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ABSTRACT

Initial work focused on the regulation of nitrite reductase, the defining reaction of denitrification as well as nitric oxide (NO) reductase. Expression of the genes encoding both proteins was controlled by NnrR. This regulator was shown to be responsive to NO. More recent work has shown NnrR function is also likely inhibited by oxygen. Therefore, it is this protein that sets the oxygen level at which nitrate respiration takes over from aerobic respiration. The gene encoding NO reductase appears to only require NnrR for expression. Expression of the gene encoding nitrite reductase is more complex. In addition to NnrR, a two component sensor regulator complex termed PrrA and PrrB is also required for expression. These proteins are global regulators and serve to link denitrification with other bioenergetic processes in the cell. They also provide an additional layer of oxygen dependent regulation. The sequencing of the *R. sphaeroides* 2.4.3 genome allowed us to identify several other genes regulated by NnrR. Surprisingly, most of the genes were not essential for denitrification. Their high level of conservation in related denitrifiers suggests they do provide a selectable benefit to the bacterium, however.

We also examined the role of nitrate reductase in contributing to denitrification in *R. sphaeroides*. Strain 2.4.3 is unusual in having two distinct, but related clusters of genes encoding nitrate reductase. One of these genes clusters is expressed under high oxygen conditions but is repressed, likely by PrrB-PrrA, under low oxygen conditions. The other cluster is expressed only under low oxygen conditions. This cluster expresses the nitrate reductase used during denitrification. The high oxygen expressed cluster encodes a protein used for redox homeostasis. Surprisingly, both clusters are fully expressed even in the absence of nitrate.

During the course of this work we found that the type strain of *R. sphaeroides*, 2.4.1, is a partial denitrifier because it has the nitrate and NO reductases but lacks nitrite reductase. Like 2.4.3 it uses NnrR to regulate NO reductase. This unexpected arrangement suggested that it may use NO reductase to detoxify NO produced in its environment. Using a green fluorescent protein based reporter system we were able to demonstrate that NO produced by a denitrifier such as 2.4.3 can induce expression of NO reductase in 2.4.1. We then went on to show that the NO produced by denitrifiers can induce a stress response in other non-denitrifying bacteria. This suggests that the NO produced during denitrification will have a significant impact on the non-denitrifiers present in the surrounding environment.

We also expanded our studies to include the denitrifier *Agrobacterium tumefaciens*. We demonstrated that the expression of the nitrite and NO reductase genes in this bacterium follows the same general scheme as in *R. sphaeroides*. We also were able to show that this bacterium would induce NO reductase in response to the NO produced by plants. Importantly, we were able to demonstrate that *A. tumefaciens* had difficulty transitioning from aerobic respiration to denitrification if the transition was sudden. This difficulty manifested as an accumulation of NO. In some conditions cells were slowly able to switch modes of respiration but in other cases NO accumulations seemed to kill the cells. The difficulty in transition appears to be due to an inability to produce enough energy once the oxygen has been completely consumed.

INTRODUCTION AND RESULTS

Denitrification is the reduction of nitrogen oxides such as nitrate to nitrogen gas (Fig. 1). This process is a critical part of the nitrogen cycle since it returns fixed nitrogen to the atmosphere. This is beneficial for mitigating nitrogen pollution but is considered detrimental from an agricultural perspective. Human perturbation of nitrogen inputs has continued apace since the late 19th century, principally due to a significant increase in the application of fixed forms of nitrogen to soils (9). Nitrate is one of the most important of the nitrogen compounds whose concentrations have increased due to human activity. Nitrate is highly soluble and is the most abundant nitrogen contaminant in ground water in the United States (10). Excess nitrate is a known health hazard (23). Current modeling of the fate of nitrate in the environment assumes denitrification occurs under anaerobic conditions. This is obviously simplistic since the denitrification pathway is multi-step and there is not one regulatory system controlling the expression of all enzymes.

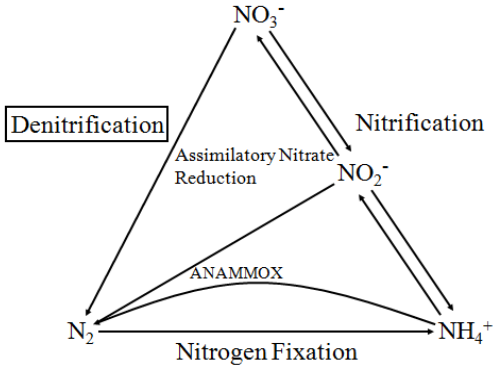

Fig. 1. Reactions in the nitrogen cycle.

