Characterization of nirV and a gene encoding a novel pseudoazurin in *Rhodobacter sphaeroides* 2.4.3

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Sequencing of the region flanking *nirK*, the gene encoding the copper-containing nitrite reductase in *Rhodobacter sphaeroides* 2.4.3, has identified two genes whose products could potentially be involved in nitrite reductase expression and activity. One of the genes has been designated *nirV*. Putative *nirV* orthologues are found in other denitrifiers, where they are also located downstream of the structural gene for nitrite reductase. The *nirV* in 2.4.3 is apparently cotranscribed with *nirK*. Inactivation of *nirV* had no effect on cell growth, or on nitrite reductase expression or activity. Downstream of *nirV* and divergently transcribed is a gene, designated *ppaZ*, encoding a protein with significant similarity to pseudoazurins from other denitrifiers. However, three of the four residues required for binding of the type I copper centre are not conserved in the deduced sequence of the protein in 2.4.3. *ppaZ* is expressed only when oxygen becomes limiting. *ppaZ* expression is dependent on both FnRL and NnrR, and a putative binding site for these proteins has been identified. Expression of *ppaZ* is also dependent on the two-component PrrB/PrrA system. Inactivation of *ppaZ* had no significant effect on cell growth or on nitrite reductase expression or activity. Expression of a maltose-binding protein–PpaZ fusion indicated that the protein could not bind copper.

Examination of the genome of the related bacterium *R. sphaeroides* 2.4.1 revealed that it encodes *ppaZ* but not *nirV* and evidence is presented suggesting that a common ancestor of 2.4.3 and 2.4.1 had both nitrite and nitric oxide reductase activity but as the strains diverged 2.4.1 lost *nirK* and *nirV*, making it incapable of nitrite reduction.

**Keywords:** photosynthetic denitrifier, denitrification, nitrite reductase, blue copper protein

INTRODUCTION

Denitrification is the reduction of nitrate to nitrogen gas. This predominantly bacterially mediated process allows bacteria to use terminal oxidants other than oxygen. To reduce nitrate completely to nitrogen gas requires four terminal reductases: nitrate reductase, nitrite reductase, nitric oxide reductase and nitrous oxide reductase (Zumft, 1997). Structural genes encoding each of these reductases have been identified from a number of denitrifiers. Characterization of these genes has demonstrated that, for all the reductases except nitrous oxide reductase, there are multiple forms of the enzyme carrying out a particular reaction. For example, there are two forms of nitrite reductase. One contains only copper as a redox-active metal (Godden *et al.*, 1991). The other type of nitrite reductase contains only haem-bound iron as its redox-active metal (Fulop *et al.*, 1995). This cytochrome-type nitrite reductase contains a c-type haem and an unusual modified haem referred to as a d-type haem. All denitrifiers characterized to date have only one of these two types of nitrite reductase.

In addition to the genes required to encode the terminal nitrogen oxide reductases there are additional genes...
required for assembly and function of the terminal reductases. For example, the haem-containing nitrite reductase requires at least seven genes for the synthesis of the novel 4d haem (Zumft, 1997). Nitrate, nitric oxide and nitrous oxide reductases have all been shown to require additional proteins for synthesis of cofactors or assembly of the finished active complex (Zumft, 1997). In most cases genes encoding accessory proteins are located in clusters of genes whose products are required for the assembly of a particular reductase.

Rhodobacter sphaeroides strain 2.4.3 expresses a copper-containing nitrite reductase encoded by nirK (Tosques et al., 1997). Analysis of chromosomal sequence downstream of nirK in R. sphaeroides 2.4.3 identified two ORFs encoding products found in other denitrifiers. One of the ORFs, which has been designated ppaZ, encodes a protein with significant similarity to pseudoazurins. Pseudoazurins are small periplasmic proteins that contain a single copper atom (Petratos et al., 1983). Biochemical studies have suggested that pseudoazurin is a direct electron donor to nitrite reductase (Kukimoto et al., 1996; Williams et al., 1995). Recent genetic evidence has confirmed this suggestion (Koutny et al., 1999). The other gene downstream of nirK encodes a product similar to NirV from Pseudomonas G-179 (Bedzyk et al., 1999). In G-179, as in R. sphaeroides 2.4.3, the nirV gene is immediately downstream of the gene encoding nitrite reductase. The role of the nirV product is unknown.

