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MOLECULAR CHARACTERIZATION OF SOIL AMMONIA-OXIDIZING BACTERIA BASED ON THE GENES ENCODING AMMONIA MONOOXYGENASE

by

Jose Javier Alzerreca

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Soil Science
(Molecular Biology)

UTAH STATE UNIVERSITY
Logan, Utah

1999

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ABSTRACT

Molecular Characterization of Ammonia-Oxidizing Bacteria Based

on the Genes Encoding Ammonia Monooxygenase

by

J. Javier Alzerreca, Master of Science

Utah State University, 1999

Major Professor: Dr. Jeanette M. Norton
Department: Plants, Soils, and Biometeorology

Ammonia-oxidizing bacteria (AOB) are chemolithotrophs that oxidize ammonia/ammonium to nitrite in a two-step process to obtain energy for survival. AOB are difficult to isolate from the environment and isolated strains may not represent the diversity in soil. A genetic database and molecular tools were developed based on the ammonia monooxygenase (AMO) encoding genes that can be used to assess the diversity of AOB that exist in soil and aquatic environments without the isolation of pure cultures. The *amo* genes have excellent potential as molecular markers; since AMO is only found in the AOB and is essential for their metabolism, AOB must carry at least one functional copy of the *amo* operon. The operon is composed of at least three genes, *amoC, amoA,* and *amoB* (encoding for the subunits AmoC, AmoA, and AmoB). The *amoC* gene was first discovered and its sequence was obtained from *Nitrosospira sp.* NpAV. The *amo* operon is found in several copies within AOB genomes in the β-subdivision but as a single copy in γ-subdivision genomes. In Southern analysis, cross-hybridization was only observed between *amo* genes within a subdivision. The γ-subdivision *amo* sequences have higher identity values to the genes encoding the related particulate methane monooxygenase than to the β-subdivision *amo* sequences. Since *amoA* encodes the subunit containing the active site, it was sequenced entirely for all the strains studied (16 *amoA* sequences total). The *amoC* and *amoB* genes were also sequenced for several strains. The *amo* genes allow for better discrimination between closely related strains than the 16S rRNA genes. In all cases, the *amo* operon consists of *amoC,* followed by a variable length intergenic region, and then by *amoAB*. The

variability in length of the intergenic region is strain specific, and is therefore potentially useful for profiling AOB communities. The *amo*-gene database was the basis for the design of conserved oligonucleotide primers for the polymerase chain reaction (PCR). These primers were used to amplify *amo* sequences from a mixed template of DNA extracted directly from soil. Results indicate that the *amo* genes are excellent molecular markers for the assessment of AOB communities in the environment.

(151 pages)

DEDICATION

This work is dedicated to Jeanette Norton, my major advisor. Her support has been far more than academic. Her patience, counseling, and teaching abilities are the characteristics that separate the exceptional professor from the common one. The quality of this work has been greatly enriched by her comments and input.

This work is also dedicated to my family and friends, who believed in me and who supported me during the entire process. I could not have completed this work without the very significant aid I received from all the above mentioned, and I thank them for their respective contributions.

ACKNOWLEDGMENTS

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Jose Javier Alzerreca.

CONTENTS

Page

ABSTRACT.. iii

DEDICATION.. v

ACKNOWLEDGMENTS.. vi

LIST OF TABLES.. ix

LIST OF FIGURES.. x

CHAPTER

1. INTRODUCTION TO AUTOTROPHIC AMMONA OXIDIZING BACTERIA............. 1

The Process of Nitrification.. 1
Biochemistry and Genetics of Ammonia Oxidation.. 2

Biochemistry of Ammonia Oxidation.. 2
Biochemistry and Molecular Biology of the AMO Enzyme.................... 3
Relationship of AMO with pMMO.. 4

Taxonomy of the Autotrophic Ammona-Oxidizing Bacteria................................ 5
Analysis of the Diversity of Ammonia-Oxidizing Bacteria in the Environment.......... 8
Rationale and Project Objectives.. 11

Development of an *amo* Gene Database.. 12
Development of a Method to Assess Ammonia-Oxidizing Bacteria............ 13

References.. 15

2. A GENE ENCODING A MEMBRANE PROTEIN EXISTS UPSTREAM OF THE *amoA/amoB* GENES IN AMMONIA-OXIDIZING BACTERIA; A THIRD MEMBER OF THE *amo* OPERON?.. 22

Abstract.. 22
Introduction.. 22
Materials and Methods.. 23

Bacterial Strains, Plasmids, and Culture Conditions............................ 23
DNA Isolation, Hybridization Analysis, Amplification and Sequencing...... 25

Results And Discussion.. 27
References.. 30

3. THE *amo* OPERON IN *Nitrosococcus,* MARINE, AMMONIA-OXIDIZING γ-PROTEOBACTERIA.. 34

Abstract.. 34
Introduction.. 35

Materials and Methods.......... 35

Bacterial Strains and DNA Isolation.......... 35
Hybridization Analysis for the *amo* Operon.......... 36
Primers and PCR Amplification.......... 38
NOC Operon Cloning, PCR Purification, and Sequencing.......... 39
C-113 Operon and 16S rDNA Sequencing.......... 40
Sequence Comparison and Analysis.......... 40

Results and Discussion.......... 41

Marine Strain C-113.......... 45
Comparison of *amo* Genes and Their Putative Expression Products.......... 45

References.......... 48

4. DIVERSITY OF AMMONIA MONOOXYGENASE OPERON IN AUTOTROPHIC AMMONIA-OXIDIZING BACTERIA.......... 52

Abstract.......... 52
Introduction.......... 53
Materials and Methods.......... 55

Bacterial Strains, Plasmids, and Culture Conditions.......... 55
DNA Isolation, Hybridization Analysis, Amplification, and Sequencing.... 55
Sequence, Phylogenetic, and Topology Analyses.......... 58
Environmental Samples.......... 59

Results and Discussion.......... 61

Copy Number and Gene Arrangement of the *amo* Operon.......... 61
Diversity of Primary Sequences of *amo* Genes and the Encoded Peptides... 63
Secondary Structures and Topologies of Deduced Amo Proteins.......... 68
Application of *amo* Primers for Environmental Samples.......... 68

References.......... 71

5. CONCLUSIONS.......... 76

References.......... 80

APPENDIXES.......... 82

Appendix A Electronic Mail Permission Letters for Chapter 2 and Chapter 3.......... 83
Appendix B Authorship Letters for Chapter 2 and Chapter 4.......... 86
Appendix C Protein Expresion Studies for *amoC*.......... 89
Appendix D *amoC* and *amoAB* Transcription Studies with RT-PCR in *Nitrosospira sp.* NpAV.......... 93
Appendix E List of Genbank Entries.......... 99
Appendix F GenBank Entries.......... 102
Appendix G Amino Acid Alignments and Pair Table for the *amo* Genes and their Deduced Peptides.......... 136

LIST OF TABLES

Table		Page
1-1	Strains cultured in the Norton laboratory for this study	7
2-1	Bacterial strains and plasmids used in this study	24
3-1	Size of the *amo* genes and the intergenic spacer between *amoC* and *amoA* (C-A I.S.) in autotrophic ammonia-oxidizing bacteria and comparison to the *pmo* genes of *Methylococcus capsulatus* (Bath)	45
3-2	Identity and similarity values for *amo* and *pmo* genes and their deduced peptides	46
4-1	Bacterial strains used in this study	56
4-2	Primer sequences for amplification of *amo* genes from environmental samples	60
4-3	The length of the *amoC* to *amoA* intergenic variable region and the length of the amplified region spanning from the end of *amoC* into *amoA* (from primer 305F to 304 reverse)	70

LIST OF FIGURES

Figure Page

1-1 Pathway of NH_3 oxidation to NO_2^- via the enzymes ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO) in *Nitrosomonas europaea*. 3

2-1 Physical map of the 4.4 kb-, 5.7 kb-, and 16.8 kb-sized *Eco*RI restriction fragments from the genome of *Nitrosospira* sp. NpAV. 26

2-2 Nucleotide sequence for the copy of *amoC3* gene and flanking regions from *Nitrosospira* sp. NpAV contained on the 4.4 kb *Eco*RI restriction fragment........................ 28

2-3 PCR amplification of ammonia oxidizer genomic DNA using the *amoC* and *amoA* specific primers.. 30

3-1 Southern blots with gDNA from three AOB strains.. 37

3-2 Physical map of the NOC *amo* operon.. 38

3-3 DNA sequence of the NOC *amo* operon.. 43

3-4 Amino acid sequence alignment for the deduced peptides from the *amoA* and *pmoA* genes....... 47

4-1 Southern blot of ammonia oxidizer genomic DNA with *amoA* probe from *N. europaea* showing the multiple copies of *amoA*.. 58

4-2 Physical map of the *amo* operon in *Nitrosospira sp.* NpAV and *Nitrosococcus oceanus* Showing the four ORFs: *amoC*, *amoA*, *amoB*, and ORF4.. 60

4-3 Low stringency Southern blot of ammonia oxidizer genomic DNA.. 61

4-4 Southern blot of ammonia oxidizer genomic DNA with *amoA* probe from *N. europaea*........... 64

4-5 Secondary structure predictions for AmoC, AmoA, and AmoB peptides from *Nitrosospira sp.* NpAV (NAV), *Nitrosomonas europaea* (NEU) and *Nitrosococcus oceanus* (NOC)............ 66

4-6 Neighbor joining unrooted tree for the deduced AmoA peptide sequences based on the Clustal W alignment.. 67

4-7 PCR products of the *amoA* internal region and the *amoC* to *amoA* intergenic region for pure culture representatives and for extracted soil DNA.. 69

CHAPTER 1

INTRODUCTION TO AUTOTROPHIC AMMONIA OXIDIZING BACTERIA

The Process of Nitrification

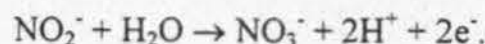
The nitrogen (N) cycle is a complex series of reactions in which N is continually recycled between the atmosphere, land, oceans, rivers, and lakes. Nitrification is defined as the conversion of reduced N in the form of ammonia (NH_3) or ammonium (NH_4^+) to oxidized N in the form of nitrite (NO_2^-) or nitrate (NO_3^-). The bacteria that mediate the nitrification process are known as nitrifiers.

Nitrification is an important process in the nitrogen cycle [1] for understanding and possibly controlling the form of N in soil. When N is in the reduced form of NH_4^+, it will tend to remain in the soil, while if oxidized to NO_2^- and NO_3^- it can readily leach out of the soil or be lost as a gas through denitrification. Therefore, nitrification may lead to N loss from soil systems. Nitrification rates are dependent upon the availability of the substrate, NH_3/NH_4^+, and by the nitrifier population. In many soils, the amount of NH_3/NH_4^+ is limited and nitrifier activity depends on the decomposition of organic nitrogen compounds [2] releasing NH_3/NH_4^+ through mineralization. In most soils, the predominant nitrifying bacteria are chemolithoautotrophs gaining metabolic energy from the oxidation of inorganic N and using CO_2 as a carbon source [3]. The heterotrophic nitrifiers are of limited importance in most environments and will not be discussed further in this thesis.

The process of nitrification occurs in two steps and each step is mediated by two different groups of nitrifying bacteria. The first step is the autotrophic oxidation of NH_3 to NO_2^- by the ammonia-oxidizing bacteria (AOB) such as those found in the *Nitrosomonas* and *Nitrosospira* genera:

$$NH_3 + O_2 + 2H^+ + 2\,e^- \rightarrow NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4e^-.$$

The second step in the process is the further oxidation of NO_2^- to NO_3^- by the nitrite-oxidizing bacteria such as those found in the *Nitrobacter* genus:



$$NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^-.$$

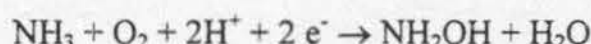
This thesis focuses on the AOB that are responsible for the first, typically rate-limiting step of nitrification. Most known AOB are found in the β-subdivision of the proteobacteria, with a few representatives in the γ-subdivision. Their habitats can be terrestrial as well as marine [4-6].

Overall, the energy obtained by the AOB from ammonia oxidation is relatively small [7]. Furthermore, because these bacteria are autotrophic, the reductant obtained during ammonia oxidation is used for the fixation of CO_2, their sole carbon source, via ribulose bisphosphate carboxylase/oxygenase [8]. Therefore, these bacteria often produce close to the minimum energy required to cover all their maintenance requirements, and typically have slow grow rates and long generation times [1]. The enzyme ammonia monooxygenase (AMO) catalyzes the first step in the ammonia oxidation and is therefore indispensable for the cells' survival; without it, no energy can be obtained.

Biochemistry and Genetics of Ammonia Oxidation

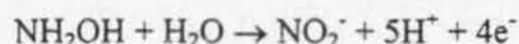
Biochemistry of Ammonia Oxidation

The AOB have an extensive cytoplasmic membrane system, in which many of the enzymes responsible for ammonia oxidation and electron transport exist. In the first step in ammonia oxidation, the membrane-bound AMO oxidizes ammonia to hydroxylamine according to the following reaction (Fig. 1-1) [9]:



$$NH_3 + O_2 + 2H^+ + 2\,e^- \rightarrow NH_2OH + H_2O$$

The next oxidation step is mediated by the periplasmic enzyme hydroxylamine oxidoreductase (HAO), this chemical reaction yields the electrons used for energy production:



$$NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4e^-$$

In *Nitrosomonas europaea*, to generate the proton motive force, two of these four electrons are transferred initially to cytochrome 554 and flow down through an electrochemical gradient made of cytochromes such as P460, c-cytochromes, and end in the terminal aa_3 oxidase, where they combine with O_2 to form H_2O [10]. Under low O_2 availability, *N. europaea* can denitrify using nitrite as the terminal electron acceptor.

With each cycle of ammonia oxidation, two electrons are returned via an unknown pathway to AMO, to regenerate more hydroxylamine. Therefore, the net gain is only two electrons [10].

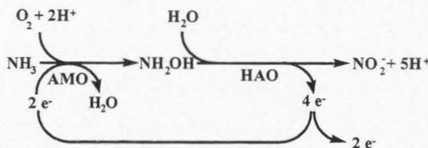


Fig. 1-1. Pathway of NH_3 oxidation to NO_2^- via the enzymes ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO) in *Nitrosomonas europaea*. Figure adapted from reference [9].

Biochemistry and Molecular Biology of the AMO
Enzyme

AMO is a membrane-bound copper-containing enzyme [9,11]. AMO is yet to be purified in an active form, its subunit composition and tertiary structure have not been fully elucidated [12]. The principal substrate for AMO is thought to be NH_3 rather than NH_4^+ [13]. If a particular ammonia oxidizer has the ability to produce urease, then an alternative substrate can be urea [14]. AMO catalyzes the oxidation of a wide variety of non-polar compounds [10], but these co-oxidations are incapable of supporting growth. AMO can also oxidize methane [8] and plays a role in the metabolism of halogenated hydrocarbons [15,16] and N_2O production [17]. Acetylene is a suicide substrate for AMO; it binds strongly to the active site and inactivates the enzyme [9,18]. C-14 labeled acetylene was used to purify the subunit containing the active site, the AmoA subunit. AMO is photosensitive and irreversibly inactivated by light [19].

In *N. europaea*, AmoA is a membrane bound 26-27 kDa peptide [19]. In addition to AmoA, at least two other subunits, AmoB and AmoC, are believed to be structural subunits of AMO [Chapter 2][19]. AmoB is an approximately 43-kDa polypeptide that co-purifies with AmoA [18]. The function of this iron-copper containing polypeptide is not known. An additional subunit, AmoC, has been proposed to be a structural component of AMO [Chapter 2][20]. In *N. europaea*, the deduced AmoC has 271 amino acid residues and is approximately 31.4 kDa in weight [20].

The AMO operon consists of at least three genes, *amoC*, *amoA,* and *amoB*, which encode for the three known subunits of the AMO enzyme [20,21]. The *amoA* and a partial *amoB* were first sequenced

from *N. europaea* using two overlapping clones [22]. This *amoA* gene consists of 831 bases followed directly by the start of *amoB*. The full-length *amoB* gene, also sequenced from two overlapping clones, is 1148 bp in *N. europaea* [23]. The *amoB* gene is found immediately downstream from *amoA* in all β-subdivision ammonia-oxidizing strains examined [Chapter 4].

N. europaea has two copies of the AMO operon in its genome [22]. It should be noted that the *N. europaea amoA* and *amoB* sequences presented above were obtained from genomic DNA and could be from either of the two *N. europaea* copies or a combination of both (chimeric sequence). *Nitrosospira sp.* NpAV has three copies of the AMO operon which are nearly identical [24]. Most ammonia oxidizer strains examined to date have several copies of the AMO operon [Chapter 4]. Only *Nitrosococcus oceanus* and marine strain C-113, both γ-subdivision strains, have a single copy [Chapter 3].

The functions of the multiple copies of the AMO operon have not been determined. One possible explanation is that several copies of the same gene are necessary to have enough enzyme product to satisfy the cells' needs. However, multiple copies of genes encoding functional proteins are unusual in prokaryotes, in contrast to the common occurrence of multiple copies of ribosomal genes. The genes encoding HAO are also present in multiple copies [25]. One copy of the gene encoding the 63-kDa subunit of HAO from *N. europaea* has been fully sequenced [26].

AMO is a member of a novel class of copper-containing monooxygenases, and its principal enzymatic function makes it unique as well as indispensable for the AOB. These properties make AMO an ideal functional marker. Only AOB possess this enzyme and the genes that encode it. Functional markers may be used to identify specific genera from an unknown conglomerate of bacteria in environmental samples. However, one other enzyme from the methane-oxidizing bacteria has been found to share many characteristics with AMO, the particulate methane monooxygenase (pMMO) [8]. To develop molecular tools for the *amo* genes, we need to take into account and compare similarities with the *pmo* genes.

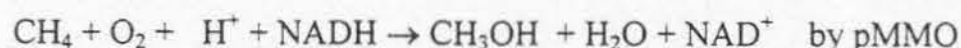
Relationship of AMO with pMMO

AMO and pMMO are related functionally and structurally [8,11,27]. The predicted amino acid sequences have a high level of identity. These enzymes may be considered analogous because both have a similarly crucial role for the cells' metabolism. AMO and pMMO share similar substrates and inhibitors,

and both may contain a tri-nuclear copper center at the active site [11,28,29]. pMMO has been purified from *Methylococcus capsulatus* (Bath) and found to also contain iron [30], and there is evidence that AMO may also contain an iron center [31].

The analogous biochemical reactions of interest mediated by these enzymes are:

$$NH_3 + O_2 + 2H^+ + 2\,e^- \rightarrow NH_2OH + H_2O \qquad \text{by AMO}$$



$$CH_4 + O_2 + H^+ + NADH \rightarrow CH_3OH + H_2O + NAD^+ \qquad \text{by pMMO}$$

While NH_3 and CH_4 are the primary substrates for AMO and pMMO, respectively, both enzymes can process a variety of substrates, including ammonia, methane, chlorinated hydrocarbons, short-chain alkanes and alkenes, de-halogenating short-chain halogenated hydrocarbons, and the hydroxylation of aromatic compounds [32-34].

Like AMO, pMMO consists of at least three membrane bound subunits: pMmoC [30,35], pMmoA (27 kDa) [27,30], and pMmoB (45-47 kDa) [27,30]. These subunits are encoded by the *pmo CAB* operon [27,30,35]. Amino acid sequences deduced from *amoA* and *pmoA* show an approximately 60% similarity and 40% identity [Chapter 4]. Based on sequence comparisons, the level of conservation between *amoA* and *pmoA* shows a stronger correlation with the phylogenetic relatedness of the organisms than with the function of the gene products; three identity groups for the α, β and γ subdivisions are observed instead of two identity groups representing AOB and methanotrophs [Chapter 3][8]. Our ability to use *amo* genes as functional markers for AOB is dependent upon our knowledge of the diversity of both *amo* and *pmo* genes in β- and γ-subdivision bacteria, since we are interested in applying these tools in soil environments where both AOB and methanotrophs may co-exist.

Taxonomy of the Autotrophic Ammonia-Oxidizing Bacteria

The ammonia-oxidizing bacteria are part of the proteobacteria (purple bacteria) found in both the β-subdivision [36-39] and the γ-subdivision [38-41]. The proteobacteria are presumed to have photosynthetic ancestry [42,43]. These β and γ-subdivision AOB groups were formerly included in the family *Nitrobacteriaceae* [44,45], defined by their ability to grow as chemolithotrophs by the oxidation of ammonia to nitrite or the oxidation of nitrite to nitrate. However, this previous classification does not

coincide with our current understanding of the phylogeny of these bacteria. The genus names of the ammonia oxidizers begin with *Nitroso*, the nitrite oxidizers begin with *Nitro*. No organism has been found to oxidize ammonia directly to nitrate. Initially, classification to the genus level for the AOB was done based on cell shape and the arrangement of the intracytoplasmic membranes. The recognized genera included: *Nitrosospira. Nitrosolobus, Nitrosovibrio, Nitrosomonas,* and *Nitrosococcus*.

The current standard for bacterial classification and phylogeny is the use of ribosomal sequences primarily the 16S rRNA. Based on 16S rRNA sequencing studies, it has been shown that the family *Nitrobacteriaceae* has several phylogenies with members belonging to four different subdivisions of the proteobacteria [36,37]. The known AOB are limited to the β- and γ-subdivisions. It has recently been proposed that the AOB in the β-subdivision of the proteobacteria be reclassified into two genera, *Nitrosomonas* and *Nitrosospira*, the latter consisting of the original genera *Nitrosospira, Nitrosolobus* and *Nitrosovibrio* [6,36]. However, Teske et al. [37] recommend further analysis before including *Nitrosolobus* into the genera *Nitrosospira*, noting that internal compartmentalization or membrane structure is distinct from the other two (both lack the extensive system observed in *Nitrosolobus* [45]). In this thesis the original genus names are retained to avoid confusion. The taxonomy of the AOB is changing rapidly and is likely to be revised frequently as new isolates are characterized.

For the determination of the AOB taxonomy, the *amo* genes are an important complement to 16S rRNA sequences [Chapter 4]. Classification using 16S rRNA sequences is generally adequate but may be insufficient for discrimination at the strain level since the 16S rRNA sequence identity between strains within AOB species is generally greater than 97%. For example, the 16S rRNA identity is 98.5% between *Nitrosospira* strain AHB1 and *Nitrosolobus multiformis* C-71[46]. For increased definition between closely related AOB strains, the intergenic spacer region between the 16S and 23S genes in the ribosomal operon may be useful [47]. Aakra et al. [47] presented a study for phylogenetic analysis utilizing the 16S-23S rDNA intergenic spacer region for AOB. They concluded that this spacer region provides phylogenetic trees that confirm previous 16S rDNA reports. However, the spacer region provides insight on recent evolutionary events, and is useful at the lower taxonomic levels. Their ribotyping experiments revealed that the AOB may all contain only one copy per genome of the *rrn* operon, and that this operon is arranged just

like the typical bacterial operons: 16S rRNA gene, spacer, 23S rRNA gene. Alternatively, for finer resolution phylogenetic analysis, the functional *amo* genes are another potential molecular tool [Chapter 4].

All reported soil AOB belong to the β-subdivision of the proteobacteria [6,36,37]. Currently, the isolated γ-subdivision AOB are all marine organisms. However, there is some limited evidence that freshwater γ-subdivision AOB may exist [48]. Eight representatives from the β-subdivision and two marine strains from the γ-subdivision (*N. oceanus* [37,43], and strain C-113) are currently maintained in the Norton laboratory and were used for the research reported in this thesis (Table 1-1).

Most previous biochemical and molecular biology studies have focused on *N. europaea*, originally isolated from sewage, and which can be grown conveniently under laboratory conditions [1]. However, it appears that *Nitrosospira spp*. may be more common in soils, based on cultured-based evidence [49-51] and PCR evidence [52]. *Nitrosospira* species as well as *Nitrosomonas* species can be seen in never-tilled soils, however, on cultivated soils *Nitrosospira* species are dominant [53]. While it is clear that a great variety of AOB exist in different environments, in soil systems, closely related strains may co-exist. The ability to differentiate between these closely related strains is necessary for a more accurate assessment of their diversity and function in the soil. Molecular markers and tools are required for this fine level of differentiation.

Table 1-1

Strains cultured in the Norton laboratory for this study

Nitrifier strain	Subdivision
Nitrosospira sp. NpAV	β
Nitrosospira briensis C-128	β
*Nitrosolobus multiformis** ATCC 25196	β
*Nitrosolobus multiformis** 24C	β
*Nitrosovibrio tenuis** NV-12	β
Nitrosomonas europaea ATCC 19178	β
Nitrosomonas eutropha C-91	β
Nitrosococcus oceanus C-107	γ
Marine strain C-113	γ

* May be reclassified in *Nitrosospira* genus.

Analysis of the Diversity of Ammonia-Oxidizing Bacteria in the Environment

The AOB are a key functional group in the terrestrial and aquatic N cycle. The diversity of these bacteria in the environments has been of interest since the beginnings of soil microbiology as a discipline [54]. Early work on nitrification in the soil generally focused at the process level, not on the diversity of the bacteria involved because of the technical difficulties involved in the isolation and maintenance of the AOB. The AOB do not form visible colonies on selective solid media and therefore must be isolated from liquid cultures. Pure cultures of AOB have extremely slow growth rates [6,7], the fastest doubling time reported being 8 hours, but more frequently within the range of 11 to 50 hours [1], compared to 30 minutes for *Escherichia coli*. In our laboratory conditions, growth of a batch culture for genomic DNA isolation takes approximately one month to achieve maximum biomass. These and other reasons such as non-standard growth media, non-standard growth conditions, and ease of heterotrophic contamination have made AOB difficult to study [55]. Some initial attempts to analyze the diversity of AOB utilized flourescent antibody-detection techniques in combination with direct microscopy to count specific strains of AOB. However, this approach has limitations since antibody production is dependent on having pure strains and the antibodies are highly specific for each strain [55]. Furthermore, any approach based on laboratory isolation cultures may not reflect the true frequencies of the different strains in soil, since isolation biases affect the outcome. A particular strain can be selected out based on the media, temperature, and other factors and this strain may not be particularly predominant in its native soil [56]. A more appropriate approach, especially for the AOB, is the use of molecular markers such as 16S rRNA and *amo* to analyze soil DNA since results are not directly impacted by the above mentioned problems.

Molecular techniques have recently been used to study the diversity of AOB and give us a better understanding of the microbial ecology of these bacteria [4-6,37,48,57,58]. The first step in order to use molecular techniques for the study of bacteria in soil is the extraction of DNA. DNA may be obtained in several ways such as cell centrifugation and lysis or by direct cell lysis from soil samples [55]. Ideally, the extracted DNA must represent as close as possible the true frequencies of the different bacterial populations in soil. After extraction, purification of DNA is generally required since soil DNA samples usually contain

impurities like humic acid, which inhibits subsequent biochemical reactions such as the polymerase chain reaction (PCR) [55]. Several DNA purification methods exist such as gel electrophoresis, glass-milk resin, and various chemical treatments [55].

Since the AOB are a small fraction of the total soil bacteria (generally less than 0.1%), we need to increase our detection sensitivity by amplifying the target DNA using PCR. PCR uses *Taq* polymerase, two specific primers (small DNA oligonucleotides that match the sequence of the target DNA at each end of the desired product) and the target template DNA. PCR amplifies exponentially the DNA between the two primers, so that a few hundred starting DNA molecules become millions. Essentially, PCR provides the way to obtain enough specific DNA for detection and analysis. PCR is amazingly fast compared to other techniques, and is practical. The greatest technical problem with PCR is the ease of cross-contamination of the initial reaction under laboratory conditions. PCR can be performed using primers specific for the AOB and purified soil DNA as a template. The specificity of the primers determines the composition of the PCR products which may be mixed. After PCR is accomplished, the products are first examined on standard agarose gels. The amplified products can later be used to generate a library of clones; alternatively, PCR products can be analyzed directly, with agarose gels or by denaturant gradient gel electrophoresis (DGGE). The goal of these analyses is to investigate the community composition of the AOB.

A clone library is made when PCR products of relatively small sizes (<6 kb) are ligated into cloning vectors. The plasmid vector, with this new inserted DNA, is then transformed into and perpetuated within a cloning cell host, such as *E. coli*. The transformed *E. coli* strains are grown and the plasmid DNA is harvested and screened for the desired inserts. These inserts are then sequenced and their relationship to each other and known strains is analyzed.

Agarose gels can be used for direct analysis if PCR products vary in size. Alternatively, PCR products can be restriction digested and the resulting patterns observed; however, in complex communities, it may be problematic to relate the patterns to individual strains. DGGE is a powerful tool designed to separate the DNA products not only by size, but also by base composition. Because double-stranded DNA denatures according to its base sequence, higher GC-content DNA will denature at higher temperatures or higher denaturant concentration than DNA with high AT content. A polyacrylamide gel is prepared with a

gradient of denaturing agent either parallel or perpendicular to the direction of the electrical field. The mixture of double stranded DNA (i.e. PCR products from mixed templates) is separated according to its stability resulting in bands representing the different strains' DNA sequences. Individual bands are presumed to be associated with specific DNA sequences, representing the diversity of the original template. The technical problems with DGGE are that setup is complicated and time consuming, and bands from DNA of different base composition may co-migrate and overlap.

Some examples of these techniques applied for the study of AOB diversity follow. They are fundamentally similar, and differ only in the PCR target. For these preliminary environmental studies of AOB, molecular markers such as rRNA and the *amo* functional genes have been used succesfully. Stephen et al. [6] used rRNA as target, Sakano and Kerkhof [48] used rRNA as well as the functional *amoA* gene, and Rotthauwe et al. [52] used exclusively the *amoA* gene.

Stephen et al. [6] performed PCR using 16S rRNA targeted primers on extracted DNA from various environments to analyze the composition of the different soil and marine samples for the β-subdivision AOB. Based on this study, the genus *Nitrosospira* separates into four general clusters: one marine-type cluster, and three soil-type clusters. It appears that marine *Nitrosospira spp.* are distinct from soil type species. The *Nitrosomonas* genus appears to also separate into three clusters, an entirely marine-type cluster, and two soil-marine type clusters. Overall, some of these clusters are preliminary, and they must be confirmed by selective experiments designed to isolate representative pure cultures. Stephen et al. [6] add that the presently available cultured species may not be representative of the diverse marine and soil AOB and suggest that more research needs to be done to obtain a more accurate representation of AOB in pure cultures. Their data also suggests that polluted environments may select for a particular AOB population, since *Nitrosomonas* type sequences were obtained only from polluted samples, compared to *Nitrosospira* type found in polluted and non-polluted samples. In general, *Nitrosospira spp.* can be found in a variety of environments including soil, freshwater, and marine [6,58].

A second study by Stephen et al. [59] utilized a combination of PCR and DGGE to analyze natural AOB composition in environmental samples that varied in pH. They used the 16S rDNA as target for the PCR experiments, and obtained products that where initially analyzed by DGGE. Although they obtained

some separation for these PCR products, they encountered the problem of not being able to relate the visualized bands with specific AOB strains. Furthermore, overlapping of bands was also observed, making recognition more difficult. To actually determine which strains were being observed, the bands had to be excised, cloned, and sequenced. To relate the PCR bands with known strains using DGGE, they further designed probes based on the limited 16S rRNA sequences available for the AOB. Using these probes they were able to distinguish the different AOB clusters, *Nitrosomonas* and *Nitrosospira*, at the various degrees they existed in the samples.

Sakano and Kerkhof [48] used molecular tools to evaluate the changes in the populations of AOB within an ammonia biofilter. Using rRNA-targeted PCR, they concluded that the diversity of heterotrophic bacteria decreased as the biofilter experiment progressed. Using *amoA* as an AOB marker, they observed a decrease in AOB diversity as well, with *Nitrosospira*-like strains being the dominating populations within the biofilter at the end of the biofilter experiment.

Rotthauwe et al. [52] investigated AOB populations from several environments including rice roots, activated sludge, freshwater and enrichment cultures. Using a conserved internal region from *amoA/B*, they obtained PCR products that only originated from AOB, without obtaining background signal from the related *pmo* gene. They found that representatives from the *Nitrosospira* and *Nitrosomonas* genera were most common. They concluded that using *amoA* as a functional marker allows for very high specificity, and high resolution between closely related populations.

While these reports show the potential of the *amo* genes for environmental study, achieving a true representation of the diverse AOB using these genes requires an iterative process of primer design and application. The more comprehensive our knowledge of the diversity of the *amo* genes, the more successfully our molecular tools will represent the environmental AOB.

Rationale and Project Objectives

The development of molecular tools based on the *amo* genes, is a first step towards determining the community composition of AOB. We believe that the *amo* genes will be excellent functional markers with several advantages over the use of 16S rRNA genes. Using 16S rRNA genes may result in the

detection of AOB strains related to known AOB (since the primers and probes are designed from them). Furthermore, they may also detect closely related strains, which are not AOB. Using the *amo* genes guarantees that only AMO-containing populations will be detected, because AMO is indispensable for their survival and only found in AOB. In the future, AMO-based tools may be used to examine environmental control of gene expression. Studies with the *amo* genes also bring us insight in the evolution of functional genes.

To use the *amo* genes in environmental analysis, two observations need to be taken in account. First, PCR primer design is critical for the success of this analysis. Depending on the goal, primers can be designed within conserved regions of the *amo* genes to amplify these segments from samples. They can be designed to detect a broad range of AOB, or they could be made to discriminate and amplify particular AOB genera. Second, in order to design any primer so it performs as we expect, we need to have a fair idea of the diversity of the AMO-genes sequences within the environment. For this reason, we need to develop an *amo* gene database from diverse representatives from the different environments, soil and aquatic.

This research consists of two main phases: 1) the creation of a genetic database of the *amo* genes from pure cultures representing members from the β- and γ-subdivisions and 2) the development of molecular tools for the evaluation of these *amo*-containing populations in environmental samples.

Development of an *amo* Gene Database

The genes for AMO (*amoC*, *amoA* and *amoB*) are functional [20], indispensable for survival, and only found in the AOB. Therefore, they are ideal molecular markers for environmental analysis of AOB. By understanding the diversity of the *amo*-gene sequences from representatives from a variety of environments, our ability to obtain the desired amplicons is increased. Developing an *amo* gene database becomes significant, because it allows the design of a variety of specific primers for PCR that can generalize, group, discriminate or individualize as desired between the various AOB strains, based on the similarities and differences of the database sequences coding (conserved) and non-coding (non-conserved) regions.

Furthermore, the PCR products from soil DNA can then be sequenced and analyzed against the *amo*-gene database for relationship to known strains. Novel strains may be detected as well. We have used

the *amo* genes, particularly *amoA*, to develop this initial database. The first objective of this research was to sequence additional representatives of the AOB such as *Nitrosospira spp.* and *Nitrosolobus spp.* Apart from the work of this research group, most full-length sequences encoding functional products were available only from *N. europaea*. We are committed to sequence *amoA* for as many nitrifier strains as possible, since AmoA is the most studied subunit of the AMO enzyme. The current database also contains *amoB* and *amoC* sequences that we and others have obtained. As time allows, additional sequences for *amoB* and *amoC* from several AOB strains will be obtained. Most strains have multiple copies of the three-gene *amo* operon, and sequencing of these individual copies has been done for several strains in our laboratory [Chapter 4][24].

