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The *cbbQ* Genes, Located Downstream of the Form I and Form II RubisCO Genes, Affect the Activity of Both RubisCOs

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***Hydrogenovibrio marinus* strain MH-110, an obligately lithoautotrophic hydrogen-oxidizing bacterium, possesses three sets of the genes for ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO): namely, two form I type (*cbbLS-1* and *cbbLS-2*) and one form II type (*cbbM*) enzymes. The *cbbQ-m* gene was located downstream of *cbbM,* and showed high similarity to other *cbbQ* genes and *nirQ/norQ* genes in denitrifying bacteria. Phylogenetic analysis of CbbQ and NirQ/NorQ indicated that CbbQ-m from *Hv. marinus* closely resembled CbbQ from *Thiobacillus intermedius* and *Thiobacillus neapolitannus* and less closely resembled NirQ and NorQ. The *cbbQ-m* gene has been shown to activate the form II RubisCO in *E. coli* cells, and the *cbbQ-t* from *Hydrogenophilus thermoluteolus* could also activate the form II RubisCO. Both *cbbQ* genes have also been shown to activate the form I RubisCO from *Hp. thermoluteolus* in *E. coli* cells. However, the activation levels of two form I RubisCOs from *Hv. marinus* were smaller than that of form I RubisCOs from *Hp. thermoluteolus.* Form II RubisCO activated by CbbQ-m (QM) was purified from *E. coli* cells. The result of the 8-anilino-1-naphthalenesulfonate binding assay and the circular dichroism spectra indicated that QM was conformationally different from Form II RubisCO that was not activated by CbbQ.** **© 1999 Academic Press**

Ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO; EC4.1.1.39) is one of two specific enzymes of the Calvin-Benson cycle. The enzyme in plants, algae, cyanobacteria, and most photo- and chemoautotrophic bacteria, is a high molecular weight protein (M_r 500,000~560,000) composed of eight large and eight small subunits (L_8S_8; form I type). The large subunit (M_r 50,000~64,000) containing the active site is encoded by the *cbbL* gene, and the small subunit (M_r 10,000~16,000) is encoded by the *cbbS* gene. The second type of RubisCO, form II type (L_x) RubisCO, encoded by the *cbbM* gene and consisting of only large subunits, has also been described in *Rhodospirillum rubrum* (L_2; 1, 2), *Gonyaulax* dinoflagellates (L_2; 3), and *Rhodopseudomonas palustris* (L_8; 4). *Rhodobacter sphaeroides* (5), *Rhodobacter capsulatus* (6), *Thiobacillus denitrificans* (7), and *Thiobacillus intermedium* (8) have one form I and one form II type RubisCO.

Hydrogenovibrio marinus strain MH-110 is an obligately lithoautotrophic, halophilic, and aerobic hydrogen-oxidizing bacterium isolated from a marine environment (9, 10). This bacterium fixes CO_2 via the Calvin-Benson cycle. In previous papers, we demonstrated that *Hv. marinus* possessed two different sets of genes for two form I type (*cbbLS-1* and *cbbLS-2*) and one form II RubisCO gene (11, 12). All of these RubisCOs have been purified from *Escherichia coli* cells harboring a set of genes for each, and some properties of the purified enzymes have been investigated (13). The form II RubisCO has been also purified from *Hv. marinus* cells (14).

The *cbbQ* gene that encodes a protein carrying an ATP-binding motif is located downstream of *cbbLS* in *Hydrogenophilus thermoluteolus* (15) (formerly known as *Pseudomonas hydrogenothermophila* (16)), in *Chromatium vinosum* (15), and in *Rhodobacter capsulatus* (17). CbbQ is thought to play an important role in the posttranslational regulation of RubisCO, because coexpression of *cbbQ* with *cbbLS* in *E. coli* affects the conformational state and the activity of RubisCO (18). The *cbbQ* genes are extremely similar to the *nirQ* genes from *Pseudomonas aeruginosa* (19) and *Pseudomonas stutzeri* (20) as well as to the *norQ* genes from *Para-*

Abbreviations used: *Hv, Hydrogenovibrio; Hp, Hydrogenophilus; cbbQ-m, cbbQ* of *Hv. marinus; cbbQ-t, cbbQ* of *Hp. thermoluteolus; cbbLS-t, cbbLS* of *Hp. thermoluteolus;* UM, CbbM that is not activated by CbbQ; QM, CbbM that is activated by CbbQ.

