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Fluorescence-Based Reporter for Gauging Cyclic Di-GMP Levels in *Pseudomonas aeruginosa*

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The increased tolerance toward the host immune system and antibiotics displayed by biofilm-forming *Pseudomonas aeruginosa* and other bacteria in chronic infections such as cystic fibrosis bronchopneumonia is of major concern. Targeting of biofilm formation is believed to be a key aspect in the development of novel antipathogenic drugs that can augment the effect of classic antibiotics by decreasing antimicrobial tolerance. The second messenger cyclic di-GMP is a positive regulator of biofilm formation, and cyclic di-GMP signaling is now regarded as a potential target for the development of antipathogenic compounds. Here we describe the development of fluorescent monitors that can gauge the cellular level of cyclic di-GMP in *P. aeruginosa*. We have created cyclic di-GMP level reporters by transcriptionally fusing the cyclic di-GMP-responsive *cdrA* promoter to genes encoding green fluorescent protein. We show that the reporter constructs give a fluorescent readout of the intracellular level of cyclic di-GMP in *P. aeruginosa* strains with different levels of cyclic di-GMP. Furthermore, we show that the reporters are able to detect increased turnover of cyclic di-GMP mediated by treatment of *P. aeruginosa* with the phosphodiesterase inducer nitric oxide. Considering that biofilm formation is a necessity for the subsequent development of a chronic infection and therefore a pathogenicity trait, the reporters display a significant potential for use in the identification of novel antipathogenic compounds targeting cyclic di-GMP signaling, as well as for use in research aiming at understanding the biofilm biology of *P. aeruginosa*.

Biofilm-forming bacteria are increasingly recognized as a serious problem in a variety of settings, from fouling of pipelines to infections of humans. Biofilms display increased tolerance toward antibiotics and the host immune system, which makes infections caused by them difficult if not impossible to eradicate (14). This inadequacy of antibiotics has led researchers to look into new strategies for combating biofilm-based infections. One such strategy is the development of antipathogenic drugs (19). The idea behind this strategy is to identify molecular targets that are important to the pathogenicity of the bacteria and to develop drugs that interfere with the actions of the target. Interference with these targets should render the bacteria more susceptible to the action of the immune system and/or antibiotics. The drugs that are developed should not affect growth of the bacteria, making resistance less likely to develop, since no direct selective pressure is exerted by the compounds.

The antipathogenic drug principle has been tested with the opportunistic pathogenic bacterium *Pseudomonas aeruginosa* as a model organism, and proof of concept has been delivered (21). This common environmental bacterium exploits weaknesses in immunocompromised individuals and people with cystic fibrosis (CF) to establish infections. In the latter case, the bacterium enters the lungs and establishes a recalcitrant and terminal infection that responds poorly to the actions of the immune system and antibiotic treatment regimens (reviewed in reference 27). This recalcitrance is believed to be a consequence of the bacteria residing in biofilms, which have been shown to shield them from the immune system and increase their tolerance toward antibiotics (1, 7). The biofilm-forming capability resulting in immune system evasion and antibiotic tolerance should therefore be recognized as a key target in the development of antipathogenic drugs against *P. aeruginosa*.

Protection from the immune system is thought to occur due to production of biofilm extracellular matrix components (35) and rhamnolipid, a cytolytic surfactant (1, 28). Rhamnolipid and the increased antibiotic tolerance, together with production of other virulence factors imposing damage to the infected tissue, have been shown to be quorum sensing dependent. Consequently, there has been substantial research within the field of developing antipathogenic drugs targeted against the LuxR-type quorum-sensing regulator LasR of *P. aeruginosa* (8). Screening for these quorum-sensing inhibitors has been aided by the development of genetic tools allowing monitoring of quorum-sensing activity with the aim of identifying compounds that interfere with the quorum-sensing regulators. Fluorescent reporters constructed as quorum-sensing-regulated promoters transcriptionally fused to *gfp* have especially been of great value in the work and have led to identification of potent inhibitors such as brominated furanones and the fungal secondary products penicillic acid and patulin (20, 21, 40).

