A New Non-Degenerate Primer Pair for the Specific Detection of the Nitrite Reductase Gene *nrfA* in the Genus *Desulfovibrio*

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Key Words

nrfA gene • Nitrite reductase • *Desulfovibrio* • PCR primers • Dissimilatory nitrate reduction to ammonia • DNRA

Abstract

Dissimilatory nitrate reduction to ammonia (DNRA) is the process in which nitrate is reduced, via nitrite, to ammonia. Bacteria known to carry out DNRA mainly originate from wastewater treatment plants, where DNRA is a relevant process. The ability to carry out DNRA is phylogenetically widespread, and the gene *nrfA*, encoding for the key enzyme of the second step of the pathway, could be used as a marker for this process. In this study we developed a new primer pair specific for *nrfA* in the genus *Desulfovibrio*. The specificity of the primer pair was tested on DNA from thirteen species of *Desulfovibrio* and DNA from two wastewater samples. PCR amplifications yielded products of the expected size (850 bp), and sequences obtained from *Desulfovibrio* strains and environmental sample clone libraries matched the *Desulfovibrio nrfA* gene. Nevertheless, we found *nrfA* gene sequences in the environmental samples that are not present in the databases. The new primer set can be used to obtain more sequences of the *nrfA* gene and improve our knowledge of the DNRA pathway in this genus, e.g. with the aim to improve the wastewater treatment process.

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Introduction

Dissimilatory nitrate reduction to ammonia (DNRA) or nitrate ammonification is an anaerobic process in which nitrate is reduced to ammonia with nitrite as an intermediate [Cabello et al., 2009; Einsle et al., 1999; Kraft et al., 2011]. The ability to carry out DNRA is phylogenetically widespread [Kraft et al., 2011]. Many sulphate-reducing bacteria (SRB) are able to perform DNRA in the presence of nitrate when sulphate concentrations are sufficiently low, although sulphate reduction is preferred over DNRA if both electron acceptors are present [Kraft et al., 2011; Marietou et al., 2009]. In DNRA, nitrate reduction to nitrite is usually performed by the periplasmic nitrate reductase NapAB, while nitrite reduction to ammonia is catalysed by the pentaheme cytochrome c nitrite reductase NrfA, without the release of any intermediates [Cabello et al., 2009; Einsle et al., 1999; Kraft et al., 2011]. In SRB, in particular in *Desulfovibrio*, nitrite reductase NrfA plays an important role in those strains able to use nitrate and nitrite as electron acceptor when sulphate is not freely available and in those bacteria capable of reducing nitrite but not nitrate [Cabello et al., 2009; Greene et al., 2003; Haveman et al., 2004; Simon, 2002]. In fact, nitrite is a very toxic compound, and an additional function of nitrite reductase is to detoxify nitrite which allows *Desulfovibrio* spp. to survive in environments containing ni-

trite up to millimolar concentrations [Cabello et al., 2009]. NrfA is present in many *Desulfovibrio* species, many of them synthesise a very active, constitutive nitrite reductase [Marietou et al., 2009; Moura et al., 2007; Simon, 2002].

In aquatic systems, a substantial proportion of nitrate could be reduced via DNRA instead of via denitrification [Dong et al., 2009]. Ammonia production could be due to the action of SRB as they are able to perform DNRA [Percheron et al., 1999], and the ammonia produced by this pathway is then retained into the aquatic system, and therefore is available for other organisms [Dong et al., 2009; Smith et al., 2007]. Moreover, when nitrate concentrations decrease, bacteria are likely to be competitively more efficient than denitrifiers [Dong et al., 2009; Percheron et al., 1999].

Bacteria known to carry out DNRA mainly originate from wastewater treatment plants and from gastrointestinal tracts of mammals [Kraft et al., 2011]. Moreover, SRB, including *Desulfovibrio* spp., are used in bioremediation of industrial wastes [Muyzer and Stams, 2008]. In wastewater besides denitrification, other processes, including DNRA, account for a relevant percentage of nitrogen chemical form conversion [Kløve et al., 2005].

