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Transcription and activities of NO_x reductases in *Agrobacterium tumefaciens*: the influence of nitrate, nitrite and oxygen availability

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Summary

The ability of *Agrobacetrium tumefaciens* to perform balanced transitions from aerobic to anaerobic respiration was studied by monitoring oxygen depletion, transcription of *nirK* and *norB*, and the concentrations of nitrite, nitric oxide (NO) and nitrous oxide in stirred batch cultures with different initial oxygen, nitrate or nitrite concentrations. Nitrate concentrations (0.2–2 mM) did not affect oxygen depletion, nor the oxygen concentration at which denitrification was initiated (1–2 μM). Nitrite (0.2–2 mM), on the other hand, retarded the oxygen depletion as it reached ≈20 μM, and caused initiation of active denitrification as oxygen concentrations reached 10–17 μM. Unbalanced transitions occurred in treatments with high cell densities (i.e. with rapid transition from oxic to anoxic conditions), seen as NO accumulation to μM concentrations and impeded nitrous oxide production. This phenomenon was most severe in nitrite treatments, and reduced the cells' ability to respire oxygen during subsequent oxic conditions. Transcripts of *norB* were only detectable during the period with active denitrification. In contrast, *nirK* transcripts were detected at low levels both before and after this period. The results demonstrate that the transition from aerobic to anaerobic metabolism is a regulatory challenge, with implications for survival and emission of trace gases from denitrifying bacteria.

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Introduction

Denitrification is a key function in the biogeochemical cycling of nitrogen, and has industrial applications in water purification and wastewater treatment. It is a major source of atmospheric nitrous oxide, and may also contribute to the emission of nitric oxide (NO; Otte *et al*., 1996). The process sustains respiratory growth when oxygen is limiting, and can be performed by a wide range of phylogenetically unrelated prokaryotes. Free energy is transduced as N-oxides are used as electron acceptors by an electron transport chain similar to that used during aerobic respiration. The genes required for denitrification include those encoding four reductases; nitrate- (Nar/Nap), nitrite- (Nir), NO (Nor) and nitrous oxide (Nos) reductase, genes encoding various ancillary proteins and a number of regulatory genes. Signals that activate transcription of the genes encoding these proteins vary between species, but generally low O_2 and the presence of nitrate or denitrification intermediates such as nitrite or NO are required for optimal gene expression. Nitrate, nitrite and nitrous oxide can be effectors for expression of their respective reductases while NO is an effector for both Nir and Nor expression (Philippot, 2002; Baek and Shapleigh, 2005; van Spanning *et al*., 2007).

For denitrification to contribute to survival in natural environments, its regulation must be effectively coordinated with other aspects of cellular metabolism. Denitrification is energetically unfavourable compared with O_2 respiration (Thauer *et al*., 1977), hence mechanisms that minimize the electron flow to N-oxides in the presence of O_2 will be energetically favourable. A minimum expression of denitrifying enzymes may be necessary, however, for survival during rapid transition from oxic to anoxic conditions (Højberg *et al*., 1997; Mazoch *et al*., 2003). Coordinated expression and activity of the different reductases involved in denitrification are necessary to avoid toxic concentrations of nitrite and NO, although moderate accumulations may be an advantage for the denitrifying bacteria by inhibiting other organisms (Choi *et al*., 2006).

However, the nature of the toxicity of the intermediates in denitrification is not simple. The toxicity of nitrite is contingent, as it appears to be a result of reactive products such as N_2O_3, NO and nitrosation products

Table 1. Overview of the initial concentrations of nitrate (NA), nitrite (NI) and O_2 in cultures of *A. tumefaciens*.

		Initial O_2 concentration (% v/v)		
		~0	1	7
Initial concentration (mM) of NO_3^- (NA) or NO_2^- (NI)	0.2	($0.2_{NA/NI}$, 0_{O2})	($0.2_{NA/NI}$, 1_{O2})	($0.2_{NA/NI}$, 7_{O2})
	0[a]	(B, 0_{O2})	(B, 1_{O2})	(B, 7_{O2})
	1	($1_{NA/NI}$, 0_{O2})	($1_{NA/NI}$, 1_{O2})	($1_{NA/NI}$, 7_{O2})
	2	($2_{NA/NI}$, 0_{O2})	($2_{NA/NI}$, 1_{O2})	($2_{NA/NI}$, 7_{O2})

KNO_3 (NA) or KNO_2 (NI) was added to serum flasks (120 ml) containing 50 ml of basal Sistrom's medium (B) to final concentrations of 0.2, 1 and 2 mM. The oxygen levels were adjusted to initial concentrations of approximately 0%, 1% and 7%.
a. Basal Sistrom's medium contains approximately 17 μM NO_3^-.

(Nedospasov *et al*., 2000; Yamasaki, 2000), although the nitrite molecule itself has been claimed to affect the membrane as well as specific enzyme functions (Stein and Arp, 1998; Jensen, 2003; Titov and Petrenk, 2003). The toxicity of NO is a result of its reactivity with transition metal proteins and O_2 as well as its ability to form adducts with amines and thiols (Zumft, 1997).

The regulation of denitrification has potentially global consequences as it can have a major impact on NO and nitrous oxide release to the atmosphere (Otte *et al*., 1996). Gaseous emissions from soils are controlled by soil physical conditions such as gas diffusion and the variable balance between the production and reduction rates of the NO and nitrous oxide by the denitrifying bacteria (Conrad, 1996). A recurring observation is a sequentially transient accumulation of the denitrification intermediates nitrite, NO and nitrous oxide during anoxic spells (Bakken and Dörsch, 2007), which is ascribed to sequential expression of genes, as observed in cultured denitrifiers during transition from oxic to anoxic conditions (Philippot *et al*., 2001). This implies that emissions of NOx from soil are controlled by the regulated expression of the four reductases, which vary between species and with environmental conditions (Cheneby *et al*., 2004; Choi *et al*., 2006). Biogeochemical modelling of NOx emissions from soils emulate the sequential expression of the reductases in various ways, and are not particularly successful in predicting the spatial and temporal variability (Bakken and Dörsch, 2007). To explore the potential role of the regulatory systems in controlling the NOx emissions, we need refined phenotypic data on the denitrification response under realistic conditions.

