ORIGINAL ARTICLE

Phenotypic and genotypic richness of denitrifiers revealed by a novel isolation strategy

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Present-day knowledge on the regulatory biology of denitrification is based on studies of selected model organisms. These show large variations in their potential contribution to NO₂⁻, NO, and N₂O accumulation, attributed to lack of genes coding for denitrification reductases, but also to variations in their transcriptional regulation, as well as to post-transcriptional phenomena. To validate the relevance of these observations, there is a need to study a wider range of denitrifiers. We designed an isolation protocol that identifies all possible combinations of truncated denitrification chains (NO₃⁻/NO₂⁻/NO/N₂O/N₂). Of 176 isolates from two soils (pH 3.7 and 7.4), 30 were denitrifiers *sensu stricto*, reducing NO₂⁻ to gas, and five capable of N₂O reduction only. Altogether, 70 isolates performed at least one reduction step, including two DNRA isolates. Gas kinetics and electron flow calculations revealed that several features with potential impact on N₂O production, reported from model organisms, also exist in these novel isolates, including denitrification bet-hedging and control of NO₂⁻/NO/N₂O accumulation. Whole genome sequencing confirmed most truncations but also showed that phenotypes cannot be predicted solely from genetic potential. Interestingly, and opposed to the commonly observed inability to reduce N₂O under acidic conditions, one isolate identified as *Rhodanobacter* reduced N₂O only at low pH.

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Introduction

The past 150 years have experienced a massive anthropogenic input of reactive nitrogen to the biosphere, leading to a global acceleration of microbial nitrogen transformations (van Groenigen *et al.*, 2015; Stokstad, 2016). One of the major processes is denitrification, by which NO₃⁻ and/or NO₂⁻ is reduced to gaseous forms of nitrogen. Complete denitrification of NO₃⁻ to N₂ involves four reduction steps. These are catalyzed by four main types of functional enzymes, the nitrate reductases Nar or Nap; the NO-generating nitrite reductases NirK or NirS; the N₂O generating nitric oxide reductases cNor, qNor, or qCuNor; and the two types of nitrous oxide reductases N₂OR (Zumft, 1997; Shapleigh, 2013). A denitrifying organism may contain both nitrate reductases, but generally only one type of the nitrite, nitric oxide, and nitrous oxide reductases. Organisms lacking one or more of the denitrification steps occur frequently in the environment (Shapleigh,

2013; Graf *et al.*, 2014), but physiological studies of truncated denitrifiers are scarce, yet they demonstrate prominent physiological and regulatory differences (Bergaust *et al.*, 2008; Roco *et al.*, 2016). Denitrification is primarily performed by facultative anaerobic bacteria, and has received substantial interest during the last decades due to its economic and environmental implications, reducing the amount of N available to the crops, and acting as the main source of the greenhouse gas N₂O (Schlesinger, 2008), which is also the most important destructor of the stratospheric ozone layer (Portmann *et al.*, 2012).

Attempts to correlate *nosZ* gene abundance, sequence diversity and/or transcript numbers with N₂O emissions from soils show variable results (Rich and Myrold, 2004; Morales *et al.*, 2010; Jones *et al.*, 2014; Liu *et al.*, 2014; Domeignoz-Horta *et al.*, 2015), demonstrating that these parameters cannot alone predict the propensity of a soil microbial community to emit N₂O. The root of the problem may be that denitrification genes in soil are not necessarily transcribed in response to anoxia, and that transcription does not necessarily lead to the production of functional enzymes. The current knowledge on the regulatory biology of denitrification is based on studies of only a few model organisms.

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Denitrification is widely spread among taxonomically diverse groups of bacteria and more fundamental understanding is needed from a wider range of organisms before we may, eventually, use metagenomics to predict the propensity of soils to emit N_2O .

Many groups of denitrifying bacteria have as yet no cultivable representatives available in biobanks (Zumft, 1997; Janssen *et al.*, 2002). Previous isolation efforts have been conducted under anoxic conditions in the presence of NO_3^- (Gamble *et al.*, 1977; Cheneby *et al.*, 2000; Heylen *et al.*, 2006; Hashimoto *et al.*, 2009). Denitrifying bacteria may, however, be unable to grow if exposed to sudden anoxia, as, if they do not have enough time to express the denitrification reductases, they may be entrapped in anoxia without energy to produce the enzymes (Højberg *et al.*, 1997; Hassan *et al.*, 2014). This will limit the isolates to the ones that manage such abrupt shifts. Moreover, the early isolation studies used full-strength media, which have been shown to inhibit a large fraction of viable soil bacteria (Olsen and Bakken, 1987). Even more importantly, the previous attempts to isolate denitrifying bacteria were not inclusive because: (1) they have not isolated organisms without NAR, as nitrite was generally not provided; (2) they have not isolated organisms with only N_2OR ; and (3) they have probably not captured organisms with only NIR since such organisms produce NO as end product and therefore will not survive in pure culture when exposed to anoxia. Moreover, NO was not measured in those studies. On the basis of this, and with the aim to isolate as broad a range of denitrifying bacteria, possessing different truncated denitrification pathways, we chose a two-pronged strategy. First, as it was also done in the study by Roco *et al.* (2016), we isolated the bacteria on low-nutrient agar plates under aerobic conditions, to allow the organisms to adapt to growth under these conditions before challenging them with a gradual transition to anaerobic respiration. Second, after re-streaking until pure cultures were obtained, single colonies were transferred to vials containing low-nutrient medium with NO_3^- and NO_2^- , and with micro-oxic headspace supplemented with N_2O . This strategy, which was a development of the one by Roco *et al.* (2016), allowed us to efficiently identify bacteria performing all four denitrification steps, as well as bacteria with all possible combinations of truncated denitrification pathways (except those carrying only Nor), and those performing DNRA (dissimilatory reduction of NO_3^- via NO_2^- to NH_4^+).

