The nitric oxide regulated *nor* promoter of *Paracoccus denitrificans*

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The promoter of the *Paracoccus denitrificans* nitric oxide reductase operon (*norCBQDEF*) has been characterized by primer extension and deletion analysis. A major transcript that is detectable only in anaerobically grown cells initiates 43.5 bp downstream of the centre of a putative binding site for the transcription factor NNR (nitrite and nitric oxide reductase regulator, which is known to regulate *nor* expression). A minor transcript initiates 121 bp upstream of the major transcript and is detectable in cells grown aerobically or anaerobically. Deletion derivatives of the *nor* promoter region were constructed and analysed *in vivo* using transcriptional fusions to the reporter gene *lacZ*. Expression patterns from promoter deletions in a wild-type strain and an *nnr* mutant confirmed that the minor transcript is NNR independent, and makes a small contribution to *nor* expression under both aerobic and anaerobic growth conditions. A deletion derivative truncated to within 7 bp of the putative NNR-binding site showed a near wild-type response to anaerobic growth, showing that no upstream DNA sequences are required for activation of the major promoter. Site-directed mutagenesis of the putative NNR-binding site confirmed that this is the major *cis*-acting sequence mediating the anaerobic inducibility of *nor* expression.

Keywords: *Paracoccus denitrificans*, nitric oxide, transcription, denitrification

INTRODUCTION

In denitrifying bacteria growing in the absence of oxygen and in the presence of nitrate or nitrite, nitric oxide (NO) is produced by a nitrite reductase (of the copper or cytochrome *cd₁* type), and is subsequently consumed by a nitric oxide reductase (Zumft, 1997; Watmough *et al.*, 1999). In those organisms that have been studied, expression of the nitrite and NO reductase genes is coordinately regulated by a transcription factor belonging to the FNR (fumarate and nitrate reductase regulator)/CRP (cAMP receptor protein) family (Tosques *et al.*, 1996; Arai *et al.*, 1995; van Spanning *et al.*, 1997; Vollack *et al.*, 1999). In *Rhodobacter sphaeroides* and *Paracoccus denitrificans*, there is good evidence that the transcription factor (designated NnrR or NNR, respectively) is activated by NO or a chemical species related to NO (Kwiatkowski & Shapleigh, 1996; Tosques *et al.*, 1996; van Spanning *et al.*, 1995, 1999).

The coordinate regulation by NO of the genes encoding the enzymes responsible for making and consuming NO may be a strategy for maintaining a low intracellular concentration of the toxic NO.

In *P. denitrificans*, NO reductase is encoded in the six-gene *norCBQDEF* operon, which has a predicted NNR (nitrite and nitric oxide reductase regulator)-binding site upstream of *norC* (de Boer *et al.*, 1996; Fig. 1). The *norC* and *norB* genes encode the enzymically active NorCB complex that can be purified from *P. denitrificans* (de Boer *et al.*, 1996; Hendriks *et al.*, 1998). The remaining four genes have unknown functions, but are required for the synthesis of an active NO reductase *in vivo* (de Boer *et al.*, 1996). Transcription of the *nor* genes is activated by NNR under anaerobic growth conditions in response to NO or a related species (van Spanning *et al.*, 1995, 1999). NNR also activates the transcription of the divergent *nirI* and *nirS* genes that are required for nitrite reductase activity (Saunders *et al.*, 1999). Another FNR family member, FnrP, activates expression of nitrate reductase in *P. denitrificans*, probably in response to anoxia and/or a redox signal (van Spanning *et al.*,

Abbreviations: FNR, fumarate and nitrate reductase regulator; NNR, nitrite and nitric oxide reductase regulator; NO, nitric oxide.

1997). NNR and FnrP appear to bind to identical or very similar DNA sequences, yet activate their target genes specifically with little or no cross-talk (van Spanning *et al.*, 1997). FnrP appears to be a true orthologue of FNR, in that it contains the cysteine residues that are conserved in FNR-like proteins and are believed to provide ligands to an oxygen/redox-sensitive iron-sulphur cluster. NNR, on the other hand, does not have these residues and so is thought to function by a different mechanism (Baker *et al.*, 1998).

