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1

2 The *Pseudomonas aeruginosa* phosphodiesterase gene *nbdA* is 3 transcriptionally regulated by RpoS and AmrZ

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5

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9 **Keywords:** *Pseudomonas aeruginosa*, phosphodiesterase, c-di-GMP, nitric oxide, transcription

10 **Abbreviations:** c-di-GMP, bis-3,5-cyclic di-guanosine monophosphate; DGC, diguanylate cyclase;
11 NO, nitric oxide; PDE, phosphodiesterase; RACE, rapid amplification of cDNA ends;

12 ABSTRACT

13 *Pseudomonas aeruginosa* is an opportunistic pathogen causing serious infections in immune
14 compromised persons. These infections are difficult to erase with antibiotics, due to the
15 formation of biofilms. The biofilm lifecycle is regulated by the second messenger molecule c-
16 di-GMP (bis-3,5-cyclic di-guanosine monophosphate). *P. aeruginosa* encodes 40 genes for
17 enzymes presumably involved in the biosynthesis and degradation of c-di-GMP. A tight
18 regulation of expression, subcellular localized function and protein interactions control the
19 activity of these enzymes. In this work we elucidated the transcriptional regulation of the
20 gene encoding the membrane-bound phosphodiesterase NbdA. We previously reported a
21 transcriptional and posttranslational role of nitric oxide (NO) on *nbdA* and its involvement in
22 biofilm dispersal. NO is released from macrophages during infections but can also be
23 produced by *P. aeruginosa* itself during anaerobic denitrification. Recently however,
24 contradictory results about the role of NbdA within NO-induced biofilm dispersal were
25 published. Therefore, the transcriptional regulation of *nbdA* was reevaluated to obtain
26 insights into this discrepancy. Determination of the transcriptional start site of *nbdA* by
27 5'-RACE and subsequent identification of the promoter region revealed a shortened open
28 reading frame (ORF) in contrast to the annotated one. In addition, putative binding sites for
29 RpoS and AmrZ were discovered in the newly defined promoter region. Employing
30 chromosomally integrated transcriptional *lacZ* reporter gene fusions demonstrated a RpoS-
31 dependent activation and AmrZ repression of *nbdA* transcription. In order to investigate the
32 impact of NO on *nbdA* transcription, conditions mimicking exogenous and endogenous NO
33 were applied. While neither exogenous nor endogenous NO had an influence on *nbdA*
34 promoter activity, deletion of the nitrite reductase gene *nirS* strongly increased *nbdA*
35 transcription independently of its enzymatic activity during denitrification. The latter supports
36 a role of NirS in *P. aeruginosa* apart from its enzymatic function.

37 IMPORTANCE

38 The opportunistic pathogen *Pseudomonas aeruginosa* possesses a network of genes
39 encoding proteins for the turnover of the second messenger c-di-GMP involved in regulating-
40 among others-the lifestyle switch between planktonic, motile cells and sessile biofilms.
41 Insight into the transcriptional regulation of these genes is important for the understanding of
42 the protein function within the cell. Determination of the transcriptional start site of the
43 phosphodiesterase gene *nbdA* revealed a new promoter region and consequently a

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shortened open reading frame for the corresponding protein. Binding sites for RpoS and AmrZ were identified *in silico* and confirmed experimentally. Previously reported regulation by nitric oxide was reevaluated and a strong influence of the moonlighting protein NirS identified.

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INTRODUCTION

The opportunistic human pathogen *Pseudomonas aeruginosa* is able to form acute and chronic infections, the latter associated with biofilm formation (1). Within biofilms, bacteria are embedded in a self-produced matrix and are highly protected against the host immune system and antibiotic treatments (2, 3). Therefore, biofilm associated infections are difficult to treat and the *P. aeruginosa* biofilm lifecycle has become a well-studied topic in the last decades. Environmental cues like changes in nutrient availability or the diatomic gas nitric oxide (NO) are able to induce biofilm dispersal by promoting a switch between the sessile and planktonic lifestyle of the bacteria (4-6). In general, the biofilm lifecycle is dependent on the second messenger bis-(3,5)-cyclic diguanosine-monophosphate (c-di-GMP). However, c-di-GMP does not only regulate the biofilm lifecycle, but rather is involved in various bacterial processes e.g., motility, secretion systems, virulence and cell cycle progression (7). The intracellular level of c-di-GMP is dependent on diguanylate cyclases (DGC) that build c-di-GMP from two molecules of GMP and c-di-GMP-specific phosphodiesterases (PDE) that hydrolyze c-di-GMP to either pGpG or GMP (8-12). DGC domains contain a conserved GGDEF motif whereas PDE domains contain either an EAL or HD-GYP motif. Typically, bacteria encode multiple DGC, PDE or tandem enzymes. Adjustment of the intracellular c-di-GMP concentration can be achieved by regulating the production of c-di-GMP modulating proteins on different levels: transcription, post-transcription and post-translation.

On the transcriptional level, control of gene expression by various transcription factors or alternative sigma factors that react to changing environmental or growth conditions allows a temporal separation of redundant PDEs or DGCs within a bacterial cell. For instance, in *E. coli* many DGC or PDE encoding genes are under the control of the alternative sigma factor RpoS (σ^S) which regulates genes for stationary growth phase or stress responses (13). Post-transcriptionally, RNA-binding proteins like CsrA of *E. coli* or RsmA of *P. aeruginosa* are able to influence the translation of DGCs or PDEs by binding to corresponding mRNAs (14, 15). Functional sequestration of DGCs and PDEs within a cell is achieved on the post-translational level. For example, PDEs with EAL-motif often require dimerization to enable c-di-GMP hydrolysis (16). Binding of GTP to the GGDEF domain of the *P. aeruginosa* RbdA enhances PDE activity of the tandem protein (17). Diguanylate cyclases can be object of product feedback inhibition by binding of c-di-GMP to the I-site of the DGC domain (18). Additionally, PDE and DGC domains are often coupled to sensory domains, which allows stimulation of activity in response to environmental signals. Binding of O_2 to the heme co-factor of the *E. coli* DosP for example is required for the proteins PDE activity (19). In the case of PA0575 of *P. aeruginosa*, binding of L-arginine to a sensory Venus flytrap (VFT) domain stimulates c-di-GMP degradation (20). Another possibility to avoid functional redundancies of c-di-GMP modulating proteins is introduced by the "fountain model". It proposes spatial sequestration of particular DCGs and PDEs within a cell and the influence on only a local c-di-GMP pool rather than on the global c-di-GMP concentration (21).

