NO-Induced Biofilm Dispersion in *Pseudomonas aeruginosa* Is Mediated by an MHYT Domain-Coupled Phosphodiesterase

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Dispersion is a process used by bacteria to successfully transit from a biofilm to a planktonic growth state and to spawn novel communities in new locales. Alterations in *bis*-(3'-5')-cyclic dimeric GMP (c-di-GMP) levels have been shown to be associated with biofilm dispersal in a number of different bacteria. The signaling molecule nitric oxide (NO) is known to induce biofilm dispersion through stimulation of c-di-GMP-degrading phosphodiesterase (PDE) activity. However, no c-di-GMP modulating enzyme directly involved in NO-induced dispersion has yet been described in the opportunistic pathogen *Pseudomonas aeruginosa*. Here, we characterized MucR (PA1727) and NbdA (PA3311, NO-induced biofilm dispersion locus A), two membrane-bound proteins with identical domain organization consisting of MHYT-GGDEF-EAL, with respect to their role in NO-induced dispersion. Inactivation of *mucR* impaired biofilm dispersion in response to NO and glutamate, whereas inactivation of *nbdA* only impaired biofilm dispersion upon exposure to NO. A specific role of NbdA in NO-induced dispersion was supported by increased PDE activity, resulting in decreased c-di-GMP levels in biofilms expressing *nbdA* upon exposure to NO, a response that was absent in the $\Delta nbdA$ strain. Moreover, increased PDE activity was mainly due to a transcriptional activation of *nbdA* upon addition of NO. Biochemical analyses of recombinant protein variants lacking the membrane-anchored MHYT domain support NbdA being an active PDE. In contrast, MucR displayed both diguanylate cyclase and PDE activity *in vitro*, which seemed regulated in a growth-dependent manner *in vivo*. This is the first description of a PDE specifically involved in NO-induced biofilm dispersion in *P. aeruginosa*.

Chronic *Pseudomonas aeruginosa* infections are characterized by the production of mucoid alginate and the formation of biofilms. These surface-associated communities of bacteria are embedded in an extracellular matrix of their own synthesis (1). Biofilm formation is a well-regulated process and occurs in stages (2, 3). Dispersion may occur during all stages of biofilm development, enabling cells leaving the biofilm to colonize new surfaces. Biofilm dispersion can be triggered by a variety of environmental cues, including nutrient availability (e.g., glutamate), heavy metals, or nitric oxide (NO) (4–6). A key player in the inverse regulation of biofilm formation and motility is the second messenger *bis*-(3'-5')-cyclic dimeric GMP (c-di-GMP). High levels of c-di-GMP promote biofilm formation and inhibit motility, whereas low levels of c-di-GMP support a planktonic lifestyle (7). Cellular levels of c-di-GMP are controlled by the opposing activity of two enzymes: diguanylate cyclases (DGC) containing a characteristic GGDEF domain and phosphodiesterases (PDE) harboring EAL or HD-GYP domains. Previous findings suggested NO-induced dispersion of *P. aeruginosa* biofilms to coincide with increased PDE activity, resulting in decreased intracellular c-di-GMP levels (4, 8). However, no protein that either directly senses or responds to NO and thereby controlling c-di-GMP turnover has been identified, raising the question of how *P. aeruginosa* senses or responds to NO. Proteins harboring heme nitric oxide/oxygen binding (H-NOX) domains have been implicated in other bacteria, including *Shewanella woodyi* and *S. oneidensis* (9, 10), to play a role in NO sensing and modulation of intracellular c-di-GMP levels. However, *P. aeruginosa* does not encode H-NOX proteins. Instead, the genome encodes two proteins containing an MHYT domain. The fairly unknown MHYT domain is a transmembrane domain of seven predicted membrane-spanning helices and proposed to possess putative sensory function for diatomic gases such as oxygen, carbon monoxide, or NO through protein-bound copper

ions (11). Highly conserved methionine and histidine residues located near the outer face of the protein are supposed to coordinate copper ions (11). In addition to harboring the MHYT domain, both proteins possess cytoplasmic GGDEF and EAL domains, suggesting a role in c-di-GMP turnover.

We investigated here the role of the membrane-anchored proteins MucR (PA1727) and NbdA (NO-induced biofilm dispersion locus A; PA3311) in NO-dependent biofilm dispersion. Our results suggest that MucR and NbdA both possess PDE activity, while MucR additionally exhibits DGC activity. Deletion of either *nbdA* or *mucR* impaired biofilm dispersion in response to NO. NbdA was found to be specific to NO-induced dispersion with exposure to NO resulting in transcriptional activation of *nbdA* and subsequently PDE activity.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. *P. aeruginosa* and *Escherichia coli* strains used in the present study are listed in Table 1. All strains were routinely grown aerobically at 37°C in Luria-Bertani (LB) medium or Vogel-Bonner minimal medium (VBMM) (12) in shake flasks at 220 rpm. Solid medium was prepared by adding 0.3 to 1.5% agar to the medium. Antibiotics were used at the following concentrations: 200 µg of gentamicin (Gm)/ml, 500 µg of carbenicillin/ml, and 100 µg of tetracycline (Tc)/ml for *P. aeruginosa* and 10 µg of Gm/ml, 10 µg of Tc/ml, and

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TABLE 1 Bacterial strains and plasmids used in this study