In wastewater treatment plants, waste-activated sludge is usually treated using an anaerobic digester as it recovers methane, a renewable energy source [Nakashimada et al., 2008]. DNRA is the main nitrate reduction pathway in anaerobic digesters and in other methanogenic environments [Percheron et al., 1999]. Unfortunately, methane production in anaerobic digesters could be inhibited by high concentration of ammonia released from waste-activated sludge [Nakashimada et al., 2008]. Mazeas et al. [2008] reported that the accumulation of ammonia nitrogen, as there is no ammonia elimination process in anaerobic condition, is a biotechnological challenge.

Nitrate-reducing pathways, such as denitrification, are very well known [Kraft et al., 2011; Mohan et al., 2004], yet, in contrast, few studies have focused on DNRA. While the protein product of the *nrfA* gene has been studied [Cabello et al., 2009; Einsle et al., 1999; Kraft et al., 2011], only a few studies are available on the genes encoding this enzyme [Kraft et al., 2011; Mohan et al., 2004]. To date, there are only few *nrfA* sequences available in nucleotide databases, mainly from pathogenic strains and therefore probably not relevant in environmental studies. As a consequence, the isolation of environmentally important species performing DNRA is an important step in order to obtain additional sequences of the *nrfA* gene [Kraft et al., 2011] and generate new knowledge about this

Table 1. Primer sequences for PCR amplification of *nrfA*

Primer	Sequence (5' → 3')
nrfA-F2	ACCTGCTGGAAGTCAARA
nrfA-R5	GTGGAAGCCACGCTGTT

R = G or A.

pathway. As the functional gene *nrfA* occurs in diverse bacterial taxa, such as gamma-, delta- and epsilon-Proteobacteria and in members of the Bacteroides, available primers are highly degenerate to target this wide range of microorganisms [Kraft et al., 2011; Mohan et al., 2004]. Unfortunately, the employment of degenerate primers makes PCR more challenging such as the need to use touchdown protocols to minimise annealing mismatches and high-fidelity Taq polymerases to improve PCR results [Mohan et al., 2004]. Nevertheless, non-specific or failed amplifications still occur.

The aim of this research was the development of a new PCR primer set to amplify *nrfA* specifically from *Desulfovibrio*, and thus provide a new tool for studying DNRA in different environmental and technological systems.

Results

Alignment of *nrfA* sequences from *Desulfovibrio* species, available from GenBank and KEGG databases, showing several consensus sequences are shown in figure 1. From this alignment, four primer pairs were designed from nucleotide sequences corresponding to the five amino acid consensus sequences in figure 1. After in silico analysis of the characteristics of the degenerate oligonucleotide, the forward primer from consensus region 2 and reverse primer from consensus region 5 were selected as primers (table 1).

Theoretical specificities of the new primer set, tested with both the BLAST tool from NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and ThermoPhyl software [Oakley et al., 2011], showed that the primers are specific, with perfect matches to *Desulfovibrio* sp. and sensitive as they matched with all available *nrfA* sequences from *Desulfovibrio* sp., although the primers also matched the very closely related *Desulfomicrobium* (data not shown).

For most of the *Desulfovibrio* strains tested, PCR amplification of the partial *nrfA* gene, using nrfA-F2 and

Fig. 1. Multiple alignment of *NrfA* sequences used to design *nrfA* primers. The sequences were retrieved from GenBank and aligned in ClustalX2. Numbered black lines represent the positions of the five consensus amino acid sequences used for designing PCR primers. The full names of the bacteria from which the sequences

were derived are *Desulfovibrio vulgaris* str. Hildenborough, *Desulfovibrio vulgaris* DP4, *Desulfovibrio aspoensis* Aspo-2, *Desulfovibrio baculatum*, *Desulfovibrio vulgaris* str. 'Miyazaki F' and *Desulfovibrio desulfuricans* ATCC 27774.

nrfA-R5 primers, resulted in a product of the predicted size (table 2).

