Novel aspects of the ecophysiology of denitrifying bacteria and their roles in N_2O emissions

Nyoppdagede aspekter rundt denitrifiserende bakteriers økofysiologi, og deres rolle i N_2O emisjoner

Philosophiae Doctor (PhD) Thesis

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“Nobody ever figures out what life is all about, and it doesn't matter. Explore the world. Nearly everything is really interesting if you go into it deeply enough.”

Richard Feynman

ABSTRACT

Environmental bacteria are in general limited by nutrient availability, and as the conditions fluctuate rapidly and unpredictably they must adapt or they will not thrive. This requires fast and adequate sensing and responding mechanisms, assuring fitness with minimum investment, as there is not much energy to be wasted. The common notion about the regulatory biology of denitrification is based on detailed studies of only a few model organisms. Although the gene regulatory networks of these organisms share some common traits, there is a substantial variation in the way organisms tackle transition from aerobic respiration to denitrification, and in the levels they accumulate intermediates. This knowledge, however, only arose from studies of model organisms and there is a need to validate its importance in the natural settings. Denitrification as a sequential reduction of nongaseous nitrate (NO_3^-) and nitrite (NO_2^-) to gaseous nitric oxide (NO), nitrous oxide (N_2O) and dinitrogen gas (N_2), is a key process in the nitrogen cycle, yet its two gaseous intermediate products have great impact on the climate (N_2O) and the chemistry of the troposphere (NO). In addition to its global forcing, N_2O is also destroys stratospheric ozone, and the emission of this gas has attracted the attention of researchers and ecologists. Agricultural soils are the main source of anthropogenic N_2O emission and in order to develop mitigation strategies, there is a need for understanding the mechanism of N_2O production and reduction. There is ample evidence, that the $N_2O/(N_2O+N_2)$ product ratio of the denitrification process depends on several factors, among which the ambient pH is a dominant driver. Other processes within the nitrogen cycle can also liberate N_2O, however, denitrification is the

largest source of it, and up to date, there is only one biological sink for this N_2O – a multicopper-dependent nitrous oxide reductase (N_2OR, NosZ). The enzyme that carries the two-electron reduction of N_2O to N_2 is located in the bacterial periplasm, where its maturation and assembly takes place. Combining facts of pH dependent N_2O reduction in natural settings and the subcellular localization of N_2OR I started to investigate the processes laying behind.

In Paper I, we have tested the relevance of the observation obtained from model denitrifying bacteria and we do see that phenomena ascribed to them are also occurring in the nature. Moreover, we see that the presence of the *nosZ* gene does not correspond with the function in over 20% of investigated organisms.

The second paper reveals the undescribed *bet-hedging* survival strategy in the denitrifying bacterium *Paracoccus denitrificans*, which improves its fitness, minimizes costs of protein expression and has great ecological influence, as the majority of the isogenic bacterial population acts as a strong sink for the N_2O greenhouse gas.

In Paper III we try to explain why acidic soils are the major source of the N_2O. To our understanding the ambient pH hampers the maturation of copper in the N_2OR enzyme, thus causes the emissions. The preliminary results, that we have obtained so far, support our hypothesis.

SAMMENDRAG

Bakterier i naturlig miljø er normalt begrenset av tilgang på substrat, og de utfordres av hyppige endringer i miljøbetingelsene. For å overleve disse endringene må de for det første ha sensorer som merker endringen, og som initierer tilpasningen. Tilpasning er normalt synonymt med produksjon av nye enzymer. Dette koster energi, som er en minimumsfaktor. Overleving (fitness) er derfor ikke bare avhengig av adekvat respons, men også at energiforbruket til dette minimeres. Denitrifiserende bakterier svarer på oksygenmangel ved å uttrykke gener som koder for et sett med enzymer som setter dem i stand til å respirere ved å redusere nitrogen-oksyder. Det regulatoriske nettverket for disse genene er undersøkt i et fåtall modellorganismer, og disse viser noen felles trekk, men betydelig variasjon, som har konsekvenser for deres produksjon av intermediater. Det er et åpenbart behov for å studere denne regulatoriske biologien i flere, og ikke minst mer økologisk relevante organismer.

Denitrifikasjon er en stegvis reduksjon av nitrogen oxyanioner (NO_3^- og NO_2^-, til nitrogen monoksid (NO), dinitrogenoksyd (N_2O) og molekylært nitrogen (N2). Prosessen har en nøkkelrolle i den globale nitrogen syklusen ved å tilbakeføre reaktivt nitrogen fra biosfæren til atmosfæren. Men den er også en kilde til utslipp av NO og N2O. NO påvirker kjemien i troposfæren, og N2O (lystgass) er en kraftig klimagass som også bidrar til nedbrytning at ozonlaget (stratosfærisk ozon). Det er derfor knyttet stor interesse til utslippet av N2O.

Dyrket jord er den viktigste kilden til utslipp av «antropogent» N2O, dvs det N_2O-utslippet som skyldes menneskers aktivitet. Det er stort behov for å finne metoder til å redusere dette utslippet, og en forutsetning for dette er

at vi skaffer oss bedre forståelse av de mekanismene som regulerer utslippet. Vi vet at denitrifikasjon er den viktigste kilden til N2O, og at $N_2O/(N_2+N_2O)$ - produktforholdet styres av pH i tillegg til en rekka andre faktorer. Enzymet N_2O reduktase (N_2OR) er naturens eneste enzym som kan katalyser reduksjon av N_2O til harmløst N_2. N_2OR er et multi-kopper enzym som fungerer i bakterienes periplasma (mellom celle membran og ytre membran), og det er her dette enzymet blir «ferdigstilt». Produksjon og funksjon av N_2OR har vært ett overordnet tema i min doktorgrad. Avhandlingen inneholder tre artikler.

Artikkel 1 omhandler isolering og karakterisering av denitrifiserende bakterier fra jord, spesielt deres genetiske repertoar for denitrifikasjon, og i hvilken grad de har trekk også tilsvarer de vi finner hos modellbakterier. I 20% av isolatene finner et misforhold mellom genotyp og fenotyp: de har *nosZ*; genet som koder for N_2OR, men de reduserer ikke N_2O.

Artikkel 2 beskriver regulering av denitrifikasjons-gener hos modellbakterien *Paracoccus denitrificans*. Her dokumenteres en spesiell type bet hedging som sikrer mulighet for anaerob respirasjon med et minimum energiforbruk til protein syntese. Strategien er spesielt interessant fordi den resulterer i minimal produksjon av N_2O fordi et flertall av cellene utrykker N2OR, som reduserer N_2O, men ikke de enzymene som produserer N_2O (NO_2^- - og NO-reduktase).

Artikkel 3 rapporterer en rekke forsøk på å finne årsaken til at denitrifiserende bakterier har vanskelig for å lage funksjonelt N_2OR ved lav pH. Hypotesen har vært at problemet er knyttet til innsetting av kobber i enzymet. De foreløpige resultatene gir støtte til denne hypotesen.

LIST OF THE PAPERS INCLUDED IN THE THESIS

Paper I

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

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Paper II

Avoiding entrapment in anoxia at minimal cost; a *bet hedging* strategy of denitrifying prokaryotes that minimize N_2O emission.

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Manuscript

Paper III

A step forward in understanding why acidic soils are significant sources of the greenhouse gas N_2O

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INTRODUCTION

Denitrification in the nitrogen cycle

Nitrogen is a major element of all living organisms. The biological nitrogen cycle comprises many redox processes involving several nitrogen species of different oxidation states, as shown in Figure 1. Dissimilatory reduction of nitrate (NO_3^-) via nitrite (NO_2^-), nitric oxide (NO) and nitrous oxide (N_2O) to environmentally neutral dinitrogen (N_2) is a major driving process of the nitrogen turnover in soils. This process, called denitrification, is performed mostly by prokaryotes, which can utilize oxidized nitrogen compounds as alternative electron acceptors in lieu of oxygen. It is worth mentioning that denitrification *sensu stricto* is a respiratory reduction of nongaseous NO_2^- to gaseous $NO/N_2O/N_2$ (Zumft, 1997) and this will be its definition throughout this thesis, unless otherwise stated.

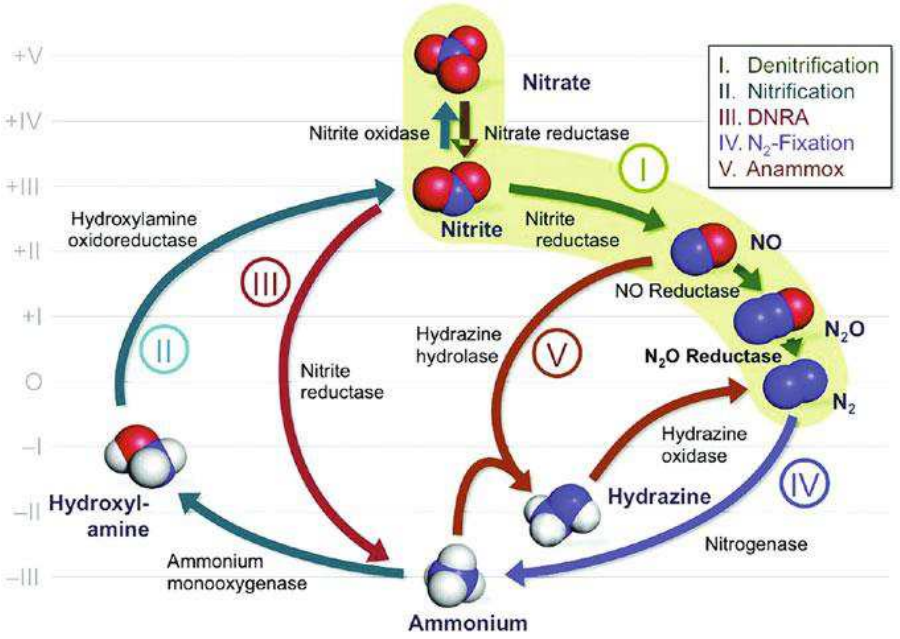

Figure 1.The biogeochemical nitrogen cycle. Reprint from Schneider et al (2014).

Humankind has been greatly affecting the nitrogen cycle, bringing it into imbalance through increasing input of reactive nitrogen into the biosphere. Escalating food demands contributed to development of artificial nitrogen fertilizers in the early XX century. Disrupted nitrogen turnover causes liberation of potentially harmful and reactive intermediates that can readily reach water and atmosphere. Especially, the gaseous compounds are prone to escape the system, as they are free intermediates. Three major biological processes that contribute to N_2O production in soils and wastewater treatment plants are nitrification, denitrification and nitrifier denitrification (Bremner 1997, Law et al 2012). Both nitrification and incomplete denitrification are responsible for N_2O emissions from soils (Fowler et al 2009, Syakila and Kroeze 2011). The nitrifier denitrification process was thought to be a significant source of N_2O, however this has recently been disproved. The new understanding of nitrifier denitrification is more of detoxification rather than respiratory role, as only ~ 1 per cent of electrons is directed to nitrifier denitrification (Hink et al 2017). There are some indications of N_2O emissions from other biologically mediated processes involved in water treatment, like anaerobic oxidation of nitrite to ammonium, called anammox. In those cases, however, the N_2O emissions are low and ascribed rather to heterotrophic activity of accompanying bacteria, than anammox process (Jetten et al 2005, Jin et al 2016, Okabe et al 2011). Denitrifying organisms protect themselves from toxic effects of NO, reducing it further to N_2O (Butland et al 2001). The N_2O emission has been recognized as major single ozone depleting emission and it is expected to remain the largest throughout the XXI century (Ravishankara et al 2009). Nitrous oxide has been under special scrutiny over the last decades, since anthropogenic

N_2O emissions have been accelerating as never before (Syakila and Kroeze 2011). N2O can be released as a byproduct from multiple sources. However, until now, there is only one biological sink for it, which is the multicopper-dependent nitrous oxide reductase (N_2OR) (Einsle and Kroneck 2004, Schneider et al 2014, Wust et al 2012, Zumft and Kroneck 2007).

Denitrification reductases, the reactants and their roles

Nitrate reductase

There are two dissimilatory nitrate reductases: the membrane bound NarG and the periplasmic NapA, both being molybdoenzymes. NarG is anchored by NarH and NarI on the electronegative side of the cell membrane, and therefore contributes to the H^+ motive force (pmf) directly. Its cytoplasmic orientation requires the presence of a transport system that delivers NO_3^- across the membrane. NapA instead reduces NO_3^- in the periplasm, thus it is not involved in generating pmf. Unlike NarG, NapA is generally expressed under aerobic conditions, suggesting a role during aerobic growth (Gavira et al 2002).

Nitrite reductase

The reduction of NO_2^- to NO is per definition the core reaction of the denitrification pathway, as it is the first reduction step that produces gas. There are two main nitrite reductases: the cytochrome cd1 dependent NirS and the copper dependent NirK. Unlike nitrate reductases, which can both be found in single organism, there is little evidence for coexistence of NirS and NirK within the same organism so far (Graf et al 2014). NirK exists as

homotrimer, with two copper centers in each monomer, whereas NirS is a homodimer with two non-covalently bound hemes (c and d_1) (Zumft 1997).

Nitric oxide reductase

Three different sub-classes of NO reductases involved in denitrification are known: cNOR, qNOR and qCu_ANOR (de Vries and Schröder 2002, Zumft 2005). The short chain NOR (cNOR, scNOR) has two integral membrane subunits and accepts electrons from cytochrome c or pseudoazurin. The q type NOR, also called the long chain, is a single subunit enzyme and receives electrons from the quinone pool, is the most popular among denitrifying bacteria and has been characterized for the first time in the hyperthermophilic Archea. Yet another, more unusual NOR hybrid which contains a Cu_A center similar to the one of cytochrome oxidases, has been described in a few Gram-positive bacteria, for example of *Bacillus azotoformans*. The enzyme receives electrons from menaquinol or from cytochrome c and its high reactivity suggests a detoxification role of this enzyme (Suharti et al 2004).

Nitrous oxide reductase

At present, two types of N_2O reductase (N_2OR) are recognized. The N_2OR found in most denitrifying bacteria was for long considered the most common type of this enzyme, although it was known that some ammonifying bacteria such as Wolinella succinogenes have an "atypical" N_2OR with an additional C-terminal mono-haem cytochrome c domain) (Simon et al 2004, Zumft and Kroneck 2007). A major difference seen between the two types was that the apoprotein of the typical N_2OR was transported to the periplasm by the Tat system, while the atypical one was transported using the Sec secretory system. More recently it was shown that the "atypical" N_2OR is

widespread among bacteria, especially in non-denitrifiers (Jones et al 2013, Sanford et al 2012), and the terms N_2OR Clade I and Clade II was proposed (Jones et al 2013). Clade I is characterized by a Tat dependent signal peptide, absence of a haem domain, and presence of the genes *nosR* and *nosX* in the *nosZ* operon, while Clade II generally is Sec dependent, often has a haem c domain, and lacks the genes *nosR* and *nosX* (Torres et al 2016). Common to both clades is that the enzyme exists as homodimer in "head to tail" orientation. Each monomer possesses two copper centers: the binuclear site Cu_A, which is responsible for electron transfer, and the tetranuclear catalytic site Cu_Z. The copper maturation of N_2OR takes place in the periplasm, which is rather unusual for the Tat secretion system. The electrons needed for the N_2O reduction originate from the quinone pool and are transferred via a bc1 complex and small soluble periplasmic transporters, such as cytochrome c_{550} or pseudoazurin, to the N_2OR (Richardson et al 2009).

Gene regulation and modularity of denitrification

Regulation of gene expression – the current understanding

The overall reulatory network for denitrifiers is quite general, possibly because organism are facing simlilar chalenges of oxygen fluctuatons and oxyanions avalabilities. They all have to be prepared for the transition from aerobic respiration to denitrification and *vice versa*. *De novo* synthesis of respiratory enzymes comes with time and energetic costs, therefore needs to be controlled. Althoug the main regulators are uniform/have their orthologues in different organisms, there is some diversity in regulatory interactions as shown in Figure 3. Denitrifying organisms are facultative

anaerobes and prefer to respire oxygen to other electron acceptors, as it yields the most energy. When oxygen becomes limiting, however, denitrifiers express reductases that allow them to sustain a respiratory metabolism and thrive under anaerobiosis by utilizing nitrogen oxyanions. Denitrification is a stepwise reaction, meaning that the product of one reductase becomes a substrate for another one. In order to avoid cytotoxic accumulations of NO_2^- and NO, the activity of the denitrification machinery must be also fine regulated (Fig. 2).

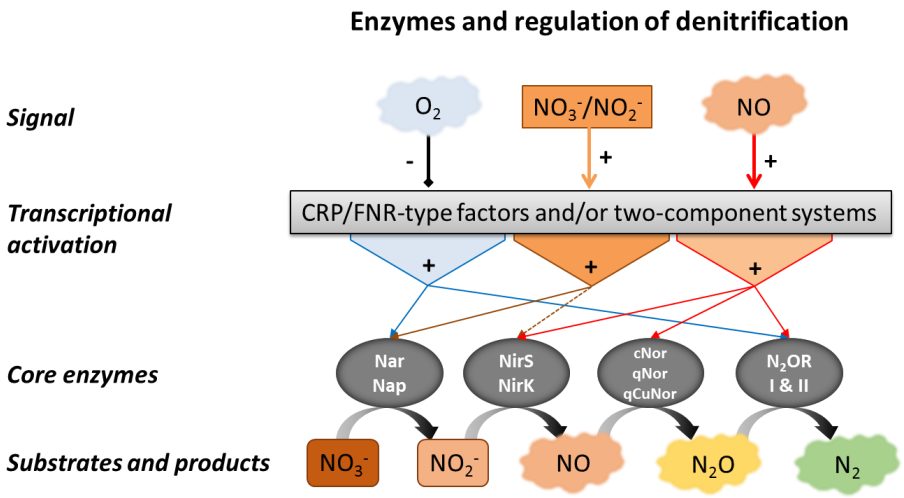

Figure 2. Summary of the regulatory roles of oxygen and nitric oxide sensors. Courtesy Linda Bergaust.

There are three main external controllers involved in the initiation or repression of transcription of the denitrification genes: oxygen, nitrate and nitric oxide (Zumft, 2002). The two most important O_2 sensors involved in denitrification are FixL and FNR. The oxygen-sensing transcriptional regulator FNR belongs to one of the superfamilies of FNR/CRP proteins. There are several orthologues of FNR, which are presumed to work in similar ways (Fig.

3), such as FnrP in *P. denitrificans*, ANR in *Pseudomonas aeruginosa* and FnrN in *Rhizobium leguminosarum* (Edwards et al 2010, Spiro 2012, Wu et al 2000). Active FNR in *E. coli* exists as a dimer with $[4Fe\text{-}4S]^{2+}$ cluster coordinated by four cysteines which, when exposed to oxygen, is converted to $[2Fe\text{-}2S]^{2+}$ cluster causing reduced tendency for dimerization of FNR, thus lowering its affinity for the DNA targets. FNR is believed to be an activator of the *nar* and *nos* operons. The FixLJ/FixK regulatory system is only reported from Alphaproteobacteria and has mostly been studied in rhizobia. The FixL protein can be both soluble and membrane associated, as found in *Bradyrhizobium japonicum* and *Sinorhizobium meliloti*, respectively (Spiro 2012). Together with its cognate response regulator FixJ, these proteins constitute a two-component regulator FixLJ. In the absence of oxygen FixJ phosphorylates FixL. This, in turn, activates the transcription of the FNR-like *fixK* gene (or similar such as *fixK2* in *Bradyrhizobium diazoefficiens*). This regulator, which itself lacks oxygen sensing, belongs to another CRP/FNR superfamily than FNR described above. The FixK transcriptional activator binds to FNR-boxes upstream several different genes needed for anoxic growth (Mesa et al 2009).

Nitrate, as the first substrate in the denitrification chain, must be detected and transported into the cytoplasm, which requires the presence of NO_3^- sensors in denitrifiers. *Pa. denitrificans* contains NarR, which activates the *nar* operon in response to nitrate/nitrite, however the mechanism of NarR interaction with either nitrate or nitrite is not known. It has been suggested that NarR may respond to NO_3^-/NO_2^- indirectly in a metal based sensing mechanism (Wood et al 2002). In *Ps. aeruginosa* and *Ps. stutzeri* we find NarXL - a two-component sensor regulator system that responds to NO_3^-

and/or NO_2^- (Spiro 2012). NarX is a sensor kinase containing two trans-membrane helices that flank the periplasmic domain, which binds nitrate and nitrite.

Nitric oxide is an intermediate product of denitrification, and due to its high reactivity and cytotoxicity, cells must handle it with care. Its apparently ubiquitous role in the regulation of denitrification is thus hardly surprising. Nitric oxide generally stimulates transcriptional activation through FNR-type regulators which, similar to the FixK-like factors, lack the cysteine motif characteristic to FNR. One such transcriptional factor is NNR in *Pa. denitrificans*, which activates transcription of *nir*, *nor* and *nos* genes encoding the NO_2^-, NO and N_2O reductases, respectively. The results from *in vivo* experiments demonstrate that NNR responds directly to NO. The exact mechanisms of NO sensing remain however elusive in all orthologues of NNR. The NNR from *Pa. denitrificans* and DNR from *Ps. aeruginosa* require haem for their NO-dependent activity.

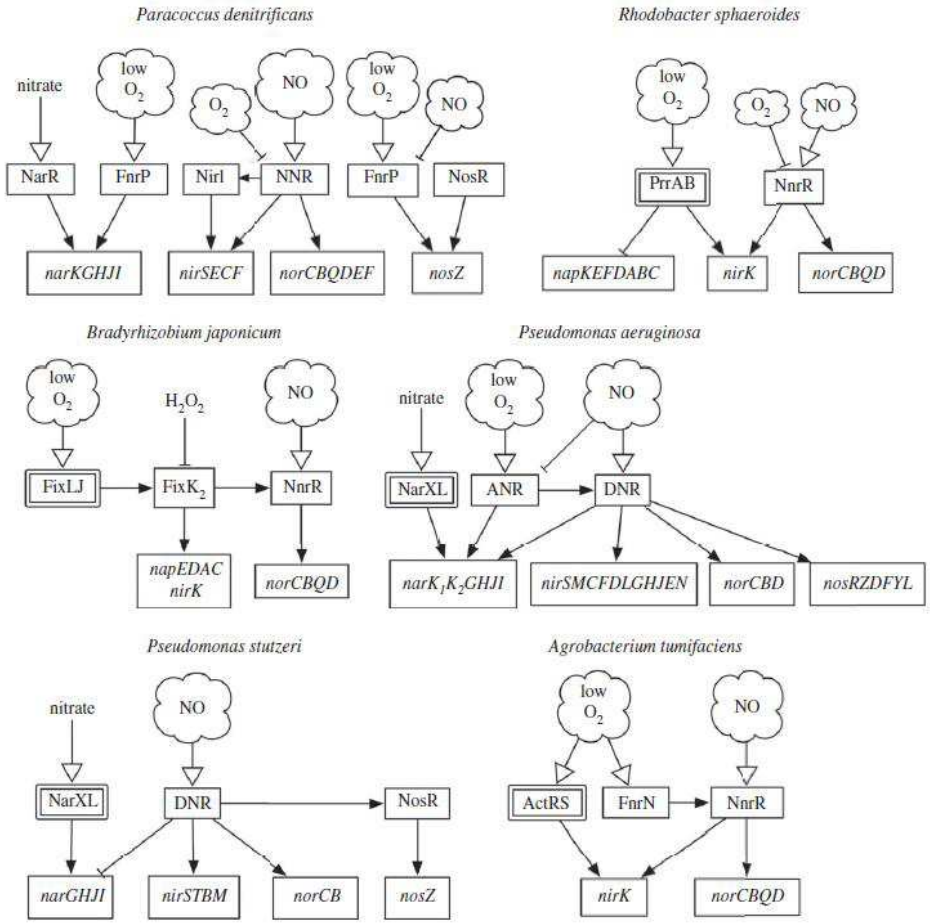

Figure 3. *Regulatory networks controlling expression of denitrification genes in a selection of model organisms. In each case, the diagram is organized into three layers, these being the regulatory signals, regulatory proteins and the structural genes. Thus, arrows between the upper and middle layers represent signalling events, while arrows within the middle layer, and between the middle and lower layers represent gene regulation. Proteins boxed by double lines are two-component systems (histidine kinase and response regulator). Genes and operons associated with denitrification include those designated nap and nar (for periplasmic and membrane-bound nitrate reductase, respectively), nir (nitrite reductase), nor (NO reductase) and nos (N_2O reductase). The nos genes are not shown for B. japonicum since their regulation is not understood, while these genes are absent from Agrobacterium tumifaciens. The ability to express N2O reductase in Rhodobacter strains is variable, and nos gene expression has not been studied in ths genus. Reprint from Sprio (2012).*

Bacterial survival strategies in fluctuating environments

Bacteria are especially ingenious when it comes to survival. They can protect themselves from hostile environmental conditions or avoid antibiotics or immune defense by creating biofilms (Stewart 2002). Soil organisms are notoriously starving for energy, thus they have developed "dormancy" strategies with low or arrested activity (Siebring et al 2014, Wood et al 2013). Yet they are very abundant and remain poised to take up all substrates that become available (Hobbie and Hobbie 2013). Denitrifiers are frequently challenged by fluctuating O_2 concentrations and anoxic spells of variable length (Marchant et al 2017), hence there is a risk that producing a full set of denitrification enzymes would impose an unnecessary metabolic burden if oxygen returns suddenly. On the other hand, when confronted with oxygen depletion, the bacteria must express a minimum of denitrification enzymes "in time", i.e. before oxygen is completely depleted, to avoid entrapment in anoxia without energy to produce denitrification enzymes (Hassan et al 2016, Højberg et al 1997). Expression of the entire denitrification proteome comes with a large metabolic investment, however, and a waste of energy in case oxygen reappears rather shortly (within hours). Thus, it can be expected that when living in nutrient limited environments bacteria cannot afford wasting energy, and the management of protein synthesis and turnover is crucial for their survival.

Truncations and denitrification gene assemblages

Organisms lacking one or more of the denitrification steps occur frequently in the environment (Graf et al 2014, Lycus et al 2017, Shapleigh 2013).