We extracted bacteria from two agricultural peat soils of contrasting pH (pH 3.7 and pH 7.4) sampled from a long-term field experiment (Liu *et al.*, 2010), where earlier studies have shown strong negative relationship between pH and $\text{N}_2\text{O}/\text{N}_2$ product ratio (Liu *et al.*, 2010; Russenes *et al.*, 2016), in accordance with findings from a wide range of other soils (Simek and Cooper, 2002; Cuhel *et al.*, 2010; van den Heuvel *et al.*, 2011; Bakken *et al.*, 2012; Russenes *et al.*,

2016). Recently, we have found that the two soils are also profoundly different regarding the transient accumulation of nitrite during anoxic incubations (an order of magnitude higher in the pH 7.4 soil), and that the low pH soil is indeed able to reduce N_2O , albeit at a slower rate and much delayed compared to the high pH soils (Lim, Bakken and Frostegård, unpublished). We hypothesized that we would isolate comparable numbers of denitrifiers from both soils and that truncated denitrification chains would be common. We also expected that detailed phenotypic analyses would reveal a variety of phenotypes, not always reflecting the genomic potential of the organisms, and with examples of 'Rapid Complete Onset (RPO)' and 'Progressive Onset (PO)' of denitrification (Liu *et al.*, 2013), as well as 'bet hedging', that is, that only a fraction of the cells switch to denitrification (Hassan *et al.*, 2014; 2016). Furthermore, we hypothesized to find some organisms in the low pH soil that were capable of reducing N_2O at low pH.

Materials and methods

Soil samples and isolation of bacteria

Soils were collected from a long-term research field site at Fureneset Rural Development Centre located in Fjaler on the western coast of Norway, where plots (8×12.5 m) had been limed to different pH levels about 40 years ago (Sognnes *et al.*, 2006). Two soils of contrasting pH were used; one from an untreated plot ($\text{pH}_{\text{H}_2\text{O}}$ 3.7, in the following referred to as 'low pH soil') and one from a plot that had received 800 m^3 shell sand ha^{-1} ($\text{pH}_{\text{H}_2\text{O}}$ 7.4, in the following referred to as 'high pH soil'). The samples were sieved (4 mm mesh size) and kept at 4°C until use (1–3 months).

Approximately 20 g of each soil were diluted 1:10 in sterile distilled water and blended in an Omni Mixer Homogenizer (Omni) for 3×1 min, speed 7. The homogenization was carried out on ice to prevent heating. The soil homogenates were then left to settle for 10 min, allowing the bigger aggregates to sediment. Appropriate dilutions of the supernatants were plated onto 1/10 TSA medium (Tryptic Soy Agar, Merck, Darmstadt, Germany), containing 1.5 g l^{-1} casein peptone and 0.5 g l^{-1} soy peptone, supplemented with 100 mg l^{-1} cycloheximide (Fluka, Steinheim, Germany) to prevent fungal growth. Bacteria from the high pH soil (series A) were plated on medium with pH 7.3. Bacteria from the low pH soil were divided in two series, in order to capture as many types of bacteria as possible. Series B was plated on 1/10 TSA medium of pH 7.3 and series C was plated on 1/10 TSA adjusted to pH 5.7 using 20 mM phosphate buffer and 1.4 mM sulfuric acid. The same buffering system was used for both pH levels, therefore the low pH was set to 5.7. The plates were incubated under oxic conditions at $22 \pm 2^\circ\text{C}$ for two weeks, and inspected regularly to

detect newly appearing colonies. Colonies were picked from dilutions that gave around 50 colonies per plate, generally the 10^{-6} dilution. Single, well-spaced colonies were selected based on morphology (size, shape, surface structure, and color), aiming at obtaining as large a variation of bacterial isolates as possible, and streaked to purity on new plates with the same medium. Total viable counts were performed at the end of the incubation, after 14 days. Pure cultures were preserved at -80°C in TSB medium (Tryptic Soy Broth, Merck) containing 15% glycerol, until further analysis. The total number of bacteria in the two soils was determined using microscopic direct counts after SybrGreen staining (Noble and Fuhrman, 1998).

Endpoint analyses for determination of phenotypes

The experimental setup was designed to identify complete or partial denitrifiers, as well as nitrate reducers and organisms performing DNRA (Figure 1). Three out of four electron acceptors used in nitrate reduction (NO_3^-) and denitrification (NO_2^- , N_2O) were provided. Bacterial cultures were raised from frozen stocks and inoculated into serum vials containing 4 ml of 1/10 TSB medium supplemented with 1 mM NaNO_3 and 1 mM NaNO_2 . The pH of the medium was 7.3 for the A and B series. For series C the medium was adjusted to pH 5.7 (as described above). The cultures were incubated with vigorous shaking to secure oxic conditions, thus preventing denitrification. When visible turbidity was reached, the vials were crimp-sealed with rubber septa and made anoxic by repeated evacuation and helium filling. Pure O_2 (1% v/v) and pure N_2O (1% v/v) was added to the headspace and the cultures were incubated at room temperature for 10 or 21 days (isolates from high and low pH soils, respectively). At the end of the incubation, headspace samples were taken to quantify NO , using a chemiluminescence NO analyser (Sievers NOA), while N_2O and N_2 were quantified by gas chromatography (Molstad *et al.*, 2007). Concentrations of NO_3^- and NO_2^- were determined by measuring the amount of NO produced by the reaction with vanadium (III) chloride in HCl at 95°C ($\text{NO}_2^- + \text{NO}_3^-$), and the reaction with sodium iodide in acetic acid at room temperature (NO_2^- only), using the purger system coupled to the Sievers Nitric oxide analyser NOA280i (Cox, 1980; Braman and Hendrix, 1989). Samples in which nitrogen imbalance was detected were investigated for ammonium concentration using a colorimetric hypochlorite method according to Bower and Holm-Hansen (1980).

