The role of two CbbRs in the transcriptional regulation of three ribulose-1,5-bisphosphate carboxylase/oxygenase genes in *Hydrogenovibrio marinus* strain MH-110

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Hydrogenovibrio marinus MH-110 possesses three different sets of genes for ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO): two form I (*cbbLS-1* and *cbbLS-2*) and one form II (*cbbM*). We have previously shown that the expression of these RubisCO genes is dependent on the ambient CO₂ concentration. LysR-type transcriptional regulators, designated CbbR1 and CbbRm, are encoded upstream of the *cbbLS-1* and *cbbM* genes, respectively. In this study, we revealed by gel shift assay that CbbR1 and CbbRm bind with higher affinity to the promoter regions of *cbbLS-1* and *cbbM*, respectively, and with lower affinity to the other RubisCO gene promoters. The expression patterns of the three RubisCOs in the *cbbR1* and the *cbbRm* gene mutants showed that CbbR1 and CbbRm were required to activate the expression of *cbbLS-1* and *cbbM*, respectively, and that neither CbbR1 nor CbbRm was required for the expression of *cbbLS-2*. The expression of *cbbLS-1* was significantly enhanced under high-CO₂ conditions in the *cbbRm* mutant, in which the expression of *cbbM* was decreased. Although *cbbLS-2* was not expressed under high-CO₂ conditions in the wild-type strain or the single *cbbR* mutants, the expression of *cbbLS-2* was observed in the *cbbR1 cbbRm* double mutant, in which the expression of both *cbbLS-1* and *cbbM* was decreased. These results indicate that there is an interactive regulation among the three RubisCO genes.

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INTRODUCTION

The Calvin–Benson–Bassham (CBB) cycle plays the most important role in assimilating CO₂ into organic carbon on earth, since all plants and cyanobacteria use this cycle to fix CO₂, as do many autotrophic bacteria. D-Ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO, EC 4.1.1.39), one of two unique enzymes in the pathway, catalyses the actual CO₂ fixation reaction. Four forms of RubisCO have been identified to date: form I, encoded by *cbbLS*, is composed of eight large (CbbL) and eight small subunits (CbbS) and is found in all plants and cyanobacteria, and in some proteobacteria; form II, encoded by *cbbM*, consists of only large subunits of varying numbers, depending on the organism examined; form III, found in archaea, is also composed of large subunits (Kitano *et al.*, 2001; Finn & Tabita, 2003); and form IV, reported recently, is referred to as RubisCO-like protein (RLP) (Hanson & Tabita, 2001; Ashida *et al.*, 2003). RLP is incapable of

catalysing the fixation of CO₂, but is likely to be involved in the regulation of sulfur metabolism in *Bacillus subtilis* (Ashida *et al.*, 2003). The distribution and function of each form of RubisCO mentioned above indicate that form I and form II are the principal forms of RubisCO that assimilate CO₂ in the atmosphere.

Hydrogenovibrio marinus strain MH-110 is an obligately lithoautotrophic, halophilic and aerobic hydrogen-oxidizing bacterium isolated from a marine environment (Nishihara *et al.*, 1989, 1991). This bacterium fixes CO₂ via the CBB cycle. We have shown that *H. marinus* possesses three types of RubisCO: two form I RubisCOs (CbbLS-1 and CbbLS-2), and a form II RubisCO (CbbM) (Fig. 1) (Chung *et al.*, 1993; Yaguchi *et al.*, 1994; Hayashi *et al.*, 1998; Yoshizawa *et al.*, 2004). The ambient CO₂ concentration affects the expression of these three RubisCO genes (Yoshizawa *et al.*, 2004). It is already known that *cbbM* is expressed constitutively, but the expression levels of *cbbM* increase at CO₂ concentrations above 2%. *cbbLS-1* is expressed at 2% and 0.03% CO₂ concentrations, but not at 15% and 0.15% concentrations. *cbbLS-2* is expressed at CO₂ concentrations below 0.15%. We have previously

Abbreviations: CBB cycle, Calvin–Benson–Bassham cycle; LTTR, LysR-type transcriptional regulator; PEP, phosphoenolpyruvate; RubisCO, D-ribulose 1,5-bisphosphate carboxylase/oxygenase.

Fig. 1. The organization of three RubisCO operons of *H. marinus* strain MH-110. The arrows indicate the size and direction of the genes. *cbbL* and *cbbS* encode large and small subunits of form I RubisCO, respectively. *cbbM* encodes form II RubisCO. *cbbR* encodes the LysR-type transcriptional regulator (LTTR). *cbbQ* and *cbbO* encode proteins thought to be involved in the post-translational activation of RubisCO. *cso* genes encode carboxysome proteins. *bfr* encodes bacterioferritin. *can* encodes carbonic anhydrase.

shown that the expression of these RubisCO genes is regulated at the transcriptional level (Yoshizawa *et al.*, 2004), although the molecular mechanism for the regulation has not yet been elucidated.

CbbR, one of the LysR-type transcriptional regulators (LTTRs), has been found in many photo- and chemoautotrophic bacteria, and has been shown to be required to activate expression of RubisCO (Windhövel & Bowien, 1991; Falcone & Tabita, 1993; Gibson & Tabita, 1993; van den Bergh *et al.*, 1993; Paoli *et al.*, 1998; Dubbs *et al.*, 2000, 2004; Vichivanives *et al.*, 2000). Although the *cbbR* genes are located upstream of genes encoding RubisCO in a divergent orientation in most bacteria, the number of *cbbR* genes is not equivalent to that of genes for RubisCO (Windhövel & Bowien, 1991; Gibson & Tabita, 1993; Meijer *et al.*, 1996; Kusian & Bowien, 1997; Smith & Tabita, 2002; Dubbs & Tabita, 2003). For example, a single *cbbR* gene product regulates two *cbb* operons in *Rhodobacter sphaeroides* (Gibson & Tabita, 1993; Smith & Tabita, 2002; Dubbs & Tabita, 2003). In *H. marinus*, the *cbbR* genes were found upstream of *cbbLS-1* and *cbbM*, and were designated *cbbR1* and *cbbRm*, respectively; however, no regulatory genes were found upstream of *cbbLS-2* (Fig. 1) (Yoshizawa *et al.*, 2004).