The present experiments were done with *Agrobacterium tumefaciens*, which belongs to the α-group of proteobacteria. It exists in free living form in soil as well as in association with plants. Analyses of denitrifying bacterial communities have demonstrated an enrichment of *Agrobacterium*-related species in the rhizosphere (Cheneby *et al*., 2004). *Agrobacterium tumefaciens* is a partial denitrifier as it lacks the genes encoding nitrous oxide reductase (Baek and Shapleigh, 2005; Rodionov *et al*., 2005). Consequently, nitrous oxide is the final product of denitrification. The experiments discussed here were designed to investigate the regulatory response of its denitrification apparatus to differing nitrite and nitrate concentrations during the transition from aerobic to anaerobic respiration. A feature of the experimental design was to allow the cultures to reach different cell densities prior to the transition to an anoxic state. This will affect the speed of transition from aerobic to anaerobic respiration as the sample with more cells will consume O_2 more quickly. Our hypothesis was that the concentrations of N-oxides, in particular nitrite and NO, would affect the expression of denitrification genes. Furthermore, we hypothesized that a rapid transition to anoxic conditions might complicate the coordinated expression of the denitrification. We monitored all relevant gases (CO_2, O_2, NO and nitrous oxide), and measured nitrite concentrations as well as transcription of *nirK* and *norB* relative to 16S rRNA levels in selected treatments. The results demonstrate that nitrite induced denitrification at much higher oxygen concentrations than when only nitrate was present. Unbalanced transitions from aerobic to anaerobic respiration occurred, particularly at high cell densities, seen as NO accumulation out of control and impeded nitrous oxide production. Several conspicuous response patterns were observed, probably reflecting a complex regulatory network of denitrification.

Results

O_2 respiration

Agrobacterium tumefaciens was inoculated into flasks containing four levels of nitrate or nitrite (0, 0.2, 1 or 2 mM) and initial O_2 concentrations of 0, 1 or 7% v/v. This yielded a total of 12 different treatments (Table 1). Cells grown under oxic conditions were used to inoculate the flasks. Preliminary experiments including three replicates of each treatment demonstrated a high level of reproducibility between parallels of all the experiments with the exception of high O_2 and high nitrite (data not shown). The reproducibility of treatments with modest additions is evident in the comparison of the NO peaks produced in

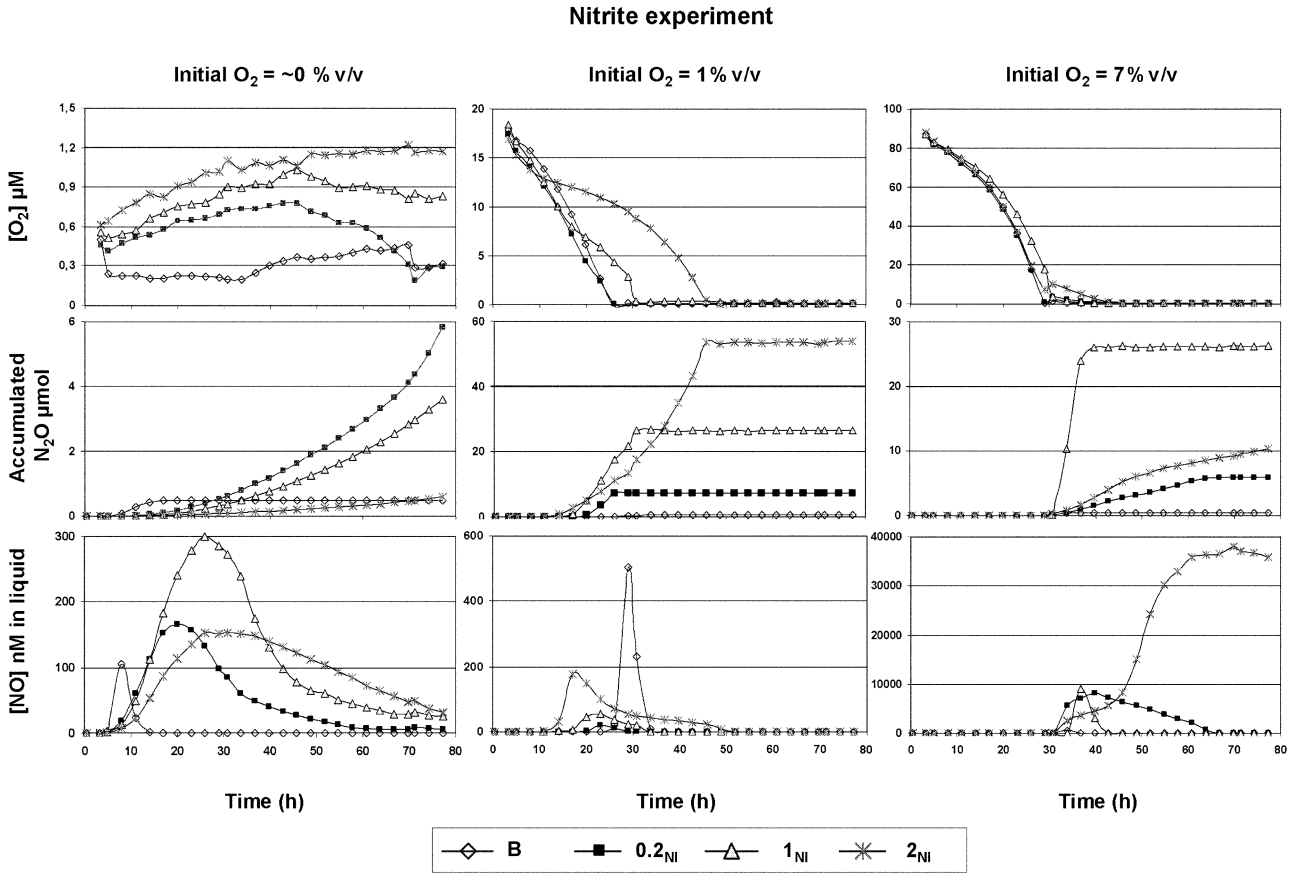

Fig. 1. General overview of gas levels in the nitrite treatments. The concentrations of O_2 (µM in bulk liquid) nitrous oxide (µmol per flask, corrected for dilution by sampling) and NO (nM in liquid) for the three initial O_2 levels are given vertically. The different nitrite concentration treatments are indicated with four curves in each graph: B, basal Sistrom's medium containing 17 µM nitrate; 0.2_{NI}, basal Sistrom's medium + 0.2 mM KNO_2; 1_{NI}, basal Sistrom's + 1 mM KNO_2; 2_{NI}, basal Sistrom's medium + 2 mM KNO_2.

the ($B,0_{O2}$) and the ($B,1_{O2}$) treatments (Figs 1 and 2). The differences in the treatments with higher nitrogen oxide additions, in particular nitrite, likely reflect the stress associated with NO accumulation, which is discussed further below.

