promoter regions covering the LTTR binding motif (T-N$_{11}$-A; Schell, 1993) present at -38 to -20 and -119 to -97 of the *cbbLS-1* and *-2* promoters, respectively (Park *et al.*, 2009), two sets of

oligonucleotide primers were synthesized. For the *cbbLS-1* promoter region, two synthetic primers, designated PH1-fwd; 5'-ATCGGACTCGTGCGGAC-3' and EXE1-rev; 5'-GCACG TCGGAGTCCTTCGG-3', corresponding to nucleotide positions -58 to -42 and 168 to 186 of the *cbbLS-1* promoter, respectively, were synthesized. To amplify the *cbbLS-2* promoter region, two primers, designated PH2-fwd; 5'-CGTCC CTTCTTCGTGCAGTG-3' and EXE2-rev; 5'-CCTCTTGG GGTGTAATCCTG-3', corresponding to nucleotides -184 to -165 and 139 to 158 of the *cbbLS-2* promoter, respectively, were synthesized. The amplified 247 and 343 bp PCR products were cloned into pGEM-T Easy (Promega) to produce subclones, pEHP1 and pEHP2, for use in the *cbbLS-1* and *-2* promoter assays, respectively. The pEHP1 and pEHP2 plasmids were digested with *Sal*I and *Sph*I and cloned into pUC18 to produce subclones, pEHP11 and pEHP21, respectively. The pEHP11 and pEHP21 plasmids were then digested with *Hin*dIII and *Sac*I and cloned into pGL3-basic (Promega) to produce subclones, pEHP12 and pEHP22, respectively. The pEHP12 and pEHP22 plasmids were next digested with *Kpn*I and *Xba*I and cloned into pGEM-7Zf(-) to produce subclones, pDO12 and pDO15, respectively. Finally, the pDO12 and pDO15 plasmids were digested with *Bst*XI and *Xba*I and cloned into pNBV1, an *E. coli*-mycobacteria shuttle vector (Howard *et al.*, 1995), to produce subclones, pDO13 and pDO16, respectively. The pDO13 and pDO16 plasmids harboring the putative promoter regions of *cbbLS-1* and *cbbLS-2* genes, respectively, were subsequently introduced separately into *Mycobacterium* sp. strain JC1 by electroporation using a Gene Pulser apparatus (Bio-Rad, USA) at 2.5 kV, 800 Ω, and 25 μF. pDO18, a promoterless vector, was used as a negative control.

Luciferase assay

Luciferase assays were performed with a luciferase assay reagent (Promega), according to the manufacturer's description (Promega). The relative luciferase activities were determined by luminometer (TD-20/20, Turner Designs, USA) for 15 sec after 3-sec interval. Activity was expressed as light units.

Overexpression and purification of the CbbR in *E. coli*

To overexpress CbbR in *E. coli* for use in the gel mobility shift assay, two oligonucleotide primers were synthesized. The primer CbbR-fwd is a 26-mer; 5'-CCGgaattcATGACC AACGCGCGATT-3' with a 9-mer extension, including an *Eco*RI site (underlined small letters), derived from amino acid sequence MTNARL, which is present at the N-terminus of CbbR in *Mycobacterium* sp. strain JC1 (GenBank accession no. FJ042653). The primer CbbR-rev is a 27-mer; 5'-CCG ctcgagTCAGGCGGTAACGGCTCC-3' with a 9-mer extension, including a *Xho*I sites (underlined small letters), derived from the stop codon TGA and amino acid sequence GAVTA, which are present at the 3'-end of the *cbbR* gene and the C-terminus of the CbbR protein, respectively, in *Mycobacterium* sp. strain JC1 (GenBank accession no. FJ 042653). The amplified 927 bp products were digested with *Eco*RI and *Xho*I and cloned into pGEX-4T1 (Promega) to produce a subclone, pEHR09. The pEHR09 plasmid harboring the complete *cbbR* gene was subsequently introduced into *E. coli* BL21(DE3) and CbbR expression was induced

with 0.5 mM IPTG at 25°C for 4 h. The overexpressed GST-CbbR fusion proteins were purified and cleaved using glutathione Sepharose 4B and thrombin protease (Pharmacia Biotech., Sweden), respectively, according to the manufacturer's instructions.

