Mechanisms of oxygen inhibition of *nirK* expression in *Rhodobacter sphaeroides*

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R. sphaeroides strain 2.4.3, when lacking the *cbb₃* oxidase, is unable to transition from aerobic respiration to denitrification using cellular respiration as a means of reducing oxygen levels. This is due to an inability to express *nirK*, the gene encoding nitrite reductase. Under certain photosynthetic conditions this strain can transition from aerobic to nitrate respiration, demonstrating that *nirK* expression can occur in the absence of a functional *cbb₃* oxidase. If oxygen levels are reduced under non-photosynthetic conditions using low-oxygen gas mixes, nitrite reductase activity is detected at wild-type levels in the strain lacking the oxidase. In addition, co-culture experiments show that incubation of the *cbb₃* deficient strain 2.4.3 with *R. sphaeroides* 2.4.1, which is *nirK* deficient but has the high-affinity *cbb₃* oxidase, restores denitrification in sealed-vessel experiments. Taken together these results indicate that high end-point O₂ levels are the reason why the strain lacking the *cbb₃* oxidase cannot transition from aerobic respiration to denitrification under certain conditions. The protein probably being affected by these O₂ levels is the transcriptional regulator NnrR.

INTRODUCTION

Denitrification is the dissimilatory reduction of nitrogen oxyanions to gaseous end products (Zumft, 1997). This process is secondary to aerobic respiration and is utilized as an alternative respiratory mode under micro-oxic and anoxic conditions. Denitrification is widespread in the biological world with eukaryotes, fungi, archaea and bacteria being able to respire fixed nitrogen oxyanions to gaseous forms (Risgaard-Petersen *et al.*, 2006; Zumft, 1997). An obligate intermediate in the denitrification pathway is nitric oxide (NO), produced upon nitrite reduction. The production of NO is believed to act directly as a signal for transcriptional regulators of denitrification genes, including the genes encoding nitrite and NO reductase (Giardina *et al.*, 2008; Kwiatkowski & Shapleigh, 1996; Spiro, 2007; Tosques *et al.*, 1996). NO is also a reactive free radical so its production must be tightly controlled. Consistent with this tight control, studies of expression of *nirK*, the gene encoding a copper-containing nitrite reductase, in some denitrifiers has revealed that its expression requires multiple regulators that integrate information about levels of oxygen and nitrogen oxides (Baek *et al.*, 2008; Bedmar *et al.*, 2005; de Bruijn *et al.*, 2006; Laratta *et al.*, 2002). For example, in *Agrobacterium tumefaciens* the *nirK* gene has two levels of regulation. *nirK* is regulated first by ActR, a global transcriptional regulator, and second by NnrR, a denitrification specific regulator (Baek *et al.*, 2008).

Rhodobacter sphaeroides strain 2.4.3 is a model for denitrification in an anoxygenic photoheterotroph. Strain 2.4.3 has a *nirK* and a cytochrome *c*-oxidizing nitric oxide

reductase (Bartnikas *et al.*, 1997; Tosques *et al.*, 1997). Previously, the NnrR in this bacterium was established as an NO-responsive Fnr/Crp-type transcriptional regulator that regulates several genes involved in denitrification, including *nirK* (Hartsock & Shapleigh, 2010; Kwiatkowski & Shapleigh, 1996; Tosques *et al.*, 1996). In addition to NnrR, the global two-component regulatory system PrrAB is required for upregulation of *nirK* since inactivation of the genes encoding these proteins drastically reduces *nirK* expression (Laratta *et al.*, 2002). PrrB is a membrane-bound histidine sensor kinase and PrrA is the response regulator. As electron flow through the aerobic respiratory chain decreases, concomitant with oxygen levels, the kinase activity of PrrB is upregulated. In turn, PrrA is phosphorylated and activates or represses hundreds of genes that are involved in the transition from aerobic to anaerobic growth (Eraso *et al.*, 2008). Importantly, the *cbb₃* oxidase is required for inhibiting the kinase activity of PrrB (Oh *et al.*, 2004). In the absence of a functional *cbb₃* oxidase, PrrB constitutively phosphorylates PrrA, resulting in significantly higher levels of phosphorylated PrrA under aerobic conditions (Ranson-Olson & Zeilstra-Ryalls, 2008). This uncouples the regulatory system from oxygen levels and allows PrrA-mediated activation or repression of genes under conditions of high O₂ (O’Gara *et al.*, 1998).

