Requirement of Nitric Oxide for Induction of Genes Whose Products Are Involved in Nitric Oxide Metabolism in *Rhodobacter sphaeroides* 2.4.3*

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During denitrification, freely diffusible nitric oxide (NO) is generated for use as a terminal electron acceptor. NO is produced by nitrite reductase (Nir) and reduced to nitrous oxide by nitric oxide reductase (Nor). Using Nir and Nor-deficient mutants of *Rhodobacter sphaeroides* 2.4.3, we have shown that the endogenous production of NO or the addition of exogenous NO induces transcription of certain genes encoding Nir and Nor. A Nor-deficient strain was found to be capable of expressing wild type levels of nirK-lacZ and norB-lacZ fusions in medium unamended with nitrogen oxides. When this experiment is performed in the presence of hemoglobin, fusion expression is eliminated. NO and the NO-generator, sodium nitroprusside, can induce expression of both fusions in a strain lacking Nir and the consequent ability to produce NO. Sodium nitroprusside cannot induce expression of nirK-lacZ in a strain lacking the transcriptional activator NmrR (nitrite and nitric oxide reductase regulator). Addition of the cyclic nucleotides cAMP and 8-bromoguanosine-cGMP does not result in expression of either fusion. These results demonstrate that denitrifying bacteria produce NO as a signal molecule to activate expression of the genes encoding proteins required for NO metabolism.

Many bacteria have the capacity to use compounds other than oxygen as terminal electron acceptors during respiration. Nitrate is a commonly used alternative electron acceptor and can be reduced to either ammonia or gaseous N-oxides.1 The anaerobic respiration of nitrate to gaseous end products, principally dinitrogen, is referred to as denitrification. Nitric oxide (NO) is produced as an obligatory intermediate during denitrification (1). NO is generated by nitrite reductase (Nir), which catalyzes the one electron reduction of nitrite (2). Nitrite is generated by the two electron reduction of nitrate, the initial oxidation of which is catalyzed by the one electron reduction of nitrite (3). The NO produced by Nir is freely diffusible, as evidenced by the ability of extracellular hemoglobin (Hb) to trap NO produced during denitrification (4). Nitric oxide reductase (Nor) reduces NO to nitrous oxide. During denitrification the activity of Nor is critical since it maintains NO concentrations in the low nanomolar range (5). Possessing an unpaired electron, NO is very reactive, particularly with transition metals that are critical to many biological processes (6). If Nor did not efficiently reduce NO, NO would rapidly accumulate to toxic concentrations. Nitrous oxide is reduced to dinitrogen in the final step in denitrification. The reduction of each N-oxide is linked to energy conservation (7).

The expression of genes whose protein products are involved in denitrification requires both low oxygen and the presence of NO-oxides (1). How the cell recognizes these environmental signals is not well understood. In particular, it is not clear how the cell regulates those enzymes directly involved in NO metabolism to mitigate the accumulation of this toxic intermediate. However, transcriptional activators have been recently discovered in denitrifiers that only activate the genes encoding Nir and Nor (8, 9). Coupling the transcription and concomitant expression of Nir and Nor through a single transcriptional activator prevents the differential and potentially deleterious expression of Nir and Nor. It is not clear what signal or signals modulate the activity of the nir and nor transcriptional activator. One possibility is NO (10). It has been shown that inactivation of Nir reduces Nor expression (11). Studies from this laboratory on the expression of *nirK* in *Rhodobacter sphaeroides* 2.4.3 show that Nir activity is required for Nir expression.1 The requirement of Nir activity for the expression of both Nir and Nor suggests that a product of Nir, possibly NO, is required for expression of Nir and Nor. However, there has been no direct evidence for this.

NO is also generated by mammals where it has a multitude of functions (12). Mammals generate NO through the oxidation of arginine in contrast to the reductive mechanisms utilized by bacteria. In mammals, NO is involved in regulating such processes as vascular relaxation and neurotransmission primarily by activation of guanylyl cyclase (13, 14). Activation of guanylyl cyclase increases the level of cyclic GMP, which then interacts with a wide range of targets (15, 16). NO is also produced by activated macrophages as part of the cell-mediated immune response to cause cytostasis and cytotoxicity (17). NO may also interact directly with certain proteins to regulate their activity. Proteins whose activity appears to be directly modulated by NO include the iron-responsive protein (18), ribonucleotide reductase (19) and ferrochelatase (20).