Different assemblages of denitrifying reductases have been reported: organisms may contain both nitrate reductases, but generally only one type of the nitrite, nitric oxide, and nitrous oxide reductases (Graf et al 2014, Lycus et al 2017, Roco et al 2016). Truncations may occur in denitrification phenotypes for several reasons: 1) absence of a gene, 2) a mutation in the functional genes encoding the reductases, or in genes involved in their regulation, 3) transcriptional regulation as well as posttranscriptional phenomena may come into play. Studying truncated denitrifiers in pure cultures under laboratory conditions could be difficult, as they may accumulate toxic intermediates, that are otherwise scavenged by neighboring cells in natural settings, for example: denitrifiers accumulating NO to toxic concentrations leading to growth stagnation and finally death of the cultures (Bergaust et al 2008). Some of the truncations, especially those that do not contribute to N_2O production, but can readily reduce it, are of special interest as they may be used in future environmental applications for mitigation of N_2O greenhouse gas emissions. Nevertheless, a true denitrifier must carry a *nir* gene. Both *nirS* and *nirK* code for reductases performing the defining reaction of denitrification, still the gene ecoding NirS is recognized as representative of canonical denitrifiers and *nirK* as being more likely associated with nitrite reducers with truncated denitrification pathways (Graf et al 2014).

Influence of the pH on soil denitrification

From the early observations till now – historical overview

The pioneering work investigating the indirect and direct influence soil pH on the denitrification process originates from the early fifties of the past century. The study by Wijler and Delwiche (1954) showed for the first time the importance of N_2O in the nitrogen cycle. The authors were capable of providing quantitative data for different nitrogen forms, which allowed them to assess the impact of wide range of pH levels on denitrification in soils. The main conclusions from these studies show the retarded denitrification process at pH below 6, as well as the increasing contribution of N_2O as an end-product with decreasing pH of the soils. They also stress the importance of NO at pH<6. Another report (Nommik 1956), clearly demonstrates that with decreasing pH in soils, the amounts of N_2O gas liberated from denitrification, increase. These two with, the support from other papers at the time led to the general acceptance of the pH as one of the major regulators of denitrification process in soils. Large improvements in instrumentations and methodology allowed researchers in the seventies to develop refined hypothesis about the subordination of N2O reduction to soils' pH. In the 1974 Focht presented the hypothesis that N_2OR enzyme may be sensitive to low pH. Finally, the summary by Knowles in 1982 yet again corroborates the major findings from the past 30 years of research: the overall denitrification is retarded at pH<6; the N_2O/N_2 product ratio of denitrification is negatively correlated with the pH of soils; the NO becomes an important factor at pH<6.

N_2O emissions from acidic soils

There is ample evidence coming from later studies supporting the same observations in field scale studies. Nevertheless, there are also some evidence from pure cultures studies of model denitrifying bacterium *Paracoccus denitrificans*, showing the same pH dependency of N_2O reduction (Bergaust et al 2010). The refined study by Bergaust revealed that lack of the N_2OR function at pH 6 is due to a posttranscriptional phenomenon, since the transcription of *nosZ* was detected at both pH 7 and pH 6. The transcription at pH 6 was twofold lower that at pH 7, still not low enough to explain the complete lack of the N_2O reduction. Similar results as those found for pure cultures studies were found in microcosms experiments of soils of different pH as well as denitrifying bacterial communities extracted from soil and exposed to different pH regimes in the laboratory (Liu et al 2010, Liu et al 2014). In fact N_2O reduction occurs in low pH soils (pH<6), as reported by van den Heuvel et al (2010) and Lycus et al (2017). The organisms capable of reducing N_2O under acidic conditions were in both studies representatives of the genus *Rhodanobacter*. Interestingly, as reported by Lycus et al (2017) the investigated *Rhodanobacter* was unable to perform full-fledged denitrification (NO_2^- to N_2) at neutral pH conditions.

Aim and outline of papers

The aim of my PhD work was to expand the current knowledge about the regulatory biology of denitrification in bacteria. I focused on revealing the mechanisms behind two intriguing phenomena introduced by Bergaust et al (2010, 2011) with potentially big impact on the N_2O emissions from these

bacteria: a drastic drop in electron flow observed during transition to anaerobic respiration, and the problem of synthesizing functional enzymes for N_2O reduction under acidic conditions. I also set out to determine if these phenomena, studied in the model denitrifier *Paracoccus denitrificans*, exist in a wider variety of ecologically relevant soil bacteria. . The research involved colleagues at NMBU, Cornell University and University of East Anglia with complementary skills.

More specifically, we:

1. Designed an isolation protocol to identify all possible combinations of truncated denitrification chains ($NO_3^-/NO_2^-/NO/N_2O/N_2$). Of 176 isolates from two soils (pH 3.7 and 7.4), 70 performed at least one of these reduction steps. Gas kinetics and electron flow calculations revealed that several features with potential impact on N_2O production, reported from model organisms, also exist in these novel isolates, including denitrification bet hedging. Most of the strains tested showed the expected inability to reduce N_2O when incubated under acidic conditions with one exception, a *Rhodanobacter* sp., which reduced N_2O only at low pH. (Paper I)
2. Developed methods to detect the expression of Nir and N_2OR in the model strain *Paracoccus denitrificans* which allowed us to demonstrate that these bacteria, when exposed to anoxia, express N_2OR in all cells while bet hedging with respect to Nir and Nor. This strategy allows them to secure anaerobic respiration with a minimum investment. Moreover, we show that the denitrification proteome is preserved in persister cells in response to oxygenation after anoxic spells. The findings add new dimensions to our understanding of how

denitrifying bacteria regulate anoxic respiration, and identifies novel regulatory traits that minimize N_2O emissions. (Paper II)

3. Developed a culturing method for *P. denitrificans* that secured stringent control over the pH experienced by the individual bacterial cells, and analyzed the extracted N_2OR from cells grown at pH 6.0 and 7.0. The results so far demonstrated the presence of N_2OR in comparable amounts in cells grown under both pH regimes, but showed absence of copper in N_2OR developed at pH 6.0. The findings corroborate our hypothesis of impaired N_2OR assembly in the periplasm under low pH conditions. (Paper III)

MATERIALS AND METHODS

The methods used are described in the enclosed papers and references therein, therefore only the core equipment and methods will be highlighted in this thesis.

ROBOTIZED INCUBATION SYSTEM AND NITRITE MEASUREMENTS (courtesy Natalie Lim):

The robotised auto-sampling system designed by our research group gives us full control throughout long sampling times. The system is also able to measure multiple gases including N_2, which is often difficult to measure due the risk of leakage from the surrounding air (Molstad et al 2007).

Sampling frequency is fully robotised and computer controlled, ensuring that the necessary gas measurements are performed continuously throughout

the day for as long as required, and in a precise and repeatable manner. Additionally, the system allows up to 40 flasks to be incubated at constant temperatures above or below ambient temperature. The robotised incubation system has also been used in a variety of experiments, ranging from pure cultures to soil samples (Bergaust, *et al.*, 2010, Falk, *et al.*, 2010).Air-tight serum flasks filled with the liquid cultures or soil samples are sealed with butyl-rubber septa and aluminium crimps, and may be made anaerobic via a series of helium washing cycles.

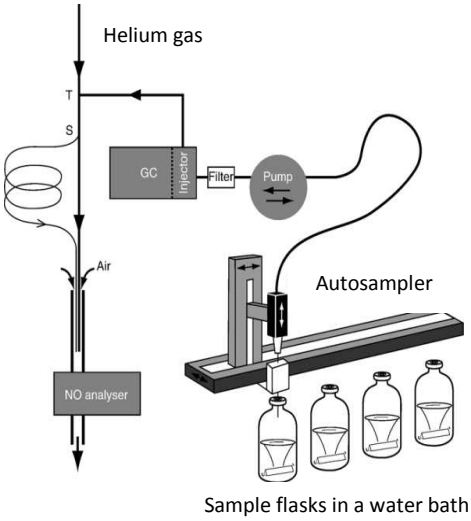

Figure 4. A schematic diagram of the robotised incubation system, and the gas flow to the gas chromatograph (GC) and NO gas analyser. The gases are sampled from flasks with a peristaltic pump and are split between a GC and an NO analyser, using helium as the carrier gas. Figure adapted from Molstad et al 2007

Nitrite measurements were made using technology developed by chemists and often used nowadays in diagnostics and research in medicine (Cox, 1980). This method accurately measures nitrite concentrations down to nanomolar levels. Nitrite quantities are measured immediately after sampling, reducing the likelihood of nitrite degradation due to storage. Samples for nitrite measurement are injected into the system via the "Injection port with septa" (see Fig. 5). The entire system is kept oxygen-free

by continuous helium flow bubbling through the reducing agent via the “Frit”. A reducing agent, 1% w/v sodium iodide (NaI) in acetic acid, immediately reduces the injected nitrite to NO and is measured by chemiluminescence using a NO analyser (labelled as ‘NOA’ in Figure 5).

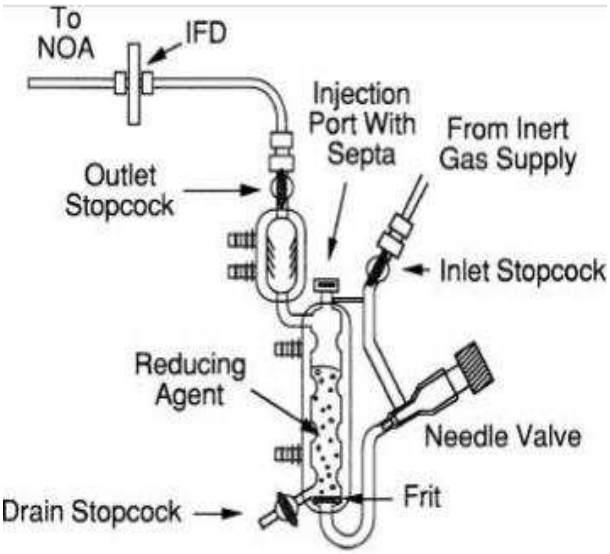

Figure 5. Nitrite measurement set up. Nitrite is reduced to nitric oxide, and is measured by the Nitric Oxide Analyser (NOA). Image from the Sievers Nitric Oxide Analyzer NOA 280i Operation and Maintenance Manual.

IMMUNOASSAYS FOR DTETECTION, VISUALISAZTION AND QUATIFICATION OF NosZ IN *Pa. denitrificans*

Recombinant partial N_2O reductase (NosZ) from *Pa. denitrificans* was custom synthetized by Cusabio Biotech. The polypeptide was then used for immunization of chickens in order to obtain polyclonal anti-NosZ antibodies. Affinity purified anti-NosZ polyclonal IgY antibodies were obtained, purified and delivered by Norwegian Antibodies. The portions of antibodies were biotinylated (Thermo Fischer Scientific) and used for detection of NosZ in

formaldehyde fixed, permeabilized cells of *Pa. denitrificans.* The bright blue-fluorescent dye Pacific Blue – Streptavidin conjugate (Thermo Fischer Scientific) was used for the visualization of NosZ by fluorescence microscopy (details in Paper II and III).

Cocktail enzyme-linked immunosorbent assay (Cocktail-ELISA) was developed based on Treder et al (2009), with some modifications. The detection was proceeded in two-step reaction: first, 96 wells plates were coated with anti-NosZ IgY, where standardized and unknown samples were incubated with biotinylated Anti-NosZ IgY antibodies followed by washing and additional incubation with avidin-HRP conjugate (Thermo Fischer Scientific) in the next step. The TMB substrate (Thermo Fischer Scientific) was used to for developing reaction, which was terminated by addition of 2M H_2SO_4.

SDS-PAGE and Western Blot were performed according to the standardized producers protocols (Biorad). Anti-NosZ IgY (Norwegian Antibodies) and rabbit anti-chicken yolk immunoglobulin polyclonal antibodies, HRP conjugated (Cusabio) were applied for detection and visualization of NosZ protein.

RESULTS AND DISCUSSION

Isolation of new denitrifiers

With the aim to validate the importance of observations generated by studies of model denitrifiers we performed a newly designed isolation program, which proved to be successful in isolating a large number of diverse soil denitrifiers originating from soils of two contrasting pHs, as shown in Figures 2 and 3 in Paper I. By the design of the protocol, we were able to capture all possible truncations of denitrification chain (except organisms reducing only NO). The majority of isolates were organisms respiring nitrate, which is not surprising, as NO_3^- is the most favorable electron acceptor after O_2. Among denitrifiers *sensu stricto*, the truncated ones were more abundant than the full-fledged ones (Fig. 1 in Paper I). We obtained 9 isolates performing NO_3^- reduction to N_2, eight of them coming from pH 7.3 soil and only one isolated from acidic soil of pH 3.8, which supports previous observations that optimal pH for the denitrification process is between 7 and 8, thus denitrifiers are thought to be more abundant in those soils. The presence of a single isolate performing the whole denitrification in the low pH soil reflects the previous observations by van den Heuvel et al (2010). Surprising and unexpected was the fact that *Rhodanobacter* sp. isolated from low pH soil was able to reduce N_2O when grown in medium of pH 5.7, however failed to do so at neutral pH. Detailed phenotypic characterization of isolated denitrifiers revealed large variations in the concentrations of accumulated intermediate products, corroborating the relevance of the observations in model denitrifiers. Interestingly, yet being rather an indication than a proof, was the observed electron flow pattern to sequential electron acceptors during transition from aerobic respiration to denitrification in these organisms. We have observed

the same patterns in fresh, non-domesticated isolates (bottom panels in Figs. 4 and 5 in Paper I) as well as in well studied organisms such as the model organism *Pa. denitrificans*, adapted to laboratory conditions, which shows a sophisticated bet hedging strategy with respect to expression of denitrification genes (Paper II).

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 our study (Paper I), where we found a mismatch between genetic potential and the corresponding reduction of N_2O 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 counteracting those that act as sources.

Bet hedging in denitrifiers as a fitness trait and its impact on N_2O reduction

By coupling dynamic modelling with a stringent experimental approach we were able to discover phenomena occurring in a well studied organism that have been readily overlooked for decades. We provide a proof for a very sophisticated trait in *Pa. denitrificans*, which improves its fitness in environments with oxygen fluctuations by minimizing the cost spent on the development of a respiratory proteome during transition from aerobic respiration to denitrification. To our knowledge, such bet –hedging has never been presented in a denitrifying bacterium. The common notion about expression of denitrification reductases is that all cells express the denitrification proteome in response to changing conditions (decreasing oxygen tension, presence of nitrate) which does not seem to be the case for *Pa. denitrificans.* We have had previous indications that *Pa. denitrificans*, as well as other denitrifiers, express N_2OR earlier than other reductases. This seemed to be a reasonable strategy as N_2O reduction conserves energy and N_2O as a gas is easily reachable if emitted by neighboring cells producing it (for the latter reason it can easily diffuse out of the system and reach atmosphere). We also have had some indications originating from scrutinized gas kinetics of denitrifying batch cultures of *Pa. denitrificans*, that a minority of the cells may express NirS during the transition from oxic conditions to anoxia as demonstrated in the Figure 1 of Paper II. These observations were supported by modelling. The model assuming low probability of initial transcription of *nirS* in *Pa. denitrificans* was able to explain the observed gas kinetics and drastic drop in electron flow towards acceptors during transition from aerobic to anaerobic respiration (Hassan et al 2014). We have also investigated the fate of the denitrification proteome after oxygen

reappearance, by exposing the anaerobically grown, actively denitrifying cells, to aerobic conditions and allowing them to grow for a certain number of generations. Cells were then tested for their denitrifying activity in an entrapment assay (described in details in Supplementary material for the Paper II). The results were surprising again, clearly indicating that the number of NirS positive cells remains constant in aerobically grown culture (Fig. 5 in Paper II). Time lapse experiment scrutinizing the fate of the denitrification proteome after exposure to oxygen revealed that, in fact, NirS (most likely together with Nor) are preserved in cells that stop growing – we call them persister cells. These persister cells retain their denitrification proteome, when the others dilute it while growing aerobically (Fig. 6 in Paper II). After sudden shortage of oxygen, cells with denitrification reductases switch to utilizing nitrogen oxyanions immediately and without the need for *de novo* synthesis of the reductases.

This bet-hedging assures the survival of the organism in the environment, where the oxygen availability changes rapidly and unpredictably. By expressing N_2OR in all cells, *Pa. denitrificans* assures the minimum energy sufficient for sustaining, although much lower, activity during short anoxic spells without the need for expression of other reductases. In case the anoxia will be prolonged, these cells can gradually develop the complete denitrification machinery. Such bet-hedging appears to be widespread among denitrifying bacteria, as judged from the diauxic nature of their transition from oxic to anoxic respiration. This adds new dimensions to our understanding of how denitrifying prokaryotes regulate anoxic respiration, and identifies novel regulatory traits that minimize N_2O emissions.

Possible explanation why acid soils emit so much N_2O

Soil denitrification accounts for up to 70% of global anthropogenic N_2O emission, with acidic soils being the major sources of this emission. The latter have been observed for several decades with yet no proposed mechanism responsible for the phenomenon. Although there are several microbial sources of N_2O, to date, the only known sink for it is an enzyme nitrous oxide reductase (N_2OR). This enzyme contains two copper sites: Cu_A similar to the one of cytochrome oxidases, and the unique tetranuclear Cu_Z site. The CuA donates electrons to the active site of the enzyme being the Cu_Z site. The assembly and copper maturation of N_2OR takes place in the periplasm and requires several accessory proteins. It has been demonstrated that Gram-negative bacteria are not capable of controlling the periplasmic pH as well as they do in the cytoplasm, indicating that under hostile ambient pH conditions the periplasmic environment can strongly be affected. This, coupled to the field observations and the results from laboratory experiments investigating the influence of pH on denitrification $N_2O/(N_2O+N_2)$ product ratio, led us to generate the hypothesis that suboptimal, low pH hampers copper maturation of N_2OR in acidic soils, thus causing N_2O fluxes from these soils as presented in Figure 1 in Paper III. Based on our experiences with the model organism *Paracocus denitrificans* and the knowledge we gained from it, we have developed a method in order to validate our hypothesis. Few very important pitfalls had to be overpassed in order to control the phenotype in *Pa. denitrificans* at pH 6: strong buffering system to assure a steady pH conditions, as denitrification *per se* requires H^+, thus contributes to the local raise of pH especially if cell aggregation comes into play; the optimum pH for growth of *Pa. denitrificans* is far from acidic, thus the yield from culture raised

at pH <6 is low. Taking the precautions, we grew *Pa. denitrificans* at low cell densities (OD_{660}<0.15), provided with vigorous stirring and 250 mM sodium phosphate buffer at pH 6.0 (such stringent control was not required at pH 7) and harvested denitrifying cells when 50% of provided nitrate was converted to N_2O, with no detected N_2 production. Cells were pelleted, rinsed and stored frozen until proteins were extracted. We extracted the periplasmic fraction of the protein from the same amounts of wet weight of cells obtained from both pH 6 and pH 7. Periplasmic extracts were size fractionated (30-300 kDa) and finally concentrated to 500 µl of total volume. These were used for analysis. The Western Blot analysis confirmed the presence of N_2OR in periplasmic extracts originating from both preps. This was also corroborated by immunocytostaining of the whole cells raised at pH 6 and pH 7, targeting the N_2OR (Fig. 3 in Paper III). The total copper concentration as well as the N_2OR concentration in both preps were assessed by ICPMS and ELISA, respectively. The results showed 104 µM vs 10 µM Cu, and 11 vs 2.7 $\mu g*ml^{-1}$ N_2OR, respectively, in the pH 7 and in the pH 6 periplasmic preps. These results gave a strong support to our hypothesis of hampered N_2OR maturation under acidic conditions. We tried to perform spectroscopic studies by EPR, however the first results were not conclusive. We could see a strong Cu signal spectrum from the pH 7 prep, but it was greatly overridden by copper-containing proteins other than N_2OR, like for example pseudoazurin. The pH 6 spectrum showed, however, no Cu signal at all, supporting our idea of lack of copper incorporation into the N_2OR apoenzyme under suboptimal low pH conditions. The EPR studies have to be performed again, including larger amounts of purified N_2OR from cells grown under both pH regimes, in order to be conclusive.

Future work on this project involves optimization and upscaling of our present protocol in order to produce sufficient amounts of cells for protein extraction and N_2OR purification. The studies will also involve the proteomic approach in order to investigate other copper containing proteins involved in N_2OR maturation. Finally, the N_2OR purified from both pH treatments will be analyzed for its activity in a methyl-viologen assay.

CONCLUSIONS AND FUTURE PERSPECTIVES

The work presented in this thesis has improved our understanding of the regulatory biology of denitrification, stressing the relevance of the observations in model organisms. Newly isolated denitrifying bacteria exhibit large phenotypic variation at the transition from aerobic to anaerobic respiration, yet they handle the intermediate products of denitrification (NO_2^-, NO and N_2O) completely differently. Although model organisms for denitrification have been scrutinized for decades, and thanks to stringent experimental approaches supported by modelling, we were still able to unravel sophisticated bet-hedging mechanisms occurring in *Paracoccus denitrificans.* This underlines the importance of the choice of relevant parameters for studying model organisms. The phenomenon discovered in *Pa. denitrificans* excellently reflects how denitrifying bacteria adapt in order to improve their fitness in fluctuating environments. It also stresses the environmental significance of this strategy, as most of the isogenic cells in a population are able to reduce the greenhouse gas N_2O, but yet do not produce it for a long time. Our studies on novel isolates strongly suggest that such bet-hedging may be spread across different taxa.

The empirical knowledge available for almost seven decades did not bring any explanation for the emissions of N_2O from acidic soils. We seem to be very close to the understanding of the mechanism laying behind it, which can help us to counteract them in the future. Our first evidences presented here support our hypothesis of a hampered N_2OR maturation at low pH. The hypothesis needs however further testing by spectroscopy and activity assays in order to be proven.

Most of my work within the thesis was performed in pure culture studies of *Paracoccus denitrificans*, which is a rather peculiar denitrifier; it expresses N_2OR prior other reductases, exhibits ingenious bet-hedging and still is being utilitarian for explaining many environmentally important phenomena.

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Paper I

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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 NO_2^-, NO, and N_2O 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 ($NO_3^-/NO_2^-/NO/N_2O/N_2$). Of 176 isolates from two soils (pH 3.7 and 7.4), 30 were denitrifiers *sensu stricto*, reducing NO_2^- to gas, and five capable of N_2O 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 N_2O production, reported from model organisms, also exist in these novel isolates, including denitrification bet-hedging and control of $NO_2^-/NO/N_2O$ 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 N_2O under acidic conditions, one isolate identified as *Rhodanobacter* reduced N_2O 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 NO_3^- and/or NO_2^- is reduced to gaseous forms of nitrogen. Complete denitrification of NO_3^- to N_2 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 N_2O generating nitric oxide reductases cNor, qNor, or qCuNor; and the two types of nitrous oxide reductases N_2OR (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 N_2O (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 N_2O 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 N_2O. 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 N_2O/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 (pH_{H2O} 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} (pH_{H2O} 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 ± 2 °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 (NO_2^-+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

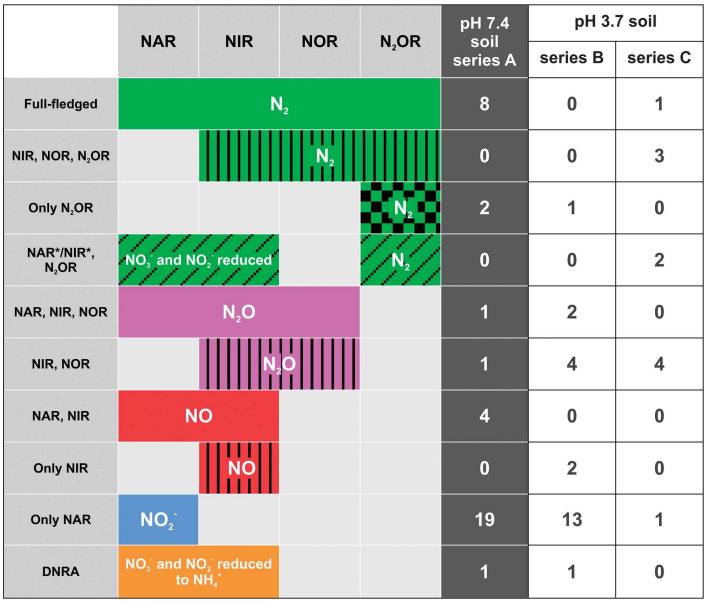


Figure 1 Phenotypic classification of new soil isolates able to perform at least one step in the dissimilatory reduction of NO_3^-, NO_2^-, NO, and N_2O. The phenotypes were designated as NAR, NIR, NOR, and N_2OR. The end points (N_2, N_2O, NO, NO_2^-, or NH_4^+) are indicated inside the colored bars. 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*, N_2OR indicates organisms which reduced NO_3^-/NO_2^-, 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 N_2O.

pH as the respective pre-cultures) with 2 mM NO_3^- for the complete denitrifiers and 1 mM NO_2^- for those lacking nitrate reductase. The air in the headspace had been replaced with He and 1% (v/v) O_2. The cultures were incubated with vigorous stirring at 23 °C in a robotized incubation system enabling the quantification of O_2, CO_2, NO, N_2O and N_2 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 NO_2^- and OD_{660} 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 NO_3^-, NO_2^-, NO, N_2O, and N_2 allowed the determination of a phenotype for each isolate able to perform at least one step in the reduction of NO_3^- to N_2 (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