In addition to being critical for reducing nitrogen loads in polluted environments denitrification also produces the gaseous nitrogen oxides nitric oxide (NO) and nitrous oxide (N_2O), both of which are greenhouse gases. Of the two gaseous nitrogen oxides liberated during denitrification N_2O is the most significant greenhouse gas. N_2O absorbs 3400 times more infrared radiation than CO_2 and has a residence time of about 12 years, much longer than that of CO_2 (27). Isotopic analysis suggests much of the 20% increase in N_2O levels in the atmosphere in the last 15 years is a consequence of agricultural practices (28).

Most denitrification is carried out by bacteria with this process serving as an alternative means of making energy under oxygen-limiting conditions. *Rhodobacter sphaeroides* is a purple photosynthetic bacterium with a diverse physiological repertoire but best known as a model bacterium for the study of photosynthesis. One strain of this bacterium, known as 2.4.3, was previously characterized as being able to grow by the anaerobic reduction of nitrogen oxides (21). We chose this bacterium to use as a model bacterium for the study of the regulation of denitrification. Given the importance of nitrate as an environmental pollutant it is essential we have a thorough understanding of all the factors that control its biological fate. When this work began it was clear denitrification occurred primarily under low oxygen conditions but little was known about how this was achieved or what environmental signals are important cues.

Our initial studies were dedicated to cloning and sequencing the genes encoding the nitrite and NO reductases from 2.4.3 (3, 25). We chose to focus on these genes since nitrite reductase is the first step in the denitrification pathway to produce a gaseous nitrogen oxide (Fig. 2). This makes it the defining step of denitrification. Since NO is toxic, NO reductase most work in concert with nitrite reductase to mitigate potential toxicity. As expected, expression of both reductases required low oxygen conditions as well as the presence of a nitrogen oxide such as nitrate or nitrite. Unexpectedly, we found that expression of both reductases was significantly decreased in a mutant lacking nitrite reductase (15). We also observed that expression of the genes encoding these proteins was near or above wild type levels in a strain lacking NO reductase in medium not supplemented with nitrate or nitrite (15). This turned out to be a consequence of the accumulation of NO produced from reduction of the trace amounts of nitrate in the medium. These two results suggested that NO is a key effector required for the expression of these two proteins. Evidence in support of this hypothesis was provided by the

observation that sodium nitroprusside, an inorganic source of NO, could stimulate nitrite and NO reductase expression in the nitrite reductase deficient strain.

The protein that served as the NO dependent regulator of expression was found to be a member of the FNR/CRP family and designated NnrR for nitrite and NO reductase regulator (26). We have studied this protein extensively but have been unable to determine its sensing mechanism. The most reasonable mechanism is via a metal center since this is a well known means of sensing NO in many organisms, including humans. However, no heme binding motifs or residues are conserved in an alignment of known NnrR proteins. We did carry out a mutagenesis analysis of the handful of residues in the sensing domain that are highly conserved (19). We found that several residues in a conserved 10 amino acid stretch were essential for function, including tyrosine 93. This tyrosine is highly conserved and is a hallmark of this group of regulators. Mutagenesis of most other residues only produced modest changes in function. While it seems likely some type of metal center is critical for function the nature of this center in NnrR remains elusive. Recent evidence discussed below suggests NnrR is also oxygen sensitive.

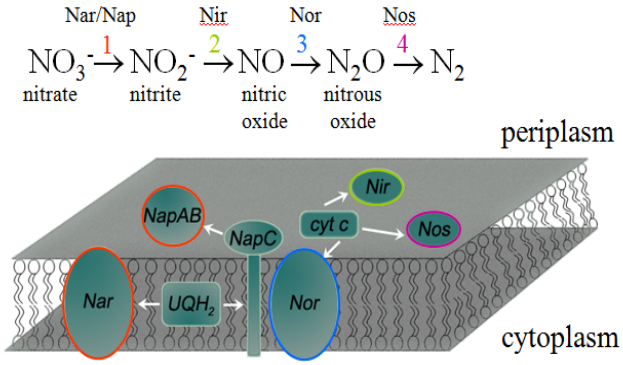

Fig. 2 Reactions of the nitrogen cycle and their topological arrangement in the cell wall of a gram negative bacterium.

Since we knew the type strain of *R. sphaeroides*, known as 2.4.1, lacked nitrite reductase we concluded it would not have an NnrR. This proved not to be the case when we showed it has both NnrR and the genes encoding NO reductase (16). This led us to conclude that NO reductase in 2.4.1 must be used for dealing with the toxicity of NO from exogenous sources. To test this we took advantage of a NO-dependent green fluorescent protein expression system we developed (29). Using this system we were able to demonstrate that the NO produced by a denitrifier can activate NO responsive regulators in a related bacterium. We were able to extend this work to demonstrate that unrelated, non-denitrifying bacteria are also impacted by the NO produced by denitrifiers (7).

