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The Anaerobic Regulatory Network Required for *Pseudomonas aeruginosa* Nitrate Respiration▽

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In *Pseudomonas aeruginosa*, the $narK_1K_2GHJI$ operon encodes two nitrate/nitrite transporters and the dissimilatory nitrate reductase. The $narK_1$ promoter is anaerobically induced in the presence of nitrate by the dual activity of the oxygen regulator Anr and the N-oxide regulator Dnr in cooperation with the nitrate-responsive two-component regulatory system NarXL. The DNA bending protein IHF is essential for this process. Similarly, *narXL* gene transcription is enhanced under anaerobic conditions by Anr and Dnr. Furthermore, Anr and NarXL induce expression of the N-oxide regulator gene *dnr*. Finally, NarXL in cooperation with Dnr is required for anaerobic nitrite reductase regulatory gene *nirQ* transcription. A cascade regulatory model for the fine-tuned genetic response of *P. aeruginosa* to anaerobic growth conditions in the presence of nitrate was deduced.

The most efficient way for the gram-negative bacterium *Pseudomonas aeruginosa* to generate energy in the absence of oxygen is through denitrification. During this process, molecular oxygen is replaced by nitrate as the terminal electron acceptor. Nitrate (NO_3^-) is reduced in four consecutive steps, via nitrite (NO_2^-), nitric oxide (NO), and nitrous oxide (N_2O) to dinitrogen (N_2). This process is vital for growth and survival under microaerobic and anaerobic conditions as found in biofilms and microcolonies of infectious *P. aeruginosa* (1, 25a). The majority of earlier investigations focused on the enzymology and regulation of nitrite (NO_2^-)-to-dinitrogen (N_2) conversion (27). Here, the regulatory network for the onset of nitrate respiration under oxygen-limiting conditions was elucidated using reporter gene fusions, strains carrying mutated regulatory genes, and site-directed mutagenesis of potential regulator binding sites.

Importance of *narGHJI*, *narXL*, *anr*, and *dnr* for anaerobic growth of *P. aeruginosa*. In order to confirm the importance of the nitrate reductase genes *narGHJI* and the regulatory genes *anr*, *dnr*, and *narXL* for the anaerobic growth of *P. aeruginosa*, knockout mutants were characterized concerning their growth behavior. *P. aeruginosa* Anr is the oxygen-sensing regulatory protein homologue to *Escherichia coli* Fnr (19, 26). Dnr of *P. aeruginosa* belongs to the Crp-Fnr superfamily of transcriptional regulators and was reported to activate transcription of the genes *nir*, *nor*, and *nos* (6, 9). In *Pseudomonas stutzeri*, DnrD was shown to detect NO (13, 22). NarXL is a nitrate-responding two-component regulatory system (14). All investigated *P. aeruginosa* mutant strains failed to grow under anaerobic nitrate respiratory conditions (data not shown). They did not reveal any growth phenotype when tested under aerobic conditions (data not shown). These experiments identify *narL*, *anr*, *dnr*, and *narG* as key players in the anaerobic growth of *P. aeruginosa*.

Transcriptional control of the *nar* locus is mediated by the $narXL$-$narK_1$ intergenic region. In *E. coli* and *P. stutzeri*, Fnr- and NarXL-dependent transcription of the *narGHJI* operon is mediated by the *narG* upstream region (8, 15, 23, 24). In contrast to these observations, inspection of the 200-bp 5′ upstream region of *narG* in *P. aeruginosa* revealed no obvious binding motifs for the Fnr homologue Anr or the nitrate response regulator NarL. Using the two PnarG1 and PnarG2 reporter gene fusions, containing 100 bp and 411 bp of the upstream region of the *narG* gene fused to the reporter gene *lacZ* in the pQF50 plasmid, respectively, we showed that these DNA fragments did not mediate transcriptional activation of P*narG-lacZ* under any of the tested aerobic and anaerobic growth conditions (Table 1). Information regarding primer sequences, construction of all reporter gene fusions, mutated promoter constructs, and various strains can be provided upon request. We failed to detect a transcriptional start site using primer extension experiments upstream of *narG* (data not shown). Additionally, the intergenic region between $narK_2$ and *narG* was successfully amplified from cDNA synthesized from mRNA extracted from anaerobically grown *P. aeruginosa* PAO1 cells, confirming that *narG* is cotranscribed with the $narK_1K_2$ genes located upstream. Consequently, the 173-bp DNA fragment localized between *narX* and $narK_1$ harbors two divergently oriented promoters (Fig. 1A). This was also recently detected using a promoter predictor program and mutational analysis (19a). The 5′ end of $narK_1K_2GHJI$ mRNA was localized at 29 bp upstream of the translational start codon of $narK_1$, and the 5′ end of the *narX* mRNA was localized at 45 bp upstream of the translational start codon of *narX*, by using primer extension analysis on an ALF model DNA sequencer (Pharmacia) (16).

