Isolation and Characterization of cbbL and cbbS Genes Encoding Form I Ribulose-1,5-bisphosphate Carboxylase/Oxygenase Large and Small Subunits in *Nitrosomonas* sp. Strain ENI-11

Ryuichi HIROTA, Junichi KATO, Hiromu MORITA, Akio KURODA, Tsukasa IKEDA, Noboru TAKIGUCHI, and Hisao OHTAKE

Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, Higashi-Hiroshima, Hiroshima 739-8527, Japan

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The *cbbL* and *cbbS* genes encoding form I ribulose-1,5-bisphosphate carboxylase/oxygenase (*RubisCO*) large and small subunits in the ammonia-oxidizing bacterium *Nitrosomonas* sp. strain ENI-11 were cloned and sequenced. The deduced gene products, CbbL and CbbS, had 93 and 87% identity with *Thiobacillus intermedium* CbbL and *Nitrobacter winogradskyi* CbbS, respectively. Expression of *cbbL* and *cbbS* in *Escherichia coli* led to the detection of *RubisCO* activity in the presence of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). To our knowledge, this is the first paper to report the genes involved in the carbon fixation reaction in chemolithotrophic ammonia-oxidizing bacteria.

**Key words:** *Nitrosomonas*; nitrification; CO₂ fixation; ribulose-1,5-bisphosphate carboxylase/oxygenase

The ammonia-oxidizing autotrophic bacteria derive their carbon for growth from CO₂ and their energy for metabolism by the oxidation of ammonia to nitrite in the process of nitrification. They use the Calvin-Benson-Bassham (CBB) reductive pentose phosphate pathway to assimilate CO₂. Three enzymatic activities are unique to the Calvin cycle: ribulose-1,5-bisphosphate carboxylase/oxygenase (*RubisCO*), phosphoribulokinase, and sedoheptulose bisphosphatase. The remainder of the cycle is driven by a series of enzymes that are generally present in both autotrophs and heterotrophs. The CBB enzyme responsible for the actual fixation of CO₂ is RubisCO. There are two distinct forms of RubisCO, form I and form II, in prokaryotic autotrophs. The form I RubisCO is a large hexadecameric enzyme (L₄S₈) composed of eight large subunits (50-55 kDa) and eight small subunits (12-18 kDa), while form II has only large subunits (L₄). Little is known about the genes involved in the CBB pathway in the chemolithotrophic ammonia-oxidizing bacteria. In this study, we cloned and sequenced the *cbbL* and *cbbS* genes encoding form I *RubisCO* large and small subunits of the ammonia-oxidizing bacterium *Nitrosomonas* sp. strain ENI-11.

*Nitrosomonas* sp. strain ENI-11 was grown aerobically at 28°C in modified Alexander medium. To clone the *cbbL* gene, a degenerate oligonucleotide primers were designed on the basis of the bacterial *cbbL* gene sequences available from GenBank. The alignment of *cbbL* nucleotide sequences was done by using the CLUSTAL W program. The forward primer CBP1 (5'-ACNTGGACCNACNGTGGACNGAY-3') and the reverse primer CBP2 (5'-RTTYTCRTCRTCYTTYTTNGTAR-3') targeted the stretches corresponding to positions 58-65 and 190-198 of the amino acid sequence of *Rhodobacter capsulatus* CbbL, respectively. PCR was done with the oligonucleotide primers and an ExTag DNA polymerase (Takara Shuzo Co. Ltd., Shiga, Japan) on a DNA thermal cycler (Perkin-Elmer). The PCR cycle included denaturation for 30 s at 94°C, primer annealing for 30 s at 55°C, and extension for 1 min at 72°C (30 cycles). Standard procedures were used for DNA manipulation, Southern hybridization, and Northern hybridization.

PCR generated a 0.4-kb DNA fragment when genomic DNA of *Nitrosomonas* sp. strain ENI-11 was used as a template (data not shown). The nucleotide sequence of the 0.4-kb PCR product showed 83% similarity to the *Thiobacillus denitrificans* *cbbL* gene. Consequently, this PCR product was used as a DNA probe for Southern hybridization. Total
**cbbl and cbbs Genes in Nitrosomonas SP. ENI-11**

Fig. 1. Restriction Map of pCB01 Containing the 3.4-kb BamHI Fragment from the *Nitrosomonas* sp. Strain ENI-11 Chromosomal DNA and the Subclone of pCLS1.

