RELATIVE ABUNDANCE OF PROTEOBACTERIAL MEMBRANE BOUND- AND PERIPLASMIC-
NITRATE REDUCTASES IN SELECTED ENVIRONMENTS

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Abstract
Dissimilatory nitrate reduction is catalyzed by a membrane bound- and a periplasmic- nitrate reductase. We set up a real-time PCR assay to quantify these two enzymes, using the narG and napA genes encoding the catalytic subunits of the two types of nitrate reductases as molecular markers. The narG and napA gene copy numbers in DNA extracted from different environments showed high variations with most numbers ranging from $2 \times 10^2$ to $6.8 \times 10^4$ copies per ng of DNA. This study provides evidence that, in soil samples, the number of proteobacteria carrying the napA gene is often as high as that of proteobacteria carrying the narG gene. The high correlation observed between narG and napA gene copy numbers in soils suggests that the ecological roles of the corresponding enzymes might be linked.
Introduction

Nitrate in the environment can either be assimilated by plants and microorganisms or reduced to nitrite by two microbial dissimilatory processes: denitrification or dissimilatory reduction of nitrate to ammonium. Nitrate reduction by denitrification is of great importance since the produced nitrite is then reduced to N\textsubscript{2}O or N\textsubscript{2} gases, which can lead to considerable nitrogen losses in agriculture and emissions of greenhouse gases (6, 13, 28). The reduction of nitrate present in contaminated water by the nitrate-reducing bacteria living in the human digestive tract is a potential health problem. As nitrite enters the blood stream, it reacts with hemoglobin to form methemoglobin, blocking oxygen transport and causing a disease commonly called "blue baby syndrome" (26). Two types of dissimilatory nitrate reductase differing in their location were characterized: a membrane-bound (Nar) and a periplasmic (Nap) nitrate reductase (2, 16, 29). The membrane-bound nitrate reductase is present in proteobacteria, firmicutes, actinobacteria and even archaea, whereas the periplasmic nitrate reductase is present only in proteobacteria (21, 24). Nitrate-reducing proteobacteria can harbour Nar or Nap, or both (18, 29). In contrast to Nar, the physiological role of Nap is still unclear and seems to vary between bacteria (11, 24). Thus, one proposed role for Nap is to support anaerobic metabolism as an alternative to Nar (1, 8). It has also been proposed that Nap facilitates the switch from aerobic respiration to denitrification (27) or scavenges nitrate in some pathogenic bacteria (23). The importance and diversity of the bacteria containing Nar has been extensively studied using both cultivation based and direct molecular approaches (3, 5, 7, 17, 19, 22). However, only a few studies have focused on bacteria containing Nap (4, 9, 25). In this study, we investigated the relative abundance of the two types of nitrate reductases in various environments using a real-time PCR-based assay.

Primer design, standard curves and real-time PCR procedures
In order to quantify the two types of nitrate reductases, a new real-time PCR assay was set up using the narG and napA genes encoding the catalytic subunits of the membrane bound- and periplasmic nitrate reductases, respectively, as molecular markers. All available sequences were aligned and narG and napA primer sets specific to the proteobacteria were designed. The degenerated narG-f: 5’-TCGCCSATYCCGGCSATGTC-3’ and narG-r: 5’-GAGTTGTACCAGTCRGCSGAYTCSG-3’ (modified after (1)) and napA4r: 5’-ACYTCRCGHGCVTRCCRCA-3’) primers were used to amplify fragments of 173 and 152 bp, respectively. Serial dilution of linearized plasmids containing the narG and napA genes from *P. aeruginosa* PAO1 were used to generate standard curves. The real-time PCR assays were carried out in a 20 µL reaction volume containing the SYBR green PCR Master Mix (ABsoluteTM QPCR SYBR® ROX Mix » Abgene France), 2 µM of each primer, 100 ng of T4 gp 32 (QBiogene, France), and 1.25 µL of template DNA (2-12.5 ng). Thermocycling conditions for narG were: 15 min at 95°C, 6 cycles consisting of 30 s at 95°C, 30 s at 63°C with a touchdown of -1°C by cycle, 40 cycles consisting of 30 s at 95°C, 30s at 58°C, 30s at 72°C, 30s at 80°C. Conditions for napA were similar except for the annealing temperature set at 61°C. All real-time PCR reactions were performed with the ABI prism 7900 (Applied Biosystem). Quantification of the 16S rRNA gene was performed as described previously (15). DNAs extracted from triplicate samples from 18 different environments such as soils (agricultural, industrial or glacier), river sediment, waters or biofilms were used as templates.