Strains and plasmids	Genotype or phenotype ^a	Source or reference
Strains		
<i>E. coli</i>		
JM83	<i>ara Δlac-pro strA thi</i> [φ80 <i>lacZ</i> ΔM15]	36
BL21(λDE3)	F [−] <i>ompZ</i> r [−] m [−] λ <i>lysPlacUV5-T7-genlPlacq-lacI</i>	37
S17-1(λ <i>pir</i>)	<i>thi pro hsdR^M recA</i> RP4-2::TcMu-Km::Tn7	38
<i>P. aeruginosa</i>		
PAO1	Wild type	39
PAO1Δ <i>mucR</i>	<i>mucR</i> ::Gm	This study
PAO1Δ <i>nbdA</i>	<i>nbdA</i> ::Tc	This study
Plasmids and vectors		
pASK-IBA7	Ap ^r ; expression vector for N-terminal fusion to Strep tag	IBA GmbH (Göttingen)
pCC2	Ap ^r ; <i>pleD</i> * from <i>Caulobacter crescentus</i> in pET11	24
pCC3396	Ap ^r ; CC3396 from <i>Caulobacter crescentus</i> in pET21	23
pEX18Ap	Ap ^r ; oriT1 <i>sacB</i> 1; gene replacement vector with MCS from pUC18	14
pBBR1MCS-2	Kan ^r ; broad-host-range vector, P _{lac}	15
pBBR1MCS-5	Gm ^r ; broad-host-range vector, P _{lac}	15
pKB8-I	Ap ^r Tc ^r ; pBSL15 derivative carrying Tc resistance of RP4	This study
pYPRUB10-I	pK18 derivative carrying Gm cassette from Tn1696	Y. Pfänder and B. Masepohl, unpublished data
pMEP01	Ap ^r ; BsaI fragment encoding GGDEF of <i>nbdA</i> in pASK-IBA7	This study
pMEP02	Ap ^r ; BsaI fragment encoding EAL of <i>nbdA</i> in pASK-IBA7	This study
pMEP03	Ap ^r ; BsaI fragment encoding GGDEF-EAL of <i>nbdA</i> in pASK-IBA7	This study
pMEP08	Ap ^r Tc ^r ; SacI-excised Tc cassette of pKB8-I flanked by EcoRI/SacI and SacI/BamHI up- and downstream regions of <i>nbdA</i> in pEX18Ap	This study
pMEP14	Gm ^r ; <i>nbdA</i> and 543 bp upstream inserted into SmaI-cut pBBR1MCS-5	This study
pKBP03	Ap ^r ; BsaI fragment encoding GGDEF-EAL of <i>mucR</i> in pASK-IBA7	This study
pKBP04	Ap ^r ; BsaI fragment encoding GGDEF of <i>mucR</i> in pASK-IBA7	This study
pKBP05	Ap ^r ; BsaI fragment encoding EAL of <i>mucR</i> in pASK-IBA7	This study
pBSP28	Kan ^r ; <i>mucR</i> and 466 bp upstream inserted into HindIII/BamHI-cut pBBR1MCS-2	This study
pSHP02	Ap ^r Gm ^r ; SacI-excised Gm cassette of pYPRUB10-I flanked by EcoRI/SacI and SacI/BamHI up- and downstream region of <i>mucR</i> in pEX18Ap	This study

^a Tc^r, tetracycline resistance; Ap^r, ampicillin resistance; Kan^r, kanamycin resistance; Gm^r, gentamicin resistance.

100 μg of ampicillin/ml for *E. coli*. *sacB* counterselection during second homologous recombination used agar plates containing 5% sucrose.

Construction of isogenic *P. aeruginosa* mutants and expression plasmids. Isogenic mutants were constructed by replacing the *mucR* and *nbdA* coding region with a resistance cassette according to the previously described *sacB*-based strategy (13). Approximately 600 bp of the flanking up- and downstream regions of *mucR* and *nbdA*, including ~50 bp of the coding region were amplified via PCR using primers listed in Table 2. Subsequently, the fragments were cloned into the BamHI-EcoRI restriction site of the suicide vector pEX18Ap (14). Finally, a Gm resistance cassette (excised from pYPRUB-10I, but originally derived from transposon Tn1696) for *mucR* and a Tc resistance cassette (PCR amplified from pKB8-I, but originally derived from the RP4) for *nbdA* was inserted into the SacI site between the flanked regions. The resultant plasmids were transformed in the *E. coli* donor strain S17-1 and transferred to PAO1 via diparental mating. Double-recombinant mutants were selected on *Pseudomonas* isolation agar (Difco) containing 200 μg of Gm/ml or 100 μg of Tc/ml, and counterselection was achieved by the addition of 5% sucrose. Mutants were confirmed by Southern blot analyses and PCR, followed by sequencing.

The coding regions for the predicted GGDEF-EAL, GGDEF, and EAL domains from MucR and NbdA (PA3311) were each cloned via BsaI restriction sites in the vector pASK-IBA7 to generate N-terminal translational fusions with Strep-Tag II (IBA GmbH, Göttingen, Germany) (for the primers used, see Table 2). The integrity of the plasmids was confirmed by restriction analysis and DNA sequencing (MWG, Ebersberg,

Germany). An *nbdA* complementation plasmid (pMEP14) was constructed by amplifying the *nbdA* gene, including 543 bp of the promoter containing upstream region using *Pfu* polymerase and ligating it into SmaI-cut pBBR1MCS-5 (15). Similarly, a *mucR* complementation plasmid (pBSP28) was constructed, including 466-bp promoter region, and ligated into HindIII/BamHI-cut pBBR1MCS-2 (15). Site-directed mutagenesis was done using a QuikChange mutagenesis kit according to the protocol supplied by the manufacturer (Stratagene, Heidelberg, Germany).

Biofilm growth and induction of dispersion. For biofilm dispersion assays, biofilms were cultivated in VBMM in once-through continuous flow tube reactor system composed of size 13 silicone tubing (Masterflex; Cole Parmer, Inc.) at 22°C for 6 days as previously described (5, 6, 16). After 6 days of biofilm growth, biofilm dispersion was induced by the sudden addition of glutamate (20 mM) or 500 μM sodium nitroprusside (SNP) as a source of NO, to the growth medium as previously described (4, 17). Dispersion was indicated by an increase in turbidity at 600 nm in the effluent from the silicone tubing. To determine whether the NO-induced dispersion response required protein synthesis, biofilms were treated for 30 min with 60 μg of tobramycin/ml prior to the induction of dispersion. Control biofilms were not treated.