The LTTR is known to recognize a specific effector molecule, which alters the ability of the LTTR to bind to the promoter region of its target gene (Schell, 1993). It has been reported that NADPH enhances the DNA-binding ability of the CbbR of *Xanthobacter flavus* (van Keulen *et al.*, 1998, 2003). NADPH also changes the binding ability of the CbbR of *Hydrogenophilus thermoluteolus* (Terazono *et al.*, 2001). In the case of *Ralstonia eutropha*, phosphoenolpyruvate (PEP) is found to increase the DNA-binding ability of the CbbR and has a negative effect on RubisCO transcription *in vitro* (Grzeszik *et al.*, 2000). It has recently been reported that some metabolites formed by the CBB cycle and some intermediates, including PEP, affect the ability of two CbbRs to bind to target promoter regions in *Rhodobacter capsulatus* (Dubbs *et al.*, 2004). The effects of these effectors have not necessarily been observed in the CbbRs of other bacteria (Grzeszik *et al.*, 2000; Terazono *et al.*, 2001; Tichi & Tabita, 2002), which suggests that alternative effectors are recognized by the CbbRs of other bacteria. The concentration of

CO₂, which is the substrate for RubisCO, affects the expression of RubisCO in many types of bacteria (Sarles & Tabita, 1983; Jouanneau & Tabita, 1986; Hallenbeck *et al.*, 1990a, b), although the effects of CO₂ on the DNA-binding ability of CbbR have not yet been reported. The expression patterns of the three RubisCOs in *H. marinus* are known to change according to the CO₂ concentration (Yoshizawa *et al.*, 2004), thus indicating that there is a regulatory mechanism that responds to the CO₂ concentration. Two CbbRs appear to be involved in such regulation, and these CbbRs could sense the CO₂ concentration or particular metabolites produced in cells exposed to various CO₂ concentrations. In this study, we report that each CbbR regulates the expression of the adjacent RubisCO gene, and that the CO₂ concentration influences the expression of one of the two *cbbR* genes. We also show that an interactive regulation between the three RubisCOs is operative in *H. marinus*.

METHODS

Bacterial strains, plasmids, media, and growth conditions.

The plasmids and bacterial strains used in this study are listed in Table 1. *Escherichia coli* strains (Yanisch-Perron *et al.*, 1985) were grown aerobically in Luria-Bertani (LB) medium at 37 °C. *H. marinus* was grown in 500 ml of inorganic medium (Yoshizawa *et al.*, 2004) bubbled continuously with a gas mixture (H₂/O₂/CO₂) at the rate of 500 ml min⁻¹ in a 1 l fermenter (Able, Tokyo, Japan) at 37 °C. The CO₂ concentration in the gas mixture was adjusted by changing the ratio of CO₂ in the gas mixture, as described previously (Yoshizawa *et al.*, 2004), i.e. the 2% CO₂ concentration was achieved with a gas mixture (H₂:O₂:CO₂=83:15:2), with the exception that the gas containing 0.03% CO₂ was a mixture of 20% H₂/80% air. The antibiotic concentrations used for *H. marinus* strains were as follows: kanamycin (Km) 15 µg ml⁻¹, tetracycline (Tc) 2 µg ml⁻¹, gentamicin (Gm) 10 µg ml⁻¹, streptomycin (Sm) 10 µg ml⁻¹. For *E. coli*, the antibiotic concentrations were as follows: ampicillin, 100 µg ml⁻¹; Km, 30 µg ml⁻¹; Tc, 12.5 µg ml⁻¹; Gm, 10 µg ml⁻¹; Sm, 15 µg ml⁻¹.

Expression of the two *H. marinus* CbbRs in *E. coli*. The two CbbRs of *H. marinus*, CbbR1 and CbbRm, were overexpressed in *E. coli* for purification. The coding region of the *cbbR1* gene was amplified by PCR from the total genomic DNA of *H. marinus* as template, using primers 5'-ATAGAGCATACATATGCAAAATTAC-3' and 5'-CTTTTACCCGGGCTATCTTGATC-3' for *cbbR1*. The PCR reaction consisted of 500 pmol of each of the oligonucleotide

