Phylogenetic analysis of nitric oxide reductase gene homologues from aerobic ammonia-oxidizing bacteria

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Abstract

Nitric oxide (NO) and nitrous oxide (N₂O) are climatically important trace gases that are produced by both nitrifying and denitrifying bacteria. In the denitrification pathway, N₂O is produced from nitric oxide (NO) by the enzyme nitric oxide reductase (NOR). The ammonia-oxidizing bacterium Nitrosomonas europaea also possesses a functional nitric oxide reductase, which was shown recently to serve a unique function. In this study, sequences homologous to the large subunit of nitric oxide reductase (norB) were obtained from eight additional strains of ammonia-oxidizing bacteria, including Nitrosomonas and Nitrosococcus species (i.e., both β- and γ-Proteobacterial ammonia oxidizers), showing widespread occurrence of a norB homologue in ammonia-oxidizing bacteria. However, despite efforts to detect norB homologues from Nitrosospira strains, sequences have not yet been obtained. Phylogenetic analysis placed nitrifier norB homologues in a subcluster, distinct from denitrifier sequences. The similarities and differences of these sequences highlight the need to understand the variety of metabolisms represented within a “functional group” defined by the presence of a single homologous gene. These results expand the database of norB homologue sequences in nitrifying bacteria.

Keywords: Ammonia-oxidizing bacteria; Nitrifier-denitrification; Nitric oxide reductase; Nitrite reductase; Nitrous oxide

1. Introduction

Ammonia-oxidizing nitrifying bacteria play several important roles in nitrogen cycling in terrestrial, freshwater, and marine environments. Ammonia-oxidizers are primarily responsible for the oxidation of ammonia (NH₃) to nitrite (NO₂⁻). This activity is important for determining the form of nitrogen (NH₄⁺, NO₃⁻, NO₂⁻) available for use by primary producers, and it facilitates the loss of nitrogen from ecosystems by denitrification. Ammonia-oxidizing bacteria also contribute directly to the flux of nitric oxide (NO) and nitrous oxide (N₂O) gases from terrestrial [1–3] and aquatic [4–8] environments. Because NO and N₂O play important roles in atmospheric chemistry, global warming, and stratospheric ozone depletion, it is important to understand the natural controls on the production of these gases [9,10].

Approaches for evaluating the contribution of ammonia-oxidizers to N₂O production include inhibition studies [1], chemical and mass balance arguments [8], environmental constraints (e.g., O₂ availability) [11], and stable isotopic analyses of N₂O [4,6,12]. The application of genetic tools to study the role of nitrifiers in N₂O production has not received much attention (but see [13]). In order to develop a genetic approach and determine appropriate target genes with which to investigate the role of nitrifiers in N₂O production in the..
environment, it is important to elucidate the pathway(s) and enzymes involved in N₂O production by nitrifying bacteria.

Evidence exists for two potential pathways for N₂O production in ammonia-oxidizing bacteria. The reduction of NO₃⁻ to N₂O is evidenced by stable isotope tracer studies that demonstrate incorporation of NO₃⁻ into N₂O [14-16] and is supported by biochemical characterization of a copper-containing nitrite reductase in *Nitrosomonas europaea* [17-19]. Homologues of nirK and norB have been fully sequenced in *N. europaea* [20], and partial nirK homologues have also been identified in several marine nitrifiers that are capable of nitrifier-denitrification [21, Casciotti and Ward, unpublished]. However, mutation of the nirK and norB genes in *N. europaea* does not eliminate its ability to produce NO and N₂O. Instead, these mutants have a lowered resistance to high levels of NO/C0₂. While it is reported that NirK and NorB are active in wild type *N. europaea* and consume nitrite and nitric oxide, respectively, the products of these reactions are not identified. It may be relevant that even though the cyanobacterium *Synechocystis* PCC 6803 does not gain energy from nitric oxide reduction and is considered non-denitrifying (uses NOR as a detoxification mechanism) the product of this enzyme reaction is still N₂O [24].