Overexpression of CbbR in *Mycobacterium* sp. strain JC1

For overexpression of CbbR in *Mycobacterium* sp. strain JC1, an acetamide-inducible vector, pMV306AC (Kang *et al.*, 2005), was used. To amplify the *cbbR* gene and place it in-frame with the His-tag coding sequence, one set of oligonucleotide primers was synthesized. The HiscbbR-fwd primer is a 23-mer; 5'-CATATGACCAACGCGCGATTGC G-3', that anneals to the first 17 nt of the *cbbR* gene and contains additional bases to generate a *Nde*I recognition site upstream of the start codon (underlined); and the HiscbbR-rev primer is a 47-mer; 5'-CAGCTGTCAGTGAT GGTGATGGTGATGGGCGGTAACGGCTCCCCGAA-3', anneals to the last 20 nt of the *cbbR* gene and contains additional 6× His coding sequences (bold) and a *Pvu*II recognition site (underlined) upstream and downstream of the stop codon, respectively. The amplified 936 bp PCR product was cloned into the pGEM-T Easy vector to produce subclone pJL1. A 1,322 bp fragment from pKO digested with *Fsp*I and *Nru*I, which contained the hygromycin resistant gene, was inserted into pMV306AC digested with *Stu*I to create subclone pJL2. A 931 bp fragment from pJL1 digested with *Nde*I and *Pvu*II was cloned into pJL2 digested with same enzymes to produce subclone pJL3. The pJL3 was then introduced into *Mycobacterium* sp. strain JC1 by electroporation using a Gene Pulser apparatus (Bio-Rad) at 2.5 kV, 1000 Ω, and 25 μF. Transformed cells were cultivated overnight at 37°C in NB supplemented with hygromycin (50 μg/ml), and a 2-ml portion of the cultures was inoculated into 200 ml hygromycin-supplemented NB. After 14 h of cultivation, the culture was supplemented with acetamide at a final concentration of 3% (w/v) and incubated for another 18 h at 37°C. Expression of His-tagged CbbR was detected by immunoblot with anti-His antibody (Santa cruz, USA) after denaturing polyacrylamide gel electrophoresis (PAGE) of cell-free extracts, following the method of Laemmli (1970). Proteins were stained with Coomassie brilliant blue R-250 (CBB) using a modification (Kim and Hegeman, 1981) of the method of Weber and Osborn (1969).

Electrophoretic mobility shift assay (EMSA)

EMSA was performed using a modification of the method described by Dhandayuthapani *et al.* (1997). The DNA probes used for EMSA analysis were prepared by digesting the pEHP1 and pEHP2 with *Not*I. The resulting 281- and 377-bp fragments containing putative promoter regions of *cbbLS-1* and *-2* genes, respectively, were labeled with [γ-^{32}P] ATP using T4 DNA polynucleotide kinase. The EMSA reaction mixture (40 μl) contained 1 ng of end-labeled DNA probe, purified CbbR (at specified concentrations), 0.1 μg of poly(dI-dC) DNA, and 8 μl of 5× binding buffer [100 mM HEPES; pH 8.0, 40% (v/v) glycerol, 5 mM EDTA, 5 mM DTT, 10 mM MgCl$_2$, and 30 mM KCl]. The mixtures were incubated for 20 min at 37°C. The native 5% (v/v) polyacrylamide gel was pre-run for 1 h prior to electrophoresis of the mixtures for 3 h at 10 V/cm in 0.5× TBE buffer (45 mM Tris base, 45 mM boric acid, and 1 mM EDTA, pH 8.0). A 10- to 360-fold excess of unlabeled DNA fragment was used for the competition experiments. Unlabeled DNA probes and 448 bp *Pvu*II fragments of pBluescript II KS(+) were used as specific competitors and non-specific competitors, respectively. To confirm the binding of CbbR to the promoter regions of *cbbLS-1* and *-2* genes, 39-mer oligonucleotides covering the T-N$_{11}$-A LTTR-binding motif (underlined) and corresponding to -48 to -10 of the *cbbLS-1* promoter (CbbR1-F; 5'-TGCG GACACGCTATGAGCAGGCTGAACAGGATTGCCTCA-3', CbbR1-R; 5'-TGAGGCAATCCTGTTCAGCCTGCTCAT AGCGTGTCCGCA-3') and to -127 to -89 of the *cbbLS-2* promoter (CbbR2-F; 5'-CACCTCACCCCACCTAGTGGAGG CCGAGGGGGAAAGCCAA-3', CbbR2-R; 5'-TTGGCTTT CCCCCCTCGGCCTCCACTAGGTGGGTGAGGTG-3') were synthesized. The CbbR1-F and CbbR2-F were then hybridized with CbbR1-R and CbbR2-R, respectively, and used for EMSA.

DNase I footprinting assay

DNase I protection assays were performed following the method of Leblanc and Moss (1994) with modifications.
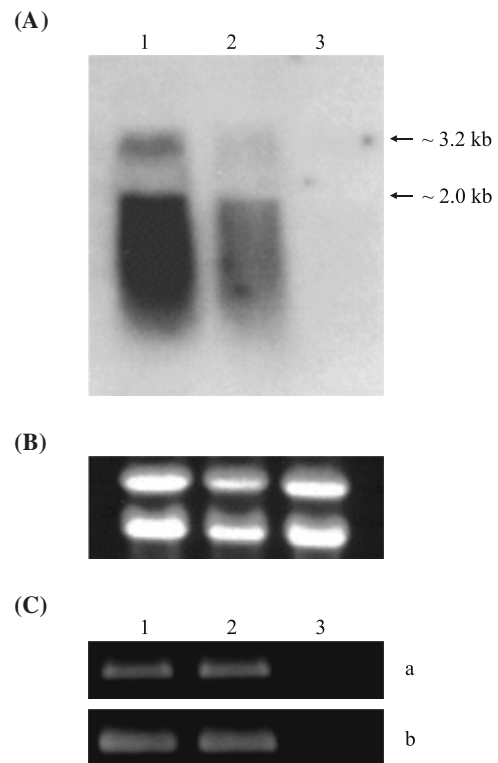