Phylogenetic analysis

Bacterial isolates that carried out at least one of the steps in the reduction of NO_3^- to N_2 were taxonomically classified based on their 16S rRNA gene sequences. Each isolate was cultivated in 1/10 TSB

medium, DNA was extracted and purified using standard procedures (QIAamp DNA Mini Kit, Qiagen, Hilden, Germany) and the 16S rRNA gene was amplified using universal eubacterial primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Baker *et al.*, 2003). Purified PCR products (Omega, Norcross, GA, USA) were Sanger sequenced (GATC, Germany) and a phylogenetic analysis of these and 16S rRNA genes from closely related isolates retrieved from the NCBI database, was performed based on the neighbor-joining method (Interior-branch test, number of bootstrap replications = 500, site coverage cutoff = 95%) using the MEGA version 6 (Tamura *et al.*, 2013).

Whole genome sequencing

A total of 12 isolates displaying complete or partial denitrification were genome-sequenced by MiSeq (Illumina, San Diego, CA, USA). Genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen), and samples were processed using the Nextera XT sample preparation kit. Sequencing was done using the MiSeq reagent kit v3 for 300×2 paired end sequencing. Adapters and bar codes were trimmed from the sequence reads before further preprocessing by Trimmomatic (quality threshold 20). Assembly was done using SPAdes with contig length set to 500 and minimum read coverage of two. Draft genomes were uploaded to the RAST server (<http://rast.nmpdr.org/>, (Aziz *et al.*, 2008) for identification of open reading frames, and scrutinized for genes encoding the nitrogen oxide reductases. Gene annotations were confirmed through BLASTp and the presence of conserved active site motifs. The selected isolates included four full-fledged denitrifiers as determined by end-point analysis, and 8 isolates lacking the last step of the denitrification pathway (N_2O to N_2), thus potentially contributing to N_2O emission. One isolate of special interest, which utilizes N_2O as sole electron acceptor during anaerobic growth, was also investigated.

Denitrification regulatory phenotypes

Denitrification Regulatory Phenotypes (DRP) of selected isolates were determined following Bergaust *et al.* (2011) and Liu *et al.* (2013). Pre-cultures were raised from frozen stocks that were inoculated into vials containing 1/10 TSB medium adjusted to pH 7.3 or pH 5.7 (for high and low pH isolates, respectively) and incubated with vigorous stirring until they reached the optical density (OD, 660 nm) of 0.1. This approach ensured that fully oxic conditions were maintained during pre-culturing, thus preventing expression of nitrogen oxide reductases prior to the experiment. When OD_{660} reached 0.1, 1 ml portions of the aerobic cultures were inoculated into new, gas tight 120 ml vials in triplicates containing 50 ml sterile medium (same

	NAR	NIR	NOR	N ₂ OR	pH 7.4 soil series A	pH 3.7 soil	
						series B	series C
Full-fledged	N ₂				8	0	1
NIR, NOR, N ₂ OR		N ₂			0	0	3
Only N ₂ OR				N ₂	2	1	0
NAR*/NIR*, N ₂ OR	NO ₃ ⁻ and NO ₂ ⁻ reduced			N ₂	0	0	2
NAR, NIR, NOR	N ₂ O				1	2	0
NIR, NOR		N ₂ O			1	4	4
NAR, NIR	NO				4	0	0
Only NIR		NO			0	2	0
Only NAR	NO ₂ ⁻				19	13	1
DNRA	NO ₃ ⁻ and NO ₂ ⁻ reduced to NH ₄ ⁺				1	1	0

Figure 1 Phenotypic classification of new soil isolates able to perform at least one step in the dissimilatory reduction of NO₃⁻, NO₂⁻, NO, and N₂O. The phenotypes were designated as NAR, NIR, NOR, and N₂OR. The end points (N₂, N₂O, NO, NO₂⁻, or NH₄⁺) are indicated inside the colored bars. DNRA designates dissimilatory nitrate reduction to ammonium. A total of 176 bacterial isolates were investigated; 99 from a soil of pH_{H2O} 7.4 (dark gray) and 77 from a soil of pH_{H2O} 3.7 (white). Bacteria from the low pH soil were isolated either on medium of pH 7.3 (Series B) or pH 5.7 (Series C). The color codes that identify the different phenotypes are used to indicate the phenotype of the isolates in the phylogenetic trees presented in Figures 2 and 3. NAR*/NIR*, N₂OR indicates organisms which reduced NO₃⁻/NO₂⁻, but with no corresponding gases detected in the end-point analysis. End-point ammonium measurements did not classify these organisms as DNRA. They were, however, capable of reduction of externally provided N₂O.

pH as the respective pre-cultures) with 2 mM NO₃⁻ for the complete denitrifiers and 1 mM NO₂⁻ for those lacking nitrate reductase. The air in the headspace had been replaced with He and 1% (v/v) O₂. The cultures were incubated with vigorous stirring at 23 °C in a robotized incubation system enabling the quantification of O₂, CO₂, NO, N₂O and N₂ in the headspace of respiring cultures (Molstad *et al.*, 2007) but with a modified GC system (Qu *et al.*, 2014). Frequent gas sampling from headspace during the transition from aerobic respiration to denitrification provided high-resolution gas kinetics which, coupled to NO₂⁻ and OD₆₆₀ measurements, allowed detailed characterization of the denitrification phenotypes of the isolates under the given conditions.

Results

Total viable counts after 14 days of incubation were $4.71 \times 10^7 \pm 8.62 \times 10^6$ ($n=10$) and $3.14 \times 10^7 \pm 3.06 \times 10^6$ ($n=7$) CFUs g⁻¹ of wet soil for the high- and low pH soils, respectively. This accounted for ~0.4% and 0.3% of the total cell number, determined by epifluorescence microscopic counts ($1.19 \times 10^{10} \pm 6.15 \times 10^9$ cells g⁻¹ soil for the high pH soil and $1.1 \times 10^{10} \pm 4.1 \times 10^9$ cells g⁻¹ soil for the low

pH soil). Altogether, 176 isolates were obtained as pure cultures. Of these, 99 isolates were from the high pH soil (series A) and 77 isolates were from the low pH soil with 50 isolates in series B (isolated on medium with pH=7.3) and 27 isolates in series C (isolated on medium with pH=5.7). End-point analysis of NO₃⁻, NO₂⁻, NO, N₂O, and N₂ allowed the determination of a phenotype for each isolate able to perform at least one step in the reduction of NO₃⁻ to N₂ (Figure 1). Isolates that could perform nitrate reduction, and/or at least one step of denitrification, were obtained in similar numbers from the low and the high pH soils, and comprised representatives of all possible truncations of the denitrification chain, except the phenotype ‘Only NOR’ which could not be detected by our isolation scheme.

