Characterization of a member of the NnrR regulon in *Rhodobacter sphaeroides* 2.4.3 encoding a haem–copper protein

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Upstream of the *nor* and *nnrR* cluster in *Rhodobacter sphaeroides* 2.4.3 is a previously uncharacterized gene that has been designated *nnrS*. *nnrS* is only expressed when 2.4.3 is grown under denitrifying conditions. Expression of *nnrS* is dependent on the transcriptional regulator NnrR, which also regulates expression of genes required for the reduction of nitrite to nitrous oxide, including *nirK* and *nor*. Deletion analysis indicated the sequence 5'-TTGCG(N₄)CACAA-3', which is similar to sequences found in *nirK* and *nor*, is required for *nnrS* expression. Mutation of this sequence to the consensus Fnr-binding sequence by changing two bases in each half site caused *nnrS* expression to become nitrate independent. Inactivation of *nnrS* did not affect nitric oxide metabolism, nor did it affect expression of any of the genes involved in nitric oxide metabolism. However, taxis towards nitrate and nitrite was affected by *nnrS* inactivation. Purification of a histidine-tagged NnrS demonstrated that NnrS is a haem- and copper-containing membrane protein. Genes encoding putative orthologues of NnrS are sometimes but not always found in bacteria encoding nitrite and/or nitric oxide reductase.

Keywords: photosynthetic denitrifier, denitrification, nitrite reductase, nitric oxide reductase, regulation

INTRODUCTION

Denitrifying bacteria have the capacity to utilize nitrate as an alternative electron acceptor (Payne, 1981). Nitrate typically serves as an alternative electron acceptor when oxygen concentrations become limiting. Denitrifying bacteria can reduce nitrate to gaseous forms of nitrogen, principally dinitrogen, in a process referred to as denitrification (Zumft, 1997). During denitrification, nitrate is reduced to nitrite, a reaction also carried out during ammonification and nitrate assimilation (Berks *et al.*, 1995). Nitrite is then reduced to nitric oxide (NO), the first gaseous nitrogen oxide to be produced, making this reaction the defining reaction of denitrification. NO is then reduced to nitrous oxide, which in turn is reduced to nitrogen gas in the final step of denitrification (Zumft,

1993). A separate enzyme catalyses each reduction in the denitrification pathway.

Most denitrifying bacteria only express the proteins required for respiration of nitrate when oxygen is limiting and oxides of nitrogen are present (Zumft, 1997). The mechanism by which denitrifiers regulate expression of the various terminal reductases is beginning to be elucidated. One aspect of the regulation of denitrification that has become apparent is that the genes encoding products required for the reduction of nitrite and NO are regulated independently of the other reductases. The concerted regulation of these processes is carried out by a member of the Fnr/CRP family of transcriptional regulators (Arai *et al.*, 1995; Tosques *et al.*, 1996; van Spanning *et al.*, 1995; Vollack & Zumft, 2001).

In *Rhodobacter sphaeroides*, the gene encoding the protein regulating expression of nitrite reductase (Nir) and nitric oxide reductase (Nor) has been designated *nnrR* (Tosques *et al.*, 1996). The *nnrR* product also

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The GenBank accession number for *nnrS* is U62403.

amplification did not introduce errors. This fragment was ligated with the *Bam*HI–*Clal* fragment from pT55, generating *nnrS*-HT, and cloned into pT7-19U. The resulting fragment containing the entire *nnrS* ORF with the additional histidine codons was cloned into pRK415 and conjugated into *R. sphaeroides*.

The procedure used to generate *R. sphaeroides* strain 3T19A, which contains a Tn-*lacZ* insertion in *nnrS*, has been described previously (Bartnikas *et al.*, 1997). The exact site of insertion was determined by cloning a fragment of DNA containing the site of insertion and sequencing from the Tn region into the *nnrS* region. Sequence analysis indicated the *lacZ* insertion is in-frame with the predicted *nnrS* ORF.

Assays for enzymic activities. β -Galactosidase activities were determined in duplicate on at least four 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.

