Transcriptional analysis of the *nirS* gene, encoding cytochrome *cd₁* nitrite reductase, of *Paracoccus pantotrophus* LMD 92.63

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The gene for cytochrome *cd₁* nitrite reductase of *Paracoccus pantotrophus*, a protein of known crystal structure, is *nirS*. This gene is shown to be flanked by genes previously recognized in other organisms to encode proteins involved in the control of its transcription (*nirI*) and the biosynthesis of the *d₁* cofactor (*nirE*). Northern blot analysis has established under anaerobic conditions that a monocistronic transcript is produced from *nirS*, in contrast to observations with other denitrifying bacteria in which arrangement of flanking genes is different and the messages produced are polycistronic. The lack of a transcript under aerobic conditions argues against a role for cytochrome *cd₁* in the previously proposed aerobic denitrification pathway in *Pa. pantotrophus*. A putative rho-independent transcription termination sequence immediately following *nirS*, and preceding *nirE*, can be identified. The independent transcription of *nirS* and *nirE* indicates that it should be possible to produce site-directed mutants of *nirS* borne on a plasmid in a *nirS* deletion mutant. The transcript start point for *nirS* has been determined by two complementary techniques, 5'-RACE (Rapid amplification of cDNA 5' ends) and primer extension. It is 29 bp upstream of the AUG of *nirS*. An anaerobox, which presumably binds Nnr, is centred a further 41.5 bp upstream of the transcript start. No standard σ^{70} DNA sequence motifs can be identified, but a conserved sequence (T-T-G/C-C-G/C-G/C) can be found in approximately the same position (–16) upstream of the transcript starts of *nirS* and *nirI*, whose products are both involved in the conversion of nitrite to nitric oxide.

Keywords: *Paracoccus pantotrophus*, nitrite reductase, transcription, denitrification, promoter

INTRODUCTION

True denitrifiers convert nitrate to gaseous nitrogen via nitrite, nitrous oxide and nitric oxide, though many other species of bacteria, including archaea, can perform

parts of the process (Zumft, 1997; Ferguson, 1994). The efficient production of nitric oxide (NO) by the nitrite reductase is important to cellular survival since build up of nitrite is toxic to the cell. It is also the first committed step of denitrification because although nitrite can be assimilated, NO cannot. The NO species is also cytotoxic, and must be immediately reduced further to the non-toxic nitrous oxide (N₂O). Thus the co-ordinated regulation of nitrite and nitric oxide reductases forms the core of the denitrification process (Zumft, 1997; Baker *et al.*, 1998; Ferguson, 1994). The conversion of nitrite to nitric oxide is carried out by one of two types of enzyme: a copper-containing nitrite reductase or a di-haem nitrite reductase which contains a *c* haem and a *d₁* haem. The latter haem has only been found in denitrifiers containing this type of nitrite reductase. Di-haem *cd₁* nitrite reductases have been

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Abbreviations: Fnr, fumarate nitrate reduction transcriptional activator; Nnr, nitrate nitrite reduction transcriptional activator; 5'-RACE, rapid amplification of cDNA 5' ends; TSS, transcript start site.

The GenBank accession number for the sequence in this paper is U75413.

characterized in *Paracoccus pantotrophus* LMD 92.63 (Moir *et al.*, 1993; Baker *et al.*, 1997) as well as in other denitrifiers including *Pseudomonas aeruginosa* (Silvestrini *et al.*, 1989), *Pseudomonas stutzeri* Zobell (Jüngst *et al.*, 1991), *Ps. stutzeri* JM300 (Smith & Tiedje, 1992), *Paracoccus denitrificans* IFO 12442 (Ohshima *et al.*, 1993), *Pa. denitrificans* PD1222 (de Boer *et al.*, 1994) and *Ralstonia eutropha* H16 (Rees *et al.*, 1997). The structural gene for nitrite reductase (*nirS*) has been fully or partially sequenced in all these organisms.