Fig. 1. Transcription of *cbbL-1* and *-2* under different growth conditions. Transcription of *cbbL-1* and *-2* in *Mycobacterium* sp. strain JC1 was analyzed by Northern blot (A) and RT-PCR (C) using total RNAs prepared from cells grown in SMB-CO (lane 1), SMB-MeOH (lane 2), and NB (lane 3). Ribosomal RNAs were used as control for Northern blot (B). RT-PCR products were obtained with primers CbbL12-F and CbbL1-R for *cbbL-1* (Fig. 1C-a) and CbbL12-F and CbbL2-R for *cbbL-2* (Fig. 1C-b).
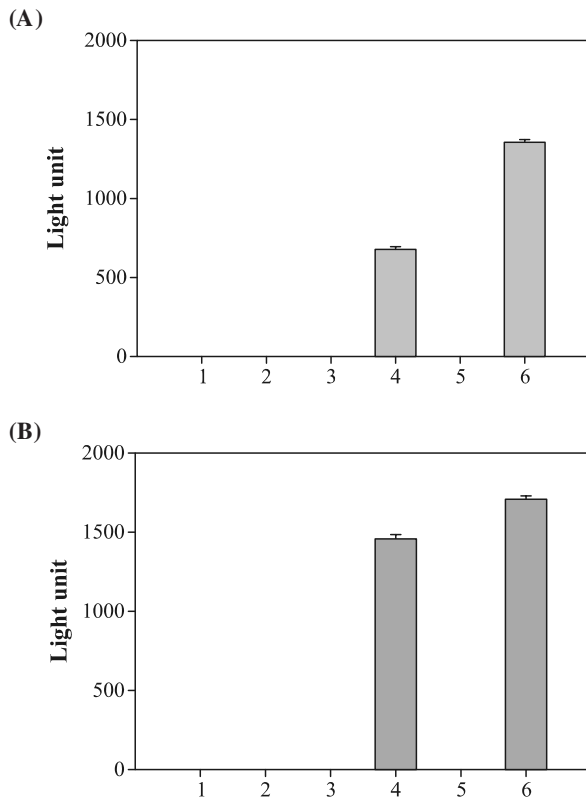
(A)

(B)

Fig. 2. Activities of *cbbLS* promoters in *Mycobacterium* sp. strain JC1. (A) Activity of *cbbLS-1* promoter. The promoter activity was assayed with cell-free extracts prepared from cells harboring pDO18 (a promoter-less vector) grown on LB (lane 1), methanol (lane 3), and CO (lane 5) and those harboring pDO13 (a vector containing a putative *cbbLS-1* promoter) grown on LB (lane 2), methanol (lane 4), and CO (lane 6). (B) Activity of *cbbLS-2* promoter. The promoter activity was analyzed with cell-free extracts prepared from cells harboring pDO18 (a promoter-less vector) grown on LB (lane 1), methanol (lane 3), and CO (lane 5) and those harboring pDO16 (a vector containing a putative *cbbLS-2* promoter) grown on LB (lane 2), methanol (lane 4), and CO (lane 6).

DNA fragments containing putative promoter regions of *cbbLS-1* and -2 genes were prepared by digesting pEHP1 and pEHP2 with *Apa*I and *Bst*XI. The resulting 351- and 447 bp fragments were end-labeled with [γ-^{32}P] ATP using T4 DNA polynucleotide kinase, and then digested with *Nco*I or *Nde*I to prepare strand-specific end-labeled DNA fragments. The strand-specific end-labeled fragments were mixed with purified CbbR in the binding buffer used for EMSA and left for 30 min at 37°C. The mixture was then treated with 5 μl of DNase I solution (10 mM CaCl$_2$, 50 mM MgCl$_2$, and 10 μg/ml DNase I) for 1 min at room temperature and the reaction was stopped by addition of 1 μl 0.5 M EDTA. After phenol-chloroform extraction, the reactants were mixed with gel-loading buffer [95% (v/v) formamide, 20 mM EDTA, 0.005% (w/v) bromophenol blue, and 0.05% (w/v) xylene cyanol FF], incubated for 5 min at 95°C, and loaded onto a 6% (w/v) polyacrylamide sequencing gel with reference sequences obtained using the method of Maxam and Gilbert (1980).