Given the indirect evidence suggesting that NO production is required for expression of the genes encoding Nir and Nor in denitrifiers, as well as the established role of NO as modulator

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1 The abbreviations used are: N-oxide, nitrogen-oxide; NO, nitric oxide; Nir, nitrite reductase; Nor, nitric oxide reductase; NmrR, nitrite and nitric oxide reductase regulator; SNP, sodium nitroprusside; Hb, hemoglobin; MOPS, 3-(N-morpholino)propanesulfonic acid; DETA, diethylenetriamine nitric oxide adduct.

2 I. E. Tosques, A. V. Kwiatkowski, J. Shi, and J. P. Shapleigh, submitted for publication.
of biological activities in eukaryotes, we have carried out a series of experiments to determine if NO is directly involved in regulation of genes involved in denitrification. We utilized the denitrifier *R. sphaeroides* 2.4.3 for these experiments. *R. sphaeroides* 2.4.3 possesses a copper-containing Nir encoded by a single gene, *nirK*, and a two subunit Nor that is encoded by the *nor* operon. The *nor* operon includes *norC* and *norB*, which encode the structural subunits, and two other genes likely required for Nor assembly. Through monitoring the expression of reporter gene fusions in denitrification mutants under various conditions, we have provided evidence that clearly shows the role of NO in modulating *nirK* and *nor* operon expression.

**MATERIALS AND METHODS**

**Bacterial Strains, Plasmids, and Growth Conditions—** *R. sphaeroides* strain 2.4.3 (ATCC17025) is a denitrifying variant of *R. sphaeroides* (21). Strains 15.12 and 11.10 are 2.4.3 derivatives that are Stb and contain Tns inserts in *norB* and *nirK*, respectively. Tns insertion in either *nirK* and *norB* eliminates, respectively, all detectable Nir and Nor activity. *N. tabacum* strain 31215 is a 2.4.3 derivative in which the *nor* operon encoding NnrR has been insertionally inactivated (8). The nature of NnrR is described below. Both the *nirK-lacZ* and *norB-lacZ* operon fusions were generated by constructing an operon fusion between the gene of interest and the *lacZ* Km carriagte from pKOK6 (22). All fusions are present in 2.4.3 in trans on the broad-host range vector pRK415 (23).

*R. sphaeroides* was cultured in MOPS-Sistrom’s medium at 30°C. MOPS-Sistrom’s medium was made by reducing the concentration of KPO₄ buffer in Sistrom’s medium (24) from 20 mM to 10 mM and substituting 20 mM MOPS as buffer to reduce potential N-oxide contamination. Nitrate, when necessary, was added to a final concentration of 12 mM. Antibiotics were added to *R. sphaeroides* cultures at the following concentrations: tetracycline 1 μg/mL, trimethoprim 30 μg/mL, streptomycin and spectinomycin, 50 μg/mL, and kanamycin 25 μg/mL. Microaerobic cultures were grown in 100 mL of MOPS-Sistrom’s medium in 250 mL flasks capped with rubber stoppers after inoculation to prevent oxygen exchange. Flasks were typically inoculated with 1.0 mL of fresh stock and incubated overnight before initiating sampling.

**Enzyme Assays—** After permeabilizing cells, β-galactosidase activities were determined as described previously by Miller (see Maniatis et al. (25)). β-Galactosidase levels represent data from at least two independent measurements. Samples from microaerobic cultures were assayed as described by Tosques et al.²

The presence of SNP, discussed below, in β-galactosidase assays caused a purple color after termination with sodium carbonate. Since this color quickly faded, all β-galactosidase assays from samples in which SNP had been added were allowed to sit for at least 20 min before taking absorbance readings.

NO, NO-producing Compounds and Cyclic Nucleotide Addition Experiments—Saturated NO solutions were prepared in 50 mM MOPS buffer (pH 7.5). Five mL of MOPS buffer were added to a 12-mL serum vial and sealed. Once the buffer was added, the vial was first evacuated and then sparged with N₂ gas. This vacuum/sparging cycle was repeated at least three times to ensure the vials contained little oxygen. The anaerobic buffer solution was usually bubbled with NO gas to make a saturated solution. Saturated NO solutions were made just prior to use.