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TABLE 1. Regulation of the $narK_1K_2GHJI$ transcription by Anr, Dnr, IHF, and NarL

P. aeruginosa strain (genotype)	Promoter construct[c]	Gene in *trans*[b]	β-Galactosidase activity (Miller units)[a]					
			+ O_2 + nitrate	+ O_2 + nitrite	+ O_2	− O_2 + nitrate	− O_2 + nitrite	− O_2 fermentative
PAO1	PnarG1	—	3	4	5	11	10	7
PAO1	PnarG2	—	9	8	6	10	10	8
PAO1	$PnarK_1$	—	11	10	12	720	390	159
PAO6261 (Δ*anr*)	$PnarK_1$	—	6	6	5	8	12	7
PAO6261 (Δ*anr*)	$PnarK_1$	*dnr*	—	—	—	134	60	53
PAO1	$PnarK_1$	*dnr*	—	—	—	761	399	329
RM536 (Δ*dnr*)	$PnarK_1$	—	11	10	9	256	195	104
RM536 (Δ*dnr*)	$PnarK_1$	*anr*	—	—	—	678	161	268
PAO1	$PnarK_1$	*anr*	—	—	—	846	200	441
PAO1	$PnarK_1$ΔAnr	—	8	6	5	19	16	21
CHA-A2 (Δ*ihfA*)[d]	$PnarK_1$	—	4	5	5	72	53	37
PAO1	$PnarK_1$ΔIHF	—	8	7	6	299	152	183
PAO9104 (Δ*narL*)	$PnarK_1$	—	4	4	5	14	10	22
PAO9104 (Δ*narL*)	$PnarK_1$	*narL*	9	8	10	630	378	126
PAO1	$PnarK_1$ΔNarL1	—	10	9	10	32	33	30
PAO1	$PnarK_1$ΔNarL2	—	11	8	11	147	107	91
PAO1	$PnarK_1$ΔNarL3	—	2	—	—	138	—	—

[a] All values are results of three independent experiments performed in triplicate. Bacterial strains were grown aerobically and anaerobically in LB medium supplemented with 10 mM sodium nitrate or 1 mM sodium nitrite as described previously (7). Arginine fermentation conditions were achieved as outlined previously (21). The β-galactosidase activities are given in Miller units and are calculated as described previously (18). Standard deviations were between 3 and 13% of given values. —, not measured.

[b] The plasmids pHA411 (*anr*) and pHA541Ω (*dnr*) provided the *anr* and *dnr* genes in *trans* as described previously (4, 5). Vector without an insert served as background control. —, no gene in *trans*.

[c] Details for the construction of tested reporter gene fusions and *P. aeruginosa* mutants will be provided upon request.

[d] CHA-A2 is not isogenic to PAO1. Due to the clear cut results obtained with this strain, the data obtained are presented.