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Besides quorum sensing, another interesting signaling system, based on the secondary messenger cyclic di-GMP (c-di-GMP), which controls important events in the biofilm developmental life cycle in *P. aeruginosa* and many other bacteria, has recently come to the forefront. The messenger is synthesized by diguanylate cyclases (DGCs) with a characteristic GGDEF domain (42) and degraded by phosphodiesterases (PDEs) with characteristic EAL or HD-GYP domains (41, 43). c-di-GMP transmits a wealth of environmental signals through various effector proteins to positively control biofilm-promoting exopolysaccharide and adhesin expression (9, 15, 17, 24, 34, 45), as well as negatively control flagellum- and type IV pilus-driven motility (23, 29, 36). Evidence has been provided that maintenance of a low level of c-di-GMP prevents biofilm formation and that provoked decreases in the intracellular level of c-di-GMP can induce dispersal of already established biofilms (e.g., see reference 18). These findings suggest that antipathogenic drugs targeting c-di-GMP signaling may be developed either to prevent biofilms from forming during infections or to disperse already established biofilms, making them susceptible to efficient treatment by coadministration of classic antibiotics that show much more potential against planktonic bacteria. A decrease in c-di-GMP levels may be achieved through inhibition of DGCs or activation of PDEs (e.g., see reference 6). Furthermore, signaling could be disrupted by inhibiting the activity of effector proteins without changing the c-di-GMP level itself.

To efficiently screen for antipathogenic drug candidates that target c-di-GMP metabolism, a valuable tool would be a reporter of the intracellular levels of c-di-GMP that functions in a manner similar to that of the previously mentioned monitors of quorum sensing. We have created two series of c-di-GMP reporters by transcriptionally fusing a c-di-GMP-responsive promoter to genes encoding green fluorescent protein (GFP). Although the two reporter series were independently constructed, one at the University of Copenhagen, Copenhagen, Denmark, and the other at the University of Washington, Seattle, WA, we publish the information about them jointly in this paper. We show that the reporter constructs are able to provide a fluorescent readout of the intracellular level of c-di-GMP in *P. aeruginosa*, distinguishing strains with different levels of c-di-GMP. Furthermore, we show that the reporters are able to identify compounds that reduce the intracellular level of c-di-GMP in *P. aeruginosa*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains and plasmids used in the study are listed in Table 1. *Escherichia coli* strains were routinely grown in LB medium and on LB agar at 37°C. *P. aeruginosa* strains were grown in LB medium or ABTG+casA minimal medium (AB medium of Clark and Maaløe [12] supplemented with 2.5 mg thiamine liter^{-1}, 0.5% [wt/vol] glucose and 0.5% [wt/vol] Casamino Acids) and on LB agar or ABTC agar (AB medium supplemented with 2.5 mg thiamine liter^{-1} and 10 mM citrate) at 37°C. For the c-di-GMP measurements, *P. aeruginosa* strains were grown in VBMM (25). Antibiotics were supplied where necessary at the following concentrations: for *E. coli*, 100 μg ampicillin ml^{-1}, 15 μg gentamicin ml^{-1}, 35 μg kanamycin ml^{-1}, and 6 μg chloramphenicol ml^{-1}; for *P. aeruginosa*, 60 μg gentamicin ml^{-1} and 200 μg carbenicillin ml^{-1}.