Results and Discussion

Expression patterns of the RubisCO genes

A culture of *Mycobacterium* sp. strain JC1 growing on CO or methanol assimilates CO$_2$, the oxidation product of both substrates, through the Calvin cycle, which uses RubisCO as a key enzyme (Kim *et al.*, 1997; Ro *et al.*, 1997; Park *et al.*, 2003). Two copies each of the structural genes *cbbLS-1* and *-2* that encode form IE RubisCO enzymes with 78.5% identity in amino acid sequence have been identified in *Mycobacterium* sp. strain JC1 (Park *et al.*, 2009).

The results of our northern blot analysis showed that RubisCO was expressed in *Mycobacterium* sp. strain JC1 cells grown on CO (Fig. 1A, lane 1) or methanol (Fig. 1A, lane 2), but not in cells grown on nutrient broth (Fig. 1A, lane 3), indicating that the genes for RubisCO are only expressed in the presence of CO$_2$. RT-PCR (Fig. 1C) analysis revealed that both copies of the *cbbLS-1* (Fig. 1C-a) and *-2* genes (Fig. 1C-b) in *Mycobacterium* sp. strain JC1 were expressed in cells grown on either CO (Fig. 1C-a, lanes 1 and 2) or methanol (Fig. 1C-b, lanes 1 and 2) as the sole source of carbon and energy, indicating that the two RubisCO genes are co-regulated under these growth conditions. These results do not coincide with a previous report that the two copies of *cbbLS* genes in *Mycobacterium* sp. strain JC1 are not be expressed simultaneously in cells growing on CO or methanol, since the peptide maps of RubisCOs prepared from CO- and methanol-grown cells were not identical (Kim *et al.*, 1997). We assume that there were experimental errors during protein purification and/or peptide mapping in the previous experiments.

The detection of 3.2 and 2.0 kb bands in the northern
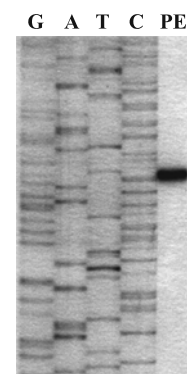

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      ┌─→+1
GGCTCGGTTGCGCCATGCGCGACGGCTGCTCGCCGACGGCGGCGAAGTAG
ACCTGGCGGCATTGTCCACGATCGAATCAGCCGATATTCTCGCCAGCCGC
GTGTTCGCGACTGCGAAGGGATCGGTCGGCCCATGACCAACGCGCGATTG
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Fig. 3. Identification of the transcriptional start site of the *cbbR* gene. The transcriptional start site of the *cbbR* gene was identified by primer-extension mapping using a ^{32}P-labeled 21-mer oligonucleotide primer which is complementary to nucleotide positions 160 to 180 bp downstream of the *cbbR* start codon. Primer extension products were analyzed (lane PE) in parallel with the sequencing ladder (lanes G, A, T, and C) primed with the same primer. The bold nucleotide "C" with arrow indicates the transcriptional start site of *cbbR* gene. The start codon of *cbbR* gene (ATG) is expressed in bold.
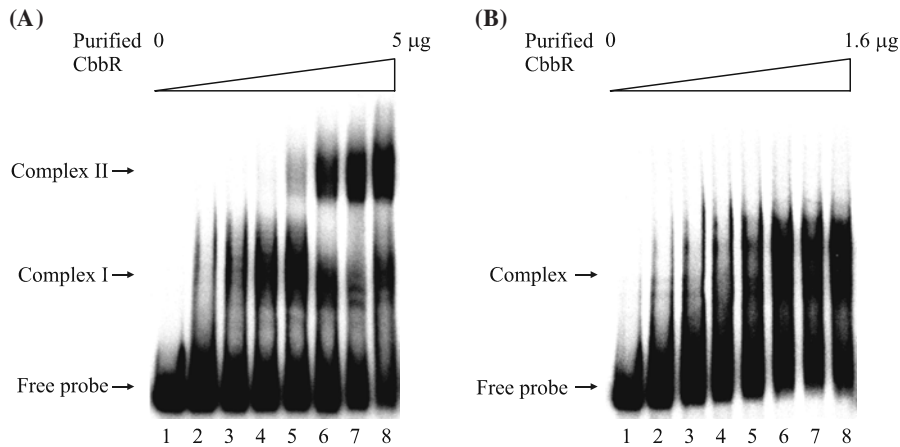
Fig. 4. EMSA for *cbbLS* promoters. (A) EMSA with *cbbLS-1* promoter region and CbbR. A 8.6-fmol of ^{32}P-labeled 281 bp *Not*I fragment prepared from pEHP1 was incubated with 0, 0.4 (0.94 pmol), 0.8, 1.6, 2.4, 3.2, 4.0, and 5.0 μg of purified CbbR (lanes 1 to 8, respectively) under the conditions described in 'Materials and Methods'. (B) EMSA with *cbbLS-2* promoter region and CbbR. A 7.1-fmol of ^{32}P-labeled 377 bp *Not*I fragment prepared from pEHP2 was incubated with 0, 0.2 (0.47 pmol), 0.4, 0.6, 0.8, 1.0, 1.2, and 1.6 μg of purified CbbR (lanes 1 to 8, respectively) under the conditions described in 'Materials and Methods'.

blot suggests that the *cbbL-1*, *cbbS-1*, and *cbbX* (Park *et al.*, 2009) may also be co-expressed in *Mycobacterium* sp. strain JC1, since the sizes of the DNA fragments covering the *cbbLS-1*, *cbbLS-2*, and *cbbLS-1cbbX* are 1,875, 1,876, and 2,883 bp, respectively. However, it is unclear whether the

2.0 kb *cbbLS* transcript is made from both *cbbLS-1* and *cbbLS-2* genes.

