JOURNAL OF BACTERIOLOGY, Aug. 1996, p. 4958–4964 Vol. 178, No. 16
0021-9193/96/$04.00+0

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

IVAN E. TOSQUES, JIARONG SHI, AND JAMES P. SHAPLEIGH*

Department of Microbiology, Cornell University, Ithaca, New York 14853

Received 22 February 1996/Accepted 17 June 1996

During denitrification, the production and consumption of nitric oxide (NO), an obligatory and freely diffusible intermediate, must be tightly regulated in order to prevent accumulation of this highly reactive nitrogen oxide. Sequencing upstream of *norCB*, the structural genes for NO reductase, in the denitrifying bacterium *Rhodobacter sphaeroides* 2.4.3, we have identified a gene, designated *nnrR*, which encodes a protein that is a member of the cyclic AMP receptor family of transcriptional regulators. Insertional inactivation of *nnrR* prevents growth on nitrite, as well as the reduction of nitrite and NO, but has no effect on reduction of nitrate or photosynthetic growth. By using *nirK-lacZ* and *norB-lacZ* fusions, we have shown that NnrR is a positive transcriptional regulator of these genes. *nnrR* is expressed at a low constitutive level throughout the growth of *R. sphaeroides* 2.4.3. These results show that NnrR is not a global regulator but is instead a regulator of genes whose products are directly responsible for production and reduction of NO. Evidence is also presented suggesting that an NnrR homolog may be present in the nondenitrifying bacterium *R. sphaeroides* 2.4.1. The likely effector of NnrR activity, as determined on the basis of work detailed in this paper and other studies, is discussed.

Denitrification is a respiratory process in which bacteria utilize nitrate and other nitrogen oxides (N oxides) as alternate electron acceptors when oxygen concentrations are limiting. During denitrification, nitrate (NO_3^-) is reduced successively, through a series of intermediates, to nitrogen gas (N_2). Briefly, NO_3^- is reduced by nitrate reductase (Nar) to nitrite (NO_2^-), NO_2^- is reduced by nitrite reductase (Nir) to nitric oxide (NO), NO is reduced by nitric oxide reductase (Nor) to nitrous oxide (N_2O) and N_2O is reduced by nitrous oxide reductase (Nos) to N_2 (23).

It has been observed that denitrifying bacteria express N oxide reductases only under microaerobic conditions in the presence of N oxides (5, 6). These studies also showed that Nar, Nir, and Nos are most strongly induced under microaerobic conditions by NO_3^-, NO_2^-, and N_2O, respectively. The expression of Nor was not monitored. The results suggest that the expression of each reductase responds somewhat specifically to the N oxide that is its substrate. A regulatory model consistent with these observations would require a sensor of oxygen tension and a set of sensors responsive to some or all of the N oxides. Recent work has begun to identify some of the regulatory proteins required for expression of N oxide reductases in various denitrifiers (2, 3, 35, 38).

When considering any regulatory scheme for expression of the N oxide reductases, it is critical that expression occur in such a way as to prevent the accumulation of NO. NO is a highly reactive, freely diffusible molecule that is an obligatory intermediate during denitrification (40). To mitigate the accumulation of NO, there must be tight control of both its production and consumption. This suggests that the expression of Nir, the source of NO, and Nor, which reduces NO, is coordinately regulated so that Nir expression cannot occur without Nor expression. The expression of Nir by itself would be lethal if NO_2^- were present in even nanomolar concentrations. It is critical, therefore, to study the regulation of these two enzymes in concert.

We have chosen to investigate the regulation of Nir and Nor in the denitrifying bacterium *Rhodobacter sphaeroides* 2.4.3. *R. sphaeroides* is a useful bacterium in which to study denitrification because of the extensive work that has been done on other components of the electron transport chain (34). The complete denitrification electron transport chain includes many of these proteins. The understanding of the organization and function of these proteins provides a valuable framework in which to place our studies of denitrification in this organism. Moreover, recent work has begun to identify and characterize the actions of a number of proteins regulating expression of electron transport proteins (7, 39).

In this paper, we report a series of experiments carried out to characterize a gene encoding a putative transcriptional activator in *R. sphaeroides* 2.4.3. During the isolation and sequencing of the genes required for Nor activity, we have identified a gene whose product is a member of the Fnr/CRP family of transcriptional regulators. Fnr and related proteins have been found to regulate many proteins whose expression is dependent on oxygen concentration (29). The best-studied member of this family is Fnr from *Escherichia coli*. This protein has N-terminal cysteine residues which have been found to be critical for responding to oxygen limitation (20, 27). Fnr-like proteins, containing the N-terminal cysteines, have been identified in other bacteria, including denitrifiers and *R. sphaeroides* (24, 39). However, there are members of this family that do not possess the N-terminal cysteines and are probably responsive to effectors other than oxygen (8). This sequence variability makes it difficult to predict a priori to which signal a member of this family responds. The deduced primary sequence of the transcriptional regulator in *R. sphaeroides* 2.4.3 does not contain N-terminal cysteines, suggesting that it may respond to effectors other than oxygen. Mutants of *R. sphaeroides* 2.4.3

* Corresponding author. Mailing address: Department of Microbiology, Wing Hall, Cornell University, Ithaca, NY 14853. Phone: (607) 255-8535. Fax: (607) 255-3904. Electronic mail address: jps2@cornell.edu.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Reference or source
Strains		
DH5αF′	Host for *E. coli* cloning: F′ ϕ80 *lacZ*Δ*m15 recA1 endA1 gyrA96 thi-1 hsdR17* (r_K^- m_V^-) *supE44 relA1 deoR* (Δ*lacZYA-argF*)*U169*	
S17-1	For conjugational transfer of plasmids from *E. coli* to *R. sphaeroides*: *recA thi pro hsdRM*$^+$ *RP4*:2-Tc:*Mu*:Km:Tn*Z*	
2.4.3	Wild-type denitrifying strain of *R. sphaeroides*	ATCC 17025
2.4.1	Wild-type nondenitrifying strain of *R. sphaeroides*	ATCC 17023
R125	*nnrR* strain of *R. sphaeroides* with chromosomal insertion of ΩSp^{r}Sm^r cassette in the upstream *Bam*HI site of *nnrR*	This study
Plasmids		
pT7/T3-18U, -19U	pT7/T3-18U and -19U have multiple cloning sites oriented oppositely; used for generation of single-stranded DNA (sequencing) and cloning in *E. coli* (Ap^r)	BRL[a]
pRK415	Broad-host-range plasmid (Tc^r)	Keen et al. (11)
pSUP202	Suicide plasmid (Tc^r Ap^r Cm^r)	Simon et al. (28)
pLU106	Derivative of pRK with *puc-lacZ* fusion (Tc^r Km^r)	S. Kaplan
pIT9	Derivative of pRK with *norB-lacZ* (*Pst*I site) transcriptional fusion (Tc^r Km^r)	This study
pJS84	Derivative of pRK with *nirK-lacZ* transcriptional fusion (Tc^r Km^r)	Tosques et al. (33)
pIT37	Derivative of pSUP-202 containing *nnrR* with ΩSp^{r}Sm^r cassette inserted at upstream *Bam*HI site	This study
pIT53	Derivative of pRK with *nnrR-lacZ* (*Bgl*II site) translational fusion (Tc^r)	This study
pIT55	Derivative of pRK containing fragment with entire *nnrR* gene (Tc^r)	This study
pIT65	Derivative of pRK containing *nnrR* and *nor-lacZ*	This study