Development of a Method to Assess Ammonia Oxidizing Bacteria

For diversity analysis, PCR can be used directly on DNA extracted from environmental samples, utilizing AOB conserved primers. To obtain a true representation of the AOB diversity within environmental samples, the key element is the design of these conserved primers [Chapter 4]. Primers should be specific enough to amplify only the *amo* genes but broad enough to include *amo* sequences from unknown but related AOB strains. Based on the *amo*-gene database from pure cultured AOB, molecular primers and probes will be designed to evaluate the community composition in selected soil samples [Chapter 4]. In general, labeled oligonucleotide-probes can be used to identify AOB [4,58,60-63], to differentiate between active and inactive components and expose spatial relationships between species [63,64].

If the conserved primers are located inside an individual *amo* gene, PCR products will be obtained that are practically the same length. Cloning the PCR products into a vector to generate a cloning library is the most common approach to analyze these products. The clones are then sequenced and the results are examined. However, the *amo* genes have been demonstrated to be difficult to clone, presumably because *amo*-coding sequences may be transcribed from the vector and translated in the host cell. These products may be detrimental and toxic to the cloning-host cell [23]. The availability of full-length sequences [Chapter 4] provides more choices for potential primer targets to generate desirable PCR products.

PCR products can also be examined directly for diversity and sequence fingerprinting using DGGE. Analysis of the different PCR products from environmental samples, by sequencing of clone libraries or DGGE analysis would require some additional time; the work on these analysis methods is ongoing in the Norton laboratory but outside the scope of this thesis.

During the development of the *amo*-gene database, we observed that it was possible to obtain PCR products of different sizes spanning the *amoC* to *amoA* intergenic region, the size being specific for each strain [Chaper 4]. The intergenic region between *amoC* and *amoA* is found in all strains studied, and *amoA* always follows *amoC* after this non-coding region [Chapter 4][21]. The size of this region is unique for each species, therefore making it an ideal location to use for PCR analysis. Using primers targeting conserved *amoC* and *amoA* regions, the PCR product-size differences can be accounted by the strains' particular intergenic region sizes. High-resolution agarose gels can be utilized to obtain better PCR band separation. This method is simple and less time consuming since it only requires PCR and gel electrophoresis. Aakra et al. [47], using rRNA intergenic regions, were able to separate closely related AOB strains, which further validates this approach.

In future applications using the *amo* genes to assess the diversity of AOB it will be desirable to evaluate the different populations' sizes. For this purpose, PCR should be quantitative. Quantitative PCR is accomplished by running a series of reactions with the sample templates spiked with known amounts of an internal standard template in a competitive PCR reaction. The frequencies of the different AOB sequences will be in direct relationship to the abundance of these sequences in the sample, thus giving us better insight of the nature of the AOB populations. The first step in devising a quantitative PCR system is the production of an appropriate internal standard. The *amo*-genes sequence information in this thesis can be used to accomplish this goal.

This thesis contains results of three investigations relating to the *amo* genes in AOB. In Chapter 2, we present the discovery and sequencing of the third gene of the *amo* operon, *amoC*, and demonstrate its presence in all the three copies of the *amo* operon in *Nitrosospira. sp.* NpAV. We further investigate if the *amoC* gene is present in other AOB. Chapter 3 contains the first sequences of the *amo* operon for two γ-subdivision strains, *N. oceanus* and strain C-113. We examined the relationship of these genes with the

representative *amo* genes from the β-subdivison AOB and the *pmo* genes from *M. capsulatus* (Bath). The diversity of the *amo* operon in a variety of AOB strains and the development and application of molecular tools to environmental samples are described in Chapter 4. The conclusions of these studies are summarized and discussed in Chapter 5.

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CHAPTER 2

A GENE ENCODING A MEMBRANE PROTEIN EXISTS UPSTREAM OF THE *amoA/amoB* GENES IN AMMONIA-OXIDIZING BACTERIA; A THIRD MEMBER OF THE *amo* OPERON? [1]

Abstract

The gene cluster encoding ammonia monooxygenase (AMO) in the chemolithotrophic soil bacterium *Nitrosospira sp.* NpAV was found to contain a third open reading frame, termed *amoC*, upstream of the genes, *amoA* and *amoB*, that encode two subunits of AMO. The *amoC* gene and its flanking regions were isolated and sequenced from a 4.4 kb *Eco*RI fragment that contains one of three copies of the ammonia monooxygenase gene cluster. The presence of this gene upstream of the other two *amoA* gene copies in *Nitrosospira* NpAV as well as upstream of *amoA* genes in the genomes of other ammonia oxidizing nitrifiers (strains in the genera *Nitrosomonas, Nitrosospira*, *Nitrosolobus,* and *Nitrosovibrio*) was confirmed by using genomic DNA, oligodeoxyribonucleotide primers, and PCR. The *amoC* gene in *Nitrosospira* sp. NpAV encodes a 270-amino-acid polypeptide of approximately 31 kDa.

Introduction

Nitrosospira sp. NpAV (NAV) is a chemolithotrophic soil bacterium that derives its metabolic energy solely from the aerobic oxidation of ammonia to nitrite. This process involves the enzymes ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO), both of which have had their physiological and biochemical characteristics extensively studied [1-9]. AMO is a membrane-bound multi-subunit enzyme responsible for the conversion of NH_3 to hydroxylamine [3]. A 27 to 30 kDa-sized membrane-associated protein which contains the active binding site of AMO [3] and a second iron-copper protein of 38 to 43 kDa have been isolated as probable AMO subunits, AmoA and AmoB,

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[1] Reprinted from FEMS Microbiology Letters, Volume 150, Klotz, M.G., Alzerreca, J., and Norton, J.M. A gene encoding a membrane protein exists upstream of the *amoA/amoB* genes in ammonia-oxidizing bacteria; a third member of the amo operon? Pages 65-73. Copyright 1997, with permission of Elsevier Science (Appendix A). See also letter on authorship (Appendix B).

respectively, from *Nitrosomonas europaea*. The sequences of the genes *amoA* and *amoB*, coding for these subunit proteins, have been determined from overlapping clones [8,10,11]. Because the *amoB* gene extends immediately downstream from the *amoA* gene in all ammonia oxidizer strains yet examined [10-17], it has been concluded that the *amoA* and *amoB* open reading frames (ORF) constitute the structural portion of the *amo* operon [10,11,18]. We have recently confirmed the presence of these two ORFs in the genome of NAV (three copies: [14]), *Nitrosomonas eutropha* (two copies: [15]), *Nitrosovibrio tenuis* (two copies: [16]), *Nitrosospira briensis* (three copies: [17]) and *Nitrosolobus multiformis* (three copies: [19]) by the isolation and sequencing of complete *amoA* ORFs and flanking sequences. The AMO enzyme is related to the particulate methane monooxygenase (pMMO) which is found in the methane oxidizers such as *Methylococcus capsulatus* (Bath) [20]. AMO and pMMO are the first representatives of a new class of copper-containing monooxygenases [20].

In our recent report on the near identity of *amoA* genes in the three copies of the *amo* operon in the genome of NAV [14] we observed that the upstream flanking regions of the three *amoA* gene copies were also remarkably similar. In search of an explanation for this result and as suggested by the finding of a putative pMmoC protein as a third subunit of the particulate methane monooxygenase [21,22], we initiated the search for a third gene in the *amo* gene clusters. In this chapter, we present the sequence for the ORF and the flanking regions of the *amoC* gene that resides upstream of the *amoA3* gene on the 4.4 kb *Eco*RI DNA fragment from the genome of NAV [14].

Materials and Methods

Bacterial Strains, Plasmids, and Culture Conditions

The bacterial strains and plasmids used in this study are summarized in Table 2-1. Ammonia oxidizers were cultivated in 500 ml static batch cultures of ATCC media 929 at 30°C with periodic pH adjustment and harvested by centrifugation after approximately 3 weeks of growth as described recently [14].

Table 2-1
Bacterial strains and plasmids used in this study

Strains	Relevant phenotype or genotype	Source or reference
Nitrosospira sp. NpAV	Wild type ammonia oxidizer from soil	E. Schmidt (Univ. of Minnesota) via M.A. Bruns (Univ. of CA, Davis)
Nitrosomonas europaea ATCC 19178	Isolated from soil	ATCC
Nitrosolobus multiformis ATCC 25196	Type strain isolated from soil	ATCC
Nitrosospira briensis C-128	Isolated from soil	S. Watson and F. Valois (Woods Hole Collection)
Nitrosospira sp. 39-19	Isolated from agricultural soil in Minnesota	E. Schmidt (Univ. of Minnesota) via M.A. Bruns (Univ. of CA, Davis)
Nitrosovibrio tenuis NV-12	Isolated from soil	S. Watson and F. Valois (Woods Hole Collection)
E. coli InvαF'	One Shot™ competent cells ϕ*lacZ*	Invitrogen®, San Diego, CA
E. coli DH5α	F^- Φ80 Δ*lacZ* M15 *recA1* Sm^R	BRL, Bethesda, MD

Plasmids		
pCR™II and pCR™2.1	Amp^R , Kan^R, *placZα*, colE1	Invitrogen®, San Diego CA
pNA10-9	PCR™II with insert of amo10 -amo9 amplicon from the 4.4 kb *Eco*RI fragment	this study
PNA101-9	PCR™II with insert of amo101-amo9 amplicon containing *amoC3* from the 4.4 kb *Eco*RI fragment	this study
PNA-5	PCR™II with insert of *amoA3* amo10-amo5 amplicon from the 4.4 kb *Eco*RI fragment	[14] Genbank U38251

DNA Isolation, Hybridization Analysis, Amplification, and Sequencing

Genomic DNA (gDNA) was isolated from the ammonia oxidizer cells following the procedure of McTavish et al. [10,11]. The gDNA was restriction digested and electrophoresed on a 0.6% agarose gel in 1x TAE. Previous analysis of NAV gDNA by Southern analysis identified three easily separable *Eco*RI fragments of approximately 4.4, 5.7 and 16.8 kb which hybridized to the *amoA* (Fig. 1 in [14]) and *amoB* gene probes. The migration distances of these sizes were determined using two molecular weight markers and then the corresponding regions were cut from the gel. The DNA was purified from these gel slices using a Geneclean® III kit (Bio 101, San Diego). In order to obtain sequences flanking the known regions, a chromosome crawl approach [23] was applied to the 4.4 kb fragment-containing fraction. Briefly, the 4.4kb *Eco*RI fraction was self ligated and then cut with *Nsi* I to linearize. The linear size-fractionated DNAs were used as templates for the polymerase chain reaction (PCR) employing DNA polymerase and oligonucleotide primers designed using our library of *amo* gene sequences. The following conditions were used for all PCR experiments: 25 cycles (1 min at 94°C, 1 min at 55°C and 4 min at 72°C) with a 7 min extension at 72°C; optimum concentrations were 2.5 mM $MgCl_2$ and 0.5 μM for primer. For nucleotide sequencing, the PCR products were gel purified, reamplified and the products were purified using the Wizard™ PCR DNA Purification System (Promega, Madison WI). These purified PCR products were directly sequenced using synthetic primers based upon the existing sequence and DNA polymerase for dideoxy dye-primer cyclo sequencing (ABI 373A, USU Biotechnology Center) following the instructions of the manufacturer. Obtained sequences were aligned (Sequencher™ 3.0, Gene Codes Corporation, Madison, WI) and sequence similarities were investigated using the NCBI Blast program [24].
The DNA sequence upstream of the *amoA3* gene up to the *Eco*RI restriction site and the targets for the primer sequences are depicted in Figure 2-1. The sequence for the *amoC3* gene was deposited in GenBank under accession number U72203. Similarly, the 5.7 kb and 16.8 kb DNA fractions from NAV were used as PCR templates to test for the existence of the *amoC2* and *amoC1* genes upstream of *amoA2* and *amoA1*, respectively. Appropriate primers, genomic DNA as the template and the PCR, were used to verify the presence of an *amoC* sequence upstream of *amoA* in the additional ammonia oxidizer strains (Table 2-1).

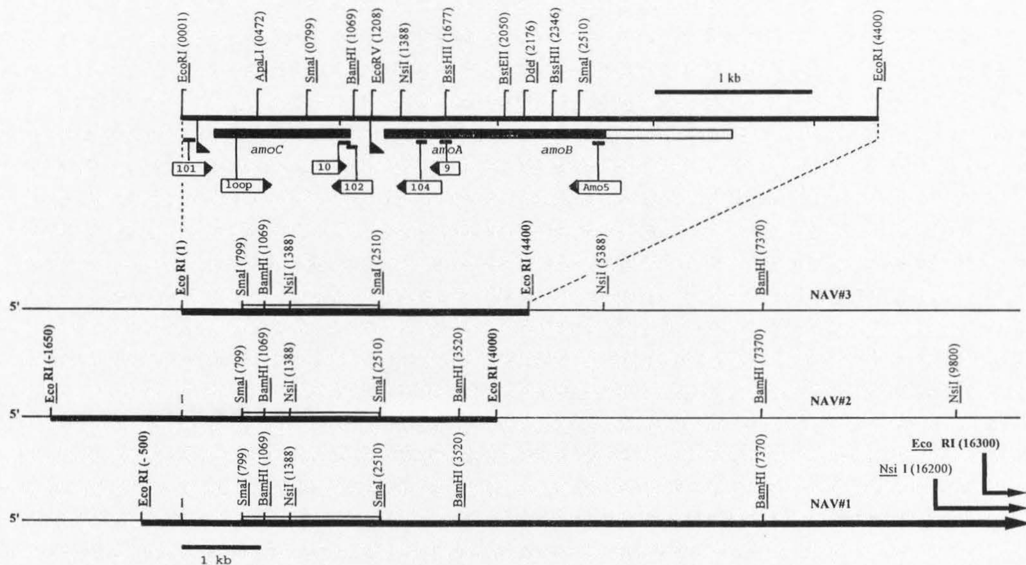

Fig. 2-1. Physical map of the 4.4 kb-, 5.7 kb-, and 16.8 kb-sized *Eco*RI restriction fragments from the genome of *Nitrosospira* sp. NpAV. The open reading frames of the *amoC*, *amoA* and *amoB* genes as well as putative ribosome binding sites are indicated. The location of oligoribonucleotide primers used for the PCR-cloning of the *amoC3* and *amoA3* genes are indicated by arrows. The restriction sites in the first 2547 bp of the enlarged caption of copy NAV#3 has been confirmed by nucleotide sequencing.

Results and Discussion

Analysis of the nucleotide sequence upstream of the *amoA* coding regions in the *amo* operon from the ammonia oxidizer strains investigated revealed the C-terminus of an ORF of surprising sequence similarity. The entire ORF and its flanking regions were sequenced from one of the three copies of the *amo* operon in NAV (Fig. 2-2). The gene was named *amoC3* in analogy to the third subunit of the pMMO enzyme in methanotrophs [22]. A BLAST search [24] of sequences accessible through NCBIs' web-server revealed similarity only to the C-terminus portion of the protein encoded in the *N. europaea* sequence (GenBank accession # L08050).

Nucleotide sequencing of the region upstream of the *amoA3* gene, restriction enzyme mapping as well as PCR product analysis allowed us to construct a physical map of the three *amo* operons in the genome of NAV (Fig. 2-2). This map will be complemented and confirmed in future experiments by continued subcloning and nucleotide sequencing. A 1,387-bp nucleotide sequence including the *amoC3* gene and flanking sequences extending from the *Eco*RI site of the 4.4 kb clone upstream into the *amoA3* gene downstream is shown in Figure 2-2. The identified translational start codon (ATG) was preceded by a purine-rich region suited for ribosome binding (S/D), an in-frame translational stop codon (TAG), and a putative *E. coli* σ^{70}-type promoter with transcriptional start point (Fig. 2-2). While this putative promoter is part of the expression clone pNA101-9 and expression of the *amoC3* gene was obtained despite its orientation against the plasmid-borne p*lacZ* promoter, the exact transcriptional start point needs to be determined in upcoming primer extension experiments. The translational stop codon defines an 813-bp *amoC3* ORF which encodes a 270-amino acid peptide with a deduced molecular mass of 31.1 kDa. The intergenic region between the *amoC3* gene and *amoA3* gene promoter contains a putative stemloop (extending from nucleotide 1064 to 1140) with a calculated free energy value of -31.4 kcal/moles. However, the DNA immediately downstream from the putative loop has a high (G+C) content, which should diminish the probability of transcriptional termination significantly [25].

Southern analysis of the linear size-fractionated DNAs used to PCR-clone the *amo* genes revealed that an *amoC* gene was present in each of the three *amo* operons in the genome of NAV (data not shown). Similarly, PCR using an upstream primer that hybridizes in *amoC* (AmoCloop) and a downstream primer

```
   1  GAATTCAGGGCGGTTCAGGGAACATGTGCGGCAGCGTAATCAATTCGTTTTGCTCTTTCGTTTTGCAGACAAACTGCTTGACTTGGCGCATCTTCGGGG

                                                +1
 100  ATAGTCTTTACGGGGTTGTGGGTATGTAATGGTTTAGTATTTAGTATTATGGCGGCGACGCATTCTTTCTGCGAGGAAGCGGGGAGAGGCGGCTAGCTG
            -35                     -10                      --> AmoC                            *
 199  CGGGCAAGCACAAGCAGCAGAGGGTACTCGATTAACGTAAGAGGAGATAAAGAAATGGCAACAACACTGGGAACATCAGGCAGTCACGCGGGGTCGAGC
   1                                                 S/D   M  A  T  T  L  G  T  S  G  S  H  A  G  S  S
 298  GGCCGCGACTACGACATGTCGCTGTGGTACGACTCCAGGTGGTATAAATTTGGACTGATCACCATGCTTGGGGTAGCGATATTCTGGATCTGGTTTCAA
  16  G  R  D  Y  D  M  S  L  W  Y  D  S  R  W  Y  K  F  G  L  I  T  M  L  G  V  A  I  F  W  I  W  F  Q
 397  CGGACCTTTGCCTACTCGCATGGCATGGACTCGATGGAGCCGGAATTTGAGAAGGTATGGATGGGGTTGTGGCGTGTGCACATGATCGTCATGCCGATC
  49  R  T  F  A  Y  S  H  G  M  D  S  M  E  P  E  F  E  K  V  W  M  G  L  W  R  V  H  M  I  V  M  P  I
 496  TTTGCGCTGGTCACCTGGGGCTGGATCTGGAAGACACGGGACACCAACCTGGACAACCTGGACCCGAAACTGGAAATCAAGCGTTACTTTTACTGGATG
  82  F  A  L  V  T  W  G  W  I  W  K  T  R  D  T  N  L  D  N  L  D  P  K  L  E  I  K  R  Y  F  Y  W  M
 595  ATGTGGCTGGGCGTATACCTGTTTGGCGTGTACTGGGGTGGCAGCTTCTTCACGGAGCAAGACGCCTCCTGGCATCAAGTGATTATCCGCGACACCAGC
 115  M  W  L  G  V  Y  L  F  G  V  Y  W  G  G  S  F  F  T  E  Q  D  A  S  W  H  Q  V  I  I  R  D  T  S
 694  TTCACCCCGAGCCATGTGGTGGTATTTTATGGCTCCTTCCCGATGTACATTGTCTGCGGCGTGGCCAGCTACCTGTACGCCATGACGCGCCTGCCGCTG
 148  F  T  P  S  H  V  V  V  F  Y  G  S  F  P  M  Y  I  V  C  G  V  A  S  Y  L  Y  A  M  T  R  L  P  L
 793  TATGCCCGGGGCACCTCGTTCCCGCTGGTGATGGCGATTGCCGGCCCGCTCATGATTCTGCCGAACGTAGGCTTGAACGAATGGGGCCATGCCTTCTGG
 181  Y  A  R  G  T  S  F  P  L  V  M  A  I  A  G  P  L  M  I  L  P  N  V  G  L  N  E  W  G  H  A  F  W
 892  TTCATGGAAGAACTGTTTAGCGCACCGCTGCACTGGGGCTTTGTGATACTGGGCTGGTCGGGCCTGTTTGCTGGCGGCATTGCGGCACAGATCATCACC
 214  F  M  E  E  L  F  S  A  P  L  H  W  G  F  V  I  L  G  W  S  G  L  F  A  G  G  I  A  A  Q  I  I  T
 991  CGCTACTCCAACCTGACCGACGTGGTCTGGAACGGACAAAGCAAAGTCATCCTCAACAACCGGATCGTCCCGTAAGGGGGATCCGTAGACCCGGCCGCC
 247  R  Y  S  N  L  T  D  V  V  W  N  G  Q  S  K  V  I  L  N  N  R  I  V  P  *-------.----....-------.--
1090  CGTTGACTCCCGCCAGAGTGGGGAATCAGCCGGGCGGACCGGCCCACCTTCGACCTGCGGCGAAGACCGGAAAAGGCACCGGCAGGGGGAAGGCGGCA
      -.---.-->         <---------.....----------.....----.----
1189  AGACAGCGGCGTGACACGATATCGGCGTGACACGCAAAAATGAACAACGGCAGCATTTATCAGTTTTAAGATCGTACGACATATTTGAGGGGAGGGCAC
       --> AmoA   -35                       -10        +1                                     *     S/D
1288  GATGAGCAGAACAGACGAAATATTAAAGGCGGCCAAGATGCCGCCGGAATCGGTAAAGATGTCCAGGATGATAGACGCGATCTATTTTCCGATTTTATG
   1   M  S  R  T  D  E  I  L  K  A  A  K  M  P  P  E  S  V  K  M  S  R  M  I  D  A  I  Y  F  P  I  L  C
```

Fig. 2-2. Nucleotide sequence for the copy of *amoC3* gene and flanking regions from *Nitrosospira* sp. NpAV contained on the 4.4 kb *Eco*RI restriction fragment (*amoC3*, Genbank Accession number U72203). Putative transcriptional ("-35" and "-10" promoter regions and start point "+1") and translational (S/D and start codon) control sequences are underlined; in-frame translational stop codons are marked by an asterisk. The start of the AmoC3 and AmoA3 ORFs are indicated with an arrow above the ATG start codons.

that hybridizes in *amoC* (Amo102) or *amoA* (Amo104 or Amo9) confirmed that one copy of *amoC* is upstream of each of the three copies of the *amoA*/*amoB* gene tandem arrangement in NAV (Figs. 2-1, 2-3). Because the C-terminus of the AmoC ORF was highly conserved in the DNA of various ammonia-oxidizer strains, PCR with primers based on the NAV *amoC3* and *amoA3* sequences were used to confirm the presence of *amoC* genes in *N. europaea*, *N. multiformis*, *N. tenuis*, *N. briensis* and *N. sp*. 39-19 (Fig. 2-3). The PCR with primers internal to *amoC* (AmoCloop and Amo102) gave products of approximately the

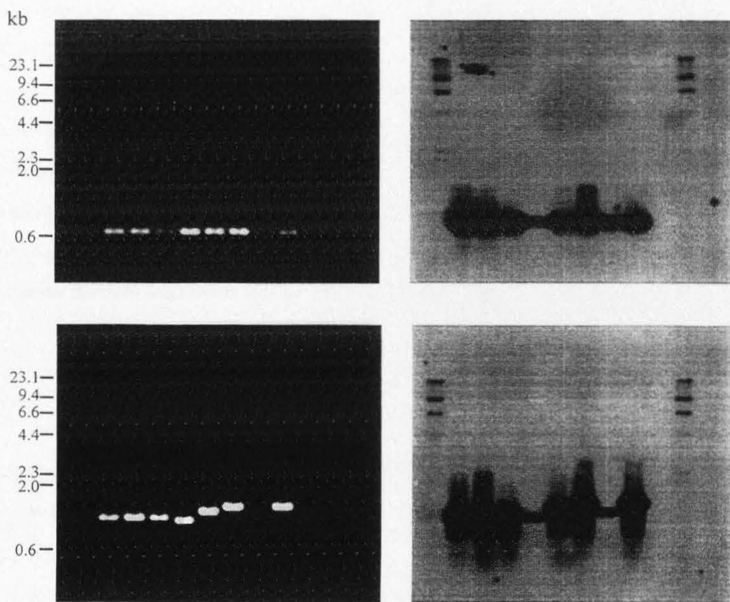

Fig. 2-3. PCR amplification of ammonia oxidizer genomic DNA using the *amoC* and *amoA* specific primers (target sites see Fig 1.) with templates as indicated. Lane 1, MW standard; lane 2, *Nitrosospira sp.* NpAV 4.4 kb *Eco*RI fragments; lane 3, *Nitrosospira sp.* NpAV 5.8 kb *Eco*RI fragments; lane 4, *Nitrosospira sp.* NpAV 16.8 kb *Eco*RI fragments; lane 5, *Nitrosomonas europaea* genomic DNA; lane 6, *Nitrosolobus multiformis* genomic DNA; lane 7, *Nitrosovibrio tenuis* genomic DNA; lane 8, *Nitrosospira briensis* genomic DNA, lane 9 *Nitrosospira sp.* 39-19 genomic DNA, lane 10 empty, lane 11 MW standard. Lanes repeated in top and bottom panels with primers AmoCloop to Amo102 (top) and AmoCloop to Amo104 (bottom). Southern analysis was performed by using the *amoC3* digoxigenin labeled probe.

same size as in NAV (612 bp). Although template concentrations were approximately equal, *N. briensis* DNA amplified weakly possibly due to primer mismatch. In the Southern blot, however, the *amoC* amplicon from *N. europaea* gave the weakest hybridization, indicating lower similarity to the DNA probe prepared from NAV *amoC3* gene. In the PCR with one primer internal to *amoC* (AmoCloop) and one primer in *amoA* (Amo104), the products from NAV were of the expected 1.1 kb size. Products in PCR assays which used genomic DNA templates from other ammonia-oxidizing bacteria yielded products of different sizes. This is attributed to the different lengths of the *amoC*/*amoA* intergenic regions in these organisms based upon our sequences of the *amoA* gene upstream regions in the respective nitrifier strains [15-17].

Protein expression studies for the putative *amoC3* gene for NAV were carried out by our collaborator, Dr. Martin Klotz. For completeness, this material is included in appendix C. To further demonstrate that the *amoC* is part of the *amo* operon, I performed reverse transcriptase PCR (RT-PCR) on total RNA from *Nitrosospira sp.* NpAV looking for evidence that the *amoC* was co-transcribed with *amoAB*, which was found. These results are reported in appendix D. Sayavedra-Soto et al. [26] further expanded this study with northern blots for NEU and RT-PCR for NEU.

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cds; and GenBank U72670. *Nitrosomonas eutropha* ammonia monooxygenase 2 subunit A (*amoA2*) gene, complete cds and ammonia monooxygenase 2 subunit B (*amoB2*) gene, partial cds.

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CHAPTER 3

THE *amo* OPERON IN *Nitrosococcus*: MARINE, AMMONIA-OXIDIZING γ-PROTEOBACTERIA[1]

Abstract

While there is an extensive database of genes encoding ammonia monooxygenase (*amo*) found in the ammonia-oxidizing β-proteobacteria, few *amo* sequences are available representing the γ-proteobacteria. We sequenced the complete *amo* operon (*amoCAB*) for *Nitrosococcus oceanus* (ATCC 19707), a marine, autotrophic, ammonia-oxidizing bacterium belonging to the γ-subdivision of the proteobacteria. An additional autotrophic, ammonia-oxidizing bacterium isolated from a marine environment (strain C-113) was identified as belonging to the *Nitrosococcus* genus by 16S rDNA analysis and its *amo* operon was sequenced. This is the first report of a full-length sequence for the *amo* operon from a γ-subdivision autotrophic ammonia-oxidizing bacterium. The *N. oceanus* and C-113 *amo* genes were 88-90% identical to each other, 49-53% identical to the *pmo* genes encoding the related particulate methane monooxygenase of *Methylococcus capsulatus* (Bath), and 39-42% identical to the *amo* genes of the β-subdivision autotrophic ammonia-oxidizing bacteria. In both *Nitrosococcus* strains the *amo* operon was found as a single copy and contained three genes, *amoC, amoA, amoB*, with intergenic spacer regions between *amoC* and *amoA* (286 bp) and between *amoA* and *amoB* (65 bp). Sequence similarity values and secondary structure predictions suggested a slightly closer relationship to the *pmo* operon of *M. capsulatus* (Bath) than to the β-subdivision *amo* operons. We conclude that the *amo* genes will be useful as functional markers in community analysis and for fine-scale phylogenetic differentiation within the γ-subdivision AOB.

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[1] Coauthored by J.J. Alzerreca, J.M. Norton, and M.G. Klotz. Reprinted from FEMS Microbiology Letters, Volume 180. The amo operon in marine, ammonia-oxidizing γ-proteobacteria. Pages 21-29. Copyright 1999, with permission of Elsevier Science (Appendix A).

Introduction

Ammonia-oxidizing bacteria (AOB) obtain metabolic energy by the preliminary oxidation of ammonia/ammonium to hydroxylamine. This vital enzymatic step is carried out by ammonia monooxygenase (AMO). This enzyme has at least three subunits, encoded by the genes *amoC*, *amoA* and *amoB* that are believed to comprise the functional *amo* operon [1]. The sequences of the *amo* genes have been studied primarily in representatives of the β-subdivision, where many of the terrestial AOB are found [2,3,4,5]. AOB have also been identified in the γ-subdivision. Currently all γ-subdivision AOB which have been isolated are from marine environments.

In the present study, fully sequenced *amo* operons from two representatives of the γ-subdivision AOB are presented. The *Nitrosococcus amo* sequences were compared to those from the β-subdivision AOB and to the *pmoC, pmoA* and *pmoB* genes encoding the evolutionarily related particulate methane monooxygenase (pMMO) enzyme [6,7] from *Methylococcus capsulatus* (Bath) (MCA), a proteobacterium in the γ-subdivision.

Materials and Methods

Bacterial Strains and DNA Isolation

Nitrosococcus oceanus strain C-107 (=ATCC19707; here abbreviated NOC) and a previously unidentified marine autotrophic ammonia-oxidizing bacterium isolated from the Red Sea (strain C-113) were obtained from F. Valois (Woods Hole Oceangraphic Institute,Woods Hole, MA, USA). NOC strain identity was confirmed by using 16S rDNA amplification and RFLP of the amplified products (M. Voytek and B. Ward, personal comunication). Both marine strains were grown for 4 to 5 weeks with no shaking, in the dark on marine media (12.5 mM $(NH_4)_2SO_4$, 3 μM Chelated iron, 0.4 μM Na_2MoO_4, 1.0 μM $MnCl_2$, 0.008 μM $CoCl_2$, 0.35 μM $ZnSO_4$, 187.5 mM NaCl, 23.3 mM $MgSO_4$, 15 mM $MgCl_2$, 7.6 mM $CaCl_2$, 7.5 mM KCl, 0.15 mM Na_2CO_3, 2.25 mM $NaHCO_3$, 0.5675 mM K_2HPO_4, and phenol red 0.25%, adjusted to pH 6.5-6.8 and autoclaved). In addition, *Nitrosospira sp.* NpAV (NAV) and *Nitrosomonas europaea* ATCC25978 (NEU) were grown as previously

described [5]. Genomic DNA (gDNA) was isolated from all strains following the procedure described by McTavish et al. [4].

Hybridization Analysis for the *amo* Operon

The initial identification of *amoA*-hybridizing fragments was determined by Southern analysis. NOC gDNA was digested with *Eco*RI and *Kpn*I, electrophoresed on a 0.6% agarose gel in 1X TAE, and blotted onto a nylon membrane [8]. We designed an *amoA* targeted primer, No1R, based on the sequence alignments from Holmes et al. [6]. A non-radioactive internal NOC-*amoA* probe (479 bp) was prepared from a NOC gDNA template using the primers A189 [6] and No1R in a PCR reaction with digoxigenin (dig)-labeled dUTP (dig-dUTP: dUTP, 1:5) according to the manufacturer's recommendations (Boehringer Mannheim Corporation, Indianapolis, IN). The digoxigenin-labeled probe was hybridized with the transferred NOC gDNA and hybridizing fragments were detected as suggested by the manufacturer (Genius™ System, Boehringer Mannheim). Molecular weights of hybridizing fragments were determined using the RFLPscan program (Scanalytics/CSPI, Billerica, MA). We identified a 3-kb *Eco*RI fragment and a 2.9-kb *Kpn*I fragment, both of which hybridized to the *amoA* probe. The identified NOC *amoA*-hybridizing fragments were isolated from the agarose [5] and used as size-fractionated templates for PCR-cloning and sequencing.

The potential for cross-hybridization between the *amo* genes from the β- and γ-subdivision proteobacteria, and the copy number of the *amo* operons in individual strains were also assessed using Southern blots (Fig. 3-1). Four probes were made: one internal *amoA* and one internal *amoC* probe each from NAV and NOC (see Fig.3-2 for NOC probes). On the NAV *amo* operon sequence (GenBank U92432), the NAV-*amoC* probe (611bp) starts at base 442 and ends on base 1052, while the NAV-*amoA* probe (675bp) begins at base 1436 and ends on base 2110. On the NOC sequence (GenBank AF047705), the NOC probes were designed from base 251 to base 858 (NOC-*amoC*, 608bp) and from base 1301 to base 1779 (NOC-*amoA*, 479bp).

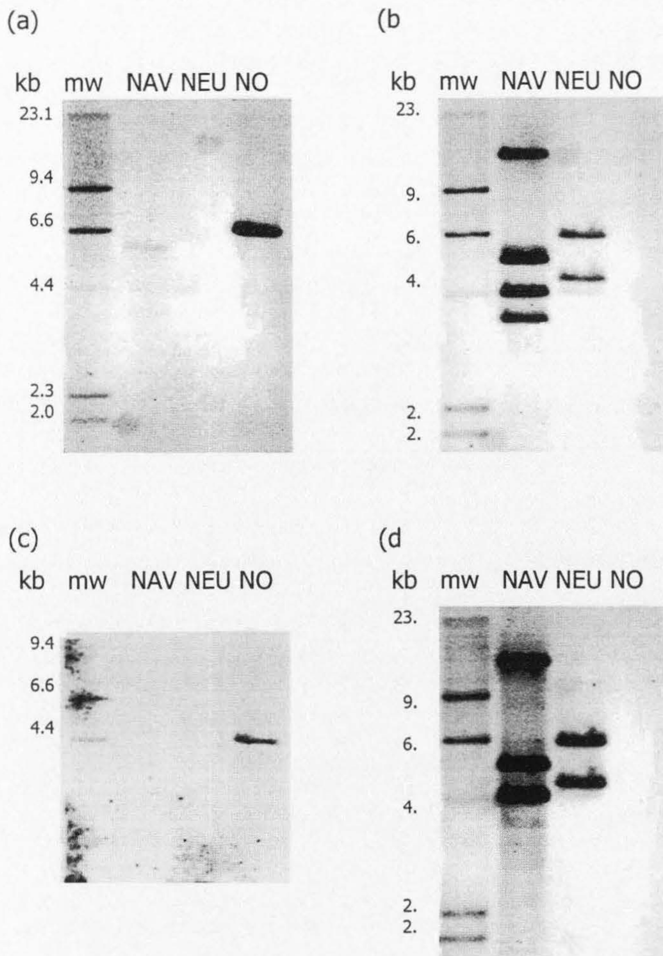

Fig. 3-1. Southern blots with gDNA from three AOB strains. Lanes are the same for all the pictures and are: mw: λ/HindIII molecular weight marker, NAV: *Nitrosospira sp. NpAV* digested with *Eco*RI, NEU: *Nitrosomonas europaea* digested with *Eco*RI, and NOC: *Nitrosococcus oceanus* digested with *Hind*III. The membranes were probed with a) an internal NOC *amoC* probe (NOC-*amoC*), b) an internal NAV *amoC* probe (NAV-*amoC*), c) an internal NOC *amoA* probe (NOC-*amoA*) and d) an internal NAV *amoA* probe (NAV-*amoA*).