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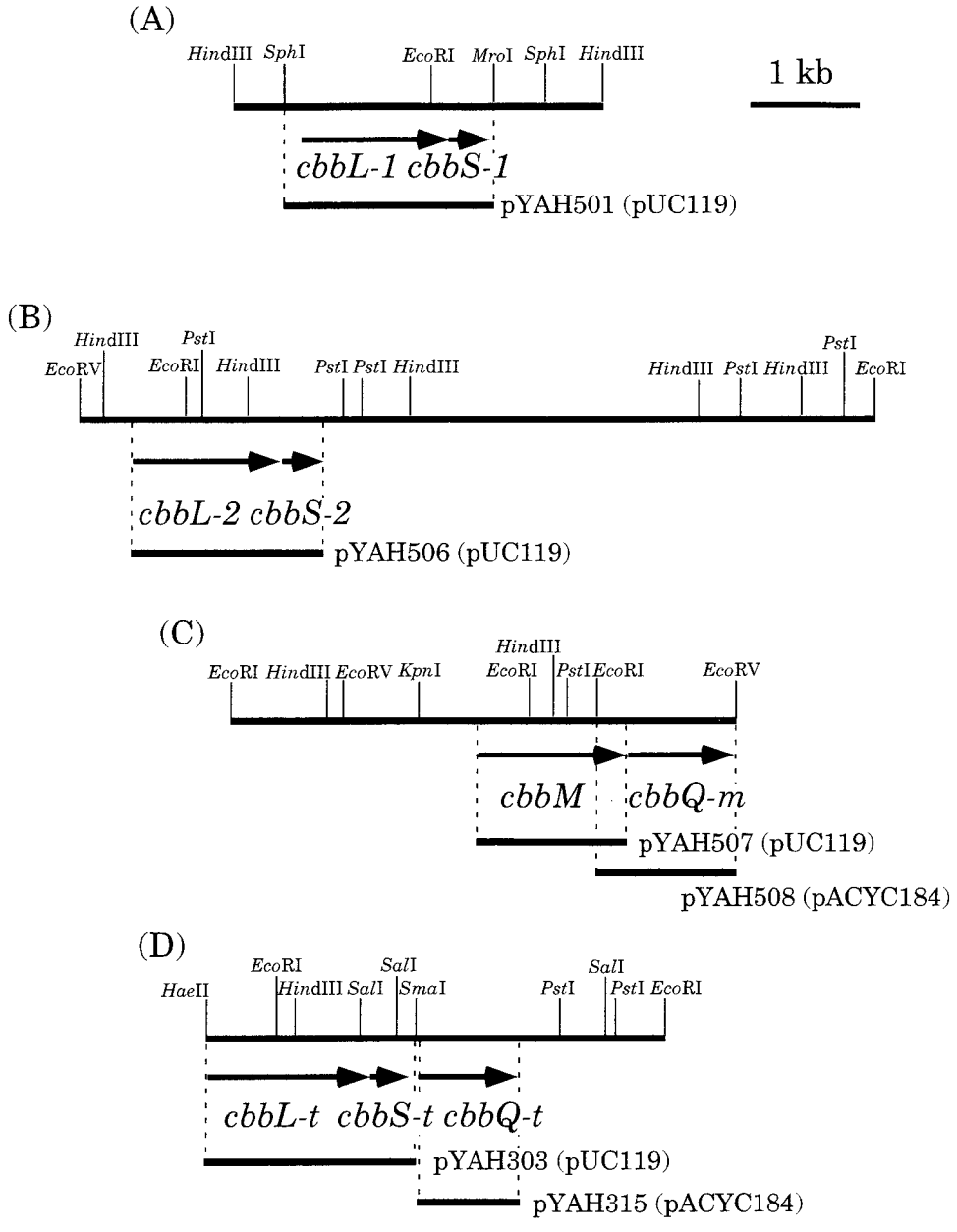

FIG. 1. Physical map of the *cbb* genes of *Hv. marinus* and *Hp. thermoluteolus*. *cbbL* and *cbbS* are coding for large and small subunits of form I RubisCO, respectively. *cbbM* is coding for form II RubisCO. *cbbQ* is coding for a putative ATP-binding protein. (A) Physical map of *cbbLS-1* of *Hv. marinus*. (B) Physical map of *cbbLS-2* of *Hv. marinus*. (C) Physical map of *cbbMQ* of *Hv. marinus*. (D) Physical map of *cbbLSQ* of *Hp. thermoluteolus*.

coccus denitrificans (21), *Rhodobacter sphaeroides* (22), and *Paracoccus halodenitrificans* (23). In these bacteria, the *nirQ* and *norQ* genes are located in the genome, within the gene cluster for denitrification. The *nirQ* gene is reportedly necessary for the posttranslational activation of nitric oxide reductase in *P. stutzeri* (20). We have previously reported that NirQ from *P. aeruginosa* can activate RubisCO from *Hp. thermoluteolus* (24).