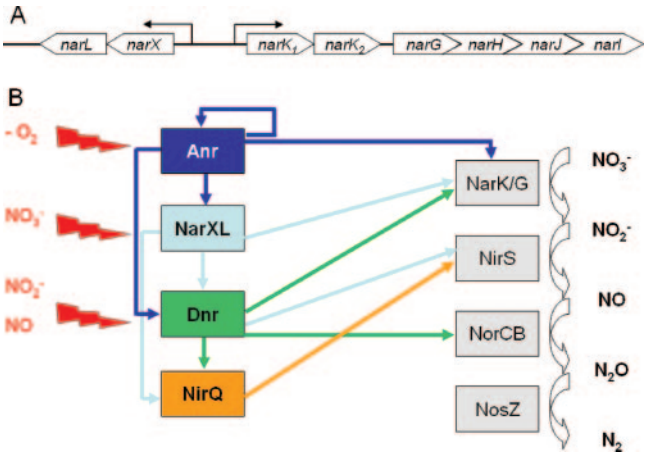

FIG. 1. (A) Schematic representation of the *nar* locus in *Pseudomonas aeruginosa*. The *narXL* and $narK_1K_2GHJI$ operons share a divergently oriented promoter region covering 173 bp. The 5′ end of the *narXL* mRNA was localized at 45 bp upstream of the translational start of *narX*. The $narK_1K_2GHJI$ genes are transcribed as an operon starting at 29 bp upstream of the $narK_1$ translational start. (B) The currently elucidated regulatory network for the onset of denitrification in *P. aeruginosa*. The major initial signal to turn on the denitrification pathway in *P. aeruginosa* is low-oxygen tension. This signal is measured by the Fe-S clusters attached to Anr (27). Anr increases the transcription of the *narXL* operon encoding a two-component regulatory system responding to the presence of nitrate. Anr and NarL cooperatively activate the *dnr* gene for the third involved regulatory system, Dnr, responding to NO (5). The fourth regulatory system, NirQ, in turn requires Dnr and NarL for its formation (4). Under low-oxygen tension conditions and in the presence of nitrate, Anr and NarL activate in concert with the DNA bending protein IHF, the $narK_1K_2GHJI$ operon encoding nitrate/nitrite transporters, and the structural genes for the respiratory nitrate reductase. The enzyme converts nitrate into nitrite. Now, both Dnr and NirQ, most likely responding to N-oxides, are essential for the gene regulatory scenario required for the formation of the three enzyme complexes that catalyze the conversion of nitrite into N_2.

Regulation of the $narK_1K_2GHJI$ promoter. A reporter gene fusion, P$narK_1$-*lacZ* containing 435 bp upstream of the translational start codon of $narK_1$ was constructed. The reporter gene activity was found to be induced 65-fold when *P. aeruginosa* was grown under anaerobic conditions in the presence of nitrate compared to that under identical but aerobic conditions. A 39-fold induction was detected when nitrate was replaced with nitrite, and still a 13.3-fold induction was present during arginine fermentative conditions (Table 1). Subsequently, the $PnarK_1$ construct was introduced into the two *P. aeruginosa* regulatory mutant strains PAO6261 (Δ*anr*) and RM536 (*dnr*::Tc). Aerobic $narK_1$ promoter activity in the wild-type and in the two mutant strains was comparably low (Table 1). No obvious $narK_1$ promoter activity was detected in the *anr* mutant strain under any of the tested anaerobic conditions. Deletion of the *dnr* gene resulted in 2.8-fold lower promoter activity levels than that in the wild-type strain under anaerobic growth conditions in the presence of nitrate. A 2-fold and 1.5-fold reduction of wild-type reporter gene activity was observed for anaerobic growth in the presence of nitrite and under fermentative growth conditions, respectively. Thus, Anr is essential, and Dnr enhances $narK_1$ activation (Fig. 1B). A regulatory cascade was proposed where Anr activates the expression of *dnr*, which in turn activates target promoters (5). To determine whether both regulators act in a coordinated manner, gene complementation assays were performed. The *anr* mutant strain carrying the P$narK_1$-*lacZ* promoter reporter gene fusion was transformed with the *dnr* gene on an expression plasmid (pHA541Ω) in *trans*. Reporter gene expression increased significantly compared to that of the *anr* mutant without *dnr* in *trans*. However, the values obtained were below those of reporter gene expression in the wild-type strain or the wild-type strain carrying a *dnr* expression plasmid (Table 1). A

minor but significant role for Dnr in the anaerobic activation of the $narK_1$ promoter was indicated, in which Dnr function was independent of Anr. Introducing an *anr* expression plasmid (pHA411) into the *dnr* mutant led to $narK_1$ promoter activity levels that were almost the same as those of the wild type (Table 1). Consequently, the $narK_1$ promoter represents another example of the dual actions of Anr and Dnr, besides those of the *P. aeruginosa hemF* and *hemN* promoters (Fig. 1B) (18). A highly conserved potential Anr/Dnr binding site (5′-TTGATTCCTATCAA-3′; conserved nucleotides in the Anr binding site are underlined) centered at −40.5 bp upstream of the 5′ end of the $narK_1$ mRNA, was detected using Virtual Footprint of PRODORIC software (www.prodoric.de). Three potential NarL binding sites, NarL1 (5′-TACCTCT-3′) at −108 bp, NarL2 (5′-TACGGCT-3′) at −113 bp, and NarL3 (5′-TACCTCC-3′) at −208 bp with respect to the $narK_1$ mRNA 5′ end, were found. One putative binding site for the DNA bending protein IHF (5′-CAATAATTTCAGCCG-3′) was proposed at −119 bp upstream of the $narK_1$ mRNA 5′ end.