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
<i>E. coli</i>		
JM109	<i>endA1 recA1 gyrA96 thi hsdR17</i> (r_k^- , m_k^+) <i>relA1 supE44</i> $\Delta(lac-proAB)$ [F7 <i>traD36 proAB lacIqZDM15</i>]	Yanisch-Perron <i>et al.</i> (1985)
S17-1 λ pir	C600::RP-4 2-(Tc::Mu) (Km::Tn7) <i>thi pro hsdR hsdM+ recA</i>	Simon <i>et al.</i> (1983)
ER2566	F ⁻ λ^- <i>fhuA2[lon] ompT lacZ::T7</i> genel <i>gal sulA11</i> $\Delta(mcrC-mrr)114::IS10$ R(<i>mcr-73::miniTn10</i>)2 R(<i>zgb-210::Tn10</i>)1 (Tet ^s) <i>endA1 [dcm]</i>	New England BioLabs
BL21(DE3)	F ⁻ <i>ompT hsdS_B</i> (r_B^- , m_B^-) <i>dcm gal</i> λ (DE3)	Novagen
<i>H. marinus</i>		
MH-110	Wild-type	Nishihara <i>et al.</i> (1991)
dR1	<i>cbbR1::Km^r</i> derivative of MH-110	This study
dRm	<i>cbbRm::Km^r</i> derivative of MH-110	This study
ddR	<i>cbbR1::Gm^r</i> , <i>cbbRm::Km^r</i> derivative of MH-110	This study
Plasmids		
pUC119	Ap ^r	TaKaRa
pHSG298	Km ^r	TaKaRa
pJP5608	Tc ^r , derivative of pJP5603 mobilizable suicide vector	Penfold & Pemberton (1992)
pHR45 Ω aac	Ap ^r , Gm ^r , derivative of pHP45 Ω containing Ω cassette encoding Gm ^r	Blondelet-Rouault <i>et al.</i> (1997)
pTYB12	Ap ^r , T7RNA polymerase-based expression vector	New England BioLabs
pGEM-3Zf(+)	Ap ^r	Promega
pRK2013	Km ^r , conjugative helper plasmid	Figurski & Helinski (1979)
pHRP309	Gm ^r , broad-host-range vector	Parales & Harwood (1993)
pTYBR1	Ap ^r , pTYB12 with a <i>NdeI</i> – <i>SmaI</i> fragment encoding <i>cbbR1</i>	This study
pUCR1	Ap ^r , pUC119 with a <i>Bam</i> HI– <i>Eco</i> RI fragment encoding <i>cbbR1</i>	This study
pUCRm	Ap ^r , pUC119 with a <i>Sall</i> – <i>XbaI</i> fragment encoding <i>cbbRm</i>	This study
pKR1	Ap ^r , Km ^r , pUCR1 with Km resistance gene insertion into the <i>BalI</i> site within <i>cbbR1</i>	This study
pKRm	Ap ^r , Km ^r , pUCRm with Km resistance gene insertion into the <i>NruI</i> site within <i>cbbRm</i>	This study
pJPKR1	Km ^r , Tc ^r , pJP5608 with a 2.2 kb <i>PvuII</i> fragment containing the <i>cbbR1</i> and Km resistance gene from pKR1	This study
pJPKRm	Km ^r , Tc ^r , pJP5608 with a 2.2 kb <i>XbaI</i> – <i>Sall</i> fragment containing the <i>cbbRm</i> and Km resistance gene from pKRm	This study
pGR1	Ap ^r , Gm ^r , pUCR1 with Ω Gm cassette insertion into the <i>BalI</i> site within <i>cbbR1</i>	This study
pJPGR1	Gm ^r , Tc ^r , pJP5608 with 2.7 kb <i>Eco</i> RI– <i>SacI</i> fragment containing the <i>cbbR1</i> and Ω Gm cassette from pGR1	This study
pCR1	Gm ^r , pHRP309 with a <i>Bam</i> HI– <i>Sall</i> fragment containing <i>cbbR1</i> and its promoter	This study
pCRm	Gm ^r , pHRP309 with an <i>Eco</i> RI fragment containing <i>cbbRm</i> and its promoter	This study
pRPAR1	Ap ^r , pGEM-3Zf(+) with an amplified <i>Eco</i> RI– <i>XbaI</i> fragment containing the last 107 codons of <i>cbbR1</i>	This study
pRPARm	Ap ^r , pGEM-3Zf(+) with an amplified <i>Eco</i> RI– <i>XbaI</i> fragment containing the last 110 codons of <i>cbbRm</i>	This study

primers, 500 ng DNA template and 2.5 units Ex Taq polymerase (TaKaRa). The amplified fragment containing *cbbR1* was ligated with the expression vector pTYB12 using the appropriate restriction sites, resulting in pTYB12. The constructed plasmid was designed to encode fusion proteins of CbbR1 with an intein and a chitin-binding domain (CBD). The recombinant fusion protein was expressed in *E. coli* strain ER2566 and purified according to the manufacturer's instructions (New England BioLabs). In brief, the cell lysate was loaded on a chitin column. The recombinant CbbR1 was excised from the intein-CBD fusion complex on the column by an autocleavage reaction from the intein moiety at 4 °C in the presence of DTT.

CbbRm was purified by fusion with His-tag. To construct an expression plasmid for His₆-CbbRm, the *cbbRm* gene was cloned into pET21c (Novagen). The construct was used to transform *E. coli* BL21(DE3) (Novagen). The transformant was cultured in LB supplemented with ampicillin at 37 °C. When the OD₆₀₀ reached 0.7, IPTG was added at a final concentration of 0.5 mM and the cells were cultured for a further 3 h. Cells were harvested by centrifugation and were suspended in a suspension buffer (20 mM sodium phosphate, pH 7.5, 1 M NaCl). The resuspended cells were broken by passing through a French pressure cell (Aminco, Lake Forest, CA) at 110 MPa. Cell extracts were obtained by centrifugation at 4 °C for 25 min at 20 000 g. The cell extracts were loaded onto a 1 ml nickel Hitrap Chelating affinity column (Amersham Pharmacia). The column was washed with 20-bed volume of suspension buffer containing 50 mM imidazole. The His-tagged CbbRm was eluted with suspension buffer containing 200 mM imidazole. The eluted proteins were dialysed twice at 4 °C in a buffer containing 50 mM HEPES (pH 7.8), 200 mM KCl, 10 mM MgCl₂, 1 mM DTT, 0.05 mM PMSF and 50% (v/v) glycerol, and the proteins were stored at -20 °C.

Preparation of cell extracts of *H. marinus*. Cells were harvested from the cultures at the late-exponential phase by centrifugation and were suspended in BEMD buffer (50 mM Bicine, 0.1 mM EDTA, 10 mM MgCl₂·6H₂O, 1 mM DTT, pH 7.8). The cells were disrupted twice using a French pressure cell (Aminco) at 110 MPa and then centrifuged for 25 min at 20 000 g at 4 °C to remove the cell debris. The protein concentrations were determined by the Bio-Rad protein assay method with BSA as standard.

Western immunoblot analyses. Ten micrograms of cell extract were resolved by SDS-PAGE and transferred to PVDF membranes (Sequi-Blot PVDF membrane, Bio-Rad) by using a semi-dry blotting system (ATTO, Tokyo, Japan). Polyclonal antibodies raised against synthetic oligopeptides encoded by *cbbS-1* or *cbbS-2* of *H. marinus*, and against form II RubisCO of *H. marinus* at a 1:1000 dilution were used to detect the three kinds of RubisCO of *H. marinus*. The blots were developed as described previously (Yoshizawa *et al.*, 2004).