The transcription of *nirS* has been shown to be activated by Fnr-like proteins. These are Nnr in the case of *Pa. denitrificans* (van Spanning *et al.*, 1997), Anr in *Ps. aeruginosa* (Arai *et al.*, 1997; Hasegawa *et al.*, 1998) and the set of regulatory proteins named DnrE, DnrS and DnrD in *Ps. stutzeri* (Vollack *et al.*, 1999). All these proteins bear a high similarity to the transcriptional activator Fnr of *Escherichia coli*, but lack the N-terminal cysteines which allow the formation of an oxygen-labile iron-sulphur cluster in Fnr (Khoroshilova *et al.*, 1995). The functions associated with oxygen sensing Fnr in *E. coli* are performed by another protein in *Pa. denitrificans*, FnrP, which is distinct from Nnr, and likewise *Ps. stutzeri* possesses FnrA. It is tempting to assume that the regulation of denitrification in *Ps. aeruginosa* and *Ps. stutzeri* is very similar to that in *Pa. denitrificans* and *Pa. pantotrophus*. However, not only is the gene organization within the *nir* loci different (Zumft, 1997; Baker *et al.*, 1998), but the environmental signals which trigger denitrification are different as well. It would appear that in *Ps. stutzeri* nitrate and the absence of oxygen are sensed, directly or indirectly, by DnrE, DnrS and DnrD (Vollack *et al.*, 1999) and these proteins then activate the *nir* and *nor* operons. In *Pa. denitrificans* and *Ps. aeruginosa*, the transcription of the Nnr or Dnr proteins is dependent on nitric oxide (van Spanning *et al.*, 1999; Arai *et al.*, 1999), and *nirS* in these organisms is thus subject to product regulation rather than the more normal substrate regulation.

In *Pa. denitrificans*, nitric oxide and Nnr also serve to activate promoters upstream of the *nor* (nitric oxide reductase) operon and *nirI* (van Spanning *et al.*, 1999), a gene encoding a putative DNA-binding protein of unknown function. The site at which Nnr binds is presumably the inverted repeat, reminiscent of an Fnr box, found upstream of *nirS*, *nirI* and *norC*. These regulatory sequences differ by only a few base pairs from the consensus *E. coli* motif for Fnr binding, and are also very similar to the anaeroboxes found upstream of *Pa. denitrificans* FnrP regulated genes. Selection between FnrP and Nnr may not solely be on the basis of the sequence of the DNA-binding site and secondary factors are presumably involved. It has yet to be shown whether *Pa. pantotrophus* has multiple Nnr-like proteins (as in *Ps. stutzeri*), but knockouts of Nnr suggest it is the major contributor to the activation of *nirS* transcription in *Pa. denitrificans* (van Spanning *et al.*, 1997).

The transcription of *nirS* in *Pa. pantotrophus* [note that this organism was formerly designated *Thiosphaera pantotropha* (Rainey *et al.*, 1999)] requires study for two

important reasons. First, *Pa. pantotrophus* has been described as an aerobic denitrifier (Arts *et al.*, 1995; Robertson *et al.*, 1995) yet Moir *et al.* (1995) failed to detect cytochrome *cd₁* in aerobically grown cells. Second, this organism has served as the source of protein for the first crystal structure of *cd₁* nitrite reductase (Fülöp *et al.*, 1995; Baker *et al.*, 1997). An understanding of how the structural gene is transcribed with the genes responsible for the manufacture of the *d₁* haem prosthetic group is essential if gene knockout strains are to be constructed, in which mutant *nirS* genes can be efficiently manufactured by the cell in a holo form. Results obtained with *Pa. pantotrophus* can be extrapolated to *Pa. denitrificans* as the two organisms are closely related to one another (Rainey *et al.*, 1999) although are distinct species. To begin to understand the activation of transcription of *nirS* we have sequenced part of the *nir* locus from *Pa. pantotrophus* LMD 92.63, determined the transcription start site of the gene and estimated the length of transcripts from the *nirS* promoter.

