Heme and nitric oxide binding by the transcriptional regulator DnrF from the marine bacterium Dinoroseobacter shibae increases napD promoter affinity

Matthias Ebert1, Peter Schweyen1, Martin Bröring1, Sebastian Laass1, Elisabeth Härtig1,1, and Dieter Jahn1

From the 1Institute of Microbiology, Technische Universität Braunschweig, Spielmannstrasse 7, D-38106 Braunschweig, the 2Institute for Inorganic and Analytical Chemistry, Technische Universität Braunschweig, Hagenring 30, D-38106 Braunschweig, the 3Institute for Molecular Biosciences, Goethe-University Frankfurt, Max-von-Laue-Str. 9, D-60438 Frankfurt, and the 4Braunschweig Integrated Centre of Systems Biology (BRICS), Technische Universität Braunschweig, Rebenring 56, D-38106 Braunschweig, Germany

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Under oxygen-limiting conditions, the marine bacterium Dinoroseobacter shibae DFL121 generates energy via denitrification, a respiratory process in which nitric oxide (NO) is an intermediate. Accumulation of NO may cause cytotoxic effects. The response to this nitrosative (NO-triggered) stress is controlled by the Crp/Fnr-type transcriptional regulator DnrF. We analyzed the response to NO and the mechanism of NO sensing by the DnrF regulator. Using reporter gene fusions and transcriptomics, here we report that DnrF selectively repressed nitrate reductase (nap) genes, preventing further NO formation. In addition, DnrF induced the expression of the NO reductase genes (norCB), which promote NO consumption. We used UV-visible and EPR spectroscopy to characterize heme binding to DnrF and subsequent NO coordination. DnrF detects NO via its bound heme cofactor. We found that the dimeric DnrF bound one molecule of heme per subunit. Purified recombinant apo-DnrF bound its target promoter sequences (napD, nosR2, norC, hemA, and dnrE) in electromobility shift assays, and we identified a specific palindromic DNA-binding site 5’-TTGATN4ATCAA-3’ in these target sequences via mutagenesis studies. Most importantly, successive addition of heme as well as heme and NO to purified recombinant apo-DnrF protein increased affinity of the holo-DnrF for its specific binding motif in the napD promoter. On the basis of these results, we propose a model for the DnrF-mediated NO stress response of this marine bacterium.

Many microorganisms can replace oxygen as terminal electron acceptor with nitrate during electron transport-dependent ATP generation. Several modes of the corresponding nitrate respiration are known. During the ecologically central process of denitrification, nitrate (NO₃⁻) is reduced via nitrite (NO₂⁻), nitric oxide (NO), and nitrous oxide (N₂O) to molecular nitrogen (N₂) (1). The intermediate nitric oxide is cytotoxic by accumulation. As a consequence, the transition process from oxygen respiration to denitrification is tightly controlled.

Many bacteria utilize so-called Crp (cAMP receptor protein)/Fnr (fumarate and nitrate reductase regulator) superfamily regulators for this purpose. This highly flexible group of transcription factors shares a low overall amino acid sequence identity (2). However, they all contain a C-terminal DNA-binding helix-turn-helix motif and an N-terminal sensory domain formed by multiple antiparallel β-strands that are connected by a long dimerization helix (2–4). According to the binding of the cofactor at the individual sensing region, a defined signal can be detected (2). Considering the localization of their binding sites, these proteins can act as activators or repressors of transcription. Binding sites upstream of or even overlapping with the −35 region of a standard bacterial promoter lead to transcriptional activation. Binding sites overlapping with or downstream of the −10 region are usually mediating gene repression (2, 5).

One prominent example of Crp/Fnr regulators is the global transcription factor Fnr of Escherichia coli forming together with the homologous proteins Anr, FnRA, FnRL, and FnRP, a major subgroup of Crp/Fnr regulators. All of them sense oxygen via an oxygen-labile [4Fe-4S] cluster (5). This subgroup possesses four conserved cysteine residues at the N or C terminus for ligation of the oxygen and NO-sensitive iron–sulfur clusters (6–11).

NsrR of Bacillus subtilis, another Fe-S cluster-containing regulator, plays a major role in NO detoxification (12). NsrR with intact Fe-S cluster represses hmp gene expression under anaerobic conditions (13). The hmp gene, encoding a flavohemoglobin, plays a central role in B. subtilis for the response to nitrosative stress (14, 15). Accumulation of NO inactivates NsrR by destabilizing the Fe-S cluster and leads to the derepression of hmp transcription.

In Pseudomonas aeruginosa another Crp/Fnr-like regulator, Dnr (dissimilatory nitrate respiration regulator), was shown to create a fine-tuned hierarchical regulation of denitrification genes like nirS, norCB, and nosR (16–19). In Pseudomonas stutzeri DnrD is involved in nitric oxide signaling and transcriptional control of denitrification genes (20).
The Dnr regulators are lacking the conserved cysteine residues of Fnr involved in iron–sulfur cluster ligation. But they still respond to nitric oxide and mediate the regulation of various denitrification genes (20). Members of this regulatory subfamily coordinate a heme cofactor (21–23). The crystal structure of the sensor domain of *P. aeruginosa* Dnr without bound heme was solved (24). Functional studies identified histidine residues 139 and 186 as axial ligands for heme binding (25). In response to the binding of the effector molecule NO, a conformational change of heme-bound Dnr was proposed, which results in efficient DNA binding (24, 26).

Conflicting data exist about the DNA-binding capabilities of the protein. An initial EMSA study showed DNA binding solely for the *P. aeruginosa* Dnr with bound heme, whereas apo-Dnr failed to bind the nirS promoter sequence (24). Fluorescence anisotropy measurements of a Texas Red-labeled nor promoter fragment bound to *P. aeruginosa* holo-Dnr revealed DNA binding with a $K_D$ of $44 \pm 9$ nM only in the presence of NO. Holo-Dnr without bound NO and the CO-bound form of the regulator failed to bind the promoter, even if the regulator is provided in a 50-fold excess over the DNA (21).

In *P. aeruginosa* the binding sites for Dnr and the Fnr analogue Anr can optically not be distinguished (27). Both regulators recognize the palindromic sequence TTGATN$_4$ATCAA. However, clear-cut Anr, Dnr, and Anr + Dnr (both regulators regulate the same gene via one binding site) regions can be defined using transcriptomics (19). The molecular mechanism underlying promoter specificity and transcriptional activation by Dnr after binding to the promoter remains to be determined.