No amplification was observed for three of the species tested, *D. fructosivorans* DSM 3604, *D. longus* DSM 6739 and *D. burkinensis* DSM 6830, while a correctly sized product was amplified from all *Desulfovibrium* spp. tested (table 2). BlastN alignments of *nrfA* gene fragment sequenced from both *Desulfovibrio* and *Desulfovibrium* isolates (table 2) and environmental samples (data not shown) confirm that the PCR product was a fragment of the *nrfA* gene. However, percentage identity for all tested species was lower than 94%, except for the 99% identity of the *nrfA* gene of *Desulfovibrium baculatum* DSM 4028, the only *nrfA* gene present in database among culture collection species used in this work.

To study *nrfA* gene differences, sequences obtained from this *Desulfovibrio/Desulfovibrium* collection, NCBI and KEGG databases and environmental sample

clones were clustered in operational taxonomic units (OTUs).

Rarefaction curve reached a plateau starting from a cutoff value of 0.14. At the 0.14 cutoff value, all *nrfA* gene sequences analysed clustered in 14 OTUs (fig. 2a). The threshold value of similarity between the 14 OTUs obtained is 86%, confirming the diversity in *nrfA* gene sequence in the *Desulfovibrio* genus.

In order to know if sequences from wastewater-digested sludge samples are present in databases, we built a Venn diagram of shared OTUs. Sequences from both wastewater samples were a single OTU group (group A) as no differences in OTU clustering of cloned sequences originated from wastewater samples were observed. The second OTU group (group B) was composed by the newly deposited *Desulfovibrio/Desulfovibrium nrfA* sequences (table 2) and previously deposited *nrfA* sequences from NCBI and KEGG databases. At the 0.14 cutoff

Fig. 2. a Rarefaction curves for *nrfA* gene sequences from *Desulfovibrio* collection, environmental sample clones and NCBI and KEGG databases. Plots at different cutoff values are shown. **b** Venn diagram of shared OTUs for the clone sequences from both wastewater digested sludge samples group (group A)

and group B, which is composed of the newly deposited *Desulfovibrio/Desulfomicrobium nrfA* sequences and previously deposited *nrfA* sequences from NCBI and KEGG databases, at a distance of 0.14.

Table 2. Results of PCR amplifications, fragment identification with BlastN and accession numbers of *nrfA* gene fragments deposited in EMBL

Microorganism tested	PCR product, bp	BlastN closest relative strain	Identity, %	Accession
<i>Desulfovibrio desulfuricans</i> ATCC 13541	850	<i>Desulfovibrio desulfuricans</i> ATCC 27774	82	HE613751
<i>Desulfovibrio desulfuricans</i> DSM 642	850	<i>Desulfovibrio desulfuricans</i> ATCC 27774	82	HE613752
<i>Desulfovibrio piger</i> DSM 749	850	<i>Desulfovibrio desulfuricans</i> ATCC 27774	80	HE613753
<i>Desulfovibrio vulgaris</i> DSM 1925	850	<i>Desulfovibrio vulgaris</i> str. 'Miyazaki F'	90	–
<i>Desulfovibrio africanus</i> DSM 2603	850	<i>Desulfovibrio desulfuricans</i> ATCC 27774	90	HE613754
<i>Desulfovibrio fructosivorans</i> DSM 3604	none			
<i>Desulfovibrio simplex</i> DSM 4141	850	<i>Desulfovibrio desulfuricans</i> ATCC 27774	90	HE613755
<i>Desulfovibrio termitidis</i> DSM 5308	850	<i>Desulfovibrio vulgaris</i> str. 'Miyazaki F'	94	HE613756
<i>Desulfovibrio longus</i> DSM 6739	none			
<i>Desulfovibrio burkinensis</i> DSM 6830	none			
<i>Desulfovibrio intestinalis</i> DSM 11275	850	<i>Desulfovibrio desulfuricans</i> ATCC 27774	90	HE613757
<i>Desulfovibrio cuneatus</i> DSM 11391	850	<i>Desulfovibrio vulgaris</i> str. 'Miyazaki F'	70	HE613758
<i>Desulfovibrio litoralis</i> DSM 11393	850	<i>Lawsonia intracellularis</i> PHE/MN1-00	68	HE613759
<i>Desulfomicrobium macestii</i> DSM 4194	850	<i>Desulfomicrobium baculatum</i> DSM 4028	88	HE613760
<i>Desulfomicrobium norvegicum</i> DSM 1741	850	<i>Desulfomicrobium baculatum</i> DSM 4028	88	HE613761
<i>Desulfomicrobium baculatum</i> DSM 4028	850	<i>Desulfomicrobium baculatum</i> DSM 4028	99	–
<i>Desulfomicrobium apsheronum</i> DSM 5918	850	<i>Desulfomicrobium baculatum</i> DSM 4028	89	HE613762
<i>Desulfomicrobium escambiense</i> DSM 10707	850	<i>Desulfomicrobium baculatum</i> DSM 4028	89	HE613763