In *P. aeruginosa*, the c-di-GMP modulating network consists of 40 DGC, PDE or tandem proteins which contain both domains (22). One of them is the NO-induced biofilm dispersion locus A (NbdA). NbdA is a three domain protein, consisting of the membrane anchored MHYT domain, a diguanylate cyclase domain with a degenerated GGDEF motif and a phosphodiesterase domain with an EAL motif (23). NbdA was shown to be a functional phosphodiesterase, lacking DGC activity (23). The MHYT domain of NbdA is predicted to be a sensory domain for diatomic gases like oxygen, NO or carbon monoxide (CO) (24). In a previous study we observed that a *nbdA* deletion mutant showed glutamate-induced biofilm dispersal, but was unable to disperse in response to nitric oxide. Additionally, we suggested transcriptional regulation of *nbdA* by NO as *nbdA* transcript levels were increased in NO-treated planktonic cells or dispersed cells after NO-induced dispersal when compared to

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99 untreated planktonic cells (23). In *P. aeruginosa* NbdA is not the only protein involved in NO-
100 induced biofilm dispersal as deletion mutants of the PDEs *rbdA* and *dipA* both display the
101 same phenotype as Δ*nbdA* (25). However, in a biofilm model on airway epithelial cells it was
102 demonstrated that the deletion of neither *nbdA*, *rbdA* nor *dipA* led to a loss of biofilm
103 dispersal in response to NO (26). These contrary findings underline, that c-di-GMP
104 modulating proteins are tightly regulated in *P. aeruginosa* and changes in environmental
105 conditions might impact expression or activity of those enzymes. Therefore, we decided to
106 reevaluate the transcriptional regulation of *nbdA* to obtain insights into this discrepancy and
107 to gain a better understanding of NbdA's role within the c-di-GMP network of *P. aeruginosa*
108 PAO1.

109

110 **MATERIAL AND METHODS**

111 **Bacterial strains and growth conditions**

112 All strains used in this study are listed in Table 1. If not stated otherwise, bacteria were
113 grown in LB medium at 37 °C. For denitrification conditions, growth medium was
114 supplemented with 50 mM KNO_3. Pseudomonas isolation agar was used for selection of
115 *P. aeruginosa* after mating procedures. Antibiotics were used in following concentrations:
116 gentamicin, 10 µg ml^{-1} (*E. coli*); 75 µg ml^{-1} (*P. aeruginosa*); tetracycline, 5 µg ml^{-1} (*E. coli*);
117 100 µg ml^{-1} (*P. aeruginosa*).

118 **Table 1: Strains and plasmids used in this study.**

Strain	Genotype	Reference
E. coli		
DH5α	F^- ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *endA1 hsdR17 deoR gyrA96 thi-1 relA1 supE44*	(27)
S17-I	*recA pro thi hsdR*$^-$*M*$^+$ Tp^r Sm^r; RP4:2 Tc::Mu-Km::Tn7/λpir	(28)
P. aeruginosa		
PAO1	wild type (wt) DSM 22644	DSMZ
wt mini-CTX1-*lacZ*	PAO1 with mini-CTX1-*lacZ* integrated at *attB* site	This work
wt p*nbdA-lacZ*	PAO1 with mini-CTX1-p*nbdA-lacZ* integrated at *attB* site	This work
Δ*rpoS*	*rpoS* deletion mutant in PAO1 background	This work
Δ*rpoS* p*nbdA-lacZ*	Δ*rpoS* with mini-CTX1-p*nbdA-lacZ* integrated at *attB* site	This work
Δ*amrZ*	*amrZ* deletion mutant in PAO1 background	This work
Δ*amrZ* p*nbdA-lacZ*	Δ*amrZ* with mini-CTX1-p*nbdA-lacZ* integrated at *attB* site	This work
Δ*nirS*	*nirS* deletion mutant in PAO1 background	This work
Δ*nirS* p*nbdA-lacZ*	Δ*nirS* with mini-CTX1-p*nbdA-lacZ* integrated at *attB* site	This work
Δ*nirF*	*nirF* deletion mutant in PAO1 background	This work
Δ*nirF* p*nbdA-lacZ*	Δ*nirF* with mini-CTX1-p*nbdA-lacZ* integrated at *attB* site	This work
Δ*norCB*	*norCB* deletion mutant in PAO1 background	This work
Δ*norCB* p*nbdA-lacZ*	Δ*norCB* with mini-CTX1-p*nbdA-lacZ* integrated at *attB* site	This work
Plasmid	**Relevant properties**	
pEXG2	Gm^r, *mob, sacB*	(29)
pEXG2-Δ*rpoS*	truncated *rpoS* with 452 bp up and 439 bp downstream	This work
pEXG2-Δ*amrZ*	truncated *amrZ* with 484 bp up and 485 bp downstream	This work
pEXG2-Δ*nirS*	truncated *nirS* with 448 bp up and 512 bp downstream	This work
pEXG2-Δ*nirF*	truncated *nirF* with 517 bp up and 491 bp downstream	This work
pEXG2-Δ*norCB*	truncated *norCB* with 452 bp up and 439 bp downstream	This work
mini-CTX1-*lacZ*	Tet^r, *lacZ*, FRT site, *attP* site, *int*	(30)
mini-CTX1-p*nbdA-lacZ*	transcriptional fusion of *nbdA* promoter to *lacZ*	This work
pDrive	PCR cloning vector	Qiagen

119

120 **Plasmid and strain construction**

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121 Oligonucleotides used for plasmid construction are listed in Table 2. Markerless deletion
122 mutants were produced as described previously with minor modifications (31). DNA
123 fragments were generated for each deletion via splicing-by-overlap extension (SOE) PCR
124 using the corresponding Up and Down primer pairs (Table 2) and integrated into the allelic
125 exchange vector pEXG2 (29). The vector was transferred from *E. coli* S17-I to *P. aeruginosa*
126 PAO1 via biparental mating. Pseudomonas isolation agar supplemented with gentamicin was
127 used to select cells which integrated the allelic exchange vector by homologous
128 recombination. Those cells were streaked twice on LB medium containing sucrose (15 %
129 w/v) to force the second crossover event. Truncation of target genes was verified via colony
130 PCR and sequencing.

131 Transcriptional *nbdA*-*lacZ* fusion was generated in the vector mini-CTX1-*lacZ* (30). A 171 bp
132 fragment of the *nbdA* promoter region and 279 bp of the coding sequence was amplified via
133 PCR and integrated in front of the promoterless *lacZ* gene encoded on the vector. The
134 transcriptional fusion and the empty vector control were transferred to *P. aeruginosa* strains
135 via biparental mating and chromosomally integrated into the *attB* site on the genome via
136 integrase-mediated chromosomal integration.