Among the 99 isolates from the high pH soil, 36 could perform at least one of the reduction steps (Figure 1). For the low pH soil, this fraction was 44% (34 out of 77 isolates; 23 from the B series and 11 from the C series). There were all in all 30 isolates that qualified as denitrifiers *sensu stricto*, that is, with the capacity to reduce nitrite to NO, 14 from the high pH soil (A) and 16 from the low pH soil (B and C combined). Altogether, nine of these isolates were full-fledged denitrifiers (5.7% of the total 176

Figure 2 Phylogenetic tree based on neighbor joining (MEGA 6, Tamura *et al.*, 2013) of 16S rRNA gene sequences. The tree shows the relationship between the bacterial isolates from the high pH soil (series A), identified as having the capacity of dissimilatory reduction of at least one of the tested N oxides, and closely related isolates retrieved from the NCBI database. The color codes correspond to the ascribed phenotypes shown in Figure 1.

isolates), capable of reducing NO_3^- to N_2 . Of these, 8 were from the high-pH soil and only one was from the low-pH soil (series C). Sequencing of the 16S rRNA genes showed that all full-fledged denitrifiers

were members of the *Proteobacteria* (Figures 2 and 3). Those that were isolated from the high pH soil represented the orders *Burkholderiales*, *Pseudomonadales* and *Rhizobiales*, whereas the isolate

from the low pH soil (isolate C04) belonged to *Burkholderiales*.

Twenty-one of the isolates classified as denitrifiers *sensu stricto* showed a truncated denitrification phenotype, meaning that they lacked the capacity to perform one or more of the reduction steps (Figure 1). Only three of these truncated denitrifiers were capable of reducing N_2O . These isolates were unable to reduce NO_3^- , but reduced NO_2^- to N_2 (phenotype 'NIR, NOR, N_2OR '). They were isolated from the low pH soil, series C, and classified as members of the genus *Rhodanobacter* (Figure 3). In addition to the twelve denitrifiers that were able to reduce N_2O (9 full-fledged and the three truncated isolates), there were three isolates classified as 'Only N_2OR ' (two from series A and one from series B). One of those in series A was identified as belonging to the order *Cytophagales* (Figure 2). The other two with this phenotype could unfortunately not be recovered from long-term storage and were therefore not sequenced.

The phenotypes 'NAR, NIR, NOR' and 'NIR, NOR' were unable to perform the last step of denitrification, and thus emitted N_2O as end product. Together, these two phenotype groups comprised 12 isolates (7%) of the total number of isolates (Figure 1). Three of them belonged to the 'NAR, NIR, NOR' phenotype, one coming from the high pH soil (order *Burkholderiales*; Figure 2) and two from the low pH soil (orders *Xanthomonadales* and *Enterobacteriales*, both series B; Figure 3). The 'NIR, NOR' phenotype was found for 8 isolates from the low pH soil (four from the B and four from the C series), and belonged to the order *Xanthomonadales* with most of them clustering with the genus *Rhodanobacter* (Figure 3). Only one isolate with this phenotype was isolated from the high pH soil (order *Flavobacteriales*; Figure 2).

The phenotype 'NAR, NIR', which reduced NO_3^- to NO but showed no production of N_2O or N_2 , was found for four isolates from the high pH soil. These isolates accumulated 0.68–2.11 μM NO (concentration in the liquid), measured at the end of the incubation. Two of the isolates were members of the orders *Burkholderiales* and *Bacilliales*, respectively, while the two others were unrecoverable from storage and thus not sequenced. None of the isolates from the low pH soil showed this phenotype. Two isolates from the low pH soil (B20 and B21) exhibited the one-step reduction of NO_2^- to NO ('Only NIR'; Figure 1), as seen from their accumulation of NO reaching up to 54.8 μM at the end of the incubation (Supplementary Materials, Supplementary Table S1). This value is based on the measured NO concentration in the headspace, assuming equilibrium between gas phase and liquid phase. One isolate (B20) in the 'Only NIR' group clustered together with the order *Xanthomonadales* and was identified as belonging to the genus *Rhodanobacter*, the other one could not be recovered from the long-term storage and was therefore not sequenced.

Bacteria capable of only NO_3^- reduction to NO_2^- (phenotype group 'Only NAR'; Figure 1), and thus not considered 'denitrifiers' *sensu stricto*, comprised the largest group with 19% and 18% of the total number of isolated isolate in the high- and low pH soil, respectively. Interestingly, 13 out of the 14 'Only NAR' isolates from the low pH soil were isolated on pH 5.7 medium (series C). The isolates from the high pH soil were taxonomically diverse, as determined from the 16S rRNA sequences. Most of them belong to *Actinomycetales*, but there are also representatives from *Bacilliales*, *Flavobacteriales* and *Burkholderiales*. Only six isolates were sequenced from the low pH soil, all of them belonging to *Actinomycetales* (Figures 2 and 3). Isolates for which the end-point analysis showed nitrogen imbalance, thus suggesting DNRA (seen as NO_3^- reduction, but no production of relevant gases), were investigated for NH_4^+ accumulation. On the basis of this, only one isolate from the high pH soil and one from the low pH soil (series B) were identified as DNRA organisms (classes *Actinobacteria* and *Bacilli*, respectively).

