

# CO<sub>2</sub>-Responsive Expression and Gene Organization of Three Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase Enzymes and Carboxysomes in *Hydrogenovibrio marinus* Strain MH-110

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*Hydrogenovibrio marinus* strain MH-110, an obligately lithoautotrophic hydrogen-oxidizing bacterium, fixes CO<sub>2</sub> by the Calvin-Benson-Bassham cycle. Strain MH-110 possesses three different sets of genes for ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO): CbbLS-1 and CbbLS-2, which belong to form I (L<sub>8</sub>S<sub>8</sub>), and CbbM, which belongs to form II (L<sub>x</sub>). In this paper, we report that the genes for CbbLS-1 (*cbbLS-1*) and CbbM (*cbbM*) are both followed by the *cbbQO* genes and preceded by the *cbbR* genes encoding LysR-type regulators. In contrast, the gene for CbbLS-2 (*cbbLS-2*) is followed by genes encoding carboxysome shell peptides. We also characterized the three RubisCOs in vivo by examining their expression profiles in environments with different CO<sub>2</sub> availabilities. Immunoblot analyses revealed that when strain MH-110 was cultivated in 15% CO<sub>2</sub>, only the form II RubisCO, CbbM, was expressed. When strain MH-110 was cultivated in 2% CO<sub>2</sub>, CbbLS-1 was expressed in addition to CbbM. In the 0.15% CO<sub>2</sub> culture, the expression of CbbM decreased and that of CbbLS-1 disappeared, and CbbLS-2 was expressed. In the atmospheric CO<sub>2</sub> concentration of approximately 0.03%, all three RubisCOs were expressed. Transcriptional analyses of mRNA by reverse transcription-PCR showed that the regulation was at the transcriptional level. Electron microscopic observation of MH-110 cells revealed the formation of carboxysomes in the 0.15% CO<sub>2</sub> concentration. The results obtained here indicate that strain MH-110 adapts well to various CO<sub>2</sub> concentrations by using different types of RubisCO enzymes.

Ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO; EC 4.1.1.39) is a key enzyme in the Calvin-Benson-Bassham (CBB) cycle. RubisCO for the CBB cycle is typically categorized into two forms. Form I RubisCO, the most common form, consists of eight large and eight small subunits in a hexadecameric (L<sub>8</sub>S<sub>8</sub>) structure. This form is widely distributed in CO<sub>2</sub>-fixing organisms, including all higher plants, algae, cyanobacteria, and many autotrophic bacteria. Form II RubisCO, on the other hand, consists of only large subunits (L<sub>x</sub>), the number of which differs among organisms. Although this form, first found in *Rhodospirillum rubrum* (31, 42), is more widespread among species than was originally thought, its existence is limited to autotrophic bacteria. In addition to these traditional form I and form II enzymes, two novel types, form III and form IV RubisCO, have been revealed by the complete genome sequences of some archaea and bacteria (1, 12, 17, 47). Even though these two forms have not been shown to be a part of the CBB cycle, form III and form IV RubisCOs are fairly well established now.

RubisCO for the CBB cycle catalyzes two different reactions: CO<sub>2</sub> fixation, in which CO<sub>2</sub> interacts with enzyme-bound ribulose-1,5-bisphosphate (RuBP) to produce 2 molecules of

3-phosphoglycerate (PGA), and O<sub>2</sub> fixation, in which O<sub>2</sub> interacts with enzyme-bound RuBP to produce 1 molecule each of PGA and 2-phosphoglycolate (PG). A RubisCO enzyme's efficiency is usually measured by the specificity factor ( $\tau$ ), which is the ratio of the rate constants for both CO<sub>2</sub> and O<sub>2</sub> fixation (29). The higher a RubisCO's  $\tau$  value is, the better the RubisCO can discern CO<sub>2</sub> from O<sub>2</sub>. This endows it with highly efficient CO<sub>2</sub> fixation and thus allows it to adjust to a lower CO<sub>2</sub> concentration. The  $\tau$  value is generally over 80 for form I RubisCO in higher plants, between 25 and 75 for form I RubisCO in bacteria, and under 20 for form II RubisCO (24, 40).

Some bacteria have been found to possess more than one set of RubisCO genes. *Ralstonia eutropha* (26) and *Chromatium vinosum* (45) have two sets of genes that encode form I enzymes, while *Halothiobacillus neapolitanus* (formerly *Thiobacillus neapolitanus*) (38), *Thiomonas intermedia* (formerly *Thiobacillus intermedius*) (41), *Thiobacillus denitrificans* (11), *Rhodobacter sphaeroides* (14), and *Rhodobacter capsulatus* (15, 35) have genes for both form I and form II enzymes. Moreover, *Acidithiobacillus ferrooxidans* (formerly *Thiobacillus ferrooxidans*) (23) and *Hydrogenovibrio marinus* (34, 48) have three different sets of RubisCO genes, two of which encode form I enzymes while the third encodes a form II enzyme.