In this report, we identified the *cbbQ* gene downstream of form II RubisCO gene in *Hv. marinus* and described the ability of *cbbQ* to change the activities of the form I and form II RubisCOs in *E. coli* cells.

MATERIALS AND METHODS

Bacterial strain, vectors and media. *E. coli* strain JM109 (25) was used as a host for all plasmids used in this paper and was routinely cultivated at 37°C in LB medium with 100 μg/ml ampicillin. 2× YT medium (25) was used for cultivation of *E. coli* for purification of CbbM. pUC119 (Takara Shuzo, Kyoto, Japan) was used for subcloning and as an expression vector. pACYC184 (26) was used for expression of CbbQ.

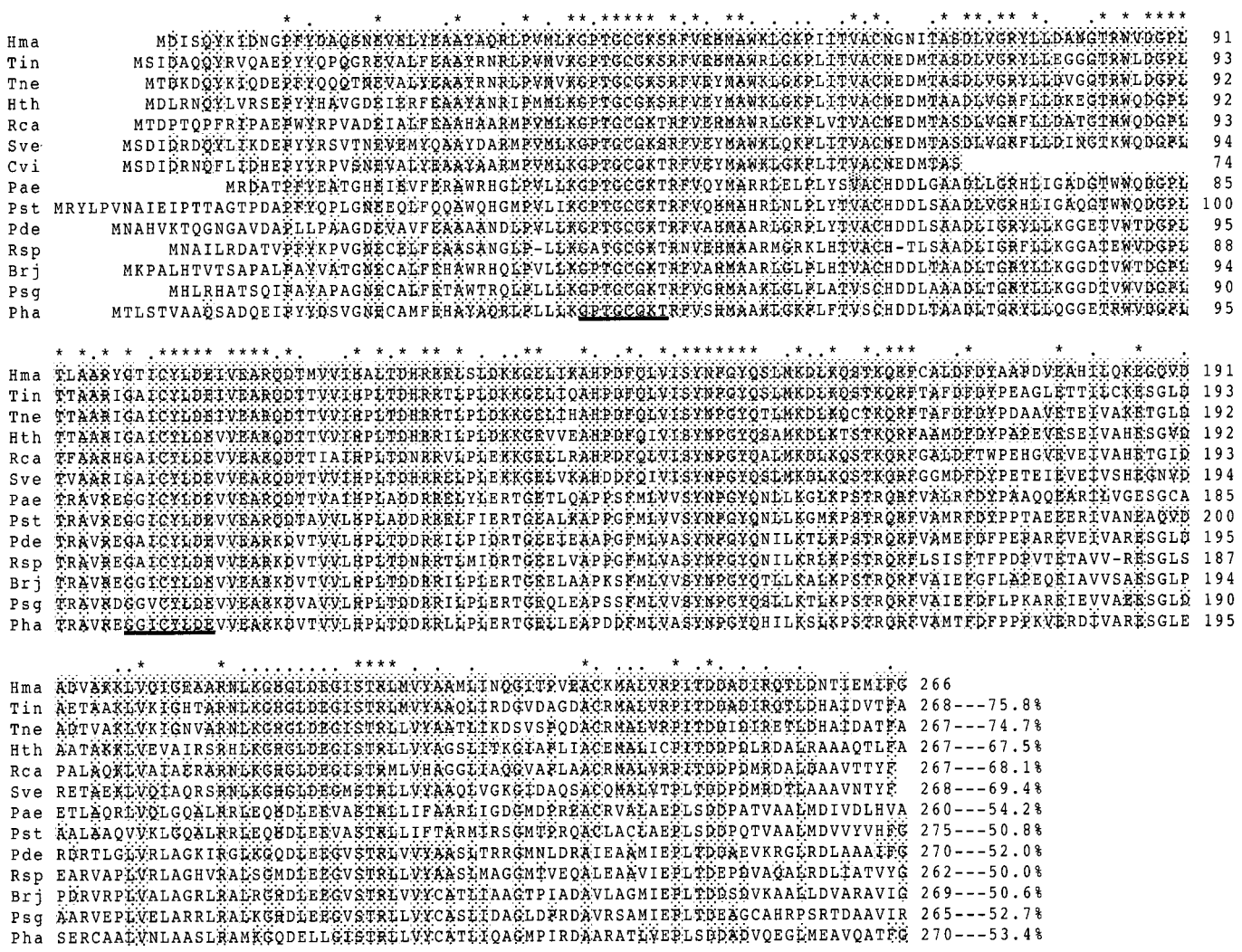

FIG. 2. Alignment of predicted amino acid sequences of CbbQ. The derived amino acid sequence of *cbbA* from *Hv. marinus* (Hma) aligned with CbbQ from *T. intermedius* (Tin), CbbQ from *T. neapolitannus* (Tne), CbbQ from *Hp. thermoluteolus* (Hth), CbbQ from *Rb. capsulatus* (Rca), CbbQ from *C. vinsum* (Cvi), NirQ from *P. aeruginosa* (Pae), NirQ from *P. stutzeri* (Pst), NorQ from *Pa. denitrificans* (Pde), CbbQ from *Rb. sphaeroides* (Rsp), NorQ from *Br. japonicum* (Brj), NorQ from *Pseudomonas* sp. G-179 (Psg), NorQ from *Pa. halodenitrificans* (Pha). Conserved amino acids in all of the sequences are indicated by asterisks, and conserved amino acids in all of the CbbQ sequences are indicated by dots. Presumed nucleotide-binding motifs are underlined. Percentages indicate the identities to the CbbQ from *Hv. marinus.*

DNA sequence. DNA sequence of the region downstream of *cbbM* was determined from both directions according to the method described previously (15). The nucleotide sequence data for *cbbQ* have been submitted to the DDBJ, EMBL, and GenBank nucleotide sequence database under the Accession Number AB025104.