Contrary to the expectations based on the model of PrrAB function, it has been observed that *nirK* is not expressed in the absence of the *cbb₃* oxidase (Laratta *et al.*, 2002). Since PrrA-P levels are high in this background it would be expected that *nirK* and *nor* expression would not be impacted by the loss of the oxidase. Here, experiments

undertaken to gain insight into the lack of *nirK* expression in the absence of the *cbb₃* oxidase are described, and a model of why *nirK* is not expressed is presented.

METHODS

Bacterial strains and culture conditions. Two strains of *Rhodobacter sphaeroides* were used in this study. Strain 2.4.3 (ATCC 17025) was used as the model denitrifying strain and the type strain 2.4.1 (ATCC 17023) was used as a non-denitrifying comparison. Mutants of the strains used in this study are listed in Table 1. The strains were cultured in Sistrom's medium at 32 °C. When necessary, medium was supplemented with 12 mM potassium nitrate to support denitrifying growth (Lueking *et al.*, 1978). The flask culture conditions used have been previously described (Tosques *et al.*, 1996). Briefly, 100 ml liquid medium was added to 250 ml Erlenmeyer flasks, which were then inoculated and sealed with a rubber stopper. Flasks were incubated at 32 °C and agitated at 250 r.p.m. Aerobic growth results in depletion of oxygen in the headspace and, for this ratio of head space to liquid, low-oxygen and denitrifying growth commences at optical densities above 0.6. Relevant antibiotics were added at the following concentrations: tetracycline, 1.0 µg ml⁻¹; kanamycin, 25 µg ml⁻¹; streptomycin, 50 µg ml⁻¹; and gentamicin, 20 µg ml⁻¹.

Escherichia coli DH5 α was used for molecular cloning and transformations. *E. coli* S17-1 was used for biparental matings. All *E. coli* cultures were grown in Luria–Bertani medium and liquid cultures were grown at 32 °C with aeration (Maniatis *et al.*, 1982). When necessary, antibiotics were added at the following concentrations: ampicillin, 100 µg ml⁻¹; tetracycline, 10 µg ml⁻¹; streptomycin, 25 µg ml⁻¹; and kanamycin, 25 µg ml⁻¹.

Construction of plasmids and strains. The strains and plasmids used in this study are listed in Table 1. For PCRs, chromosomal DNA was isolated from *R. sphaeroides* strain 2.4.3 using the Puregene DNA Isolation kit. All oligonucleotide primers were purchased from Integrated DNA Technologies (IDT). Standard methods were used

for restriction digests, ligations and biparental conjugations. Transformations were done using the TSS chemical method (Chung *et al.*, 1989).

Δ ccoN mutant construct. A *cbb₃* oxidase mutant was made by deleting *ccoN*, which encodes the catalytic subunit. This was achieved by double recombination, resulting in the insertion of a streptomycin-resistance cassette. To allow for a double-cross-over event, two flanking regions of *ccoN* were amplified and the amplicons were cloned together into pUC19, using either *Pst*I/*Bam*HI or *Bam*HI/*Eco*RI. A streptomycin-resistance cassette was inserted into the *Bam*HI site; this process utilized the *aadA*⁺ cassette from pHP45 Ω (Prentki & Krisch, 1984). The entire construct was then cloned into the *Eco*RI and *Pst*I sites of the suicide vector pSUP202-1 (Simon *et al.*, 1983). The construct was transformed into *E. coli* S17-1 and conjugated into *R. sphaeroides* 2.4.3. Transconjugants were selected on streptomycin medium and subsequently screened to confirm tetracycline sensitivity. The insertion in *ccoN* was then verified by PCR.