Purification of NnrS-HT. The purification of histidine-tagged NnrS followed the basic protocol developed for the purification of the *aa₃*-type cytochrome oxidase of *R. sphaeroides* (Mitchell & Gennis, 1995). Cells were grown microaerobically in media containing nitrate. They were harvested by centrifugation, resuspended in 50 mM Tris buffer at pH 8.0 and frozen at -20°C . The cells were then broken by passage three times through a French pressure cell at 18000 p.s.i. (124.2 MPa). Cell debris was removed by centrifugation at 17000 *g* for 20 min. Membranes were pelleted by centrifuging the supernatant at 160000 *g* for 90 min. The membranes were resuspended in 10 mM Tris buffer pH 8.0 with 40 mM KCl at a ratio of 8 ml buffer per litre cell culture harvested. Membrane proteins were solubilized using lauryl maltoside (Anatrace) added as a powder to a final concentration of 1% along with phenylmethylsulfonyl fluoride at 0.5 mM. This solution was stirred for 15 min at 4°C . Unsolubilized debris was removed by centrifuging the membranes at 160000 *g* for 45 min. The supernatant was removed and imidazole added to a concentration of 10 mM. Ni-NTA resin (Qiagen) was then added to the solution at a ratio of about 3 ml resin per litre culture harvested. This was stirred for 1 h at 4°C . The mixture was then poured into a column of appropriate dimensions and washed with 100 ml 10 mM Tris buffer pH 8.0 with 40 mM KCl and 0.1% lauryl maltoside (TKL buffer) plus 10 mM imidazole. This was followed by 40 ml TKL buffer plus 20 mM imidazole. The protein was eluted with TKL buffer plus 100 mM imidazole and all the fractions with colour were pooled. The coloured fractions were then loaded on a column of Q-Sepharose (Sigma) pre-equilibrated with 10 mM Tris buffer at pH 7.8 and 0.1% lauryl maltoside. The column was then washed with several volumes of equilibration buffer. Bound protein was eluted using 10 mM Tris buffer at pH 7.8 and 0.1% lauryl maltoside with 300 mM NaCl. All the fractions with colour were pooled. Purified protein was prepared for SPS-PAGE as described by Peiffer *et al.* (1990). The yield of NnrS-HT was about 0.6 mg protein per litre cell culture.

Spectroscopic analyses. Optical spectra were recorded using a Beckman DU 640 spectrophotometer in a single-beam mode. Carbon monoxide binding was assessed by blowing CO over the surface of the sample for up to 10 min. Samples were then sealed and their spectra recorded. X-band EPR was recorded

with an ER-200 IBM Bruker X-band spectrophotometer as described previously (Olesen *et al.*, 1998). Copper content was measured by inductively coupled plasma (ICP) emission spectroscopy. Pyridine haemochrome analysis was carried out by the method of Berry & Trumpower (1987).

RESULTS

Sequence analysis of *nnrS*

Sequence analysis of the region upstream of the *nor* operon and *nnrR* (Tosques *et al.*, 1996) indicated a strongly predicted ORF divergently transcribed from *nnrR* (Fig. 1). The ORF is 1194 bases in length, has a G + C content of 71.4 mol% and, as discussed above, has been designated *nnrS*. There are 208 bp between the putative translation start sites of *nnrR* and *nnrS*. A possible ribosome-binding site, AGGAGG, is centred 9 bases upstream of the putative translation start.

The deduced amino acid sequence of the protein encoded by *nnrS* is 397 aa in length with a molecular mass of 41.4 kDa. The amino acid content is heavily biased toward hydrophobic residues. The predicted sequence contains 32% alanine and leucine residues, for example. Hydropathy analysis predicts 12 membrane-spanning regions, suggesting that NnrS is an integral membrane-bound protein (not shown). There are no recognizable sequence motifs in the primary sequence.