Gel shift assays. A DNA fragment containing the promoter region of *cbbLS-1*, *cbbLS-2*, *cbbM*, or *orf90-cbbRm* was amplified by PCR using the genomic DNA of *H. marinus* as a template with the following primers: 5'-TACGAATTCGTTGTGCC-3' and 5'-TCTTTTGGCTCATACTCTGGCATCC-3' for *cbbLS-1*; 5'-CCGCAAAAGAATTCACC-3' and 5'-CGTTAACCACAGAAGCTTCTTC-3' for *cbbLS-2*; 5'-CGAATTGGGATCCTAACTTACCC-3' and 5'-AGTGAATTCAGCGTAACG-3' for *cbbM*; and 5'-AGAAATTGCATGCTGTACG-3' and 5'-GTGGCATAGATTACATGCATTG-3' for *orf90-cbbRm*. These amplified fragments were inserted into pUC119 at the appropriate restriction sites and sequenced to confirm that no mutation was introduced during the PCR. The DNA fragments containing each promoter region were excised from the constructed plasmids by digestion with the appropriate restriction enzymes. The length of each fragment was as follows: *cbbLS-1*, 357 bp; *cbbLS-2*, 293 bp; *cbbM*, 146 bp; and *orf90-cbbRm*, 227 bp. The fragments were

labelled by either filling the recessed end with [α -³²P]dATP (Amersham Pharmacia) using Klenow enzyme or phosphorylating the end of the fragments with [γ -³²P]ATP (Amersham Pharmacia) using T4 polynucleotide kinase. The labelling reaction using Klenow enzyme was performed in a 50 μ l mixture containing 50 ng of the DNA fragment, 100 μ M dCTP, 100 μ M dGTP, 100 μ M dTTP, 20 μ Ci [α -³²P]dATP, 4 U Klenow enzyme, 10 mM Tris/HCl (pH 7.5), 7 mM MgCl₂ and 0.1 mM DTT. After the reaction mixture had been incubated at 37 °C for 1 h, 10 μ M dNTPs were added to the mixture, which was then incubated for another 5 min. The labelling reaction using T4 polynucleotide kinase was done in a 50 μ l mixture containing 100 ng of the DNA fragment, 20 μ Ci [γ -³²P]ATP, 10 U T4 polynucleotide kinase, 50 mM Tris/HCl (pH 8.0), 10 mM MgCl₂ and 5 mM DTT. The mixture was incubated at 37 °C for 1 h and then heated at 90 °C for 10 min to inactivate kinase. The labelled DNA was purified by using SUPREC-02 (TaKaRa), and stored at -20 °C. Purified CbbR1 or CbbRm was incubated with the labelled DNA fragments (5000 c.p.m.) in a binding buffer containing 10 mM Tris/HCl (pH 8.0), 50 mM NaCl, 0.5 mM DTT, 10% (v/v) glycerol, 50 μ g ml⁻¹ poly(dI-dC).poly(dI-dC) (Amersham Pharmacia) and 50 μ g ml⁻¹ BSA at room temperature for 30 min. The reaction mixture was applied to a 4% non-denaturing acrylamide gel in Tris/glycine buffer and run at 10 V cm⁻¹. The gel was subsequently dried and visualized using a Bio Imaging Analyser (Fujifilm, Tokyo, Japan) or autoradiographed with intensifying screens at -80 °C.

DNA manipulations. Routine DNA manipulations, including chromosomal DNA isolation, plasmid preparation, restriction endonuclease digestion, agarose gel electrophoresis, fragment ligation and bacterial transformation were performed according to standard methods (Sambrook *et al.*, 1989). Southern blotting procedures were carried out with a Hybond-N membrane (Amersham Pharmacia). The hybridized DNA was detected by a staining reaction, as described previously (Yoshizawa *et al.*, 2004).

Construction of *H. marinus cbbR* mutant strains. To construct the *cbbR1* and *cbbRm* mutants, a coding region of the *cbbR1* or *cbbRm* gene was amplified by PCR using the total DNA of *H. marinus* as template with the following primers: 5'-GCTGGTACCTACATTGTTTGGCC-3' and 5'-TAAGGGAATTCTAATAACAAAATCACC-3' for *cbbR1*; 5'-CAAAAATCTAGACGATGTCG-3' and 5'-GATTATTAGTATAGACGTCGAC-3' for *cbbRm*. The amplified fragments containing the *cbbR1* and *cbbRm* genes were cloned into pUC119, resulting in pUCR1 and pUCRm, respectively. A 1.2 kb *PvuII*-*StuI* fragment from pHSG298, encoding a Km resistance gene, was inserted into the unique *BalI* and *NruI* sites within *cbbR1* and *cbbRm*, respectively. The DNA fragment containing *cbbR1::Km^r* or *cbbRm::Km^r* of the resulting plasmids was excised by digestion with *EcoRI*/*BamHI* or *SphI*/*XbaI*, and was cloned into a suicide vector pJP5608 (Penfold & Pemberton, 1992), resulting in pJPKR1 or pJPKRm. These plasmids were transferred to *H. marinus* strain MH-110 from *E. coli* strain S17-1 λ pir (Simon *et al.*, 1983) by transconjugation as follows. One millilitre of overnight culture of *E. coli* donors and 1.5 ml of overnight culture of *H. marinus* recipients were harvested and washed with sterilized water and 0.5 M NaCl, respectively. The cells of *H. marinus* and *E. coli* were suspended with 100 μ l 0.5 M NaCl and spotted onto membrane filters (0.45 μ m pore size, Milipore) on a plate of inorganic medium (Yoshizawa *et al.*, 2004). The plates were incubated at 37 °C for 1 day in a desiccator (Tokyo Glass Kikai, Tokyo, Japan) after the gas in the desiccator was replaced with a gas mixture (H₂:O₂:CO₂=75:10:15). Transconjugants on the membrane were suspended in 1 ml 0.5 M NaCl and spread on the inorganic plate containing Km. The Km^r colonies were screened for sensitivity to the plasmid-encoded Tc resistance in order to select strains in which the *cbbR* genes were disrupted by double homologous recombination. One and two Km^r

Tc^s strains were obtained from *cbbR1* and *cbbRm* transconjugants, respectively. The disruption of each *cbbR* gene was confirmed by Southern hybridization. The obtained *cbbR1* and *cbbRm* mutant strains, designated dR1 and dRm, respectively, were used for the subsequent experiments. To construct a *cbbR1 cbbRm* double mutant strain, the *cbbR1* gene of strain dRm was disrupted. A 1.8 kb *Sma*I fragment encoding the Gm resistance gene from pHP45 Ω ac (Blondelet-Rouault *et al.*, 1997) was inserted into the unique *Bal*I site in the *cbbR1* gene on pUCR1. The *Eco*RI-*Xba*I fragment containing the disrupted *cbbR1* gene on the constructed plasmid was excised and cloned into pJP5608, resulting in pJPGR1. *E. coli* strain S17-1 λ pir was used to transfer pJPGR1 into *H. marinus* strain dRm. Four hundred Gm^r colonies were screened for Tc sensitivity and two Gm^r Tc^s clones were obtained. Double homologous recombination was confirmed by Southern hybridization. One of the obtained Gm^r Tc^s strains was designated ddR and was used in subsequent experiments.