Next, nucleotide exchanges (5′-TTGATTCCTATCAA-3′ to 5′-TCGATTCCTACTTA-3′) were introduced into the Anr box consensus motif ($PnarK_1$ΔAnr). Putative regulator binding sites were mutated using a QuikChange mutagenesis kit (Stratagene, Amsterdam, The Netherlands) or via crossover PCR (12) (details can be provided on request). No obvious promoter activity was detected under any of the tested conditions (Table 1). These results confirm the importance of the putative Anr binding site centered at −40.5 bp upstream of the transcriptional start for anaerobic induction of $narK_1$. The P*narK*1-*lacZ* reporter gene fusion was next introduced into the IHF (CHA-A2) and *narL* (PAO9104) mutant strains. The β-galactosidase activities were found to be significantly reduced (10-fold and 51-fold, respectively) for both mutants, indicating the involvement of both proteins in $narK_1$ transcription (Table 1 and Fig. 1B). Complementation of the *narL* mutant with a *narL* expression plasmid (pRK-LM) nearly restored wild-type-level promoter activity (Table 1). To verify the importance of a predicted IHF binding motif within the $narK_1$ promoter, the motif was mutated from 5′-CAATAATTTCAGCCG-3′ to 5′-*GGGG*AATTTCAGCCG-3′ ($PnarK_1$ΔIHF). The 2.4-fold decrease observed for the promoter activity of wild-type *P. aeruginosa* strain PAO1 carrying a P$narK_1$ΔIHF-*lacZ* fusion indicated the importance of the predicted IHF binding motif in $narK_1$ promoter activation (Table 1). The failure to completely eliminate $narK_1$ promoter activity with the introduced mutations might be due to residual IHF binding capacity of the mutated site or to an additional as-yet-unknown second IHF binding site in the $narK_1$ promoter. Additionally, the IHF protein can be replaced by less binding sequence-specific HU proteins (11, 17). The potential NarL binding sites NarL1 and NarL3 were correctly oriented for $narK_1$ activation; NarL2 was oriented in the opposite direction. Mutagenesis of the NarL1 binding site ($PnarK_1$ΔNarL1) resulted in a nearly total loss of reporter gene activities under denitrifying conditions (Table 1). Mutagenesis of NarL2 ($PnarK_1$ΔNarL2) decreased $narK_1$ promoter activity down to a level which was in the range of that of the IHF mutant. Since the NarL2 and the IHF binding motifs overlap, secondary effects of the NarL2 mutagenesis on the IHF binding site cannot be excluded. Mutagenesis of the third NarL binding site, NarL3 ($PnarK_1$ΔNarL3), abolished reporter gene activities, as shown in Table 1. Consequently, NarL1 and NarL3 are at least important for the anaerobic induction of the $narK_1$ promoter.

P. aeruginosa possesses a second dissimilatory nitrate-reducing system localized in the periplasm, encoded by the *napEFDABC* operon. No obvious influence of tested regulators, oxygen tension and nitrate or nitrite availability on *napEFDABC* promoter activity was detected using a *napE* promoter reporter gene fusion (Table 1). Clearly, *napEFDABC* expression is not coregulated with the onset of denitrification.