A draft of the genome sequence of *R. sphaeroides* strain 2.4.1, which is closely related to strain 2.4.3, has recently become available (<http://www.jgi.doe.gov>). Examination of the 2.4.1 genome sequence indicates that, as in 2.4.3, the *nnrS* gene is located upstream of *nor* and *nnrR*. There are 211 bp between the translation start codons of *nnrS* and *nnrR* in 2.4.1. The DNA sequences of the *nnrS* ORFs from the strains, as well as the primary sequences of their products, are 80% identical. There is also a second gene in 2.4.1 whose product has limited identity to NnrS (not shown). The gene is located within a cluster of genes whose products are related to genes involved in cobalamin synthesis. The product of this gene is only about 22% identical to NnrS but is of similar length and degree of hydrophobicity and contains stretches of amino acids that are conserved in all members of this family of proteins (not shown).

Comparison of the deduced NnrS sequence with entries in the databases identified several bacteria that contain putative *nnrS* orthologues. Alignment of these sequences identified several regions within this group of proteins that are highly conserved and define this family of proteins. Overall, the N-terminus of the proteins is most highly conserved but there are short stretches of sequences in the C-terminus that are also highly conserved. Most of the bacteria containing a putative NnrS orthologue are proteobacteria that encode Nir and/or Nor. An exception is *Vibrio cholerae*, which can not respire NO, but encodes a protein with similarity to NnrS. Most non-denitrifiers, including *E. coli* and *Bacillus subtilis*, do not encode obvious orthologues of *nnrS*.

Fig. 2. β -Galactosidase activity of *nmrS-lacZ* in various *R. sphaeroides* 2.4.3 strains under different conditions. (a) Expression of the in-frame chromosomal fusion of 3T19A. (b) Expression from plasmid pTB1. The solid bar represents data from cultures grown microaerobically with nitrate, the cross-hatched bar represents cultures grown microaerobically and the bar with no fill represents cultures grown aerobically.

Inactivation and expression of *nmrS*

The *nmrS* ORF was disrupted using Tn-*lacZ* (Manoil, 1990), generating strain 3T19A. Southern blot analysis of chromosomal DNA indicated that 3T19A contained an insertionally inactivated *nmrS* (not shown). The mutant could grow anaerobically by photosynthesis or denitrification. Nitrite reductase activity, which is critical for expression of Nir and Nor, was similar to wild-type and there was no accumulation of nitrite when cells of 3T19A were grown microaerobically with nitrate. Consistent with this result, expression levels of a plasmid-borne copy of *nirK-lacZ* in 3T19A were similar to that observed in wild-type 2.4.3 (not shown).

Analysis of the insertion site of the Tn-*lacZ* indicated that the *lacZ* inserted into the *nmrS* ORF at base 688. This location would place the *lacZ* in-frame with the predicted *nmrS* reading frame. To confirm this, β -galactosidase activity of 3T19A was monitored under various growth conditions (Fig. 2a). There was little detectable β -galactosidase activity in cells of 3T19A cultured aerobically. Activity could be detected in cells grown microaerobically in medium unamended with nitrate. Maximal activity was observed in cells cultured microaerobically in medium amended with nitrate (Fig. 2a).

The pattern of expression of *nmrS* is very similar to the pattern of expression of both *nirK* and the *nor* operon (Bartnikas *et al.*, 1997; Tosques *et al.*, 1997). All three genes show limited expression aerobically, a significant increase under microaerobic conditions and maximal expression under microaerobic conditions in medium amended with nitrate. Previous work has shown that *nirK* and *nor* operon expression is dependent on NnrR (Tosques *et al.*, 1996). Given the similarities in expression patterns of *nirK*, *nor* and *nmrS*, it seems likely

that *nmrS* is a member of the NnrR regulon. To determine if *nmrS* expression is dependent on NnrR, the *nmrS-lacZ* construct pTB1 was conjugated into wild-type and the NnrR-deficient strain R125 and its expression monitored under various growth conditions (Tosques *et al.*, 1996). Since there was no increase in *nmrS-lacZ* expression under microaerobic conditions in R125, NnrR is required for expression of *nmrS* (Fig. 2b). As expected, maximum expression of the plasmid-borne copy of *nmrS-lacZ* in the wild-type strain occurred in medium containing nitrate when oxygen was limiting (Fig. 2b).

