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# Tuning the modular *Paracoccus denitrificans* respirome to adapt from aerobic respiration to anaerobic denitrification

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## Summary

**Bacterial denitrification is a respiratory process that is a major source and sink of the potent greenhouse gas nitrous oxide. Many denitrifying bacteria can adjust to life in both oxic and anoxic environments through differential expression of their respiromes in response to environmental signals such as oxygen, nitrate and nitric oxide. We used steady-state oxic and anoxic chemostat cultures to demonstrate that the switch from aerobic to anaerobic metabolism is brought about by changes in the levels of expression of relatively few genes, but this is sufficient to adjust the configuration of the respirome to allow the organism to efficiently respire nitrate without the significant release of intermediates, such as nitrous oxide. The regulation of the denitrification respirome in strains deficient in the transcription factors FnrP, Nnr and NarR was explored and revealed that these have both inducer and repressor activities, possibly due to competitive binding at similar DNA binding sites. This may contribute to the fine tuning of expression of the denitrification respirome and so adds to the understanding of the regulation of nitrous oxide emission by denitrifying bacteria in response to different environmental signals.**

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## Introduction

Denitrifying bacteria play an important role in determining the fate of reactive nitrogen in both terrestrial and aquatic environments, especially when oxygen ($O_2$) is limiting (Thomson *et al.*, 2012). These bacteria can switch between respiring $O_2$ and nitrate ($NO_3^-$) using a process known as denitrification in which two $NO_3^-$ anions are reduced via nitrite ($NO_2^-$), nitric oxide (NO) and nitrous oxide ($N_2O$) to di-nitrogen ($N_2$) in a series of reactions that consume 10 electrons in total (Reactions 1–4).

$$2NO_3^- + 4e^- + 4H^+ \xrightarrow{\text{Nar}} 2NO_2^- + 2H_2O \quad \text{(Reaction 1)}$$

$$2NO_2^- + 2e^- + 4H^+ \xrightarrow{\text{Nir}} 2NO + 2H_2O \quad \text{(Reaction 2)}$$

$$2NO + 2e^- + 2H^+ \xrightarrow{\text{Nor}} N_2O + H_2O \quad \text{(Reaction 3)}$$

$$N_2O + 2e^- + 2H^+ \xrightarrow{\text{Nos}} N_2 + H_2O \quad \text{(Reaction 4)}$$

The enzymes catalysing these reactions serve as termini in a branched respiratory network. The network comprises: primary dehydrogenases which extract electrons by oxidizing both organic and inorganic substrates; reductases that transfer electrons to terminal electron acceptors; and electron-transfer proteins that mediate electron transfer between the primary dehydrogenases and reductases. Together these proteins comprise the sub-proteome that make up the various respiratory pathways which we have termed the '*respirome*' (Fig. 1). It is hypothesized that the transition between aerobic and anaerobic respiration (denitrification) would require the differential expression of some elements of the respirome for growth and energy conservation.

A full appreciation of bacterial bioenergetics requires an understanding of both the regulation of respirome biosynthesis and electron flux through the respiratory pathways. These factors will not only influence the degree to which free energy released by the oxidation of electron donors is either captured as a proton motive force or dissipated, but also control the levels of some reactive chemical species that can be harmful to cellular processes. For example,

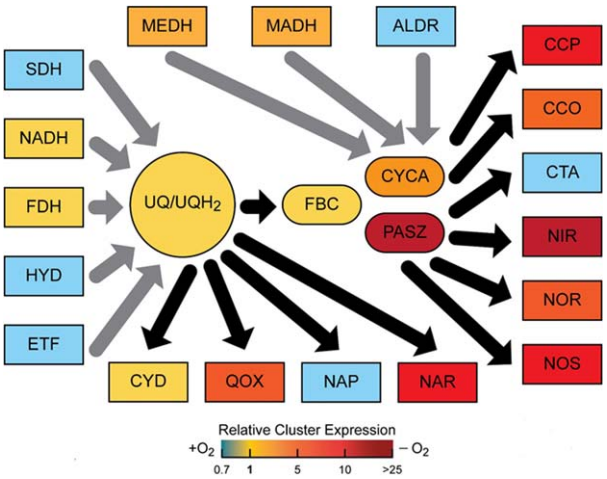


**Fig. 1.** Relative gene expression profile of the respiratory network of *P. denitrificans* under the aerobic ($+O_2$) and anaerobic ($-O_2$) continuous culture incubations ($n = 3$, ±SE).
The capitalized abbreviation denotes a component of the respirome and the colour of the box indicates the relative levels of expression of the gene cluster encoding that complex. Abbreviations: SDH, succinate dehydrogenase; NADH, nicotinamide adenine dinucleotide dehydrogenase; FDH, formate dehydrogenase; HYD, hydrogenase; ETF, electron transport flavoprotein; MEDH the methanol dehydrogenase; MADH the methylamine dehydrogenase; ALDR, aldose reductase; UQ/UQH$_2$, the ubiquinone pool; FBC the cytochrome $bc_1$ complex, CYCA, cytochrome $c_{550}$; PASZ, pseudoazurin; QOX, cytochrome $ba_3$ oxidase; CCP, the cytochrome c peroxidase; CCO, cytochrome $cbb_3$ oxidase, CTA, cytochrome $aa_3$ oxidase; NAR, dissimilatory nitrate reductase; NAP, periplasmic nitrate reductase; NIR, nitrite reductase; NOR, nitric oxide reductase; NOS, nitrous oxide reductase. Black solid arrows (↗) indicate the electron output and the grey solid arrows (↗) the electron input. The colours indicate the relative levels of expression of the gene clusters encoding each component in response to the aerobic-anaerobic transition based on the micro-array.

during anaerobic $NO_3^-$ respiration, the NO radical generated by the $NO_2^-$ reductase (Nir, Reaction 2) is a potent cytotoxin that can cause nitrosative damage and cell death (Poole, 2005). Although NO can be consumed by the action of the NO reductase (Nor, Reaction 3), elevated levels of the product, $N_2O$, exert a cytotoxic effect at the level of the vitamin $B_{12}$ pool (Sullivan *et al.*, 2013). Therefore, under ideal conditions, the NO-generating, NO-consuming and $N_2O$-consuming processes (Reactions 2–4) should function in concert to form inert $N_2$ gas and prevent the release of harmful intermediates of denitrification. As well as being a respiratory substrate and a cytotoxin, $N_2O$ is also a potent greenhouse gas (Thomson *et al.*, 2012). Consequently, a fuller understanding of synthesis and consumption of $N_2O$ at the cellular level is an essential element in developing mitigation strategies that can limit its atmospheric levels.

The importance of bacterial denitrification to the global nitrogen cycle and its influence on other biogeochemical cycles, such as the carbon and sulphur cycles, is reflected by the level of detail in which we understand the structure and mechanism of each terminal reductase and the intermediate electron carriers (Richardson, 2008). The mode of respiration employed by the *P. denitrificans* respirome is known to be influenced by a number of environmental signals, including $O_2$, NO and $NO_3^-$ (Zumft, 1997; Baker *et al.*, 1998). Moreover, a number of studies have established roles in *P. denitrificans* for three members of the CRP family of transcriptional activators, FnrP, NnrR and NarR, whose primary effectors are $O_2$ for FnrP, NO for NnrR and $NO_3^-/NO_2^-$ for NarR (Van Spanning *et al.*, 1997; 1999; Wood *et al.*, 2001; Veldman *et al.*, 2006; Bouchal *et al.*, 2010; Spiro, 2012). However, comparatively little is known about the interplay between these regulatory networks. Here, we report differences in the transcriptomes of *P. denitrificans* respiring under either aerobic or anaerobic denitrifying conditions alongside targeted metabolic measurements. Together these data provide new insight into how the respirome is remodelled in response to the transition between aerobic and anaerobic conditions.