The experiments described in this paper were designed to study the role of pseudoazurin and the nirV product during nitrogen oxide reduction in R. sphaeroides. Expression studies showed that pseudoazurin is expressed during anaerobic growth. However, the role of this protein is unclear because three of the four amino acid residues required for copper ligation are not conserved in the pseudoazurin encoded by 2.4.3. Inactivation of the gene encoding pseudoazurin did not cause any detectable phenotypic changes. Analysis of the expression of nirV indicated that it is cotranscribed with nirK and is therefore a member of the NnrR regulon, which includes nirK and the genes encoding nitric oxide reductase (Tosques et al., 1996). Inactivation of nirV also did not cause detectable phenotypic changes to cells cultured under low-oxygen conditions.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** Escherichia coli strain DH5α was used as the maintenance strain for plasmids. E. coli S17-1 was used as donor for matings (Simon et al., 1983). R. sphaeroides 2.4.3 (ATCC 17023) and R. sphaeroides 2.4.1 (ATCC 17023) are the wild-type strains used in this study. Strains 11.10 (Tosques et al., 1997) and R125 (Tosques et al., 1996) are NnrR-deficient and NnrR-deficient strains of 2.4.3, respectively. Strain R213 is an FnrL-deficient strain of R. sphaeroides 2.4.3 constructed as described below and R214 is an FnrL- and NnrR-deficient strain of 2.4.3 (J. P. Shapleigh, unpublished). Strains 5.11 and 14.3 were isolated as a result of a screen for transposon mutants unable to grow with nitrate as electron acceptor (I. E. Tosques & J. P. Shapleigh, unpublished). The transposon insertion site has been mapped in each strain; in 14.3 the insertion was at base pair 135 of the prrB ORF and in 5.11 the insertion was at base pair 358 of the prrB ORF. Plasmid pRK415, a broad-host-range plasmid, was used for transferring genes from E. coli to R. sphaeroides (Keen et al., 1988). Plasmids pSUP202 and pJP5603 were used for gene inactivation (Simon et al., 1983).

E. coli strains were grown in LB medium (Maniatis et al., 1982). Rhodobacter strains were grown in Sistrom’s medium at 30°C (Leuking et al., 1978). Nitrate was added to Rhodobacter cultures to a final concentration of 12 mM. Other culture conditions and amendments were as described previously (Tosques et al., 1997). Procedures for growing wild-type and mutant R. sphaeroides microaerobically, which permits expression of genes regulated by NnrR, are described elsewhere (Tosques et al., 1996).

Taxis assays were similar to previously described R. sphaeroides taxis assays (Gauden & Armitage, 1995). Cells for taxis assays were grown microaerobically in medium unamended with nitrate. After growth, cells were concentrated twofold in Sistrom’s medium by centrifugation and then diluted in Sistrom’s agar such that the final agar concentration was 0.4%. Cells resuspended in agar were poured into Petri plates and a plug containing either 0.5 M nitrate or 0.3 M nitrite in 2% Sistrom’s agar was inserted in the centre of the plate. The plates were cooled for 5–10 min and then placed in an anaerobic jar and incubated under a N₂ atmosphere.

**DNA manipulation and sequencing.** Chromosomal DNA was isolated from 2.4.3 using the Puregene system (Gentra Systems). Plasmid isolations were done using the alkaline lysis method (Birnboim & Doly, 1979). Standard methods were used for restriction digests, agarose gel electrophoresis, and ligations. Southern hybridizations were carried out as described previously (Toffanin et al., 1996). Transformations were done using TSS (Chung et al., 1989). Plasmids were moved into 2.4.3 by conjugation. Biparental matings were carried out with E. coli S17-1 as the donor.

The 30 kb EcoRI fragment encoding nirV and ppaZ was originally isolated from a lambda library of 2.4.3 DNA (Fig. 1a). Some of this fragment has been sequenced previously.
(Tosques et al., 1997). Both strands of the remaining unsequenced regions were sequenced at the Cornell University BioResource Center. Fragments were generated for sequencing using available restriction sites or by PCR. Sequence comparisons were carried out using the BLAST program (Altschul et al., 1990) available at each of the URLs listed in the text. Preliminary sequence data were obtained from the DOE Joint Genome Institute (JGI) at http://www.jgi.doe.gov/.

The nirV–lacZ construct was generated by isolating a 1.1-kb XhoI–BamHI fragment containing most of the nirV ORF and 408 bp upstream of the putative nirV start (Fig. 1a). This fragment was ligated, along with the lacZ–Kn’ cassette of pKOK6 (Kokotek & Lotz, 1989) digested with BamHI, into pRK415 that had been digested with BamHI and SalI. A second lacZ fusion to nirV, designated nirV68-<lacZ> was generated by ligating an approximately 6.5 kb HindIII–BamHI fragment and the lacZ–Kn’ cassette of pKOK6 digested with BamHI into pRK415 digested with HindIII and BamHI. The 6.5 kb HindIII–BamHI fragment contains approximately 5.0 kb of DNA upstream of the nirK translation start.

The pppZ–lacZ construct was generated by ligating a 0.55 kb EcoRI–StuI fragment into pUC19 (Fig. 1a). This fragment contained 426 bp upstream of the putative pppZ translation start. This construct was digested with EcoRI and PstI to remove the insert, which was ligated, along with the lacZ–Kn’ cassette of pKOK6 digested with PstI, into pRK415 (Fig. 1). The pppZ1–lacZ construct was generated by amplifying a DNA fragment with the upstream primer 5’-GGCTACACGCGGAAAGTCCG-3’ and the downstream primer 5’-GGCTACACGCGGAAAGTCCG-3’. The amplicon was restricted with KpnI and BamHI and ligated with the pKOK6 lacZ–Kn’ cassette digested with BamHI into pRK415. The pppZ2–lacZ construct was generated using the same strategy as for pppZ1–lacZ but was amplified with the upstream primer 5’-GGCTACACGCGGAAAGTCCG-3’ and the downstream primer 5’-GGCTACACGCGGAAAGTCCG-3’. The amplicon was restricted with KpnI and BamHI and ligated with the pKOK6 lacZ–Kn’ cassette digested with BamHI into pRK415. The pppZ2–lacZ construct was generated by isolating a 650 bp BamHI–PstI fragment containing approximately 200 bp upstream of the putative pppZ translation start (Fig. 1a). This fragment was ligated, along with the lacZ–Kn’ cassette of pKOK6 digested with PstI, into pRK415 digested with PstI and BamHI. The nirK–lacZ construct has been previously described (Tosques et al., 1997). All the fusions are transcriptional.