The presence or absence of all the relevant genes encoding N-oxide reductases was determined by whole genome sequencing of the 12 isolates that were phenotypically classified as capable of reducing NO_3^- or NO_2^- to N_2O or N_2 (thus phenotypes 'Full-fledged'; NIR, NOR, N_2OR and 'NAR, NIR, NOR', Figure 1), and one isolate capable of sole reduction of N_2O to N_2 (Figure 6). Of the seven sequenced isolates able to reduce NO_3^- to NO_2^- , four carried *narG* but not *napA* while one isolate, belonging to the genus *Bradyrhizobium*, carried *napA* but not *narG*. Thus, none of the isolates carried both these genes. In addition, three of the isolates lacking *narG* and *napA* carried *nasA*, which is another gene for nitrate reductase involved in nitrate assimilation (Luque-Almagro *et al.*, 2011). The sequencing results confirmed the presence in all 12 denitrifiers of genes encoding the defining reaction of denitrification (NO_2^- reduction to NO), catalyzed by the product of a *nir* gene (Zumft, 1997). Approximately 10 of these isolates carried the *nirK* gene coding for a Cu containing nitrite reductase (Zumft, 1997). One *Rhodanobacter* isolate (C06) from the low pH soil had an ~240 bp larger *nirK* gene than the others, containing a cytochrome C domain at the 3' end. The *nirS* gene, encoding the *cd*₁ nitrite reductase (Zumft, 1997), was identified in only two isolates from the high pH soil, representing the genera *Hydrogenophaga* and *Pseudomonas*. Genes encoding respiratory NO reductases were detected in all the isolates for which NO reduction to N_2O was detected (12 out of the 13 sequenced isolates). As predicted from the phenotypic analyses, no *nor* genes were found in isolate A40 (*Algoriphagus*). Generally, the isolates carrying a respiratory *nor* had either the *qnor* or the *cnor* type, and these were mainly divided between the low and high pH soil isolates, respectively. The only exception was isolate

Figure 3 Phylogenetic tree based on neighbor joining (MEGA 6, Tamura *et al.*, 2013) of 16S rRNA gene sequences. The tree shows the relationship between the bacterial isolates from the low pH soil (series B and C), identified as having the capacity of dissimilatory reduction of at least one of the tested N oxides, and closely related isolates retrieved from the NCBI database. The color codes correspond to the ascribed phenotypes shown in Figure 1.

C04 (genus *Polaromonas*), which in addition to two versions of *qnor*, had a large *norB* gene which was placed downstream of a gene annotated as cytochrome c oxidase polypeptide. The atypical *norB* gene encoded a protein apparently carrying a ~130 aa C-terminal extension as compared to the normal length NorB found in the *Pseudomonas* isolate (A25). The aa sequences of the cNor in C04 cluster with proteins of similar size in *Rhodanobacter* species (NCBI BLASTp, 85 and 84% identities for

putative NorC and NorB, respectively), but were not found within the present collection. Some of the other isolates from the low pH soil did however also have double copies of *qnor*. Nine genomes harbored a *nosZ* gene which encodes a nitrous oxide reductase (*N₂OR*): eight of them were classified as *nosZ* clade I, and one was identified as belonging to *nosZ* clade II (Jones *et al.*, 2013)—the isolate A40 assigned to the genus *Algoriphagus*. However, only six of these isolates exhibited *N₂OR* activity under the

conditions tested (pH 5.7 or pH 7.3 for isolates from low and high pH soil, respectively). Of the low pH soil isolates able to reduce N_2O , one was identified as belonging to the genus *Rhodanobacter* (C01) while one belonged to *Polaromonas* (C04) (Figure 3).

Denitrification regulatory phenotypes of selected isolates are presented in Figure 4 (high pH soil) and Figure 5 (low pH soil). Nitrate reduction was initiated as the O_2 concentration in the medium reached $\sim 5 \mu\text{M}$ for most isolates, but the *Rhodanobacter* isolates C01 and C05 started to denitrify at $16\text{--}17 \mu\text{M}$ O_2 , while the *Rhodanobacter* isolate C06 depleted virtually all O_2 before any gas production could be detected. The isolates show clear differences in the accumulation of intermediates, revealing a large diversity in the regulation of denitrification in these organisms. Among the isolates from the high pH soil, an isolate of *Hydrogenophaga* (A37) accumulated NO_2^- as long as NO_3^- was present in the medium. In contrast a *Bradyrhizobium* isolate (A49) exhibited a rapid onset of all denitrification genes in response to decreasing O_2 concentration, seen as an early increase in N_2 with no detectable NO_2^- , and only transient and low accumulation of NO (maximum amount 7 nmol vial^{-1} , corresponding to 5 nM concentration in the liquid) and N_2O (maximum $30 \mu\text{mol N vial}^{-1}$). The N_2O accumulation in the *Bradyrhizobium* culture never exceeded 40% of the total N provided, and at the end of the incubation all nitrogen was recovered as N_2 . Isolate A25, ascribed to the genus *Pseudomonas*, accumulated 100% of the provided N oxides as N_2O before reducing this further to N_2 . Isolate A23, clustering with *Polaromonas* (family *Comamonadaceae*; order *Burkholderiales*) instead accumulated N_2O as the end-product of denitrification with no detection of N_2 . The absence of a *nosZ* gene in this organism was confirmed by whole genome sequencing (Figure 6).