To visualize the biofilm architecture and the dispersion response, biofilms were grown for 6 days in flow cells, and confocal images were acquired as previously described (2, 3). Quantitative analyses of confocal images of flow cell-grown biofilms prior to and after the induction of dispersing conditions were performed using COMSTAT (18).

TABLE 2 Primer used in this study

Primer ^a	Sequence (5'–3') ^b
Expression plasmids (<i>nbdA</i>)	
PA3311_GGDEFwdBsaI	GCGGTCTCTTATCAGAAGAACTGCGCGTTGTTGCGCCCG
PA3311_revBsaI	GCGGTCTCTTATCAGGCCTGGTTCAGGCTGCGCAG
PA3311_EALfwdBsaI	GCGGTCTCAGCGCCAGGAAGTGCAGATGGAGGAGGAACTGC
PA3311_GGDEFrevBsaI	GCGGTCTCTTATCAGAAGAACTGCGCGTTGTTGCGCCCG
Expression plasmids (<i>mucR</i>)	
PA1727_GGDEFwdBsaI	ATGGTAGGTCTCAGCGCCTCGCACGAGCCAACCGCGA
PA1727_revBsaI	ATGGTAGGTCTCATATCAGGCGACGCTGGCGAGCAACT
PA1727_EALfwdBsaI	ATGGTAGGTCTCAGCGCGAACAGTTGCAACTACTGCATGAC
PA1727_GGDEFrevBsaI	ATGGTAGGTCTCATATCAGAAGAAGCAGTAGCCGTTCCGT
<i>nbdA</i> deletion plasmid	
PA3311_EcoRIfwd	GCGAATTCTCGGGGCGTAGGTGGAATAG
PA3311_SacIrev	GCGAGCTCACCTGCAAGTTCGGCATG
PA3311_SacIfwd	GCGAGCTCGTTCACCTCGACGCGCGTCC
PA3311_BamHIrev	GCGGATCCGAAAGCGGCAGCCTGGCGATC
<i>nbdA</i> complementation	
PA3311com fwd	GCGGATCCACACCGAGCAACAGCAGATAG
PA3311com rev	GCAAGCTTGAACCTCAGGCCTGGTTCAGG
<i>mucR</i> deletion plasmid	
PA1727_EcoRIfwd	GCGAATTCCGCGCGAAACTGGCCCGCTGC
PA1727_SacIrev	GCGAGCTCCGAGAGCGGTGTAGGACGCC
PA1727_SacIfwd	GCGAGCTCCCTGCTCGGCAGGCCGATG
PA1727_BamHIrev	GCGGATCCGCGAATGTCCGGGCGGGTC
<i>mucR</i> complementation	
PA1727com fwd	GCGGATCCAGAAAGTCGATGGGATGGATGC
PA1727com rev	GCAAGCTTTTCAGGCGACGCTGGCGAG
Site-directed mutagenesis	
QC PA3311 AGDEF→GGDEF	CTGGGTGGCGCGCTTCGGCGGGCGACGAGTTCTGC
QC MucR GGDEF→AGDEF	GGACACCATCGCCCGCTCGCCGGCGACGAGTTCGTCC
qRT-PCR	
PA3311_RT_for	GTCGACGGTACGTGC
PA3311_RT_rev	GCAGAACGCCCCGATC
<i>mreB</i> -forward	CTGTCTGATCGACCTGGG
<i>mreB</i> -reverse	CAGCCATCGGCTCTTCG
HDA1	GACTCCTACGGGAGGCAGCAGT
HDA2	GTATTACCGCGGCTGCTGGCAC

^a QC, QuikChange primer. Only the forward primer sequences are shown.^b Mutated and/or changed bases are indicated in boldface.

qRT-PCR. Quantitative real-time reverse-transcription PCR (qRT-PCR) was used to determine the expression levels of *nbdA* using 1 µg of total RNA isolated from wild-type (WT) *P. aeruginosa* grown planktonically, as a biofilm (prior to and after the induction of dispersion) and from dispersed cells after exposure to NO and glutamate. Isolation of mRNA and cDNA synthesis was carried out as previously described (19, 20). qRT-PCR was performed using an Eppendorf MasterCycler ep realplex (Eppendorf AG, Hamburg, Germany) and a Kapa SYBR Fast qPCR kit (Kapa Biosystems, Woburn, MA), with the oligonucleotides listed in Table 2. *mreB* was used as a control. The stability of *mreB* levels were verified by 16S RNA abundance using the primers HDA1 and HDA2 (21). Relative transcript quantitation was accomplished using ep realplex software (Eppendorf AG) by first normalizing the transcript abundance (based on the threshold cycle value) to *mreB*, followed by determining the transcript abundance ratios. Melting-curve analyses were used to verify specific single product amplification.

Recombinant protein production and purification. For overproduction of the particular protein domains, *E. coli* BL21 (λDE3) carrying the respective plasmid was subcultured from overnight cultures into 1 liter of LB medium. The cells were grown at 37°C with shaking. Protein production was induced with anhydrotetracycline (AHT, 0.2 µg/ml) at an optical density at 578 nm of ca. 0.4 to 0.6, and the cultures were further incubated at 20°C (GGDEF and GGDEF-EAL) or 30°C (EAL). After incubation for 16 h, the cells were harvested by centrifugation at 6,000 × g. The pellets were resuspended in lysis buffer (20 mM Tris-HCl [pH 8.0], 250 mM KCl, 0.1 mM EDTA, 0.1% [vol/vol] Triton X-100) and lysed by sonication (Branson sonicator W250; 10 times for 30 s each time, 50% amplitude, plane titan-metal probe, 13 mm in diameter). Lysates were clarified by centrifugation for 1 h at 18,000 × g, and the proteins were purified by using Streptactin Sepharose according to the instructions supplied by the manufacturer (IBA GmbH, Gottingen, Germany). The purity and protein yield were confirmed by SDS-PAGE. Prior to further use, purified pro-

teins were dialyzed overnight against the assay buffer containing 75 mM Tris-HCl (pH 7.8), 250 mM NaCl, and 25 mM KCl, and the proteins were stored at 4°C on ice. The concentration of the recombinant protein variants was determined using the molar extinction coefficient (ϵ_{280}) calculated from the deduced amino acid composition (22). The expression and purification of PleD* and CC3396 of *C. crescentus* were performed as previously described using the expression plasmids pCC2 and pCC3396, respectively (23, 24).