To disrupt nirV two fragments were amplified that contained nirV along with flanking DNA. The upstream fragment was amplified using the oligonucleotides 5’-GGCTACACGCGGAAAGTCCG-3’ and 5’-GGCTACACGCGGAAAGTCCG-3’. The downstream fragment was amplified using the oligonucleotides 5’-GGCTACACGCGGAAAGTCCG-3’ and 5’-GGCTACACGCGGAAAGTCCG-3’. These two amplicons were purified, the upstream amplicon digested with PstI and BamHI and the downstream amplicon digested with EcoRI and BamHI. A BamHI-digested antibiotic-resistance cassette of pPH455 (Prentki & Krisch, 1984), encoding streptomycin/spectinomycin resistance (St'/Spc'), was also isolated. These fragments were ligated into pSAlp2 that had been digested with PstI and EcoRI. Cells were selected on media containing tetracycline, and streptomycin plus spectinomycin, and appropriate constructs were confirmed by restriction digest. The construct was transformed into E. coli S17-1. After conjugation of this plasmid into 2.4.3, exconjugants were isolated that were tetracycline sensitive (Tc’) and St'/Spc’.

Inactivation of nirV was confirmed by Southern hybridization using genomic DNA isolated from the mutant probed with a digoxigenin-labelled fragment containing the entire nirV ORF plus about 100 bp of flanking DNA. The NirV-deficient strain was designated strain R351. Inactivation of pppZ was carried out by cloning a 2.8 kb EcoRI fragment, which contains pppZ and flanking DNA, into the vector pJP5603. This was digested with StuI, which cuts at position 123 of the pppZ ORF, and ligated with the streptomycin/spectinomycin-resistance cassette of pUI1638 (Eraso & Kaplan, 1994) digested with EcoRV. After conjugation of this plasmid into 2.4.3, exconjugants that were Tc’ and St’/Spc were isolated. The pppZ disruption was likewise confirmed via Southern hybridization of a labelled DNA fragment encompassing the entire pppZ ORF to genomic DNA isolated from the mutant.

The gene encoding FnRl (fnrl) was inactivated by using the following oligonucleotides to amplify a fragment of the fnrl ORF using 2.4.3 chromosomal DNA as template: 5’-GGCGGTACACGCGGAAAGTCCG-3’ and 5’-GGCGGTACACGCGGAAAGTCCG-3’. These oligonucleotides were designed based on the fnrl sequence from R. sphaeroides 2.4.1 (Zeilstra-Ryalls & Kaplan, 1995). The amplicon was digested with EcoRI and PstI, sites that were added by sequences on the oligonucleotides, and ligated into the suicide plasmid pJP5603 digested with the same enzymes. This construct was conjugated into 2.4.3 and kanamycin-resistant single crossovers were isolated.

The pMAL-pppZ construct was generated by amplifying a 385 bp fragment containing the bulk of pppZ except the for the signal sequence. The oligonucleotides used for the amplification were 5’-GGCGGTACACGCGGAAAGTCCG-3’ and 5’-GGCGGTACACGCGGAAAGTCCG-3’. These oligonucleotides were designed based on the fnrl sequence from R. sphaeroides 2.4.1 (Zeilstra-Ryalls & Kaplan, 1995). The amplicon was digested with EcoRI and BamHI and ligated into the pMAL-C2 vector (New England Biolabs) digested with the same enzymes. The pMAL-nirV fusion was constructed by amplifying an 822 bp fragment containing all of nirV except the signal sequence. The oligonucleotides used for the amplification were 5’-GGCGGTACACGCGGAAAGTCCG-3’ and 5’-GGCGGTACACGCGGAAAGTCCG-3’. The amplicon was digested with EcoRI and BamHI and ligated into the pMAL-C2 vector digested with the same enzymes. Expression and purification of the fusions were carried out as described previously (Olesen et al., 1998).

Assays. β-Galactosidase activities were determined in duplicate on at least three independently grown cultures as previously described (Tosques et al., 1996). Cells removed from stoppered flasks were not kept anaerobic but were used immediately for assays. Samples were taken at various times during growth and the highest values obtained before the cells stopped growing were used to determine the reported values. Copper measurements were carried out using the protocol described by Felsenfeld (1960). MBP-NirV and MBP-PpaZ were purified from cells grown in medium containing 100 μM CuSO₄. Nitrite reductase was assayed by measuring the rate of nitrite disappearance of washed whole cells incubated at 30 °C in Sistrom’s medium (Stewart & Parales, 1988).

RESULTS

Sequence analysis of the region downstream of nirK

Analysis of approximately 2 kb of sequence downstream of the structural gene for nitrite reductase, nirK, in R. sphaeroides 2.4.3 revealed two ORFs (Fig. 1a). Immediately downstream of nirK and in the same orientation was an ORF encoding a protein with significant similarity to the product of nirV of Pseudomonas strain

pppZ and nirV in R. sphaeroides
R. JAIN and J. P. SHAPLEIGH

(a) Alignment of the deduced amino acid sequences of NirV from R. sphaeroides 2.4.3 (2.4.3), Pseudomonas G-179 (G179) (Bedzyk et al., 1999) and Rps. palustris (R. pal.) (http://www.jgi.doe.gov/tempweb/JGI_microbial/html/index.html). * indicates residues present in all three sequences. Boxes contain residues identified by sequence comparisons as present in proteins other than NirV orthologues (see text for details).