## Results and discussion

### *Comparison of steady-state aerobic and anaerobic denitrifying cultures of* P. denitrificans

Continuous cultures were established in which *P. denitrificans* was allowed to grow to a steady-state biomass under aerobic conditions. Cultures were initially grown under batch culture conditions at 100% air-saturation (236 μM dissolved oxygen) for ~20 h (Fig. 2A). The system was then switched to continuous culture with a dilution rate ($D$) of 0.05 $h^{-1}$. Sufficient aeration was maintained so as to sustain aerobic metabolism in the steady-state culture. The reservoir medium feed contained 20 mM $NO_3^-$ and 5 mM succinate and was designed to establish an electron donor-limited (succinate), electron acceptor-sufficient (oxygen or nitrate) continuous culture. In continuous cultures the original biomass is washed out of a bioreactor exponentially; $x_t/x_0 = e^{-Dt}$ (where $x_0$ = biomass at time 0 and $x_t$ = biomass at time $t$), thus, at $D = 0.05$ $h^{-1}$ a period of ~50 h is required before the bioreactor is dominated by newly generated cells. The continuous culture experiments ran for ~100 h to be confident of achieving steady-state. In the aerobic continuous culture nitrate levels remained constant at ~20 mM throughout the experimental period (Fig. 2B). This indicated that neither denitrification nor assimilatory $NO_3^-$ reduction pathways were operational and that the steady-state culture was sustained only by aerobic respiration. Succinate was not detectable (<10 μM) in the bioreactor under conditions of biomass steady-state (50–120 h; Table 1).

In order to establish a steady-state anaerobic-denitrifying culture, the bioreactors were set up identically to the aerobic cultures except that after the 20 h aerobic batch

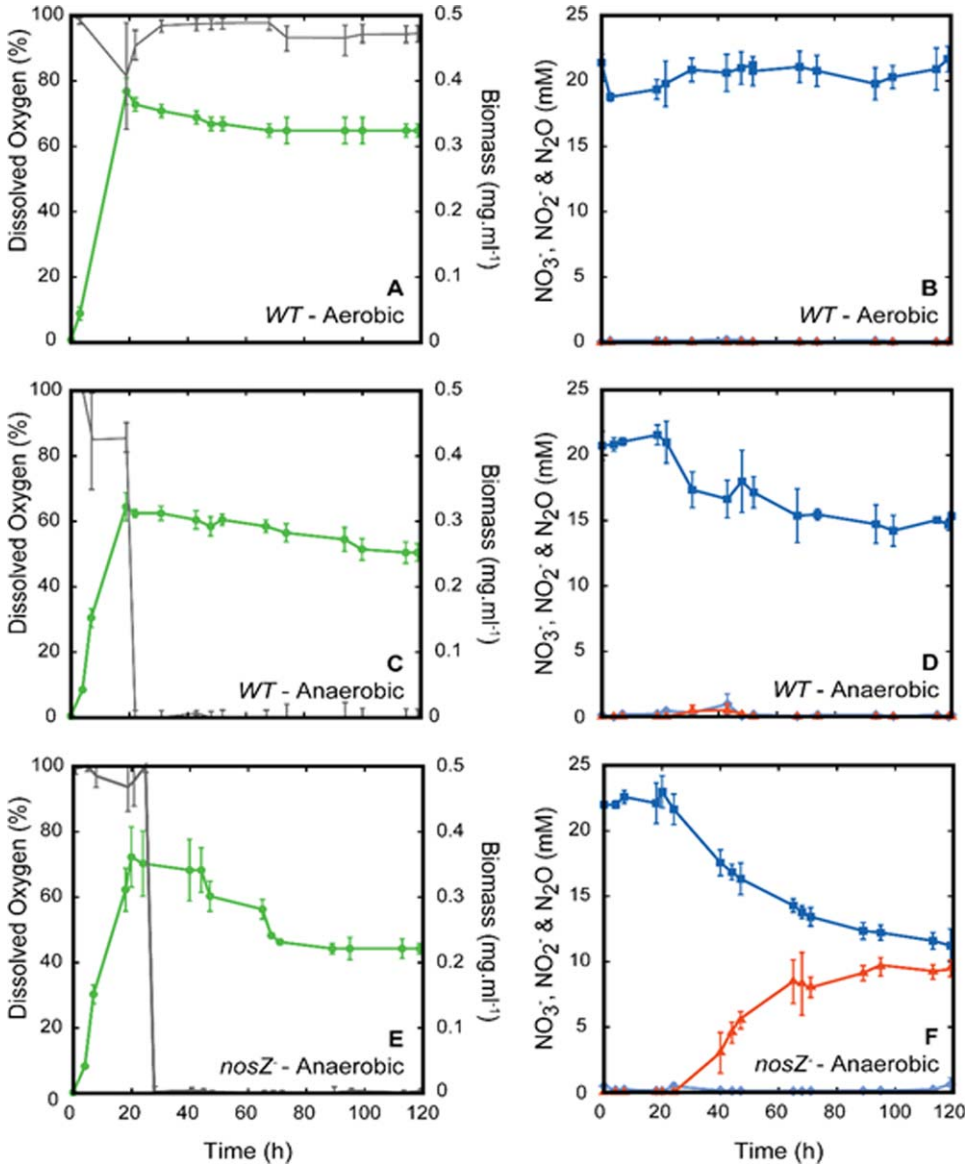


**Fig. 2.** Growth and nitrate metabolism during continuous culture of *P. denitrificans*. Panel A and B illustrate aerobic incubation of the wild-type (WT) strain (PD1222), C and D anaerobic incubation of the wild-type strain, E and F anaerobic incubation of the *nosZ*$^-$ strain (PD102.21). The dissolved oxygen and biomass concentration through the continuous culture incubation are denoted with a plain grey line (−) and a green solid circle line (●) respectively (panels A, C and E). The concentration of nitrate, nitrite and nitrous oxide through the continuous culture incubation are denoted with a blue solid square line (■), a light blue diamond line (◆) and an orange triangle line (▲) respectively (panels B, D and F). The $N_2O$ concentration is given as N-$N_2O$ in the culture.

growth period the air supply was switched off and the culture switched to the continuous mode (Fig. 2C). The dissolved oxygen rapidly decreased to 0% air saturation within ∼1 h of the air-supply being switched off and a transition period began of ∼40 h, during which time the $NO_3^-$ levels in the bioreactor decreased as the culture switched from aerobic to anaerobic respiration (denitrification) (Fig. 2D). The biomass was comparable to the aerobic cultures (Table 1) and

**Table 1.** The rates of nitrate and nitrite consumption ($[NO_3^-]_c$, $[NO_2^-]_c$) and nitrite and nitrous oxide production ($[NO_2^-]_p$, $[N_2O]_p$) in *P. denitrificans* strains PD1222 aerobic and PD1222, PD102.21 *nosZ*$^-$, PD2921 (*fnrP*$^-$), PD7721 (*nnrR*$^-$) and PD2345 (*narR*$^-$) anaerobic continuous cultures ($n = 3$ independent biological replicates ±SE).