As part of an effort aimed at understanding NO-regulated gene expression in *P. denitrificans*, the promoter region of the NO reductase operon (*nor*) has now been studied and this paper reports the presence of two transcription-start sites. The major start site is active only in cultures grown anaerobically (with nitrate) and is positioned 43.5 bp downstream of the putative NNR-binding site, making this a class II (Busby & Ebright, 1999) factor-dependent promoter (as is also the case for the *nirI* and *nirS* promoters; Saunders *et al.*, 1999). *In vivo* analysis of deletion derivatives of the promoter, and of point mutations in the putative factor-binding site, has confirmed the location of the NNR-binding site and shown that NNR is the only regulatory protein with a significant role in controlling expression of the NO reductase.

METHODS

Bacterial strains, plasmids and growth media. The plasmids used were pUC18 for routine cloning (Yannisch-Perron *et al.*, 1985) and the broad-host-range promoter probe vector, pMP220 (Spaink *et al.*, 1987). *Escherichia coli* strain JM83 [*ara* Δ(*lac-proAB*) *rpsL* Δ80 *lacZ* ΔM15] was used for all routine DNA manipulations, and S17-1 (*thi pro hsdR recA* integrated RP4-2 Tc::Mu Km::Tn7) was used as the donor for conjugations with *P. denitrificans*. The *P. denitrificans* strains used were Pd1222 (de Vries *et al.*, 1989) and its isogenic *nmr* mutant Pd 77.71 (van Spanning *et al.*, 1995). *E. coli* strains were grown in Lennox (L) broth and *P. denitrificans* strains were grown in a defined medium containing succinate as the sole carbon and energy source (Harms *et al.*, 1985) and supplemented with 50 mM nitrate for anaerobic growth. Antibiotics were used at the following concentrations (μg ml⁻¹): ampicillin, 100; tetracycline, 0.5 (*P. denitrificans*) or 12.5 (*E. coli*); spectinomycin, 25. Aerobic cultures (20 ml in 250 ml flasks) of *P. denitrificans* were shaken at 250 r.p.m.; anaerobic cultures were grown in standing bottles filled to the top, in both cases at 30 °C. β-Galactosidase was assayed in duplicate according to the method of Miller (1992) on at least three independently grown exponential-phase cultures. *E. coli* was made competent using 0.1 M CaCl₂, 10% (v/v) glycerol and was transformed using the method of Pope & Kent (1996). Plasmids were introduced into *P. denitrificans* by conjugation (Shearer *et al.*, 1999).

Primer extensions. Total RNA was isolated from *P. denitrificans* using RNeasy spin columns (Qiagen) according to the manufacturer's instructions. RNA integrity was checked by agarose gel electrophoresis and concentrations were determined by measuring the absorbance at 260 nm. RNA (10 or 20 μg) was added to a 1.5 ml microfuge tube containing 10 μl 10 mM DTT, 0.1 M KCl, 4 mM Tris/HCl (pH 7.9), 40

units RNasin (Promega) and 0.2 pmol ³²P-end-labelled primer RNA1 (5'-CGTAGATATGGCTGTGCACGGTCAATGC-GC-3'). The tube was heated to 80 °C for 5 min, incubated at 30 °C for 3 h and then cooled on ice. Then 4 μl 5× MMLV reverse transcriptase buffer, 2 μl dNTP mix (50 mM), 2 μl DTT (0.1 M), 0.5 μl actinomycin D and 200 units MMLV reverse transcriptase were added and the volume made up to 20 μl with water. The reaction was incubated at 37 °C for 1 h and stopped by addition of 4 μl Sequenase stop solution (Amersham-Pharmacia). Products (5 μl) were separated on an 8% sequencing gel alongside a sequencing ladder generated using primer RNA1.

