Transcription of the \textit{amoC}, \textit{amoA} and \textit{amoB} genes in 
\textit{Nitrosomonas europaea} and \textit{Nitrosospira} sp. NpAV

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Abstract

Nitrifying bacteria such as \textit{Nitrosomonas europaea} and \textit{Nitrosospira} sp. NpAV use ammonia monooxygenase (AMO) for oxidation of their primary growth substrate, ammonia. Two polypeptides of AMO are coded for by contiguous genes, \textit{amoA} and \textit{amoB}, which are preceded by a third gene, \textit{amoC}. The \textit{amoCAB} clusters are present in multiple copies in nitrifying bacteria of the \textit{L} subdivision. These bacteria also have one \textit{amoC} copy that is not adjacent to a copy of \textit{amoAB}. The seven known \textit{amoC} genes in different nitrifiers code for similar polypeptides (>68%). Reverse transcriptase-polymerase chain reactions and Northern blots indicated that \textit{amoC} from the \textit{amoCAB} cluster is contained on a transcript with \textit{amoAB}. Two other transcripts were detected with \textit{amo} probes and may be a product of processing of the \textit{amoCAB} mRNA or independent transcripts. 

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1. Introduction

\textit{NH}_3\textsuperscript{oxidizing} bacteria such as \textit{Nitrosomonas europaea} and \textit{Nitrosospira} sp. NpAV transform \textit{NH}_3\textsuperscript{oxidizing} bacteria such as \textit{Nitrosomonas europaea} and \textit{Nitrosospira} sp. NpAV transform NH\textsubscript{3} to NO\textsubscript{2}\textsuperscript{−}. These bacteria are obligate chemolithotrophs as they are entirely dependent upon NH\textsubscript{3} oxidation for energy generation and obtain all of their carbon for growth from CO\textsubscript{2} [1–3]. The oxidation of NH\textsubscript{3} to NO\textsubscript{2}\textsuperscript{−} is carried out in two steps [4]. First, NH\textsubscript{3} is oxidized to hydroxylamine (NH\textsubscript{2}OH) in a reaction catalyzed by the membrane bound enzyme ammonia monooxygenase (AMO). The NH\textsubscript{2}OH is subsequently oxidized to NO\textsubscript{2}\textsuperscript{−} by means of the periplasmic enzyme hydroxylamine oxidoreductase (HAO) [5]. The genes coding for two of the putative structural proteins of AMO (\textit{amoA} and \textit{amoB}) have been isolated and sequenced [6–8].

All autotrophic NH\textsubscript{3} oxidizing bacteria that have been examined (e.g. \textit{N. euopea}, \textit{Nitrosospira multiformis}, \textit{Nitrosococcus oceanus}, \textit{Nitrosospira} sp. NpAV) possess similar \textit{amo} genes [8–10]. Most
NH₃ oxidizing bacteria have multiple copies of the genes coding for AMO e.g.: two copies of amoAB have been identified in the genome of N. europaea [6] and Nitrosospira sp. NpAV contains at least three copies [10]. Within an individual strain the amo copies are nearly identical [10]. The amoA and amoB genes are always contiguous and preceded by a third gene, amoC [11].

In N. europaea, we have used amoA mutants to show that both copies of amoA are expressed and that neither one is essential for growth [12]. Nevertheless, a mutation in one amoA copy resulted in strains that grew more slowly than the wild-type cells or N. europaea strains with a mutation in the other copy suggesting distinct metabolic roles for each copy [12].

The subunit proteins of AMO are similar to those of the particulate methane monoxygenase (pMmo) in methanotrophs. The pMmo encoding genes (pmo) are present in more than one copy as well [13]. The similarity also extends to the putative subunit compositions, the inhibitor profiles and the broad (though not identical) substrate ranges [9,11,14]. The pMmo genes of M. capsulatus are transcribed into a polycistronic mRNA of 3.3 kb [15]. While we previously have shown that the genes encoding the two putative structural AMO proteins, AmoA and AmoB, are co-transcribed in the same mRNA, we wished to determine whether amoC was also part of the same transcriptional unit and thus a member of the amo operon. In this paper we also report the gene sequences and the deduced amino acid sequences from three gene copies of amoC in N. europaea and two additional amoC sequences from Nitrosospira sp. NpAV.

2. Materials and methods

2.1. Strains and cell cultures

E. coli cells were grown in Luria Bertani media as described [16]. The strains of E. coli used were DH5α, JM109 and TOP10 (Invitrogen, Carlsbad, CA). N. europaea cells and Nitrosospira sp. NpAV were grown in batch cultures as described [10,17]. Cell cultures (1.0 to 1.5 l) in late logarithmic phase were harvested for nucleic acid extraction.