Construction of Copenhagen c-di-GMP reporter plasmids. Construction of reporter plasmids was conducted using standard molecular cloning techniques and the *E. coli* cloning strain DH5α. The *cdrA* (*PA4625*) promoter from *P. aeruginosa* PAO1 was amplified using the primers P-cdrA-up-XbaI and P-cdrA-dn-RBSII-SphI (sequences are available upon request), with the latter including the sequence for an optimized ribosomal binding site (RBSII). The amplified promoter was inserted between the XbaI and SphI sites in the empty expression vector pJBA23, creating plasmid pRYB1. The sequences coding for stable GFP (*gfp*) and unstable GFP [*gfp*(ASV), where ASV refers to a peptide tail as described in reference 2] were excised from plasmids pJBA25 and pJBA113, respectively, using SphI and HindIII digestion and inserted between the corresponding sites in pRYB1, creating pRYB1*gfp* and pRYB1*gfp*(ASV). The plasmids were sequenced using primers P-cdrA-fwd, P-cdrA-rev, Gfp-seq-int(+), and Gfp-seq-int(−) to ensure that no mutations were found in the *cdrA* promoter, the ribosomal binding site, or the GFP genes. To create the final *P. aeruginosa*-compatible reporter plasmids, the reporter cassettes from pRYB1*gfp* and pRYB1*gfp*(ASV) were excised using NotI digestion and inserted in NotI-digested pUCP22-Not and pBK-miniTn7-ΩGm, creating pCdrA::*gfp*^{C}/pCdrA::*gfp*(ASV)^{C} and pTn7CdrA::*gfp*^{C}/pTn7CdrA::*gfp*(ASV)^{C} (where C represents Copenhagen), respectively. Clones were analyzed by XbaI and BglII restriction analysis to select plasmids that harbored the *cdrA* promoter in the opposite direction than the inherent *lac* promoter found on pUCP22-Not and pBK-miniTn7-ΩGm (pUC19 derived).

Construction of Seattle c-di-GMP reporter plasmids. Standard molecular cloning techniques were used in the construction of reporter plasmids in *E. coli* strain DH5α. The plasmid-based reporters pCdrA::*gfp*^{S} and pCdrA::*gfp*(ASV)^{S} (where S represents Seattle) were constructed by amplifying a region comprising 381 bp upstream of the *cdrA* start codon and 22 bp of the coding sequence using primers PA4625 reporter up and PA4625 reporter down and inserting it into the BamHI sites of the promoterless GFP expression vectors pMH487 and pMH489, respectively. The correct orientation was verified by PCR.

pMH487 and pMH489 were created by inserting an RNase III splice site in XbaI- and SphI-doubly digested pMH305 and pMH391 (20), respectively. The insertion deleted an RBS site lying directly upstream of *gfp* in the two vectors. pMH305 was created by inserting a NotI-digested RBSII-*gfp*(Mut3)-T_0-T_1 fragment from pJBA25 into NotI-digested pUCP22NotI.

Construction of Δ*pel*Δ*psl* and Δ*wspF*Δ*pel*Δ*psl* background strains. The *pelA*, *pslBCD*, and *wspF* deletions made in *P. aeruginosa* PAO1 to create the background strains for the c-di-GMP reporters were conducted as follows. For the *pelA* and *pslBCD* deletions, the deletion vectors pMPELA and pMPSL-KO1, respectively, were used. The plasmids were introduced into *P. aeruginosa* PAO1 and derivatives through triparental mating using pRK600 as a helper plasmid to facilitate the conjugal transfer of the plasmids. Conjugants displaying double-crossover events creating the desired gene deletion were directly selected for by plating of conjugation mixtures on ABTC agar plates supplemented with 5% (wt/vol) sucrose and gentamicin. Deletions were verified by colony PCR using the primer pairs pelA-excis-Up/pelA-excis-Dn and pslBCD-Up/pslBCD-Dn for the *pelA* and *pslBCD* deletions, respectively. Untagged mutants were created by Flp-mediated excision of the FRT-Gm-FRT cassette (where FRT is the Flp recombination target and Gm is a gentamicin resistance gene) by introducing pFlp2 into the mutants as described previously (25). Untagged mutants were cured for pFlp2 and verified by colony PCR using the aforementioned primer pairs. For the *wspF* deletion, the deletion vector pΔwspF was introduced into *P. aeruginosa* by biparental mating and single-crossover conjugants were selected by growth on ABTC agar supplemented with gentamicin. Double-crossover events were promoted by planktonic growth in LB medium without antibiotic selection, and the corresponding mutants were screened for by means of their wrinkly colony morphology. The correct deletion was verified by colony PCR using the primer pair wspF_fwd and wspF_rev.