The cell density increased during the oxic phase in proportion to the amount of oxygen consumed. The initial cell density was 5×10^7 ml^{-1} and the final cell densities for the three initial oxygen levels were 8.5, 43 and 250×10^7 ml^{-1}. As expected, the oxygen consumption rates during the oxic phase increased linearly with the amount of oxygen consumed as a result of the increase in cell number. As a result, the maximum oxygen consumption rates, observed just prior to oxygen depletion, were six to seven times higher in the 7% treatment compared with the 1% treatment.

The concentrations of O_2, nitrous oxide and NO throughout the incubations of the nitrite treatments are shown in Fig. 1, and the nitrate treatments in Fig. 2. In a balanced transition from aerobic to anaerobic respiration, it would be expected that oxygen consumption would increase as a function of cell density until the point at which nitrogen oxide reduction begins, and that there would only be limited accumulation of nitrogen oxide intermediates. This pattern only occurred in the nitrate treatments without added O_2 (Fig. 2). In nearly all of the other treatments, the gas measurements indicated the transition from aerobic to anaerobic respiration was impaired. Moreover, there were differences in denitrification patterns for cells grown with nitrate compared with nitrite. The presence of nitrite interfered with the O_2 consumption in ways which were clearly dependent on the nitrite and O_2 concentrations, but apparently also on NO production and concentration.

In the treatments not supplemented with O_2 after helium flushing, which had initial O_2 concentration of about 0.5 µM, O_2 consumption seemed to be blocked by nitrite from the very beginning of the incubation. This resulted in a gradual increase in O_2 concentration as a result of the addition of O_2 at each sampling (see *Experimental procedures*). The blocking of O_2 consumption occurred before any significant amounts of NO were detected.

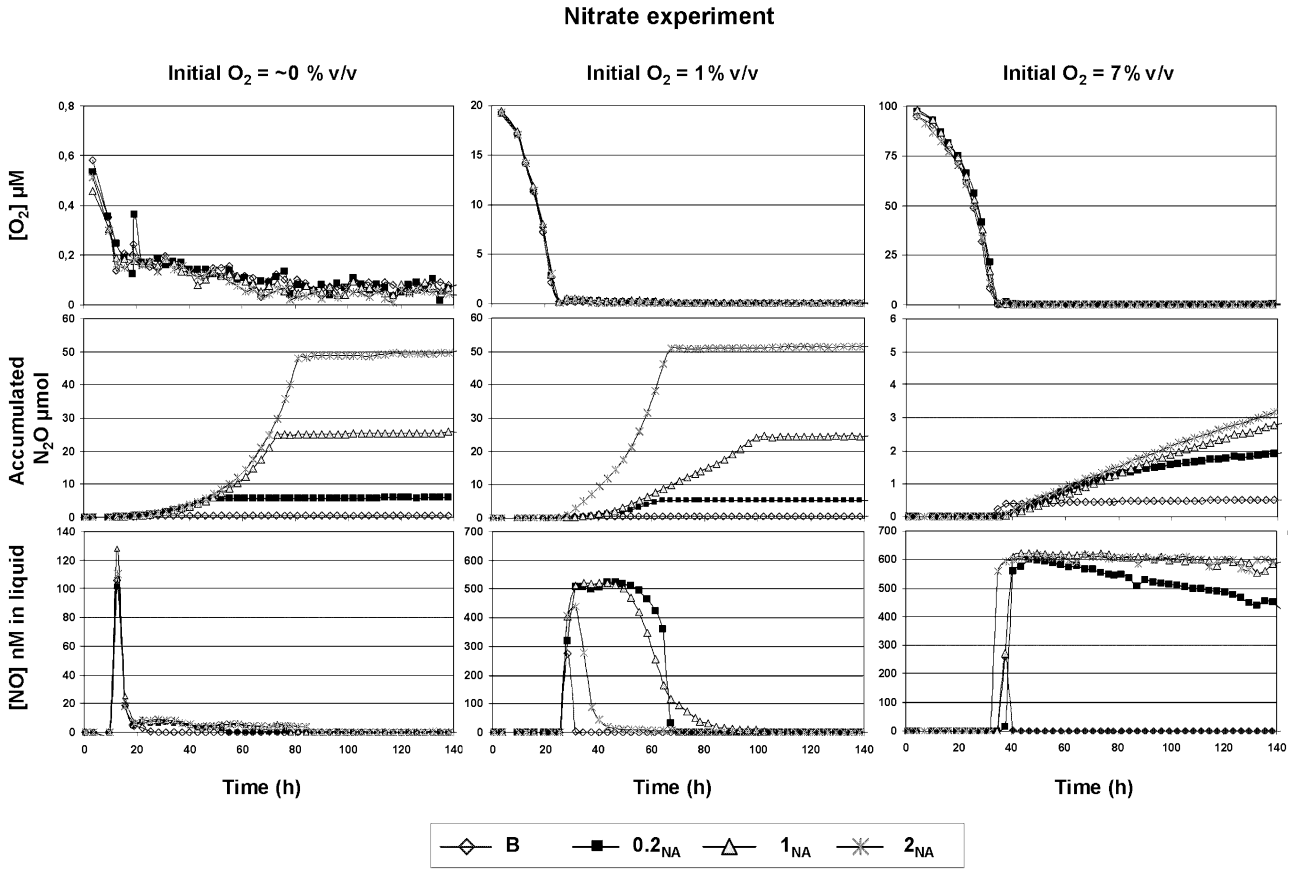

Fig. 2. General overview of gas levels in the nitrate treatments (see above). As in Fig. 1, the concentrations of O_2, nitrous oxide and NO for the three initial O_2 levels are given vertically. Nitrate treatments are indicated with four curves in each graph: B, basal Sistrom's medium containing 17 µM nitrate; 0.2_{NA}, basal Sistrom's medium + 0.2 mM KNO_3; 1_{NA}, basal Sistrom's + 1 mM KNO_3; 2_{NA}, basal Sistrom's medium + 2 mM KNO_3.

In the treatments with 1% v/v initial O_2 concentration (Fig. 1, middle panel), O_2 consumption during the first 10 h of incubation was the same in all the samples, indicating that nitrite was not inhibiting aerobic respiration. However, after this, there was a clear nitrite concentration-dependent transient retardation of the O_2 consumption, which coincided closely with the transient accumulation of NO (seen more in detail in Fig. 3). In the treatments with 7% v/v initial O_2 (Fig. 1, right panel), a retardation of O_2 consumption was observed only in the 2 mM nitrite treatment.

In contrast to the nitrite treatments, the addition of nitrate, regardless of the concentration, had no observable effect on the O_2 consumption (Fig. 2). It is noteworthy that in all the nitrate treatments, the onset of significant NO accumulation did not occur before O_2 was nearly depleted.