Five isolates from the low pH soil were whole genome sequenced (Figure 6). The denitrification regulatory phenotypes of four of these are shown in Figure 5. Three of them (isolates C01, C05, and C06), which were classified as members of the genus *Rhodanobacter*, were incapable of reducing NO_3^- but readily reduced NO_2^- . Genome analysis confirmed the absence of genes encoding dissimilatory nitrate reductase, and the presence of a *nirK* gene (Figure 6). The two other isolates, designated B06 and C04, started to respire NO_3^- when O_2 concentrations became low ($\sim 10 \mu\text{M}$ in the liquid phase), but did not respire the NO_2^- produced until most, or all, of the NO_3^- was depleted. The isolate C04, for which the closest match was the genus *Polaromonas* within the order *Burkholderiales*, accumulated significant amounts of NO reaching a maximum of $225 \text{ nmol (vial}^{-1})$, which corresponds to $0.15 \mu\text{M}$ in the liquid. Similar amounts of NO were detected in two of the *Rhodanobacter* cultures (C01 and C05). The *Polaromonas* isolate C04 accumulated up to 80% of the provided N oxyanions as N_2O , before reducing it

further to N_2 . The isolate B06, clustering with *Rahnella* (Figure 3) within the family *Enterobacteriaceae*, gradually accumulated NO until the concentration exceeded $2.5 \mu\text{M}$ in the liquid ($3.7 \mu\text{mol vial}^{-1}$). Reduction of NO to N_2O was detectable in the *Rahnella* culture, but came to a halt when the concentration of NO reached $0.5 \mu\text{M}$, which apparently inhibited respiration, seen as inhibited CO_2 production (Supplementary materials, Supplementary Figure S1). This was contrary to the *Rhodanobacter* isolate C06, which accumulated tenfold higher concentration of NO compared with *Rahnella* sp., but still continued respiration (seen as further reduction of NO, and CO_2 evolution). All three investigated isolates of the genus *Rhodanobacter* were equipped with a *nosZ* clade I gene. Yet, all three accumulated N_2O to different levels. The *Rhodanobacter* isolate C01, which was the only one capable of N_2O reduction at pH 5.7, accumulated 80% of the provided nitrogen as N_2O before reducing it further to N_2 . This was in contrast to the other two *Rhodanobacter* isolates, C05 and C06, which were unable to reduce N_2O under the conditions tested. Of these, isolate C05 reduced 100% of the provided N to N_2O in $< 60 \text{ h}$, whereas C06 only converted 9% of the N to N_2O during 100 h incubation. The O_2 consumption rate, on the other hand, was approximately three times higher in C06 than in C05 (Figure 5).

The analysis of high-resolution gas kinetics, coupled with frequent measurements of NO_2^- , allowed estimations of the electron flow to O_2 and to the various N-oxide electron acceptors (Figures 4 and 5). Differences in electron flow patterns reveal variations in the way denitrifying organisms regulate the transition from aerobic to anaerobic respiration and reflect how large a proportion of cells in a population switches to denitrification upon depletion of oxygen (Hassan *et al.*, 2014). The smooth patterns of total electron flow seen for isolates A25 (*Pseudomonas* sp.) and A37 (*Hydrogenophaga* sp.) from the high pH soil (Figure 4), and for isolates C01 and C05 (both *Rhodanobacter* sp.) from the low pH soil (Figure 5), suggest that all cells in the cultures switched to denitrification in response to anoxia. Contrary to this, a drastic drop in electron flow after the O_2 depletion, as exhibited by the *Bradyrhizobium* isolate A49 (Figure 4), suggests that only a marginal fraction of the aerobic culture managed the transition to denitrifying respiration. The pattern for the low pH soil isolate C04 (*Polaromonas* sp.; Figure 5) revealed yet another phenomenon, where the electron flow dropped twice; first in response to O_2 depletion, and second in response to NO_3^- depletion (accumulated as NO_2^-).

Discussion

The proposed isolation strategy allowed us to capture organisms capable of nitrate reduction only,

Figure 4 Denitrification regulatory phenotypes of selected isolates from the high pH soil. Aerobically prepared bacterial cultures were inoculated into sealed 120 ml medical vials containing 50 ml 1/10 TSB medium of pH 7.3 supplemented with 2 mM KNO₃. Headspace atmosphere after repeated helium flushing was adjusted with pure O₂ to 1% concentration (v/v) prior inoculation. The vials were incubated at 22 °C with vigorous stirring. Gas kinetics (O₂, NO, N₂O, N₂), NO₂ concentrations and OD₆₆₀ were monitored by frequent sampling from headspace and liquid phase, respectively. Error bars represent standard deviation for three replicates. Bottom graphs depict the calculated rates of electron flow to O₂, nitrogen oxides (NO_x) and the total electron flow.

denitrification *sensu stricto* (reduction of nitrite to gaseous N), and/or DNRA (reduction of nitrite to ammonium) from both soils, representing not only a wide phenotypic but also a large taxonomic diversity. Most of them belonged to four phyla: *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Actinobacteria*. The protocol includes some significant improvements compared to most previously used strategies for isolation of denitrifiers. Most importantly, the truncated phenotypes listed in Figure 1 would not be captured by traditional isolation schemes as: (1) Organisms incapable of reducing nitrate (14 of the 30 isolates) would not be isolated using schemes that only provide nitrate (Gamble *et al.*, 1977; Cheneby *et al.*, 2000; Hashimoto *et al.*, 2009). Heylen *et al.* (2006) did compare media containing NO₃⁻ and NO₂⁻ for isolation of denitrifiers

from activated sludge, but the effect was not clear. (2) Organisms without NOR would not be detected by any of the old approaches, none of which measured NO. Moreover, NO accumulation can result in denitrification coming to a halt long before all NO₃⁻/NO₂⁻ is reduced (Bergaust *et al.*, 2008). (3) Organisms unable to grow on high strength media would not be captured. The nutrient concentration is an important factor, as demonstrated by Hashimoto *et al.* (2009). This is relevant for Gamble *et al.* (1977), but not for Heylen *et al.* (2006) and Cheneby *et al.* (2000) who used medium strength media, comparable to ours.

To compare the frequency of denitrifying organism with that found by others, we must consider the techniques and criteria used. Cheneby *et al.* (2000) enumerated and isolated denitrifying bacteria by

Figure 5 Denitrification regulatory phenotypes of selected isolates from the low pH soil. Aerobically prepared bacterial cultures were inoculated into sealed 120 ml medical vials containing 50 ml 1/10 TSB medium of pH 5.7 supplemented with 2 mM KNO₃ for isolates B06 and C04, and 1 mM KNO₂ for the *Rhodanobacter* isolates C01, C05, and C06. Headspace atmosphere after repeated helium flushing was adjusted with pure O₂ to 1% initial concentration (v/v) prior inoculation. The vials were incubated at 22 °C with vigorous stirring. Gas kinetics (O₂, NO, N₂O, N₂), NO₂⁻ concentrations and OD₆₆₀ were monitored by frequent sampling from headspace and liquid phase, respectively. Error bars represent standard deviation for three replicates. Bottom graphs depict the calculated rates of electron flow to O₂, nitrogen oxides (NO_x) and the total electron flow.