In an effort to identify other genes required for nitrite and NO reduction in 2.4.3 we isolated transposon mutants unable to grow via denitrification on nitrite supplemented medium. While a number of different genes were identified in this search one gene showed up as a site of insertion in four separate mutants (17). This gene was shown to be *prrB*, which encodes the sensor part of a two component sensor regulator pair. Subsequent work showed that PrrB and PrrA, its cognate partner, were directly required for expression of *nirK*, the gene encoding nitrite reductase (17). Expression of the genes encoding NO reductase do not seem to require PrrB-PrrA but this is somewhat difficult to determine since any mutation that impacts nitrite reductase expression also affects expression of other genes under control of NnrR since the production of NO is eliminated.

Work by others has demonstrated that loss of the high oxygen affinity *cbb*$_3$ terminal oxidase leads to a higher level of the active form of PrrA (24). Since we had concluded that PrrB-PrrA were the sole O_2 sensitive regulators of *nirK* expression the loss of *cbb*$_3$ should result in *nirK* expression occurring at normal or perhaps higher levels of O_2 if NO were present. Unexpectedly though we found that *nirK* expression was significantly lower in the *cbb*$_3$ mutant when it was grown using standard conditions (17). Standard growth conditions means that cells growing in sealed vessels consume oxygen through respiration to levels low enough to allow the denitrification genes to be expressed. Further work revealed *nirK* expression could be restored

to wild type when cells were grown in medium sparged with high purity nitrogen gas. By testing growth under a variety of conditions it was found that the *cbb*$_3$ mutant will grow aerobically at oxygen levels ~2% or greater and via denitrification at oxygen levels lower than 0.2% (12). This strain cannot grow in the dark at the intermediate oxygen concentrations because the loss of the high affinity terminal oxidase presents aerobic respiration and because some aspect of denitrification is inhibited. Importantly, growth can occur in the middle range of oxygen concentrations under photosynthetic conditions. Taken together these results indicate there is oxygen dependent regulation of nitrite and NO reductase expression that does not involve PrrB-PrrA. Since this unusual regulatory pattern is denitrification specific the most likely candidate for the oxygen sensitive factor is NnrR. Repeated attempts to generate a more oxygen resistant form of NnrR proved unsuccessful for unknown reasons.

Given our focus on NO we were interested when it was reported that a protein known as cytochrome c prime (c') could reduce NO to nitrous oxide (8). In addition, it had previously been reported that this protein, which is normally highly expressed under low oxygen conditions in *R. sphaeroides,* was repressed by the addition of nitrate to the medium (22). Our experiments with c' in 2.4.3 revealed that it was actually more highly expressed during denitrification (6). We found no evidence for this protein having NO reductase activity. Nevertheless, c' was found to be capable of binding the NO produced during denitrification. These results suggest to us that c' is important in sequestering NO during denitrification in the environment when available nitrogen oxides are in low amounts (6).

When the genome of 2.4.3 was sequenced by the DOE it became possible to identify additional genes regulated by NnrR. This was feasible because our previous work had characterized the consensus NnrR binding site (4). Additional occurrences of this sequence in the genome were searched using web based tools and an additional four sites were identified (11). One of these was upstream of a gene encoding a copper protein which is required for passing electrons to nitrite reductase. We showed in prior work that another protein known as cytochrome c_2 can also donate electrons to nitrite reductase although the gene encoding c_2 is not regulated by NnrR (18). This was somewhat unexpected since we had previously found that *R. sphaeroides* has another copy of a gene encoding this copper protein which contains mutations that prevent it from binding copper (14). Oddly enough this disabled version is the form located adjacent to the gene encoding nitrite reductase while the paralog used during denitrification is located on a different chromosome. Another pair of genes, designated *norE* and *norF,* are exclusively regulated by NnrR. The function of the proteins encoded by these genes is unclear but using a taxis assay we described in a previous paper (20) we were able to show that loss of these proteins made the cells more sensitive to NO (11). Interestingly, these genes are not found in 2.4.1 even though this strain also has a NO reductase. The function of the other genes identified in 2.4.3 as being regulated by NnrR was not able to be determined.

R. sphaeroides 2.4.3 is an excellent model organism in many respects but one of the unusual aspects of this bacterium is that it grows poorly as a denitrifier under strictly anoxic conditions. This has required that we develop culture conditions that allow us to generate sufficient biomass of denitrification induced cells. These culture techniques have proven to be quite beneficial for us though and many of the results discussed above have taken advantage of these techniques. Nevertheless, we felt it would be useful to have a denitrifier that could grow well under anoxic conditions. To this end we undertook work with the plant pathogen *Agrobacterium tumefaciens*. This bacterium was chosen because it grows rapidly, had a sequenced genome, was in the same phylogenetic group as *R. sphaeroides* and would grow under anoxic conditions. Our initial studies revealed that the genes encoding nitrite and nitric oxide reductase were regulated in a manner similar to the same genes in *R. sphaeroides* with an NnrR-like protein being critical (2). As with 2.4.3 exogenous sources of NO induced expression. These exogenous sources included NO produced by plants. Further work revealed that orthologs of PrrB and PrrA, termed ActR and ActS were required for expression of *nirK* (1).