Nitrogen oxide respiration

Microoxic conditions. As mentioned above, the microoxic treatments with nitrate were the only samples that exhibited a respiratory transition consistent with a smooth switch from aerobic to anaerobic respiration. In the analogous treatments containing nitrite (Fig. 1, left panel), there was an inverse relationship between the nitrite concentration and the levels of nitrous oxide produced over the course of the incubation. These cultures showed a transient NO accumulation which lasted much longer than NO pulses in most other nitrite treatments. Thus, in the samples not supplemented with O_2, the presence of nitrite caused a severe restriction of not only O_2 consumption but also of nitrite and NO reduction. The degree of inhibition was proportional to the nitrite concentration.

The inhibitory effect of nitrite treatments on cellular metabolism was further confirmed by the CO_2 measurements (not shown). At the end of the incubations, the accumulated CO_2 in the nitrate treatments not supplemented with O_2 were 6, 18, 63 and 110 µmol CO_2 per flask in the 0, 0.2, 1 and 2 mM nitrate treatments respectively. In comparison, the final accumulated CO_2 concentrations in the corresponding nitrite treatments were 4, 7, 4 and 1 µmol CO_2 per flask respectively. Thus, the metabolic activity of the cells as evidenced by CO_2 production

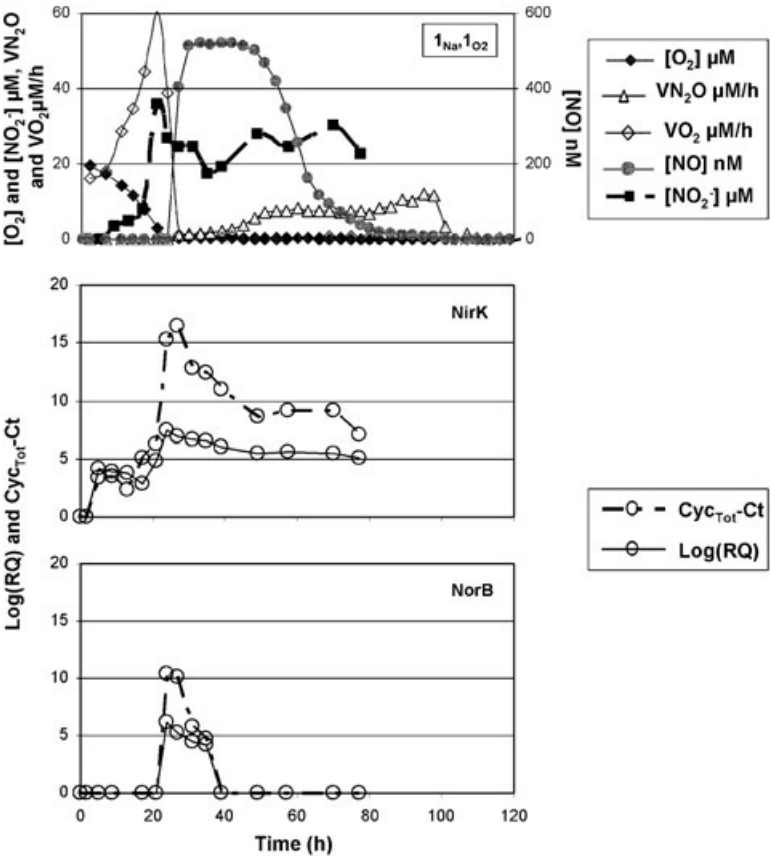

Fig. 3. Rates, gas levels and expression of *nirK* and *norB* in treatment (1_{NA}, 1_{O2}). The rates and gas levels are given in the upper panel while *nirK* and *norB* expression relative to 16S as well as the absolute expression of *nirK*/*norB* as given by Cyc_{tot}-Ct are shown in the lower panels.

increased with increasing nitrate concentrations, whereas the higher initial concentrations of nitrite inhibited CO_2 production.

1% O_2 treatments. Overlays of the various progress curves from the 1_{Na}, 1_{O2} treatment (Fig. 3, top panel) permits a determination of the sequence of events leading to the production of N_2O under these conditions. Nitrate reductase activity, inferred by the production of nitrite, was detected before the available O_2 was consumed. Nitrite was initially detected after 8 h and reached a maximum concentration of about 35 µM immediately before NO accumulation commenced. The concentration of O_2 at the onset of nitrite production was approximately 15–18 µM. As the processes of denitrification continued, the level of nitrite was kept at concentrations between approximately 20–30 µM.

Figure 4, upper panel allows a closer inspection of the kinetics of O_2 versus nitrite reduction in the (1_{NI},1_{O2}) treatment. O_2 consumption dominated during the first 18 h of growth although there was a small production of NO during this time. This was followed by a period where there was an obvious but transient downshift in O_2 consumption, which coincided with a rapid increase in nitrite reduction to NO and further reduction of NO to nitrous oxide. A subsequent increase in O_2 consumption coincided with the reduction of the NO concentration. This apparent transient downshift in O_2 consumption in response to the onset of rapid nitrite reduction was also evident in the other 1% O_2 treatments containing nitrite (Fig. 1).

Analysis of the results for the 1% initial O_2 concentration also allowed a precise determination of the oxygen concentration at which NO and nitrous oxide were first produced (Table 2). In the absence of nitrite, NO and nitrous oxide were first detected at $\leq$ 0.61 µM O_2. In the presence of 0.2 mM nitrite, NO and nitrous oxide were first detected at 12.6 and 7.9 µM O_2 respectively; and these critical oxygen concentrations increased with increasing nitrite concentrations. Another conspicuous effect of nitrite is that it seems to increase the time lag between detection of NO and that of nitrous oxide (last column, Table 2). Inspection of the data for the 7% oxygen treatment shows the same response pattern, but the estimates of critical O_2 concentrations were difficult to obtain because of rapidity of the transition to anoxic conditions in these treatments relative to the sampling frequency.