METHODS

Materials. Restriction endonucleases and T4 ligase were supplied by New England Biolabs. Sequenase was supplied by Amersham International. AMV reverse transcriptase was obtained from a variety of sources including Promega. Oligonucleotides were synthesized by V. Cooper of the Dyson Perrins Chemistry Laboratory, University of Oxford.

Bacterial strains, media and vectors. RNA and DNA were isolated from *Paracoccus pantotrophus* LMD 92.63. *Escherichia coli* JM83 or DH5 α was used for routine cloning. *Pa. pantotrophus* was grown on either the minimal medium described by Robertson & Kuenen (1983) or Luria-Bertani (LB) medium. LB was also used for the growth of *E. coli*. Plasmid pTNIR3 was kindly supplied by T. de Boer, Bio-Centrum Amsterdam, Vrije Universiteit, the Netherlands (T. de Boer, R. J. M. van Spanning & A. H. Stouthamer, unpublished results).

Extraction of total RNA from *Pa. pantotrophus*. Total RNA was isolated from cells of *Pa. pantotrophus* LMD 92.63 using a method based on that of Cuypers & Zumft (1993). Cultures (50 ml) were grown to mid-exponential phase, harvested by centrifugation and lysed in 4 ml lysis buffer (1% SDS, 1 mM EDTA). The lysate was extracted with 4 ml phenol/chloroform, saturated with 2 M sodium acetate (pH 4.0) and centrifuged at 10000 *g* for 10 min at 4 °C. The aqueous phase was then re-extracted with 4 ml chloroform and recovered by centrifugation. Propan-2-ol (0.8 vol.) was added and the mixture was incubated on ice for 30 min, then centrifuged at 15000 *g* for 15 min at 4 °C. The precipitated nucleic acids were redissolved in 200 μ l 1 $\times$ concentration DNase buffer (40 mM Tris/HCl, pH 8.0; 10 mM NaCl; 6 mM CaCl₂) containing 100 units RNase-free DNase and incubated at 37 °C for 30 min. After DNase treatment, the mixture was extracted once more with an equal volume of phenol/chloroform and the RNA was ethanol-precipitated by incubating at -70 °C, followed by centrifugation at 15000 *g* for 15 min, 4 °C. The pellet was washed with 100 μ l ice-cold 70% (v/v) ethanol, air-dried and redissolved in 100 μ l RNase-free water. RNA quality was analysed by the absorbance ratio $A_{260}:A_{280}$ and by formaldehyde-agarose gel electrophoresis.

Northern blotting. Northern blotting was used to detect

specific mRNA transcripts from denitrification genes in *Pa. pantotrophus*. The method used closely followed that of Engler-Blum *et al.* (1993). The membrane was pre-hybridized for 1 h at 68 °C in a high SDS phosphate buffer (250 mM sodium phosphate, pH 7.2; 1 mM EDTA; 20 %, w/v, SDS). A digoxigenin-labelled dsDNA probe was hybridized to the RNA in 10 ml of the same buffer at 68 °C overnight. The membrane was washed three times for 20 min each at 65 °C in 50 ml H-wash buffer (20 mM sodium phosphate, pH 7.2; 1 %, w/v, SDS; 1 mM EDTA), then once for 5 min in W-wash buffer (100 mM maleic acid, pH 8.0; 3 M NaCl; 0.3 %, v/v, Tween 20) and blocked for 1 h in blocking buffer (W-wash buffer plus 0.5 %, w/v, blocking reagent; Roche). Digoxigenin antibody conjugate was diluted 1:10000 in blocking buffer and the membrane was incubated with 20 ml of this buffer for 30 min. The membrane was then washed four times for 10 min each with W-wash buffer and equilibrated for 5 min in substrate buffer (100 mM Tris/HCl, pH 9.5; 100 mM NaCl; 50 mM MgCl₂). Hybridized probe was detected by chemiluminescence, using the alkaline phosphatase substrate CSPD (Roche) diluted 1:100 in 1 ml substrate buffer and pipetted onto the membrane. Following a 5 min incubation in the dark, excess solution was removed and the membrane was incubated in a sealed hybridization bag at 37 °C for 15 min. The membrane was then exposed to X-ray film (Kodak), initially for 1 h, or until exposed bands appeared on the film.