We purified ActR and used it to determine its binding site in *nirK* and to confirm that it does not bind to the promoter region of the gene encoding NO reductase.

Since it was obvious that oxygen and NO levels were key to the expression of the genes encoding nitrite and NO reductase we undertook a study to examine the levels of these compounds as *A. tumefaciens* transitioned from oxygen to nitrate respiration. This was done in collaboration with Prof. Lars Bakken of the Department of Plant and Environmental Sciences, Norwegian University of Life Sciences, Ås, Norway. This was a multi-factorial experiment with variations in both oxygen and nitrate concentrations (5). While variations in nitrate did impact transitions the variations in oxygen were more critical. Cells starting with very low levels of oxygen had no trouble switching respiratory modes. Interestingly, the onset of denitrification was preceded by a pulse of NO which fits with our understanding of NnrR-dependent regulation as discussed above (5). NO reductase quickly reduced the NO produced during the burst preventing it from accumulating to lethal levels. In contrast to what occurred at low oxygen, cells grown at higher oxygen had difficulty making the transition to nitrate respiration. This was manifested by the slow production of nitrous oxide as well as the accumulation of NO. The temporary burst of NO was replaced with long term NO exposure. In some cases the NO was eventually consumed and nitrous oxide production commenced consistent with onset of nitrate respiration. The difficulty cells in the higher oxygen cultures have in making the respiratory transition most likely arises from the speed of oxygen consumption (5). In a rapidly growing culture the time it takes for the conditions to shift from oxygen concentrations being too high to allow NnrR function to too low to support aerobic respiration is very short. This means that when oxygen runs out the cells have no way to make the energy necessary to produce the proteins required for denitrification. The NO arises from the low level, constitutive nitrite reductase expression required for the NO production necessary to activate NnrR. Since NO reductase is solely regulated by NnrR it is not expressed until essentially all oxygen is gone and if this happens rapidly the protein cannot be made.

The sequencing of the 2.4.3 genome revealed that it contains two clusters of genes encoding a periplasmic nitrate reductase. One cluster, termed *onap*, is nearly identical to the genes in closely related bacteria. The other, termed *rnap*, is more frequently found in bacteria such as *Escherichia coli* and its relatives. Additional work by us has shown that the *onap* cluster is expressed when oxygen concentrations are high but repressed when oxygen is low (13). *rnap* expression is the converse, only being expressed when oxygen is low (13). Surprisingly, nitrate did not enhance expression of either cluster. Aerobic cells were found to produce nitrate reductase when grown in liquid medium; however, it is not active under these conditions until cells reach stationary phase. Interestingly, we found that expression of *onap* increased about 3-4 fold in cells growing on solid medium and this was reflected by an increase in nitrate reduction under these conditions. Given this behavior in 2.4.3, as well as insight gained from work done by others it can be concluded this enzyme is required for redox homeostasis. This is why it was designated o for overflow. The other enzyme reduces nitrate more efficiently under denitrifying conditions and is required for respiration, hence its designation as *rnap*. Sequence analysis indicates *rnap* is almost certainly under control of a member of the FNR family of transcriptional regulators. However, attempts to identify this regulator have so far proved fruitless. *onap* is repressed by PrrA/PrrB (13). However, the regulation underlying its enhanced expression on solid medium remains unclear.

CONCLUSION

We have unraveled a great many of the details underlying regulation of genes critical for denitrification in *R. sphaeroides*. In most cases we identified the precise signal used to control expression and the proteins responsible for detecting these molecules to develop a much more detailed, realistic model of how denitrifiers control nitrate respiration. A model for our current understanding is shown in Fig. 3. Not surprisingly the regulation is more complex than

anticipated. The gene encoding nitrite reductase is under the most complex control. This makes sense given it produces NO, which is an important signal molecule as well as a toxic free radical. Some aspects of the regulation are specific for denitrification while others, in particular PrrB-PrrA are more global regulators linking denitrification gene expression with expression of other genes involved in energy metabolism. Oxygen is a key regulator and it is interesting that the protein responsible for detecting NO, a key nitrogen oxide signal, is also controlled by oxygen. Its sensitivity to oxygen seems to be set to allow induction of certain denitrification genes when oxygen respiration becomes energy limiting. This makes it likely aerobic denitrification does not occur in most of our standard denitrification model organisms. While the model works well on paper our work has also shown that it does not work well in the 'real world' if there is a sudden shift between aerobic and anaerobic modes of growth. This suggests these sudden shifts are rare in the environment but may also help explain why isolating denitrifiers from the environment may only yield those strains capable of surviving this sudden shift while most denitrifiers may struggle to grow.