137 **Table 2: Primers used in this study.**

Primer	Sequence (5'→3')
deletion mutant construction	
rpoS-Up-F	GCGCCTGCAGCAAGCTCCAGCATCTGGAGCGCTG
rpoS-Up-R	CTCCTGGAGCCCGGCATCGCGCTGAAGCGCCTGCGG
rpoS-Down-F	GATGCCGGGCTCCAGGAGGAG
rpoS-Down-R	GCGCCTCGAGTCGGCCGTTTTGCCTCAAACGGAA
rpoS-seqF	CTAGAATCGCGCGCGCTTAGCCG
rpoS-seqR	TGCTCGGGGCCGTCGTCTGTTC
amrZ-Up-F	GCGCAAGCTTGATGCACCGATCAACGC
amrZ-Up-R	CTCCGCATCGTGTGCGGTAGGAGTTGCCTGTTTCA
amrZ-Down-F	GCACACGATGCGGAG
amrZ-Down-R	GCGCGAATTCTCAGGTTGACCAGCAGAAC
amrZ-seqF	ACCCAGCACGTCGAT
amrZ-seqR	GGAATGACTCCGGGCT
nirS-Up-F	GCGCCTGCAGAAGAGGACAGGCGAACGTCAGCGC
nirS-Up-R	GGCACCTTGCTCGCCTCGCGGCTGATCACCCCGACCGGTAAG
nirS-Down-F	CGAGGCGAGCAAGGTGCCCAC
nirS-Down-R	GCGCCTCGAGCCAGGTAGCAGATACCGCCTTCGCG
nirS-seqF	TGAGGAGAAGCGGCGCGAGGGGA
nirS-seqR	GTAGTCGAAGCGCAACGCGACGAAACG
nirF-Up-F	GCGCCTGCAGCAGGTAGCGCAGGTGTTGCCG
nirF-Up-R	AGCCAGCAGCCGCCCTTGCCGAGCGGCATCTTCTTCAGCCAC
nirF-Down-F	CAAGGGCGGCTGCTGGCTACA
nirF-Down-R	GCGCCTCGAGCGCCGAAGCGGAACTCGCG
nirF-seqF	ATGGCCACATCGGCAGGCGAC
nirF-seqR	GCTCCCCCCTACGAGGAACCGTG
norCB-Up-F	GCGCAAGCTTTGCTGGCGCCGGTGTTATACGC
norCB-Up-R	TAGGCGACCAGGCCGATGAGCCCGCCGAAATAGATGTTCCTGGCC
norCB-Down-F	CTCATCGGCCTGGTCGCCTACC
norCB-Down-R	GGGGAATTCATTTCCAGTTCGGCGTCTGCCGC
norCB-seqF	TCATCGGCGACGGCATGGACC
norCB-seqR	ATCGGTTGCAGCAGCACGTGG
pEXG2-seqF	CGACCTCATTCTATTAGACTCTCGTTTGGATTGC
pEXG2-seqR	GTTCGCTCGCGTATCGGTGATTCATTCTG
p*nbdA*-*lacZ* fusion	
p*nbdA*-lacZ-F	GCGCGAATTCATGCCTTTTCTCCCCGGGAAAATGC
p*nbdA*-lacZ-R	GCGCGGATCCCAGGTCGTAGCGCAGGGCGA
lacZ-rev	GGATTTCCTTACGCGAAATACGGG
seq3miniCTX*lacZ*	ATCCACCGGCGCGCGTAATACG
RT-PCR	
nbdA-RT1-F	TTCCTCGACCTCGATCACTT
nbdA-RT1-R	TGGGTTCCTTCATCTTCTGC
recA-RT1-F	GACCGAGGCGTAGAACTTCA
recA-RT1-R	CAACTGCCTGGTCATCTTCA
5'-RACE primer	
SP1_*nbdA*:	ATGGCTGGTAGTACTGGGTGGC

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SP3_*Bgl*II_*nbdA*	TAAGATCTGGCGAATAGGTGCAGTTCTG
Oligo (dT) anchor primer	GACCAGATCTATCGATGTCGACTTTTTTTTTTTTTTTTTV*
anchor-1 primer	GACCAGATCTATCGATGTCGACT

138 * V= A,C or G

139 **β-Galactosidase assay**

140 For measurements of promoter activity the β-galactosidase assay protocol of Miller (32) was
141 modified as follows. Overnight cultures containing the promoter-*lacZ* fusion were diluted to
142 an OD_{600} of 0.01 in 20 ml LB with respective antibiotics. In exponential (4 h) and early
143 stationary growth phase (7 h) OD_{600} was measured and cells of 100 µl culture were harvested
144 by centrifugation. Cells were resuspended in 800 µl Z-buffer (60 mM Na_2HPO_4 x $7H_2O$;
145 40 mM NaH_2PO_4 x H_2O; 10 mM KCl; 1 mM $MgSO_4$ x $7H_2O$; 50 mM β-mercaptoethanol). To
146 permeabilize the cells 25 µl of 0.1 % (w/v) sodium-dodecyl sulfate (SDS) and 25 µl
147 chloroform were added. After 5 min incubation 200 µl of 4 mg/ml *ortho*-nitrophenyl-β-D-
148 galactosid (ONPG) were added to the mixture at 37 °C to start the reaction. When
149 β-galactosidase activity was indicated by a color change due to the formation of the yellow
150 colored product *ortho*-nitrophenol, the reaction was stopped by addition of 500 µl 1 M
151 Na_2CO_3. Cell debris was precipitated by centrifugation and product formation was measured
152 in the supernatant at OD_{420}. The activity of β-galactosidase was calculated as follows: Miller
153 units (MU) = (OD_{420} / (OD_{600} * volume * incubation time)) * 1000.

154 **RNA extraction and semi quantitative RT-PCR**

155 Bacteria were grown to exponential and early stationary phase as described above. To
156 prevent RNA degradation, cells of an equivalent of OD_{600} = 1 in 1 ml were mixed with 100 µl
157 RNA stop solution (5 % (v/v) phenol in ethanol). Cell pellets were stored at -80 °C or used
158 directly for RNA extraction. RNA was extracted by enzymatic lysis according to Qiagen RNA
159 protect handbook and further purified with the RNeasy Plus Mini kit (Qiagen) following the
160 suppliers instruction. Extracted RNA was transcribed into cDNA using ProtoScript II Reverse
161 Transcriptase (New England Biolabs) according to the manufacturers' protocol. 2.5 ng of
162 cDNA were used as template for 25 µl semi quantitative RT-PCR reaction. Used primers are
163 listed in Table 2.

