**INTRODUCTION**

*Nitrosomonas europaea* is an aerobic ammonia-oxidizing bacterium that participates in the C and N cycles and hence is involved in events that affect the environment (Bock *et al*., 1986). As an obligate chemolithotroph *N. europaea* derives all the reductant required for energy and biosynthesis from the oxidation of ammonia (NH$_3$) to nitrite (NO$_2^-$). This bacterium utilizes CO$_2$ as its predominant carbon source, and is an obligate chemolithotroph, deriving all the reductant required for energy and biosynthesis from the oxidation of ammonia (NH$_3$) to nitrite (NO$_2^-$). This bacterium fixes carbon via the Calvin–Benson–Bassham (CBB) cycle via a type I ribulose bisphosphate carboxylase/oxygenase (RubisCO). The RubisCO operon is composed of five genes, *cbbLSQON*. This gene organization is similar to that of the operon for ‘green-like’ type I RubisCOs in other organisms. The *cbbR* gene encoding the putative regulatory protein for RubisCO transcription was identified upstream of *cbbL*. This study showed that transcription of *cbb* genes was upregulated when the carbon source was limited, while *amo*, *hao* and other energy-harvesting-related genes were downregulated. *N. europaea* responds to carbon limitation by prioritizing resources towards key components for carbon assimilation. Unlike the situation for *amo* genes, NH$_3$ was not required for the transcription of the *cbb* genes. All five *cbb* genes were only transcribed when an external energy source was provided. In actively growing cells, mRNAs from the five genes in the RubisCO operon were present at different levels, probably due to premature termination of transcription, rapid mRNA processing and mRNA degradation.

Autotrophic nitrifiers assimilate CO$_2$ via the Calvin–Benson–Bassham (CBB) cycle. Genetic information about the enzyme that catalyses CO$_2$ fixation in *N. europaea* was revealed by the sequence of its genome (Chain *et al*., 2003). The DNA sequence suggests that the enzyme is a type I ribulose bisphosphate carboxylase/oxygenase (RibisCO). Most of the genes encoding the enzymes for a complete CBB cycle are present in the genome. The two missing genes are those encoding sedoheptulose 1,7-bisphosphatase (EC 3.1.3.37) and NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.13). However, fructose 1,6-bisphosphatase (EC 3.1.3.11) in *N. europaea* may function primarily for sedoheptulose 1,7-bisphosphate hydrolysis in the CBB cycle, rather than for fructose 1,6-bisphosphate hydrolysis in gluconeogenesis (Yoo & Bowien, 1995). NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase is apparently replaced by an NADH-dependent enzyme (EC 1.2.1.12) (Chain *et al*., 2003).

There are two recent reports on the *cbb* genes of ammonia-oxidizing bacteria. In one, Hirota *et al.* (2002) cloned and sequenced the *cbbLS* genes of *Nitrosomonas* ENI-11 and expressed functional RubisCO activity in *Escherichia coli*. In the other, Utáker *et al.* (2002) cloned and sequenced the *cbbLS* genes of *Nitrosospira* sp. isolate 40KI and showed its functionality in a *cbb*-deletion strain of *Ralstonia eutropha*. However, to date there are no reports describing the transcription patterns and regulation of the *cbb* genes in ammonia-oxidizing bacteria. Instead, most research has focused on the genetic makeup and the regulation for the

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**Abbreviations:** AMO, ammonia monooxygenase; HAO, hydroxylamine oxidoreductase; PRK, phosphoribulokinase; RubisCO, ribulose bisphosphate carboxylase/oxygenase.

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Received 24 September 2003
Revised 30 January 2004
Accepted 5 March 2004
utilization of ammonia in \textit{N. europaea} and other ammonia-oxidizing bacteria (Arp et al., 2002; Hommes et al., 1998, 2001; Klotz & Norton, 1995, 1998; Norton et al., 1996). The two key enzymes in this process, ammonia monoxygenase (AMO) and hydroxylamine oxidoreductase (HAO), catalyse the sequential oxidation of \( \text{NH}_3 \) to \( \text{NO}_2^- \). The genetic loci for AMO and HAO are in multiple copies in nitrifiers. In \textit{N. europaea} there are two gene copies for AMO and three gene copies for HAO (compared to a single gene copy for RubisCO). Mutation of the different gene copies of AMO and HAO indicated that no copy was indispensable, although some phenotypes were different from the wild-type (Hommes et al., 1996, 1998; Stein et al., 2000). Transcript analysis and mutagenesis studies suggested that the transcription of the \textit{amoCAB} operon may be regulated by more than one promoter (Hommes et al., 2002; Stein et al., 2000). In this work, the transcription and regulation of \textit{cbb} genes in response to major nutrients and environment factors were characterized.

