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Characterization of the Nitric Oxide Reductase-Encoding Region in *Rhodobacter sphaeroides* 2.4.3

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A gene cluster which includes genes required for the expression of nitric oxide reductase in *Rhodobacter sphaeroides* 2.4.3 has been isolated and characterized. Sequence analysis indicates that the two proximal genes in the cluster are the Nor structural genes. These two genes and four distal genes apparently constitute an operon. Mutational analysis indicates that the two structural genes, *norC* and *norB*, and the genes immediately downstream, *norQ* and *norD*, are required for expression of an active Nor complex. The remaining two genes, *nnrT* and *nnrU*, are required for expression of both Nir and Nor. The products of *norCBQD* have significant identity with products from other denitrifiers, whereas the predicted *nnrT* and *nnrU* gene products have no similarity with products corresponding to other sequences in the database. Mutational analysis and functional complementation studies indicate that the *nnrT* and *nnrU* genes can be expressed from an internal promoter. Deletion analysis of the regulatory region upstream of *norC* indicated that a sequence motif which has identity to a motif in the gene encoding nitrite reductase in strain 2.4.3 is critical for *nor* operon expression. Regulatory studies demonstrated that the first four genes, *norCBQD*, are expressed only when the oxygen concentration is low and nitrate is present but that the two distal genes, *nnrTU*, are expressed constitutively.

Denitrification is the reduction of nitrate (NO_3^-) to gaseous intermediates, principally nitrogen gas. Nitric oxide (NO), an obligatory intermediate during denitrification, is generated from the one-electron reduction of nitrite (NO_2^-) (41). NO reduction is coupled to energy generation (18, 29). NO is also a well-known cytotoxic compound, so its production during denitrification has the potential of causing significant cell damage. To mitigate the toxicity of NO, its steady-state concentration during denitrification is maintained at low-nanomolar levels (11). The protein responsible for NO reduction, NO reductase (Nor), catalyzes the reaction $2NO + 2H^+ \rightarrow N_2O + H_2O$. Nitrous oxide ($N_2O$) is an inert, nontoxic intermediate that is frequently the terminal product of denitrification (42). Nor has been purified and shown to be a heterodimeric membrane protein (9, 14, 16). Metal analysis has shown that it contains only iron in stoichiometric amounts. Recently, the Nor structural genes have been characterized, and sequence analysis revealed that Nor was related to the cytochrome *c* oxidase superfamily (36). In particular, Nor is most closely related to the heme *b*-containing oxidases, which are expressed under conditions of low oxygen concentration. It has been suggested that Nor was the original member of this family and that the other members arose by modifying the Nor structure (26).

The genetic organization of the region of the chromosome encoding the *nor* structural genes varies among denitrifiers. In *Pseudomonas stutzeri*, the two structural genes form a distinct transcriptional unit (43). In *Pseudomonas aeruginosa*, the two structural genes and a third gene, encoding a product of unknown function, apparently form an operon (3). The nonstructural gene in the *P. aeruginosa* operon is also found immediately downstream of the *nor* operon in *P. stutzeri*, but in the latter denitrifier it is independently transcribed. In *Paracoccus denitrificans*, the *nor* operon apparently consists of six genes, which include homologs of the three genes in the *P. aeruginosa* operon (8). Regulation of *nor* expression appears to be under the control of the *nnrR* product in *P. denitrificans* and *Rhodobacter sphaeroides* 2.4.3 and an apparent functional homolog of NnrR in *P. aeruginosa* (2, 35, 37). NnrR, which is a member of the Fnr/Crp family of transcriptional regulators, regulates both Nor and NO_2^- reductase (Nir) expression but does not appear to be involved in regulation of other aspects of denitrification. In *R. sphaeroides* 2.4.3, expression of Nir and Nor is dependent on Nir activity, suggesting that NO production is critical for expression of proteins required for NO metabolism (34). Recent work has provided additional evidence that NO is an effector for expression of *nirK*, the Nir structural gene, and *nor* (20).

The coding properties and genetic organization of the *R. sphaeroides* 2.4.3 *nor* operon and a phenotypic analysis of *nor* mutants are described in this paper. Sequence analysis suggests that the *nor* operon consists of six open reading frames (ORFs), the first two of which are the *nor* structural genes. Mutational analysis indicates that the first four genes in the operon are required for production of an active Nor complex. The last two genes are apparently required for expression of both Nir and Nor and have been designated *nnrT* and *nnrU*, respectively. The *nnr* designation was developed by Van Spanning to indicate genes whose products appear to be involved in expression of Nir and Nor (37). *nnrT* and *nnrU* can be expressed from an internal promoter, since insertional inactivation within the first four genes does not cause phenotypic changes consistent with loss of expression of the two downstream genes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *Escherichia coli* DH5α was used as a maintenance strain for plasmids. *E. coli* S-17-1 was used as a donor for matings (30). *R. sphaeroides* 2.4.3 (ATCC 17025) is the wild-type strain. Strain 15.12 is a Nor-deficient mutant of 2.4.3 described previously (20). Plasmid pT7/T3-18U or 19U (Bethesda Research Laboratories) was used for cloning as well