| Strain | $O_2$ | OD | $[NO_3^-]_c$ μmol.g$^{-1}$.h$^{-1}$ | $[NO_2^-]_p$ μmol.g$^{-1}$.h$^{-1}$ | $[NO_2^-]_c$ μmol.g$^{-1}$.h$^{-1}$ | $[N_2O]_p$ μmol.g$^{-1}$.h$^{-1}$ |
|---|---|---|---|---|---|---|
| PD1222 | + | 0.61 ± 0.03 | 76 ± 19.5 | 12 ± 6.6 | 65 ± 19.8 | 0.0 ± 0.01 |
| PD1222 | – | 0.52 ± 0.02 | 1215 ± 274 | 18 ± 9.2 | 1196 ± 283 | 0.2 ± 0.03 |
| PD10221 | – | 0.41 ± 0.02 | 2551 ± 235 | 85 ± 54.2 | 2466 ± 202 | 2142.5 ± 220.66 |
| PD2921 | – | 0.60 ± 0.05 | 717 ± 46 | 3 ± 2.8 | 714 ± 45 | 0.1 ± 0.01 |
| PD7721 | – | 0.40 ± 0.00 | 4553 ± 81 | 4774 ± 160 | −221 ± 122 | 0.1 ±0.01 |
| PD2345 | – | 0.40 ± 0.00 | 1188 ± 66 | 1.2 ± 0.6 | 1187 ± 66 | 0.2 ±.0.22 |

succinate was not detectable (<10 μM) during the steady-state (60–120 h) and the steady-state nitrate level was ~15 mM, reflecting that the cultures were succinate-limited and $NO_3^-$-sufficient. Notably, neither $NO_2^-$ nor $N_2O$ accumulated above low micromolar levels in the steady-state cultures. Thus, the cellular rate of $NO_2^-$ reduction by Nir (Reaction 2) closely matched the rate of $NO_2^-$ production from $NO_3^-$ reduction by Nar (Reaction 1) and likewise, the rate of $N_2O$ production by Reactions 1 to 3 (Nar, Nir and Nor) was matched by the rate of $N_2O$ consumption by Reaction 4 (Nos; Table 1). This suggests that the denitrification process in the steady-state culture was optimized for complete reduction of nitrate to $N_2$.

The absence of any significant accumulation of $NO_2^-$ or $N_2O$ in the chemostat systems suggested that a balanced denitrification respirome was operating. However, as a control for $N_2O$ production in the experimental system we determined the physiological consequences of removing the terminal $N_2O$-removing step (Fig. 2E and F). This was done by determining the growth and metabolite levels in a steady-state anaerobic-denitrifying culture at 30°C pH 7.5 of a strain of *P. denitrificans*, PD102.21 which lacks a functional copy of *nosZ* (Bergaust *et al.*, 2012). NosZ is the catalytic subunit of the $N_2O$ reductase which binds 12 copper ions per functional homodimer and if absent the organism is unable to reduce $N_2O$ to $N_2$ (Felgate *et al.*, 2012). As was the case for the WT cultures, the dissolved $O_2$ concentration in the bioreactor rapidly decreased to 0% air saturation within ~1 h of the air-supply being switched off and succinate was not detectable (<10 μM) in the biomass steady-state phase (60–120 h). After the period of transition associated with the shift from aerobic to anaerobic metabolism the biomass in the steady-state was only slightly less than in the WT cultures (Table 1), however, the $NO_3^-$ levels in the bioreactor continued to decrease over the lifetime of the experiment with ~10 mM $NO_3^-$ remaining in the vessel after 120 h (Fig. 2F). Interestingly, there was no $NO_2^-$ accumulation in the vessel, but $N_2O$ emissions increased to ~4.9 mM $N_2O$ (equivalent to ~9.8 mM 'N') an amount that represents ~98% recovery of nitrogen (Eqs. 1–3). These differences are best understood in terms of the $NO_3^-$ consumption quotient of the *nosZ*$^-$ strain under anaerobic conditions which is 2551 μmol $NO_3^-$ $g^{-1}.h^{-1}$, approximately twice that of the WT culture (Table 1), indicating that loss of the final step of denitrification (Eq. 4) is being compensated for by increased electron flux through the preceding steps (Eqs. 1–3).

### *Differential expression of the* P. denitrificans *respirome under anaerobic denitrifying conditions compared to aerobic conditions*

Analysis of the *P. denitrificans* genome sequence enables the construction of a core respirome (Fig. 1). The only quinol for which there is a complete biosynthetic pathway is ubiquinone-10 [UQ (ubiquinone); $E_{m7.0} \sim +80$ mV]. Hence, the entire respiratory network is benzoquinone-dependent. This is in contrast to enteric bacteria such as *E. coli* which can also synthesise menaquinone (MQ; $E_{m7.0} \sim -70$ mV) to facilitate both low potential napthoquinone- and benzoquinone-dependent respiratory systems that are present. In *P. denitrificans* electron input into the UQ pool from organic compounds can take place via formate, succinate, NADH, aldose sugars, α-glycerophosphate and flavoprotein (linked to the catabolism of straight- and branched-chain fatty acids) dehydrogenases. Electrons can also feed into the respiratory system from the single carbon compounds ($C_1$) via methanol and methylamine dehydrogenases, but in this case electrons flow into the system upstream of UQ directly into the higher potential cytochrome/cupredoxin pool ($E_{m7.0}$ +200 – +300 mV) that includes cytochrome $c_{550}$ and pseudoazurin. These two pools are linked via the cytochrome $bc_1$ complex. The core respirome can use six different terminal electron acceptors: $O_2$ (for which there are four oxidases, cytochrome $aa_3$, cytochrome $ba_3$, cytochrome $cbb_3$ and cytochrome *bd*); hydrogen peroxide; $NO_3^-$ (for which there are two reductases) $NO_2^-$, NO and $N_2O$. Thus, *P. denitrificans*, in common with many facultative denitrifying bacteria, has evolved for life at the aerobic – anaerobic interface. In addition to being able to derive electrons from organic electron donors the respiratory network also includes systems for obtaining electrons from hydrogen and inorganic sulphur compounds (Fig. 1). The respiratory pathways are distributed across the cytoplasmic membrane in a way that facilitates vectorial charge separation and allows electron transfer to be coupled to the generation of a transmembrane proton-electrochemical gradient (Richardson, 2008).

Having established steady-state chemostat cultures under aerobic and anaerobic conditions at identical dilution rates and similar steady-state biomass levels, it was possible to compare the differential expression of the respirome through global transcriptional analysis. The expression levels of just 200 out of ~5100 genes (4%) of the *P. denitrificans* genome were ≥ 2-fold (≥ 95% significance) changed in the anaerobic denitrifying compared to the aerobic steady-state cultures; 157 genes upregulated and 43 genes downregulated (Supporting Information Fig. 1). These 200 genes are clustered into a number of putative operons so that the change in growth conditions elicits even fewer discrete transcriptional responses. Such a highly specific response is consistent with the concept of 'respiratory flexibility' which proposes efficient feedback to environmental change through minimal remodelling of the respirome by the addition or removal of the minimum number of enzymatic modules to a core respiratory system (Richardson, 2008) (Fig. 1).

**Table 2.** Relative expression values of selected genes from the microarray and RT-qPCR investigations of the aerobic and anaerobic treatment ($n = 3$ biological replicates ±SE).

| | | Microarray | | RT-qPCR | |
|---|---|---|---|---|---|
| Gene_ID | Annotation | Average | ±SE | Average | ±SE |
| Pden_4233 | *narG* | 7.11 | 0.58 | 18.70 | 4.03 |
| Pden_2487 | *nirS* | 128.76 | 23.69 | 854.00 | 420.55 |
| Pden_4219 | *nosZ* | 24.04 | 8.93 | 135.60 | 15.16 |
| Pden_1689 | *nodA* | 9.25 | 7.34 | 23.10 | 9.12 |
| Pden_2483 | *norB* | 9.15 | 1.86 | 14.30 | 0.84 |
| Pden_4721 | *napA* | 0.54 | 0.11 | 0.80 | 0.14 |
| Pden_5107 | *qoxB* | 4.01 | 1.02 | 43.57 | 8.60 |
| Pden_4222 | *pasZ* | 53.59 | 21.63 | 296.93 | 108.70 |
| Pden_1937 | *cycA* | 2.02 | 0.41 | 7.00 | 3.01 |

Relative expression values are based on the ratio of anaerobic to aerobic gene expression.