164 **Determination of transcriptional start sites by 5'-RACE**

165 *P. aeruginosa* cells were inoculated 1:100 from an overnight culture in LB medium and
166 incubated 5 h at 37 °C. Total RNA isolation was performed as previously described (33).
167 Primers used for 5'-RACE are listed in Table 2. cDNA was synthesized at 42 °C for 60 min
168 with M-MLV-RT (Promega) using gene-specific primer SP1_*nbdA.* The cDNA was treated
169 with shrimp alkaline phosphatase (New England Biolabs) and purified with MinElute kit
170 (Qiagen). A deoxyadenosine tail was added to the 3' end of the cDNA using terminal
171 transferase (Thermo Fisher Scientific). Second-strand synthesis was performed with an oligo
172 (dT) anchor primer. The obtained double-stranded DNA was amplified with the anchor-1
173 primer and nested gene-specific primer SP3_*Bgl*II *nbdA*. The resulting PCR product was
174 purified (MinElute kit, Qiagen) and cloned in pDrive using the Qiagen PCR cloning kit
175 (Qiagen). For the determination of the transcriptional start site 10 individual clones were
176 sequenced.

177

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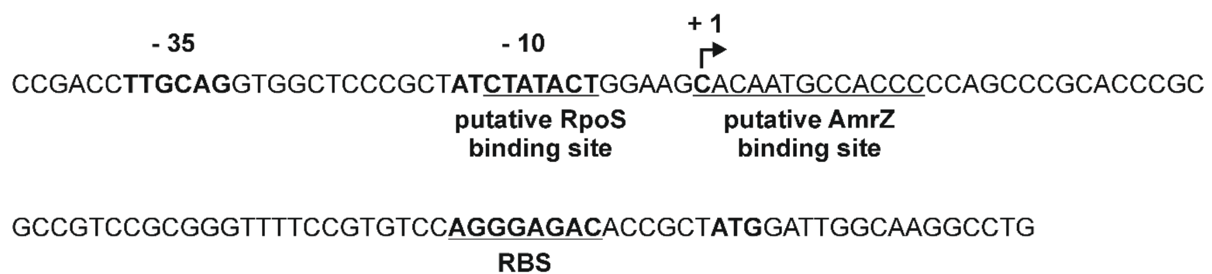
178 **RESULTS**

179 **Determination of transcriptional start site of *nbdA* reveals a regulatory region with**
180 **RpoS and AmrZ binding sites**

181 Automated annotation of the genome sequence of PAO1 predicted the open reading frame
182 (ORF) for *nbdA* to code for 783 amino acid residues. Sequence alignments of the translated
183 sequence with proteins containing N-terminal MHYT-domains revealed a long N-terminal
184 extended region for NbdA. This incited us to analyze the gene region in more detail. In close
185 proximity to the annotated start codon no ribosomal binding site (RBS) could be detected. A
186 5'-RACE PCR experiment revealed the transcriptional start site of *nbdA* 103 nucleotides
187 downstream of the computationally annotated translation start (Fig. 1). The nearest potential
188 translational start site is 170 nucleotides downstream of the previously annotated translation
189 start and possesses a bona fide ribosome binding site. This results in a shorter ORF coding
190 for a 726 amino acid protein whose N-terminus aligns well with the N-termini of other MHYT-
191 domain proteins. With the new defined transcriptional start, the *nbdA* promoter region was
192 analyzed. Conserved binding sites for the alternative sigma factor RpoS (σ^S) and the
193 transcription factor AmrZ were identified (34, 35).

194



195

196 **Fig. 1:** Reannotation of the promoter region of *nbdA* with the experimentally determined transcriptional start site. 5'-RACE PCR
197 experiments revealed transcriptional start site of the *nbdA* gene with a C (+1), 171 nucleotides downstream from the previously
198 annotated ORF start site (Pseudomonas database (36)). The promoter region of *nbdA* was reanalyzed and reveals deduced
199 binding sequences for the alternative σ-factor RpoS and the transcriptional regulator AmrZ.

200

201 The binding motif for RpoS lies in the -10 region of the *nbdA* promoter. The predicted AmrZ
202 binding sequence covers the transcriptional start site of *nbdA* indicating a repressor function.
203 In order to investigate the role of the alternative sigma factor RpoS and the transcription
204 factor AmrZ on *nbdA* expression, the promoter region of *nbdA* was transcriptionally fused to
205 the reporter gene *lacZ* and integrated in the φCTX attachment site of PAO1 wt, Δ*rpoS* and
206 Δ*amrZ*. Activity of the β-galactosidase in the respective strains was determined in
207 exponential (4 h) and early stationary (7 h) growth phase. In the wt strain, a 4-fold increase in
208 *nbdA* transcription was observed when cells entered the early stationary phase, which
209 suggests transcriptional activation by RpoS. Deletion of *rpoS* resulted in a loss of *nbdA*
210 promoter activity in both, exponential and stationary growth phase (Fig. 2), confirming the
211 role of RpoS as transcriptional activator of *nbdA*. In the Δ*amrZ* strain a strong increase of
212 *nbdA* promoter activity was observed in both, exponential and early stationary growth phase
213 (Fig. 2). AmrZ is therefore likely acting as a transcriptional repressor for *nbdA*.

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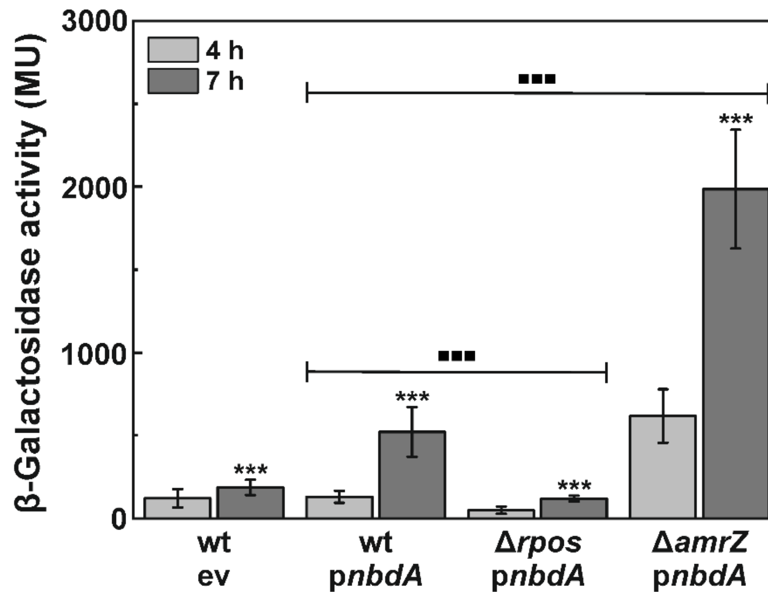

214

215 **Fig. 2:** Transcription of *nbdA* is activated by RpoS and repressed by AmrZ. Promoter activity was determined with integrated
216 p*nbdA*-*lacZ* fusions in late exponential (4 h) and early stationary (7 h) growth phase in wild type (wt) and deletion mutants Δ*rpoS*
217 and Δ*amrZ*. Additionally, the wt with integrated empty vector (ev) was tested for background β-galactosidase activity. The assay
218 was performed in triplicates. Significant changes between 4 and 7 h samples are marked with *. Significant changes in *nbdA*
219 expression levels between deletion mutant strains and the corresponding wt sample are marked with ■ (*** $P < 0.001$,
220 determined by Student´s T-test).