Construction of Δ*fleQ* strain. The pEX18Gm::Δ*fleQ* deletion vector was constructed in the following way. Overlap extension PCR using the primer pairs fleQ-1s/fleQ-1a and fleQ-2s/fleQ-2a was used to amplify a DNA fragment with a 1,482-bp in-frame deletion of the *fleQ* coding se-

TABLE 1 Strains and plasmids used in the study[a]

Strain or plasmid	Relevant genotype and/or characteristics	Reference or source
Strains		
P. aeruginosa PAO1		
Wild type		46
Δ*pel*	Δ*pelA*	This study
Δ*pel*Δ*psl*	Δ*pelA* Δ*pslBCD*	This study
Δ*wspF*	Δ*wspF*	This study
Δ*wspF*Δ*pel*	Δ*wspF* Δ*pelA*	This study
Δ*wspF*Δ*pel*Δ*psl*	Δ*wspF* Δ*pelA* Δ*pslBCD*	This study
Δ*fleQ*	Δ*fleQ*	This study
Δ*pel*Δ*psl* Tn7CdrA::*gfp*^{C}	Δ*pelA* Δ*pslBCD* mini-Tn*7*-P_{cdrA}-RBSII-*gfp*(Mut3)-T_0-T_1	This study
Δ*wspF*Δ*pel*Δ*psl* Tn7CdrA::*gfp*^{C}	Δ*wspF* Δ*pelA* Δ*pslBCD* mini-Tn*7*-P_{cdrA}-RBSII-*gfp*(Mut3)-T_0-T_1	This study
Δ*pel*Δ*psl* Tn7CdrA::*gfp*(ASV)^{C}	Δ*pelA* Δ*pslBCD* mini-Tn*7*-P_{cdrA}-RBSII-*gfp*(ASV)-T_0-T_1	This study
Δ*wspF*Δ*pel*Δ*psl* Tn7CdrA::*gfp*(ASV)^{C}	Δ*wspF* Δ*pelA* Δ*pslBCD* mini-Tn*7*-P_{cdrA}-RBSII-*gfp*(ASV)-T_0-T_1	This study
P_{BAD}PA1120	*araC*-P_{BAD}::PA1120 integrated into the *attB* site	This study
E. coli		
DH5α	Classic cloning strain	Lab collection
S17-1 λpir	Classic cloning and conjugation strain	Lab collection
Plasmids		
pJBA23	pUC18Not-RBSII-T_0-T_1 Amp^r	2
pJBA25	Source of *gfp* (Mut3), Amp^r	2
pJBA113	Source of *gfp* (ASV), Amp^r	2
pUCP22Not	*E. coli-Pseudomonas* shuttle vector, Amp^r Gm^r	22, 48
pBK-miniTn7-ΩGm	Mini-Tn*7*-ΩGm delivery vector, Amp^r Gm^r	32
pRYB1	pUC18Not-P_{cdrA}-RBSII-T_0-T_1, Amp^r	This study
pRYB1*gfp*	pUC18Not-P_{cdrA}-RBSII-*gfp*(Mut3)-T_0-T_1, Amp^r	This study
pRYB1*gfp*(ASV)	pUC18Not-P_{cdrA}-RBSII-*gfp*(ASV)-T_0-T_1, Amp^r	This study
pCdrA::*gfp*^{C}	pUCP22Not-P_{cdrA}-RBSII-*gfp*(Mut3)-T_0-T_1, Amp^r Gm^r	This study
pCdrA::*gfp*(ASV)^{C}	pUCP22Not-P_{cdrA}-RBSII-*gfp*(ASV)-T_0-T_1, Amp^r Gm^r	This study
pTn7CdrA::*gfp*^{C}	mini-Tn*7*-P_{cdrA}-RBSII-*gfp*(Mut3)-T_0-T_1 delivery vector, Amp^r Gm^r	This study
pTn7CdrA::*gfp*(ASV)^{C}	miniTn7-P_{cdrA}-RBSII-*gfp*(ASV)-T_0-T_1 delivery vector, Amp^r Gm^r	This study
pΔwspF	*wspF*-knockout vector, Gm^r	24
pMPELA	*pelA*-knockout vector, Amp^r Gm^r	45
pMPSL-KO1	*pslBCD*-knockout vector, Amp^r Gm^r	31
pFlp2	Source of Flp2 recombinase, Amp^r	25
pUX-BF13	Plasmid providing Tn*7* transposase genes in *trans*, Amp^r	4
pRK600	Mobilization plasmid for Tn7-tagging of *Pseudomonas* strains	30
pMH305	pUCP22Not-RBSII-*gfp*(Mut3)-T_0-T_1, Amp^r Gm^r	This study
pMH391	pUCP22Not-RBSII-*gfp*(ASV)-T_0-T_1, Amp^r Gm^r	20
pMH487	pUCP22Not-RNase III-*gfp*(Mut3)-T_0-T_1, Amp^r Gm^r	This study
pMH489	pUCP22Not-RNase III-*gfp*(ASV)-T_0-T_1, Amp^r Gm^r	This study
pCdrA::*gfp*^{S}	pUCP22Not-P_{cdrA}-RBS-CDS-RNaseIII-*gfp*(Mut3)-T_0-T_1, Amp^r Gm^r	This study
pCdrA::*gfp*(ASV)^{S}	pUCP22Not-P_{cdrA}-RBS-CDS-RNase III-*gfp*(ASV)-T_0-T_1, Amp^r Gm^r	This study
pEX18Gm	Allelic exchange vector, Gm^r	25
pEX18Gm:: Δ*fleQ*	*fleQ* in-frame deletion vector, Gm^r	This study
pJN1120	Plasmid containing *tpbB* (PA1120) fused to the arabinose-inducible AraC-P_{BAD} promoter, Gm^r	23
pDONRminiCTX2	Gateway-compatible mini-CTX2, Tc^r Cm^r	Joe J. Harrison
pDONRminiCTX2::P_{BAD}PA1120	3.1-kb fragment encoding the *araC* regulator, *araBAD* promoter region fused to the DGC *tpbB* from pJN1120	This study