***nirK* overexpression construct.** For overexpression of *nirK*, an existing overexpression construct (pWLNIR) fusing *prnB'* and *nirK* was moved into a new vector background, pBBR1-MCS5, by using *Hind*III and *Eco*RI. This new overexpression vector was termed pAKHNIR.

Enzyme assays. Relative gene expression levels for *lacZ* fusions were measured by determining β -galactosidase activity (Maniatis *et al.*, 1982). Activity was determined based on three independent cultures, and the mean values were calculated. Nir activity was determined using a colorimetric Nir assay (Laratta *et al.*, 2002). Activity was determined for at least three independently grown cultures, and the mean values were calculated. Error bars represent one standard deviation in the figures. Oxygen uptake was measured using a Clark-type oxygen electrode.

Special culture conditions. For denitrifying growth at high r.p.m., cells were grown in sealed flasks as previously described (Tosques *et al.*, 1996, 1997) at 250 r.p.m. For denitrifying growth at low r.p.m., flasks were grown in the dark on a bench-top rotary shaker at 50 r.p.m. For photo-denitrifying growth at high and low r.p.m., flasks

Table 1. Bacterial stains and plasmids used in this study

Strain or plasmid	Genotype or description*	Reference or source
Strains		
S17-1	For conjugational transfer of plasmids: <i>recA thi pro hasdRM</i> ⁺ <i>RP4</i> :2-Tc:Mu:Km:TnZ	
2.4.3	Wild-type strain of <i>R. sphaeroides</i>	ATCC 17025
2.4.1	Wild-type strain of <i>R. sphaeroides</i>	Type strain
Δ ccoN	2.4.3 derivative with Ω Sm/Sp in <i>ccoN</i> , deletion mutant	This study
Plasmids		
pUC19	Used for cloning in <i>E. coli</i> DH5 α (Ap ^R)	Yanisch-Perron <i>et al.</i> (1985)
pBBR1MCS-5	Broad-host-range plasmid (Gm ^R)	Kovach <i>et al.</i> (1995)
pSUP202-1	Mobilizable suicide vector (Tc ^R Ap ^R Cm ^R)	Simon <i>et al.</i> (1983)
pHP45 Ω	Source of <i>aadA</i> ⁺ -Sm ^R cassette (Ap ^R Sm ^R)	Prentki & Krisch (1984)
pNir-298	pRK415 with <i>nirK-lacZ</i> transcriptional fusion containing 298 bp of the <i>nirK</i> promoter (Tc ^R Km ^R)	Laratta <i>et al.</i> (2002)
ccoNKO	pSUP202-1 with <i>ccoN::</i> Ω <i>aadA</i> ⁺ (Tc ^R Sm ^R)	This study
pUI2803H303A	pRK415 with <i>ccoNOQP</i> , <i>ccoN</i> mutation H303A (Tc ^R)	Oh (2006)
pWLNIR	pRK415 with <i>prnB'-nirK</i> fusion (Tc ^R)	Laratta <i>et al.</i> (2002)
pAKHNIR	pBBR1MCS-5 with <i>prnB'-nirK</i> fusion (Tc ^R)	This study

*Tc, tetracycline; Km, kanamycin; Sm, streptomycin; Ap, ampicillin; Gm, gentamicin; Cm, chloramphenicol.

were grown next to an incandescent light source at the r.p.m. mentioned above. For micro-oxic growth, plates were incubated in a polycarbonate anaerobic jar with the atmosphere three times evacuated and replaced with high-purity (minimum 99.995 %) N₂ from Airgas.

