

The transcription factor DNR from *Pseudomonas aeruginosa* specifically requires nitric oxide and haem for the activation of a target promoter in *Escherichia coli*

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Pseudomonas aeruginosa is a well-known pathogen in chronic respiratory diseases such as cystic fibrosis. Infectivity of *P. aeruginosa* is related to the ability to grow under oxygen-limited conditions using the anaerobic metabolism of denitrification, in which nitrate is reduced to dinitrogen via nitric oxide (NO). Denitrification is activated by a cascade of redox-sensitive transcription factors, among which is the DNR regulator, sensitive to nitrogen oxides. To gain further insight into the mechanism of NO-sensing by DNR, we have developed an *Escherichia coli*-based reporter system to investigate different aspects of DNR activity. In *E. coli* DNR responds to NO, as shown by its ability to transactivate the *P. aeruginosa* *norCB* promoter. The direct binding of DNR to the target DNA is required, since mutations in the helix–turn–helix domain of DNR and specific nucleotide substitutions in the consensus sequence of the *norCB* promoter abolish the transcriptional activity. Using an *E. coli* strain deficient in haem biosynthesis, we have also confirmed that haem is required *in vivo* for the NO-dependent DNR activity, in agreement with the property of DNR to bind haem *in vitro*. Finally, we have shown, we believe for the first time, that DNR is able to discriminate *in vivo* between different diatomic signal molecules, NO and CO, both ligands of the reduced haem iron *in vitro*, suggesting that DNR responds specifically to NO.

INTRODUCTION

Pseudomonas aeruginosa is one of the most important opportunistic pathogens; it is a Gram-negative bacterium which colonizes the inflamed lungs of cystic fibrosis patients, causing persistent infections (Yoon *et al.*, 2006). *P. aeruginosa* is able to grow in the absence of oxygen, through anaerobic metabolism, which is important for infectivity and for the formation of biofilm (Hassett *et al.*, 2002; Barraud *et al.*, 2006), a surface-associated antibiotic-resistant microbial community (Singh *et al.*, 2000). The molecular race between host and pathogen thus includes strategies that are centred on the ability of *P. aeruginosa* to survive under oxygen-limited conditions; cells lying near the edge of the mucous layer rapidly deplete oxygen (O₂), creating a gradient where O₂ drops to very low levels. Under these microaerobic conditions *P. aeruginosa* grows in thick biofilms probably employing both microaerobic

respiration and the denitrifying redox chain (Yoon *et al.*, 2002; Alvarez-Ortega & Harwood, 2007; Platt *et al.*, 2008). The complete denitrification pathway involves four enzymes: nitrate reductase, nitrite reductase, nitric oxide reductase (NOR) and nitrous oxide reductase, operating sequentially to reduce nitrate to dinitrogen gas via nitrite (NO₂⁻), nitric oxide (NO) and nitrous oxide (N₂O) (Zumft, 1997). The expression and the activity of the NIR and NOR enzymes are tightly controlled because it is mandatory for the bacteria to keep the concentration of intracellular NO below cytotoxic levels, to limit nitrosative stress.

In *P. aeruginosa* the denitrification pathway is regulated by redox signalling, through a cascade of transcription factors; in particular, the global oxygen-sensing regulator ANR (anaerobic regulation of arginine deaminase and nitrate reduction) (Galimand *et al.*, 1991), a homologue of the *Escherichia coli* oxygen sensor FNR protein, activates, under anaerobic conditions, the gene coding for the transcription factor DNR (dissimilatory nitrate respiration regulator), which, in the presence of N-oxides, promotes the expression of the *nir*, the *nor* and the *nos* genes (Arai *et al.*, 1995, 1997, 1999, 2003).

Abbreviations: ALA, 5-aminolaevulinic acid; CRP, cAMP receptor protein; DNR, dissimilatory nitrate respiration regulator; FNR, fumarate and nitrate reductase regulator; HTH, helix–turn–helix; SNP, sodium nitroprusside; β-gal, β-galactosidase.

The DNR transcription factor belongs to the CRP/FNR (cAMP receptor protein/fumarate and nitrate reductase regulator) superfamily of regulators (Zumft, 2002; Körner *et al.*, 2003). The members of this superfamily are usually homodimers, each monomer being formed by three domains (McKay & Steitz, 1981): (i) an N-terminal sensing domain (also referred to as the effector domain) with the typical fold of the cAMP-binding domain of CRP; (ii) a long dimerization α -helix recruited to form the dimer interface; and (iii) a C-terminal DNA-binding domain that contains a helix–turn–helix (HTH) motif.