Key differences in the transcriptional levels of specific operons between the aerobic and anaerobic denitrifying cultures are summarized in Fig. 1 and presented in full in Supporting Information Fig. 1. A selection of the changes were independently verified by RT-qPCR of representative functional genes (Table 2 and Fig. 3). On the electron output side of the respirome, the genes responsible for the synthesis and activity of the denitrification system (Fig. 3) are located in the *narGHJI* (Pden_4233-4237), *nirXI-SECFDHGN* (Pden_2485-2495), *norCBQDEF* (Pden_2479-2484) and *nosCRZDFYLX* (Pden_4214-4222) gene clusters that were upregulated; ~10-, 120-, 15- and 20-fold respectively. The operons encoding two of the terminal oxidases (de Gier *et al*., 1994), cytochrome $cbb_3$ oxidase (*ccoNOPQ*; Pden_1845-1849) and the ubiquinol-dependent cytochrome $ba_3$ oxidase (*qoxABC;* Pden_5106-5108), were also upregulated ~4-fold to 6-fold, whilst levels of the transcript of the gene encoding the respiratory cytochrome *c* peroxidase (*ccp*; Pden_0893) was increased 12-fold. The level of expression of cytochrome $aa_3$ oxidase (*ctaEGBC*; Pden_4317-4321) and cytochrome *bd* oxidase (*cydAB*; Pden_4010-4011) did not change significantly.

On the electron input side of the respirome, the expression of genes encoding a number of the primary dehydrogenases responsible for electron input, such as succinate dehydrogenase (*sdhCDAB*; Pden_0567-0570), formate dehydrogenase (*fdnGHI*; Pden_2829-2827), NADH dehydrogenase (*nuoABCDEFGHIJKLMN*; Pden_2250-2231), hydrogenase (*hupTUVFALECDFGHJK*; Pden_3093-3106 and *hypABCDEF*; Pden_3107-3113), methylamine dehydrogenase (*mauRFBEDACJGMN*; Pden_4728-4738) and sulphite dehydrogenase (*soxSVW-XYZABCDEFGH*; Pden_4147-4160), did not change significantly. However, methanol dehydrogenase operon (*mxaFJRSACKL*; Pden_2293-2003) was induced anaerobically by ~3-fold to fivefold. In addition, a second copy for *sdhDCB* (Pden_3020-3022) lacking a gene for the catalytic flavo-protein subunit was weakly upregulated.

The electron transfer between the input and output sides of the respirome is mediated by the cytochrome $bc_1$ complex (*fbcFBC*; Pden_2305-2307), which is responsible for electron transfer between the UQ pool and the terminal reductases and is common to both aerobic respiration and anaerobic denitrification (Richardson, 2008). Consistent with this dual role, level of expression of the *fbcFBC* operon was unchanged between aerobic and anaerobic conditions. The gene (*cycA;* Pden_1937) encoding a heme-dependent electron acceptor, cytochrome $c_{550,}$ was also similarly expressed either under aerobic and anaerobic conditions. By contrast, the expression of pseudoazurin (*pasZ*; Pden_4222), a cupredoxin, which serves as an alternative electron acceptor for the cytochrome $bc_1$ complex and co-substrate for cytochrome *c* peroxidase,

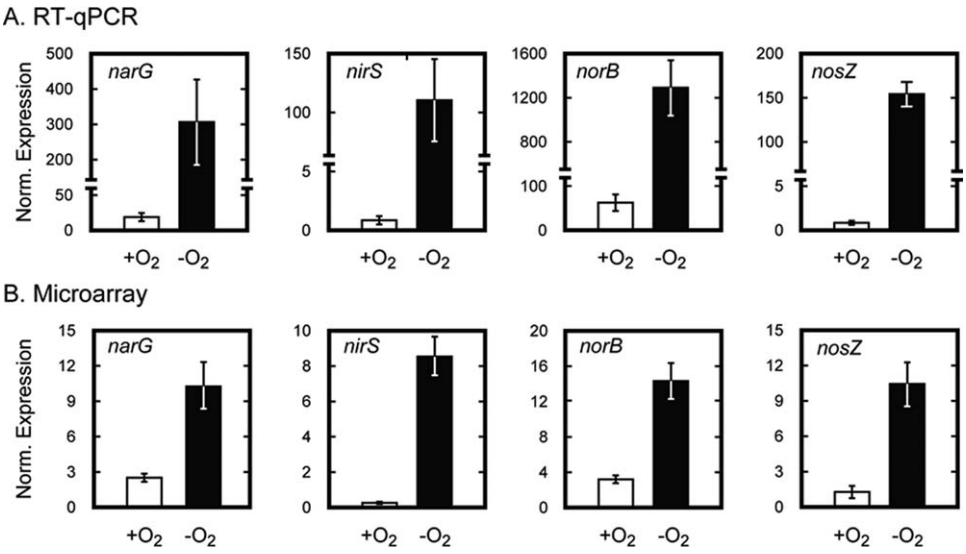


**Fig. 3.** Expression of denitrification functional genes; nitrate reductase (*narG*), nitrite reductase (*nirS*), nitric oxide reductase (*nirB*) and nitrous oxide reductase (*nosZ*). Panels demonstrate the average normalized gene expression based on (A) RT-qPCR and (B) on microarray investigations of the aerobic ($+O_2$) and anaerobic ($-O_2$) continuous culture incubations ($n = 3$, ±SE).

Nir, Nor and Nos (Moir and Ferguson, 1994; Pearson *et al.*, 2003; Paes de Sousa *et al.*, 2007) was strongly upregulated (~55-fold) under anaerobic conditions compared to aerobic conditions. This observation suggests that pseudoazurin, rather than cytochrome $c_{550}$, serves as the principle electron transfer shuttle between the cytochrome $bc_1$ complex and Nir, Nor and Nos in anaerobic denitrification. However, the presence of both cytochrome $c_{550}$ and pseudoazurin in the anaerobic respirome is consistent with previous studies that showed both the *cycA* and *pasZ* are needed to be inactivated in order to abolish denitrification in *P. denitrificans* (Pearson *et al.*, 2003); an observation that suggests some degree of functional overlap between the two electron transfer proteins.

Finally, we consider the relative expression of a number of stress-related proteins in anaerobic versus the aerobic cultures. Levels of the periplasmic $NO_3^-$-reductase (*napAB*, Pden_4719-4723) (Ellington *et al.*, 2003), involved in redox balancing during aerobic metabolism remained unchanged and absolute expression levels were low. However, a gene encoding a flavohemoglobin-dependent nitric oxide dioxygenase *(nodA*; Pden_1869) involved in nitrosative stress (Hartop *et al.*, 2017) is highly expressed in anaerobic-denitrifying conditions along with the adjacent gene (Pden_1690) that encodes a putative nitric oxide responsive repressor of the NsrR family (Spiro, 2007; Vine and Cole, 2011). This observation is consistent with the cells experiencing a degree of nitrosative stress during denitrification, most probably associated with production of intracellular reactive nitrogen species such as the NO radical. Several putative nitrosative/oxidative stress responsive genes were also significantly upregulated under denitrifying conditions including a second *nsrR* homologue located as part of a putative NO-responsive peroxidase/alkylhydroperoxidase gene cluster (Pden_3024-3027) and an *E.coli ytfE* homologue (Pden_4224) putatively involved in the repair of NO damaged iron-sulfur cluster proteins (Vine and Cole, 2011) and clustered with genes encoding a number of putative repair/biosynthesis proteins (Pden_4225-4230). The above suggests an instrumental role of NO acting concurrently as a substrate, stressor, signalling and regulatory molecule in *P. denitrificans* under denitrifying conditions. Additional general stress response comes from the observation that expression of the gene encoding a putative universal stress protein (*uspA*, Pden_1849) is increased by 8-fold under denitrifying conditions (Supporting Information Fig. S2), whilst expression of the DNA methylase *dnpII* (Pden_2068) that may be involved in stress-related DNA protection/repair is upregulated 13-fold.