2.2. DNA manipulations

Genomic and plasmid DNA preparations, DNA restriction digests, Southern and Northern hybridizations, and other standard DNA manipulations were performed as described [12]. A total genomic library of N. europaea previously prepared was screened for some of the ORFs studied [18]. DNA probes were labeled by using a random priming kit (Promega Corporation, Madison, WI) and [α³²P]dCTP (DuPont NEN Products, Wilmington, DE) or by using a chemiluminescent labeling kit (Genius system, Boehringer Mannheim). The hybridization signals were visualized using a phosphorimager (Molecular Dynamics, Sunnyvale, CA). Restriction and DNA modifying enzymes were from Promega Corporation and United States Biochemical (Cleveland, OH) except for Taq DNA Polymerase from PE Applied Biosystems (Branchburg, NJ). Oligonucleotides were synthesized at the Center for Gene Research and Biotechnology, Central Services Laboratory, Oregon State University or commercially obtained. Cloning of PCR-obtained DNA fragments was performed using commercially available kits from Invitrogen, Promega or Stratagene (La Jolla, CA).

The transcript for amoC was evaluated by Northern hybridizations and by the reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was isolated as described [12,18]. For RT-PCR the RNA was treated 15 min at 37°C with RQ1 RNase-free DNase (1 U µg⁻¹ of template DNA), phenolchloroform and ethanol precipitated. The RT-PCR reaction was carried out with a kit from PE Applied Biosystems. The absence of genomic DNA in the RNA samples was determined in a control reaction with no reverse transcriptase.

DNA sequencing was performed using dideoxynucleotides-dye primer chemistry in a BioDynamics sequencing instrument at the Center for Gene Research Central Laboratory, Oregon State University and at the Utah State University Biotechnology Center, using a Perkin-Elmer ABI373A automated sequencer.
3. Results

3.1. Isolation and characterization of amoC genes that precede amoAB genes

In *N. europaea*, Southern hybridizations with probes for amoC-1, amoC-2 or amoA demonstrate that amoC-1 and amoC-2 are located in the same DNA fragments as amoA-1 and amoA-2 respectively (Figs. 1 and 2). Two hybridizing fragments were observed in the digests with EcoRI and HindIII, because these sites flank and are located outside of the two copies of the amo gene cluster in the *N. europaea* genome (Fig. 1). The fragments highlighted in the EcoRI digest of *N. europaea* DNA were the same sizes as those previously reported using an amoA probe [6]. In the BanHI digest two fragments were produced from an internal site in amoC-1 or amoC-2 and another external site which was specific for each copy (Fig. 2 and [11]). In Nitrosospira sp. NpAV, four copies of amoC were detected (Fig. 2), but only three of these were found on the same fragments with amoAB [11]. In both bacteria, all identified amoA copies were preceded by an amoC gene (this work and [11]).

The nucleotide sequence of each copy of amoC adjacent to the two amoAB genes in *N. europaea* was determined. The sequence of approximately 80% of the open reading frame (ORF) including the termination codon of one of the two *N. europaea* amoC genes was determined from a cloned fragment obtained by the polymerase chain reaction (PCR). The PCR fragment was obtained using the primers CL2 and amo102 based on the known Nitrosospira sp. NpAV amoC sequence [11]. The remnant 5’-end sequence (including 74 nucleotides upstream the start codon) of amoC in *N. europaea* was obtained using the single primer approach [19]. Based on the sequence upstream from the start codon, we proceeded to obtain copy specific clones for sequencing. We used two mutant *N. europaea* strains; each strain has a single insertion of a cassette conferring resistance to kanamycin into one of the two amoA copies [12]. The copy specific fragments were obtained by PCR using primers encompassing the kanamycin cassette and the newly found upstream DNA sequence of amoC.