Phylogenetic analysis of CbbQ. The CbbQ amino acid sequences of *Hv. marinus* were multiply-aligned using CLUSTAL W ver. 1.6 (27). After being multiply-aligned, the multiple sequence alignment was corrected manually when necessary. A phylogenetic tree was constructed from evolutionary distance data by applying the algorithm of the neighbor-joining method (28). To evaluate the robustness of the branches of the inferred tree, the bootstrap resampling method of Felsenstein (29) was used with 1,000 replications.

Accession numbers of the sequences used for phylogenetic analysis of the CbbQ/NirQ/NorQ type proteins are as follows: *Bacillus subtilis* (YojN), AF026147; *Bradyrhizobium japonicum* (NorQ), AJ132911; *Hp. thermoluteolus* (CbbQ), D30764; *Pa. denitrificans* (NorQ), U28078; *Pa. halodenitrificans* (NorQ), AB010889; *P. aeruginosa* (NirQ), D37883; *Pseudomonas* sp. G-179 (NorQ), AF083948; *P. stutzeri* (NirQ), Z17423; *Rb. capsulatus* (CbbQ), L82000; *Rb. sphaeroides* (NorQ), AF000233; *T. intermedius*, AF012127; *T. neapolitannus*, AF046932.

Construction of the plasmid including cbbL, cbbS, cbbM, and cbbQ genes. The vectors used for the expression of *cbbLS-1*, *cbbLS-2*, and *cbbM* were pYAH501, pYAH506, and pYAH507, respectively. pYAH501 was constructed by ligating the *Sph*I-*Mro*I region into the *Sph*I and *Hin*cII sites of pUC119 after the *Mro*I end was blunted by Klenow fragment (Fig. 1A). pYAH506 and pYAH507 were constructed by ligating the *Nde*I-*Sac*I fragment of pJS305 and pJE225 (13) into the *Hin*cII and *Sac*I sites of pUC119 after the *Nde*I ends were blunted by Klenow fragment, respectively (Fig. 1B and C). pYAH508 was constructed by ligating the *Eco*RI-*Eco*RV region into the *Eco*RI and *Sca*I sites of pACYC184 (Fig. 1C). The constructions of pYAH303 (18), pYAH315 (24), and pACYC184Cm$^-$ (24) were described previously (Fig. 1D). Recombinant DNA manipulations were carried out as described by Sambrook *et al.* (25).