Regardless of what the specific functions of nirK and norB are in ammonia-oxidizing bacteria the presence of these genetic sequences in ammonia-oxidizing bacteria and the potential for different uses and controls relative to denitrifying bacteria complicates the interpretation of functional gene diversity in mixed environmental samples. Genes encoding nitrite reductase (nirK and nirS) have been widely used to study denitrifier diversity due to the central role of nitrite reductase in the denitrification pathway [13,25-29]. The gene nirS has not been identified in nitrifying bacteria, but nirK is found in both nitrifying and denitrifying bacteria [20,21]. The high diversity of nirK and its ambiguous distinction between nitrifiers and denitrifiers, however, makes it difficult to distinguish the relative abundance or diversity of these functional groups on the basis of this gene. In a recent study by Avrahami et al. [13], many of the nirK sequences cloned from a grassland soil in Germany were most closely related to the nirK sequence of the cultivated strain TA-921i-NH4, an estuarine ammonia oxidizer. The functional designation of these sequences is therefore somewhat ambiguous and highlights the potential challenges in interpreting the functional significance of nirK sequences.

Nitric oxide reductase (NOR) presents an alternative target for detection of denitrifiers in environmental studies that may be simpler to interpret from the standpoint of functional diversity. The enzyme nitric oxide reductase (NOR), which produces N₂O from NO, is a member of the heme-copper oxidase family, which includes cytochrome c oxidases [30]. The nitric oxide reductase found most commonly in denitrifying bacteria is a membrane-bound dimer of subunits encoded by the genes norB and norC [31]. NorB contains the active site, which consists of a b-type heme prosthetic group as well as a non-heme iron [32]. Additional heme groups in NorC and NorB are thought to mediate electron transport from soluble cytochrome c to the catalytic site [33]. A second form of nitric oxide reductase, that also produces N₂O, has been found in several Bacteria and Archaea [31,34,35,37]. This alternate form of nitric oxide reductase accepts electrons from reduced quinol rather than cytochrome c, and it lacks the NorC subunit [31]. Instead, the catalytic subunit (homologous to NorB) has an N-terminal extension that is hypothesized to mediate electron transfer from quinol [36]. This alternate form of NOR has been termed qNOR by some authors [31,37] and has been given the gene designation of norZ in *R. eutropha* [34] or qnorB in other organisms [37]. In this paper, NorB will refer to the common cytochrome c-oxidizing NOR and NorZ will refer to the quinol-oxidizing form, with gene designations norB and norZ, respectively.

Braker and Tiedje [37] explored the distribution of norB in cultured denitrifying strains, and in environmental samples demonstrated its promise as a functional gene marker for denitrification. In the current study, the presence and diversity of norB gene homologues among several cultured ammonia-oxidizing bacteria was investigated using the polymerase chain reaction (PCR) and DNA sequencing. The goal was to examine the distribution of norB-like sequences among ammonia oxidizers and their relationship to denitrifier norB sequences. This work is a necessary precursor to studying the expression of norB and the role of nitric oxide reductase in the metabolism of nitrifiers other than *N. europaea*. The results presented here indicate that several ammonia-oxidizing bacteria possess a norB homolog similar enough to published denitrifier norB genes that they have been detected using degenerate PCR primers based primarily on denitrifier norB sequences.

2. Materials and methods

2.1. Bacterial culture conditions and DNA extraction

*Nitrosomonas europaea* and *Nitrosomonas eutropha* were grown in Walker medium with deionized water [38], *Nitrosomonas* sp. C-113a, *Nitrosomonas* sp. C-45, *Nitrosomonas* sp. URW, *Nitrosomonas* sp. NO3W, and *Nitrosoccus oceani* were grown in medium with full strength seawater using the recipe given by Ward [39] (W medium). *Nitrosomonas marina* was grown in W medium made up in 50% seawater. Cultures were grown
The PCR primers used in this study are given in Table 1. Primers for the norB gene (norB1, norB2, norB6, norB7, and norB8) are degenerate and were designed to amplify a diverse range of norB sequences. These primers were based on conserved regions of the norB gene from Pseudomonas fluorescens (AF197467), Pseudomonas stutzeri (Z28384), Pseudomonas aeruginosa (AE004489), Paracoccus denitrificans (AB014090), Halomonas halodenitrificans (AB010889), Bradyrhizobium japonicum (AJ132991), Rhodobacter sphaeroides (AF000233), Achromobacter cycloclastes (AJ298324), and N. europaea (www.jgi.doe.gov). The design of primer norB3 was based only on norB-like sequences obtained from ammonia-oxidizing bacteria using the norB2–norB6 primer pair and has several mismatches with known denitrifier norB sequences in the target region and is therefore biased towards ammonia oxidizing bacteria. Primers norB3 and norB1 were used in a second phase of PCR amplification to extend the length of nitrifier norB sequences for phylogenetic analysis. The expected norB2–norB6 fragment is 395 bp and the expected norB1–norB3 fragment is 236 bp, and the combined norB1–norB6 sequence is 587 bp.