Previous studies indicated that the expression of both forms of RubisCO is correlated with CO<sub>2</sub> concentration. In *R. sphaeroides*, synthesis of both form I and form II RubisCOs was augmented when the bacterium was moved from heterotrophic

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(malate as a carbon source) to autotrophic ( $H_2$  with 1.5%  $CO_2$ ) growth conditions. But the promotion of form I RubisCO synthesis is higher than that of form II. Probably the form I enzyme must be expressed in a low- $CO_2$  concentration to support growth (25). In *H. neapolitanus*, both form I and form II RubisCOs were synthesized when the organism was cultured in air supplemented with 5%  $CO_2$ , but when it was cultured in air alone, the expression of form I RubisCO increased and that of form II decreased (4). The disruption of a form I RubisCO gene results in the promotion of form II RubisCO gene expression, but the mutant is unable to grow in atmospheric  $CO_2$  concentrations (4).

In autotrophic bacteria, a RubisCO gene, whether it encodes a form I or a form II enzyme, is generally clustered with CBB cycle-related genes, such as *cbpP*, the gene for phosphoribulokinase, and *cbpF*, the gene for fructose-1,6-bisphosphatase (13, 22, 30). In some cases, the RubisCO genes are clustered with the genes for carboxysome shell peptides. A carboxysome is a polyhedral organelle in which RubisCOs are sequestered, and it plays an important role in  $CO_2$  fixation (37, 39). This gene organization has been examined in thiobacilli (5). In many cases, the regulatory gene *cbpR* is located upstream of the RubisCO gene in the opposite orientation (40). *cbpR* encodes a LysR-type transcriptional regulator that induces the transcription of the RubisCO gene and other *cbp* genes.

*H. marinus* strain MH-110 is an obligately lithoautotrophic hydrogen-oxidizing bacterium isolated from a marine environment (32, 33). As stated above, this organism possesses three different sets of RubisCO genes: *cbpLS-1* and *cbpLS-2* encode the form I enzymes CbbLS-1 and CbbLS-2, respectively, and *cbpM* encodes the form II enzyme CbbM. Until now, CbbM was purified from *H. marinus*, while CbbLS-1 and CbbLS-2 were purified by using the heterologous expression system of *E. coli* (6, 21). It has been revealed that each of the three RubisCOs has different properties in vitro. The specificity factors ( $\tau$ ) of CbbLS-1, CbbLS-2, and CbbM were determined to be 26.6, 33.1, and 14.8, respectively, suggesting that RubisCOs are adapted to different  $CO_2$  concentrations (21, 49). The structural genes of the three RubisCOs were cloned, and their nucleotide sequences were determined (34, 48). The *cbpQ*-type gene, which is similar to the *nirQ/norQ* gene of denitrifying bacteria, was found downstream of *cbpM* (18). However, the complete structure of each of the three RubisCO gene clusters remains to be investigated. In this study, we clarified the organization of the *cbpLS-1*, *cbpLS-2*, and *cbpM* gene clusters in strain MH-110 and characterized the three RubisCOs in vivo by examining their expression profiles in different  $CO_2$  concentrations.

#### MATERIALS AND METHODS

**Bacterial strains, plasmids, and culture conditions.** *E. coli* strains DH5 and JM109 were used as hosts for the Charomid 9-36 (Nippon Gene, Toyama, Japan) and pUC119 vectors, respectively. *E. coli* strains were routinely grown in Luria-Bertani medium at 37°C with 50  $\mu$ g of ampicillin/ml. *H. marinus* was cultivated under atmospheric pressure consisting of  $H_2$ ,  $O_2$ , and  $CO_2$  (75:15:10, vol/vol/vol) at 37°C in an inorganic medium as described previously (33). For large-scale cultures, 50 ml of precultivated cells was inoculated into a 1-liter fermentor (BMJ-1; Able, Tokyo, Japan) containing 0.5 liters of medium and was cultivated at 37°C with a constant supply (gas flux, 0.5 liters/min) of a gas mixture consisting of either (i) 70%  $H_2$ , 15%  $O_2$ , and 15%  $CO_2$ , (ii) 83%  $H_2$ , 15%  $O_2$ , and 2%  $CO_2$ ,

(iii) 85%  $H_2$ , 15%  $O_2$ , and 0.15%  $CO_2$ , or (iv) 20%  $H_2$  and 80% air (equivalent to 0.03%  $CO_2$ ).

**Cloning and DNA sequencing.** Standard protocols were employed for DNA manipulation and cloning (36). Restriction and DNA-modifying enzymes were purchased from Takara Bio (Kyoto, Japan) and Toyobo (Osaka, Japan). For Southern hybridizations, digested DNA was separated by 1% agarose gel electrophoresis and blotted onto a Hybond-N nylon membrane (Amersham Pharmacia, Little Chalfont, Buckinghamshire, United Kingdom). The DNA probes were nonradioactively labeled with digoxigenin. A 618-bp SphI-HindIII fragment of plasmid pJN1 (34), a 455-bp KpnI-EcoRI fragment of pJS1 (34), and a 1,375-bp EcoRI-HindIII fragment of pYAH508 (18) were used as probes in the cloning of the downstream regions of *cbpLS-1*, *cbpLS-2*, and *cbpM*, respectively. A 775-bp HindIII-BamHI fragment of pCM1 (Fig. 1) was used as a probe for the cloning of the region farther downstream of *cbpM*. To clone the upstream regions, 776-, 893-, and 853-bp PCR fragments were used for the probes. The nucleotide sequences for PCR amplification were as follows: 5'-GCTGGATCC TACATTGGTTTTGCC-3' and 5'-TAAGGGAATTCTAATAACAAATCAC C-3' for *cbpLS-1*, 5'-CTATATCAAGGATCCAGATC-3' and 5'-CGTTAACC ACATAAGCTTCTTC-3' for *cbpLS-2*, and 5'-CGAATTGGGATCCTAACTT ACCC-3' and 5'-GAAGTCATAACAAGCTTTCGCG-3' for *cbpM*.

