Expression of a putative nitrite reductase and the reversible inhibition of nitrite-dependent respiration by nitric oxide in *Nitrobacter winogradskyi* Nb-255

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Summary
The nitrite oxidizing Alphaproteobacterium, *Nitrobacter winogradskyi*, primarily conserves energy from the oxidation of nitrite (NO$_2^-$) to nitrate (NO$_3^-$) through aerobic respiration. Almost 20 years ago, NO-dependent NADH formation was reported to occur in both aerobic and anaerobic cell suspensions of *N. winogradskyi* strain ‘agilis’, suggesting that NO oxidation might contribute to energy conservation by *Nitrobacter*. Recently, the *N. winogradskyi* Nb-255 genome was found to contain a gene (Nwin_2648) that encodes a putative copper-containing nitrite reductase (NirK), which may reduce NO$_2^-$ to NO. In this study, the putative nirK was found to be maximally transcribed under low O$_2$ (between zero and 4% O$_2$) in the presence of NO$_2^-$.

Transcription of nirK was not detected under anaerobic conditions in the absence of NO$_2^-$ or in the presence of NO$_3^-$ and pyruvate. Although net production of NO could not be detected from either aerobically grown or anaerobically incubated cells, exogenous NO was consumed by viable cells and concomitantly inhibited NO$_2^-$-dependent O$_2$ uptake in a reversible, concentration dependent manner. Both NO$_2^-$-dependent O$_2$ uptake and NO consumption were inhibited by 1 mM cyanide suggesting involvement of cytochrome oxidase with NO consumption. Abiotic consumption of NO was measured, yet, both the rates and kinetics of NO transformation in buffer alone, or by heat killed, or cyanide-treated cells differed from those of viable cells. In light of this new information, a modified model is proposed to explain how NirK and NO manage electron flux in *Nitrobacter*.

Introduction
As a chemolithoautotroph, *Nitrobacter winogradskyi* conserves energy from the oxidation of NO$_2^-$ and fixes carbon dioxide as a carbon source (Bock *et al*., 1986; Bock *et al*., 1991). NO$_2^-$ oxidation is an aerobic process catalysed by nitrite oxidoreductase (NXR), which converts NO$_2^-$ to NO$_3^-$ (Yamanaka and Fukumori, 1988). The reaction catalysed by NXR is reversible (Sundermeyer-Klinger *et al*., 1984), and *N. winogradskyi* can convert NO$_3^-$ to NO$_2^-$ under anaerobic conditions when given pyruvate or glycerol as an energy source (Kiesow, 1964; Freitag *et al*., 1987). Although the terminal products of denitrification in *N. winogradskyi* are reported to be nitric oxide (NO) and nitrous oxide (N$_2$O) (Freitag *et al*., 1987; Ahlers *et al*., 1990), the mechanism of N$_2$O production remains unknown as NO reductase genes were not identified in the *N. winogradskyi* genome (Starkenburg *et al*., 2006). Nonetheless, a protein from *Nitrobacter vulgaris* that copurified with NXR was shown to reduce NO$_2^-$ to NO (Ahlers *et al*., 1990). Furthermore, Freitag and Bock (1990) measured NO-dependent NADH formation in both aerobic and anaerobic cell suspensions, which led to the hypothesis that NO may be an intermediate of the nitrite oxidation system (Bock *et al*., 1991; Poughon *et al*., 2001). Because NADH is synthesized via a reverse flow of electrons from NO$_2^-$ oxidation (Sewell and Aleem, 1969; Yamanaka and Fukumori, 1988), NO should be a more favourable electron donor than NO$_2^-$ to generate reductant for biosynthesis given the less positive redox potential of the NO$_2^-$ / NO couple ($E'_0 = +387$ mV) relative to the NO$_3^-$ / NO$_2^-$ couple (+420 mV).