DNA probes used in this study were made from internal PCR products of *nirS* or *nirE*. The 141 bp *nirS* probe was amplified from genomic *Pa. pantotrophus* DNA using primers NIRSF (CTGGCCCTTGTCTTGGGC) and 228R (TACGTCCTGCTGTGCAAGGT), which were subsequently labelled with dUTP-digoxigenin-alkaline phosphate conjugate (Roche). A 419 bp probe for *nirE* was made in the same manner using the primers 2431F (AGACGGTGACGAACGGGG) and 2849R (GGGCACATAGTCGACGGG).

Primer extension analysis. Primer extension was used to determine the 5' end of mRNA transcripts. The experiment is divided into three stages: oligonucleotide labelling, primer-RNA annealing and cDNA extension. Ten picomoles of oligonucleotide was end-labelled in a 20 µl reaction exactly as described in Sambrook *et al.* (1989). The labelled oligonucleotide was redissolved in 100 µl TE buffer and could be stored at -20 °C for up to 1 week. One microlitre (approx. 10⁵ c.p.m.) of labelled primer was annealed to 40 µg heat-denatured RNA in 20 µl annealing buffer (2 mM Tris/HCl, pH 7.9; 250 mM KCl; 0.2 mM EDTA) at 50 °C for 90 min. The annealed primer RNA was then ethanol-precipitated, redissolved in 20 µl reverse transcription mix (50 mM Tris/HCl, pH 8.3; 75 mM KCl; 3 mM MgCl₂; 10 mM DTT; 1.3 mM each dNTP; 20 units RNasin inhibitor; 50 µg actinomycin D ml⁻¹; 33 units AMV reverse transcriptase) and incubated at 37 °C for 2 h. cDNA synthesis was stopped with 25 mM EDTA. The mixture was treated with RNase at 37 °C for 20 min, extracted with phenol/chloroform, ethanol-precipitated and redissolved in 4 µl TE buffer. Six microlitres of sequencing stop solution was added and the labelled cDNA was run on a 6 % acrylamide sequencing gel alongside a reference sequencing ladder generated using the same primer.

5'-RACE (Rapid amplification of cDNA 5' ends). 5'-RACE was used as an alternative method to determine the 5' end of mRNA transcripts. The procedure used closely followed that of Frohman (1993). Ten micrograms of total RNA was reverse-transcribed using the *nirS* sequence specific primer 284R (5'-GACATCCTGCTGCGCAAGGT-3'). The mixture was then diluted with TE buffer, excess primer and nucleotides were removed by centrifugation through a Centricon-100 filter and

the cDNA was recovered on a Millipore spin filter in 10 µl TE buffer. Addition of the poly-dATP tail and the two subsequent nested PCR amplifications were performed exactly as described by Frohman (1993), using the forward primers QTF, Q0F and Q1F (detailed in Frohman, 1993) and the sequence-specific reverse primers 242R (5'-ATAGTCGGTGC GCGTCTT-3') and 228R (5'-TCTTGTGGTCCTCCAGCG-3'). PCR products were analysed by electrophoresis on 1.2 % agarose gels, cloned into the pGEM-T vector and sequenced using M13 universal primers.

Sequencing. Sequencing was performed manually. The standard Sequenase protocol (Amersham International) was modified by the addition of DMSO to the reaction to aid the resolution of stops caused by the high G + C mol % content of the DNA.

Alignment of sequences and DNA analysis. Sequence data were analysed using GCG9 (Genetics Computer Group, USA) and the GenBank and EMBL databases held at Oxford University Molecular Biology Data Centre, Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, UK).