PCR amplification reactions using the norB2–norB6 primer pair contained 50 mM KCl, 10 mM Tris base (pH 8.0), 2.5 mM MgCl₂, 100 μM each dNTP, 100 pmol each primer, 0.5 μl DNA (10–50 ng), and 1 μl Taq polymerase in total reaction volume of 50 μl reaction, and the remaining constituents were the same as for norB2–norB6 reactions. The PCR amplifications were carried out using the following thermal cycle: 95 °C for 5 min, then 80 °C for 1 min, followed by 25 cycles of 50 °C for 1.5 min, 72 °C for 2 min, 94 °C for 1 min, then a final annealing at 50 °C for 1.5 min and extension at 72 °C for 5 min.

Amplification of norB gene fragments from Nitrosomonas cryotolerans, was achieved using a nested PCR approach with norB1–norB8 followed by norB2–norB7. The PCR protocol for the norB1–norB8 amplification consisted of: 94 °C for 4 min, then 25 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. The nested reaction with norB2–norB7 was carried out with 1 μl of the initial stage in a 50 μl reaction with the following protocol: 94 °C for 4 min, then 35 cycles of 94 °C for 1 min, 52 °C for 1 min, and 72 °C for 1 min. A final extension at 72 °C was carried out for 7 min.

### 2.3. Cloning and sequencing

Amplification products of the expected sizes were excised from a 1% agarose gel, extracted using the QiaQuick Gel Extraction Kit (Qiagen), and cloned using the TOPO-TA Cloning Kit (Invitrogen). Transformants were selected and screened for plasmid inserts by PCR as previously described [21]. Inserts from 7–10 clones from each cloning experiment were sequenced on both strands using the BigDye Kit (Applied Biosystems) with vector primers T7 and M13 (reverse). Cycle-sequencing reactions were precipitated according to the manufacturer’s instructions and sequenced on an ABI310 genetic analyzer.

### 2.4. Sequence analysis and phylogenetic comparison

Sequences of norB1–norB3 and norB2–norB6 or norB2–norB7 products from each organism were assembled into a continuous section using AutoAssembler version 1.4.0 (Perkin-Elmer) (10–20 sequences per assembly). Amino acid sequences for NorB were deduced from consensus norB sequences for each organism, and were aligned using ClustalW [41]. A neighbor-joining tree was constructed for NorB amino acid sequences using distance matrix analysis in PAUP* 4.0, and 100 bootstrap replicates were performed.

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### Table 1

**PCR primers for norB**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Position</th>
<th>Direction</th>
<th>Sequence</th>
<th>Degeneracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>norB1</td>
<td>400–419</td>
<td>Forward</td>
<td>5’-CGNGARTTYCTSGARCARCC-3’</td>
<td>Degeneracy</td>
</tr>
<tr>
<td>norB2</td>
<td>592–610</td>
<td>Forward</td>
<td>5’-GACAARHWVTAYTGGTGGT-3’</td>
<td>192-fold</td>
</tr>
<tr>
<td>norB3</td>
<td>616–635</td>
<td>Reverse</td>
<td>5’-CCYTCVACCAGASATGCAC-3’</td>
<td>12-fold</td>
</tr>
<tr>
<td>norB6</td>
<td>967–986</td>
<td>Reverse</td>
<td>5’-TGCAKSARR RCCCC ABA ABCCC-3’</td>
<td>144-fold</td>
</tr>
<tr>
<td>norB7</td>
<td>1000–1019</td>
<td>Reverse</td>
<td>5’-CCRTGGSTRWARWARTTSAC-3’</td>
<td>256-fold</td>
</tr>
<tr>
<td>norB8</td>
<td>1051–1069</td>
<td>Reverse</td>
<td>5’-CRTADGCVCCRWAGAAVGCG-3’</td>
<td>216-fold</td>
</tr>
</tbody>
</table>

* Numbering based on complete norB gene sequence from Pseudomonas stutzeri (Z28384).
Parsimony and maximum likelihood analyses were performed on the same alignment using PROTPARS and PROTML from PHYLIP version 3.6b.