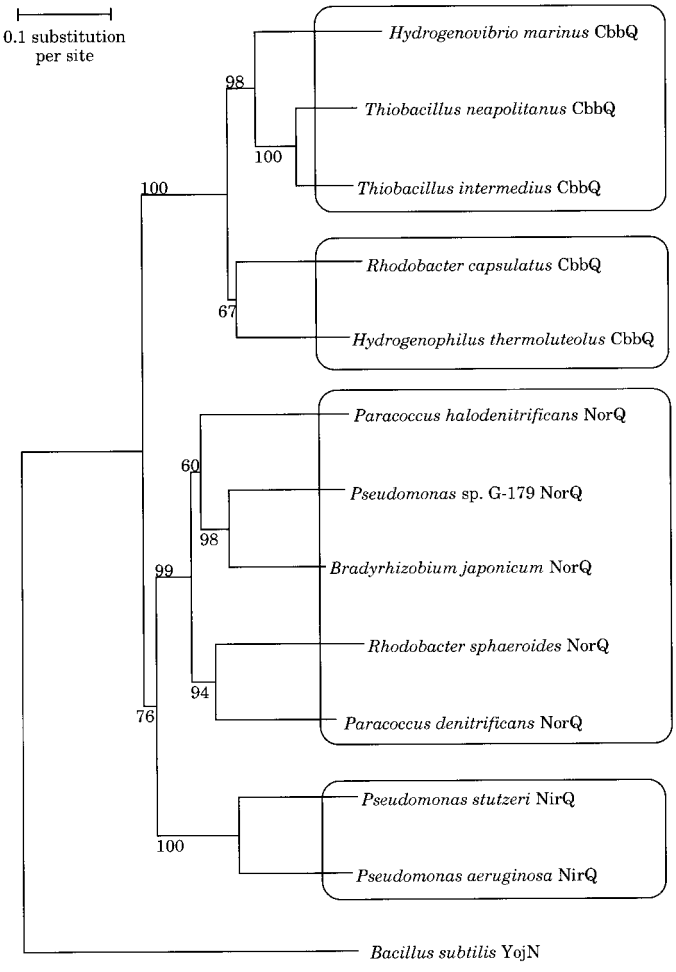

FIG. 3. Phylogenetic tree based on amino acid sequences of the CbbQ/NirQ/NorQ proteins, constructed by neighbor-joining. CbbQs are used in experiment of the expression are underlined. The number on the branches are confidence limits (expressed as percentages) estimated by a bootstrap analysis performed with 1,000 replications. Confidence limits less than 60% are not shown.

Preparation of cell extract. *E. coli* cells harboring plasmids were grown at 37°C in 150 ml of LB-medium with 100 μg/ml ampicillin and 25 μg/ml tetracycline. Cells were suspended in 10 ml of the buffer A (bicine 10 mM, EDTA 0.1 mM, $MgCl_2 \cdot 6H_2O$ 10 mM, 2-mercaptoethanol 1 mM, monoiodoacetic acid 1 mM, pH 7.5) and disrupted twice by passage through a French Pressure Cell at 110 MPa. The cell extract was centrifuged at 10,000 × g for 1 h to remove cell debris.

RubisCO assay. The enzyme assay for RubisCO was performed using $NaH^{14}CO_3$ as described previously (13, 18). One unit of enzyme activity was defined as the amount catalyzing the fixation of 1 μmol CO_2 per min at 30 or 50°C.

Protein concentration was measured using Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Lab., Richmond, CA) with bovine serum albumin as a standard.

Polyacrylamide gel electrophoresis and Western immunoblot analysis. Cell extract was separated by electrophoresis in a 12.5% polyacrylamide gel (PAGE) in the presence of sodium dodecyl sulfate (SDS). The other methods for PAGE and Western immunoblot analysis were described previously (13).

TABLE 1

RubisCO Activity in Cell Extract of *E. coli* Harboring *cbbM* or *cbbMQ*

Plasmid	Gene[a]	RubisCO activity[b] (U/mg)
pYAH507 + pACYC184Cm$^-$	*cbbM*	0.091
pYAH507 + pYAH315	*cbbM, cbbQ-t*	0.144
pYAH507 + pYAH508	*cbbM, cbbQ-m*	0.135
pYAH508	*cbbQm*	<0.01

[a] *cbbQ-t* is the *cbbQ* from *Hp. thermoluteolus* and *cbbQ-m* is the *cbbQ* from *Hv. marinus.*

[b] The data are presented the average of three repetitions for each experiment. The enzyme assay for RubisCO was performed by using $NaH^{14}CO_3$ at 30°C. One unit of enzyme activity is defined as the amount of catalyzing the fixation of 1 μmol CO_2 per min.

Purification of form II RubisCO enzymes from recombinant E. coli cells. *E. coli* cells harboring plasmid were grown at 37°C in 2 liters of 2× YT-medium (25) with 100 μg/ml ampicillin and 25 μg/ml tetracycline. The purification method for form II RubisCO that was not activated by CbbQ was described previously (13). The purification method for form II RubisCO that was activated by CbbQ was used same procedure excepted that the enzymes were eluted with a linear gradient of 0–1 mM NaCl with buffer A on Q-Sepharose and Mono Q columns (Pharmacia, Uppsala, Sweden). The purified protein was monitored by SDS-PAGE.

Fluorescence measurement. Fluorescence measurement was performed as described previously (18), with the exception that the buffer A was used, and incubation was carried out 10 min at 30°C. A spectrum of the buffer was subtracted from each protein spectrum.

Measurement of CD spectra. Circular dichroic (CD) spectra of RubisCO proteins were measured on a JASCO automatic recording spectropolarimeter, Model J 720, from 320 to 200 nm.