The denitrification proteins are rich in iron (hemes and iron sulphur centres) and this is perhaps reflected by a low level upregulation of ferrous iron-uptake proteins under denitrifying conditions, for example, Fep (Pden_3525-3529) and Efe (Pden_1733-1735) and outer-membrane TonB type iron receptors (Pden_4174 & 4103). Finally, we note that, whilst denitrification as respiratory process is associated with the inner membrane and periplasm, some outer membrane proteins, Pden_3636 (an *ompW* homologue) and Pden_2411 (a *tolC* homologue) are also upregulated under the anaerobic denitrifying conditions. OmpW has also been implicated in anaerobic metabolism in *E. coli* and that may play role in the influx or efflux of dicarboxylates into or out of the cell (Xiao *et al.*, 2016).

### *The impact of the $O_2$-responsive Fnr, the NO-responsive Nnr and nitrogen oxyanion–responsive NarR, on expression of the denitrification apparatus of the respirome*

The present study considered the expression of these three transcription activators FnrP, NnrR and NarR. Each was expressed under aerobic and anaerobic steady-state conditions at similar levels and that the absolute levels of expression followed the order *fnrP* (Pden_1850) > *nnrR* (Pden_2478) > *narR* (Pden_4238; Fig. 4). The higher copy number of the *fnrP* transcript relative to *nnrR* and *narR* may be required to deliver a global response, whilst the lower copy number of the *narR* and *nnrR* transcripts may argue for tighter and more specific regulation. The performance of *fnrP*$^-$, *nnrR*$^-$ and *narR*$^-$ strains under anaerobic denitrifying conditions in the continuous culture conditions

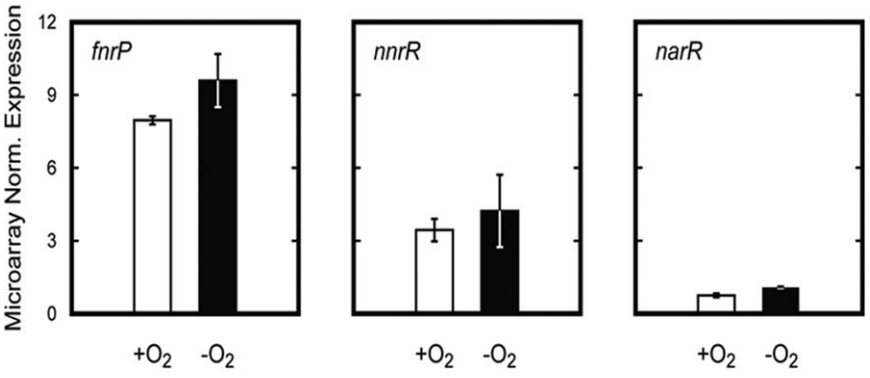


**Fig. 4.** Expression of denitrification transcriptional regulator genes responsive to oxygen (f*nrP*), to nitric oxide and nitrite (*nnrR*) and to nitrate (*narR*).
The average relative gene expression is based on the microarray profiles of the aerobic ($+O_2$) and anaerobic ($-O_2$) continuous culture incubations ($n = 3$, ±SE).

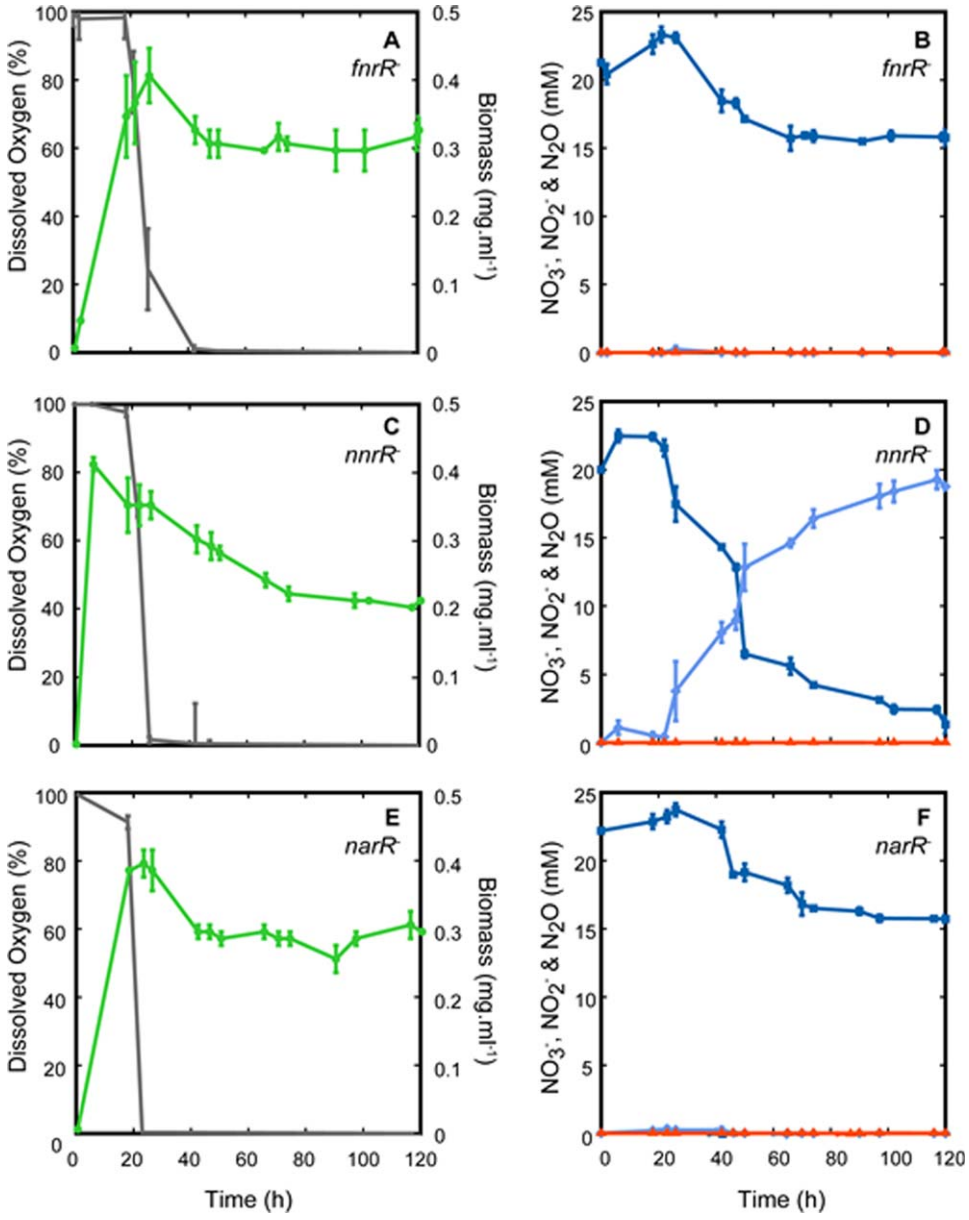


**Fig. 5.** Anaerobic growth and nitrate metabolism under continuous culture of *P. denitrificans* strains deficient in *fnrP*, *nnrR* and *narR*. Panel A and B illustrate the anaerobic growth and nitrate metabolism of the *fnrP*$^-$ strain (PD2921), panel C and D the *nnrR*$^-$ strain (PD7721) and panel E and F the *narR*$^-$ strain (PD2345) of *P. denitrificans*. The dissolved oxygen and biomass concentration through the continuous culture incubation are denoted with a plain grey line (−) and a green solid circle line (●) respectively (panels A, C and E). The concentration of nitrate, nitrite and nitrous oxide through the continuous culture incubation are denoted with a blue solid square line (■), a light blue diamond line (◆) and an orange triangle line (▲) respectively (panels B, D and F). The $N_2O$ concentration is given as N-$N_2O$ in the culture.

established for the WT was therefore investigated (Fig. 5 and Table 3).