N-oxide sensors belong to different subgroups of the CRP/FNR superfamily, namely the DNR, NnrR and FNR subgroups (Rinaldo *et al.*, 2006), which recognize the same consensus sequence in their target promoters, the FNR box (TTGATN₄ATCAA) (Eiglmeier *et al.*, 1989; Hoeren *et al.*, 1993; Hasegawa *et al.*, 1998); these proteins contain a highly conserved amino acid sequence motif Glu–SerArg (E–SR), directly involved in the interaction with the FNR box (Green *et al.*, 2001). Another transcription factor belonging to the E subgroup of the CRP/FNR superfamily, i.e. NssR from *Campylobacter jejuni*, has been recently described to act, perhaps indirectly, as a NO sensor (Elvers *et al.*, 2005).

Previous studies, carried out in *P. aeruginosa* PAO1 with the *nirS*, the *norC* and the *nosZ* promoters fused to the *lacZ* reporter gene, showed that the DNR transcription factor responds *in vivo* to N-oxides (Arai *et al.*, 1999, 2003). A similar response to NO *in vivo* has been reported for the DNR homologue NNR in *Paracoccus denitrificans* (Van Spanning *et al.*, 1999) and, for the same regulator, also in *E. coli* using the FNR-dependent *E. coli melR* promoter (Hutchings *et al.*, 2000).

Understanding of the biochemical mechanisms used to respond to specific redox signals (such as NO) is crucial in order to dissect the *in vivo* function of a sensor protein. The molecular mechanism underlying the activity of DNR is still elusive, mainly because a reliable system to probe the NO-dependent DNA binding of this regulator has not been available. Therefore to investigate in detail the NO-dependent activity of *P. aeruginosa* DNR, we have studied the DNR-mediated transcriptional activity in *E. coli*, a species which is unable to carry out denitrification. A β -galactosidase (β -gal) assay was performed under anaerobic conditions in the presence of N-oxides, using the putative DNR target promoter *norCB* (hereinafter *nor*) from *P. aeruginosa*. The choice of this promoter is supported by previous evidence showing that in *P. aeruginosa*, in the presence of N-oxides, *nor* is more strongly activated by DNR than *nirS* (Arai *et al.*, 1999). In the first part of this paper we report the successful development of this system, and the characterization of the NO-dependent activity of DNR in *E. coli*.

To gain further insight into the mechanism of NO-sensing by DNR or other homologous transcription factors, in the second part of the paper we describe how the system

developed in *E. coli* was successfully used to investigate different aspects of the DNR activity. We have demonstrated the specificity of the DNR–DNA interaction, analysing both the transcriptional activity of DNR mutants and the effect of mutations in the FNR box of the *nor* promoter. Since spectroscopic and structural evidence and *in vitro* functional assays suggested the involvement of haem in DNR activity (Giardina *et al.*, 2008), we have also confirmed the importance of this cofactor in the mechanism of DNR-mediated activation of the *nor* promoter in *E. coli*.

Finally, in order to test whether DNR responds specifically to NO, we investigated its activity in the presence of carbon monoxide (CO).

METHODS

Plasmid construction and mutagenesis. The *dnr* gene was cloned into the pACYc184 vector (Biolabs) under the constitutive *tet* promoter; an extra *EcoRV* restriction site and the ribosome-binding site were also added, yielding the plasmid pACY-DNR. The *nor–lacZ* reporter system was obtained by cloning the *nor* promoter from the *P. aeruginosa* PAO1 genome first into the *EcoRI* and *BamHI* sites of the pUC18 vector, yielding the plasmid pUC-NOR and subsequently into the *EcoRI* and *BamHI* sites of the pRS415 vector, yielding the plasmid pRS-NOR. The *nor* promoter fragment used in this work includes 150 bp upstream of the ATG of the *norCB* gene (Fig. 1). Site-directed mutagenesis was done using the QuikChange mutagenesis kit (Stratagene) according to the manufacturer's instructions, with pACY-DNR or pUC-NOR as a template. Two mutants were obtained in the HTH region of DNR: N152stop, previously described (Giardina *et al.*, 2008), which lacks the entire HTH domain, and E193D, which has a substitution (Glu to Asp) in position 193. The mutations introduced in the FNR box of the *nor* promoter were as follows: a double mutation in which the GA bases in positions 3 and 4 have been substituted with AG (TTAGTN₄ATCAA, named *nor1*), and a single mutation in which the A base in position 4 has been changed to a G (TTGGTN₄ATCAA, named *nor2*). The *nor1* and *nor2* fragments were then inserted into the *EcoRI* and *BamHI* sites of the pRS415 vector, yielding, respectively, plasmids pRS-NOR1 and pRS-NOR2.