221

222 As there is a sharp oxygen gradient present in biofilm macrocolonies (37), O_2 might also
223 have an impact on the expression of genes active in biofilms. Although there is no hint for an
224 FNR-like, ANR, or DNR regulator binding site in the promoter region, we tested *nbdA*
225 promoter activity also under anaerobic conditions. Induction of the *nbdA* promoter was
226 observed when cultures reached stationary phase in all tested strains, similarly to the aerobic
227 growth conditions (Fig. 3). Overall, the values for promoter activity under oxygen limitation
228 were significantly lower than in aerobic conditions. The *nbdA* promoter activity in the *amrZ*
229 deletion strain was significantly increased compared to the wild-type background. Therefore,
230 AmrZ seems to repress *nbdA* transcription similarly in aerobic and anaerobic growth
231 conditions.

232

233

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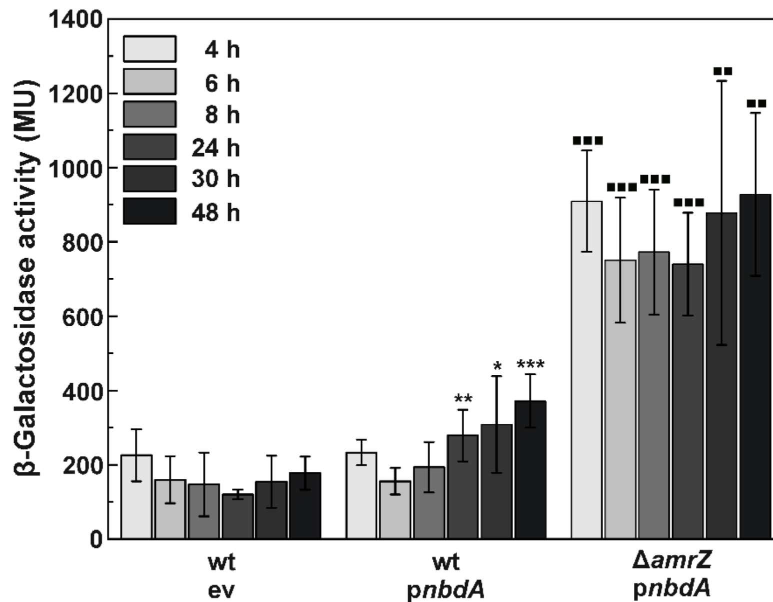

234

235 **Fig. 3:** Under oxygen limitation, transcription levels of *nbdA* are induced in stationary growth phase. PAO1 wt and Δ*amrZ*
236 containing an integrated p*nbdA*-*lacZ* fusion and the empty vector control (ev) were grown anaerobically for 48 h in LB medium
237 with 50 mM $NaNO_3$. Inoculation of cultures was performed aerobically therefore the first hours of growth were required to
238 consume remaining oxygen. Expression levels were determined by β-galactosidase assays in triplicates. Significant changes in
239 *nbdA* transcription levels of wt samples to the 6 h sample are marked with *. Significant changes in *nbdA* transcription level of
240 Δ*amrZ* samples to corresponding wt levels are marked with ■. (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, determined by Student's T-
241 test).

242

243 Impact of nitric oxide on the transcription of *nbdA*

244 We previously reported increased amounts of *nbdA* transcript in dispersed cells after NO-
245 induced biofilm dispersal compared to untreated planktonic cells and suggested a NO-
246 dependent transcriptional regulation of *nbdA* (23). In the light of divergent results on the role
247 of *nbdA* in NO-induced biofilm dispersion (23, 26) we wanted to clarify the regulation of *nbdA*
248 in response to endogenous and exogenous NO. During infections, host macrophages
249 release exogenous NO in order to eradicate bacteria (38). However, under anaerobic
250 conditions, *P. aeruginosa* is able to form endogenous NO during denitrification. Within the
251 denitrification process, nitrite is reduced by the nitrite reductase NirS into NO, which is then
252 further reduced to nitrous oxide by NorCB (39, 40). For its enzymatic activity, NirS requires
253 the incorporation of a heme d_1 cofactor which is synthesized by NirF (41). Interruption of the
254 denitrification pathway by deletion of the *norCB* gene leads to the accumulation of intrinsic
255 NO under denitrifying conditions (4). A Δ*norCB* strain containing the *nbdA* promoter *lacZ*-
256 fusion was used to analyze the effect of endogenous NO on *nbdA* transcription. Deletion
257 mutants of *nirS* and *nirF*, both unable to form endogenous NO, served as negative controls.
258 The denitrification deficient strains showed normal growth under aerobic conditions in LB
259 medium complemented with KNO_3 (Fig. 4A). In contrast, under anaerobic denitrifying
260 conditions the growth of PAO1 Δ*nirS* and Δ*nirF* was reduced compared to the wt PAO1 (Fig.
261 4B). The Δ*norCB* strain was no longer able to grow. For the analysis of *nbdA* transcription,
262 the strains containing the *nbdA* promoter *lacZ*-fusion were grown under aerobic/microaerobic
263 conditions and ß-galactosidase assays were performed with samples of the exponential (4 h)
264 and early stationary growth phase (7 h) (Fig. 4C). Compared to the wt, the *nirS* deletion had
265 a severe activating effect on *nbdA* expression in both, exponential and early stationary
266 growth phase. Surprisingly, *nbdA* transcription in the Δ*nirF* strain, which produces an
267 enzymatically inactive NirS, was not as high as in the Δ*nirS* strain but comparable to the level
268 of transcription in the wt background. The transcription of *nbdA* in the Δ*norCB* strain is

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269 slightly decreased compared to the wt background. Due to the impaired growth in anaerobic
270 conditions of Δ*norCB* strain, we could not test for the effect of accumulation of endogenous
271 NO on *nbdA* transcription. In order to confirm the findings for the Δ*nirS* strain, a semi
272 quantitative RT-PCR experiment was performed with cDNA of wt and deletion mutant in both
273 tested growth phases (Fig. 4D). While the control PCR with *recA* primers showed equally
274 strong bands for all samples, *nbdA* expression in the wt in exponential growth phase was
275 weaker than in early stationary growth phase. In the *nirS* deletion strain, there was more
276 transcript of *nbdA* detectable than in wt, which is consistent to the findings of the β-
277 galactosidase assay.