The NO-generator SNP was purchased from Sigma. Diethylenetriamine/nitric oxide adduct (DETA) was purchased from Research Biochemicals International. SNP was prepared as a 50 mM solution in 50 mM MOPS buffer (pH 7.5). DETA was prepared as a 50 mM solution in 10 μL NaOH. SNP and DETA solutions were prepared just before use and maintained at room temperature.

The membrane permeable cGMP analog 8-bromoguanosine-cGMP (Sigma) was prepared as a 5 mM solution and cAMP (Sigma) as a 50 mM solution, both in deionized water. Both cyclic nucleotides were freshly prepared at room temperature but stored briefly on ice until addition.

For experiments testing NO and NO generating compounds, cultures of strain 11.10 *nirK-lacZ* and *norB-lacZ* were grown microaeroically (as described above) in unamended MOPS medium to an absorbance of 0.5 at 600 nm. Then, 10-mL aliquots of the original 100-mL cultures were removed from the sealed flasks with a syringe and added to 12-mL serum vials. Upon addition of cells, vials were crimp-sealed and incubated on a 100°C shaker for 45–60 min to allow the cells to deplete any oxygen present in the cultures. After the preincubation, solutions containing compounds to be tested as inducers were added to appropriate concentrations using gas-tight syringes to minimize oxygen contamination. Vials were either returned to the incubator or placed over incandescent light. Samples for enzyme assay were removed periodically until activity began to diminish. The reported activities are the maximal levels observed under a particular condition.

**Hb Experiments—** Human Hb was obtained from Sigma. Hb was prepared as a 1 mM solution in 50 mM MOPS buffer (pH 7.5). Hb was added to 3 mL of buffer in a 12-mL serum vial that was then sealed and bubbled with N₂ gas for 5 min to reduce the oxygen concentration. Hb solutions were stored at 4°C until use.

Cells for these experiments were prepared as described above for testing NO and NO-generating compounds. After the preincubation, Hb was added to vials in concentrations ranging from 1 to 5 μM. Concentrations greater than 5 μM interfered with the assay for β-galactosidase activity. One-mL samples were removed from vials for assay and spectrophotometric scan. Samples for spectrophotometric work were added to cuvettes being flushed with N₂ gas and quickly sealed to limit O₂ contamination. Individual cultures of 11.10 and 15.12 with Hb were sampled periodically to track the formation of reduced Hb and HbNO over the course of 6 h. Samples were scanned from 450 to 700 nm using a Beckman DU-650 spectrophotometer.

**RESULTS**

**Expression of nirK and norB Fusions in a Nor-deficient Strain—** Experiments with a Nor-deficient mutant of *R. sphaeroides* 2.4.3, strain 11.10, demonstrated that Nir activity is an obligate requirement for the transcriptional activation of *nirK*². Compared to wild type, 11.10 displays abated levels of *nirK-lacZ* expression when grown microaerobically with N-oxides. Since 11.10 has been shown to be unable to produce NO (26), it is possible the reduction in *nirK-lacZ* expression is due to the cell using NO as a signal to activate *nirK* transcription. If NO production is required for Nir and Nor expression, the insertional inactivation of the operon encoding Nor should not have a significant effect on gene transcription as NO can be generated by this mutant. We have isolated a strain, 15.12, in which a transposon is present in *norB*, which encodes one of the subunits of Nor. When this strain is grown microaeroically, the addition of nitrite results in the generation of high levels of NO (26). To monitor gene expression in a Nor-deficient background, *nirK-lacZ* and *norB-lacZ* fusions were conjugated separately into 15.12, and β-galactosidase synthesis was measured in microaerobic cultures.

Expression of *nirK* and *norB* fusions in 15.12 was initially measured in microaerobic cultures lacking exogenous N-oxides. Under these conditions, *nirK-lacZ* expression in 15.12 is 15-fold higher than wild type cells grown without exogenous N-oxides and 2-fold higher than wild type cells cultured microaerobically with 12 mM nitrate added to the medium (Fig. 1). Expression of *norB-lacZ* increases 5-fold relative to wild type grown in the absence of exogenous N-oxides, but is 2-fold lower than wild type cultured with N-oxides (Fig. 1). The large increases in induction observed in 15.12, particularly with *nirK-lacZ*, were surprising given that no N-oxides were added to the medium. In wild type cells, expression of *nirK-lacZ* was also observed in unamended medium, but was only one-sixth of the level in cultures amended with 12 mM nitrate (Fig. 1). Nir-deficient strains show no increase in *nirK-lacZ* expression above basal levels in unamended medium demonstrating that Nir activity is obligatory for induction of *nirK* under these conditions.² Since Nir is functional in 15.12, the *nirK-lacZ* expression observed is likely due to Nir produced by the reduction of trace amounts of N-oxides present in unamended medium. The critical difference between 15.12 and wild type under these conditions is that any NO produced during denitrification in 15.12 cannot be enzymatically reduced, resulting in low turnover of...