7% O_2 treatments. The nitrate treatments all gave evidence of having significant difficulty in switching to anaerobic respiration. None of the treatments converted all of the added nitrate to N_2O. The rate of accumulation of

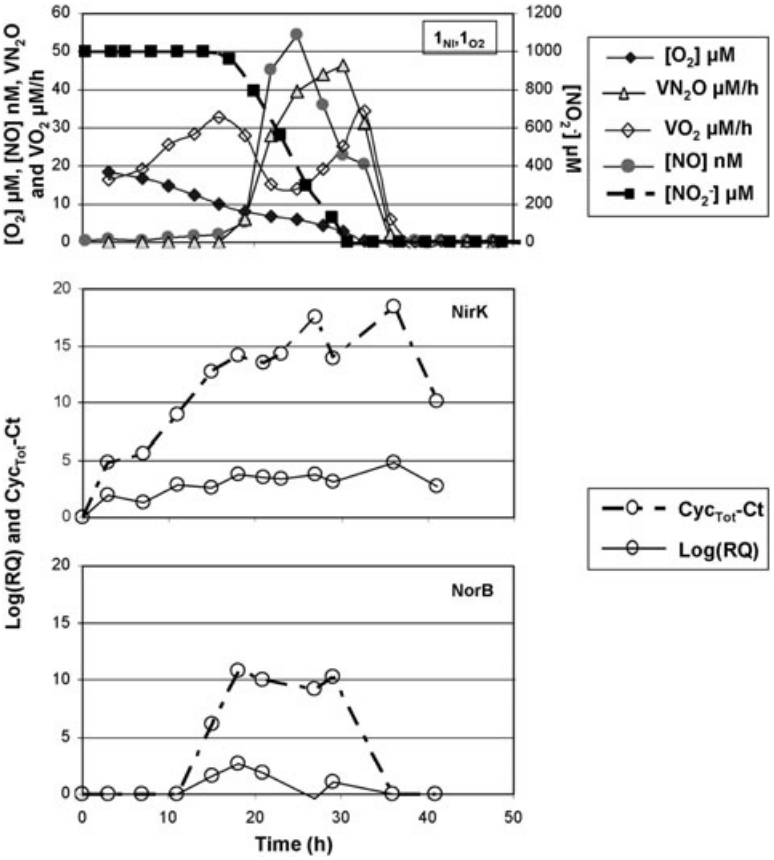

Fig. 4. Rates, gas levels and expression of *nirK* and *norB* in treatment (1_{NI},1_{O2}). The rates and gas levels are given in the upper panel while *nirK* and *norB* expression relative to 16S as well as the absolute expression of *nirK* and *norB* as given by Cyc_{tot}-Ct are shown in the lower panels.

N_2O was slower than in the samples without added O_2, despite having much higher cell numbers. As with other nitrate treatments, a burst of NO production occurred as the O_2 levels approached zero. However, in samples with added nitrate, the NO levels were not significantly reduced as the incubation progressed.

Table 2. Oxygen concentrations in the medium (µM) at the time of the first detection of NO and nitrous oxide, and time lag (hours) between the first detection of NO and that of nitrous oxide (hours).

	µM O_2 at first detection of		
	NO	N_2O	Δt^a
mM NO_2^-			
0[b]	0.52	0.33	3
0.2	12.6	7.9	6
1	15.2	10.7	6
2	17.2	14.2	6
mM NO_3^-			
0[b]	0.12	0.47	–3
0.2	0.41	0.41	0
1	0.46	0.61	–3
2	0.38	0.54	–3

Data for the treatment with 1% initial oxygen.
a. Time lag between detectable production of NO and nitrous oxide. Negative values imply that nitrous oxide was detected before NO. Resolution is 3 h, i.e. the time between gas sampling.
b. 17 µM NO_3 in basal Sistrom's medium, no nitrate or nitrite added.

The pattern of gas production in the nitrite treatments was significantly different from in the nitrate treatments (Fig. 1). In both the 0.2 mM and 2 mM treatments, N_2O production was very slow. In the 1 mM sample, the nitrite was quickly reduced to N_2O. In each treatment, NO accumulated to very high levels. Both the 0.2 mM and 1 mM treatments had maximal NO concentrations of about 10 µM, but the NO levels persisted in the 0.2 mM treatment. NO levels in the 2 mM sample reached 40 µM, indicating a severe imbalance in Nir and Nor activities.

Real-time polymerase chain reaction

The expression of *nirK* and *norB* was assessed in both the (1_{NA},1_{O2}) and the (1_{NI},1_{O2}) treatments. Figures 3 and 4 show the expression of *nirK* and *norB* (lower panels) as well as the O_2 concentration and O_2 consumption rates in these treatments and the concentration of the different denitrification intermediates (upper panels). Gene expression is presented both relative to 16S rRNA levels [log(RQ), estimated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001)] and as absolute levels of *nirK* and *norB* expressed by the total number of polymerase chain reaction (PCR) cycles 40-Ct (Cyc_{Tot}-Ct). No expression of *nirK* or *norB* was detected in the cells at the time point immediately following inoculation (time 0). In the culture treated

with nitrite, there was an almost immediate increase in the *nirK* mRNA concentration. *nirK* expression then increased gradually during the first 20 h of incubation and levelled out after this. Nitrite concentrations began to decrease after about 15 h and approached zero after approximately 30 h. While the consumption of nitrite did result in a decrease in *nirK* expression as shown by 40-Ct, the expression did not decrease to the levels found early in the incubation. Expression of *norB* showed a quite different pattern. Its expression stayed below detectable levels during the first 11 h of incubation and was first detected after 15 h, concomitant with an increase in the NO level. Between 30 and 35 h, expression of *norB* unexpectedly decreased to below detectable levels. The production rate of N_2O increased between 16 and 18 h and reached a peak at approximately 31 h, after which it decreased rapidly, coincident with the decrease in *norB* expression.

In the nitrate treatment, a low level of *nirK* expression was detected 3 h after inoculation (Fig. 3), and stayed at this low level during the first 20 h of incubation. After this, a rapid increase in *nirK* expression was seen concomitant with a depletion of O_2 and a rapid increase in nitrite concentration. At about the same time, there was a peak in the nitrite level immediately followed by the rapid accumulation of NO. *norB* mRNA was detected after 24 h, coinciding with the increase in NO concentration and immediately followed by N_2O production. As seen with the nitrite treatments, *norB* expression decreased to below detectable levels at about 39 h. This decrease occurred in spite of the presence of large amounts of NO in the surrounding medium. NO levels did eventually decrease but this did not happen until after 50 h of incubation.

Discussion

The main focus of this work was to elucidate the impact of nitrate, nitrite and O_2 concentrations on *A. tumefaciens* as it undergoes a transition from aerobic to anaerobic respiration. The gas analyses provided insight into the nature of the factors controlling transition. One of the important observations was that it was not always easy for the cells to make a successful shift between respiratory pathways. In some treatments, aerobic respiration was inhibited, making the transition more difficult. This was particularly true in the nitrite treatments (Fig. 1). In contrast, nitrate had no obvious effect on O_2 consumption.

The high initial concentrations of nitrite in the nitrite treatments had a clear impact on O_2 consumption (Fig. 1). There was, however, no immediate inhibition of O_2 consumption upon inoculation (Fig. 4), and therefore a direct effect of nitrite is unlikely. The inhibition is instead likely to be caused by some activity not found in the inoculum. One candidate for this is nitrite reductase, which generates NO leading to inhibition of the terminal oxidases (Carr and Ferguson, 1990). In treatments supplemented with O_2, those with higher initial nitrite levels begin NO production earlier, resulting in an earlier decrease in O_2 consumption (Fig. 1).