[a] BRL, Bethesda Research Laboratories.

which are unable to express the regulator have no detectable Nir or Nor activity. This phenotype is identical to the phenotype of an NnrR-deficient strain of *Paracoccus denitrificans* (35). Because of the phenotypic similarities, we have chosen to use the terminology of van Spanning et al. (35) and refer to the gene we have identified as *nnrR*, which encodes the transcriptional regulator NnrR (for nitrite and nitric oxide reductase regulator).

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Please refer to Table 1 for a list of the plasmids and strains used in this study. *E. coli* DH5α was used as a maintenance strain for plasmids. *E. coli* S17-1 was used as a donor for matings. *R. sphaeroides* 2.4.3 (ATCC 17025) and 2.4.1 (ATCC 17023) are wild-type denitrifying and nondenitrifying strains, respectively. Strain R125 is an *R. sphaeroides* 2.4.3 derivative which is Sp^r Sm^r because of the insertion of an Sp^r Sm^r Ω element (9) into the upstream *Bam*HI site of *nnrR* (Fig. 1). Plasmids pT7/T3-18U and pT7/T3-19U (Bethesda Research Laboratories) were used for cloning as well as synthesis of single-stranded DNA. Plasmid pRK415 is a broad-host-range plasmid used for transferring genes from *E. coli* to *R. sphaeroides* (11). pLU106 is a pRK construct with a *puc-lacZ* fusion and was kindly provided by Sam Kaplan (University of Texas Health Science Center at Houston) (16). The construction of pIT9 (pRK *norB-lacZ*), pJS84 (pRK *nir-lacZ*), pIT53 (pRK *nnrR-lacZ*), pIT55 (pRK *nnrR*), and pIT37 (pSUP-202 *nnrR*::Ω; used for inactivating *nnrR*) is discussed in Table 1, Fig. 1, and below.

E. coli strains were grown in LB medium (19). *Rhodobacter* strains were grown in Sistrom's medium at 30°C (18). NO_3^- and NO_2^- were added to *Rhodobacter* cultures to final concentrations of 1 g liter^{-1} and 0.5 g liter^{-1}, respectively. Antibiotics were added to *E. coli* cultures at the following concentrations: ampicillin, 100 μg ml^{-1}, tetracycline, 25 μg ml^{-1}; trimethoprim, 30 μg ml^{-1}; kanamycin, 25 μg ml^{-1}; and streptomycin-spectinomycin, 25 μg ml^{-1}. Antibiotics were added to *Rhodobacter* cultures at the following concentrations: tetracycline, 1 μg ml^{-1}; trimethoprim, 30 μg ml^{-1}; kanamycin, 25 μg ml^{-1}; and streptomycin-spectinomycin, 50 μg ml^{-1}. To induce the *Rhodobacter* N oxide reductases, cells were cultured in a 250-ml Erlenmeyer flask containing 100 ml of medium. After inoculation, the flask was capped with a rubber stopper. Cells grown in this manner, referred to as microaerobically grown cells, rapidly consume the oxygen in the flask, leading to expression of anaerobically inducible proteins. To avoid removing the stopper, samples for enzymatic assay were withdrawn with a syringe. Cultures grown aerobically were cultured in 50 ml of Sistrom's medium in a flask capped with a foam stopper. Photosynthetic cultures were grown in 20-ml screw-cap tubes filled with Sistrom's medium. Tubes were incubated on their side over incandescent lights.

DNA manipulation and sequencing. Plasmid isolation was done by the alkaline lysis method (4). Standard methods were used for restriction endonuclease digestions, agarose gel electrophoresis, and ligations (19). Plasmids were moved into *R. sphaeroides* 2.4.3 by conjugation. Biparental matings were carried out with *E. coli* S17-1 as the donor. Sequence was obtained from single-stranded DNA by the chain termination method with materials and protocols from the Sequenase version 2.0 kit (U.S. Biochemicals) and ^{35}S-dATP from Amersham. Both strands of the region encoding *nnrR* were sequenced. Database searches were performed with the BLAST programs (1). To construct the *nnrR-lacZ* fusion, a translational fusion was made by ligating the *lacZ* fragment of pLKC481 (32) (without the gene coding for kanamycin resistance) to the *Bgl*II site in *nnrR* (Table 1). To

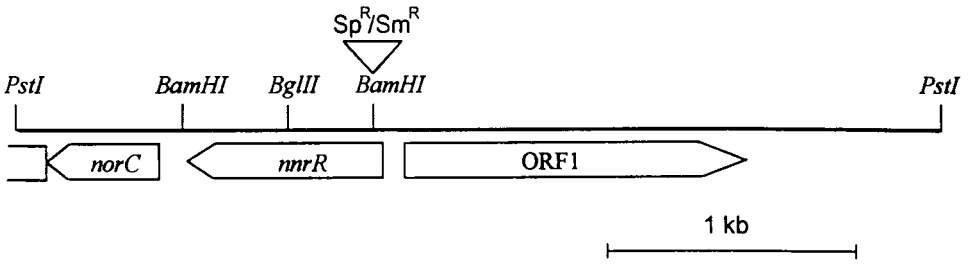

FIG. 1. Schematic representation of the region of the chromosome of *R. sphaeroides* 2.4.3 containing *nnrR*. Also shown is the location of *norC*, a portion of *norB* (immediately downstream of *norC*), and ORF1, an open reading frame indicated by sequence analysis to be upstream of *nnrR* (27a). The *Pst*I site in *norB* was used for construction of a *norB-lacZ* fusion, and as indicated, the 5′ *Bam*HI site in *nnrR* was used to construct the *nnrR*::Ω element used for insertional inactivation of *nnrR*. Also shown is the *Bgl*II site used for construction of the *nnrR-lacZ* fusion.

construct the *nirK*- and *norB-lacZ* fusions, the *lacZ*-Km^r cassette of pKOK6 (13) was used in both cases.