PCR methods. The *nor* promoter fragments (*norP175*, *norP133*, *norP98* and *norP57*) were generated using the reverse primer *norP1B* (5'-CGGGCGTAGATATGGCTGTGCACG-3') and either P175 (5'-TTTGCCTGATGCCGCCCAAGGCCGC-3'), P133 (5'-GTCTGCCGGGTGCCGTATCGAGCT-3'), P98 (5'-CGCAGGACGCGGAATTTCCGGACA-3') or P57 (5'-CCTTCACTTGACTTTCATCAATGAG-3'). Each reaction contained 25 ng template DNA (pEG8HI; van Spanning *et al.*, 1997), 2 μl each primer (25 μM), 5 μl *Pwo* buffer (MgCl₂ included), 1.5 μl dNTP mix (50 mM), 0.5 μl *Pwo* (5 units μl⁻¹) in a total volume of 50 μl. Reaction conditions were: 94 °C for 5 min, then 25 cycles of 94 °C for 30 s, 65 °C for 30 s, 72 °C for 30 s, followed by 72 °C for 5 min. Products were separated on a 1.5% agarose gel, purified using a Qiaex Gel extraction kit (Qiagen) and ligated into *Sma*I-cut, phosphatase-treated pUC18 (Pharmacia). Ligation reactions were then used to transform competent JM83 and plated on L agar + X-Gal and ampicillin. The orientations of the inserts were determined by colony PCR and then by sequencing of both strands. Each fragment was excised with *Kpn*I and *Xba*I and ligated into pMP220 cut with the same enzymes, to generate plasmids designated *pnorP57*, *pnorP98*, *pnorP133*, *pnorP175* and *pnorP133NN* (see Fig. 1b). For colony PCR, white colonies were picked and transferred to a 0.2 ml PCR tube (the toothpick was then used to inoculate 5 ml L broth + ampicillin). The tubes were microwaved at full power (1000 W), with the lids open, for 1 min. PCR mix contained (for eight reactions) 20 μl *Taq* buffer, 12 μl dNTP mix (50 mM), 16 μl each primer (5 μM), 6 μl MgCl₂ (50 mM), 1 μl *Taq* and 129 μl H₂O; 25 μl was added to each tube. The cycling conditions were as described above and products were separated on a 1.5% agarose gel. The primers used were the pUC18 Universal primer and the appropriate promoter-specific forward primer (P57, P98, P123 or P175).

PCR mutagenesis. To incorporate single or double point mutations into the *nor* promoter, appropriate complementary primers were used in an amplification reaction with plasmid DNA as the template. The template DNA was removed by treatment with *Dpn*I (which digests only methylated DNA) and the remaining DNA was used to transform JM83. Each reaction mix contained: 25 ng template DNA (the P133 promoter cloned in pUC18), 5 μl *Pwo* buffer (MgCl₂ included), 1.5 μl dNTP mix (50 μM), 4 μM each primer (NN1, 5'-TTGCCTTCACTTGACTTTCATCAGTGAGCGACTC-3' and NN2, 5'-GAGTCGCTCACTGCTGAAAGTCAGGTG-AAGGCAA-3', or TT1, 5'-CGACTCACGCGCGCCGGA-CAGT-3' and TT2, 5'-ACTGTCCGGCGCGCGTGAGTC-G-3'), 0.5 μl *Pwo* (5 units μl⁻¹), in a total volume of 50 μl. Reaction conditions were: 94 °C for 5 min, then 25 cycles of 94 °C for 30 s, 67 °C for 30 s, 72 °C for 10 min, followed by 72 °C for 15 min. The reaction mixtures were transferred to 1.5 ml tubes and treated with 10 units *Dpn*I for 30 min at 37 °C, then 72 °C for 30 min. After cooling on ice, each reaction was treated with 2 units T4 DNA ligase for 1 h and

then used to transform competent JM83. Control reactions contained no primers. Mutant DNAs were sequenced using an ABI Prism automated sequencer. Other general recombinant DNA techniques were as described by Sambrook *et al.* (1989).

RESULTS AND DISCUSSION

Transcript analysis of the *nor* promoter

Primer extension analysis using total RNA isolated from nitrate respiring *P. denitrificans* Pd1222 consistently revealed the presence of two transcription-start sites (Fig. 1a). The apparently major transcript initiates at a site designated P1 downstream of the putative NNR-binding site. A second start site, P2, is upstream of the NNR-binding site and 121 bp upstream of P1 (Fig. 1b). The proposed NNR-binding site is centred 43.5 bp upstream of P1. Primer-extension analysis with total RNA from aerobically grown cells detected only the P2 transcript, at a somewhat lower level than in anaerobically grown cells (Fig. 1a). The additional products seen in Fig. 1a, indicative of mRNAs apparently initiating