Multiple copies of *cbbLS* genes in a bacterium have been shown to be expressed in various species-specific patterns (Jouanneau and Tabita, 1986; Gibson *et al.*, 1991; Gibson
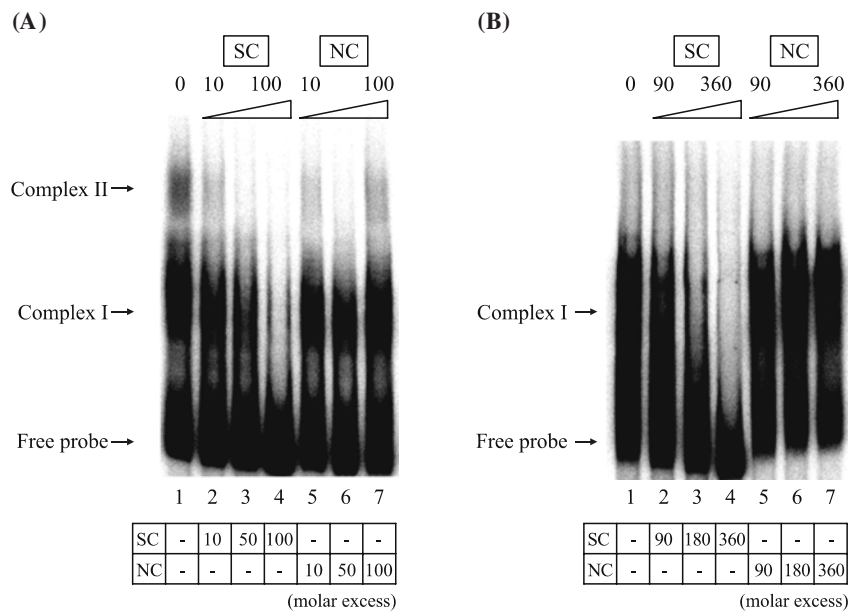

Fig. 5. EMSA for *cbbLS* promoters in the presence of competitors. (A) Competition for *cbbLS-1* promoter. Purified CbbR (7.54 pmol) was incubated with 8.6 fmol of ^{32}P-labeled 286 bp *Not*I fragments from pEHP1 in the absence (lane 1) and presence of 10, 50, and 100-fold molar excess of cold 286 bp *Not*I fragments (specific competitor [SC]; lanes 2 to 4, respectively) or those of cold 448 bp *Pvu*II fragments from pBluescript II KS[+] (non-specific competitor [NC]; lanes 5 to 7, respectively) under the conditions described in 'Materials and Methods'. - : no competitor. (B) Competition for *cbbLS-1* promoter. Purified CbbR (3.14 pmol) was incubated with 7.1 fmol of ^{32}P-labeled DNA fragments from pEHP2 in the absence (lane 1) and presence of 90, 180, and 360-fold molar excess of cold 342 bp *Not*I fragments (specific competitor [SC]); lanes 2 to 4, respectively) or those of cold 448 bp *Pvu*II fragments from pBluescript II KS[+] (non-specific competitor [NC]; lanes 5 to 7, respectively) under the conditions described in 'Materials and Methods'. - : no competitor.

and Tabita, 1993; Kusian and Bowien, 1995; Smith and Tabita, 2002; Dubbs *et al.*, 2004; Toyoda *et al.*, 2005). To measure the expression patterns of the two copies of the *cbbLS* genes in *Mycobacterium* sp. strain JC1, the expression levels of each copy of both RubisCO genes were determined in a promoter assay using the luciferase reporter gene. The promoter of *cbbLS-2* gene in the CO- (1,711 units) and methanol-grown (1,470 units) cells was 1.26- and 2.17-fold more active than the promoter of the *cbbLS-1* gene, respectively (Fig. 2). These results indicate that *cbbLS-2* is expressed more strongly than *cbbLS-1* in *Mycobacterium* sp. strain JC1 under both chemoautrophic and methylotrophic growth conditions. It was also observed that the promoter activities of *cbbLS-1* (1,358 units) and *-2* in CO-grown cells were 2.0- and 1.16-fold higher than those of *cbbLS-1* (679 units) and *-2* in methanol-grown cells, respectively, indicating that the RubisCO of *Mycobacterium* sp. strain JC1 is more strongly expressed in cells growing on CO than in cells growing on methanol. Cells harboring pDO13 (a vector containing a putative *cbbLS-1* promoter) or pDO16 (a vector containing a putative *cbbLS-2* promoter) exhibited no significant luciferase activity during growth on LB. Cells harboring pDO18 (a promoterless vector) grown on LB, CO, and methanol also did not exhibit a significant level of luciferase activity.