**Evaluation of assay specificity and sensitivity**

The functional gene pipeline interactive tool ([http://flyingcloud.cme.msu.edu/fungene/](http://flyingcloud.cme.msu.edu/fungene/)) was used for *in silico* evaluation of the primer specificity. Searches for the narG and napA primer sequences among 36 and 40 narG and napA sequences from complete genomes of
proteobacteria showed that 78, 70, 86 and 86% did not exhibit any mismatch with the narG-f, narG-r, V17m and napA4r primers, respectively. Primer specificity was further confirmed experimentally using a collection of 19 strains (Table 1). Four firmicutes and one actinomycete nitrate reducing strains were selected as negative controls and 14 nitrate reducing strains belonging to the alpha, beta and gamma proteobacteria were selected as positive controls. None of Gram+ nitrate reducers, which were used as negative controls, gave an amplicon. For Proteobacteria, an absence of PCR products with both narG and napA primers was only recorded with Alcaligenes faecalis 8750. Sequence analysis of 84 and 79 narG and napA real-time PCR products from agricultural soils (csa and Yvet), glacier soil (Rotm), cave biofilm (Pad) and river phototrophic biofilm (Gar) revealed all sequences were related to the narG or napA genes. A high diversity of the sequences of the real time PCR products was observed with identities as low as 67% for narG and 69% for napA to the sequences used for the design of the primers (Figs 3 and 4, supplemental materials). This indicates that our newly developed real time PCR systems are suitable for general detection of proteobacterial nitrate reductase genes. However, 3 out of 84 narG sequences fell into a cluster containing only narG from actinobacteria indicating that the designed narG primers were not entirely specific to proteobacteria.

The detection limit of our assay was around 10 copies per ng of template DNA and no signal was detected in the no template control (NTC). The PCR efficiencies of the narG and napA real-time PCR assays were 86 and 83%, respectively. Genomic DNA from Pseudomonas aeruginosa PAO1, for which the theoretical 16S rRNA, narG and napA gene copy numbers per ng of DNA were calculated, was used as external control. The presence of PCR inhibitors co-extracted with DNA was tested as described previously (12) and did not reveal any significant inhibition. Therefore, our assays based on novel primer sets narG-f-narGr- and
V17m-napA4r provide an efficient and sensitive method to quantify either Nar or Nap in environmental samples.

Quantification of narG and napA genes

To compare with accuracy the number of genes in the different environments, results were expressed as gene copy numbers per ng of extracted DNA. Quantification of the narG and napA genes revealed high variations between environments with most numbers ranging from $2 \times 10^2$ to $6.8 \times 10^4$ copies per ng of DNA (Fig. 3). The highest copy numbers for both narG and napA genes were observed in the river sediment samples. The napA gene copy numbers were lower than those of narG in most of the freshwater samples, whereas similar narG and napA gene copy numbers were observed in all soil samples except the Yvetot soil and the Rotmoosfermer glacier soil (Fig. 1). The numbers of 16S rRNA genes were between 1 to 3 logs higher than those of narG or napA. However, up to 12 copies of 16S rRNA may be found in the same bacterial genome (10), while only one napA copy and a maximum of three narG copies have been identified in the same strain (18). Hence our study provides evidence that in soils, the number of proteobacteria containing periplasmic nitrate reductase is similar to the number of proteobacteria with the membrane-bound nitrate reductase. This is consistent with the results of Roussel-Delif et al. (25) and Carter et al. (4) which showed that a large proportion of nitrate-reducing Gram negative isolates contain Nap. Unfortunately, it is not possible to conclude from our study whether the similar narG and napA copy numbers in many samples resulted from a majority of nitrate reducers possessing both types of nitrate reductase or from similar numbers of bacteria possessing either Nar or Nap. The narG from proteobacteria were mainly targeted in our assay whereas these are also present in Gram positive bacteria and archaia in contrast to napA. Therefore the fact that the narG gene copy
numbers were either similar to or higher than the napA gene copy numbers indicates that the
Nar is probably predominant in the environment.