Figure 6 Genetic potential for denitrification. Whole genomes sequenced (MiSeq) and scrutinized for denitrification functional genes (RAST). *Large *nirK* containing 3'-localized cytochrome C domain. **Large *norBC* containing a cytochrome C domain. ****nosZ* clade II. Genomes were deposited in NCBI, and are available under given accession numbers.

most probable number technique in the presence of NO₃⁻, using N₂O production in the presence of acetylene as the criterion for denitrification. Thus, they would capture only organisms possessing NAR, NIR, and NOR, with or without N₂OR, but would overlook the phenotypes 'NIR, NOR, N₂OR';

'Only N₂OR'; 'NIR, NOR'; 'Only NIR' or 'DNRA' listed in Figure 1. They found that the frequency of denitrifiers in different soils ranged from 0.9 to 4.7% of the total number of aerobes (which were also enumerated by most probable number). In comparison, our culture collection contained altogether 12

isolates with NAR, NIR, NOR $\pm$ N₂OR, which was 6.8% of the total number of isolates. Thus, our frequency is higher than the highest value recorded by Cheneby *et al.* (2000), suggesting that our isolation strategy is at least as inclusive as their most probable number scheme. Higher frequencies of denitrifying bacteria (9.7%) were reported by Gamble *et al.* (1977). This is, however, the percentage of cells growing anaerobically, and thus not comparable with our results since the number of viable anaerobes is lower than the number of viable aerobes in most soils (Cheneby *et al.*, 2000). In addition to the isolates in the studies by Gamble *et al.* (1977) and Cheneby *et al.* (2000), our isolation scheme also captured isolates lacking NAR but with the ability to reduce nitrite to NO, thus performing the defining step of denitrification. If all the phenotypes that had 'NIR' are included, they comprised 16% of all the isolates in our study.

Taken together, this suggests that isolation on low nutrient plates under fully oxic conditions, followed by a gentle transition to anaerobic respiration in liquid medium, as done in the present study, was a successful strategy that allowed us to capture a large number of diverse truncated and complete denitrifiers. The implementation of a gentle transition is in line with the observations by Højberg *et al.* (1997), showing that an abrupt decrease in oxygen may leave denitrifiers entrapped in anoxia without energy, and thus unable to produce colonies. Another important factor which apparently increased the number of different isolates was the adjustment of pH of the medium for the isolates from the low pH soil. This may reflect that many bacteria have a relatively narrow pH range for growth (Bååth, 1996). It is somewhat surprising that many organisms from the low pH soil grew well on neutral pH medium, but were not found among the isolates on the low pH plates. One explanation could be that bacteria live in microsites with locally increased pH.

The frequent occurrence of truncated denitrification pathways among the isolates is in line with the vast information found in databases about the genetic potential of denitrifying bacteria (Shapleigh, 2013; Graf *et al.*, 2014). Full-fledged denitrifiers were almost exclusively found in the high pH soil, with only one out of nine isolates being from the low pH soil, series C (Figure 1) and none from the B series (bacteria from low pH soil spread on high pH medium). A truncated denitrification phenotype can be due to the absence of, or mutations in, the functional genes encoding the reductases, or genes involved in their regulation. In addition, transcriptional regulation as well as post-transcriptional phenomena may come into play. The end-point analysis in which all possible electron acceptors for denitrification were provided, except NO, allowed us to identify all different combinations of truncated denitrification

phenotypes, apart from the tentative phenotype 'Only NOR' (Figure 1).

Organisms that carry a N₂O reductase, but lack one or more of the other denitrification genes, are of particular interest since they are potential sinks for the greenhouse gas N₂O. The 'NIR, NOR, N₂OR' phenotype was found in only three organisms isolated from the low pH soil, all belonging to the genus *Rhodanobacter*. If living in a complex system, these bacteria would use NO₂⁻, as well as N₂O, produced by other members of the microbial community. They could thus act both as a source and a sink for N₂O. Three isolates were sole N₂O reducers (phenotype 'Only N₂OR'), leaving nitrate and nitrite untouched in the end-point analysis. As such, they will not produce any N₂O, but can reduce N₂O produced by other organisms. Unfortunately, only one of them was viable after storage in -80 °C. Genome sequencing confirmed that this isolate, belonging to the genus *Algoriphagus* in the phylum *Bacteroidetes*, carried a *nosZ* gene (clade II) but lacked the other denitrification genes. Such organisms should be searched for since they are interesting as components in plant growth promoting bacterial inocula, where their role would be to reduce N₂O emissions from cultured fields (Gao *et al.*, 2016).

Altogether twelve isolates were found which had N₂O as end-point (Figure 1). This phenotype was explained by lack of the *nosZ* gene for the two isolates from the high pH soil (A23 and A02), and also for one of the *Rhodanobacter* isolates (B04) from the low pH soil, although this latter contained the *nosL* gene which may be a remnant from a more complete operon. Three of the whole genome sequenced isolates from the low pH soil were unable to reduce N₂O at neither low nor high pH, yet they did carry the *nosZ* gene. They all belonged to the genus *Rhodanobacter*, reported to be dominant members of various denitrifying communities in low pH environments (van den Heuvel *et al.*, 2010; Green *et al.*, 2012). The inability of *nosZ*-carrying bacteria to reduce N₂O at acidic pH, despite producing *nosZ* transcripts at similar levels as *nirS* transcripts, was reported for *Paracoccus denitrificans* (Bergaust *et al.*, 2010). Similar results were obtained from intact soil and extracted soil bacterial communities (Liu *et al.*, 2010; 2014). This suggests a general, post-transcriptional effect, most likely caused by unsuccessful assembly of the N₂O reductase at low pH (Bakken *et al.*, 2012). One of the *Rhodanobacter* isolates (C01) did, however, reduce N₂O at low pH, an interesting observation that calls for detailed biochemical investigations that may shed new light on the regulation of denitrification and N₂O emissions in low pH soils.