[a] See text for details. r, resistant; Amp, ampicillin; Gm, gentamicin; Cm, chloramphenicol; Tc, tetracycline.

quence. The fragment was inserted in the allelic exchange vector pEX18Gm (25). pEX18Gm::Δ*fleQ* was introduced in *P. aeruginosa* by *E. coli* S17-1 λpir-mediated biparental mating. The deletion was created using a standard method for two-step allelic exchange as described by Schweizer and Hoang (44) and was confirmed by PCR using the primers fleQ-os and fleQ-as flanking the deletion fragment.

Construction of P_{BAD}::*tpbB* CTX chromosomal insertion. The integration plasmid pDONRminiCTX2::P_{BAD}PA1120 was engineered for the conditional expression of the DGC TpbB (PA1120) and inserted into the chromosome to construct the *P. aeruginosa* strain P_{BAD}PA1120. PCR was used to amplify from pJN1120 (23) a 3.1-kb fragment comprising the *araC*-P_{BAD} arabinose-inducible expression cassette (39) fused to *tpbB*. The PCR primer pair used, BBctxP_{BAD}::1120attB1 and BBctxP_{BAD}::1120attB2, contains attb1 and attb2 sites that allow recombination into the pDONRminiCTX2 integration plasmid (Joe Harrison, unpublished) using BP Clonase (Invitrogen), as suggested by the manufacturer. The resulting plasmid was introduced into *P. aeruginosa* by electroporation (10), where it integrates into the *attB* locus and the mini-CTX plasmid

backbone is excised upon expression of Flp recombinase via pFLP2, as described by Hoang et al. (26).