**METHODS**

**Media, bacterial cultures, determination of enzyme activities and materials.** \textit{N. europaea} (ATCC 19178) was grown in batch cultures as previously described (Ensign et al., 1993; Stein & Arp, 1998). Cells were harvested from 3-day-old or mid- to late-exponential phase cultures by centrifugation. The cells were washed in \( \text{NH}_4^-\)-free, \( \text{CO}_2^-\)-free buffer to remove residual growth medium. The \( \text{NH}_3 \) deprivation (starvation) treatments were prepared by incubating cells in \( \text{NH}_4^-\)-free, \( \text{CO}_2^-\)-free medium for 1 h before an energy source was added. The total RNA was isolated from cells for the hybridization experiments by the guanidinium isothiocyanate-phenol-chloroform extraction method (Sambrook et al., 1989). Prior to electrophoresis, RNA was stained with \( \sim 5 \mu \text{g} \) ethidium bromide ml\(^{-1}\) in the loading buffer. The RNA was blotted onto Nytran membranes (Schleicher & Schuell BioScience). Probes for hybridization were generated by PCR with primers specific for each gene and labelled by random priming (Prime-a-Gene Labelling System, Promega) with \( \left[ \gamma^32\right] \text{PdCTP} \) (3000 Ci mmol\(^{-1}\), 110 TBq mmol\(^{-1}\); ICN). Hybridization was carried out as described by Sambrook et al. (1989) and Sayavedra-Soto et al. (1998). Images and relative signal densities were obtained by phosphorimaging and ImageQuant softwares as described by the manufacturer (Molecular Dynamics).

**DNA preparation, restriction digests and agarose gel electrophoresis.** DNA was prepared from bacteria as described by Sambrook et al. (1989). The recovery of DNA fragments was carried out with a commercial kit (Qiagen). PCR was performed with Taq DNA polymerase (Promega). RT-PCR was done with M-MLV reverse transcriptase (Promega) according to the manufacturer’s instructions, with a 30°C extension temperature. DNA templates for RT-PCR were treated with RQ1 DNase (Promega) or ‘DNA-free’ DNase (Ambion) multiple times until no DNA product was detected by Taq DNA polymerase in a PCR with any of the \textit{cbb} primers used. The primers used in the PCR and RT-PCR experiments are listed in Table 1.

The start of transcription was determined using a commercial kit (GeneRacer; Invitrogen). Briefly, the RNA oligonucleotide provided in the kit was ligated with RNA ligase to the 5’-ends of the mRNA pool as directed by the manufacturer. The corresponding cDNA was then made with a \textit{cbbL}-specific reverse primer, followed by PCR amplification of the chimeric DNA fragment with the \textit{cbbL} reverse primer and the kit’s forward primer. Twelve chimeric fragments were cloned and sequenced. The start of transcription was at the nucleotide where ligation of the 5’-end of the mRNA and the oligonucleotide occurred.

**RESULTS**

**\textit{N. europaea} \textit{cbb} operon characterization**

The putative RubisCO operon in this bacterium is composed of five genes, namely \textit{cbbLSQQ} and a fifth gene here designated \textit{cbbN}. This composition was deduced from the nucleotide sequence of the genome of \textit{N. europaea} and gene similarity comparisons. In this operon, \textit{cbbL} and \textit{cbbS} code for the RubisCO large and small subunits respectively. The genes \textit{cbbQ} and \textit{cbbO} encode proteins that are expected to be involved in the processing, folding, assembling, activation and regulation of the RubisCO complex enzyme as in other organisms (Baxter et al., 2002; Hayashi et al., 1997, 1999). \textit{cbbN} has the same orientation as \textit{cbbLSQQ}. The intergenic region between \textit{cbbO} and \textit{cbbN} is only 20 bp, in which no apparent promoter could be inferred from the sequence, implying that it is transcribed from the same \textit{cbb} promoter. \textit{cbbN} encodes a hypothetical protein of 101
Table 1. Primers used for the amplification of cbb and other genes and for RT-PCR