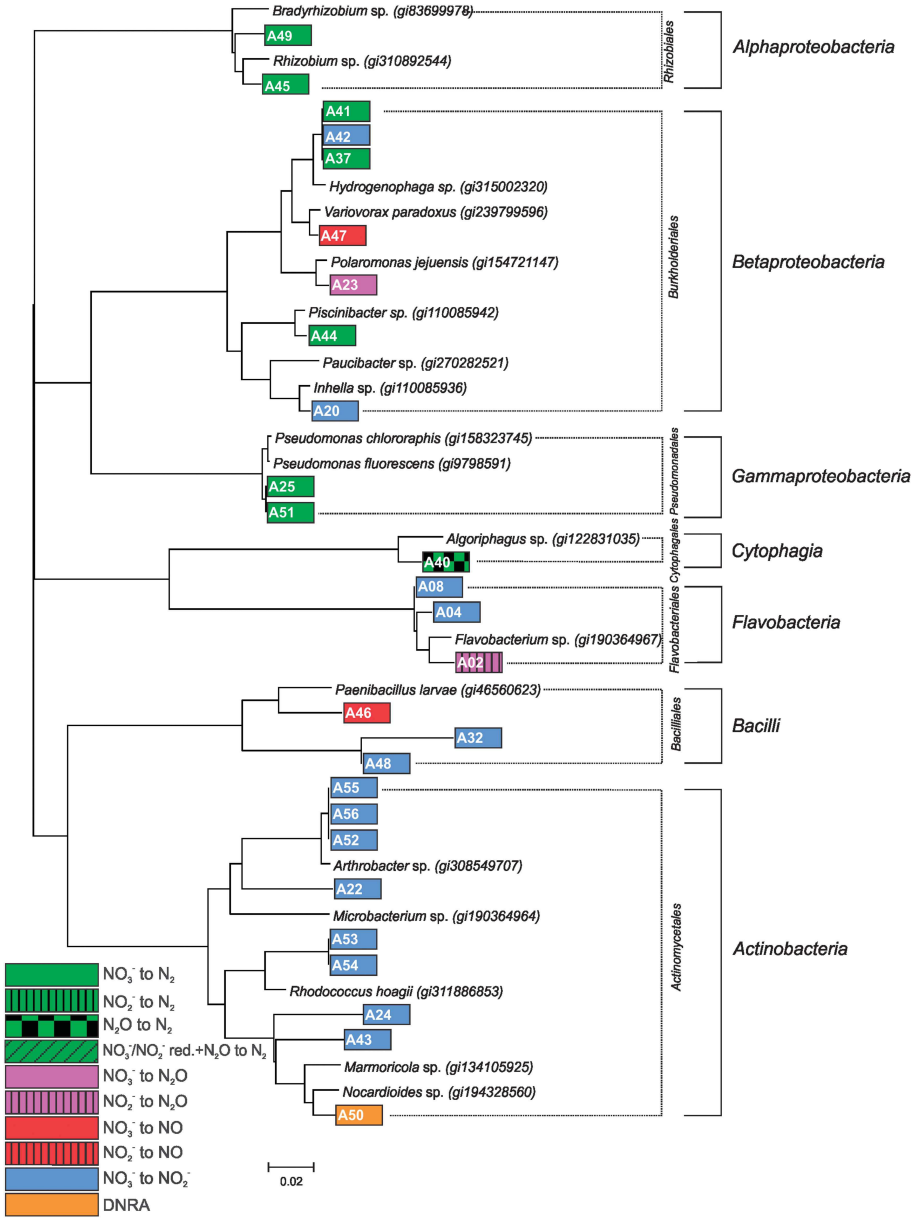


Figure 2 Phylogenetic tree based on neighbor joining (MEGA 6, Tamura *et al*., 2013) of 16S rRNA gene sequences. The tree shows the relationship between the bacterial isolates from the high pH soil (series A), identified as having the capacity of dissimilatory reduction of at least one of the tested 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*, *Pseudomonales* 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 *Burkholderiale*s; Figure 2) and two from the low pH soil (orders *Xanthomonadales* and *Enterobacteriale*s, 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 endpoint 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

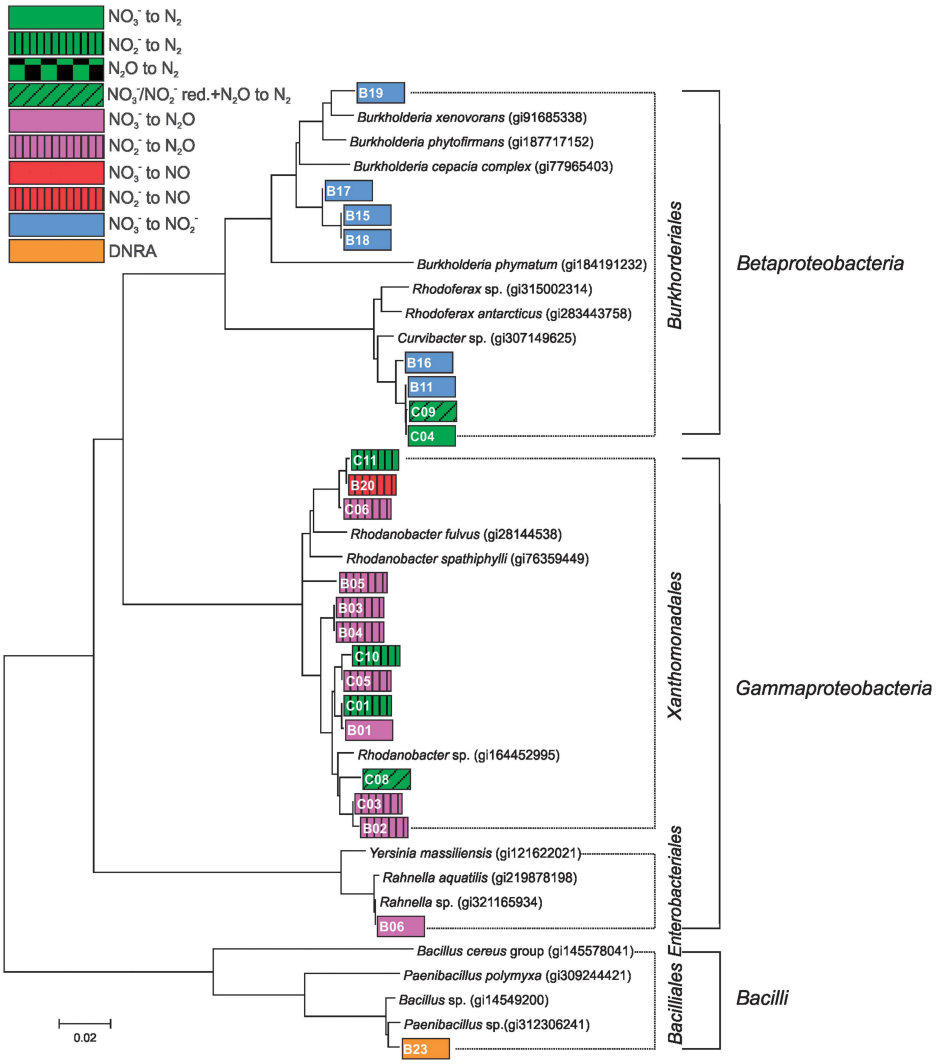


Figure 3 Phylogenetic tree based on neighbor joining (MEGA 6, Tamura *et al*., 2013) of 16S rRNA gene sequences. The tree shows the relationship between the bacterial isolates from the low pH soil (series B and C), identified as having the capacity of dissimilatory reduction of at least one of the tested 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 CO4 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 (N_2OR): 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 N_2OR 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 ~5 μM for most isolates, but the *Rhodanobacter* isolates C01 and C05 started to denitrify at 16–17 μM 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 μmol 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 (~10 μM 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 nmol ($vial^{-1}$), which corresponds to 0.15 μM 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 μM in the liquid (3.7 μmol $vial^{-1}$). 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 μM, 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 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, 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,

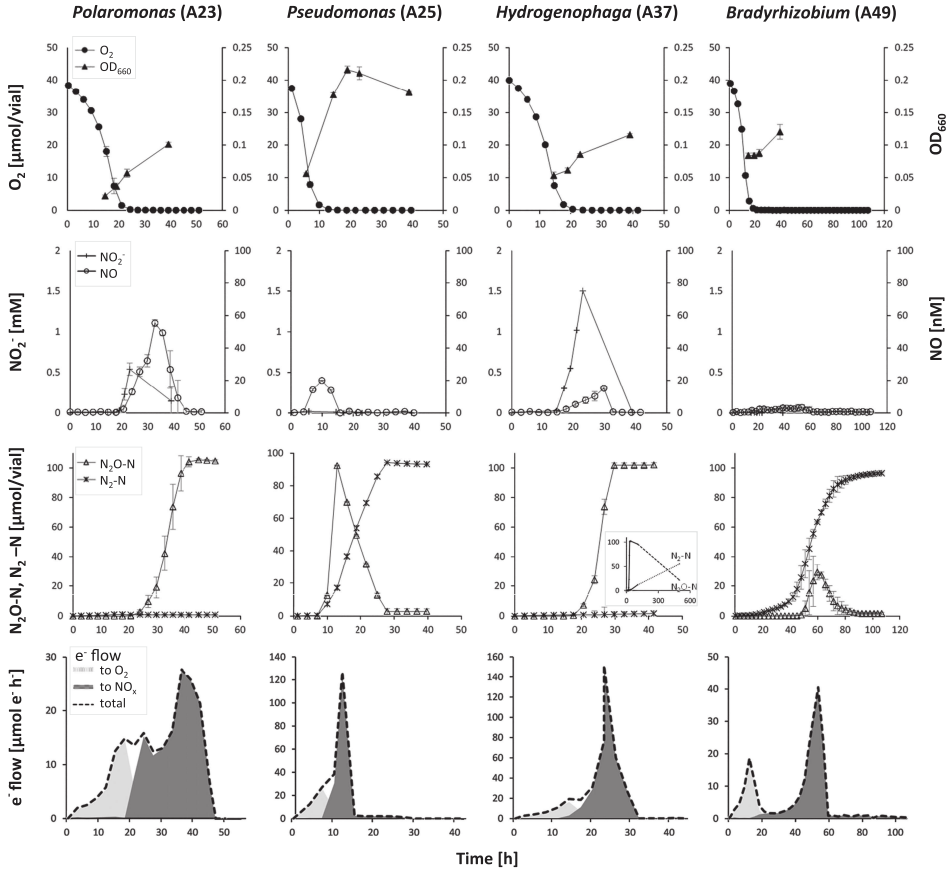


Figure 4 Denitrification regulatory phenotypes of selected isolates from the high pH soil. Aerobically prepared bacterial cultures were inoculated into sealed 120 ml medical vials containing 50 ml 1/10 TSB medium of pH 7.3 supplemented with 2 mM KNO_3. Headspace atmosphere after repeated helium flushing was adjusted with pure O_2 to 1% concentration (v/v) prior inoculation. The vials were incubated at 22 °C with vigorous stirring. Gas kinetics (O_2, NO, N_2O, N_2), NO_2^- concentrations and OD_{660} 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 O_2, 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 NO_3^- and NO_2^- 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 NO_3^-/NO_2^- 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

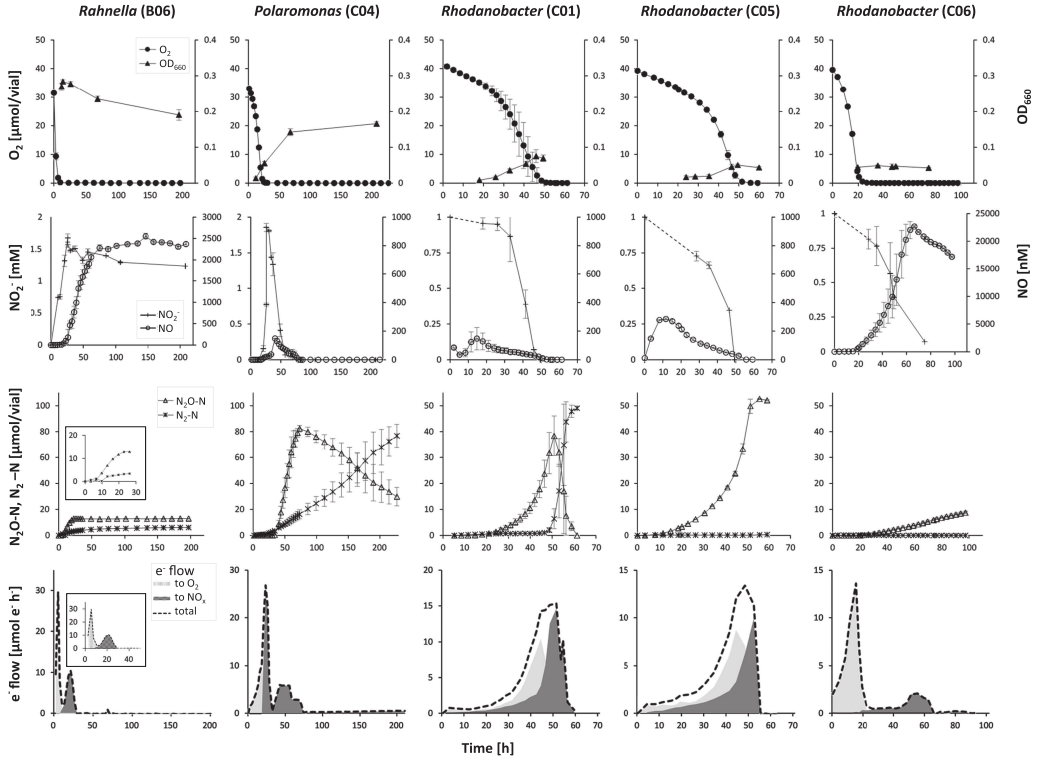
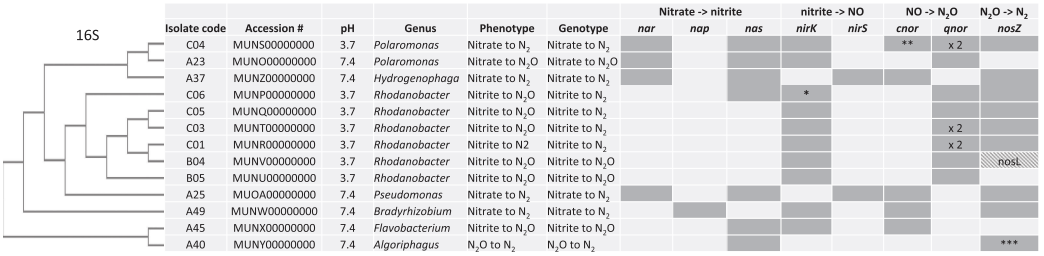


Figure 5 Denitrification regulatory phenotypes of selected isolates from the low pH soil. Aerobically prepared bacterial cultures were inoculated into sealed 120 ml medical vials containing 50 ml 1/10 TSB medium of pH 5.7 supplemented with 2 mM KNO_3 for isolates B06 and CO4, and 1 mM KNO_2 for the *Rhodanobacter* isolates C01, C05, and C06. Headspace atmosphere after repeated helium flushing was adjusted with pure O_2 to 1% initial concentration (v/v) prior inoculation. The vials were incubated at 22 °C with vigorous stirring. Gas kinetics (O_2, NO, N_2O, N_2), NO_2^- concentrations and OD_{660} 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 O_2, nitrogen oxides (NO_x) and the total electron flow.



Isolate code	Accession #	pH	Genus	Phenotype	Genotype	Nitrate -> nitrite *nar*	Nitrate -> nitrite *nap*	Nitrate -> nitrite *nas*	nitrite -> NO *nirK*	nitrite -> NO *nirS*	NO -> N_2O *cnor*	NO -> N_2O *qnor*	N_2O -> N_2 *nosZ*
C04	MUNS00000000	3.7	*Polaromonas*	Nitrate to N_2	Nitrate to N_2						**	x 2	
A23	MUNO00000000	7.4	*Polaromonas*	Nitrate to N_2O	Nitrate to N_2O								
A37	MUNZ00000000	7.4	*Hydrogenophaga*	Nitrate to N_2	Nitrate to N_2								
C06	MUNP00000000	3.7	*Rhodanobacter*	Nitrite to N_2O	Nitrite to N_2				*				
C05	MUNQ00000000	3.7	*Rhodanobacter*	Nitrite to N_2O	Nitrite to N_2								
C03	MUNT00000000	3.7	*Rhodanobacter*	Nitrite to N_2O	Nitrite to N_2							x 2	
C01	MUNR00000000	3.7	*Rhodanobacter*	Nitrite to N2	Nitrite to N_2							x 2	
B04	MUNV00000000	3.7	*Rhodanobacter*	Nitrite to N_2O	Nitrite to N_2O								nosL
B05	MUNU00000000	3.7	*Rhodanobacter*	Nitrite to N_2O	Nitrite to N_2O								
A25	MUOA00000000	7.4	*Pseudomonas*	Nitrate to N_2	Nitrate to N_2								
A49	MUNW00000000	7.4	*Bradyrhizobium*	Nitrate to N_2	Nitrate to N_2								
A45	MUNX00000000	7.4	*Flavobacterium*	Nitrite to N_2O	Nitrite to N_2O								
A40	MUNY00000000	7.4	*Algoriphagus*	N_2O to N_2	N_2O to N_2								***

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 NO_3^-, using N_2O 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 N_2OR, but would overlook the phenotypes 'NIR, NOR, N_2OR'; 'Only N_2OR'; '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 ± N_2OR, 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 N_2O reductase, but lack one or more of the other denitrification genes, are of particular interest since they are potential sinks for the greenhouse gas N_2O. The 'NIR, NOR, N_2OR' 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 NO_2^-, as well as N_2O, produced by other members of the microbial community. They could thus act both as a source and a sink for N_2O. Three isolates were sole N_2O reducers (phenotype 'Only N_2OR'), leaving nitrate and nitrite untouched in the end-point analysis. As such, they will not produce any N_2O, but can reduce N_2O 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 N_2O emissions from cultured fields (Gao *et al.*, 2016).

Altogether twelve isolates were found which had N_2O 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 N_2O 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 N_2O 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 N_2O reductase at low pH (Bakken *et al.*, 2012). One of the *Rhodanobacter* isolates (C01) did, however, reduce N_2O at low pH, an interesting observation that calls for detailed biochemical investigations that may shed new light on the regulation of denitrification and N_2O emissions in low pH soils.

All the *Rhodanobacter* isolates lacked genes coding for the dissimilatory NO_3^- reductases (Nar or Nap). Such organisms have probably been overlooked in isolation studies where NO_3^-, but not NO_2^-, 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 counteracting those that act as sources.

Conflict of Interest

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on The ISME Journal website (http://www.nature.com/ismej)

Supplementary material to article "Phenotypic and genotypic richness of denitrifiers revealed by a novel isolation strategy" by Lycus et al.

Table S1. Phenotypic classification of new soil isolates able to perform at least one step in the dissimilatory reduction of NO_3^-, NO_2^-, NO and N_2O. 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. The cultures were incubated with vigorous shaking to secure oxic conditions, thus preventing denitrification. Most cultures were incubated in small vials (12 ml total volume), except for some cases where 120 ml vials were used (indicated with [a)], see below). 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, liquid and headspace samples were taken to quantify NO_3^-, NO_2^- , NO, N_2O and N_2. For samples in which nitrogen imbalance was detected, the concentration of NH_4^+ was also measured. The designated phenotypes follow Table 1 (in the article).

Series A: Bacterial isolates from high pH soil (pH 7.4) isolated on agar plates (1/10 Tryptic Soy Agar, pH 7.3)
Series B: Bacterial isolates from low pH soil (pH 3.8) isolated on agar plates (1/10 Tryptic Soy Agar, pH 7.3)
Series C: Bacterial isolates from low pH soil (pH 3.8) isolated agar plates (1/10 Tryptic Soy Agar, pH 5.7)

"Initial" indicates the initial amounts of provided NO_3^-, NO_2^- and N_2O at the beginning of the incubation; rows with bright grey background are those where initial NO_3^-/ NO_2^- was measured. Rows with dark grey indicate samples for which NO_3^- measurements are not provided.
Delta values (Δ) represent net consumption/production of indicated component.
NAR; NIR; NOR; NOS; DNRA – represent respectively: nitrate, nitrite, nitric oxide, nitrous oxide reduction seen as consumption/production of relevant intermediates.

Statistical analysis: all measurements were done in triplicates, except those marked [b)] (see below), and One-way ANOVA test was performed (*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$).

Criteria for the end-point phenotypic classification (see also Table 1 in the article):
Nitrate reduction: more than 50% of the provided NO_3^- was reduced and recovered as $NO_2^-/NO/N_2O/N_2$
Nitrite reduction: more than 50% of the provided NO_2^- was reduced and recovered as $NO/N_2O/N_2$
Nitric oxide reduction: more than 50% of the provided NO_3^- and/or NO_2^- was reduced and recovered as N_2O/N_2
Nitrous oxide reduction: more than 50% of the provided N_2O was reduced to N_2

Organisms were classified as DNRA when more than 75% of NO_3^- and/or NO_2^- was reduced, but less than a 50% percent of total N was recovered as a gas.
[a)] Cultured in 120 ml vials (50 ml medium plus 70 ml headspace); [b)] Culture lost from the collection, not viable after long-term storage; statistical analysis: a two-sample t-test on the summarized data was performed. The tests were performed at 0.05 and 0.01 significance.

Full-fledged denitrifiers from pH 7.4 soil, series A: 8 isolates (A25, A37, A41, A44, A45, A49, A57 and A51)

Sample	NO3-	ΔNO3-	NO2-	ΔNO2-	NO	ΔNO	N2O	ΔN2O	N2	ΔN2						N sum
	µmol	µmol	µmol	µmol	nmol	nmol	µmol	µmol	µmol	µmol	**NAR**	**NIR**	**NOR**	**NOS**	**DNRA**	
Initial	4	0.0	4	0.0	0.0	0.0	6.2	0.0	0.0	0.0						14.2
A25	***0.1	-3.9	***0.0	-4.0	0.3	0.3	0.0	-6.2	***22.8	22.8	+	+	+	+	-	22.9/14.2
Initial [a)]	50	0.0	50	0.0	0.0	0.0	82.3	0.0	0.0	0.0						182.3
A37[a)]	***0.0	-50	***0.0	-50	0.0	0.0	***0.0	-92	***178.0	178.0	+	+	+	+	-	178/192
A41 [a)]	***1.6	-48.4	***1.7	-48.3	0.8	0.8	***63.1	-19.2	*68.8	68.8	+	+	+	+	-	135.2/182.3
A44 [a)]	***0.0	-50	***0.0	-50	2.6	2.6	***3	-89	***188	188	+	+	+	+	-	193/192
A45 [a)]	***1.7	-48.3	***1.7	-48.3	0.5	0.5	***0.0	-82.3	***108.2	108.2	+	+	+	+	-	111.5/182.3
A49 [a)]	***0.0	-50	***0.0	-50	0.0	0.0	***59.9	-22.4	***77.0	77	+	+	+	+	-	136.8/182.3
A57[b)]	no data	no data	0.0	-4.5	0.0	0.0	0.0	-7.3	**14.5	14.5	+	+	+	+	-	14.5/16.1
A51[b)]	no data	no data	0.0	-4.1	0.0	0.0	0.0	-7.4	***17.9	17.9	+	+	+	+	-	17.9/16.1

Full-fledged denitrifiers from pH 3.7 soil, series C: 1 isolate (C04)

Sample	NO3-	ΔNO3-	NO2-	ΔNO2-	NO	ΔNO	N2O	ΔN2O	N2	ΔN2						N sum
	µmol	µmol	µmol	µmol	Anmol	nmol	µmol	µmol	µmol	µmol	**NAR**	**NIR**	**NOR**	**NOS**	**DNRA**	
Initial	4.5	0	4	0	0	0	6.2	0	0	0						14.2
C04	***0	-4.5	***0.0	-4.0	0.1	0.1	***2.5	-3.7	***18.1	18.1	+	+	+	-	-	21.2

NIR, NOR, N_2OR (nitrite to N_2) from pH 3.7 soil, series C: 3 isolates (C01, C10 and C11)

Sample	NO3-	ΔNO3-	NO2-	ΔNO2-	NO	ΔNO	N2O	ΔN2O	N2	ΔN2						N sum
	µmol	µmol	µmol	µmol	Anmol	nmol	µmol	µmol	µmol	µmol	**NAR**	**NIR**	**NOR**	**NOS**	**DNRA**	
Initial	4.5	0	4	0	0	0	6.2	0	0	0						14.2
C01	4.5	0.0	***0.0	-4	0	0	*1.7	-4.6	9.9	9.9	-	+	+	+	-	16.1

C10	4.4	0.0	***0.0	-4	0.0	0.0	1.6	-4.6	7.7	7.7	-	-	-	+	+	13.7/14.2
C11	4.5	0.0	***1.6	-2.4	***77.1	77.1	***0.1	-6.2	7.2	7.2	-	-	-	+	+	13.5/14.2

Only N_2OR (nitrous oxide reducers only) from pH 7.4 soil, series A: 2 isolates (A40 and A60)

Sample	NO3-	ΔNO3-	NO2-	ΔNO2-	NO	ΔNO	N2O	ΔN2O	N2	ΔN2						N sum
	µmol	µmol	µmol	µmol	nmol	nmol	µmol	µmol	µmol	µmol	**NAR**	**NIR**	**NOR**	**NOS**	**DNRA**	
Initial	4		4.1		0		7.3									
A40	no data	no data	3.3	ns	0.0	0.0	***0.0	-6.2	no data	***6.0	-	-	-	+	-	
A60 [b)]	no data	no data	4.3	ns	0.0	0.0	***0.6	-6.7	no data	*5.9	-	-	-	+	-	

Only N_2OR (nitrous oxide reducers only) from pH 3.7 soil, series B: 1 isolate (B22)

Sample	NO3-	ΔNO3-	NO2-	ΔNO2-	NO	ΔNO	N2O	ΔN2O	N2	ΔN2						N sum
	µmol	µmol	µmol	µmol	nmol	nmol	µmol	µmol	µmol	µmol	**NAR**	**NIR**	**NOR**	**NOS**	**DNRA**	
Initial	4		4.1		0		7.3									
B22 [b)]	no data	no data	4.7	ns	0.0	0.0	***0.0	-7.3	no data	**9.9	-	-	-	+	-	

NAR*/NIR*, N_2OR (nitrate/nitrite reduction, but no gas produced; plus N_2O reduction) from pH 3.7 soil, series C: 2 isolates (C08 and C09)

Sample	NO3-	ΔNO3-	NO2-	ΔNO2-	NO	ΔNO	N2O	ΔN2O	N2	ΔN2						N sum
	µmol	µmol	µmol	µmol	nmol	nmol	µmol	µmol	µmol	µmol	**NAR**	**NIR**	**NOR**	**NOS**	**DNRA**	
Initial [a)]	50		50		0		92									192
C08 [a)]	***0	-50	***0.0	-50	2	2	***0.0	-92	***90	90	-	-	-	+	- 0.22 µmol NH4+	92

C09 [a)]	***0	-50	***0.0	-50	2	2	***0.0	-92	***104	104	-	-	-	+	- 0.17 µmol NH4+	106

NAR, NIR, NOR (nitrate reducers to N_2O) from pH 7.4 soil, series A: 1 isolate (A23)

Sample	NO3-	ΔNO3-	NO2-	ΔNO2-	NO	ΔNO	N2O	ΔN2O	N2	ΔN2						N sum
	µmol	µmol	µmol	µmol	nmol	nmol	µmol	µmol	µmol	µmol	**NAR**	**NIR**	**NOR**	**NOS**	**DNRA**	
Initial	4	0	4	0	0	0	6.2	0	0	0						14.2
A23	***0.5	-3.5	***2.9	-1.1	0.5	0.5	***9.5	3.2	5	5	+	+	+	-	-	17.9

NAR, NIR, NOR (nitrate reducers to N_2O) from pH 3.7 soil, series B: 2 isolates (B01 and B06)

Sample	NO3-	ΔNO3-	NO2-	ΔNO2-	NO	ΔNO	N2O	ΔN2O	N2	ΔN2						N sum
	µmol	µmol	µmol	µmol	nmol	nmol	µmol	µmol	µmol	µmol	**NAR**	**NIR**	**NOR**	**NOS**	**DNRA**	
Initial			4.2		0.0		7.3									
B01 [b)]	no data	no data	**0.5	-3.7	0.0	0.0	13.3	*6.0	ns	ns	+	+	+	-	-	
Initial	5		4				6.32									15.3
B06	***0.11	-4.89	***0.00	-4	**0.07	0.07	***9.41	3.1	***4.83	4.83	+	+	+	-	-	14.4