***In vivo* β -galactosidase assay.** Plasmids pACY-DNR and pRS-NOR were transformed into *E. coli* TOP10 (Invitrogen) [F[−] *mcrA* Δ (*mrr–hsdRMS–mcrBC*) ϕ 80*lacZ* Δ M15 Δ *lacX74 nupG recA1 araD139 Δ (*ara–leu*)7697 *galE15 galK16 rpsL*(Str^R) *endA1* λ [−]] or *E. coli* C600 [F[−] *tonA21 thi-1 thr-1 leuB6 lacY1 glnV44 rfbC1 fhuA1* λ [−] (*hemA::kan*)] (Δ *hemA*) (a kind gift of Cécile Wandersman, Institut Pasteur, Paris, France). The TOP10 strain is phenotypically FNR⁺, as tested in a control experiment with an FNR-dependent reporter fusion (pRW50 carrying the *FF-pmelR* fragment) (Wing *et al.*, 1995) (data not shown). The rich medium for bacterial growth was Luria–Bertani (LB) broth [tryptone (10 g l^{−1}), yeast extract (5 g l^{−1}), NaCl (10 g l^{−1})], supplemented with 0.4% (w/v) glucose (LG broth) and 0.4 mM ALA (5-aminolaevulinic acid hydrochloride), as indicated.*

DNR activity was tested by the β -gal assay at least in duplicate according to the method of Miller (1972) on at least two independently grown cultures. The aerobic cultures were grown in 50 ml medium in 250 ml flasks at 37 °C to OD₆₀₀ 0.4 and were shaken at 200 r.p.m. Anaerobic growth was carried out by transferring 10 ml of the aerobic samples from the flasks to filled tubes sealed with a rubber cap. After 30 min of growth to remove the residual oxygen, selected samples were induced with either 10 μ M

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1      GCCGCTGCTGATGGCCCTGGCGGTGGGCGCTGCGCTGTACCTGGCGGGATGAATAACTG      60
      
61      AGTAAATCGTTCGGAATCTTTGATTGCCATCAAGCGGGTTCGCCGCCGCCGCTTCCTAGA      120
      
      
121      ATCGCCGCACCACCCATTCCAGGAGGCCGGATGTCCGAGACCTTTACCAAAGGC-----      174
      

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Fig. 1. Sequence of the *P. aeruginosa* *nor* promoter fragment used in this study (150 bp). The putative binding site for the DNR protein is boxed and the bases of the consensus FNR box are highlighted in bold; the initial part of the coding sequence of the *norCB* gene, not included in the PCR fragment, is shown in grey and the translational start point is underlined.

aqueous NO from a saturated solution (2 mM) or 2 mM nitrite using a gas-tight syringe or, if indicated, with 5% (v/v) CO gas; the cultures were then incubated without shaking for 1 h before β -gal was assayed. For the N152stop mutant, the growth temperature was switched from 37 °C to 20 °C after 1 h of growth. For the Δ *hemA* strain, the rich medium was supplemented with ALA when indicated.

For Western blot analysis, cultures were grown under the same conditions as used for the β -galactosidase assays; cells harvested and disrupted by boiling in the loading buffer (1.50 mM Tris/HCl pH 6.8, 300 mM DTT, 6% SDS, 0.3% bromophenol blue, 30%, v/v, glycerol) were separated by SDS-PAGE and Western blot analysis was carried out. The DNR protein was detected using a polyclonal rabbit antibody (Davids Biotechnologies) and a goat anti-rabbit IgG (Sigma) alkaline phosphatase-conjugated secondary antibody.