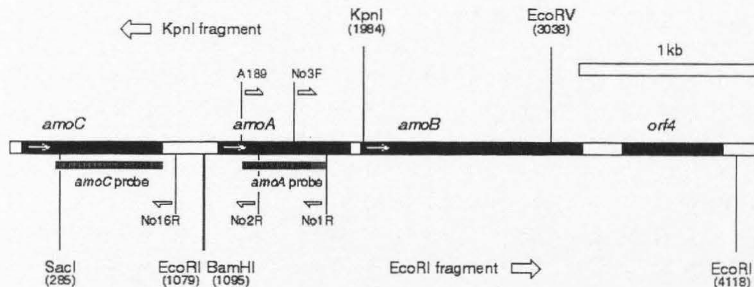

Fig. 3-2. Physical map of the NOC *amo* operon. The three gene operon is presented with selected primers and the *Eco*RI and *Kpn*I fragments used for sequencing. The *Eco*RI fragment was fully sequenced. The arrows on the fragments show the direction of sequencing.

The dig-labeled probes were made by performing PCR with specific primers on the appropriate gDNA template in the presence of dig- labeled dUTP as described above.

The gDNA from three strains (NOC, NAV, and NEU) was restriction digested, with *Hin*dIII, *Eco*RI, and *Eco*RI, respectively, electrophoresed and blotted onto nylon membranes. Duplicate membranes were prepared. Both membranes were analyzed by hybridization with the dig-labeled probes described above. Initially, one membrane was probed with NAV-*amoC*, and the other with NOC-*amoC*, and the hybridized fragments were detected. These membranes were stripped and re-probed, one with NAV-*amoA* and the other with NOC-*amoA*, respectively. Hybridization (65°C, overnight), detection of the hybridized fragments, and membrane stripping for reproving were done following the manufacturer's recommendations (Genius System, Boehringer Mannheim).

Primers and PCR Amplification

The following primers were used for this study (lower case bases represent mismatches in respect to the NOC sequence): AmoNo1R; CAgCGTACCTTTTTCAACCA, AmoNo3F; GACGGCGGTAGTGGGTGGATTA, AmoNo2R; CAACCGATACAATGGGAACTAC and AmoNo16R; TCTATTCAACCCTTCGCTAACT (Fig. 3-2). Amplify (version 2.53β for the

Macintosh) [9] was used to aid in primer design and evaluation. Primers were prepared commercially (Genemed Synthesis Inc., San Francisco, CA) and PCR was performed in 0.2mL thin-wall PCR tubes using the Ericomp Delta-Cycler I system (San Diego, CA). The 50μL PCR reactions contained 0.5μM of each primer, 2.5mM $MgCl_2$, 0.25mM dNTP mix, 1X Taq polymerase buffer B [20mM Tris-HCl (pH 8.0 at 25°C), 100mM KCl, 0.1mM EDTA, 1mM DTT, 50% glycerol, 0.5% Tween 20 and 0.5% Nonidet P40], approximately 100ng template DNA, and 2.5U Taq polymerase (Promega, Madison, WI). The PCR conditions to obtain the desired products were an initial 4 min denaturation step at 94°C, followed by 25 cycles of a one min denaturing step at 94°C, a one min annealing step at 55°C and a four min extension step at 72°C. A final extension step of 1 hour at 72°C completed the PCR, which is recommended for more efficient cloning [10].

NOC Operon Cloning, PCR Purification and Sequencing

The 479bp amplicon obtained from NOC gDNA with primers A189 and AmoNo1R was cloned into the vector pCR2.1 using the TA Cloning® kit (Invitrogen, Carlsbad, CA), following the manufacturer's instructions. The clones were screened by *Eco*RI restriction digest analysis and a single clone was found to contain the desired insert (clone NOC15B). The plasmid was purified and sequenced using the vector primers, by dideoxy dye-primer cyclo sequencing (ABI 373A, USU Biotechnology Center) following the instructions of the manufacturer. To extend the sequence from this first partial *amoA*, we used inverse PCR as described by Triglia et al. [11], on size-fractionated NOC gDNA previously identified as containing the *amo* operon. We excised the *Eco*RI (3kb) and *Kpn*I (2.9kb) fragments from an agarose gel and purified them with the GeneClean® III kit (BIO 101, La Jolla, CA).

The size-fractionated templates were self-ligated (assay volume 80μL), using 4U of T4 DNA Ligase (Boehringer Mannheim) following manufacturer's recommendations. PCR was performed with 5 μL of these ligation reactions as templates, using the same conditions described above. Two primers, AmoNo2R and AmoNo3F, were designed from the NOC15B sequence, facing against each other, for amplification with the 3kb *Eco*RI template PCR. Amplification resulted in a 2.9kb product, which was

column purified (PCR SELECT® II columns, 5 Prime →3 Prime, Boulder, CO) following the manufacturer's instructions. The purified PCR product was sequenced as described above. Obtained sequences were edited and aligned using Sequencher, version 3.1 for the Macintosh (GeneCodes, Madison, WI).

Sequencing of the PCR product revealed that the *Eco*RI fragment contained only the *amoA* and *amoB* genes. The *Eco*RI site was located immediately upstream of the *amoA* gene. To obtain the missing *amoC* gene sequence, inverse PCR was performed again on a gel-purified and self-ligated 2.9kb *Kpn*I fragment, using primers AmoNo16R and AmoNo3F. The PCR product was purified and sequenced.

Most of the NOC sequences were obtained by walking upstream and downstream directly on the purified PCR product, since attempts to clone it failed. It appears that cloned fragments of NOC *amo* genes were toxic to the *E. coli* host [12]. After completion of the sequence, a clone containing the complete *amoA* and *amoB* genes was obtained by PCR cloning as described previously for NAV [13] and NEU [1].

C-113 Operon and16S rDNA Sequencing

PCR products were initially obtained from C-113 gDNA using NOC based primers. Subsequently, we designed C-113 specific PCR primers and the obtained amplicons were column purified for direct sequencing as described above. In order to obtain the partial 16S rDNA sequence, PCR was performed on C-113 gDNA with primers pBf and pFr, which are conserved for the 16S rDNA of eubacteria [14] using PCR conditions as described above. A 1028-bp amplified product was cloned into pCR2.1. The clone, NMA28C, was found to contain the desired partial 16S rDNA sequence, and was fully sequenced as described above.

Sequence Comparison and Analysis

Completed sequences were analyzed for identity values with existing *amo* and *pmo* genes from the GenBank database using the LaserGene package (DNASTAR, Madison, WI) [15]. The predicted secondary structures of the deduced peptides were analyzed using LaserGene, NCBI's

BLAST server (http://www.ncbi.nlm.nih.gov/) and the ClustalW alignment program (http://www.ddbj.nig.ac.jp/htmls/E-mail/clustalw-e.html). Analysis of the C-113 16SrDNA was performed using the Ribosomal Database Project [16].

Results and Discussion

It is well documented that AOB in the β-subdivision proteobacteria have multiple copies of the *amo* operon [4,5]. Consequently, we examined the genome of NOC, an autotrophic AOB from the γ-subdivision of the proteobacteria, for the number of *amo* operon copies and their potential cross-hybridization with *amo* genes from two representatives from the β-subdivision proteobacteria using Southern analysis and *amoC* as well as *amoA* probes designed from NOC and NAV *amo* operon sequences (Fig. 3-1). NOC gDNA digested with *Hin*dIII contained a single band of 6.7 kb that hybridized to the NOC-*amoC* and *amoA* probes (Fig. 3-1A and C). Digests with other restriction enzymes (data not shown) confirmed that NOC has only one copy of the operon. The NAV-*amoC* probe revealed four distinct bands of 3.8, 4.4, 5.7 and 16.8 kb in the NAV lane (Fig. 3-1B), corresponding to the four copies of *amoC* within the NAV genome [1]. The probe hybridized strongly with the two *amoC* copies of the NEU genome (Fig. 3-1B, lane 3). The NAV-*amoC* probe did not hybridize with the NOC gDNA, and no bands were seen (Fig. 3-1B, lane 4).

While the NOC-*amoA* probe hybridized only to NOC gDNA, the NOC-*amoC* probe revealed a weak band (5.8 kb) in the Southern blot of NAV gDNA (Fig. 3-1A). This may have been due to hybridization of the NOC-*amoC* probe to a remnant copy of an *amoC* gene that is more similar to the NOC *amoC*. Further analysis of this putative fifth *amoC* gene copy in NAV is in progress.

The membrane probed with the internal NAV-*amoA* probe presented bands only in the NAV and NEU lanes (Fig. 3-1D). Previous reports that a partial *amoA* gene amplified from *N. oceanus* (ATCC 19707) gDNA hybridized with an internal *amoA* probe from *N. europaea*, and that the NOC sequence was essentially identical to this probe sequence [17], were not substantiated by our study. We were unable to match the reported probe with our NOC operon sequence; furthermore, submitting the probe for a BLAST alignment returned none of our NOC sequences. In additional experiments, a NEU-*amoA* probe was used in

a Southern analysis with gNOC DNA. No cross-hybridization was observed between the NEU probe and DNA from gNOC (Norton, unpublished data) confirming our results with the NAV-*amoA* probe. Our observations indicate that NOC has only one copy of the *amo* operon and no non-operon *amoC* genes in its genome. This is in contrast to the AOB from the β-subdivision, which have multiple copies of the *amo* operon [5] and additional non-operon copies of the *amoC* gene [1].

The first complete sequence of the *amo* operon from NOC is presented in Figure 3-3. The *amo* operon consists of three genes: *amoC* encoding a 262-amino-acid peptide, *amoA* encoding a 248-amino-acid peptide, and *amoB* encoding a 417-amino-acid peptide. Putative promoters and transcriptional start sites and ribosomal binding sites have been identified upstream of *amoC* and *amoA* (Fig. 3-3). A strong transcriptional terminator is found downstream of *amoB* (-118.8 KJ/mol). During the sequencing of the *Eco*RI fragment, a fourth open reading frame was found following the *amo* transcriptional terminator, which has similarity to ORF 4 in NAV. Our results indicate that NOC has a three-gene operon that matches the corresponding *amo* operon for the β-subgroup AOB.

The AOB from both the β and γ subdivision have an intergenic region between *amoC* and *amoA* which has a size specific to each AOB (Table 3-1). The 286-bp intergenic region between the NOC *amoC* and *amoA* genes is within the range (163-445 bp) of the known β-group intergenic distances; the different intergenic-region sizes, as well as gene sizes for all strains studied, are summarized in Table 3-1. Preliminary experiments have demonstrated that these variances in spacer length may provide a practical approach for screening gDNA samples with PCR, with the primer pair enclosing the variable intergenic region [18]. In contrast to β-subdivision AOB, which have contiguous *amoA* and *amoB* genes, the NOC *amoA* and *amoB* genes are separated by a 65-bp intergenic region. Interestingly, the *pmoA* and the *pmoB* genes in MCA are also separated by spacers in both *pmo* operons [7].

Marine Strain C-113

We sequenced a partial 16S rDNA clone from strain C-113 and this 16S rDNA sequence was deposited into GenBank (Accession number AF153343). Sequence analysis identified this strain as belonging to the γ-subdivision of the proteobacteria [16]. Furthermore, the C-113 16S rDNA sequence was

```
#1     CCGGTGTTTA ATGGCTGGTC AAGTGTCATC AACTATAACA ATGGTTTCAG TCAAATAGGA GGGTATCAAT
                 -10          +1                                               S/D
#71    ATGGCTGCAA CTAGTAGAGC TGTAGCGCAA GGAGTAGCCG AGAAAGAAAC TGCTGATTTT TTTGCCTGGC
       M  A  A  T   S  R  A   V  A  Q   G  V  A  E   K  E  T   A  D  F   F  A  W  R
#141   GTGGCATGTG GTTAGCGGTC GCGGCCTTTT TCGCGTTATA CATCTGTGTG CGTTGGTATG AAGGTGTTTA
        G  M  W   L  A  V   A  A  F  F   A  L  Y   I  C  V   R  W  Y  E   G  V  Y
#211   TGGATGGAAG TATGGTTTAG ATGCCTTTTT GCCGGAATTC CAAACATACT GGATGAATCT GCTGTACATT
       G  W  K   Y  G  L  D   A  F  L   P  E  F   Q  T  Y  W   M  N  L   L  Y  I
#281   GAGCTCGTTG TTGAGGCACT GGCAACAGCA GCCCTGGTTT CTTACCTAAT TAAGACCCGG GATCGCAATA
       E  L  V  V   E  A  L   A  T  A   A  L  V  S   Y  L  I   K  T  R   D  R  N  M
#351   TGGAAGCCAT GACTCCCCGC GAGGAGGTGC GCCGTTACTG CACCCTTTAT ATGTGGTGGG TTGTTTATGG
        E  A  M   T  P  R   E  E  V  R   R  Y  C   T  L  Y   M  W  W  V   V  Y  G
#421   CGTTGGGTTA TTTTGGGGCG CGAGCTTCTT TACCGAGCAG GATGGTGCTT GGCATCAGAC CGTAGTTCGG
        V  G  L   F  W  G  A   S  F  F   T  E  Q   D  G  A  W   H  Q  T   V  V  R
#491   GATACAGACT TCACCCCAAG CCATATTATT GAGTTCTACA TGAGCTACCC GATCTACGTA ATGGTAGGTT
       D  T  D  F   T  P  S   H  I  I   E  F  Y  M   S  Y  P   I  Y  V   M  V  G  L
#561   TAGGTTCGTT CATGTATGCT AAGACCCGTA TTCCTTATTT TGCAAAGGGC TGGTTTGTAC CCTACCTCAT
        G  S  F   M  Y  A   K  T  R  I   P  Y  F   A  K  G   W  F  V  P   Y  L  M
#631   GTTGGTGTTT GGACCCTTTA TGATTTTCCC GAATGTGGGT TTGAATGAAT GGGGTCACAC CTTCTGGTTT
       L  V  F   G  P  F  M   I  F  P   N  V  G   L  N  E  W   G  H  T   F  W  F
#701   ATGGAGGAGC TGTTTGTGGC ACCTCTGCAC TGGGGTTTTG TATTCTTTGC TTGGTTTATC CTTGCAGTAT
       M  E  E  L   F  V  A   P  L  H   W  G  F  V   F  F  A   W  F  I   L  A  V  F
#771   TCGGGGTGTT TCTGCAGGTT CAGCCCCGTA TGAAGGAATT GATCGGCAGA GAGCTGCAGC AGAGCGAGGA
        G  V  F   L  Q  V   Q  P  R  M   K  E  L   I  G  R   E  L  Q  Q   S  E  D
#841   CTATGCCCGT AGCTGATATT TTAGATTATC ATATTTGATT TCAGTTAGCG AAGGGTTGAA TAGAAGGGCG
       Y  A  R   S  *                                                          ----
#911   GTCGCAATCT CCGGAATAGT TAATGAGATC GTGGCCGCCT TTTGTATAA AGTGCTGTAC AGAAATTTTA
       ----------------->   <---------------------
#981   TTAGGGATAC GCTTATTTAT ATCGGGTTGA AAGAAATGGA AGTAAGCAAC CTAATGAGAA AGTAGCTGTT
                        -40          -35                          -10    +1
#1051  AAAATAGGTG GCAGGAAGCA TACCTTTAGA ATTCATGGAT TTCTGGATCC GATGAATGAA TAATCATAAA
                                     EcoRI                 AmoA
#1121  TTAGTTTAAA TAGAACCCTT AATATTTTTA GGAGGGCAGG TTTAATGAGT GCACTTACGT CTGCGGTTCG
                                       S/D             M  S   A  L  T  S   A  V  R
#1191  TACACCGGAG GAAGCTGCTA AAGTCTTTAG AACGTTGGAT TTTATAGCGT TAGGGGCTTT TTTTATGATT
        T  P  E   E  A  A  K   V  F  R   T  L  D   F  I  A  L   G  A  F   F  M  I
#1261  TTGCTGGCTT CCCACCACGT TCACGTCATG CTTTTAATGG GGGACTGGGA CTTCTGGGTT GACTGGAAAG
       L  L  A  S   H  H  V   H  V  M   L  L  M  G   D  W  D   F  W  V   D  W  K  D
#1331  ACCGGCGTTT TTGGGTCACC GTAGTTCCCA TTGTATCGGT TGCTTACCCC GCTGCTGCAC AGGCGTTTTT
        R  R  F   W  V  T   V  V  P  I   V  S  V   A  Y  P   A  A  A  Q   A  F  F
#1401  TTGGGAGAAG TTCCGCCTTC CCTTTGGTGC TACCCTTGTA ACGCTAGGGG TGTTAGCAGG TGAGTGGGCT
        W  E  K   F  R  L  P   F  G  A   T  L  V   T  L  G  V   L  A  G   E  W  A
#1471  AACCGCTACT TTAATTTCGT AGGGTTTACC TATTTTCCGA TTAATTTCGT ATGGCCGACC ATTCTGCTGC
       N  R  Y  F   N  F  V   G  F  T   Y  F  P  I   N  F  V   W  P  T   I  L  L  P
#1541  CCATGGCGCT GTTCTTGGAT GCCATGCTTG CGATTTCCAA GAGCTACGGC TTGACGGCGG TAGTGGGTGG
        M  A  L   F  L  D   A  M  L  A   I  S  K   S  Y  G   L  T  A  V   V  G  G
#1611  ATTAATGTAC GGTTTGTTGA TGTATCCCGC TAACTGGCCG CTGCTTTCGG CATTCCATGT GCCTGCTGAG
        L  M  Y   G  L  L  M   Y  P  A   N  W  P   L  L  S  A   F  H  V   P  A  E
#1681  TACAATGGAG TAGTAATGTC TTTGGCCGAT ATTATGGGCT ACCAATACGT CCGGACCGGT ACTCCTGAGT
       Y  N  G  V   V  M  S   L  A  D   I  M  G  Y   Q  Y  V   R  T  G   T  P  E  Y
#1751  ATATCCGGAT GGTTGAAAAA GGTACGTTGA AAACGTTTGG TAAGGACGTG GTCCCGGTTT CAGCTTTCTT
        I  R  M   V  E  K   G  T  L  K   T  F  G   K  D  V   V  P  V  S   A  F  F
#1821  CTCGGGTTTT GTTGCCATGG TAATGTACTT TGTGTGGCAC TTTGTTGGCC GCTGGTTCTC CAAGGACTAC
        S  G  F   V  A  M  V   M  Y  F   V  W  H   F  V  G  R   W  F  S   K  D  Y
#1891  CATATCAACC AATTGTAAAA TCGTTTGTGA TAATGAGGAT AATAATTCTA AAATTCTAAA ATACAAAAAG
       H  I  N  Q   L  * AmoB
#1961  AGAGACGGAG AACATGAAAG GTACCCATAT AACAAATCGA GCCAGAAAGT GGCTAGCCAT TGGGTTTACT
           S/D      M  K  G   T  H  I   T  N  R   A  R  K  W   L  A  I   G  F  T
```

Fig. 3-3. DNA sequence of the NOC *amo* operon. The sequence also includes a fourth ORF, of unknown function.

```
#2031  GCGGTGATAG CCTCAAGCGT TTTCTATATT CCGACAGTAG CTGCCCATGG CGAGAAGGCG CAGGCAGCTT
        A  V  I  A   S  S  V   F  Y  I   P  T  V  A   A  H  G   E  K  A   Q  A  A  F
#2101  TCCTGCGCAT GCGGACAATC CATTGGTATG ACATGGTATG GTCCAAGGAT ACCATTGCGG TTAATGAGAC
         L  R  M   R  T  I   H  W  Y  D   M  V  W   S  K  D   T  I  A  V   N  E  T
#2171  CTATACCATA AGCGGGAAGT TCCGGGTTTT TGAGGATTGG CCGGAAGCAG TCGAAAAACC CCATGTATCC
        Y  T  I   S  G  K  F   R  V  F   E  D  W   P  E  A  V   E  K  P   H  V  S
#2241  TTTTTAAATG CGGGTCAACC TGGTCCAGTC ACGGCTCGGC TTACTTCCTA CGTCAATGGT ATGTTCGTCC
        F  L  N  A   G  Q  P   G  P  V   T  A  R  L   T  S  Y   V  N  G   M  F  V  P
#2311  CTCGTTCGAT AGGTCTTGAA TTGGGCGGCG ATTACGATTT TGAGATGACG ATGCAAGGGC GCCGTCCTGG
         R  S  I   G  L  E   L  G  G  D   Y  D  F   E  M  T   M  Q  G  R   R  P  G
#2381  GACGTGGCAT GTTCATACCT TGCTAAATGT CCAAGGAGGG GGGCCGCTCA TCGGTCCAGG TAAATACATC
        T  W  H   V  H  T  L   L  N  V   Q  G  G   G  P  L  I   G  P  G   K  Y  I
#2451  ACCATTACCG GAGATATGGC TGATTTTGAA AACAAAATCA CGGATCTGAC CGGTAATACG GTCAACCTGG
        T  I  T  G   D  M  A   D  F  E   N  K  I  T   D  L  T   G  N  T   V  N  L  E
#2521  AAACCATGGC CACGGGCACG GTTATTGGTT GGCATCTGTC CTGGTACGTT CTTGGTATCG CCTGGATTGG
         T  M  A   T  G  T   V  I  G  W   H  L  S   W  Y  V   L  G  I  A   W  I  G
#2591  GTGGTGGGCC CGCCGTCCCA TGTTCTTGCC CCGCTACATG AAAATAAAGG CGGGCAAGGC TAATGATCTA
        W  W  A   R  R  P  M   F  L  P   R  Y  M   K  I  K  A   G  K  A   N  D  L
#2661  TTAACTGCCC AGGACAAAAA ATTGACTATA GGCGTCCTTG TGGGCGTCCT GCTCATTATT TTGTTCGGCT
        L  T  A  Q   D  K  K   L  T  I   G  V  L  V   G  V  L   L  I  I   L  F  G  S
#2731  CCAAGAATGC TGAGGATAAA TTCCCAGTCA CCATTCCGTT GCAGGCTGGG CTGCTGGGCA CTATTGACTC
         K  N  A   E  D  K   F  P  V  T   I  P  L   Q  A  G   L  L  G  T   I  D  S
#2801  CTTGCCGGTG GATTATAATT CGATGGTAAG CGCTAACGTG CTTAAGGCTA ACTATCGGGT GCCGGGGCGG
        L  P  V   D  Y  N  S   M  V  S   A  N  V   L  K  A  N   Y  R  V   P  G  R
#2871  ACTATCAGCA TGACGGTTGA AATCACTAAC CATACTGACC AGGTGATTTC TATTGGCGAG TTCAATACTG
        T  I  S  M   T  V  E   I  T  N   H  T  D  Q   V  I  S   I  G  E   F  N  T  G
#2941  GGGGCATTCG ATTCATGAAT GCAAATGTGC GGGTTGATGA GACGGATTAT CCTGAGGAGT TGTTGGCACC
         G  I  R   F  M  N   A  N  V  R   V  D  E   T  D  Y   P  E  E  L   L  A  P
#3011  GGAAGGGTTG GAAGTGAGTC AACAGGATAT CGCTCCAGGT GAAACCGTAG TTGTTGACAT CTCCGCCACC
        E  G  L   E  V  S  Q   Q  D  I   A  P  G   E  T  V  V   V  D  I   S  A  T
#3081  GATGCCGCCT GGGAAGTTCA GCGTATGGCC GACGTCATTT ATGATCCAGA CAGCCGCTTT GCGGGCTTGA
        D  A  A  W   E  V  Q   R  M  A   D  V  I  Y   D  P  D   S  R  F   A  G  L  I
#3151  TCTTCTTCGT TGATCCAGAG GGGAATGAGA TTCCGATACC TATCGGCGGT CCATTAGTTC CCACGTTTGT
         F  F  V   D  P  E   G  N  E  I   P  I  P   I  G  G   P  L  V  P   T  F  V
#3221  TTAGAAAGAA GTATCTTTAA CTATTTATGT TGCATGTGGT GTACGACAGC AATATGGTAG ATTAAACTGT
        *
#3291  TCCCATTGAA TTAAACACCG GCCCCTTGTC CCATGGGTGG ACGAAGGGCC GGTTGTTTGC TGTCCTTTCC
                        ---------------->        <----------------
#3361  ATTTTTTTTT TTATCTTGTG GCTCGGTCTT TTCAGGCTCT GAAGCTGCGT TTTAGTTAAT CCCTCAGAGC
                  ORF4                   -35                        -10      +1
#3431  AAAAAGGTAT TAGCTTATGA AAAAAGTACT ACTTAGCTGC CTTGTATCGT TTGGATTATT GTTGCTAAAT
        S/D              M  K   K  V  L   L  S  C   L  V  S  F   G  L  L   L  L  N
#3501  ACTTCCGCCA TGGCCCATGG GGGAGGAGGA ATTGAAACAG ATCAGTGCGT TGTCAATATC GGTAATTACC
        T  S  A  M   A  H  G   G  G  G   I  E  T  D   Q  C  V   V  N  I   G  N  Y  R
#3571  GAATGCATTT TTTTGCTTAC CAACCGGAAA CTTCTGGTGG TGAAGAGCTG TGCTGGGACT TGCCGATGAC
         M  H  F   F  A  Y   Q  P  E  T   S  G  G   E  E  L   C  W  D  L   P  M  T
#3641  GGGAAGCACC ATTTTAGTGT TTGATTTAGT GGATAGGATA TTGCGTGATC GGCCGGTTGA GGTTCGGATC
        G  S  T   I  L  V  F   D  L  V   D  R  I   L  R  D  R   P  V  E   V  R  I
#3711  GTTGAAGAGC AGAAAACGGC CGCTGGCCCG TCAAATTATA ATCCCATAGT AGAGCGTCCG GTTCAAAAAT
        V  E  E  Q   K  T  A   A  G  P   S  N  Y  N   P  I  V   E  R  P   V  Q  K  Y
#3781  ATCCAAAGGG AACTATTGAG CTTGATACCG ATTTTACCAA AGCAGGAGAA TACACTGCGG TAGTTATTCT
         P  K  G   T  I  E   L  D  T  D   F  T  K   A  G  E   Y  T  A  V   V  I  L
#3851  TGGTGGTGAT CAACCGATGG TCTTCAAAGC ACTCTTCGAG TGGGTCTCCA GGGAGAGCAG ACCGTTCAGT
        G  G  D   Q  P  M  V   F  K  A   L  F  E   W  V  S  R   E  S  R   P  F  S
#3921  GGATCGCCTC GATCGCTGGC GGTGCTTCTT ATTTTGGGGC TTATTTTTTG GTATAGTCGC CGTGGCGGAA
        G  S  P  R   S  L  A   V  L  L   I  L  G  L   I  F  W   Y  S  R   R  G  G  K
#3991  AAGAGGCCAA GGCATCCTAG TTTCAATCCA ATTGGAATTG GATACTTTAA ATAGTATTCA CAGCGTTTAG
         E  A  K   A  S  *
#4061  AACATAATAG AAGGAAGTTT CCCCGGCATT CCATTATCCA AATAATGGAA TGCCGGGGAA TTC
                                                                   EcoRI
```

Fig. 3-3. Continued.

nearly identical to 16S rDNAs from *Nitrosococcus oceanus* type strain C-27 and NOC strain C-107 (used in this study), differing only by 1 and 2 bases, respectively, out of 976 aligned bases. The ability of strain C-113 to grow autotrophically on ammonium marine media and the 16S rDNA sequence data identified the C-113 strain as belonging to the *Nitrosococcus* genus. We have sequenced 98% of *amoC* and the complete *amoA* and *amoB* genes for strain C-113 (GenBank AF153344). All three *amo* genes are 88-90% identical to the corresponding NOC genes (Table 3-2). The intergenic region between *amoC* and *amoA* from these strains is 77% identical. The observed 88-90% identity for the *amo* genes allows improved strain discrimination compared to the 99% identity values obtained for 16S rDNA for these γ-subdivision AOB. The *amo* genes also provide better discrimination for the β-subdivision strains.

Comparison of *amo* Genes and Their Putative
Expression Products

The comparison of identities and similarities for the *amo* and *pmo* genes and their deduced peptide sequences is shown in Table 3-2. MCA's *pmo* genes have been proposed to be homologous to the *amo* genes [6,7,19] and thus have been included in the analysis. An alignment of the deduced amino acid sequences is presented in Figure 3-4. The highest identity value at the DNA level is between NOC and C-113 at 90% for *amoB* and *amoC*. This level of identity (84-90%) in *amo* genes is also typical between representatives from the same genus found in the β-proteobacteria. For example, NEU and *Nitrosomonas*

Table 3-1
Size of *amo* genes and the intergenic spacer between *amoC* and *amoA* (*C-A* I.S.) in autotrophic ammonia-oxidizing bacteria and comparison to the *pmo* genes of *Methylococcus capsulatus* (Bath)

	amoC	*amoA*	*AmoB*	*C-A* I.S.
Nitrosococcus oceanus	786	744	1251	286
Nitrosospira sp. NpAV	813	825	1248	223
Nitrosomonas europaea	825	831	1304	163
C-113 (*Nitrosococcus sp.*)	-	741	1260	286
*Methylococcus capsulatus (Bath)**	783	744	1245	154

* = Sizes correspond to the *pmo* enzyme subunits.

eutropha (NET) have 88% identity in *amoA*. The lowest identity/similarity values were between NOC and NAV/NEU, with 38% identity at the DNA level (*amoC*) and between NOC and NEU with 37% similarity at the protein level (*amoC*). Both NOC and strain C-113 have higher identity values (49-55%) with MCA than with the β-subdivision AOB. Identity/similarity values are lowest between β and γ-subdivision AOB.

To analyze the secondary structure for the deduced peptides, the Kyte-Doolittle hydrophilicity [20], and the Chou-Fasman [21] Alpha, Beta, and Turn regions plots were obtained for all NOC Amo peptides (data not shown). When two functional proteins are compared as enzymatic analogs, a hydrophilicity analysis of the peptide sequences can be useful. The Kyte-Doolittle hydrophilicity plots for the NAV *amoA* and the NOC *amoA* are similar. The same domain motifs (transmembrane-hydrophobic regions) that appear in the NAV plots have similar corresponding motifs within the NOC *amoA* plots. NOC *amoC* and *amoA* have six transmembrane spanning domains, whereas NOC *amoB* has two transmembrane domains of high certainty. We observed that NOC and NAV Amo peptides share most of the general features that make them very likely to acquire the same tridimensional folding when active and associated with the membrane.

Our results show that the two ammonia-oxidizing strains, NOC and strain C-113, belong to the γ-subdivision and have a single copy of the *amo* operon. All members of the β-subdivision that have been examined contain at least two operon copies. The analysis of *amo*-genes from β- and γ-subdivision AOB

Table 3-2

Identity and similarity values for *amo* and *pmo* genes and their deduced peptides.