NIR, NOR (nitrite reducers to N_2O) from pH 7.8 soil, series A: 1 isolate (A02)

Sample	NO3-	ΔNO3-	NO2-	ΔNO2-	NO	ΔNO	N2O	ΔN2O	N2	ΔN2						N sµm
	µmol	µmol	µmol	µmol	nmol	nmol	µmol	µmol	µmol	µmol	**NAR**	**NIR**	**NOR**	**NOS**	**DNRA**	
Initial	4	0	4	0	0	0	6.2	0	0	0						14.2
A02	3.7	-0.3	***0.6	-3.4	***28.9	28.9	***8.3	2	**5.5	5.5	-	+	+	-	-	18.2

NIR, NOR (nitrite reducers to N_2O) from pH 3.7 soil. Series B: 4 isolates (B02, B03, B04 and B05); Series C: 4 isolates (C02, C03, C05 and C06)

Sample	NO3-	ΔNO3-	NO2-	ΔNO2-	NO	ΔNO	N2O	ΔN2O	N2	ΔN2						N sum
	µmol	µmol	µmol	µmol	nmol	nmol	µmol	µmol	µmol	µmol	**NAR**	**NIR**	**NOR**	**NOS**	**DNRA**	
Initial			4.2		0.0		7.3									
B02 [b)]	no data	no data	**2.0	-2.2	0.0	0.0	9.3	**1.9	ns	ns	-	+	+	-	-	
B03 [b)]	no data	no data	**2.5	-1.7	0.0	0.0	*8.8	1.4	ns	ns	-	+	+	-	-	
Initial			2.8		0.0		7.6									
C02 [b)]	no data	no data	**0.0	-2.8	0.0	0.0	*10.5	2.9	ns	ns	-	+	+	-	-	
Initial	4	0	4	0	0	0	6.2	0	0	0						14.2
B04	***3.8	-0.2	***0.0	-4	0	0	***9.9	3.6	5.4	5.4	-	+	+	-	-	19.1
B05	***4.8	0.8	***0.0	-4	2.9	2.9	***10.3	4.1	5	5	-	+	+	-	-	20.1
C03	4.9	0.0	0.0	-4.0	0.1	0.1	9.7	3.5	5.5	5.5	-	+	+	-	-	20.1
C05	4.8	4.8	***0.0	-4	0.2	0.2	10.1	3.9	5.4	5.4	-	+	+	-	-	20.3
C06	4.7	0.0	***0.0	-4.0	0.1	0.1	7.2	1.0	8.3	8.3	-	+	+	-	-	20.2

NAR, NIR (nitrate/nitrite reducers, NO accumulating) from pH 7.4 soil, series A: 4 isolates (A26, A46, A47 and A59)

Sample	NO3-	ΔNO3-	NO2-	ΔNO2-	NO	ΔNO	N2O	ΔN2O	N2	ΔN2						N sum
	µmol	µmol	µmol	µmol	nmol	nmol	µmol	µmol	µmol	µmol	**NAR**	**NIR**	**NOR**	**NOS**	**DNRA**	
Initial [a)]	50	0	50	0	0	0	82.3	0	0	0						182.3
A59 [b)]	***0.0	-50	***97.0	47	***123.8	123.8	***64.1	-18.2	*17.2	17.2	+	-	+ 1.03 µM	-	-	178.3
A46 [a)]	***0.0	-50	***89.5	39.5	***252.7	252.7	***66.4	-15.9	*15.8	15.8	+	-	+ 2.11 µM	-	-	171.9
A47 [a)]	***0.0	-50	***84.2	34.2	***82.1	82.1	***71.9	-10.4	*17.2	17.2	+	-	+ 0.68 µM	-	-	173.4
Initial	4	0	4	0	0	0	6.2	0	0	0						14.2
A26 [b)]	***0.0	-4	***8.1	4.1	*14.8	14.8	6.3	0	4.6	4.6	+	-	+ 1.23 µM	-	-	19.1

Numbers in the column NOR indicate the NO concentration measured at the end of the incubation, assuming the equilibrium between liquid and gas phase.

Only NIR (nitrite reducers, NO accumulating) from pH 3.7 soil, series B: 2 isolates (B20, B21)

Sample	NO3-	ΔNO3-	NO2-	ΔNO2-	NO	ΔNO	N2O	ΔN2O	N2	ΔN2						N sum
	µmol	µmol	µmol	µmol	nmol	nmol	µmol	µmol	µmol	µmol	**NAR**	**NIR**	**NOR**	**NOS**	**DNRA**	
Initial	4	0	4	0	0	0	6.2	0	0	0						14.2
B20	4.6	0.0	**2.8	-1.2	***178.1	178.1	6.7	0.4	4.9	4.9	-	+	+ 14.83 µM	-	-	
B21	***3.9	-0.1	***1.9	-2.1	***657.2	657.2	6.4	0.2	5.3	5.3	-	+	+ 57.75 µM	-	-	

Numbers in the column NOR indicate the NO concentration measured at the end of incubation, assuming the equilibrium between liquid and gas phase.

Only NAR (nitrate reducers only) from pH 7.4 soil, series A: 19 isolates (A04, A08, A13, A20, A22, A24, A28, A32, A34, A39, A42, A43, A48, A52, A53, A54, A55, A56 and A58)

Sample	NO3-	ΔNO3-	NO2-	ΔNO2-	NO	ΔNO	N2O	ΔN2O	N2	ΔN2						N sum
	µmol	µmol	µmol	µmol	nmol	nmol	µmol	µmol	µmol	µmol	**NAR**	**NIR**	**NOR**	**NOS**	**DNRA**	
Initial	4.0		4.5		0.0		7.3									
A22 [b)]	no data	no data	***7.4	3.3	0.0	0.0	8.0	ns	ns		+	-	-	-	-	
A28 [b)]	no data	no data	***8.0	3.9	0.0	0.0	8.3	ns	ns		+	-	-	-	-	
A39 [b)]	no data	no data	**6.3	2.2	0.1	0.1	8.8	ns	ns		+	-	-	-	-	
A43 [b)]	no data	no data	***9.2	4.7	0.0	0.0	7.4	ns	ns		+	-	-	-	-	
A48 [b)]	no data	no data	***9.9	5.4	0.0	0.0	7.5	ns	ns		+	-	-	-	-	
A58 [b)]	no data	no data	***8.8	4.2	0.0	0.0	7.8	ns	ns		+	-	-	-	-	
Initial	4	0	4	0	0	0	6.2	0	0	0						14.23
A04	***0.0	-4	***7.9	3.9	0.6	0.6	6.3	0	4.9	4.9	+	-	-	-	-	19.1
A08	***0.0	-4	***7.7	3.7	1	1	6.5	0.1	4.8	4.8	+	-	-	-	-	19.0
A13	***2.0	-2	***5.6	1.6	0.7	0.7	6	-0.4	4.9	4.9	+	-	-	-	-	18.6
A20	***0.0	-4	***8.1	4.1	0.4	0.4	6.3	0	4.9	4.9	+	-	-	-	-	19.3
A24	***0.0	-4	***8.1	4.1	0.8	0.8	6.1	-0.3	4.8	4.8	+	-	-	-	-	19.0
A32	***1.6	-2.4	***5.3	1.3	0.9	0.9	6.3	0	4.5	4.5	+	-	-	-	-	17.6
A34	3.5	-0.5	**4.2	0.2	0.3	0.3	6.4	0	4.5	4.5	+	-	-	-	-	18.5

A55	***0.9	-3.1	***7.2	3.2	0.1	0.1	6.0	0.3	5.0	5.0	+	-	-	-	-	
A56	***0.0	-4	***9.5	5.5	0.1	0.1	1.4	-4.8	7	7	+	-	-	-	-	17.9
Initial [a)]	50	0	50	0	0	0	82.3	0	0	0						182.3
A42 [a)]	***0.0	-50	***92.7	42.7	1.8	1.8	***75.2	-7.2	**12.9	12.9	+	-	-	-	-	180.7
A52 [a)]	***0.0	-50	***104.6	54.6	***0.1	0.1	41.2	-41.1	38.7	38.7	+	-	-	-	-	184.4
A53 [a)]	***0.0	-50	***104.7	54.7	***0.3	0.3	***33.0	-49.3	39.2	39.2	+	-	-	-	-	176.9
A54 [a)]	***2.1	-47.9	***103.7	53.7	9.8	9.8	40.6	-41.7	37.9	37.9	+	-	-	-	-	184.3

Only NAR (nitrate reducers only) from pH 3.7 soil. Series B: 13 isolates (B07, B08, B09, B10, B11, B12, B13, B14, B15, B16, B17, B18 and B19); Series C: 1 isolate (C07)

Sample	NO3-	ΔNO3-	NO2-	ΔNO2-	NO	ΔNO	N2O	ΔN2O	N2	ΔN2						N sum
	µmol	µmol	µmol	µmol	nmol	nmol	µmol	µmol	µmol	µmol	**NAR**	**NIR**	**NOR**	**NOS**	**DNRA**	
Initial	4.0		4.0		0.0	0.0	7.3									
B07 [b)]	no data	no data	***9.0	4.8	0.0	0.0	7.2	ns	ns		+	-	-	-	-	
B08 [b)]	no data	no data	***8.6	4.4	0.0	0.0	7.5	ns	ns		+	-	-	-	-	
B09 [b)]	no data	no data	***8.6	4.5	0.0	0.0	7.2	ns	ns		+	-	-	-	-	
B10 [b)]	no data	no data	***8.8	4.6	0.0	0.0	7.3	ns	ns		+	-	-	-	-	
B11 [b)]	no data	no data	***5.8	1.6	0.0	0.0	9.6	ns	ns		+	-	-	-	-	
B12 [b)]	no data	no data	***9.0	4.8	0.0	0.0	8.7	ns	ns		+	-	-	-	-	
B13 [b)]	no data	no data	***8.1	3.9	0.0	0.0	7.8	ns	ns		+	-	-	-	-	
B14 [b)]	no data	no data	***9.4	5.2	0.0	0.0	7.6	ns	ns		+	-	-	-	-	
Initial	4	0	4	0	0	0	6.2	0	0	0						14.2
B15	***1.7	-2.3	3.6	-0.4	4.3	4.3	6.4	0.2	5.4	5.4	+	-	-	-	-	17.1
B16	***0.0	-4	***5.2	1.2	16.4	16.4	6.3	0	6.2	6.2	+	-	-	-	-	17.7
B17	***0.0	-4	***8.3	4.3	21.3	21.3	6.4	0.1	5.4	5.4	+	-	-	-	-	20.2
C07	***0	-4	***9.0	5	5.7	5.7	1.5	-4.7	7.6	7.6	+	-	-	-	-	18.1

B18	***0.0	-4	***8.6	4.6	***36.9	36.9	5.5	-0.8	5	5	+	-	-	-	-	19.1
B19	***0.0	-4	***8.4	4.4	***29.8	29.8	6.3	0.1	5.5	5.5	+	-	-	-	-	

DNRA (nitrate/nitrite reduction to ammonia) from pH 7.4 soil, series A: 1 isolate (A50)

Sample	NO3-	ΔNO3-	NO2-	ΔNO2-	NO	ΔNO	N2O	ΔN2O	N2	ΔN2						N sum
	µmol	µmol	µmol	µmol	nmol	nmol	µmol	µmol	µmol	µmol	**NAR**	**NIR**	**NOR**	**NOS**	**DNRA**	
Initial [a)]	50	0	50	0	0	0	82.3	0	0	0						182.3
A50 [a)]	***0.0	-50	***0.0	-50	0	0	***78.6	-3.7	37.6	37.6	+	+	+	-	+	116.2

DNRA (nitrate/nitrite reduction to ammonia) from pH 3.7 soil, series B: 1 isolate (B23)

Sample	NO3-	ΔNO3-	NO2-	ΔNO2-	NO	ΔNO	N2O	ΔN2O	N2	ΔN2						N sum
	µmol	µmol	µmol	µmol	nmol	nmol	µmol	µmol	µmol	µmol	**NAR**	**NIR**	**NOR**	**NOS**	**DNRA**	
Initial	4		4.1				7.3									
B23 [b)]		no data	***0.0	-4.2			7.4	ns	ns	ns	-	-	-	-	+	

STERILE MEDIUM CONTROLS (endpoint):

Small vials (12 ml)

Sample	NO3-	ΔNO3-	NO2-	ΔNO2-	NO	ΔNO	N2O	ΔN2O	N2	ΔN2	N sum
	µmol	µmol	µmol	µmol	nmol	nmol	µmol	µmol	µmol	µmol	
TSB pH 5.7	2.4	-1.6	3.5	-0.5	4.4	4.4	6.2	0.0	6.0	6.0	18.1
TSB pH 7.3	3.3	-0.7	4.0	0.0	0.2	0.2	6.5	0.2	5.2	5.2	19.0

Large vials (120 ml)

Sample	NO3-	ΔNO3-	NO2-	ΔNO2-	NO	ΔNO	N2O	ΔN2O	N2	ΔN2	N sum
	µmol	µmol	µmol	µmol	nmol	nmol	µmol	µmol	µmol	µmol	
TSB pH 7.3	40.6	-9.4	49.0	-1.0	0.6	0.6	40.3	-42.0	31.3	31.3	161.2

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Supplementary Fig S1

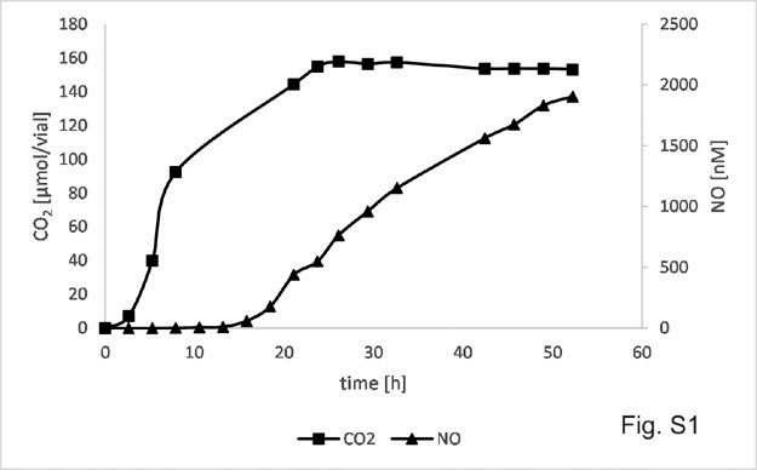

Fig. S1

Paper II

Avoiding entrapment in anoxia at minimal cost; a *bet hedging* strategy of denitrifying prokaryotes that minimize N_2O emission.

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Abstract

To sustain respiratory metabolism in anoxia, denitrifying prokaryotes (DP) express denitrification enzymes NAR, NIR, NOR and N_2OR, catalyzing NO_3^-→NO_2^-→NO→N_2O→N_2. We demonstrate that the model strain, *Paracoccus denitrificans* secures anaerobic respiration with a minimum investment by sequential expression and cell differentiation: In response to oxygen depletion, all cells express N_2OR, whereas only a minority express NIR. Moreover, NIR is preserved in *persister cells* in response to oxygenation after anoxic spells. This phenotypic heterogeneity effectively reduces the metabolic burden of enzyme expression needed to avoid entrapment in anoxia, while making *P. denitrificans* a strong sink for N_2O. We find that similar regulatory phenomena appear to be widespread among DP, as judged from the diauxic nature of their transition from oxic to anoxic respiration. This adds new dimensions to our understanding of how DP regulate anoxic respiration, and identifies novel regulatory traits that minimize N_2O emissions.

Introduction

Denitrifying organisms use nitrogen oxides as terminal electron acceptors to sustain respiration in the absence of oxygen. This metabolism plays a key role in the global nitrogen cycle, returning reactive nitrogen from the biosphere to the atmosphere (Galloway *et al.* 2004). Although the final product of denitrification is harmless N_2, fractions are emitted to the atmosphere as N_2O, and the increasing emission of N_2O over the last decade is primarily due to denitrification, ultimately driven by the anthropogenic escalation of the global nitrogen cycle (Schlesinger 2009). The concerns over climate forcing and destruction of stratospheric ozone by N_2O (Ravishankara 2009) have fueled increasing interest in the ecology and physiology of denitrifying organisms in general, with a strong emphasis on the phenomena that determine their N_2O production.

Denitrifying organisms emit N_2O because it is a free intermediate in their stepwise reduction of nitrate to N_2, catalyzed by the four metallo-enzymes NAR, NIR, NOR and N_2OR (Fig.1), which are encoded by *nar, nir, nor* and *nos* gene clusters, respectively. These genes are widespread among prokaryotes in soils, sediments and biofilms (Shapleigh 2012), and analyses of bacterial genomes have revealed that ~30 % of the genomes containing the *nos* genes lacked genes encoding NIR (NirS or NirK, Graf *et al.* 2014). Such "truncated denitrifiers" have attracted much attention because they are net sinks for N_2O, whereas organisms equipped with NIR, NOR, NOR and N_2OR are both sinks and sources. This was taken to suggest that the abundance of the structural gene, *nosZ*, could predict the propensity of a denitrifying community to emit N_2O, but the search for evidence has not been successful. Since genome analyses show that approximately 70% of all genomes with *nosZ* also carry the genes for NIR and NOR, it stands to reason that the regulation of denitrification in these organisms plays an important role in controlling N_2O emission.

Regulatory networks controlling the transcription of denitrification genes have been unraveled for a number of organisms (van Spanning *et al.* 2007, Zumft and Kroneck 2007). A common feature is the role of oxygen as a superordinate repressor. This is plausibly a strong fitness trait because oxygen respiration is energetically favorable over denitrification (Chen and Strous 2013), hence producing denitrification enzymes under oxic conditions would be a useless metabolic burden. Organisms in soils, biofilms and surface layers of sediments are frequently challenged by fluctuating O_2 concentrations and anoxic spells of variable length (Marchant *et al.* 2017). When confronted with oxygen depletion, they must express a minimum of denitrification enzymes "in time", i.e. before oxygen is completely depleted, to avoid entrapment in anoxia without energy to produce denitrification enzymes (Højberg *et al.* 1997, Hassan *et al.* 2016a). Expression of the entire denitrification proteome would be a waste of energy in all cases of "false alarm", i.e. if oxygen reappears within hours. Thus, they have a regulatory dilemma, which has its parallel in other organisms that are forced by substrate depletion to express new enzymes. This was modelled by Chu (2017),

who concluded that leaky repression is an optimal adaptation. In the case of denitrification, this would mean a leaky oxygen-repression of at least one denitrification gene.

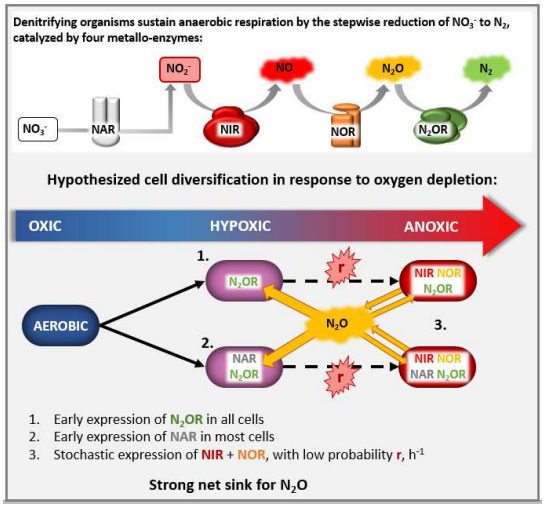

Figure 1. *Hypothesized cell diversification of* P. denitrificans *in response to oxygen depletion, corroborated by modelling the diauxic electron flow kinetics (Fig 2). The model assumptions are that all cells express N_2OR, while the expression of NAR and NIR+NOR are stochastic; occurring with a specific probability which is very low for NIR+NOR, but with a positive feedback via NO (Hassan et al. 2014, 2016a). Cells without NIR (but with N_2OR) can respire by reducing the N_2O produced by the cells with NIR. Thus, the entire population effectively avoids entrapment in anoxia.*

Experiments with the model strain *Paracoccus denitrificans* have provided some kinetic evidence for leaky repression of NAR- and N_2OR, but not of NIR- and NOR-expression (Qu *et al.* 2016). Moreover, *P. denitrificans* displays a conspicuous depression of respiratory electron flow in response to oxygen depletion, and this *diauxie* was taken to suggest that only a fraction of the cells express NIR in time. Modelling lent strong support to the hypothesis that the phenomenon could be ascribed to a low probability for the initiation *nirS* transcription, but with a positive feedback via NO and the NO sensor NNR (Hassan *et al.* 2016a).

Inspired by the fact that a similar *diauxie* in the transition from oxic to anoxic respiration is observed in other denitrifying organisms (Hassan *et al.* 2016a, Lycus *et al.* 2017), we have investigated the mechanisms in more detail in *P. denitrificans*, using a chromosomal *mCherry-NirS* fusion to trace NirS (carrying the catalytic site), and immunocytostaining to trace N_2OR.

Results

To enable a tracing of NirS, we constructed a strain carrying a chromosomal *mCherry-nirS* fusion, which enables us to track NirS positive cells. The insertion of the *mCherry* gene was done by homologous recombination as described in Supplementary Material 1.2. The denitrification phenotype of the *mCherry-NirS* strain was tested by comparing with the parent strain regarding oxygen consumption (and growth rate), oxygen concentration at

which denitrification is initiated, anoxic growth rate, and the apparent probability for cells to express NirS (using the model of Hassan *et al.* 2016a). The results are summarized in Fig 2, showing that a) the denitrification phenotype of the *mCherry-NirS* construct is very similar to the wild type at all temperatures tested, b) the apparent probability for NirS expression (r) increases with temperature for both strains as predicted by the Arrhenius equation ($V=A*e^{-Ea/RT}$) with very high apparent activation energy, c) the NO concentration-maximum increases with temperature for both strains.

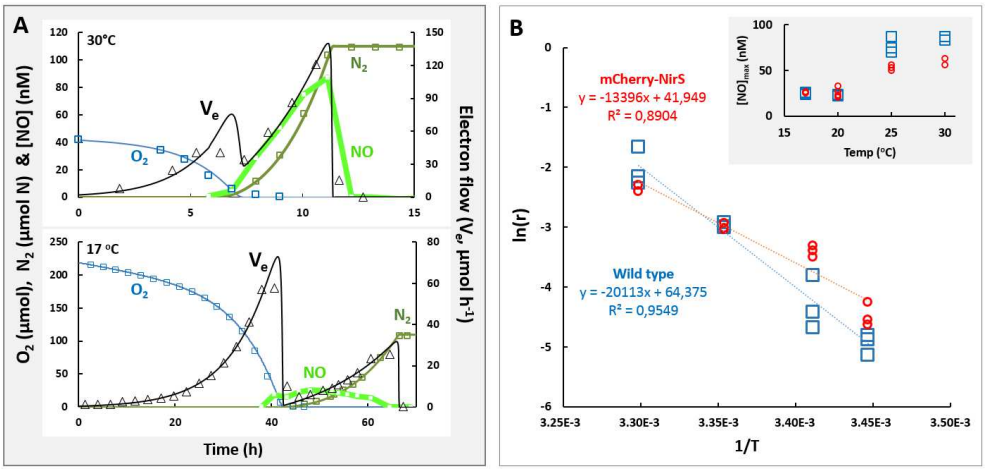

Figure 2. *Denitrification phenotype of the* mCherry-NirS *strain and the wild type as a function of temperature. The A panels show gas- and electron flow kinetics, measured and modelled (Hassan* et al. *2016a) for single vials at 30 and 17 °C. The characteristic depression in electron flow rate after oxygen depletion at 17 °C is hardly detectable at 30 °C. The experiment was performed with 3-6 replicate vials at different temperature, and the probability for NirS expression (r, h^{-1}) was estimated for each individual vial, and ln(r) plotted against the inverse temperature (°K, Panel B), showing reasonably linear decline (linear regression shown in panel). The estimated apparent activation energy (and 95% CI) is 111 (± 26) and 166 (± 35) kJ mol^{-1} for the* mCherry-NirS *strain and the wild type, respectively ($p<0.01$ for the difference between strains). The maximum NO concentrations at the different temperatures (nM NO) is shown in the inserted panel. The entire dataset and the model fits are shown in Figure S4 and the model fitting (to estimate r for individual vials) is shown in Figure S5.*

To detect N_2OR in single cells, we developed an immunofluorescence staining method for NosZ, enabling the visualization of N_2OR in individual cells (Supplementary material 1.3; Figure S1 and S2).

To differentiate between growing and non-growing cells, we tried a number of published methods to track growth, either by positively staining of growing cells, or by detecting growth as a dilution of stain, but none of them worked for *P. denitrificans* under our experimental conditions (See Supplementary Material 4), and we decided to design our own method. Our short-name for the method is FITCT, which stands for *Fluorescein Isothiocyanate Cell Tracking*. In short, cells are exposed for 10 minutes to fluorescein

isothiocyanate (which binds covalently to proteins), and after removal of excess FITC the cells are inoculated into fresh medium and their growth is monitored. The staining was found to have negligible effects on the phenotype with respect to oxic and anoxic respiration and growth (Table S2), and the fluorescence of cells was reduced by 50 % for each cell division, while non-growing cells retained the fluorescence (Figures S9 and S10).