RESULTS AND DISCUSSION

In vivo activation of DNR by N-oxides in the heterologous *E. coli* system

The effect of N-oxides on the activation by DNR of the *nir*, *nor* and *nos* genes has been previously demonstrated in *P. aeruginosa* (Arai *et al.*, 1999, 2003). The primary aim of our work was to characterize the NO-dependent DNR transcriptional activity in a heterologous model organism such as *E. coli*, which is unable to carry out denitrification. Plasmid pRS-NOR, carrying the *P. aeruginosa* *nor* promoter (Fig. 1) was transformed into *E. coli* TOP10, with or without plasmid pACY-DNR, harbouring the *dnr* gene under the control of a constitutive *tet* promoter. A β -gal assay according to the method of Miller (1972) was carried out under aerobic and anaerobic conditions in the presence of different N-oxides.

As shown in Fig. 2, DNR was able to transactivate the *nor* promoter in *E. coli* under anaerobic conditions and in the presence of 10 μ M NO. The maximal activation of the *nor* promoter was observed at 10 μ M NO, testing a range of NO concentrations from 5 μ M to 50 μ M; the increase of the NO concentration from 10 μ M to 50 μ M had no influence on the cell growth but slightly decreased the DNR activation (data not shown). An activation of the *nor* promoter under anaerobic conditions comparable to that obtained with NO gas was observed using 100 μ M sodium nitroprusside (SNP), a nitrosylating agent and a source of NO⁺ (data not shown).

The activation observed in the presence of 2 mM nitrite (Fig. 2) can be explained since it is known that, in *E. coli*, nitrite is a source of NO, due to the activity of nitrite reductase, which reduces nitrite to ammonia (Corker & Poole, 2003). Nitrite is not directly involved in the activation of DNR since no activity was observed in air in the presence of 2 mM nitrite, a condition in which NO is not produced (Darwin *et al.*, 1993).

As shown in Fig. 2, DNR is unable to activate the *nor* promoter under aerobic conditions. We also tested the

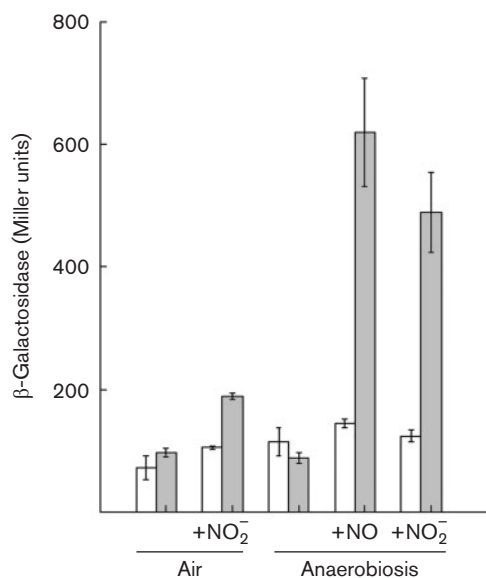


Fig. 2. Activation of the *P. aeruginosa* *nor* promoter requires DNR and N-oxides. The DNR protein was expressed constitutively in *E. coli* TOP10 containing plasmid pRS-NOR, in which the *nor* promoter is fused to *lacZ* (grey bars). As a control, the β -galactosidase activity was assayed under the same experimental conditions in *E. coli* TOP10 containing only the pRS-NOR vector (white bars). Cultures were grown aerobically and anaerobically in LG medium to exponential phase, and induced, as indicated, by the addition of 10 μ M NO or 2 mM sodium nitrite. β -Galactosidase activity was measured 1 h after induction in duplicate in three independently grown cultures; mean values are shown, together with experimental error bars (SD).

activity of the protein in air in the presence of SNP; the use of SNP as a source of NO⁺ under aerobic conditions has been previously reported for the NorR protein (Hutchings *et al.*, 2002). No DNR activity was observed in air in the presence of SNP (data not shown), in agreement with the physiological role of DNR, which, in *P. aeruginosa*, is involved in the regulation of denitrification, an alternative respiratory metabolism activated under oxygen-limiting conditions (Zumft, 1997).

Our results show that DNR is able to activate its target promoter in *E. coli*, suggesting that the transcriptional machinery of this bacterium can be efficiently used with a *P. aeruginosa* promoter such as the *nor* promoter. A similar NO-dependent transactivation in *E. coli* was obtained with the NNR protein from *Paracoccus denitrificans*; in the latter case, however, an FNR-dependent *E. coli* promoter was used (Hutchings *et al.*, 2000).