3. Results and discussion

3.1. PCR primer development

The goal of this study was to compare the diversity and relationship of norB gene homologues in ammonia-oxidizing bacteria to those found in denitrifying bacteria. In order to accomplish this, norB primers were developed prior to the recent publication of a similar set of norB primers [37]. The primers described here were based on a similar group of full-length norB sequences that are available in GenBank, and they target nearly identical conserved regions compared with the cnorB1F and norB1 (this study) target nearly identical regions, with one base change in their overlapping region. Primer norB1 extends three bases longer in the 5′ direction compared with cnorB1F. Primers cnorB2F and norB2 target identical regions, with relatively minor differences in degrees of degeneracy. The Braker and Tiedje [37] primers generally, but not always, allow for greater degeneracy in target sequence. Primers cnorB6R and norB6 target overlapping regions with some differences in degeneracy choices. Primers cnorB7 and norB7 are separated by about 10 bp but do not overlap. NorB8 is farther downstream than any of the primers described previously. Finally, our internal norB3 primer is specifically biased towards nitrifier sequences, having been designed as an internal primer from alignments of only the nitrifier norB homologues that were the focus of this study. Overall, the similarity in these independently designed primer sets for norB is noteworthy, and something may be learned from the similarities and differences of the two primer sets. For example, we also had the greatest success with norB2-norB6 amplification, strengthening the recommendation of Braker and Tiedje [37] for the use of these target regions for norB amplification. One significant difference in the primer design, that may be important for the work described here, is that the norB sequence from N. europaea, the only nitrifier norB sequence available prior to this study, was included in the alignment used to design primers in this study but apparently not in the previous design [37]. This may account for some of the different choices made in designing the two sets of primers. The use of norB primers specifically targeted for nitrifier norB sequences may provide a method of differential amplification and detection of nitrifier and denitrifier norB sequences in complex environmental samples.

3.2. PCR amplification of norB gene homologues

Sections of the norB gene were successfully amplified from nine ammonia-oxidizing bacteria: N. europaea, N. eutropha, N. marina, Nitrosomonas sp. strains C-113a, URW, NO3W, TA-921i-NH4, N. cryotolerans, and Nitrosococcus oceani. These strains include a variety of soil, freshwater, and marine ammonia-oxidizers from both β- and γ-subdivisions of the Proteobacteria. Several norB primer combinations were also tested with genomic DNA from Nitrosospira briensis, Nitrosospira tenuis, and Nitrosospira multiformis. Thus far, while products of the approximately correct size have been obtained from Nitrosospira species, sequence analysis has shown them to be non-specific products.

3.3. NorB phylogenetic analysis

With the caveat that among nitrifiers, norB has thus far only been fully sequenced and shown to be functional in N. europaea [20, 23], we have translated the PCR products sequenced from nitrifier species into amino acid sequences for phylogenetic analysis. Alignment of the translated nitrifier norB homologues (169 amino acids) with published NorB sequences and the homologous NorZ protein is relatively unambiguous, with only a few gaps required for the optimal alignment. There are 12 predicted membrane-spanning sections in the NorB protein [31], and the region amplified by norB2 and norB6 primers includes the sixth, seventh, and eighth membrane-spanning sections. The positions of three conserved histidine residues (H-207, H-258, and H-259 in P. stutzeri) that are involved in binding non-heme iron at the active site [30], as well as a conserved glutamate residue (E-211) that is required for enzyme activity [42] are conserved in the nitrifier NorB homologues.

To include newer database sequences in phylogenetic analysis, an alignment of 97 AA was used to generate a neighbor-joining tree based on a pairwise distance matrix analysis of NorB-like sequences (Fig. 1). The topology of a neighbor-joining tree based on norB/Z nucleic acid sequences gives the same groupings as in the amino acid-based tree and does not offer any additional phylogenetic resolution (not shown). Only NorB-type protein sequences are included in this comparison, except for the NorZ-type protein from Synechocystis PCC 6803 [24] used as the outgroup.

The NorB-like sequences have been assigned to six groups, identified in Fig. 1 and listed in Table 2, to facilitate the discussion. Each numbered group is supported by robust bootstrap values (filled circles in Fig. 1) and represents sequences that are >75% identical to each other. All of the major groupings discussed below are also supported by parsimony analysis. Supplemental physiological and phylogenetic information about the organisms included in Fig. 1 is given in Table 2.
The sequences from ammonia-oxidizing bacteria form a coherent cluster (group 1) that is separate from denitrifier sequences (groups 2–6) (Fig. 1). The nitrifier cluster is supported by a bootstrap value of 65% by distance analysis but is unresolved by parsimony. This group contains NorB-like sequences from a diverse range of ammonia-oxidizing bacteria, including *Nitrosomonas* species from the β-subdivision, and *N. oceani*, a representative of the
γ-subdivision nitrifiers. Among group 1 nitrifiers, sequence identity is high (77–100%) compared to identities between group 1 and group 2 (64–74%), group 3 (61–73%), group 4 (59–66%), group 5 (20–30%), and group 6 (19–26%). Of interest is the placement of environmental clone cRCR1 (CAD45417) from the Red Cedar River. Of interest is the placement of environmental clone cRCR1 (CAD45417) from the Red Cedar River. Of interest is the placement of environmental clone cRCR1 (CAD45417) from the Red Cedar River. Of interest is the placement of environmental clone cRCR1 (CAD45417) from the Red Cedar River.