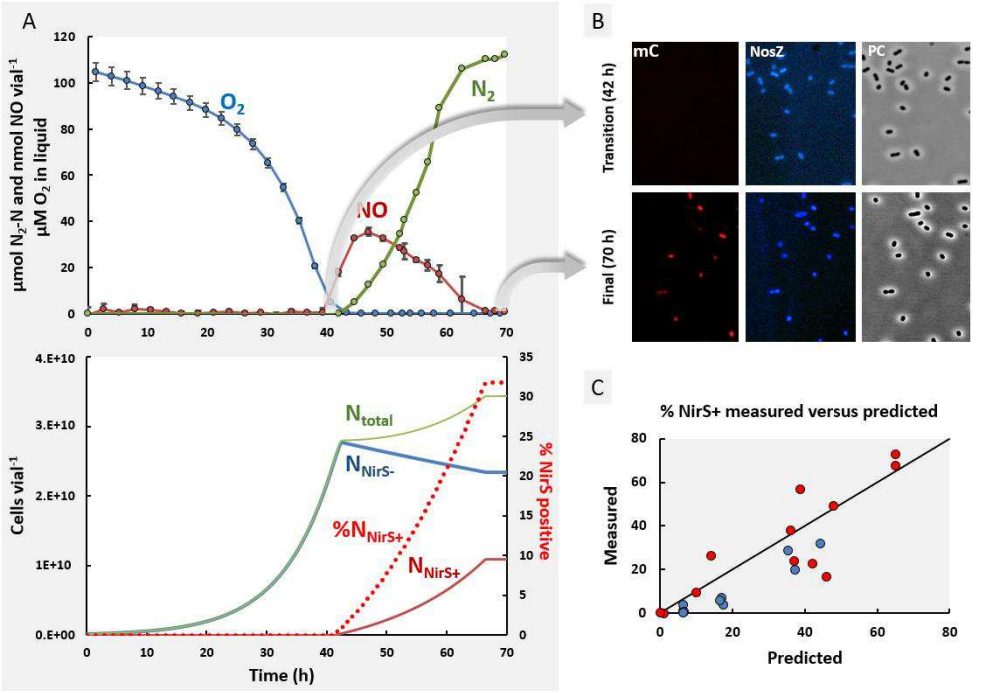

Figure 3: *Gas kinetics and expression of NirS and N_2OR in* P. denitrificans *carrying mCherry-NirS during the transition from aerobic respiration to denitrification (17°C). Panel A (top) shows the depletion of O_2 followed by accumulation of NO and recovery of the initial 2 mM NO_2^- as N_2. N_2O was in the low nanomolar range throughout the incubation. The bottom panel A depicts the population dynamics predicted by the model; N is the number of cells in the vial, subscripts indicating cells with* NirS *(*NirS+*) or not (*NirS-*); percent cells with* NirS *(dotted line) is plotted against right axis). Panel B shows microscopic images of cells immunocytostained for NosZ, taken at the time of oxygen depletion (42 h, upper row) and at depletion of e^- acceptors (70 h, lower row). The images, from left to right, show the mCherry fluorescence (mC), N_2OR immunofluorescence (NosZ), and phase contrast (PC). All cells stained positive for N_2OR at the time of transition, while none were positive for NirS. In the late sample (70 h), all cells stained positive for N_2O reductase and a high fraction showed mCherry-NirS fluorescence. Several samples were taken at different times throughout the anoxic phase, and the fraction of mCherry-NirS positive cells were enumerated. Panel C shows the recorded frequencies of mCherry-NirS positive cells, plotted against the model predictions for an experiment with 10% acetylene (red circles, see Fig 4 for further details) and without acetylene (blue, see Figure S7 for further details).*

These tools allowed us to stringently test the hypothesized cell diversification summarized in Figure 1. A stochastic initiation of NirS expression, leading to two subpopulations was

convincingly verified by the observed fraction of red fluorescent, i.e. mCherry-NirS positive, cells throughout batch cultivation at 17°C (Fig. 3). The fraction of NirS positive cells increased as predicted by the model, which assumes a low probability for the initiation of *nirS* transcription once the repression by oxygen is relieved, and that the NirS positive cells grow exponentially throughout the anoxic phase. Moreover, the immunocytostaining of NosZ demonstrated convincingly that all cells expressed N_2OR (Fig 3).

A direct demonstration of anaerobic growth by the cells with NirS and restricted growth of cells lacking NirS was provided by using FITC-stained cells of the *mCherry-NirS* strain as inoculum for batch cultures with initially near-zero oxygen concentrations (to minimize the dilution of FITC by aerobic growth). While the FITC signal was clearly reduced in the cells with NirS (red fluorescence), it was retained in the cells that lacked NirS (Fig 4).

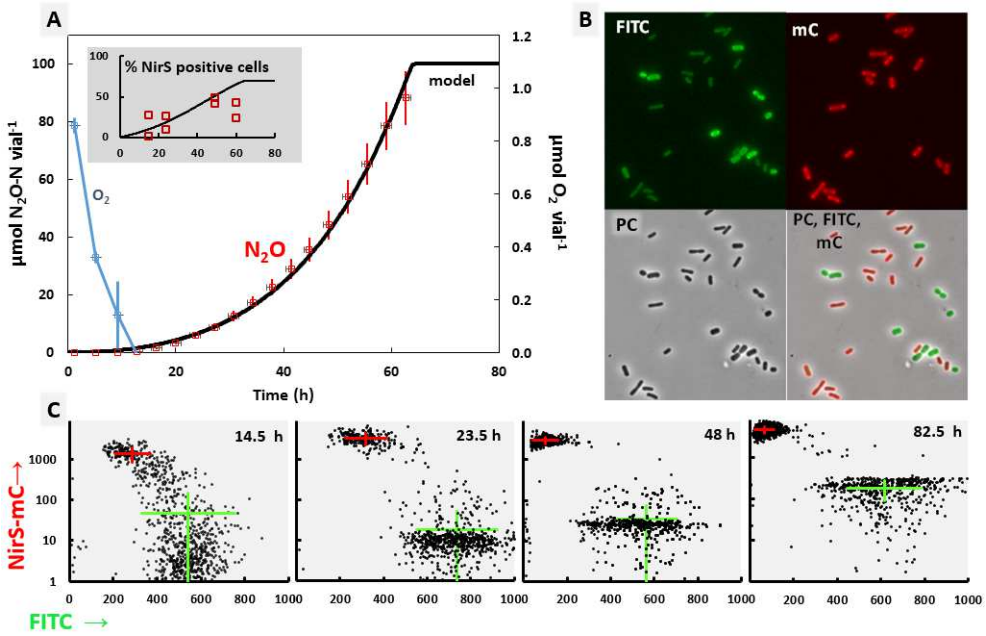

Figure 4: *Anaerobic growth by the subpopulation with NirS, visualized by FITC stained cells of the mCherry-NirS strain. Cells stained with FITC were transferred to near-anaerobic vials with Sistrom's medium (2 mM NO_2^-) and 10% acetylene in the headspace. Panel A shows gas kinetics (measurement as red squares and model as black line), inserted panel: observed frequency of mCherry-NirS positive cells (fluorescence>250) with the modelled frequency (black line). Panel B shows micrographs of cells after 48 h; same frame in all four squares (FITC-florescence, mCherry fluorescence (mC), phase contrast (PC) and a combination of all). Panel C shows single cell fluorescence distribution throughout. Note that the scale for mC-fluorescence is logarithmic (linear plots of the entire dataset are shown in Figure S6). The crossed lines show the average and standard deviation of the fluorescence intensity for two populations: red cross for cells with mC>500, green cross for cells with mC<500. An equivalent experiment without acetylene showed similar results (Figure S7).*

Our model (Hassan *et al.* 2016a) assumed a stochastic initiation of *nirS* transcription, with a very low probability, which then turns autocatalytic by NO via the NO-sensor NNR. In theory, this could imply that NO produced by the first few cells that express NirS would induce *nirS* transcription in the rest of the population, but this is evidently not the case. A tentative explanation is that the bulk concentrations of NO are too low (10-30 nM in the liquid), due to the high-affinity NO-reductase of the actively denitrifying cells (Hassan *et al.* 2016b). A crude test of this was conducted by injecting NO to the culture at the time of oxygen depletion, and the result was that 100% of cells expressed NirS (strong mCherry-NirS fluorescence) and fast reduction of nitrite to N_2 (result not shown). Another prediction of the model is that in the absence of any usable electron acceptor, no cells would be able to express NirS due to lack of metabolic energy; hence NirS-free cells would be entrapped in anoxia even in the presence of nitrite. However, if provided with N_2O, they would have the energy to express NirS, even in the absence of nitrogen oxyanions, albeit to a very low level due to lack of the positive feedback loop via NO and NNR. To test this, we used aerobically raised, FITC-stained *mCherry-NirS* cells to inoculate anaerobic vials with a medium that was effectively stripped for nitrogen oxyanions (see Bergaust *et al.* 2012), and provided them with N_2O as the only electron acceptor (controls without N_2O were included). The results demonstrated that practically all cells were able to grow by reducing N_2O, as evidenced by dilution of the FITC-fluorescence, while cells not provided with N_2O did not (Figure S8, S9). The cells provided with N_2O expressed NirS, but only to a level <10 % of that in actively denitrifying cells. Thus, although the results corroborated our hypotheses, the apparent low NirS expression in 100 % of the cells provided with N_2O was unexpected. It could be taken to suggest a regulatory effect of high concentrations of N_2O, since the N_2O concentration spiked to ~300 μM each time a new dose of N_2O was injected. To our knowledge, however, no such regulatory effect of N_2O has been proven.

Fate of denitrification enzymes during oxic spells

Little is known about the fate of the denitrification enzymes once oxygen returns. They could either be diluted by aerobic growth, degraded, or localized in ageing cells by asymmetric distribution among daughter cells, as has been demonstrated both with cytoplasmic (Lindner *et al.* 2008, Macara and Mili 2008) and periplasmic proteins (Scribano *et al.* 2014).

As a first approach to investigating the fate of the denitrification proteome, we designed an "entrapment assay" in which cells without intact nitrite reductase would be unable to grow: the cells were transferred to anoxic media without nitrogen oxyanions, to which nitrite was added subsequently. The kinetics of nitrite reduction was used to estimate the fraction of cells with intact nitrite reductase. Cells without NirS (raised through >10 generations of aerobic growth) were proven unable to initiate anaerobic respiration in this assay, while cells with NirS (anaerobically raised cells) were active immediately. We used this assay to assess the fate of a denitrification proteome during aerobic growth. Briefly, *P. denitrificans* was raised by many generations of anaerobic growth on nitrite, ensuring that all cells

expressed a full set of denitrification enzymes. These denitrifying cultures were then exposed to fully oxic conditions in medium without nitrogen oxyanions and allowed to grow by aerobic respiration through a variable number of generations (up to 40 h; ~12 generations). At intervals, these cells were tested with the entrapment assay (a graphical overview of the experiment is shown in Figure S3).

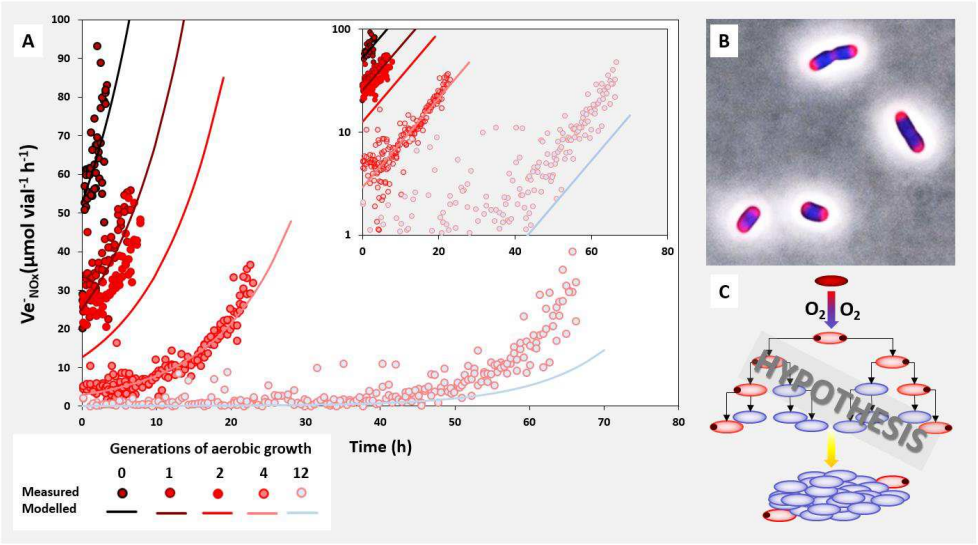

Figure 5: Asymmetric distribution of NirS to daughter cells during aerobic growth? *Anaerobically raised cells were grown aerobically, and after 1-12 generations they were tested for their ability to switch to anaerobic respiration in the entrapment assay (sudden anoxia, ensuring that cells without NIR are effectively entrapped in anoxia). Panel A shows the electron flow to N-oxides (Ve-$_{NOx}$) during the entrapment assay for vials inoculated with cells after 0, 1, 2, 4 and 12 generations of aerobic growth (inoculation density was identical in all vials), together with the predicted rates (lines) for each, assuming asymmetric distribution of NirS during aerobic growth (all NirS migrating to one daughter cell, Panel C). Insert shows the same data on log scale. Panel B shows cells with mCherry-NirS localized at the cell poles and even distribution of N_2OR (blue) in anaerobically grown cells exposed to oxygen (the apparent absence of N_2OR at the cell pole is due to the strong mCherry-fluoresence (See Figure S14). Panel C shows the hypothesized distribution NirS during aerobic growth, which was rejected by the time lapse imaging of cells growing aerobically (Figure 6).*

The experiment had three alternative outcomes, depending on what the cells do with NirS during aerobic growth: 1) If cells actively degrade NirS, we would observe a rapid decline in the initial denitrification rate throughout the first generations of aerobic growth, 2) If NirS is not actively degraded, but evenly distributed among daughter cells, its concentration would decline by dilution (possibly accelerated by active degradation (Scribano *et al.* 2014)), and this would imply that the number of cells switching to denitrification during the entrapment assay would increase during aerobic growth until the NirS content reached a critically low concentration, 3) if NirS is not actively degraded, but localized to the old poles of the cells during aerobic growth, the number of cells able to switch to denitrification in the

entrapment assay would be practically constant through generations of aerobic growth. The result (Fig 5) provided convincing evidence for the latter; the estimated number of cells that were able to switch to denitrification was essentially constant throughout the aerobic growth phase. The first microscopy of anaerobically raised cells after exposing them to oxygen apparently corroborated this interpretation (Figure 5) and we were convinced that this was a parallel to the preferential localization of certain virulence proteins at the old cell poles of *Shigella flexneri* (Scribano *et al.* 2014).

We investigated this further by time lapse microscopy of anaerobically raised cells during aerobic growth on agar pads, and discovered conspicuous patterns (Figure 6): In practically all cells, mCherry-NirS migrated to the cell poles within minutes. Some cells did not grow at all, and in these cells NirS remained at the poles. The cells that did grow, first redistributed their NirS to the entire periplasm, and started to grow, diluting their NirS by even distribution among daughter cells. However, some cells within microcolonies of growing cells "decided" to stop growing after 1-3 generations, and in these cells, NirS migrated back to the poles.

This pattern provides a plausible explanation to the observed result of the entrapment assay. The arrested growth by some of the cells qualifies for the term *persister cells*, because they are cells that retain their NirS so as to be able to tackle sudden anoxia, while the rest of the population would only be able to switch to denitrification if oxygen is depleted gradually.

Control experiments were conducted to exclude artifacts regarding the migration to the cell poles. A strain in which mCherry is expressed in response to taurine and transported to the periplasm was constructed (Supplementary Material 1.2.2); demonstrating an even distribution of mCherry throughout the periplasm and no migration to the cell pole in response to oxygen (Figure S11). Thus, the migration of mCherry-NirS to the cell pole in response to oxygen is clearly due to a property of NirS, not mCherry. Cells inactivated by NaN_3 showed no migration to the pole (Figure S12), hence the migration depends on some metabolic integrity. To exclude artifacts created by the agar pad conditions, we corroborated the polar localization by transferring anaerobically grown cells to aerobic vials which were sampled for microscopy (fixed by formalin immediately after sampling). Samples taken throughout the first 30 minutes confirmed the rapid migration to the poles, and samples taken after 2-5 generations of aerobic growth demonstrated that the *persister cells* had NirS localized at the cell poles. We also observed migration to the cell poles under anoxic conditions, but only in response to depletion of electron acceptors (Figure S13).

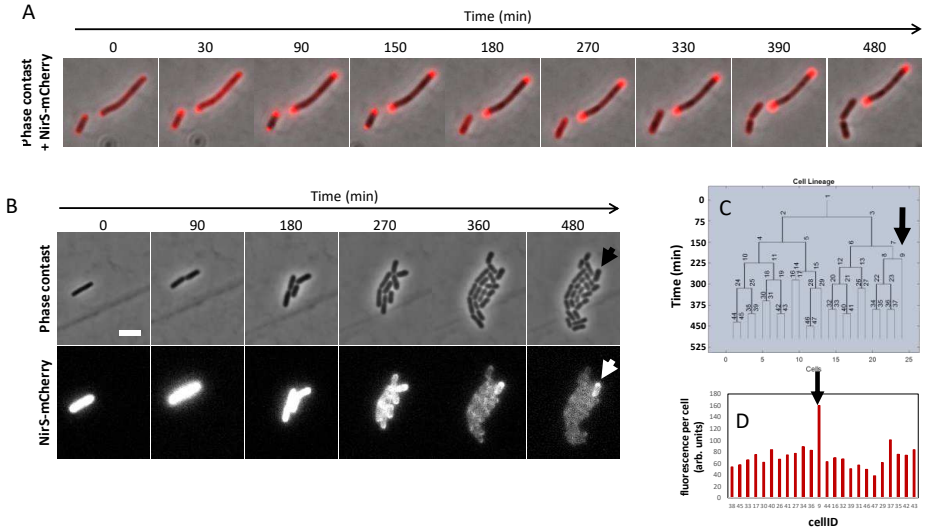

Figure 6. *Time lapse photos of cells with intact NirS during aerobic growth on agar slabs. Panel A shows the time lapse of a single and a doublet cell which failed to grow aerobically and retained mCherry-NirS at the poles. Redistribution occurred in the single cell after 120 min, and it eventually divided. Panel B shows a growing cell, with even distribution of mCherry-NirS, growing fast from the very beginning. However, a single cell among the third generation cells stopped growing, hence retained mCherry-NirS while the rest of the population continued to dilute NirS by growth (illustrate by the cell lineage (C) and mCherry intensity of individual cells (D). mCherry-NirS migrated to the poles in the cell that stopped growth after 3 generations. More time lapse images are shown in Figure S16.*

Discussion

"Yes; but you must wager. It is not optional. You are embarked. Which will you choose then?"
Blaise Pascal -Note 233, Pensées

Prokaryotes are no longer seen as simple vesicles with evenly distributed macromolecules, growing by symmetric binary fission, producing phenotypes infallibly determined by their genes. Instead, we have become increasingly aware of their complexity and intricate inter- and intracellular organization, equipped with networks whose "decisions" are ambiguous (Ackermann 2013). Cell diversification in isogenic cultures has been described in a wide variety of prokaryotes, and the phenomenon is ascribed to noise and bistability of the regulatory networks (Veening *et al.* 2008). Well-documented cases are endospore formation, chemotaxis, expression of genes for substrate utilization (*lac* operon in *E. coli*), and the formation of *persister cells* (Lewis 2007). Some such phenomena are termed *bet-hedging* because the population spreads the risks when responding to fluctuating conditions, in effect

accepting a penalty for a fraction of the population, in exchange for a long-term fitness advantage for the entire population (de Jong *et al.* 2011).

P. denitrificans displayed a clear bet-hedging strategy with respect to the expression of NirS + NOR when challenged with imminent anoxia. The hypothesized mechanism was a constant, low probability of initial NirS expression, but with a positive feedback loop via NO and NNR (Hassan 2014, 2016a), and this is strongly supported by our experimental results. Moreover, we observed a strong effect of temperature on the probability for NirS-expression (Fig 2). While raising new questions concerning NirS regulation in this organism, it coincidently explains why the phenomenon has gone undiscovered until now: In general, the respiratory apparatus of *P. denitrificans* has been studied at temperatures ≥ 30°C, and at this high temperature, the phenomenon is indeed almost undetectable. This underscores the importance of conducting physiological experiments under environmentally relevant conditions.

The regulation of NirS-expression in *P. denitrificans* can be seen as a clever energy-conserving strategy, i.e. minimizing the cost of protein synthesis in all cases when oxygen quickly reappears. It could prove fatal, however, if it results in complete entrapment of the majority of cells in long-term anoxia. This penalty is evidently avoided by a leaky repression of *nosZ* (and possibly the *nar* genes). By expressing N_2OR in 100% of the population in response to hypoxic conditions, *P. denitrificans* ensures continued respiratory growth, albeit slow, by scavenging N_2O produced by other cells in the population or the community. Should the anoxic spell be prolonged, these cells will eventually express NirS using the energy from N_2O respiration.

Thus, *P. denitrificans* can be predicted to act as a strong N_2O sink in temperate environments with frequent fluctuations in O_2 availability. As such, our observations have environmental implications, and a crucial question is whether these regulatory traits are anecdotal, confined to *P. denitrificans* only. This appears not to be the case: several denitrifying bacteria have displayed diauxie during transition from oxic to anoxic conditions (refs in Hassan *et al.* 2014, 2016a), and several newly isolated strains from soil displayed a clear depression of the respiratory electron flow during the transition from oxic to anoxic conditions (Lycus *et al.* 2017).

Subcellular localization of cytoplasmic proteins has been described in a range of bacteria, and it is evident that the organisation of proteins is subject to spatiotemporal regulation. Polar localization of proteins serves a number of purposes, and is involved e.g. in asymmetric cell division, modulation of the cell cycle, chemotaxis and motility (Davis and Waldor, 2013), and shedding of useless/damaged proteins (Tyedmeyer *et al.* 2010). A number of mechanisms governing polar localization of proteins in the cytoplasm have been described (Laloux and Jacobs-Wagner, 2014). However, only a few examples of spatially organised periplasmic proteins have been reported (e.g. Scribano *et al.* 2014). We have demonstrated spatiotemporal variation of NirS localization in *P. denitrificans* (Fig 5 and 6, Time lapse movie

SM). NirS was evenly distributed during anoxic growth, but migrated to the poles in response to depletion of electron acceptors (nitrogen oxides), and if cells were transferred to oxic conditions. Here, we observed a conspicuous oscillation of NirS during the first minutes after exposure to oxygen, finally settling in the poles in those cells that failed to grow aerobically, while those growing showed a more even distribution. The non-growing cells evidently retained NirS, enabling them to switch to anoxic respiration in response to sudden disappearance of oxygen (the entrapment assay), while those growing (and diluting their NirS) were not.

Spatiotemporal organization of cytoplasmic proteins is often intimately linked to cell cycle and proton motive force (Strahl and Hamoen 2010), and it is reasonable to assume that periplasmic enzymes associating with the membrane or membrane bound factors, may be governed by similar rules. To our knowledge, NirS is not involved in cell division or its controlling factors, but it is likely to interact intimately with the other denitrification enzymes, such as the membrane-embedded NorBC during active denitrification (Borrero-de Acuña *et al.* 2016). It is feasible that this interaction is dependent on the electrogenic state of the membrane, and that NOR and NirS thus engage in a "capture and release" cycle driven by the proton motive force (pmf). Speculating further, once detached from NOR, NirS may diffuse passively to the poles and/or interact weakly with a secondary partner with polar localization. A link between the proton motive force and NirS localization is also supported by the observation of migration of NirS to the cell poles under anoxic conditions in response to exhaustion of NO_x (Figure S13). In contrast, there was no evidence of polar organisation of N_2OR under any of the conditions tested (Fig 3 & 5, Fig S14). If *P. denitrificans* resembles *Ps.* aeruginosa with respect to respirasome assembly, N_2OR may form a complex with the integral membrane protein NosR rather than NorBC (Borrero-de Acuña *et al.* 2016). This could explain the divergent localization response of NosZ and NirS.

Much like rapid transitions from aerobic to anaerobic growth, the abrupt return of oxygen can be viewed as a crisis with profound regulatory challenges. In order to grow, cells must reassemble their aerobic respirasome, and this may require *de novo* protein synthesis dependent on existing energy reserves. Thus, the conservation of NirS in non-growing persister cells, may be a result of ATP depletion, i.e. "entrapment in oxia" in a minority of cells fully invested in an anaerobic lifestyle.

Conclusion

Bet-hedging with respect to NirS+NOR, coupled with early and complete onset of N_2OR expression, bear environmental implications because organisms with this regulatory set-up become strong net sinks of N_2O. Moreover, at the risk of unduly anthropomorphizing non-sentient organisms, such phenotypic heterogeneity can be seen as an ingenious strategy for safeguarding ones interests without exhaustive investments. Placing wagers on multiple near-future outcomes nullifies the risk on population level, at acceptable fitness costs to individual cells.

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Supplemental Material for

Avoiding entrapment in anoxia at minimal cost; a *bet hedging* strategy of denitrifying prokaryotes that minimize N_2O emission.

Lycus P, Soriano-Laguna M, Kjos, M, Richardson D, Gates A, Milligan DA, Frostegård Å, Bergaust L, Bakken LR.

This document includes
Materials and Methods
Supplementary text with Figures S1-S16 and Tables S1&S2
References

Contents

1.1 Growth conditions, kinetics and modelling

1.1.1 Growth conditions and preparation of inocula

All cultivation was done in Sistrom's minimal medium containing (g L^{-1}): K_2HPO_4 3.48, NH_4Cl 0.195, succinic acid 4.00, L-glutamic acid 0.10, L-aspartic acid 0.04, NaCl 0.50, nitrilotriacetic acid 0.20, $MgSO_4$ * $7H_2O$ 0.30, $CaCl_2$ *$7H_2O$ 0.015 and $FeSO_2$*$7H_2O$ 0.007. In addition, trace elements and vitamins were added (final concentration, g L^{-1}) EDTA (triplex 3) 0.001765, $ZnSO_4$ * $7H_2O$ 0.01095 g, $FeSO_4$ * $7H_2O$ 0.005, $MnSO_4$ * H_2O 0.00154, $CuSO_4$*$5H_2O$ 0.000392, $CoCl_2$ * $2H_2O$ 0.00014, H_3BO_3 0.000114, nicotinic acid 0.0010, thiamine HCl 0.0005, biotin 0.000010. Nitrite (normally 2 mM) was added, either before or after inoculation. Despite the absence of added nitrate, the medium contained traces of nitrate (5-10 µM), and for experiment where the absence of nitrogen oxyanions (NO_3^-, NO_2^-) was essential, these traces were removed by anoxic incubation with 10^7 cells of *P. denitrificans* over night, followed by filter-sterilization and autoclaving (Bergaust *et al.* 2012).

1.1.2 Gas kinetics, measurements and calculations

All growth experiments for determination of the kinetics of respiratory metabolism were performed in 120 mL serum vials sealed with butyl rubber septa and aluminum crimp caps, containing 50 mL medium and a polytetrafluoroethylene (PTFE) coated magnet. The headspace air was replaced with He or He + O_2 (different proportions), and the vials were placed in the thermostated water-bath of the incubation robot which monitors the headspace concentrations of O_2, CO_2, NO, N_2O and N_2. The measured gas concentrations were used to calculate net rates of O_2 consumption and reduction of NO_2^-, NO and N_2O for each time interval, taking the dilution by sampling and the marginal leakage of N_2 and O_2 into account, as explained in detail by Molstad *et al*. (2007).

1.1.3 Modelling recruitment to denitrification

The model is explained in detail by Hassan *et al.* (2016). In short, it assumes a constant but low probability (r, h^{-1}) for the initiation of *nirS*-transcription, when [O_2] ≤ 10 µM. It further assumes that once *nirS* transcription is initiated, it becomes autocatalytic resulting in a fast expression of both NirS and NOR.