Assays for enzymatic activities. β-Galactosidase activity was determined on at least two independently grown cultures as previously described (19). Cells removed from stoppered flasks were not kept anaerobic but were used immediately for assays. Nir activity was assayed by monitoring the disappearance of NO_2^- in the presence of the electron donor methyl viologen essentially as previously described (31). If necessary, the cells were washed to remove NO_2^- present in the growth medium. The presence of NO_2^- in the growth medium was determined by taking small aliquots of cells (typically 10 μl), adding them to 900 μl of phosphate buffer (pH 7.5), and without adding an electron donor, assaying for NO_2^- (31). To assay β-galactosidase and Nir activities, samples were taken at various times during the growth cycle. The reported activities are the maximal values unless otherwise noted. Nor activity was assayed by measuring the production of N_2O with a gas chromatograph (26).

SNP experiments. Cells for use in these experiments were grown microaerobically in Sistrom's medium unamended with N oxides to an A_{600} of approximately 0.50. Then, 10-ml aliquots of culture were transferred to 12-ml serum vials. The serum vials were crimp sealed with rubber stoppers, and the cells were then shaken for 30 to 45 min at 30°C to permit the cells to lower the O_2 concentration. After the preincubation, sodium nitroprusside (SNP) was added, with a syringe, to a final concentration of 0.5, 1, or 2 mM. The serum vials were incubated for 2 to 3 h, and then their contents were assayed for β-galactosidase activity as described by Kwiatkowski and Shapleigh (15).

Nucleotide sequence accession number. The nucleotide sequence of the *nnrR* gene has been deposited on the GenBank database under accession number U62403.

RESULTS

Cloning and sequence analysis of *nnrR*. In the process of isolating and sequencing the operon encoding the Nor complex, we identified an open reading frame immediately upstream of, and transcribed in the same direction as, *norC* (Fig. 1). Database comparisons of the translation product of this open reading frame indicated that it encoded a protein homologous to members of the Fnr/CRP family of proteins. Determining the exact size of the open reading frame was difficult because of the lack of an ATG codon near the expected 5′ end of the predicted open reading frame. Since GTG has been found as a translational initiation codon in *Rhodobacter* species, the codon at position 181 in the sequence was chosen as the putative translation start site (37). This translational starting point was chosen on the basis of sequence alignments and the occurrence of potential start codons at the 5′ end of the open reading frame. Given this translation start site, the open reading frame, designated *nnrR* (see below), is 720 bases in length and has a G+C content of 68.4% (Fig. 2).

A poor Shine-Dalgarno sequence precedes the putative translation start site (Fig. 2). A sequence, centered 96 bp upstream of the translation start site, 5′-TGGCCGGTTGCTTGCA-3′, has homology to the RpoN (σ^{54}) consensus binding motif, 5′-TGGCAC(N_5)TTGC(A/T)-3′ (21). Starting at position 179, there is a sequence, 5′-TTGTG(N_4)CGCAA-3′, which has homology to the consensus binding motif of Fnr from *E. coli*, 5′-TTGAT(N_4)ATCAA-3′ (29). The putative binding motif in the *nnrR* sequence overlaps the translation start site since the GTG in the upstream half site is the putative translation start codon. An identical Fnr-like binding motif is centered 42.5 bp upstream of the *norC* transcription start site in *R. sphaeroides* 2.4.3 (27a). The *nirK* promoter contains a site 43.5 bases upstream of the *nirK* transcript start site with only a single nucleotide difference from the sequences in *nnrR* and *norC* (33). While there is no direct evidence that these sites are binding motifs for a transcriptional regulator, sequence conservation and location relative to the start of transcription strongly suggest their involvement in regulation. The location of the putative binding site in *nnrR* suggests that NnrR might negatively regulate its own synthesis. This is similar to the promoter region of *fnr* from *E. coli*, which contains an Fnr binding motif that is likely to be involved in negative autoregulation (30).

```
   1 GGCCGCCTCCTCGTGCAAAGAGAGGACGGCGGCTGGTGCCGCCGCCCTCCGAGGTGCCGG

  61 CCGTTCCGGGACGCGGGTGGCCGGTTGCTTGCAGGTACGACGGTCAGGCTCTGCTGTGGG
                      RpoN

 121 CCGGTGCCTGATCTGTTCCCGTCGCTCACTCTCTTGCCTCAGGACCGGCAGACGCTCATT

   1  M  L  P  Q  I  R  N  G  S  V  N  K  L  D  E  T  L  L  T  R
 181 GTGCTGCCGCAAATTCGGAACGGATCCGTGAACAAACTCGACGAAACCCTTCTGACCAGG
       NnrR              BamHI

  21  L  P  P  F  S  L  L  D  R  P  Q  I  R  T  I  L  D  Q  A  T
 241 CTGCCGCCCTTCTCGCTGCTCGATCGTCCGCAGATCCGCACCATCCTGGATCAGGCCACG

  41  S  R  R  Y  D  E  G  S  T  V  F  G  E  G  M  A  A  D  R  F
 301 TCGCGCCGCTACGACGAGGGCAGCACGGTCTTTGGCGAGGGGATGGCGGCGGACCGGTTC

  61  Y  L  L  L  D  G  T  I  R  V  V  R  T  T  P  T  G  E  Q  I
 361 TACCTGCTGCTCGACGGCACGATCCGCGTGGTGCGCACCACCCCCACCGGCGAGCAGATC

  81  I  A  L  H  I  G  P  G  Q  L  F  G  I  A  P  A  L  A  R  D
 421 ATCGCGCTGCATATCGGACCGGGGCAGCTGTTCGGGATCGCTCCGGCGCTGGCGCGCGAC

 101  T  Y  P  A  T  A  V  A  A  A  E  C  V  T  L  S  W  P  V  R
 481 ACCTATCCGGCGACGGCGGTGGCCGCGGCCGAGTGCGTCACGCTCTCCTGGCCCGTGCGG

 121  L  W  G  D  F  V  A  S  Y  P  G  F  A  T  E  S  N  R  T  L
 541 CTCTGGGGGGATTTCGTCGCCTCCTATCCGGGCTTTGCGACCGAGAGCAACCGGACGCTC

 141  G  A  R  L  G  E  M  Q  N  R  I  T  E  L  A  T  Q  Q  V  E
 601 GGCGCGCGGCTGGGGGAAATGCAGAACCGGATCACCGAGCTTGCGACGCAGCAGGTCGAG

 161  Q  R  V  A  A  C  L  L  R  M  V  N  Q  S  G  R  K  V  A  T
 661 CAGCGGGTCGCGGCCTGCCTCCTGCGCATGGTCAACCAGTCCGGGCGGAAGGTCGCGACC

 181  G  I  E  I  S  F  P  I  T  R  Q  N  I  S  E  M  T  G  T  T
 721 GGAATCGAGATCTCGTTCCCGATCACGCGGCAGAACATCTCGGAGATGACGGGCACGACG
           BglII
 201  L  H  T  V  S  R  L  L  S  A  W  E  R  E  G  I  V  E  S  T
 781 CTCCACACGGTTTCGCGGCTTCTCTCGGCCTGGGAGCGCGAGGGAATCGTCGAGAGCACG

 221  R  R  R  I  V  V  T  A  P  H  R  L  V  V  L  S  G  P  A  G
 841 CGCCGGCGCATCGTCGTCACCGCCCCGCACCGGCTGGTGGTGCTGAGCGGGCCTGCCGGA

 241  Q  V  *
 901 CAGGTCTGAGGCGGCCGGGGCGCGGGCTTCTCAGATCCCGGCCAGAAGACGCAGGGCGCG

 961 ACGGAACTCGTCCTCGTCCAGCCCGTATTCGGCGCAGGCTTCCACGACCGTGTGGAAGGG
```