DNA-DNA hybrids on the membranes were detected by a staining reaction involving nitroblue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate, and alkaline phosphatase conjugated to anti-digoxigenin Fab fragments (Boehringer Mannheim, Mannheim, Germany). Based on the Southern blot analyses, gene libraries were constructed. Positive clones were identified by colony hybridizations with the same probes used in the Southern hybridizations. pLS1 and pJO1 carry a 5.4- and a 5.2-kb EcoRI fragment upstream and downstream of *cbpLS-1*, respectively. pUP2 and pJT1 carry a 4.3-kb EcoRI fragment and a 3.1-kb KpnI fragment upstream and downstream of *cbpLS-2*, respectively. pMB1 and pCM1 carry a 7.8- and a 4.2-kb BamHI fragment upstream and downstream of *cbpM*, respectively. pCN1 carries a 6.0-kb HindIII fragment farther downstream of pCM1 (Fig. 1). A Big Dye Terminator cycle sequencing kit (Perkin-Elmer Japan, Applied Biosystems Division) was used for dideoxy chain-termination, and an ABI PRISM model 377 DNA sequencer (Applied Biosystems, Foster City, Calif.) was used for DNA sequence determination. Oligonucleotide primers were purchased from Sawady (Tokyo, Japan).

**Preparation of CFE.** *H. marinus* cells cultivated in different  $CO_2$  concentrations were harvested by centrifugation and resuspended in BEMD buffer (50 mM Bicine, 0.1 mM EDTA, 10 mM  $MgCl_2$ , and 1 mM dithiothreitol [pH 7.8]). The cells were disrupted by passing the suspension twice through a French pressure cell at 110 MPa. Cell debris was removed by centrifugation at 100,000  $\times g$  and 4°C for 1 h, and the supernatant was used as cell extracts (CFE). Protein concentrations were determined by the Bradford assay (Bio-Rad, Hercules, Calif.), with bovine serum albumin as a standard.

**PAGE and Western blot analysis.** CFE were separated by 15% polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-15% PAGE). The proteins were then transferred to a polyvinylidene difluoride membrane (Sequi-Blot PVDF membrane; Bio-Rad) in a Trans-Blot electrophoretic transfer cell (Bio-Rad). Western blotting was performed using anti-RubisCO antibodies and, as a secondary antibody, horseradish peroxidase-conjugated, blotting grade, affinity-purified goat anti-rabbit immunoglobulin G (IgG) (H+L) (Bio-Rad). The desired proteins were detected with an HRP-1000 immunostaining kit (Konica, Tokyo, Japan). Antibodies that could specifically distinguish between CbbLS-1 and CbbLS-2 were raised against synthetic oligopeptides that have sequences specific to small subunits of CbbLS-1 and CbbLS-2, respectively. The sequences of oligopeptides are PSRLSDPTSRKAC for CbbS-1 and EFTA DEIYDQIVC for CbbS-2. The oligopeptides and antisera were prepared by Takara Bio. The anti-CbbM antibody was generated against the purified enzyme from strain MH-110.

**RNA isolation and RT-PCR.** Total RNA was isolated from bacterial cells by using ISOGEN (Nippon Gene), which is based on the acid guanidine thiocyanate phenol-chloroform extraction method. The reaction mixture for reverse transcription (RT) was prepared on a half scale of the two-step RT-PCR protocol by using the mRNA Selective PCR kit (version 1.1; Takara Bio). Two micrograms of total RNA was used for the RT reaction, which was conducted at 50°C for 15 min using synthetic oligonucleotide primers. Primers (with sequences in parentheses) were as follows: L12-RT (5'-CTGGCATGTGCCATACGTGG-3') for *cbpLS-1* and *cbpLS-2* and M-RT (5'-AGTAGGTTTCATGCCGTACC-3') for *cbpM*.