Oxygen dilution experiments. Cells were grown in 125 ml serum vials on solid slants of Siström's medium. Solid medium slants were made using 15 ml culture medium. After inoculation the vials were sealed and evacuated for 40 s. The vials were then flushed with N₂ gas for 45 s. Vials were equilibrated to atmospheric pressure using a water-filled syringe to allow trapped gas to escape without allowing atmospheric exchange. Leaving the gas-escape syringe in place, the desired amount of air was added back using a gas-tight syringe. Finally the gas-escape syringe was removed and the vials were incubated at 32 °C in the dark. For each percentage of oxygen in the headspace the following volumes of air were added back: 2 %, 11.1 ml; 1.3 %, 7.4 ml; 0.67 %, 3.7 ml; 0.45 %, 2.5 ml; 0.22 %, 1.2 ml; 0.13 %, 0.73 ml; 0.07 %, 0.36 ml and <0.07 %, no addition. The 'no addition' condition was also the micro-oxic condition used, since the N₂ gas used contained enough oxygen to allow growth under dark conditions on medium lacking nitrate. The atmospheric control (20 % oxygen) was evacuated and then allowed to equilibrate with atmospheric air using an open syringe.

RESULTS

nirK expression in a *ccb₃* oxidase mutant

The expression of *nirK* in a *ccoN* mutant background was previously studied using a strain in which *ccoN* was inactivated via Campbell insertion of a suicide plasmid (Laratta *et al.*, 2002). More recent work found that this insertion was unstable following prolonged incubation under micro-oxic conditions, allowing the restoration of *ccb₃* activity. Therefore, a second *ccoN* mutant was made in which 700 bases of *ccoN* were deleted to eliminate the possibility of the restoration of *ccb₃* activity. This strain was designated Δ *ccoN*. As with the previous *ccoN* mutant, Δ *ccoN* could not grow under micro-oxic conditions. Unlike the previous mutant, however, Δ *ccoN* did not regain the ability to grow under these conditions, even after prolonged incubation. Like other *ccb₃* mutants of *R. sphaeroides* (O'Gara *et al.*, 1998), Δ *ccoN* had elevated pigmentation under oxic conditions (data not shown).

As expected, *nirK* was not expressed in Δ *ccoN* cells grown under non-photosynthetic conditions in sealed flasks that

were made micro-oxic by the respiratory activity of the growing cells. Under these conditions the cells lacked detectable Nir activity (Fig. 1), nitrite levels of >3 mM were observed, and the nitrite was not consumed even after incubation for up to 72 h (data not shown). However, when Δ *ccoN* was grown under photosynthetic conditions in sealed vials that were made micro-oxic via cell respiration, Nir activity was detected during active growth and no nitrite accumulated in the medium (Fig. 1). Cells from these cultures did not regain the ability to grow under low-oxygen conditions. Wrapping the vials in foil to prevent photosynthetic growth restored the Nir-deficient phenotype of Δ *ccoN*.

Besides the difference in light conditions, photosynthetic cultures were also grown at slower agitation rates than cultures growing under non-photosynthetic conditions. To test if the restoration of Nir activity was influenced by agitation rate, cells were grown under photo-denitrifying conditions at the faster agitation rate of the dark experiments. Under these conditions Δ *ccoN* had no Nir activity (Fig. 1) and nitrite accumulated to high levels. These results show that the Nir-deficient phenotype is conditional. Manipulation of culture conditions can restore *nirK* expression and Nir activity, indicating that *ccb₃* activity or expression is not obligatory for *nirK* expression.

Impact of constitutive *nirK* expression in a Δ *ccoN* background

The simplest interpretation of the phenotype caused by inactivation of *ccoN* is that the loss of *ccb₃* affects the function of one of the regulatory proteins required for *nirK* expression. However, since Nir activity is required for *nirK* expression (Tosques *et al.*, 1997) it cannot be excluded that it is Nir assembly or activity that is being affected in Δ *ccoN*. To test this possibility, the plasmid pAKHNIR, which contains *nirK* under control of a constitutive promoter, was conjugated into Δ *ccoN*. This expression system has been used previously to demonstrate that the loss of Nir activity in a mutant of *R. sphaeroides* 2.4.3 lacking both cytochromes *c₂* and *c_Y* was due to a change in *nirK* expression, not to the loss of electron donors to Nir (Laratta *et al.*, 2006). Unexpectedly, Δ *ccoN* with pAKHNIR