	A. *amoA*					B. *amoB*					C. *amoC*				
	NOC	C113	MCA	NAV	NEU	NOC	C113	MCA	NAV	NEU	NOC	C113	MCA	NAV	NEU
NOC		88	55	44	44		90	49	39	40		90	53	38	38
C113	93		54	41	42	93		49	39	39	94		54	42	42
MCA	61	59		53	49	50	52		46	41	56	56		48	43
NAV	42	41	47		72	38	39	40		64	38	46	43		75
NEU	43	42	48	84		38	39	40	73		37	46	40	85	

DNA Percent Identity in upper triangle, Amino Acid Percent Similarity in lower triangle.

and the *pmo* genes from γ-subdivision methane-oxidizing bacteria leads us to propose that these lineages may have trifurcated early in evolution and have evolved independently since then [22]. We suggest that the *amo* operon sequences from γ-subdivision AOB (NOC strain C-107 and strain C-113) will be useful for the identification of target sequences for γ-subdivision AOB community analysis in the same manner as *amo* operon sequences from NAV and other AOB [23-25] have been used for the analysis of β-subdivision

```
   NOCamoA.seq  MSAL.TS....AVRTPEEAAKVFRTLDFIALGAFFMILLASHHVHVMLLMGDWDFWVDWK
 C-113amoA.seq  MSAL.TS....AVRTPEEAAKVSRTLDFLALGAFFGILLASHHVHVMLLMGDWDFWVDWK
  MCApmoA1.seq  MSAA.QS....AVRSHAEAVQVSRTIDWMALFVVFFVIVGSYHIHAMLTMGDWDFWSDWK
  NAVamoA1.seq  MS..RTDEILKAAKMPPESVKMSRMIDAIYFPILCILLVGTYHMHFMLLAGDWDFWLDWK
  NEUamoA1.seq  VSIFRTEEILKAAKMPPEAVHMSRLIDAVYFPILIILLVGTYHMHFMLLAGDWDFWMDWK

                61                                                       120
   NOCamoA.seq  DRRFWVTVVPIVSVAYPAAAQAFFWEKFRLPFGATLVTLGVLAGEWANRYFNFVGFTYFP
 C-113amoA.seq  DRRFWVTVVPIVSVAYPAAAQAFFWEKFRLPFGATLVTLGLLAGEWANRYFNFVGFTYFP
  MCApmoA1.seq  DRRLWVTVTPIVLVTFPAAVQSYLWERYRLPWGATVCVLGLLLGEWINRYFNFWGWTYFP
  NAVamoA1.seq  DRQWWPVVTPIVGITYCATIMYYLWVNYRLPFGATLCIVCLLVGEWLTRFWGFYWWSHYP
  NEUamoA1.seq  DRQWWPVVTPIVGITYCSAIMYYLWVNYRQPFGATLCVVCLLIGEWLTRYWGFYWWSHYP

                121                                                      180
   NOCamoA.seq  INFVWPTILLPMALFLDAMLAISKSYGLTAVVGGLMYGLLMYPANWPLLSAFHVPAEYNG
 C-113amoA.seq  INFVWPTILLPMALFLDAMLAISKSYGLTAVVGGLMYGLLMYPANWPLLSAFHVPAEYNG
  MCApmoA1.seq  INFVFPASLVPGAIILDTVLMLSGSYLFTAIVGAMGWGLIFYPGNWPIIAPLHVPVENNG
  NAVamoA1.seq  INFVLPSTMIPGALIMDTVMLLTRNWMITALVGGGAFGLLFYPGNWPIFGPTHLPLVAEG
  NEUamoA1.seq  INFVTPGIMLPGALMLDFTLYLTRNWLVTALVGGGFFGLLFYPGNWPIFGPTHLPIVVEG

                181                                                      240
   NOCamoA.seq  VVMSLADIMGYQYVRTGTPEYIRMVEKGTLKTFGKDVVPVSAFFSGFVAMVMYFVWHFVG
 C-113amoA.seq  VVMSLADVAGYQYVRTGTPEYIRMVEKGTLRTFGKDVVPVSAFFAGFVAMVMYFVWHFVG
  MCApmoA1.seq  MLMSIADIQGYNYVRTGTPEYIRMVEKGTLRTFGKDVAPVSAFFSAFMSILIYFMWHFIG
  NAVamoA1.seq  VLLSLADYTGFLYVRTGTPEYVRLIEQGSLRTFGGHTTVIAAFFSAFVSMLMFCVWWYFG
  NEUamoA1.seq  TLLSMADYMGHLYVRTGTPEYVRHIEQGSLRTFGGHTTVIAAFFSAFVSMLMFTVWWYLG

                241                                 277
   NOCamoA.seq  RWFSKDYHINQL.........................
 C-113amoA.seq  RWFSKDYSVDQC.........................
  MCApmoA1.seq  RWFSNERFLQST.........................
  NAVamoA1.seq  KLYCTAFYYVKGPRGRVTMKNDVTAYGEEGFPEGIK.
  NEUamoA1.seq  KVYCTAFFYVKGKRGRIVHRNDVTAFGEEGFPEGIK.
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Fig. 3-4. Amino acid sequence alignment for the deduced peptides from the *amoA* and *pmoA* genes.

AOB [2]. Primers that amplify both the β and γ-subdivision AOB *amo* genes have been designed where these different sequences are most conserved [6]. However, this region is also conserved in *pmoA* and therefore these primers could amplify genes from methane-oxidizing bacteria, such as MCA. On the other hand, based on our complete sequences from two γ-AOB, it appears that the C terminal region of AmoA peptides is not conserved because it is truncated in the γ-subdivision representatives, making it a likely target for primers and probes specific for discrimination between β- and γ-subdivision AOB. Furthermore, this region would allow for discrimination between the *amoA* of NOC and the *pmoA* of MCA. While additional complete *amo* sequences from γ-subdivision AOB will substantiate the usefulness of this region for environmental analysis, our results indicate that the *amo* genes will be useful for fine-scale phylogenetic differentiation within the γ-subdivision AOB.

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CHAPTER 4

DIVERSITY OF AMMONIA MONOOXYGENASE OPERON IN AUTOTROPHIC AMMONIA-OXIDIZING BACTERIA [1]

Abstract

The autotrophic ammonia oxidizing bacteria use the essential enzyme ammonia monooxygenase (AMO) to transform ammonia to hydroxylamine. The *amo* operon consists of at least three genes: *amoC*, *amoA*, and *amoB*; *amoA* encodes the sub-unit containing the putative enzyme active site. The use of the *amo* genes as functional markers for ammonia-oxidizing bacteria in environmental applications requires knowledge of the diversity of the *amo* operon on several levels: 1) the copy number of the operon in the genome, 2) the arrangement of the three genes in an individual operon, and 3) the primary sequence of the individual genes. We present a database of *amo* gene sequences for pure cultures of ammonia-oxidizing bacteria representing both the β- and the γ-subdivision of the proteobacteria in the following genera: *Nitrosospira* (7 strains), *Nitrosomonas* (4 strains), and *Nitrosococcus* (2). We observed that the *amo* operon was found in multiple (2-3), nearly identical copies in the β-subdivision representatives but in single copies in the γ-subdivision ammonia oxidizers. The analysis of the deduced amino acid sequence revealed strong conservation for all three AMO peptides for both primary and secondary structure. The molecular systematic analysis of the *amoA* sequence database revealed clear patterns of relatedness in which the clustering was congruent to recent analysis based on the 16S rRNA sequences. For the *amoA* gene within the β-subdivision, nucleotide identity values are approximately 85% within the *Nitrosomonas* or the *Nitrosospira* groups but approximately 75% when comparing between these groups. We identified conserved regions in *amoA* and *amoC* which are targets sites for the construction of primers for PCR amplification of *amo* DNA isolated from pure cultures, enrichments, and the soil environment. The intergenic region between *amoC* and *amoA* is variable in length and is used to profile the community of ammonia-oxidizing bacteria in environmental samples.

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[1] Co-authored by J.J. Alzerreca, J.M. Norton, and M.G. Klotz. See Appendix B.

Introduction

Nitrification is a key process in the global nitrogen (N) cycle resulting in N loss from ecosystems, eutrophication of surface and groundwater, and the production of atmospherically active trace gases. Nitrification determines the form and mobility of inorganic N in soils and therefore influences N availability to plants and N loss by leaching out of the root zone. Ammonia oxidation to nitrite is the first step in the process of nitrification and is mediated primarily by the chemolithotrophic ammonia-oxidizing bacteria (AOB). Taxonomically, the cultured AOB are found in both the β- and the γ-subdivisions of the proteobacteria. The difficulty of isolating pure cultures and the low growth rates of the autotrophic ammonia oxidizers have slowed progress in characterizing their natural populations.

Recently, molecular tools have been developed based on 16S rRNA sequences to examine the diversity of uncultured AOB from the β-proteobacteria in both terrestrial and marine habitats [1-4]. While 16S rRNA sequences are the standard for taxonomic and phylogenetic investigations, insight into phenotypic characteristics of the ammonia oxidizers requires further characterization on the physiological and molecular level. Importantly, the high overall similarity of the partial ammonia oxidizer 16S rDNA sequences obtained from environmental DNA samples means that there are relatively few informative sites and discrimination on the strain or ecotype level may be difficult using this target [1]. For example, the overall 16S rDNA identity is at least 98.5% between *Nitrosospira* strain AHB1 and *Nitrosolobus multiformis* C-71 [5] and among seven new isolates from the *Nitrosospira* genus identity ranged from 97.4% to 100% [6]. These high identity values mean that designing discriminatory oligonucleotide probes and primers for environmental ammonia oxidizer strains may be difficult based on 16S rDNA sequences.

Ammonia monooxygenase (AMO) is a membrane-bound multiple subunit protein which is responsible for the conversion of ammonia to hydroxylamine [7]. AMO encoding genes are excellent molecular markers for ammonia oxidizers because of the enzyme's essential role in the metabolism of the autotrophic AOB from both the β- and the γ-subdivisions of the proteobacteria. Previously, few full-length sequences for genes encoding functional products have been characterized in AOB, all of these being from one organism *Nitrosomonas europaea* (NEU) [8-10]. In this study, we present a database of full-length

gene sequences encoding ammonia monooxygenase (AMO) from pure cultures of terrestrial and marine ammonia oxidizers. Based on the *amo* sequences available in GenBank (this study, [11]), internal portions of amoA have been amplified and cloned from environmental samples, thereby substantiating the suitability of *amoA* as a functional marker for AOB [4,11].

The AMO enzyme has not yet been isolated in a purified, active form, although *in vitro* activity of cell lysates can be maintained [12]. A 27 to 30 kDa-sized membrane-associated protein which contains the active binding site of AMO [7] and a second iron-copper protein of 38 to 43 kDa have been isolated as probable AMO subunits, AmoA and AmoB, respectively, from NEU. The sequences of two genes, *amoA* and *amoB*, coding for these subunit proteins have been determined from NEU [8,9]. The sequences of the *amoA* and *amoB* genes from additional ammonia oxidizer strains are presented in this paper. An ORF upstream of the *amoA* gene in all AOB examined to date has been designated as the *amoC* gene [13]. In *Nitrosospira sp.* NpAV (NAV), the *amoC* gene encodes an approximately 31 kDa membrane protein [13]. The *amoA*, *amoB* and *amoC* genes comprise the functional AMO operon [14]. The *amo* operon exists as multiple (two or three), nearly identical copies in all the ammonia oxidizer strains that have been examined to date (this paper, [15]) except for *Nitrosococcus* strains, which contain a single operon copy (this paper). The AMO enzyme in AOB is similar to the particulate methane monooxygenase (pMMO) which is found in the methane-oxidizing bacteria [16]. Both enzymes are members of a new class of integral membrane, iron/copper-containing monoxygenases [16]. The pMMO is also believed to be comprised of three subunits encoded in the genes *pmoA*, *pmoB*, and *pmoC* [17-19].

Structural genes encoding enzymatic functions have been used previously in environmental investigations of methane and methanol oxidation [20-23], hydrogen metabolism in *Desulfovibrio* [24,25], and in pollutant degradation [26,27]. Several of these studies illustrate the potential diversity found in functional genes [22,26]. Therefore, successful use and interpretation of results from experimental systems using functional genes as targets requires knowledge of the range of diversity of the target gene and a database of gene sequences. Furthermore, the sequence database from pure culture representatives is the first step towards developing the molecular tools for quantifying gene expression in the environment [24]. This paper presents a database for the *amo* genes of AOB which may be used as the basis for construction

of DNA and oligonucleotide probes and primers with specificity at the subdivision, clade, genus, and species levels. The identified highly conserved regions of the *amo* genes are suited as probes or PCR primers for the ammonia oxidizer functional group. The designed primers were successfully used to amplify portions of the *amo* operon from DNA extracted from environmental samples.

Materials and Methods

Bacterial Strains, Plasmids, and Culture Conditions

The bacterial strains and plasmids used in this study are summarized in Table 4-1. Ammonia oxidizers were cultivated and harvested as described previously [15]. Marine strains were grown in an artificial seawater media (12.5 mM $(NH_4)_2SO_4$, 187.5 mM NaCl, 7.5 mM KCl, 23.3 mM $MgSO_4$, 15 mM $MgCl_2$, 7.6 mM $CaCl_2$, 0.15 mM Na_2CO_3, 2.25 mM $NaHCO_3$, 0.5675 mM K_2HPO_4, 3 μM chelated iron, 1.0 μM $MnCl_2$, 0.4 μM Na_2MoO_4, 0.35 μM $ZnSO_4$, 0.008 μM $CoCl_2$, and 0.25% Phenol red, adjusted to pH 6.5-6.8).

DNA Isolation, Hybridization Analysis, Amplification, and Sequencing

Genomic DNA (gDNA) was isolated from the ammonia oxidizer cells following the procedure of McTavish et al. [8]. The gDNA was restriction digested, electrophoresed on a 0.8% agarose gel in 1x TAE, and blotted to a nylon membrane. The *amoA* DNA probes purified from plasmids were prepared by random labeling with digoxigenin or, alternatively, prepared using PCR with digoxigenin-UTP (Boehringer Mannheim). Initially, a NEU *amoA* probe of 792 bp was used for the hybridizations to identify *amo* similar genes. The probes were hybridized to the membrane and the hybridizing fragments detected following the manufacturer recommendations (Genius™ System, Boehringer Mannheim). Molecular weights of hybridizing fragments were determined using the RFLPscan program (Scanalytics/CSPI, Billerica, MA).

The gDNA was used as template for PCR. The Ericomp Delta-Cycler I system (San Diego, CA) was used with 0.2-mL thin-wall PCR tubes from Robbins Scientific Corporation (Sunnyvale, CA) for the PCR. The 50-μL PCR reactions contained 0.5 μM of each primer, 2.5 mM $MgCl_2$, 0.25mM dNTP mix, 1X

Taq polymerase buffer B [20 mM Tris-HCl (pH 8.0 at 25°C), 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol, 0.5% Tween 20 and 0.5% Nonidet P40], 100 ng template DNA, and 2.5 U Taq Polymerase.

Table 4-1

Bacterial strains used in this study

Strains (abbreviation)	Relevant phenotype or genotype	Genbank Accessions for *amo*	Source or reference
Nitrosospira sp. NpAV (NAV)	isolated from agricultural soil in Minnesota, USA	U92432, U72981, U38250, U38251, AF016003, U20644	E. Schmidt (Univ. of Minnesota) via Bruns
Nitrosospira briensis C-128 (NBR)	isolated from soil same as ATCC 25971	U76553	S. Watson and F. Valois (Woods Hole)
Nitrosospira sp. 39-19 (N39)	isolated from agricultural soil in Minnesota, USA	AF006692, AF016002	E. Schmidt (Univ. of Minnesota) via Bruns,
Nitrosovibrio tenuis NV-12 (NVT)	isolated from soil	U76552	S. Watson and F. Valois (Woods Hole)
Nitrosolobus multiformis ATCC 25196 (NMU)	type strain isolated from soil in Surinam, South America	U15733, U89833, U91603	ATCC
Nitrosolobus multiformis 24-C (N24)	isolated from agricultural soil in Minnesota, USA		E. Schmidt (Univ. of Minnesota) via Bruns
Nitrosomonas europaea ATCC 19178 (NEU)	isolated from soil, reported to be same strain as ATCC 25978	L08050, U96187	ATCC
Nitrosomonas eutropha C-91 (NET)	type strain sewage	U72670, U51630	S. Watson and F. Valois (Woods Hole)
Nitrosomonas sp. JL21	ammonia sensitive from activated sludge		Suwa et al. 1997 [43]
Nitrosomonas sp. AL212	ammonia sensitive from activated sludge		Suwa et al. 1997 [43]
Nitrosomonas sp. GH22	ammonia insensitive from activated sludge		Suwa et al. 1997 [43]
Nitrosococcus sp. C-113 (N113)	marine strain isolated from Red Sea		S. Watson and F. Valois (Woods Hole)
Nitrosococcus oceanus C-107 (NOC)	marine strain, same strain as ATCC 19707	U96611	S. Watson and F. Valois (Woods Hole)

PCR reagents were obtained from Promega (Madison, WI). The PCR conditions to obtain the desired products were an initial 4-min denaturation step at 94°C, followed by 25 cycles of a 1-min denaturing step at 94°C, a 1-min annealing step at 55°C, and a 4-min extension step at 72°C. A final extension step of 1 hour at 72°C completed the PCR, which is recommended for more efficient cloning [28].

The oligonucleotide primers for PCR were designed using published NEU [8,29] and our library of *amo* sequences. These primers were evaluated with the Amplify© program [30]. The PCR product was ligated into pCR™II plasmid and transformed into One Shot™ competent *Escherichia coli* cells according to the manufacturer's directions (TA Cloning® System, Invitrogen, San Diego, CA). The DNA sequences were obtained from purified plasmids (Wizard™, Promega Inc.; Madison, WI) containing the double-stranded insert templates, using M13F and M13R and synthetic primers based on the existing sequence and DNA polymerase for dideoxy dye-primer cyclo sequencing (ABI 373A, USU Biotechnology Center) following the manufacturer's instructions.

To obtain specific copies of the *amo* genes from the genome, gDNA was restriction digested and then size fractionated to obtain DNA specific to an individual copy (Fig. 4-1) [15]. This size-fractionated DNA was then used as a PCR template. For NAV, *Nitrosolobus multiformis* (NMU), and *Nitrosococcus oceanus* (NOC), we obtained sequences flanking the known regions using a chromosome crawl approach [31] on the size-fractionated DNA. The self-ligated size-fractionated DNAs were used as templates for the polymerase chain reaction (PCR) employing DNA polymerase and oligonucleotide primers designed to bind to the known regions of the gene based on our library of *amo* gene sequences. The PCR then proceeded from the known regions into the unknown flanking sequences (inverse PCR). When possible, PCR products were cloned, and plasmids were purified and sequenced as described above. However, many PCR products (especially those containing full ORF and promoter regions) were difficult to clone and maintain in *E. coli*; therefore, these products were sequenced directly. For direct sequencing, the PCR products were gel purified, reamplified and the products were purified using commercial kits.

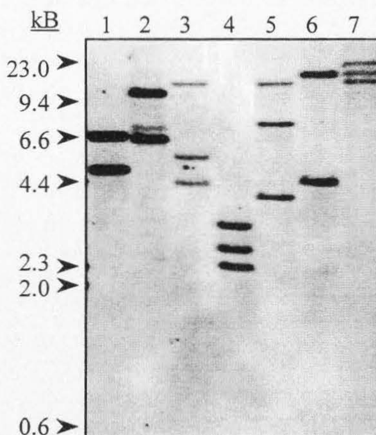

Fig. 4-1. Southern blot of ammonia oxidizer genomic DNA with *amoA* probe from *N. europaea* showing the multiple copies of *amoA*. Lane 1. *Nitrosomonas europaea, Eco*RI; 2. *Nitrosomonas eutropha, Eco*RI; 3. *Nitrosospira sp.* NpAV, *Eco*RI; 4. *Nitrosospira briensis, Eco*RI; 6. *Nitrosospira sp.* 39-19, *Kpn*I; 6. *Nitrosovibrio tenuis Kpn*I; 7. *Nitrosolobus multiformis* ATCC 25196, *Sac I.*

Sequence, Phylogenetic, and Topology Analyses

Obtained DNA sequences were contig aligned (Sequencher 3.0, Gene Codes Corporation, Ann Arbor, MI) and sequence similarities were investigated using the NCBI Blast program [32]. The sequences are deposited in GenBank under the accession numbers given in Table 4-1. Deduced amino acid sequences were aligned manually or using the Clustal W Program (clustalw@nig.ac.jp; or Lasergene™, DNASTAR™, Madison, WI). Amino acid similarity scores are based on the PAM 250 scoring matrix [33]. Although the use of the deduced protein rather than DNA sequences results in some loss of information, this approach avoids the problems associated with unequal rates of change for the third base position (silent substitutions) as compared to replacement substitutions in the first and second positions [34]. Furthermore the protein encoding regions are the most biologically relevant aspect of the gene sequences available and

can also be used to compare similar proteins that may not be unambiguously aligned at the nucleotide level [34]. The aligned amino acid sequences were processed by heuristic parsimonial analyses using PAUP version 3 [35]. In order to minimize the possibility that the algorithm would detect a local parsimony (potential monophyly of clusterings comprised of more than one species), 200 bootstrap replicates were generated. A 50% majority-rule consensus tree was constructed from parsimony replicates by using nearest-neighbor interchange branch-swapping during stepwise addition of the closest sequence. The deduced peptide sequences were also analyzed for membrane topology [36,37] and secondary structures [38] using Protean™ software (Lasergene™, DNASTAR™ Inc., Madison, WI).

Environmental Samples

Soil (0-30 cm) was sampled pre-planting from an replicated agricultural field plot (Cache Co. UT) that had been planted to silage corn and received various treatments of dairy wastes the previous year in a related project (Shi and Norton, unpublished). The soil treatments are replicated 4 times, 2 replicates of 4 selected treatments are shown as examples. The waste treatments shown include: 1) control plots with no nitrogen added, 2) soils treated with 200 kg N ha^{-1} as ammonium sulfate, 3) composted dairy waste at 100 Mg ha^{-1}, and 4) liquid dairy waste at 200 m^3 ha^{-1}. The waste treatment levels were chosen to apply approximately 200 kg ha^{-1} available N over the season. DNA was extracted from soil using a repriprocating disruptor (Fast Prep™ Instrument, Bio 101, Vista, CA) and the manufacturer's lysing matrix and isolation procedures for soil (FastDNA® Spin® Kit for Soil, Bio 101, Vista, CA). The isolated DNA was further purified before amplification using gel electrophoresis and collection of the high molecular weight DNA (>15kB). The following PCR conditions were used for environmental samples: Ericomp DeltaCycler I thermal cycler (Ericomp, Inc., San Diego, CA) 42 cycles (1 min at 94°C, 1 min at 52°C, and 4 min at 72°C) with a 7-min extension at 72°C; optimum concentrations were 2.5 mM $MgCl_2$ and 0.5 µM for primer. The primers used are shown in Table 4-2 and Figure 4-2. For nested PCR, the PCR reactions were purified before reamplification using columns (PCR Select®II, 5 Prime > 3 Prime, Inc., Boulder, CO).

Table 4-2

Primer sequences for amplification of *amo* genes from environmental samples.

Primer number	Target site and gene in *Nitrosospira sp.* NpAV GenBank accession U92432	Primer length (bp)	Sequence
301F*	1436-1461 *amoA*	26	GACTGGGACTTCTGGCTGGACTGGAA
302R	2084-2110 *amoA*	27	TTTGATCCCCTCTGGAAAGCCTTCTTC
304R	1316-1342 *amoA*	27	TAYCGCTTCCGGCGGCATTTTCGCCGC
305F	1012-1035 *amoC*	24	GTGGTTTGGAACRGICARAGCAAA
306R	2239-2236 *amoB*	25	CGTTCCACTGGATGGTAAGCATVCG

* F indicates forward primer, R indicates reverse

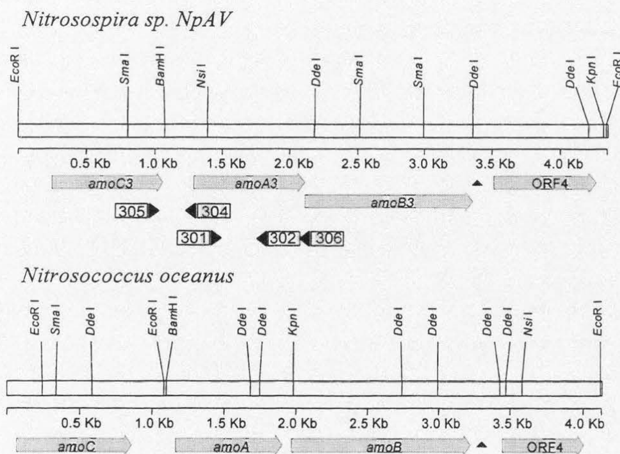

Fig. 4-2. Physical map of the *amo* operon in *Nitrosospira sp.* NpAV and *Nitrosococcus oceanus* showing the four ORFs: *amoC, amoA*, *amoB,* and ORF4. The target sites for various primers and the putative transcriptional terminator (•) for the *amo* operon are shown.

Results and Discussion

Copy Number and Gene Arrangement of the
amo Operon

All representatives of the β-subdivision AOB have multiple copies of the *amo* operon (examples are shown in Fig. 4-1). The restriction enzymes used for each strain do not cut within the ORF of *amoA*; in most cases the hybridizing fragment contains the whole operon. *Nitrosomonas* generally have two copies of the complete *amo* operon in the genome while *Nitrosospira* generally have three copies with the exception of *Nitrosospira tenuis* (NVT) (two copies). In NEU and NAV, additional copies of *amoC* have been found which are not associated with *amoA/B* [14]. In the γ-subdivision, for the strains examined, there is only one identified copy of the *amo* operon (Fig. 4-3). The observed variability in copy number needs to be

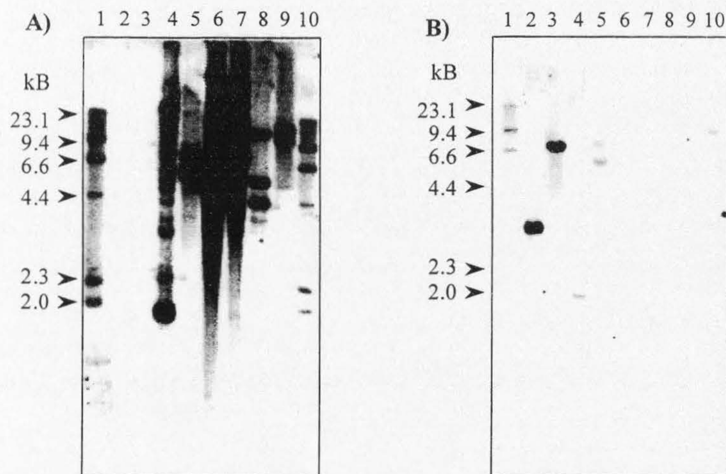

Fig. 4-3. Low stringency Southern blot of ammonia oxidizer genomic DNA. A) *amoA* probe from *Nitrosomonas europaea* and B) *amoA* probe from *Nitrosococcus oceanus*. All lanes contain equivalent amounts (~2 μg) of genomic DNA. Lane 1 MW marker, 2. *N. oceanus, Eco* RI; 3. *N. oceanus, Hin*d III; 4. *Nitrosolobus multiformis, Eco* RI; 5. *N. multiformis, Hin*d III; 6. *N. europaea, Eco R*I; 7. *N. europaea, Hin*d III; 8. *Nitrosospira sp.* NpAV, *Eco* RI; 9. *Nitrosospira sp.* NpAV, *Hin*d III; 10. MW marker.

accounted for if the *amo* target is to be used to quantify AOB. At present, the signal strength should be reported as *amo* copies, although if the community composition is known it would be possible to calculate an approximate bacterial cell number. The variation in copy number of the *amo* operon which we have observed is less than the variation in the copy number of 16S rDNA sequences in bacterial genomes [39]. The authors are unaware of any published information on the variability of *rrn* copy number in the AOB.

The physical maps of the *amo* operon are compared for NAV and NOC in Figure 4-2. In all cases observed the *amo* operons consists of *amoC* followed by a variable length intergenic region, then *amoA*, then *amoB*. Putative promoters have been identified upstream of both *amoC* and *amoA* and the full-length *amoCAB*, *amoAB*, and *amoC* transcripts confirmed in NAV and NEU [14]. In the β-subdivision AOB, *amoA* is followed immediately by *amoB,* in several cases with a 1-bp overlap and a shift in the reading frame. In the γ-subdivision bacteria, NOC, and *Methylococcus capsulatus* (Bath) (GenBank Accession U94337 [40]), there is an intergenic region between *amoA/pmoA* and *amoB/pmoB* of 65 bp and 106 bp, respectively. There is a strong transcriptional terminator following *amoB* (for example the stem loop found at position 3375-3416 in U92432 from NAV with Gibbs free energy value of -118.8 kJ/mol). An additional ORF4 encoding a membrane protein of unknown function has been identified following the *amoB* terminator in both β- and γ-subdivision AOB.

Within individual AOB strains observed to date, the multiple copies of the complete *amo* operon are nearly identical (this study, [15]), including the non-coding intergenic regions. It has been proposed that the near identity of *amo* operon copies is the result of rectification and that paralogy has most likely occurred in the common ancestor before the onset of orthologous evolution [41]. The near identity has been found to extend to regions beyond the terminator into, and possibly beyond, the ORF4, and we are currently investigating the extent of this replicated sequence in NAV. We reported recently that AOB genomes also contain copies of *amo*C genes that are not members of the *amoCAB* operons [14]. While the *amoC*-4 copy in NAV is nearly identical to the other three operon copies, the identified *amoC*-3 copy in NEU shared only 68% identity with the other two operon copies [14]. NAV also contains another locus which hybridized to an intragenic *amoC* probe from NOC. In contrast, all copies of *amoA* so far identified in AOB are members of the complete *amoCAB* operon structure described above. These observations

suggest that primer sets designed for environmental samples should include at least one target in *amoA* or *amoB* so as to focus on the complete *amo* operons in the genome.

Diversity of Primary Sequences of *amo* Genes and the Encoded Peptides

The sequence data available from this project have been submitted to GenBank database under the accession numbers listed in Table 4-1. A quick reference list of these and other sequences is included in Appendix D, and sequences are also available full length in appendix E. The largest differences in *amo* genes exist between the *amo* genes found in the γ- and the β-subdivision AOB. For example, the *amoC*, *amoA*, and *amoB* genes of NOC have identity values of 42-45% with the *amoC*, *amoA*, and *amoB* genes of the β-subdivision representatives (see Appendix G). The *amoC* to *amoA* and *amoA* to *amoB* intergenic regions were excluded from this analysis because of ambiguous alignment. The observed level of sequence identity is reflected in our observation that even under low stringency conditions there is no visible hybridization signal from genomic NOC DNA when probed with a NEU *amoA* probe (Fig. 4-3). Previous reports that the *amoA* gene from *N. oceanus* ATCC 19707 (=Watson strain C-107) was 100% identical to the NEU *amoA* in the internal region sequenced [42] were not supported by our observations. Sinigalliano et al. [42] also contended that PCR primers and hybridization probes targeting the *amoA* gene based on the *amoA* sequence of NEU should be both sensitive and general enough to detect a broad range of AMO-containing bacteria including those in the γ-subdivision such as NOC. Under our experimental conditions we were unable to amplify any *amo* genes from NOC using the primers suggested by Sinigalliano et al. [42] and observed no visible cross-hybridization between the NEU probe and NOC gDNA (Fig. 4-3). Nevertheless, there are conserved regions of *amoA* between the γ- and the β-subdivision representatives (see below), some of which have been previously used to amplify both the *amoA* and *pmoA* genes [16]. The partial sequence for *amoA* available for *N. oceanus* strain NCIMB 11848 (=Watson strain C-107, GenBank accession # U31652) now appears to be a contaminating *pmoA* sequence (C. Murrell, personal communication 1998). An additional marine AOB strain (C-113) was characterized by partial 16S rDNA sequencing (GenBank accession # AF153343) and was determined to be a γ proteobacterium closely related to *N. oceanus* strain C-107 (partial 16S rDNA identity 973/975 (>99%)). The identity level for the

complete *amoA* genes of these two strains of *Nitrosococcus* was 88% (see electronic supplement). The fact that *Nitrosococcus* strains with >99% identity in their 16S rDNA sequences have only 88% identity in their *amoA* genes substantiates the strength of using *amo* as a functional gene marker for strain differentiation in the γ-subdivision AOB.