FnrP binds an oxygen sensitive iron sulphur (FeS) cluster and is active under anoxic conditions when the FeS cluster is intact (Spiro, 2012). Thus, the primary role of FnrP is to sense falling $O_2$ concentrations and initiate transcription of the denitrification pathway; FnrP binding motifs have been found upstream of *cco*, *pasZ*, *nar* and *nos* clusters. Initiation is achieved through increased expression of the terminal oxidases cytochrome *cbb*$_3$ and cytochrome *ba*$_3$, which remove any remaining $O_2$ and so increase the pool of active FnrP, that works in concert with NarR to activate the *nar* operon and initiate $NO_3^-$ reduction. The *fnrP*$^-$ strain (PD2921) (Van Spanning *et al.* 1997) could establish similar biomass steady-state levels to the WT (Fig. 5A). However, a major difference between the *fnrP*$^-$ strain and the WT was the rate of $O_2$ consumption following the cessation of aeration in the continuous culture system. The *fnrP*$^-$ strain required ~5-times longer than the WT strain to consume the oxygen in the medium during the transition to anaerobiosis (Fig. 6). This observation suggests that the regulation of oxygen respiration becomes dysfunctional during the oxic-anoxic transition. Assessing the oxygen depletion curves suggested that the *fnrP*$^-$ strain has a much higher apparent whole cell Monod constant ($K_s$) for oxygen than the WT. In the transcriptomic studies of the FnrP-deficient cultures, we noted a number gene of particular interest that were upregulated or downregulated anaerobically, relative to that observed in WT cultures (Table 3). Anaerobic expression of the quinol-dependent

**Table 3.** Relative expression values, compared to WT, of selected genes in the *fnrP*⁻, *nnrR*⁻ and *narR*⁻ strains determined by RTqPCR.

| | | FNR | | NNR | | NarR | |
|---|---|---|---|---|---|---|---|
| Gene ID | Annotation | Av. | SE | Av. | SE | Av. | SE |
| Pden_4721 | *napA* | 0.47 | 0.20 | nd | – | 0.21 | 0.01 |
| Pden_4233 | *narG* | 0.09 | 0.04 | 2.94 | 0.81 | 0.19 | 0.10 |
| Pden_2487 | *nirS* | 13.46 | 6.21 | 0.01 | 0.00 | 4.79 | 2.46 |
| Pden_2483 | *norB* | 3.88 | 1.91 | 0.16 | 0.04 | 1.05 | 0.22 |
| Pden_4219 | *nosZ* | 6.19 | 3.17 | 0.36 | 0.17 | 3.07 | 1.38 |
| Pden_4222 | *pasZ* | 4.97 | 1.72 | 0.12 | 0.05 | 6.87 | 1.19 |
| Pden_1937 | *cycA* | 11.52 | 4.60 | 6.38 | 1.89 | 9.08 | 1.93 |
| Pden_3028 | *ctaDI* | 0.39 | 0.08 | nd | – | nd | – |
| Pden_5108 | *cyoA* | 3.09 | 0.61 | nd | – | nd | – |
| Pden_1846 | *ccoQ* | 2.18 | 0.16 | nd | – | nd | – |
| Pden_4011 | *cydBA* | 1.67 | 0.08 | nd | – | nd | – |
| Pden_4449 | *nasC* | nd | – | nd | – | 0.94 | 0.13 |

Expression values were normalized to the expression of polB and the relative ratio R is calculated using the anaerobic treatment of the PD1222 strain as a reference based on the Pfaffl method ($n = 3$ biological replicates). nd = not determined.

$ba_3$ oxidase (Pden_5108) increased relative to that of the low $K_m$ cytochrome $cbb_3$ oxidase (Pden_1846) compared to WT cultures (Table 3). This may therefore serve to increase competition between low and high $K_m$ oxidase systems, hence the increase in the apparent $K_s$. Thus, FnrP may play an important role in the aerobic-anaerobic transition by repressing expression of the low affinity oxidase. Once in anaerobic steady-state, the *fnrP*⁻ strain consumed nitrate at slightly lower rates than the WT (Table 1), but no significant release of denitrification intermediates nitrite or $N_2O$ were observed. However, there were some very striking changes in expression of the denitrification genes in the *fnrP*⁻ strain compared to the WT. Expression of *nar* decreased by ~10-fold, compared to WT. However, expression of the *nir, nor, nos*, *paz* and *cycA* genes increased markedly (~4-fold to 13-fold; Table 3). This suggests that the presence of active FnrP protein serves to repress expression of these genes in the WT strain.

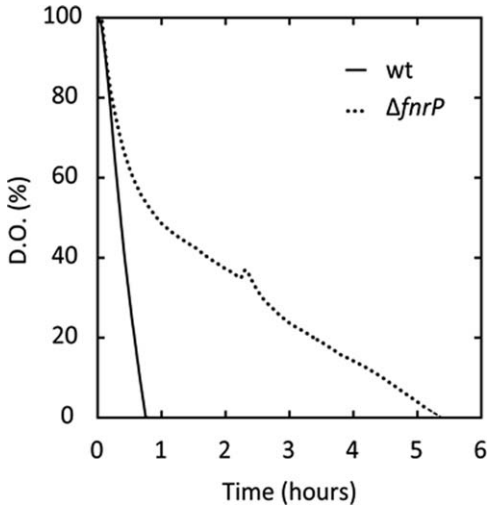


**Fig. 6.** Dissolved oxygen (D.O.) tension profiles of continuous cultures of the wild-type (WT) and mutant *fnrP*⁻ strain of *P. denitrificans* when the air-supply was shut-off at 0 h. D.O. is presented as % air saturation.

Some of the genes that are upregulated in anaerobic denitrifying conditions have previously been reported to be regulated by the transcriptional regulator NnrR, which is proposed to respond to intracellular NO, positively activating transcription when the ligand is bound. The *nnrR* strain displayed lower $\mu_{max}$ in anaerobic batch cultures (~0.15 $h^{-1}$ compared to the WT (~0.35 $h^{-1}$). However, this was still above the $D$ 0.05 $h^{-1}$ at which the bioreactor was operating and so we were able to establish comparable steady-state nitrate-respiring cultures with this strain (Fig 5B). It was notable, though, that the anaerobic steady-state phenotype of the *nnrR* strain (PD7721) (Van Spanning *et al.* 1997)) was markedly different from the WT. During the transition from aerobic to anaerobic metabolism (20–50 h), nitrite accumulated stoichiometrically with nitrate consumption. In steady-state (50–120 h) the specific rate of nitrate consumption was ~4–5 fold higher than the WT, with residual nitrate concentration in the chemostat falling to ~1 mM, compared to ~15 mM in the WT (Table 1). However, the total electron acceptor consumption of ~40 mM electron equivalents was only ~20% less that the ~50 mM electron equivalents utilized by the WT. Thus, the denitrification network was compensating for loss of Reactions 2–4 by increasing electron flux through the protonmotive Reaction 1.

The genetic basis of this re-tuning of flux through the respiratory network was apparent from RT-qPCR analysis. Most notable was a strong decrease in *nirS* (~100-fold) and *norB* (~100-fold) expression, but an increase in *nar* expression (~2-fold; Table 3). An increase in Nar relative to Nir/Nor would lead to a change of electron flux at the level of the Q-pool. Notably, *pazS* encoding the electron shuttle pseudoazurin (Pden_4222), was also strongly downregulated in the *nnrR*⁻ background (Table 3). This reflects tight regulation by NO, via Nnr, of the subcomponent of the respiratory network downstream of the cytochrome $bc_1$ complex that serves to generate and consume the cytotoxic NO, the Paz-dependent Nir and Nor systems. It was also notable that expression of the alternative electron shuttle CycA (cytochrome $c_{550}$) increased substantially in the *nnrR*⁻ strain, suggesting a hitherto unrecognized repression of this gene by NnrR that may compensate for the loss of pseudoazurin. This was also observed for the expression of cytochrome $cbb_3$ oxidase, which is known to have a low NO reductase activity. The *nos* locus is divergently transcribed from *pazS* and presumably is under the control of the same *cis*-operator

given the presence of a single palindromic putative FnrP-/NnrR-binding site.