RESULTS AND DISCUSSION

Characterization of the *nirS* locus

We have previously reported the DNA sequence and the derivation of the amino acid sequence of the coding region of *nirS* from *Pa. pantotrophus* (Baker *et al.*, 1997). Sequencing of the *Pa. pantotrophus nir* locus was carried out on plasmid pTNIR3 isolated from *Pa. pantotrophus* LMD 92.63. The arrangement of ORFs (Fig. 1) derived from sequencing of one or both strands was the same as that found in *Pa. denitrificans* PD1222 (de Boer *et al.*, 1994) but differences were noted in the restriction map (Fig. 1). The gene encoding the structural protein (*nirS*) was followed by *nirE* (encoding S-adenosyl-L-methionine uroporphyrinogen III methyltransferase) and preceded by *nirI*, which is divergently transcribed and proposed to encode a transcriptional regulator of nitrite reductase expression in *Pa. denitrificans* (van Spanning *et al.*, 1997). Sequencing was carried out on both strands in the regions indicated in Fig. 1 so as to check the protein sequence deduced during the determination of the crystal structure of cytochrome *cd₁* nitrite reductase (Fülöp *et al.*, 1995; Baker *et al.*, 1997) and to identify regulatory sequences upstream of *nirS*. Overall, the sequence reported here (accession no. U75413) has 93.5 % identity to the equivalent region of PD1222; further sequence information showed that *nirE* is followed by *nirC*, while *norC* and *norB*, encoding nitric oxide reductase, are considerably upstream of *nirS* (Fig. 1).

Nucleotide sequence of DNA flanking *nirS*

The DNA upstream of *nirS* contained only one stretch of sequence with a previously observed motif, an anaerobox in the same relative position to the initiation codon as was noted by de Boer *et al.* (1994). The DNA between this anaerobox and the start of the *nirS* gene was devoid of any clear -10 or -35 sequences that might indicate the σ^{70} -dependency of *nirS* transcription, although we have previously argued that the genus

Paracoccus pantotrophus* LMD 92.63** Paracoccus denitrificans* Pd 1222**

Fig. 1. Restriction map of the *nir* loci of *Pa. pantotrophus* LMD 92.63 and *Pa. denitrificans* PD1222 (de Boer *et al.*, 1994), showing the region sequenced (U75413). Labels marked with an asterisk refer to database accession numbers. Experimentally determined restriction sites are: B, *Bam*HI; E, *Eco*RI; H, *Hind*III; N, *Not*I; P, *Pst*I; S, *Sma*I; Sp, *Sph*I. Shaded genes indicate those completely sequenced on both strands. Parts of *nirC*, *norC* and *norB* for *Pa. pantotrophus* were also sequenced on both strands.

Paracoccus has a constitutive promoter consensus much like that of all the other eubacteria (Baker *et al.*, 1998).

The *nirS* stop codon is not immediately followed by the initiation codon of the adjacent gene *nirE*. Instead, there is a 73 bp intergenic region, which contains an inverted repeat which would form a strong stem-loop, followed by a region rich in adenosine and thymine (Fig. 2). These

features indicate a rho-independent terminator, but in other denitrifiers *nirS* is co-transcribed with the genes required for d_1 biosynthesis (Härtig & Zumft, 1999; Kawasaki *et al.*, 1997). To determine if *nirS* of *Pa. pantotrophus* is transcribed separately or as an operon including at least *nirE*, Northern blots were used to determine the size of transcript(s) arising from the *nirS* promoter.

Fig. 2. Nucleotide sequences of parts of the *nirS* locus. Two nucleotide sequences are shown: the top sequence is from *Pa. pantotrophus* (accession no. U75413); the lower from *Pa. denitrificans* (accession no. U05002). Non-coding regions of U75413 have not been previously discussed but the *nirS* coding region appears in full elsewhere (Baker *et al.*, 1997), and thus only a few codons from either end of the gene are shown. Nucleotides underlined in the intergenic regions indicate differences between *Pa. pantotrophus* and *Pa. denitrificans*. Transcript start sites are indicated as circled nucleotides. Boxed nucleotides indicate motifs discussed in the text. The transcript start sites of *nirS* (this work) and *nirI* (Saunders *et al.*, 1999) have been experimentally determined.