Complementation of *cbbR* mutants. Broad-host-range plasmids, pCR1 and pCRm, were constructed by cloning of the *cbbR1* fragment from pUCR1 and the *cbbRm* fragment from pUCRm, respectively, to pHRP309 (Parales & Harwood, 1993). These plasmids were transferred from *E. coli* JM109 to the *cbbR* mutants by the triparental mating method with *E. coli* HB101 containing the mobilizable helper plasmid pRK2013 (Figurski & Helinski, 1979). The conditions of conjugation were the same as those described above.

RNase protection assays. The total RNA was isolated from bacterial cells by using ISOGEN (Nippon Gene, Tokyo, Japan), as described previously (Yoshizawa *et al.*, 2004). ³²P-labelled RNA probes for the RNase protection assays were generated by *in vitro* transcription using a MAXIscript kit (Ambion). The DNA fragment containing either the last 107 codons of *cbbR1* or the last 110 codons of *cbbRm* was amplified by PCR from the genomic DNA as a template with the following primers: 5'-GGAAAAGAATTCTGGTATTCG-3' and 5'-CGACTTCTAGAACAGCACTG-3' for *cbbR1*; and 5'-AGAGAAATTCGGATCCGGTATCC-3' and 5'-CCAATCTAGAAATTCGGTAAATTTTCG-3' for *cbbRm*. The amplified fragments were cloned into pGEM-3Zf(+), resulting in pRPAR1 for *cbbR1* and pRPARm for *cbbRm*. The *cbbR1* probe, a 411 bp fragment containing the last 107 codons of *cbbR1*, was generated by transcription with the SP6 promoter using *Eco*RI-digested pRPAR1 as template. The *cbbRm* probe, a 440 bp fragment containing the last 110 codons of *cbbRm*, was generated by transcription with the SP6 promoter using *Bam*HI-digested pRPARm as template. Transcription reactions contained 500 μ M ATP, GTP and CTP, and 5 μ l [α -³²P]UTP [800 Ci mmol⁻¹ (29600 GBq mmol⁻¹), 10 mCi ml⁻¹ (370 MBq mmol⁻¹); Amersham Pharmacia]. The reactions were carried out for 15 min with SP6 RNA polymerase. After the transcription reaction, full-length transcripts were recovered after separation on an 8 M urea/5% polyacrylamide gel. RNase protection assays were carried out using the RPAIII kit (Ambion); 10 μ g total RNA was used for the reactions with the *cbbR1* riboprobes. Together with the *cbbRm* riboprobes, 20 μ g total RNA was used for the reactions. Unhybridized probes were digested with a 1:100 dilution of RNase A/RNase T₁ mix. The radiolabelled probes were separated on an 8 M urea/5% polyacrylamide gel and were visualized using a Bio Imaging Analyser (Fujifilm) and by autoradiography.

Primer extension. Primer extension mapping of the transcription start sites of *cbbLS-1*, *cbbLS-2*, *cbbM*, *cbbR1* and *cbbRm* was performed by using Primer Extension System-AMV Reverse Transcriptase (Promega). The oligonucleotide primers used in the experiments were as follows: 5'-GTCTTTGCCATTTTTCACCTCTTG-3' for *cbbLS-1*, 5'-CTAGAGGAGTGTAGTCTGGTGTCC-3' for *cbbLS-2*, 5'-TCATGTTGGCGGTGATCACAAG-3' for *cbbM* and 5'-GCGGGCGACAGATTCAAAAATACG-3' for *cbbR1*. To map the transcription start site of *cbbRm*, the oligonucleotides cbbRm-1

(5'-TGTTGGCGATACACTCAAAAACC-3') and cbbRm-2 (5'-GATAATTATCGTTGATTTCGTCG-3') were used. The primers were end-labelled with [γ -³²P]ATP (Amersham Pharmacia). One microlitre of labelled primer and 5 μ l AMV primer extension 2 $\times$ buffer (100 mM Tris/HCl, pH 8.3, 100 mM KCl, 20 mM MgCl₂, 1 mM spermidine, 2 mM GTP, CTP, ATP and TTP) were added to 5 μ l (20 μ g) total RNA. The extension reaction mixtures were incubated at 58 °C for 20 min to anneal the primer to the RNA before cooling the reactions to room temperature for 10 min in a TaKaRa PCR thermal cycler MP. A mixture of 2 mM sodium pyrophosphate, AMV primer extension 1 $\times$ buffer and AMV reverse transcriptase (0.05 U μ l⁻¹) was added to the annealed reactions and incubated at 42 °C for 30 min. The primer extension products thus obtained were analysed in a 5% sequencing gel in parallel with DNA sequencing reactions carried out using the same primers as those used in the primer extension experiments. The plasmids carrying the promoter regions were used as templates for the sequencing reactions.

RESULTS

Binding of CbbR1 and CbbRm to the promoter regions of the *cbb* genes

Given that CbbR1 and CbbRm belong to the LTTR family, these CbbRs could bind to the promoter regions of RubisCO genes and activate their expression in *H. marinus* MH-110. To examine the ability of both CbbR1 and CbbRm to bind to these regions, gel shift assays were performed. CbbR1 was expressed in *E. coli* as a fusion protein with an intein containing a chitin-binding domain that was removed by treatment with DTT on a chitin column during purification. CbbRm was expressed as a His-tagged protein in *E. coli* and was purified using Ni²⁺ affinity chromatography. The DNA fragments containing the promoter regions of *cbbLS-1*, *cbbLS-2* and *cbbM* were end-labelled with ³²P and incubated with CbbR1 or CbbRm. The gel shift assays revealed that CbbR1 and CbbRm were able to bind to the promoter regions of *cbbLS-1* and *cbbM*, respectively (Fig. 2). Excess amounts of the unlabelled fragment eliminated the shifted bands, indicating that the band-shift by CbbR1 or CbbRm was sequence-specific. The results also indicated that CbbR1 and CbbRm bound to the promoter regions of other RubisCO operons with lower affinity. However, excess amounts of the unlabelled fragments of the other promoters did not completely inhibit the binding of CbbR1 to the *cbbLS-1* promoter or of CbbRm to the *cbbM* promoter. These results indicated that CbbR1 and CbbRm primarily regulate the expression of *cbbLS-1* and *cbbM*, respectively. CbbR1 gave two shifted bands for the *cbbLS-1* promoter and the ratio of the upper shifted band to the lower one was increased with an increase in protein concentrations. CbbRm also gave two shifted bands for the *cbbM* promoter at higher protein concentrations. It has been shown that CbbR binds to the target promoter with two subsites and forms a dimer of dimers on the promoter in some bacteria (Kusano & Sugawara, 1993; Kusian & Bowien, 1995; van Keulen *et al.*, 1998, 2003). Thus, CbbR1 and CbbRm of *H. marinus* probably bind to each target promoter as a dimer of dimers.