<table>
<thead>
<tr>
<th>Primer for</th>
<th>Sequence (5’–3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Northern hybridization probes (forward/reverse)*</td>
<td></td>
</tr>
<tr>
<td>cbbL</td>
<td>CGGATATTCTGGCTTGCTTC/ACCACGTTACCTGAGTGGAG</td>
</tr>
<tr>
<td>cbbS</td>
<td>AAAAGTCTTGGACGACAT/CAGGTTACACCTGAGTGGAG</td>
</tr>
<tr>
<td>cbbO</td>
<td>TGGCCAGTCTGTGCTTCGAG/ACGTTACACCTGAGTGGAG</td>
</tr>
<tr>
<td>cbbN</td>
<td>TAATCCGGAGTCTGCAGCAG/TCAGTTACACCTGAGTGGAG</td>
</tr>
<tr>
<td>cbbR</td>
<td>TGCTGCTATAACCTTTACTTG/CAGGTTACACCTGAGTGGAG</td>
</tr>
<tr>
<td>Carbonic anhydrase (NE1926)</td>
<td>CAGGGGATACAGCTGTTGAA/GCAATCACCTGACTCCGTTT</td>
</tr>
<tr>
<td>Succinate dehydrogenase</td>
<td>GTTCGGAGCCATGAATATC/CAGGATACACCTGAGTGGAG</td>
</tr>
<tr>
<td>Glutamate dehydrogenase</td>
<td>CATCTGATGCTTGCTTGAG/ACGTTACACCTGAGTGGAG</td>
</tr>
<tr>
<td>Phosphoribulokinase (NE1801)</td>
<td>GCCAGGTTACACCTGAGTGGAG/ACGTTACACCTGAGTGGAG</td>
</tr>
<tr>
<td>Sulfate transporter (NE1927)</td>
<td>TTTCGGGTACACCTTCTGCTT</td>
</tr>
<tr>
<td>For RT-PCR (FW, forward; RVS, reverse)</td>
<td></td>
</tr>
<tr>
<td>cbbL-FW2</td>
<td>CACTGGGAAGCTTGGTTGGA</td>
</tr>
<tr>
<td>cbbS-FW2</td>
<td>AGTTGGGAATCCCGGCTTGT</td>
</tr>
<tr>
<td>cbbQ-FW2</td>
<td>GATGTCATGCACTGCTGAG</td>
</tr>
<tr>
<td>cbbQR-VS2</td>
<td>CATCTGATGCTTGCTTGAG</td>
</tr>
<tr>
<td>cbbQ-RVS3</td>
<td>TTGTCGATACACCTTCTGCTT</td>
</tr>
<tr>
<td>cbbO-RV2</td>
<td>TGACAACTCGTACGGAGG</td>
</tr>
<tr>
<td>cbbO-RV3</td>
<td>GGGGGCTGTACAGGAGG</td>
</tr>
<tr>
<td>For transcript analysis</td>
<td></td>
</tr>
<tr>
<td>cbbL (reverse)</td>
<td>GTGGGCGGATACACCTTCTGCTT</td>
</tr>
<tr>
<td>cbbL (reverse, nested)</td>
<td>TGCTGACATTTACGTAATCTCCTTG</td>
</tr>
</tbody>
</table>

*For amoA and hao primers, see Hommes et al. (2001).

amino acids and has no similarity to known annotated genes. A putative regulatory gene, cbbR, was found 194 bases upstream of the start codon of cbbL and is transcribed in the opposite direction. The deduced amino acid sequence of N. europaea cbbR is most similar to that of Thiobacillus denitrificans (NCBI: AF307090) and Allochromatium vinosum (formerly Chromatium vinosum) (Viale et al., 1991). In other species, cbbR is identified as a LysR-type regulatory gene (Shively et al., 1998). The cbb genes, including cbbR, are contained in a 6581 nucleotide DNA fragment (Fig. 1A). A stem–loop structure was identified in the intergenic region between cbbS and cbbQ with a calculated free energy (ΔG°) of −155 kJ mol⁻¹. The stem–loop is formed by 101 of the 119 nucleotides that form the intergenic region and may have a regulatory or processing role.