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³ J. Shi and J. P. Shapleigh, unpublished results.

² J. E. Tosques and J. P. Shapleigh, unpublished data.
NO under low oxygen concentrations. Nor activity in wild type cells increases NO turnover, lowering its steady state concentration and consequently decreasing nirK-lacZ expression in unamended medium.

Expression of the fusions in 15.12 in medium amended with N-oxides was also monitored. Under these conditions, the maximal level of expression of both fusions is significantly lower than when grown in unamended medium (Fig. 1). The lower levels of expression are probably a consequence of NO accumulating to cytotoxic levels. When 15.12 is grown microaerobically with nitrate, growth stops at an $A_{600}$ of ~0.6. Wild type cells grown under identical conditions typically reach an $A_{600}$ of >1.0. This low cell yield in 15.12 is consistent with NO becoming cytotoxic before maximal expression of the fusions is achieved. The higher levels of expression observed in unamended medium suggest that the trace amounts of N-oxides in the medium permit NO accumulation to levels optimal for gene expression without the toxicity observed in amended medium. The observation that Nor-deficient strains can achieve high levels of nirK-lacZ and norB-lacZ expression, whereas Nir-deficient strains cannot, provides further evidence demonstrating that NO production is obligatory for induction of these genes.

Inclusion of Hb Prevents nirK-lacZ Expression in 15.12—It has been shown that NO produced during denitrification can be effectively scavenged by extracellular hemoglobin (4). Hemoglobin makes an effective scavenger because it has a very high affinity for NO (27) and because it can be added at very high concentrations, effectively outcompeting other proteins with the capacity to bind NO. Another advantage of using Hb is that the HbNO complex is stable and can be identified spectrophotometrically. Since Hb is such an effective scavenger it should be possible to test the hypothesis that NO production is critical for expression of the genes encoding Nir and Nor. If freely diffusible NO is required for expression of genes encoding proteins required for NO metabolism, the presence of Hb should reduce or prevent their expression.

To determine the effect of hemoglobin on nirK expression, Hb was added to cells of 15.12 nirK-lacZ in unamended medium. Unamended medium was used to minimize the amount of NO produced during the experiment. Hb was added to sealed vials containing uninduced cells in concentrations ranging from 1 mM to 5 mM; as predicted, the inclusion of Hb prevents nirK-lacZ expression above basal level at all concentrations (Table I). Negative controls lacking Hb show significant induction, 15-fold higher than in aerobic cultures, but 5-fold less than microaerobic 15.12 cultures not transferred to smaller vials (Table I). Induction could be detected 2–3 h after samples had been transferred to vials. The reason for the decrease in induction of the controls relative to larger cultures is unclear, but is likely associated with the limited growth that occurs after cells are transferred to the vials. As an additional control, expression of nirK-lacZ was monitored in the Nir-deficient 11.10 under similar conditions. Basal levels of nirK-lacZ expression are observed in 11.10 with and without Hb, as expected (data not shown).

Samples from vials containing 15.12 and 11.10 cultured in the presence of Hb for several hours were analyzed spectrophotometrically for the presence of HbNO. Spectra from cultures of Nir-deficient cells are very similar to the spectrum of reduced, uncomplexed Hb, with absorption maxima in the $a$-region of the spectrum at 555 nm (Fig. 2, spectrum A) (4). This was expected; cellular respiration would have decreased the oxygen concentration and 11.10 is incapable of producing NO (26). Hb from the Nor-deficient cultures has absorption maxima at 547 and 570 nm (Fig. 2, spectrum B). This is consistent with the formation of the HbNO complex in the 15.12 samples (4). It is possible this HbNO complex could have been formed as a consequence of a chemical reaction between nitrite and Hb (4). However, this seems unlikely since the HbNO complex was not
observed in experiments with 11.10. These experiments demonstrate that unamended medium contains N-oxides that are reduced to NO in a Nir-dependent reaction, and since oxygen concentrations are low and NO is diffusible, a stable HbNO complex is formed. This confirms that nirK-lacZ and norB-lacZ induction in a Nor-deficient strain in unamended medium is a result of NO being produced by reduction of trace levels of N-oxides present in unamended medium. The elimination of nirK-lacZ expression by Hb further demonstrates that NO production is obligatory for induction of genes encoding proteins required for NO metabolism.