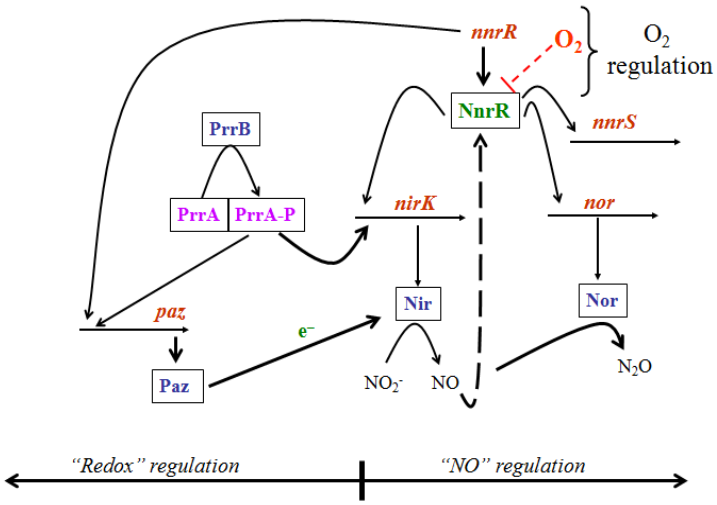

Fig. 3 Model of regulation of nitrite and NO reductase expression in R. sphaeroides and by extension other related proteobacterial denitrifiers. *paz* encodes the copper proteins that passes electrons to nitrite reductase. *nnrS* is a protein of unknown function. For simplicity not all genes regulated by NnrR are shown.

One of the more unexpected aspects of this work that was revealed with the sequencing of *R. sphaeroides* genomes was the common occurrence of partial denitrification. While the strain we work with, 2.4.3, encodes a complete denitrification chain the type strain has half a denitrifying electron transport chain. Another strain named 2.4.9 has only gene, NO reductase. Recently another *R. sphaeroides* genome has been made available and it has three of the four genes, lacking nitrate reductase. The physiological and environmental reasons for these unusual arrangements are not clear at this point. These results do demonstrate though that while we have learned much about regulation of these genes, the roles they play in the survival of organisms in the environment is complex and not obligatorily linked to anaerobic respiration. We have shown NO reductase can be used for mitigating NO toxicity and nitrate reductase can be used for redox homeostasis. This explains some partial denitrification patterns but not all suggesting additional physiological roles for this important group of enzymes.

CITED REFERENCES

1. **Baek, S. H., A. Hartsock, and J. P. Shapleigh.** 2008. *Agrobacterium tumefaciens* C58 uses ActR and FnrN to control nirK and nor expression. J Bacteriol **190:**78-86.
2. **Baek, S. H., and J. P. Shapleigh.** 2005. Expression of nitrite and nitric oxide reductases in free-living and plant-associated *Agrobacterium tumefaciens* C58 cells. Appl Environ Microbiol. **71:**4427-4436.
3. **Bartnikas, T. B., I. E. Tosques, W. P. Laratta, J. Shi, and J. P. Shapleigh.** 1997. Characterization of the region encoding the nitric oxide reductase of *Rhodobacter sphaeroides* 2.4.3. . J. Bacteriol. **179:**3534-3540.