The organization of the N. europaea cbb operon is similar to that in All. vinosum (Viale et al., 1989), Acidithiobacillus ferrooxidans (formerly Thiobacillus ferrooxidans) (Kusano & Sugawara, 1993; Kusano et al., 1991), Methylbacterium capsulatus (Tichi & Tabita, 2002) and Rhodobacter capsulatus (Baxter et al., 2002; Paoli et al., 1998). In these organisms, cbbLSQ is a common block, a feature of ‘green-like’ type I RubisCO genes. The term ‘green-like’ type I RubisCO implies similarity to the RubisCOs in plants and green algae (Utaker et al., 2002). The N. europaea cbbLSQ block is similar to the Rb. capsulatus cbbI operon (Vichivavives et al., 2000) and Hydrogenophilus thermoluteolus (Terazono et al., 2001). A sequence database search revealed that N. europaea CbbL is most similar to that of Ac. ferrooxidans (91% amino acid sequence) (Heihorst et al., 2002), T. denitrificans (90%) (Hernandez et al., 1999), All. vinosum (87%) (Viale et al., 1989) and Hydrogenophaga pseudoalvus (86%) (Lee & Kim, 1998). In contrast, the N. europaea CbbR protein shares only 85% amino acid identity with that of Nitrosomonas sp. strain ENI-11 (Hirota et al., 2002), and has much lower identity to that in other nitrifying bacteria (e.g. ≤83% for Nitrobacter vulgaris, GenBank accession no. L2285) and some Nitrosospira species (Utaker et al., 2002). N. europaea CbbS is most similar to that of T. denitrificans (83%) and Ac. ferrooxidans (79%) (Pulgar et al., 1991). As with CbbL, N. europaea CbbS showed only 53% amino acid sequence similarity to the CbbS of both Nitrosomonas sp. strain ENI-11 (Hirota et al., 2002) and Nitrobacter vulgaris. CbbQ is similar to nirQ, a denitrification gene in Pseudomonas species (Yokoyama et al., 1995). The N. europaea CbbO shares 50% amino acid identity to the putative Ac. ferrooxidans CbbO-like protein (AJ133725.1), and 40% to the H. thermoluteolus CbbO (Hayashi et al., 1997). CbbO has moderate similarity to the probable denitrification protein NorD (AE004489.1). In H. thermoluteolus, cbbY is downstream of cbbLSQ.
However, *H. thermoluteolus cbbY* and *N. europaea cbbN* are different (771 bp vs 303 bp respectively) (Hayashi & Igarashi, 2002; Hayashi et al., 2000; Terazono et al., 2001). It is also worth noting that *cbbY* in other species is not immediately downstream of *cbbQ* and is in a different transcriptional unit (Gibson & Tabita, 1997).

The predicted molecular masses for *N. europaea* Cbb proteins are as follows (kDa): CbbR, 34.9; CbbL, 52.9; CbbS, 13.8; CbbQ, 29.8; CbbO, 88.4; CbbN, 11.4. The molecular masses of *N. europaea* CbbL and CbbS are typical of those RubisCOs in most autotrophic bacteria.

**Analysis of the cbb promoter and intergenic regions**

The intergenic region between *cbbR* and *cbbL* is 194 bp in *N. europaea*, compared to 213 bp in *Nitrosomonas* sp. strain ENI-11 (Hirota et al., 2002), 182 bp in *Ral. eutropha* (Kusian et al., 1995), 226 bp in *All. vinosum* (Viale et al., 1989) and 144 bp in *Ac. ferrooxidans* (Pulgar et al., 1991). The nucleotide sequence upstream of *cbbL* in *N. europaea* does not show significant similarity to those of the above-mentioned species. No putative promoter between other *cbb* genes could be inferred by visual inspection and promoter predicting programs (e.g. http://www.fruitfly.org/seq_tools/promoter.html). Furthermore, the intergenic spaces between *cbbQ* and *cbbO* (41 bp), and between *cbbO* and *cbbN* (20 bp), are smaller than the considered 50 bp minimum in most promoter-predicting programs (however, the possibility of a promoter overlapping the upstream gene cannot be discarded).

Since none of the alignments with other autotrophic bacteria provided convincing evidence for promoter and transcriptional start sites of the *N. europaea cbb* operon, we proceeded to determine experimentally the 5′ end of the *cbb* transcript using a commercial kit (see Methods). Of the 12 chimeric clones sequenced, five did not contain any *cbbL* sequence and seven revealed two potential transcriptional start sites: two clones showed a thymidine, 79 bases upstream of the ATG start codon of *cbbL*, and five clones showed a guanine, 83 bases upstream of the ATG start codon of *cbbL* (Fig. 2). The putative promoter region at −10 (TATAGT) and −35 (TTTAAC) bases shows similarity to the *E. coli σ70* consensus sequence (Fig. 2). The −35 region shows similarity to that in other autotrophic bacteria such as *Xanthobacter flavus* and *Ral. eutropha* (TTTANN) (reviewed by Shively et al., 1998). Two possible start sites for the transcription of the *cbb* genes were also identified in *Nitrospumonas* sp. ENI-11 (Hirota et al., 2002). A feature of the regulatory regions of the RubisCO genes in other bacteria is the AT-rich boxes found upstream of the *cbb* operon (Schell, 1993). In *N. europaea*, an AT-rich element (50 ATs out of 56 bp) can be readily identified in the intergenic region of *cbbR* and *L* (Fig. 2). It is known that CbbR belongs to the LysR-type regulators. LysR regulators bind to DNA sequences with T/A-(N)11/12-A/T inverted repeats (Goethals et al., 1992; Schell, 1993; Xu & Tabita, 1994). In *N. europaea* several such symmetrical repeats exist in the intergenic region of *cbbR* and *cbbL* (Fig. 2).