Construction of c-di-GMP reporter strains. The plasmid-based reporters pCdrA::*gfp*^{C} and pCdrA::*gfp*(ASV)^C were introduced into the *P. aeruginosa* Δ*pel*Δ*psl* and Δ*pel*Δ*psl*Δ*wspF* background strains by means of biparental mating. pCdrA::*gfp*^{S} and pCdrA::*gfp*(ASV)^S were introduced into the background strains by electroporation (10). The background strains were tagged with the mini-Tn*7* reporter constructs from pTn7CdrA::*gfp*^{C} and pTn7CdrA::*gfp*(ASV)^C by means of four-parental mating with *E. coli* DH5α harboring the delivery vectors as donor strains and with *E. coli* strains harboring pUX-BF13 and pRK600 as helper strains.

Colony morphology and fluorescence intensity analysis. The colony morphology and fluorescence intensity of the Copenhagen reporter strains were analyzed by streaking the strains on minimal medium plates and growing them for 20 h. The colonies were visualized using a Zeiss LSM710 confocal microscope equipped with a ×10 objective, a UV lamp, GFP filter sets, and a monochrome camera. Morphologies were visualized by bright-field microscopy, and fluorescence was visualized by epifluorescence microscopy. Pictures were taken using AxioVision software and combined using a standard graphics program.

Shake flask-based c-di-GMP reporter assay. The Copenhagen reporter strains were assayed by growth in shake flasks in the following way. Strains were inoculated from fresh streaks on ABTC agar with gentamicin into a 50-ml shake flask with 15 ml ABTG+casA supplemented with gentamicin. After overnight (ON) growth at 185 rpm, the strains were diluted to an optical density at 600 nm (OD_{600}) of 0.03 into 100 ml ABTG+casA supplemented with gentamicin in a 250-ml shake flask and grown for an additional 24 h at 185 rpm, with samples for OD_{600} and fluorescence measurements being withdrawn at regular intervals. Fluorescence was measured as arbitrary fluorescence intensity units (FIU) on a Turner Quantech digital filter fluorometer (Thermo Scientific) using a 490-nm narrow-band excitation filter, a 515-nm sharp-cut emission filter, and the raw fluorescence mode with gain code 11. For the sodium nitroprusside (SNP; Sigma) experiment, 250 μM, 31.25 μM, 2 μM, and 0.25 μM final concentrations were made from a stock solution of 50 mM SNP dissolved in Milli-Q water. Shake flasks containing SNP were incubated in the dark due to the light-sensitive nature of SNP.

Microtiter plate-based c-di-GMP reporter assay. Microtiter plate-based assays with the Copenhagen reporter strains were carried out as follows: ON cultures of the strains were prepared as described above for the shake flask experiments (see above) and diluted to an OD_{600} of 0.03 in 100-ml shake flasks with 30 ml ABTG+casA supplemented with gentamicin. Growth was measured manually until the OD_{600} reached 0.5, at which point 300 μl of the cultures was added to the wells of a black-welled 96-well microtiter plate (Nunc) and incubated in a Victor^2 (Perkin Elmer) plate reader heated to 37°C and set up to measure OD_{450} and green fluorescence (arbitrary GFP units) every 30 min for approximately 22 h. The plates were shaken in an orbital pattern (3-mm diameter) at normal speed for 10 min before and after each round of measurements to optimize growth. For the SNP experiment, 2-fold serial dilutions starting from 250 μM SNP were made from a 50 mM stock of SNP dissolved in Milli-Q water. To maintain the OD_{600} at 0.5 for the pregrown reporter strains, the cell culture was used to make the serial dilutions.