The gene of either copy of amoC in *N. europaea* was 813 nucleotides in length encoding 271 amino acids to form a 31.4 kDa polypeptide with a pI of 6.58. In *N. europaea*, amoC terminated 163 nucleotides upstream of amoA. The sequences of the 74 nucleotides upstream from the start codon and the intergenic regions were identical between the two gene copies of *N. europaea*. The amoC gene copies in *N. europaea* were 99% identical and encoded identical peptides. In comparison (Fig. 3), the 270 amino acids of the deduced AmoC-2 and AmoC-3 polypep-
tides from Nitrosospira sp. NpAV have a calculated mass of 31.2 kDa and a pI of 6.65. The amoC-amoA intergenic region was 212 nucleotides in Nitrosospira sp. NpAV [11]. The two deduced AmoC polypeptides coded by amoC-2 and amoC-3 from Nitrosospira sp. NpAV differed in two amino acids. The deduced polypeptides from both N. europaea amoC gene copies were 87% identical to the deduced amoC-2 and amoC-3 gene products in Nitrosospira sp. NpAV and 44% identical to the polypeptides deduced from pmoC-1 and pmoC-2 in M. capsulatus or from amoC in N. oceanus (Fig. 3 and [11]). The predicted structure of AmoC in N. europaea revealed some of the same features as the AmoC of Nitrosospira sp. NpAV [11]. The amino acids from position 200 to 230 were 95% identical in AmoC and pMmoC which suggests that this helix forming motif is important for AmoC and pMmoC function.

3.2. Isolation and characterization of amoC genes that are not succeeded by amoAB genes

Southern blots were also performed using the amoC probe from Nitrosospira sp. NpAV with genomic DNA from several strains of ammonia oxidizing bacteria from the β subdivision including Nitrosospira sp. NpAV, N. multiformis, N. briensis, N. tenuis and Nitrosomonas eutropha (data not shown). In all cases an additional band hybridized to the amoC probe that was not detected by the amoA probes. The genomic library of N. europaea was then screened and a KpnI-EcoRI DNA fragment containing amoC-3 was cloned and sequenced. Southern analysis with a probe specific for this amoC copy of N. europaea (Fig. 1) revealed that the DNA fragment that was not associated with the amoA containing fragments (Fig. 2). The polypeptide encoded by amoC-3 was 68% identical to that encoded by amoC-1 or amoC-2 in N. europaea. The calculated molecular mass was 31.7 kDa with a pI of 6.63. A PCR fragment containing amoC-4 in Nitrosospira sp. NpAV was sequenced. AmoC-4 calculated molecular mass was 31.2 kDa with a pI of 6.8. The polypeptide coded by amoC-4 in Nitrosospira sp. NpAV was almost identical to the amoC-2, amoC-3 gene products and 85% identical to the amoC-1, amoC-2 products in N. europaea.
3.3. amoC is a member of an amoCAB operon

The available nucleotide sequence of amoC in *N. europaea* and in *Nitrosospira* sp. NpAV provided a means to determine if amoC is part of the amoAB operon. Northern blots with probes for amoA and amoB using total RNA isolated from *N. europaea* have shown that both genes are part of the same operon [20]. In *M. capsulatus* the pmo genes are in a similar arrangement to *N. europaea* and are co-transcribed in a polycistronic mRNA of 3.3 kb [15]. Because of the contiguous location of amoC to amoAB, the size of the large transcript identified in *N. europaea* Northern blots and the similarities to the pmo genes, seemed likely that amoC was also present on the transcript which contained amoAB [11,20]. To examine whether amoC was part of the amoAB operon in *N. europaea* and Nitrosospira sp. we performed reverse transcriptase-PCR (RT-PCR) and Northern hybridizations.

The RT-PCR was done using primers complementary to amoC and either amoA or amoB and *N. europaea* and *Nitrosospira* sp. NpAV total RNA. The amplified fragments were obtained using forward primer CL2 and antisense primers located in amoA or amoB (Fig. 1). RT-PCR products were produced from *N. europaea* and *Nitrosospira* sp. NpAV total RNA corresponding to the predicted sizes (Fig. 4). These amplified cDNAs confirmed that amoC was co-transcribed with amoA. Because we had not determined in *Nitrosospira* sp. NpAV if amoB was part of the same operon, we performed an additional reaction using primer CL2 and a primer within the amoB gene near the start codon. While the product yield was relatively low compared to the reactions producing shorter fragments, we were able to show...
that amoC was part of an amoCAB operon in Nitrospira sp. NpAV as well. This result was confirmed using primers in amoA and amoB which also gave strong products (data not shown). Thus, with RT-PCR we demonstrated that the transcripts from the amo operon in N. europaea and Nitrosospira sp. NpAV contained amoC, amoA, and amoB.