In the present work, we used the model to estimate the probability for NIR expression (r, h^{-1}) by fitting the model to measured gas kinetics, using the same model as Hassan *et al.* (2016), but reprogrammed in R. The experiments were done at 17, 20, 25 and 30°C (Figure S4). Low initial oxygen concentrations (~40 µmol $vial^{-1}$) in experiments at temperatures ≥20 °C were chosen to secure adequate resolution of the kinetics (using higher initial oxygen concentrations would result in very fast reduction of nitrite). The model was fitted to measured O_2 and N_2 only (fitting the model to measured NO is essentially inconsequential

for the estimated recruitment rate), and for each vial individually. The estimated r for each vial was treated (statistically) as independent observations of r.

We used the Metropolis Markov chain Monte Carlo algorithm in R (Geyer and Johnson 2017) to carry out two consecutive estimations based on measured O_2 and N_2 for individual vials. Initial headspace O_2 and initial cell number were first estimated based on the O_2 data. The output values from this estimation were used to estimate the initial background N_2 present before estimation denitrification and the recruitment rate (the probability for cells to express NirS). These were based on N_2 measurements starting from two sampling points before O_2 had declined to below 0.5% partial pressure, and before full recovery of NO_2^- as N_2.

A regression model was used to describe the negative correlation between the natural log of recruitment rate and inverse temperature, and calculate the apparent activation energy of recruitment (Fig 2B). To determine whether there were strain-specific differences, the model included terms for the main effect of strain and the interaction between strain and inverse temperature. This analysis was superior to one in which the interaction term was omitted (*F*-test; $p<0.01$).

1.2 Strain construction

1.2.1 The *mCherry-NirS* strain

Unmarked insertion mutants were generated in *P. denitrificans* PD1222 using the mobilizable multi-purpose cloning vector pK18mobsacB (Schäfer *et al*. 1994). A fragment of 999 bps flanking the 5' end of *nirS* was amplified by PCR, using primers NirS_F and NirS_R (see table below), and cloned into pJET1.2 (Thermo Scientific). Then, a SacI restriction site was introduced downstream of the *nirS* Sec signal peptide by inverse PCR using primers SacI_F and SacI_R. This SacI site was subsequently used to clone a fragment of 708 bps containing mCherry gene and primers NirS_F and NirS_R were used to check for the orientation of the fragment. Then the whole construct of 1707 bps was digested with XmaI and PstI and subcloned into pK18mobsacB. This pK18mobsacB derivative plasmid was then conjugated into *P. denitrificans* wild type. Single cross over recombination events were initially selected by plating the cells from the conjugation into LB with spectinomycin and kanamycin and identified by colony PCR. The insertional mutation was finally resolved by growing the bacteria in a modified LB media (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 4 g L^{-1} NaCl and 6 % (w/v) sucrose) which forced the bacteria to undergo a second recombination event where the plasmid was excised from the chromosome. Double recombinants were screened by colony PCR and the PCR products were sequenced for confirmation.

Table S1. Primers and plasmids

PRIMERS		
NAME	**SEQUENCE**	
NirS_F	aaCCCGGGGCCGGAATAGCCGGTGGT	
NirS_R	aaCTGCAGGAACAGGTTTTCCAGATC	
SacI_F	aaGAGCTCGAGGATCACAAGACCAAG	
SacI_R	aaGAGCTCGAGTGCGGCGGCAGGATC	
mCsoI_F	aaCATATGAGACAAAGGACCCCATTC	
mCsoI_R	aaCATATGTTTGTAAAGTTCATCCAT	
PLASMIDS		
NAME	**CHARACTERISTICS**	**REFERENCE**
pJET21	Used for subcloning	*Thermo Scientific*
pK18*mobsacB*	Mobilizable multi-purpose cloning vector	(Schäfer *et al*. 1994)
pLMB509	Taurine inducible plasmid	(Tett *et al*. 2012)
pMJSL01	pJET21 with *nirS* 5' fragment	This work
pMJSL02	pJET21 with *nirS* 5' fragment and SacI site	This work
pMJSL03	pJET21 with *nirS* 5' fragment and *mCherry*	This work
pMJSL04	pK18*mobsacB* with *nirS* 5' fragment and *mCherry*	This work
pMJSL05	pLMB509 with a soluble periplasmic *mCherry*	This work

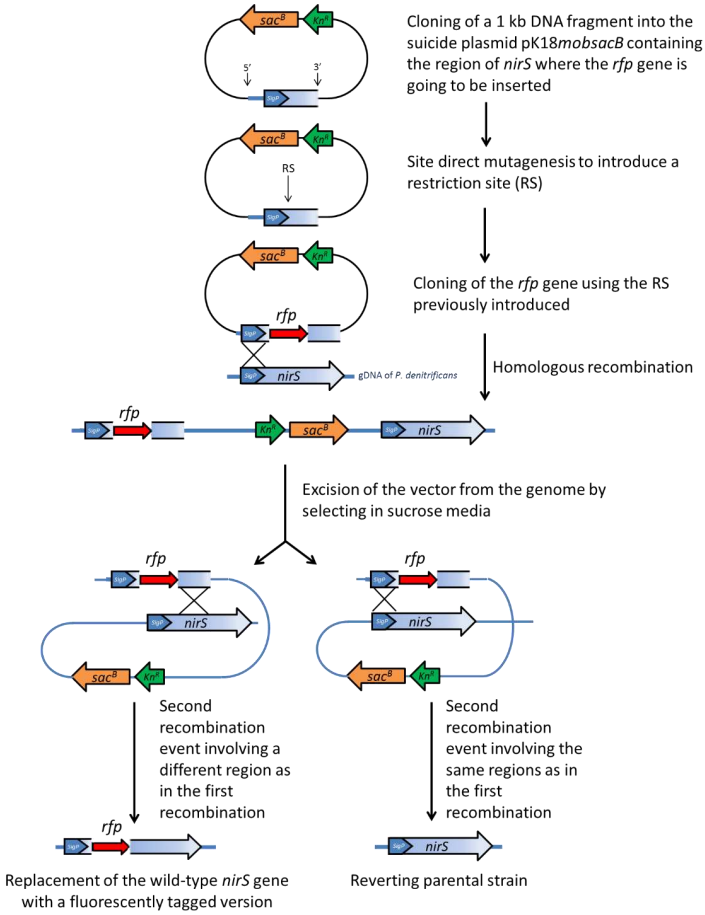


Figure S1: The scheme demonstrating generation of the *mCherry-NirS* mutant in PD1222.

1.2.2 Strain with periplasmic mCherry

As a negative control to test the localization of naked mCherry protein in the periplasm, a genetic construct was generated for the expression of a soluble periplasmic mCherry protein exported through the Sec system. For this purpose, a fragment of 907 bps was amplified by PCR from *P. denitrificans mCherry-NirS* genomic DNA using primers mCsol_F and mCsol_R. The PCR product was then cloned into pMJSL05 which is a derivative of the taurine inducible plasmid pLMB509 for alpha-proteobacteria (Tett *et al.* 2012).

1.3 Immunofluorescence staining of N_2OR

Wild type and the mCherry-NirS mutant of the same strain of *P. denitrificans* PD1222 were grown under denitrifying conditions in minimal Sistrom's medium as described above. Cells were harvested at specified time points and fixed in 3.8% formalin at room temperature for 30 min. Aerobically grown inoculum was used as a negative control (with no expression of N_2OR). Preserved cells were stored at $4^{o}C$ until analyzed. All the incubations were performed at room temperature avoiding light exposure. The samples were incubated for 1 hour and for 30 min. with antibodies and Pacific Blue conjugate, respectively. Each step of immunocytochemical staining was followed by fivefold washing with phosphate-buffered saline containing 0.05% Tween20 (PBS-T). Blocking buffer contained PBS-T and 1% BSA (Fluka). Samples were analyzed immediately after staining. Affinity purified primary polyclonal chicken anti-NosZ IgY antibodies (Norwegian Antibodies) were biotinylated (Thermo Fischer) and used for detection of N_2O reductase (NosZ) in formaline fixed and permeabilized cells of the *mCherry-NirS* strain and wild type. Secondary labeling with streptavidin Pacific Blue conjugate (Thermo Fischer) was used for visualization and localization of the NosZ-IgYab by fluorescence microscopy, as described in section 1.5. The exposure time was 1000 ms for both mCherry and Pacific Blue.

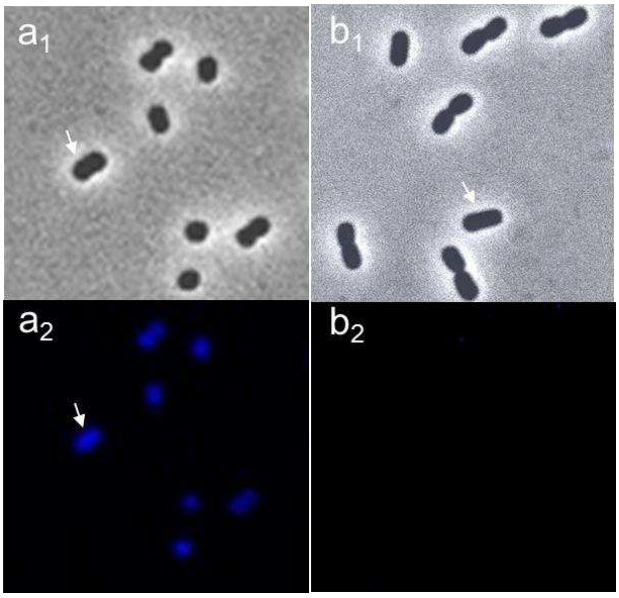


Figure S2: The immunocytostaining of WT PD1222. Upper panel (a_1, b_1)– phase contrast, lower panel (a_2, b_2)– fluorescence microscopy. Presence of N_2OR in actively denitrifying cells (a) and its absence in aerobically grown inoculum (b).

1.4 FITC cell Tracking (FITCT)

The procedure for staining cells with FITC for tracking growth (dilution of the FITC signal) is:

Staining solution:

- Dissolve FITC (Fluorescein isothiocyanate, Sigma Aldrich) in dimethyl sulfoxide (Sigma Aldrich), 1 mg mL^{-1}
- Dilute 1:4 in Sistrom's medium (i.e. the medium used for cultivating the bacteria)
- Filter the solution (0.2 μm). Prepare staining solution immediately before staining.

Staining:

- Concentrate cells by centrifugation to reach a cell density of 1-5E9 mL^{-1}. Use low temperature (5 ^{0}C) to avoid anoxia in the pellet. To effectively disperse the cells, pump 5 times through a syringe with narrow needle.
- Mix cell suspensions with staining solution (proportions: 0.375 mL staining solution 1.5 mL cell suspension), by pumping in and out of a syringe 5 times, and then stir continuously (15 mL Falcon tubes placed in a rotator) for 10 min.
- Wash cells by 3 min centrifugation in Eppendorf tubes, resuspend in fresh medium, and repeat.
- Final suspension must be stirred continuously until used to inoculate. Minimize the time before use.

Tracking:

- Cells that grow will dilute the FITC signal accordingly, while cells that do not grow will remain strongly fluorescent. By quantifying the FITC fluorescence from individual cells (either by confocal microscopy or flow cytometry), the number of generations can be assessed.

1.5 Fluorescence microscopy and time lapse imaging

Fluorescence microscopy was performed on a Zeiss AxioObserver with ZEN Blue software. Images were acquired with an ORCA-Flash4.0 V2 Digital CMOS camera (Hamamatsu Photonics) through a 100x phase-contrast objective. A HXP 120 Illuminator (Zeiss) was used as a fluorescence light source. The exposure times were 1000 ms for mCherry and 750 ms for FITC.

Image analysis was performed using the ImageJ plugin MicrobeJ (Ducret *et al*. 2016). Cell outlines were determined using the phase contrast images. The FITC and mCherry signal intensities were extracted from the corresponding fluorescent images. The data was

exported and further plotting and data analysis was performed in Excel and RStudio. Images were prepared for publication using ImageJ (http://rsb.info.nih.gov/ij/).

Time-lapse fluorescence imaging of cells on agar pads

Cells of the mCherry-NirS mutant were grown under denitrifying conditions (as described above) for several batch generations assuring the expression of NirS in 100% of the cells. This was accomplished by sequential sub culturing from one to another anoxic vial containing 2 mM nitrite.

Agarose pads for the time-lapse experiment were prepared freshly, as described by Skinner *et al.* (2013), with some modifications. The gene frames (ABgene; 1.7 x 2.8 cm) were used to generate agarose pads. Approximately 1/3 volume of the frame was filled with agarose in order to provide a sufficient O_2 reservoir for maintaining aerobic conditions during the incubation. Further, the pads were sliced with a sterile scalpel to improve the aeration of the agarose. One μL of denitrifying culture was inoculated onto the agarose pad just before the start of the experiment. The frame was sealed with a cover slip and the slide was incubated in a temperature controlled chamber during the experiment. Images (phase contrast and mCherry fluorescence) were acquired with regular time intervals (1 min or 15 min) through a 100x phase-contrast objective.

Time-lapse experiments were analysed using the Matlab-based package SuperSegger (Stylianidou *et al*. 2016) and the ImageJ plugin MicrobeJ (Ducret *et al*. 2016). Segmentation of cells at the different time points in the growing microcolonies was performed using SuperSegger with standard settings and cell lineage trees were extracted. The segmentation patterns were then used to guide manual segmentation of microcolonies using MicrobeJ. Intensity values of single cells were then extracted from the corresponding fluorescent images. The data was exported and further plotting and data analysis was performed in Excel and RStudio. Images were prepared for publication using SuperSegger and ImageJ (http://rsb.info.nih.gov/ij/).

1.6 The fate of denitrification enzymes during aerobic growth, *entrapment assay*

As a first approach (before constructing the *mCherry-NirS* mutant), we conducted a kinetic experiment to explore the fate of the denitrification enzymes during aerobic growth. Cells with denitrification enzymes (raised by anaerobic growth) were transferred to fully aerated medium and allowed to grow aerobically through several generations, and tested for their ability to switch to denitrification when exposed to "sudden anoxia" (*entrapment assay*), as outlined in Fig S3

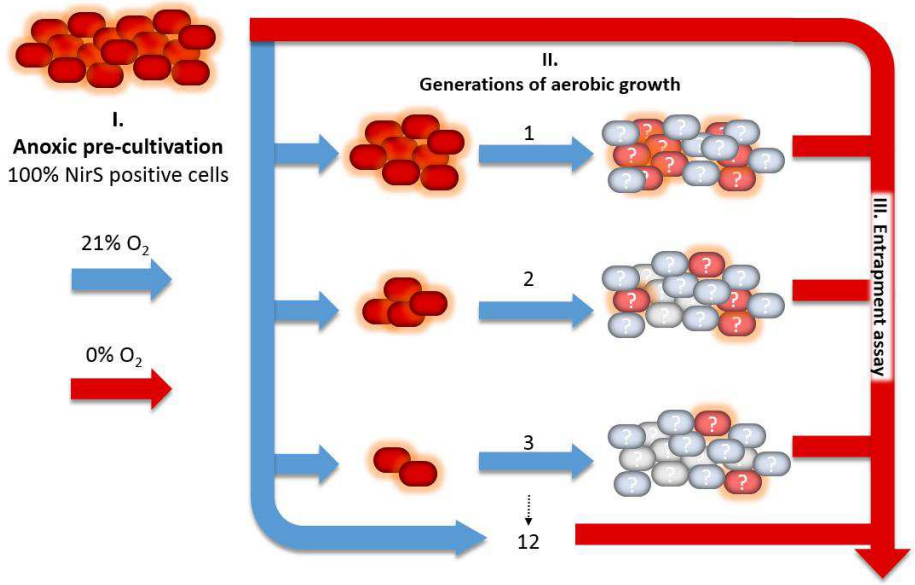


Figure S3: Outline of the experiment to explore the fate of denitrification enzymes during aerobic growth. (I) *P. denitrificans* was raised by anaerobic growth on nitrate, ensuring that all cells expressed a full set of N-oxide reductases. Different volumes of this pre-culture were transferred to fully oxic medium without nitrogen oxyanions (II). Once the aerobic cultures reached cell densities equal to the anoxic pre-culture (after 1…12 generations), cells were transferred to anoxic media without nitrogen oxyanions, to which nitrite was added after depletion of the traces of oxygen present (III, entrapment assay). This was to ensure entrapment in anoxia of any cell without intact nitrite reductase. The kinetics of their reduction of nitrite was used to estimate the fraction of cells with intact nitrite reductase.

In detail:

I. *Preparation of denitrifying culture:* 500 µL of glycerol stock was inoculated into a serum vial containing basal Sistrom's medium, and the culture was incubated aerobically at 25°C until OD_{660} reached ~0.150. This aerobic pre-culture was used to inoculate four anoxic vials with 50 mL Sistroms with 4 mM KNO_3. These cultures were incubated at 20°C, and the accumulation of N-oxides was monitored by headspace sampling every 2 hours. When all the available nitrate had been recovered as N_2, the cultures were pooled (~3.4 x 10^8 cells mL^{-1}; OD_{660} = 0.277) and then transferred to fresh medium, either in new anoxic vials (step III), or fully aerated vials (step II).

II. *Aerobic growth:* A series of oxic vials with 25 (n=2), 40 (n=2), 48 (n=2) and 50 (n=4) mL basal Sistroms medium (without nitrogen oxyanions) were inoculated with 25, 10, 2 and 0.2 or 0.02 mL denitrifying culture, respectively (from step I), to a final

volume of 50 mL in all. All cultures were allowed to grow aerobically at 20°C until reaching cell densities similar to that in the denitrifying pre-culture. After a variable number of generations, when OD_{660} slightly exceeded that in the anoxic inoculum, duplicate vials were pooled and cell density adjusted to ~3.4 x 10^8 cells mL^{-1}. These cultures were then transferred to experimental vials for a denitrification activity assay (step III).

III. *Entrapment assay*: 25 mL of pooled aerobic cultures from step II were transferred to 25 mL anoxic medium without nitrogen oxyanions (n=3) and He-rinsed as previously described (Molstad *et al.* 2007). When the cells had consumed the initial traces of O_2 (118 ± 93 ppmv), He-sparged KNO_2 (0.1M) was injected through the septum to an initial concentration of 2 mM. Subsequent nitrite reduction to N_2 was monitored through frequent headspace sampling (every ~13 minutes) until NO_x depletion.

Importantly, this scheme ensured that the initial cell density in the *entrapment assay* (step *III*) was approximately the same for all vials, regardless of aerobic legacy. By ensuring that residual O_2 (initial headspace [O_2] = 118 ± 93 ppmv) was effectively depleted in the absence of any nitrogen oxyanions (nitrite was added after oxygen depletion), we forced the cells to rely on existing N-oxide reductases to initiate denitrification (and growth) once spiked with nitrite.

We expected a gradual reduction of the NirS content of all cells, faster if degraded actively than if diluted by growth only; which would imply that the denitrification kinetics in the *entrapment assay* would be more or less unaffected by the number of generations of aerobic growth until the NirS concentration became critically low. This expectation based on the assumption that any cell with a minimum of NirS would be able to initiate denitrification during the entrapment assay, synthesize more NirS within minutes, and initiate normal anaerobic growth. The result, however, strongly suggested that the number of cells able to switch to denitrification was practically constant during aerobic growth. This suggested asymmetric distribution of NirS during aerobic growth; i.e. that at each aerobic cell division, all NirS is allocated to one of the daughter cells. The results were analyzed accordingly (Fig 5).

2 Supplementary texts, figures and tables

2.1 Temperature dependency of recruitment (experiments & modelling, Fig 2)

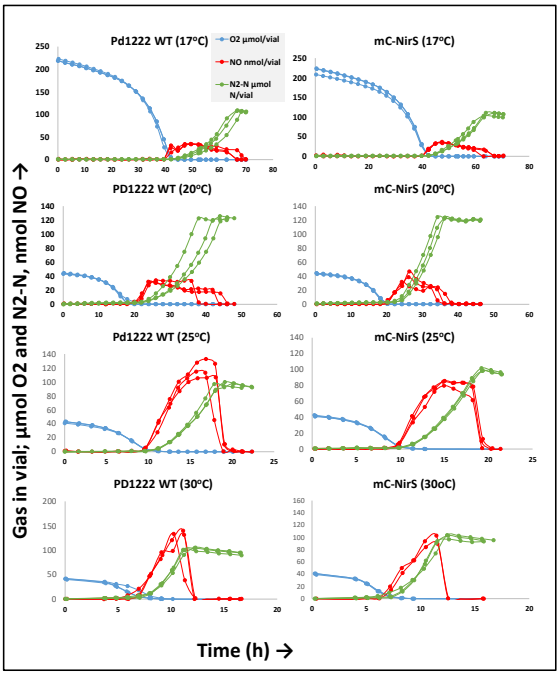


Figure S4: Gas kinetics throughout transition from oxic to anoxic conditions for the wild type (*P. denitrificans* PD1222) and the mCherry-NirS construct (mC-NirS). The initial O_2 concentrations were ~7% in headspace (220 µmol vial^{-1}) in the experiment at 17°C, but only 1% in all other experiments. **TagS9**

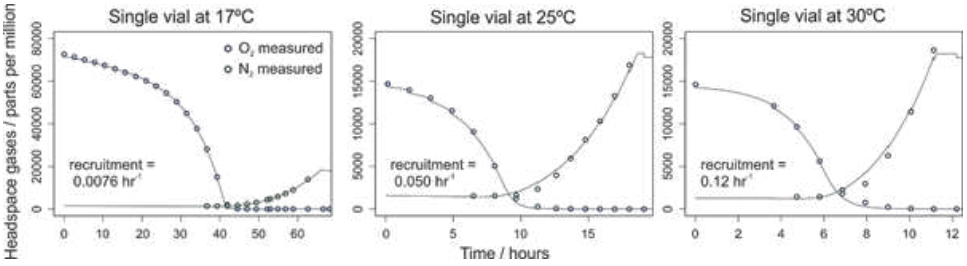


Figure S5: Estimation of recruitment rate by fitting model to measured gas kinetics. The panels show measured gas kinetics for single vials, with fitted model and the estimated probability for NirS expression (r, h^{-1}). The estimated r for individual vials at each temperature are used to make the temperature response panel in Figure 2. The model output was also used to assess differences in phenotype during growth at 17°C (t-test; n=6). The maximum rate of electron flow to O_2 did not differ between strains (p=0.95). Estimated recruitment rates to be 7.2 x 10^{-3} h^{-1} (± 1.5 x 10^{-3} h^{-1}, 95% CI) for PD1222 and 1.2 x 10^{-2} h^{-1} (± 2.9 x 10^{-3} h^{-1}, 95% CI) for *mCherry-NirS* (p < 0.05).

2.2 Temporal development of NirS expression and FITC fluorescence (supplementary to fig 3 and 4)

Two experiments were run to investigate the kinetics of NirS expression as observed (microscopy) versus that predicted by the model. In the first experiment, 10 vials containing 50 mL Sistroms with 2 mM NO_2^- and 10 % acetylene in the headspace (near-anoxic) were inoculated with 2.3E9 FITC-stained cells. Incubation temperature was 17 ^{0}C. Headspace gas concentrations were monitored, and samples (5 mL) were taken for microscopy at intervals (2 vials sampled each time). The result is summarized in Fig S6.

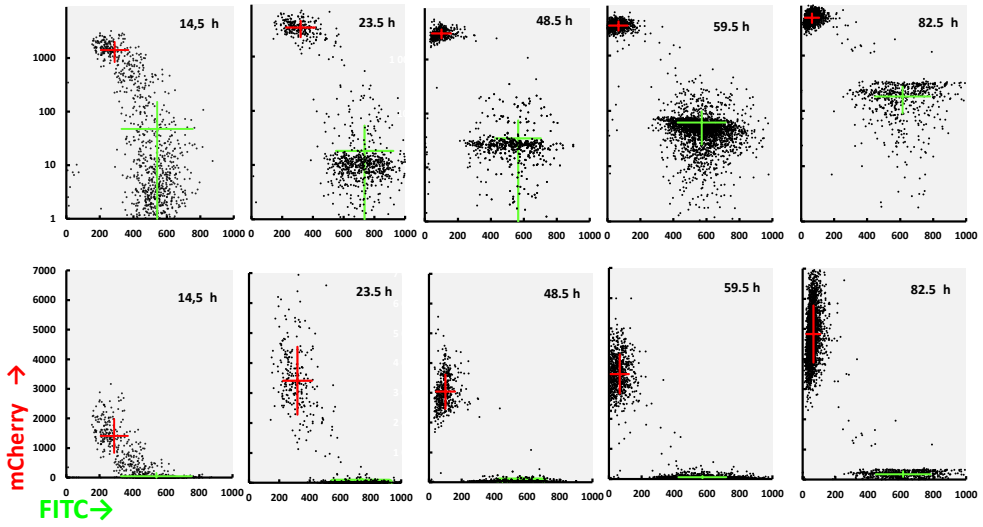


Fig S6. NirS expression and FITC dilution by growth (2mM NO_2^- with 10% acetylene in headspace). Supplementary data to Fig 2. Top panel shows scatter plots of single cells FITC- and mCherry intensities as plotted in Fig 4 (logarithmic scale for mCherry), including the sample taken after 59.5 h. The lower panel shows the same data, but with linear scale for mCherry. In both, the average and standard deviation are indicated by red cross for cells with mCherry>500 and green cross for cells with mCherry<500. **TagS2**

The second experiment was designed similarly to the entrapment assay (see 1.6): Vials with Sistroms without nitrogen oxyanions were inoculated with 2.28E9 FITC-stained cells per vial; temp=17^{o}C. Nitrite was added (to reach 2 mM) 2 hours later. The production of N_2 was extremely slow (below detection limit) during the first 50 h, and then increased exponentially with an apparent growth rate of 0.05 h^{-1} (Fig S7 AB), which is the anaerobic growth rate of the organisms at 17 ^{o}C. Extrapolation of the rate of N_2 back to time zero

suggest that the number of cells able to switch to denitrification was only 0.8E5 cells = 0.036% of the inoculum. The estimated cell numbers (Fig S7D) with NirS (NirS+) and without NirS (NirS-) were calculated by measured N_2-production, growth yield (= 1.7E13 cells per mol electrons (Bergaust *et al*. 2010), and partitioning of N_2O according to the size of the two populations.