The marine bacterium *Dinoroseobacter shibae* DFL12$^T$ (DSM 16493), as one representative of the highly abundant *Roseobacter* group of marine bacteria, possesses a fine-tuned hierarchical network for the adaptation to anaerobic conditions (28). One Fnrl and three Dnr regulators (DnrD, DnrE, and DnrF) mediate the transition from aerobic to anaerobic conditions by activating denitrification genes in the presence of nitrate (28).

In *D. shibae* nitrate gets reduced to nitrite using a periplasmic dissimilatory nitrate reductase (*nap*-type), a periplasmic cd$_1$-type nitrite reductase (*NirS*), a membrane-localized NO reductase (*NorCB*), and a periplasmic N$_2$O reductase (*NosZ*) (29). All of these enzymes as well as corresponding maturation and regulatory proteins are encoded in a large denitrification gene cluster.

In a previous study, we determined the role of Fnrl and the three Dnr regulators (DnrD, DnrE, and DnrF) for their response to anaerobiosis in the presence of nitrate (28). Fnrl and DnrD were identified as key players for the regulation of the transition process.

Here, we investigated the regulatory function of DnrF. Because the toxic gas NO is produced during denitrification and released, the organism requires a fast and effective stress response. It must include detection of NO and a subsequent gene regulatory scenario that leads to a stop of NO production and enhances NO consumption. DnrF was identified as the NO stress regulator of *D. shibae*, where NO is detected via a bound heme. Repression of the nitrate reductase genes by DnrF prevented further NO production, although the parallel induction of the NO reductase genes ensures the consumption of the harmful NO$_2$-oxide.

**Results**

**Defining NO stress in *D. shibae***

During the process of denitrification, several dangerous N-oxides, including NO, are generated as intermediates. From previous experiments we knew that the *nirS* gene, encoding the enzyme nitrite reductase, responsible for NO generation, is controlled by NO. To determine the role of sublethal NO concentrations as signal for gene expression in *D. shibae*, we grew the wild-type strain DFL12$^T$ harboring a nirS-lacZ reporter gene fusion under aerobic conditions until an optical density at 578 nm of 0.5 was reached. Subsequently, the cells were shifted to anaerobic conditions. Depletion of oxygen was determined using a PreSense Fibox 3 trace assay in combination with a PtS3-type oxygen sensor (detection limit 15 ppb dissolved oxygen, 0–100% oxygen). When all oxygen was consumed by the culture, we added NO-saturated water to final NO concentrations of 50, 1000, 10,000, and 70,000 nM and monitored growth and *β*-galactosidase activities from the *nirS-lacZ* fusion after 15, 30, and 60 min (Fig. 1). These highly different NO concentrations were used because in the literature quite diverse NO concentrations were employed to study NO-dependent transcriptional changes. In *P. stutzeri* 50 nM NO caused an inducing effect on *nirS* gene expression. In contrast, in *P. aeruginosa* 10,000 nM were used to induce *norC-lacZ* reporter gene expression (20, 30), and in Gram-positive organisms like *B. subtilis* survival of 50,000 nM NO can be observed (31).

After shifting the cells to gas tide serum flasks, oxygen consumption occurred within 15 min. Because of the loss of an electron acceptor, growth stopped after the anaerobic shift indicating a major metabolic crisis for *D. shibae* (32). After addition of 50, 1000, and 10,000 nM NO, a slight decrease in optical density was recorded, but after that the cell density remained stable. However, 70,000 nM NO leads to a significant drop in cell density indicating cell death and cell lysis (Fig. 1A). For *β*-galactosidase assays, samples were taken 0, 15, 30, and 60 min after the addition of indicated NO concentrations (Fig. 1B). At time point 0, background levels of 4–6 Miller units were measured for all cultures. Without addition of NO, *β*-galactosidase activity increased up to 9 Miller units after 15 min, 69 Miller units after 30 min, and 79 Miller units after 60 min (Fig. 1B). This NO-independent increase of *nirS-lacZ* expression is due to an anaerobic induction mediated by Fnrl (28). Addition of 50 nM NO led to *β*-galactosidase activities of 16 Miller units after 15 min, 93 Miller units after 30 min, and 119 Miller units after 60 min. The presence of 50 nM NO increased the *nirS-lacZ* reporter gene expression by a factor of 1.5. Addition of 1000 or 10,000 nM resulted in *β*-galactosidase activities comparable with the untreated anaerobic culture. The NO concentration of 70,000 nM led to a loss in activity presumably caused by the cell death generated by the toxic effect of NO. Thus, 50 nM was identified as the NO concentration with best gene-inducing effect without severe toxic effects at the same time.

**NO-dependent gene expression in *D. shibae***

The anaerobic NO-dependent global gene expression was investigated by DNA arrays comparing transcripts from *D. shibae* wild-type cells grown anaerobically in the presence and...
Regulon of the NO stress regulator DnrF

To determine the role of the DnrF regulator in mediating NO-dependent regulation, its regulon was defined by comparing the transcriptome of the wild-type strain with the corresponding mutant strain DS004 (ΔdnrF). A large NO-dependent regulon was found for DnrF with 365 induced and 199 repressed genes compared with wild type, indicating a major regulatory function of DnrF (supplemental Table S2). The comparison of the NO-mediated response of the wild-type strain and the DnrF regulon using a heat map analysis revealed significant overlap in the regulation of the denitrification gene cluster (Fig. 2). The nirDAGHBC operon, encoding the dissimilatory periplasmic nitrate reductase, was found to be repressed by DnrF, which was indicated by the increased transcription in the ΔdnrF mutant strain compared with the wild type (Fig. 2). This finding is in accordance with previous observations (28). This repression is mediated via the palindromic sequence 5′-TTGATN₅ATCAA-3′, which is located 96.5 bp upstream of the napD translational start site (Fig. 5B). Furthermore, a DnrF-dependent activation was found for the nirSECDFGHJN operon, indicated by a reduced transcription in the ΔdnrF mutant strain compared with the wild type (Fig. 2). Interestingly, only nirG and nirH exhibited a significant alteration in gene expression but lacked an assignable regulator-binding site. Moreover, a significant activation was found for the norC/B genes encoding nitric-oxide reductase. A corresponding potential DnrF regulator-binding site was found 67.5 bp upstream of the translational start site (Fig. 2). However, no significant alternation in transcription of the nosRZDFLYX operon dependent on DnrF was found after NO treatment. Overall, the observed regulatory scenario with an NO-dependent repression of the NO-producing system (nitrate reductase Nap) and an induction of the NO-consuming enzyme (NO reductase NorCB) finally identifies DnrF as NO stress regulator of D. shibae.