Transcription of the *cbbR* gene
It is interesting that the *cbbR* gene is located in the same orientation downstream of the *cbbLS-1* genes in *Mycobacterium* sp. strain JC1 (Park *et al.*, 2009) since the *cbbR* genes of all autotrophic organisms are located in the opposite orientation immediately upstream of one of the *cbb* operons (Kusian and Bowien, 1997). The identity of deduced amino acid sequences between the CbbR of *Mycobacterium* sp. strain JC1 and those of *Hydrogenophilus thermoluteolus* (GenBank accession no. BAA95688) and *Cupriavidus taiwanensis* (GenBank accession no. CAQ72294) was 33%.

Primer extension revealed that the transcriptional start site of the *cbbR* gene was the G located 110 bp upstream of the *cbbR* start codon (Fig. 3). The consensus sequences of known promoters in other bacteria (Gomez and Smith, 2000) were not found in the -10 and -35 regions, suggesting that a new type of promoter sequence may work for the *cbbR* gene in *Mycobacterium* sp. strain JC1. Northern blot analysis revealed that *cbbR* was transcribed into a monocistronic mRNA (data not shown).

Binding of CbbR to the promoter regions of *cbbLS-1* and *-2* genes
Gel mobility shift assays indicated that CbbR bound to the *cbbLS-1* and *-2* promoter regions in *Mycobacterium* sp. strain JC1 (Fig. 4). Two and one binding complexes were formed in the promoter regions of *cbbLS-1* (Fig. 4A) and *cbbLS-2* (Fig. 4B), respectively. The number of binding complex in the promoter region of *cbbL-2* did not change even when the amount of CbbR added into the reaction mixture was increased up to 5 μg (data not shown). The specificity of binding of CbbR to the promoter regions of *cbbLS-1* and *-2* was measured in competitive EMSA assays using excessive amounts of specific and non-specific competitors

(Fig. 5A and B).

It has been reported that the CbbRs of bacteria usually bind to nucleotide positions 80 to 1 bp upstream of the transcriptional start site (Van Keulen *et al.*, 1998; Dubbs *et al.*, 2000; Vichivanives *et al.*, 2000; Dubbs and Tabita, 2003; Dubbs *et al.*, 2004; Dangel *et al.*, 2005). In the case of *R. sphaeroides*, CbbR binds to the *cbb*$_{II}$ promoter between positions -61 and +1 of the promoter (Dubbs and Tabita, 2003). Foot-printing experiments revealed that CbbR bound to positions -3 to -59 bp of the *cbbLS-1* promoter, with a hypersensitive site between -20 and -42 bp (Fig. 6A). In the case of the *cbbLS-2* promoter, CbbR was found to bind from -119 to -78 bp relative to the transcriptional start point of the operon, with a hypersensitive site from -101 to -90 bp (Fig. 6B). These results indicate that the CbbR in *Mycobacterium* sp. strain JC1 uses a similar mechanism as other bacterial CbbRs to regulate the expression of *cbbLS-1* and suggest that the expression of *cbbLS-2* may be regulated by CbbR in a different way from what has been previously reported.

The expression of RubisCO in cells grown on CO and methanol, that produce CO_2 as an oxidation product of CO and methanol, respectively (Fig. 1), but not in cells grown
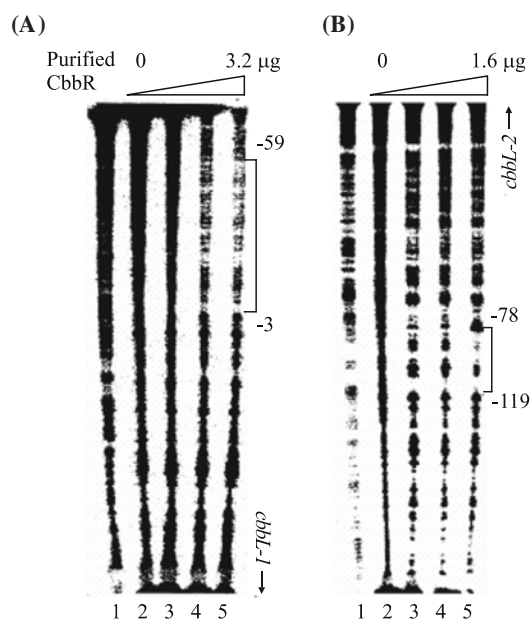