Synthesis of c-di-GMP and α - 32 P-labeled c-di-GMP. c-di-GMP was synthesized enzymatically as described previously (23). α - 32 P-labeled c-di-GMP and unlabeled c-di-GMP were produced by using the constitutive active diguanylate cyclase PleD* (24). A total of 10 μ l of α - 32 P-labeled GTP (400 Ci/mmol) was incubated with 100 μ g of PleD* for 3 h at 30°C in a total volume of 100 μ l. The protein was heated for 5 min at 95°C and then centrifuged for 5 min at 12,000 $\times$ g. The purity of the labeled c-di-GMP was confirmed by separation via thin-layer chromatography (TLC) in 1.5 M KH_2PO_4 -(NH_4) SO_4 (1.5:1 [vol/vol]) and used without further purification.

Unlabeled c-di-GMP was produced similarly by incubating 100 μ M GTP with 100 μ g of PleD* at 37°C for 16 h in a total volume of 1 ml. The quality of the synthesized c-di-GMP was confirmed via high-pressure liquid chromatography (HPLC).

Analysis of DGC and PDE activity by HPLC: enzymatic assays. DGC and PDE assays were performed as previously described (23). Enzyme activity assays containing 10 μ M protein were started either by the addition of 100 μ M GTP for DGC activity or by the addition of 100 μ l of clarified, unlabeled c-di-GMP (~17 μ M) for PDE activity tests in a total volume of 1 ml (DGC) or 600 μ l (PDE). Aliquots were withdrawn at regular time intervals. The reaction was stopped by heating the reaction mixture for 5 min at 95°C, followed by a centrifugation step for 5 min at 12,000 $\times$ g. The clarified supernatant was filtered (Phenex-NY 4-mm syringe filters; Phenomenex, Aschaffenburg, Germany) and separated via reversed-phase HPLC. A Phenomenex Gemini NX 5 μ C $_{18}$ reversed-phase column was used at 37°C with 10 mM triethylammonium bicarbonate buffer containing 7.5% (vol/vol) methanol as a mobile phase. A flow rate of 1 ml/min was used on an Agilent 1100 series system with detection at 254 nm.

Analysis of PDE activity of NbdA by polyethyleneimine-cellulose chromatography. To test for a possible regulatory function of the GGDEF domain on the EAL domain of NbdA, the method described by Christen et al. was used (23). The PDE reaction was performed with 5 μ M protein in the absence or presence of 100 μ M cold GTP. The reaction was started by addition of 1 μ l of clarified 32 P-labeled c-di-GMP (~2.5 μ M, after the removal of residual [α - 32 P]GTP using alkaline phosphatase [NEB, Beverly, MA]) in a total volume of 50 μ l. Reactions were incubated for 2 h at 37°C; at regular time intervals, aliquots were removed, and the reaction was stopped by heating to 95°C. Samples were diluted 2:1 (vol/vol) with a mobile phase containing saturated NH_4SO_4 and 1.5 M KH_2PO_4 (pH 3.6; 1:1.5 [vol/vol]) and centrifuged at 15,000 $\times$ g for 5 min. Subsequently, 2 μ l was spotted onto Polygram CEL 300 polyethyleneimine-cellulose TLC plates. The plates were developed in mobile phase, dried, and exposed to a storage PhosphorScreen.

Extraction and quantification of cyclic di-GMP. c-di-GMP was extracted in triplicate from wild-type (WT) and mutant *P. aeruginosa* strains grown planktonically and as biofilms using heat and ethanol precipitation essentially as previously described (17, 20). Biofilms were grown under continuous-flow conditions for 5 days and subsequently harvested by pinching the tube, resulting in the biofilm biomass to be extruded from the inner surface of the biofilm tube reactor. Extracted c-di-GMP was dried using a SpeedVac and resuspended in 10 mM ammonium bicarbonate buffer. Samples (10 or 20 μ l) were analyzed using an Agilent 1100 HPLC apparatus equipped with an autosampler, degasser, and detector set to 253 nm and then separated using a reversed-phase C $_{18}$ Targa column (2.1 by 40 mm; 5 μ m) at a flow rate of 0.2 ml/min with the following gradient: 0 to 9 min, 1% B (i.e., 1% solvent B and 99% solvent A); 9 to 14

min, 15% B; 14 to 19 min, 25% B; 19 to 26 min, 90% B; and 26 to 40 min, 1% B (A, 10 mM ammonium acetate; B, 10 mM ammonium acetate in methanol) (17). Commercially available c-di-GMP was used as a reference for the identification and quantification of c-di-GMP in cell extracts. Moreover, the identity of HPLC-eluted c-di-GMP was confirmed by tandem mass spectrometry using a QStar mass spectrometer (Applied Biosystems) by detecting the following c-di-GMP fragments: 691.1, 151.9, 691.1, 248.0, 691.1, and 539.8 m/z as described by Thormann et al. (25).

PDE activity. The PDE activity of cell extracts obtained from planktonic and biofilm cells was determined using the synthetic chromogenic substrate *bis*(*p*-nitrophenyl) phosphate (bis-pNPP; Sigma-Aldrich) essentially as previously described (26, 27). The specific PDE activity was determined by measuring the release of *p*-nitrophenol (pNP) at 405 nm. An extinction coefficient for pNP of 1.78×10^4 M $^{-1}$ cm was used. Controls without extracts were included to account for any nonenzymatic bis-pNPP hydrolysis.

