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 it requires for energy and biosynthesis from the oxidation of ammonia \( \text{(NH}_3 \text{)} \) to nitrite \( \text{(NO}_2^- \text{)} \) (Winogradsky, 1931). This bacterium is an autotroph that utilizes \( \text{CO}_2 \) as its primary carbon source, and is an obligate autotroph that participates in the C and N cycles. N. europaea 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, \text{cbbLSQON}. This gene organization is similar to that of the operon for ‘green-like’ type I RubisCOs in other organisms. The \text{cbbR} gene encoding the putative regulatory protein for RubisCO transcription was identified upstream of \text{cbbL}. This study showed that transcription of \text{cbb} genes was upregulated when the carbon source was limited, while \text{amo}, \text{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 \text{amo} genes, \text{NH}_3 was not required for the transcription of the \text{cbb} genes. All five \text{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 \( \text{CO}_2 \) via the Calvin–Benson–Bassham (CBB) cycle. Genetic information about the enzyme that catalyses \( \text{CO}_2 \) 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 (RubisCO). 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 \text{cbb} genes of ammonia-oxidizing bacteria. In one, Hirota et al. (2002) cloned and sequenced the \text{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 \text{cbbLS} genes of Nitrosospira sp. isolate 40KI and showed its functionality in a \text{cbb}-deletion strain of Ralstonia eutropha. However, to date there are no reports describing the transcription patterns and regulation of the \text{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 monoxygenase; HAO, hydroxylamine oxidoreductase; PRK, phosphoribulokinase; RubisCO, ribulose bisphosphate carboxylase/oxygenase.
utilization of ammonia in *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 NH₃ to NO₂⁻. The genetic loci for AMO and HAO are in multiple copies in nitrifiers. In *N. europaea* there are two gene copies for AMO and three gene copies for HAO (compared to a single gene copy for Rubisco (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 *amoCA*B 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 *cbb* genes in response to major nutrients and environment factors were characterized.

**METHODS**

**Media, bacterial cultures, determination of enzyme activities and materials.** *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 NH₄Cl-free, CO₂-free buffer to remove residual growth medium. The NH₃ depletion (starvation) treatments were prepared by incubating cells in NH₄Cl-free, CO₂-free medium for 16 h in order to deplete most mRNAs and to avoid the toxic effects of accumulated metabolites (Stein & Arp, 1998). For the experiments where cells in stationary phase were necessary, the cells were harvested 3 days after reaching the maximum OD₆₀₀ (~0.07). Because the pH of the medium in the cultures decreases as nitrite accumulates, NH₃ becomes limiting in stationary phase (depriving the cells of the growth substrate). It is known that the mRNAs of AMO and HAO are induced upon incubation in NH₄Cl and decrease to negligible amounts upon approximately 1 day of starvation (Sayavedra-Soto et al., 1996). The induction experiments involving gases were carried out in bottles sealed with grey butyl septa. In the O₂ limitation experiments requiring the inactivation of AMO, acetylene was injected at 100 mbar through septa by injecting pure O₂ and CO₂ as necessary. The headspace CO₂ and O₂ levels were determined using a Shimadzu GC8A gas chromatograph equipped with a thermal conductivity detector and a 4 ft (1.2 m) Porapak Q column. In experiments requiring the inactivation of AMO, acetylene was injected at 2% (v/v) and allowed to equilibrate with the cell suspension in NH₄Cl-free medium for 1 h before an energy source was added. Induction of mRNA transcription and enzyme activities was commonly done by incubation with the inducing agent at 30°C on a rotary shaker at 100 r.p.m. for 2 h. Nitrite concentration was determined colorimetrically using the Griess reagent (sulfanilamide and *N*-naphthylethylenediamine) (Hageman & Huckleby, 1971).

**Nucleic acid manipulation and hybridization.** RNA was isolated as described by Reddy & Gilman (1993) and Vangnai et al. (2002). Briefly, 250 μl acid phenol, 250 μl chloroform, SDS to 1% and sodium acetate to 0.3 M were added to 500 μl cell suspension in buffer (2 mM MgCl₂ and 50 mM NaH₂PO₄, pH 7.5). The cell suspension was mixed thoroughly and centrifuged for 5 min at 16,000 g. The total RNA was precipitated with ethanol and resuspended in diethylpyrocarbonate (DEPC)-treated water. When necessary, cells were resuspended in a solution of NaN₃ (1 mM) and aurintricarboxylic acid (1 mM) (Reddy & Gilman, 1993), or in a commercial RNA stabilizer solution (RNAlatel; Ambion), to prevent mRNA changes and degradation during sample preparation. For Northern hybridization analysis, total RNA was resolved in denaturing 1:2% agarose gels (Sambrook et al., 1989). Prior to electrophoresis, RNA was stained with ~5 μg 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 [γ⁻³²P]dCTP (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 digestions and agarose gel electrophoresis were done 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 50°C extension temperature. RNA templates for RT-PCR were treated with RQI 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 *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 *cbbL*-specific reverse primer, followed by PCR amplification of the chimeric DNA fragment with the *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.