Fig. 3. Northern blot of total RNA from *Pa. pantotrophus* using a *nirS*-derived probe. RNA from aerobically (lane 1) or anaerobically (lane 2) grown *Pa. pantotrophus* was detected by chemiluminescence. The relative positions of the *E. coli* 23S (2904 bases) and 16S (1542 bases) rRNA species are shown on the left of the blot, and the sizes of the RNA molecular mass markers (New England Biolabs) are shown on the right.

Northern blot studies

mRNA from nitrate-grown *Pa. pantotrophus* blotted and hybridized to an internal *nirS* DNA probe showed that *nirS* occurs on a monocistronic transcript about 1825 bp in length (Fig. 3). This size was estimated by comparison with an RNA molecular mass marker (Fig. 3). The quality of the RNA preparation was assessed by comparison with 16S and 23S rRNA of *E. coli*, rather than that of *Pa. pantotrophus*. The *E. coli* rRNA was used because the 23S rRNA of *Pa. pantotrophus* appeared to spontaneously and reproducibly degrade into discrete RNA fragments, one of which was the same size as the 16S rRNA molecule (data not shown). *E. coli* preparations prepared in tandem did not behave in this manner. The apparent degradation may be indicative of an 'intervening sequence' (Gregory *et al.*, 1996). The decay of the 23S rRNA made the estimation of total RNA quality in the preparation (normally gauged by the relative amounts of 23S and 16S rRNA) difficult to assess.

The 1825 bp RNA band hybridizing to *nirS* did not appear under aerobic conditions, once again casting doubt over the ability of *Pa. pantotrophus* to denitrify aerobically using *cd*₁ nitrite reductase. Aerobic reduction of nitrate has been well documented, for example, in *Pa. denitrificans* (Sears *et al.*, 1997), but true aerobic denitrification with the generation of nitrogen as the end product remains in general controversial. Experiments that support the existence of aerobic

denitrification in whole cells of *Pa. pantotrophus* (Arts *et al.*, 1995; Robertson *et al.*, 1995) have not yet been supported by molecular approaches to the problem. The present Northern blot studies indicate that little if any *nirS* mRNA is produced, showing that the observation by Moir *et al.* (1995) that *cd*₁ nitrite reductase could not be detected by Western blotting in aerobically grown *Pa. pantotrophus* cells results from lack of transcription from the *nirS* gene. Nitrite reductase plays a crucial part in denitrification (Zumft, 1997) and deletion mutants of *nirS* in *Pa. denitrificans* and *Pa. pantotrophus* are unable to denitrify (de Boer *et al.*, 1994; N. F. W. Saunders, S. C. Baker & S. J. Ferguson, unpublished results); thus the molecular basis for any aerobic nitrite reduction is currently obscure. Although some authors maintain that extended laboratory cultivation of *Pa. pantotrophus* leads to the loss of the aerobic denitrification (reviewed by Stouthamer *et al.*, 1997), a complete reversal in the properties of a multi-component regulatory system (comprising at least an NO sensor, Nnr and NirI) is improbable.

The monocistronic nature of the *nirS* transcript agreed with the presence of a putative downstream transcript termination structure (Fig. 2), and no larger band could be seen even if the Northern blot was overexposed. The length of the *nirS* transcript suggests that transcription terminates around 55 bp upstream of the *nirE* translation initiation codon, within 10 bp of the stem-loop structure found in the *nirS*–*nirE* intergenic region. This supports the proposal that this structure does indeed function as a rho-independent terminator. When the same RNA preparation was blotted to a *nirE* probe (which hybridized successfully to a plasmid-borne *nirE* gene) no hybridization was seen to any RNA molecule, confirming that the previously observed transcript did not contain *nirE* mRNA. This observation suggests that the *nirE* transcript was in an abundance too low to be visualized by the methods used.