Southern blots using the *amoA* gene probe from NEU showed that the *amoA* genes from all investigated strains within the β-subdivision AOB cross-hybridized (Fig. 4-1). Hybridization signals were stronger with probes corresponding to the same genus (Fig. 4-1), i.e., within *Nitrosomonas* or within the *Nitrosospira* group (including the former *Nitrosolobus, Nitrosovibrio* genera). Comparison of sequences and blots from within one species such as NMU (Fig. 4-4) showed equivalent hybridization strengths for the two representative strains but still exhibited restriction fragment length polymorphism and significant sequence divergence (88% identity). From our observations for both the γ- and the β-subdivision AOB, we conclude that the comparison of *amo* genes offers a finer level resolution (to the strain level) than 16S rDNA sequences.

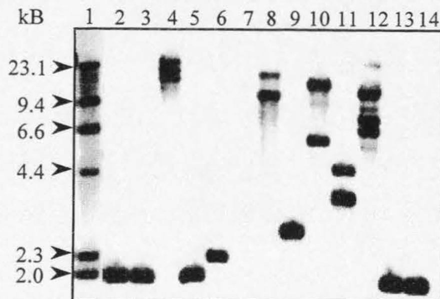

Fig. 4-4. Southern blot of ammonia oxidizer genomic DNA with *amoA* probe from *N. europaea*. Lane 1. MW marker Lanes 2-6 *Nitrosolobus multiformis* ATCC 25196: *Eco*R I, *Eco*R I+*Sac* I, *Sac* I, *Eco*R I +*Sma* I, *Sma* I; Lane 7. Empty; Lanes 8-14 *N. multiformis* 24C: *Bam*H I, *Bam*H I+ *Eco*RI, *Eco*R I, *Eco*R I +*Sac* I, *Sac* I , *Eco*R I +*Sma* I; *Sma* I.

The amino acid alignments for the deduced Amo peptides as well as an amino acid/DNA pair table are available as appendix G. The unusual start codon GTG is used in the *Nitrosomonas amoA* sequences so far examined. This is somewhat puzzling in that the genomes of *Nitrosomonas* strains tend to contain less (G+C) than their relatives in the *Nitrosospira* cluster [41]. Analysis of the peptide sequences revealed several interesting differences and similarities between *amo* operons of *Nitrosospira* and *Nitrosomonas*. First, the third and fourth amino acids in AmoA from *Nitrosomonas* are deleted in all *Nitrosospira* AmoA peptides examined to date. Interestingly, the environmental strains AL212 and JL212, which are classified as *Nitrosomonas* based on 16S rDNA [43] and have a GTG start codon in the AmoA ORF, also have this two amino acid deletion. Secondly, the amino acids from position 51 to 62 (GDWDFWXDWKDR, X=L, M, V, S) and from position 196-206 (YVRTGTPEYVR) in the alignment are strongly conserved in all AmoA and pMmoA sequences examined. These regions are predicted to form portions of periplasmic loops (Fig. 4-5 and text below, [44]) and we chose the first region as the target site for the conserved internal *amoA* forward primer, 301. Thirdly, amino acid residues 96 and 97 of the NEU AmoC peptide are deleted in AmoC peptides from NAV. While it had a *Nitrosospira*-typical deletion in AmoA, the environmental *Nitrosomonas* strain AL212 lacks this deletion in its AmoC peptide although overall its AmoC is more similar to those found in *Nitrosospira*.

Following the alignment of amino acid sequences of complete AmoA and pMmoA proteins, unrooted phylogenetic trees were constructed by parsimony methods based on the amino acid sequences of 20 peptides (Fig. 4-6). Trees were also constructed for the available AmoC and AmoB peptides (data not shown), which had a similar topology to the AmoA tree. The tree reveals a trifurcation between the AmoA from β-subdivision AOB, the AmoA from γ-subdivision AOB and the PmoA from the γ-subdivision methane oxidizer, *M. capsulatus* (Bath). Recent suggestions that the β-subdivision proteobacteria may actually be a subgroup of the γ-subdivision proteobacteria (Schleifer ASM 1998) and our (G+C) analysis [41] coincide with our observations (Fig. 4-6). We have also observed that the *Nitrosospira* AmoA peptides cluster closer together with shorter branch lengths, suggesting that *Nitrosospira* Amo sequences may have evolved at the slowest pace [41].

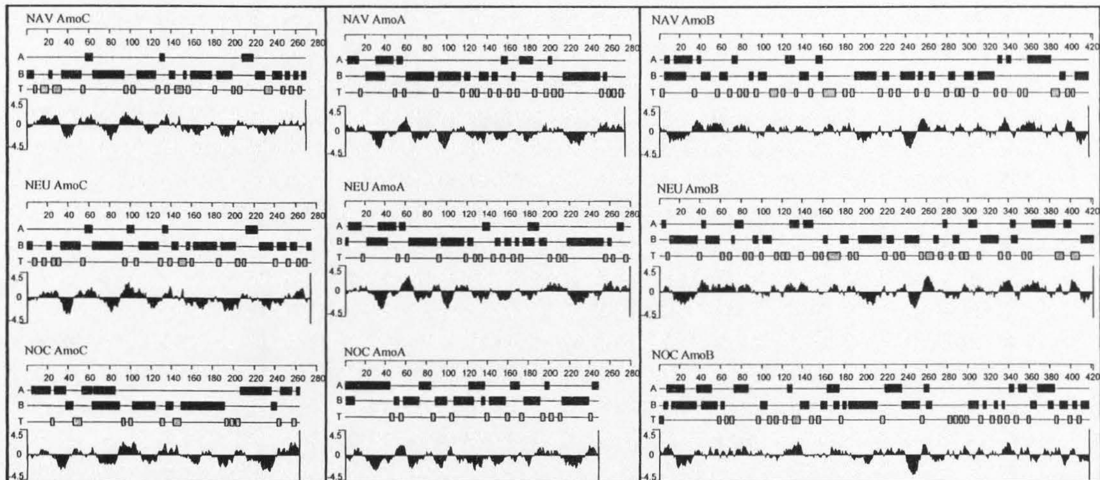

Fig. 4-5. Secondary structure predictions for AmoC, AmoA, and AmoB peptides from *Nitrosospira sp.* NpAV (NAV), *Nitrosomonas europaea* (NEU) and *Nitrosococcus oceanus* (NOC). The predictions for alpha (A), beta (B) and turn (T) regions as predicted by Chou [38] and the Kyte-Doolittle hydrophobicity plot [36] indicating membrane topology are shown.

The general topology of the branch representing the AOB in the β-subdivision is similar to trees based on 16S rDNA [6,43,45]. However, the AmoA sequences from the two ammonia-sensitive strains, AL212 and JL21, form a branch intermediate between the type culture *Nitrosomonas* and the *Nitrosospira* branches primarily due to the deletion of the amino acids in positions 3 and 4 in the alignment as discussed above.

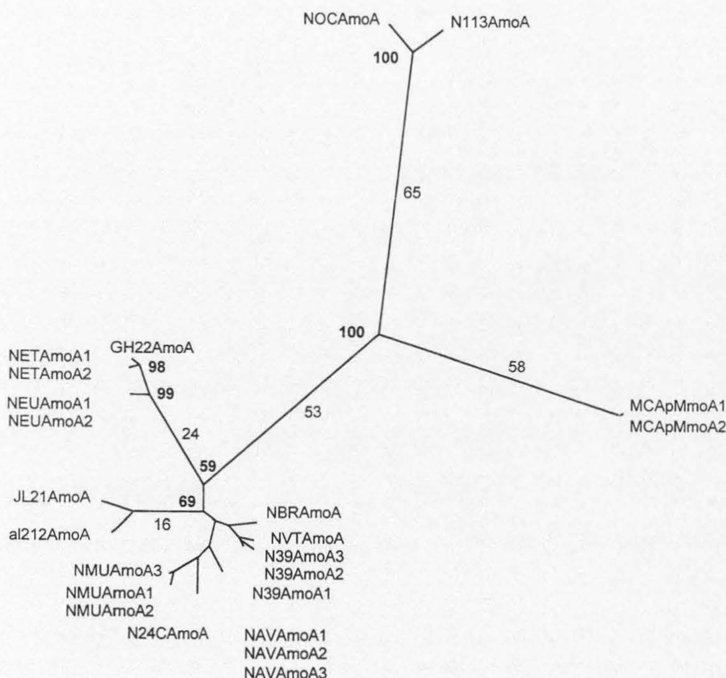

Fig. 4-6. Neighbor joining unrooted tree for the deduced AmoA peptide sequences based on the Clustal W alignment. The 50% majority-rule consensus tree was constructed from parsimony replicates by using nearest-neighbor interchange branch-swapping during stepwise addition of the closest sequence. For strain abbreviations see Table 4-1, MCA= *Methylococcus capsulatus* (Bath) (copy 2, [40]; copy 1, [17]).

Secondary Structures and Topologies of Deduced Amo Proteins

The secondary structures for the deduced AmoC, AmoA, and AmoB subunit proteins from NAV, NEU and NOC are shown in Figure 4-5. The best predictions indicate that both AmoC and AmoA are integral membrane proteins consisting of six transmembrane alpha-helices. Because these helices may be bundled in the membrane, only the side chains exposed to the exterior of the bundle need to be hydrophobic, interior-facing chains may form a hydrophilic pore through the membrane. The transmembrane helices are connected with generally short hydrophilic loops exposed to the aqueous environment: however, the periplasmic loops between the first and second and the fifth and sixth transmembrane domains in AmoA (from approximately residue 51-62 and 165-218 in the alignment, respectively) are predicted to be longer and consists of the highly conserved amino acids described above. In general, amino acids which interact with each other or the periplasmic environment are more conserved than the hydrophobic positions interacting with the membrane. The AmoB peptide is predicted to consist of two membrane spanning domains and long periplasmic or cytoplasmic regions. Detailed amino acid analysis revealed that each of the three Amo/pMmo subunit proteins contain three conserved histidine residues accessible to the periplasm which could form peripheral copper binding sites as suggested for pMmo by Nguyen et al. [19].

Application of *amo* Primers for Environmental Samples

The availability of the sequence database and alignments allowed us to construct primer sets targeting the *amo* operon of the AOB in the β proteobacteria (Table 4-2, Fig. 4-2). While primers may be developed that amplify *amoA* from both the β and the γ AOB, these will generally be so broad as to amplify *pmoA* as well. We suggest that primers be constructed specifically to differentiate between the *amo* and the *pmo* genes in the γ-subdivision after a more extensive database is available. The primer pair of 301-302 amplifies a core region of 675 bp from *amoA* from both pure cultures and soil DNA templates without a nested PCR (Fig. 4-7A,C). Because the length of this amplicon is identical in all β AOB examined, differentiation must be based on further analysis by restriction length polymorphism, denaturing gradient

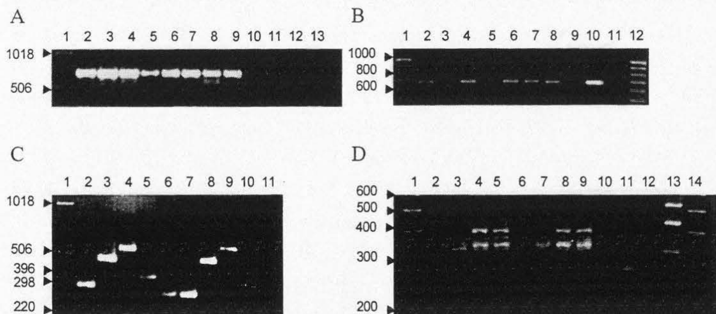

Fig. 4-7. PCR products of the *amoA* internal region (A and B) and the *amoC* to *amoA* intergenic region (C and D) for pure culture representatives and for extracted soil DNA. A) Lane 1 MW, lanes 2-12 products using primers 301-302; lanes 2-10. template genomic DNA from *Nitrosospira sp.* NpAV, *Nitrosolobus multiformis* 24C, *Nitrosospira sp.* 39-19, *Nitrosospira briensis*, *Nitrosomonas eutropha*, *Nitrosomonas europaea*, *Nitrosolobus multiformis*, *Nitrosovibrio tenuis*, *Nitrosococcus oceanus*, 11. positive control, 12. no DNA control, 13. MW standard. B) Lane 1 MW, lanes 2-11 products using primers 301-302, lanes 2-9. template extracted soil DNA. Soil treatments are 2 & 3: control no N added; 4 & 5 ammonium sulfate fertilized; 6 & 7 high rate compost added, 8& 9, high rate liquid waste; 10. positive control, 11. no DNA control, 12. MW standard. C) Lane 1 MW, lanes 2-11 products from primers 305-304, templates 2-9 same as panel A, 10. positive control, 11. negative control. D) Lane 1, MW, Lanes 2-13 products from primers 305-304 in a nested PCR, 2-9. templates as in panel B, 10. negative nested control, 11. positive control *N. europaea*, 12. negative control, 13. genomic DNA from *N. tenuis*, *N. multiformis*, and *Nitrosospira sp.* NpAV, 14. MW standard.

gel electrophoresis (DGGE), or sequencing of clone libraries. Hastings et al. [46,47] have previously used nested PCR to amplify an internal portion of *amoA* from DNA extracted from soil but their primers were specific for NEU and did not amplify even the closely related NET. We have designed primers that will minimize template bias within the β AOB based on our database. The primer pair 305-306 is useful for obtaining the full-length *amoA* gene and flanking regions for sequence analysis. The primer pair 305-304 amplifies from the end of *amoC* across the intergenic region and into *amoA* from both pure cultures and soil DNA templates (Fig. 4-7B and D). The length of the *amoC-A* intergenic region and the predicted length of the 305-304 amplicon for pure cultures are shown in Table 4-3. The shortest intergenic lengths were found for representatives of the *Nitrosomonas* genera. This intergenic primer set will be useful for profiling the AOB in environmental samples and for screening libraries of longer clones before sequencing in order to select representatives from different groups (i.e. *Nitrosomonas* versus *Nitrosospira*).

Table 4-3
The length of the *amoC* to *amoA* intergenic variable region and the length of the amplified region spanning from the end of *amoC* into *amoA* (from primer 305F to 304 reverse).

Strain	Length of amoC to amoA intergenic region (bp)	Length of 305F-304R amplicon (bp)
Nitrosospira sp. NpAV	223	331
Nitrosospira briensis C-128	263	371
Nitrosospira sp. 39-19	445	553
Nitrosovibrio tenuis NV-12	427	535
Nitrosolobus multiformis 25196	323	431
Nitrosolobus multiformis 24-C	261	369
Nitrosomonas europaea 19178	163	277
Nitrosomonas eutropha C-91	173	287
Nitrosomonas sp. AL212	174	282
Nitrosomonas sp. JL21	173	281
Nitrosomonas sp. GH22	173	287
Nitrosococcus oceanus C-107	308	NA
Nitrosococcus sp. C-113	286	NA

NA=no amplification since these are γ subdivision ammonia oxidizing bacteria not found in soil.

We applied this intergenic primer set in a nested PCR (305-302 initially followed by 305-304) and detected changes in the community composition of the AOB related to soil treatments without cloning or sequencing (Fig. 4-7D). The *amo* intergenic length profiles created using this combination of primers were consistent for the two field-replicated soil samples and are different based on the N fertilization regime. All samples, including the untreated soil, had two bands (355 and 339 bp) close to the amplicon size in NAV (331 bp). The soils treated with animal wastes or ammonium sulfate fertilizer showed additional bands, some which are closer in size to *Nitrosomonas.* The soils treated with large amounts of available NH_4^+ at one time (Fig. 4-7D lanes 3, 4 and 7, 8) have a strong band closer to the NMU length (431 bp). Overall, the profiles of the intergenic length allow us to conclude that 1) several types of AOB coexist in these soils, 2) the dominant types represent different types of *Nitrosospira*, 3) representatives from *Nitrosomonas* appear in the waste treated and the fertilized soils, and 4) changes in ammonium availability results in different AOB community structure. The observation that nitrosomonads were detected in the waste treated or

heavily fertilized plots is similar to observations using 16S rDNA targeted probes in soils which had received swine manure [46]. Additional work is ongoing to include competitive internal standards to develop a quantitative PCR system. Further characterization of the diversity of the *amo* operon in environmental samples will extend our ability to link the structure of the ammonia oxidizer community to the functional controls of nitrification.

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CHAPTER 5

CONCLUSIONS

Ammonia-oxidizing bacteria (AOB) are chemolithotrophs that oxidize ammonia/ammonium (NH_3/NH_4^+) to nitrite (NO_2^-) in a two-step enzymatic process to obtain the energy they require for survival. The membrane-bound ammonia monooxygenase (AMO) enzyme carries out the first rate-limiting step in this reaction. For this reason, the AOB must carry at least one functional copy of the *amo* operon within their genome. The overall hypothesis of this research was that *amo* genes would be useful markers for the analysis of the diversity of AOB. At the initiation of this project, only one *amoA* and a partial *amoB* sequence from *Nitrosomonas europaea* were available [1]. In this project, a collection of *amo* sequences from diverse AOB strains was obtained. These sequences were essential for the rational design of tools to analyze the diversity of AOB present in the environment.

The *amo* operon was initially thought to be made of two genes, *amoA* and *amoB*. The *amoA* gene is presumed to contain the sequence for the active site. In Chapter 2 we identified a third gene, *amoC*, upstream of *amoA* and *amoB* in *Nitrosospira sp.* NpAV. We found *amoC* to be present in all AOB examined and later observed *amoC* to be contained on a single transcript with *amoAB* [2]. Therefore, *amoC* is the third member of the AMO operon. However, the *amoC* gene function remains unknown. AmoC may act as a component that holds the other subunits in place, since the AmoA subunit is susceptible to frequent inactivation and probably needs frequent replacement. We started our *amoC* study in *Nitrosospira sp.* NpAV and after Southern analysis and PCR, we identified four copies, three of them within the three *amo*-operon copies that this strain carries. The additional fourth copy is identical to the others but independently located within the genome [2]. *N. europaea* has two, nearly identical, *amo*-operon copies, and we found *amoC* to be within each one of them. Furthermore, we also found an additional independent *amoC* copy within the genome; however, it is not identical to the other two operon-*amoC* copies [2].

We have also identified a fourth open reading frame (ORF4) following *amoB* in all strains examined. However, we believe that it is not part of the *amo* operon since there is a strong transcription terminator following *amoB*. The presence of ORF4 in all examined strains and secondary structure analysis suggests that it may encode a membrane protein with a possible role in the ammonia oxidation.

While the identified soil AOB are found in the β-subdivision, at least one AOB genus, *Nitrosococcus*, belongs to the γ-subdivision. In Chapter 3 we presented the first *amo* operon sequences for two *Nitrosococcus* strains, *N. oceanus* and strain C-113. Our work on these two marine strains revealed that they do share many similarities with the β-subdivision AOB but also have interesting differences. In Southern analysis experiments, we observed the *amo* genes cross-hybridize only within a subdivision (β or γ), but not across the β- and γ-subdivisions. Cross-hybridization is observed at various levels between *Nitrosomonas* and *Nitrosospira* (*Nitrosovibrio, Nitrosolobus*) strains and between *Nitrosococcus* strains.

We have consistently observed that all AOB strains examined in the β-subdivision have at least two copies of the AMO operon. In contrast, the marine AOB studied belonging to the γ-subdivision have a single copy. The significance of the number of copies of the *amo* operon in the physiology of AOB remains to be investigated.

Analysis of the *N. oceanus amo* sequences revealed thay they shared a higher sequence identity with the genes for the particulate methane monooxygenase (pMMO) from *Methylococcus capsulatus* (Bath) than with the *amo* genes from the β-subdivision representatives. We also observed that the strains examined in the γ-subdivision have an intergenic region between *amoA* and *amoB* similar to the intergenic separation between *pmoA* and *pmoB*. Within the β-subdivision strains, *amoB* follows immediately to *amoA*, and sometimes with a one base overlap. Phylogenetic analysis of AMO from the β- and γ-subdivisions and pMMO results in an unrooted tree with a trifurcation, indicating an early divergent event in evolution of these proteins.

Although the *amo* operon consists of at least three genes (*amoC*, *amoA*, and *amoB* for both the β- and γ-subdivision AOB), we have focused on the sequencing of *amoA* for all strains examined. Whenever possible, we have also obtained the sequence of the *amoB* and *amoC* genes. For most strains we have also sequenced *amoA* from one or more specific copies of the genome.

Our results on Chapter 4 indicate that the *amo* genes offer an important complement to the analysis of 16S rRNA sequences for the assessment of the diversity of the AOB. The *amo* genes were revealed to have the ability to differentiate between closely related strains which may be difficult to

separate based on 16S rRNA sequence analysis. Our *amo* gene database is presented and we demonstrate how the use of the aligned sequences allows us to identify conserved regions of the *amo* genes useful as targets for PCR primers. These primers are used in the amplification of *amo* sequences from DNA isolated from the soil environment. Several recent studies on the diversity of AOB in the environment have used our *amo* sequences from GenBank as the basis for the development of primers and for comparison to their environmental *amo* sequences [3-5]. This substantiates the importance of our contributions of *amo* sequences to the international database.

Our *amo* database allowed for the design of molecular tools for the analysis of the diversity of *amo* genes in soil DNA. Primers 301-302 were designed within the *amoA* gene, and are useful for the initial detection of *amo*-containing populations. To further obtain complete *amoA* sequences and flanking regions for these populations, primers 305-306 were designed. Primers 305-304 amplify the intergenic region after *amoC* and before *amoA*. These primers were designed to quickly characterize environmental samples since these PCR products are of different sizes and provide a useful profile on agarose gels. Since the size of this intergenic region is species dependent, several bands are often observed representing different AOB present in the environment. It then becomes a matter of matching these PCR products with PCR products made with known pure cultures by size. We have accomplished sufficient resolution to separate these PCR bands with high concentration agarose gels. Using these molecular tools, we have observed profile changes based on the N treatments given to the soil. We conclude that molecular tools based on the *amo* genes provide an efficient method to screen and evaluate the AOB community in environmental samples. However, this iterative screening process is not only useful for profile analysis. It also provides a way to detect new strains (new *amoA* variations) to further expand the database, and hence provide more information to refine the current primers. It remains to fine-tune the primers in order to get more useful data from the PCR products, and to broaden their reach. On the other hand, new primers can also be designed to exclude non-desired sequences. This and other possibilities are attainable, as the *amo* gene database grows.

We further analyzed the deduced amino acid sequences from the subunits for AMO for all strains and also compared them to the pMMO amino acid sequences. For AmoA and pMmoA, the amino acids from position 51 to 62 and from position 196 to 206 (numbering as in the Clustal alignment, see appendix

G) are strongly conserved in all sequences examined. These regions are predicted to form portions of periplasmic loops. Within the β-subdivision AOB, we observed some consistent differences when the amino acid sequences were compared between *Nitrosospira* and *Nitrosomonas*. For example, when *Nitrosospira sp.* NpAV and *N. europaea* species were compared, we observed amino acid deletions for *Nitrosospira sp* NpAV in the AmoA (number 3 and 4) and the AmoC (number 96 and 97) peptide sequences. However, two environmental *Nitrosomonas* strains, AL212 and JL212, also have the *Nitrosospira*-like AmoA deletions.

The predictions from secondary structure analysis indicate that AmoC and AmoA are integral membrane proteins consisting of six transmembrane alpha-helices. Because these helices may be bundled in the membrane, only the side chains exposed to the exterior of the bundle need to be hydrophobic. Interior-facing chains may form a hydrophilic pore through the membrane. The transmembrane helices are connected with generally short hydrophilic loops exposed to the aqueous environment; however, the periplasmic loops between the first and second (~ residues 51-62) and the fifth and sixth (~ residues 165-218) transmembrane domains in AmoA are predicted to be longer and consist of highly conserved amino acids. In general, amino acids which interact with each other or the periplasmic environment are more conserved than the hydrophobic positions interacting with the membrane. The AmoB peptide is predicted to consist of two membrane spanning domains and long periplasmic or cytoplasmic regions.

AMO and pMMO are members of a novel class of monooxygenases that share many similar features. When the *pmoC* gene was discovered and sequenced for the methanotrophs, this implied that a third gene could also exist for AMO. When the *pmoC* gene information was released, it further encouraged the search for the probable *amoC* gene, which was quickly found and sequenced. Both AMO and pMMO have at least three subunits, exhibit high amino acid sequence similarity (61-59% between *amoA* vs. *pmoA* both from γ-representatives and ~48% between *amoA* from β-representatives vs. *pmoA* from γ-representatives) and contain iron [6,7]. We observed that both AMO and pMMO have three conserved periplasmic histidines, and since these enzymes are also known to be associated with copper, these histidines may have a role in copper binding.

Ideally, the molecular tools based on the *amo* genes developed in this project will allow us to obtain a better picture of the environmental AOB community than previously possible. We have demonstrated that DNA can be directly extracted from soil, further purified, and then *amo* genes from unknown soil AOB can be amplified using our primers. This direct approach eliminates the need for selective isolation procedures and their inherent biases.

We have found the genes encoding AMO to be excellent functional markers for the AOB for two reasons: 1) *amo* genes are always and only found in AOB and 2) *amo* sequences offer better resolution of closely related AOB strains than 16S rDNA markers. We have studied the AMO operon genes extensively for representative AOB strains, and our library of these *amo*-gene sequences has allowed us to develop a method that when used on environmental samples, results in the retrival of *amo* sequences from a fairly broad range of AMO-containing bacteria. Finally, we have significantly increased the number of *amo* sequences from pure culture strains available in the GenBank database.

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APPENDIXES

Appendix A. Electronic Mail Permission Letter

for Chapter 2 and Chapter 3

FCR/hw/mar99.255
25 March 1999

Javier Alzerreca
Utah State University

Dear Mr Alzerreca

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14 December 1999

Mr Jose Javier Alzerreca
c/o Jeanette Norton
Department PSB
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Dear Mr Alzerreca

***FEMS Microbiology Letters**, Vol 180, 1999, pp 21-29. Alzerreca et al. "The amo operon in marine, ammonia-oxidizing ...."*

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Appendix B. Authorship Letters for

Chapter 2 and Chapter 4

UofL® University of Louisville, Kentucky

Department of Biology and Center for Genetics and Molecular Medicine

Life Sciences Building
Louisville, KY 40292

October 12, 1999

Dr. Noelle E. Cockett
Interim Dean of Graduate Studies
Utah State University
Logan, Utah 84322

Dear Dr. Cockett,

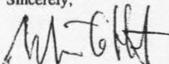
This letter is to attest that Mr. Javier J. Alzerreca is a major author of the published article:

> Klotz, M.G., J. Alzerreca, and J.M. Norton. 1997. A gene encoding a membrane protein exists upstream of the *amoA/amoB* genes in ammonia-oxidizing bacteria; a third member of the *amo* operon? FEMS Microbiol. Letters 150:65-73.

as he has performed the majority of the experiments that were reported in this paper.

Please do not hesitate to contact me if you have further questions.

Sincerely,



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October 20, 1999

Dr. Noelle Cockett
Interim Dean of Graduate Studies
Utah State University
Logan UT 84322

Dear Dr. Cockett,

This letter is to attest that Mr. Jose Javier Alzerreca is the major author of his thesis chapter IV that is an unpublished article. The article will be submitted for publication in a revised form sometime in the next year. Javier performed the majority of the experimentation described in this chapter.

Sincerely,

Jeanette Norton
Associate Professor of Soil Microbiology

Appendix C. Protein Expresion Studies for *amoC*

As deduced from the obtained nucleotide sequence, the *amoC3* gene in NAV encodes a neutral (pI=6.7) 270-amino-acid polypeptide of approximately 31.1 kDa (Fig. B1). Topological analysis of the predicted primary structure revealed 6 membrane-spanning domains (Fig. B2). Analysis of the AmoA and AmoB proteins from *N. europaea* [1] and NAV [2] also revealed multiple membrane-spanning domains; 6 predicted domains for AmoA and two domains for AmoB. As in the case of AmoA, the AmoC3 peptide did not contain an N- terminal signal sequence [3] suggesting that membrane incorporation occurs without the chaperoning aid of the SecA protein. Instead, the AmoC protein could serve itself as a chaperoning locator helping the AmoA and AmoB protein subunits to integrate into the membrane properly. AmoC can be expressed independently from other AMO subunits [4, 5].

Furthermore, Sayavedra-Soto et al. [6] suggested that the AmoA subunit, which carries the catalytic site of ammonia oxidation [7], is frequently inactivated and needs to be replaced by *de novo* synthesized protein. In such a scenario, AmoC could serve as a director which supervises AmoA - AmoB interactions in order to maintain AMO functionality

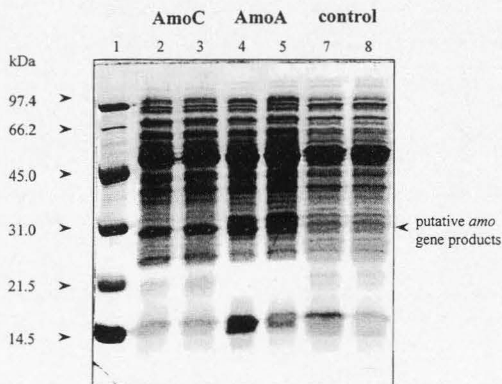

Fig. B1. Polyacrylimide showing the size and presence of the putative *amoC* gene product.

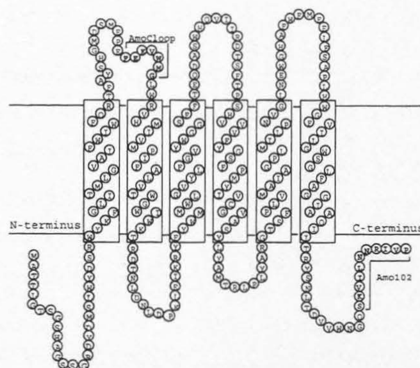

Fig. B2. Proposed membrane topology of the *amoC3* gene product of *Nitrosospira* NpAV. The membrane spanning domains were obtained by using the algorithm of Sipos and von Heijne (1993). The calculation was based on a full window of 21, a core window of 11 and a critical length of 60 from the complete sequence of 270 amino acids.

To study the expressibility of the cloned *amoC3* gene, the SDS-PAGE profiles of fluids from *E. coli* strain DH5α carrying recombinant plasmids pNA101-9 (Fig. B1; Lanes 2 and 3), (pNA-5) (Fig. B1; Lanes 4 and 5) and pNA10-9 (Fig B1; Lanes 6 and 7) were compared and analyzed. The gel reveals the presence of a unique, approximately 30 kDa single protein band (putative AmoC3) in both the total lysate and protoplasmic fluids from the strain containing pNA101-9, a double band at approximately 30 kDa (putative AmoA3, [5]) in the fluids from the strain containing pNA-5 and no unique bands in the fluids from the control strain containing pNA10-9. Both polypeptides, AmoA3 and AmoC3, have an approximately 6 kDa smaller molecular mass based upon the standards used in SDS-PAGE than their masses calulated from the deduced primary structures. The unique additional band of approximately 14.5 kDa in the total lysate from clone DH5α (pNA-5) (Fig. B1, lane 4), may represent an expression product unique to the complementary strand of the DNA insert in pNA10-5.

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Appendix D. *amoC* and *amoAB* Transcription Studies with RT-PCR in *Nitrosospira* sp. NpAV

1. Materials and methods

1.1 Strains and cell cultures.

Nitrosospira sp. NpAV were grown in batch cultures as described [1, 2]. Cell cultures (1.0 to 1.5 l) in late logarithmic phase were harvested for nucleic acid extraction.

1.2 DNA manipulations.

Genomic and plasmid DNA preparations, DNA restriction digests, Southern and Northern hybridizations, and other standard DNA manipulations were performed as described [3].

The transcript for *amoC* was evaluated by the reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was isolated as described [3, 4]. For RT-PCR the RNA was treated 15 min. at 37°C with RQ1 RNAse-free DNAse (1u/µg of template DNA), phenol-chloroform 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 dideoxy-nucleotides-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.

2. Results

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

In *Nitrosospira* sp. NpAV, four copies of *amoC* were detected, but only three of these were found on the same fragments with *amoAB* [5]. In both bacteria, all identified *amoA* copies were preceded by an *amoC* gene (this work and [5]).