Statistical analysis. A Student *t* test was performed for pairwise comparisons of groups, and multivariate analyses were performed by using a one-way analysis of variance (ANOVA), followed by an *a posteriori* test using Sigma Stat software. All experiments were performed at least in triplicate.

RESULTS

Several reports have linked NO-induced biofilm dispersion to PDE activity (8, 17). To identify proteins controlling c-di-GMP turnover in response to NO, we searched the *Pseudomonas* genome database for proteins combining c-di-GMP modulating activity with putative gas sensor input domains, in particular the largely unexplored MHYT domain predicted to possess putative gas sensor function (11). The *P. aeruginosa* genome encodes two proteins containing MHYT domains that in addition also possess GGDEF and EAL domains. These proteins were identified as the membrane-anchored proteins MucR (PA1727) and NbdA (NO-induced biofilm dispersion locus A; PA3311). NbdA is a hypothetical protein of unknown function. MucR, on the other hand, was shown to be a positive regulator of alginate biosynthesis by being an active DGC. Furthermore, MucR is proposed to interact with the alginate biosynthesis protein Alg44, which contains a c-di-GMP-binding PilZ domain essential for alginate biosynthesis (28, 29).

Mutations in *nbdA* and *mucR* abolish the dispersion response of *P. aeruginosa* biofilms to NO. To determine whether NbdA and MucR play a role in NO sensing, biofilms of mutant strains lacking functional *nbdA* and *mucR* were tested for the capability to disperse upon exposure to NO. Biofilm dispersion was induced by a sudden addition of SNP as a source of NO to the growth medium. Dispersion was indicated by an increase of the medium turbidity in the effluent of biofilm reactors as dispersion involved the release of single cells from the biofilm and reversion to the planktonic mode of growth. Untreated biofilms and biofilms treated with glutamate were used as controls. Under the conditions tested, $\Delta nbdA$ and $\Delta mucR$ mutant biofilms did not disperse in response to NO (Fig. 1). Although $\Delta nbdA$ biofilms dispersed in response to glutamate, *mucR* mutant biofilms appeared to be impaired in glutamate-induced dispersion (Fig. 1), suggesting that NbdA, which is produced in the $\Delta mucR$ strain, plays a specific role in NO-induced dispersion.

The dispersion response was further investigated and visualized by confocal microscopy. Addition of glutamate to the growth medium resulted in dispersion of *P. aeruginosa* PAO1 WT biofilms, as indicated by the reduction of attached biofilm biomass, which coincided with microcolonies appearing hollowed out or partly eroded. The average biomass decreased from ca. 16 μm^3 /

FIG 1 Inactivation of *nbdA* impairs dispersion in response to NO, while inactivation of *mucR* impairs biofilm dispersion in response to both glutamate and SNP (NO). *P. aeruginosa* WT (A) and strains inactivated in *nbdA* (B) and *mucR* (C) were grown in a tube reactor for 6 days in VBMM, and then biofilm dispersion was induced by the addition of 20 mM glutamate and 500 μ M SNP. Biofilm effluents were collected in 1-min intervals, and the turbidity recorded at 600 nm. The effluent of untreated *P. aeruginosa* WT and mutant biofilms was used as controls. Experiments were repeated at least in triplicate.

μm^2 to $<5 \mu\text{m}^3/\mu\text{m}^2$ following dispersion (Table 3 and Fig. 2A). At the same time, the average thickness of the biofilms formed by WT decreased from $\sim 28 \mu\text{m}$ to an average of $<8 \mu\text{m}$ (Table 3). Similar results were obtained upon NO-induced dispersion, which coincided with a 10-fold decrease in the biofilm biomass (Table 3 and Fig. 2B). In contrast, no difference in the biofilm architecture of *nbdA* and *mucR* mutant biofilms was noted upon treatment with NO (Table 3 and Fig. 2B). Similarly, the addition of glutamate had no effect on the biofilm architecture of ΔmucR biofilms, whereas the same treatment resulted in an ~ 2 -fold reduction in the biofilm biomass and average biofilm height in the ΔnbdA strain. Moreover, biofilm dispersion was apparent by voids and microcolonies appearing hollow (Table 3 and Fig. 2A).

Cellular c-di-GMP levels are influenced by deletion of *mucR* and overexpression of *nbdA*. Since deletion of *nbdA* and *mucR*

had an impact on the dispersion response and overall biofilm architecture, we next tested whether this is due to changes in the intracellular c-di-GMP pool. Inactivation of *nbdA* did not alter c-di-GMP levels of cells grown planktonically and as biofilms compared to WT. However, statistically significant reduced c-di-GMP levels were observed compared to WT upon overexpression of *nbdA* regardless of growth conditions (Fig. 3). These findings pointed toward a PDE activity of NbdA.

Analysis of c-di-GMP levels of the ΔmucR mutant strain suggested MucR to modulate c-di-GMP levels in a growth mode-dependent manner. Although inactivation of *mucR* resulted in reduced c-di-GMP levels under planktonic growth conditions, increased c-di-GMP levels were observed under biofilm growth (Fig. 3). The findings suggest that both domains (GGDEF and EAL) of MucR are active *in vivo* in a lifestyle-dependent manner.

TABLE 3 Percent change in total biofilm biomass and average thickness obtained by COMSTAT quantitative analysis of the biofilm structure of *P. aeruginosa* PAO1 and mutant strains upon exposure to glutamate and nitric oxide^a

Dispersion type and strain	% Change	
	Total biomass (μm ³ /μm ²)	Avg thickness (μm)
After induction of dispersion by glutamate		
PAO1	28.8	32.5
Δ <i>nbdA</i> mutant	53.2	50.6
Δ <i>mucR</i> mutant	95.2	97.5
After induction of dispersion by nitric oxide		
PAO1	10.9	11.8
Δ <i>nbdA</i> mutant	95.9	81.6
Δ <i>mucR</i> mutant	97.0	100.2

^a The total biofilm biomass and average thickness detected after the induction of dispersion are expressed as percentages compared to untreated samples. COMSTAT analysis was carried out using biofilms grown in triplicate with at least six images per replicate.