Because several of the observed DnrF-regulated genes might also be regulated by the DnrD regulator, the corresponding mutant strain (DS002 (ΔdnrD)) was analyzed under NO-inducing conditions, and genes of overlapping regulons were defined. The NO-dependent DnrD regulon consists of 17 genes and is given in supplemental Table S1. In total, 22 genes belong to the overlapping regulon of DnrD and DnrF (supplemental Table S1). Thus, a specific DnrF regulon was determined, which is composed of a total of 80 genes. Because not all genes exhibit a potential DnrF-binding site, a significant portion of indirect regulatory scenarios can be deduced from the DnrF-dependent repression of various transcriptional regulator genes like zntR, an ATP-dependent transcriptional regulator (Dshi_1881) and an XRE family transcriptional regulator (Dshi_2488), which exhibited a potential DnrF-binding site in their promoter sequences. However, most potential DnrF-binding sites were mainly found in promoters of genes of unknown function (Dshi_0391, Dshi_0625, Dshi_2048, Dshi_2148, and Dshi_3542). Furthermore, a striking influence of DnrF on numerous genes encoding transport systems was observed. A direct activation of an S-adenosylmethionine uptake transporter (Dshi_2148), a sulfite exporter tauE/safe (Dshi_0205), sugar transporter systems (Dshi_0488/Dshi_1808), a periplasmic dicarboxylate
transporter (dctQ/dctM), and cation/H⁺ antiporter (mnhC/mnhG) were found. However, significant repression by DnrF was found for the genes of two ABC transporters (znuC/Dshi_1421/Dshi_2434). The DnrF-binding site in the promoter of Dshi_1421 encoding a potential ribose ABC transporter indicated direct regulation. An impact on the general stress response was previously shown for periplasmic transporter (dctQ/dctM) and cation/H⁺ antiporter (33). Only a few genes of the general stress response, including the exonuclease (urvB), a cytochrome c peroxidase gene with potential DnrF-binding site (Dshi_2749), and the cell division factor (mraZ) were found controlled by DnrF. A significant activation was found for a potential quorum-sensing gene (Dshi_3013) and the plasmid-encoded replication protein C (repC). A significant role of quorum-sensing genes and plasmid-encoded genes in the general stress response was previously shown (33, 34). Alternative metabolic strategies like arginine fermentation via ornithine carbamoyltransferase (arcB), acetate utilization via acetate-CoA ligase (acsA), or control of the central metabolism via the malic enzyme (maeb) were found to be regulated by DnrF. This makes sense in the light of the metabolic problems occurring after the utilization of residual oxygen amounts (32). Nevertheless, regulation by DnrF was observed for numerous other genes like hisD, encoding a histinol dehydrogenase. Moreover, a direct regulation via a potential DnrF-binding site in the promoter was found for a gene encoding a potential hemolysin (Dshi_2763) and the gene encoding a cobalamin synthesis protein P47K (Dshi_2592). The advantage in regulating these genes under nitrosative stress conditions remains unclear.

**DnrF senses NO via bound heme**

The result of the transcriptome analyses pointed toward a role of DnrF in mediating the observed NO-dependent gene regulation in *D. shibae*. For thorough biochemical characterization of the DnrF protein, the transcription factor was heterologously produced as a His-tagged fusion protein in *E. coli* and chromatographically purified to apparent homogeneity under strict anaerobic conditions. The ability of DnrF to bind heme was determined spectroscopically by titration of heme to the DnrF apoprotein under anaerobic conditions. A stoichiometric binding of approximately 1 mol of heme/mol of subunit of DnrF was observed (Fig. 3A). A mostly dimeric state of the purified DnrF protein was determined by gel-permeation chromatography. DnrF has a calculated molecular mass of 26,260 Da. Apo-DnrF, holo-DnrF, and holo-DnrF treated with NO eluted when analyzed under anaerobic conditions as two peaks with relative molecular masses of ~52,000 ± 3000 and 106,000 ± 5000 Da (Fig. 3B). In all analyzed cases the major oligomeric form observed was dimeric. The higher-ordered form of DnrF detected corresponded to a tetramer, most likely as dimer of dimers. The detected amounts of this form were decreasing from apo- via holo-holo-DnrF with NO. Higher-ordered complexes were also observed during the electrophoretic mobility shift assays (EMSAs) experiments (Fig. 4). Perhaps these higher ordered complexes represent a DNA-bound storage form of inactive DnrF.

UV-visible absorption spectroscopic analysis of the purified apo-DnrF protein failed to detect absorption indicative of a bound heme (Fig. 3, B and C). Free heme has an UV-visible absorption spectrum with maxima at 365 and 385 nm. When heme was added to apo-DnrF in a stoichiometric range, the resulting holo-DnrF with bound heme in its ferric form showed a Soret peak at 417 nm (Fig. 3C). Reduction of the bound heme to its ferrous form by the addition of dithionite changed the corresponding absorption maxima of the Soret, α, and β peak to 427, 530, and 560 nm, respectively (Fig. 3, B and C). However, shoulders at 360 and 392 nm indicated residual amounts of unbound heme in the reaction (Fig. 3C). Because the heme molecule is not covalently bound to the DnrF protein, an equilibrium reaction between the bound and unbound state was assumed. These recorded spectra are similar to those of heme proteins with histidine residues as axial ligands, like the heme regulator HtrR of *Lactococcus lactis* (35). In Dnr of *P. aeruginosa*, an essential function in heme coordination was assumed for His-187 (25). However, an amino acid sequence alignment of DnrF from *D. shibae* with Dnr of *P. aeruginosa* and DnrD from *P. stutzeri* failed to detect a conserved histidine residue, which may be involved in heme coordination. Even the previously experimentally determined histidine residues of *P. aeruginosa* Dnr His-167 and His-187 were not conserved in *D. shibae* Dnr (25, 36). The addition of 1% NO in gaseous form to holo-DnrF altered the absorption maximum of the Soret
peak from 427 to 393 nm, and the solution became red (Fig. 3C) (24, 25). Furthermore, a blur of the Q bands was observed due to vibrational excitations. To investigate the NO-dependent alteration in the absorption spectra of DnrF, electron paramagnetic resonance (EPR) measurements were performed. Thirty \( \mu \text{M} \) heme-reconstituted DnrF were analyzed in its ferric, ferrous and NO-bound state at 4–5 K in an X-band EPR spectrometer (Fig. 3D). Data plotting for heme-reconstituted DnrF versus pure heme revealed comparable spectra showing an axial EPR resonance with a value of 6.0. The ferric heme displayed a low-spin Fe(II) nitrosyl complex \([\text{FeNO}]^7\) (Fig. 3D) (37). The exposure of ferrous heme containing DnrF to 1% NO gas mixture resulted in a broad signal with an isotropic g value of g = 2.05. The resonance is consistent with a five-coordinated, low-spin Fe(II) nitrosyl complex \([\text{FeNO}]^7\) (Fig. 3D) (37).