To further corroborate that amoC was part of the amo operon we performed Northern hybridizations with probes for amoC, amoA, and amoB. In N. europaea total RNA blots, all three amo gene probes highlighted an RNA fragment of approximately 3.5 kb (Fig. 5) which demonstrated that the amo operon contains three cistrons: amoC, amoA and amoB. The Northern hybridizations of the amoA and amoB probes to a 3.5 kb sized RNA were consistent with previous results [20]. As described previously [12,20], Northern hybridizations of N. europaea total RNA with amoA or amoB probes also highlighted a fragment of approximately 2.4 kb in size. While this fragment was not observed using an amoC probe, Northern hybridizations with amoC probes detected an additional 1.1 kb fragment which was not detected with the amoA and amoB probes. Conclusive Northern hybridizations using total RNA isolated from Nitrospira sp. NpAV the 1.1 kb transcript clearly, but failed to detect the 3.5 and 2.4 kb transcripts. We used specific probes for amoC-3 in N. europaea to test for transcription as well. However, we could not detect the induction of a specific mRNA for amoC-3 in Northern hybridizations, even upon NH$_4^+$ induction [20]. On the other hand, RT-PCR yielded the cDNA of amoC-3 thus demonstrating its transcription in N. europaea.

N. europaea cells incubated as a sediment (following centrifugation) or in spent medium for at least 12 h at 4°C in the absence of substrate did not contain the 3.5 kb mRNA detectable with amoA or amoB probes [20,21]. However, the 1.1 kb mRNA (amoC-1, amoC-2) could be detected with the amoC probe even after 72 h of similar incubations (data not shown). Independent of the age of the culture harvested, the 1.1 kb fragment was at higher concentrations than the 3.5 or 2.4 kb fragments in most Northern blots (Fig. 5).

Incubation of Nitrosospira sp. NpAV cells in ammonia for up to 4 h did not induce detectable amounts of amo RNA in Northern hybridizations, contrary to what is observed in N. europaea cells. When N. europaea was incubated for 2 h

**Fig. 5.** Phosphorimages of N. europaea RNA blots hybridized to probes for amoC, amoA, and amoB. Radiolabeled probes were produced and used as described in the text.
in the presence of NH₃ it produced amo mRNA amounts easily detected by Northern hybridizations with probes for either amoC, amoA or amoB (Fig. 5 and [20]).

4. Discussion

Comparisons of the deduced polypeptides from the nucleotide sequence of amoC indicated a high degree of similarity between the ammonia oxidizers studied here (Fig. 3). However, the upstream flanking nucleotide sequences in N. europaea, Nitrosospira sp. NpAV and M. capsulatus did not show areas of similarity that could point to key regions for the differential regulation of the gene copies [12]. As such the nucleotide sequences offer no explanation for the differential expression of both operons.

By using an amoC probe derived from N. europaea we produced Northern blots showing a transcript of the size as the one previously described using amoA or amoB probes [20]. In N. europaea the detection of the mRNA for amoCAB was obtained using probes of moderate specific activity (2 × 10⁹ cpm µg⁻¹ of DNA). On the other hand, Nitrosospira sp. NpAV Northern hybridizations were more challenging. Because the relatively long generation time of Nitrosospira sp. NpAV (72 h) the levels of the amo mRNA probably are at much lower levels than the levels in N. europaea which has a shorter generation time (8 h) at similar growth stages. RT-PCR was more sensitive in detecting the full amoCAB transcript in Nitrosospira sp. NpAV (Fig. 4). Because of the similarity among nitriﬁers, the RT-PCR experiments and the results obtained with N. europaea RNA Northern hybridizations, we conclude that in Nitrosospira sp. NpAV, amoC is also part of the amo operon as proposed recently [11]. The genes coding for the three AMO subunit polypeptides are co-transcribed from each of the multiple amoCAB operons in N. europaea and in Nitrosospira sp. NpAV; therefore, the similarities among ammonia oxidizing bacteria (including methanotrophs) can be extended from the level of gene product similarity to the transcriptional level. Northern blots with an amoC-3 specific probe failed to detect a transcript, nevertheless its mRNA was detected by RT-PCR and specific amoC-3 primers, demonstrating its transcription. The expression of amoC-3 in N. europaea was not enhanced in NH₃ incubations; it appears that its expression is at much lower levels than the expression levels of amoC-1 or amoC-2 under the conditions of our experiments.

We consistently have observed the 2.4 kb RNA with probes for amoA and amoB in N. europaea but not with amoC probes. The evidence suggests that this RNA contains amoA and amoB sequence. Whether the 2.4 kb and the 1.1 kb fragments are the result of the processing of the full amoCAB mRNA remains to be determined. In Nitrosospira sp. NpAV there is evidence for the assumption that the amoAB genes can be expressed independently of the amoCAB promoter, because the amoA and amoC genes were expressed in E. coli from indigenous promoters located in the amoC to amoA intergenic region and directly upstream of the amoC gene, respectively [11]. The nature of the amoC copies, including the amoC copies not followed by amoAB in nitriﬁers need still to be characterized. Mutagenesis of amoC genes in ammonia oxidizers may help to elucidate the role of these polypeptides.

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