Further physiological investigations into the metabolism of NO by NO$_2^-$-oxidizing bacteria have not been described. A recent comparative analysis of three genomes of *Nitrobacter* (Starkenburg *et al*., 2008) indicated that each of...
them contain a gene encoding a putative copper containing nitrite reductase (NirK), which may be responsible for the production of NO (Ahlers et al., 1990). Interestingly, the Nitrobacter nirK is orthologous to nirK in the ammonia oxidizing Betaproteobacterium, Nitrosomonas (Cantera and Stein, 2007a), and more distantly related to the NirK encoded in the genome of Nitrobacter's close alphaproteobacterial relative, Rhodopseudomonas (Larimer et al., 2004). In recent years, considerable effort has been expended to understand the role of NirK in ammonia oxidizers (Beaumont et al., 2002; 2004; 2005; Schmidt et al., 2004; Cantera and Stein, 2007a,b). Unlike an archetypal nitrite reductase that is expressed under anaerobic conditions and functions in denitrification (Berks et al., 1995; Zumft, 1997), the expression of NirK by Nitrosomonas europaea is mainly controlled by the concentration of NO2− and pH, but not by NO or O2 (Beaumont et al., 2004). NirK is active and expressed under aerobic conditions, and studies completed using a nirK-deficient strain of N. europaea demonstrated that nirK is required for optimal growth and cell yield during the aerobic oxidation of ammonia (Schmidt et al., 2004; Cantera and Stein, 2007b).

In this study, the expression of the putative nirK (Nwin_2648) by N. winogradskyi was investigated, and the factors that control its expression were explored. In addition, the effect of NO on the nitrite oxidation system was examined and a role is proposed for NirK and NO in the energetics system of N. winogradskyi.

### Table 1. Effect of O2, pH and NO2− on nirK expression.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Oxygen (%)</th>
<th>pH</th>
<th>NO2− (mM)</th>
<th>NO3−/pyruvate (mM)</th>
<th>NO2− consumed (mM)</th>
<th>nirK expression</th>
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<td>26.2</td>
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Batch cultures of Nitrobacter winogradskyi NB255 (ATCC 25391) were grown lithoautotrophically in chemically defined medium (750 μM MgSO4, 30 μM FeSO4·7H2O, 200 μM CaCl2, 1 μM CuSO4, 200 mM Na2MoO4, 500 mM MnCl2, 175 mM ZnSO4, and 4.2 μM CoCl2) containing NaNO2 (30 μM) and buffered using 20 μl ml−1 of a phosphate buffer stock solution (480 mM KH2PO4, 42 mM NaH2PO4, pH 7.8). In early log phase, cells were harvested by centrifugation, washed twice and re-suspended in sterile phosphate buffered base medium (pH 7.5) to a final concentration of 0.35 OD600 (25 μg cell protein ml−1). After 3 h of treatment, RNA was extracted from cell suspensions using a RNeasy Mini kit (Qiagen Sciences, Maryland, USA) and cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad Laboratories) according to the manufacturer’s instructions. nirK and 16S rRNA were amplified by quantitative PCR in 50 μl reactions using a Bio-Rad quantitative PCR detection system (Bio-Rad Laboratories, Hercules, CA) with the following cycle parameters; cycle 1: 95°C for 30 s, cycles 2–36: step 1 – 95°C for 30 s, step 2 – 55°C for 30 s, step 3 – 72°C for 40 s, cycle 37: 95°C for 60 s, cycle 38: 55°C for 1 min, cycles 39–120: initial set point temperature was 55°C and was increased by 0.5°C every 10 s. The following primers were used for amplification; NWinrK-F 5′-AAGACCTTCTCCTACGTTCC-3′; NW16s-975F 5′-GGAGCATGGACGAGAGG-3′; NW16s-1157R 5′-GTAAGGGCCATGAGG-3′.