To amplify the cDNA produced from RNA by the RT reaction, PCR was performed according to the protocol. As a template, 5  $\mu$ l of the RT product was used. The forward and reverse primers for RubisCO genes (with nucleotide sequences in parentheses) were as follows: L1-F (5'-TGGATGCCAGAGTAT

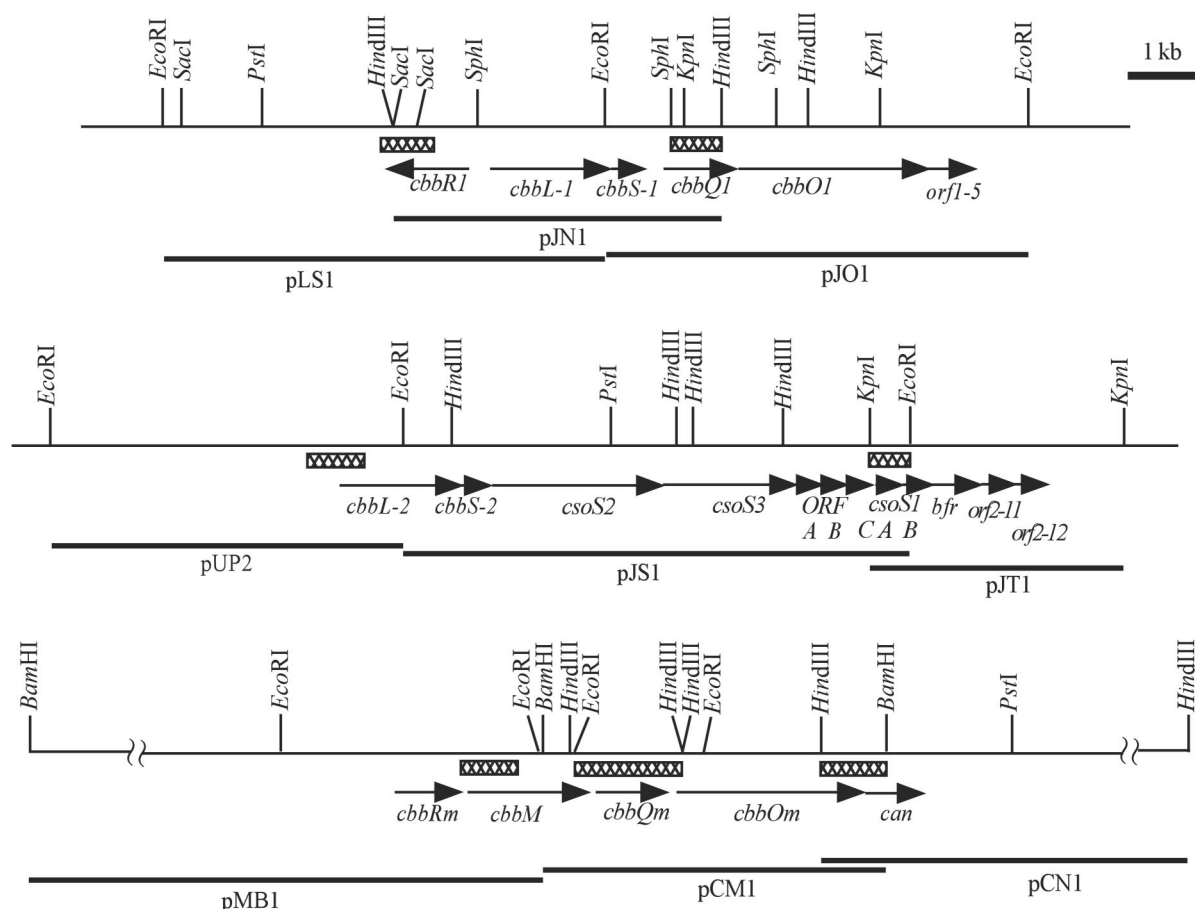


FIG. 1. Physical maps of the RubisCO gene clusters of *H. marinus*. *cbbL* and *cbbS* encode large and small subunits of form I RubisCO, respectively. *cbbM* encodes form II RubisCO. *cbbR* encodes a LysR-type transcriptional regulator. *cbbQ* and *cbbO* encode proteins involved in posttranslational activation of RubisCO. *cso* genes encode carboxysomal proteins. *bfr* encodes bacterioferritin. *can* encodes carbonic anhydrase. Crosshatched boxes indicate positions of probes used for cloning.

GAGCC-3') and L1-R (5'-GTCACGCATGATGTCGATCC-3') for *cbbLS-1*; L2-F (5'-GACACCAGACTACTCCTC-3') and L2-R (5'-CGAAACCTAGCGTAGAAGCG-3') for *cbbLS-2*; and M-F (5'-TTCACCTCGTGGTGTGATG C-3') and M-R (5'-CGAGCAAGCTTCATGTAGCA-3') for *cbbM*.

The PCR condition was 30 cycles of amplification, each cycle consisting of a denaturing step for 1 min at 85°C, an annealing step for 1 min at 45°C, and an extension step for 2 min at 72°C. The PCR products were separated on a 1% agarose gel. After being stained with ethidium bromide, the gel was exposed to a UV illuminator.

**Electron microscopy.** Cells were prefixed with 2.5% glutaraldehyde overnight and postfixed with 1% osmium tetroxide for 1.5 h at room temperature. After dehydration in an ethanol series and then in propylene oxide, the cells were embedded into Quetol 812 epoxy resin (Nissin EM, Tokyo, Japan), and the resin was polymerized at 55°C overnight. Blocks were cut into ultrathin sections with a diamond knife on a microtome. Microscopic observation was performed using a transmission electron microscope (TEM) (JEM-1010; JEOL Hightech Co. Ltd., Tokyo, Japan).