NarR is required for maximal expression of *narGHJI* and is active in the presence of either nitrate or nitrite. In the *narR*$^-$ strain under steady-state conditions the extracellular $NO_3^-$ concentration was ~16 mM and the concentration of $NO_2^-$ and $N_2O$ in the reaction vessel was below detection limits throughout, thus, displaying similar physiological features to the WT strain. Notably, though, the reduction of $NO_3^-$ did not coincide with $O_2$ depletion (~20 h), but was severely delayed, beginning after ~40 h (Fig. 5E and F). During this lag period there was no net consumption of $NO_3^-$. This indicates a phenotype that is unable to respire on $NO_3^-$ and required a prolonged anoxic period to adapt and alter the regulatory network to respond to the external stimuli. The *narR*$^-$ strain had a biomass quotient of 0.16 $g.l^{-1}.h^{-1}$ which was 0.04 $g.l^{-1}.h^{-1}$, higher than that of the WT strain, once $NO_3^-$ respiration was initiated. The $NO_3^-$, $NO_2^-$ and $N_2O$ consumption quotients were comparable to the WT control.

In the *narR*$^-$ strain there were some surprising and hitherto unrecognized changes associated with the loss of the regulator, which had been previously only associated with the regulation of *narGHI*. Expression of both *nar* and *nap* was downregulated, compared to the WT strain, but neither was completely switched off (Table 3), suggesting NarR acts to activate expression of both the periplasmic and membrane-bound $NO_3^-$ reductase systems. Given the relatively low observed levels of expression of *nar* and *nap*, we examined the *nas* gene cluster which encodes the assimilatory $NO_3^-$ reductase. Expression of the *nasC* (Pden_4449) gene remained unchanged in the *narR*$^-$ strain, indicating the lag-time associated with $NO_3^-$ utilisation is presumably linked to the slower rate of synthesis of a functional NarGHI complex, rather than the action of an additional $NO_3^-$ reductase. In addition to the $NO_3^-$ reductases, expression of *nir* and *nos* were upregulated (5-fold and 3-fold), compared to the WT, as were *cycA* and *pazS* (9-fold and 6-fold) encoding the electron transfer proteins cytochrome $c_{550}$ and pseudoazurin respectively (Table 3). As with the *fnrP*$^-$ and *nnrR*$^-$ strains, this suggests that NarR can serve to both induce (*nar*, *nap*) and repress (*nir*, *nos*, *pasZ*, *cycA*) components of the denitrification respirome.

In conclusion, this is the first study to compare a complete transcriptome derived from a steady state culture of a denitrifying bacterium, *P. denitrificans*, respiring $NO_3^-$ anaerobically with one obtained from an aerobic steady-state culture. The use of controlled steady-state chemostat cultures minimizes differential gene expression brought about by changes in parameters such as growth, rate, cell densities or pH that can be associated with gene expression studies in batch cultures. Interrogation of the data sets suggests that the switch from aerobic to anaerobic metabolism is brought about by changes in the levels of expression of relatively few genes, but this is sufficient to adjust the configuration of the respirome to allow the organism to efficiently respire $NO_3^-$ without the significant release of intermediates, such as the potent greenhouse gas $N_2O$. The fine-tuning of the denitrification network may require that, in addition to being transcriptional activators of distinct genes of the system, FnrP, NnrR and NarR may also serve as repressors competing with each other for binding sites upstream of target genes. This could explain why all three of the *fnrP*$^-$, *nnR*$^-$ and *narR*$^-$ strains displayed both increased and decreased expression of denitrification genes, relative to the WT. Take, for example, the increased expression of some NnrR-regulated denitrification network genes (*nir* and *paz*) observed when FnrP is absent. FnrP and NnrR recognize very similar binding sites and so could compete for the same site, but with only NnrR able to form a productive transcription initiation complex. The absence of such competition in the *fnrP*$^-$ strain would lead to increased frequency of a productive transcription initiation complex being formed by NnrR. Such cross-talk between the three key transcriptional regulators of denitrification would allow exquisite transcriptional control of the system and will be further investigated using direct binding assays in future experiments.

## Materials and methods

### *Bacterial strains and culture techniques*

All strains of *P. denitrificans* used were stored as stock cultures at −80°C in LB-glycerol (1:1 ratio) vials. Starter cultures were obtained from a single colony and were incubated in LB with the appropriate antibiotic compound until $OD_{600}$=0.6 and subsequently incubated minimal medium until $OD_{600}$=0.6. Pure cultures of bacteria were cultured in minimal medium (MM) based on a minimal salt solution (Vishniac and Santer, 1957). The MM consisted of 29 mM di-sodium orthophosphate ($Na_2HPO_4$), 11 mM potassium di-hydrogen orthophosphate ($KH_2PO_4$), 5.4 mM ammonium chloride ($NH_4Cl$), 0.4 mM magnesium sulfate ($MgSO_4$), 20 mM sodium nitrate ($NaNO_3$) and 5 mM sodium succinate ($Na_2C_4H_6O_4$). Trace elements were added to a final concentration of 342 μM ethylene di-amine tetra-acetic acid (EDTA), 15 μM zinc sulfate ($ZnSO_4.7H_2O$), 51 μM manganese chloride ($MnCl_2.4H_2O$) 36 μM iron sulfate ($FeSO_4.7H_2O$), 12 μM ammonium molybdate ($(NH_4)_6Mo_7O_{24}.4H_2O$), 13 μM copper sulfate ($CuSO_4.5H_2O$) and 13.5 μM cobalt chloride ($CoCl_2.6H_2O$). All chemicals were supplied by Fisher Scientific, UK.

Continuous culture of the bacterial strains in continuous stirred tank reactors (CSTR) was essentially as described previously (Felgate *et al.*, 2012). Briefly, the culture volume occupied 1.5 L out of 2.5 L total vessels capacity (BioFlo 310, New Brunswick Scientific, USA). The growth medium, the vessel, the probes and the tubing were sterilized by autoclaving. The bioreactor incubation lasted ~120 h and was separated in two phases. First, the starter culture increased in biomass aerobically for ~20 h in batch mode. Second, sterile nutrient

solution was injected continuously (80 ml.h$^{-1}$) initiating a continuous culture incubation with a dilution rate of 0.05 h$^{-1}$. Air saturation was either maintained at 100% or 0% air saturation by adjusting the air supply depending on the conditions required. Typically, continuous cultures were incubated at 30°C with 200 r.p.m. agitation and the pH was maintained at 7.5 with the addition of 1M NaOH and 0.1M $H_2SO_4$.Gas and liquid samples were taken aseptically throughout the incubation period. The pH, gas mixture, agitation, temperature and feeding rate were automatically controlled and adjusted to the desired levels with the embedded reactor software (New Brunswick Scientific, USA). We assumed that stable conditions in the CSTR were reached when no change in bacterial biomass was observed spectrophotometrically at 600 nm (Biowave, UK).