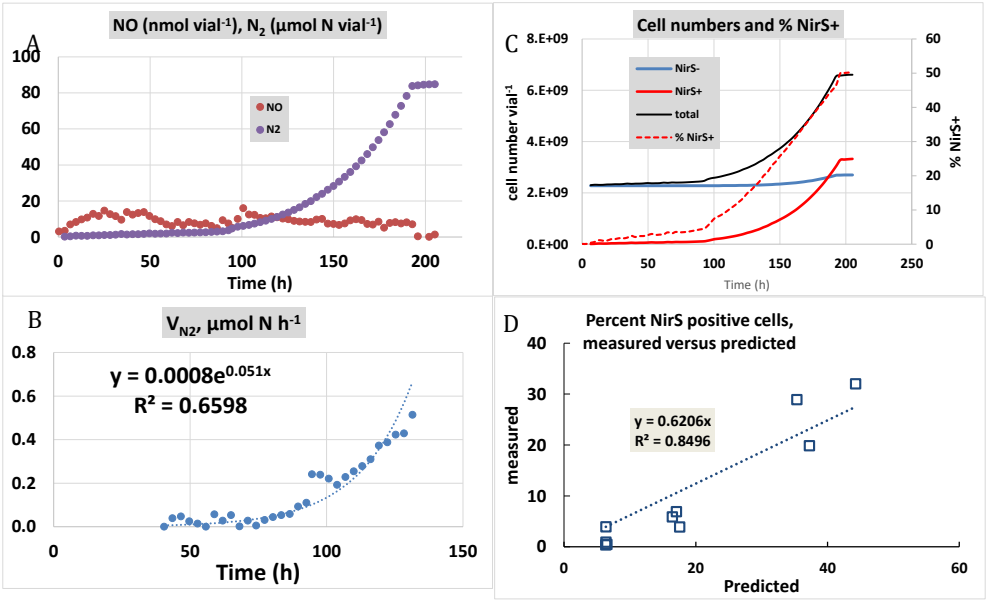


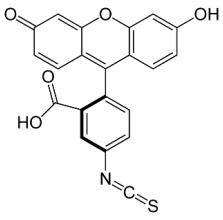
Fig S7 Gas kinetics and NirS expression during anoxic respiration, medium without nitrogen oxyanions, NO_2^- added ~2 h after inoculation. Panel A shows NO (nmol vial^{-1}) and N_2 (µmol N vial^{-1}). Panel B shows the rate of N_2 production for 40- 130 h, and the fitted exponential function. Panel C shows the calculated populations (cell numbers per vial for cells with and without NirS) calculated based on the measured N_2 production (Yield=1.7E13 cells per mol electrons) and a partitioning of N_2O between the two populations (NirS+ and NirS-) according to population size. Panel A-C are results for a single vial (two replicate vials gave very similar results). Panel C shows the relationship between predicted frequency of NirS positive cells and that observed by fluorescence microscopy. **TagS1**

2.3 Development and testing of FITCT on phenotype and cell tracking

Several published methods for tracking growing/non-growing cells were tested, without much success:

- The BONCAT procedure for positively staining growing cells (Hatzenpichler *et al.* 2014) did not work for our purpose with *P. denitrificans* because the staining was ineffective, requiring the use of very high (1 mM) concentrations of AHA (azidohomoalanine, an L-methionine surrogate), which caused apparent inhibition of our strain.
- The Nalidixic acid inhibition (metabolically active cells swell due to prevention of cell division), used in microbial ecology to count metabolically active cells (Joux and LeBaron 1997). This did not work well with *P. denitrificans* because of marginal increase in cell size of metabolically active cells. No clear distinction between growing and non-growing.
- Mitotracker green (MTG, Molecular Probes Inc) is a hydrophobic fluorochrome that stains cell membranes. In theory, growing cells would dilute the MTG signal, while non-growing cells would remain fluorescent. While this would possibly work for short-term experiments, it did not for ours: non-growing cells (in stationary phase cultures) lost most of their fluorescence over-night (even the MTG absorbed in PHB-granules inside the cells was lost).
- Cell tracker green (CTG, Molecular Probe Inc) is fluorescein diacetate. This is non-fluorescent until cleaved by esterase enzymes, and the fluorescein then becomes entrapped in the cytoplasm. It became a popular vital stain for fungal hyphae four decades ago, and was also found to stain most, but not all bacteria (Lundgren 1981). It would be excellent for our purpose (using the reduction of the fluorescence as a measure for growth). Unfortunately, *P. denitrificans* was one of those that do not take up FDA, as was the case for 20% of soil organisms tested by Lundgren (1981).

We then developed our own method, based on fluorescein isothiocyanate (FITC). FITC is a fluorescent dye (fluoresceine) with a thiocyanate group that binds covalently to proteins by reactions with amine and sulfhydryl groups. In theory, FITC staining could interfere with the metabolism of the cells since the covalent bonding to FITC modifies proteins (Weingart *et al*. 1999, Pucket and Barton 2009). We therefore minimized the exposure to FITC, and tested the effect of the staining on the physiology of the strain.



Development of the protocol:

In the first experiments, we used a range of FITC concentrations (5,15, 30 µg FITC mL^{-1}) staining for 1 h, and one with 230 µg FITC mL^{-1} which was stained for 30 seconds only (effective staining time = 3.5 min since centrifugation took 3 min). All treatments resulted in strongly fluorescing cells, somewhat stronger for the short staining at 230 µg FITC mL^{-1}. Neither of the staining methods affected growth, tested by monitoring OD during a 16 h aerobic incubation; the growth rates were all within ± 10 % of that in an unstained control.

Based on this, we concluded that staining for 10 min with a FITC concentration of 50 µg FITC mL^{-1} during staining (i.e. the staining protocol as described above) would secure strong fluorescence and minimize the risk for inhibition of the metabolism.

Testing FITCT on the physiology of *P. denitrificans*

We tested the effect of the staining on the respiratory metabolism in *P. denitrificans*, using Pd1222 wild type and the *mCherry-NirS* strain. The two strains were raised under strict oxic conditions, mid log phase cells were concentrated by centrifugation to 1.5E9 cells mL^{-1} for the wild type and 1.39 cells mL^{-1} for the *mCherry-NirS* strain.

These cell suspensions were stained according to the protocol: 6 mL cell suspension mixed with 1.5 mL FITC staining solution (final FITC concentration 50 µg mL^{-1}), distributed in 4 1.5 mL Eppendorf tubes, staining for 10 min; then washed twice in Sistrom's medium. The final pellets were dispersed in 0.75 mL Sistrom's medium, and used immediately to inoculate the test vials (10^{10} cells per vial). Control cells (not stained) were treated exactly the same way (but without FITC).

The test vials were 120 mL serum vials with 50 mL Sistroms without nitrogen oxyanions, and 0.5 % O_2 in the headspace. Nitrite was injected after the first gas sampling (0.1 mL 1 M KNO_2, i.e 100 µmol $vial^{-1}$). The vials were incubated at 17 ^oC (precooled to this temperature prior to inoculation) and monitored for gas concentrations in the headspace by frequent sampling. The results (Table S2) indicate a marginal inhibition of the initial oxygen consumption, but no consistent effect on the subsequent denitrification phenotype (in response to oxygen depletion), apart from a slight reduction in the maximum concentration of NO.

The growth rate under full aeration was also tested by measuring OD in vigorously stirred suspensions of cells (stained and not stained) for a period of 16 h. The OD increased exponentially both for stained and unstained cells, and the estimated specific growth rate of the stained cells was not significantly different from unstained cells (growth rate of stained cells = 101 +/- 3 % of the unstained cells).

Table S2: Physiological effects of FITC staining of cells. Two strains (Pd1222= WT, and the mCherry-NirS construct =mC), stained (FITC) or unstained (control), were incubated at 17°C in vials with 0.5% O_2 in headspace and monitored for O_2 depletion and subsequent anaerobic denitrification. TagS10

Strain and treatment	O_2 consumption, first 5 hours (fmol O_2 $cell^{-1}$ h^{-1})	[O_2] (µM) when [NO]≥2 nM	$[NO]_{max}$, nM	Anoxic growth rate (h^{-1})
WT control	0.12	2.7	35	0.064
WT FITC	0.11	3.1	27	0.072
mC control	0.12	3.7	27	0.062
MC FITC	0.11	2.7	25	0.064

Testing the dilution of FITC by growth

To evaluate the FITCT as a method to determine growth, FITC stained cells of the *mCherry-NirS* strain were grown anaerobically, and monitored for gas kinetics, OD and fluorescence of individual cells. In short, mid log phase cells from an aerobic culture were concentrated by centrifugation, stained with FITC (according to protocol) and used as inoculum (1 mL, OD_{660}=1.82, 2.28E9 cells) injected to anaerobic 120 mL serum vials containing 50 mL Sistrom's medium without nitrogen oxyanions (stripped for oxyanions, as described by Bergaust *et al*. 2012), and with ~1% N_2O in the headspace. The vials were placed in the incubation robot at 17 ^{0}C, stirred continuously, and provided with more N_2O by 3 repeated injections (100 µmol N_2O-N each time) throughout the first 50 h. Then nitrite was injected (0.1 mL 1M NO_2^- = 100 µmol per vial), and the incubation was continued until all the NO_2^- had been reduced to N_2 (Fig S8). As controls, we included 3 vials without N_2O in headspace (and no nitrite injections).

Liquid sample (5 mL) were taken after 13, 24, 36 and 48 h, for measurement of OD (1 mL) and for fluorescence microscopy (3.6 mL, fixed with formalin). To avoid gas pressure to drop by the liquid sampling, 5 mL pure helium was injected prior to each sampling. The dilution by sampling was taken into account when estimating cumulated N_2-production, and the cell density was estimated from the calculated electron flow, assuming a growth yield of $1.9*10^{13}$ cells per mol electrons (as determined for *P. denitrificans* by Bergaust *et al*. (2010) for anaerobic growth in the same medium as used here).

The results are summarized in Fig S9, demonstrating that FITC is diluted in proportion with growth, while it is retained in non-growing cells (the control without electron acceptor).

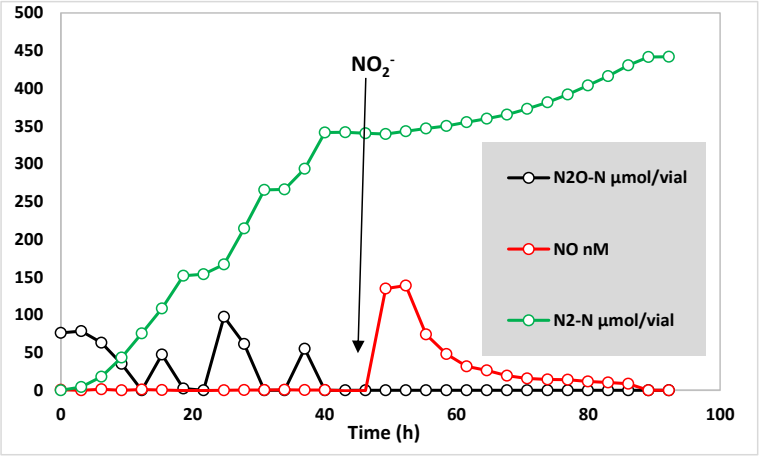


Figure S8: Gas kinetics in a single vial with anaerobically grown *P. denitrificans mCherry-NirS* strain, first provided with N_2O in headspace (repeated injections), then 100 µmol NO_2^- after 50 h (indicated by arrow). The N_2O and NO concentrations are as measured, while the N_2 is the cumulated N_2 production, taking leakage and dilution by sampling into account (see Molstad *et al*. 2007). **TagS8**

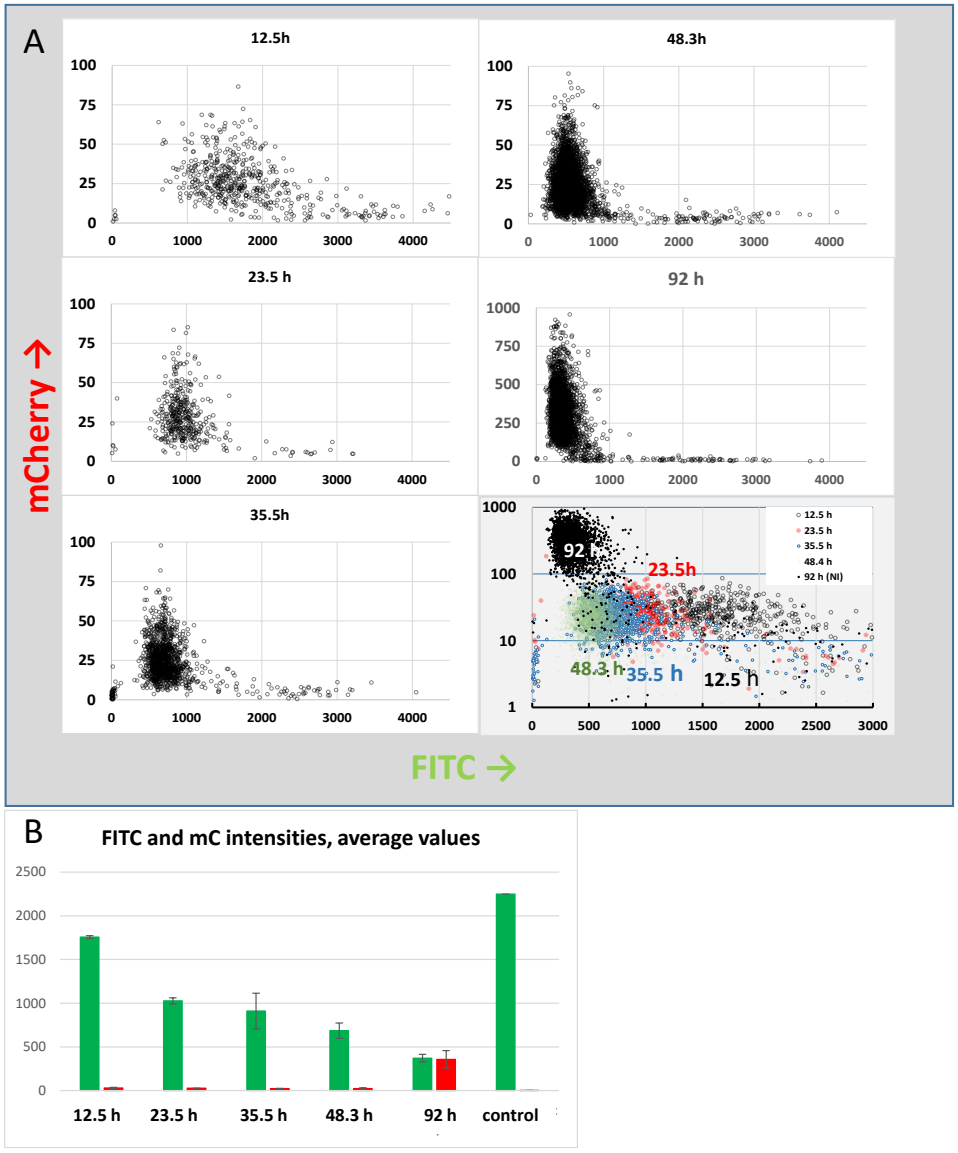


Figure S9. Fluorescence intensities throughout the incubation (see Figure S8). Panel A shows the distribution of fluorescence intensity at different time of sampling (indicated in each panel) and the shaded panel shows the collection of all data in one graph (logarithmic scale for mCherry). Panel B shows the average FITC- (green bars) and mCherry fluorescence, also including the control vial (no electron acceptors) sampled at the end of the incubation (92 h). *TagS3*

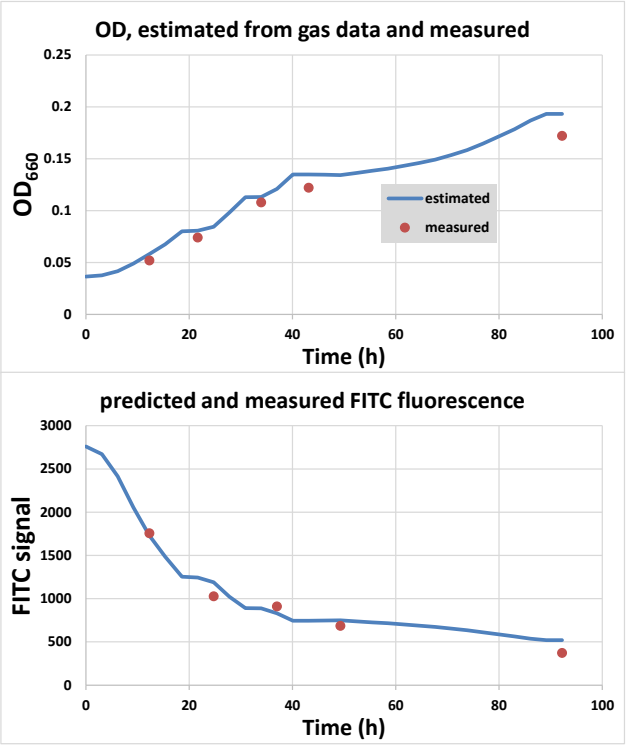


Figure S10: Predicted and measured OD_{660} and FITC intensity throughout the anoxic growth by N_2O-reduction (for the first 50 h), and nitrite reduction (50-92 h). Top panel shows the OD_{660} as predicted by the gas kinetics (cell growth estimated as a function of cumulated electron flow to NOx), and that measured. The lower panel shows the predicted FITC signal (anchored in the first measurement, i.e. 12.5 h). TagS4

2.4 Migration of NirS to the cell poles; control experiments.

The observed migration of mCherry-NirS could be due to properties of mCherry (rather than NirS), or a more general migration of all proteins to the pole. This was tested by experiments with the construct expressing periplasmic mCherry. We also wanted to assess whether the migration is dependent on metabolic activity. This was tested by observing the localization of mCherry-NirS in anaerobically raised cells which were inactivated by injection of 1% NaN_3 prior to sampling and exposure to O_2. The results demonstrate that the observed migration of mCherry-NirS to the poles is due to properties of NirS (not mCherry), and requires some metabolic integrity of cells (Figures S11 and S12).

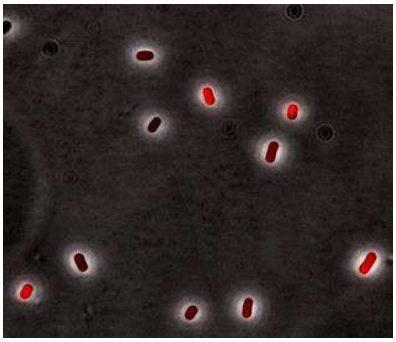


Figure S11: The expression of mCherry equipped with a Sec export signal peptide on an expression plasmid, induced with 1 mM taurine. Cells grown anaerobically in Sistrom's medium at pH 7 with initial 2 mM nitrite. Just before NO_2^- depletion, cells were transferred to fresh aerobic medium and grown for 1 generation aerobically, with vigorous stirring under ambient oxygen tension.

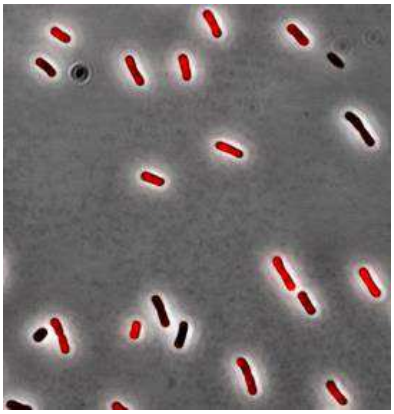


Figure S12: *mCherry-NirS* mutant grown anaerobically in Sistrom's medium at pH 7 with 2 mM initial nitrite. Just before NO_2^- depletion cells were inactivated with 1% NaN_3 and incubated for 1 generation time aerobically, with vigorous stirring under ambient oxygen tension

There is circumstantial evidence for a link between NirS polarization and active respiration. In addition to sudden exposure to oxygen, the depletion of NOx under anoxic conditions dramatically increases the frequency of polarized NirS in the mCherry-NirS strain (Fig S12). This may indicate that even distribution of NirS in the periplasm is dependent on membrane potential.

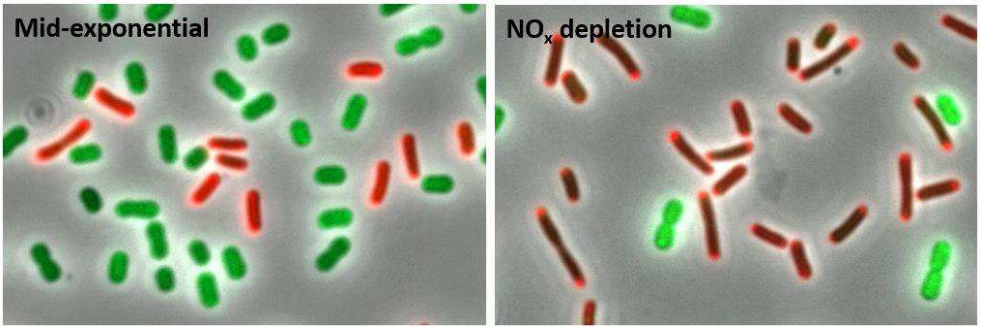


Figure S13: FITC-stained *mCherry-NirS* mutant sampled during anaerobic growth (left panel) and under anoxia after depletion of e^- acceptors. Cells were fixed immediately (formalin) when sampled. Both samples were from the same vial.

2.5 Even distribution of N_2OR.

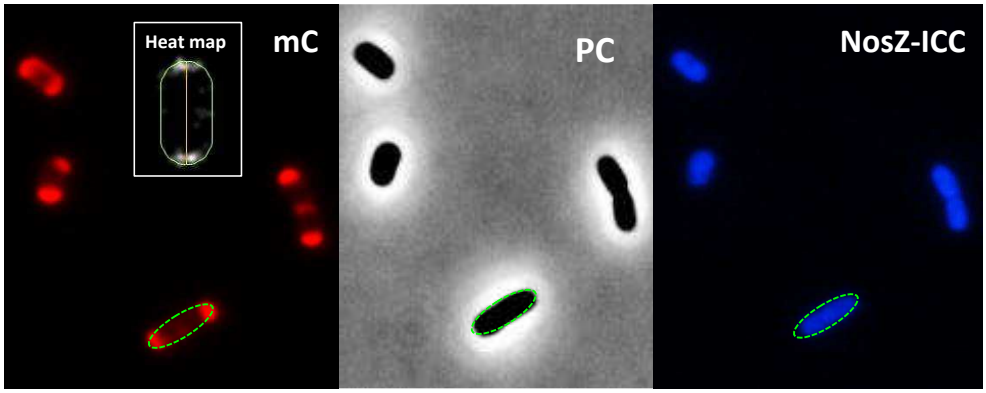


Figure S14. Migration of NirS to the cell pole, leaving N_2OR behind in response to oxygen. The three panels show the same cells with mCherry-fluorescence (mC), in phase contrast (PC) and NosZ immunofluorescence (NorZ-ICC). The approximate outline of the cell (from phase contrast, green dashed oval) is projected onto the fluorescence images. The cells were actively denitrifying when sampled, then exposed to oxygen, fixed (formalin), and cyto-stained (N_2OR). Inserted panel in mC shows the heat-map of mC fluorescence (average of 150 cells analysed)

2.6 Localization of mCherry-NirS in periplasm during denitrification

To verify that mCherry-NirS is localized in the periplasm, we grew the *mCherry-NirS* strain under oxic and anoxic (with 2 mM NO_3^- under anoxic conditions), and investigated the localization of the mCherry fluorescence in DAPI-stained cells, using confocal microscopy and Z-stacking. This confirmed that mCherry-NirS is localized in the periplasm (Figure S15).

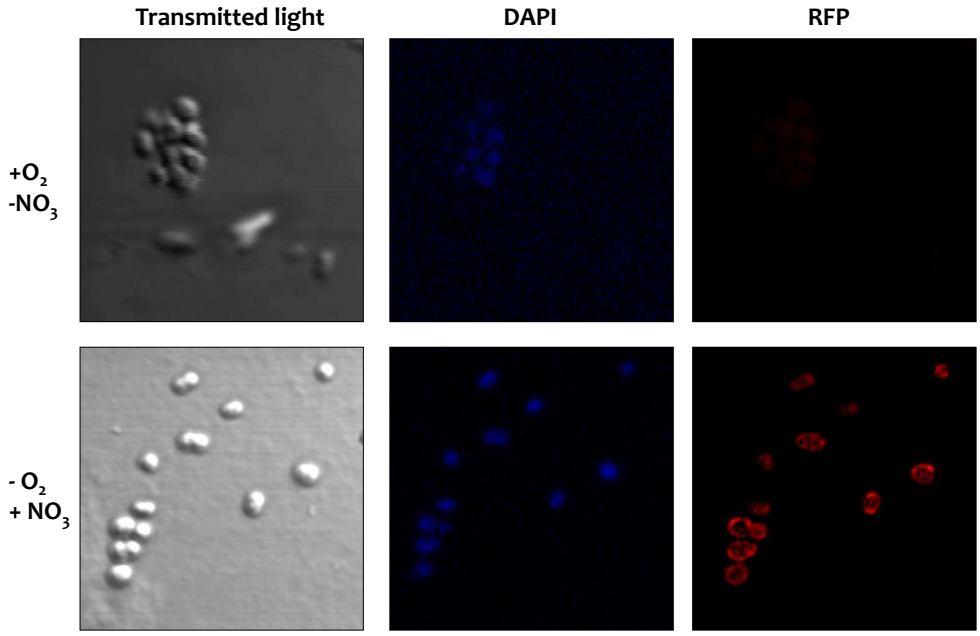


Figure S15. Localization of mcherry-NirS in the periplasm. Cells were grown at 30°C, under fully oxic conditions (without nitrate) or under anoxic conditions (with 2 mM nitrate). The cells were DAPI-stained and visualized by confocal microscopy, using Z-stacking to visualize the localization of mCherry-NirS. The strain with *mCherry-Sec* (see chapter 1.2.2) showed the same periplasmic localization of mCherry fluorescence.