**Functional identification of the DnrF-binding site**

The transcriptome analyses comparing *D. shibae* wild type and the corresponding \( \Delta \text{dnrF} \) mutant strain also led to the identification of several genes showing a clear-cut DnrF-dependent regulation (Fig. 2). Five of them, namely *hemA3, nosR2, norC, dnrE*, and *napD*, were selected to study their DnrF-binding properties to the corresponding promoters using EMSA. As a reference, the *nosR1* promoter was chosen. Initial experiments were performed under aerobic conditions. Double-stranded promoter fragments of 75 bp in length containing the putative DnrF-binding sequences with nucleotide sequence identity to the palindromic sequence 5’-TTGATN4ATCAA-3’
were labeled with digoxigenin and incubated with increasing amounts of purified His-tagged apo-DnrF. This palindromic sequence was considered to be specifically recognized by Dnr regulators (38). The equilibrium dissociation constants ($K_d$) of apo-DnrF/promoter fragment were estimated by non-linear regression according to the Hill equation: $y = [\text{DnrF}] / (K_d + [\text{DnrF}])$. By using the nosR1 promoter fragment only a faint retarded DNA–DnrF complex was observed at DnrF concentration above 64 nM (Fig. 4A). Because of the poor binding of DnrF to the promoter DNA the determination of a specific dissociation constant was not possible. This weak interaction of DnrF with the nosR1 promoter fragment may be due to the improper palindromic sequence 5’-TTGATGTCATGGG-3’ lacking the conserved half-site motif at the 3’ end (conserved bases are given in bold). In contrast, binding of apo-DnrF to the hemA3 promoter fragment already occurred at concentrations higher than 8 nM (Fig. 4B). A dissociation constant ($K_d$) of 44.18 nM was calculated. Here, a higher degree of conservation of the potential DnrF-binding sequence was observed (5’-TTGACGTGGTAAA-3’). An even better binding affinity of DnrF was found for the nosR2 promoter that was localized directly upstream of the nor operon (Fig. 4C). A $K_d$ of 14.7 nM was determined. Moreover, a second retarded complex became visible at concentrations higher than 64 nM (Fig. 4C). The second complex found in the EMSA may represent a higher-ordered complex, possibly consisting of a dimer of dimers (Fig. 3B). Next, analysis of the affinity of DnrF for the norC promoter yielded a $K_d$ of 11.95 (Fig. 4D). Similar to the results of the nosR2 promoter analysis, a higher-ordered complex was observed at higher DnrF concentrations of 32 nM. Binding studies using the dnrE promoter sequence revealed a DnrF/DNA interaction already at concentrations of 2 nM and a higher ordered complex above concentrations of 32 nM DnrF (Fig. 4E). Quantitative analyses revealed a dissociation constant of DnrF for the dnrE promoter of 10.68 nM. Binding studies of DnrF to the napD promoter sequences resulted in the highest affinity of DnrF for a promoter sequence. Efficient DNA binding occurred already at concentrations of 2 nM DnrF with a calculated dissociation constant $K_d$ of 9.8 nM. Again, the second higher-ordered complex occurred at a DnrF concentration of 32 nM. The high affinity of DnrF to the napD promoter fragment led to a complete shift of the employed DNA fragment in the EMSA, and at DnrF concentrations higher than 128 nM free DNA was no longer detectable (Fig. 4F).

To determine the role of the potential binding site 5’-TT-GATTCGATCAA-3’ centered at position −96.5 with respect to the translational start site of napD, the binding sequence was mutated to 5’-GCGATCTCGATGC-3’ in the context of the 75-bp napD promoter sequence and was used in EMSA studies. DnrF formed a stable complex with the wild-type napD promoter sequence, whereas no DNA binding of DnrF was observed for the mutated napD promoter sequence (Fig. 5A, lanes 2 and 3). In addition, competition experiments were performed by adding increasing amounts of unlabeled wild-type and mutated napD promoter DNA to the binding assay. Addition of the wild-type napD in a 10-, 50-, and 100-fold molar excess completely abolished DNA binding to the labeled napD promoter fragment. The shifted complex disappeared already with a 50-fold molar excess (Fig. 5A, lanes 4–6). In contrast, even the highest amount of the mutated napD promoter DNA

Figure 4. EMSA of the transcriptional regulator DnrF and its promoters in the denitrification gene cluster. For EMSA 4 nM of a 75-bp-long DIG-labeled nosR1 (A), hemA (B), nosR2 (C), norC (D), dnrE (E), and napD (F) promoter fragments, respectively, were incubated without (lane 1) and with increasing amounts of apo-DnrF protein starting from 2 nM (lane 2), 4 nM (lane 3), 8 nM (lane 4), 16 nM (lane 5), 32 nM (lane 6), 64 nM (lane 7), and up to 128 nM (lane 8). The formed protein–DNA complexes were separated on a 8% non-denaturing polyacrylamide gel. Retarded protein–DNA complexes were labeled I and II.
used as a competitor for the binding of DnrF to the labeled napD promoter fragment failed to compete efficiently (Fig. 5A, lanes 7–9). These experiments clearly demonstrate that DnrF is regulating napD expression via the proposed DnrF-binding site 5’-TTTGATCTCGATCAA-3’ at position −96.5 with respect to the translational start site.