between P1 and P2, were not seen consistently in all reactions and so are probably non-specific products resulting either from premature termination of the primer extension, or from non-specific annealing of the primer to another mRNA. Identification of the transcription-start sites of four NNR-dependent promoters from *P. denitrificans* and *Paracoccus pantotrophus* (this work; Saunders *et al.*, 1999, 2000) allowed inspection of the sequences for possible promoter elements and *cis*-acting sequences. It has been suggested that NNR-regulated promoters may be targets for RNA polymerase containing an alternative sigma factor (Baker *et al.*, 1998) or for regulation by additional transcription factor(s) (Saunders *et al.*, 2000). The rationale behind these proposals comes from the fact that both FnrP and NNR are believed to recognize the same binding site at their target promoters in *P. denitrificans* yet activate their target genes specifically. The factor binding sites upstream of the FnrP-regulated *ccoN* promoter and the NNR-regulated *norC* promoter of *P. denitrificans* have identical core-binding motifs,

Fig. 1. (a) Primer-extension analysis using 10 (lanes 1 and 3) or 20 μ g (lanes 2 and 4) of total RNA isolated from anaerobic (lanes 1 and 2) and aerobic (lanes 3 and 4) cultures of *P. denitrificans* Pd1222. Reactions were run alongside a sequencing ladder generated using the same primer (RNA1). The major (P1) and minor (P2) transcripts are marked, and the sequence around P1 is highlighted. The nucleotide sequence around P2 was determined from a similar gel that was run further to separate the sequence ladder. (b) The *norC* promoter sequence with both transcript-start sites marked. The nucleotides at which transcription initiates are underlined. The putative NNR-binding site (TTGAC---ATCAA) is boxed, the AT-rich sequence is indicated with a dotted line and the 10 bp palindrome is indicated with a double-headed arrow. The 5' limits of the deletion derivatives are indicated, along with the names of the plasmids in which they were cloned. Plasmid numbers indicate the number of nucleotides upstream of P1 incorporated in the promoter-*lacZ* fusion.

<i>norP1</i> (Pd)	ttttgccttcacTTGACtttcATCAAtgagcgactcacgcTTGCgagacagtgggtcttatC	+1
<i>nirS</i> (Pd)	cgcacotcgccTTAAcAaagGTCAAagccccgcgcgcgaTTGCgcccaggaacggcgtG	
<i>nirI</i> (Pd)	gcgcgcggggctTTGACctttGTTAAggccgaggtgcggcatTTGCgcatcagcgggacG	
<i>nirS</i> (Pp)	gccgcaccccgTTAAcAatgTCAAagccttgccgcgcaaaTCCGcagcaggaacggcgtG	+1

Fig. 2. Alignment of the three known NNR-dependent promoters from *P. denitrificans* (Pd) from the *norC*, *nirI* and *nirS* genes. Note that *nirI* and *nirS* are divergently transcribed, with a single NNR-binding site in the intergenic region (Saunders *et al.*, 1999). Also shown is the *nirS* promoter from *P. pantotrophus* (Pp; Saunders *et al.*, 2000). Sequences are aligned at their NNR-binding sites and the conserved TTGC sequence is underlined.

and yet there does not appear to be any cross-talk between the two regulators (van Spanning *et al.*, 1997).

Promoter alignments

All four promoters appear to be class II, with the factor-binding site centred 41.5–43.5 bp upstream of the transcription-start site, which is the preferred spacing for FNR-dependent promoters (Wing *et al.*, 1995). Otherwise, the alignment of the *nirI*, *nirS* and *norC* promoters reveals very little similarity (Fig. 2). Only one sequence appears to be conserved, a TTGC motif positioned 14–20 bp upstream of each transcript start site, as recently noted by Saunders *et al.* (2000) for the *nirS* promoters of both *P. pantotrophus* and *P. denitrificans*. This sequence, and its position, are reminiscent of the conserved –12 recognition sequence for the alternative sigma factor, σ^N (Wang *et al.*, 1999). However, the NNR-dependent promoters have only weak matches to the –24 element consensus, the second σ^N recognition element (Barrios *et al.*, 1999), and so are unlikely to be dependent on RNA polymerase containing σ^N . Neither is there any obvious similarity to the –10 and –35 recognition elements for the major (σ^{70} type) sigma factor found in many promoters from other bacteria, although it has been proposed that these motifs are likely to be conserved in the α -*Proteobacteria* (Gruber & Bryant, 1997; Baker *et al.*, 1998). Hence, the role of the TTGC motif, if any, is uncertain and was tested by mutagenesis (see below).