Fig. 1. Nir activity of 2.4.3 and Δ *ccoN* under denitrifying (DN) and denitrifying-photosynthetic (DN, PH) conditions. Under each condition, cultures were grown for at least 48 h at two different mixing rates; high (250 r.p.m.) and low (50 r.p.m.).

showed very slow growth in nitrate-supplemented medium, even under oxic conditions (Fig. 2). The slow growth of the ΔccoN strain was nitrate dependent since growth was not slowed in medium lacking nitrate (Fig. 2). Nir activity could be detected in cells of the ΔccoN strain grown in medium without nitrate. Using a methyl-viologen-dependent assay Nir activity was 10-fold higher in ΔccoN with pAKHNIR than without (Fig. 3).

pAKHNIR was mobilized into several other strains of *R. sphaeroides* 2.4.3 to determine if any had the nitrate-dependent growth inhibition shown by ΔccoN . Strains lacking either Nor or NnrR showed no significant growth inhibition in nitrate-supplemented medium until cultures reached optical densities consistent with the onset of denitrification (data not shown). Once these cultures reached optical densities where denitrification genes are typically induced, growth stopped. A strain in which *cycA*, which encodes cytochrome *c*₂, an important electron donor to *cbb*₃, was deleted also showed no growth inhibition in the presence of nitrate (Daldal *et al.*, 2001).

Influence of O₂ on the growth of ΔccoN

The slow growth of ΔccoN with pAKHNIR in the presence of nitrate is consistent with Nir activity producing NO, which inhibits respiration. This suggests that the loss of the *cbb*₃ oxidase does not prevent Nir assembly or function. Therefore, the inhibition of *nirK* expression in ΔccoN is most probably due to an impact on a regulatory factor. PrrA is known to be required for *nirK* expression but the increase in PrrA-P levels seen in a *cbb*₃ mutant would seem unlikely to inhibit *nirK* expression (Laratta *et al.*, 2002).

NnrR is the other known regulator of *nirK* expression. Current evidence suggests NnrR activity is controlled by NO (Kwiatkowski & Shapleigh, 1996). Members of the *cbb*₃ family have been shown to have NO reductase activity (Forte *et al.*, 2001) so loss of this oxidase might lead to an increase in NO levels. Previous work has shown that the loss of Nor also leads to accumulation of NO but this does

Fig. 2. Growth of ΔccoN with a plasmid (pAKHNIR) that constitutively expresses *nirK*. Cultures were grown in the dark in stoppered flasks with 10 mM nitrate (■) or without nitrate (◆). Optical density at 600 nm was monitored over time.

Fig. 3. Nir activity of ΔccoN and ΔccoN carrying a plasmid (pAKHNIR) that constitutively expresses *nirK* (cK). Cultures were grown for 24 h in the dark in stoppered flasks with 10 mM nitrate. Black bars represent whole-cell Nir activity, which measures endogenous electron flow to Nir. Grey bars represent Nir activity using the artificial electron donor methyl viologen as an overall indicator of Nir presence and functionality.

not inhibit NnrR function since *nirK* and *nor* are still expressed in a strain lacking Nor activity (Bartnikas *et al.*, 1997; Kwiatkowski & Shapleigh, 1996). Therefore, it seems unlikely that changes in NO levels are responsible for the observed changes in *nirK* expression.