Regulation of the *narXL promoter*. To study the regulation of the *narXL* promoter, a P*narX-lacZ* fusion carrying 206 bp of the *narX* promoter region (PnarX) was constructed. A moderate 1.7- to 2.0-fold promoter induction was observed during anaerobic nitrate and nitrite respiratory and fermentative conditions (Table 2). The aerobic constitutive *narXL* transcription was independent of all regulators tested (Table 2). Moreover, *P. aeruginosa* NarXL did not autoregulate its own gene expression and did not require IHF for expression (8, 20). However, *P. aeruginosa narX* promoter activity was slightly reduced under any of the tested anaerobic conditions in the case of a missing Anr or Dnr regulator (Table 2 and Fig. 1B). To distinguish between cascade regulation and dual activities by Anr and Dnr, the *dnr* gene was expressed in *trans* from a plasmid in the *anr* mutant PAO6261 carrying the P*narX-lacZ* reporter gene fusion. As a control, *anr* was expressed in the *dnr* mutant RM536 harboring the same fusion. In both experiments, full anaerobic expression was not restored, indicating a dual function for both regulators at the *narXL* promoter. For weaker Anr-dependent promoters in *P. aeruginosa*, Anr half-site reactivity has been reported (14, 25). Therefore, both half sites of the potential Anr binding site centered at −60.5 bp upstream of the *narX* mRNA 5′ end were mutated independently, from 5′-TTGATTCCTATCAA-3′ to 5′-T*GA*ATTCCTATCA A-3′ in PnarXΔAnr1 and to 5′-TTGATTCCTA*AG*AA-3′ in PnarXΔAnr2. Decreased reporter gene activities of both mutated *narX* reporter gene fusions were at the levels observed for the reporter gene fusions with the intact *narXL* promoter tested with the regulatory mutants PAO6261 (Δ*anr*) and RM536 (*dnr*::Tc), respectively (Table 2). These results demonstrated that both half sites of the Anr/Dnr binding sequence are involved in anaerobic *narX* transcriptional activation. The location of the potential Anr/Dnr binding site at −60.5 bp might provide an explanation for the moderate 1.7-fold induction of the *narX* promoter under anaerobic conditions.

The NarL regulator and *dnr*, *nirQ*, *anr*, *nirS*, and *norC* transcription. Transcriptional *lacZ* fusions of the regulatory genes *dnr*, *nirQ*, and *anr* and the structural genes *nirS* (encoding nitrite reductase) and *norC* (encoding nitric-oxide reductase cytochrome *c* subunit) were introduced into *P. aeruginosa* PAO1 and the *narL* mutant strain PAO9104. In agreement with previous findings, the P*dnr-lacZ* fusion was found to be induced by Anr under anaerobic conditions (5). Moreover, *dnr* transcription was highly dependent on NarL (Table 2 and Fig. 1B). In agreement, highly conserved NarL binding sites were localized, centered at 189 bp and 114 bp upstream of the translational start point. The ATP-binding protein NirQ from *P. aeruginosa* activates respiratory nitrite reductase activity (10). The expression of the P*nirQ-lacZ* fusion was found to be strongly induced under anaerobic conditions. Like P*dnr-lacZ*

TABLE 2. Anaerobic induction of the *napE* and *narXL* promoters by NarL, IHF, Anr, and Dnr and NarL influence on the expression of P*dnr-lacZ,* P*nirQ-lacZ,* P*anr-lacZ,* P*nirS-lacZ,* and P*norC-lacZ*

P. aeruginosa strain	Promoter construct[c]	Gene in *trans*[b]	β-Galactosidase activity (Miller units)[a]					
			+ O_2 + nitrate	+ O_2 + nitrite	+ O_2	− O_2 + nitrate	− O_2 + nitrite	− O_2 fermentative
PAO1	PnapE	—	352	410	361	291	305	310
PAO6261 (Δ*anr*)	PnapE	—	402	576	408	350	361	338
RM536 (Δ*dnr*)	PnapE	—	238	253	262	286	284	239
PAO9104 (Δ*narL*)	PnapE	—	322	313	292	400	396	320
PAO1	PnarX	—	293	269	251	504	539	519
PAO9104 (Δ*narL*)	PnarX	—	220	225	216	523	426	580
CHA-A2 (Δ*ihfA*)[d]	PnarX	—	300	310	285	526	549	530
PAO6261 (Δ*anr*)	PnarX	—	246	259	241	367	239	336
PAO6261 (Δ*anr*)	PnarX	*anr*	272	—	—	622	—	—
PAO6261 (Δ*anr*)	PnarX	*dnr*	272	—	—	372	—	—
RM536 (Δ*dnr*)	PnarX	—	259	280	257	259	291	282
RM536 (Δ*dnr*)	PnarX	*dnr*	257	—	—	582	—	—
RM536 (Δ*dnr*)	PnarX	*anr*	298	—	—	398	—	—
PAO1	PnarXΔAnr1	—	194	216	200	280	320	316
PAO1	PnarXΔAnr2	—	241	269	228	331	393	365
PAO1	Pdnr	—	7	8	5	210	132	122
PAO9104 (Δ*narL*)	Pdnr	—	8	6	7	12	14	15
PAO1	PnirQ	—	17	22	12	327	427	60
PAO9104 (Δ*narL*)	PnirQ	—	8	6	8	32	53	24
PAO9104 (Δ*narL*)	PnirQ	*dnr*	11	12	10	5	12	7
PAO1	Panr	—	103	114	105	233	293	322
PAO9104 (Δ*narL*)	Panr	—	81	93	92	191	206	268
PAO1	PnirS	—	12	23	29	470	852	268
PAO9104 (Δ*narL*)	PnirS	—	15	14	15	321	627	331
PAO9104 (Δ*narL*)	PnirS	*dnr*	13	—	—	521	—	—
PAO1	PnorC	—	18	28	25	1053	1642	493
PAO9104 (Δ*narL*)	PnorC	—	22	17	18	480	1627	307
PAO9104 (Δ*narL*)	PnorC	*dnr*	25	—	—	880	—	—