Other interesting features of the *nor* promoter region include an AT-rich sequence positioned immediately upstream of the factor-binding site, and a 10 bp palindrome further upstream (Fig. 1b). The AT-rich sequence (67 mol% A + T) is typical for *P. denitrificans* DNA (23 mol% A + T) and may represent an additional regulatory feature or promoter element. It is positioned where the C-terminal domains of the RNA polymerase α subunits would contact the DNA, according to the class II model of transcription activation (Busby & Ebright, 1999). The 10 bp palindrome is the only significant symmetrical sequence detected in the *nor* upstream region and is similar to a sequence found in the divergent *nirI* and *nirS* promoters (Saunders *et al.*, 1999).

The transcript start site of the *nor* operon of *Pseudomonas stutzeri* has also been determined and is

positioned 40.5 bp downstream of a binding site for a regulatory protein of the FNR family (Zumft *et al.*, 1994). Transcription initiation from this promoter is independent of σ^N (Härtig & Zumft, 1998). At least in these respects, the *nor* promoters of *Pse. stutzeri* and *Par. denitrificans* appear to be similar.

In vivo analysis of the *nor* promoter

To investigate the *norC* promoter further, a series of 5' deletions was constructed by PCR. Deletions were designed with the aim of identifying any regions of DNA that may be required for promoter activity and regulation. Each promoter fragment was cloned into the low-copy-number promoter-probe vector pMP220 and the resulting constructs were conjugated into *P. denitrificans* Pd1222. A Pd1222 derivative containing a single-copy chromosomal *norP-lacZ* fusion (van Spanning *et al.*, 1997) was used to check that there were no major copy-number effects associated with the use of pMP220. The largest promoter construct (in *pnorP175*) contains both the P1 and P2 start sites, with 54 bp of DNA upstream of P2 (Fig. 1b). The second deletion (in *pnorP133*) contains P1 and is truncated immediately upstream of the P2 start site, having only 12 bp of DNA upstream of P2. This deletion was designed to remove the RNA polymerase-binding site presumably associated with P2. Subsequent truncations removed the palindrome (*pnorP98*) and the AT-rich sequence (*pnorP57*) in turn (Fig. 1b). The full-length plasmid-borne construct (*pnorP175*) exhibited only approximately twofold higher activity than the chromosomal-borne, single-copy fusion and was regulated in a similar fashion (Fig. 3). In both cases there is a substantial increase in promoter activity in cultures grown under anaerobic, denitrifying conditions. Removal of the sequences immediately upstream of P2 (*pnorP133*) had a relatively small effect, but caused a greater decrease in *nor* promoter activity under anaerobic conditions than under aerobic conditions (Fig. 3). This suggests that the P2 transcript makes a small contribution to the total *nor* promoter activity, which is more significant under anaerobic conditions; this conclusion is consistent with the results of the mRNA analysis. Removal of the 10 bp palindrome (in *pnorP98*) had no significant effect on *nor* promoter activity. Removal of the AT-rich sequence (in *pnorP57*) caused a further small decrease in the aerobic activity of the promoter, and a small increase in

Fig. 3. Histogram showing β -galactosidase activities for each *nor* promoter-*lacZ* fusion. Plasmid *pnorP133NN* is identical to plasmid *pnorP133* except for an altered (NN) NNR-binding site. Anaerobic cultures are represented by spotted bars; aerobic cultures are represented by white bars.

anaerobic activity, compared to the previous two deletions (Fig. 3). This suggests that the AT-rich sequence may have a negative effect on transcription from *norP1*. The *pnorP57* construct contains a promoter deleted to within 7 bp of the putative NNR-binding site, yet retains the anaerobic inducibility of the full-length *nor* promoter. Thus, the P1 promoter is the most important contributor to the activation of the *nor* promoter, which presumably occurs through NNR binding to the putative binding site retained in *pnorP57*.