All the *Rhodanobacter* isolates lacked genes coding for the dissimilatory NO₃⁻ reductases (Nar or Nap). Such organisms have probably been overlooked in isolation studies where NO₃⁻, but not NO₂⁻, was provided. In the present study, most of the

isolates that lacked NAR were from the low pH soil. It can be speculated that this is a way for organisms living in acidic environments to avoid being exposed to high concentrations of toxic N oxides. Nitrite produced from the reduction of NO_3^- would be released into the environment and, if not being taken up again for reduction, chemical decomposition of NO_2^- could result in high concentrations of toxic NO in the vicinity of the cells (Bancroft *et al.*, 1979; van Cleemput and Samater, 1996). The fact that 14 isolates from the low pH soil were classified in the phenotype group 'Only NAR' contradicts, however, this explanation and raises the question if these organisms have other ways to protect themselves from toxic nitrogenous compounds. One possibility could be that they are equipped with two or several NO reductases as seen in some of the sequenced isolates from the low pH soil, which carried two copies of *qnor* (Figure 6). Alternatively, they exist in close assemblages with other cells in their natural environment, which carry NO reductases and thus keep NO concentrations low. The latter may explain the presence of several isolates with the phenotype 'NAR, NIR' or 'Only NIR', with NO as end point product of denitrification. This phenotype, which was also found in a study by Falk *et al.* (2010), is most likely dependent on other organisms and will not survive when grown in anaerobic batches as pure cultures.

The denitrification regulatory phenotypes of selected isolates demonstrate a large variation in the way different denitrifying bacteria handle the transition from aerobic to anaerobic respiration (Figures 4 and 5). Yet, assembling the denitrification machinery comes with an energetic cost. Moreover, production and consumption of the toxic intermediates NO_2^- and NO should ideally be synchronized to avoid accumulation of these compounds, which requires strict regulatory control. The regulatory network of denitrification has only been studied in detail for a few model organisms, revealing a large number of transcriptional regulator enzymes and ancillary factors (Zumft, 1997; Kunak *et al.*, 2004; Wunsch *et al.*, 2005; Zumft and Kroneck, 2007; Bergaust *et al.*, 2012). Although denitrifying organisms may harbor similar regulatory proteins, their regulatory phenotypes may be profoundly different. Some organisms such as isolate C04 (and most likely A37) in this study, reduce all the available NO_3^- to NO_2^- before further reduction to gaseous end-products. Such a progressive onset of the denitrification enzymes was earlier found among some isolates of *Thauera* (Liu *et al.*, 2013), and appeared to be caused by a strict, NO_3^- dependent transcriptional repression of *nirS* encoding NO_2^- reductase. The details for this mechanism remain, however, to be elucidated. This strategy may be dangerous in a low pH environment, since it may lead to high NO concentrations due to chemical decomposition of NO_2^- . On the other hand, it may be an adequate 'bet hedging' strategy: if oxygen returns before the cells

have produced the entire denitrification proteome, they would save energy. Control of the gaseous intermediates NO and N_2O also varied grossly between the isolates; while some had strict control of NO, reaching maxima of 10–20 nmol vial⁻¹, others produced 0.2–3.5 μmol NO (B06, C04, C05 and C06). Despite such high levels, most isolates managed to reduce the NO, albeit slowly, except B06 which was apparently inactivated (or killed) by NO. The N_2O levels varied from organisms showing a transient accumulation of 30 μmol vial⁻¹ (A49), to those that reduced all N oxides to N_2O before further reduction to N_2 (A25 and A37) thus being potentially strong N_2O sources in an environmental setting.

The large variation in the regulatory biology of the isolates is also reflected in the electron flow kinetics (bottom graphs in Figures 4 and 5). Several organisms performed a smooth transition from aerobic to anaerobic respiration, while some (A23 and A49) showed a dip in electron flow when transferring to using N oxide as electron acceptor. The latter is consistent with findings for *P. denitrificans*, where apparently only a fraction of the cells in the population switch to denitrification. This can be regarded as a bet-hedging strategy, which is expected to increase the chances of survival of the population; if the anoxic period is long-lasting it is advantageous to produce a complete denitrification proteome, but if the anoxic spell is short it may be more energy saving to only produce one or two of the reductases (Hassan *et al.*, 2014). From an environmental point of view such bet-hedging populations, in which the major fraction of cells produce only NAR, or NAR + N_2OR , as is the case of *P. denitrificans*, will be strong sinks for N_2O produced by other organisms (Hassan *et al.*, 2016).

The contribution of molecular techniques to the field of microbial ecology has greatly improved the insight into the structure and genetic potential of complex microbial consortia. Yet, this cannot replace physiological and biochemical studies of organisms grown in pure cultures under controlled conditions. As pointed out by (Rocca *et al.*, 2015), the correlation between gene abundance and the corresponding process is often weak. This was also demonstrated in the present study, where we found a mismatch between the genetic potential and observed N_2O reduction in 23% of the isolates. The discrepancies between genetic potential and measured function are most plausibly the results of transcriptional and metabolic regulation of the denitrification genes and their products, demonstrating that the presence of a gene cannot alone be used to predict a function. The large variation in the regulation of denitrification found among the isolates pinpoints the need for more detailed knowledge about a wider range of denitrifiers than that obtained from model bacteria, and the present study contributes to this by finding new representative organisms to include in ecophysiological studies of

denitrification. The long-term, practical goal of these efforts should be to find ways to mitigate the gas emissions from these organisms by stimulating phenotypes that act as sinks for N_2O , and counter-acting those that act as sources.

Conflict of Interest

The authors declare no conflict of interest.

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