The *nor* promoter fragment used in this study includes 150 bp upstream of the ATG of the *norCB* gene; this fragment contains the canonical recognition motif for the transcriptional activator protein FNR, named the FNR box, centred -64.5 bases upstream of the ATG (Fig. 1). Interestingly, no activation of the *nor* promoter was observed in the TOP10 strain containing only plasmid pRS-NOR under anaerobic conditions (Fig. 2), suggesting that the endogenous FNR protein is unable to recognize this promoter. A preliminary *in silico* analysis of the *P. aeruginosa* *nor* promoter (identifying a good candidate for a -10 sequence centred around base 119.5) and its comparison with the *norC* promoter of *Pseudomonas stutzeri* (in which the transcriptional start site has been mapped: Zumft *et al.*, 1994) suggest that the FNR box could be located as in class II promoters; this hypothesis will require to be further confirmed experimentally. Interestingly, however, the region located between the palindromic TTGAT motif of the consensus FNR box in the *nor* promoter has a high GC content (Fig. 1), possibly explaining the lack of activation, given that it is known that FNR favours an AT-rich motif against a GC-rich motif in this region (Scott *et al.*, 2003). We cannot exclude, however, that other features of the *nor* promoter may preclude productive interaction between FNR and the *E. coli* transcriptional machinery. Interestingly, although the *E. coli* FNR protein seems unable to activate the *nor* promoter (this work), the DNR protein is able to recognize in *P. aeruginosa* an FNR-dependent *E. coli* promoter (Hasegawa *et al.*, 1998), making the scenario more complicated.

DNR binds specifically the *nor* promoter

To demonstrate the specificity of the DNR-*nor* promoter interaction in the *E. coli* system described above, we performed the β -gal assay with different mutants which have mutations in the HTH DNA recognition motif of the DNR protein or in the FNR box of the *nor* promoter.

The inability of the N152stop mutant, which lacks the last 75 amino acids and hence the entire DNA-binding domain of DNR (Giardina *et al.*, 2008), to transactivate the *nor* promoter under anaerobiosis in the presence of NO (Fig. 3a) suggests that the NO-dependent activation of the *nor* promoter requires the direct binding of DNR to the target DNA. To further analyse the DNR-DNA interaction, we focused our attention on the Glu-193 residue of the protein, a position highly conserved in the CRP/FNR superfamily (Green *et al.*, 2001); this glutamate residue was shown to be involved in the direct interaction of the FNR protein with the FNR box sequence on the target promoter (Spiro *et al.*, 1990). In agreement with the predicted involvement of this glutamate in binding the FNR box, the Glu 193 to Asp (E193D) variant of the DNR protein was unable to activate the *nor* promoter (Fig. 3a) under anaerobiosis in the presence of NO. This result confirms the specificity of the interaction of DNR with the *nor* promoter. The lack of activation by the two DNR mutants in the *E. coli* strain is not due to the lack of expression of the mutated proteins, which are correctly produced in the bacterium as verified by Western blotting (data not shown).

We also probed the DNR-DNA interaction by producing specific base substitutions in the FNR box of the *nor* promoter. Two mutants were produced: the first one carries an inversion of two bases in the FNR box (TTAGT-N₄TTGAT) (*nor1*) and the second one carries a single base change (A₄ to G) (TTGGTN₄TTGAT) (*nor2*). The corresponding plasmids (see Methods) were transformed into an *E. coli* strain harbouring the plasmid pACYDNR and the β -gal activity under anaerobic conditions with or without NO gas was measured. As shown in Fig. 3(b), DNR was unable to activate both mutant promoters under anaerobiosis in the presence of NO. These results confirm again the specificity of the interaction between DNR and the target DNA in the *E. coli* system. Moreover we have demonstrated, to our knowledge for the first time, that a single nucleotide substitution in the FNR box is sufficient to completely disrupt the activity of the DNR protein on the *nor* promoter. The sensitivity of the FNR box to the A₄ to G mutation in the consensus sequence was shown also for the *narGHJI* promoter, an *E. coli* FNR target (Walker & DeMoss, 1992).