Mutagenesis of the *nmrS* regulatory region

Studies on two other members of the NnrR regulon, *nirK* and *nor*, have demonstrated that a sequence about 70 bases upstream of the start of translation and similar to the consensus Fnr-binding site, 5'-TTGAT(N₄)ATCAA-3' (Spiro, 1994), is required for transcriptional activation (Bartnikas *et al.*, 1997; Tosques *et al.*, 1997). In *nirK* this sequence is 5'-TTGCG(N₄)CGCAA-3' and in *nor* this sequence is the same except the C in the first putative half-site is a T. Centred 186 bp upstream of the putative *nmrS* translation start is the sequence 5'-TTGCG(N₄)CACAA-3', which is similar to the sequences identified upstream of *nirK* and *nor*. This sequence is the site previously identified upstream of *nmrR* (Tosques *et al.*, 1996). To determine if this sequence is required for expression of *nmrS*, transcriptional *lacZ* fusions were generated which contain different lengths of upstream DNA (Fig. 1). The fusion used as a reference is pTB1, which contains 739 bases upstream of the putative translational start of *nmrS*. The next-largest fusion (pTB2), which is delimited by the *Bam*HI site upstream of *nmrS*, contains 208 bases upstream of *nmrS* and includes the potential activator-binding site plus an additional 15 bp (Fig. 1). The maximal expression of pTB2 was slightly reduced compared to pTB1 but expression was still induced about 40-fold when cells were shifted from aerobic growth to microaerobic growth in media amended with nitrate (Table 1). pTB2 was not expressed above background levels in strain R125. Plasmid pTB3 contains a fragment with only 166 bp upstream of *nmrS* and consequently lacks the putative regulatory sequence (Fig. 1). There was no detectable expression of pTB3 above background levels under any condition tested (Table 1). These results show that the DNA sequence between -166 and -208 relative to the putative *nmrS* translation start, which includes the sequence motif found in other NnrR-regulated genes, is necessary for *nmrS* transcription.

To provide further evidence that the putative NnrR-binding site upstream of *nmrS* is a binding site for a transcriptional activator, the putative target sequence was mutated into a consensus Fnr-binding site, 5'-TTGAT(N₄)ATCAA-3'. This construct, designated pTB4, is identical to pTB2 except for the four base-pair changes within the putative NnrR-binding site (Fig. 1).

Table 1. Expression of *nnrS-lacZ* constructs in wild-type and NnrR-deficient strains of *R. sphaeroides* 2.4.3

pTB2 and pTB3 are truncated versions of pTB1 (see Methods).

Construct	2.4.3*		2.4.3*	R125†	R213‡
	+O ₂	-O ₂	-O ₂ , +NO ₃ ⁻	-O ₂ , +NO ₃ ⁻	-O ₂ , +NO ₃ ⁻
pTB1	43 ± 9	273 ± 38	1451 ± 57	23 ± 6	ND
pTB2	ND	202 ± 41	938 ± 61	30 ± 8	ND
pTB3	ND	6 ± 3	11 ± 5	ND	ND
pTB4	84 ± 13	3099 ± 418	3149 ± 446	2473 ± 278	276 ± 49

* Wild-type *R. sphaeroides*.

† NnrR deficient.

‡ FnrL deficient.

ND, Not done.

Fig. 3. (a, b) Taxis of *nnrS* mutant 3T19A to plugs of nitrate (a) and nitrite (b). (c, d) Taxis of 3T19A containing the gene encoding the NnrS-HT *in trans* to plugs containing either nitrate (c) or nitrite (d). All cultures were incubated anaerobically, and the agar plug contained either 50 g potassium nitrate l⁻¹ or 25 g sodium nitrite l⁻¹.

In the wild-type strain, the expression of pTB4 was no longer dependent on nitrate but was dependent on the oxygen concentration in the medium, as expected for FnrL-dependent expression (Table 1). Likewise, ex-

pression was not significantly affected in the NnrR-deficient strain but was significantly decreased in the FnrL-deficient strain. The shifting of *nnrS* to the FnrL regulon by mutagenesis of this site strongly supports the

conclusion that this site is the binding site of the transcriptional activator that activates *nnrS* expression.