(b) Alignment of the amino acid sequences of pseudoazurin from R. sphaeroides 2.4.3 (2.4.3), R. sphaeroides 2.4.1 (2.4.1) (http://www.jgi.doe.gov), Thiosphaera pantotropha (Tpan) (Leung et al., 1997) and Alcaligenes faecalis (Afaec) (Yamamoto et al., 1987). * indicates residues present in each sequence in the alignment. Residues in bold (and marked with a dot above the alignment) are highly conserved in pseudoazurins but not in the deduced sequences of pseudoazurins from R. sphaeroides. Residues shown to bind the copper of the type I copper centre are boxed.

G179 (Bedzyk et al., 1999) (Fig. 2a). Proteins with significant identity to NirV were also found in the genomes of Rhodopseudomonas palustris and Sinorhizobium meliloti. In each of these bacteria nirV is located immediately downstream of a gene encoding a copper-containing nitrite reductase. In Neisseria meningitidis and Neisseria gonorrhoeae, genes whose products have similar similarity to NirV are located downstream of genes encoding copper-containing nitrite reductases (http://wit.mcs.anl.gov/WIT2/). Since the nitrite reductases in the Neisseria spp. are divergent from nitrite reductases in typical denitrifiers, it is not unexpected that the NirV proteins have limited similarity as well. Stretches of residues in NirV extending from residue 126 to 133 and from 193 to 198 appear to be motifs found in a number of other proteins (Fig. 2a). Proteins with these motifs include carF from Pectobacterium carotovorum (McGowan et al., 1997) and xylR from Bacillus steatorrhophilus (Cho & Choi, 1995), as well as many uncharacterized ORFs from sequenced bacterial genomes, including genes encoding putative S/T protein kinases from Chlamydia species (Stephens et al., 1998). No particular function has been assigned to this region in any known protein. The gap between the nirK ORF and the putative translation start of nirV is 210 bp. There is a ribosome-binding site 9 bp upstream of the putative initiation codon. The ribosome-binding site upstream of nirK is also 9 bp upstream of the translation start. There is no obvious sequence similar to the NnrR or FnrL consensus binding sites in the region between nirK and nirV. The deduced NirV protein contains a region at its N-terminus suggestive of a signal sequence (Fig. 2a). A basic residue is found at position 3 followed by an approximately 20 amino acid stretch rich in hydrophobic residues.

Downstream of nirV but transcribed in the opposite direction is a gene encoding a protein with significant similarity to pseudoazurins from various sources (Fig. 1a). The 2.4.3 pseudoazurin is the same length as other pseudoazurins and contains many of the residues conserved in this family of copper-containing proteins (Fig. 2b). However, the 2.4.3 pseudoazurin is unusual in that it is missing a number of the residues that are...
conserved in the pseudoazurin family. In particular, three of the four residues involved in copper ligation are not present (Fig. 2b). In the 2.4.3 pseudoazurin, residue 65 is a phenylalanine while in all other pseudoazurins this residue is a histidine that binds copper. Residues 103 and 106 in 2.4.3 PpaZ are serine and tyrosine, respectively, but in other pseudoazurins are the copper-binding residues cysteine and histidine (Inoue et al., 1986). There are several other conserved residues that are not present in the 2.4.3 pseudoazurin. One particularly significant change is the deletion of an aspartic acid residue found in all other pseudoazurins characterized to date. For reference, the gap left by the loss of this residue is coincident with residue 117 of the complete A. faecalis pseudoazurin sequence (Hormel et al., 1986).

Since the pseudoazurin encoded by 2.4.3 does not appear to be capable of copper binding, the gene has been designated ppaZ for pseudopseudoazurin. There is a ribosome-binding site located 8 bp upstream of the putative translation start. A region with similarity to the FnrL and NnrR binding sites is located 79 bp upstream of the putative translation start. The sequence of this region is 5'-TTGATGCAGCGCAA-3'. The TTGAT sequence is identical to half sites found in the FnrL consensus sequence (Spiro, 1994) and the CGCAA sequence is identical half sites found in the FnrR consensus sequence (Frasquet et al., 1997). A similar site has been observed upstream of bhmN of R. sphaeroides 2.4.3, which is located downstream of the operon encoding nitric oxide reductase (T. B. Bartnikas & J. P. Shapleigh, unpublished).