**Nucleotide sequence accession numbers.** The nucleotide sequence data reported in this paper have been submitted to the DDBJ, EMBL, and NCBI nucleotide sequence databases under accession numbers AB122069, AB122070, and AB122071.

## RESULTS

**Organization of the RubisCO gene clusters.** The genes for RubisCO are usually clustered with the genes for other CBB cycle enzymes in autotrophic bacteria (27). In the case of strain

MH-110, *cbbQm*, which was identified downstream of *cbbM*, had been the only gene found in the flanking region of the three RubisCO genes (18). To clarify the organization of the RubisCO gene clusters, we cloned the upstream and downstream regions of each RubisCO gene and determined the nucleotide sequences of the cloned fragments (Fig. 1). *cbbLS-1* is followed by the genes designated *cbbQ1*, *cbbO1*, and *orf1-5*. Translated sequences of *cbbQ1* and *cbbO1* have 74 and 38% amino acid identity with those of *cbbQ* and *cbbO* of *Hydrogenophilus thermoluteolus* TH-1, respectively. The *cbbQ*-type genes encode putative ATP-binding proteins and are similar to the *norQ/nirQ*-type genes (7, 50). The *cbbO* genes are similar to the *norD* genes (20, 22). The *norQ/nirQ* and *norD* genes are located in the vicinity of the genes for cytochrome *bc*-type nitric oxide reductase of denitrification bacteria. These genes are required for the expression of functional nitric oxide reductase or anaerobic growth by denitrification (7, 19). Preliminary experiments suggested that the *cbbQ* and *cbbO* gene products are involved in the posttranslational activation or conformational change of RubisCO, but the physiological functions of these gene products are still unclear (18, 20). *orf1-5* encodes a protein of 178 amino acids that is not homologous to any protein in the protein databases. As with the



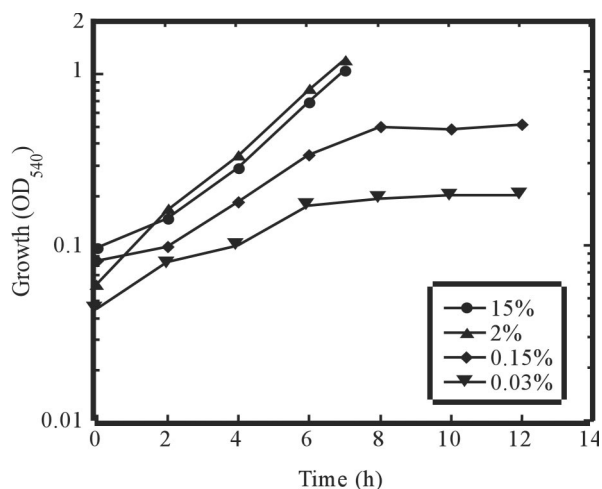


FIG. 2. Growth of *H. marinus* in a CO<sub>2</sub> concentration of 15, 2, 0.15, or 0.03%. Growth was monitored by measuring the optical density of the cultures at 540 nm.

RubisCO gene clusters of other autotrophic bacteria, the *cbbR*-type gene, encoding a member of the LysR family of transcriptional regulators, is located upstream of *cbbLS-1* in the opposite direction and is designated *cbbR1*. *cbbLS-2* is followed by *csoS2*, *csoS3*, *orfA* and *orfB*, and *csoS1C*, *csoS1A*, and *csoS1B*, each of which encodes a carboxysome shell peptide. Translated sequences of the genes are 26, 44, 74, 49, 92, 88, and 90% identical to those of the corresponding genes of *H. neapolitanus* (2, 3, 10). These genes are followed by *bfr*, which encodes a putative bacterioferritin, and two unknown open reading frames (ORFs), namely, *orf2-11* and *orf2-12*. *cbbLS-2* and the genes that follow it are assembled in an operon-like structure, suggesting that carboxysomes are formed under the conditions under which CbbLS-2 is expressed. The *cbbR*-type gene was not found in the upstream region of *cbbLS-2* (data not shown). In the downstream region of *cbbM*, a *cbbO*-type gene, which is designated *cbbOm*, is newly located downstream of *cbbQm*, thus revealing the existence of two sets of *cbbQO* genes in strain MH-110. Translated sequences of *cbbQm* and *cbbOm* have 69 and 32% identity with those of *cbbQ1* and *cbbO1*, respectively. The *can* gene, encoding carbonic anhydrase (EC 4.2.1.1), was found downstream of *cbbOm*. Carbonic anhydrase catalyzes the hydration-dehydration of CO<sub>2</sub>-HCO<sub>3</sub><sup>-</sup> and has been shown to be essential for the growth of *R. eutropha* at ambient CO<sub>2</sub> concentrations (28), but its role in strain MH-110 remains to be investigated. Another *cbbR* gene, designated *cbbRm*, was found in the upstream region. This gene lies in the same orientation with *cbbM*, in contrast to *cbbR1*, which is located in the opposite orientation to *cbbLS-1*. CbbRm shares 32% identity with CbbR1. The other genes found in the seven cloned fragments are not likely to be involved in CO<sub>2</sub> fixation (data not shown).