Total cell protein was estimated by the biuret method (Gornall et al., 1949) and the protein composition was analysed by PAGE as described by Hyman & Arp (1993).

**RESULTS**

*N. europaea* *cbb* operon characterization

The putative Rubisco operon in this bacterium is composed of five genes, namely *cbbLSQO* and a fifth gene here designated *cbbN*. This composition was deduced from the nucleotide sequence of the genome of *N. europaea* and gene similarity comparisons. In this operon, *cbbL* and *cbbS* code for the Rubisco large and small subunits respectively. The genes *cbbQ* and *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). *cbbN* has the same orientation as *cbbLSQO*. The intergenic region between *cbbO* and *cbbN* is only 20 bp, in which no apparent promoter could be inferred from the sequence, implying that it is transcribed from the same *cbb* promoter. *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>
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<tr>
<td><strong>Northern hybridization probes (forward/reverse)</strong></td>
<td></td>
</tr>
<tr>
<td>cbbL</td>
<td>CGGATATCTTGCGTTCAC/CCACCGTACGAGTGGAC</td>
</tr>
<tr>
<td>cbbS</td>
<td>AAAAGTCTGTTGACGTC/TAATACCATTTGGCTTC</td>
</tr>
<tr>
<td>cbbQ</td>
<td>TGAGCGATGTTATCGAGC/AAATACCATTTGGCTTC</td>
</tr>
<tr>
<td>cbbO</td>
<td>CACTCGCTGTGCTATG/ACATTTGGCTTC</td>
</tr>
<tr>
<td>cbbN</td>
<td>TTATCGGAGTGATCAGG/TCAAGGATTCGCTTC</td>
</tr>
<tr>
<td>cbbR</td>
<td>TGCGCTATACATCAATCATC/GTTCGCTATCC</td>
</tr>
<tr>
<td>Carbonic anhydrase (NE1926)</td>
<td>CAGGGCTACACAGTTGAA/GCAAATCCGACATCCGTGTT</td>
</tr>
<tr>
<td>Succinate dehydrogenase</td>
<td>GTTCGAGGCGCATGATATC/GCACAGTGGATGATGT</td>
</tr>
<tr>
<td>Glutamate dehydrogenase</td>
<td>CATCTCGTGATCGTCGCA/GAGACCATACCATCC</td>
</tr>
<tr>
<td>Phosphoribulokinase (NE1801)</td>
<td>GCTGGAAGCCGTTTACGT/GTGATCCGTGATATCC</td>
</tr>
<tr>
<td>Sulfate transporter (NE1927)</td>
<td>TTTCGCCGGATATTATGCT/AGCCCATACATTTCGCGT</td>
</tr>
<tr>
<td><strong>For RT-PCR (FW, forward; RVS, reverse)</strong></td>
<td></td>
</tr>
<tr>
<td>cbbL-FW2</td>
<td>CACTGGGAAGCTTGGC/AGATGGTTC</td>
</tr>
<tr>
<td>cbbS-FW2</td>
<td>AGTTCTGGGAATC/CGCTGTTT</td>
</tr>
<tr>
<td>cbbQ-FW2</td>
<td>GATGTCTCATGAGC/CTCGTGTC</td>
</tr>
<tr>
<td>cbbQ-RVS2</td>
<td>CATCGCACTGAT/CTCGGACAA</td>
</tr>
<tr>
<td>cbbQ-RVS3</td>
<td>TTGTGCATCAA/AGGAAACCC</td>
</tr>
<tr>
<td>cbbO-RVS2</td>
<td>TGACACATGCGTC/CCGACCT</td>
</tr>
<tr>
<td>cbbO-RVS3</td>
<td>GTCTCGATCTCCGTCGAGAT/CCGACCT</td>
</tr>
<tr>
<td><strong>For transcript analysis</strong></td>
<td></td>
</tr>
<tr>
<td>cbbL (reverse)</td>
<td>GTGCGGATATTACCGTTA/ACCCACGTCT</td>
</tr>
<tr>
<td>cbbL (reverse, nested)</td>
<td>TGCTGACATTTACGTAAT/CTCGCT</td>
</tr>
</tbody>
</table>

*For amoA and hao primers, see Hommes et al. (2001).
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 (Hirot a 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*. The putative promoter region at −10 (TATAGT) and −35 (TTTAAC) bases 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 *Nitrosomonas* sp. ENI-11 (Hirot a 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.