**Regulation of napD gene expression by DnrF in vivo**

To demonstrate regulation of target gene expression by DnrF in vivo, a napD-lacZ reporter gene fusion was tested in *D. shibae*. For this purpose, a 165-bp DNA fragment spanning napD promoter sequences from −145 to +20 with respect to the translational start site was cloned upstream of the lacZ reporter gene in the plasmid pBBRoblacZ (Fig. 5B). The plasmid carrying the napD-lacZ reporter gene fusion was transformed into the *D. shibae* wild-type and the *dnrF* mutant strain (DS004 (ΔdnrF)). The resulting *D. shibae* strains were grown under aerobic and anaerobic denitrifying conditions, and β-galactosidase activities were determined from samples taken in the mid-exponential growth phase. Under aerobic conditions, only very low β-galactosidase activities close to the detection limit were measured for the wild-type and the *dnrF* mutant strain (Fig. 5C). Under anaerobic conditions, a 10-fold increase of reporter gene expression up to 139 Miller units was detected for the wild-type strain. However, in the *dnrF* mutant strain a 32-fold increased expression up to 2690 Miller units was determined (Fig. 5C). These results clearly demonstrated a DnrF-dependent repression of the napD-lacZ reporter gene under denitrifying growth conditions.

**Heme binding and NO coordination increase DNA-binding affinity of DnrF**

The regulator protein DnrF in its apo-form is already able to bind efficiently to its palindromic target sequence (5’-TTTGATCTCGATCAA-3’) within the napD promoter. To investigate the consequences of heme and subsequent NO binding to the regulator, DNA-binding affinities of apo-DnrF and holo-DnrF with bound heme in the reduced state and in the presence of NO were determined using EMSA experiments. The anaerobically purified regulator DnrF was incubated with the napD fragment as described above. With increasing amounts of apo-DnrF, two shifted complexes were identified (Fig. 6A). The amounts of shifted complexes were quantified, and binding affinity was deduced by calculating the equilibrium dissociation constant (*K*<sub>D</sub>). For apo-DnrF, a *K*<sub>D</sub> value of 8 ± 1.5 nM protein was determined (Fig. 6, A and D). Addition of heme in the ferrous state resulted in a slightly increased binding affinity to a *K*<sub>D</sub> value of 4.7 ± 1.3 nM (Fig. 6, B and D). However, addition of NO in gaseous form further increased the binding affinity to a *K*<sub>D</sub> value of 1.6 ± 0.4 nM (Fig. 6, C and D). Thus, NO sensing by heme-bound holo-DnrF resulted in a 5-fold increased binding affinity of the regulator to the DNA.

**Discussion**

Under anaerobic conditions, the utilization of nitrate as a terminal electron acceptor is essential for the survival and growth of *D. shibae* (32). Corresponding reduction processes lead to the production of the toxic *N*-oxide NO. Thus, a stringent NO-triggered stress response is needed. Recent investigations of the *D. shibae* adaptation strategies to anaerobic nitrate-reducing...
conditions revealed a fine-tuned transcriptional regulatory network composed of four different Crp/Fnr-type regulators. Besides one oxygen-sensing FnrL homologue, a set of three potentially NO-sensing Dnr regulators were identified. Initial transcriptome analysis of D. shibae grown under anaerobic, denitrifying conditions identified DnrD as a major denitrification regulator. Here, we addressed the function of DnrF. We essentially were answering the following question. How does D. shibae deal with NO as an intermediate of denitrification? B. subtilis possesses the NO stress regulator NsrR, sensing NO via a bound [4Fe-4S] controlling a large detoxification machinery to avoid NO accumulation (14, 15) and that enabled B. subtilis to survive amounts of 50 nM NO (31). However, for D. shibae a sublethal NO concentration of 10 μM was determined. This is in accordance with other members of the Alphaproteobacteria, like Rhodobacter sphaeroides (39) and Paracoccus denitrificans (40, 41). Both organisms possess Dnr homologues called NnrR and Nnr, respectively. An essential role of those regulators under nitrosative stress conditions was demonstrated (39, 40, 42). However, their detailed biochemical function remains to be determined. With respect to the D. shibae DnrF regulon, the major findings were the repression of the genes for the nitrate reductase (nap) to prevent further NO2− production and subsequent NO formation together with the induction of the NO-consuming NO reductase (nor). Production of NO reductase is also a strategy of several pathogenic bacteria to withstand NO produced by macrophages of the human immune system (43). Interestingly, only one of the two genes encoding potential flavohemoglobins, Dshi_1666, is under the control of DnrD and DnrF under anaerobic conditions. Biochemical analyses of D. shibae DnrF using UV-visible and EPR spectroscopy revealed a non-covalent coordination of NO to heme and the binding of NO by forming a low-spin Fe2+ nitrosyl complex (FeNO)7. The distal and proximal ligands of the heme iron of DnrF are unknown. Amino acid sequence alignments of Dnr from various Pseudomonas species revealed a conserved stretch of 26 amino acids, of which 15 residues were assumed to form an apolar pocket to accommodate the heme molecule (24). This amino acid stretch is missing in D. shibae DnrF. Moreover, conserved histidine residues were functionally identified to be responsible for NO coordination in P. aeruginosa Dnr (25). All Dnr regulators of D. shibae lack the corresponding conserved histidine residues.

The palindromic sequence 5′-TTGATN₆ATCAA-3′ upstream of the napD gene was shown to be recognized specifically already by apo-DnrF. The reconstitution of apo-DnrF with heme leads to a significant increase in binding affinity of DnrF. Additional coordination of NO to holo-DnrF increased the binding affinity. This is in contrast to findings of P. aeruginosa Dnr, where apo-Dnr failed to bind the nirS promoter sequence (24). Fluorescence anisotropy measurements of holo-Dnr revealed DNA binding with a KD of 44 ± 9 nM only in the presence of NO. Free holo-Dnr and the CO-bound form of the regulator failed to bind the promoter fragment (21). In contrast, DnrF is binding already in its apo-form, which enables D. shibae to have a fast and precise stress response to NO (Fig. 7). Generation of holo-DnrF via heme binding to apo-DnrF increased DNA affinity. Heme binding might induce already the structural changes to Dnr responsible for the observed increase in DNA-binding affinity. In the presence of NO, the DNA affinity of holo-DnrF was found further increased. This resulted in either strong gene repression, like in the case of the nap