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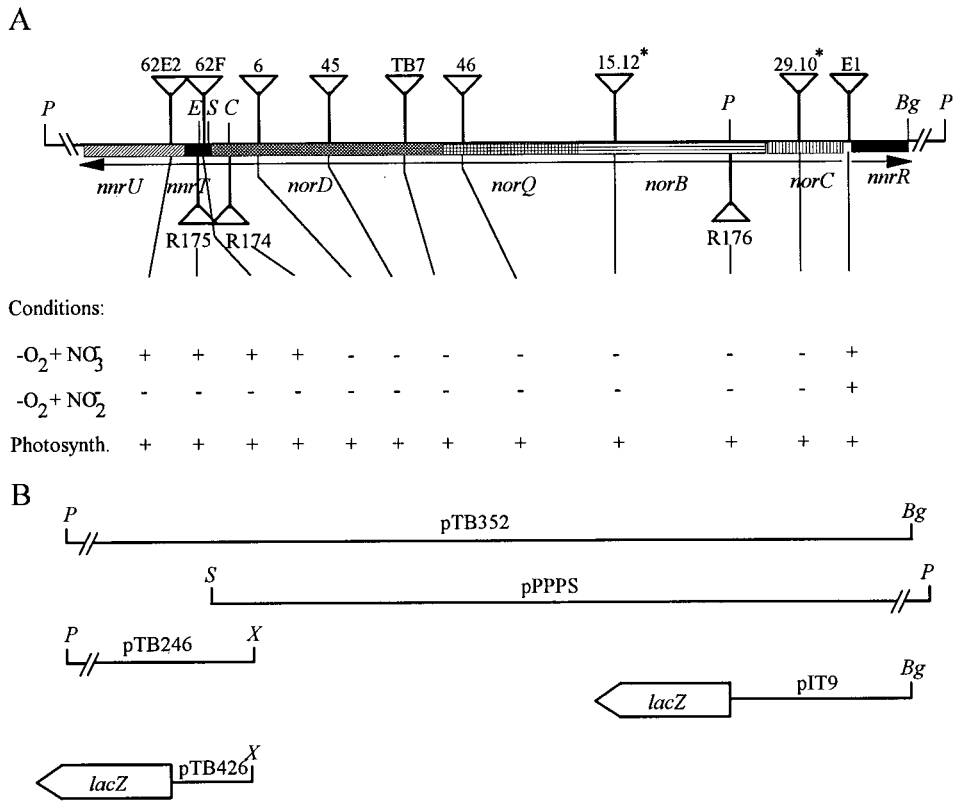

FIG. 1. (A) Schematic representation of the region of the chromosome of *R. sphaeroides* 2.4.3 containing the *nor* operon and *nnrR* and phenotypes resulting from insertional inactivation of particular genes. Boxes and arrows indicate the locations and orientations of deduced ORFs. The site of insertion in the various mutants is indicated by a triangle above the appropriate ORF. Strains containing Tn insertions are those with triangles above the ORFs, and among these, those with Tn*5* (Tp^r) are indicated by an asterisk; all others contain Tn-*lacZ*. Strains with Ω interposon insertions are indicated by triangles below the ORFs. The capacity to grow (+, wild-type growth; −, no growth) under different conditions is shown for each strain. *E*, *S*, *C*, *P*, and *Bg*, locations of some of the *Eco*RI, *Sph*I, *Cla*I, *Pst*I, and *Bgl*II sites, respectively, within this region. (B) DNA fragments used to generate important plasmid constructs. The fragments are aligned below the *nor* region shown in panel A. The designation given to constructs is shown above the corresponding fragment.

as expression of single-stranded DNA in *E. coli*. Plasmid pRK415, a broad-host-range plasmid, was used for transferring genes from *E. coli* to *R. sphaeroides* (17). Plasmids pSUP202 and pJP5603 were used as suicide vectors (25, 30). *E. coli* strains were grown in Luria-Bertani medium (22). *Rhodobacter* strains were grown in Sistrom's medium at 30°C. Procedures for growing wild-type and mutant *R. sphaeroides* strains anaerobically and microaerobically are described elsewhere (35).

DNA manipulation and sequencing. Chromosomal DNA was isolated from strain 2.4.3 by using the Puregene system (Gentra Systems). Plasmid isolations were done by the alkaline lysis method (6). Standard methods were used for restriction digests, agarose gel electrophoresis, and ligations. Southern hybridizations were carried out as described previously (33). Transformations were done with TSS (7). Plasmids were moved into 2.4.3 by conjugation. Biparental matings were carried out with *E. coli* S17-1 as the donor.

DNA sequencing was done with 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 (Amersham). Both strands of the region encoding *nor* were sequenced by using several different strategies, including making subclones for sequencing with the M13 reverse-sequencing primer, using custom primers to sequence gaps, and sequencing clones derived from partial *Sau*3AI digests with the M13 reverse-sequencing primer. Database searches of sequences were performed with the BLAST programs (1).