Previous work has suggested that NO is an important signal for the expression of *nirK* and *nor* (Baek and Shapleigh, 2005). The results presented here demonstrate that NO is required for maximal expression of *nirK* and *nor*. However, it is obvious that *nirK* and *nor* are not under identical transcriptional control. In the (1_{NI},1_{O2}) treatment *nirK* expression is detectable in the first sample after inoculation (Fig. 4). In the nitrite treatments, there may be some NO production from the dismutation of HNO_2 even at near neutral pH, which could explain the early onset of *nirK* expression. This nitrite-dependent activation of NnrR has been observed previously and was ascribed to chemical generation of NO (Tosques *et al*., 1997). If this was the case, then *norB* expression should follow a similar pattern, but it does not. There is a longer delay between inoculation and detectable *norB* expression than with *nirK*. In the nitrate treatments, *nirK* expression also occurs before detectable production of NO (Fig. 3). As with the nitrite treatment, *norB* expression in the nitrate treatments does not occur until NO is detected. Recent work has demonstrated that *nirK* expression requires a two-component system that is not directly required for the expression of *nor* (Baek *et al*., 2007). This two-component system may be responsible for the expression of *nirK* observed soon after inoculation. The observation that the *norB* mRNA level dropped below the detection limit, while NO continued to be present at high concentrations (Fig. 3) is more difficult to explain. Current evidence indicates that NnrR is the only transcription factor required for *nor* expression (Baek *et al*., 2007). This suggests that *nor* expression may require additional factors or that the regulation of NnrR activity is more complicated than currently predicted. It is also important to note that nitrate reductase activity is first detected at significantly higher levels of O_2 than nitrite reductase activity (Fig. 3). The pattern of reductase activity observed here results in a sequential production of denitrification intermediates as the O_2 concentration decreases.

One of the more unexpected differences among the treatments was the variation in NO concentrations generated during the incubation. The concentration of O_2 and presence of nitrite have been previously reported to influence the level of NO accumulation in denitrifiers (Goretski and Hollocher, 1990; Goretski *et al*., 1990). The present results show a more nuanced picture of NO accumulation, being dependent not only on the electron acceptor concentrations, but also on the speed of transition from aerobic to anaerobic respiration which is directly controlled by the cell density. In the high O_2 treatments containing nitrite (Fig. 1), NO concentrations reached $\geq$ 10 μM. These levels of NO

are about three orders of magnitude higher than the steady-state level found for *Pseudomonas stutzeri* (Zafiriou *et al*., 1989; Goretski *et al*., 1990). The high NO concentrations seemed to depress metabolic activity as evidenced by a reduced production of N_2O and CO_2. Later experiments with a range of initial oxygen concentrations and nitrite concentrations have confirmed that high NO concentrations are reached in cultures with nitrite if the initial oxygen concentrations are $\geq$ 7%. Furthermore, the ability of these cultures to respire oxygen (after purging with He and injection of O_2) showed that all cultures, which had experienced $\geq$ 3 μM NO, had lower respiration rates (30–90%) than they had during the initial oxic phase. In contrast, the respiration rates invariably increased through the first, second and third oxic phases for cultures in which NO levels had not exceeded 0.2 μM (Lars Bakken and Peter Dörsch, unpublished).

In the nitrate treatments with high O_2, it is interesting that once NO production commences, it accumulates to 500–600 nM, and this correlates with an almost complete arrest of cellular activity (Fig. 2). In comparison, the (1_{NI},7_{O2}) sample accumulated 10 μM NO, but this was consumed before the end of the incubation. This indicates that NO concentrations $>$ 0.5 μM do not permanently disable respiration. Therefore, it seems likely that there were different processes leading to respiratory inhibition in the nitrate and nitrite treatments with high O_2. In the samples with nitrate, it is possible that the rapid O_2 respiratory rate resulted in consumption of available terminal oxidants before the shift to anaerobic respiration occurred. This inability to respire would mean that the cells would not have the energy required to produce proteins required to cope with microoxic conditions. It has previously been reported that expression of the denitrification genes requires O_2 (Højberg *et al*., 1997; Mazoch *et al*., 2003). This difficulty in transition does not occur in the nitrite treatments. A possible explanation is that the NO produced from Nir and from dismutation of nitrite slows O_2 respiration, particularly at low O_2 levels, effectively decelerating the aerobic–anaerobic transition and allowing the expression of the denitrification proteins.

While an inability to shift respiratory modes may explain the observed response in the nitrate treatments with 7% O_2, it does not explain why NO concentrations remain stable. NO likely accumulates as the order of gene expression previously discussed causes *norB* expression to occur after nitrate and nitrite reductase expression causing an imbalance in the cells ability to produce nitrite and reduce NO. However, something must prevent further accumulation once the NO concentrations reach 500–600 nM. One possibility is that nitrate reductase activity is inhibited, which would then limit Nir turnover. Nitrate reductase contains [Fe-S] centres and, in other proteins, these prosthetic groups are known to be affected by NO (Wink and Mitchell, 1998). Inhibition of nitrate reductase would also explain why the nitrite treatments could reach NO levels much higher than 600 nM as they do not require nitrate reductase for nitrite production.

If O_2 is required for the production of denitrification proteins, how do the cells in the nitrate treatments unsupplemented with O_2 express denitrification proteins with no difficulty? While these samples are low in O_2, they are not anoxic. Measurements of O_2 concentrations show that initially there was 600 nM O_2 in the medium, and each injection introduced 20 nmol of O_2 per flask. The cultures quickly reduced the oxygen concentration to about 200 nM, at which time there was a spike of NO followed by production of N_2O. As the cell numbers were low, particularly in comparison with the numbers in the 7% O_2 samples, each cell likely had sufficient O_2 to allow for a stable aerobic–anaerobic transition. It is not unreasonable to suggest that O_2 respiration took place even at these low levels as *A. tumefaciens* contains a cbb_3 oxidase, and these oxidases are known to have a K_m for O_2 in the low nM range (Preisig *et al*., 1996). Additional support for the importance of this O_2 respiration in the nitrate treatments unsupplemented with O_2 is provided by comparison with the equivalent nitrite treatments. The O_2 concentrations in the samples either were steady or increased, indicating that respiration was inhibited, as seen in some of the other nitrite treatments. Unable to respire, the cells should have difficulty expressing the denitrification proteins. As predicted, the limited production of N_2O from all the samples shows that induction of the denitrification proteins under these conditions was difficult.