The presence of *Pa. pantotrophus nirS* as a monocistronic mRNA is in contrast to the *nirSMCF-DLGHJEN* nitrite reductase operon of *Ps. aeruginosa*, in which a single transcript provides both the structural nitrite reductase gene and the genes for *d*₁ haem biosynthesis (Kawasaki *et al.*, 1997). Northern blot analysis of the *nirSTM* operon of *Ps. stutzeri* (ZoBell) showed that two transcripts originated from the *nirS* promoter, one containing *nirS* alone, the other *nirS*, *T* and *M* (Härtig & Zumft, 1999). In neither pseudomonad is the *nirS* gene followed by a terminator such as that identified in *Pa. pantotrophus* (Fig. 2).

Initiation of transcription of *nirS*

The transcription start site (TSS) of *nirS* was initially determined by primer extension analysis (Fig. 4), and this suggested a guanidine 29 bp upstream of the translation initiation codon. Since high GC content mRNA (*Pa. pantotrophus* has a genome of approximately 66 mol% G + C; Rainey *et al.*, 1999) has proved to be susceptible to artefactual termination of the primer

Fig. 4. Primer extension product (lane marked cDNA) synthesized from *nirS* mRNA, compared with a sequencing ladder generated from the *nirS* gene using the same primer. Part of the sequence upstream of *nirS* is shown on the left of the sequencing ladder and the G residue indicating the transcription start site is labelled with an asterisk.

extension reaction (S. C. Baker & S. J. Ferguson, unpublished results), the TSS was further analysed using 5'-RACE. This again showed that, under denitrifying conditions, the *nirS* transcript commenced from a guanidine 29 bp upstream of the translation initiation codon (Fig. 5).

The promoter region of *nirS* indicated by these transcription studies had few identifiable sequence motifs. Despite the presence of a clear anaerobox (TTAA-CaatgGTCAA) similar to the binding site of *E. coli* Fnr (TTGAT-N₄-ATCAA; Gunsalus & Park, 1994) centred at -41.5 from the TSS, no clear -10 motif could be seen, which might have indicated the footprint of σ^{70} RNA polymerase. NirS could only be expressed in *E. coli* when a *tac* promoter, rather than the *Pa. pantotrophus nirS* promoter, was used to drive expression (S. C. Baker, N. F. W. Saunders & S. J. Ferguson, unpublished results). Thus it appears that Fnr plus the RNA polymerases available in *E. coli* cannot recognize the *nirS* promoter. It is likely that *Pa. pantotrophus* contains transcription factors that recognize the context of the anaerobox as being Nnr- rather than Fnr-dependent. In Fnr-regulated genes of organisms such as *E. coli*, the -35 motif for this RNA polymerase is replaced by the sequence for the binding of the regulatory protein when an Fnr-type protein acts as an activator (Spiro & Guest,

Fig. 5. Sequence of the cloned PCR product synthesized from *nirS* mRNA by 5'-RACE. The PCR product generated using the primers Q1F and 228R (see text for details) was cloned into the vector pGEM-T and sequenced using the M13F universal primer. The Q1F primer can be read from the sequence and is followed by 11 T residues, representing the poly-A tail that was added to the cDNA following its synthesis from the *nirS* mRNA. The residue immediately following the poly-T sequence is a G residue (marked with an asterisk). This residue indicates the 5' end of the mRNA and is the same residue as that identified by primer extension.

1990), so one would not necessarily expect to see a σ^{70} -35 recognition sequence in the *nirS* promoter.

An Fnr-like box, equivalent to that seen in *Pa. denitrificans* (Fig. 2), has previously been suggested to be the binding site of Nnr (van Spanning *et al.*, 1997). Nnr is in the Fnr/Crp family of proteins and is induced in *Pa. denitrificans* during denitrification. Insertion of a kanamycin cassette into the gene encoding Nnr leads to a nitrite reductase minus phenotype, characterized by the absence of a NirS polypeptide, but conversely an Fnr mutant is unaffected in nitrite reduction (de Boer *et al.*, 1996). The combination of these results and the presence of an Fnr-like motif in an appropriate position upstream of the TSS of *nirS* strongly suggests that TTAACaatgGTCAA is the binding site of Nnr.