The *nor* operon region was isolated from a lambda library containing inserts of strain 2.4.3 DNA by using a 500-bp *Kpn*I-*Bgl*II fragment from *norB* of *P. stutzeri* as a heterologous probe essentially as described previously (28). A 5.9-kb *Pst*I fragment, containing most of the *nor* operon, was isolated from the lambda library and sequenced. The 5′ end of the operon was isolated on a 3.7-kb *Pst*I fragment from strain R98. R98 contains an insertion of an Ω interposon into the *nor* operon. Chromosomal DNA from R98 was digested with *Sph*I and cloned into pT7/T3-18U. By selecting for Sp^r, a clone which contained approximately 6.0 kb of DNA upstream of *norC*, including the 3.7 kb *Pst*I fragment, was isolated. The 3.7-kb *Pst*I fragment and a 3.7-kb *Pst*I-*Sph*I fragment from the 6.0-kb fragment were ligated together to generate pPPPS (Fig. 1B). A 1.3-kb *Bgl*II-*Pst*I fragment from the 3.5-kb *Pst*I fragment and the entire 5.9-kb *Pst*I fragment were ligated to generate pTB352. A 3.4-kb *Xho*I-*Pst*I fragment from the 5.9-kb *Pst*I fragment was used to generate pTB246. The construction of the *norB-lacZ* transcriptional fusion plasmid pIT9 has been previously described (35).

The strategy used to generate strains 29.10 and 15.12, which contain Tn*5* (Tp^r) insertions, was described by Tosques et al. (35). To generate the transposon (Tn) insertion used to construct *R. sphaeroides* strains containing Tn-*lacZ* (23), either the 5.9-kb *Pst*I fragment or the 3.7-kb *Pst*I fragment was mutagenized by being placed in *trans* in *E. coli* CC170, which contains a single chromosomal copy of Tn-*lacZ* (24). By selection for enhanced Kn^r, cells in which the Tn-*lacZ* had moved to the plasmid were isolated. Restriction mapping was used to localize the site of Tn insertion. The sites of insertion of the Tn in strains E1, 46, 62F, and 62E2 were confirmed by sequence analysis. Strains R176, R174, and R175 were constructed by insertion of an Ω interposon into the *Pst*I site in *norB*, the *Cla*I site in *norD*, and the *Eco*RI site in *nnrT*, respectively (Fig. 1A).

Deletion analysis of the regulatory region upstream of *norC* was carried out by using PCR to generate fragments of DNA of appropriate sizes and with useful restriction sites. The following oligonucleotides, together with the downstream primer 5′-GGCCGTCGACGCTATGAAGAAGAGCGATCCC-3′, which corresponds to residues +68 to +48 downstream of the putative translation start of *norC*, were used as upstream primers to generate the fragments used in the construction of, in order, pIT78, pIT77, pIT73, and pIT76: 5′-GGGGTACCCCGGCCAGCGGTCGAAGAGCC-3′, 5′-GGGGTACCTTCTTTGTGATCCCGCAACCTC-3′, 5′-GGGGTACCCCTCTCCGGCGCCGGCGCGGGCTAGAGGAG-3′, and 5′-GGGGTACCGATCCCGCAACCTCTCCGGCG-3′. The underlined regions indicate portions of the oligonucleotide containing a GC cap and restriction site. Appropriate fragments were initially cloned into pT7-18U and then cloned into pRK415, along with the *lacZ*-Kn^r cassette from pKOK6 (19), to generate the *norC-lacZ* fusion plasmids.

Primer extension. RNA isolation and primer extension of mRNA were performed as previously described (35) except that a synthetic oligonucleotide, 5′-GGTGGAAACCACATAATTGTG-3′, which is complementary to the coding strand in positions +97 to +117 relative to the putative start of *norC* translation, was used in the primer extension assay.

Assays for enzymatic activities. β-Galactosidase activities were determined for at least three independently grown cultures as previously described (35). Standard deviations were never larger than 15% of the reported value for any assay. Cells removed from stoppered flasks were not kept under anaerobic conditions but were used immediately for assays. Assays for Nir activity and the presence of NO_2^- in the culture medium were carried out as described by Tosques et al. (34). To assay β-galactosidase and Nir activities, samples were taken at various times during the growth cycle. The reported activities are the maximal values obtained before cells stopped growing. Nor activity was assayed gas chromatographically by measuring the production of N_2O (27).

Nucleotide sequence accession numbers. The sequence coding for the relevant regions of the *nor* operon (Fig. 2) has been submitted to the GenBank database (accession no. AF000233).

RESULTS

Characterization of the region encoding the *nor* genes. By using a fragment of *norB* from *P. stutzeri*, a 5.9-kb *Pst*I fragment was isolated from a lambda library containing fragments of strain 2.4.3 DNA, and sequence analysis showed that it contained part of an ORF with significant homology to *norB* from *P. stutzeri*, along with a number of other ORFs (Fig. 1A). By taking advantage of strain R98, which contains an Ω interposon in a region downstream of *norB*, a 3.7-kb *Pst*I fragment encoding the remaining portion of *norB* and all of *norC* was isolated. Subsequent characterization of the 3.7-kb *Pst*I fragment has shown that it also encodes *nnrR*, which is critical for *nor* and *nirK* expression (39). Analysis of the sequence downstream from the 3′ end of *nnrR* indicates that there are six ORFs that appear to constitute an operon (Fig. 1A). In order, the genes in this region have been designated *norC*, *norB*, *norQ*, *norD*, *nnrT*, and *nnrU*. The organization of these six genes into a multicistronic unit is inferred from the short distances between all six ORFs (Fig. 2). The largest distance between ORFs is a 13-bp gap between *norC* and *norB*. The putative termination codons and translation start sites of *norB* and *norQ*, as well as *nnrT* and *nnrU*, overlap (Fig. 2).