2.2 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 [6]. 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 [7]. Because of the contiguous location of *amoC* to *amoAB*, seemed likely that *amoC* was also present on the transcript which contained *amoAB* [5, 6]. To examine whether *amoC* was part of the *amoAB* operon in *Nitrosospira* sp. NpAV we performed reverse transcriptase-PCR (RT-PCR).

The RT-PCR was done using primers complementary to *amoC* and either *amoA* or *amoB* and *Nitrosospira* sp. NpAV total RNA. The amplified fragments were obtained using forward primer CL2 and antisense primers located in *amoA* or *amoB*. RT-PCR products were produced from *Nitrosospira* sp. NpAV total RNA corresponding to the predicted sizes (Fig. C1). 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 *Nitrosospira* sp. NpAV as well. This result was confirmed using primers in *amoA* and *amoB* which also gave strong products. Thus, with RT-PCR we demonstrated that the transcripts from the *amo* operon in *Nitrosospira* sp. NpAV contained *amoC*, *amoA*, and *amoB*.

RT-PCR for the *amo* operon

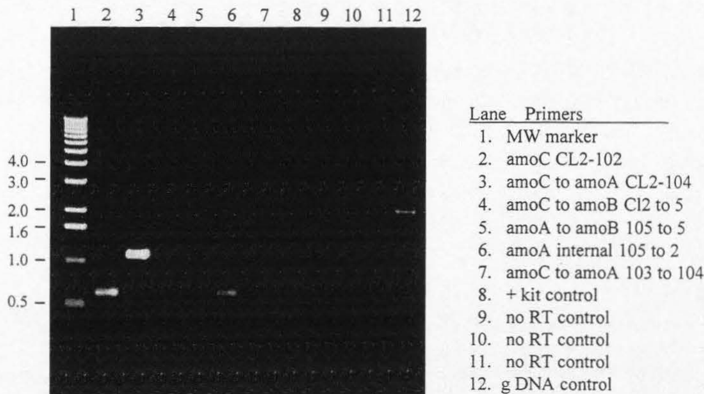

Fig. C1. Agarose gels showing the amplified DNA fragments produced by the reverse transcriptase-polymerase chain reaction from total RNA. Primers chosen to produce amplicons containing *amoC, amoCA* or *amoCAB* were used. Lane 1: DNA marker in kb. The following lanes show the DNA amplified from *N. europaea* (lanes 2-4) and from *Nitrosospira sp.* NpAV (lanes 5-7). Lane 8: + kit control, lanes 9-11: no RT control, lane 12: + gDNA control. The reactions used primer CL2 and the following primers: lane 2: primer 102 for internal *amoC* amplicon; lane 3: primer Ne, *amoC* to N terminus *amoA* amplicon; lane 4: primer CT *amoC* to C terminus *amoA* ; lane 5 primer 102 internal *amoC* amplicon; lane 6 primer 104, *amoC* to middle *amoA* amplicon; lane 7: primer 5 *amoC* to N terminal region *amoB*.

3. Discussion

After performing RT-PCR experiments we conclude that in *Nitrosospira* sp. NpAV, *amoC* is also part of the *amo* operon as proposed recently [5]. The *amoC* gene mRNA was detected by RT-PCR and specific *amoC-3* primers, demonstrating its transcription. The genes coding for the three AMO subunit polypeptides are co-transcribed from each of the multiple *amoCAB* operons in *Nitrosospira* sp. NpAV and *N. europaea* [8]; therefore, the similarities among ammonia-oxidizing bacteria (including methanotrophs) can be extended from the level of gene product similarity to the transcriptional level.

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 [5]. 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.

Acknowledgments

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Appendix E. List of GenBank Entries

List of GenBank Entries to date (with Javier Alzerreca as author):

AF016003: *Nitrosospira sp. NpAV* ammonia monooxygenase operon copy 2; ammonia monooxygenase 2 subunits C (amoC2), A (amoA2), and B (amoB2) genes, complete cds.

U92432: *Nitrosospira sp. NpAV* ammonia monooxygenase operon copy 3: ammonia monooxygenase 3 subunits C (*amoC3*), A (*amoA3*) and B (*amoB3*) genes, complete cds.

AF071774: *Nitrosospira sp. NpAV* ammonia monooxygenase subunit C-4 (amoC-4) gene, complete cds.

AF032438: *Nitrosospira sp. NpAV* ammonia monooxygenase operon copy 1: ammonia monooxygenase 1 subunit C (amoC1) gene, partial cds, and ammonia monooxygenase subunits A (amoA1) and B (amoB1) genes, complete cds.

AF016002: *Nitrosospira sp. Np39-19* ammonia monooxygenase subunit A2 (*amoA2*) gene, complete cds.

AF006692: *Nitrosospira sp. Np39-19* ammonia monooxygenase subunit A3 (*amoA3*) gene, complete cds.

AF042170: *Nitrosospira sp. Np39-19* ammonia monooxygenase subunit C1 (amoC1) gene, partial cds; ammonia monooxygenase subunit A1 (amoA1) gene, complete cds; and ammonia monooxygenase subunit B1 (amoB1) gene, partial cds.

U96611: *Nitrosococcus oceanus* ammonia monooxygenase subunit A (*amoA*) gene, complete cds.

AF047705: *Nitrosococcus oceanus* ammonia monooxygenase subunit A (amoA) and ammonia monooxygenase subunit B (amoB) genes, complete cds; and unknown gene.

U96187: *Nitrosomonas europaea* ammonia monooxygenase C1 (*amoC1*) gene, partial cds.

AF073793: *Nitrosomonas europaea* ammonia monooxygenase 3 subunit C (amoC3) gene, complete cds.

U91603: *Nitrosolobus multiformis* ammonia monooxygenase subunit AmoC1 (*amoC1*) gene, partial cds, ammonia monooxygenase subunit AmoA1 (*amoA1*) gene, complete cds, and ammonia monooxygenase subunit AmoB1 (*amoB1*) gene, partial cds.

AF042171: *Nitrosolobus multiformis* ammonia monooxygenase subunit AmoA (amoA) gene, complete cds.

U89833: *Nitrosolobus multiformis* ammonia monooxygenase operon copy 3: ammonia monooxygenase subunit AmoC3 (*amoC3*) gene, partial cds, ammonia monooxygenase subunit AmoA3 (*amoA3*) gene, complete cds and ammonia monooxygenase subunit AmoB3 (*amoB3*) gene, partial cds.

U76552: *Nitrosovibrio tenuis* ammonia monooxygenase subunit A (*amoA*) gene, complete cds.

U76553: *Nitrosospira briensis* ammonia monooxygenase subunit A (*amoA*) gene, complete cds.

AF153344: C-113) ammonia monooxygenase subunit A

(amoA) and ammonia monooxygenase subunit B (amoB) genes, complete cds.

AF153343: Unidentified ammonia-oxidizing bacteria (strain C-113) partial 16S rRNA sequence.

Appendix F. GenBank Entries

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            complete cds.
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NID         g2654877
VERSION     AF016003.1  GI:2654877
KEYWORDS    .
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            Eubacteria: Proteobacteria: beta subdivision: Ammonia-oxidizing
            bacteria: Nitrosospira.
REFERENCE   1  (bases 1037 to 1861)
  AUTHORS   Klotz.M.G. and Norton.J.M.
  TITLE     Sequence of an ammonia monooxygenase subunit A-encoding gene from
            Nitrosospira sp. NpAV
  JOURNAL   Gene 163 (1). 159-160 (1995)
  MEDLINE   96001263
REFERENCE   2  (bases 1037 to 1861)
  AUTHORS   Norton.J.M.. Low.J.M. and Klotz.M.G.
  TITLE     The gene encoding ammonia monooxygenase subunit A exists in three
            nearly identical copies in Nitrosospira sp. NpAV
  JOURNAL   FEMS Microbiol. Lett. 139 (2-3). 181-188 (1996)
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            genes in ammonia oxidizing bacteria: a third member of the amo
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  JOURNAL   FEMS Microbiol. Lett. 150 (1). 65-73 (1997)
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  TITLE     Diversity of the genes encoding ammonia monooxygenase in
            autotrophic ammonia oxidizing bacteria
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  AUTHORS   Klotz.M.G. and Norton.J.M.
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  JOURNAL   Submitted (24-JUL-1997) Department of Biology. University of
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LOCUS       NSU92432     4341 bp    DNA             BCT       02-MAY-1997
DEFINITION  Nitrosospira sp. NpAV ammonia monooxygenase operon copy 3: ammonia
            monooxygenase 3 subunits C (amoC3). A (amoA3) and B (amoB3) genes.
            complete cds.
ACCESSION   U92432 U72981 U38251
NID         g2062745
VERSION     U92432.1  GI:2062745
KEYWORDS    .
SOURCE      Nitrosospira sp. NpAV.
  ORGANISM  Nitrosospira sp. NpAV
            Eubacteria: Proteobacteria: beta subdivision: Ammonia-oxidizing
            bacteria: Nitrosospira.
REFERENCE   1  (bases 1190 to 2179)
  AUTHORS   Klotz.M.G. and Norton.J.M.
  TITLE     Sequence of an ammonia monooxygenase subunit A-encoding gene from
            Nitrosospira sp. NpAV
  JOURNAL   Gene 163 (1). 159-160 (1995)
  MEDLINE   96001263
REFERENCE   2  (bases 1190 to 2179)
  AUTHORS   Norton.J.M.. Low.J.M. and Klotz.M.G.
  TITLE     The gene encoding ammonia monooxygenase subunit A exists in three
            nearly identical copies in Nitrosospira sp. NpAV
  JOURNAL   FEMS Microbiol. Lett. 139 (2-3). 181-188 (1996)
  MEDLINE   96275898
REFERENCE   3  (bases 1 to 1230)
  AUTHORS   Klotz.M.G.. Alzerreca.J. and Norton.J.M.
  TITLE     A gene encoding a membrane protein exists upstream of the amoA/amoB
            genes in ammonia-oxidizing bacteria: a third member of the same
            operon?
  JOURNAL   FEMS Microbiol. Lett. (1997) In press
REFERENCE   4  (bases 1 to 4341)
  AUTHORS   Norton.J.M.. Alzerreca.J. and Klotz.M.G.
  TITLE     Sequence of the complete ammonia monooxygenase operon copy #3 from
            Nitrosospira sp. NpAV
  JOURNAL   Unpublished
REFERENCE   5  (bases 1 to 4341)
  AUTHORS   Klotz.M.G.
  TITLE     Direct Submission
  JOURNAL   Submitted (06-MAR-1997) Department of Biology. University of
            Colorado. 1200 Larimer Street. CB171. Denver. CO 80217-6433. USA
COMMENT     On May 2. 1997 this sequence version replaced gi:1698851
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FEATURES             Location/Qualifiers
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LOCUS       AF071774      813 bp    DNA             BCT       30-OCT-1998
DEFINITION  Nitrosospira sp. NpAV ammonia monooxygenase subunit C-4 (amoC-4)
            gene. complete cds.
ACCESSION   AF071774
NID         g3777538
VERSION     AF071774.1  GI:3777538
KEYWORDS    .
SOURCE      Nitrosospira sp. NpAV.
  ORGANISM  Nitrosospira sp. NpAV
            Bacteria: Proteobacteria: beta subdivision: Ammonia-oxidizing
            bacteria: Nitrosospira.
REFERENCE   1  (bases 1 to 813)
  AUTHORS   Sayavedra-Soto.L.A.. Hommes.N.G.. Arp.D.J.. Alzerreca.J.J..
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            Norton,J.M. and Klotz,M.G.
  TITLE     Transcription of the amoC, amoA and amoB genes in Nitrosomonas
            europaea and Nitrosospira sp. NpAV
  JOURNAL   FEMS Microbiol. Lett. 167, 81-88 (1998)
REFERENCE   2  (bases 1 to 813)
  AUTHORS   Norton,J.M., Alzerreca,J.J. and Klotz,M.G.
  TITLE     Diversity of the genes encoding ammonia monooxygenase in
            autotrophic ammonia oxidizing bacteria
  JOURNAL   Unpublished
REFERENCE   3  (bases 1 to 813)
  AUTHORS   Alcerreca,J.J., Norton,J.M. and Klotz,M.G.
  TITLE     Direct Submission
  JOURNAL   Submitted (11-JUN-1998) Department of Biology, University of
            Colorado, 1200 Larimer Street, CB171, Denver, CO 80217-3364, USA
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DEFINITION  Nitrosospira sp. NpAV ammonia monooxygenase operon copy 1: ammonia
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            monooxygenase subunits A (amoA1) and B (amoB1) genes, complete cds.
ACCESSION   AF032438 U38250
NID         g2641609
VERSION     AF032438.1  GI:2641609
KEYWORDS    .
SOURCE      Nitrosospira sp. NpAV.
  ORGANISM  Nitrosospira sp. NpAV
            Eubacteria; Proteobacteria; beta subdivision; Ammonia-oxidizing
            bacteria; Nitrosospira.
REFERENCE   1  (bases 1 to 2631)
  AUTHORS   Klotz,M.G. and Norton,J.M.
  TITLE     Sequence of an ammonia monooxygenase subunit A-encoding gene from
            Nitrosospira sp. NpAV
  JOURNAL   Gene 163 (1), 159-160 (1995)
  MEDLINE   96001263
REFERENCE   2  (bases 1 to 2631)
  AUTHORS   Norton,J.M., Low,J.M. and Klotz,M.G.
  TITLE     The gene encoding ammonia monooxygenase subunit A exists in three
            nearly identical copies in Nitrosospira sp. NpAV
  JOURNAL   FEMS Microbiol. Lett. 139 (2-3), 181-188 (1996)
  MEDLINE   96275898
REFERENCE   3  (bases 1 to 2631)
  AUTHORS   Klotz,M.G., Alzerreca,J. and Norton,J.M.
  TITLE     A gene encoding a membrane protein exists upstream of the amoA/amoB
            genes in ammonia oxidizing bacteria: a third member of the amo
            operon?
  JOURNAL   FEMS Microbiol. Lett. 150 (1), 65-73 (1997)
  MEDLINE   97306641
REFERENCE   4  (bases 1 to 2631)
  AUTHORS   Norton,J.M., Alzerreca,J.J. and Klotz,M.G.
  TITLE     Diversity of the genes encoding ammonia monooxygenase in
            autotrophic ammonia oxidizing bacteria
  JOURNAL   Unpublished
REFERENCE   5  (bases 1 to 2631)
  AUTHORS   Klotz,M.G., Cambronne,E.D., Hickman,C.D., Sawaged,L.S. and
            Norton,J.M.
  TITLE     Direct Submission
  JOURNAL   Submitted (31-OCT-1997) Department of Biology, CB 171, University
            of Colorado, 1200 Larimer Street, Denver, CO 80217-3364, USA
COMMENT     On Nov 23, 1997 this sequence version replaced gi:1151086.
FEATURES             Location/Qualifiers
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RBS                  274..279
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CDS                  287..1111
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                     monooxygenase 2 subunit A and to ammonia monooxygenase 3
                     subunit A encoded by the sequences in GenBank Accession
                     Numbers U20644 and U92432 respectively"
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                     /transl_table=11
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                     MLLAGDWDFWLDWKDRQWWPVVTPIVGITYCATIMYYLWVNYRLPFGATLCIVCLLVG
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                     PGNWPIFGPTHLPLVAEGVLLSLADYTGFLYVRTGTPEYVRLIEQGSLRTFGGHTTVI
                     AAFFSAFVSMLMFCVWWYFGKLYCTAFYYVKGPRGRVTMKNDVTAYGEEGFPEGIK"
gene                 1098..2414
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RBS                  1098..1103
                     /gene="amoB1"
CDS                  1111..2358
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                     /note="similar to Nitrosospira sp. NpAV ammonia
                     monooxygenase 3 subunit B and to ammonia monooxygenase 2
                     subunit B encoded by the sequences in GenBank Accession
                     Numbers U92432 and AF016003 respectively"
                     /codon_start=1
                     /transl_table=11
                     /product="ammonia monooxygenase 1 subunit B"
                     /protein_id="AAB86882.1"
                     /db_xref="PID:g2641612"
                     /db_xref="GI:2641612"
                     /translation="MNAKNLFKLGVIGLYGMATLALSTLDISPAAAHGERSQEPFLRM
                     RTIQWYDMKWGPDTTKVNDFATMTGKFHLAEDWPRAVGKPGRAFFNVGSPSPVFVRLS
                     TKLNGEPTYISGPLEIGRDYAFEVKLKARIPGRHHMHAMVNIKDAGPIAGPAAWMNIT
                     GSWDDFTNPVKLLTGETIDTETFNFNNGIFWHLLWLGLGCFWIGYYVARPMFLPRSRV
                     LLAYGDDLLLDPMDKKVAWIVLIATFGIVWGGYRYTETKHPYTVPIQAGESKVQPM
                     PVKPNPIAIKVTHANYDVPGRALRVTMSVTNSGDTAYRIGEFTTAGVRFINKVGLKHL
                     DRNYPKELVATGLSFDNDAPIQPGETREVKMVAKDALWEVQRLMALLGDPESRFGGL
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terminator           2373..2414
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BASE COUNT      587 a    784 c    761 g    499 t
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       61 taagggggat ccgtagaccc ggccgcccgt taactcccgc cagagtgggg gaatcagccg
      121 ggcggaccgg cccaccttcg acctgcggcg aagaccggaa aaggcaccgg caggggggaag
      181 gcggcaagac agcggcgtga cacgatatcg gcgtgacacg caaaaatgaa caacggcagc
      241 atttatcagt tttaagatcg tacgacatat ttgaggggag ggcacgatga gcagaacaga
      301 cgaaatatta aaggcggcca agatgccgcc ggaatcggta aagatgtcca ggatgataga
      361 cgcgatctat tttccgattt tatgcatcct gctggtaggc acctaccaca tgcacttcat
      421 gctgctggca ggcgactggg acttctggct tgactggaaa gaccgccaat ggtggccggt
      481 tgtcaccccc attgtaggca tcacctactg tgccaccatc atgtactacc tgtgggtgaa
      541 ctaccgcctg ccgtttggcg cgacgctatg tatcgtctgc ctgctggtag gcgaatggct
      601 gacccgcttc tggggtttct actggtggtc gcactacccg atcaacttcg tcctgccctc
      661 caccatgatt ccgggcgcgc tcatcatgga caccgtcatg ctgctcacgc gcaactggat
      721 gatcacggcc ctggtaggcg gtggcgcatt tggcctgctg ttttatccgg gcaactggcc
      781 gatattcgga ccgacgcacc tgccgctggt ggccgaaggc gttctgctgt cgctggctga
      841 ctacaccggc ttcctgtatg tccgcacggg caccccggaa tacgtacggc tgatcgaaca
      901 aggctccctg cgcaccttcg gtggtcacac cacggtgatt gcggccttct tctccgcctt
      961 cgtctccatg ctcatgttct gcgtctggtg gtactttggc aaactctact gcaccgcgtt
     1021 ctactacgtc aaaggcccgc gtggccgggt taccatgaag aacgacgtca ccgcctatgg
     1081 cgaagaaggg tttccggagg ggatcaaata atgaacgcaa agaacctgtt caaactgggg
     1141 gtcattggcc tctatggcat ggccaccttg gccctgagca cccttgacat ctcgccggcg
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     1261 gacatgaaat ggggccccga caccaccaag gtcaacgact tcgccaccat gaccggcaaa
     1321 ttccacctgg ccgaagactg gccgcgcgcg gtgggcaaac cgggacgggc gttcttcaac
     1381 gtaggcagcc ccagcccggt attcgtacgg ctctccacca aactgaacgg cgaaccgacc
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     1501 cgcatcccgg gacgccacca catgcacgcc atggtcaaca tcaaggacgc aggcccgatt
     1561 gccggccctg ccgcctggat gaacatcacc ggcagctggg atgacttcac caacccggtg
     1621 aaactgctga caggcgagac catcgacacc gagaccttca acttcaacaa cggcatcttc
     1681 tggcacttgt tgtggttggg actgggctgt ttctggatag gctactacgt tgcccggccc
     1741 atgttcctgc cgcgcagccg ggtgttgctg gcctatggag acgacctgct gctggacccg
     1801 atggacaaga aggttgcgtg gatcgtactg attgcgacct tcggcattgt ctggggcggc
     1861 taccgctata cggaaaccaa acacccgtac acggtgccca tccaagcggg tgaatcgaag
     1921 gtacaaccga tgccggtgaa acccaacccc atcgcgatca aagtgacgca cgccaactat
     1981 gacgtcccgg gccgcgcact gcgcgtgacc atgagcgtca ccaacagtgg cgacaccgct
     2041 tatcgtatag gcgagttcac caccgcgggc gtgcgcttca tcaacaaagt ggggctcaag
     2101 cacctggacc gcaactatcc gaaggaactg gtagccacgg gcctgtcgtt tgacaatgac
     2161 gcgccgatcc aacctggcga gacgcgtgaa gtcaagatgg tagcgaagga tgcactgtgg
     2221 gaagtacagc gtctgatggc gctgctgggc gacccggaaa gccgctttgg cggattgctg
     2281 atgacctgga gcgattcagg cgaccgcaac atcaacagca tagccggcgc agtcataccg
     2341 gtcttcacca agctctaagc tacaaagcat gacaccggtc cgccaccgtc gccagacgga
     2401 aaggtggatc ggtgtttttt aatgtgccag tgaaacaacc cataccttcg gaatagccgg
     2461 ggggacgaag aaaagtgaac catcgtcata tccaaacgga agaacccgct cgtggttagg
     2521 caaatgaaac aggcaggcgc tgcggttctc attctggttg ggcgctata cgccggcgca
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LOCUS       AF016002      825 bp    DNA             BCT       22-SEP-1997
DEFINITION  Nitrosospira sp. Np39-19 ammonia monooxygenase subunit A2 (amoA2)
            gene, complete cds.
ACCESSION   AF016002
NID         g2425027
VERSION     AF016002.1  GI:2425027
KEYWORDS    .
SOURCE      Nitrosospira sp. Np39-19.
  ORGANISM  Nitrosospira sp. Np39-19
            Eubacteria; Proteobacteria; beta subdivision; Ammonia-oxidizing
            bacteria; Nitrosospira.
REFERENCE   1  (bases 1 to 825)
  AUTHORS   Shiozawa,T.L., Norton,J.M., Alzerreca,J.J. and Klotz,M.G.
  TITLE     Direct submission
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 825)
  AUTHORS   Norton,J.M., Alzerreca,J.J. and Klotz,M.G.
  TITLE     Diversity of the genes encoding ammonia monooxygenase in
            autotrophic ammonia oxidizing bacteria
  JOURNAL   Unpublished
REFERENCE   3  (bases 1 to 825)
  AUTHORS   Shiozawa,T.L., Norton,J.M., Alzerreca,J.J. and Klotz,M.G.
  TITLE     Direct Submission
  JOURNAL   Submitted (24-JUL-1997) Department of Biology, CB 171, The
            University of Colorado, 1200 Larimer Street, Denver, CO 80217-3364,
            USA
FEATURES             Location/Qualifiers
     source          1..825
                     /organism="Nitrosospira sp. Np39-19"
                     /strain="Np 39-19"
                     /note="Strain source: Dr. Ed Schmidt, University of
                     Minnesota"
                     /db_xref="taxon:61907"
     gene            1..825
                     /gene="amoA2"
     CDS             1..825
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                     protein, encoded by Genbank Accession Number U20644"
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                     PGNWPIFGPTHLPLVVEGVLLSVADYTGFLYVRTGTPEYVRNIEQGSLRTFGGHTTVI
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BASE COUNT      160 a    246 c    225 g    194 t
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      121 cacatgcact tcatgctgct ggcaggagac tgggacttct ggctggactg gaaagaccgt
      181 caatggtggc cggtggtaac cccgatcgtg ggcatcacct actgtgccgc catcatgtac
      241 tacctgtggg tgaactaccg gctcccgttt ggcgcgaccc tgtgcatcgt gtgcctgctg
      301 tcgggtgaat ggctgacccg ctactggggc ttctactggt ggtcgcacta ccccatcagc
      361 ttcgtatttc cctccaccat gataccgggt gccctggtca tggacacggt catgctgctc
      421 acgcgcaact ggatgatcac cgcgctggtt ggcggcggcg cctttggctt cttgttctac
      481 cctggcaact ggcccatctt tggcccgacc cacctgccgc tggtagtgga aggcgtactg
      541 ctgtcggtag ctgactacac cggcttcctg tatgtacgta ccggcacgcc tgagtatgtc
      601 cgcaacatcg aacaaggctc gctgcgcacc tttggcggcc acaccacggt cattgccgca
      661 ttctttgccg ccttcatctc catgctcatg ttctgcatct ggtggtactt tggcaaactc
      721 tactgcaccg cgttcttcta cgtcaaaggc caccgcggcc gtgtcaccat gaaggacgac
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LOCUS       AF006692      825 bp    DNA             BCT       05-AUG-1997
DEFINITION  Nitrosospira sp. Np39-19 ammonia monooxygenase subunit A3 (amoA3)
            gene. complete cds.
ACCESSION   AF006692
NID         g2266997
VERSION     AF006692.1  GI:2266997
KEYWORDS    .
SOURCE      Nitrosospira sp. Np39-19.
  ORGANISM  Nitrosospira sp. Np39-19
            Eubacteria; Proteobacteria; beta subdivision; Ammonia-oxidizing
            bacteria; Nitrosospira.
REFERENCE   1  (bases 1 to 825)
  AUTHORS   Norton,J., Alzerreca,J.J. and Klotz,M.G.
  TITLE     Diversity of the genes encoding ammonia monooxygenase in
            autotrophic ammonia oxidizing bacteria
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 825)
  AUTHORS   Shiozawa,T.L., Norton,J.M., Alzerreca,J.J. and Klotz,M.G.
  TITLE     Direct Submission
  JOURNAL   Submitted (04-JUN-1997) Plants, Soils & Biometereology, Utah State
            University, Logan, UT 84322, USA
FEATURES             Location/Qualifiers
     source          1..825
                     /organism="Nitrosospira sp. Np39-19"
                     /strain="39-19"
                     /db_xref="taxon:61907"
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                     Minnesota"
     gene            1..825
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     CDS             1..825
                     /gene="amoA3"
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                     Accession number U92432"
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                     PGNWPIFGPTHLPLVVEGVLLSVADYTGFLYVRTGTPEYVRNIEQGSLRTFGGHTT
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      361 ttcgtatttc cttccaccat gataccgggt gccctggtca tggacacggt catgctgctc
      421 acgcgcaact ggatgatcac cgcgctggtt ggcggcggcg cctttggcct cttgttctac
      481 cctggcaact ggcccatctt tggcccgacc cacctgccgc tggtagtgga aggcgtactg
      541 ctgtcggtag ctgactacac cggcttcctg tatgtacgta ccggcacgcc tgagtatgtc
      601 cgcaacatcg aacaaggctc gctgcgcacc tttggcggcc acaccacggt cattgccgca
      661 ttctttgccg ccttcatctc catgctcatg ttctgcatct ggtggtactt tggcaaactc
      721 tactgcaccg cgttctttta cgtcaaaggc caccgcggcc gtgtcaccat gaagaacgac
      781 gtcactgcat ttggcgaaga aggctttcca gaggggataa aataa
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LOCUS       AF042170     1628 bp    DNA             BCT       02-JUL-1998
DEFINITION  Nitrosospira sp. Np39-19 ammonia monooxygenase subunit C1 (amoC1)
            gene, partial cds; ammonia monooxygenase subunit A1 (amoA1) gene,
            complete cds; and ammonia monooxygenase subunit B1 (amoB1) gene,
            partial cds.
ACCESSION   AF042170
NID         g3282752
VERSION     AF042170.1  GI:3282752
KEYWORDS    .
SOURCE      Nitrosospira sp. Np39-19.
  ORGANISM  Nitrosospira sp. Np39-19
            Eubacteria; Proteobacteria; beta subdivision; Ammonia-oxidizing
            bacteria; Nitrosospira.
REFERENCE   1  (bases 1 to 1628)
  AUTHORS   Norton,J.M., Alzerreca,J.J. and Klotz,M.G.
  TITLE     Diversity of the genes encoding ammonia monooxygenase in
            autotrophic ammonia-oxidizing bacteria
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1628)
  AUTHORS   Shiozawa,T.L., Norton,J.M., Alzerreca,J.J. and Klotz,M.G.
  TITLE     Direct Submission
  JOURNAL   Submitted (09-JAN-1998) Department of Plants, Soils and
            Biometeorology, Utah State University, Logan, UT 84322, USA, and
            Department of Biology, University of Colorado, 1200 Larimer Street,
            CB 171, Denver, CO 80217-3364, USA
FEATURES             Location/Qualifiers
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                     /db_xref="taxon:61907"
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                     Minnesota"
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     CDS             <1..63
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                     /db_xref="PID:g3282753"
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                     /translation="LTDVVWNGQSKVILNNRIVP"
     gene            565..1332
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     CDS             565..1332
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                     /note="AmoA1; putative membrane protein; similar to
                     Nitrosospira sp. Np39-19 AmoA3 and AmoA2 proteins encoded
                     by GenBank Accession Numbers AF006692 and AF016002.
                     respectively; N-terminal truncation by 18 amino acid
                     residues;  similar to Nitrosospira sp. NpAV AmoA proteins
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                     /transl_table=11
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                     /db_xref="GI:3282754"
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                     LLSVADYTGFLYVRTGTPEYVRNIEQGSLRTFGGHTTVIAAFFAAFISMLMFCIWWYF
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                     /gene="amoB1"
     CDS             1333..>1628
                     /gene="amoB1"
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                     protein encoded by GenBank Accession Number AF032438"
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                     /transl_table=11
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                     /protein_id="AAC25056.1"
                     /db_xref="PID:g3282755"
                     /db_xref="GI:3282755"
                     /translation="MNAKNLFKLGVVGLYGMATLAMTLALDISPAAAHGERSQEPFLR
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BASE COUNT      374 a    500 c    464 g    289 t      1 others
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    181 cccggagagg agcggctgca cagcagaccc aggaagacca ccgcgccccg actgacccca
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    361 aagcagcaca aaaagcgaca gcgcaggtgc ggcccggcca ggcccaagag gcggagagac
    421 aacaatgcag cacgagcgaa agaccacggc agcacgaaca cattattcat gtatcaagat
    481 cgtagacata cttgagggga gggtgcgatg agcagaacag acgaaatact aaaggcggcc
    541 aaaatgccgc ccgaagcagt atagatgtcc aggatgatag acgcggtata tttcccgatt
    601 ctatgcatcc tgctggttgg gacctaccac atgcacttca tgctgctggc aggagactgg
    661 gacttctggc tggactggaa agaccgtcaa tggtggccgg tggtaacccc gatcgtgggc
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    781 gcgaccctgt gcatcgtgtg cctgctgtcg ggtgaatggc tgacccgcta ctggggbttc
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    901 ctggtcatgg acacggtcat gctgctcacg cgcaactgga tgatcaccgc cctggttggc
    961 ggcggcgcct ttggcctctt gttctaccct ggcaactggc ccatctttgg cccgacccac
   1021 ctgccgctgg tagtggaagg cgtactgctg tcggtagctg actacaccgg cttcctgtat
   1081 gtacgtaccg gcacgcctga gtatgtccgc aacatcgaac aaggctcgct gcgcaccttt
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   1201 tgcatctggt ggtactttgg caaactctac tgcaccgcgt tcttctacgt caaaggccac
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   1381 atggccaccc tggccatgac cctggcgctg gacatctcgc ctgcggcggc acacggcgag
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   1501 cccgagacca ccaaggtcaa cgacttggcc agcatgaccg gcaagttcca cctggccgag
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   1621 cccgtata
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LOCUS       NOU96611      744 bp    DNA             BCT       18-MAY-1997
DEFINITION  Nitrosococcus oceanus ammonia monooxygenase subunit A (amoA) gene,
            complete cds.
ACCESSION   U96611
NID         g2104719
VERSION     U96611.1  GI:2104719
KEYWORDS    .
SOURCE      Nitrosococcus oceanus.
  ORGANISM  Nitrosococcus oceanus
            Eubacteria; Proteobacteria; gamma subdivision; Nitrosococcus.
REFERENCE   1  (bases 1 to 744)
  AUTHORS   Alzerreca,J.J., Norton,J.M. and Klotz,M.G.
  TITLE     Sequence analysis of ammonia monooxygenase operons in nitrifying
            bacteria
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 744)
  AUTHORS   Alzerreca,J.J., Norton,J.M. and Klotz,M.G.
  TITLE     Direct Submission
  JOURNAL   Submitted (05-APR-1997) Dept. of Plants, Soils and Biometeorology,
            Logan, UT 84322; and Department of Biology, University of Colorado,
            1200 Larimer Street, CB 171, Denver, CO 80217-3364, USA
FEATURES             Location/Qualifiers
     source          1..744
                     /organism="Nitrosococcus oceanus"
                     /strain="C-107"
                     /note="isolated from seawater by F. Valois and S.W. Watson
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                     (Woods Hole Collection)"
                     /db_xref="taxon:1229"
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                     /gene="amoA"
                     /note="similar to Methylococcus capsulatus methane
                     monooxygenase subunit PmoA2, encoded by Genbank Accession
                     Number U94337, and to Nitrosomonas europaea acetylene
                     binding protein (ammonia monooxygenase subunit A), PIR