Thick arrows indicate the ORFs and the direction of transcription. The approximate location and orientation of primers CBP1 and CBP2 are indicated by thin arrows. The hybridization probe is shown by a horizontal bar. Abbreviations for restriction sites: B, BamHI; C, ClaI; E, EcoRI; HII, HindII; N, NotI; S, SalI; P, Pst1. The nucleotide sequence of the *Nitrosomonas* sp. strain ENI-11 *cbbl* region reported here has been deposited in GSDB, DDBJ, EMBL, and NCBL nucleotide sequence databases under accession number AB061373.

Genomic DNA from *Nitrosomonas* sp. strain ENI-11 was digested with various restriction enzymes and electrophoresed on a 1.0% agarose gel. The probe strongly hybridized to a 3.4-kb BamHI fragment. A size-fractionated library of BamHI fragments of *Nitrosomonas* sp. strain ENI-11 genomic DNA (3.0 to 4.0 kb in size) was created with a pMW119 vector (Nippon Gene Co., Toyama, Japan) and Epicurian Coli SURE (Stratagene). Plasmid DNAs, isolated from recombinant colonies, were screened with the 0.4-kb PCR product as a probe. One positive plasmid, designated as pCB01, was obtained.

Restriction endonuclease analysis showed that pCB01 contained a 3.4-kb BamHI fragment of the *Nitrosomonas* sp. strain ENI-11 chromosomal DNA. Nucleotide sequence analysis showed that the 3.4-kb BamHI fragment contained part of two open reading frames (ORFs) (cbbr and cbss) and two whole ORFs (cbbl and cbbs) (Fig. 1). The predicted product of *cbbl* had 93% amino acid identity with *Thiobacillus intermedium* K12 CbbL (GenBank accession number AF046933), while that of *cbbs* showed 87% identity with *Nitrobacter winogradskyi* CbbS (GenBank accession number AF109914), respectively. A 401-bp truncated *cbbr* was located 200 bp upstream of *cbbl*. The truncated *cbbr* showed 62% nucleotide sequence identity with the 5'-portion of *Chromatium vinosum* rbcR, which encodes a LysR-type transcriptional regulator. The *cbbr* gene was divergently transcribed from *cbbl*. The 5'-portion of *cbss* was also located 77 bp downstream of *cbbs*. The truncated *cbss* showed 70% nucleotide sequence identity with the 5'-portion of *T. intermedium* K12 *cbso* (GenBank accession number AF046933) which encodes a carboxysome polypeptide.

To investigate expression of *cbbl* and *cbbs* in *Escherichia coli*, a 2.1-kb ClaI-NotI fragment, which contained the entire *cbbl* and *cbbs*, was excised from pCB01 and ligated to ClaI- and NotI-digested pBluescript II KS(+) (Stratagene). The resulting plasmid, designated pCLS1, was introduced into *E. coli* MV1184 by transformation. The *E. coli* transformants were grown at 37°C with shaking for 10 h in 2 × YT with or without 0.1 mM isopropyl-β-d-thiogalactopyranoside (IPTG). Cells were harvested by centrifugation, washed twice with BEMD buffer (50 mM N,N-bis(2-hydroxyethyl)glycine (Bicine), 0.1 mM EDTA, 10 mM MgCl2·6H2O, and 1 mM dithiothreitol [pH 7.8]), and disrupted by sonication at 0°C. The crude extract was used for enzyme assays. Rubisco activity was assessed by the method described previously. One unit of enzyme activity was defined as the amount of enzyme which catalyzed the fixation of 1 pmol of CO2 per min at 37°C. Rubisco activity was 1112 units per mg protein in *E. coli* MV1184 (pCLS1) grown in the presence of 0.1 mM IPTG and undetectable in the same strain grown in the absence of IPTG. The control strain *E. coli* MV1184 (pBluescript II KS(+)) showed no significant Rubisco, regardless of the presence or absence of IPTG.