Analysis of *fleQ* regulation and *PA1120* induction in Seattle reporter strains. The activity of the Seattle reporter strains was assayed in the following way. Strains were inoculated from fresh streaks on LB agar supplemented with gentamicin into 16-mm test tubes with 3 ml LB broth supplemented with gentamicin. After growth overnight at 250 rpm, the strains were diluted in triplicate (100 μl into 3 ml LB medium supplemented with gentamicin in a 16-mm test tube) and grown to mid-logarithmic growth phase (approximately 2.5 h). Arabinose was also supplemented when indicated at a final concentration of 0.2% (wt/vol) in studies where the conditional expression of the DGC TpbB was evaluated. Samples were concentrated 2 times by centrifugation, and the supernatant was removed. Bacterial pellets were suspended in phosphate-buffered saline and aliquoted into a Costar 96-well black clear-bottom microtiter plate (Corning) and measured for fluorescence (excitation = 485 nm, emission = 535 nm) and optical density (595 nm) using a Tecan GENios plate reader. Data are presented as relative fluorescent units (RFU), which are arbitrary fluorescence intensity units corrected for cell density.

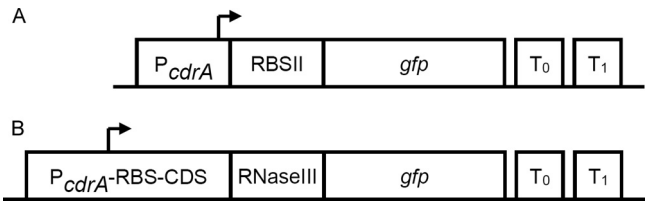

FIG 1 Schematic drawing of the Copenhagen (A) and Seattle (B) c-di-GMP reporter cassettes. (A) The cassette consists of a transcriptional fusion between the promoter from *cdrA* (P_{cdrA}) and a gene encoding either stable GFP or unstable GFP (both designated *gfp* in this figure) with an optimized ribosomal binding site (RBSII). The transcriptional fusion is followed by two transcriptional terminators (T_0 and T_1). (B) The cassette consists of a transcriptional fusion between the promoter from *cdrA*, including the native RBS and parts of the coding sequence (P_{cdrA}-RBS-CDS) and a gene encoding either stable GFP or unstable GFP (*gfp*). The fusion is linked via an RNase III splice site and is followed by two transcriptional terminators (T_0 and T_1). In addition, both cassettes have flanking NotI restriction sites and a chloramphenicol resistance gene interspersed between the two terminators (omitted for clarity). Individual elements are not drawn to scale.

Nucleotide extraction and quantitative c-di-GMP measurements. Nucleotides were extracted as previously described (13). Briefly, 990 μl from a culture grown to mid- to late exponential phase was spiked with 10 μl of 10 mM 2-chloro-AMP (Biolog) as internal standard and immediately treated with 70% perchloric acid (Sigma) to a final concentration of 0.6 M. The pHs of the soluble fractions containing nucleotides were adjusted with $KHCO_3$ as previously described (23), prior to liquid chromatography-tandem mass spectrometry (LC-MS/MS) measurements of the c-di-GMP levels. The protein-containing precipitates were quantified by the Bradford assay (Bio-Rad) and used to normalize c-di-GMP measurements.

RESULTS

Construction of c-di-GMP-responsive reporters. For construction of the c-di-GMP reporters, we first set out to identify a suitable gene that was highly responsive to fluctuations in the intracellular level of c-di-GMP in *P. aeruginosa*. One gene, *cdrA* (*PA4625*), encoding a large adhesin (9) was found to be highly upregulated in response to increased levels of c-di-GMP (9, 24). We expected the *cdrA* promoter to be a good candidate as part of a transcriptional fusion with *gfp* that could act as a reporter of c-di-GMP levels in *P. aeruginosa*.

The exact location of the *cdrA* promoter, including the −35, −10, and other regulatory sequences, is unknown, so for the Copenhagen reporters, we arbitrarily chose to amplify the region of the genome from 23 to 221 bp upstream of the start codon. The gap between the amplified sequence and the start codon of *cdrA* was used to omit the native ribosomal binding site (RBS) in the reporter constructs. Instead, we used an artificial RBS to enhance translation from the *gfp* mRNA in order to increase the signal related to c-di-GMP-responsive transcription from the promoter.