**Effect of CO<sub>2</sub> concentration on the growth of *H. marinus*.** In order to determine the effect of CO<sub>2</sub> availability on cell growth, strain MH-110 was cultivated in different CO<sub>2</sub> concentrations: 15, 2, 0.15, and 0.03% (Fig. 2). At CO<sub>2</sub> concentrations of 15 and 2%, strain MH-110 showed short doubling times, 2.2 and 1.9 h, respectively. This indicates an adequate CO<sub>2</sub> supply in

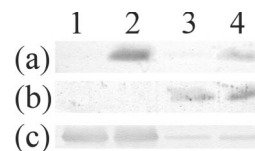


FIG. 3. Immunoblot analyses of CbbLS-1, CbbLS-2, and CbbM RubisCOs. CFE (3  $\mu$ g) were resolved on an SDS-15% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. An anti-CbbLS-1 oligopeptide antibody (a), an anti-CbbLS-2 oligopeptide antibody (b), and an anti-form II RubisCO antibody raised against CbbM from *H. marinus* (c) were used. CFE were prepared from 15% (lane 1), 2% (lane 2), 0.15% (lane 3), or 0.03% (lane 4) CO<sub>2</sub> cultures.

the medium. At a CO<sub>2</sub> concentration of 0.15%, however, the doubling time increased to 5.1 h, making CO<sub>2</sub> availability the limiting factor. Strain MH-110 was able to grow even at a CO<sub>2</sub> concentration of 0.03% (in the 20% H<sub>2</sub>-80% air mixture), but the doubling time increased further, to 11.0 h, and the optical density at 540 nm reached about 0.2 at maximum.

**Effect of CO<sub>2</sub> concentration on expression of RubisCO enzymes.** The expression patterns of the three RubisCO enzymes at the different CO<sub>2</sub> concentrations were determined by an immunological method. Immunoblotting was performed with three different antibodies that recognize CbbLS-1, CbbLS-2, and CbbM, respectively. Since CbbLS-1 and CbbLS-2 have high homology, anti-CbbLS-1 and anti-CbbLS-2 antibodies were raised against synthetic oligopeptides that have unique sequences designed from N-terminal amino acid sequences of CbbS-1 and CbbS-2, respectively. The prepared antibodies were tested for cross-reactivity between CbbLS-1 and CbbLS-2 by using recombinant enzymes expressed in *E. coli* cells, and no cross-reactivity was observed (data not shown). An anti-form II RubisCO antibody was raised against purified form II RubisCO from *H. marinus* (Fig. 3). When the bacterium was cultivated at a CO<sub>2</sub> concentration of 15%, only the form II RubisCO, CbbM, was expressed. When cultivated at a CO<sub>2</sub> concentration of 2%, CbbLS-1 and CbbM were expressed. In the 0.15% CO<sub>2</sub> culture, CbbLS-2 and CbbM were expressed. In the 0.03% CO<sub>2</sub> culture, all three RubisCOs were expressed. These results indicate different properties of the three RubisCOs in vivo.

**Effect of CO<sub>2</sub> concentration on transcription of the RubisCO genes.** The expression pattern of RubisCO at the transcriptional level was examined by RT-PCR. Oligonucleotide primer sets constructed for PCR amplification of each RubisCO were shown not to hybridize to other RubisCO genes (data not shown). The RT-PCR results showed that only *cbbM* was expressed at a CO<sub>2</sub> concentration of 15%, *cbbLS-1* and *cbbM* were expressed at 2% CO<sub>2</sub>, *cbbLS-2* and *cbbM* were expressed at 0.15% CO<sub>2</sub>, and all three genes were expressed at 0.03% CO<sub>2</sub> (Fig. 4). These results were consistent with those of the immunological analyses, indicating that the expression of the three RubisCOs was regulated at the transcriptional level.

**Formation of carboxysomes at low CO<sub>2</sub> concentrations.** Cells that were cultivated at CO<sub>2</sub> concentrations of 15, 2, or 0.15% were harvested for electron microscopic observations (Fig. 5). Many polyhedral particles approximately 100 nm long, showing the typical shape and approximate size of carboxysomes, were observed in cells grown at a CO<sub>2</sub> concentration of

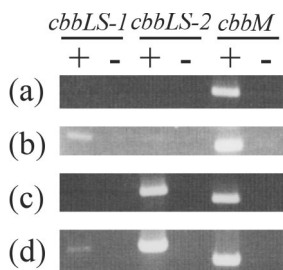


FIG. 4. Analyses by RT-PCR of expression of the three RubisCO genes. RNA was isolated from cells grown in a CO<sub>2</sub> concentration of 15% (a), 2% (b), 0.15% (c), or 0.03% (d). +, RT was carried out before PCR; -, PCR without the RT reaction.

0.15%. They were scarcely found in cells grown at a CO<sub>2</sub> concentration of 15 or 2%.