RESULTS AND DISCUSSION

Sequence Analysis and Identification of the Downstream Region of cbbM

The nucleotide sequence was determined for a 1.4-kb fragment of DNA downstream of *cbbM* (data not shown). An ORF of 798 nucleotides was located there and was preceded by a reasonable ribosome binding site. A search for amino acid homology with other proteins showed that this protein had high identity to CbbQ, NirQ, and NorQ proteins. The two nucleotide-

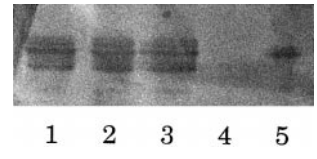

FIG. 4. Western immunoblot analysis of cell extract of *E. coli.* Lane 1, cell extract of *E. coli* harboring pYAH507 and pACYC184Cm$^-$; lane 2, cell extract of *E. coli* harboring pYAH507 and p315; lane 3, cell extract of *E. coli* harboring pYAH507 and pYAH508; lane 4, *E. coli* harboring pYAH508. Lane 5, purified UM. 5 μg protein was electrophoresed in lanes 1, 2, 3, and 4 on (B).

binding domain had high similarity among these proteins (Fig. 2).

Phylogenetic Analysis of the CbbQ/NirQ/NorQ Type Proteins

A phylogenetic tree of the CbbQ/NirQ/NorQ type proteins was constructed using the neighbor-joining method (Fig. 3). Phylogenetic analysis indicated that CbbQ from *Hv. marinus* resembled CbbQ from other bacteria more closely than NirQ, NorQ, and YojN. Bootstrap values were indicated and strongly supported that these proteins should be clustered into four major groups. The four groups were classified by the location of the genes encoding the proteins; the first group genes (*cbbQ*) was located downstream of *cbbM*, the second group (*cbbQ*) was located downstream of *cbbLS*, the third group (*norQ*) was located downstream of *norCB* encoding nitric oxide reductase, and the last group (*nirQ*) was located upstream of *nirS* encoding nitrite reductase. YojN had lower identity to the other proteins, and had no nucleotide-binding motif. YojN seemed to be different from the CbbQ/NirQ/NorQ type proteins. The sequences of the large subunits of form I RubisCO are clustered into two major groups, designated as Red-like and Green-like (30, 31). The RubisCOs (including *cbbLS-1* from *C. vinosum*) encoded by the genes located upstream of *cbbQ* have been located in the form II group and the Green-like group (17, 30, 31). On the other hand, *cbbX* genes encoding another ATP-binding protein have been located downstream of *cbbLS* genes in *Ralstonia eutropha* (32), *Rhodobacter sphaeroides* (33), and *Xanthobacter flavus* (34), as well as downstream of RubisCO genes in a red alga, *Antithamnion* sp. (35), and in *Porphyra purpurea* (36). In *Rb. sphaeroides*, a *cbbX* deletion mutation cannot grow photoautotrophically (33). The RubisCOs encoded by the genes located upstream of *cbbX* have been located in the Red-like group (30). These finding indicate that the RubisCO genes (*cbbLS* and *cbbM*) and neighboring genes were acquired by horizontal gene transfers. However, the RubisCOs (*cbbLS-1* and *cbbLS-2* from *Hv. marinus*, *cbbLS-2* from *C. vinosum*, and RubisCO genes from plant, green algae, and cyanobacteria) encoded by the genes not located upstream of *cbbQ* were located in the Green-like group.

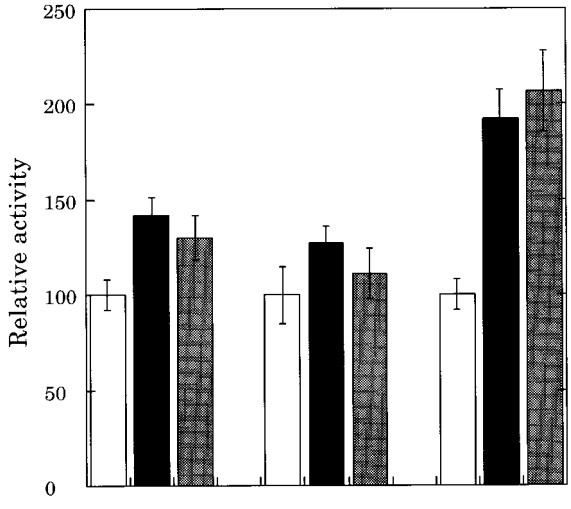

FIG. 5. Relative RubisCO activity in cell extract harboring form I RubisCO genes and *cbbQ*. Cell extract of *E. coli* harboring column 1, pYAH501 and pACYC184Cm$^-$; column 2, pYAH501 and pYAH315; column 3, pYAH501 and pYAH508; column 4, pYAH506 and pACYC184Cm$^-$; column 5, pYAH506 and pYAH315; column 6, pYAH501 and pYAH508; column 7, pYAH303 and pACYC184Cm$^-$; column 8, pYAH303 and pYAH315; column 9, pYAH303 and pYAH508. The data in column 7 and 8 were taken from our previous results (24). The values are presented the average of three repetitions for each experiment. The relative activity refers to the percentage of each RubisCO activity of cell extract harboring pACYC184Cm$^-$. Standard error bars are shown. The enzyme assays for RubisCO were performed at 30°C (columns 1–6) or 50°C (columns 7–9).

