ELSEVIER

FEMS Microbiology Ecology 52 (2005) 197–205

FEMS MICROBIOLOGY Ecology

www.fems-microbiology.org

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

Karen L. Casciotti *,[1], Bess B. Ward

Department of Geosciences, Princeton University, Princeton, NJ 08540, USA

Received 16 June 2004; received in revised form 15 September 2004; accepted 3 November 2004

First published online 23 November 2004

Abstract

Nitric oxide (NO) and nitrous oxide (N_2O) are climatically important trace gases that are produced by both nitrifying and denitrifying bacteria. In the denitrification pathway, N_2O 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 (NH_3) to nitrite (NO_2^-). This activity is important for determining the form of nitrogen (NH_4^+, NO_2^-, NO_3^-) 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 (N_2O) gases from terrestrial [1–3] and aquatic [4–8] environments. Because NO and N_2O 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 N_2O production include inhibition studies [1], chemical and mass balance arguments [8], environmental constraints (e.g., O_2 availability) [11], and stable isotopic analyses of N_2O [4,6,12]. The application of genetic tools to study the role of nitrifiers in N_2O 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 N_2O production in the

* Corresponding author. Tel.: +1 508 289 3738, fax: +1 508 457 2013.

E-mail address: kcasciotti@whoi.edu (K.L. Casciotti).

[1] Present address: Woods Hole Oceanographic Institution, MS 8, Woods Hole, MA 02543, USA.

0168-6496/$22.00
doi:10.1016/j.femsec.2004.11.002

environment, it is important to elucidate the pathway(s) and enzymes involved in N_2O production by nitrifying bacteria.

Evidence exists for two potential pathways for N_2O production in ammonia-oxidizing bacteria. The reduction of NO_2^- to N_2O is evidenced by stable isotope tracer studies that demonstrate incorporation of NO_2^- into N_2O [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 N_2O. Instead, these mutants have a lowered resistance to high levels of NO_2^- and NO [22,23]. 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 N_2O [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 N_2O 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 N_2O, 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 *Nitrosococcus 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