Fig. 6. DNase I footprinting of *cbbLS* promoter regions. (A) DNase I footprinting of *cbbLS-1* promoter. The 351 bp *Apa*I/*Bst*XI double-stranded target DNA fragments from pEHP1 were end-labeled with [γ-^{32}P]ATP using T4 DNA polynucleotide kinase and incubated with 0, 0.8, 1.6, 3.2 μg of purified CbbR (lanes 2 to 5, respectively) in 50 μl of binding buffer under the conditions described in 'Materials and Methods'. (B) DNase I footprinting of *cbbLS-2* promoter. The 447 bp *Apa*I/*Bst*XI fragments from pEHP2 were incubated with 0, 0.4, 0.8, 1.6 μg of purified CbbR (lanes 2 to 5, respectively) in 50 μl of binding buffer as described in 'Materials and Methods'. The numbers delimiting the central binding regions on both A and B mark the positions relative to the transcriptional start site of the *cbbL* gene. To locate the DNase I foot-print C+T specific degradation of the DNA fragments was performed for both analyses (lane 1).
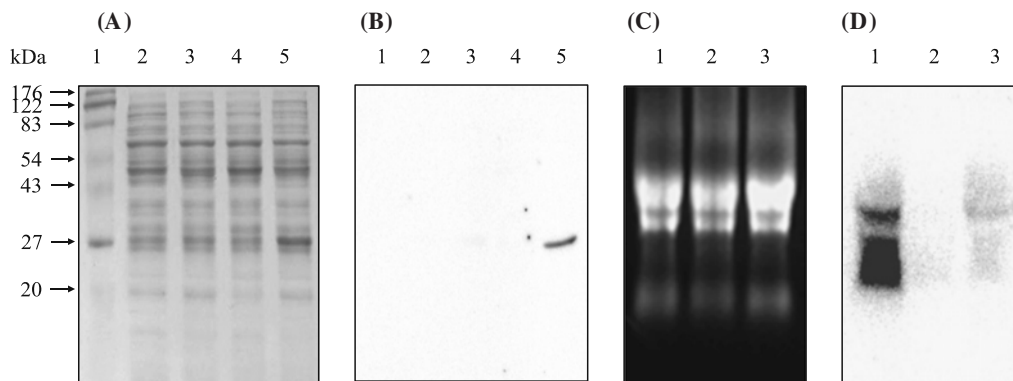
Fig. 7. Role of CbbR in the transcription of RubisCO genes. The role of CbbR in the transcription of *cbbLS* was analyzed with total RNA prepared from cells of *Mycobacterium* sp. strain JC1 grown in NB. (A and B) Expression of CbbR in cells grown in NB. Expression of CbbR in cells harboring pJL3 (lanes 4 and 5) or not (lanes 2 and 3) was analyzed by CBB staining (A) or immunoblot (B) after denaturing PAGE of cells-free extracts prepared from cells grown in NB in the absence (lanes 2 and 4) or presence (lanes 3 and 5) of acetamide as described in 'Materials and Methods'. Lane 1, protein size markers. (C and D) Effect of CbbR on the transcription of *cbbLS* genes. Transcription of *cbbLS* genes in cells growing on methanol (lane 1) and those harboring pJL3 growing in NB in the absence (lane 2) or presence (lane 3) of acetamide was analyzed by Northern blot (D) using total RNA prepared from the cells and the 515 bp *cbbL-1* DNA fragment obtained by PCR using CbbL12-F and CbbL1-R primers as described in 'Materials and Methods'. Ribosomal RNAs were used as control for Northern blot (C).

in NB, and the specific binding of CbbR to the promoter regions of *cbbLS-1* and *-2* genes suggests that the RubisCO genes in *Mycobacterium* sp. strain JC1 are positively regulated by CbbR, as is the case in other organisms (Schell, 1993). To test this hypothesis, we examined the changes in the transcription of RubisCO genes in cells growing in NB in the presence of excessive amount of CbbR. We could not observe the changes in cells defective in the production of CbbR because constructing the CbbR-negative mutant of *Mycobacterium* sp. strain JC1 proved too difficult. We found that overexpression of *cbbR* in *Mycobacterium* sp. strain JC1 growing in NB induced the expression of *cbbLS* (Fig. 7B,

lane 5; 7D, lane 3), whereas no detectable *cbbLS* transcripts are normally produced during growth in this medium (Fig. 1A, lane 3; Fig. 7D, lane 2). These results indicate that CbbR also acts as a positive regulator of RubisCO genes in *Mycobacterium* sp. strain JC1. The *cbbLS* transcript levels in the NB-grown cells overexpressing CbbR were lower than those of methanol-grown cells, probably due to the rapid degradation of the *cbbLS* transcripts in the absence of CO_2.