**Fig. 1.** The RubisCO gene organization in *N. europaea*. (A) Arrows indicate the orientation of the genes. The numbers are DNA segment lengths in bp for genes (upper) and intergenic regions (lower). (B) Detection of intergenic regions by RT-PCR. The bars indicate the regions amplified by RT-PCR. The amplified products were resolved on an agarose gel. The ‘PCR only’ lane is a control reaction performed without reverse transcriptase to test for RNA purity. Lanes 1 to 5 correspond to the amplified cDNA fragments located in the areas indicated by the bars above. The numbers below the bars are the size of the fragments in kb. Lane 6 is a DNA size marker.
The nucleotide sequence upstream of N. europaea cbbL. The two putative starts of transcription are in bold (+1/+1). The numbers −10 and −35 indicate the deduced promoter region. An AT-rich region with at least 89% AT composition is boxed. The T/A-N11−14-A/T symmetry sequences match the inverted arrowheads in the lines next to the nucleotide sequence. The ribosome-binding site for cbbL is labelled as RBS. The cbbL and cbbR start codons (ATG) are indicated in bold italics at both ends of the sequence.

 Profiles of cbb mRNA induction and decay

The induction profiles of cbbL and cbbS were determined in NH3-deprived cells upon transfer to growing conditions, and were compared to the induction profile of hao. NH3-deprived cells had low levels of detectable hao and RubisCO mRNAs 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

Fig. 3. Northern hybridizations showing the level of transcription of the cbb genes in (i) induced cells (actively growing) and (s) starved cells (ammonia-deprived). The far left lane is an RNA ladder. The experiment was repeated in triplicate with similar trends. Equivalent amounts of RNA were loaded in all lanes in the gel as estimated by staining with ethidium bromide.

http://mic.sgmjournals.org
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\textsubscript{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 \textit{N. europaea} cells to acetylene (C\textsubscript{2}H\textsubscript{2}), a potent inactivator
of AMO, prevents NH\textsubscript{3} use as an energy source. Cells
depleted of mRNA were transferred to growth medium in
the presence of C\textsubscript{2}H\textsubscript{2} and tested for cbbLS gene transcrip-
tion. In the presence of NH\textsubscript{3} and C\textsubscript{2}H\textsubscript{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 pres-
ence of C\textsubscript{2}H\textsubscript{2} with and without NH\textsubscript{3}. When NH\textsubscript{3} oxidation
is inhibited, HA, the product of NH\textsubscript{3} oxidation, can be
used as energy source by \textit{N. europaea} cells. In the presence
of HA, the cbbL and cbbS mRNAs were detected regardless
of the absence or presence of NH\textsubscript{3}, as is evident in the
C\textsubscript{2}H\textsubscript{2} treatments (Fig. 5). In agreement with what
previously was reported (Sayavedra-Soto et al., 1996), amo
was transcribed in media containing NH\textsubscript{3} and C\textsubscript{2}H\textsubscript{2} (not shown). In these cells, NH\textsubscript{3} served as a signal to turn on the
transcription of amo, presumably at the expense of internally
reserved energy sources. However, in our experi-
ments with cbbL and cbbS, NH\textsubscript{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 (●), amoA (△) 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.

**Fig. 5.** Dependence of cbbLS genes transcription on energy source. Late-exponential-phase
\textit{N. europaea} cells were washed and starved overnight, then transferred to NH\textsubscript{3}-free medium
with or without C\textsubscript{2}H\textsubscript{2} and allowed to equilibrate for 1 h. The
medium was then supplemented with NH\textsubscript{4}\textsuperscript{+} (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). In the absence of added Na₂CO₃ in the medium (i.e. air CO₂ only), the levels of the cbbL and cbbS mRNAs were approximately sevenfold higher than that in the medium with full Na₂CO₃. These results suggest that a similar response would be observed in the environment regardless of whether CO₂ or HCO₃⁻ was the predominant carbon source.

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 chemooauto-troph. 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; Kuslan 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 insertion 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
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. ferroxidans* (Kusano et al., 1991). In support of an mRNA processing alternative, the consensus cleavage site sequence of RNase E, (G/A)ATT(A/T) (Ehretsman et al., 1992), was identified in the first three intergenic regions of RNase E, RNase G and RNase III. RNase III acts on nuclease 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 *Ral. eutropha* and *X. flavus* (Kusano & Bowien, 1995; Shively et al., 1998; van Keulen et al., 2003). Thus the unique 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 eutalis* (formerly *Pseudomonas eutali cus*) (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 \(\mu\)M 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 carbon-limiting conditions than under carbon-saturated conditions and CO$_2$ fixing capacity in the obligate chemolithoautotroph (Bath).

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