4. **Bartnikas, T. B., Y. Wang, T. Bobo, A. Veselov, C. P. Scholes, and J. P. Shapleigh.** 2002. Characterization of a member of the NnrR regulon in *Rhodobacter sphaeroides* 2.4.3 encoding a heme-copper protein. Microbiol. **148:**825-833.
5. **Bergaust, L., J. Shapleigh, A. Frostegard, and L. Bakken.** 2008. Transcription and activities of NOx reductases in *Agrobacterium tumefaciens*: the influence of nitrate, nitrite and oxygen availability. Environ. Microbiol. **10:**3070-3081.
6. **Choi, P. S., V. M. Grigoryants, H. D. Abruña, C. P. Scholes, and J. P. Shapleigh.** 2005. Regulation and function of cytochrome c' in *Rhodobacter sphaeroides* 2.4.3. J. Bacteriol. **187:**4077-4085.
7. **Choi, P. S., Z. Naal, C. Moore, E. Casado-Rivera, H. D. Abruña, J. D. Helmann, and J. P. Shapleigh.** 2006. Assessing the impact of denitrifier-produced nitric oxide on other bacteria. Appl. Environ. Microbiol. **72:**2200-2205.
8. **Cross, R., D. Lloyd, R. K. Poole, and J. W. B. Moir.** 2001. Enzymatic removal of nitric oxide catalyzed by cytochrome c' in *Rhodobacter capsulatus*. J Bacteriol **183:**3050-3054.
9. **Galloway, J. N., F. J. Dentener, D. G. Capone, E. W. Boyer, R. W. Howarth, S. P. Seitzinger, G. P. Asner, C. C. Cleveland, P. A. Green, E. A. Holland, D. M. Karl, A. F. Michaels, J. H. Porter, A. R. Townsend, and C. J. Vöosmarty.** 2004. Nitrogen Cycles: Past, present, andfFuture. Biogeochemistry **70:**153-226.
10. **Green, C. T., L. J. Puckett, J. K. Bohlke, B. A. Bekins, S. P. Phillips, L. J. Kauffman, J. M. Denver, and H. M. Johnson.** 2008. Limited occurrence of denitrification in four shallow aquifers in agricultural areas of the United States. J. Environ. Qual. **37:**994-1009.
11. **Hartsock, A., and J. P. Shapleigh.** 2010. Identification, functional studies, and genomic comparisons of new members of the NnrR regulon in *Rhodobacter sphaeroides*. J. Bacteriol. **192:**903-911.
12. **Hartsock, A., and J. P. Shapleigh.** submitted. Mechanisms of oxygen inhibition of *nirK* expression in *Rhodobacter sphaeroides*. Microbiol.
13. **Hartsock, A., and J. P. Shapleigh.** submitted. Physiological roles for two periplasmic nitrate reductases in *Rhodobacter sphaeroides* 2.4.3. J Bacteriol.
14. **Jain, R., and J. P. Shapleigh.** 2001. Characterization of *nirV* and a gene encoding a novel pseudoazurin in *Rhodobacter sphaeroides* 2.4.3. Microbiol **147:**2505-2515.
15. **Kwiatkowski, A., and J. P. Shapleigh.** 1996. Requirement of nitric oxide for induction of genes whose products are involved in nitric oxide metabolism in *Rhodobacter sphaeroides* 2.4.3. J. Biol. Chem. **271:**24382-24388.
16. **Kwiatkowski, A. V., W. P. Laratta, A. Toffanin, and J. P. Shapleigh.** 1997. Analysis of the role of the *nnrR* gene product in the response of *Rhodobacter sphaeroides* 2.4.1 to exogenous nitric oxide. J. Bacteriol. **179:**5618-5620.
17. **Laratta, W. P., P. S. Choi, I. E. Tosques, and J. P. Shapleigh.** 2002. Involvement of the PrrB/PrrA two-component system in nitrite respiration in *Rhodobacter sphaeroides* 2.4.3: evidence for transcriptional regulation. J. Bacteriol. **184:**3521-3529.
18. **Laratta, W. P., M. J. Nanaszko, and J. P. Shapleigh.** 2006. Electron transfer to nitrite reductase of *Rhodobacter sphaeroides* 2.4.3: examination of cytochromes c2 and cY. Microbiol. **152:**1479-1488.
19. **Laratta, W. P., and J. P. Shapleigh.** 2003. Site-directed mutagenesis of NnrR: a transcriptional regulator of nitrite and nitric oxide reductase in *Rhodobacter sphaeroides*. FEMS Microbiol. Lett. **229:**173-178.
20. **Lee, D. Y., A. Ramos, L. Macomber, and J. P. Shapleigh.** 2002. The taxis response of various denitrifying bacteria to nitrate and nitrite. Appl. Environ. Microbiol. **68:**2140-2147.
21. **Michalski, W., and D. J. D. Nicholas.** 1988. Identification of two new denitrifying strains of *Rhodobacter sphaeroides*. FEMS Microbiol. Lett. **52:**239-244.

22. **Michalski, W. P., D. J. Miller, and D. J. D. Nicholas.** 1986. Changes in cytochrome composition of *Rhodopseudomonas sphaeroides f. sp. denitrificans.* Biochim. Biophys. Acta **849:**304-315.
23. **Organization, World Health** 2004. Guidelines for drinking water quality. 3rd ed, vol. I-Recommendations. Geneva.
24. **Ranson-Olson, B., and J. H. Zeilstra-Ryalls.** 2008. Regulation of the *Rhodobacter sphaeroides* 2.4.1 *hemA* gene by PrrA and FnrL. J. Bacteriol. **190:**6769-6778.
25. **Tosques, I. E., A. V. Kwiatkowski, J. Shi, and J. P. Shapleigh.** 1997. Characterization and regulation of the gene encoding nitrite reductase in *Rhodobacter sphaeroides* 2.4.3. J. Bacteriol. **179:**1090-1095.
26. **Tosques, I. E., J. Shi, and J. P. Shapleigh.** 1996. Cloning and characterization of *nnrR*, whose product is required for the expression of proteins involved in nitric oxide metabolism in *Rhodobacter sphaeroides* 2.4.3. J. Bacteriol. **178:**4958-4964.
27. **Working Group I.** 2001. IPCC third assessment report: climate change 2001: the scientific basis. p. 239-288. vol. The Scientific Basis. Cambridge University Press., Cambridge, UK.
28. **Wolff, E., and R. Spahni.** 2007. Methane and nitrous oxide in the ice core record. Philos. Transact. A Math Phys. Eng. Sci. **365:**1775-1792.
29. **Yin, S., M. Fuangthong, W. P. Laratta, and J. P. Shapleigh.** 2003. Use of a green fluorescent protein-based reporter fusion for detection of nitric oxide produced by denitrifiers. Appl. Environ. Microbiol. **69:**3938-3941

Publications citing funding from this grant (this includes references cited in the list above).