FIG. 2. Nucleotide sequence of a fragment of DNA from *R. sphaeroides* 2.4.3 containing *nnrR*. Putative RpoN and NnrR binding sites have been underlined. The *Bam*HI site used for insertional inactivation of *nnrR* and the *Bgl*II site used for construction of the *nnrR-lacZ* translational fusion have also been underlined. The numbers on the left indicate the position number of the first nucleotide or amino acid on each line.

The protein encoded by *nnrR* is 242 residues in length and has a mass of 26,539 Da. Alignments of the deduced protein sequence of NnrR with other proteins by BLAST show that it has the highest sequence similarity to NtcA, which is involved in nitrogen regulation in *Synechococcus* and *Anabaena* species (10, 36). It has close to the same level of similarity to FixK, which is required for expression of a number of genes whose products are involved in nitrogen fixation and anaerobic respiration (8), from various diazotrophs and Fnr from *E. coli*. None of these proteins has greater than 25% identity with NnrR, however. NnrR is 22% identical to NtcA from *Synechococcus* species, 22% identical to Fnr from *E. coli*, and 21% identical to FixK from *Bradyrhizobium japonicum* (Fig. 3). NnrR from *R. sphaeroides* 2.4.3 shows less identity and similarity to NnrR from *Paracoccus denitrificans* (35) and Dnr from *Pseudomonas aeruginosa* (3), both denitrifying bacteria, than to the NtcA and Fnr proteins discussed above (Fig. 3). This is surprising given the similar phenotype obtained when the genes encoding NnrR from both denitrifiers as well as Dnr are inactivated (3, 35). Despite the low overall level of identity of NnrR with other related proteins, sequence alignments do definitively assign this protein to the Fnr/CRP family, however (Fig. 3). The conservation of key residues in the helix-turn-helix domain at the C terminus as well as several glycine residues in a centrally located and structurally important β-roll

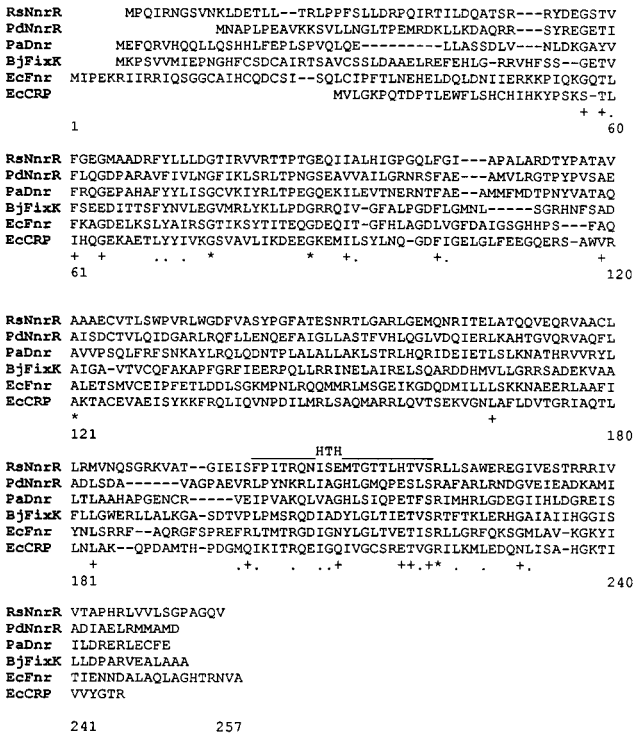

FIG. 3. Alignment of amino acid sequence of *R. sphaeroides* NnrR (RsNnrR) with sequences of *Paracoccus denitrificans* NnrR (PdNnrR), *Pseudomonas aeruginosa* Dnr (PaDnr), *B. japonicum* FixK (BjFixK), *E. coli* Fnr (EcFnr), and *E. coli* CRP (EcCRP). Identical amino acids in all six sequences are marked with an asterisk. Plus signs and dots indicate residues conserved in at least five of the six sequences and conserved substitutions, respectively. Numbers coincide with the RsNnrR sequence. HTH indicates the helix-turn-helix domain.

structure establishes the relatedness. One other important observation concerning the primary structure is the lack of cysteine residues at the N terminus.