Figure 6. Increased binding affinity of DnrF to the napD promoter upon addition of heme and NO. A, EMSA analyses of apo-DnrF (black curve in D), B, holo-DnrF with ferrous heme (blue curve in D). C, holo-DnrF with ferrous heme + NO (green curve in D). Increasing amounts of purified DnrF (lane 1, none; lane 2, 2 nM; lane 3, 4 nM; lane 4, 8 nM; lane 5, 16 nM; lane 6, 32 nM) were titrated against 4 nM napD promoter fragment. To obtain holo-DnrF, equimolar amounts of apo-DnrF and ferric heme were pre-incubated. The reduction of holo-DnrF was achieved by addition of 2 mM dithionite. NO coordination was reached by incubation with gaseous NO. Retarded DnrF complexes are labeled with I and II. The dissociation constants (KD) were calculated by non-linear regression according to the Hill equation. D, binding assays were conducted under anaerobic conditions using native 8% polyacrylamide gel. The percentage of DnrF bound DNA from the supplied promoter fragment was plotted against the employed amount of the different DnrF forms. Error bars represent the observed standard deviation of three biological replicates.
NO stress regulator DnrF of Dinoroseobacter shibae

![Figure 7. Model of the NO-dependent gene regulation by DnrF.](image)

The transcriptional regulator DnrF is per se able to bind to the palindromic DNA sequence 5’-TTGA\_N\_TCAA-3’. The coordination of ferrous heme (blue, DnrF) and subsequent binding of NO under nitrosative stress conditions (green, DnrF) are presumed to cause structural rearrangements of the regulator. As a consequence, the DNA-binding affinity and transcriptional transactivation via interaction with the RNA polymerase (Pol) are controlled. Transcriptional activation (green arrow) or repression (red arrow) is dependent on the position of the DnrF-binding site within the controlled promoter.

Figure 7

operon, or gene activation as observed for the other tested DnrF-dependent promoters. The regulatory activity of NO-bound holoheme DnrF is finally dependent on the localization of the corresponding binding site in the target promoter as observed for regulators of the Crp/Fnr class (2, 5). In the case of the nosR2 promoter, the binding site is located at position -41.5 with respect to the transcriptional start site, a typical site for transcriptional activation (44). Transcriptional repression of the nap promoter might be due to blocking of the corresponding promoter at the -10 region. Furthermore, DnrF also differentially modulates the transcriptional transactivation as observed for the nor operon (Fig. 2) most likely via differential interaction with the RNA polymerase upon NO. As summarized in Fig. 7, a fast, DnrF-controlled and solely denitrification enzyme triggered response stop NO production and leads to the consumption of residual NO. Interestingly, potential flavohemoglobin genes for NO detoxification were not induced. These observations describe a novel bacterial strategy for the detoxification of the hazardous N-oxide NO.

**Experimental procedures**

**Bacterial strains, media, and growth conditions**

Cultures of the type strain *D. shibae* DFL12\(^T\), Δ*dnrD* mutant strain DS002, and Δ*dnrF* mutant strain DS004 (Table 1) were grown in marine-bouillon (MB, Roth, Karlsruhe, Germany) at 30 °C in flasks shaking at 200 rpm in the dark or on MB plates solidified with 1.5% agar. For *D. shibae* mutant strains, the DS002 and DS004 medium was supplemented with 80 μg/ml gentamycin. *E. coli* strains were routinely grown in lysogenic broth (LB) supplemented with the appropriate antibiotics and amino acids at 37 °C and shaking at 200 rpm (Table 1) (45). To investigate the growth behavior under aerobic and anaerobic conditions, the *D. shibae* strains were grown in artificial seawater medium (SWM)\(^2\) (46) supplemented with 16.9 mM succinate in flasks shaking at 200 rpm. For nitrosative stress experiments, 25 mM pyruvate was added, and incubation was performed in serum flasks sealed with rubber stoppers and shaking at 100 rpm (26). NO-saturated water solution was prepared according to Moore *et al.* (31).

**Growth curve and shift experiments**

For growth experiments, a pre-culture of the appropriate *D. shibae* strain was inoculated in SWM supplied with 16.9 mM succinate and grown overnight at 30 °C and 200 rpm in the dark. Next, 125 ml of the main culture was inoculated to an OD\(_{578}\) of 0.05 in SWM supplied with 16.9 mM succinate in a 1-liter baffled flask. After reaching an OD\(_{578}\) of ~0.5, the cultures were shifted to anaerobic conditions. For anaerobic cultivation, the rubber stopper sealed serum flask was used and 25 mM pyruvate was added. Oxygen tension was measured every 5 min using a PreSense Fibox 3 LCD trace version 7 and an oxygen sensor type PST3 with an accuracy of ±0.15% (PreSense, Regensburg, Germany). After 30 min of anaerobic cultivation, NO saturated water was added, under strict anaerobic conditions, for final amounts of 50,1000,10,000, and 70,000 mM NO (31). Samples were taken for RNA preparation after 30 min of anaerobic starvation and 30 min after injection of appropriate amounts of NO-saturated water solution.

**RNA preparation and DNA microarray**

RNA preparation following DNA array analysis was performed as described elsewhere (28, 32). Generated data have been deposited in the NCBI Gene Expression Omnibus (47) and are accessible by Geo Series accession number GSE95560.

**Promoter-lacZ reporter gene fusions**

A 165-bp napD DNA fragment corresponding to promoter sequences from position -145 to +20 with respect to the translational start was PCR-amplified using primers EH689 and EH670 (Table 2) together with the LIC tail sequences and cloned by ligation-independent cloning into pBBRLIC-LacZ resulting in plasmid pBBrnapD-lacZ as described previously (28).