1. **Baek, S. H., A. Hartsock, and J. P. Shapleigh.** 2008. *Agrobacterium tumefaciens* C58 uses ActR and FnrN to control *nirK* and *nor* expression. J. Bacteriol. **190:**78-86.
2. **Baek, S. H., G. Rajashekara, G. A. Splitter, and J. P. Shapleigh.** 2004. Denitrification genes regulate *Brucella virulence* in mice. J. Bacteriol. **186:**6025-6031.
3. **Baek, S. H., and J. P. Shapleigh.** 2005. Expression of nitrite and nitric oxide reductases in free-living and plant-associated *Agrobacterium tumefaciens* C58 cells. Appl. Environ .Microbiol. **71:**4427-4436.
4. **Bartnikas, T. B., I. E. Tosques, W. P. Laratta, J. Shi, and J. P. Shapleigh.** 1997. Characterization of the region encoding the nitric oxide reductase of *Rhodobacter sphaeroides* 2.4.3. J. Bacteriol. **179:**3534-3540.
5. **Bartnikas, T. B., Y. Wang, T. Bobo, A. Veselov, C. P. Scholes, and J. P. Shapleigh.** 2002. Characterization of a member of the NnrR regulon in *Rhodobacter sphaeroides* 2.4.3 encoding a heme-copper protein. Microbiol. **148:**825-833.
6. **Bergaust, L., J. Shapleigh, A. Frostegard, and L. Bakken.** 2008. Transcription and activities of NOx reductases in *Agrobacterium tumefaciens*: the influence of nitrate, nitrite and oxygen availability. Environ. Microbiol. **10:**3070-3081.
7. **Choi, P. S., V. M. Grigoryants, H. D. Abruna, C. P. Scholes, and J. P. Shapleigh.** 2005. Regulation and function of cytochrome c' in *Rhodobacter sphaeroides* 2.4.3. J Bacteriol **187:**4077-4085.
8. **Choi, P. S., Z. Naal, C. Moore, E. Casado-Rivera, H. D. Abruña, J. D. Helmann, and J. P. Shapleigh.** 2006. Assessing the impact of denitrifier-produced nitric oxide on other bacteria. Appl. Environ. Microbiol. **72:**2200-2205.
9. **Hartsock, A., and J. P. Shapleigh.** 2010. Identification, functional studies, and genomic comparisons of new members of the NnrR regulon in *Rhodobacter sphaeroides*. J. Bacteriol. **192:**903-911.
10. **Hartsock, A., and J. P. Shapleigh.** submitted. Mechanisms of oxygen inhibition of *nirK* expression in *Rhodobacter sphaeroides*. Microbiol.