**Sequence analysis of R. sphaeroides strain 2.4.1**

The genome sequence of the closely related 2.4.1 strain of *R. sphaeroides* has recently become available (http://www.jgi.doe.gov; follow the link to the *Rhodobacter* page. Examination of the available contigs indicates that 2.4.1 also contains a gene encoding a putative pseudoazurin (Fig. 1b). The deduced protein sequence is 88% identical to the ppaZ gene product, with the 2.4.1 product being one residue shorter because of a deletion in the hydrophobic stretch of the putative signal sequence (Fig. 2b). Significantly, three of the residues required for copper ligation in other pseudoazurins are not conserved in the 2.4.1 sequence. In both 2.4.1 and 2.4.3 the residue at position 65, which in other pseudoazurins is a copper-binding histidine, is a phenylalanine encoded by the codon TTC. The switch from histidine, encoded by CAT or CAC, to phenylalanine requires a two-base change assuming usage of the more frequently used CAC codon. A single base change was required to make residues 103 and 106 encode serine and tyrosine, respectively, in both 2.4.1 and 2.4.3, instead of the cysteine and histidine found in the identical positions in other pseudoazurins. The conversion of residues 103 and 106 to hydroxyl-containing amino acids, which are potential metal-binding ligands, makes it possible that a metal could be bound to PpaZ. However, PpaZ is distinct from other pseudoazurins because it lacks a binding site for a type 1 copper centre. As in 2.4.3, the sole copper-binding residue remaining in the 2.4.1 pseudoazurin is the methionine at position 110. A potential FnrL or NnrR binding site is located 69 bp upstream of the putative translation start.

Examination of the region downstream of ppaZ in 2.4.1 revealed that there is no nirK or nirV as in 2.4.3. Instead there is an approximately 500 bp region that does not appear to contain any ORFs, followed by a region encoding a protein that is similar to Srl1485 from *Synechocystis* sp. strain PCC 6803 (Fig. 1b). The region immediately upstream of nirK in 2.4.3 also encodes a protein with significant identity to srl1485. The srl1485 protein is related to proteins with phosphatidylglycerol-4-phosphate 5-kinase activity (not shown). Alignment of the available sequence from within the srl1485 ORFs from the two strains shows there is > 85% sequence identity until a few residues upstream of the termination codon (not shown). There is < 35% sequence identity in the region immediately downstream of srl1485. A similar pattern is seen in the region encoding ppaZ. There is significant sequence identity throughout the ppaZ ORFs, but upon reaching the termination codon the identity of the sequences from the two strains decreases significantly. There is an approximately 220 bp region immediately upstream of the putative translation start of ppaZ in 2.4.3 that shares little identity with the same region in 2.4.1 (not shown). However, distal to this stretch there is a significant level of identity between the available sequences from the two strains (not shown). In 2.4.1 the region upstream of ppaZ is predicted to encode a protein with significant identity to the 2-isopropylmalate synthase from *N. meningitidis*. Assuming conservation of gene order in 2.4.1 and 2.4.3 the sequence analysis indicates that nirK, nirV and ppaZ appear to constitute the nitrite reductase gene cluster in 2.4.3.

**Expression of nirV**

The 210 bp gap between nirK and nirV could be sufficient to allow nirV to be regulated independently of nirK. To test this a nirV-lacZ clone was made that included the entire intergenic region plus 198 bases of the 3' end of the nirK ORF. Expression of this construct in 2.4.3 was assessed microaerobically in medium with or without added nitrate. There was essentially no β-galactosidase activity detectable under any culture condition (not shown). One explanation for this lack of expression is that nirK and nirV are cotranscribed. To test this a construct, designated nirV–lacZ, was made that included the intergenic region, all of nirK and approximately 500 bp of DNA upstream of the nirK translation start. There was detectable β-galactosidase expression using the nirV–lacZ fusion, with expression of 482 Miller units of activity in cells grown in nitrate-amended medium under microaerobic conditions. Cells grown under identical conditions but in medium lacking nitrate had fivefold lower β-
The importance of NnrR was confirmed by the lack of consistent with a requirement for NnrR for expression. galactosidase activity. This decrease in expression is consistent with a requirement for NnrR for expression. The observation that expression from \textit{ppaZ–lacZ} fusion, designated \textit{ppaZ–lacZ} construct in the NnrR-deficient strain R124. The growth conditions are indicated on the x axis: \textit{O}_2, cells cultured aerobically; \textit{O}_2, cells cultured microaerobically; \textit{O}_2 + \textit{NO}_3, cells grown micro-aerobically in medium amended with 12 mM nitrate.

Expression of \textit{ppaZ}

The presence of a possible NnrR or FnrL binding site upstream of the \textit{ppaZ} ORF suggests that this gene will be preferentially expressed under low-oxygen conditions. This was tested by constructing a \textit{lacZ} fusion, designated \textit{ppaZ–lacZ}, that included about 400 bp upstream of the putative translation start. Maximal expression of \textit{ppaZ–lacZ} occurred when cells were cultured microaerobically in unamended Sistrom’s medium (Fig. 3). When the strain was cultured in nitrate-amended medium, expression of the \textit{ppaZ–lacZ} fusion was reduced by about 15%. Expression of the fusion under aerobic conditions was about 25-fold lower than when cells were incubated under limiting oxygen (Fig. 3). This expression pattern is consistent with FnrL functioning as the primary regulator of \textit{ppaZ}. To test this, expression of \textit{ppaZ–lacZ} was monitored in R213, an FnrL-deficient strain of 2.4.3 (Fig. 3). Expression in this strain was about 22-fold lower than in the wild-type strain. Under identical conditions, expression in R125, an NnrR-deficient strain of 2.4.3, was only about 2-fold less than wild-type (Fig. 3). When the FnrL-deficient strain was grown in medium containing nitrate, expression of the fusion increased about threefold relative to nitrate-unamended medium. This suggests that NnrR may regulate \textit{ppaZ} expression if FnrL is not active. Expression in strain R214, a 2.4.3 strain that lacks both FnrL and NnrR, was about 38-fold lower than expression in the wild-type strain in nitrate-unamended medium and did not increase when nitrate was present in the medium (Fig. 3).