The position of a single Fnr-like box between the *nirS* transcription start site (this work) and that of *nirI* (Saunders *et al.*, 1999) suggests that, unusually, a single motif controls the transcription of *nirS* and *nirI* in a bi-directional manner. The *nirI* promoter has previously been shown to be Nnr-dependent (van Spanning *et al.*, 1999). This may have implications in the co-regulation of these two genes, as sterically the binding of a holo RNA polymerase to enable transcription of *nirS* would prevent transcription towards *nirI*.

Pa. pantotrophus promoter regions controlled by Nnr (the regions upstream of *nirS* and *nirI*) were aligned with respect to their TSSs to identify common motifs between them. It is striking that the sequence T-T-G/C-C-G/C-G/C occurs in the region -14 to -19 with respect to the transcription start site in both the *nirS* and *nirI* promoter regions. This motif differs considerably from the σ^{70} -like -10 motif (TATAAT in *E. coli*; Hawley & McClure, 1983) or any other known RNA polymerase motifs and cannot be found in the same position upstream of any other transcript start sites of the alpha *Proteobacteria* (as judged by a GCG FINDPATTERNS examination of prokaryotic sequence databases using T-T-G/C-C-G/C-G/C-N₂₀₋₁₀₀-ATG as a search term and subsequently examining sequence annotations for defined transcript start sites within the search results). The one promoter recovered in this search was that of *Pa. denitrificans* *norC*, again positively regulated by Nnr. This suggests that there may be other DNA-binding protein(s) involved in the Nnr-dependent transcription of *nirS* and *nirI*, but the motif at -16 cannot be found in the promoter regions of *nirK* [encoding an Nnr-controlled copper-containing nitrite reductase (Bartnikas *et al.*, 1997; Tosques *et al.*, 1997)] of the closely related bacterium *Rhodobacter sphaeroides*. Such motifs are also absent from *Ps. stutzeri* *nirS* and *dnrS* (the latter gene is the functional equivalent of *Pa. denitrificans* *nnr*) promoters, which conform more closely to the σ^{70} -mediated *E. coli* Fnr consensus promoter sequence with a clear -10 hexamer (Vollack *et al.*, 1999).

Although *Pa. pantotrophus* and *Pa. denitrificans* are very closely related, albeit separate, species (Rainey *et al.*, 1999), the *nirS* promoter regions of the two organisms do differ from one another (Fig. 2), and a notable feature is that one motif has retained its structure while altering its sequence. In *Pa. pantotrophus*, an imperfect inverted repeat (TTGCGCGC-AA) appears between the Nnr box and the T-T-G/C-C-G/C-G/C motif discussed above. This inverted repeat also appears in the *Pa. denitrificans* *nirS* promoter region, but the flanking TT/AA nucleotides are replaced with G and C so that the sequence appears as CCG-CGCGCGG. The position of this sequence may indicate the binding site of another protein, but the purpose of retaining the integrity of the repeat between species remains obscure. Such inverted repeats cannot be found in the upstream region of *nirI*.

The monocistronic nature of transcription from the promoter upstream of *Pa. pantotrophus* *nirS*, and the presence of a rho-independent terminator in the *nirS*-*nirE* intergenic region, suggest that genes downstream of *nirS* are transcribed from a separate promoter. A gap of approximately 70 bp between the termination codon of *nirS* and the initiation codon of *nirE* is a plausible location for this promoter. A sequence much like that seen in the *nirS* and *nirI* promoters (TTCGCG) can be seen in a plausible position relative to the *nirE* translation start codon (40 bp upstream), but no binding site

for Nnr can be distinguished. Extensive attempts to determine the *nirE* TSS experimentally by both techniques used for *nirS* in this paper were unsuccessful, perhaps because of the low abundance of *nirE*-hybridizing mRNA noted during Northern blotting. Since *nirE* and the genes downstream encode proteins involved in the production of the specialized *d*₁ haem, fewer transcripts would be required relative to the structural gene for nitrite reductase (*nirS*). Thus the presence of two promoters to control the structural gene and accessory proteins may provide a means of co-regulating the abundance of nitrite reductase polypeptide with its specialized *d*₁ haem.

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