### *Construction of NarR mutant* (PD2345)

An unmarked deletion mutant (PD2345) in *narR* (Pden_4238) was constructed by allelic replacement using pK18*mobsacB* [26]. PD2345 was made by PCR-amplifying and ligating the 5′ and 3′ flanking regions, 600 and 826 bp in size, respectively, using primers designed to contain specific restriction enzyme sites (Supporting Information Table S1). The resulting insert was cloned in to pK18*mobsacB*. Each suicide plasmid was verified by sequencing and conjugated into *P. denitrificans* PD1222 via tri-parental mating using helper plasmid pRK2013, selecting for single crossover recombination events using the appropriate antibiotics. These primary kanamycin resistant transconjugants were then grown to stationary phase in the absence of kanamycin, and double cross-over recombination events were selected by serial dilution and plating cells onto a modified nutrient-agar recipe (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.4% (w/v) NaCl and 1% (w/v) agar) supplemented with 6% w/v sucrose. Sucrose resistant deletion mutants that had lost kanamycin resistance were then screened by PCR, using primers external to the deletion. PCR products spanning the deletion were then sequenced for confirmation.

### *Dissolved oxygen determinations*

Dissolved $O_2$ (DO) in the CSTR was monitored automatically with a polarographic electrode probe (InGold, Mettler Toledo). After overnight polarization DO probes were calibrated at 0 and 100% levels in $N_2$- and air-saturated medium respectively. DO readings were normalized and $O_2$ concentration was calculated in μM as the percentage of $O_2$ saturation using the Henry's law and adjusting the proportionality constant ($K_{H0}$) to 30°C.

### *Nitrate and nitrite analyses*

The concentration of $NO_3^-$ and $NO_2^-$ were determined with a high performance liquid chromatography system (HPLC; Dionex ICS-900) equipped with an IonPac AS22 2 mm column (Dionex, UK) and a conductivity detector (Dionex, UK). The eluent solution consisted of 1.8 mM sodium carbonate ($Na_2CO_3$) and 1.7 mM sodium bicarbonate ($NaHCO_3$). A sulphuric acid solution (0.1% per volume) was used as a regenerant. Chemical standards of 0.1–30 mM $NO_3^-$ and $NO_2^-$ were used to determine concentrations in liquid media. Selected samples taken at the later stage of continuous culture under aerobic and anaerobic states were analysed for succinate concentration in a similar HPLC system as above equipped with an IonPac ICE-AS6 9 mm column and eluted with 0.4 mM heptafluorobutyric acid. Chemical standards of 0.1–6 mM sodium succinate were used to determine concentrations in liquid media.

### *Nitrous oxide analyses*

Bioreactor headspace concentrations of $N_2O$ were determined by gas chromatography (Clarus 500, Perkin Elmer) using a 30 m elite-Plot Q column with inner diameter of 0.53 mm with $^{63}$Ni electron capture detector (ECD). The carrier gas was zero-grade $N_2$ and the make-up gas a 5% methane ($CH_4$) in argon (Ar) mixture supplied by BOC, UK. $N_2O$ gas standards (Standard Gases, UK) were used and ranged from 0.4 to 10 000 ppm. The total $N_2O$ concentration was calculated applying the Henry's gas solubility law and the van't Hoff equation to adjust the proportionality constant to the desired temperature as in Felgate *et al*. (2012).

### *Extraction of bacterial DNA and RNA*

*P. denitrificans* PD1222 genomic DNA was extracted using a Genomic DNA extracting kit and 100/G columns (Qiagen) from 10 ml stationary phase cells according to manufacturer's specifications. For total RNA extractions, 30 ml of cells were withdrawn from steady state chemostat cultures (cell density ~0.2 – 0.3 mg biomass ml$^{-1}$) and added to 12 ml of ice-cold 5% phenol in ethanol (v/v) solution, and incubated on ice for 30 min to stabilize RNA and prevent degradation. Cells were then pelleted and stored at −80°C until RNA was isolated, using SV Total RNA isolation kit (Promega) according to manufacturer's specifications. Trace DNA contamination was removed using Turbo DNA-free™ Dnase (Ambion) and this was confirmed by PCR amplification of RNA samples using MyFi™ DNA polymerase (Bioline) according to manufacturer's specifications. Nucleic acids were quantified spectrophotometrically using a Nanodrop 2000 (Thermo Scientific), and integrity of RNA samples was analysed using an Experion™ Automated Electrophoresis platform (BioRad) using RNA StdSens chips (BioRad) according to manufacturer's specifications.

### *Transcriptional analyses; techniques and analyses*

Total RNA from *P. denitrificans* (2 μg) was reverse-transcribed to cDNA using Superscript II™ reverse transcriptase and random primers (Invitrogen) in a final volume of 20 μl, according to the manufacturer's specifications. Following these reactions, cDNA was diluted 1:5 with $H_2O$ prior to use in qPCR reactions. Primers (Supporting Information Table S2) were designed using Primer3 Plus web software, to amplify products between 100 and 150 bp, with a Tm of ~ 60°C and used at a final concentration of 0.4 μM (Untergasser *et al*., 2007). Real-time quantification of transcripts was done using SensiFAST™ SYBR No-ROX kit (Bioline) and a CFX96™ real-time PCR detection system (BioRad), according to manufacturer's

specifications. Each reaction was done in triplicate, and transcript amounts were quantified from three RNA preparations isolated from independent biological replicates. Standard curves and amplification efficiencies were determined using serially-diluted genomic DNA (10-fold) from a stock concentration of 100 ng.$\mu l^{-1}$. The relative fold-change values were calculated using amplification efficiencies, as described previously (Pfaffl, 2001).

### *Microarray techniques and analyses*

High density, custom-made array slides (Oxford Gene Technology, UK) representing the 5.24 Mbp genome of *P. denitrificans* PD1222 were used as described in Sullivan *et al*. (2013). RNA was extracted from each replicate, quantified and quality-checked from experimental samples after ~100 h of continuous cultivation in CSTR. The RNA was labelled during reverse transcription with the blue CY5 dye (Amersham) and was mixed with a reference sample of genomic DNA labelled in parallel with the red CY3 dye (Amersham). The reference sample was genomic DNA from *P. denitrificans* (PD1222) cultured overnight in LB medium. After hybridization and the appropriate washing and drying steps, each array was scanned (GenePix 4000A, Axon Instruments) and separate images for each fluor (532 and 635 nm) and the fluorescence ratio were acquired for all target elements using the GenePix software (Axon Instruments). All array datasets were normalized within blocks and between arrays using the Babar R script (Alston *et al*., 2010) and anti-$\log_2$ transformed prior to further analysis with GeneSpring 7.3 software (Aligent). Microarray data are available through the NCBI GEO database accession number GSE97959.

### *Statistical and bioinformatic analyses*

Analytical data were measured twice from each replicate and standard error was used to indicate the uncertainty around the estimate of the mean measurement. Microarray transcriptional data were analysed for significance and enriched applying the Volcano statistical test ($p \leq 0.1$, $\geq$2-fold relative expression; GeneSpring 7). Sequence databases and genome annotation of *P. denitrificans* (PD1222) were accessed through the NCBI Entrez server (www.ncbi.clm.nih.gov/Entrez) with accession numbers CP000489 (2.85 Mb), CP000490 (1.73 Mb) and CP000491 (0.65 Mb). The *P. denitrificans* respirome was constructed from analysis of the genome annotations and additionally by aligning amino-acid sequences of respiratory proteins of the *E. coli* (Unden and Bongaerts, 1997; Constantinidou *et al*., 2006) and *Rhodobacter sphaeroides* proteomes (Dufour *et al*., 2010), and searching for selected amino-acid sequence motifs in the proteome of *P. denitrificans* through the Universal Protein Portal (UniProt) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) genome server. Gene clusters of significantly expressed genes were queried in the KEGG genome server with a gap size of 0 and SW score threshold of 100 against all organisms.

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## Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Table S1.** Oligonucleotide primers used for the construction and verification of PD2345 mutation in *n*arR (pden_4238).
**Table S2.** Primers used for real-time qPCR
Supplementary Fig. 1. Heat map from DNA microarray analyses of *P. denitrificans* PD1222 grown in chemostats anaerobically with nitrate as electron acceptor or aerobically. Colours indicate Log2 of normalized expression values under either condition. Genes were selected based on at least a twofold change in expression with $p \leq 2$.