***nnrR* is required for Nir and Nor activities.** To determine what role NnrR plays in regulating denitrification, we insertionally inactivated *nnrR*. This was done by inserting an Ω element (9) in the upstream *Bam*HI site of *nnrR*, creating R125. Wild-type *R. sphaeroides* 2.4.3 grows anaerobically with N oxides as terminal electron acceptors and under photosynthetic conditions. R125 grows under all of the same conditions, except under anaerobic conditions with NO_2^- as the sole electron acceptor (Table 2). When NO_3^- was supplied to R125 as the electron acceptor, the organism could grow anaerobically in the dark but there was a near quantitative accumulation of NO_2^-. The effect of *nnrR* inactivation on growth with N_2O could not be determined since strain 2.4.3 will not grow with N_2O as the sole electron acceptor. Both the lack of growth under anaerobic conditions when NO_2^- is the electron acceptor and the accumulation of NO_2^- in the medium when NO_3^- is the electron acceptor indicate that the *nirK* gene product is not active in R125. Nar activity is not affected by the absence of NnrR since cells can grow anaerobically with NO_3^- as an electron donor. To confirm the absence of Nir and test for the expression of Nor, cells grown microaerobically with NO_3^- were tested for Nir and Nor activities. As expected, there was no measurable Nir activity, and Nor was inactive as well. Since R125 is wild type in all other respects, it seems that NnrR is not a global regulator; rather, it is a specific regulator of Nir and Nor expression. R125 could be restored to wild type by complementation with a plasmid containing the 3.5-kb *Pst*I fragment shown in Fig. 1.

Expression of *nirK-lacZ*, *norB-lacZ*, and *puc-lacZ* in an *nnrR*-deficient strain. The expression of *nirK-lacZ* and *norB-lacZ* fusions in R125 was used to establish that transcription of *nirK* and *norCB* is affected by *nnrR* inactivation (Fig. 4). The maximal expression of *nirK-lacZ* in wild-type cells under anaerobic conditions in medium containing NO_3^- or NO_2^- is about 2,500 U (33). Under similar conditions, *nirK-lacZ* expression in R125 shows a maximal expression of about 100 U. This is the same level as that found during aerobic growth in wild-type or R125 cells. A similar pattern was observed in *nnrR*-deficient cells containing *norB-lacZ* (Fig. 4). The maximal expression of *norB-lacZ* in R125 was the same as aerobic levels of *R. sphaeroides* 2.4.3 or R125. Inactivation of *nnrR* completely eliminates the transcriptional activation of both *nirK* and *norB* fusions.

As an additional test to confirm the specificity of NnrR for genes whose products are required for NO_2^- or NO reduction, we monitored the expression of *puc-lacZ* in wild-type strain 2.4.3 (Table 3). The *puc* operon encodes light-harvesting complexes that are highly expressed when cells are grown under conditions favorable for photosynthesis (17). The fusion used was constructed with the *puc* genes from *R. sphaeroides* 2.4.1 (16). Wild-type strain 2.4.3 showed high levels of *puc-lacZ* expression when grown under conditions favorable for photosynthesis (Table 3). *puc-lacZ* expression in R125 grown under conditions favorable for photosynthesis was nearly identical to that observed in the wild type.

Expression of the *nnrR-lacZ* fusion in wild-type and *nnrR*-deficient strains. NnrR could become functional as a consequence of direct interaction with an effector or an increase in its expression. To test if the latter of these possibilities occurs, an *nnrR-lacZ* fusion was constructed. A translational fusion between the *Bgl*II site of *nnrR* and the *Bam*HI site of the *lacZY* cassette from pLKC481 was constructed in the broad-host-range vector pRK415 (11, 32). This construct contains 2.0 kb of DNA upstream of the *nnrR* open reading frame. In wild-type strain 2.4.3 cells grown microaerobically with NO_3^-, the ex-

TABLE 2. Growth characteristics of *nnrR*-deficient *R. sphaeroides* R125 and of R125 containing *nnrR* in *trans* on pIT55[a]

Growth conditions[b]	Growth characteristics of strain					
	R125			R125 + pIT55		
	With no N oxides	With NO_3^-	With NO_2^-	With no N oxides	With NO_3^-	With NO_2^-
Dark, $+O_2$	+	+	+	+	+	+
Dark, $-O_2$	−	+	−	−	+	+
Light, $-O_2$	+	+	+	+	+	+

[a] NO_3^- and NO_2^- were added at 10 and 0.5 mM, respectively. No NO_2^- was reduced in the cultures grown with NO_2^- under conditions favorable for photosynthesis.
[b] $-O_2$, cells were grown microaerobically.

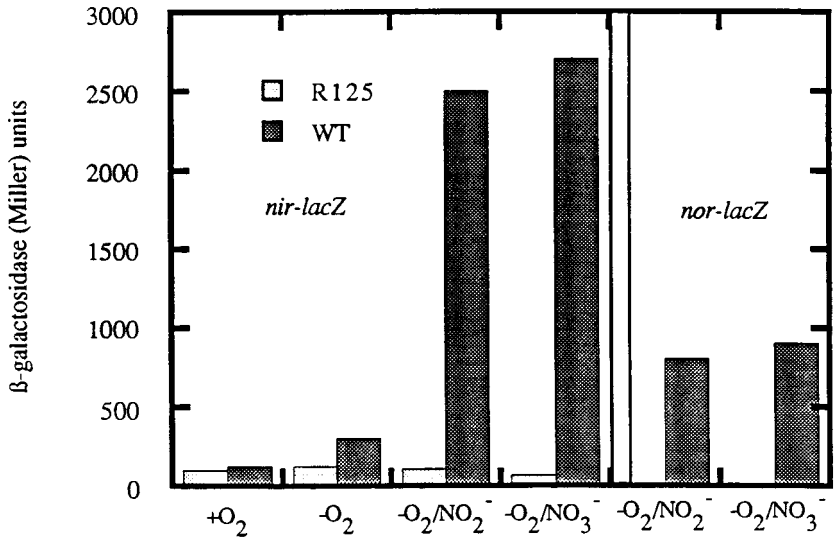

FIG. 4. β-Galactosidase activities of *nirK-lacZ* and *norB-lacZ* in *nnrR*-deficient (R125) and wild-type (WT) backgrounds under various growth conditions. $-O_2$ indicates cultures grown microaerobically. NO_3^- and NO_2^- were added to final concentrations of 10 and 0.75 mM, respectively. *norB-lacZ* expression in R125 was undetectable under aerobic anaerobic conditions, with or without N oxides.

pression of *nnrR-lacZ* went from about 200 U during the initial aerobic phases of growth down to less than 50 U in the later, anaerobic phase of growth (Fig. 5). Decreasing expression was also observed in cultures without NO_3^- (data not shown), possibly because of N oxides that contaminate unamended media (15, 33). Since this decrease in expression is suggestive of negative autoregulation, the expression of *nnrR-lacZ* in strain R125 grown with or without NO_3^- was monitored. In this strain, irrespective of the growth conditions, the levels of expression of the fusion did not decrease upon shifting from aerobic to anaerobic growth (Fig. 5).