Addition of NO to 11.10 Induces nirK and nor Operon Expression—Since the presence of freely diffusible NO is required for nirK and nor operon induction, it should be possible to induce these genes by the addition of exogenous NO to cultures. Neither the Nor-deficient mutant 15.12 or wild type 2.4.3 can be used for NO addition experiments since these strains have been shown to be inducible when grown microaerobically in the absence of exogenous N-oxides. Therefore, the Nir-deficient strain was employed as it is incapable of inducing nirK-lacZ expression under microaerobic conditions in unamended medium. Any change observed in gene expression upon the addition of NO to 11.10 should be a result of that addition.

The concentration of NO that is optimal for nirK and nor operon expression is likely similar to the steady-state levels encountered as nitrite is reduced to NO and then to nitrous oxide. The steady state concentration of NO produced by denitrifiers has been found to range from 1 to 65 nM (28). For these experiments, NO was added at slightly higher concentrations to reduce manipulations of NO stock solutions. Subdivided samples of 11.10 containing either the nirK-lacZ or norB-lacZ reporter fusion were amended with 0.20 μM to 4.0 μM NO. The addition of NO results in increased levels of β-galactosidase expression in cultures with either fusion (Fig. 3). Maximum expression is observed within 30 min after the addition of NO. The maximum observable level of nirK-lacZ expression is 12-fold greater than basal, or aerobic, activity in 2.4.3, but one-third of wild type expression observed microaerobically with N-oxides (Fig. 3). The highest level of norB-lacZ expression detected is less than one-half of wild type with N-oxides (Fig. 3). Maximum levels of induction are generally observed at NO concentrations near 1.0 μM, though levels of expression vary considerably between samples. Concentrations of NO less than 1.0 μM typically result in either very limited induction or no induction at all, whereas concentrations exceeding 1.0 μM often result in cell lysis. Once NO has been added and induction observed, further additions of NO did not increase β-galactosidase expression.

To dismiss the possibility that NO was reacting with oxygen in these cultures forming nitrite, which then triggered gene expression, equimolar amounts of NO and nitrite were added separately to individual cultures of 11.10 nirK-lacZ or norB-lacZ. NO and nitrite were added to final concentrations of 1.0, 2.0, and 4.0 μM. At all concentrations, induction is only observed in 11.10 samples which received NO (data not shown). As previously observed, the presence of small amounts of nitrate/nitrite (<1 μM) does not induce expression of either nirK or the nor operon in 11.10.

TABLE I
Effect of Hb on expression of nirK-lacZ in the Nor-deficient mutant 15.15

| Condition     | β-Galactosidase activity |
|---------------|--------------------------|
| Hb            | 80 Miller units          |
| Without Hb    | 1150 Miller units        |

Fig. 2. Absorption spectra of Hb present in cultures of strain 11.10 (scan A) and strain 15.12 (scan B). Cultures were grown microaerobically in MOPS-Sistrom’s medium unamended with N-oxides. Hb was present at a concentration of 1 μM. Scans were referenced against an identical sample lacking Hb. Scan B was manually displaced from scan A for clarity.
addition of SNP, samples were either incubated over incandescent light or agitated to facilitate the release of NO. Induction of both nirK-lacZ and norB-lacZ fusions was observed in the presence of SNP, though not to wild type levels (data not shown). However, unlike when adding NO in solution, expression increases with time, typically peaking after 3–4 h in dark cultures and 7–8 h in cultures incubated over light. The peak level of induction correlates with the amount of SNP added.

It was observed during these experiments that, like certain eukaryotic tissues, R. sphaeroides appears to promote the release of NO from SNP. Cultures in which SNP had been added without a reducing agent were found to induce lacZ expression to levels comparable with, and sometimes exceeding, those observed in cultures containing reducing agents. Further experiments were carried out without reducing agents, and since cells were tolerant of mM concentrations of SNP, 2 mM SNP was added to all cultures. Cultures were incubated under both light and dark conditions, as before. Under these conditions, near wild type levels of induction are observed for both fusions (Fig. 3). The level of nirK-lacZ expression with SNP is 90% of the maximum wild type level while the level of norB-lacZ expression is nearly 100% of the maximum wild type level (Fig. 3). The highest levels of expression were found in cultures incubated over light for more than 10 h. The inability to achieve wild type levels of induction in earlier experiments was attributed to low concentrations of SNP; NO production diminished before full activity was reached.