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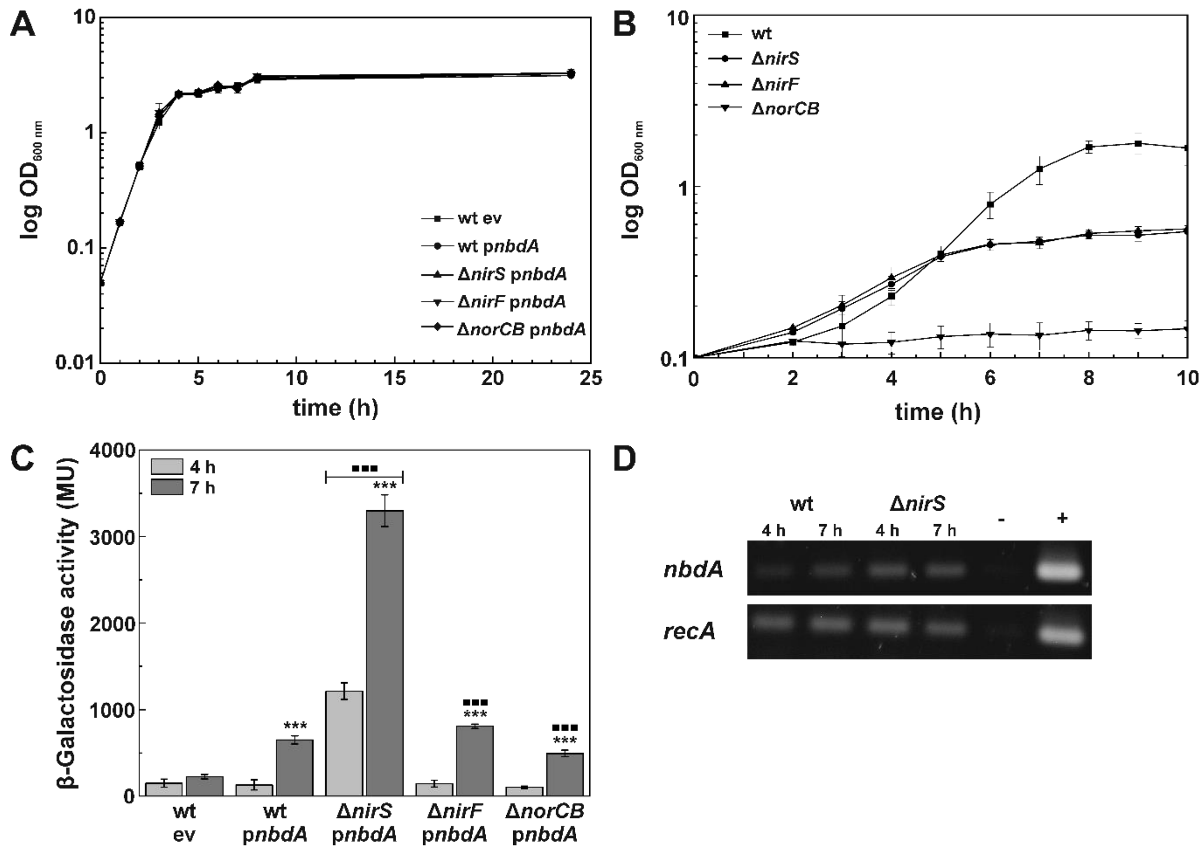

281 **Fig. 4:** *P. aeruginosa* wild type (wt), Δ*nirS*, Δ*nirF*, and Δ*norCB* strains harboring a transcriptional p*nbdA lacZ*-fusion and the wt
282 containing the "empty vector" control (ev) were grown in LB supplemented with 50 mM KNO_3. [**A**] Growth in glass culture flasks
283 was monitored during 24 h in three biological replicates. [**B**] Anaerobic growth in LB medium supplemented with 50 mM KNO_3 of
284 the wt and deletion mutants was analyzed in sealed bottles for 10 h in biological triplicates. [**C**] β-galactosidase activity was
285 determined from cells aerobically/ microaerobically grown to exponential (4 h) and early stationary phase (7 h). All assays were
286 performed in triplicates. Significant changes between 4 and 7 h samples are marked with *. Significant changes in *nbdA*
287 expression levels between deletion mutant strains and the corresponding wt sample are marked with ■. (*** $P < 0.001$,
288 determined by Student's T-test). [**D**] Semi quantitative RT-PCR for *nbdA* transcript and the control *recA* was performed. RNA
289 was extracted from wt and Δ*nirS* strains after 4 and 7 h growth in LB medium.

290 In addition to the influence of intrinsic nitric oxide on *nbdA* transcription, the effect of
291 exogenous NO was investigated. Therefore, the PAO1 wt harboring the *nbdA-lacZ* fusion
292 was grown with increasing amounts of the NO donor sodium nitroprusside (SNP) and *nbdA*
293 promoter activity was determined by β-galactosidase activity (Fig. 5A). Low concentration of
294 added SNP to the growth medium had no effect on *nbdA* promoter activity, whereas the
295 addition of 500 µM SNP led to a decrease of *nbdA* transcription. This effect is comparable to
296 the observed decrease of *ndbA* transcription in the NO-accumulating strain Δ*norCB*. As
297 *P. aeruginosa* is able to detoxify nitric oxide via flavohemoglobin (42, 43) under aerobic
298 conditions, the influence of short-term NO stress on *nbdA* transcription was analyzed.

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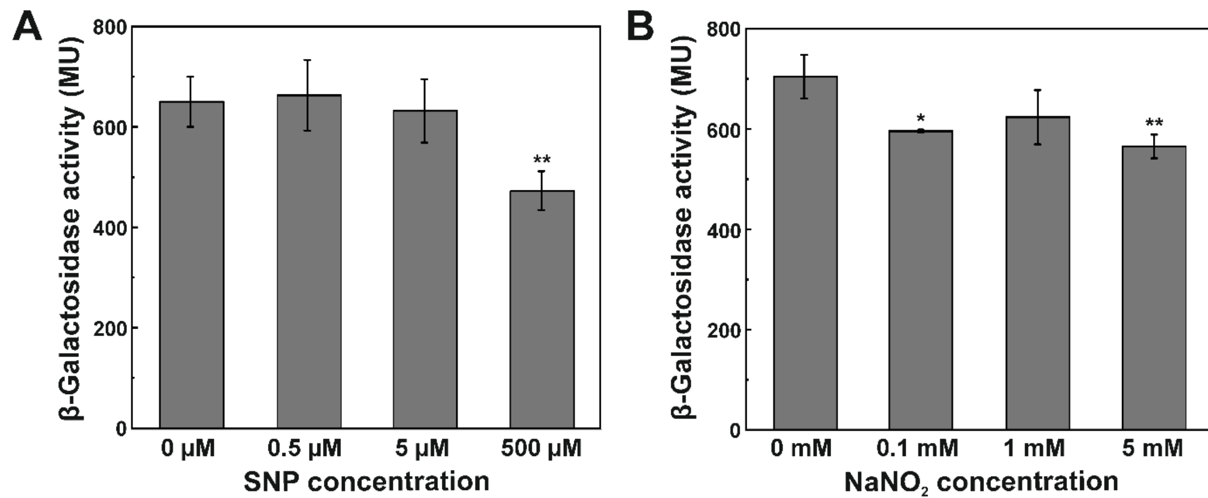
299 Therefore, PAO1 wt *nbdA-lacZ* was grown to stationary phase in LB and then stressed for 30
300 min by the addition of 500 µM SNP. Compared to the untreated control, no changes in the
301 *nbdA* promoter activity were observed (data not shown).