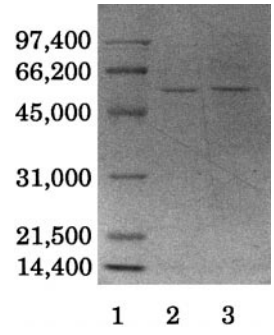

FIG. 6. Purification of QM from *E. coli*. Lane 1, marker proteins (phosphorylase, 97,400; bovine serum albumin, 66,200; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; and hen egg white lysozyme,14,400). Lane 2, the purified UM. Lane 3, the purified QM. QM and UM indicate recombinant form II RubisCOs purified from *E. coli* harboring cbbM-cbbQ and cbbM, respectively. All purified proteins were separated by SDS-PAGE (12.5%) gel).

Effect of cbbQ Expression on Form II RubisCO Activity in the E. coli Cell

We abbreviate the *cbbQ* of *Hp. thermoluteolus* as *cbbQ-t* and the *cbbQ* of *Hv. marinus* as *cbbQ-m*. pUC vector was used for the expressions of *cbbLS* and *cbbM*, and pACYC vector was used for the expression of *cbbQ*. pACYC184Cm$^-$ (24) was used as a negative control of pYAH315 and pYAH508. The replication origin of pACYC184 is compatible with that of the pUC vectors (26); therefore the plasmids derived from pUC vector are able to co-exist with pACYC derivatives in *E. coli* cells. The cell extract of *E. coli* JM109 harboring both pYAH507 and pACYC184Cm$^-$ showed RubisCO activity of 0.091 unit/mg protein (Table 1). This activity was about 1.6-fold and 1.5-fold greater when pYAH315 or pYAH508 was used than when pACYC184Cm$^-$ was

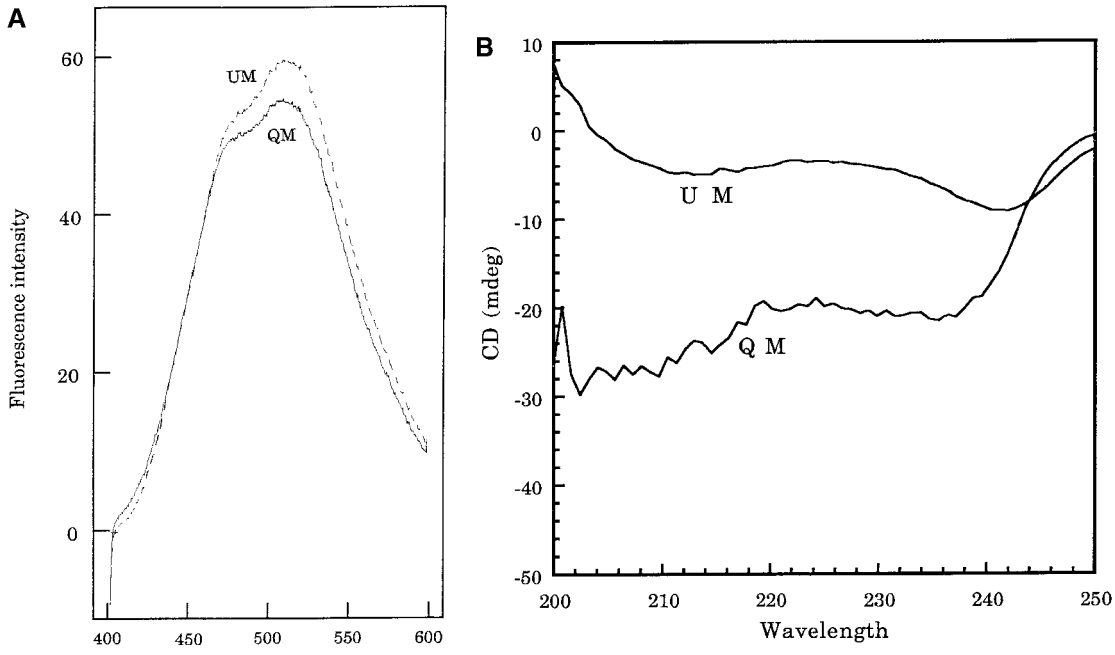

FIG. 7. Conformational differences between UM and QM. QM and UM indicate recombinant form II RubisCOs purified from *E. coli* harboring *cbbM-cbbQ* and *cbbM*, respectively. (A) Fluorescence emission spectra of ANS in 0.02 mg of CbbM proteins. (B) CD spectra of CbbM proteins. Proteins were diluted in the buffer A, pH 7.5, to 0.025 mg/ml and scanned from 250 to 200 nm.