Previous work using a heterologous expression system has suggested that a member of the DNR/Nnr family is O₂ sensitive (Lee *et al.*, 2006). *nirK* expression is most inhibited in ΔccoN in sealed vessel experiments in which O₂ levels are reduced by cellular respiration. Under these conditions, the loss of *cbb*₃ will affect the end-point level of O₂ in the cultures. The two major respiratory oxidases in *R. sphaeroides* 2.4.3 have different affinities for oxygen. The *cbb*₃ oxidases have been shown to have a *K_m* for O₂ that is in the low nanomolar range while the *aa*₃ oxidase is at the low micromolar level (Preisig *et al.*, 1996; Riistama *et al.*, 2000). Loss of the *cbb*₃ oxidase will thus result in a higher end-point O₂ level inside the sealed incubations used in this work. Therefore, it is not unreasonable to suggest that the lack of *nirK* expression in ΔccoN could be due to the inhibition of NnrR by O₂. To test if this residual O₂ is responsible for inhibition of *nirK* expression a series of experiments was carried out in sealed vials with a range of headspace oxygen concentrations established using gas mixtures (Fig. 4). As expected, the wild-type strain 2.4.3 grew in the dark under O₂ concentrations ranging from atmospheric (20 %) down to the sample with no added O₂. Even at the lowest O₂ concentrations growth was not nitrate dependent. Nevertheless, wild-type cells began to denitrify at approximately 2 % oxygen and below, as indicated by the onset of Nir activity. In agreement with these results, *nirK* expression could be detected in cultures grown with ≤1 % O₂ (data not shown).

ΔccoN grew in vials with oxygen levels ranging from atmospheric to 2 % and this growth was not nitrate dependent. Unlike the wild-type strain, however, no growth was observed in the vials for incubations up to 10 days if O₂ levels were <2 % or >0.2 %, irrespective of the presence of

Fig. 4. Growth of 2.4.3 and $\Delta ccoN$ under a range of established oxygen concentrations. Oxygen concentration is represented as a percentage, with atmospheric oxygen considered 20 %. For each oxygen concentration, growth, Nir activity and *nirK-lacZ* expression were assessed. Expression values are in Miller units (MU).

nitrate. Growth was observed with O₂ levels <0.2 % after 5 days but only if nitrate was present, demonstrating that growth was due to denitrification. Cells of the *ccoN* mutant grown under these low O₂ conditions had wild-type levels of Nir activity (Fig. 4) and *nirK* and *nor* expression. One possible explanation for the lack of growth under intermediate O₂ conditions is that there is no oxidase expressed due to the absence of the *cbb₃* gene and a decrease in expression of the genes encoding the *aa₃* oxidase (Pappas *et al.*, 2004). However, cells of $\Delta ccoN$ grown at 0.07 % O₂ on nitrate-containing medium consumed O₂ at the same rate as the wild-type strain grown under identical conditions.

To further test the role of O₂ in controlling the denitrifying ability of $\Delta ccoN$, a co-culture experiment was carried out. Growing $\Delta ccoN$ in a sealed flask together with a strain containing an oxidase with an O₂ affinity equivalent to that of the *cbb₃* in strain 2.4.3 should restore *nirK* expression and Nir activity in $\Delta ccoN$ if end-point O₂ levels are critical. Conveniently, the *R. sphaeroides* type strain 2.4.1 has an aerobic respiratory chain identical to 2.4.3 but lacks *nirK* (Kwiatkowski *et al.*, 1997). Co-cultures with either a 1:3 or 3:1 ratio of wild-type 2.4.3 or $\Delta ccoN$ to 2.4.1 cells were used for these experiments. Initial experiments done in the dark indicated that 2.4.1 out-competed $\Delta ccoN$ by at least 30:1 based on c.f.u. ml⁻¹ values determined at the end of the experiment (data not shown). The poor growth of $\Delta ccoN$ under these conditions is consistent with the inability of $\Delta ccoN$ to efficiently respire at low O₂ concentrations. To overcome this problem the co-culture experiments were carried out under high-agitation photosynthetic conditions, which were shown to prevent *nirK* expression in $\Delta ccoN$ (Fig. 1). When cultures were grown under these conditions the ratios of $\Delta ccoN$ to 2.4.1 did not change significantly during the course of the experiment. As expected, no nitrite accumulated in the co-cultures and the cells showed near wild-type levels of Nir activity (Fig. 5).