If SNP addition is equivalent to NO generation during denitrification, it follows that anything that disrupts the cells ability to respond to NO should also disrupt the ability to respond to SNP. We have recently identified a gene, nnrR, that encodes a putative transcriptional activator of nirK and the nor operon (8). Inactivation of this gene causes cells to be unable to induce nirK and nor operon expression, even in the presence of NOxides. A similar gene has been identified in the denitrifier Paracoccus denitrificans (9). The addition of 2.0 mM SNP to microaerobic cultures of a strain in which the gene encoding NnrR has been insertionally inactivated did not lead to an increase in nirK-lacZ expression above basal levels (data not shown). This result indicates that SNP is acting through the same trans-acting factors as NO. It also demonstrates that the NnrR protein is necessary for the NO-dependent activation of both nirK and nor operon transcription.

One other NO-producing compound, DETA, was tried as an inducer for nirK and nor operon expression in 11.10. This compound was chosen because, once in solution, it spontaneously liberates NO at a moderate rate (31, 32). DETA was added to microaerobic cultures of 11.10 at concentrations ranging from 0.25 to 2 mM. Upon addition of DETA, all cultures were incubated in the dark. Maximum levels of nirK-lacZ and norB-lacZ expression obtained with DETA are approximately 2-fold less than observed levels with NO and more than 5-fold less than levels achieved with SNP under similar conditions (Fig. 3). Maximum induction is observed within 4 h after addition.

Cyclic Nucleotides and Gene Expression—In eukaryotes, a major route by which NO exerts its cellular effects is modulating the levels of cGMP (14). NnrR is a member of the Fnr/Crp family of activators (8). Members of this family have been shown to increase gene expression in response to increases in the intracellular concentration of the cyclic nucleotide cAMP (33). It is therefore possible that NO is exerting its effects in 2.4.3 by modulating levels of cyclic nucleotides. To test if cyclic nucleotides are involved, cAMP and the membrane permeable cGMP analog, 8-bromoguanosine-cGMP (34) were added to uninduced, microaerobic cultures of 11.10 norB-lacZ and β-galactosidase expression followed over time. The cGMP analog was added at concentrations ranging from 2.5 to 250 μM; cAMP was added to a final concentration of 1 mM. No induction of norB-lacZ was observed in any of the 11.10 cultures with either cAMP or the cGMP analog (data not shown).

**DISCUSSION**

Taking advantage of both Nir and Nor-deficient strains of R. sphaeroides 2.4.3 we have provided clear evidence that freely diffusible NO must be present for the induction of nirK and the nor operon in the denitrifier R. sphaeroides 2.4.3.
previously shown that nirK-lacZ expression is significantly reduced in a Nir-deficient strain, which is incapable of producing NO, grown under microaerobic conditions. Experiments described in this report show that a Nir-deficient strain is capable of wild type expression of both nirK-lacZ and norB-lacZ under conditions where NO is prevented from accumulating to toxic concentrations. If a similar experiment is performed in the presence of Hb, the endogenously generated NO becomes complexed with Hb and the fusions are no longer expressed. This experiment is analogous to experiments with the Nir-deficient strain as availability of NO is severely restricted in both instances. Experiments with these two classes of mutants lead to the same conclusion; elimination of NO, either by disruption of its production or removal by scavenger, eliminates nirK and norB induction.

We have also utilized the Nir-deficient strain of 2.4.3 to show that NO and NO-generating compounds induce nirK and nor expression. The NO-generator SNP produces the highest levels of induction in these types of experiments. The addition of SNP to uninduced cultures appears to most closely mimic the conditions optimal for expression. The variation in levels of induction achieved by the addition of different forms of NO probably reflects the difficulty in balancing NO levels optimal for expression versus levels that result in cytotoxicity. The decrease in nirK and norB fusion expression observed in the Nir-deficient strain in medium containing exogenous nitrate demonstrates that excessive NO is deleterious to gene expression. The low levels of expression experienced with the NO-generator DETA are probably due to the slow generation of NO by this compound. The near wild type expression of Nor-deficient strains in unamended medium was fortuitous. If N-oxide concentrations in unamended medium were different, induction in a Nor-deficient strain might have been so low as to have been overlooked.