**Gene transcription of cbb operon**

We wanted to determine if all five genes for the RubisCO operon were transcribed in *N. europaea* under normal growing conditions. We detected their cDNAs using RT-PCR (Fig. 1B) and their mRNAs in Northern hybridizations.
RNA ladder. The experiment was repeated in triplicate with starved cells (ammonia-deprived). The far left lane is an cbbB mRNA long enough to include all five components of RubisCO was not detected consistently by Northern hybridization. Again, this result suggests that the cbb mRNA is actively processed or it degrades rapidly, if indeed it is transcribed from a single promoter.

The transcription of the genes for RubisCO was compared to the transcription of key genes for (a) energy-harvesting enzymes – AMO, HAO, glyceraldehyde 3-phosphate dehydrogenase and succinate dehydrogenase, (b) carbon metabolism – the anion transporter NE1927 (speculated to transport sulfate or $\text{HCO}_3^{-}$) and phosphoribulokinase (PRK), and (c) N metabolism – glutamate dehydrogenase and glutamate synthase. The genes for these enzymes were all transcribed in growing cells but not in NH$_3$-deprived cells (data not shown). The mRNA for AMO was detected in low amounts in NH$_3$-deprived cells (see below). This result differed from a previous observation where no amo transcript was detected in starved cells (Sayavedra-Soto et al., 1996). The preparation of NH$_3$-deprived cells in this study (at 30 °C for 16 h) was different from the previous study, in which sedimented cells were incubated for a day at 4 °C to deplete mRNA. Apparently, incubation in NH$_3^+$-free medium allowed the cells to keep a detectable level of amo mRNA. In contrast, the mRNAs of cbb and all other genes we examined were not detected either in stationary phase cells or in NH$_3$-deprived cells (data not shown).

Profiles of cbb mRNA induction and decay

The induction profiles of cbbL and cbbS were determined in NH$_3$-deprived cells upon transfer to growing conditions, and were compared to the induction profile of hao. NH$_3$-deprived cells had low levels of detectable hao mRNA by Northern hybridization. These cells, upon transfer to fresh medium, produced the mRNAs for cbbL and cbbS within 0-5 h (Fig. 4A). The mRNA of hao increased as previously reported (Sayavedra-Soto et al., 1996). In these induction experiments, the levels of the mRNAs of cbbL and cbbS reached a maximum level at around 2 h and decreased by 4 h (Fig. 4A). To determine the biological half-life of the mRNAs, time-course depletion experiments were conducted (by following the net
decrease in mRNA in cells deprived of energy source but with no RNA synthesis inhibitors). Messages of \(cbbL\) and \(cbbS\) declined much faster than those of \(amo\) and \(hao\) (Fig. 4B, and blots not shown). After 16 h starvation, messages from \(cbbL\) and \(cbbS\) were depleted to about 5% of initial levels, while the mRNAs for \(amo\) and \(hao\) were more abundant compared to those of \(cbbL\) and \(cbbS\) (Fig. 4B).

**Ammonia as a signal for gene transcription**

NH\(_3\) is thought to be the main signal to induce the transcription of \(amo\) in addition to providing energy for all cellular functions (Hyman & Arp, 1995; Sayavedra-Soto et al., 1996). We wanted to determine whether it is a signal for Rubisco gene transcription as well. Exposure of \(N.\ europaean\) cells to acetylene (C\(_2\)H\(_2\)), a potent inactivator of AMO, prevents NH\(_3\) use as an energy source. Cells depleted of mRNA were transferred to growth medium in the presence of C\(_2\)H\(_2\) and tested for \(cbbLS\) gene transcription. In the presence of NH\(_3\) and C\(_2\)H\(_2\), the mRNAs of \(cbbL\) and \(cbbS\) were not transcribed (Fig. 5). To ensure that the cells had sufficient energy to carry out transcription, hydroxylamine (HA) was supplied to the cell in the presence of C\(_2\)H\(_2\) with and without NH\(_3\). When NH\(_3\) oxidation is inhibited, HA, the product of NH\(_3\) oxidation, can be used as energy source by \(N.\ europaean\) cells. In the presence of HA, the \(cbbL\) and \(cbbS\) mRNAs were detected regardless of the absence or presence of NH\(_3\), as is evident in the C\(_2\)H\(_2\) treatments (Fig. 5). In agreement with what previously was reported (Sayavedra-Soto et al., 1996), \(amo\) was transcribed in media containing NH\(_3\) and C\(_2\)H\(_2\) (not shown). In these cells, NH\(_3\) served as a signal to turn on the transcription of \(amo\), presumably at the expense of internally reserved energy sources. However, in our experiments with \(cbbL\) and \(cbbS\), NH\(_3\) was not required as a signal and internally reserved energy sources were not sufficient for their transcription. The genes for carbon fixation were all transcribed at detectable levels as long as an energy supply was available. The levels of \(cbbL\) and \(cbbS\) mRNA in

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**Fig. 4.** Time-course for \(cbbL\) and \(cbbS\) induction and decay. (A) Northern hybridization showing the time-course for the induction of \(cbbL\), \(cbbS\) and \(hao\). Exponential-phase cells were washed and starved overnight, then induced in normal culture medium. (B) Time-course for the depletion of \(cbbL\) (○), \(cbbS\) (●), \(amo\) (▲) and \(hao\) (▲) mRNAs. Mid-exponential-phase cells were transferred to ammonia-free medium and incubated at 30°C. Transcript levels were determined by densitometry of the signals in the Northern blots.