Mutant isolation

Attempts were made to isolate mutants of $\Delta ccoN$ that could grow via nitrate-dependent respiration at O₂ levels between 0.2 and 2.0 %. Both spontaneous and UV-induced mutagenesis were used in an effort to generate mutants. Various selective conditions were tried, including photo-synthetic conditions, low-oxygen conditions without nitrate and transitions from no-nitrate to nitrate-containing medium. After repeated attempts, involving incubation times of up to 3 months, no strains of $\Delta ccoN$ were isolated that would grow in nitrate-containing medium at these O₂ levels.

DISCUSSION

nirK expression requires the direct or indirect involvement of multiple regulators that integrate information about environmental conditions (Laratta *et al.*, 2002; Tosques *et al.*, 1997). In strain 2.4.3, nitrate is the initial substrate for denitrification, making all downstream denitrification

Fig. 5. Nir activity of co-cultures of strains 2.4.3 or $\Delta ccoN$ with strain 2.4.1. In all cases cultures were grown for 36 h under photosynthetic-denitrifying conditions. Co-cultures were started in two different ratios, 3:1 and 1:3, which represent the starting quantity of 2.4.3:2.4.1 or $\Delta ccoN$:2.4.1.

regulation dependent on the expression of nitrate reductase. Nitrate reductase is upregulated by an unknown mechanism in response to decreasing oxygen levels (A. Hartsock & J. P. Shapleigh, unpublished). The nitrite produced by nitrate reduction is then reduced to NO by the low levels of Nir produced by basal levels of *nirK* expression. NO then activates DNA binding by NnrR, which is necessary but not sufficient for expression of *nirK* (Kwiatkowski & Shapleigh, 1996; Tosques *et al.*, 1996, 1997). *nirK* expression also requires activation by PrrA-P (Laratta *et al.*, 2002). Production of PrrA-P occurs when O₂ levels are low enough to limit turnover of the *cbb₃* oxidase (Oh *et al.*, 2004). A decrease in O₂ is not required to produce high levels of PrrA-P in cells lacking the *cbb₃* oxidase (O’Gara *et al.*, 1998). If there are no additional layers of regulation involved it would be predicted that *nirK* expression should occur at higher O₂ levels in a *ccoN* mutant than in the wild-type. The contradictory observation that the loss of the *cbb₃* oxidase reduced *nirK* expression suggested there are additional uncharacterized factors required for *nirK* expression, which motivated this study.

The experiments described here suggest that the conditional *nirK* expression phenotype in $\Delta ccoN$ is a consequence of the loss of a high-affinity oxidase which, under certain growth conditions, leaves O₂ levels so high as to prevent *nirK* expression. The restoration of Nir activity in $\Delta ccoN$ cells grown in vials whose atmosphere has been sparged with a low-O₂ gas shows that *nirK* expression can be readily restored in this strain and that *cbb₃* activity is not obligatory for *nirK* expression. *cbb₃* is only required for *nirK* expression when aerobic respiration is being used to lower O₂ concentrations during growth.

The likely oxygen-sensitive component that is being affected by the loss of *cbb₃* is NnrR. The loss of the *cbb₃* oxidase does not affect photosynthetic growth (O’Gara *et al.*, 1998), suggesting that a factor specifically associated with denitrification is being affected. Since nitrate reductase is functional in $\Delta ccoN$, this indicates that the factor being affected is either Nir or Nor specifically or some factor involved in regulation of *nirK* as well as *nor*. The activities of Nir and Nor are not O₂ sensitive, suggesting it is the regulation of their expression that is affected (Morley *et al.*, 2008). Since PrrA is functional in $\Delta ccoN$, this leaves NnrR as the probable O₂-sensitive component. This conclusion is supported by previous work which suggested that the regulator of the genes encoding the NO-forming nitrite reductase and NO reductase in *Paracoccus denitrificans*, which, like NnrR, is also a member of the FNR family, is O₂ sensitive (Lee *et al.*, 2006). Having regulators of this type be sensitive to O₂ ensures that NO production does not occur until O₂ levels are extremely low. This reduces the possibility of the formation of reactive nitrogen oxides that can arise due to the interaction of NO with various oxygen species (Hughes, 2008; Patel *et al.*, 1999). This layer of O₂-dependent regulation also ensures that denitrification is secondary to O₂-dependent regulation at

a wide range of O₂ levels. However, while O₂ concentrations must be low for *nirK* expression it is notable that it can be expressed at O₂ levels that are high enough to support aerobic respiration via the *cbb₃* oxidase (Fig. 4). This makes denitrification a micro-oxic process and suggests cells can co-respire oxygen and nitrate.