The NO-dependent expression of nirK and the nor operon suggests the following model of gene regulation. For this discussion it is assumed that nitrate is present under all conditions. Under aerobic conditions nitrate reductase is not expressed, and as a consequence, NO cannot be produced, preventing nirK and the nor operon expression. As oxygen concentrations decrease, nitrate reductase is expressed and nitrite begins to accumulate. It has been previously shown that nitrate reductase expression occurs at higher oxygen concentrations than Nir expression (35, 36). The accumulation of nitrite does not directly induce nirK and nor operon expression since it is not an effector. However, the combination of low oxygen concentrations and nitrite accumulation is favorable for the production of NO. Since Nir generates NO, Nir must be expressed at some level even in the absence of its apparent effector, NO. While this NO-independent expression has not been directly demonstrated, we have shown that in the absence of the transcriptional regulator NnrR, nirK-lacZ expression is about 80 units under all conditions but that norB-lacZ expression is undetectable under the same conditions (8). This suggests that under noninducing conditions, when NnrR is not active, nirK is expressed but the nor operon is not, permitting the cells to produce NO if nitrite is present. It is important to note that even low levels of Nir should be sufficient to induce NO-dependent nirK and nor operon expression since NO is an effective inducer at nanomolar concentrations. Once NO begins to accumulate, expression of both nirK and the nor operon is increased in an NO-dependent manner. The concerted activity of Nir and Nor results in steady-state concentrations of NO in the nanomolar range that are sufficient for continued gene expression until nitrite is exhausted (28).

The critical factor in regulating gene expression in this model is oxygen concentration. High oxygen concentrations increase the autoxidation of NO, limiting NO-dependent expression (37). It is this interaction with oxygen that makes NO such a useful effector. It would be futile for cells to express Nir and Nor under conditions where NO would be autoxidized rapidly. If NO is stable enough to serve as an effector, oxygen concentrations should be low enough for NO metabolism to proceed without interference. It is unclear if NO is the direct activator of gene expression in 2.4.3 or if it functions as a second messenger, as in eukaryotes. The absence of nirK-lacZ expression in response to addition of cyclic nucleotides suggests NO does not modulate activity of a nucleotide cyclase in 2.4.3. It is possible NO may interact with NnrR. NnrR is a member of a family of transcriptional activators that includes proteins known to directly interact with small molecules such as oxygen and carbon monoxide (38, 39). Work is currently underway to determine if NnrR is a NO-responsive transcriptional activator.

While NO-dependent nirK and nor operon expression in 2.4.3 has many obvious differences with NO-mediated gene regulation in eukaryotes, it is similar in one important aspect: NO is endogenously generated by a specific protein to modulate cellular processes. In denitrifiers, NO is generated by the reduction of nitrite catalyzed by Nir, whereas in eukaryotes NO is generated by the oxidation of arginine by NO synthase (40). The generation of NO by a specific protein is an important characteristic since organisms incapable of generating NO also modulate gene expression in response to NO exposure. An example of this is the SoxR system in E. coli, which is incapable of denitrification (41). SoxR regulates proteins involved in response to superoxide-generating agents, including NO, by activating transcription of soxS, and the increase in SoxS leads to expression of proteins required for coping with oxidative stress (42). The SoxR-mediated response to NO is part of the response to radicals and not specific for NO, nor does the response activate any proteins that directly metabolize NO (43). In both denitrifiers and eukaryocytes, specific conditions induce the production of NO, which is used as a signal to elicit the desired biological response.

The use of NO as a signal molecule is probably common to most denitrifiers. Genes encoding products that only regulate the genes encoding Nir and Nor expression have been discovered in other denitrifiers (9, 44). The regulation of these two genes by a single transcriptional activator, which does not regulate genes involved in other steps in denitrification, is consistent with the regulatory scheme discussed above. The use of NO as a signal molecule by both denitrifiers and eukaryotes probably arose for the same reason; NO is highly reactive so only small concentrations are required to regulate cellular responses. However, long before eukaryocytes evolved and placed so many important cellular processes under NO control, bacteria had developed regulatory systems to take advantage of the unique chemistry of this molecule.

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