NbdA and MucR are both active PDEs. In order to confirm the proposed enzymatic activities, the EAL domains of both NbdA and MucR were recombinantly produced in *E. coli*, purified to near homogeneity (Fig. 4), and assayed for PDE activity. A 10 μM concentration of the single EAL domain of NbdA converted 17 μM c-di-GMP to pGpG after incubation for 10 min (Fig. 5A). The identity of pGpG was confirmed by using the active PDE CC3396 from *C. crescentus* and comparing the retention times by HPLC (23). In contrast to previous findings (28, 29), MucR also showed PDE activity. Analysis of the single EAL-domain of MucR indi-

cated that it is PDE active but at a slower rate than the EAL domain from the NbdA counterpart (Fig. 5B). When we assayed the two-domain variant of MucR consisting of GGDEF-EAL, the PDE activity was still observed but at an even slower rate. After 60 min, all c-di-GMP was converted to pGpG (Fig. 5C).

MucR but not NbdA is an active DGC. In addition to the EAL domain, NbdA and MucR both harbor N-terminally located GGDEF domains. To determine whether NbdA and MucR also possess DGC activity, the recombinant two-domain variants and the single GGDEF-domains were used in DGC assays. GTP served as the substrate in DGC activity tests and the formation of c-di-GMP was monitored via HPLC. Control experiments used PleD*, a constitutive active form of PleD from *C. crescentus* with known DGC activity (24). MucR was found to display DGC activity with 10 μM the GGDEF domain of MucR completely converting 100 μM GTP to c-di-GMP within 60 min (Fig. 6A). Interestingly, the two-domain variant GGDEF-EAL of MucR showed a much faster turnover to c-di-GMP since complete turnover of GTP was achieved after 10 min (Fig. 6B). Therefore, the EAL domain might have a positive impact on DGC activity. Although MucR was shown to possess PDE activity (see Fig. 5C), no further conversion of the produced c-di-GMP to pGpG was observed in assays started with GTP (Fig. 6B). Either GTP is inhibiting PDE activity or PDE turnover is so slow that we were unable to detect any pGpG in the time frame of our experiment.

In contrast, no DGC activity was noted for the GGDEF and the GGDEF-EAL domain of NbdA. Even after a 60-min incubation, no turnover of GTP was detected (Fig. 6D and E). This is consistent with NbdA harboring an imperfect AGDEF motif, whereas MucR possesses a conserved GGDEF motif.

Site-directed mutagenesis of the GGDEF motif of MucR to the

FIG 2 NbdA and MucR are involved in NO-induced biofilm dispersion response of *P. aeruginosa*. The Δ*nbdA* mutant was unable to respond to the addition of NO, whereas the Δ*mucR* mutant failed to disperse upon addition of glutamate and NO. Confocal scanning laser microscopy images show the same *P. aeruginosa* PAO1, Δ*nbdA* and Δ*mucR* mutant biofilms before and after the induction of dispersion by glutamate (A) and SNP (NO) (B). Biofilms were stained with the Live/Dead BacLight viability stain (Invitrogen Corp.). White scale bars, 100 μm.

FIG 3 Cellular c-di-GMP levels are influenced by the deletion of *mucR* and the overexpression of *nbdA*. C-di-GMP levels were determined in cells grown planktonically (A) and as biofilms (B). *, Significantly different from wild-type ($P < 0.05$) as determined by ANOVA and Sigma Stat. Strains designated Δ nbdA/nbdA and Δ mucR/mucR contain the complementation plasmids pMEP14 and pBSP28, respectively.

one of NbdA resulted in loss of DGC activity of the MucR variant (Fig. 6C). A substitution of AGDEF by GGDEF in NbdA, however, resulted in a GGDEF domain with DGC activity. After incubation for 40 min, nearly all GTP was converted to c-di-GMP (Fig. 6F).

FIG 4 Purification of truncated recombinant protein variants used in the present study. SDS-PAGE of affinity purified cytoplasmic domains. Lane 1, GGDEF-EAL of NbdA; lane 2, GGDEF of NbdA; lane 3, EAL of NbdA; lane 4, GGDEF-EAL of MucR; lane 5, GGDEF of MucR and lane 6, EAL of MucR. Lane M, molecular size marker.

PDE activity of NbdA is stimulated by GTP. Although many PDE are inhibited by GTP (8), imperfect GGDEF motifs have recently been implicated in having regulatory impact on neighboring EAL domains by binding GTP and thereby stimulating the PDE (23). We therefore hypothesized that this might be true for NbdA as well. To test this possibility, the two-domain variant (AGDEF-EAL) of NbdA was assayed in the presence or absence of GTP. In contrast to the isolated EAL domain, the AGDEF-EAL protein variant did not show any PDE activity. Only the addition of GTP restored PDE activity of the two-domain variant (Fig. 5D), indicating that the AGDEF domain inhibits the PDE activity of the EAL domain, which is restored by the addition of GTP.