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**Fig. 5.** Dependence of \(cbbLS\) genes transcription on energy source. Late-exponential-phase \(N.\ europaean\) cells were washed and starved overnight, then transferred to NH\(_3\)-free medium with or without C\(_2\)H\(_2\) and allowed to equilibrate for 1 h. The medium was then supplemented with NH\(_3\)\(^+\) (ammonium sulfate, 25 mM), or hydroxylamine (HA, 1 mM), or both. After incubation for 2 h, cells were harvested for RNA extraction and Northern analysis (see Methods). The bottom panel shows the rRNAs stained with ethidium bromide to show equivalent amounts in the samples in the analysis. The experiment was repeated three times and yielded similar trends.
the HA treatment were higher than those in the treatments that contained NH₃. In these treatments, faint hybridization signals were detected for transcripts long enough to contain up to five cbb genes (Fig. 5).

**Effect of CO₂ levels on transcription**

The transcription of cbbL, cbbS, amo and hao in response to gaseous CO₂ (no Na₂CO₃ added to the medium) was studied. The cbbL and cbbS mRNAs were detected at higher levels in the treatments with low CO₂. The cbbL message level was over five times higher at air (0-03 %) CO₂ than at 3 % CO₂ (Fig. 6). In contrast, the amo and hao mRNA levels increased as the CO₂ levels increased; the message levels were about eight- and threefold higher, respectively, at 3 % CO₂ than at 0-03 %. This response was similar when different levels of Na₂CO₃ were added to the medium (not shown). The transcription levels of cbbL and cbbS decreased as the carbonate (Na₂CO₃) levels in the medium increased. As with CO₂, the amo and hao mRNA levels and those of the anion transporter (NE1927) and PRK, increased as the concentration of Na₂CO₃ increased (data not shown). The transcription levels of these genes could be detected even at an O₂ level as low as 0-2 %.

**Effect of O₂ levels on cbb mRNA levels**

The presence of O₂ is another major factor for the growth and metabolism of N. europaea, an aerobic chemoautotroph. Transcription of cbbL, cbbS, amoA and hao in response to three O₂ levels (0-2 %, 2 % and air) was studied by transferring cells to fresh medium in sealed bottles with controlled O₂ levels. When the O₂ level was lower, all four genes, amo, hao, cbbL and cbbS, were transcribed at lower levels (blots not shown). The highest transcription was observed at 21 % O₂ (air level). The transcription of these genes could be detected even at an O₂ level as low as 0-2 %.

**DISCUSSION**

Six contiguous genes in N. europaea were identified as cbb genes based on similarity to the RubisCO genes in other organisms, of which cbbL and cbbS encode RubisCO (Baxter et al., 2002; Hayashi et al., 1997, 1999). Five of these genes (cbbLSQON) appear to form an operon. A cbbLSQON operon is suggested by the production of cDNAs containing the intergenic regions between all the five genes (Fig. 1B) and the observed mRNA fragments of appropriate sizes to contain any combination of contiguous genes in the cbb operon (Figs 5 and 6). Although we were not able to detect a transcript long enough to include all five messages, the presence of a single promoter for all five genes in the cbb operon is not unprecedented. For example, the transcription of the cbb operons in Ac. ferrooxidans, X. flavus,Ral. eutropha and Rhodobacter sphaeroides are transcribed from a single promoter (Kusano et al., 1991; Kusian et al., 1995; Schäferjohann et al., 1996). In Ral. eutropha, X. flavus (Meijer et al., 1991) and Rb. sphaeroides a single promoter was demonstrated by insertional mutations in their cbb genes. The mutations prevented the transcription of cbb genes downstream from the insertion, suggesting that the cbb operons in these bacteria are indeed large (e.g. in Ral. eutropha could be 15 kb) (Gibson & Tabita, 1997; Meijer et al., 1991; Schäferjohann et al., 1995; Windhovel & Bowien, 1990).