Effect of *nnrS* inactivation on chemotaxis to nitrite

Since *nnrS* is expressed when nitrate is used as a terminal electron acceptor, its product is likely to be involved in nitrogen oxide metabolism but it is obviously not essential for denitrification when cells are cultured in liquid medium. An alternative method for assessing the ability of *R. sphaeroides* strains to utilize a particular respiratory substrate is to assess their ability to show taxis towards the substrate. Chemotaxis towards a particular electron acceptor in *R. sphaeroides* is directly dependent on the ability to respire using that compound as an electron acceptor (Gauden & Armitage, 1995). If NnrS is involved in nitrogen oxide metabolism, it is possible that NnrS-deficient mutants exhibit changes in taxis towards nitrogen oxides that are not discernible using liquid cultures.

The 3T19A strain did not show obvious taxis towards a nitrate plug (Fig. 3a), but taxis towards a plug containing nitrite was observed (Fig. 3b). The 3T19A strain expressing a hexahistidine-modified version of NnrS (see below) demonstrated taxis towards plugs containing nitrate (Fig. 3c). Transconjugants of 3T19A complemented with wild-type *nnrS* showed a similar response (not shown). Interestingly, the 3T19A cells formed a much narrower band surrounding the nitrite-containing plug than did complemented 3T19A (compare Figs 3b and 3d). The mean distance from the nitrite plug to the inside edge of the ring of cells was similar in 3T19A and the complemented strain but the width of the ring of 3T19A cells was consistently smaller by at least 50%. The response of the complemented 3T19A is identical to what has been observed in taxis experiments with the wild-type strain (D. Y. Lee and others, unpublished).

Purification of the *nnrS* product

The high proportion of hydrophobic residues suggests that NnrS, like Nor, is membrane bound. Examination of the alignment of putative NnrS orthologues reveals that there are several histidines that are highly conserved in this set of sequences (not shown). Some of these histidines are also located in regions of high sequence conservation. For example, the sequence WHXHEM-(L/I)(F/W)G appears to be a uniquely identifying motif for this set of proteins. Conserved histidines in membrane-bound proteins are sometimes utilized as ligands for metal centres, as in Nor and the related haem-copper oxidases (Zumft, 1997). NnrS was purified to determine if it contained metal centres.

NnrS was purified by affinity-based techniques after a hexahistidine tag was added to its C-terminus (generating NnrS-HT). Membranes of cells carrying a plasmid-borne copy of *nnrS*-HT were detergent solubilized and purified using a two-step procedure. SDS-

Fig. 4. Absorption spectra of protein purified from the 2.4.3 strain expressing NnrS-HT. The spectrum indicated by the solid line is the absorbance of the as-isolated protein. The spectrum indicated by the large dashes is the absorbance of the protein reduced by addition of sodium ascorbate. The spectrum indicated by the small dashes is the absorbance of the protein reduced by addition of sodium dithionite.

PAGE analysis of the material eluted from the second column revealed one major band of 25 kDa and a few minor bands (not shown). If the sample was heated, the 25 kDa band disappeared and bands of much higher molecular mass appeared (not shown). This behaviour is consistent with the predicted hydrophobic nature of NnrS and likely explains why the major band has a smaller molecular mass than the predicted weight of NnrS. Hydrophobic proteins frequently run at smaller than predicted values (Hosler *et al.*, 1992). In a control experiment, no 25 kDa band was found after the extraction and purification of membranes from cells lacking NnrS-HT. To further demonstrate that the copy number of the *nnrS*-HT directly correlated with the amount of NnrS-HT purified, *nnrS*-HT was crossed into the chromosome to determine expression levels from a single copy of *nnrS*-HT. A 25 kDa protein was purified from the single-copy strain but at only about 25% of the yield from cells containing a plasmid-borne copy of *nnrS*-HT (not shown).