### Results and discussion

**Expression of nirK**

Experiments were conducted to determine if nirK was expressed by N. winogradskyi, and if the transcription of nirK was affected by pH, NO2− and/or O2. Cell suspensions of N. winogradskyi from lithoautotrophically grown batch cultures were exposed to different levels of O2 in the presence of NO2− for 3 h (Table 1). An effect of O2 limitation was apparent as NO2− consumption declined in response to decreasing amounts of O2. For example, NO2− oxidizing cells exposed to 10% O2 consumed 9.4 mM NO2− while cells given 2% O2 only consumed 2.1 mM NO2−. With respect to nirK expression, and in contrast to Western blot analysis of NirK expression in N. europaea (Beaumont et al., 2004), O2-limited N. winogradskyi cells contained significantly higher amounts of nirK mRNA than O2 replete cells. Compared with cells exposed to 20% O2, nirK expression increased 4.8, 20.3 and 34.3-fold when the initial O2 concentration was reduced to 10%, 4% and 2% respectively. In cells exposed to NO2− in the absence of O2, nirK was transcribed 20-fold more than by cells exposed to both NO2− and 20% O2, indicating effective induction of nirK by NO2− in the absence of O2. The expression of nirK under completely anaerobic conditions in the absence of NO2− was significantly reduced compared with both aerobic cell suspensions and anaerobic cell suspensions containing...
NO$_3^-$ (Table 1, experiments 1 and 2). When *N. winogradskyi* was incubated anaerobically in the presence of NO$_3^-$ and pyruvate, the amount of nirK mRNA was threefold to 50-fold lower than in cells exposed to 20% O$_2$ and 30 mM NO$_3^-$. In addition, the effect of pH on the expression of nirK under aerobic conditions was assessed (Table 1, Experiment 3). While cells consumed less NO$_3^-$ at pH 6.6, nirK expression was not significantly different over the pH range of 6.6–8.2. A similar pH shift (8.2–7.2) had been shown previously to increase NirK expression in *N. europaea* (Beaumont et al., 2004).

In summary, nirK was maximally expressed under low O$_2$ conditions in the presence of NO$_2^-$. Curiously, in the absence of NO$_2^-$ and O$_2$, nirK expression declined significantly below the levels of nirK mRNA detected in anaerobic, NO$_2^-$ containing cell suspensions, suggesting that nirK expression under anaerobic conditions is NO$_2^-$ dependent. It is possible that *N. winogradskyi* could express NirK and generate NO after a longer period of anaerobic incubation in the presence of pyruvate and NO$_3^-$ if NO$_2^-$ accumulated to a critical induction level. In support of this hypothesis, an active, copper-containing nitrite reductase was isolated from *Nitrobacter vulgaris* after anaerobic growth (Ahlers et al., 1990) and some loss of N via N$_2$O was detected under anaerobic conditions at low (0.3–0.6 mM) nitrite concentrations (Freitag and Bock 1990). Nevertheless, we have observed that NO$_2^-$ cannot serve as sole terminal electron acceptor for anaerobic growth of *N. winogradskyi* and others have demonstrated that strict anaerobic growth on glycerol and nitrate as the electron donor and electron acceptor, respectively, cannot be sustained because as NO$_3^-$ accumulates to >0.5 mM, growth is inhibited (Bock et al., 1986; Freitag et al., 1987).

Furthermore, treatment of cultures with 2 mM NO$_3^-$ at the onset of culturing completely inhibited anaerobic growth (Freitag et al., 1987). Thus, if NirK is expressed during anaerobic, denitrifying conditions, the rate of NO$_2^-$ reduction to NO by NirK must be significantly slower than the rate of NO$_3^-$ reduction to NO$_2^-$ by NXR. Taken together, these data suggest that nitrate reductase only plays a minor role in a classical denitrifying energetics pathway in *N. winogradskyi* and appears to function primarily during nitrate oxidation under an O$_2$-limited environment.

**NO consumption**

Because the results indicated that nirK was transcribed under both aerobic and O$_2$-limited conditions, experiments were carried out to determine if the increase in nirK mRNA correlated with an increase in NirK enzyme activity by measuring NO production. In short, NO was not detected in the aqueous solutions of cells grown in fully aerobic conditions or in cells harvested after 12 h exposure to O$_2$-limited conditions (data not shown). Nonetheless, we observed that 2 µM of exogenously supplied NO was quickly consumed by cell suspensions of *N. winogradskyi* at a rate of 64 nmol min$^{-1}$ (mg protein)$^{-1}$. The rate of NO transformation by suspensions of heat-killed cells (Fig. 1A) was identical to the rate of transformation that occurred upon addition of NO to phosphate buffer without cells (≤27 nmol min$^{-1}$ (mg protein)$^{-1}$), indicating that the maximum rate of NO transformation was dependent upon viable cells, and that abiological consumption could also occur at a significant rate.