To determine if the sequence identified as a possible FnrL or NnrR binding site is required for expression, a \textit{lacZ} fusion containing the putative FnrL binding site plus 5 bases upstream of the site was constructed. When the wild-type 2.4.3 containing this construct, designated \textit{ppaZ1–lacZ}, was cultured under low oxygen it retained \textit{β}-galactosidase activity but it was about 15-fold lower than 2.4.3 with \textit{ppaZ–lacZ} cultured under identical conditions (Table 1, Fig. 3). In contrast, a construct that was 5 bp longer than \textit{ppaZ1–lacZ} but with a 1 bp deletion in the downstream half site of the putative binding site (see Methods) showed less than 5 units of activity under identical conditions. A third fusion, designated \textit{ppaZ3–lacZ}, with 115 bp upstream of the putative FnrL binding site, also showed a decrease in expression compared to \textit{ppaZ–lacZ}, having about 60% of the activity of the longer construct (Table 1, Fig. 3). The \textit{ppaZ3–lacZ} construct also showed relatively lower expression than \textit{ppaZ–lacZ} in the FnrL-deficient strain but there was no significant difference between the two fusions in the NnrR-deficient strain (Table 1). Taken together, these results are consistent with the putative binding site with similarity to the FnrL and NnrR consensus binding sites being critical for expression of \textit{ppaZ}.

The observation that expression from \textit{ppaZ3–lacZ} was consistently lower than that from \textit{ppaZ–lacZ}, even though both constructs contain significant lengths of DNA upstream of the putative FnrL or NnrR binding site, suggested that NnrR and FnrL might not be the only proteins involved in regulating \textit{ppaZ} expression. The PrrB/A sensor–regulator complex has been shown to be involved in expression of a number of genes that are preferentially expressed under low-oxygen conditions (Eraso & Kaplan, 1994). Several \textit{prrB} mutants in 2.4.3 have been isolated as a result of screening for mutants unable to grow anaerobically on nitrite-amended medium (I. E. Tosques & J. P. Shapleigh, unpublished). Expression of \textit{ppaZ–lacZ} in two of these mutants, strains 5.11 and 14.3, was about 10-fold lower than in the wild-type strain (Table 2, Fig. 3). Expression of \textit{ppaZ3–lacZ} also decreased in strain 5.11 (Table 2). The truncated fusion showed about fourfold lower expression in 5.11 than in wild-type. It is interesting the \textit{ppaZ–lacZ} and \textit{ppaZ3–lacZ} constructs have the same level of expression in 5.11 (Table 2). In the PrrB-deficient background expression is being driven primarily by FnrL-dependent transcription and since both constructs retain the putative FnrL binding site, it is not unexpected that their \textit{lacZ} expression levels are similar. The difference in \textit{lacZ} expression from these two constructs in the wild-type strain may reflect less efficient PrrB/PrR-dependent transcription in the shorter \textit{ppaZ3–lacZ} construct.
Galactosidase activities were determined in duplicate on at least three independently grown samples of R. sphaeroides PrrB-deficient strains of R. sphaeroides. To learn more about the role of the inactivation of ppaZ

Strains were grown in Sistrom's medium. Nitrate was added to a final concentration of 12 mM. 2.4.3 is the wild-type strain, R213 is the FnrL-deficient strain and R125 is the NnrR-deficient strain. β-Galactosidase activities were determined in duplicate on at least three independently grown samples and are shown as means ± SD. See Fig. 3 for the activity of the ppaZ–lacZ fusion in 2.4.3.

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<tr>
<th>Fusion</th>
<th>Strain/condition</th>
<th>β-Galactosidase activity (Miller units)</th>
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<tbody>
<tr>
<td>ppaZ1–lacZ</td>
<td>2.4.3/low oxygen</td>
<td>220 ± 55</td>
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<td>ppaZ1–lacZ</td>
<td>2.4.3/low oxygen + nitrate</td>
<td>235 ± 41</td>
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<tr>
<td>ppaZ3–lacZ</td>
<td>R213/low oxygen</td>
<td>284 ± 28</td>
</tr>
<tr>
<td>ppaZ3–lacZ</td>
<td>R213/low oxygen + nitrate</td>
<td>416 ± 10</td>
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<tr>
<td>ppaZ3–lacZ</td>
<td>R125/low oxygen</td>
<td>1524 ± 21</td>
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<table>
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<tr>
<th>Fusion</th>
<th>Strain/condition</th>
<th>β-Galactosidase activity (Miller units)</th>
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<tr>
<td>ppaZ–lacZ</td>
<td>14.3/low oxygen</td>
<td>331 ± 115</td>
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<tr>
<td>ppaZ–lacZ</td>
<td>5.11/low oxygen</td>
<td>414 ± 164</td>
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<td>ppaZ–lacZ</td>
<td>5.11/low oxygen + nitrate</td>
<td>283 ± 85</td>
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<td>ppaZ3–lacZ</td>
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<td>491 ± 7</td>
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<td>ppaZ3–lacZ</td>
<td>5.11/low oxygen + nitrate</td>
<td>326 ± 4</td>
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Table 1. Expression of β-galactosidase activity from ppaZ1–lacZ and ppaZ3–lacZ fusions in different strains grown under various conditions