DNR activity requires haem biosynthesis

The mechanism whereby DNR can sense the signal molecule is not yet understood; it is certain, however, that DNR, which lacks conserved cysteines, is unable to form an Fe-S centre that can interact with the signal molecule as reported for the O₂ sensor FNR (Khoroshilova *et al.*, 1995, 1997; Green *et al.*, 1996; Jordan *et al.*, 1997). Moreover, spectroscopic evidence supports the hypothesis that haem is involved in NO sensing; in fact the DNR-haem complex is stable and can bind NO in the ferrous state to yield a pentacoordinate derivative (Giardina *et al.*, 2008). In

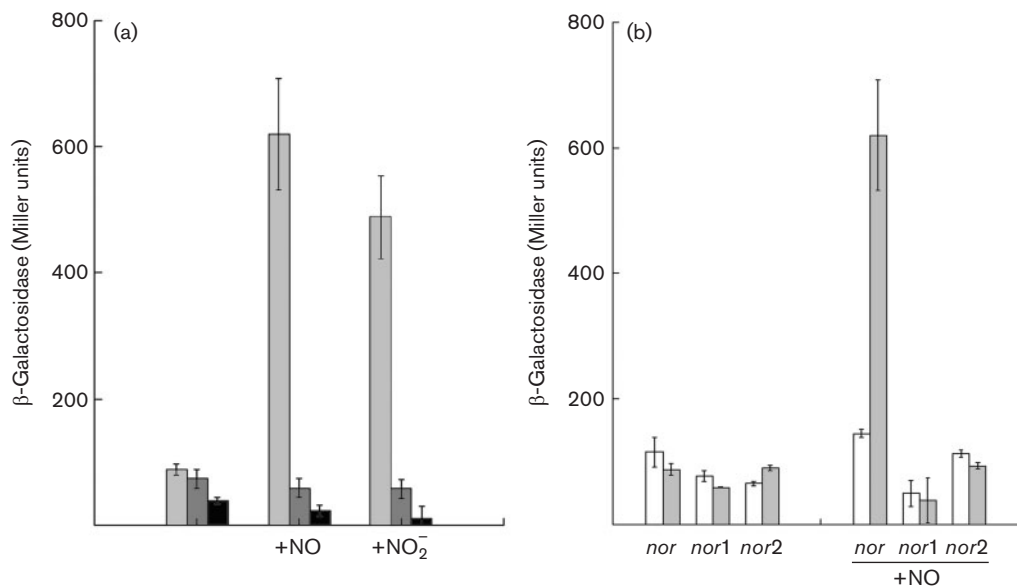


Fig. 3. Activation of the *nor* promoter requires the direct binding of DNR. (a) The N152stop protein, lacking the DNA-binding domain (dark grey bars) or the E193D protein (black bars) was expressed constitutively in *E. coli* TOP10 together with plasmid pRS-NOR and the β -galactosidase activity was measured. For comparison, the β -galactosidase activity of the wild-type protein is also shown (grey bars). (b) Plasmid pRS415 containing the wild-type *nor* promoter or the mutant promoters *nor1* (FNR box: TTAGTN₄ATCAA) or *nor2* (FNR box: TTGGTN₄ATCAA) was expressed constitutively in *E. coli* TOP10 together with pACY-DNR (grey bars). As a control, the β -galactosidase activity was measured under the same experimental conditions in *E. coli* TOP10 containing only the plasmids with the wild-type and mutant promoters (white bars). Cultures were grown anaerobically in LG medium to exponential phase, and induced, as indicated, by the addition of 10 μ M NO. β -Galactosidase activity was measured 1 h after induction in duplicate in three independently grown cultures; mean values are shown, together with experimental error bars (sd).

addition, the only evidence in the literature in which a member of the DNR class of regulators binds the target DNA *in vitro* was obtained by an electrophoretic mobility shift assay using the *P. aeruginosa* DNR protein bound to haem (Giardina *et al.*, 2008). The hypothesis of a haem-based sensing is reasonable also in the light of a similar haem-based mechanism well known for the *Rhodospirillum rubrum* CooA protein (Shelver *et al.*, 1997); in this case the CO molecule binds to the haem iron and triggers a conformational change, thus regulating the transcriptional activity (Lanzilotta *et al.*, 2000). These findings suggest that a haem-based mechanism is the most likely to explain the NO-dependent regulation of the function of DNR.

To demonstrate that the haem is required for DNR activity *in vivo*, we measured the NO-dependent activation of the *nor* promoter in a haem-deficient (Δ *hemA*) *E. coli* strain, which lacks the HemA protein involved in the production of ALA, a key intermediate in the biosynthesis of haem (Harris *et al.*, 1993). A similar approach was successfully employed to show that haem biosynthesis is essential for the sensing activity of NNR from *Paracoccus denitrificans* (Lee *et al.*, 2006).