The small contribution of P2 to the *nor* promoter was confirmed by conjugating *pnorP175* and *pnorP133* into an *nnr* mutant, Pd77.71 (van Spanning *et al.*, 1999). In anaerobic cultures (which in the case of this strain grow very poorly), 270 units of β -galactosidase activity were recorded for Pd77.71 (*pnorP175*) while Pd77.71 (*pnorP133*) had only 70 units of activity. This confirms that the *norP2* is NNR independent, and makes only a small contribution to *nor* promoter activity under anaerobic conditions. In aerobic cultures of the NNR mutant, *pnorP175* and *pnorP133* directed similarly low β -galactosidase activities (90 and 70 units respectively).

The high anaerobic activity of the shortest promoter construct (*pnorP57*) is not consistent with *norP1* being dependent on σ^N , since in all known cases σ^N has an absolute requirement for an additional transcription factor (upstream activator) bound to a remote site 100 bp or more upstream of the transcript start (Shingler, 1996). This conclusion was further supported by the finding that alteration of the TTGC motif to TTTT had no significant effect on *nor* promoter activity in the full-length construct (results not shown). Thus, *norP1* cannot be σ^N dependent, since at σ^N -dependent

promoters the GC nucleotides of the -12 element are essential for promoter activity (Barrios *et al.*, 1999). Mutation of the TTGC motif to GCGC resulted in a 20% reduction in promoter activity anaerobically, demonstrating that this motif, though conserved at a similar position at all four NNR-dependent promoters, is not essential for promoter activation (results not shown).

To show unequivocally that the suggested NNR-binding site does indeed play this role, the sequence (TTGACtttcATCAA) was altered to the neutral NN sequence (CTGACtttcATCAG), which is recognized by neither FNR nor CRP of *E. coli* (Spiro *et al.*, 1990), and which it was assumed would not be recognized by NNR. Mutagenesis was undertaken on the promoter fragment containing only 12 bp of DNA upstream of P2 (Fig. 1a) since in the fusion vector containing this fragment (*pnorP133*) only P1 is active anaerobically (Fig. 3). Introduction of the NN sequence into the putative NNR-binding site (in *pnorP133NN*) eliminated promoter activity under anaerobic conditions and abolished the residual aerobic activity seen in *pnorP57* (Fig. 3). Hence, *norP1* activity is completely dependent on the putative NNR-binding site and it is very likely that NNR recognizes the same sequence, or a very similar sequence, as does *E. coli* FNR. These results also suggest that at least some of the *nor* promoter activity in aerobic cultures depends on NNR, indicating that there may be some active NNR present in aerobic cells. Attempts to confirm the NNR-DNA interaction biochemically using purified protein in gel-retardation assays (in the presence and absence of NO generators) have so far proved inconclusive.

Concluding remarks

The physiological role of the minor NNR-independent promoter of the *nor* operon is unclear. Transcription from this promoter was detected both aerobically and anaerobically, *in vivo* and *in vitro*, and is consistent with previous reports of a low level of activity of the *nor* promoter in aerobic cultures (van Spanning *et al.*, 1999). Furthermore, there is substantial residual NOR activity in an *nnr* mutant, and the NorC polypeptide is detectable in aerobically grown cells (van Spanning *et al.*, 1997). All of these observations point towards there being a significant NNR-independent activity of the *nor* promoter under both aerobic and anaerobic conditions. Aerobic expression of NO reductase may safeguard cells against sudden exposure to NO, and may have a role in the reported ability of *P. denitrificans* to denitrify in the presence of oxygen (Davies *et al.*, 1989). However, the low level of aerobic activity observed with the chromosomal fusion strain and with the plasmid-borne fusions was reduced but not completely abolished by removal of *norP2* and must therefore be partly due to *norP1*. Mutation of the NNR-binding site completely abolished both anaerobic and aerobic activity of *norP1*, which suggests that at least some of the activity detected aerobically is NNR dependent. The key to understand-

ing NO signalling and NNR-dependent regulation in *P. denitrificans* lies in identifying the sigma factor(s) which recognize NNR-dependent promoters and on elucidating the exact mechanism of NNR activation by NO.

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