The G+C content for the 5,984 bp that comprises this region is 68%, which is similar to the chromosomal average for *R. sphaeroides*. There is a Shine-Dalgarno sequence immediately preceding *norC* (Fig. 2). Centered at position −69.5 relative to the putative start of translation of *norC* is the sequence 5′-TTGTG(N_4)CGCAA-3′, which has similarity to the consensus binding site sequence of the transcriptional activator Fnr (Fig. 2) (32). Preceding the translation start site of *nnrT*, and within *norD*, is a putative Shine-Dalgarno sequence (Fig. 2). Sequence analysis did not identify any other regulatory sequences, including possible transcriptional terminators, between the putative translation start of *norC* and the putative translation termination codon of *nnrU*. Inverted repeats that could function as transcriptional terminators are found between *nnrR* and the *nor* operon and also between *hemN* and *regX* (5). *hemN* is transcribed in the same direction as the *nor* operon, and its putative start of translation is 74 bp from the end of *nnrU*. *regX* is divergently transcribed from *hemN*, and its putative translation termination site is 49 bp from the end of *hemN*.

Features of the ORFs in the *nor* operon. *norC* is 444 bp in length and encodes a product of 147 amino acid residues with a molecular mass of 16.3 kDa. The deduced primary sequence of *norC* has greater than 45% identity with translated sequences of *norC* genes from other denitrifiers. Hydropathy analysis suggests that NorC has one membrane-spanning region near its N terminus. The deduced primary sequence contains a CXXCH motif, indicating that NorC is a cytochrome *c* binding protein.

norB is 1,341 bp in length and encodes a product of 446 residues with a molecular mass of 49.7 kDa. Hydropathy plots suggest that NorB contains 12 membrane-spanning helices. Sequence alignments reveal significant identity between the deduced *norB* primary sequence and *norB* products from other denitrifiers as well as cytochrome *c* oxidases from various bacteria. In particular, there are six histidine residues that are conserved in alignments of NorB with subunit I of cytochrome *c* oxidases (data not shown). These histidine residues have been shown to be ligands of the prosthetic groups associated with subunit I (13), and their conservation suggests that they fulfill a similar function in NorB.

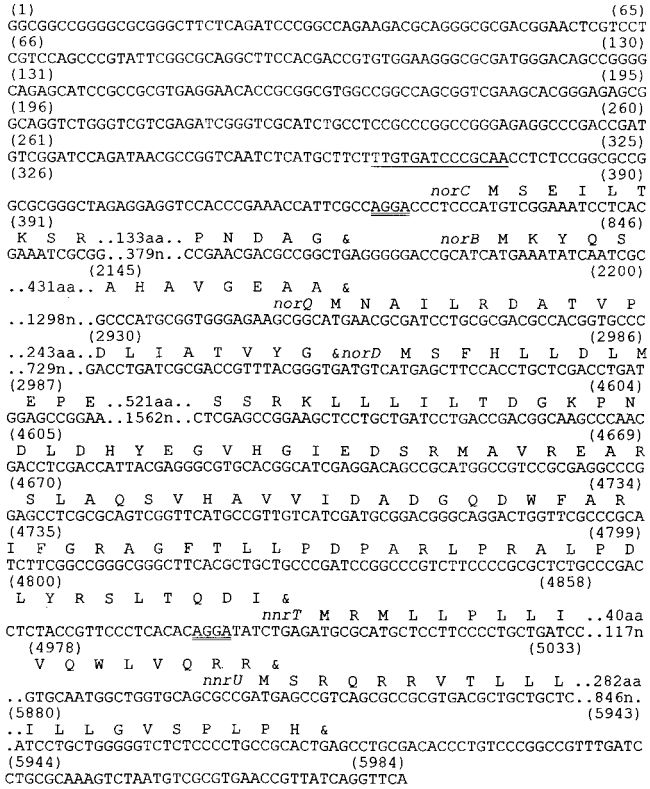

FIG. 2. Nucleotide and deduced primary amino acid sequences of relevant regions of the *nor* operon. The sequence includes DNA upstream of the operon, intergenic regions, and the region between the putative transcription stop of *nnrU* and the translation start of *hemN*. The length of sequence (n, nucleotides; aa, amino acids) omitted within each ORF is indicated. The numbers in parentheses are derived from the complete *nor* sequence. An extended sequence upstream of the *norD-nnrT* intergenic region is indicated because it likely contains sequences critical for *nnrT* regulation. The underlined region indicates sequence having identity to the recognition sequence of Fnr. Shine-Dalgarno sequences are indicated by double underlines.

norQ is 789 bp in length and encodes a product of 262 residues with a molecular mass of 28.3 kDa. The *norQ* product is a largely hydrophilic, presumably cytoplasmic protein of unknown function. Sequence analysis indicates that NorQ contains two putative nucleotide binding motifs, as found in other NorQ homologs (15). In other denitrifiers, *norQ* homologs are always present near the region which encodes Nor, but not always as part of the *nor* operon. In the pseudomonads, *norQ* is located near *norCB* in a region containing genes whose products are required for Nir assembly (4, 15). Interestingly, the *norQ* product of strain 2.4.3 has 54% identity with the *cbbQ* product from *Pseudomonas hydrogenothermophila*, which is not required for denitrification (40). Little is known about the physiological role of the *cbbQ* product, but its similarity to NorQ indicates that NorQ belongs to a family of proteins not exclusively required for denitrification.

