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Denitrification regulatory phenotype, a new term for the characterization of denitrifying bacteria

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Abstract

Current knowledge of denitrification is based on detailed studies of a limited number of organisms. In most cases the importance of these paradigm species in natural ecosystems is questionable. Detailed phenotypic studies of a wider range of prokaryotes, both type strains and dominant denitrifiers isolated from complex systems, will aid the generation of more sophisticated mathematical models for the prediction of NO and N_2O emission to the environment. However, in order to facilitate the comparison of a vast range of prokaryotes, phenotypic experiments and functional characteristics included should be standardized. In the present paper, we discuss the term DRP (denitrification regulatory phenotype) for describing a set of phenotypic traits and experimental conditions for the characterization of denitrifying organisms. This is exemplified by the contrasting DRP characteristics of the two well-studied denitrifiers *Paracoccus denitrificans* and *Agrobacterium tumefaciens*.

Introduction

Microbial life and proliferation in environments such as soil is filled with challenges. Frequent fluctuations in the availability of carbon, nutrients and electron acceptors are drivers of the evolution of a myriad of survival strategies. Denitrification is the dissimilatory stepwise reduction of NO_x (nitrate/nitrite) to molecular N_2 performed by a wide variety of prokaryotes under oxygen limitation [1]. This process is of great environmental interest since it is a major contributor of NO (nitric oxide) and N_2O (nitrous oxide) to the atmosphere. NO is a highly reactive toxic free radical and its emission is a contributing factor in the acidification of soil and water systems through the formation of HNO_2. It is a signal molecule in a plethora of different regulatory networks and its reduction and detoxification is an important survival mechanism across phyla [2,3]. The reduction of N_2O to N_2 is performed by the copper-containing enzyme, N_2OR (N_2O reductase), which is encoded by *nosZ* and found only in prokaryotes [4]. It is the only currently known enzyme in existence filling this function; without it, our atmosphere, and hence life on Earth, would be very different. N_2O is a powerful greenhouse gas with a GWP (global warming potential) 310 times greater than that of CO_2 and it is also recognized as a major regulator of stratospheric ozone levels [5]. Since the industrialization and introduction of modern agricultural practices, the atmospheric level of N_2O has increased from below 270 ppb (parts per billion) to 319 ppb and it is still steadily rising by approx. 0.3 % every year [6].

Key words: *Agrobacterium tumefaciens*, denitrification, denitrification regulatory phenotype (DRP), nitric oxide, nitrous oxide, *Paracoccus denitrificans*.

Abbreviations used: CRP, cAMP receptor protein; DRP, denitrification regulatory phenotype; FNR, fumarate and nitrate reduction regulator; GWP, global warming potential; N_2OR, N_2O reductase; NO_x, nitrate/nitrite; ppb, parts per billion.

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A soil system's propensity to emit N_2O is influenced by a series of parameters such as pH and the availability of oxygen and/or NO_x. Although many of the observations made in complex systems concur with regard to major trends such as the negative relationship between soil pH and the N_2O/N_2 product ratio of denitrification, most of the underlying mechanisms remain to be described [7,8]. Comprehensive studies of phenotype and gene expression in pure cultures, consortia and microbial communities are needed in order to understand more completely the dynamics of biologically driven N_2O and NO emissions to the environment.

DRP (denitrification regulatory phenotype)

Current knowledge of denitrification is based on detailed studies of a few paradigm species, most of which belong to the α- and γ-proteobacteria [1]. The type of information gathered has been arbitrary in the sense that there are no standardized guidelines for the characterization of denitrifiers and little is known about any link between phylogeny and function. Early phenotypic data were derived from fairly crude experiments with excessively dense cultures and consequently a poor control of parameters, such as formation of aggregates and O_2 availability [9]. A recent study of phenotypic response patterns in *Agrobacterium tumefaciens* during transition to denitrification clearly illustrated the importance of treatment effects on observed phenotype [10]. This points to the need for detailed and precise datasets describing the influence of a number of selected parameters on denitrification in different organisms. Such studies should include not only already well known model strains, but also organisms that are isolated from environments where they are shown to play key roles as denitrifiers. This will pave the way

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for the generation of more sophisticated mathematical models of microbial processes in soil. However, for such comparative studies to be of real scientific value, it is essential to define the traits by which we characterize new organisms. Recently, we introduced the term DRP for this purpose [12]. DRP encompasses traits which are all probably of consequence to a given microbe's survival and contribution of NO_x to the environment, under a defined set of conditions.