Experiments were also conducted to determine if NO consumption was linked to respiration by using O$_2$ and NO-specific electrodes inserted into the same assay chamber. When NO$_2^-$ was added to cell suspensions of *N. winogradskyi*, O$_2$ was quickly consumed (Fig. 1B). After the addition of 2 µM NO, however, NO$_2^-$-dependent O$_2$ uptake was severely inhibited. When NO was completely consumed, O$_2$ uptake was immediately restored close to the initial rate (≥90%). The time interval of NO-dependent inhibition of O$_2$ consumption was concentration dependent over a range of 0.5–32 µM NO, and O$_2$ uptake always resumed after the NO was consumed (data not shown). These results were verified by following the simultaneous consumption of NO and O$_2$ using gas inlet mass spectrometry (Fig. 1C and D). After the addition of 3.5 µM NO to cells respiring NO$_2^-$, O$_2$ uptake was inhibited for over 3 min (Fig. 1D). NO consumption was also monitored in the absence of O$_2$. After O$_2$ was depleted by NO$_2^-$-dependent O$_2$ consumption, the time required to consume 7 µM NO increased by 50% compared with replete oxygen conditions suggesting that O$_2$ directly, or indirectly, affects NO consumption.

Because O$_2$ uptake was inhibited by NO, experiments were conducted to determine if NO was inhibiting the major proteins involved in the NO$_2^-$ oxidizing system. O$_2$ uptake and NO consumption were monitored in cells treated with either azide, a selective inhibitor of NXR (Aleem and Sewell, 1981; Tanaka et al., 1983; Ginestet et al., 1998), or N,N-diethyldithiocarbaminate (DDC), an inhibitor of copper-containing nitrite reductase (Zumft, 1997). Although 20 µM azide reduced NO$_2^-$-dependent O$_2$ uptake rate by 85%, NO was still consumed at a rate comparable to untreated viable cells (data not shown). DDC did not affect NO$_2^-$-dependent O$_2$ uptake or the consumption of NO. In contrast, both NO$_2^-$-dependent O$_2$ uptake and NO consumption were inhibited by 1 mM cyanide (Fig. 1C). The NO consumption curve of cyanide-treated cells mimicked abiotic NO consumption that occurred in buffer alone or in the presence of heat killed cells (Fig. 1A).

Because cyanide-treated cells did not consume NO, and cyanide was previously shown to inhibit cytochrome aa$_3$ oxidase of *N. winogradskyi* strain ‘agilis’ (Yamanaka et al., 1985), it is possible that the arrest of NO$_2^-$-dependent O$_2$
uptake may be the result of NO binding to cytochrome oxidase. Others have reported that the binding of NO to cytochrome oxidase is reversible (Cooper, 2002) and that the rate of NO association is up to eight orders of magnitude faster than the rate of its dissociation when cytochrome oxidase is reduced (Blackmore et al., 1991; Sarti et al., 2000). This is consistent with our observation that μM concentrations of NO arrested NO\textsubscript{2}\textsuperscript{-}-dependent O\textsubscript{2} uptake within seconds, yet, the consumption of NO and restoration of O\textsubscript{2} uptake required several minutes (Fig. 1D). The concentration of free NO in denitrifying bacteria is kept low (1–30 nM) (Zumft, 1993; 1997), and NO accumulation was shown to be lethal to a NO reductase deficient mutant of the denitrifier, P. stutzeri (Zumft, 1993). Although NO is a known toxicant and reacts with many cellular targets, including haem, iron-sulfur, and copper containing proteins (Zumft, 1993), even after a long-term exposure to excess NO (125 mM for 90 min), N. winogradskyi remained viable and NO\textsubscript{2}\textsuperscript{-} could be oxidized after removal of NO (S.R. Starkenburg, unpubl. results). Thus, N. winogradskyi appears to be well equipped to mitigate the negative impacts of NO.