norD is 1,869 bp in length and encodes a protein of 622

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NnrT
1          10         20         30         40         50
MRMLPLLILV PGIAMAATFD VPTPAAQTAA AEFWFAVAAG SFVVALAAVQ WLVQRR

NnrU
1          10         20         30         40         50
MSRQRRVTLL LYPFGAGART VNLFFASLIL SWVGLPVLSP AWSVLGGMML GIPASHLFAG
HIVRLMERAE TRLDRLDRIC GGACGLLRIA PAAGAARRAR AADRPSGPRL YFALYGTVSL
LVLAWVIVAA GRAPYVELWP QTPAPRWVPN VTSPIFWTLV VLGIRLPWPW TLGGQRAARF
DPDRPGFAAV TRHPLLIALM LWSFAHLFPN GDLAHVILFG SFFGLSLAAI PMFDARARRA
LPHCEAWDAF RATSIFSLRP LLNPEWLKQG EDHLIPRLLA IVAFWATALA LHPILLGVSP
LPH
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FIG. 3. Deduced amino acid sequences of the *nnrT* and *nnrU* gene products.

FIG. 4. Primer extension analysis of the *nor* operon. Total RNA was isolated from cells grown microaerobically in the presence of nitrate. The primer was a 21-mer that was complementary to sequence 97 bp after the first base of the putative *norC* translation start. The reaction product was electrophoresed on a sequencing gel next to a DNA ladder produced by sequencing with the primer used in the extension reactions.

residues with a molecular mass of 68.5 kDa. In all of the denitrifiers characterized to date, the *norD* ORF is adjacent to *norCB* genes. NorD, like NorQ, appears to be cytoplasmically located, due to its hydrophilic nature and the lack of a secretory presequence. The *norD* product has no obvious similarity with other sequences in the database, aside from the *norD* homologs of other denitrifiers. There are no obvious sequence motifs in the deduced NorD sequence. The only distinguishing feature of NorD is that it contains 21% arginine residues by weight, which is an unusually high percentage for a cytoplasmic protein.

The last two ORFs in the putative *nor* operon encode products without obvious homologs in other denitrifiers. The codon usage of *nnrT* strongly reflects the high G+C bias of the *R. sphaeroides* genome, consistent with it being a transcribed ORF. It encodes a putative protein of 56 residues with a molecular mass of 6.1 kDa (Fig. 3). The *nnrT* product has a high percentage of hydrophobic residues, and hydropathy analysis suggests that it contains two membrane-spanning helices (data not shown). Sequence analysis of other denitrifiers has identified ORFs in close proximity to the *nor* region that encode small hydrophobic proteins. For example, ORF3 in *P. aeruginosa* encodes a product of 85 residues (4), and *norF* from *P. denitrificans* encodes a product of 77 residues (8). Neither of these gene products has any significant similarity to the *nnrT* product.

nnrU is 912 bp in length and encodes a product that is 303 residues long and has a molecular mass of 33.4 kDa (Fig. 3). The *nnrU* product, like that of *nnrT*, is hydrophobic, and hydropathy analysis suggests that it has seven transmembrane-spanning regions (data not shown). The *nnrU* product has no significant similarity with the ORF2 product from *P. aeruginosa* (4) or with the *norE* product of *P. denitrificans* (8); both of these are hydrophobic proteins similar to the subunit III of cytochrome *c* oxidases and have been suggested to be important for Nor function. Sequence comparison with the entire database also failed to yield any proteins with significant identity to the *nnrU* product. Neither the *nnrT* nor the *nnrU* product has recognizable structural motifs that might indicate the role they play during denitrification.

Primer extension analysis. To determine the transcriptional start of the *nor* operon, total RNA was isolated from cells grown microaerobically in medium amended with NO_3^-. By using a primer that hybridizes to the 5′ end of *norC*, primer extension on the isolated RNA was carried out. Under these conditions, only one transcript was identified (Fig. 4). The 5′ end of this transcript is located 27 bp upstream of the putative start of translation and 42.5 bp downstream of the putative activator binding site. In both the *nor* operon and *nirK* regulatory regions, the spacing between the putative activator binding site and the transcript start is about 40 bp (Fig. 5). This distance is typical of genes whose expression is dependent on activators in the Fnr family (38). There are other conserved sequences in the alignment that may be binding sites of RNA polymerase.

Expression of the *nor* operon. Studies of the expression of the *nor* operon by using a *norB-lacZ* transcriptional fusion plasmid have been previously reported (20). As expected, *nor* is maximally expressed under microaerobic conditions in the presence of NO_3^-. Nearly identical levels of expression were found under all culture conditions with a *norQ-lacZ* transcriptional fusion plasmid (data not shown). To confirm that the putative promoter sequence centered at position −42.5 relative to the start of transcription is critical for *nor* expression, a series of fusion constructs that contained different lengths of upstream DNA was made. The wild-type reference was the *norB-lacZ* construct used in previous studies, which contains 521 bp upstream of the start of transcription. Constructs containing 82 and 177 bp upstream of the transcript start showed expression comparable to that of the wild type (Table 1). A construct with 51 bp upstream, which includes the entire promoter sequence but no additional upstream DNA, showed only 3% of the wild-type activity. Constructs with less than 51 bp of upstream sequence showed less than 1% of wild-type activity.