The 2.1-kb *ClaI-NotI* fragment of pCLS1 contained the 113-bp upstream region of *cbbl* (Fig. 1). To find whether the indigenous *cbbl* promoter exists in this region, we did a primer extension analysis of RNA from *Nitrosomonas* sp. strain ENI-11. The synthetic fluorescence-labeled oligonucleotide CBBLI(5'-Cy5-GGCGCTGATAGGTTTTGCTTGC-3') was used as a primer and the product of primer extension experiments was analyzed by the method described previously. Thus, two transcriptional initiation sites of *cbbl* were found 117 and 126 bp upstream of *cbbl*, respectively (Fig. 2). *E. coli* σ70-like promoter sequences were found 125 to 158 bp upstream from the translation initiation codon of *cbbl* (Fig. 2B). It has been shown that CbbR activates transcription of both *cbbl* and *cbbs* in *Rhodobacter sphaeroides*. A potential CbbR binding motif, TNA-N7,s-TNA, was also found immediately upstream of the transcription initiation sites of *cbbl* of *Nitrosomonas* sp. strain ENI-11. Rubisco activity was detected with *E. coli* MV1184.
Fig. 2. Primer Extension Analysis (A) and Nucleotide Sequence of the \textit{cbb\textit{LS}} Promoter Region (B).

(A) The upper panel shows the electropherogram of the upstream region of \textit{cbb\textit{L}}. The DNA sequencing reaction was done by using the fluorescence-labeled oligonucleotide \textit{CbbLL1} and \textit{pCB1}. The lower panel shows the electropherogram of primer extension products obtained with RNA isolated from \textit{Nitrosomonas} sp. strain ENI-11 as the template and the oligonucleotide \textit{CbbLL1} as the primer. The retention times of the major extension products are shown. (B) Transcriptional initiation sites of \textit{cbb\textit{L}} are shown in boldface. The \textit{cbb\textit{LS}} promoter regions (−35 and −10) are underlined. The putative \textit{CbbR} binding sites (\textit{TNA}-N₂-TNA) are boxed. The location of the \textit{CbbL} restriction site used for construction of \textit{pCLS1} is double underlined.

(p\textit{CLS1}) only when it was grown in the presence of 0.1 mM IPTG. This result is likely due to the lack of the \textit{cbb\textit{L}} promoters in the 2.1-kb \textit{Cbl-NotI} fragment of \textit{pCLS1}. Northern hybridization was done with RNA from \textit{Nitrosomonas} sp. strain ENI-11 by using the 0.4-kb PCR product as a probe. Only a 2.1-kb transcript was observed (Fig. 3). The results of the Northern hybridization and primer extension experiment led us to conclude that \textit{cbb\textit{L}} and \textit{cbb\textit{S}} form an operon.

The amino acid sequence of \textit{Nitrosomonas} sp. strain ENI-11 \textit{CbbL} was compared with those reported previously with other bacteria. The putative ami-

no acid sequences of \textit{Nitrosomonas europaea} \textit{CbbL} and \textit{CbbS} were also obtained from JGI Microbial Sequencing Homepage (http://spider.jgi-psf.org/JGI_microbial/html/nitrosomonas_homepage.html). The ENI-11 \textit{CbbL} protein was closely related to those from \textit{T. intermedium} K12 (93% identity) in the β subclass of the Proteobacteria and \textit{Nitrobacter winogradskyi} (91% identity) in the α subclass of the Proteobacteria. However, the ENI-11 \textit{CbbL} was less related to the putative \textit{N. europaea} \textit{CbbL} protein (85% identity). This was unexpected because their close relationship had been shown based on 16S rRNA and the genes involved in nitritification.\(^{10,22}\) The putative \textit{N. europaea} \textit{CbbL} protein was closely related to \textit{Hydrogenophaga pseudoflava} \textit{CbbL}\(^{20}\) and \textit{Chromatium vinosum} \textit{RbcA}.\(^{24}\) The difference in the amino acid sequence of \textit{CbbS} was more striking between the two \textit{Nitrosomonas} species (53% identity). In addition, the gene organization of the ENI-11 \textit{cbb\textit{LS}} region was more similar to that of \textit{Thiobacillus ferrooxidans} (GenBank accession number AF129925) in the γ subclass of the Proteobacteria than that of \textit{N. europaea} (data not shown). All known chemolithotrophic ammonia-oxidizing bacteria are obligate chemolithotrophs, which are completely dependent on CO\textsubscript{2} fixation.\(^{25}\) Since they obtain all of their energy for growth from the oxidation of ammonia to nitrite, it is not surprising that the genes involved in the ammonia oxidation are highly conserved between \textit{Nitrosomonas} sp. strain ENI-11 and \textit{N. europaea}. RubisCO, the key enzyme of the CBB cycle, is also essential for their growth, however, the amino acid sequences of \textit{CbbL} and \textit{CbbS} are less conserved. Taken together, it seems possible that ENI-11 and \textit{N. europaea} \textit{cbb} genes were
acquired from different ancestral organisms via horizontal gene transfer.

References