302



303

304 **Fig. 5:** Analysis of transcriptional activity of *nbdA* promoter in response to exogenic NO and NO_2 sources. Reporter strain PAO1
305 wt::p*nbdA-lacZ* was grown to early stationary phase (7 h) in medium containing increasing amounts of the NO-releasing
306 compound sodium nitroprusside (SNP) [**A**] or $NaNO_2$ [**B**]. β-galactosidase activity was measured in triplicates (* $P < 0.05$; ** $P <$
307 0.01, determined by Student's T-test).

308 In order to figure out whether the strong increase of *nbdA* expression in the Δ*nirS* strain was
309 based on nitrite accumulation due to interrupted denitrification (4), β-galactosidase assays
310 were performed with different amounts of nitrite in the growth medium (Fig. 5B). None of the
311 tested nitrite concentrations had a comparable effect on the *nbdA* promoter as the deletion of
312 *nirS*. The addition of nitrite to the medium rather decreased expression of *nbdA* slightly,
313 probably due to bacteriostatic effect of nitrite.

314

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316 **DISCUSSION**

317 In this study we analyzed the transcriptional regulation of *nbdA* coding for the
318 phosphodiesterase NbdA, involved in the c-di-GMP modulating network in *P. aeruginosa*.
319 Determination of the transcription initiation site of *nbdA* by 5'-RACE revealed an erroneous
320 annotation of the ORF in the databases. A new promoter region was identified, containing
321 putative binding sites for RpoS and AmrZ. Gene expression of *nbdA* was shown to be
322 activated in stationary growth phase by the alternative sigma factor RpoS (σ^S). A further level
323 of regulation is introduced through the repression by the ribbon-helix-helix transcription factor
324 AmrZ. Oxygen limitation, supplementation with nitrite, and endogenous or exogenous nitric
325 oxide did not affect the transcription of *nbdA.* Surprisingly, deletion of the nitrite reductase
326 NirS showed a strong activating effect on *nbdA* transcription, while a strain with an
327 enzymatically inactive NirS (Δ*nirF*) showed no transcriptional changes.

328 The sigma factor RpoS is known as the master regulator of gene expression during
329 stationary growth phase. Furthermore, it is responsible for the activation of genes in
330 response to different stresses, e.g. starvation, heat, oxygen or osmotic stress (44-46). In
331 some Proteobacteria, RpoS is additionally involved in regulation of virulence genes, quorum
332 sensing and motility (47-50). In *E. coli*, RpoS has been shown to play an important role in
333 biofilm maturation, architecture and density (51-53). This link is partly due to the involvement
334 of RpoS in the c-di-GMP regulatory network of *E. coli*. In the *E. coli* K12 strains MC4100 and
335 W3110 a great subset of GGDEF/EAL-domain encoding genes was identified to be under
336 control of RpoS (54, 55). Similarly, in *Pseudomonas* sp. several genes related to biofilm
337 formation, maturation and architecture were shown to be regulated by RpoS (56, 57). A
338 global analysis of *P. aeruginosa* PA14 revealed that 30 out of 40 genes encoding for c-di-
339 GMP modulating enzymes, are either transcriptionally activated or repressed by RpoS (Table
340 3 and references therein). Thus, similar to *E. coli*, RpoS-dependent regulation significantly
341 affects the c-di-GMP network of *P. aeruginosa*. RpoS regulated genes are often subject to
342 further regulatory mechanisms. Activator or repressor proteins might be involved, as well as
343 post-transcriptional regulation. The *psl* operon coding for matrix polysaccharide biosynthesis
344 genes in *P. aeruginosa* is controlled transcriptionally by RpoS and post-transcriptionally by
345 RsmA (58). In *P. putida* KT2440 the exopolysaccharide cluster *pea* is activated by RpoS and
346 repressed by AmrZ (59). Actually, when we evaluated and compared the data of the PA14
347 RpoS regulon (35) and the PAO1 AmrZ regulon (34) we found 18 out of 40 genes encoding
348 for c-di-GMP modulating enzymes in *P. aeruginosa* presumably regulated by both proteins,
349 RpoS and AmrZ (Table 3, (34, 35)).

350 The transcriptional regulator AmrZ controls a large regulon containing 398 gene regions in
351 PAO1. Transcription of *amrZ* itself is in a great extend dependent on the alternative sigma
352 factor AlgT (σ^{22}) which is known to regulate coversion to mucoidity and stress responses in
353 *P. aeruginosa* (60, 61). AmrZ was shown to regulate genes important for *P. aeruginosa*
354 virulence, including type IV pili, extracellular polysaccharides, and the flagellum (34). It
355 particularly influences genes required for alginate production and twitching-motility (34, 62-
356 64). Within the c-di-GMP network of *P. aeruginosa*, AmrZ activates transcription of 14 genes
357 and represses 10 genes encoding GGDEF/EAL-domain proteins ((34), Table 3). With these
358 numbers, AmrZ appears to be one of the major regulators for genes coding for c-di-GMP
359 modulating enzymes in *P. aeruginosa*, possibly affecting the cellular c-di-GMP level. This
360 role for AmrZ was previously also observed in *P. fluorescens* F113, where the cellular c-di-
361 GMP level was affected by AmrZ through the regulation of a complex network of genes
362 encoding DGCs and PDEs (65). From our work we conclude that *nbdA* transcription is
363 repressed by AmrZ during aerobic as well as anaerobic planktonic growth while a condition
364 in which the *nbdA* promoter is de-repressed remains uncertain. Repression through AmrZ is
365 described to be dependent on the C-terminus mediated tetramerization of the protein (66). In

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366 some cases, e.g. *pilA* repression, the expression level of AmrZ plays an important role for its
367 function, as binding efficiency of AmrZ to different promoter regions differs (64). Additionally,
368 a competition of the activator RpoS and the repressor AmrZ upon binding to the *nbdA*
369 promoter might be possible.

370 **Table 3: Genes coding for c-di-GMP modulating proteins in PAO1 and PA14 and their association to RpoS or AmrZ**
371 **regulon. Data extracted from Schulz 2015, and Jones 2014.**

372 RpoS regulon was analyzed in *P. aeruginosa* PA14 via mRNA profiling of Δ*rpoS* vs. wt (35). AmrZ regulon was measured via
373 RNA-Seq experiments in a PAO1 *amrZ* complementation strain vs. Δ*amrZ* (34). GGDEF: diguanylate cyclase domain, EAL:
374 phosphodiesterase domain, GGDEF-EAL: tandem diguanylate cyclase – phosphodiesterase domain, HD-GYP
375 phosphodiesterase domain.
376 + activation; - repression; none: is not part of the indicated regulon.