Mutagenesis of *nor* operon region. Previous studies have shown that insertional inactivation of *norB* results in the loss of Nor activity and prevents anaerobic growth on NO_3^- or NO_2^- (21). To assess the functions of the other genes in the *nor* operon, Tn mutagenesis was used to introduce insertions throughout the entire *nor* region. Figure 1A shows the phenotypes generated by Tn insertion in a particular gene. There were two distinct phenotypes observed in strains carrying mutations in the *nor* operon. Insertion in the *norC*-through-*norD* region prevented anaerobic growth with either NO_3^- or NO_2^-

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      5'
rsnir AGCTTGTTGTGGCAACCGCAAACTCCGGCCTCTCCAGAGGATCTACCGATCGGGTCATAGG
rsnor GCTTCTTTTGTGATCCCGCAACCTCTCCGGCGCCGGCGCGGGCTAGAGGAGGTCCACCCGA
                                                              3'
rsnir CATCATCCATCGAAGGAGAGAACCCGATG
rsnor AACCATTCGCCAGGACCTCCCATG
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FIG. 5. Alignment of regulatory regions of *nirK* (rsnir) and the *nor* operon (rsnor) from *R. sphaeroides* 2.4.3. Regions with similarity to the Fnr recognition sequence are underlined. The direction and transcription start site for each sequence are indicated by arrows. The ATG at the end of each sequence is the putative start of translation.

TABLE 1. Expression of β-galactosidase activity from transcriptional fusion plasmids containing different lengths of upstream DNA

Construct	β-Galactosidase activity[a]	
	Miller units	% of wild-type activity
pIT9 (wild type)	951	100.0
pIT78 (−177)[b]	913	96.0
pIT85 (−82)	913	96.0
pIT77 (−51)	25	3.0
pIT76 (−43)	10	1.0
pIT73 (−33)	2	0.2

[a] Activities were determined in duplicate for at least three independently grown samples. Strains were grown microaerobically in Sistrom's medium amended with 12 mM nitrate. There is less than 5 U of activity in the wild type with vector alone.

[b] Numbers in parentheses represent the distance (in nucleotides) upstream of the start of transcription of *norC*. The sequence with similarity to the Fnr recognition sequence spans nucleotides −51 to −37.

as the sole terminal electron acceptor. In contrast, strains carrying mutations in *nnrT* or *nnrU* could grow anaerobically with NO_3^- but not with NO_2^-. Strains with mutations in the *nnrT* or *nnrU* region had no detectable Nir activity under any growth conditions (data not shown). One strain, E1, which contained a Tn insertion located at the extreme 3′ end of *nnrR*, had a wild-type phenotype. The phenotype of E1 indicates that *nnrR* and the *nor* operon do not form a transcriptional unit.

It has recently been shown that an unexpected consequence of *norB* inactivation is that *norB* mutants can induce *nirK-lacZ* expression in unamended Sistrom's medium 10-fold higher than wild-type cells grown under the same conditions (20). This high-level induction in unamended medium provides a useful assay for assessing the ability of a cell to express Nir but not Nor, since the induction is a consequence of NO accumulation. The expression of *nirK-lacZ* in unamended medium in various mutant strains is shown in Table 2. Insertional inactivation of *norB*, *norQ*, and *norD* conferred the ability to induce high-level *nirK-lacZ* expression in unamended medium. In contrast, inactivation of *nnrU* or *nnrT* reduced the expression of *nirK-lacZ* in unamended medium to near-background levels, as also observed for *nirK* mutants (34).

It has previously been shown that Nir activity is required for maximal expression of *nirK-lacZ* and *norB-lacZ* in wild-type cells grown microaerobically in medium amended with NO_3^- (34). Under microaerobic conditions in medium amended with NO_3^-, *nirK-lacZ* expression was about 3% of the wild-type level in both 62E2 and 62F. *norB-lacZ* expression was only about 4% of the wild-type level in both strains. These levels are similar to those measured in a *nirK* mutant (34). It can also be seen that *norB*, *norQ*, and *norD* mutants exhibit reduced levels of *nirK-lacZ* expression in amended medium (Table 2). However, since *nirK-lacZ* expression is observed in unamended medium (Table 2), the decrease in expression is probably due to NO toxicity. This conclusion is supported by the early cessation of cell growth and eventual cell lysis seen in *nor* mutants grown microaerobically in amended medium (20).

TABLE 2. Expression of β-galactosidase from either *nirK-lacZ* or *norB-lacZ* fusions in various mutant strains grown under different conditions[a]

Strain	β-Galactosidase activity (Miller units) from:		
	nirK-lacZ		*norB-lacZ* (amended medium)
	Amended medium	Unamended medium	
2.4.3	2,427	411	1,184
R176	243	2,636	ND[b]
15.12	285	5,118	239
46	453	5,369	321
6	325	2,164	384
R174	231	42	ND
62F	157	44	72
62E2	243	56	78

[a] Cells were grown microaerobically in medium amended with 12 mM NO_3^- or in unamended medium. See Fig. 1 for the locations of the mutations in each strain.

[b] ND, assay not performed.