The purified protein has a reddish colour. Fig. 4 shows the as-isolated and ascorbate- and dithionite-reduced spectra of the concentrated protein. The spectra show the presence of a *b*-type cytochrome, with the reduced sample having absorption maxima at 427.0 nm, 529.5 nm and 560.0 nm. There is no evidence of a *c*-type cytochrome, consistent with sequence analysis of NnrS. Near-complete reduction of the haem by addition of ascorbate indicates that the haem has a positive redox potential. Dithionite-reduced protein was incapable of binding CO. Pyridine haemochrome analysis indicated the presence of protoporphyrin IX at a ratio of 1.7 mol per mol NnrS.

As expected, metal analysis of the purified protein revealed that iron was present in NnrS with a ratio of

Fig. 5. EPR spectrum of the as-isolated protein isolated from a strain expressing the NnrS-HT obtained with an IBM-Bruker ER-200 X-band EPR (9.51 Hz) and APD Cryogenics LTR-3 Helitran helium flow system operating at 13 K. Microwave power was 2 mW, field modulation was 0.0032 T and the EPR frequency was 9.513 GHz. T, tesla ($= 10^4$ gauss).

about 1.5 mol Fe per mol NnrS. In addition to iron, copper was also found in the NnrS sample at a ratio of about 1 mol Cu per mol NnrS. More details of the nature of the iron and copper in the protein can be obtained from the EPR spectrum of Fig. 5. There is an EPR signal in the $g = 5\text{--}7$ region (0.100–0.135 T) that is typical of high-spin ferric haem with rhombic distortion (Salerno *et al.*, 1996). In the g -value region of 2.3–2.0, a signal typical of type 2 copper (Solomon *et al.*, 1992) was noted with respective $g_{\parallel}$ - or $A_{\parallel}$ -values of 2.23 and 0.02 T. When the protein was oxidized with a molar excess of ferricyanide, this copper signal increased in intensity, indicating the copper signal was not adventitious copper (not shown). The metal analysis and the corroborating EPR evidence of high-spin ferric haem and type 2 copper signals show that NnrS is a haem–copper protein expressed when *R. sphaeroides* is producing NO. There were two additional EPR signals whose origin was not as clear as the high-spin ferric haem or type 2 copper. Notably, there was a free radical signal at $g = 2.00$ with hyperfine splitting of 0.0015 T, and there was a broad feature at $g \sim 1.65$. Both these latter signals diminished upon ferricyanide oxidation (not shown). The $g \sim 1.65$ signal was highly temperature dependent and disappeared upon ferricyanide oxidation. A small signal near $g = 4.0$ was present; this is a signal typical for a small amount of adventitious ferric iron.

The purified protein could not produce nitrous oxide from either nitrite or NO (not shown). It was also incapable of reducing oxygen. Addition of nitrite to reduced protein did not lead to oxidation of the protein. Therefore, it seems unlikely that NnrS is involved in the metabolism of nitrite or NO. This is consistent with the phenotypic analysis of the NnrS-deficient strain 3T19A.

DISCUSSION

A gene, designated *nmrS*, whose expression is dependent on NnrR, has been identified in *R. sphaeroides* 2.4.3. Previously, only *nirK* and *nor* operon expression had been shown to be dependent on NnrR in *R. sphaeroides* 2.4.3 (Bartnikas *et al.*, 1997; Tosques *et al.*, 1997). The expression pattern of *nmrS* was nearly identical to what has been observed for *nirK* and *nor*. The likely NnrR-binding site utilized for *nmrS* activation was identified as being centred 186 bp upstream of the putative translation start site. The NnrR-binding site upstream of *nmrS* is further upstream of the translation start site than the NnrR-binding sites in either *nirK* or the *nor* operon (Bartnikas *et al.*, 1997; Tosques *et al.*, 1997). This location, however, does permit *nmrR* and *nmrS* to share an overlapping regulatory region with a single NnrR-binding site. This arrangement would indicate that as inducing conditions arise, a transcriptional activator binds and represses *nmrR* expression (Tosques *et al.*, 1996) and activates *nmrS* expression.