Among the species tested, *nrfA* was known to be present only in *D. baculatum* DSM 4028.

value, cloned sequences from group A fell into 7 OTUs, while group B sequences clustered in 10 OTUs. The number of OTUs shared between the two groups is 3; therefore, 4 OTUs from wastewater samples group (group A) are unique (fig. 2b).

Discussion

In wastewater treatment, DNRA is a relevant process and accounts for up to 34% of nitrogen conversion between its chemical forms [Kløve et al., 2005]. Waste-activated sludge is usually reduced and stabilised by anaerobic digestion technology [Nakashimada et al., 2008] as in the case of wastewater treatment plant under investigation. Methane produced in anaerobic digesters is inhibited by ammonia [Percheron et al., 1999]. In certain conditions, such as at high chemical oxygen demand/nitrogen ratios, when glycerol or glucose is added, reduction reactions occur principally via the DNRA pathway [Wong and Lee, 2011].

Molecular analyses of nitrite reductase can be used to investigate the genetic potential of the studied environment [Dong et al., 2009]. To date, nitrite reductases from SRB have been mainly studied as proteins, and only a few sequences of *nrfA* are present in databases [Almeida et al., 2003; Kraft et al., 2011; Moura et al., 2007; Pereira et al., 2000]. Despite this, *nrfA* has been used as gene marker for studying DNRA because it shows conserved regions both in nucleotide and amino acid sequences [Kraft et al., 2011; Smith et al., 2007].

In this study, we designed specific primers for the genus *Desulfovibrio* that provide a valuable new tool to analyse *nrfA* that complements the existing *nrfA* primers which are either highly specific or highly degenerate. Primers designed by Greene et al. [2003] and Haveman et al. [2004] did not target *Desulfovibrio* very well at all. Mohan et al. [2004] designed primers based on the alignment of six *nrfA* sequences similar to the *E. coli* *nrfA* sequence including the *nrfA* genes from *Sulfurospirillum deleyianum* and *Wolinella succinogenes*, but they did not include *nrfA* sequences from *Desulfovibrio*. In contrast, primers presented here are specific for *Desulfovibrio* genus because they have been designed using five to six nitrite reductase amino acid sequences present in both GenBank and KEGG databases, and PCR specificity has been further increased using only one degenerate nucleotide. It should be noted that although new primers showed a high percentage of exact matches to *nrfA* sequences of the *Desulfovibrio* genus, they may match the *nrfA* sequence of

other phylogenetic groups. The strength of these primers makes them a very good candidate also for qPCR assays for the study of DNRA importance and the DNRA/denitrification pathway ratio of nitrate reduction in wastewater treatment processes.

Moreover, results obtained from OTU clustering showed that even using these specific primers, there is a high diversity in *nrfA* sequences, and there are OTUs not represented by any sequence present in the databases, confirming that more work should be done to study the *nrfA* gene and DNRA pathway in *Desulfovibrio*.

In conclusion, the new primers presented here could be used to study DNRA or nitrite detoxification in *Desulfovibrio* and related SRB and to select and control the *nrfA* active *Desulfovibrio* community in a wastewater treatment plant in order to improve the treatment process. In addition, the use of this new primer set will help the isolation of more *nrfA* sequences from both culture collections and environmental samples.