[a] All values are results of three independent experiments performed in triplicate. Bacterial strains were grown aerobically and anaerobically in LB medium supplemented with 10 mM sodium nitrate or 1 mM sodium nitrite as described previously (7). Arginine fermentation conditions were achieved as outlined previously (21). The β-galactosidase activities are given in Miller units and are calculated as described previously (18). Standard deviations for the experiment shown were between 3 and 12%. —, no gene in *trans*.

[b] The *anr* and *dnr* genes were cloned in a vector compatible with the *lacZ* fusion vector pQF50 and cotranscribed where indicated. Vector without an insert served as background control. —, not measured.

[c] Details for the construction of tested reporter gene fusions and *P. aeruginosa* mutants will be provided upon request.

[d] CHA-A2 is not isogenic to PAO1. Due to the clear cut results obtained with this strain, the data obtained are presented.

expression, P*nirQ-lacZ* transcription was found to be dependent on NarL (Table 2 and Fig. 1B). Arai et al. described Anr- and Dnr-dependent *nirQ* expression (4). Since our experiments showed that expression of *dnr* in turn is also dependent on NarL, we investigated whether the observed NarL dependence of the *nirQ* promoter was caused by an indirect effect via a NarL-dependent decrease of Dnr levels. The *nirQ* promoter activities were still found to be low even in the presence of a high Dnr concentration, which confirmed a direct effect of NarL on *nirQ* expression (Fig. 1B). In agreement with these observations, the *nirQ* promoter harbored potential NarL binding sites centered at positions 97 bp and 4 bp upstream of the translational start site. Clearly, Dnr- and NarL-dependent NirQ formation provides the regulatory link between nitrate and nitrite respiration. We did not observe NarL dependence of *anr* expression (Table 2). The nitrite reductase *nirS* gene expression occurred under anaerobic conditions and was induced by nitrate and nitrite (3). Anr mediates this transcriptional activation indirectly via the induction of *dnr* expression in the regulatory cascade, as outlined previously (2, 4). Anaerobic P*nirS-lacZ* induction in the *narL* mutant in the presence of nitrate or nitrite was only about 1.4-fold lower than that in wild-type PAO1 (Table 2). Therefore, the effect of NarL on *nirS* transcription seemed to be of an indirect nature, most likely via the missing induction of *dnr* (Fig. 1B). Consequently, *dnr* expression in *trans* complemented the observed expression phenotype. Additionally, the *narL* mutant, expressing the structural genes for the nitrate reductase at a low level, failed to efficiently convert nitrate into nitrite. However, high nitrite levels are important for *nirS* induction as well. Almost identical results were obtained for the analysis of a P*norC-lacZ* fusion in a *narL* mutant. Transcription of *norC* is also dependent on Anr and Dnr and the presence of nitrite (2). The observed strong nitrite-dependent induction is not completely understood. Either *dnr* transcription is activated via formed N-oxides, or an additional as-yet-unknown system is involved. Obtained results clearly showed that *norC* transcription is not directly regulated by NarL (Table 2 and Fig. 1B). Again, an indirect effect via NarL-dependent *dnr* expression was concluded.

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