Interestingly, a high correlation coefficient of 0.9 between the narG and napA gene copy
numbers was calculated for the soil and sediment samples whereas this coefficient was only
0.4 in samples from river biofilms and water samples (Fig. 2). In contrast, the correlation
between the narG or napA and the 16S rRNA gene copy numbers did not exceed 0.5. The
physiological role of Nap is still unclear and probably varies between strains (11, 21, 24). The
high correlation observed between the numbers of narG and napA genes but not with 16S
rRNA suggests that the ecological role of Nap in the majority of soil proteobacteria might be
similar or complementary to that of Nar. Our results also showed that the abundance of the
two types of nitrate reductases differed between environments, which could be due to the
selection of nitrate-reducers in some habitats. Since detection of functional genes is only a
weak hint of the presence of the corresponding activity (20), investigation of the relative
contribution of the two types of nitrate reductases to the total nitrate reduction activity in the
different environments is of interest. Unfortunately, the activity of the two types of nitrate
reductase is simultaneously monitored by the nitrate reduction assay developed by Kandeler
(14), and the activity of Nar and Nap can be distinguish only on bacterial isolates (4). In the
future, integrated studies are needed to compile further information on the physiology,
diversity and distribution of nitrate-reducers for a more comprehensive understanding of
nitrate reduction in the environment.

**Nucleotide sequence accession numbers.** The GenBank accession numbers for the
sequences reported here are EF217059 to EF217221.

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REFERENCES


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Table 1. Bacterial strains used in this study to test specificity of the *narG* and *napA* primers

<table>
<thead>
<tr>
<th>Strains or isolates</th>
<th>Phylum</th>
<th>Nitrate reductase activity</th>
<th>Nitrate reductase enzyme</th>
<th>Q-PCR narG</th>
<th>Q-PCR napA</th>
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<tr>
<td><em>Bacillus senegalensis</em> M1518</td>
<td>Firmicute</td>
<td>+</td>
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<td>-</td>
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<td><em>Bacillus cereus</em> M944 ND</td>
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<td>+</td>
<td>Nar, ND</td>
<td>-</td>
<td>-</td>
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<tr>
<td><em>Bacillus thuringiensis</em></td>
<td>Firmicute</td>
<td>+</td>
<td>Nar, ND</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Bacillus megaterium</em></td>
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<td>+</td>
<td>Nar, ND</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Streptomyces bluensis</em></td>
<td>Actino</td>
<td>+</td>
<td>ND, ND</td>
<td>-</td>
<td>-</td>
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<td><em>Agrobacterium tumefaciens</em></td>
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<td>+</td>
<td>-, Nap</td>
<td>-</td>
<td>+</td>
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<tr>
<td><em>Bradyrhizobium japonicum</em> 562</td>
<td>α-Proteo</td>
<td>+</td>
<td>ND, ND</td>
<td>-</td>
<td>+</td>
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<td><em>Bradyrhizobium japonicum</em> USDA 110</td>
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<td>+</td>
<td>-, Nap</td>
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<td>-, Nap</td>
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<td>+</td>
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<td><em>Rhizobium meliloti</em> 50</td>
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<td>Nar, Nap</td>
<td>+</td>
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<td>-</td>
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<td><em>Alcaligenes eutrophus</em> H16</td>
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<td>+</td>
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<tr>
<td><em>Achromobacter cycloclastes</em> ATCC 21921</td>
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<td>+</td>
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<td>+</td>
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<tr>
<td><em>Escherichia coli</em> JM109</td>
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<td>+</td>
<td>Nar, Nap</td>
<td>+</td>
<td>+</td>
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<tr>
<td><em>Pseudomonas denitrificans</em> CCUG 2519</td>
<td>γ-Proteo</td>
<td>+</td>
<td>ND, ND</td>
<td>+</td>
<td>-</td>
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<tr>
<td><em>Pseudomonas fluorescens</em> C7R12</td>
<td>γ-Proteo</td>
<td>+</td>
<td>Nar, -</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

ND: Not determined
- : not present
Fig. 1. Abundance of 16S rRNA, narG and napA genes expressed as gene copy numbers per ng of extracted DNA, analyzed from three independent replicates per site. Error bars indicate standard deviation. S indicates significant differences ($P<0.05$) between the narG and napA gene copy numbers (Student test) for each site.

Fig. 2. Correlation between narG and napA gene copy numbers in the soil and sediment samples (white triangles) and in water and biofilm samples (black circles). Each replicate of the 18 selected environments are plotted.
$y = 0.8817x + 0.2657$

$R^2 = 0.9005$

$y = 0.6922x + 0.3486$

$R^2 = 0.4035$