The phenotypic differences between strains carrying mutations in the *norC*-through-*norD* region and strains with *nnrT* or *nnrU* mutations demonstrate that these regions encode genes whose products have markedly different roles during denitrification. *nnrU* and perhaps *nnrT* are required for expression of both *nirK* and *nor*, while *norC* through *norD* appear to be required only for the expression of an active Nor complex. The observation that insertions in this region can give rise to two distinct phenotypes also indicates that *nnrT* and *nnrU* can be expressed independently of the four preceding genes. If there were only one regulatory region controlling expression of all six genes, an insertion in *norB*, for example, should also inactivate *nnrT* and *nnrU* because of polarity. A lack of polar effects can be seen by comparing *nirK-lacZ* expression in strain 15.12 grown microaerobically in unamended medium with *nirK-lacZ* expression in 62E2 grown under identical conditions (Table 2). It is possible that the phenotypic differences arise because the Tn insertions are not being completely polar. To test this, strongly polar Ω interposons were used to introduce insertions in several regions of the *nor* operon, and the resulting mutants were grown under various conditions. Strain R176, which carries an interposon in *norB*, was phenotypically identical to strains with Tn insertions in *norB* (Fig. 1A). In particular, R176 was unable to grow with NO_3^-, and when grown microaerobically in unamended medium, it was able to induce high-level expression of *nirK-lacZ* (Fig. 1A; Table 2). This phenotype indicates that expression of *nnrT* and *nnrU* is not eliminated when upstream genes are insertionally inactivated.

Insertion of an interposon into a *Cla*I site 129 bp from the 3′ end of *norD* did not result in a phenotype identical to that seen with Tn mutations in *norD*, however. Strain R174, containing the interposon in the *Cla*I site, could grow anaerobically with NO_3^- and exhibited only background levels of *nirK-lacZ* expression under all growth conditions (Fig. 1A; Table 2). One significant difference between R174 and strains with Tn insertions in *norD* is that all Tn insertions mapped upstream of an *Xho*I site 272 bp from the putative translation start of *nnrT*. Given that the R176 phenotype confirms polarity of the Tn insertions, it seems likely that the phenotype of R174 is a consequence of the location of the insertion close to the putative translation start of *nnrT*. Strain R175, which contains an Ω interposon in *nnrT*, was phenotypically identical to 62F (Fig. 1A).

Complementation analysis. If the *nnrT* and *nnrU* genes are expressed from an internal promoter, it should be possible to complement mutations in the *nor* genes by supplying the *norC*-through-*norD* region in *trans*. Strains 46, containing an insertion in *norQ*, and R176, containing a *norB* insertion, could be restored to wild type by pPPPS, which does not encode *nnrT* or *nnrU*. This is consistent with the conclusion that *nnrT* and *nnrU* can be expressed from a promoter within the *nor* operon.

Similar experiments were done to determine if *nnrT* and *nnrU* mutants could be complemented with fragments lacking the upstream *nor* genes. Unexpectedly, strains 62E2, 62F, and

R175 could not be complemented with any fragment. pTB246, which contains 272 bp upstream of *nnrT*, could not restore the ability of 62E2 or 62F to grow with NO_2^- as the terminal electron acceptor. A similar result was obtained with pTB352, which contains all of the genes in the *nor* operon. The presence of pTB352 in strain 46 restored the capacity to grow anaerobically with NO_3^- and NO_2^-, demonstrating that this construct is capable of complementing *norQ* mutants but not *nnrT*- or *nnrU*-deficient mutants.

Expression of *nnrU-lacZ*. The lack of polarity of insertions in *norB* through *norD* suggests that there is a promoter internal to the *nor* operon that is used for expression of *nnrT* and *nnrU*. To evaluate the regulation of *nnrT* and *nnrU* independent of the *norC* regulatory region, an *nnrU-lacZ* transcriptional fusion plasmid was constructed. The upstream DNA in this construct is the 272 bp between the putative start of *nnrT* translation and the *Xho*I site in *norD*. Expression of *nnrU-lacZ* was monitored under different conditions, and 930 U of activity was measured in aerobically cultured cells. Expression decreased 31%, to 639 U, in cells cultured under microaerobic conditions in unamended medium. Inclusion of NO_3^- in microaerobic cultures decreased expression to 334 U. These data demonstrate that transcriptional activation of *nnrT* and *nnrU* can be achieved without the regulatory region upstream of *norC*, as indicated by phenotypic analysis.

DISCUSSION

A region of the *R. sphaeroides* 2.4.3 chromosome containing six genes has been characterized and found to encode products required for denitrification. By generating a series of mutants with mutations in this region, we have been able to show that four of these genes, *norC*, *norB*, *norQ*, and *norD*, are specifically required for the production of an active Nor complex. Nor-deficient strains have unique phenotypes that readily distinguish them from strains having mutations in genes required for expression of both Nir and Nor. These four genes, which when inactivated give rise to Nor-deficient phenotypes, have homologs in other denitrifiers. *norC* and *norB* are structural genes. *norC* encodes a cytochrome *c*-containing subunit responsible for transferring electrons to the *norB*-encoded protein, which, by its homology to subunit I of cytochrome *c* oxidases, likely contains the active site (43). The physiological roles of the *norQ* and *norD* products have not been elucidated, but the phenotypic similarity of the *norC* and *norB* mutants to the *norQ* and *norD* mutants demonstrates that the latter two genes encode products whose function appears to be limited to the production of an active Nor complex.