Methods and standard conditions

The phenotypic analyses were conducted in semi-automatic incubation systems developed by us [11]. In general they consist of a thermostatic water bath holding 15 (or 44 in a new version) stirred cultures and an autosampler connected to a gas chromatograph and an NO analyser. Experiments are performed under an He/O_2 atmosphere allowing for the quantification of the end-product of denitrification, N_2, as well as O_2, CO_2, NO and N_2O. Nitrite measurements are carried out separately in liquid, frequently in conjunction with the quantification of *nir*, *nor* and *nos* transcripts by real-time PCR [12]. The typical batch experiment starts with a headspace O_2 concentration of 1–5 % by vol., and the gas kinetics are monitored through oxygen depletion (by respiration) and the subsequent phase of anoxic respiration.

In order for comparative studies of large numbers of different organisms to be feasible, a set of parameters and experimental conditions need to be defined. These are not self-evident, however, due to the nearly endless variety of denitrifying prokaryotes. Ideally, all strains should be grown in a defined medium with one or a few different carbon sources under identical pH and temperature conditions. Every experimental culture should be inoculated with the same number of cells from thin inocula free of aggregates and with no prior expression of the denitrification proteome. Since this is not realistic, the second choice is to standardize the conditions to the greatest extent possible. One such step would be normalizing the aerobic respiration rates (seen as O_2 reduction rate) of inocula, i.e. to ~0.1 mol·flask^{-1}·h^{-1}, thereby ensuring that the rate of oxygen consumption towards the end of the oxic phase is the same for all cultures. Initial electron acceptor concentrations are easily controlled and should, during screening, be adjusted to levels that do not give rise to the accumulation of toxic concentrations of intermediates. Previous experiences with *Paracoccus denitrificans* [12] and *A. tumefaciens* [10], as well as other organisms belonging to the α- and β-proteobacteria, indicate that 1 % by vol. initial O_2 and 2 mM NO_3^- or NO_2^- at near neutral pH are conditions that generally facilitate successful transitions and completion of denitrification. The choice of medium and incubation temperature must be based on the strain studied.

Basic characteristics

The number of traits determining the survival and responses of an organism is nearly infinite. In order to facilitate a comprehensive comparison between organisms, it is necessary to limit the number of properties included. Table 1 lists the traits included under the DRP umbrella. Only a select series of phenotypic traits, the 'basic characteristics', are suggested as being mandatory for the first screening of strains. All of these factors are associated with how prokaryotes handle transitions between oxic and anoxic conditions in the presence of NO_x and can be divided into two categories: (i) responses to variations in O_2 and consequences to energy conservation; and (ii) accumulation of intermediates. Table 2 and Figure 1 describe two contrasting denitrifiers, *A. tumefaciens* and *P. denitrificans.*

Table 1 | **List of trials included under the term ORP**

Transitions between oxic and anoxic conditions	
Basic characteristics	**Additional characteristics**
$[O_2]$ at first detection of NO_x	Gene expression
Accumulation of NO_2^- *	Effects of range of initial $[O_2]$ and $[NO_x]$
NO_{max}	
N_2O_{max}	Cell yield from NO_x
Electron flow to O_2 and NO_x	pH effects
Relative growth rates (μ_{anoxic}/μ_{oxic})	F_{den}
Response to O_2 pulse	N_2O reduction rate

*When nitrate is initial NO_x

Response to variations in O_2 and consequences to energy conservation

An organism's ability to respond to changes in the availability of electron acceptors, and to switch effectively between respiration strategies, is of importance to survival for several reasons. First, there are differences in the ATP yield of aerobic respiration and denitrification [13] and, in order to maximize energy conservation, it is essential to direct the flow of electrons to O_2 whenever possible. Secondly, although repressing NO_x respiration in the presence of O_2 is energetically favourable, being able to switch to denitrification before O_2 is completely depleted is a prerequisite for continued growth in organisms that are unable to use other electron acceptors or generate ATP by fermentation. Several of the traits defined in Table 1, and exemplified in Figure 1 and Table 2, are descriptive of this challenge. The O_2 concentration at which denitrification is initiated (seen as a first appearance of NO) is highly variable between strains and treatments (Figure 1, left-hand panels, and Table 2) and can be taken as an indication of the O_2 affinity of the apparatus and, in a sense, the efficiency of energy conservation. However, it is also a measure of the ability to respond early enough to avoid entrapment in anoxia without denitrification enzymes. The risks involved in late induction are illustrated by the inspection of the electron flows and growth rates during oxic and anoxic phases. We have observed, for example, that cultures of *P. denitrificans* that were exposed to fast oxygen depletion showed a dramatic fall in electron flow. This was followed by an apparent growth-related exponential increase in electron flow to NO_x

Table 2 | Basic characteristics of the DRPs of *A. tumefaciens* compared with *P. denitrificans* when grown in Sistrom's medium at pH 7 and monitored for respiration and accumulation of intermediates (NO_2^-, NO, N_2O and N_2)

Abbreviation used: nd, not determined.