Can NnrR activate *nirK*- and *norB-lacZ* expression in the nondenitrifying bacterium *R. sphaeroides* 2.4.1 upon addition of NO? Previous work with *nirK* expression in *R. sphaeroides* 2.4.3 has suggested that NirK and NorCB expression is dependent on the production of NO (15, 33). Given that other regulators, like Fnr and CooA, interact with small molecules, we decided to test if interaction of NnrR with NO leads to activation of the NnrR (29). To test this, a pRK derivative containing the complete *nnrR* gene and *norB-lacZ* was constructed. This construct is similar to the *norB-lacZ* construct described above except it contains an additional 2.0 kb upstream of *nor* which contains *nnrR*. This construct was conjugated into *R. sphaeroides* 2.4.1, which is a nondenitrifying variant. Since strain 2.4.1 lacks Nir und Nor activity, we reasoned that it would also lack other genes encoding products for NO metabolism, including *nnrR*. This strain was then incubated with SNP, and levels of LacZ activity were monitored. SNP is an NO-generating compound that we have found induces near-wild-type levels of *nirK-lacZ* or *norB-lacZ* expression in *R. sphaeroides* 2.4.3 variants that are unable to produce NO because of inactivation of *nirK* (15). Using SNP and the *nnrR*-containing *norB-lacZ* clone in strain 2.4.1 gives us an in vivo system in which the direct interaction of NnrR and NO can be assessed. As can be seen in Table 4, addition of SNP does induce significant expression of *norB-lacZ* in strain 2.4.1. This induction does not occur without addition of SNP. To confirm that this induction was induced by NnrR produced from the plasmid, expression of the *norB-lacZ* construct lacking the *nnrR* open reading frame was monitored in strain 2.4.1. Unexpectedly, SNP addition induced nearly identical levels of *norB-lacZ* expression in this strain, except in the culture containing 2 mM SNP, in which expression was significantly lower than that in the strain containing *nnrR* and *norB-lacZ* (Table 4). Therefore, this experiment is inconclusive as to the interaction of NO and NnrR. It is significant, however, in that it indicates that the ability to respond to NO generation is not limited to denitrifying variants of *R. sphaeroides*. NO is a toxic free radical, and perhaps the response to NO in nondenitrifiers is part of a generalized response to free radicals, as is observed in *E. coli* (22).

TABLE 3. Effect of *nnrR* inactivation on expression of β-galactosidase activity from *puc-lacZ*

Nitrogen oxide present[a]	β-Galactosidase activity[b] in:	
	Wild-type *puc-lacZ*	R125 *puc-lacZ*
None	8,906	7,953
NO_3^-	9,516	7,596
NO_2^-	8,503	8,107

[a] All cultures were grown under conditions favorable for photosynthesis; if necessary, NO_3^- or NO_2^- was added to a final concentration of 10 and 0.75 mM, respectively.

[b] β-Galactosidase activities (in Miller units) represent the averages of the maximal activities of at least two separate cultures.

DISCUSSION

In this study, we have shown that *nnrR*, which is located immediately upstream of the start site of the operon encoding Nor, is required for expression of both Nir and Nor in *R. sphaeroides* 2.4.3. The complete lack of induction of *nirK*- and *norB-lacZ* fusions in strain R125 suggests that NnrR is a transcriptional regulator. This conclusion is supported by the deduced sequence of NnrR, which indicates that it is a member of the Fnr/CRP family of transcriptional regulators. The best studied of this type of regulator is *E. coli* Fnr, which is a global regulator (29). NnrR is not the strain 2.4.3 equivalent of Fnr, however, because (i) it lacks N-terminal cysteines that have

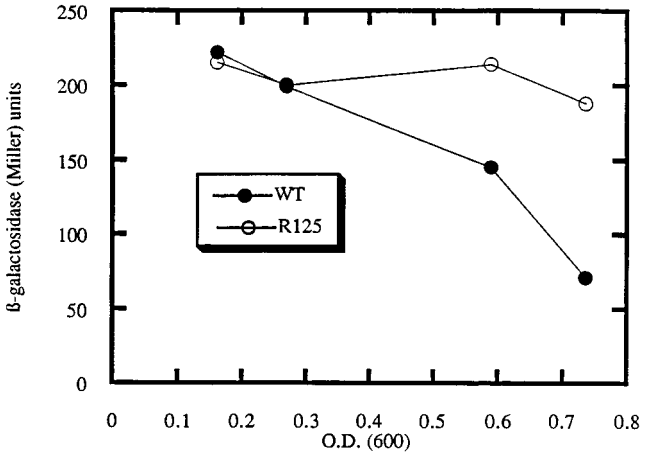

FIG. 5. Difference in β-galactosidase activity of *nnrR-lacZ* in wild-type (WT) and *nnrR*-deficient (R125) cells as a function of cell growth. Both strains were grown microaerobically in the presence of 10 mM NO_3^-. Each datum point represents the average of duplicate assays of three independent measurements. The standard deviation was never more than 25 U in any of the measurements.

been shown to be critical for signal sensing in Fnr and (ii) it is apparently involved in the regulation of only a few genes. A more typical Fnr homolog has recently been isolated from *R. sphaeroides* 2.4.1 (39). Southern blots with genomic DNA from strain 2.4.3 and a probe derived from *fnrL* from strain 2.4.1 gave a single, strongly hybridizing band whose size was inconsistent with it being *nnrR* (27a).