                     Accession Number A49853. N. oceanus has only one ammonia
                     monooxygenase amoA gene."
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                     /db_xref="PID:g2104720"
                     /db_xref="GI:2104720"
                     /translation="MSALTSAVRTPEEAAKVFRTLDFIALGAFFMILLASHHVHVMLL
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                     WPLLSAFHVPAEYNGVVMSLADIMGYQYVRTGTPEYIRMVEKGTLKTFGKDVVPVSAF
                     FSGFVAMVMYFVWHFVGRWFSKDYHINQL"
BASE COUNT      137 a    161 c    203 g    243 t
ORIGIN
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       61 ttggatttta tagcgttagg ggcttttttt atgattttgc tggcttccca ccacgttcac
      121 gtcatgcttt taatggggga ctgggacttc tgggttgact ggaaagaccg gcgtttttgg
      181 gtcaccgtag ttcccattgt atcggttgct taccccgctg ctgcacaggc gtttttttgg
      241 gagaagttcc gccttccctt tggtgctacc cttgtaacgc taggggtgtt agcaggtgag
      301 tgggctaacc gctactttaa tttcgtaggg tttacctatt ttccgattaa tttcgtatgg
      361 ccgaccattc tgctgcccat ggcgctgttc ttggatgcca tgcttgcgat ttccaagagc
      421 tacggcttga cggcggtagt gggtggatta atgtacggtt tgttgatgta tcccgctaac
      481 tggccgctgc tttcggcatt ccatgtgcct gctgagtaca atggagtagt aatgtctttg
      541 gccgatatta tgggctacca atacgtccgg accggtactc ctgagtatat ccggatggtt
      601 gaaaaaggta cgttgaaaac gtttggtaag gacgtggtcc cggtttcagc tttcttctcg
      661 ggttttgttg ccatggtaat gtactttgtg tggcactttg ttggccgctg gttctccaag
      721 gactaccata tcaaccaatt gtaa
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LOCUS       AF047705     3347 bp    DNA             BCT       02-JUL-1998
DEFINITION  Nitrosococcus oceanus ammonia monooxygenase subunit A (amoA) and
            ammonia monooxygenase subunit B (amoB) genes, complete cds; and
            unknown gene.
ACCESSION   AF047705
NID         g3282844
VERSION     AF047705.1  GI:3282844
KEYWORDS    .
SOURCE      Nitrosococcus oceanus.
  ORGANISM  Nitrosococcus oceanus
            Eubacteria; Proteobacteria; gamma subdivision; Nitrosococcus.
REFERENCE   1  (bases 1 to 3347)
  AUTHORS   Alzerreca,J.J., Norton,J.M. and Klotz,M.G.
  TITLE     Sequence analysis of ammonia monooxygenase operons in nitrifying
            bacteria
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  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 3347)
  AUTHORS   Alzerreca,J.J., Norton,J.M. and Klotz,M.G.
  TITLE     Direct Submission
  JOURNAL   Submitted (11-FEB-1998) Department of Plants, Soils and
            Biometeorology, Utah State University, Logan, UT 84322, USA, and
            Department of Biology, University of Colorado, 1200 Larimer Street,
            CB171, Denver, CO 80217-3364, USA
FEATURES             Location/Qualifiers
     source          1..3347
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                     /strain="C-107"
                     /db_xref="taxon:1229"
     terminator      131..173
     gene            377..1132
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     RBS             377..382
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     CDS             389..1132
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                     monooxygenase subunit A, GenBank Accession Number U96611;
                     Nitrosococcus oceanus has only one ammonia monooxygenase
                     amoA gene."
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                     /transl_table=11
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                     /db_xref="GI:3282845"
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                     NRYFNFVGFTYFPINFVWPTILLPMALFLDAMLAISKSYGLTAVVGGLMYGLLMYPAN
                     WPLLSAFHVPAEYNGVVMSLADIMGYQYVRTGTPEYIRMVEKGTLKTFGKDVVPVSAF
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     gene            1183..2567
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                     /gene="amoB"
     CDS             1198..2448
                     /gene="amoB"
                     /note="similar to Methylococcus capsulatus methane
                     monooxygenase subunit PmoB2, GenBank Accession Number
                     U94337, and to Nitrosospira sp. NpAV ammonia monooxygenase
                     subunits B, GenBank Accession Numbers AF032438, AF016003
                     and U92432"
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                     /transl_table=11
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                     /db_xref="GI:3282846"
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                     LTSYVNGMFVPRSIGLELGGDYDFEMTMQGRRPGTWHVHTLLNVQGGGPLIGPGKYIT
                     ITGDMADFENKITDLTGNTVNLETMATGTVIGWHLSWYVLGIAWIGWWARRPMFLPRY
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                     GenBank Accession Number U92432"
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                     /db_xref="PID:g3282847"
                     /db_xref="GI:3282847"
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                     PIVERPVQKYPKGTIELDTDFTKAGEYTAVVILGGDQPMVFKALFEWVSRESRPFSGS
                     PRSLAVLLILGLIFWYSRRGGKEAKAS"
BASE COUNT      809 a    680 c    887 g    971 t
ORIGIN
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       61 ggactatgcc cgtagctgat atttaagatt atcatatttg attttcagtt agcgaagggt
      121 tgaatagaag ggcggtcgca atctccggaa tagttaatga gatcgtggcc gccttttttgt
      181 ataaagtgct gtacagaaat tttattaggg atacgcttat ttatatcggg ttgaaagaaa
      241 tggaagtaag caacctaatg agaaagtagc tgttaaaata ggtggcagga agcatacctt
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      361 ccttaatatt tttaggaggg caggtttaat gagtgcactt acgtctgcgg ttcgtacacc
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      481 gattttgctg gcttcccacc acgttcacgt catgctttta atgggggact gggacttctg
      541 ggttgactgg aaagaccggc gtttttgggt caccgtagtt cccattgtat cggttgctta
      601 ccccgctgct gcacaggcgt tttttggga gaagttccgc cttccctttg gtgctaccct
      661 tgtaacgcta ggggtgttag caggtgagtg ggctaaccgc tactttaatt tcgtagggtt
      721 tacctatttt ccgattaatt tcgtatggcc gaccattctg ctgcccatgg cgctgttctt
      781 ggatgccatg cttgcgattt ccaagagcta cggcttgacg gcggtagtgg gtggattaat
      841 gtacggtttg ttgatgtatc ccgctaactg gccgctgctt tcggcattcc atgtgcctgc
      901 tgagtacaat ggagtagtaa tgtctttggc cgatattatg ggctaccaat acgtccggac
      961 cggtactcct gagtatatcc ggatggttga aaaaggtacg ttgaaaacgt ttggtaagga
     1021 cgtggtcccg gtttcagctt tcttctcggg ttttgttgcc atggtaatgt actttgtgtg
     1081 gcactttgtt ggccgctggt tctccaagga ctaccatatc aaccaattgt aaaatcgttt.
     1141 gtgataatga ggataataat tctaaaattc taaaatacaa aaagagagac ggagaacatg
     1201 aaaggtaccc atataacaaa tcgagccaga aagtggctag ccattgggtt tactgcggtg
     1261 atagcctcaa gcgttttcta tattccgaca gtagctgccc atggcgagaa ggcgcaggca
     1321 gctttcctgc gcatgcggac aatccattgg tatgacatgg tatggtccaa ggataccatt
     1381 gcggttaatg agacctatac cataagcggg aagttccggg tttttgagga ttggccggaa
     1441 gcagtcgaaa aaccccatgt atccttttta aatgcgggtc aacctggtcc agtcacggct
     1501 cggcttactt cctacgtcaa tggtatgttc gtccctcgtt cgataggtct tgaattgggc
     1561 ggcgattacg attttgagat gacgatgcaa gggcgccgtc ctgggacgtg gcatgttcat
     1621 accttgctaa atgtccaagg agggggccg ctcatcggtc caggtaaata catcaccatt
     1681 accggagata tggctgattt tgaaaacaaa atcacggatc tgaccggtaa tacggtcaac
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     1861 aaggcgggca aggctaatga tctattaact gcccaggaca aaaaattgac tataggcgtc
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     1981 gtcaccattc cgttgcaggc tgggctgctg ggcactattg actccttgcc ggtggattat
     2041 aattcgatgg taagcgctaa cgtgcttaag gctaactatc gggtgccggg gcggactatc
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2161 actgggggca ttcgattcat gaatgcaaat gtgcgggttg atgagacgga ttatcctgag
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2281 gtagttgttg acatctccgc caccgatgcc gcctgggaag ttcagcgtat ggccgacgtc
2341 atttatgatc cagacagccg ctttgcgggc ttgatcttct tcgttgatcc agaggggaat
2401 gagattccga tacctatcgg cggtccatta gttcccacgt ttgtttagaa agaagtatct
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2701 tcgtttggat tattgttgct aaatacttcc gccatggccc atgggggagg aggaattgaa
2761 acagatcagt gcgttgtcaa tatcggtaat taccgaatgc atttttttgc ttaccaaccg
2821 gaaacttctg gtggtgaaga gctgtgctgg gacttgccga tgacgggaag caccatttta
2881 gtgtttgatt tagtggatag gatattgcgt gatcggccgg ttgaggttcg gatcgttgaa
2941 gagcagaaaa cggccgctgg cccgtcaaat tataatccca tagtagagcg tccggttcaa
3001 aaatatccaa agggaactat tgagcttgat accgatttta ccaaagcagg agaatacact
3061 gcggtagtta ttcttggtgg tgatcaaccg atggtcttca aagcactctt cgagtgggtc
3121 tccagggaga gcagaccgtt cagtggatcg cctcgatcgc tggcggtgct tcttattttg
3181 gggcttattt tttggtatag tcgccgtggc ggaaaagagg ccaaggcatc ctagtttcaa
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3301 gtttccccgg cattccatta tccaaataat ggaatgccgg ggaattc
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LOCUS       NEU96187      606 bp    DNA              BCT       03-JUN-1997
DEFINITION  Nitrosomonas europaea ammonia monooxygenase C1 (amoC1) gene,
            partial cds.
ACCESSION   U96187
NID         g2149937
VERSION     U96187.1  GI:2149937
KEYWORDS    .
SOURCE      Nitrosomonas europaea.
  ORGANISM  Nitrosomonas europaea
            Eubacteria; Proteobacteria; beta subdivision; Ammonia-oxidizing
            bacteria; Nitrosomonas.
REFERENCE   1  (bases 1 to 606)
  AUTHORS   Alzerreca,J.J., Norton,J.M. and Klotz,M.G.
  TITLE     AmoC, a third product of the ammonia monooxygenase operon in
            Nitrosomonas europaea
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 606)
  AUTHORS   Alzerreca,J.J.
  TITLE     Direct Submission
  JOURNAL   Submitted (02-APR-1997) Plants, Soils & Biometeorology, Utah State
            University, UMC 4820, Logan, UT 84322, USA
FEATURES             Location/Qualifiers
     source          1..606
                     /organism="Nitrosomonas europaea"
                     /strain="ATCC 25978"
                     /db_xref="taxon:915"
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                     similar to the Nitrosospira sp. NpAV ammonia monooxygenase
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                     LYAMTRLPLFSRGISFPLVMAIAGPLMILPNVGLNEWGHAFWFMEELFSAPLHWGFVV
                     LGWAGLFQGGVAAQIITRYSNLTDVVWNNQSKEILNNRIVA"
BASE COUNT      131 a    130 c    165 g    180 t
ORIGIN
        1 tggcgtgtac atatggcaat catgccgctg tttgcgctgg ttacctgggg ttggatcctg
       61 aaaacgcgtg atacgaaaga gcaattggat aatctggatc ccaaactgga aatcaaacgc
      121 tacttctact acatgatgtg gctgggtgta tacatttttg gtgtttactg ggtggtagc
      181 ttcttcacgg agcaagatgc ctcctggcac caagtgatta ttcgtgatac cagtttcacg
      241 ccaagtcacg tagtggtgtt ttacggatca ttcccgatgt acatcgtttg cggtgttgca
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      421 catgctttct ggttcatgga agagttgttc agcgcaccac tgcactgggg atttgtagtg
      481 ttgggctggg cgggtctgtt ccagggtggt gttgcagctc agatcattac ccgttattcc
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      601 gcttaa
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LOCUS       AF073793      880 bp    DNA             BCT       24-OCT-1998
DEFINITION  Nitrosomonas europaea ammonia monooxygenase 3 subunit C (amoC3)
            gene. complete cds.
ACCESSION   AF073793
NID         g3284009
VERSION     AF073793.1  GI:3284009
KEYWORDS    .
SOURCE      Nitrosomonas europaea.
  ORGANISM  Nitrosomonas europaea
            Bacteria: Proteobacteria: beta subdivision: Ammonia-oxidizing
            bacteria: Nitrosomonas.
REFERENCE   1  (bases 1 to 880)
  AUTHORS   Sayavedra-Soto.L.A.. Hommes.N.G.. Alzerreca.J.J.. Arp.D.J..
            Norton.J.M. and Klotz.M.G.
  TITLE     Transcription of the amoC. amoA and amoB genes in Nitrosomonas
            europaea and Nitrosospira sp. NpAV
  JOURNAL   FEMS Microbiol. Lett. 167 (1). 81-88 (1998)
REFERENCE   2  (bases 1 to 880)
  AUTHORS   Sayavedra-Soto.L.A.. Hommes.N.G.. Alzerreca.J.. Arp.D.J..
            Norton.J.M. and Klotz.M.G.
  TITLE     Direct Submission
  JOURNAL   Submitted (22-JUN-1998) Botany. Oregon State University. Cordley
            Hall 2082. Corvallis. OR 97331. USA
FEATURES             Location/Qualifiers
     source          1..880
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           PSHIPLFYGSFPVYIIMGIAMIIYAKTRLPLYNKGWSFPLIMVVAGPLMSLPNVGLNEWGHAFWFMEELFSAPLHWGFVILAW
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BASE COUNT      203 a    210 c    232 g    235 t
ORIGIN
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       61 tgataaaccg gcttacgaca aatcagaatg gtttgacgcc aagtactaca aatacggcct
      121 gctgcccatt ctgggcatag cggtattctg ggtatggtat cagcgtacgt ttgcctattc
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LOCUS       NMU91603     1487 bp    DNA             BCT       14-APR-1997
DEFINITION  Nitrosolobus multiformis ammonia monooxygenase subunit AmoC1
            (amoC1) gene, partial cds, ammonia monooxygenase subunit AmoA1
            (amoA1) gene, complete cds, and ammonia monooxygenase subunit AmoB1
            (amoB1) gene, partial cds.
ACCESSION   U91603
NID         g1935033
VERSION     U91603.1  GI:1935033
KEYWORDS    .
SOURCE      Nitrosolobus multiformis.
  ORGANISM  Nitrosolobus multiformis
            Eubacteria; Proteobacteria; beta subdivision; Ammonia-oxidizing
            bacteria; Nitrosolobus.
REFERENCE   1  (bases 1 to 1487)
  AUTHORS   Norton,J.M., Alzerreca,J. and Klotz,M.G.
  TITLE     The ammonia monooxygenase operon in Nitrosolobus multiformis
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1487)
  AUTHORS   Klotz,M.G.
  TITLE     Direct Submission
  JOURNAL   Submitted (28-FEB-1997) Department of Biology, University of
            Colorado, 1200 Larimer Street, CB 171, Denver, CO 80217-3363, USA
FEATURES             Location/Qualifiers
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source          1..1487
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                /strain="ATCC25196; C-71"
                /db_xref="taxon:1231"
gene            <1..50
                /gene="amoC1"
CDS             <1..50
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                /note="putative membrane protein; similar to Nitrosospira
                NpAV AmoC3 protein, encoded by GenBank Accession number
                U72981"
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                /translation="KVIPNNRIVPYDKAF"
CDS             113..337
                /function="putative regulator of amo gene expression"
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                /translation="MRPAVTQPGRLKQCEAAAPGAKGEGSRRIREQQGSDKGFIQSCR
                PLLNHEVPGNSRQRHQGLQGWQAVEQHGND"
gene            374..1198
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CDS             374..1198
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                monooxygenase acetylene-binding protein, encoded by
                GenBank Accession Number L08050"
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                /db_xref="GI:1935036"
                /translation="MSRTDEILKAAKMPPEAVKMSRMIDVIYFPILCILLVGTYHMHF
                MLLAGDWDFWLDWKDRQWWPVVTPIVGITYCAAIMYYLWVNYRLPFGATLCIVCLLVG
                EWLTRFWGFYWWSHYPMNFVFPSTMIPGALVMDTVLLLTRNWMITALVGGGAFGLLFY
                PGNWTIFGPTHLPLVAEGVLLSVADYTGFLYVRTGTPEYVRLIEQGSLRTFGGHTTVI
                ASFFSAFVSMLMFTVWWYFGKVYCTAFYYVKGARGRVSMKNDVTAFGEEGFAEGIK"
gene            1199..>1487
                /gene="amoB1"
CDS             1199..>1487
                /gene="amoB1"
                /note="similar to Nitrosomonas europaea ammonia
                monooxygenase protein AmoB, encoded by GenBank Accession
                Number L08050"
                /codon_start=1
                /transl_table=11
                /product="ammonia monooxygenase subunit AmoB1"
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                     /protein_id="AAB51761.1"
                     /db_xref="PID:g1935037"
                     /db_xref="GI:1935037"
                     /translation="MKNLFKKSIAGVCSLAALGLALTLDIQPAAAHGERSQEPFLRMR
                     TIQWYDMKWGPETTKVNDIATMTGKFHLAEDWPRAVGKPGRAFFNVGSPSPV"
BASE COUNT      322 a    416 c    441 g    308 t
ORIGIN
        1 gtaaagtcat tcccaataac cgcatcgtac cgtacgacaa ggcgttttaa ggactgactc
       61 gctgacacac gcataagcag caatagccgc atcgcctccg aacaggagag cgatgcggcc
      121 agcggtaaca cagccgggca gactcaagca atgcgaagcg gcggcacctg gagcaaaagg
      181 ggaagggagt cgccggatcc gggagcaaca aggaagcgac aagggattta tacagtcatg
      241 tagacccttg ttgaatcacg aggttcccgg gaattcgcgg caacgtcatc agggactgca
      301 aggatggcaa gcagtagagc agcacggcaa cgattgatat gttttagtcg cgtaacattt
      361 gagggagggt gcgatgagca gaacagatga aatactgaag gcggcgaaga tgccgcccga
      421 agcggtaaag atgtccagga tgatagacgt gatttacttc ccgattctat gcatcctgct
      481 ggttggaacc taccacatgc acttcatgct gctggcgggt gactgggact tctggcttga
      541 ctggaaggac cggcaatggt ggcctgtggt aaccccaatt gtagggatca cctactgtgc
      601 cgcgatcatg tactacctgt gggtgaacta ccgcctgccg tttggagcca cactgtgcat
      661 cgtgtgcctt ctggtgggtg aatggctgac ccgtttctgg ggtttctact ggtggtcgca
      721 ctaccccatg aactttgtat tcccctccac catgattccc ggcgcgctgg tgatggacac
      781 cgtcctgctt ctgacgcgca actggatgat cacggcactg gttggcggcg gcgcctttgg
      841 tttgttgttc tatcctggca actggaccat cttcgggccg acccacctgc cgctggtggc
      901 agaaggcgtg ctgctctcgg tagccgacta cacgggcttt ctgtatgtcc gtaccggcac
      961 ccctgagtac gtgcgactga tcgaacaagg gtcactgcgc acctttggcg gtcacaccac
     1021 cgttatcgcc tccttcttct ccgcgttcgt ctccatgctc atgttcaccg tctggtggta
     1081 ctttggcaag gtctactgca ccgccttcta ctatgtcaag ggcgcgcgcg gccgtgtcag
     1141 catgaagaac gacgtgacag catttggcga agaaggcttt gccgagggga tcaaataaat
     1201 gaagaacctg ttcaagaaga gtatagccgg ggtatgcagc cttgccgccc tgggactggc
     1261 gctgacgctc gacatccagc cggcggcggc ccacggggaa cgctcgcagg agccgttcct
     1321 gcgcatgcgt accatccagt ggtatgacat gaaatggggc cccgagacca ccaaggtcaa
     1381 cgacatagcc accatgaccg gcaaattcca cctggccgaa gactggccgc gcgcggtggg
     1441 caagcctgga cgcgccttct tcaacgtagg cagccccagc ccggtgt
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LOCUS       AF042171       825 bp    DNA             BCT       02-JUL-1998
DEFINITION  Nitrosolobus multiformis ammonia monooxygenase subunit AmoA (amoA)
            gene. complete cds.
ACCESSION   AF042171
NID         g3282756
VERSION     AF042171.1  GI:3282756
KEYWORDS    .
SOURCE      Nitrosolobus multiformis.
  ORGANISM  Nitrosolobus multiformis
            Eubacteria; Proteobacteria; beta subdivision; Ammonia-oxidizing
            bacteria; Nitrosolobus.
REFERENCE   1  (bases 1 to 825)
  AUTHORS   Norton,J.M., Alcerreca,J.J. and Klotz,M.G.
  TITLE     Diversity of the genes encoding ammonia monooxygenase in
            autotrophic ammonia-oxidizing bacteria
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 825)
  AUTHORS   Alzerreca,J.J., Norton,J.M. and Klotz,M.G.
  TITLE     Direct Submission
  JOURNAL   Submitted (09-JAN-1998) Department of Biology, University of
            Colorado, 1200 Larimer Street, CB171, Denver, CO 80217-3364, USA
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FEATURES             Location/Qualifiers
     source          1..825
                     /organism="Nitrosolobus multiformis"
                     /strain="Nm24C"
                     /db_xref="taxon:1231"
     gene            1..825
                     /gene="amoA"
     CDS             1..825
                     /gene="amoA"
                     /note="putative membrane protein: similar to Nitrosospira
                     sp. NpAV AmoA proteins. encoded by Genbank Accession
                     Numbers U38250. U20644 and U38351."
                     /codon_start=1
                     /transl_table=11
                     /product="ammonia monooxygenase subunit AmoA"
                     /protein_id="AAC25057.1"
                     /db_xref="PID:g3282757"
                     /db_xref="GI:3282757"
                     /translation="MSRTDEIIRAAKMPPEAVKMSRMIDVIYFPILCILLVGTYHMHF
                     MLLAGDWDFWLDWKDRQWWPVVTPIVGITCYAAIMYYLWVNYRLPYGATLCIVCLLVG
                     EWLTRFWGFYWWSHYPINFVFPSTMIPGALIMDTVMLLTRNWMITALIGGGAFGLLFY
                     PGNWPIFGPTHLPLVAEGVLLSVADYTGFLYVRTGTPEYVRLIEQGSLRTFGGHTTVI
                     AAFFSAFVSMLMFTVWWYFGKVYCTAFFYVKGPRGRISMKNDVTAYGEEGFPEGIK"
BASE COUNT      158 a    241 c    228 g    198 t
ORIGIN
        1 atgagcagaa ccgatgagat tatacgggcg gcgaaaatgc cgcctgaagc agtaaagatg
       61 tccaggatga tagacgtgat ttacttcccg attctatgca tcctgctggt tgggacctac
      121 cacatgcact tcatgctgct ggcgggagac tgggacttct ggcttgactg gaaagaccgt
      181 caatggtggc ctgtcgtgac ccccatcgta gggatcacct gctatgcggc catcatgtac
      241 tacctgtggg tgaactaccg gctgccctat ggcgccaccc tgtgcatcgt gtgccttttg
      301 gtaggcgaat ggctgacgcg tttctgggga ttttactggt ggtcgcacta ccccatcaac
      361 ttcgtcttcc cctccaccat gattcctgga gcactgatca tggacaccgt catgctgctc
      421 acccgcaact ggatgatcac ggcactgatc ggaggcggcg cattcggtct gctgttctac
      481 cctggcaact ggcccatctt tggcccgacc cacctgccgc tggtcgctga aggcgtgctg
      541 ctgtcggtag ccgactacac cggctttttg tatgtacgca ccggcacccc tgagtacgtg
      601 cgcctgatcg aacaagggtc gctacgaacc tttggcgggc acaccaccgt gattgccgca
      661 ttcttctccg cattcgtctc catgctcatg ttcaccgtct ggtggtactt tggcaaggtc
      721 tactgcaccg ccttcttcta cgtgaagggc ccgcgtggac gcatctccat gaagaacgac
      781 gtgaccgcgt atggcgaaga agggtttccg gaggggatca aataa
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LOCUS       NMU89833     1487 bp    DNA             BCT       04-MAR-1997
DEFINITION  Nitrosolobus multiformis ammonia monooxygenase operon copy 3:
            ammonia monooxygenase subunit AmoC3 (amoC3) gene. partial cds.
            ammonia monooxygenase subunit AmoA3 (amoA3) gene. complete cds and
            ammonia monooxygenase subunit AmoB3 (amoB3) gene. partial cds.
ACCESSION   U89833
NID         g1858014
VERSION     U89833.1  GI:1858014
KEYWORDS    .
SOURCE      Nitrosolobus multiformis.
  ORGANISM  Nitrosolobus multiformis
            Eubacteria: Proteobacteria: beta subdivision: Ammonia-oxidizing
            bacteria: Nitrosolobus.
REFERENCE   1  (bases 1 to 1487)
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  AUTHORS   Norton,J.M., Alzerreca,J. and Klotz,M.G.
  TITLE     The ammonia monooxygenase operon in Nitrosolobus multiformis
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1487)
  AUTHORS   Klotz,M.G.
  TITLE     Direct Submission
  JOURNAL   Submitted (14-FEB-1997) Department of Biology, University of
            Colorado, 1200 Larimer Street, CB171, Denver, CO 80217-3364, USA
FEATURES             Location/Qualifiers
     source          1..1487
                     /organism="Nitrosolobus multiformis"
                     /strain="ATCC 25196; C-71"
                     /note="ammonia monooxygenase operon copy 2 has GenBank
                     Accession Number U15733"
                     /db_xref="taxon:1231"
                     /clone="NMU8C"
     gene            1..50
                     /gene="amoC3"
     CDS             <1..50
                     /gene="amoC3"
                     /note="putative membrane protein; similar to Nitrosospira
                     sp. NpAV ammonia monooxygenase C3 protein encoded by
                     GenBank Accession Number U72981"
                     /codon_start=3
                     /transl_table=11
                     /product="ammonia monooxygenase subunit AmoC3"

                     /protein_id="AAB48532.1"
                     /db_xref="PID:g1858015"
                     /db_xref="GI:1858015"
                     /translation="KVILNNRIVPYDKAF"
     CDS             113..337
                     /function="putative regulator of amo gene expression"
                     /note="ORF2"
                     /codon_start=1
                     /transl_table=11
                     /protein_id="AAB48533.1"
                     /db_xref="PID:g1858016"
                     /db_xref="GI:1858016"
                     /translation="MRPAVTQPGRLKQCEAAAPGAKGEGSRRIREQQGSDKGFIQSCR
                     PLLNHEVPGNSRQRHQGLQGWQAVEQQGND"
     gene            374..1198
                     /gene="amoA3"
     CDS             374..1198
                     /gene="amoA3"
                     /note="similar to Nitrosomonas europaea ammonia
                     monooxygenase acetylene-binding protein encoded by GenBank
                     Accession Number L08050"
                     /codon_start=1
                     /transl_table=11
                     /product="ammonia monooxygenase subunit AmoA3"
                     /protein_id="AAB48534.1"
                     /db_xref="PID:g1858017"
                     /db_xref="GI:1858017"
                     /translation="MSRTDEILKAAKMPPEAVKMSRMIDVIYFPILCILLVGAYHMHF
                     MLLAGDWDFWLDWKDRQWWPVVTPIVGITYCAAIMYYLWVNYRLPFGATLCIVCLLVG
                     EWLTRFWGFYWWSHYPMNFVFPSTMIPGALGMDTVLLLTRNWMITALVGGGAFGLLFY
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                     PGNWTIFGPTHLLLVAEGVLLSVADYTGFLYVRTGTPEYVRLIEQGSLRTFGGHTTV
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     gene            1199..1487
                     /gene="amoB3"
     CDS             1199..>1487
                     /gene="amoB3"
                     /note="similar to Nitrosomonas europaea ammonia
                     monooxygenase protein AmoB encoded by GenBank Accession
                     Number L08050"
                     /codon_start=1
                     /transl_table=11
                     /product="ammonia monooxygenase subunit AmoB3"
                     /protein_id="AAB48535.1"
                     /db_xref="PID:g1858018"
                     /db_xref="GI:1858018"
                     /translation="MKNLFKKSIAGVCSLAALGLALTLDIQPAAAHGERSQEPFLRMR
                     TIQWYDMKWGPETTKVNDIATMTGKFHLAEDWPRAVGKPGRAFFNVGSPSPV"
BASE COUNT      323 a    414 c    443 g    307 t
ORIGIN
        1 gcaaagtcat cctcaataac cgcatcgtac cgtacgacaa ggcgttttaa ggactgactc
       61 gctgacacac gcataagcag caatagccgc atcgcctccg aacaggagag cgatgcggcc
      121 agcggtaaca cagccgggca gactcaagca atgcgaagcg gcggcacctg gagcaaaagg
      181 ggaagggagt cgccggatcc gggagcaaca aggaagcgac aagggattta tacagtcatg
      241 tagaccctta ttgaatcacg aggttcccgg gaattcgcgg caacgtcatc agggactgca
      301 aggatggcaa gcagtagagc agcagggcaa cgattgatat gttttagtcg cgtaacattt
      361 gagggagggt gcgatgagca gaacagatga aatactgaag gcggcgaaga tgccgcccga
      421 agcggtaaag atgtccagga tgatagacgt gatttacttc ccgattctat gcatcctgct
      481 ggttggagcc taccacatgc acttcatgct gctggcgggt gactgggact tctggcttga
      541 ctggaaggac cggcaatggt ggcctgtggt aaccccgatt gtagggatca cctactgtgc
      601 cgcgatcatg tactacctgt gggtgaacta ccgcctgccg tttggagcca cactgtgcat

      661 cgtgtgcctt ctggtgggtg aatggctgac ccgtttctgg ggtttctact ggtggtcgca
      721 ctaccccatg aactttgtat tcccctccac catgattccc ggcgcgctgg ggatggacac
      781 cgtcctgctt ctgacgcgca actggatgat cacggcactg gttggcggcg gcgcctttgg
      841 tttgttgttc tatcctggca actggaccat cttcgggccg acccacctgc tgctggtggc
      901 agaaggcgtg ctgctctcgg tagccgacta cacgggcttt ctgtatgtcc gtaccggcac
      961 ccctgagtac gtgcgactga tcgaacaagg gtcactgcgc acctttggcg gtcacaccac
     1021 cgttatcgcc tccttcttct ccgcgttcgt ctccatgctc atgttcaccg tctggtggta
     1081 ctttggcaag gtctactgca ccgccttcta ctatgtcaag ggcgcgcgcg gccgtgtcag
     1141 catgaagaac gacgtgacag catttggcga agaaggcttt gccgaggggа tcaaataaat
     1201 gaagaacctg ttcaagaaga gtatagccgg ggtatgcagc cttgccgccc tgggactggc
     1261 gctgacgctc gacatccagc cggcggcggc ccacggggaa cgctcgcagg aaccgttcct
     1321 gcgcatgcgt accatccagt ggtatgacat gaaatggggc cccgagacca ccaaggtcaa
     1381 cgacatagcc accatgaccg gcaaattcca cctggccgaa gactggccgc gcgcggtggg
     1441 caagcctgga cgcgccttct tcaacgtagg cagcccaagc ccggtgt
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LOCUS       NTU76552      887 bp    DNA             BCT       16-DEC-1996
DEFINITION  Nitrosovibrio tenuis ammonia monooxygenase subunit A (amoA) gene,
            complete cds.
ACCESSION   U76552
NID         g1732263
VERSION     U76552.1  GI:1732263
KEYWORDS    .
SOURCE      Nitrosovibrio tenuis.
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  ORGANISM  Nitrosovibrio tenuis
            Eubacteria: Proteobacteria: beta subdivision: Ammonia-oxidizing
            bacteria: Nitrosovibrio.
REFERENCE   1  (bases 1 to 887)
  AUTHORS   Norton,J.M., Alzerreca,J. and Klotz,M.G.
  TITLE     Diversity of genes encoding ammonia monooxygenase in ammonia
            oxidizing bacteria
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 887)
  AUTHORS   Norton,J.M. and Klotz,M.G.
  TITLE     Nitrosovibrio tenuis amoA
  JOURNAL   Unpublished
REFERENCE   3  (bases 1 to 887)
  AUTHORS   Norton,J.M.
  TITLE     Direct Submission
  JOURNAL   Submitted (25-OCT-1996) Plants, Soils and Biometeorology, Utah
            State University, Logan, UT 84322, USA
FEATURES             Location/Qualifiers
     source          1..887
                     /organism="Nitrosovibrio tenuis"
                     /strain="NV-12"
                     /note="isolated from soil received from the Woods Hole
                     collection of S. Watson and F. Valois"
                     /db_xref="taxon:1233"
     gene            39..863
                     /gene="amoA"
     CDS             39..863
                     /gene="amoA"
                     /note="ammonia oxidizing membrane protein; acetylene
                     binding protein"
                     /codon_start=1
                     /transl_table=11
                     /product="ammonia-monooxygenase subunit A"
                     /protein_id="AAB38710.1"

                     /db_xref="PID:g1732264"