Table 2. β-Galactosidase activity from ppaZ–lacZ and ppaZ3–lacZ fusions in two different PrrB-deficient strains of R. sphaeroides 2.4.3: 5.11 and 14.3

Inactivation of ppaZ and nirV

To learn more about the role of the ppaZ and nirV gene products during growth under oxygen-limiting conditions, both genes were disrupted by insertional inactivation. The nirV ORF was partially deleted and replaced with a drug-resistance cassette. The strain lacking NirV, designated R351, showed the same growth rates as wild-type under microaerobic conditions and nitrite did not accumulate in nitrate-amended medium. In R. sphaeroides the taxis response to terminal oxidants is dependent on the ability of the cells to respire the oxidant (Gauden & Armitage, 1995). The taxis response of R351 to nitrate and nitrite was the same as wild-type, further demonstrating the lack of effect of nirV inactivation on nitrogen oxide reduction (not shown). Since nitrite reductase activity is linked to nirK expression (Tosques et al., 1997), nirK–lacZ expression was monitored in R351 to determine if there were any detectable changes in nitrite reductase activity as a consequence of nirV inactivation. Not unexpectedly, expression in the mutant was similar to expression of the fusion in the wild-type strain (not shown). As expected, nitrite reductase activity in whole cells of R351 was the same as wild-type (data not shown).

Since nirV has only been found in strains encoding copper-containing nitrite reductases it is possible that NirV assists in assembly of an active nitrite reductase. To test if NirV might be required as a copper chaperone, wild-type and NirV-deficient cells were grown in medium containing the chelator diethyldithiocarbamate (DDC). DDC has been shown to effectively inhibit the activity of copper-containing nitrite reductases by removal of copper from the assembled protein (Shapleigh & Payne, 1985). If NirV is a copper chaperone, it might be expected that loss of this protein might more severely affect assembly of nitrite reductase in cells grown in medium in which copper insertion into the protein has been negatively affected. The effect on nitrite reductase activity was assessed by monitoring expression of the nirK–lacZ fusion. Expression of the fusion was monitored in 2.4.3 and R351 cultured in media containing 0 to 50 μM DDC. While the expression of the fusion decreased as the DDC concentrations increased there was no significant difference in expression between
MBP–PpaZ clone was colourless and a colorimetric copper assay showed no evidence of bound copper. Similar results were obtained with the MBP–NirV fusion. Incubation of the MBP–PpaZ fusion with extracts of 2.4.3 cells grown in nitrate-amended medium did not result in copper binding to the fusion.

**DISCUSSION**

All *R. sphaeroides* strains characterized to date have genes that encode NnrR and nitric oxide reductase. In both 2.4.3 and 2.4.1 the *nnrR* and *nor* genes are located within a cluster of photosynthetic genes, suggesting that the last common ancestor of 2.4.1 and 2.4.3 also encoded NnrR and Nor (Choudhary & Kaplan, 2000; Kwiatkowski et al., 1997). Comparison of the 2.4.3 and 2.4.1 genomes has shown that both strains also contain *ppaZ*. Examination of the arrangement of genes flanking *ppaZ* revealed that the location of *ppaZ* within the chromosome is the same in both strains. However, the gene content is different since 2.4.1 lacks *nirK* and *nirV*. In their place is an approximately 500 bp region of apparently non-coding DNA (Fig. 1b). Beyond this region is an ORF > 90% identical to the coding region immediately upstream of *nirK* in 2.4.3. The regions upstream of *ppaZ* are highly similar in both strains, with no apparent change in gene order. One scenario leading to the difference in gene content in the two strains is that *nirK* and *nirV* were acquired by 2.4.3 via horizontal transfer and recombined into the region of the chromosome near *ppaZ*. Another scenario is that a common ancestor of 2.4.3 and 2.4.1 contained *nirK* and *nirV* but as the strains diverged 2.4.1 lost *nirK* and *nirV*. This latter scenario seems more reasonable for two reasons. First, when *nirK* is supplied in trans in 2.4.1, the 2.4.1 strain becomes phenotypically indistinguishable from 2.4.3 (D. Y. Lee & J. P. Shapleigh, unpublished) The physiological equivalence of the two strains is consistent with 2.4.1 having been a complete denitrifier but as it evolved it lost that trait by the loss of *nirK*. Second, it has been shown that pseudoazurin is involved in nitrite reduction in other denitrifiers so the clustering of the structural gene for nitrite reductase with the gene for pseudoazurin is unlikely to be coincidental (Koutny et al., 1999; Kukimoto et al., 1996; Williams et al., 1995). It seems likely that *ppaZ*, *nirK* and *nirV* constituted a cluster of genes involved in nitrite reduction in a common ancestor of most *R. sphaeroides* strains but somewhere in the evolution of this strain it lost *nirK* and *nirV* and consequently the capacity to reduce nitrite. Why nitrite reductase was lost but not the capacity to reduce nitric oxide is puzzling.