Fig. 2. Binding of CbbR1 and CbbRm to the promoter region of the *cbb* genes. (a) Gel shift assays using CbbR1 with DNA fragments containing the promoter regions of *cbbLS-1*, *cbbLS-2*, *cbbM* and *cbbRm*. The labelled fragments were incubated with purified CbbR1. The protein concentrations used for the *cbbLS-2*, *cbbM* and *cbbRm* promoter regions were as follows (in ng per 20 μ l reaction mixture): 0 (lane 1), 10 (lane 2), 50 (lane 3), 250 (lane 4). For the *cbbLS-1* promoter region, the protein concentrations were as follows (in ng per 20 μ l reaction mixture): 0 (lane 1), 5 (lane 2), 10 (lane 3), 20 (lanes 4, 7–9), 50 (lane 5), 100 (lane 6). A 50-fold molar excess of unlabelled fragment of the *cbbLS-1* (lane 7), *cbbLS-2* (lane 8) or *cbbM* (lane 9) promoter was added to the mixture prior to the addition of labelled fragments. (b) Gel shift assays using CbbRm with DNA fragments containing the promoter regions of *cbbLS-1*, *cbbLS-2*, *cbbM* and *cbbRm*. The labelled fragment was incubated with purified CbbRm. The protein concentrations used for *cbbLS-1*, *cbbLS-2* and *cbbRm* promoter regions were as follows (in ng per 20 μ l reaction mixture): 0 (lane 1), 10 (lane 2), 50 (lane 3), 250 (lane 4). For the *cbbM* promoter region, the protein concentrations were as follows (in ng per 20 μ l reaction mixture): 0 (lane 1), 5 (lane 2), 10 (lane 3), 20 (lanes 4, 7–9), 50 (lane 5), 100 (lane 6). A 50-fold molar excess of unlabelled fragment of the *cbbLS-1* (lane 7), *cbbLS-2* (lane 8) or *cbbM* (lane 9) promoter was added to the mixture prior to the addition of labelled fragments. The open and filled arrow boxes indicate free DNA probe and DNA–protein complex, respectively. Asterisks indicate contaminated non-specific bands.

To identify and analyse the promoter regions of each *cbb* operon and of the two *cbbR* genes, the transcription start sites of each *cbb* gene were determined (Fig. 3). The transcription start sites of the *cbbLS-1* and *cbbLS-2* operons were located at positions 103 bp and 37 bp, respectively, upstream of the first gene of each operon. *cbbM* had two transcriptional start points at positions –129 bp and –123 bp. The transcription start site of *cbbR1* was located at position –56 bp. The size of a product of the primer extension experiment using oligonucleotide *cbbRm*-1 exceeded 300 nt (data not shown). An ORF search revealed that a 270 bp gene, designated *orf90*, was located upstream of *cbbRm* in the same direction as *cbbRm*. A primer extension experiment using *cbbRm*-2, which was specific to *orf90*, revealed that the transcription start sites of the *cbbRm*

operon were located at positions 41 and 42 bp upstream of *orf90*. The physiological role of the *orf90* gene product was unclear, because no homologous sequence could be found in public databases.

Characterization of *cbbR1* and *cbbRm* mutant strains

To examine the physiological roles of CbbR1 and CbbRm, their genes were disrupted by homologous recombination. The *cbbR1* gene disruptant, strain dR1, showed a similar growth pattern to that of the wild-type strain MH-110 under all CO₂ conditions examined (data not shown). Western immunoblot analyses using antibodies against each RubisCO revealed that CbbLS-1 was not expressed in

Fig. 3. Mapping of the transcription initiation sites of the *cbbLS-1*, *cbbLS-2*, *cbbM*, *cbbR1* and *cbbRm* promoters. Primer extension experiments were performed using [5'-³²P]-labelled oligonucleotides. Total RNA extracted from cells grown under 2% CO₂ was used to identify (a) the *cbbLS-1*, (c) *cbbM*, (d) *cbbR1* and (e) *cbbRm* transcriptional initiation sites. Total RNA extracted from cells grown under 0.15% CO₂ was used to identify (b) the *cbbLS-2* transcriptional initiation site. Arrows indicate the transcription initiation sites. Lanes A, C, G and T show the sequencing reaction using the same primer. Lane RT shows the primer extension product.

strain dR1 under any of the CO₂ conditions (Fig. 4b). The expression levels of CbbM and CbbLS-2 were not affected by the mutation. These results indicated that the expression of CbbM alone is enough for growth at higher CO₂ concentrations. Transformation of strain dR1 with the recombinant *cbbR1* gene by pCR1 restored the expression of CbbLS-1 (data not shown). CbbLS-1 was not expressed at 15% CO₂ concentration in the wild-type strain MH-110 in a previous study (Yoshizawa *et al.*, 2004), but slight expression was observed in this work (Fig. 4a). The reason for the difference was not certain, but a small difference in the culture conditions seemed to affect the expression of CbbLS-1 at this CO₂ concentration.