The mRNAs of cbbL and cbbS were the most abundant, while the other cbb mRNAs were detected consistently at low levels. Similar results were observed in other autotrophic bacteria (English et al., 1992; Kusano et al., 1991; Meijer et al., 1991). In Ral. eutropha this was interpreted as a premature transcriptional termination at a sequence resembling a terminator structure downstream of the cbbLS genes (Schäferjohann et al., 1996). Indeed in N. europaea premature termination of transcription is likely to occur, since a stem–loop structure could be formed in the intergenic region between cbbS and cbbQ with a calculated free energy (ΔG°) of −155 kJ mol⁻¹ (not shown). This

![Fig. 6. Effect of CO₂ levels on the transcription of amoA, hao, cbbL and cbbS in N. europaea. Exponential-phase cells were washed and incubated in N-free, carbonate-free medium overnight. The cells were then induced for 2 h in growth medium with various CO₂ levels, and harvested for RNA isolation and Northern analysis (see Methods). The numbers at the bottom of the blot are the relative signal intensities. The images shown are representative of experiments done in triplicate with duplicate samples.](http://mic.sgmjournals.org)
predicted stem–loop structure appears more stable than that in *Ral. eutropha*, in which the free energy is $-102$ kJ mol$^{-1}$ (Schäferjohann *et al.*, 1996). Potential hairpin structures were also identified downstream of the *cbbLS* genes in *T. denitrificans* and *X. flavus* (Hernandez *et al.*, 1996; Pulgar *et al.*, 1991). The spatial conformation of an mRNA is known to affect its stability or longevity (Grunberg-Manago, 1999). Different structures of *N. europaea* *cbb* mRNAs may have contributed to the different levels of abundance that we observed.

Although a transcription terminator immediately downstream *cbbL* was identified in some species (Valle *et al.*, 1988), an examination of the intergenic sequence (63 bp) between *cbbL* and *cbbS* in *N. europaea* failed to identify a potential transcriptional terminator. This result with *N. europaea* is similar to what has been reported for *Ac. ferrooxidans* (Kusano *et al.*, 1991). In support of an mRNA processing alternative, the consensus cleavage site sequence of RNase E, (G/A)ATT(A/T) (Ehretsmann *et al.*, 1992), was identified in the first three intergenic regions in the *cbb* operon in *N. europaea*. A cleavage of the mRNA containing *cbbL* and *cbbS* may also occur. RNA processing in bacteria can occur by means other than RNase E (Calin-Jageman & Nicholson, 2003; Otsuka *et al.*, 2003). In the *N. europaea* genome sequence, a number of endoribonuclease genes were identified (Chain *et al.*, 2003), including RNase E, RNase G and RNase III. RNase III acts on double-stranded RNAs. These results and sequence analyses suggest that a complex processing of the *cbb* mRNA may be involved in the regulation of transcription and function, and is affected by either excision/cleavage or differential degradation.

The intergenic region between *cbbR* and *cbbL* should contain promoters in opposite directions, for both *cbbR* and *cbbLSQON*. CbbR is a LysR-type transcriptional regulator (Schell, 1993). CbbR is believed to be involved in autoregulation of its own transcription as in *Ac. ferrooxidans* (Kusano *et al.*, 1993). CbbR is a LysR-type transcriptional regulator (Schell, 1993). CbbR is believed to be involved in autoregulation of its own transcription as in *Ac. ferrooxidans* (Kusano *et al.*, 1993). CbbR is involved in the transcription and function of the Calvin cycle operon (van Keulen *et al.*, 1996). Potential hairpin features of the intergenic region (i.e. AT-rich region and T-(N)$_n$-A inverted repeats) may allow binding of CbbR and other potential regulators in both orientations, possibly with different affinities (Fig. 2). The AT-rich element upstream of the *rbc* (RubisCO) gene in *Synechococcus* sp. PCC 7002 was required for CO$_2$-dependent repression (Onizuka *et al.*, 2002). In their mobility-shift assay, a strong signal of a repressor binding to the AT-box was observed in extracts from cells cultured at 15% CO$_2$, but only a weak signal from cells cultured at 1% CO$_2$. It was suggested that the AT-rich element was involved in the negative regulation of the *rbc* transcription in response to CO$_2$ levels (Onizuka *et al.*, 2002). We do not know whether the AT box plays a similar role in the regulation of the *cbb* gene transcription in *N. europaea*, but our results indicate that high CO$_2$ can repress its transcription.