The role of the *ppaZ* product is obscure, particularly given its inability to bind copper. The regulation of *ppaZ* suggests that the protein is expressed whenever oxygen concentrations are low (Table 1). This, as well as the persistence of a copy of *ppaZ* in 2.4.1, suggests that the function of PpaZ is not limited to when nitrite reduction is occurring. In this context it is significant that many of the positively charged residues shown to be involved in pseudoazurin binding with nitrite reductase...
are present in PpaZ (Kukimoto et al., 1995). It is possible then that PpaZ could bind to a number of electron-transport proteins. However, since PpaZ is unlikely to be redox active, the role of PpaZ in modulating electron flow is difficult to predict.

DNA sequence analysis suggests that the sequence changes that resulted in the loss of the ability of the ppaZ product to bind copper occurred before the divergence of 2.4.1 and 2.4.3. This is because the sequence changes leading to the loss of copper-binding residues are identical in both strains. Interestingly, the high identity of the 2.4.3 and 2.4.1 ppaZ products shows that there have been only minor sequence changes in PpaZ since the divergence of the two strains. The level of similarity of PpaZ from the two strains is similar to the levels of similarity found in other electron-transport proteins. This suggests that the primary sequence of PpaZ is constrained because it is functionally active in both strains and playing a similar physiological role.

Genome sequencing of the related bacterium Rhodobacter capsulatus has revealed that it also encodes a pseudoazurin, but unlike R. sphaeroides it retains all four of the copper-binding residues (not shown). This indicates that the modifications found in PpaZ are not widespread among photosynthetic bacteria. The sequence differences between PpaZ and other pseudoazurins are also not related to R. sphaeroides 2.4.3 having a copper nitrite reductase, since both Alcaligenes faecalis and Achromobacter cycloclastes have copper-containing nitrite reductases and copper-binding pseudoazurins (Godden et al., 1991; Inoue et al., 1999; Kakutani et al., 1981a, b).

The complex regulation of ppaZ is also consistent with its encoding a functional protein with a role in the physiology of R. sphaeroides under low-oxygen conditions. At least three different regulatory proteins are involved in the activation of ppaZ expression. The related proteins FnrL and NnrR likely bind at the 5’-TTGATGCAGCGCAA-3’ sequence. There is no other sequence upstream of ppaZ with significant identity to the Fnr consensus binding site 5’-TTGATNNNNNTCAAA-3’, with N representing any base (Spiro, 1994). The change in the downstream half site in the ppaZ region to a modified consensus does not apparently affect the binding of FnrL to the DNA. This region also appears to be the target of NnrR since there is no sequence upstream of ppaZ with similarity to the NnrR consensus, which is 5’-TTG(C/T)GNNNNG(A/G)-CAAA-3’. However, the affinity of NnrR for this site does not appear to be as great as for its consensus, given the relatively low levels of expression of ppaZ–lacZ in an FnrL-deficient strain (Fig. 3).

Expression of ppaZ also requires the two-component PrrB/A system (Table 2). The expression of a hemN–lacZ fusion, which, like ppaZ, is regulated by both FnrL and NnrR, and has an identical binding site sequence in its promoter region, is not affected by prrB inactivation (R. Jain & J. P. Shapleigh, unpublished). The observation that a gene with many regulatory similarities to ppaZ is unaffected by PrrB inactivation suggests that the PrrB-deficient changes in expression of ppaZ are not likely to be a result of the physiological changes in the cell resulting from inactivation of this global regulatory protein. Rather, this suggests that phosphorylated PrrA likely binds to the ppaZ promoter region in concert with either FnrL or NnrR to activate gene transcription. Since there is no consensus sequence for the binding site of this family of proteins, it is not possible to determine if a PrrA-P binding site exists by sequence examination (Du et al., 1998). This type of dependence on multiple transcription factors has been found in other genes regulated by PrrB/A (Oh et al., 2000).

As with PpaZ, the role of NirV is unclear. However, given that 2.4.1 has lost both nirK and nirV it seems reasonable that NirV is required for some role related to nitrite reduction. The proteins with the highest similarity to NirV are only found in bacteria containing copper-containing nitrite reductases. This further supports a role for NirV during nitrite reduction. Given these observations it might be expected that NirV would be some type of copper chaperone. Alignment of closely related NirV sequences from bacteria with nitrite reductases reveals that there are several cysteine, histidine and acidic residues that are completely conserved and could potentially be copper-binding residues (Fig. 2a). However, these residues do not conform to known copper-binding motifs and could be involved in other functions (Koch et al., 1997). The lack of effect of nirV inactivation on growth in copper-limited medium is also inconsistent with a copper-binding function for nirV. However, it is possible that other proteins compensate for the loss of NirV under these conditions.

There are at least two bacteria with copper-containing nitrite reductases that do not appear to encode an accompanying nirV. One of these is Rps. palustris. In this bacterium one of its two copper-containing nitrite reductases lacks an accompanying nirV. While it is possible that the single nirV is sufficient, preliminary experiments indicate that the regulation of the two nitrite reductases is different, suggesting that the nirK lacking a downstream nirV can be expressed independently of the single copy of nirV (D. Y. Lee & J. P. Shapleigh, unpublished). Nitrosomonas europaea encodes a nitrite reductase similar to those found in Neisseria spp., but unlike the situation in the neisseriae there is no nirV orthologue downstream of the nitrite reductase structural gene (http://wit.integrated.genomics.com/IGwit/). That there are bacteria that encode copper-containing nitrite reductases but lack nirV is further indication that the function of NirV is not essential for nitrite reductase activity.

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REFERENCES


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