                     /db_xref="GI:1732264"
                     /translation="MSRTDEILKAAKMPPEAVKMSRMIDAVYFPILCILLVGTYHMHF
                     MLLAGDWDFWLDWKDRQWWPVVTPIVGITYCAAIMYYLWVNYRLPFGATLCIVCLLAG
                     EWLTRFWGFYWWSHYPMSFVFPSTMIPGALVMDTVMLLTRNWMITALVGGGAFGLLFY
                     PGNWPIFGLTHLPLVVEGVLLSVADYTGFLYVRTGTPEYVRNIEQGSLRTFGGHTTVI
                     AAFFAAFISMLMFTIWWYFGKLYCTAFFYVKGHRGRVTMKNDVTAFGEEGFPEGIK"
BASE COUNT      184 a    239 c    254 g    210 t
ORIGIN
        1 gttatcaaga tcgtagacac atttgagggg agggcgcgat gagcagaaca gatgagatac
       61 taaaggcggc gaagatgccg ccggaagcag taaagatgtc caggatgata gatgcggtgt
      121 atttcccgat tctatgcata ctgctggttg gaacctacca catgcacttc atgctgctgg
      181 cgggagactg ggacttctgg ctggactgga aagaccgtca atggtggccg gtggtaaccc
      241 cgattgtggg gatcacctat tgtgccgcca tcatgtacta cctgtgggtg aactaccggc
      301 tgccgtttgg ggccacactg tgcatcgttt gcctgctggc gggtgaatgg ctgacacgct
      361 tctggggttt ttactggtgg tcgcactacc ccatgagctt cgtgtttccc tccaccatga
      421 taccgggtgc gctggtgatg gacaccgtca tgctgctcac gcgcaactgg atgatcaccg
      481 ccctggtagg cgggggcgcc ttcgggttat tgttctaccc tggcaactgg cccatttttg
      541 gcctgaccca cctgccgctg gtcgtggaag gcgtactgct gtcggtagcc gactacacgg
      601 gcttcctgta tgtgcgtacc ggcacgcctg aatacgtccg caatatcgaa caaggctcgc
      661 tgcgcacgtt tggaggccac accaccgtca ttgccgcatt ctttgccgcg ttcatctcca
      721 tgctcatgtt caccatctgg tggtactttg gcaaactcta ctgcaccgca ttcttctacg
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      781 tgaaaggcca tcgcggccgc gtcaccatga agaacgacgt taccgcattt ggcgaagaag
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LOCUS       NBU76553      883 bp    DNA             BCT       16-DEC-1996
DEFINITION  Nitrosospira briensis ammonia monooxygenase subunit A (amoA) gene,
            complete cds.
ACCESSION   U76553
NID         g1732261
VERSION     U76553.1  GI:1732261
KEYWORDS    .
SOURCE      Nitrosospira briensis.
  ORGANISM  Nitrosospira briensis
            Eubacteria; Proteobacteria; beta subdivision; Ammonia-oxidizing
            bacteria; Nitrosospira.
REFERENCE   1  (bases 1 to 883)
  AUTHORS   Norton,J.M., Alzerreca,J. and Klotz,M.G.
  TITLE     Diversity of genes encoding ammonia monooxygenase in ammonia
            oxidizing bacteria
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 883)
  AUTHORS   Norton,J.M. and Klotz,M.G.
  TITLE     Nitrosospira briensis amoA
  JOURNAL   Unpublished
REFERENCE   3  (bases 1 to 883)
  AUTHORS   Norton,J.M.
  TITLE     Direct Submission
  JOURNAL   Submitted (25-OCT-1996) Plants, Soils and Biometeorology, Utah
            State University, Logan, UT 84322, USA
FEATURES             Location/Qualifiers
     source          1..883
                     /organism="Nitrosospira briensis"
                     /strain="C-128"
                     /note="isolated from soil received from the Woods Hole
                     collection of S. Watson and F. Valois"
                     /db_xref="taxon:35799"

     gene            36..860
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     CDS             36..860
                     /gene="amoA"
                     /note="ammonia oxidizing membrane protein; acetylene
                     binding protein"
                     /codon_start=1
                     /transl_table=11
                     /product="ammonia-monooxygenase subunit A"
                     /protein_id="AAB38709.1"
                     /db_xref="PID:g1732262"
                     /db_xref="GI:1732262"
                     /translation="MSRTDEILKAAKMPPEAVKMSRMIDAVYFPILCILLVGTYHMHF
                     MLLAGDWDFWLDWKDRQWWPVVTPIVGITYCAAIMYYLWVNYRLPFGATLCVVCLLTG
                     EWLTRYWGFYWWSHYPINFVFPSTMIPGALVMDTVMLLTRNWMITALVGGGAFGLLFY
                     PGNWPIFGPTHLPLAAEGVLLSVADYTGFLYVRTGTPEYVRNIEQGSLRTFGGHTTVI
                     ASFFAAFVSMLMFCLWWYFGKLYCTAFFYVKGTRGRVTMKNDVTAFGEEGFPEGIK"
BASE COUNT      180 a    270 c    232 g    201 t
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ORIGIN
        1 gttttaagat cgtaaacatt tgaggggagg gtgcgatgag cagaaccgat gaaatactga
       61 aggcggccaa gatgccgccg gaagcagtaa agatgtccag gatgatagac gcggtatatt
      121 tcccgattct atgtatcctc ctggttggaa cctaccacat gcacttcatg ctgctggcag
      181 gcgactggga cttctggctg gactggaaag accgccaatg gtggccggta gtgaccccca
      241 tcgtgggcat cacctattgc gccgccatca tgtactacct gtgggtcaac taccggctgc
      301 ccttcggcgc caccctctgc gtcgtctgcc tgctgaccgg cgaatggctg acccgctact
      361 ggggcttcta ctggtggtcg cactacccca tcaacttcgt cttcccctcc accatgatac
      421 cgggcgcact ggtcatggac accgtcatgc tgctcacccg caactggatg atcacagccc
      481 tggttggcgg aggcgcattc ggactcctgt tctacccggg taactggccc atctttggcc
      541 cgacccacct gccgctggca gccgaaggcg tattgttgtc ggttgctgac tacaccggct
      601 tcctgtacgt tcgcaccggc acccccgagt acgtacgcaa catcgaacaa ggctcactca
      661 gaacctttgg cgggcacacc accgtcatcg cctcattctt tgccgccttc gtctccatgc
      721 tcatgttctg cctctggtgg tacttcggca aactttactg caccgcattc ttctacgtca
      781 agggaacccg tggccgtgtc accatgaaga acgatgtcac cgcatttggg gaagaaggct
      841 tcccggaggg gatcaaataa tgggaattca acaacttatc aag
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LOCUS       AF153343     1028 bp    DNA             BCT       25-OCT-1999
DEFINITION  Nitrosococcus sp. C-113 16S ribosomal RNA gene, partial sequence.
ACCESSION   AF153343
KEYWORDS    .
SOURCE      Nitrosococcus sp. C-113.
  ORGANISM  Nitrosococcus sp. C-113
            Bacteria; Proteobacteria; gamma subdivision; Nitrosococcus.
REFERENCE   1  (bases 1 to 1028)
  AUTHORS   Alzerreca,J.J., Norton,J.M. and Klotz,M.G.
  TITLE     The amo operon in marine, ammonia oxidizing gamma proteobacteria,
            autotrophic ammonia oxidizing bacteria
  JOURNAL   FEMS Microbiology Letters 180:21-29
REFERENCE   2  (bases 1 to 1028)
  AUTHORS   Alzerreca,J.J., Norton,J.M. and Klotz,M.G.
  TITLE     Direct Submission
  JOURNAL   Submitted (21-MAY-1999) Plants, Soils and Biometeorology, Utah
            State University, Logan, UT 84322, USA
FEATURES             Location/Qualifiers
     source          1..1028
                     /organism="Nitrosococcus sp. C-113"
                     /strain="C-113"
                     /db_xref="taxon:105559"
     rRNA            1..1028
                     /product="16S ribosomal RNA"
BASE COUNT      252 a    227 c    352 g    197 t
ORIGIN
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      121 ggctaatacc gcataatctc taaggaggaa agcgggggac cgaaaggcct cgcgctggag
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      481 gcggtaatac ggagggtgcg agcgttaatc ggaattactg ggcgtaaagg gcgcgtaggc
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      721 aagcgagggg agcaaacagg attagatacc ctggtagtcc acgccctaaa cgatgagaac
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      901 gcatgtggtt taattcgatg caacgcgaag aaccttacct ggccttgaca tccccggaac
      961 tttgctgaga ggcgaaggtg ccttcgggag ccgggagaca ggtgctgcat ggctgtcgtc
     1021 agctcgta
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LOCUS       AF153344     2929 bp    DNA             BCT       25-OCT-1999
DEFINITION  Nitrosococcus sp. C-113 ammonia monooxygenase subunit A (amoA) and
            ammonia monooxygenase subunit B (amoB) genes. complete cds.
ACCESSION   AF153344
KEYWORDS    .
SOURCE      Nitrosococcus sp. C-113.
  ORGANISM  Nitrosococcus sp. C-113
            Bacteria; Proteobacteria; gamma subdivision; Nitrosococcus.
REFERENCE   1  (bases 1 to 2929)
  AUTHORS   Alzerreca.J.J.. Norton.J.M. and Klotz.M.G.
  TITLE     The amo operon in marine. ammonia oxidizing gamma proteobacteria.
            autotrophic ammonia oxidizing bacteria
  JOURNAL   FEMS Microbiology Letters 180:21-29
REFERENCE   2  (bases 1 to 2929)
  AUTHORS   Norton.J.M.. Alzerreca.J.J.. Suwa.Y. and Klotz.M.G.
  TITLE     Diversity of ammonia monooxygenase operon in autotrophic
            ammonia-oxidizing bacteria
  JOURNAL   Unpublished
REFERENCE   3  (bases 1 to 2929)
  AUTHORS   Alzerreca.J.J.. Norton.J.M. and Klotz.M.G.
  TITLE     Direct Submission
  JOURNAL   Submitted (21-MAY-1999) Plants. Soils and Biometeorology. Utah
            State University. Logan. UT 84322. USA
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                     /strain="C-113"
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     CDS             590..1333
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                     TTRLTSYINGMFVPRSIGLELGGDYEFEMTMQGRRPGTWHVHTLLNVQGGGPLIGPGK
                     YITITGDMADFENKVTDLTGNTVNLETMATGTVIGWHLFWYVLGIAWIVWWARRPMFL
                     PRYMRVEAGEANDLVTAQDKKVTIGVLVGVLLIVLFGYKSAEEKFPVTIPLQAGLLGT
                     IEPLPVDYNSMVSAKVLKANYRVPGRTITMTVELTNHTDQVLSIGEFNTGGIRFMNAN
                     VRSDDTGYPEELLAPEGLEMSQQDIAPGETVVVDISATDAAWEVQRMADVIYDPDSRF
                     AGLIFFIDPEGNEIPIPVGGPLVPTFV"
BASE COUNT      666 a    610 c    777 g    857 t     19 others
ORIGIN
        1 gtaggtttag gttcgttcat gtatgctaag acccgtattc cttattttgc aaagggctgg
       61 tctgtaccct acctcatgtt ggtgtttgga ccctttatga tcttcccgaa tgtggggctg
      121 aatgaatggg gtcacacctt ctggtttatg gaaaagctat ttgtaccacc tctgcattgg
      181 ggttttgtgt tctttgcctg gtttatcctt gcggtattcg gcgtattctt gcaggtgcaa
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      301 tgagatattt aaatttaatt tccagctgat ttaaagggtt gagtaaaagg gcggtcgcaa
      361 tctccagaat agttaatgag atggcgaccg cctctttgta taaatagatt tattattaag
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      721 aatgggkgac tgggatttct gggttgattg gaaagaccgg cgcttttggg tcaccgtggt
      781 tcccatcgta tcggtagctt atcctgctgc cgcacaggcg tttttctggg agaagttccg
      841 ccttcccttt ggcgctaccc tggtaacgct aggattgtta gcgggtgagt gggctaaccg
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     1381 taatagagag atggagaaca tgaaaggtac ccatataaca aatcgagcca aaaaatggct
     1441 agccattggg tttactgcgg tgatagcctc aagcgttttc tatattccga cagtggccgc
     1501 ccatggtgag aaggcgcagg cagccttcct gcgcatgcgg acaattcatt ggtatgacat
     1561 ggtatggtcc aaggatacca ttgcggttaa tgagacctat accataagcg gcaagttccg
     1621 ggtttttgag gattggccgg aagcagtcga agtaccccat gtgtcctttt taaatgcggg
     1681 tcaacccgga ccagtcacaa cccggctcac ttcttatatc aatggtatgt tcgtccctcg
     1741 gtcgataggc cttgaattag gcggcgatta cgagtttgag atgacgatgc aagggcgccg
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     1861 tccaggtaaa tacatcacca ttactggaga tatggcagac tttgagaaca aagtcacaga
     1921 tctaaccggt aatacggtca acctggaaac catggccacg ggtacggtta ttggctggca
     1981 tctattctgg tacgtacttg gtatcgcctg gatcgtgtgg tgggcccgtc gccccatgtt
     2041 cttgccccgc tacatgagag tagaggcggg agaggctaat gatctagtga ccgcccaaga
     2101 caaaaaagtg actatcggtg ttcttgtggg cgtcctgctc attgttttgt tcggctataa
     2161 gagtgctgag gaaaaattcc cagtcactat tcctttgcaa gctgggctgc tgggcactat
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     2281 tcgggtgccg ggcggacta tcaccatgac ggttgagctc actaatcata ctgatcaggt
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     2461 ggatatcgct ccgggtgaaa ctgtggttgt tgatatttcc gccaccgatg ctgcttggga
     2521 agtgcagcgt atggctgacg ttatttacga tccagacagc cgctttgcgg gattgatatt
     2581 ctttattgat ccagagggga atgagattcc gatacctgtc ggtggtccat tagttcctac
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2761 gctggacgaa gggccggttg tttgttggtc gtcctttcca tttctttttt taccttgtgg
2821 ctcagtcttt tcaggctctg gagctgcgtt ttaatcaact cctcagggca aaaaggtatt
2881 ggcttatgaa aaaaatacta cttagctgct tcctatcgtt taggattgc
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Appendix G. AMO Amino Acid Alignments

and Pair Table for the *Amo* Genes

and their Deduced Peptides

Alignments and pair table for the following strains (abbreviation):

Nitrosospira sp. NpAV (NAV), *Nitrosospira briensis* C-128 (NBR) *Nitrosospira sp.*39-19 (N39), *Nitrosovibrio tenuis* NV-12 (NVT), *Nitrosolobus multiformis* ATCC 25196 (NMU), *Nitrosolobus multiformis* 24-C (N24), *Nitrosomonas europaea* ATCC 19178 (NEU), *Nitrosomonas eutropha* C-91 (NET), *Nitrosomonas sp.* JL21(JL21), *Nitrosomonas sp.* AL212 (AL212), *Nitrosomonas sp.* GH22 (GH22), *Nitrosococcus sp.* C-113 (N113), *Nitrosococcus oceanus* C-107 (NOC), *Methylococcus capsulatus* (Bath) (MCA).

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Clustal W Alignment 11/98 Klotz, Norton and Alzerreca.
AmoC complete peptides

NEUC1   MATTLGTS-S ASSVSSRG-Y DMSLWYDSKF YKFGMITMLL VAIFWVWYQR YFAYSHGMDS MEPEFDRVWM
NEUC2   MATTLGTS-S ASSVSSRG-Y DMSLWYDSKF YKFGMITMLL VAIFWVWYQR YFAYSHGMDS MEPEFDRVWM
NEUC3   MATSILKDKT AQQVTDKPAY DKSEWFDAKY YKYGLLPILG IAVFWVWYQR TFAYSHGMDS MEPDFDRIWM
NAVC2   MATTLGTSGS HAGSSGRD-Y DMSLWYDSRW YKFGLITMLG VAIFWIWFQR TFAYSHGMDS MEPDFEKVWM
NAVC3   MATTLGTSGS HAGSSGRD-Y DMSLWYDSRW YKFGLITMLG VAIFWIWFQR TFAYSHGMDS MEPDFEKVWM
NAVC4   MATTVETSGS HAGSSGRD-Y DMSLWYDSRW YKFGLITMLG VAIFWIWFQR TFAYSHGMDS KEPDFEKVWM
NOCC    MAAT--SRAV HQGVAEKE-T ADFFAWRGMW L--AVAAFFA LYICVRWYEG VYGWKYGLDA FLPEFQTYWM
N113C   NNNNNNNNNN NQGVAEKE-T ADLFAWRGMW L--AVAAFFL FYIGVRWYEG VYGWKYGLDA FSPEFQTYWM
MCAC1   MAAT--TIGG HAA-A--E-- APLL--DKKW LTFALAIYTV FYLWVRWYEG VYGWSAGLDS FAPEFETYWM
MCAC2   MAAT--TIGG HAA-A--E-- APLL--DKKW LTFALAIYTV FYLWVRWYEG VYGWSAGLDS FAPEFETYWM

NEUC1   NLWRVHMAIM PLFALVTWGW ILKTRDTKEQ LDNLDPKLEI KRYFYYMMWL GVYIFGVYWG GSFFTEQDAS
NEUC2   NLWRVHMAIM PLFALVTWGW ILKTRDTKEQ LDNLDPKLEI KRYFYYMMWL GVYIFGVYWG GSFFTEQDAS
NEUC3   GLWRVQMVVI ALAAFSIWGW LLKTRNTAEQ LASLTPKQEI KRYFYFMMWL GVYIFAVYWG SSFFTEQDAS
NAVC2   GLWRVHMIVM PIFALVTWGW IWKTRNT--N LDNLDPKLEI KRYFYWMMWL GVYIFGVYWG GSFFTGQDAS
NAVC3   GLWRVHMIVM PIFALVTWGW IWKTRNT--N LDNLDPKLEI KRYFYWMMWL GVYIFGVYWG GSFFTEQDAS
NAVC4   GLWRVHMIVM PIFALVTWGW IWKTRNT--N LDNLDPKLEI KRYFYWMMWL GVYIFGVYWG GSFFTEQDAS
NOCC    NLLYIELVVE ALATAALVSY LIKTRN-R-N MEAMTPREEV RRYCTLYMWW VVYGVGLFWG ASFFTEQDGA
N113C   NLLYIELVVE ALATAALVSY LIKTRD-R-N MEAMTPREEL RRYCTLYMWW VVYGVGLFWG ASFFTEQDGA
MCAC1   NFLYTEIVLE IVTASILWGY LWKTRN-R-N LAALTPREEL RRNCTHLVWL VAYAWAIYWG ASYFTEQDGT
MCAC2   NFLYTEIVLE IVTASILWGY LWKTRN-R-N LAALTPREEL RRNCTHLVWL VAYAWAIYWG ASYFTEQDGT

NEUC1   WHQVIIRDTS FTPSHVVVFY GSFPMYIVCG VATYLYAMTR LPLFSRGISF PLVMAIAGPL MILPNVGLNE
NEUC2   WHQVIIRDTS FTPSHVVVFY GSFPMYIVCG VATYLYAMTR LPLFSRGISF PLVMAIAGPL MILPNVGLNE
NEUC3   WHQVIIRDTS FTPSHIPLFY GSFPVYIIMG IAMIIYAKTR LPLYNKGWSF PLIMVVAGPL MSLPNVGLNE
NAVC2   WHQVIIRDTS FTPSHVVVFY GSFPMYIVCG VATYLYAMTR LPLYARGTSF PLLMAIAGPL MILPNVGLNE
NAVC3   WHQVIIRDTS FTPSHVVVFY GSFPMYIVCG VATYLYAMTR LPLYARGTSF PLVMAIAGPL MILPNVGLNE
NAVC4   WHQVIIRDTS FTPSHVVVFY GSFPMYIVCG VATYLYAMTR LPLYARGTSF PLVMAIAGPL MILPNVGLNE
NOCC    WHQTVVRDTD FTPSHIIEFY MSYPIYVMVG LGSFTYAKTR IPYFAKGWFV PYLMLVFGPF MIFPNVGLNE
N113C   WHQTVVRDTD FTPSHIIEFY MSYPIYVMVG LGSFTYAKTR IPYFAKGWSV PYLMLVFGPF MIFPNVGLNE
MCAC1   WHQTIVRDTD FTPSHIIEFY LSYPIYIITG FAAFIYAKTR LPFFAKGISL PYLVLVVGPF MILPNVGLNE
MCAC2   WHQTIVRDTD FTPSHIIEFY LSYPIYIITG FAAFIYAKTR LPFFAKGISL PYLVLVVFPF MILPNVGLNE

NEUC1   WGHAFWFMEE LFSAPLHWGF VVLGWAGLFQ GGVAAQIITR YSNLTDVVWN NQSKEILNNR IV-A-
NEUC2   WGHAFWFMEE LFSAPLHWGF VVLGWAGLFQ GGVAAQIITR YSNLTDVVWN NQSKEILNNR IV-A-
NEUC3   WGHAFWFMEE LFSAPLHWGF VILAWAALFQ GGLAIQLITR YSNLVDVEWN KQDRAILDDV VTTP-
NAVC2   WGHAFWFMEE LFSAPLHWGF VILGWAGLFA GGIAAQIITR YSSLTDVVWN GQDRVILNNR IV-P-
NAVC3   WGHAFWFMEE LFSAPLHWGF VILGWAGLFA GGIAAQIITR YSNLTDVVWN GQDRVILNNR IV-P-
NAVC4   WGHAFWFMEE LFSAPLHWGF VILGWAGLFA GGIAAQIITR YSNLTDVVWN GQDRVILNNR IV-P-
NOCC    WGHTFWFMEE LFVAPLHWGF VFFAWFILAV FGVFLQ--VQ PRMK-EL--I GRELQQSEDY ARS--
N113C   WGHTFWFMEK LFVPPLHWGF VFFAWFILAV FGVFLQ--VQ GRMK-EL--L GRELQQSEDY ARS--
MCAC1   WGHTFWFMEE LFVAPLHYGF VIFGWLALAV MGTLTQ--TF YRFA-Q-GGL GQSLCEAVDE GLIAK
MCAC2   WGHTFWFMEE LFVAPLHYGF VIFGWLALAV MGTLTQ--TF YSFA-Q-GGL GQSLCEAVDE GLIAK
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Clustal W Alignment 11/98 Klotz, Norton and Alzerreca.
AmoA complete peptides

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 N39amoA1       ....................MSRMIDAVYFPILCILLVGTYHMHFMLLAGDWDFWLDWK
  NBRamoA       MS..RTDEILKAAKMPPEAVKMSRMIDAVYFPILCILLVGTYHMHFMLLAGDWDFWLDWK
  NVTamoA       MS..RTDEILKAAKMPPEAVKMSRMIDAVYFPILCILLVGTYHMHFMLLAGDWDFWLDWK
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 NMUamoA3       MS..RTDEILKAAKMPPEAVKMSRMIDVIYFPILCILLVGAYHMHFMLLAGDWDFWLDWK
 N24CamoA       MS..RTDEIIRAAKMPPEAVKMSRMIDVIYFPILCILLVGTYHMHFMLLAGDWDFWLDWK
 NEUamoA1       VSIFRTEEILKAAKMPPEAVHMSRLIDAVYFPILIILLVGTYHMHFMLLAGDWDFWMDWK
AL212amoA       VS..RTDEIIAAAKMPPEAVKMSRYIDAVYFPILCILLVGTFHMHFMLLAGDWDFWLDWK
 JL21amoA       VT..RTDEIIAAAKMPPEAVRMSRYIDAVYFPILCILLVGTYHMHFMLLAGDWDFWLDWK
 NETamoA2       VSIFRTEEILKAAKMPPEAVHMSRLIDAVYFPILVVLLVGTYHMHFMLLAGDWDFWMDWK
 NETamoA1       VSIFRTEEILKAAKMPPEAVHMSRLIDAVYFPILVVLLVGTYHMHFMLLAGDWDFWMDWK
 GH22amoA       VSIFRTEEILKAAKMPPEAVHMSRLIDAVYFPILVVLLVGTYHMHFMLLAGDWDFWMDWK
  NOCamoA       MSAL.TS....AVRTPEEAAKVFRTLDFIALGAFFMILLASHHVHVMLLMGDWDFWVDWK
 N113amoA       MSAL.TS....AVRTPEEAAKVSRTLDFLALGAFFGILLASHHVHVMLLMGDWDFWVDWK
 MCApmoA1       MSAA.QS....AVRSHAEAVQVSRTIDWMALFVVFFVIVGSYHIHAMLTMGDWDFWSDWK
 MCApmoA2       MSAA.QS....AVRSHAEAVQVSRTIDWMALFVVFFVIVGSYHIHAMLTMGDWDFWSDWK

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 N39amoA1       DRQWWPVVTPIVGITYCAAIMYYLWVNYRLPFGATLCIVCLLSGEWLTRYWGFYWWSHYP
  NBRamoA       DRQWWPVVTPIVGITYCAAIMYYLWVNYRLPFGATLCVVCLLTGEWLTRYWGFYWWSHYP
  NVTamoA       DRQWWPVVTPIVGITYCAAIMYYLWVNYRLPFGATLCIVCLLAGEWLTRFWGFYWWSHYP
 NAVamoA3       DRQWWPVVTPIVGITYCATIMYYLWVNYRLPFGATLCIVCLLVGEWLTRFWGFYWWSHYP
 NAVamoA2       DRQWWPVVTPIVGITYCATIMYYLWVNYRLPFGATLCIVCLLVGEWLTRFWGFYWWSHYP
 NAVamoA1       DRQWWPVVTPIVGITYCATIMYYLWVNYRLPFGATLCIVCLLVGEWLTRFWGFYWWSHYP
 NMUamoA1       DRQWWPVVTPIVGITYCAAIMYYLWVNYRLPFGATLCIVCLLVGEWLTRFWGFYWWSHYP
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 N24CamoA       DRQWWPVVTPIVGITCYAAIMYYLWVNYRLPYGATLCIVCLLVGEWLTRFWGFYWWSHYP
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AL212amoA       DRQWWPVVTPIAGIMYCAALMYYLWVNYRLPFGATLCIVCLLVGEWLTRYWGFYWWSHYP
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 NETamoA2       DRQWWPVVTPIVGITYCSAIMYYLWVNYRQPFGATLCVVCLLIGEWLTRYWGFYWWSHYP
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 GH22amoA       DRQWWPVVTPIVGITYCSAIMYYLWVNYRQPFGATLCVVCLLIGEWLTRYWGFYWWSHYP
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 N113amoA       DRRFWVTVVPIVSVAYPAAAQAFFWEKFRLPFGATLVTLGLLAGEWANRYFNFVGFTYFP
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 MCApmoA2       DRRLWVTVTPIVLVTFPAAVQSYLWERYRLPWGATVCVLGLLLGEWINRYFNFWGWTYFP

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 N39amoA2       ISFVFPSTMIPGALVMDTVMLLTRNWMITALVGGGAFGFLFYPGNWPIFGPTHLPLVVEG
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  NBRamoA       INFVFPSTMIPGALVMDTVMLLTRNWMITALVGGGAFGLLFYPGNWPIFGPTHLPLAAEG
  NVTamoA       MSFVFPSTMIPGALVMDTVMLLTRNWMITALVGGGAFGLLFYPGNWPIFGLTHLPLVVEG
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 N24CamoA       INFVFPSTMIPGALIMDTVMLLTRNWMITALIGGGAFGLLFYPGNWPIFGPTHLPLVAEG
 NEUamoA1       INFVTPGIMLPGALMLDFTLYLTRNWLVTALVGGGFFGLLFYPGNWPIFGPTHLPIVVEG
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AL212amoA    INFVLPSTMIPGALMLDTIMLLTGDWLITALLGGAFWGLFFYPGNWPIFGPTHLPLVVEG
 JL21amoA    LNFVLPSTMIPGALMMDTIMLLTGNWLVTALLGGGFFGLFFYPGNWPIFGPTHLPVVVEG
 NETamoA2    LNFVTPGIMLPGAFMLDFTMYLTRNWLVTALVGGGFFGLMFYPGNWPIFGPTHLPIVVEG
 NETamoA1    LNFVTPGIMLPGALMLDFTMYLTRNWLVTALVGGGFFGLMFYPGNWPIFGPTHLPIVVEG
 GH22amoA    LNFVTPGIMLPGALMLDFTMYLTRNWLVTALVGGGFFGLMFYPGNWPIFGPTHLPIVVEG
  NOCamoA    INFVWPTILLPMALFLDAMLAISKSYGLTAVVGGLMYGLLMYPANWPLLSAFHVPAEYNG
 N113amoA    INFVWPTILLPMALFLDAMLAISKSYGLTAVVGGLMYGLLMYPANWPLLSAFHVPAEYNG
 MCApmoA1    INFVFPASLVPGAIILDTVLMLSGSYLFTAIVGAMGWGLIFYPGNWPIIAPLHVPVENNG
 MCApmoA2    INFVFPASLVPGAIILDTVLMLSGSYLFTAIVGAMGWGLIFYPGNWPIIAPLHVPVEYNG


             181                                                      240
 N39amoA3    VLLSVADYTGFLYVRTGTPEYVRNIEQGSLRTFGGHTTVIAAFFAAFISMLMFCIWWYFG
 N39amoA2    VLLSVADYTGFLYVRTGTPEYVRNIEQGSLRTFGGHTTVIAAFFAAFISMLMFCIWWYFG
 N39amoA1    VLLSVADYTGFLYVRTGTPEYVRNIEQGSLRTFGGHTTVIAAFFAAFISMLMFCIWWYFG
  NBRamoA    VLLSVADYTGFLYVRTGTPEYVRNIEQGSLRTFGGHTTVIASFFAAFVSMLMFCLWWYFG
  NVTamoA    VLLSVADYTGFLYVRTGTPEYVRNIEQGSLRTFGGHTTVIAAFFAAFISMLMFTIWWYFG
 NAVamoA3    VLLSLADYTGFLYVRTGTPEYVRLIEQGSLRTFGGHTTVIAAFFSAFVSMLMFCVWWYFG
 NAVamoA2    VLLSLADYTGFLYVRTGTPEYVRLIEQGSLRTFGGHTTVIAAFFSAFVSMLMFCVWWYFG
 NAVamoA1    VLLSLADYTGFLYVRTGTPEYVRLIEQGSLRTFGGHTTVIAAFFSAFVSMLMFCVWWYFG
 NMUamoA1    VLLSVADYTGFLYVRTGTPEYVRLIEQGSLRTFGGHTTVIASFFSAFVSMLMFTVWWYFG
 NMUamoA2    VLLSVADYTGFLYVRTGTPEYVRLIEQGSLRTFGGHTTVIASFFSAFVSMLMFTVWWYFG
 NMUamoA3    VLLSVADYTGFLYVRTGTPEYVRLIEQGSLRTFGGHTTVIASFFSAFVSMLMFTVWWYFG
 N24CamoA    VLLSVADYTGFLYVRTGTPEYVRLIEQGSLRTFGGHTTVIAAFFSAFVSMLMFTVWWYFG
 NEUamoA1    TLLSMADYMGHLYVRTGTPEYVRHIEQGSLRTFGGHTTVIAAFFSAFVSMLMFTVWWYLG
AL212amoA    VLLSVADYTGFLYVRTGTPEYVRLIEQGSLRTFGGHTTVIAAFFSAFVSMLMFCVWWYFG
 JL21amoA    VLLSIADYTGFLYVRTGTPEYVRLIEQGSLRTFGGHTTVIAAFFSAFVSMLMFCVWWYFG
 NETamoA2    TLLSMADYMGHLYVRTGTPEYVRHIEQGSLRTFGGHTTVIAAFFAAFVSMLMFAVWWYLG
 NETamoA1    TLLSMVDYMGHLYVRTGTPEYVRHIEQDSLRTFGGHTTVIAAFFAAFVSMLMFAVWWYLG
 GH22amoA    TLLSMADYMGHLYVRTGTPEYVRHIEQGSLRTFGGHTTVIAAFFAAFVSMLMFAVWWYLG
  NOCamoA    VVMSLADIMGYQYVRTGTPEYIRMVEKGTLKTFGKDVVPVSAFFSGFVAMVMYFVWHFVG
 N113amoA    VVMSLADVAGYQYVRTGTPEYIRMVEKGTLRTFGKDVVPVSAFFAGFVAMVMYFVWHFVG
 MCApmoA1    MLMSIADIQGYNYVRTGTPEYIRMVEKGTLRTFGKDVAPVSAFFSAFMSILIYFMWHFIG
 MCApmoA2    MLMSIADIQGYNYVRTGTPEYIRMVEKGTLRTFGKDVAPVSAFFSAFMSILIYFMWHFIG

             241                                 277
 N39amoA3    KLYCTAFFYVKGHRGRVTMKNDVTAFGEEGFPEGIK.
 N39amoA2    KLYCTAFFYVKGHRGRVTMKDDVTAFGEEGFPEGIK.
 N39amoA1    KLYCTAFFYVKGHRGRVTMKNDVTAFGEEGFPEGIK.
  NBRamoA    KLYCTAFFYVKGTRGRVTMKNDVTAFGEEGFPEGIK.
  NVTamoA    KLYCTAFFYVKGHRGRVTMKNDVTAFGEEGFPEGIK.
 NAVamoA3    KLYCTAFYYVKGPRGRVTMKNDVTAYGEEGFPEGIK.
 NAVamoA2    KLYCTAFYYVKGPRGRVTMKNDVTAYGEEGFPEGIK.
 NAVamoA1    KLYCTAFYYVKGPRGRVTMKNDVTAYGEEGFPEGIK.
 NMUamoA1    KVYCTAFYYVKGARGRVSMKNDVTAFGEEGFAEGIK.
 NMUamoA2    KVYCTAFYYVKGARGRVSMKNDVTAFGEEGFAEGIK.
 NMUamoA3    KVYCTAFYYVKGARGRVSMKNDVTAFGEEGFAEGIK.
 N24CamoA    KVYCTAFFYVKGPRGRISMKNDVTAYGEEGFPEGIK.
 NEUamoA1    KVYCTAFFYVKGKRGRIVHRNDVTAFGEEGFPEGIK.
AL212amoA    KLYCTAFFYVKGERGRISMKNDVTAFGEKGFAQGIK.
 JL21amoA    KLYCTAFYYVKGERGRISMKNDVTAFGEKGFAQGIK.
 NETamoA2    KVYCTAFFYVKGKRGRIVQRNDVSAFGEEGFPEGIK.
 NETamoA1    KVYCTAFFYVKGKRGRIVQRNDVTAFGEEGFPEGIK.
 GH22amoA    KVYCTAFFYVKGKRGRIVQRNDVTAFGEEGFPEGIK.
  NOCamoA    RWFSKDYHINQL.........................
 N113amoA    RWFSKDYSVDQC.........................
 MCApmoA1    RWFSNERFLQST.........................
 MCApmoA2    RWFSNERFLQST.........................
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Clustal W Alignment 11/98 Klotz, Norton and Alzerreca.
AmoB complete peptides

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NAVB1      MNAKNLFKLG---------VIGLYGMATLALS-TLDISPAAAHGERSQEPFLRMRTIQWYDMKWGPDTTKVND
NAVB2      MNAKNLFKLG---------VIGLYGMATLALS-TLDISPAAAHGERSQEPFLRMRTIQWYDMKWGPDTTKVND
NAVB3      MNAKNLFKLG---------VIGLYGMATLALS-TLDISPAAAHGERSQEPFLRMRTIQWYDMKWGPDTTKVND
NEUB       MGIKNLYK--R-GVMGLYGV-A-YAVAALAMTVTLDVSTVAAHGERSQEPFLRMRTVQWYDIKWGPEVTKVNE
NOCB       MKGTHITN--R---ARKWLAIG-FTAVIASSVFYI--PTVAAHGEKAQAAFLRMRTIHWYDMVWS-DTIAVNE
N113B      MEN--MK-GTHITNRAKKWLAIGFTAVIASSVFYI--PTVAAHGEKAQAAFLRMRTIHWYDMVWSKDTIAVNE
MCAB1      M--KTIKD--R--IA-KWSAIGLLSAVAATA-FYA--PSASAHGEKSQAAFMRMRTIHWYDLSWSKEKVKINE
MCAB2      M--KTIKD--R--IA-KWSAIGLLSAVAATA-FYA--PSASAHGEKSQAAFMRMRTIHWYDLSWSKEKVKINE

NAVB1      FATMTGKFHLAEDWPRAVGKPGRAFFNVGSPSPVFVRLSTKLNGEPTYISGPLEIGRDYAFEVKLKARI
NAVB2      FATMTGKFHLAEDWPRAVGKPGRAFFNVGSPSPVFVRLSTKLNGEPTYISGPLEIGRDYAFEVKLKARI
NAVB3      FATMTGKFHLAENWPRAVGKPGRAFFNVGSPSPVFVRLSTKLNGEPTYISGPLEIGRDYAFEVKLKARI
NEUB       NAKITGKFHLAEDWPRAAAQPDFSFFNVGSPSPVFVRLSTKINGHPWFISGPLQIGRDYEFEVNLRARI
NOCB       TYTISGKFRVFEDWPEAVEKPHVSFLNAGQPGPVTARLTSYVNGMFVPRSIGLELGGDYDFEMTMQGRR
N113B      TYTISGKFRVFEDWPEAVEVPHVSFLNAGQPGPVTTRLTSYINGMFVPRSIGLELGGDYEFEMTMQGRR
MCAB1      TVEIKGKFHVFEGWPETVDEPDVAFLNVGMPGPVFIRKESYIGGQLVPRSVRLEIGKTYDFRVVLKARR
MCAB2      TVEIKGKFHVFEGWPETVDEPDVAFLNVGMPGPVFIRKESYIGGQLVPRSVRLEIGKTYDFRVVLKARR

NAVB1      PGRHHMHAMVNIKDAGPIAGPAAWMNITGSWDDFTNPVKLLTGETIDTETFNFNNGIFWHLLWLGLGCF
NAVB2      PGRHHMHAMVNIKDAGPIAGPAAWMNITGSWDDFTNPVKLLTGETIDTETFNFNNGIFWHLLWLGLGCF
NAVB3      PGSHHMHTMVNIKDAGPIAGPAAWMNITGSWDDFTNPVKLLTGETIDTETFNFNNGIFWHLLWLGLGCF
NEUB       PGRHHMHAMLNVKDAGPIAGPGAWMNITGSWDDFTNPLKLLTGETIDSETFNLSNGIFWHVVWMSIGIF
NOCB       PGTWHVHTLLNVQGGGPLIGPGKYITITGDMADFENKITDLTGNTVNLETMATGTVIGWHLSWYVLGIA
N113B      PGTWHVHTLLNVQGGGPLIGPGKYITITGDMADFENKVTDLTGNTVNLETMATGTVIGWHLFWYVLGIA
MCAB1      PGDWHVHTMMNVQGGGPIIGPGKWITVEGSMSEFRNPVTTLTGQTVDLENYNEGNTYFWHAFWFAIGVA
MCAB2      PGDWHVHTMMNVQGGGPIIGPGKWITVEGSMSEFRNPVTTLTGQTVDLENYNEGNTYFWHAFWFAIGVA

NAVB1      WIGYYVARPMFLPRSRVLLAYGDDLLLDPMDKKVAWIVLIATFGIVWGGYRYTETKHPYTVPIQAGESK
NAVB2      WIGYYVARPMFLPRSRVLLAYGDDLLLDPMDKKVAWIVLIATFGIVWGGYRYTETKHPYTVPIQAGESK
NAVB3      WIGYYVARPMFLPRSRVLLAYGDDLLLDPMDKKVAWIVLIATFGIVWGGYRYTETKHPYTVPIQAGESK
NEUB       WIGVFTARPMFLPRSRVLLAYGDDLLMDPMDKKITWVLAILTLALVWGGYRYTENKHPYTVPIQAGQSK
NOCB       WIGWWARRPMFLPRYMKIKAGKANDLLTAQDKKLTIGVLVGVLLIILFGSKNAEDKFPVTIPLQAGLLG
N113B      WIVWWARRPMFLPRYMRVEAGEANDLVTAQDKKVTIGVLVGVLLIVLFGYKSAEEKFPVTIPLQAGLLG
MCAB1      WIGYWSRRPIFIPRLLMVDAGRADELVSATDRKVAMGFLAATILIVVMAMSSANSKYPITIPLQAGTMR
MCAB2      WIGYWSRRPIFIPRLLMVDAGRADELVSATDRKVAMGFLAATILIVVMAMSSANSKYPITIPLQAGTMR

NAVB1      VQPMPVKPNP--IAIKVTHANYDVPGRALRVTMSVTNSGDTAYRIGEFTTAGVRFINKVGLKHLDRNYPK
NAVB2      VQPMPVKPNP--IAIKVTHANYDVPGRALRVTMSVTNSGDTAYRIGEFTTAGVRFINKVGLKHLDRNYPK
NAVB3      VQPMPVKPNP--IAIKVTHANYDVPGRALRVTMSVTNSGDTAYRIGEFTTAGVRFINKVGLKHLDRNYPK
NEUB       VAALPVAPNP--VSIVITDANYDVPGRALRVTMEVTNNGDIPVTFGEFTTAGIRFINSTGRKYLDPQYPR
NOCB       TIDSLPVDYNSMVSANVLKANYRVPGRTISMTVEITNHTDQVISIGEFNTGGIRFMNANVRVDET-DYPE
N113B      TIEPLPVDYNSMVSAKVLKANYRVPGRTITMTVELTNHTDQVLSIGEFNTGGIRFMNANVRSDDT-GYPE
MCAB1      GMKPLELPAPT-VSVKVEDATYRVPGRAMRMKLTITNHGNSPIRLGEFYTASVRFLDSDVYKDTTG-YPE
MCAB2      GMKPLELPAPT-VSVKVEDATYRVPGRAMRMKLTITNHGNSPIRLGEFYTASVRFLDSDVYKDTTG-YPE

NAVB1      ELVATGLSF-DNDAPIQPGETREVKMVAKDALWEVQRLMALLGDPESRFGGLLMTWSDSGDRNINSIAG
NAVB2      ELVATGLSF-DNDALIQPGETREVKMEAKDALWEVQRLMALLGDPESRFGGLLTTWSDSGDRNINSIAG
NAVB3      ELVATGLSF-DNDAPIQPGETREVKMEAKDALWEVQRLMALLGDPESRFGGLLMTWSDSGDRNINSIAG
NEUB       ELIAVGLNF-DDESAIQPGQTKELKMEAKDALWEIQRLMALLGDPESRFGGLLMSWDAEGNRHINSIAG
NOCB       ELLAPEGLEVSQQ-DIAPGETVVVDISATDAAWEVQRMADVIYDPDSRFAGLIFFVDPEGNEIPIPIGG
N113B      ELLAPEGLEMSQQ-DIAPGETVVVDISATDAAWEVQRMADVIYDPDSRFAGLIFFIDPEGNEIPIPVGG
MCAB1      DLLAEDGLSVSDNSPLAPGETRTVDVTASDAAWEVYRLSDIIYDPDSRFAGLLFFFDATGNRQVVQIDA
MCAB2      DLLAEDGLSVSDNSPLAPGETRTVDVTASDAAWEVYRLSDIIYDPDSRFAGLLFFFDATGNRQVVQIDA

NAVB1      AVIPVFTKL
NAVB2      AVIPVFTKL
NAVB3      AVIPVFTKL
NEUB       PVIPVFTKL
NOCB       PLVPTFV--
N113B      PLVPTFV--
MCAB1      PLIPSFM--
MCAB2      PLIPSFM--
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Pair table of Nucleotide Identity for *amoA* and amino acid similarity for AmoA (Norton, Klotz and Alzerreca 11/98)

% NUCLEOTIDE IDENTITY

% AMINO ACID SIMILARITY

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22		
1	***	99.0	99.2	89.5	92.1	86.5	86.3	86.4	85.9	86.1	85.7	85.5	72.5	75.8	78.4	71.8	72.0	72.5	42.1	41.5	53.6	53.6	1	N39 amoA
2	97.5	***	99.5	89.9	93.1	87.0	86.8	86.9	86.9	87.0	86.7	85.9	72.8	76.6	78.8	72.1	72.4	72.8	42.7	42.2	54.0	54.0	2	N39 amoA
3	98.0	98.8	***	90.1	93.1	87.2	87.0	87.1	86.3	86.5	86.1	86.3	73.4	76.4	79.7	72.7	72.9	73.4	40.3	39.8	52.0	52.0	3	N39 amoA
4	94.9	95.6	96.1	***	87.3	86.2	86.1	86.1	84.2	84.2	83.9	85.6	72.4	72.6	75.2	71.8	71.8	72.2	41.7	42.3	51.2	51.2	4	NBR amoA
5	96.4	97.1	97.7	94.9	***	85.2	85.1	85.1	87.9	88.0	87.6	86.3	73.1	76.6	79.4	71.6	71.9	72.4	42.5	41.9	51.9	51.9	5	NVT amoA
6	92.0	92.7	93.4	93.5	92.7	***	99.9	99.9	86.4	86.4	86.2	86.3	72.2	76.6	77.8	70.1	70.2	70.8	44.4	41.9	53.0	53.0	6	NAV amoA
7	92.0	92.7	93.4	93.5	92.7	99.6	***	99.8	86.3	86.3	86.1	86.2	72.1	76.5	77.7	70.1	70.2	70.8	44.1	41.7	52.8	52.8	7	NAV amoA
8	92.0	92.7	93.4	93.5	92.7	99.6	99.6	***	86.3	86.3	86.1	86.2	72.1	76.5	77.7	69.9	70.1	70.7	44.2	41.8	53.1	53.1	8	NAV amoA
9	90.9	91.6	91.8	93.1	93.1	93.8	93.8	93.8	***	99.9	99.5	88.4	73.8	75.6	77.0	70.7	70.9	71.2	43.7	42.3	52.8	52.8	9	NMU amoA
10	90.9	91.6	91.8	93.1	93.1	93.8	93.8	93.8	99.6	***	99.6	88.4	73.7	75.8	77.1	70.7	70.9	71.2	43.7	42.3	53.0	53.0	10	NMU amoA
11	89.8	90.5	90.6	92.0	92.0	93.1	93.1	93.1	98.5	98.5	***	88.1	73.2	75.2	76.5	70.2	70.4	70.7	43.5	42.1	52.7	52.7	11	NMU amoA
12	90.2	90.5	91.4	91.3	91.3	93.8	93.8	93.8	93.8	93.8	93.1	***	71.4	74.7	76.1	69.5	69.7	70.1	43.5	42.5	53.4	53.4	12	N24C amoA
13	82.9	83.6	84.8	84.4	83.6	83.3	83.3	83.3	83.6	83.6	82.9	82.9	***	73.1	73.7	87.8	88.2	88.1	44.1	42.7	48.8	48.8	13	NEU amoA
14	86.5	87.3	88.3	87.3	86.5	87.6	87.6	87.6	86.9	86.9	86.2	87.6	82.2	***	85.7	72.4	72.5	72.8	43.8	41.8	47.0	47.0	14	AL21 2
15	86.2	86.9	88.7	86.9	86.5	88.4	88.4	88.4	87.6	87.6	86.9	87.3	83.3	94.9	***	73.2	73.2	73.6	43.7	42.3	49.6	49.6	15	JL21 amoA
16	81.8	82.5	83.6	83.3	82.5	81.5	81.5	81.5	81.1	81.1	80.4	80.7	96.0	80.7	82.5	***	99.4	99.2	42.6	42.2	47.7	47.7	16	NET amoA
17	81.8	82.5	83.6	83.3	82.5	81.5	81.5	81.5	81.1	81.1	80.4	80.7	96.0	80.7	82.5	98.2	***	99.3	42.5	41.8	47.2	47.2	17	NET amoA
18	82.5	83.3	84.4	84.0	83.3	82.2	82.2	82.2	81.8	81.8	81.1	81.5	96.8	81.5	83.3	98.9	98.9	***	43.4	41.7	48.3	48.3	18	GH22 amoA
19	40.3	40.7	39.1	41.5	40.7	42.3	42.3	42.3	41.9	41.9	41.5	41.5	43.1	41.9	41.1	41.1	40.7	41.5	***	88.4	55.4	55.5	19	NOC amoA
20	41.9	42.3	40.7	43.5	42.3	42.7	42.7	42.7	42.3	42.3	41.9	41.9	43.5	42.7	41.9	42.3	41.9	42.7	94.8	***	55.2	55.4	20	N113 amoA
21	46.8	47.2	46.0	48.0	46.4	46.8	46.8	46.8	46.8	46.8	46.4	48.0	48.0	47.6	47.6	46.8	46.0	46.8	60.8	61.3	***	99.9	21	MCA pmoA
22	46.8	47.2	46.0	48.0	46.4	46.8	46.8	46.8	46.8	46.8	46.4	48.0	48.0	47.6	47.6	46.8	46.0	46.8	61.9	61.7	99.2	***	22	MCA pmoA