In the absence of an exogenous energy source, the presence of NH$_3$ can turn on *amo* transcription by using energy reserves in the cells (Hyman & Arp, 1995; Sayavedra-Soto *et al.*, 1996). *N. europaea* preferentially directs its internal energy reserves for the synthesis of *amo* mRNA in the presence of NH$_3$ and C$_2$H$_2$ included experimentally to block oxidation of NH$_3$ (Sayavedra-Soto *et al.*, 1996). In contrast, NH$_3$ was not a signal to turn on *cbbLS* gene transcription. Rather, an exogenous energy source was required (Fig. 5). Because NH$_3$ is the sole energy source for ammonia-oxidizing bacteria, regulation of major pathways by NH$_3$ is expected. However, the energy status of the cell appeared to be the key factor for the transcription of the *cbb* genes and NH$_3$ itself was not required. *N. europaea* cells did not commit their internal energy reserves to the transcription of the *cbb* genes (Fig. 5). *N. europaea* uses limited internal energy for the transcription of *amo* and *hao*, which are directly involved in energy harvesting (Hyman & Arp, 1995; Sayavedra-Soto *et al.*, 1996). In the absence of any exogenous energy source *cbbL* and *cbbS* mRNAs were depleted faster than *amo* and *hao* mRNAs (Fig. 4B), further indicating the dependence of *cbb* message levels on the cellular energy status. The CBB cycle is an intensely energy-consuming process; thus it is not surprising that transcription of the *cbb* operon is dependent upon the presence of an abundant energy source.

The RubisCO gene transcription in *N. europaea* is responsive to the available carbon levels, with low carbon levels resulting in the highest transcription (Fig. 6), perhaps through transcriptional derepression. Increases in the levels of RubisCO synthesis and activity under CO$_2$ limitation have also been documented for other microorganisms. For example, in the facultative autotrophic bacteria *Ralstonia oxalaticus* (formerly *Pseudomonas oxalaticus*) (Dijkhuizen & Harder, 1979) and *Ral. eutropha* (formerly *Alcaligenes eutrophus*) (Friedrich, 1982), the Calvin cycle was less repressed with limited carbon source (C$_1$ compounds such as formate). Growth of the obligate autotroph *T. neapolitanus* in a chemostat under CO$_2$ limitation caused increased activity of the Calvin cycle (Beudeker *et al.*, 1980). However, complete carbon starvation did not induce the Calvin cycle in the facultative autotroph *Ral. eutropha* (Friedrich, 1982). In other organisms such as cyanobacteria and algae, carbon-concentrating mechanisms have been identified (Shibata *et al.*, 2001; Xiang *et al.*, 2001). In contrast, PRK mRNA in *N. europaea* increased with elevated HCO$_3^-$ /CO$_2$, presumably due to the increase in the total carbon fixed.

In *N. europaea*, the highest *cbb* transcription levels were observed with atmospheric levels of CO$_2$. Atmospheric CO$_2$ content is about 0.03%, which equilibrates to approximately 9 µM CO$_2$ in water at 30°C. In spite of the high *cbb* gene transcription, *N. europaea* grew poorly in media lacking Na$_2$CO$_3$ and where C was available only from the atmosphere (data not shown), and the cultures exhibited a long lag phase (~5 days, compared to 1 day in carbonate-containing medium). Cells incubated in such
medium made much more RubisCO mRNA than cells grown in medium containing carbonate. Genes for ammonia metabolism (amo, hao) showed the opposite trend. T. neapolitanus had higher carbon-fixing capacity per unit of total cell protein in chemostat cultures under carbon-limiting conditions than under carbon-saturated conditions (Beudeker et al., 1980). As seen in our experiments, N. europaea cells appeared to respond to carbon limitation by prioritizing more resources for synthesizing more RubisCO mRNA, and presumably RubisCO enzyme as well. CO$_2$ levels in natural habitats may fluctuate frequently, so the responsiveness of the transcription of genes for carbon fixation to CO$_2$ levels may have adaptive value. The observed high cbb gene transcription with limited CO$_2$ may be an adaptation of the cells to scavenge the limited CO$_2$ (0.03% in air). Regardless of the cbb gene transcription, more efficient carbon fixation appears to be correlated with the CO$_2$ levels in the media, as is evidenced by higher growth rates of N. europaea in the presence of higher available carbon in the medium (data not shown). Carbonic anhydrase is required for Ral. eutropha to grow at atmospheric CO$_2$ concentrations (Kusian et al., 2002). Genes for potential carbonic anhydrase and an anion transporter (NE1927) in N. europaea were transcribed similarly under low- and high-carbon conditions (blots not shown), suggesting that they may not be functioning as a CO$_2$/HCO$_3^-$-concentrating mechanism. The apparent lack of a CO$_2$-concentrating mechanism is supported by the observation of poor growth of N. europaea cultures in HCO$_3^-$-free medium exposed to air. The responsiveness of cbb gene transcription to carbon levels may also be a reflection of the lack of a specific CO$_2$-concentrating mechanism in N. europaea. It will be interesting to see how other ammonia-oxidizing bacteria, such as those with carboxysomes, respond to low C levels.

ACKNOWLEDGEMENTS

This research was supported by the Office of Science (BER), US Department of Energy grant no. DE-FG03-01ER63149 and the Oregon Agricultural Experimental Station.

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