### DISCUSSION

In this work, we determined the gene organizations of the three RubisCO gene clusters of *H. marinus* strain MH-110. We found two sets of the *cbbQO* and *cbbR* genes in the *cbbLS-1* and *cbbM* gene clusters, the genes for carboxysome shell peptides in the *cbbLS-2* gene cluster, and the carbonic anhydrase gene in the *cbbM* gene cluster. Other genes found in the cloned fragments were not likely to be involved in CO<sub>2</sub> fixation. It is worth noting that despite the high similarity of nucleotide sequences between *cbbLS-1* and *cbbLS-2* (34), the organization of the *cbbLS-1* gene cluster is totally different from that of the *cbbLS-2* gene cluster; *cbbLS-1* clustered with the *cbbR* and *cbbQO* genes, and *cbbLS-2* clustered with carboxysome genes in an operon-like structure. It is also interesting that *cbbLS-1* and *cbbM*, which encode different forms of RubisCO, have similar gene clusters, with the *cbbR* gene upstream and the *cbbQO* genes downstream. This pattern of distinct RubisCO gene clusters for *cbbLS-1* versus *cbbLS-2* and similar clusters for *cbbLS-1* and *cbbM* may not be the result of simple gene duplication in the ancestor of *H. marinus*, as suggested previously (34), or of lateral gene transfer, as proposed in the case of *A. ferrooxidans* ATCC 23270 (23). Rather, it appears that

complicated phenomena of gene duplication, lateral gene transfer, and reorganization have occurred during evolution. One possible interpretation of such gene clusters is that *cbbLS-1* was reproduced by gene duplication of *cbbLS-2*, which had been introduced by lateral gene transfer, and the gene reorganization was followed with the *cbbR* and *cbbQO* genes of the *cbbM* cluster, which the ancestor of strain MH-110 might have originally possessed.

RubisCO and other CBB cycle-related genes are usually clustered in various chemo- and photoautotrophic bacteria (27). Strain MH-110 is unique in that the genes for the other CBB cycle enzymes are not clustered with the RubisCO genes. This is probably related to the fact that strain MH-110 is an obligatory autotroph. In facultative autotrophs, the CBB cycle enzymes must be uniformly regulated by the availability of suitable carbon and energy sources; clustering of the genes as a single operon or a few operons might be advantageous for uniform regulation. On the other hand, the CBB cycle is constitutively active, and uniform regulation is not necessary in obligatory autotrophs. This is probably the reason why the genes for the other CBB enzymes are not cotranscribed with the RubisCO genes as an operon in strain MH-110. Another probable reason is that, because the transcription of each of the three RubisCO genes varies according to the CO<sub>2</sub> concentration as mentioned below, cotranscription of the other *cbb* genes with one of the three RubisCO genes might be unsuitable for keeping the balance of total CBB cycle activity at various CO<sub>2</sub> concentrations. In either case, analysis of the other *cbb* genes will be necessary in order to understand the regulation of the whole CBB cycle in strain MH-110.

Also, the three RubisCOs were characterized by examining their expression in vivo in environments with different CO<sub>2</sub> availabilities. It has been suggested previously that, in a microbe which has genes for both form I and II RubisCOs, form II and form I enzymes are predominantly expressed at high and low CO<sub>2</sub> concentrations, respectively (25, 40). Nevertheless, this hypothesis has not been tested in detail. This is the first report that three RubisCO enzymes of *H. marinus* are differentially expressed depending on the CO<sub>2</sub> concentration, and it was revealed that expression was complexly regulated by the

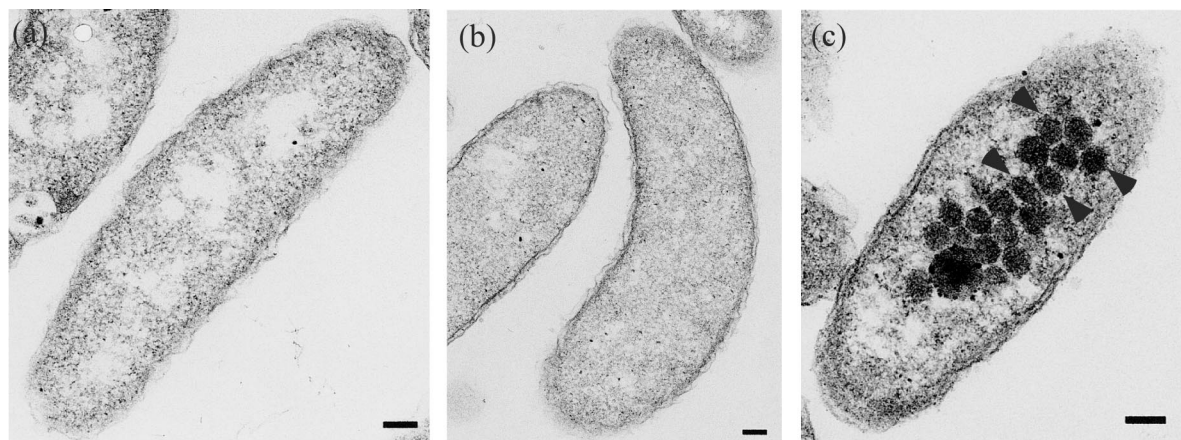


FIG. 5. Electron micrographs of *H. marinus* grown in a CO<sub>2</sub> concentration of 15% (a), 2% (b), or 0.15% (c). Arrowheads indicate carboxysomes. Bar, 100 nm.