Analysis of the upstream regions of *cbbLS-1* revealed the presence of two inverted repeats (underlined), CCTCACCC AGGAGG and CTATGAGCAGGCTGAACAG, which were
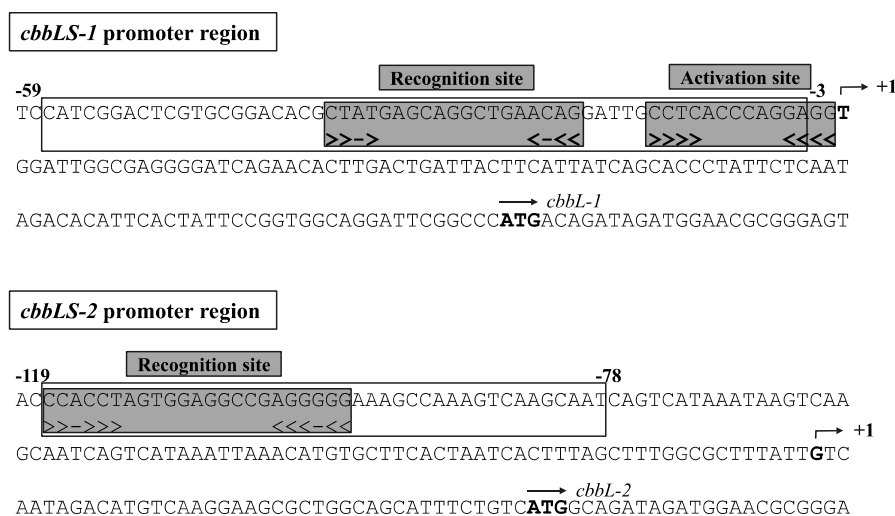

Fig. 8. Promoter regions of *cbbLS* operons. The large boxes enclose the nucleotides that are protected from DNase I digestion, as indicated by footprinting analysis in Fig. 6. The sequences in the small shaded boxes that include inverted repeats may be the putative regions that actually interact with CbbR. The numbers delimiting the central binding region mark the positions relative to the transcriptional start site of the *cbbL* gene. The transcriptional start sites and translational start codons of *cbbL-1* and *-2* genes were expressed in bold.
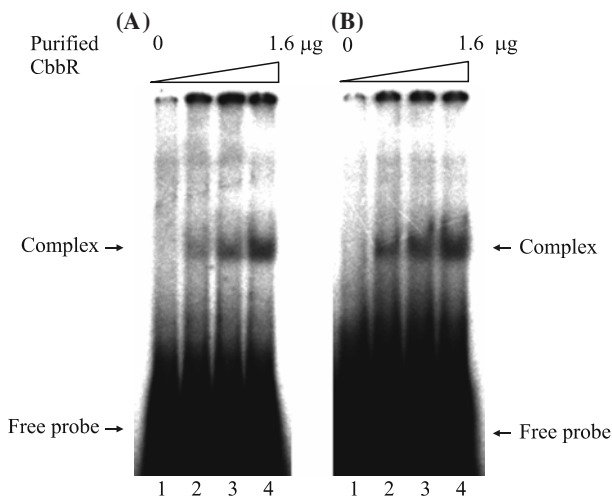
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Fig. 9. Identification of the binding regions for CbbR in the *cbbLS* promoter regions. EMSA with CbbR and dimerized 39-mer oligonucleotides containing the putative LTTR binding motif (T-N_{11}-A) in the upstream region of *cbbLS-1* (A) and *-2* (B) was performed as described in 'Materials and Methods'. A 7.5-fmol of ^{32}P-labeled DNA was incubated with 0, 0.1 (0.24 pmol), 0.4, and 1.6 μg of purified CbbR (lanes 1 to 4, respectively).

identified 1 to 14 and 20 to 38 bp upstream of the *cbbL-1* transcriptional start site, respectively (Fig. 8). The latter repeat was highly homologous to the LTTR binding motif (T-N_{11}-A; Schell, 1993) that is regarded as the CbbR recognition site. This presence of two putative binding sites (Fig. 4A) suggests that CbbR may bind to the two regions [recognition and activation sites (Schell, 1993)] in the *cbbL-1* promoter. Analysis of the upstream regions of *cbbL-2* also identified a LTTR motif (underlined), CCCTAGTGGAGG CCGAGGGGG, which was located from 119 to 97 bp upstream of the transcriptional start site of the gene (Fig. 8), suggesting that CbbR may also bind to a recognition site in the *cbbL-2* promoter region. EMSA with CbbR and dimerized 39 mer oligonucleotides containing the putative LTTR binding motif (recognition site) from the upstream regions of *cbbLS-1* and *-2* revealed that CbbR was able to bind the inverted repeats (Fig. 9).

The *cbb* operon is regulated by CbbR, which binds to the LTTR motifs of *cbb* promoter regions (Schell, 1993). Our results that the CbbR from a single *cbbR* gene specifically bound to the promoter regions of both *cbbLS-1* and *-2* genes in *Mycobacterium* sp. strain JC1 suggest that a CbbR from a single *cbbR* gene regulates both *cbbLS* operons in *Mycobacterium* sp. strain JC1, as has been shown in *R. sphaeroides* and *C. necator* (Gibson and Tabita, 1993; Kusian and Bowien, 1995).

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