Experimental Procedures

Primer Design

In order to find conserved domains, amino acid and nucleotide nitrite reductase (NrfA) sequences of five *Desulfovibrio* and one *Desulfomicrobium* strains available in both GenBank [Benson et al., 2011] and KEGG [Kanehisa et al., 2002] gene databases were aligned using ClustalX2 [Larkin et al., 2007, see fig. 1]. PCR primers were then designed using Primer3 [Rozen and Skaletsky, 2000], asking the software to design primer sets inside the consensus sequences determined in the multiple sequence alignment above. A single degeneracy based on this alignment was added manually (table 1). The in silico coverage and specificity of the new primer sets were tested using both the NCBI BLAST tool (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and ThermoPhyl, a software that assesses the specificity and sensitivity of PCR primer sets [Oakley et al., 2011]. Finally, hetero-/homodimer formation and primer stability of the novel primer sets were evaluated using OligoAnalyzer 3.1 software (<http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/>); parameters setting for primer characteristic determination were 0.2 μ M primer, 1.5 mM Mg^{2+} salt and 0.2 mM dNTPs, and the ΔG temperature was 60°C.

Desulfovibrio Strains

DNA from thirteen *Desulfovibrio* and five *Desulfomicrobium* strains obtained from both the American Type Culture Collection (ATCC; Manassas, Va., USA) and the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) were used for testing the specificity of the *nrfA* PCR assays.

Environmental Sample Collection and DNA Extraction

Two samples of digested sludge were collected from a wastewater treatment plant in the Lombardy region (Italy), which treats mainly urban and a smaller part of industrial wastes and where

we have previously found bacteria of the *Desulfovibrio* genus. Digested sludge (2 ml) was centrifuged and the supernatant discarded, and genomic DNA extracted using the PowerSoil® DNA Isolation Kit (MO BIO, Carlsbad, Calif., USA) according to the manufacturer's instruction. The yield and purity of DNA extracts were estimated spectrophotometrically, and samples were stored at -20°C.

PCR Conditions

PCRs were performed with 1 × PCR buffer, 1.5 mM MgCl₂, 0.2 mM dNTP mix, 0.2 μM of each newly designed primer and 1 U Taq DNA polymerase (GoTaq, Promega) in 25 μl PCR reaction. The cycling program consisted in an initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s and extension at 72°C for 2 min, and a final extension at 72°C for 10 min. All PCR products were checked by ethidium bromide-stained agarose gel electrophoresis.

Cloning and Analysis of *nrfA* Sequences

nrfA DNA fragments obtained by PCR amplification were ligated into pGEM-T Easy Vector (Promega Italia) and then transformed into *Escherichia coli* JM109 cells. Transformants were selected by blue/white screening. Cloned inserts were reamplified using the vector-encoded primers M13 forward and reverse (32 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 105 s), and the resulting PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Sequencing was performed for 3 PCR clones from each strain using Applied Biosystems Big Dye Terminator v3.1 cycle sequencing kit and run on the 3130xl Genetic Analyzer (Applied Biosystems, Foster City, Calif., USA). Cloned insert sequence identity was confirmed using the BLAST tool. Sequences obtained from *Desulfovibrio* collection, environ-

mental sample clones and NCBI and KEGG databases were aligned in the ClustalX2 program [Larkin et al., 2007], and a distance matrix was generated using the DnaDist program in the Phylip package [Felsenstein, 1993] using default parameters. Distance matrices were used in the Mothur program [Schloss et al., 2009] to assign OTUs, construct rarefaction curve and calculate richness estimates using the default parameters. OTUs were assigned using a 14% cutoff value.

Nucleotide Sequence Accession Numbers

Partial *nrfA* sequences from *Desulfovibrio* and *Desulfomicrobium* strains that were not previously available in the databases have been deposited in EMBL under accession numbers from HE613751 to HE613763. Cloned insert sequences obtained from the digested sludge have been deposited in EMBL under accession numbers from HE977572 to HE977582.

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