2.7 Time lapse images

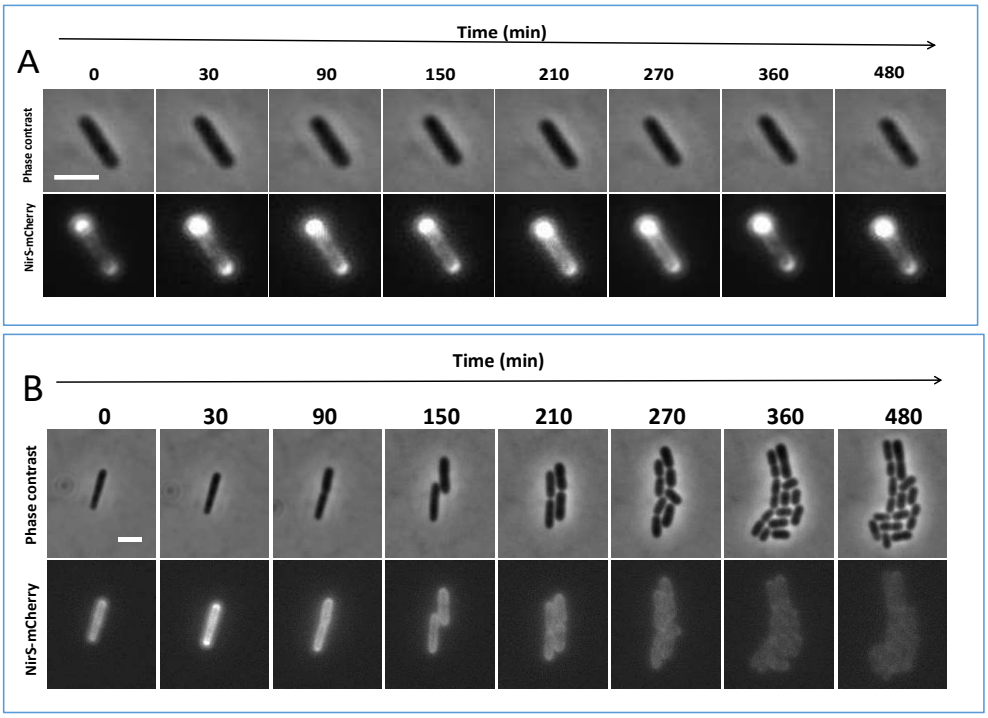


Figure S16 Time lapse images of growing and non-growing cells. Panel A shows a non-growing cell (permanently polar localization of mCherry-NirS). Panel B shows a growing cell, which was transiently polar (30 min) prior to onset of growth.

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Paper III

A step forward in understanding why acidic soils are significant sources of the greenhouse gas N_2O

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Several microbially mediated nitrogen-transformations generate nitrous oxide (N_2O), a powerful greenhouse gas and destructor of the stratospheric ozone layer [1]. Denitrification, the four-step reduction of nitrate to dinitrogen performed by a range of microorganisms, is the dominant source in most ecosystems[1]. The only known biological sink is the enzyme N_2O reductase (N_2OR), executing the reduction of N_2O to harmless N_2. Observations dating from the 1950s onward show high N_2O emissions from acidic soils[2, 3] , but no common understanding of the phenomenon has been reached, and its potential significance for mitigating N_2O emission was not recognized. Compiled results from investigations of soils from different parts of the world provide compelling evidence that the N_2O/N_2 product ratio of denitrification is pervasively controlled by pH[4, 5] Detailed studies of the model denitrifying bacterium *Paracoccus denitrificans*, and of complex soil microbial communities, demonstrated a post-transcriptional hampering of the synthesis of N_2OR at pH ≤6[6-8]. The problem appears to be general for most soil bacteria. The N_2OR is a metalloenzyme structured as a homodimer containing twelve copper atoms. Each monomer has a binuclear Cu_A site for electron transfer and a tetranuclear Cu_Z site catalyzing N_2O reduction[9]. Copper insertion into the apoprotein takes place in the periplasm, and we hypothesize that this insertion is hindered by low pH. To test this, we extracted N_2OR from actively denitrifying *P. denitrificans* cells grown under strictly controlled pH regimes (pH 6.0 and 7.0). Immunological detection demonstrated the presence of N_2OR in comparable amounts in cells grown under both conditions, yet no N_2O reduction took place at pH 6.0. Quantification of Cu by ICP-MS spectrometry suggested lack of copper insertion at pH 6, lending some support to our hypothesis of impaired N_2OR assembly under low pH conditions.

N_2O – environmental issues

Nitrous oxide (N_2O) is a greenhouse gas with strong effects on global warming, which also depletes the stratospheric ozone layer [10, 11]. The anthropogenic N_2O emissions have accelerated for over a century and are steadily rising, with agricultural soils being a major source [12]. This has been driven by the increasing demand for nitrogen fertilizers for more efficient food production, which has led to a significant escalation of the nitrogen cycle at a global scale [13-15]. Denitrification, the process by which microorganisms reduce nitrate to dinitrogen, is the dominant source of N_2O in soils, but the process also holds a clue to mitigate the N_2O emissions because the majority of these organisms are equipped with nature's only enzyme that can reduce N_2O to harmless N_2.

Denitrification

Denitrification is a trait found among a wide range of bacteria, and in some Archaea and fungi. Complete denitrification of nitrate to dinitrogen involves four reductases; nitrate reductase (NAR), nitrite reductase (NIR), nitric oxide reductase (NOR), and nitrous oxide reductase (N_2OR). Many organisms have a truncated denitrification pathway where one or more of the enzymes are missing, either due to lack of the functional gene [16, 17], or another essential gene in the operon [18-20], or because transcriptional regulation or post-transcriptional mechanisms prevent the expression of the functional enzymes transiently or permanently [6, 19]. Transcriptional regulators sensing oxygen depletion, presence of nitrate/nitrite and nitric oxide are common to most denitrifiers. Yet, the regulatory networks of denitrification are diverse and may vary even between closely related organisms [21], resulting in a range of regulatory phenotypes [19, 22]. A consequence of this is that these organisms differ substantially not only in their denitrification end-point products (NO, N_2O or N_2), but also in their transient accumulation of NO and N_2O products during anoxic spells. A fraction of this NO and N_2O will diffuse through the soil and be released to the atmosphere. This underscores the importance of kinetics: an organism (or a microbial community) may be perfectly able to reduce NO_3^- all the way to N_2 but still cause N_2O emission from the soil if N_2O-reduction lags behind the activity of the other denitrification enzymes, since N_2O will diffuse to the atmosphere before being reduced. The diffusion is fast in drained soils (most agricultural soils are drained), but very slow in flooded

soils and wetlands. This is probably the reason for low N_2O emissions from wetland as observed by Audet, Hoffmann [23]: in such systems the transiently produced N_2O will be retained in the soil, and eventually reduced to N_2.

The importance of pH for denitrification product stoichiometry; empirical evidence and hypothesis for the mechanism

Denitrification in soil is controlled by several abiotic factors including low O_2 concentrations and availability of nitrogen oxides and carbon substrates, as well as pH, moisture and temperature [24]. Of these, pH emerges as a strong regulator of denitrification product stoichiometry [1, 25], where acidic pH apparently hampers the reduction of N_2O to N_2. A number of studies from the 1950s and onward report that the N_2O/N_2 product ratio of denitrification increases with decreasing soil pH [2, 3]. The reason for this effect of pH remained obscure for a long time, and the potential significance for mitigating N_2O emissions (e.g. by liming soils) was largely ignored [25].

Figure 1a shows a compilation of our data from various studies, in which gas kinetics of soils sampled across Europe and Asia, and covering a wide range of pHs, was analyzed under standardized anoxic incubation conditions [4, 5]. This demonstrates a strong negative correlation between soil pH and $N_2O/(N_2O+N_2)$ product ratio, which not only corroborates earlier observations, but provides compelling evidence that pH pervasively controls the N_2O/N_2 product ratio of denitrification in a wide variety of soils. This would imply that N_2O emission from soils would be enhanced by soil acidification, and hence mitigated (reduced) by agronomic operations that increase the soil pH such as liming or biochar applications. Numerous field experiments with biochar provide compelling evidence for such mitigation [26] , and so do the less numerous field experiments with liming (reviewed by Qu, Wang [5]).

A first clue to an explanation of the phenomenon was provided from detailed studies of the model bacterium *Paracoccus denitrificans* [6]. In this strain, *nosZ* (encoding N_2OR), together with *nar* (encoding a membrane-bond NAR), is transcribed earlier than the *nir* and *nor* genes (encoding NIR and NOR) in response to decreasing O_2 concentrations [27]. Such early transcription of *nosZ* upon O_2 depletion was shown in cells incubated both at pH 7.0 and at pH 6.0 [6]. The pH 7.0 cultures readily reduced all provided NO_3^- to N_2 within 25 h after O_2 depletion. Reduction of NO_3^-

took place also in the pH 6.0 cultures, albeit at a slower rate, but in this case, no reduction of N_2O was detected until after 60 h. For comparison, when cells were allowed to develop the denitrification proteome under neutral pH and then were shifted to medium with pH 6.1, the N_2O reduction rate was only reduced by 45% compared to at pH 7 [6]. This demonstrated a strong post-transcriptional hampering of the making of N_2OR, but not of the other denitrification reductases . A 45% reduction in activity of functional N_2OR (pH 6 versus pH 7) is consistent with results by Fujita, Chan [28] in a study of recombinant, purified N_2OR (derived from *Achromobacter cycloclastes*), where they found approximately 50 % lower N_2O reduction activity at pH 6 compared to at pH 7. They attributed this to effects of $[H^+]$ both on the reactivity and activation of N_2OR, one important factor probably being the intramolecular electron transfer.

Experiments with soils [7] and bacteria extracted from soils [8] demonstrated essentially identical post-transcriptional effects of pH on the making of N_2OR: the denitrifying communities (intact soil or after extraction) showed immediate transcription of *nosZ* upon O_2 depletion at all pHs, but at low pH there was no N_2O reduction until after > 30 h [7, 8]. When the extracted soil bacteria were allowed to express N_2OR at pH 7 before being transferred to pH 6 medium, they readily reduced N_2O at pH 6. These PCR-based quantifications of transcripts were followed up by metatranscriptome analyses of the same soils, which demonstrated that the *nos*Z transcripts emanated from a wide range of taxonomically diverse bacteria (Lim, Bakken, Shapleigh and Frostegård, unpublished), pointing to a general problem to produce functional N_2OR at acidic pH.

Taken together, these results suggest that while the intact N_2OR is relatively robust to low pH, it is the making of the enzyme that is the sensitive step. As illustrated in Fig. 1b, assembly of the N_2OR takes place in the periplasm of Gram-negative cells [29]. The apo-N_2OR is synthesized in the cytoplasm where pH is regulated to neutral. It is transported to the periplasm, generally via the twin-arginine translocation pathway (Tat), where the final assembly and maturation takes place. This requires a set of accessory, transport and cofactor insertion proteins [30, 31]. The pH in the periplasm is strongly influenced by the pH in the environment surrounding the cells [32]. We hypothesize that the unsuccessful assembly of N_2OR at low pH is due to failure of Cu insertion into the active sites of the apoprotein in the periplasm (Fig. 1b).

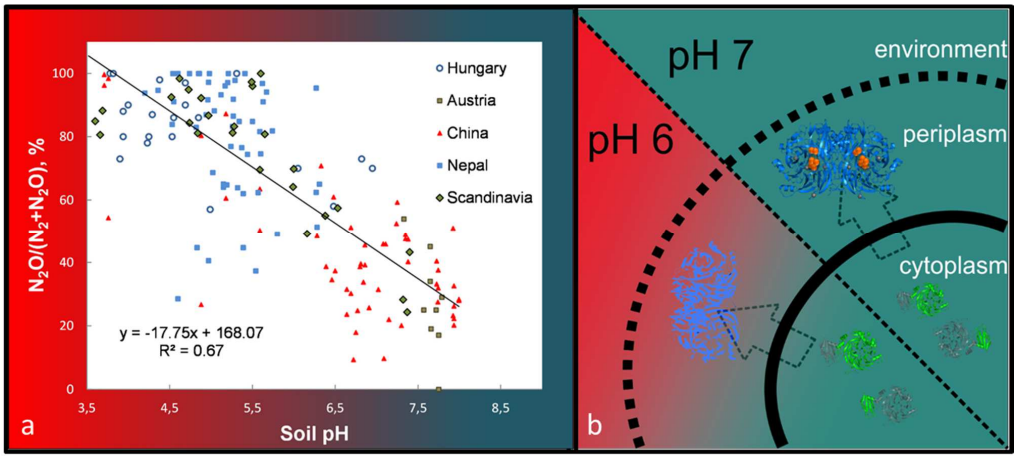

Fig. 1 A. Relationship between soil pH and $N_2O/(N_2O+N_2)$ product ratio for soils from different geographical regions in Europe, Asia and Australia. Compilation of gas kinetics data from microcosm experiments by NMBU Nitrogen Group. **B.** Illustration of the work hypothesis which is the basis for the present study. The apo-N_2OR is synthesized in the cytoplasm where pH is neutral. It is transported to the periplasm where the final assembly and maturation takes place. The pH in the periplasm is not well controlled by the bacteria and is therefore strongly influenced by the external pH. We hypothesize that insertion of Cu into the active sites of the apo-N_2OR is hampered at pH 6 and below, leading to non-functional N_2OR enzymes.

N_2OR, the only sink for N_2O

The N_2OR is the only hitherto known enzyme that can reduce N_2O, accomplished through a two-electron reduction of N_2O to harmless dinitrogen gas [33]. The enzyme consists of two multicopper sites: Cu_A, being an electron donor site, similar to the one of cytochrome c oxidase, and a unique tetranuclear Cu_Z (Fig. 2). The latter, which is the active site of the enzyme where N_2O reduction takes place, possesses either one or two sulfide bridges [9, 33-36]. The enzyme exists as a "head to tail" oriented homodimer, ensuring sufficient distance for electron transfer from the Cu_A site of one monomer to the Cu_Z site of the other monomer.

The genes that code for these supporting proteins are organized in an operon and conserved in most of the organisms that carry *nosZ* gene encoding the N_2OR. The roles and mechanisms of many of the *nos* family proteins have been revealed and described [31]. Important for the present work is the demonstration *in vi*tro that the Cu_A site can acquire copper spontaneously from the solution, whereas the Cu_Z site requires involvement of several putative maturation factors [29, 31].

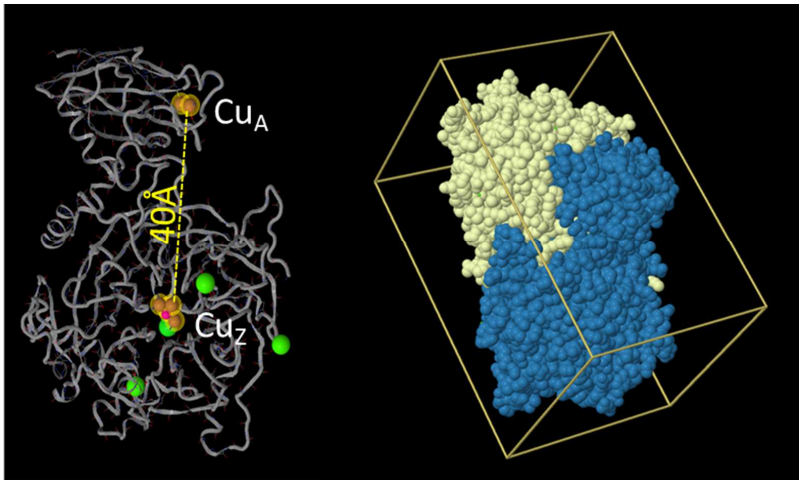

Fig. 2. Model of the N_2OR enzyme generated by RSCB PDB (http://www.rcsb.org/pdb/home/home.do). N_2OR from *P. denitrificans* (1FWX). The subunit structure shows the positioning of the copper centers Cu_A and Cu_Z and the head to tail orientation of the monomers in the functional N_2OR homodimer.

The approach

Here, we set out to determine 1) if N_2OR was present in the periplasm of *P. denitrificans* cells raised at pH 6 and 2) when present, if the copper content in N_2OR from pH 6 is comparable to the one produced by cells grown at pH 7? The task may seem trivial, but as it turned out there were several pitfalls related to the need for efficient control of pH in the cultures incubated at pH 6.0. The reason is that nitrite reduction consumes H^+ ions in the periplasm, leading to a local pH increase in the vicinity of the cells [37]. Thus, if pH is not strictly controlled to pH 6.0 by strong

buffering, individual cells may experience higher pH, especially if they are allowed to form aggregates, which will result in functional N_2O reduction in those cells. Another complicating factor was that *P. denitrificans* does not grow at pH below ca 5.7 [6]. There was thus only a narrow "window" in which the experimental requirements were fulfilled. In our first trials, cells were grown anaerobically in 7 l fermenters with continuous stirring and automatic adjustment of pH to 6.0. This inevitably resulted in production of N_2 from functional N_2OR, pointing to the need for extremely stringent culturing conditions. The solution was to grow the cultures in 3 l batches with vigorous stirring and strong buffering (phosphate buffer, 250 mM), and never allowing the cell density to exceed an OD_{660} of 0.15. Frequent monitoring of gases showed that under these conditions, no N_2O reduction took place. However, to obtain enough cell material, this culturing scheme demanded the production of > 300 l cell culture.

The presence of N_2OR enzymes in bacteria from both pH regimes was verified by immunocytostaining. The periplasmic protein fraction was extracted from the cells and, after size fractionation, the N_2OR presence was determined by Western Blot. Quantification of N_2OR was done by Enzyme-Linked Immunosorbent Assay (ELISA) and Cu content was determined by inductively coupled plasma mass spectrometry (ICP-MS). The details of the protocols are found under Materials.

Results and discussion

Nitrous oxide reductase (NosZ) is present in cells grown at pH 6.0

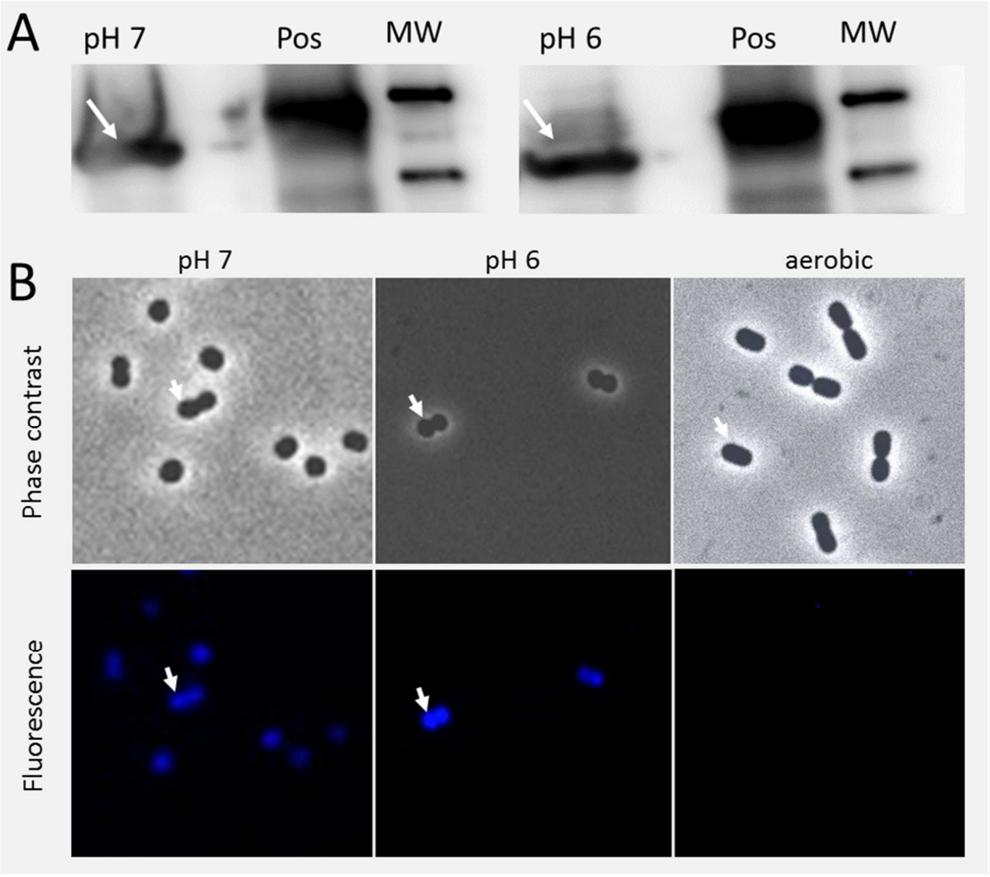

Fig. 3. Presence of N_2OR in denitrifying *Paracoccus denitrificans* cells grown at different pH regimes. **A.** Western blot analysis of the periplasmic protein fraction (size fractionated; fraction 30-300 kDa used) originating from cells grown under denitrifying conditions in mineral medium. N_2OR was present in

denitrifying cells from both pH regimes (arrows). A custom synthesized partial N_2OR from *P. denitrificans* (1FWX) was used as positive control (Pos); (MW) molecular weight marker MagicMark™ XP Western Protein Standard. **B.** Microphotographs of cells visualized by phase contrast (upper panel) and by fluorescence immunocytostaining (lower panel) of N_2OR in denitrifying (pH 7 and pH 6) and aerobically grown *P. denitrificans* cells.

Estimations of copper content in N_2OR from pH 7 and pH 6 grown cells

Periplasmic extracts from *P. denitrifican*s cells, grown under denitrifying conditions at pH 7.0 *vs* pH 6.0, were size fractionated (30-300 kDa) and the total copper concentration was measured using inductively coupled plasma mass spectrometry (ICP-MS). This revealed a difference of one order of magnitude between the samples, with 104 µM Cu in pH 7 samples and 10 µM in pH 6 samples. The concentration of N_2OR proteins in the same samples, assessed by ELISA assay, was four times higher in the pH 7 extracts than in the pH 6 ones (11 and 2.7 $\mu g*ml^{-1}$ respectively). It should be noted that the extracts were size fractionated (30-300 kD), but not further purified, thus both of them most certainly contained other Cu carrying molecules. Yet, there was approximately 2.6 times more moles of Cu mol^{-1} N_2OR in the pH 7.0 samples compared to pH 6.0 samples.

These preliminary results provide a first indication of Cu deficiency in N_2OR enzymes from pH 6.0 cells, thus supporting our hypothesis that lack of N_2O reduction at low pH is due to impaired assembly of the N2OR.

Materials and methods

Bacterial cultures

Paracoccus denitrificans DSM 314 was grown aerobically in modified Sistrom's medium at pH 7.3±0.05 with vigorous stirring for 10 generations at low cell density ($OD_{660} \leq 0.05$) to ensure the absence of N_2OR in the cells from earlier anoxic events. From this culture, 1 ml portions were

snap frozen in liquid nitrogen in the presence of 15 % glycerol and stored at -80^{o}C, used as inoculum in all further experiments.

The cultivation of cells for protein extraction basically followed the description by Bergaust et al (2010) but with upscaling to 3 l cultures, and with stronger buffering to ensure stable pH. Cultures were raised from the frozen stocks grown under strictly controlled conditions in modified Sistrom's medium at two pH levels, 5.9±0.05 and 7.3±0.05, in the presence of 250 mM sodium phosphate buffer (NaH_2PO_4 and Na_2HPO_4). The headspace atmosphere in the incubation bottles was replaced with helium and 5% (volume) of pure O_2, and 5 mM KNO_3^- was provided to each 3 liters batch culture. To prevent cell aggregation, the cultures were stirred vigorously. The pH 5.9 cultures never reached OD_{660} of 0.15. The cultures were monitored by frequent gas sampling for measurements of O_2, N_2O and N_2 to verify the absence of functional N_2OR at pH 6. Cells were harvested when 50% of the provided nitrate was converted to N_2O (pH 5.9) and N_2 (pH 7.3). Cells were pelleted and rinsed with PBS (with pH adjusted to the pH of the cultures). Pellets were snap frozen and stored in liquid nitrogen until proteins were extracted.

Extraction of the periplasmic protein fraction

Twenty grams of frozen cells (wet weight), raised at pH 5.9±0.05 and 7.3±0.05, were thawed and used for extraction. Spheroplasts were generated as described previously [38] with little modifications of the incubation times. The integrity of spheroplasts was checked by microscopy, to identify optimal conditions for spheroplasts generation (data not provided). Cell debris were removed by double centrifugation at 50 000 g for 40 min. Supernatants containing the water-soluble periplasmic fraction of proteins were saved for further purification. Our approach involved size filtration of the crude protein fraction using Viva Spin vertical membranes (Sartorius) of defined molecular weight cutoffs of 30 and 300 kDa. This fraction, containing N_2OR (ca 130 kDa), was collected and the samples were concentrated to the final volume of 500 µl on Viva Spin 10 kDa.

Anti-NosZ antibodies

Antigen: customized partial N_2OR from *P. denitrificans* was obtained from Cusabio Biotech (https://www.cusabio.com/) and used for immunization of chickens (Norwegian Antibodies, http://www.nabas.no/contact/). Eggs were collected and the total fraction of yolk IgY was extracted. The antibodies were affinity purified (using the same antigen as for immunization-N_2OR) in order to obtain anti-N_2OR specific polyclonal antibodies.

Western Blot

Size separated protein extracts were separated by gel electrophoresis (SDS-PAGE) and analyzed by Western Blotting following the protocol provided by the producer (Smart WB, Bio-Rad). The primary anti-NosZ antibodies were used for targeting the N_2OR protein in periplasmic extracts. Secondary goat anti-chicken IgY, HRP conjugated polyclonal antibodies (Cusabio Biotech) were used for detection of IgY-NosZ complexes. WB were read by Azure Biosystems C400 imaging sytem.

NosZ immunocytostaining

Antigen affinity purified primary polyclonal chicken anti-NosZ IgY antibodies (Norwegian Antibodies) were biotinylated (Thermo Fischer) and used for detection of N_2O reductase (NosZ) in formaline fixed and permeabilized cells of *Paracoccus denitrificans*. Labeling with streptavidin-Pacific Blue conjugate (Thermo Fischer) was used for visualization and localization of the NosZ-IgYab by fluorescence microscopy. Cells were grown in Sistrom's medium as described above, harvested and fixed in 3.8% formalin at room temperature for 30 min. Preserved cells were stored at 4^{o}C until cytostained and analyzed. Each step of immunocytochemical staining was followed with 5 times washing with phosphate-buffer saline with 0.05% Tween20 (PBS-T). Blocking buffer contained PBS-T and 1% BSA. The samples were incubated (room temperature) for 1 hour with anitbodies and 30 min with Pacific Blue. Fluorescence microscopy was performed immediately after staining.

Quantification of total copper amount:

Portions of periplasmic extracts were investigated for total copper amount using Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

Quantification of N_2OR:

A cocktail ELISA (Enzyme-Linked Immunosorbent Assay) was developed in order to quantify the amount of N_2OR in analyzed periplasmic extracts. Anti-NosZ antibodies were used for coating MaxiSorp flat bottom ELISA plates (Nunc). The plates were then washed and blocked. Diluted periplasmic samples of unknown concentrations of N_2OR as well as custom synthesized N_2OR standard (Cusabio) were applied to separate wells (5 replicates each). After addition of 100 µl of biotinylated anti-NosZ IgY to each well, the plates were incubated at room temperature for 3 h. They were then washed with PBS-Tween20, after which 200 µl of avidin-conjugated HPR was added to each well, followed by another hour of incubation. The plates were washed again and 200 µl of TMB substrate (Thermo Fischer) was added to each well. The reaction was allowed to develop for 30 min, and then 100 µl of 2M H_2SO_4 was added to each well in order to stop the reaction. Plates were read immediately in Absorbance Reader 800 TS (BioTek) at 405 nm wavelength and the amounts of N_2OR were calculated based on a standard curve obtained from custom synthesized partial N_2OR from *P. denitrificans* of the nominal concentration 1 mg/ml.

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