**Recombinant DnrF production**

The dnrF (Dshi_3270) gene of *D. shibae* DFL12\(^T\) was PCR-amplified from *D. shibae* genomic DNA using primers oPT163 and oPT164 containing NdeI and BamHI restriction sites, respectively (Table 2). The resulting PCR product was digested using Ndel and BamHI and ligated into the equally treated vector pET14b (Novagen, Darmstadt, Germany). The resulting vector pET14DnrF was

\(^2\)The abbreviations used are: SWM, seawater medium; DIG, digoxigenin.
**Table 1**

Strains and plasmids used in this study

| Strain                  | Description                          | Source/Ref. |
|-------------------------|--------------------------------------|-------------|
| D. shibae strains       |                                      |             |
| DFL12                   | Wild type                            |             |
| DSO01                   | ΔforL-:aacC1 (Gm<sup>r</sup>)         | 28          |
| DSO02                   | ΔforD-:aacC1 (Gm<sup>r</sup>): GFP    | 28          |
| DSO04                   | ΔforF-:aacC1 (Gm<sup>r</sup>)         | 28          |
| D516                    | DFL12<sup>r</sup> pBBR1napD-lacZ, Cm<sup>r</sup> | This work   |
| D5104                   | ΔforE-:aacC1 (Gm<sup>r</sup>): pBBR1nirS-lacZ, Cm<sup>r</sup> | 28          |
| E. coli strains         |                                      |             |
| DH10b                   | F<sup>−</sup> endA1 recA1 galE15 galK16 supG rpsL ΔlacX74 Φ80lacZ2ΔM15 araD139 Δ(arac-leu)769<sup></sup> merC Δ(mrr-hsdRMS-mcrBC) λ<sup><a></sup> </sup> | Invitrogen |
| ST18                    | E. coli S17–ΔmenA thi pro hsdR-M-chromosomal integrated | 52          |
| BI21(DE3) pLysS         | F<sup>−</sup> ompT gal dcm lon hsdSB (B<sup>−</sup> M<sup>−</sup>) Δ(DE3) pLysS (cm<sup>r</sup>) | Stratagene |

**Plasmids**

- pET14DnrF
- pBRRLIC-lacZ
- pBBR1napD-lacZ

**Table 2**

Oligonucleotides used in this study

| Name                   | Sequence (5’ to 3’)<sup>a</sup> |
|------------------------|----------------------------------|
| EH689                  | CCGCGGCTTCCCGAGCTTCGCGGTGATGAGC  |
| EH670                  | GTCCTCTTCCCGACACGAAACGAAACACACAAATC |
| oP163                  | GTCG [†] CTA [†] GACCTGACTAATCAGG |
| nosR<sup>+</sup>        | GTAACGCTGGCGCTGCGGCTGCGGCGGGGCGGGGCGG |
| nosR<sup>−</sup>-rev    | GTAACGCGCTGCAGCGCCTGCGTGCGGCGGGGCGG |
| napD                   | CTAGTGGTACTCGGGCGGGGCGGGGCGGGGCGG |
| napD<sup>−</sup>-rev    | CTAGTGGTACTCGGGCGGGGCGGGGCGGGGCGG |
| dnrE                   | CCCGCAGCCAAAACGACCACGCCCTGGATACGATGAGG |
| dnrE<sup>−</sup>-rev    | CCCGCAGCCAAAACGACCACGCCCTGGATACGATGAGG |
| hema                   | ACCCTGTCGACACCAAGGCGGGCGGGGCGGGGGGCGG |
| hema<sup>−</sup>-rev    | ACCCTGTCGACACCAAGGCGGGCGGGGCGGGGGGCGG |
| nosR<sup>+</sup>        | GACAGCATGCCTGCGGGGGGCGGGGCGGGGCGG |
| nosR<sup>−</sup>-rev    | GACAGCATGCCTGCGGGGGGCGGGGCGGGGCGG |
| norC                   | GACAGCATGCCTGCGGGGGGCGGGGCGGGGCGG |
| norC<sup>−</sup>-rev    | GACAGCATGCCTGCGGGGGGCGGGGCGGGGCGG |

<sup>a</sup> Dnr-binding sites are underlined; base exchanges are given in bold letters; restriction sites are given in italic letters

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used for production of the N-terminal His-tagged DnrF fusion protein. For heterologous production of DnrF, the E. coli BL21 (DE3) pLysS strain was used. For the production of the Histagged DnrF protein, pET14DnrF carrying the E. coli cells was grown from a starting OD<sub>578</sub> of 0.05 in 500 ml of LB medium containing 100 μg/ml ampicillin in a 1-liter flask. Incubation was carried out at 37 °C with shaking at 200 rpm. After reaching an OD<sub>578</sub> of 0.5–0.6, production of His-tagged DnrF protein was carried out at 37 °C with shaking at 200 rpm. After reaching 30 mM imidazole, the bound proteins were eluted by adding 1 ml of elution buffer (100 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 20 mM imidazole). A French press (19,200 g) was used for Heme titration to apo-DnrF and binding of NO

To investigate heme binding in vitro, 10 μM His-tagged DnrF was used. For determination of heme-binding properties, heme solution was freshly prepared. For this purpose 10 mg of heme granulate were incubated in 1 ml of 100 mM NaOH solution for 30 min. The mixture was dissolved by addition of 1 ml of 1 M NO stress regulator DnrF of Dinoroseobacter shibae

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<sup>TM</sup> Carl Roth GmbH + Co. KG, Karlsruhe, Germany. The purified protein was stored at −20 °C. Protein fractions were analyzed by SDS-PAGE (48, 49). Protein concentrations were determined using the Beer-Lambert law as well as the Bradford reagent (Sigma) according to the manufacturer’s instructions. BSA and ovalbumin were used as reference proteins. UV-visible spectra were recorded on a Jasco V-650 spectrophotometer (Jasco Inc., Easton, MD) using a high precision cell with path length of 10 mm (Hellma Analytics, Müllheim, Germany), and all steps were performed under strict anaerobic conditions. In initial experiments, the His tag was cleaved off and removed. However, in UV-visible spectroscopy, the heme-binding gel filtration, and EMSA analyses, we did not observe any difference in behavior between the His-tagged and the tag-free DnrF. Consequently, all experiments were performed using DnrF-His in accordance with Karnaukhova et al. (50).
**NO stress regulator DnrF of Dinoroseobacter shibae**

Tris-HCl, pH 7.6 solution. Residual solid matter was sedimented by centrifugation (10 min at 13,000 x g). The supernatant was filtered using a 0.2-μm filter tip. Solution was measured at OD_{260 nm} and molar concentration was determined using the Beer-Lambert law. Heme titration was performed under reducing conditions by using 16 nm purified apo-DnrF and stepwise addition of 2 μM heme up to a final concentration of 28 μM. Molar ratios were determined (Fig. 3A). Reduced derivative was obtained by addition of 2 mM sodium dithionite. The NO-bound derivative was achieved by injecting 0.5 ml of 1% gaseous NO. NO dilution occurred within a N₂-saturated atmosphere. All steps were performed under strictly anaerobic conditions in a Coy anaerobic chamber (Coy, Grass Lake, MI).