Plasmid pRS-NOR was transformed into the *E. coli* Δ *hemA* strain with or without plasmid pACY-DNR, and cells were

grown in the presence or in the absence of 0.4 mM ALA, under anaerobiosis with or without NO. To evaluate the basal activity of the *nor* promoter, we also measured the β -gal activity in the *E. coli* Δ *hemA* strain containing only plasmid pRS-NOR.

The results (Fig. 4) show that only in the presence of exogenous ALA is a NO-dependent DNR activity comparable to that seen in the TOP10 strain observed; this demonstrates that in *E. coli* haem is required to support the activation of the *nor* promoter by DNR *in vivo*, confirming previous evidence obtained *in vitro*.

DNR discriminates between different diatomic gases

Given that haem is essential for DNR activation we tested whether DNR is able to discriminate between different gases. We have previously shown that the reduced form of the DNR–haem complex is able to bind both NO and CO *in vitro* (Giardina *et al.*, 2008). In particular, the adduct of NO with the haem–DNR complex is pentacoordinate, whereas the CO-bound species is hexacoordinate. Therefore, we used the *E. coli* system developed here to carry out a β -gal assay under anaerobic conditions in the

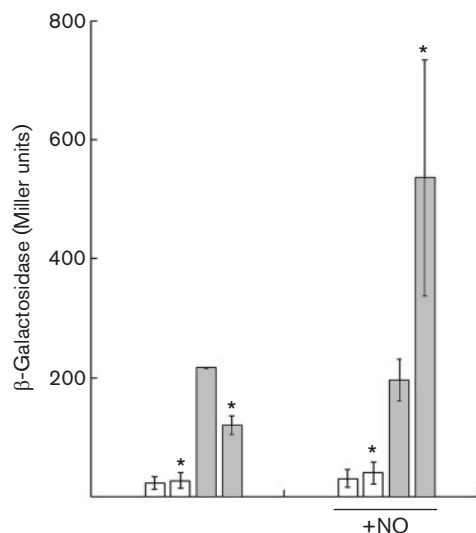


Fig. 4. Haem dependence of DNR-mediated β -galactosidase activity. The DNR protein was expressed constitutively in the $\Delta hemA$ *E. coli* strain together with plasmid pRS-NOR (grey bars). As a control, the β -galactosidase activity was assayed under the same experimental conditions in the $\Delta hemA$ *E. coli* strain containing only the pRS-NOR vector (white bars). Cultures were grown anaerobically in LG medium to exponential phase, and induced, as indicated, by the addition of 10 μ M NO; samples marked with asterisk on the graph refer to cultures grown in the presence of 0.4 mM ALA. β -Galactosidase activity (Miller, 1972) was measured 1 h after induction (grey bars) in duplicate in three independently grown cultures; mean values (Miller units) are shown, together with experimental error bars (SD).

presence of CO. The very low (25.5 ± 3 Miller units) β -gal activity measured under these conditions shows that DNR is inactive in the presence of CO, suggesting that the protein responds differently to the two diatomic signal molecules, i.e. NO and CO. These results are in agreement with our previous *in vitro* evidence that the haem coordination state of the NO and CO complexes in DNR is different (Giardina *et al.*, 2008). In conclusion, NO is the specific and unique signal required for DNR function; this selective activation in response to a particular ligand is shared with other haem-based gas sensors (e.g. CooA and H-NOX domains) (Andrew *et al.*, 2001; Boon & Marletta, 2005; Gilles-Gonzalez & Gonzalez, 2005).

Conclusions

We have shown in this work that the *P. aeruginosa* DNR protein is active in a heterologous system (*E. coli*) and can use the transcriptional apparatus of this bacterium to bind specifically to the *P. aeruginosa* *nor* promoter in order to regulate its activation. Moreover, we have shown that haem is the cofactor mediating the NO-dependent DNR regulation in *E. coli*; this finding reinforces the *in vitro* results previously obtained by our group. We have demonstrated,

we believe for the first time, that DNR is able to discriminate between different diatomic signal molecules, NO and CO.

We can therefore conclude that the heterologous system described has shed new light on the features of the *P. aeruginosa* DNR protein and, in the future, will allow us to assay the activity of site-directed mutants targeting residues putatively involved in haem binding and in NO-sensing.

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