The conversion of the NnrR- to a Fnr-binding site by mutagenesis of the NnrR recognition sequence demonstrates that DNA binding and activation by FnrL and NnrR must be mechanistically similar, as has previously been shown for Fnr and cAMP receptor protein (Zhang & Ebright, 1990). In addition, this demonstrates that Fnr and NnrR, both of which have been implicated as being involved in regulating denitrification in other bacteria (van Spanning *et al.*, 1997; Ye *et al.*, 1995), have distinctly different DNA targets in *R. sphaeroides*. In the related denitrifier *Paracoccus denitrificans*, binding-site preferences for Fnr and NnrR are difficult to discern from an analysis of the regulatory regions of genes that are expressed anaerobically (van Spanning *et al.*, 1997). This has led to the suggestion that binding discrimination is not only sequence dependent but also occurs through other mechanisms. In *R. sphaeroides*, NnrR and FnrL apparently respond to different effectors and then bind to related, but distinct, sequence motifs.

Phenotypic analysis demonstrated that NnrS is not essential for the respiration of nitrogen oxides. However, disruption of *nmrS* did affect taxis towards nitrate and nitrite, demonstrating a role for NnrS during denitrification. The reason for the changes in taxis are unclear, but likely arises from subtle changes in nitrogen oxide reduction that can not be detected in the other phenotypic assays. However, the link between NnrS and nitrogen oxide reduction is supported by the observation that NnrS-like proteins are found almost exclusively in bacteria encoding Nir and/or Nor. Bacteria such as *E. coli* that contain an ammonia-producing Nir do not encode NnrS orthologues. In *R. sphaeroides*, *Sinorhizobium meliloti* (<http://sequence.toulouse.inra.fr/meliloti.html>) and *Pseudomonas stutzeri* (Glockner & Zumft, 1996) the genes encoding the *nmrS* orthologues are clustered with other genes encoding products required for nitrite or NO reduction during denitrification. Interestingly, in *Pseudomonas aeruginosa* the putative *nmrS* orthologue is located adjacent to a gene whose

product is similar to a gene encoding a flavohaemoprotein (Stover *et al.*, 2000). Related flavohaemoproteins in *E. coli* and *Salmonella typhimurium* have been shown to be required for resistance to NO and have been characterized as NO dioxygenases (Gardner *et al.*, 1998; Membrillo-Hernandez *et al.*, 1999).

Purification of NnrS-HT demonstrated that NnrS is a membrane-bound haem-copper protein. Analysis of the sequence suggests it may have 12 membrane-spanning regions. In these regions there are histidines that are conserved throughout the NnrS family. Given the lack of other conserved metal-liganding residues in the NnrS alignment, it seems reasonable to suggest the conserved histidine residues in NnrS from *R. sphaeroides* 2.4.3 are the likely ligands of the copper and haem prosthetic groups. Examination of the alignment of putative NnrS orthologues from denitrifiers reveals four histidine residues present in every sequence. Three of these histidine residues are likely ligands for the type 2 copper. It is unknown how many other amino acid ligands are required since it is uncertain how many haems are present in NnrS. Metal and haem analysis suggested more than 1 mol haem per mol NnrS. However, the EPR spectrum in the low-field $g \sim 6$ region is indicative of a single high-spin ferric haem, which are frequently 5-coordinated. This haem could be ligated by the remaining histidine. However, there are 5-coordinate haem proteins, particularly those showing rhombicity in their EPR spectra, which have ligands other than histidine. Notably, there is cysteine sulfur in the case of cytochrome P450 (Dawson & Sono, 1987) and NO synthase (McMillan *et al.*, 1996), and there is a tyrosine oxygen in the case of catalase (Murthy *et al.*, 1981). The involvement of these residues as metal ligands is not supported by the alignment, assuming any liganding residue would be conserved in a majority of sequences. Irrespective of the exact nature of the haem(s) and copper in NnrS, the presence of metal centres indicates that NnrS likely catalyses oxidation-reduction chemistry on an as yet unidentified substrate produced when the cells are respiring nitrogen oxides.

ACKNOWLEDGEMENTS

We are grateful to Reiko Akakura for construction of the R213 and R214 strains. T. Bartnikas and T. Bobo were supported by a Howard Hughes Undergraduate Research Fellowship during part of this work. This work was supported by the Department of Energy (95ER20206).

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Received 31 July 2001; revised 31 October 2001; accepted 20 November 2001.