Since NnrR does not regulate Nar, it is likely that it satisfies regulatory constraints specific for Nir and Nor. One important factor to consider that could have a significant impact on Nir and Nor activity is the autooxidation of NO by oxygen (12). If oxygen concentrations are too high when Nir is expressed, its product, NO, could be diverted into a bioenergetically useless reaction instead of being reduced by Nor to N_2O. Therefore, Nir and Nor should be expressed only when oxygen concentrations are very low. In contrast, since oxygen does not react with NO_3^- or N_2O, the only constraint on Nar or Nos expression is for NO_3^- or N_2O respiration to be bioenergetically advantageous. Since it is unlikely that the oxygen concentration at which NO_3^- or N_2O respiration becomes advantageous is also the same concentration required for optimal Nir and Nor expression, the regulatory control of Nir and Nor is independent of the regulation of the other N oxide reductases. This conclusion is supported by studies monitoring expression of Nar, Nir, and Nos in *Pseudomonas stutzeri* (14). In this denitrifier it was found that Nir is the most sensitive of the N oxide reductases to oxygen concentrations. Cells grown in 17% oxygen had 70% of the anaerobic levels of Nar but only 20% of the anaerobic levels of Nir. It should be pointed out that an NnrR homolog has not been identified in *P. stutzeri*. However, it has been observed that Nir and Nar expression is not coupled in this denitrifier, consistent with the presence of an NnrR homolog (14).

nnrR is transcribed at a low, apparently constitutive level and may be negatively autoregulated under low-oxygen conditions. If our assignment of the translation start codon is correct, negative autoregulation of NnrR could occur through the presence of a binding site for the product of the gene overlapping the translation start site (Fig. 2). This type of regulation is very similar to the regulation of *fnr* in *E. coli* (30). These similarities suggest that NnrR is activated by interaction with some effector. Indirect evidence suggests that oxygen is not an effector. We have previously shown that cells of strain 2.4.3 lacking Nir are unable to express *nirK*- or *norB-lacZ* to wild-type levels (33). For example, Nir-deficient cells grown microaerobically in medium containing 12 μM NO_3^- have the same level of *nirK-lacZ* expression as when they are grown aerobically. Wild-type cells grown under identical conditions exhibit eightfold-higher *nirK-lacZ* expression than those grown aerobically. This indicates that a product of Nir, not anoxia, is an absolute requirement for Nir expression. Since SNP addition can elicit near-wild-type levels of *nirK*- and *norB-lacZ* expression in a Nir-deficient strain under microaerobic conditions, it is a reasonable hypothesis that NO is the product of Nir required for Nir and Nor expression (15). Since NO is freely diffusible, it is also not unreasonable to suggest that if NO were an effector, it might interact directly with NnrR (25). By homology to Fnr and Crp, the effector binding domain of NnrR is probably in its N terminus. However, there are no obvious sequence motifs, such as Cys residues that might ligate an Fe-S center, present in the N terminus of NnrR that could suggest a mechanism by which NO and NnrR might interact.

One puzzling aspect of this study was the expression of *norB-lacZ* in the nondenitrifying bacterium *R. sphaeroides* 2.4.1 in response to SNP addition. There is no obvious requirement for an NnrR homolog in strain 2.4.1. It is possible that SNP activates expression of genes whose products are involved in a stress response to the presence of free radicals and that an NnrR homolog is the transcriptional activator of this response. SNP-dependent gene expression is not an artifact in *R. sphaeroides*. The activation of *nor-lacZ* expression by SNP in strain 2.4.3 does not occur in an NnrR-deficient strain (15).

TABLE 4. β-Galactosidase activity of *R. sphaeroides* 2.4.1 containing pIT9 (*nnrR*) or pIT65 (*nnrR norB-lacZ*)[a]

SNP concentration (mM)	β-Galactosidase activity[b] in strain 2.4.1 containing:	
	pIT65 (*nnrR norB-lacZ*)	pIT9 (*norB-lacZ*)
0.0	32	14
0.5	558	561
1.0	757	605
2.0	2,014	1,336

[a] Cells were grown microaerobically in media lacking N oxides and then transferred to small vials. Then, after preincubation to reduce oxygen, SNP was added to the indicated levels. Cultures were incubated with SNP 2 h before β-galactosidase activity was determined.

[b] Activities (in Miller units) represent the averages of at least two separate cultures.

ACKNOWLEDGMENTS

We thank Steve Winans and Clay Fuqua for helpful discussions and donations of strains. We also thank Sam Kaplan for the *puc-lacZ* construct and for supplying data on *fnrL* in *R. sphaeroides* 2.4.1 before publication.

This work was supported by the Department of Energy (grant 95ER20206).

REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Anthamatten, D., B. Scherb, and H. Hennecke.** 1992. Characterization of a *fixLJ*-regulated *Bradyrhizobium japonicum* gene sharing similarity with the *Escherichia coli fnr* and *Rhizobium meliloti fixK* genes. J. Bacteriol. **174:**2111–2120.
3. **Arai, H., Y. Igarashi, and T. Kodama.** 1995. Expression of the *nir* and *nor* genes for denitrification of *Pseudomonas aeruginosa* requires a novel CRP-FNR-related transcriptional regulator, DNR, in addition to ANR. FEBS Lett. **371:**73–76.

4. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
5. **Coyne, M. S., and J. M. Tiedje.** 1990. Induction of denitrifying enzymes in oxygen-limited *Achromobacter cycloclastes* continuous culture. FEMS Microb. Ecol. **73:**263–270.
6. **Cuypers, H., and W. G. Zumft.** 1993. Anaerobic control of denitrification in *Pseudomonas stutzeri* escapes mutagenesis of an *fnr*-like gene. J. Bacteriol. **175:**7236–7246.
7. **Eraso, J. M., and S. Kaplan.** 1994. *prrA*, a putative response regulator involved in oxygen regulation of photosynthetic gene expression in *Rhodobacter sphaeroides*. J. Bacteriol. **176:**32–43.
8. **Fischer, H.-M.** 1994. Genetic regulation of nitrogen fixation in rhizobia. Microbiol. Rev. **58:**352–386.
9. **Frey, J., and H. M. Krisch.** 1985. Omega mutagenesis in Gram-negative bacteria: a selectable interposon which is strongly polar in a wide range of bacterial species. Gene **36:**143–150.
10. **Frías, J. E., A. Mérida, A. Herrero, J. Martín-Nieto, and E. Flores.** 1993. General distribution of the nitrogen control gene *ntcA* in cyanobacteria. J. Bacteriol. **175:**5710–5713.
11. **Keen, N. T., S. Tamaki, D. Kobayashi, and D. Trollinger.** 1988. Improved broad-host-range plasmids for DNA cloning in Gram-negative bacteria. Gene **70:**191–197.
12. **Kharitonov, V. G., A. R. Sundquist, and V. S. Sharma.** 1994. Kinetics of nitric oxide autooxidation in aqueous solution. J. Biol. Chem. **269:**5881–5883.
13. **Kokotek, W., and W. Lotz.** 1989. Construction of a *lacZ*-kanamycin-resistance cassette, useful for site-directed mutagenesis and as a promoter probe. Gene **84:**467–471.
14. **Körner, H., and W. G. Zumft.** 1989. Expression of denitrification enzymes in response to the dissolved oxygen level and respiratory substrate in continuous culture of *Pseudomonas stutzeri*. Appl. Environ. Microbiol. **55:**1670–1676.
15. **Kwiatkowski, A., and J. P. Shapleigh.** 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., in press.
16. **Lee, J. K., and S. Kaplan.** 1992. Isolation and characterization of *trans*-acting mutations involved in oxygen regulation of *puc* operon transcription in *Rhodobacter sphaeroides*. J. Bacteriol. **174:**1158–1171.
17. **Lee, J. K., P. J. Kiley, and S. Kaplan.** 1989. Posttranscriptional control of *puc* operon expression of B800-850 light-harvesting complex formation in *Rhodobacter sphaeroides*. J. Bacteriol. **171:**3391–3405.
18. **Leuking, D. R., R. T. Fraley, and S. Kaplan.** 1978. Intracytoplasmic membrane synthesis in synchronous cell populations of *Rhodopseudomonas sphaeroides*. J. Biol. Chem. **253:**451–457.
19. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
20. **Melville, S. B., and R. P. Gunsalus.** 1990. Mutations in *fnr* that alter anaerobic regulation of electron transport-associated genes in *Escherichia coli*. J. Biol. Chem. **265:**18733–18736.
21. **Merrick, M. J.** 1993. In a class of its own—the RNA polymerase sigma factor sigma-54 (sigma-N). Mol. Microbiol. **10:**903–909.
22. **Nunoshiba, T., T. DeRojas, J. S. Wishnok, S. R. Tannenbaum, and B. Demple.** 1993. Activation by nitric oxide of an oxidative-stress response that defends *Escherichia coli* against activated macrophages. Proc. Natl. Acad. Sci. USA **90:**9993–9997.
23. **Payne, W. J.** 1981. Denitrification. John Wiley & Sons, New York.
24. **Sawers, R. G.** 1991. Identification and molecular characterization of a transcriptional regulator from *Pseudomonas aeruginosa* PAO1 exhibiting structural and functional similarity to the FNR protein of *Escherichia coli*. Mol. Microbiol. **5:**1469–1481.
25. **Schmidt, H. H. H. W., and U. Walter.** 1994. NO at work. Cell **78:**919–925.
26. **Shapleigh, J. P., K. J. P. Davies, and W. J. Payne.** 1987. Detergent inhibition of nitric oxide reductase. Biochim. Biophys. Acta **911:**334–340.
27. **Sharrocks, A. D., J. Green, and J. R. Guest.** 1990. *In vivo* and *in vitro* mutants of Fnr, the anaerobic transcriptional regulator of *E. coli*. FEBS Lett. **270:** 119–122.

27a. **Shi, J., and J. Shapleigh.** Unpublished data.

28. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram negative bacteria. Bio/Technology **1:**784–791.
29. **Spiro, S.** 1994. The FNR family of transcriptional regulators. Antonie Leeuwenhoek **66:**23–36.
30. **Spiro, S., and J. R. Guest.** 1987. Regulation and over-expression of the *fnr* gene of *Escherichia coli*. J. Gen. Microbiol. **133:**3279–3288.
31. **Stewart, V., and J. Parales, Jr.** 1988. Identification and expression of genes *narL* and *narX* of the *nar* (nitrate reductase) locus in *Escherichia coli* K-12. J. Bacteriol. **170:**1589–1597.
32. **Tiedeman, A., and J. M. Smith.** 1988. *lacZY* gene fusion cassettes with *kan* resistance. Nucleic Acids Res. **16:**3587.
33. **Tosques, I. E., A. V. Kwiatkowski, J. Shi, and J. P. Shapleigh.** Characterization and regulation of the gene encoding nitrite reductase in *Rhodobacter sphaeroides* 2.4.3. Submitted for publication.
34. **Trumpower, B. L., and R. B. Gennis.** 1994. Energy transduction by cytochrome complexes in mitochondrial and bacterial respiration: the enzymology of coupling electron transfer reactions to transmembrane proton translocation. Annu. Rev. Biochem. **63:**675–716.
35. **van Spanning, R. J. M., A. P. N. DeBoer, W. N. M. Reijnders, S. Spiro, H. V. Westerhoff, A. H. Stouthamer, and J. van der Oost.** 1995. Nitrite and nitric oxide reduction in *Paracoccus denitrificans* is under the control of NnrR, a regulatory protein that belongs to the FNR family of transcriptional activators. FEBS Lett. **360:**151–159.
36. **Vega-Palas, M. A., F. Madueño, A. Herrero, and E. Flores.** 1990. Identification and cloning of a regulatory gene for nitrogen assimilation in the cyanobacterium *Synechococcus* sp. strain PCC 7942. J. Bacteriol. **172:**643–647.
37. **Wu, L. F., and M. H. J. Saier.** 1991. Differences in codon usage among genes encoding proteins of different function in *Rhodobacter capsulatus*. Res. Microbiol. **142:**943–950.
38. **Ye, R. W., D. Haas, J.-O. Ka, V. Krishnapillai, A. Zimmermann, C. Baird, and J. M. Tiedje.** 1995. Anaerobic activation of the entire denitrification pathway in *Pseudomonas aeruginosa* requires Anr, an analog of Fnr. J. Bacteriol. **177:**3606–3609.
39. **Zeilstra-Ryalls, J. H., and S. Kaplan.** 1995. Aerobic and anaerobic regulation in *Rhodobacter sphaeroides* 2.4.1: the role of the *fnrL* gene. J. Bacteriol. **177:**6422–6431.
40. **Zumft, W. G.** 1993. The biological role of nitric oxide in bacteria. Arch. Microbiol. **160:**253–264.