Current evidence suggests that *norQ* might be involved in some aspect of Nor assembly. In *P. stutzeri*, inactivation of the *norQ* homolog, which is designated *nirQ* because of its proximity to other *nir* genes, results in the expression of an inactive Nor complex (15). Studies with cytochrome *c* oxidase have shown that the absence of cofactors critical for oxidase activity also results in expression of an inactive protein (12). If *nirQ* is involved in regulation of *nor* expression, as originally suggested, it seems unlikely that Nor would be expressed. The lack of effect on expression, but absence of activity, is more consistent with the absence of some factor necessary for Nor activity. The phenotypic effects caused by *nirQ* inactivation in *P. stutzeri* are also important because *nirQ* is not part of the *nor* operon. In strain 2.4.3 the phenotypic effects of *norQ* inactivation could have arisen due to inactivation of *norD*. However, it is obvious that *norQ* homologs have an essential role in producing an active Nor complex. We have recently found that inactivation of *norQ* in strain 2.4.3 does not prevent Nor expression (37a). It is also possible that the *norD* product is involved in assembly, given its obligatory linkage with the *nor* structural genes and the observation that its inactivation results in a loss of Nor activity.

The organization of the four genes exclusively required for Nor activity in *R. sphaeroides* is identical to that of the first four genes of the *nor* operon in *P. denitrificans* (9). Sequence alignments of the *norCBQD* gene products from these two bacteria also show high similarity. This similarity is consistent with their close phylogenetic relationship (39). It is interesting, however, that similarity in gene organization or in primary sequence does not extend to include the regions downstream of the *norD* gene. In *P. denitrificans norD* is followed by *norE*, which encodes a protein with similarity to subunit III of cytochrome *c* oxidases (9). Based on the similarity of Nor and the oxidases, it has been suggested that the Nor complex might contain a third subunit, which is encoded by *norE*. It is frequently observed that the subunit III of oxidases is lost during purification (10, 31), which might explain why Nor purifies as a dimer. However, the conclusion that Nor is a three-subunit complex is not supported by our characterization of the *nor* region in strain 2.4.3. No gene that encodes a protein related to subunit III in oxidases was found. The *nnrU* product of 2.4.3 is somewhat similar in size to the *norE* product, and it is probably a membrane-associated protein, but sequence comparisons did not identify any protein in the database with significant similarity to this protein. Moreover, insertional inactivation of *nnrU* prevents expression of *nirK* and *nor*, which is not the phenotype expected of a Nor-deficient strain.

The phenotypic differences between strains containing mutations in *norB* through *norD* and those containing mutations in the *nnrT* and *nnrU* region are most likely due to the difference in the toxicities of NO and NO_2^-. We have shown that the *norB* mutant 15.12 accumulates large amounts of NO in the presence of NO_3^-, consistent with the *norB* product being required for NO reduction (24). Since NO is highly toxic, *norB* mutants, and strains containing mutations in the other *nor* genes, cannot grow anaerobically in the presence of either NO_3^- or NO_2^-. *nnrT* or *nnrU* mutants do not produce NO because Nir is inactive, permitting them to grow anaerobically in the presence of NO_3^-.

Phenotypic analysis as well as functional complementation and promoter deletion studies demonstrated that expression of the *norC*-through-*norD* region is dependent on the regulatory region upstream of *norC*. The promoter sequence upstream of *norC* has been found in front of *nirK* and *nnrR* (34, 35). Inactivation of *nnrR* affects expression of all four of these genes, suggesting that NnrR is the primary regulator of this set of genes. Even though sequence analysis strongly suggests that *nnrT* and *nnrU* are in an operon with the rest of the *nor* genes (Fig. 2), transcription of *nnrT* and *nnrU* cannot be totally dependent on the *norC* promoter. Insertional inactivation within *nnrT* or *nnrU* prevents *nirK* and *nor* expression. With one exception, insertion of either Tns or Ω interposons upstream of *nnrT* or *nnrU* does not prevent *nirK* or *nor* operon expression. This lack of polarity indicates that *nnrT* and *nnrU* can be expressed from an internal promoter. This conclusion is supported by the expression of *nnrU-lacZ*, which indicates that *nnrU* is variably expressed under all growth conditions, and by the presence of a Shine-Dalgarno sequence preceding *nnrT*. The only other gene involved in NO_2^- or NO reduction that we have found to be expressed under all conditions is *nnrR* (35). Since *nnrR*, *nnrT*, and *nnrU* are all expressed constitutively and, when inactivated, cause the same phenotypic changes, perhaps they work together in regulating NO metabolism.

Strain R174 was an apparent exception to the observation that insertion within *nor* does not result in a Nir-deficient

phenotype. In this strain the Ω interposon insertion is 123 bp upstream of the putative start of *nnrT* translation. All other insertions in *norD* are at least 200 bp further upstream and did not prevent expression of Nir. Given the proximity of the insertion in R174 to the putative translation start of *nnrT*, it seems likely that this insertion is located within the internal promoter required for *nnrT* and *nnrU* expression. Sequence comparisons of this region with other DNA sequences in the database did not reveal any identity to regulatory sequences in related bacteria.

It is unclear why *nnrT* and *nnrU* mutations could not be complemented. Mutations in the *nor* genes were readily complemented, demonstrating that the complementation problems arise only in those mutants unable to express *nnrT* and *nnrU*. Insertions in *hemN*, which lies immediately downstream of *nnrU*, can also be complemented. It is significant that R174 also could not be complemented. Since this mutation apparently lies within the promoter region, there should be no product expression, eliminating the possibility that a dominant mutant allele is disrupting NnrT or NnrU function.

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