PAO1 gene locus	PA14 gene locus	name	domain(s)	RpoS regulation	AmrZ regulation
PA0169	PA14_02110	*siaD*	GGDEF	-	-
PA0285	PA14_03720		GGDEF-EAL	none	-
PA0290	PA14_03790		GGDEF	none	none
PA0338	PA14_04420		GGDEF	-	-
PA0575	PA14_07500	*rmcA*	GGDEF-EAL	+	none
PA0847	PA14_53310		GGDEF	+	none
PA0861	PA14_53140	*rbdA*	GGDEF-EAL	+	none
PA1107	PA14_50060	*roeA*	GGDEF	+	-
PA1120	PA14_49890	*tpbB*	GGDEF	+	none
PA1181	PA14_49160		GGDEF-EAL	+	+
PA1433	PA14_45930		GGDEF-EAL	+	none
PA1727	PA14_42220	*mucR*	GGDEF-EAL	+	-
PA1851	PA14_40570		GGDEF	+	none
PA2072	PA14_37690		GGDEF-EAL	+	+
PA2133	PA14_36990		EAL	+	none
PA2200	PA14_36260		EAL	+	none
PA2567	PA14_31330		GGDEF-EAL	none	-
PA2572	PA14_30830		HD-GYP	+	+
PA2870	PA14_26970		GGDEF	+	+
PA3177	PA14_23130		GGDEF	none	+
PA3258	PA14_21870		GGDEF-EAL	none	none
PA3311	**PA14_21190**	***nbdA***	**GGDEF-EAL**	**+**	**-**
PA3343	PA14_20820	*hsbD*	GGDEF	+	-
PA3702	PA14_16500	*wspR*	GGDEF	+	+
PA3825	PA14_14530		EAL	+	+
PA3947	PA14_12810	*rocR*	EAL	+	none
PA4108	PA14_10820		HD-GYP	+	none
PA4332	PA14_56280	*sadC*	GGDEF	none	-
PA4367	PA14_56790	*bifA*	GGDEF-EAL	+	-
PA4396	PA14_57140		GGDEF	none	none
PA4601	PA14_60870	*morA*	GGDEF-EAL	+	-
PA4781	PA14_63210		HD-GYP	+	+
PA4843	PA14_64050	*gcbA*	GGDEF	none	+
PA4929	PA14_65090		GGDEF	+	-
PA4959	PA14_65540	*fimX*	GGDEF-EAL	+	+
PA5017	PA14_66320	*dipA*	GGDEF-EAL	none	-
PA5295	PA14_69900	*proE*	GGDEF-EAL	+	none
PA5442	PA14_71850		GGDEF-EAL	-	-
PA5487	PA14_72420	*dgcH*	GGDEF	+	none
*	PA14_59790	*pvrR*	EAL	none	none

377 * gene not present in PAO1

378

379 Effects of endogenous or exogenous nitric oxide on *nbdA* expression

380 In our previous study we observed elevated transcription levels of *nbdA* in cells dispersed
381 from biofilms after NO-treatment when compared to planktonically grown cells in RT-qPCR
382 experiments (23). Therefore, we suggested transcriptional regulation of *nbdA* by NO in this
383 biofilm model. For *P. aeruginosa* two NO-responsive transcriptional regulators, FhpR and
384 DNR are described. DNR is a heme-containing CRP/FNR type regulator that specifically
385 activates denitrification genes under anaerobic conditions (67, 68). Transcription of the

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386 second NO responsive regulator in *P. aeruginosa*, FhpR, is σ^{54}-dependent and activates
387 flavohemoglobin expression under aerobic conditions for the detoxification of NO in the cell
388 (42, 43). When analyzing the reannotated *nbdA* gene and promoter region, no similarity with
389 either the FhpR or DNR consensus binding site was detected (69). Therefore, a direct
390 influence of NO on *nbdA* transcription was unlikely. These findings in addition to the
391 contradictory results in the literature concerning the involvement of NbdA in NO-induced
392 biofilm dispersal of *P. aeruginosa* (23, 26) led to the reevaluation of the transcriptional
393 regulation of *nbdA* by NO. In this study, no direct stimulation of *nbdA* promoter activity by
394 NO, neither by addition of exogenous NO nor by accumulation of intrinsic NO in
395 planktonically grown cells was observed. The previously observed induction of *nbdA*
396 expression in our qRT-PCR experiments (23) might be due to more complex regulatory
397 processes during biofilm formation and dispersal. From the present data, we conclude that
398 *nbdA* expression in planktonic cells is not directly induced by NO at the transcriptional level.

399 **Effect of the nitrite reductase NirS on *nbdA* promoter activity**

400 In this study, we observed a strong increase in the *nbdA* transcription level when the nitrite
401 reductase NirS was deleted. At first, we assumed that the upregulation of *nbdA* expression
402 might be due to accumulation of intrinsic nitrite from interrupted denitrification. However,
403 addition of nitrite to the growth medium did not change *nbdA* promoter activity. Further, the
404 Δ*nirF* strain producing an enzymatically inactive NirS protein (41) did not enhance *nbdA*
405 transcription. Therefore, we suggest that the presence of the periplasmic protein NirS affects
406 *nbdA* transcription independently of its enzymatic activity. The moonlighting role of NirS was
407 previously described for the type III secretion system in *P. aeruginosa* (70). Additionally, NirS
408 was shown to affect flagellum biogenesis by the formation of a complex with the flagellar
409 subunit FliC and the chaperone DnaK (4, 60, 71). Suggesting this complex role for NirS
410 besides denitrification in *P. aeruginosa*, the increase of *nbdA* promoter activity in the Δ*nirS*
411 strain is probably derived from a global regulatory change in the cell.
412 All in all, we were able to reannotate the *nbdA* gene and revealed consensus sequences for
413 the alternative sigma factor RpoS and the transcription factor AmrZ within the *nbdA* promoter
414 region. Our data confirmed RpoS as activator and AmrZ as repressor for *nbdA* transcription,
415 however, no transcriptional regulation by endogenous or exogenous NO or nitrite was
416 observed in planktonically grown cells.
417

418 **AUTHORS STATEMENTS**

419 **Authors and contributors**

420 KG, SZ, NFD conceived the study, KG and SZ performed experiments, KG and SZ analyzed
421 the data, KG wrote first draft of manuscript, all authors revised and approved the final version
422 of the manuscript.

423 **Conflict of interests**

424 The authors declare no conflict of interest.

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