used, respectively. The activity was not detected when the cell was transformed only with pYAH315 (24) or pYAH508 (Table 1). These results clearly indicated that both CbbQ-t and CbbQ-m activated the form II RubisCO from *Hv. marinus*. SDS-PAGE of the cell extracts and immunological detection of RubisCO expressed in *E. coli* showed that the expression level of the enzyme protein was not affected by the existence of the *cbbQ-t* or *cbbQ-m* gene (Fig. 4). Both CbbQs are thought to mediate the posttranslational activation of form II RubisCO at an almost equal level. The activity was about two-fold greater when *cbbLS* genes from *Hp. thermoluteolus* were coexpressed with *cbbQ-t* or *nirQ* than when only *cbbLS* genes were expressed (24). The activation level of form II RubisCO by both CbbQ was smaller than that of form I RubisCO (CbbLS-t) by CbbQ-t. CbbQ-t has 68 and 53% identity to CbbQ-m and NirQ from *P. aeruginosa*. The proteins possessing up to 50% identity seem to be able to substitute for CbbQ-t functionally. Because CbbQ could activate form II RubisCO that does not possess a small subunit, CbbQ seemed to react to large subunits of RubisCO.

Effect of cbbQ Expression on Form I RubisCO Activity in the E. coli Cell

We also investigated whether *cbbQ-t* and *cbbQ-m* could activate form I RubisCOs in *E. coli* cells (Fig. 5). Three kinds of form I RubisCO genes, *cbbLS1* and *cbbLS-2* from *Hv. marinus* and *cbbLS* from *Hp. thermoluteolus*, were used in this study. We abbreviate *cbbLS* from *Hp. thermoluteolus* as *cbbLS-t*. We determined that no *cbbQ* gene was located in the downstream regions of *cbbLS-1* and *cbbLS-2* (data not shown). The activity in cell extracts of *E. coli* harboring *cbbLS-t* with *cbbQ-t* or *cbbQ-m* was two-fold greater than that in extracts harboring only *cbbLS-t*. Both CbbQs seemed to activate CbbLS-t posttranslationally. However, the activation level of CbbLS-1 or CbbLS-2 by *cbbQ* was smaller than that of CbbLS-t. CbbL-t has 79.0, 76.8 and 29.5% identity to CbbL-1, CbbL-2, and CbbM, respectively. CbbQ seemed to activate the RubisCOs that are encoded by the gene(s) located upstream of *cbbQ* genes. The activation by CbbQ had a specificity to RubisCO.

Purification of Form II RubisCO Activated by CbbQ-m

The recombinant form II RubisCO was purified so that detailed properties could be examined. Here we refer to the RubisCOs produced in the *E. coli* cells harboring *cbbM-cbbQ* and *cbbM* as QM and UM (unaffected CbbM), respectively. The purification procedure of UM was described previously (13). QM and UM were indistinguishable from each other in the denaturing gel (Fig. 6). QM was inactivated during Q-Sepharose chromatography. The activity of purified QM was quite low (data not shown). The inactivation of UM during Q-Sepharose chromatography had been also observed previously (13). The *cbbO* gene is located

downstream of *cbbLSQ* from *Hp. thermoluteolus*, and affects the activation and stabilization of RubisCO (18). Another gene may be required for activating CbbM.

8-Anilino-1-naphthalenesulfonate (ANS) has been widely used as a fluorescent probe for the identification of conformational changes in proteins including RubisCO proteins (37). Addition of RubisCO to a solution of ANS caused a large enhancement of the fluorescence intensity of the dye and a blue shift of the emission maximum from 510 to 472 nm (Fig. 7A). UM enhanced ANS fluorescence, as compared to QM, suggesting that the conformation of UM is different from that of QM. The structural integrity of the two RubisCO enzymes was further examined using circular dichroism (CD) (Fig. 7B). CD provides information regarding the relative amounts of helix, sheet, and random-coil structures present in a protein (38), and it has been used to measure the conformational change of RubisCO (39). There were differences was observed in the CD spectra of the UM and QM, indicating that the UM and QM differed in protein conformation. CbbQ seemed to mediate the conformational change in CbbM.

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