11. **Hartsock, A., and J. P. Shapleigh.** submitted. Physiological roles for two periplasmic nitrate reductases in *Rhodobacter sphaeroides* 2.4.3. J. Bacteriol.
12. **Jain, R., and J. P. Shapleigh.** 2001. Characterization of *nirV* and a gene encoding a novel pseudoazurin in *Rhodobacter sphaeroides* 2.4.3. Microbiol **147:**2505-2515.
13. **Kwiatkowski, A. V., W. P. Laratta, A. Toffanin, and J. P. Shapleigh.** 1997. Analysis of the role of the *nnrR* gene product in the response of *Rhodobacter sphaeroides* 2.4.1 to exogenous nitric oxide. J. Bacteriol. **179:**5618-5620.
14. **Laratta, W. P., P. S. Choi, I. E. Tosques, and J. P. Shapleigh.** 2002. Involvement of the PrrB/PrrA two-component system in nitrite respiration in *Rhodobacter sphaeroides* 2.4.3: evidence for transcriptional regulation. J. Bacteriol. **184:**3521-3529.
15. **Laratta, W. P., M. J. Nanaszko, and J. P. Shapleigh.** 2006. Electron transfer to nitrite reductase of *Rhodobacter sphaeroides* 2.4.3: examination of cytochromes c2 and cY. Microbiol. **152:**1479-1488.
16. **Laratta, W. P., and J. P. Shapleigh.** 2003. Site-directed mutagenesis of NnrR: a transcriptional regulator of nitrite and nitric oxide reductase in *Rhodobacter sphaeroides*. FEMS Microbiol. Lett. **229:**173-178.
17. **Lee, B., O. M. Usov, V. M. Grigoryants, W. K. Myers, J. P. Shapleigh, and C. P. Scholes.** 2009. The role of arginine-127 at the proximal NO-binding site in determining the electronic structure and function of 5-coordinate NO-heme in cytochrome c' of Rhodobacter sphaeroides. Biochemistry **48:**8985-8993.
18. **Lee, D. Y., A. Ramos, L. Macomber, and J. P. Shapleigh.** 2002. The taxis response of various denitrifying bacteria to nitrate and nitrite. Appl. Environ. Microbiol. **68:**2140-2147.
19. **Mitchell, D. M., J. P. Shapleigh, A. M. Archer, J. O. Alben, and R. B. Gennis.** 1996. A pH-dependent polarity change at the binuclear center of reduced cytochrome *c* oxidase detected by FTIR difference spectroscopy of the CO adduct. Biochemistry **35:**9446-9450.
20. **Mitchell, D. M., Y. Wang, J. O. Alben, and J. P. Shapleigh.** 1998. Fourier transform infrared analysis of membranes of *Rhodobacter sphaeroides* 2.4.3 grown under microaerobic and denitrifying conditions. Biochimica et Biophysica Acta **1409:**99-105.
21. **Naal, Z., J.-H. Park, S. Bernhard, J. P. Shapleigh, C. A. Batt, and H. D. Abruna.** 2002. Amperometric TNT biosensor based on the oriented immobilization of a nitroreductase maltose binding protein fusion. Anal. Chem. **74:**140 -148.
22. **Olesen, K. O., A. Veselov, Y. Zhao, Y. Wang, B. Danner, C. P. Scholes, and J. P. Shapleigh.** 1998. Spectroscopic, kinetic and electrochemical characterization of heterologously expressed wild type and mutant forms of copper-containing nitrite reductase from *Rhodobacter sphaeroides* 2.4.3. Biochemistry **37:**6086-6094.
23. **Shapleigh, J.** 2009. Dissimilatory and Assimilatory Nitrate Reduction in the Purple Photosynthetic Bacteria, p. 623-642. *In* C. N. Hunter, F. Daldal, M. C. Thurnauer, and J. T. Beatty (ed.), The Purple Phototrophic Bacteria, vol. 28. Springer Science.
24. **Shapleigh, J. P.** 2007. The denitrifying prokaryotes, p. 769-792. *In* M. Dworkin (ed.), The Prokaryotes:A Handbook on the Biology of Bacteria: Volume 2: Ecophysiology and Biochemistry. Springer-Verlag, New York, NY.
26. **Tosques, I. E., A. V. Kwiatkowski, J. Shi, and J. P. Shapleigh.** 1997. Characterization and regulation of the gene encoding nitrite reductase in *Rhodobacter sphaeroides* 2.4.3. J. Bacteriol. **179:**1090-1095.
27. **Tosques, I. E., J. Shi, and J. P. Shapleigh.** 1996. Cloning and characterization of *nnrR*, whose product is required for the expression of proteins involved in nitric oxide metabolism in *Rhodobacter sphaeroides* 2.4.3. J. Bacteriol. **178:**4958-4964.
28. **Usov, O. M., P. S. Choi, J. P. Shapleigh, and C. P. Scholes.** 2005. ENDOR investigation of the liganding environment of mixed-spin ferric cytochrome c'. J. Am. Chem. Soc. **127:**9485-9494.

29. **Usov, O. M., P. S. Choi, J. P. Shapleigh, and C. P. Scholes.** 2006. ENDOR of NO-ligated cytochrome c'. J. Am. Chem. Soc. **128:**5021-5032.
30. **Usov, O. M., Y. Sun, V. M. Grigoryants, J. P. Shapleigh, and C. P. Scholes.** 2006. EPR-ENDOR of the Cu(I)NO complex of nitrite reductase. J. Am. Chem. Soc. **128:**13102-13111.
31. **Veselov, A., K. Olesen, A. Sienkiewicz, J. P. Shapleigh, and C. P. Scholes.** 1998. Electronic structural infromation from Q-band ENDOR on the Type 1 and Type 2 copper liganding environment in wild type and mutant products of copper-containing nitrite reductase. Biochemistry **37:**6095-6105.
32. **Yin, S., M. Fuangthong, W. P. Laratta, and J. P. Shapleigh.** 2003. Use of a green fluorescent protein-based reporter fusion for detection of nitric oxide produced by denitrifiers. Appl. Environ. Microbiol. **69:**3938-3944.
34. **Zhao, Y., D. A. Lukoyanov, Y. V. Toropov, K. Wu, J. P. Shapleigh, and C. P. Scholes.** 2002. Catalytic function and local proton structure at the Type 2 copper of nitrite reductase: the correlation of enzymatic pH dependence, conserved residues and proton hyperfine structure. Biochemistry **41:**7464-7474.

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