The *cbbRm* gene disruptant, strain dRm, also grew at the same rate as MH-110 under all CO₂ conditions examined

(data not shown). Western immunoblot analyses revealed that the expression levels of *cbbM* at CO₂ concentrations above 2% were significantly decreased in strain dRm (Fig. 4c). However, CbbM expression was not completely abolished by the disruption of *cbbRm*, indicating that the transcription of *cbbM* was basically constitutive and not totally dependent on CbbRm. In contrast, a significant increase in CbbLS-1 was found at CO₂ concentrations above 2% in this mutant. These results indicated that the increased expression of CbbLS-1 complemented the decrease in CbbM and supported growth at higher CO₂ concentrations. CbbLS-1 was also expressed at 0.15% CO₂ concentration in strain dRm, although it was not expressed in MH-110. The expression level of CbbLS-1 at 0.03% CO₂ concentration was also higher in strain dRm than in MH-110. The disruption of the *cbbRm* gene had no effect on the expression

Fig. 4. Western immunoblots of (a) *H. marinus* wild-type strain MH-110, (b) *cbbR1* strain dR1, (c) *cbbRm* strain dRm and (d) *cbbR1 cbbRm* double mutant strain ddR using antibodies for CbbS-1 (left), CbbS-2 (centre) and CbbM (right). Cell-free extracts (approx. 10 µg total protein) of *H. marinus* strains grown at 15% (lane 1), 2% (lane 2), 0.15% (lane 3) and 0.03% (lane 4) CO₂ concentrations were used. The samples from the wild-type strain and each mutant were analysed on the same gel to allow a quantitative comparison. Panel (a) shows a representative result from each analysis.

of *cbbLS-2* under any of the CO₂ conditions. The expression patterns of CbbM and CbbLS-1 in dRm were the same as those of MH-110 when transformed with the recombinant *cbbRm* gene by pCRm (data not shown).

Induction of *cbbLS-2* expression at higher CO₂ concentrations by disruption of the two *cbbR* genes

The *cbbR1* gene in strain dRm was disrupted in order to construct the *cbbR1 cbbRm* double mutant strain ddR. Although strain ddR grew at the same rate as MH-110 at CO₂ concentrations below 0.15%, the strain showed impaired growth at CO₂ concentrations above 2% (Fig. 5). The relative growth rate of ddR was decreased to 57 and 72% of the wild-type strain at 15 and 2% CO₂ concentrations, respectively. The expression of each RubisCO in strain ddR under all CO₂ conditions was analysed by Western blotting. As

expected, the levels of CbbLS-1 and CbbM were identical to those in strains dR1 and dRm, respectively, i.e. no CbbLS-1 expression and decreased CbbM expression (Fig. 4d). It was of note that CbbLS-2 was synthesized in strain ddR even at CO₂ concentrations above 2% at which CbbLS-2 was not expressed in the wild-type. The amount of CbbLS-2 in strain ddR at higher CO₂ concentrations was comparable to that at lower CO₂ concentrations. The impaired growth of strain ddR at higher (≥2%) CO₂ concentrations must be due to the lack of CbbLS-1 and to the simultaneous reduction of CbbM, thus indicating that CbbLS-2 is inappropriate for optimum growth under high CO₂ conditions.

Expression analyses of *cbbR1* and *cbbRm*

To investigate the relationship between the expression patterns of the three RubisCO genes in *H. marinus* at various CO₂ concentrations and those of *cbbR* genes, the expression

Fig. 5. Growth profiles of the wild-type and the *cbbR1 cbbRm* mutant ddR. The wild-type and strain ddR were cultivated under four CO₂ conditions (percentage CO₂ concentrations shown). ●, Wild-type; ▼, ddR. The data are representative of duplicate experiments.

Fig. 6. Ribonuclease protection analyses of the *cbbR1* and *cbbRm* transcripts in wild-type strain MH-110 under different CO₂ conditions. (a) The *cbbR1* transcript was observed using a 411 bp riboprobe containing the last 107 codons of *cbbR1*. The expected size of the RNase-protected fragments of this *cbbR1* transcript was 321 bp. (b) The *cbbRm* transcript was detected with a 440 bp riboprobe containing the last 110 codons of *cbbRm*. The expected size of the RNase-protected fragments of this transcript was 330 bp. The *cbbR1* or *cbbRm* probe was hybridized to yeast RNA in the absence (lane 1) or presence (lane 2) of added RNase A/T1. The probes were hybridized with RNA preparations from cells grown at 15% (lane 3), 2% (lane 4), 0.15% (lane 5) or 0.03% (lane 6) CO₂. Ten (a) and 20 µg (b) total RNA were used to detect the *cbbR1* and *cbbRm* transcripts, respectively. The open and filled arrowheads indicate the size of undigested probe and a protected band, respectively. Results are representative of three independent experiments with similar results.

of *cbbR1* and *cbbRm* was analysed by RNase protection assays. Riboprobes containing the last 107 and 110 codons of CbbR1 and CbbRm, respectively, were used to detect the 3' ends of *cbbR1* and *cbbRm* transcripts, but not to detect the 5' ends of their truncated transcripts. The assays revealed that the amount of the *cbbR1* transcripts increased when the CO₂ concentration was higher than 2% (Fig. 6), indicating that *cbbR1* is regulated by another regulator that is able to respond to changes in the CO₂ concentration. CbbR1 is divergently encoded upstream of *cbbLS-1*. The gel shift assay showed that CbbR1 bound to the overlapping promoter region between *cbbLS-1* and *cbbR1*, suggesting that the expression of CbbR1 is subjected to autoregulation. Negative autoregulation has also been demonstrated for the LTTR including CbbR (Schell, 1993; Vichivanives *et al.*, 2000; van Keulen *et al.*, 2003). The amount of the *cbbRm*

transcripts did not vary significantly under any of the CO₂ conditions. CbbRm is encoded upstream of *cbbM* in the same direction, and the gel shift assay showed that neither CbbR1 nor CbbRm bound to the promoter region of *cbbRm* (Fig. 2), indicating that the expression of CbbRm is not regulated by either of the two CbbRs.