Exposure to NO correlates with increased PDE activity and decreased c-di-GMP levels in the presence of NbdA. Previously published studies (8) indicated NO-induced dispersion to coincide with a significant decrease in cellular c-di-GMP levels. Since NbdA appeared to play a specific role in NO-induced dispersion, with NbdA being an active PDE, we next sought to determine whether NbdA contributes to the reduction of c-di-GMP levels

FIG 5 The EAL domains of MucR and NbdA both harbor PDE activity. HPLC elution profiles of PDE reaction products. Reactions were started by addition of 100 μ M c-di-GMP and reaction products subsequently separated on a Gemini NX RP-18 column (Phenomenex) using 10 mM triethylammonium bicarbonate and 7.5% methanol (vol/vol) at a flow rate of 1 ml/min. Substrate and products were monitored at 254 nm using an Agilent Technologies 1100 system diode array detector. Peaks representing c-di-GMP (**) and pGpG (***) are assigned. Reaction products of PDE assay using the EAL domain of NbdA (A) and MucR (B) and the one of MucR in combination with the adjacent GGDEF-domain (C). The PDE activity of NbdA is stimulated by GTP (D). TLC on polyethyleneimine-cellulose plates was used to investigate the influence of GTP on PDE activity of the two-domain variant (AGDEF-EAL) of NbdA. In addition to radiolabeled α - 32 P-labeled c-di-GMP, 100 μ M cold GTP was added to one set of experiments. At different time points, aliquots were removed, and the reaction products were separated by TLC using saturated NH_4SO_4 and 1.5 M KH_2PO_4 (pH 3.6) (1:1.5 [vol/vol]). TLC plates were developed using a PhosphoScreen.

upon the addition of NO. Treatment of PAO1 biofilms with NO resulted in an $\sim$ 40% decrease in cellular c-di-GMP levels compared to untreated biofilms (Fig. 7A). Moreover, the decrease in c-di-GMP levels correlated with an elevated PDE activity in total cell extracts (Fig. 7B). In contrast, no difference in c-di-GMP levels or PDE activity was noted upon the addition of NO to Δ nbdA mutant biofilms, indicating that changes in c-di-GMP levels and PDE activity were NbdA dependent but not MucR dependent. Complementation of the Δ nbdA strain resulted in WT behavior (Fig. 7). Under planktonic conditions, however, no increase in PDE activity was observed upon exposure to NO in cell extracts obtained from Δ nbdA/nbdA or Δ nbdA strains (data not shown). The findings suggested that NbdA contributes to decreased c-di-GMP levels and elevated PDE activity in a growth mode-dependent manner in the presence of NO.

nbdA is transcriptionally regulated upon NO exposure. Since NO had an impact on PDE activity and cellular c-di-GMP levels in biofilms in the presence of NbdA but not MucR, we sought to determine whether NO acts by transcriptionally activating nbdA expression or posttranslationally by stimulating PDE activity. In

this regard, it has been noted that some bacteria are known to modulate gene expression upon NO exposure since it is not only a signaling molecule but can also cause nitrosative stress (8, 30). We therefore tested whether nbdA expression is regulated by NO addition. Relative expression levels of nbdA were determined using quantitative real-time PCR (qRT-PCR). Compared to untreated planktonic cells, nbdA transcript levels were already 4-fold increased in biofilms. Although the addition of glutamate had only slight effects on nbdA expression, NO-dispersed cells from biofilms showed significantly induced nbdA levels (Fig. 8). These findings suggest that nbdA is transcriptionally regulated by NO, resulting in elevated transcript levels in response to NO treatment. To test whether these elevated transcript levels ultimately result in the protein synthesis of NbdA that enhances PDE activity upon NO exposure, translation was inhibited prior to NO treatment. The addition of tobramycin to biofilms abolished the effect of NO on cellular c-di-GMP levels and PDE activity (Fig. 7), suggesting that the response of *P. aeruginosa* upon NO is regulated at the transcriptional level or through the control of RNA stability. Whether NO is additionally involved in a posttranslational modulation of

FIG 6 MucR but not NbdA is an active DGC. HPLC elution profiles of DGC reaction products of cytoplasmic domains of NbdA and MucR, respectively. Assays were started by the addition of 100 μ M GTP, and reaction products were subsequently separated on a Gemini NX RP-18 column (Phenomenex) using 10 mM triethylammonium bicarbonate and 7.5% methanol (vol/vol) at a flow rate of 1 ml/min. Substrate and products were monitored at 254 nm using an Agilent Technologies 1100 system diode array detector. Peaks representing GTP (*) and c-di-GMP (**) are assigned. The reaction products of DGC assays using the GGDEF domains of NbdA and MucR with or without the adjacent EAL domain were examined. (A) MucR_GGDEF; (B) MucR_GGDEF-EAL; (C) MucR_AGDEF-EAL; (D) NbdA_AGDEF; (E) NbdA_AGDEF-EAL; (F) NbdA_GGDEF-EAL.

NbdA's PDE activity will have to await the availability of purified full-length protein.

DISCUSSION

Nitric oxide is a signaling and defense molecule of major importance in biological systems (30). In addition, NO is produced during anaerobic respiration of nitrate in a process called denitrification in *P. aeruginosa*. In 2006, Barraud et al. (4) showed that NO-induced biofilm dispersal is dependent on nitrite reductase NirS, an enzyme involved in the production of NO during denitrification. The mechanism of NO-induced biofilm dispersion was further linked to increased cellular PDE activity and reduced levels of c-di-GMP (8). However, the PDE involved in this process were

not identified. In other bacteria, such as *Shewanella woodyi*, *S. oneidensis* MR-1, or *Legionella pneumophila*, H-NOX (heme nitric oxide binding) proteins were shown to be involved in NO sensing and modulation of c-di-GMP turnover through interaction with DGC and PDE (9, 10, 31). Moreover, in *S. woodyi*, NO-bound H-NOX was found to stimulate the PDE activity while inhibiting the DGC activity of a bifunctional enzyme consisting of a GGDEF and an EAL domain (9).