One other interesting pattern was that there was always a spike of NO that preceded the production of N_2O in those treatments where the aerobic–anaerobic transition was induced with little or no delay. This spike of NO early in the transition to anaerobic respiration has also been observed in *Neisseria meningitidis* (Anjum *et al*., 2002). The obligatory nature of this NO pulse suggests that it is a critical part of the denitrification signalling pathway. The source of the NO is most likely nitrite reductase consistent with its relatively early expression (Figs 3 and 4). Nir activity was probably limited before the pulse occurred by a combination of O_2 respiration, diverting electrons away from nitrite reductase and limited nitrite levels. Once nitrite reductase became active, NO was produced and NnrR was activated, leading to Nor expression and NO consumption. The NO pulse also likely inhibited O_2 respiration forcing electrons down the denitrification pathway, facilitating the aerobic–anaerobic transition.

Several groups have worked on the mRNA levels of denitrification genes under different conditions. Results suggest that there are large differences between species. *Paracoccus denitrificans* has been found to synthesize only very low levels of mRNAs for the denitrification en-

zymes under aerobic conditions (Baumann *et al*., 1996), while *Pseudomonas aeruginosa* has been found to synthesize significant amounts of the gene encoding nitrite reductase in the presence of O_2 (Zennaro *et al*., 1993). *Agrobacterium tumefaciens* seems to resemble *P. denitrificans* in that *nirK* mRNA is undetectable with present techniques in the absence of nitrite or nitrate under aerobic conditions. A basal level of *nirK* transcription is likely though, as NO is thought to be necessary for the induction of NnrR (Rodionov *et al*., 2005). However, as indicated earlier, when looking at the expression profiles in the two treatments, nitrite seems to be a dominating regulator of initiation of *nirK*. This seems particularly clear in the nitrate-treated culture (Fig. 3), as no NO was detected during the first 24 h and the onset of *nirK* mRNA production appeared to be perfectly synchronized with the appearance of nitrite from nitrate reductase activity.

A number of the housekeeping genes previously chosen as internal references in real-time PCR have been shown to be expressed at varying levels depending on the growth stage of the bacteria (Vandecasteele *et al*., 2001; Eleaume and Jabbouri, 2004). In this work, 16S rRNA was chosen as an endogenous control. As expected, it was found to be expressed at a higher level than *nirK* in *A. tumefaciens*. However, the expression level appeared to be far from stable. In order to assess the suitability of 16S rRNA as an internal standard, the same concentration of cDNA was added to each PCR reaction. An appropriate standard would in this case yield relative expression profiles [Log(RQ)] highly similar to those depicting absolute expression (Cyc_{Tot}-Ct). Our results show that 16S rRNA is unsuitable as an endogenous standard in this type of experiment, and a method using standard curves for absolute quantification will be developed for future experiments.

The patterns observed in these experiments are complex and the explanations provided here are almost certainly incomplete. The consistent dependency of NO accumulation on cell density and consequently on the rate of O_2 depletion, combined with its non-linear response to nitrite concentrations, suggest that one or several destabilizing feed forward inhibition/induction patterns are involved, such as those proposed by Kucera (1992) and Kunak and colleagues (2004). A more complete understanding of the response patterns observed can probably be achieved by mathematical modelling of the reaction rates and kinetics of gene expression, which are ongoing.

Experimental procedures

Bacterial strain and medium

Agrobacterium tumefaciens C58 (ATCC 33970) was grown in Sistrom's medium (Sistrom, 1962; Lueking *et al*., 1978), with an initial pH of 7.0. The growth medium contained (g l^{-1}): K_2HPO_4 3.48 g, NH_4Cl 0.195 g, succinic acid 4.00 g, L-glutamic acid 0.10 g, L-aspartic acid 0.04 g, NaCl 0.50 g, nitrolotriacetic acid 0.20 g, $MgSO_4$ * $7H_2O$ 0.30 g, $CaCl_2$ * $7H_2O$ 0.015 g and $FeSO_2$ * $7H_2O$ 0.007 g. In addition, trace elements and vitamins were added in g l^{-1}: EDTA (triplex 3) 0.001765 g, $ZnSO_4$ * $7H_2O$ 0.01095 g, $FeSO_4$ * $7H_2O$ 0.005 g, $MnSO_4$ * H_2O 0.00154 g, $CuSO_4$ * $5H_2O$ 0.000392 g, $Co(NO3)_2$ * $6H_2O$ 0.000248 g, H_3BO_3 0.000114 g, nicotinic acid 0.0010 g, thiamine HCl 0.0005 g, biotin 0.000010 g. pH was brought to 7.0 with 10 M KOH and the medium was autoclaved for sterility.

Experimental design and growth conditions

Two separate experiments were performed in which the growth medium was supplemented with four different concentrations (0, 0.2, 1 and 2 mM) of KNO_2 and KNO_3 respectively (Table 1). The medium was distributed into 120 ml serum flasks; 50 ml in each. One triangular magnetic stirring bar (Cowie 25 × 8 mm, VWR International) was added to each unit before the flasks were autoclaved and sealed tightly with rubber septa and aluminium caps. A semi-automated system was used to remove the natural atmosphere in each bottle and replace it with Helium (He) by six cycles of evacuation and He-filling. During this process, the medium in each flask was stirred at 950 r.p.m. to ensure proper gas exchange between liquid and gas phases. The excess pressure from the final He-filling was relieved using a water-filled syringe where the piston was removed. A gas-syringe was used to remove different volumes of He from head spaces and replace it with the same volumes of pure O_2 (0%, 1% and 7% v/v), prior to inoculation of the flasks. The different nitrate/nitrite – O_2 treatments are described in Table 1.

The incubation was done on a robotized incubation system designed for measuring gas kinetics in cultures of denitrifying bacteria (Molstad *et al*., 2007). The system consists of a thermostatic water bath with positions for 15 serum bottles (120 ml) with magnetic stirring, and three positions for flasks without stirring (blanks with calibration gas). Automated gas sampling through the rubber septa is done by an autosampler and a peristaltic pump connected to the gas sampling loop of a Varian CP 4700 micro GC with a 10 m poraPLOT U and a 20 m molsieve 5A column (in parallel), each equipped with a thermal-conductivity detector. The vent line from the injection loop of the GC is coupled to a T piece with He flow (Sitaula *et al*., 1992), and the He flow which is carrying the sample gas overflow (from the vent line during sample pumping) is led into the inlet tube of a chemoluminescence NO_x analyser (Model 200A, Advanced Pollution Instrumentation, USA). After each injection, the peristaltic pump is reversed, returning the same gas volume (residual sample gas plus He from the T-piece) as that removed, thus sustaining approximately one atmosphere pressure. Each sampling involves a constant dilution of the head space with He (1.9 ml per injection), and minor leaks of air (60 nmol N_2 and 20 nmol O_2 per injection) which is measured (in blanks) and taken into account when calculating rates of gas production and consumption. A Python (http://www.python.org/) program controls the sampling system, triggered the GC, integrated the NO peaks and organized the data.