DISCUSSION

In a previous report, we demonstrated that the ambient CO₂ concentration affects the expression of three RubisCOs in *H. marinus* (Yoshizawa *et al.*, 2004). The present study clarified in part the complicated regulatory mechanism of RubisCO gene expression by the analysis of the roles played by two LTTRs, CbbR1 and CbbRm. The results of the gel shift assays demonstrated that CbbR1 and CbbRm of *H. marinus* bound with higher affinity to the promoter regions of *cbbLS-1* and *cbbM*, respectively (Fig. 2), indicating that these genes are mainly under the control of their adjacently encoded regulators. We also found that CbbR1 and CbbRm bound to the promoter regions of the other RubisCO operons. However, the interaction was weak compared to that observed between CbbR1 and the *cbbLS-1* promoter or CbbRm and the *cbbM* promoter, suggesting that the binding of CbbR1 and CbbRm to the non-cognate promoter has a minor role *in vivo*.

We introduced genetic engineering in *H. marinus* and constructed knockout mutants of the *cbbR* regulatory genes. CbbR1 seems to be essential for the transcription of *cbbLS-1*, because the *cbbR1* mutant strain dR1 lost the ability to synthesize CbbLS-1 under all CO₂ conditions. The expression of the other RubisCOs was not influenced by the mutation (Fig. 4). Strain dR1 grew at the same rate as the wild-type strain under all CO₂ conditions, indicating that under all CO₂ conditions, CbbLS-1 does not have a crucial role for growth. The *cbbRm* mutant strain dRm also showed the same growth profiles as those of the wild-type strain. No activation of CbbM expression at higher CO₂ concentrations ($\geq 2\%$) was observed in the mutant. However, a significant increase in CbbLS-1 was observed under these conditions, suggesting that the increase in CbbLS-1 functionally compensated for the decrease in CbbM. A similar compensatory effect has also been observed in the RubisCO gene mutants of *R. sphaeroides* and *R. capsulatus* (Gibson *et al.*, 1991; Paoli *et al.*, 1998). The RNase protection assays revealed that under all conditions the expression level of *cbbRm* did not change drastically in strain MH-110 (Fig. 6), indicating that the activation of *cbbM* expression at higher CO₂ concentrations ($\geq 2\%$) required some factor that acts cooperatively with CbbRm. The *cbbR1 cbbRm* double knockout strain ddR showed poor growth at higher CO₂ concentrations ($\geq 2\%$) (Fig. 5). The expression levels of CbbLS-1 and CbbM were decreased in the strain (Fig. 4). Interestingly, the expression of CbbLS-2 was observed at higher CO₂ concentrations ($\geq 2\%$) in strain ddR. These results indicated that the high-level expression of CbbLS-1 or CbbM is necessary for optimal growth at higher

CO₂ concentrations, and that CbbLS-2 does not functionally compensate for the lack of CbbLS-1 and CbbM.

Disruption of *cbbRm* led to the high-level expression of CbbLS-1 at higher CO₂ concentrations, and the disruption of both *cbbR1* and *cbbRm* caused the expression of CbbLS-2 at higher CO₂ concentrations. These results indicated that *H. marinus* has a hierarchical interactive regulatory mechanism among the three RubisCOs. *R. capsulatus* possesses two *cbb* operons, and the expression of each operon is activated by the cognate CbbR regulators (Vichivanives *et al.*, 2000). An interactive regulation of the two *cbb* operons by the cross-reactions of the CbbR regulators is indicated in *R. capsulatus*. The cross-reaction of the CbbR regulators might be also operative in the interactive regulation of RubisCOs in *H. marinus*. CbbRm might act as a repressor for the *cbbR1* promoter, and both CbbR1 and CbbRm might act as repressors for the *cbbLS-2* promoter. However, because the affinity of the CbbR regulators to the non-cognate promoters was lower than that to the cognate promoters, the cross-reaction by the CbbRs seems to be insufficient to explain the interactive regulation. Disruption of *cbbR1* or *cbbRm* caused a significant decrease in their cognate RubisCO enzymes, which might change the flux of the CBB cycle. Another possible explanation for the cross-regulation is that an accumulation of certain intermediates or a change in certain cellular states caused by the loss of CbbLS-1 and/or CbbM is the trigger for the induction of another RubisCO. It has been reported that the DNA binding of CbbR is altered in the presence of certain metabolites in other bacteria (van Keulen *et al.*, 1998, 2003; Grzeszik *et al.*, 2000; Terazono *et al.*, 2001; Dubbs *et al.*, 2004). In *R. capsulatus*, the binding of both CbbRI and CbbRII to their cognate promoter was enhanced in the presence of certain metabolites formed by the CBB cycle, as well as other intermediates, including ribulose-1,5-bisphosphate, 3-phosphoglycerate and PEP (Dubbs *et al.*, 2004). These results indicated that the CBB cycle-related metabolites act as the signals for the expression of RubisCO enzymes, thus supporting the explanation mentioned above.

The growth and expression of *cbbLS-2* at lower CO₂ concentrations ($\leq 0.15\%$) was not affected by the disruption of *cbbR1* and/or *cbbRm*, suggesting that growth at the lower CO₂ concentrations might primarily be supported by CbbLS-2. The *cbbLS-2* genes are followed by genes encoding carboxysome, a polyhedral inclusion body that concentrates CO₂ for RubisCO, which is included in the body. Carboxysome, which is conserved in all cyanobacteria and some chemoautotrophic bacteria, is a part of the CO₂-concentrating mechanism (CCM), which is induced by CO₂ limitation and has been studied intensively in cyanobacteria and thiobacilli (Badger & Price, 2003; Cannon *et al.*, 2003). Because RubisCO has a low affinity for CO₂ and a slow catalytic rate, it requires the CCM under low-CO₂ conditions. Since the synthesis of carboxysome is clearly an energetic and metabolic burden for the cell, the expression of the *cbbLS-2* operon containing carboxysome

genes must be strictly regulated. The threshold CO₂ concentration for the induction of *cbbLS-2* and carboxysome is somewhere between 2 and 0.15%. However, the CO₂ molecule is not likely to be the direct effector for the expression of *cbbLS-2*, because *cbbLS-2* was expressed even at higher CO₂ concentrations in strain ddR. Change in the expression levels at concentrations between 2 and 0.15% was also found in the *cbbR1* transcript and the CbbM protein. Their regulation was opposite to that of *cbbLS-2*. These results suggest that drastic changes in the cellular states that affect the expression of the *cbb* genes occur at CO₂ concentrations between 2 and 0.15%. Identification of the sensing signals for the CbbRs may contribute to a more complete understanding of the network responsible for the regulation of RubisCO expression in *H. marinus*.

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