Character	Treatment	*A. tumefaciens*	*P. denitrificans*
$[O_2]$ (μM) in liquid at appearance of detectable NO	1 mM NO_3^-	0.46	0.95
	2 mM NO_3^-	0.38	0.46
	1 mM NO_2^-	15.2	1.15
	2 mM NO_2^-	17.2	2.0
$[NO_2^-]_{max}$ (μM)	1 mM NO_3^-	36	nd
	2 mM NO_3^-	nd	1990
$[NO]_{max}$ (nM) in liquid	Low cell density*		
	1 mM NO_3^-	3216	14.5
	2 mM NO_3^-	1332	19.5
	1 mM NO_2^-	54	10.3
	2 mM NO_2^-	176	14.3
	High cell density*		
	1 mM NO_3^-	9600‡	14.4
	2 mM NO_3^-	8800	14.5
	1 mM NO_2^-	8100	17.1
	2 mM NO_2^-	38 000	16.4
Ratio between anoxic and oxic growth rate (μ_{anoxic}/μ_{oxic})†	1 mM NO_3^-	0.90	0.61§
	2 mM NO_3^-	0.90	0.57§
	1 mM NO_2^-	0.78	0.58
	2 mM NO_2^-	0.78	0.57

*Cell density reached at the time of oxygen depletion, 'low cell density' $\sim 10^8$ cells·ml^{-1}, 'high cell density' $\sim 5 \times 10^8$ cells·ml^{-1}.

†Oxic growth rates were determined in cultures with initial concentration of $O_2 = 7$ vol% in headspace. Anoxic growth rates were determined in cultures with initial oxygen near zero.

‡The high NO concentrations apparently inhibited further denitrification, less than 50 % of the added NO_x was reduced to N_2O.

§The ratio is calculated based on electron flows derived from the measurement of gaseous intermediates, thus excluding the first reduction step of denitrification. In organisms which accumulate large amounts of nitrite during nitrate reduction (i.e. *P. denitrificans*), this way of calculating μ_{anoxic}/μ_{oxic} will underestimate the ratio when nitrate is the NO_x initially available.

until the depletion of electron acceptors (Figure 1, *P. denitrificans*). This observation gave rise to the hypothesis that under these circumstances only a fraction of the population (F_{den}) induced denitrification early enough to avoid being trapped in anoxia without denitrification enzymes. The existence of subpopulations, expressing different phenotypes, in a pure culture is not new [14], but has to our knowledge not been described previously during denitrification. Thus further evidence, based on microscopic techniques or flow cytometry measurements for the differentiation of active and inactive cells, is needed in order to verify the existence of F_{den} as a valid parameter in the characterization of denitrifiers. In contrast with *P. denitrificans*, *A. tumefaciens* did not show the same fall in electron-transport rate upon transition to anoxic respiration. It appears that the earlier onset of denitrification in relation to oxygen depletion ensured that nearly all of the cells were able to switch to denitrification.

