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 cd$_1$ 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.,
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 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 nirS). NNR and FnrP appear to bind to identical or very similar sites, specifically with little or no cross-talk (van Spanning et al., 1997). NNR and FnrP appear to bind to identical or very similar sites, 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.

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 (Spink et al., 1987). Escherichia coli strain JM83 [ara ara lac–proAB] rpsL, 58° lac ZAM15] was used for all routine DNA manipulations, and S17-1 [ihi pro bsd rcm 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 nor mutant Pd 77.71 (van Spanning et al., 1995). E. coli strains were grown in L broth and P. denitrificans strains were grown in a defined medium containing succinate as the sole carbon source supplemented with 50 mM nitrate for anaerobic growth. M. I. HUTCHINGS and S. SPIRO

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′-CGTAGATATGCGTTCGACTGCAATGC-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-Pharma). 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 norP18 (5′-CGGCCGATATGCCGCTGCAATGC-3′) and either P175 (5′-TTTGCCTATGCGCCCAAGGCGGC-3′), P133 (5′-GTCCTGCCGCTGCTGATCGC-3′), P98 (5′-CGAGCGACGGAGATTCCGCGCA-3′) or P57 (5′-CCCTACGGTCTATTGAC-3′). Each reaction contained 25 ng template DNA (pEBGHI; van Spanning et al., 1997), 100 µl of 5× PCR 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 Qiagen Gel extraction kit (Qiagen) and ligated into Smal-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 KpnI and XbaI and ligated into pMP220 cut with the same enzymes, to generate plasmids designated norP57, norP98, norP133, norP175 and norP133NN (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 DpnI (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′-TTTGCCTATGCGCCCAAGGCGGC-3′ and NN2, 5′-GATGGTCCTACTGTTGAAAGTCAAGTGAATTCCGACA-3′), 0.5 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).
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.](image)

(a) Primer-extension analysis using 10 (lanes 1 and 3) or 20 µg (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.
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 sequence. The alignment of the 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. (1999). The TTGC motif is the only upstream region and is similar to a sequence found in the intergenic region (Saunders et al., 1999). Also shown is the nirS promoter from 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.

In *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 (pnorP57) caused a further small decrease in the aerobic activity of the promoter, and a small increase in...
The high anaerobic activity of the shortest promoter construct (pNorP133) had only 70 units of activity. This confirms that at least some of the activity detected in pNorP133 directed similarly low β-galactosidase activity 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.

**ACKNOWLEDGEMENTS**

We thank Rob van Spanning for generously providing strains, Neil Shearer and Ray Dixon for useful discussions and Wendy Leung for help in collecting the β-galactosidase data. This work was supported by a BBSRC grant.

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Received 28 April 2000; revised 6 July 2000; accepted 12 July 2000.