**Gel-permeation chromatography**

The native molecular mass of DnrF was determined on an Äkta purifier system equipped with a Superdex™ 200 increase 10/300 GL column (GE Healthcare, Solingen, Germany). The column was equilibrated with 100 mM Tris-HCl, pH 7.5, 150 mM NaCl buffer at a flow rate of 0.5 ml per min. For calibration, gel filtration markers kit (M, of 12,400 (cytochrome c), 29,000 (carbonic anhydrase), 66,000 (albumin), 150,000 (alcohol dehydrogenase), and 200,000 (Amylase)) (Sigma) was used (supplemental Fig. S1) according to the manufacturer's instructions. An amount of 200 μl of DnrF at a concentration of 160 μM was injected. Elution was monitored at 280 nm for DnrF protein, at 417 nm for bound ferric heme (apo-DnrF), 427 nm for bound ferrous heme (holo-DnrF), and 398 nm for bound NO/heme in the case of NO/heme/DnrF.

**EMSA**

The extent of DNA binding of apo-DnrF, heme-reconstituted DnrF, and NO-bound DnrF was assessed using EMSA. Strand-specific promoter fragments with a central localization of the potential DnrF-binding site were synthesized (Metabion, Planegg, Germany). For analyses of the nosR1 (Dshi_0686) promoter, oligonucleotides nosR1 and nosR1-rev were used, spanning the 5’-region −32 to +43 (Table 2). For analyses of the hemA3 (Dshi_3190) promoter, oligonucleotides hemA and hemA-rev were used, spanning the promoter region −101 to −26 (Table 2). For analyses of the dnrE promoter, oligonucleotides dnrE and dnrE-rev were used, spanning the promoter region −116 to −41 (Table 2). For analyses of the nosR2 promoter, oligonucleotides nosR2 and nosR2-rev were used, spanning the promoter region −78 to −3 (Table 2). For analyses of the norC promoter, oligonucleotides norC and norC-rev were used, spanning the promoter region −111 to −36 with respect to the translational start (Table 2). For analyses of the napD promoter, a fragment spanning from −135 to −60 resulting in oligonucleotides napD and napD-rev was used (Table 2). In addition to the native napD promoter fragment, a mutated napD promoter fragment was synthesized, exhibiting base exchanges at position −106/−105 from TT to GC and at position −91/−90 from AA to GC, respectively. For the resulting napDmu promoter fragment, the oligonucleotides napDmu and napDmu-rev were used (Table 2). For the annealing reaction, 10 pmol of the complementary oligonucleotides were heated for 5 min at 95 °C in 10 mM Tris-HCl, pH 8, 1 mM EDTA, and 100 mM NaCl and then stepwise cooled down (5 °C steps, every 10 min) to room temperature. The resulting double-stranded DNA fragments were DIG-labeled using the DIG gel shift kit, 2nd generation, following the manufacturer’s instructions (Roche Applied Science, Basel, Switzerland). Unincorporated nucleotides were removed using the nucleotide removal kit from Qiagen (Hilden, Germany). Indicated amounts of DnrF (2, 4, 8, 16, 32, 64, 128, and 256 nM) were incubated with 4 nM DIG-labeled DNA fragments in 20 μl of binding buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM DTT, 5% glycerol, 10 mM NaCl, 1 mM MgCl₂, 0.1 mg/ml BSA). For competition experiments, the appropriate unlabeled DNA was added in amounts of 40 nM (10-fold excess), 200 nM (50-fold excess), and 400 nM (100-fold excess). Incubation was carried out for 20 min at room temperature. Subsequently, the mixture was loaded onto an 8% polyacrylamide gel and electrophoresed for 55 min in 1× Tris borate/EDTA buffer at 100 V. Then, the gel was blotted for 2 h onto a positively charged nylon membrane, followed by immunodetection. For this purpose, the membrane was washed in 100 mM maleic acid, 150 mM NaCl, 175 mM NaOH, and 0.3% Tween 20 and incubated for 40 min in 1× blocking solution (Roche Applied Science). Immunodetection was carried out by the addition of 0.375 units of anti-digoxigenin-AP Fab fragments (Roche Applied Science) and incubated for 30 min. After two washing steps, the pH of the membrane was increased by adding detection buffer (100 mM Tris-HCl, pH 9.5, and 100 mM NaCl). The luminescence reaction was initiated by coating the membrane with 0.25 mM CDP-Star and incubated at 37 °C for 15 min in darkness. Luminescence was measured using a high resolution camera (Photometrics, Cool SNAP HQ²) in darkness. Quantitative analyses were made by using Gelscan Version 6.0 (BioSciTec, Frankfurt am Main, Germany).

**Electron paramagnetic resonance spectroscopy**

X-Band EPR spectra were recorded with a Bruker EMX spectrometer equipped with an Oxford ESR900 gas flow cryostat. Helium was used as cooling gas. The spectra were measured at the stated temperature and frequency. The samples are prepared under nitrogen atmosphere in a glove box and filled in thin wall quartz EPR sample tubes (inner diameter 4 mm) 707-SQ-250 M manufactured by Wilmad-LabGlass (Vineland, NJ).

**Author contributions**—M. E. provided substantial contributions to the conception and design of the work, data collection, data analysis and interpretation, drafting the article, critical revision of the article, and final approval of the version to be published. P. S. contributed to the conception and design of the work, data collection, data analysis, and interpretation. M. B. contributed to the conception and design of the work, data collection, data analysis, and interpretation. S. L. contributed to conception and design of the work, data collection, data analysis, and interpretation. E. H. provided substantial contributions to the conception and design of the work, data analysis and interpretation, drafting and critical revision to the article, and final approval of the version to be published.

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**Table 2**

| Promoter Region | Oligonucleotides | Concentration (nM) |
|-----------------|-----------------|-------------------|
| nosR1           | nosR1-rev        | 2, 4, 8, 16, 32, 64, 128, 256 |
| hemA3           | hemA-rev         | 2, 4, 8, 16, 32, 64, 128, 256 |
| dnrE            | dnrE-rev         | 2, 4, 8, 16, 32, 64, 128, 256 |
| norC            | norC-rev         | 2, 4, 8, 16, 32, 64, 128, 256 |
| napD            | napD-rev         | 2, 4, 8, 16, 32, 64, 128, 256 |

**Figure 3A**

Gelscan Version 6.0 (BioSciTec, Frankfurt am Main, Germany).
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