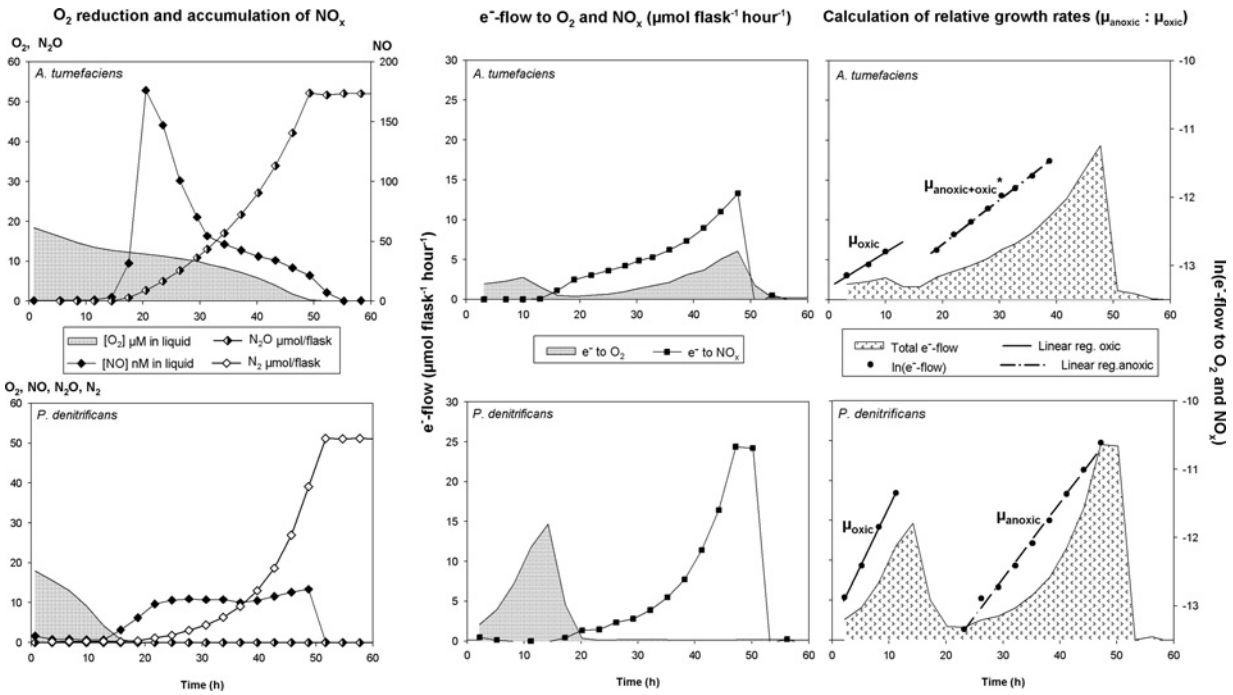
The exponential increase in electron flows during oxic and anoxic respiration is an indirect measure of growth and may be taken as an indication of energy conservation using O_2 or NO_x as electron acceptors. However, absolute values for growth rates (μ) depend on parameters such as optimal pH, temperature and carbon sources and hence are not directly comparable between strains. This motivates the inclusion of the μ_{anoxic}/μ_{oxic} ratio as an indication of the relative energy conservation in a given strain using the two respiration strategies, which in turn facilitates comparisons between strains growing under a range of conditions. Figure 1 (right-hand panels) illustrate how growth rates (μ) are calculated based on electron flow to O_2 and NO_x during aerobic and anaerobic respiration. Preliminary calculations of relative oxic and anoxic growth rates are performed under the standard set of conditions (1 % initial O_2 and 2 mM NO_2^-) during initial screening (Figure 1) and, in that respect, μ_{anoxic}/μ_{oxic} is a basic DRP character. However, in order to ensure accurate assessments of relative growth rates during oxic and anoxic respiration, the electron flow to O_2 and NO_x should be investigated separately in dedicated cultures of selected strains (Table 2).

Accumulation of intermediates

Denitrification is the dominant biological source of N_2O and an important contributor of NO to the environment. Detailed phenotypic studies of *A. tumefaciens* and *P. denitrificans*

Figure 1 | Gas kinetics and electron flow to O_2 and NO_x in *A. tumefaciens* and *P. denitrificans* at pH 7 and 1 % initial O_2 and 2 mM NO_2^-

Left-hand panels show O_2 depletion, transient NO accumulation and N_2O or N_2 production during 60 h of incubation. Middle panels show the derived electron flows to O_2 and NO_x, and the right-hand panels demonstrate the calculation of growth rates based on total electron flow (log-transformed) during the oxic and anoxic phase. In *A. tumefaciens*, the apparent increase in growth rate during the anoxic phase is a result of early induction of denitrification and simultaneous oxygen and NO_x respiration.



have shown that the accumulation of intermediates during denitrification is highly variable across species and that the dynamics of the transition from oxic to anoxic conditions in some instances can be closely linked to the availability of electron acceptors [10,12]. This is clearly shown in Figure 1 (left-hand panels) and Table 2, which, taken together, suggest that denitrifiers may be found within a wide array of phenotypes. In addition to estimations of O_2 concentration at induction of denitrification and subsequent NO and N_2O accumulation, the response to injections of O_2 during denitrification, mimicking fluctuating conditions in the environment, should be assessed during screening. A strain's tendency to denitrify in the presence of O_2 is probably an indicator of its propensity to emit N_2O, since N_2OR is apparently more sensitive to O_2 than the other NO_x reductases ([4] and B. Liu, Å Frostegård and L.R. Bakken, unpublished work).