An attempt was made to identify the products of NO transformation by N. winogradskyi. Using gas inlet mass spectrometry, the aqueous concentrations of NO\textsubscript{2}, N\textsubscript{2}O, N\textsubscript{2}O\textsubscript{3}, N\textsubscript{2}O\textsubscript{4} were measured before and after NO exposure. The concentrations of the aforementioned gases did not change significantly compared with abiotic controls (data not shown). As the presence of O\textsubscript{2} influenced the rate of NO consumption, the oxidative conversion of NO to NO\textsubscript{2} and NO\textsubscript{3} was compared in the presence and absence of living cells. When NO gas was added to the head space of bottles containing buffer alone and incubated for 30 min, NO was oxidized exclusively to NO\textsubscript{2} (Fig. 2) as previously observed (Ignarro et al., 1983). In contrast, in the presence of viable cells of N. winogradskyi, most of the NO was converted to NO\textsubscript{3} (89%) and the minority was recovered as NO\textsubscript{3} (11%). Samples taken from bottles in which NO was incubated with either heat killed or cyanide-treated cell suspensions contained a similar amount of NO\textsubscript{2} as the buffer control but did not contain NO\textsubscript{3}. The amount of NO recovered as NO\textsubscript{2} + NO\textsubscript{3} in supernatants from incubations with viable cells was 15–20% more than the NO\textsubscript{2} measured in bottles containing either phosphate buffer alone, or heat killed and cyanide-treated cells. One possible interpretation of this result is that as NO\textsubscript{2} is transformed to NO\textsubscript{3} by biological oxidation, the rate of chemical oxidation of NO to NO\textsubscript{3} is accelerated thereby increasing the overall transformation of NO to NO\textsubscript{2} + NO\textsubscript{3}. If this interpretation is true, it implies that NO\textsubscript{2} transformation to NO\textsubscript{3} may have occurred even while NO\textsubscript{2}-dependent O\textsubscript{2} uptake was inhibited by NO. Alternatively, it still remains a possibility that NO-derived NO\textsubscript{2} was biologically oxidized to NO\textsubscript{3} only after much of the
We hypothesize that *Nitrobacter* produces NO via NirK to manage the redox state of the cell and reversibly control cytochrome oxidase activity when low O₂ concentrations become unfavourable for O₂-dependent NO₂⁻ oxidation (Fig. 3B) and while NO₂⁻ might still accumulate via ammonia oxidation (Laanbroek and Gerards, 1993). Regulation of mitochondrial respiration by NO via reversible inhibition of cytochrome oxidase was proposed several years ago (Brown, 1999; 2000; Cooper, 2002), and a recent report indicates that *Staphylococcus aureus*, in response to inhibition of respiration by NO, maintains redox balance by upregulating lactate dehydrogenase to consume reducing equivalents generated from glucose oxidation (Richardson *et al.*, 2008). With regard to *Nitrobacter*, if cytochrome oxidase is inhibited by NO under low O₂ conditions (and if we assume that the turnover rate of NXR is not completely inhibited by NO), more electrons generated from NO₂⁻ oxidation could be channelled through reverse electron flow for CO₂ fixation and/or the reductive biosynthesis of the storage product, poly-β-hydroxybutyrate. Indeed, it was shown previously that *N. hamburgensis* contained significantly higher quantities of PHB granules in the anaerobic zone of a biofilm compared with cells located in the aerobic zone (Freitag *et al.*, 1987). We also observed that NO was consumed at a slower rate through abiotic mechanisms, and resulted in the formation of NO₂⁻ [turnover of NO by cytochrome oxidase could also result in the formation of NO₂⁻ (Torres *et al.*, 2000)]. Taken together, these data indicate that the NO-dependent stimulation of NADH synthesis by *Nitrobacter* under aerobic conditions reported by Freitag and Bock (1990) could simply be a result of the metabolism of NO₂⁻ produced from the auto-oxidation of NO. Further investigation into the properties of NirK, and the production and consumption of NO by *Nitrobacter* are

![Graph](image)

**Fig. 3.** Model of NirK Function and NO metabolism in *N. winogradskyi*. In the presence of O₂ (A), most electrons are directed toward respiration (note the relative size of dotted arrow indicating the amount of electron flux). Under low oxygen conditions (B), NirK expression is proposed (Fig. 3A).
warranted in an attempt to resolve this long-standing phenomenon of NO\textsubscript{2}− metabolism.

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Starkenburg, S.R., Chain, P.S., Sayavedra-Soto, L.A.,