Group 2 contains NorB sequences (89–98% identical) from a variety of denitrifying bacteria in the α- and β-subdivisions of the Proteobacteria, many of which are plant symbionts or pathogens that can also fix N₂. All of the denitrifiers in group 2 possess the copper-type nitrite reductase (NirK) (Table 2).

Group 3 contains NorB sequences (78–79% identical) from atypical denitrifiers in the γ-subdivision. Both Roseobacter denitrificans and Rhodobacter sphaeroides are capable of phototrophic growth. The NorB from R. denitrificans is also unusual in that it possesses Cu₉₅ in the active site (as cytochrome oxidase does), rather than Fe₉, as is seen in other known NorB proteins [43]. This is surprising considering the high sequence similarity of NorB from R. denitrificans to other NorB sequences and suggests that relatively few alterations are required to change the binding specificity of the active site metal.

Group 4 consists mainly of denitrifiers with the heme-type nitrite reductase (NirS). Magnetococcus MC-1 is
also included in this group, but it does not possess a readily recognizable nirK or nirS gene (as revealed by searches of its genome). There is no Nir information available for Halomonas halodenitrificans nor for the environmental clones that make up group 5.

3.4. Comparison to amoA distribution

The amoA gene, encoding the α subunit of ammonia monooxygenase has been used widely to study nitrifier diversity and phylogenetic relationships. Based on amoA sequences, the nitrifier species from which we obtained norB sequences are distributed throughout the major groups of Nitrosomonas and Nitrosococcus species. Conspicuously absent from the norB dataset, however, are sequences from the Nitrosospira clade, which are important players in many environments [44,45]. The reason for our inability to amplify a norB product from Nitrosospira species, whether it is due to primer mismatch or absence of the gene is unknown at this time. Nitrosospira species are known to produce N2O with yields similar to Nitrosomonas species [46,47], although production of N2O by Nitrosospira briensis is less sensitive to C2H2 and O2 levels compared with N. europaea [48]. It is surprising, though, to have successfully amplified a norB-like sequence from N. oceani but not from the more closely related β-subdivision nitrifiers. Full genome sequencing of Nitrosospira species will provide the most definitive answer to this question, but PCR-based efforts to obtain a norB product are ongoing.

3.5. Contrast to NirK phylogeny for nitrifiers and denitrifiers

The phylogenetic relationships based on NorB sequences shown in Fig. 1 provide an interesting comparison to phylogenies based on nitrite reductase (NirK) sequences. While NirK-like sequences from some ammonia-oxidizing bacteria are not phylogenetically distinguishable from denitrifier sequences [21], the NorB-like sequences that have been discovered thus far are distinguishable. The overlap between nitrifier and denitrifier nirK homologues creates ambiguity as to the functional significance of nirK sequences cloned from the environment. Based on the nitrifier NorB-like sequences reported here, which form a single coherent group distinct from denitrifier sequences, NorB may provide a less ambiguous and more broadly applicable assessment of denitrifier diversity. The nitrifier sequences reported here are obtained from several of the known groups of ammonia-oxidizing bacteria, although additional nitrifier sequences, in particular from the Nitrosospira lineage, will add greater confidence to the nitrifier/denitrifier distinction.

The role of NOR in N2O production by marine nitrifiers, and thus the significance of nitrifier norB sequences detected in the environment, is yet to be determined but these are important avenues for future research. The sequences obtained in this study provide some insight into the potential metabolic diversity hidden within nitrifier diversity based on partial norB gene sequences. These results also provide a good foundation for future investigation of the role of the denitrification pathway in the metabolism of various marine nitrifying bacteria and the role of ammonia-oxidizers in denitrification and N2O production.

3.6. Sequence Accession Numbers

GenBank Accession Nos. AY139082-AY139089 were assigned to the norB sequences from C-113a, N. eutropha, Nitrosomonas marina, N. oceani, NO3W, TA-921i-NH4, URW, and N. europaea, respectively. The Nitrosomonas cryotolerans norB sequence has been assigned the GenBank Accession No. AY654283.

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