Additional characteristics

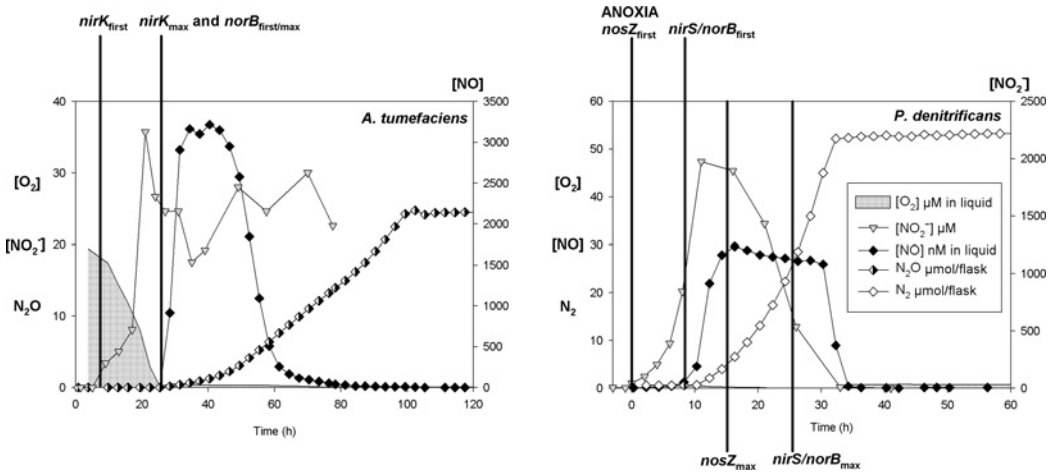
The properties listed above are relatively easily described through simple batch incubation experiments and should all be included when screening new strains. However, there are a number of other factors that fall under the term DRP, but which, for practical reasons, cannot be included for every single strain. Molecular techniques such as real-time PCR are costly, time-consuming and, even more importantly, sequence-dependent. Detailed studies of the effect of initial electron acceptor concentrations and pH require dedicated experiments and would present a bottleneck if performed on all strains. Thus it is more realistic to select a limited number of representatives for gene expression analysis and evaluation of treatment effects.

The reductases involved in denitrification are subject to transcriptional regulation by O_2, nitrate, nitrite and NO through two-component systems or factors belonging to the FNR (fumarate and nitrate reduction regulator)/CRP (cAMP receptor protein)-type regulators. However, the details of regulation have only been described in a few paradigm species and there seems to be a wide spectrum of regulatory phenotypes [15–19]. In the experimental setup described in the present paper, high-resolution phenotypic data are the basis for selective sampling and quantification of *nir*, *nor* and *nos* transcripts. The potential of this scheme is illustrated in Figure 2, comparing the timing of transcription against gas measurements in *A. tumefaciens* and *P. denitrificans*. These and similar datasets give valuable indications regarding the regulation of transcription and may guide subsequent experimental designs.

pH has emerged as one of the master variables in soil, being a major determinant of soil chemistry and most probably

Figure 2 | Timing of transcription during transition to denitrification in *A. tumefaciens* (left-hand panel, *nirK* and *norB*) and *P. denitrificans* (right-hand panel, *nirS*, *norB* and *nosZ*) at pH 7

A. tumefaciens was initially supplied with 1% O_2 and 1 mM NO_3^-, whereas *P. denitrificans* was subjected to sudden near-anoxia (0 h) and 2 mM initial NO_3^-.



driving both the selection of microbial species and shifts in community structure. The increased net N_2O emission observed in low pH soils [8], however, cannot be explained by selection pressure and community structure alone. We have observed loss of N_2O reduction at low pH in pure cultures, extracted cells and soils despite the presence of *nosZ* transcripts [7,12]. On the basis of dedicated phenotypic experiments, we found that this apparent discrepancy is most probably explained by inhibitory mechanisms directed at the reductase specifically, primarily by misfolding in the periplasm as a result of low pH and partly by inhibition of the Cu_A-centre of N_2OR by H^+ [12,20]. The observed pH effect on N_2OR in *P. denitrificans* is probably the result of an easily disturbed periplasmic pH. Apart from a study on *Escherichia coli*, demonstrating poor resilience to changes in external pH [21], little is known about the regulation of periplasmic pH in Gram-negative bacteria. The observations made during our incubation experiments can by no means be taken as direct measurements of periplasmic pH regulation or lack thereof. However, investigating the effect of pH on the denitrification apparatus, and specifically on N_2OR in a range of strains, is interesting due to the environmental implications of pH-derived effects on N_2O reduction.

Conclusion

The combination of sophisticated phenotypic analyses and molecular techniques is a powerful tool in characterizing denitrifying organisms. With the introduction of the concept of DRP we have developed parameters and traits which allow standardized and relatively high-throughput comparative studies of pure cultures and communities, supporting the generation of more realistic models of microbial processes in complex systems.

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