The head space concentration of each gas was used to estimate the gas concentrations in the liquid., assuming equi-

librium between head space and liquid phase (Molstad *et al.*, 2007). For oxygen, we found it necessary to take diffusion rates between head space and liquid phase into account, to achieve correct estimates of O_2 concentrations ($[O_2]$) in the liquid phase (see Molstad *et al.*, 2007 for details). The estimated $[O_2]$ is that of the bulk liquid, which is not necessarily equal to that at the cell surface of respiring cells. To assess the cell surface concentration of O_2, we used the equation for substrate flux towards spheres (Berg, 1993)

$$F = 4\pi D([O_2] - [O_2]_s)r \quad (1)$$

Where D is the diffusion coefficient for O_2 in water (2×10^{-5} cm^2 s^{-1}), $[O_2] = O_2$ concentration in bulk liquid (mol O_2 cm^{-3}), $[O_2]_s$ is O_2 concentration at cell surface, r is the cell radius (cm) and F is the O_2 flux towards the cell, i.e. respiration mol O_2 cell^{-1} s^{-1}.

Solving for Cs, we get:

$$[O_2]_s = [O_2] - F/4\pi Dr \quad (2)$$

Equation 2 was thus used to estimate $[O_2]_s$ by letting the estimated O_2 consumption divided by the number of cells estimate F. The calculation was based on a cell diameter 0.5×10^{-4} cm. The calculation is based on the assumption that the cells are fully dispersed, which was confirmed by microscopy. The error introduced by assuming spherical cells is marginal: the flux per area of cell surface for a rod-shaped cell with dimensions 1×2 is approximately 30% lower than that to a sphere with the same diameter. The results of these exercises demonstrated that the O_2 concentrations at the cell surface were never below 95% of that in the bulk liquid. The only concentrations reported are thus the bulk liquid concentrations, as these values were very close to what the single cell in the suspension is exposed to.

Preparation of inocula

Inocula of fully dispersed cells which had not been exposed to anoxic conditions was ensured by using overnight cultures grown aerobically with vigorous stirring (50 ml medium per flask, stirred at 950 r.m.p. with triangular magnetic stirring bar) at 15°C. The medium was 1/4 strength Sistrom's medium, to ensure low cell densities (hence lower risk for anoxic conditions and accumulation of toxic metabolites compared with full strength medium). The cell density of these late exponential cultures (microscopic counts) was approximately 5×10^9 cells ml^{-1}, of which 0.5 ml was injected per experimental flask (injected with syringe through the rubber septum), resulting in initial cell density of 5×10^7 cells ml^{-1}.

Real-time PCR

The expression of *nirK* and *norB* relative to 16S rRNA was analysed in treatments (1_{NA},1_{O2}) and (1_{NI},1_{O2}) using two-step reverse transcription real-time PCR. In order to facilitate sufficient yields from RNA extraction, flasks were subjected to the (1_{NA},1_{O2}) or the (1_{NI},1_{O2}) treatments and grown in parallel with the gas experiment and under the same conditions in a separate thermostatic water bath. Samples for real-time PCR were acquired regularly during the experiments by treating the entire liquid volumes of 50 ml with RNAprotect™ Bacteria Reagent from Qiagen. RNA extraction was performed using the RNeasy® mini-kit from Qiagen. To prevent any DNA contamination, 25 µl of each extract was treated with 0.5 µl RNase free DNase (119 U µl^{-1}) at 37°C for 3 h prior to cDNA synthesis. The cMaster® RT kit from Eppendorf and random hexamers from Applied Biosystems were used for cDNA synthesis and the resulting amounts of cDNA were measured in a Nanodrop system from Eppendorf. Real-time PCR was performed using the RealMasterMix Probe Rox from Eppendorf and custom TaqMan® Gene Expression Assay (Applied Biosystems) with FAM-labelled MGB probes (16S rRNA: forward primer: 16SRDNA-78F CAGCAGCCGCGGTAATAC; reverse primer: 16SRDNA-78R TGCTCCCCACGCTTTCG; MGB probe: 16SRDNA-78M2 FAM-CTTTACGCCCAGTAA TTC. NorB: forward primer: ATNORB-700F CGGTCTCGC CCTCTTTTCG; reverse primer: ATNORB-700R TGCGC CGATCCAGTAATAGTG; MGB probe: ATNORB-700M2 FAM CCGGTGCCGAGAATG. NirK: forward primer: NIRK_ AGROT-600F GTGCATCAGGACGACTACGT; reverse primer: NIRK_AGROT-600R TGCTGCAGCTCGTTGGTATC; MGB probe: NIRK_AGROT-600M1 FAM-ACCCTCATCAA CCCC) (Applied Biosystems). Reactions were run in a HT9700 and the data were analysed using the SDS 2.2.2 software, both from Applied Biosystems.

As indicated, both NirK and 16S rRNA were quantified by real-time PCR. Relative real-time PCR is not routinely used in microbiology, as finding a suitable endogenous control in bacterial systems has proven difficult. The three housekeeping genes *gyrA*, *gmk* and 16S are among the genes previously suggested as internal standards. Both the metabolic housekeeping genes *gyrA* and *gmk* were found to be inappropriate as internal standards over a range of growth stages in *Staphylococcus aureus*, while 16S rRNA gave the most reliable results (Eleaume and Jabbouri, 2004). Based on this, 16S rRNA was used as an internal standard to quantify *nirK* and *norB* expression. The purpose of the internal control is to normalize for differences in RNA amounts added to the reverse transcription reaction (Livak and Schmittgen, 2001). In order to assess the stability of 16S rRNA levels over time and under different conditions, the amount of added cDNA to each PCR reaction was kept constant for these experiments. The expression of *nirK* relative to 16S was assessed using the $2^{-\Delta\Delta Ct}$ method where $2^{-\Delta\Delta Ct}$ is the amount of target normalized to an endogenous control and relative to a calibrator (Livak and Schmittgen, 2001).

With each extraction of samples for real-time PCR, a sample was also extracted from the (1_{NA},1_{O2}) treatment and the concentration of nitrite determined. The nitrite levels were measured in accordance with ISO 6777-1984 (International Organisation for Standardization, 1984).

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