Several proteins involved in the dispersion of *P. aeruginosa* biofilms have been identified (5, 17, 32). Among them, the chemotaxis transducer BdlA was found to be essential for NO-induced biofilm dispersion (5). BdlA contains a heme cofactor bound in its first Per-Arnt-Sim domain, which was shown to be

FIG 7 Exposure of biofilms to NO results in reduced c-di-GMP levels (A) and increased PDE activity (B). Five-day-old biofilms of PAO1 and designated mutant strains were treated with water (control) and SNP (NO) prior to c-di-GMP extraction (A) and PDE activity assays using total cell extract (B). Tobramycin (Tob) was added prior to SNP (NO) treatment to inhibit translation.

important for biofilm dispersion (33). Although the protein lacks GGDEF and EAL domains, *bdlA* deletion led to the accumulation of c-di-GMP levels in biofilms (5). In addition, DipA, an active PDE, is known to be involved in *P. aeruginosa* biofilm dispersion

(17). However, none of these proteins was found to be specific to NO as an inducer for dispersion. In the present study, we identified two previously undescribed players in NO-mediated biofilm dispersion, NbdA and MucR. Both proteins are predicted to be

FIG 8 NO-induced dispersion of *P. aeruginosa* biofilms correlates with increased *nbdA* expression. The fold change in *nbdA* mRNA levels in WT *P. aeruginosa* biofilm, remaining biofilm postdispersion, and dispersed cells compared to untreated planktonic growth conditions was assessed by qRT-PCR analysis. *, significantly different from untreated biofilms and NO-treated planktonic cells ($P < 0.05$), as determined by ANOVA and Sigma Stat.

FIG 9 Model for the transcriptional regulation of *nbdA* through NO. (A) In wild-type biofilms, *nbdA* is only weakly expressed, resulting in only a few NbdaA proteins that do not significantly contribute to the overall cellular concentration of c-di-GMP since the inhibition of protein biosynthesis by tobramycin (tob) treatment does not change the level of c-di-GMP. (B) NO induced expression of *nbdA*, resulting in elevated NbdaA proteins which lead to reduced cellular c-di-GMP levels. Tobramycin (tob) treatment inhibits *de novo* synthesis of NbdaA; therefore, c-di-GMP levels are not changed. (C) A strain lacking *nbdA* does not express *nbdA* at all, and the cellular c-di-GMP levels are in the same range as for wild-type biofilms, supporting the very weak expression of *nbdA* under “normal” conditions.

localized in the cytoplasmic membrane and harbor domains linked to gas sensing and c-di-GMP modulating activity. However, while deletion of *nbdA* and *mucR* impaired NO-induced biofilm dispersion, only NbdaA appeared to be specific to the NO-induced dispersion response. Although MucR was shown to play a role in biofilm dispersion in response to NO and glutamate, exposure to NO in the presence of MucR but the absence of NbdaA did not result in increased PDE activity. It is therefore likely that Δ *mucR* mutant biofilms are in general impaired in biofilm dispersal. While our findings indicate MucR to play no specific role in NO-induced dispersion, our data indicate MucR to be one of the few proteins harboring both EAL and GGDEF domains that possess both DGC and PDE activity. This observation is in contrast to earlier reports that only described a DGC activity for MucR (28, 29). However, our phenotypic analyses are in good agreement with the biochemical data, suggesting a dual function. The COMSTAT analysis revealed that the average and maximum thickness, as well as the total biomass, of the *mucR* mutant biofilm was reduced compared to the WT. This phenotype is indicative of the lack of an active DGC. Under planktonic growth conditions, however, MucR was clearly shown to be an active PDE. We therefore propose that MucR is a PDE under planktonic growth conditions but acting as a DGC in biofilms.

Based on our biochemical data, NbdaA is an active PDE which requires GTP for full activity. In addition, our data showed that the stimulation of PDE activity upon NO treatment is mainly due to a transcriptional activation of *nbdA*. Considering that cellular c-di-GMP levels and the overall biofilm architecture were not affected in the strain lacking *nbdA*, these findings indicate that under “normal” conditions *nbdA* is only weakly expressed and possible only a low amount of NbdaA protein molecules is present (Fig. 9). Therefore, deletion of *nbdA* does not result in significant

changes of cellular c-di-GMP levels because the contribution of NbdaA activity under these conditions is only small. Upon NO exposure, however, transcription of *nbdA* is significantly induced, leading to elevated transcript levels and subsequently enhanced protein synthesis. In turn, NbdaA accumulation leads to increased PDE activity (Fig. 9). Inhibiting protein synthesis before NO treatment did not lead to increased PDE activity and changes in cellular c-di-GMP levels, indicating that *de novo* protein synthesis is required for the observed response.

Our results are thus in contrast with previous studies (8) that suggested an NO-dependent posttranslational activation of cellular PDE activity. However, at this point we still cannot rule out a stimulatory effect of NO toward NbdaA's PDE activity. An additional NO sensing role of NbdaA is supported by a bioinformatics study, suggesting that the membrane-integrated MHYT-domain serves as a copper-binding gas sensor domain (11). Based on this prediction, the N-terminal MHYT-domain might sense NO through a bound metal ion (29). Although diatomic gases are predominantly perceived by heme-based sensor proteins (30), protein bound metal ions can serve a similar function. While the ethylene receptor ETR1 from *Arabidopsis thaliana* utilizes a copper cofactor for gas binding (34), the transcription factor NorR from *E. coli* possesses a non-heme iron center to sense NO (35).

Increased *nbdA* expression after NO treatment, and thus increased NbdaA levels, may be sufficient to account for the observed increase in PDE activity to achieve the rapid dispersion response. Considering that, in addition to NbdaA, both BdlA and DipA play a role in NO-induced dispersion, it is also possible that NbdaA interacts with DipA and/or BdlA. The latter itself lacks PDE activity but possesses a bound heme-cofactor that also could be involved in NO sensing (5).

Overall, our data suggest a specific role of NbdaA in NO-induced

biofilm dispersion. This is correlated with increased cellular PDE activity upon exposure to NO and concurrent reduced c-di-GMP levels. Although our data support an NO-dependent transcriptional activation of *nbdA*, a posttranslational stimulation of PDE activity cannot be ruled out yet. To our knowledge, this is the first description of a PDE transcriptionally activated by NO and thereby involved in NO-induced biofilm dispersion in *P. aeruginosa*.

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