While *cbb₃* oxidase activity was needed for *nirK* expression if cell respiration was being used to reduce O₂ levels in cultures growing in the dark, this was not always the case for cells growing photosynthetically. This may indicate that in addition to being O₂ sensitive, the activation of NnrR function also requires ATP synthesis. In cultures growing in the dark, once O₂ levels reach concentrations where the *aa₃* oxidase is no longer active there is no longer rapid ATP synthesis due to the absence of the *cbb₃* oxidase. This is evidenced by the overgrowth of strain 2.4.1 in the co-culture experiments. Cells grown in the light can generate ATP via photosynthesis after O₂ levels are reduced. A lack of ATP synthesis alone, however, cannot account for the conditional *nirK* expression phenotype in $\Delta ccoN$. The importance of O₂ sensitivity is demonstrated by the observation that there is no expression of *nirK* in $\Delta ccoN$ at O₂ levels where *aa₃* is functional. The impact of increased agitation on *nirK* expression in cells growing photosynthetically also suggests O₂ plays a role in inhibiting *nirK* expression under these conditions. Rapid agitation would vigorously mix O₂ throughout the culture medium. Slower agitation and the consequent poorer mixing of O₂ may provide conditions where NnrR is functional and the ATP also necessary for its assembly or activation is available from photosynthesis. Another possibility is that the light used to allow photosynthetic growth is causing photolysis of an NO adduct that would otherwise be preventing oxygen consumption.

An unexpected observation made during these experiments was the nitrate sensitivity of the $\Delta ccoN$ strain containing pAKHNIR. Since this phenotype requires both the constitutively expressed *nirK* and the presence of nitrate in the medium, it is likely that it is due to the constitutively expressed Nir producing NO, which inhibits respiration. This was unexpected because a wild-type strain with the same *nirK* expression plasmid is not nitrate sensitive and also because strains lacking Nor activity can grow in the presence of nitrate at high O₂ levels (Bartnikas *et al.*, 1997; Tosques *et al.*, 1996). Therefore, the only strain that is affected at high O₂ concentrations is the one lacking *cbb₃* activity with pAKHNIR. One possible explanation for this is that the *cbb₃* oxidase is NO resistant or is capable of mitigating NO toxicity. The latter possibility is consistent with the observation that some *cbb₃* oxidases have NO reductase activity (Forte *et al.*, 2001). While the *cbb₃* in strain 2.4.3 may have some NO reductase activity, it is not capable of reducing NO at rates fast enough to keep up with NO production by Nir during denitrification (Bartnikas *et al.*, 1997). Strains in which *nor* has been disrupted stop growing when Nir becomes active even if *cbb₃* is present, demonstrating the importance of Nor

under denitrification conditions (Bartnikas *et al.*, 1997). It is also possible that an aerobic mechanism for mitigating NO toxicity is disrupted in *ΔccoN*, given the global nature of the PrrBA regulon (Eraso *et al.*, 2008).

Experiments described here and in previous publications indicate that oxygen control of *nirK* expression, and consequently denitrification, in *R. sphaeroides* 2.4.3 is achieved through both PrrBA and NnrR. The activity of these two regulatory systems is directly or indirectly influenced by the *cbb₃* oxidase. This suggests a general design feature in denitrifiers whereby the O₂ level at which the expression of genes encoding products involved in NO production and reduction is adjusted to correlate with the *K_m* of the highest affinity O₂-reducing oxidase in the respiratory chain. If true aerobic denitrification does occur, it may require a bacterium lacking high-affinity oxidases.

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