CO<sub>2</sub> concentration. The expression pattern was in good accordance with the specificity factor ( $\tau$ ) of each RubisCO. The  $\tau$  value, determined by calculating the ratio of the rate constants for both carboxylase and oxygenase reactions, indicates each RubisCO's efficiency at distinguishing between CO<sub>2</sub> and O<sub>2</sub> (29). A high  $\tau$  value for RubisCO means that the enzyme can selectively assimilate CO<sub>2</sub> in spite of the existence of O<sub>2</sub>, and thus it is better adjusted to a lower CO<sub>2</sub> concentration. Under the CO<sub>2</sub>-rich condition (15%), only CbbM, which has the lowest  $\tau$  value (14.8) of the three RubisCOs, was expressed in strain MH-110. Decreasing the CO<sub>2</sub> concentration to 2% triggered the expression of CbbLS-1, which has the middle  $\tau$  value (26.6) of the three. When the CO<sub>2</sub> concentration was decreased from 2 to 0.15%, CbbLS-1 disappeared, and CbbLS-2, which has the highest  $\tau$  value (33.1), took its place. Moreover, the amount of CbbM decreased at that concentration. At the extremely low CO<sub>2</sub> concentration (0.03%), CbbLS-1 reappeared, and thus all three RubisCOs were expressed. Electron microscopic observation showed the formation of carboxysomes at the 0.15% CO<sub>2</sub> concentration. This was the concentration at which CbbLS-2 was expressed, suggesting that the genes for CbbLS-2 and carboxysome shell peptides in the *cbbLS-2* gene cluster are transcribed as an operon. CbbLS-2 is probably sequestered in carboxysomes that help the enzyme fix CO<sub>2</sub> at low CO<sub>2</sub> concentrations, as in the case of cyanobacteria and thiobacilli such as *H. neapolitanus* (37).

CbbM and CbbLS-2 turned out to be subjected to one-stage regulation by CO<sub>2</sub>. CbbM was expressed in large quantities at high CO<sub>2</sub> concentrations, and in diminished quantities at low CO<sub>2</sub> concentrations. The threshold was somewhere between 2 and 0.15%. CbbLS-2, on the other hand, was not expressed at high CO<sub>2</sub> concentrations but rather was induced at low CO<sub>2</sub> concentrations. The threshold was also somewhere between 2 and 0.15%. This fact may suggest that this organism may undergo a dynamic change of gene expression when CO<sub>2</sub> is downshifted from 2 to 0.15%. It has been reported recently for the cyanobacterium *Synechocystis* sp. strain PCC 6803 that a CO<sub>2</sub> downshift induced changes in global gene expression and a dramatic up-regulation of genes involved in inducible CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> uptake systems (46). Similar changes may occur in strain MH-110 as well. The regulation of CbbLS-1 was more complicated. It was not expressed at a CO<sub>2</sub> concentration of 15 or 0.15% but was expressed at 2 and 0.03% CO<sub>2</sub>, suggesting that the external CO<sub>2</sub> concentration alone did not directly regulate expression. Rather, the cytoplasmic CO<sub>2</sub> concentration or some kinds of CBB cycle intermediates may affect the expression of CbbLS-1. It seems that *cbbLS-1* is expressed when CO<sub>2</sub> fixation activity by CbbLS-2 and/or CbbM is not enough for cell growth. To confirm the role of *cbbLS-1* and to further examine the regulation of expression of the three RubisCO genes, it is necessary to knock out each RubisCO gene. A method for constructing isogenic mutants is now under way.

The RT-PCR analyses showed that the expression of the three RubisCOs was regulated at the transcriptional level. The LysR-type transcriptional regulator, *cbbR*, is encoded upstream of both the *cbbLS-1* and *cbbM* genes, as in other autotrophic bacteria (40), but not upstream of *cbbLS-2*. Neither CbbR1 nor CbbRm is likely to sense a CO<sub>2</sub> molecule directly, since the signal molecule for CbbR has been reported to be

NADPH in *Xanthobacter flavus* and *H. thermoluteolus*, or phosphoenolpyruvate in *R. eutropha* (16, 43, 44). In the case of *R. sphaeroides*, which has two distinct *cbb* gene clusters, *cbb<sub>I</sub>* and *cbb<sub>II</sub>*, CbbR is not encoded in the *cbb<sub>II</sub>* gene cluster, but the CbbR encoded in the *cbb<sub>I</sub>* gene cluster regulates both of the *cbb* clusters (8). In addition to CbbR and RegA, the latter of which is the response regulator of the *regA-regB* two-component regulatory system, two unidentified proteins bind to the promoter region of *cbb<sub>II</sub>* in this bacterium (9). It is not certain whether CbbR1 and CbbRm regulate the *cbbLS-2* gene cluster in strain MH-110. However, the CO<sub>2</sub> concentration-responsive regulation of the three RubisCO genes should be interrelated by the action of multiple regulators, including CbbR1 and CbbRm. Future work will focus on clarification of the role of the two CbbR regulators and identification of the other regulators that control the expression of the RubisCO genes.

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