We created cassettes (Fig. 1A) consisting of the *cdrA* promoter fused to the artificial RBS and *gfp*. Because the kinetics of c-di-GMP-induced transcription from the *cdrA* promoter were unknown, we chose to construct fusions encoding either the stable GFP (Mut3) protein or the unstable GFP (ASV) protein (2). In

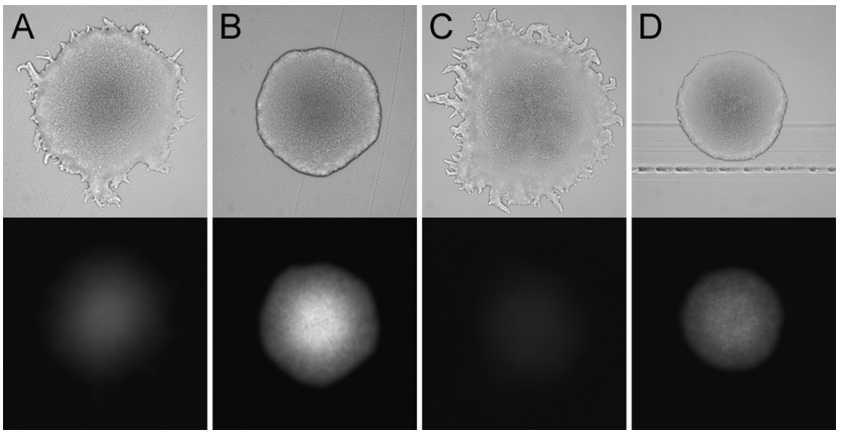

FIG 2 Colony morphology (top) and fluorescence intensity (bottom) of the plasmid-based reporter strains. (A) *P. aeruginosa* PAO1 Δ*pel*Δ*psl*/pCdrA::*gfp*^{C} (normal c-di-GMP level, stable GFP); (B) Δ*wspF*Δ*pel*Δ*psl*/pCdrA::*gfp*^{C} (increased c-di-GMP level, stable GFP); (C) Δ*pel*Δ*psl*/pCdrA::*gfp*(ASV)^{C} (unstable GFP). (D) Δ*wspF*Δ*pel*Δ*psl*/pCdrA::*gfp*(ASV)^{C} (unstable GFP). Shown are 20-h-old colonies.

these constructs, *gfp* is situated at an optimal distance from the RBS to further enhance translation (2). In addition, two transcriptional terminators, interspersed by a chloramphenicol resistance gene, are located downstream of *gfp* to efficiently terminate transcription, minimizing unnecessary read-through from RNA polymerase.

pUCP22Not and pBK-miniTn7-ΩGm were used as final destination vectors for the reporter cassettes to create plasmid-based and chromosome-integrated reporters, respectively. pUCP22Not contains a *lac* promoter directly upstream of the multiple-cloning site (MCS), and as *P. aeruginosa* does not contain *lacI*, the promoter allows read-through and expression of *gfp* even in the absence of c-di-GMP, leading to a false signal. Therefore, the pUCP22-Not-based reporters pCdrA::*gfp*^{C} and pCdrA::*gfp*(ASV)^{C} were constructed such that the inserted cassettes had the coding sequence of *gfp* situated on the complementary strand with regard to the direction of the *lac* promoter. pBK-miniTn7-ΩGm is a pUC-based vector that does not replicate in *P. aeruginosa*. It carries a mini-Tn*7* transposon without the transposase genes and allows stable chromosomal tagging at a neutral locus in the genome of *P. aeruginosa* when transposase genes are provided transiently in *trans*.

Two genetic backgrounds were chosen for the reporter constructs, a wild-type *P. aeruginosa* PAO1 strain and an isogenic *wspF* deletion mutant. Deletion of *wspF* results in elevated levels of c-di-GMP in *P. aeruginosa* through the continuous activation of the DGC WspR, a component of the Wsp signal transduction system (24). A Δ*wspF* strain containing the c-di-GMP reporter is predicted to have an increased level of fluorescence compared to the wild-type strain and could be used as the target strain for inhibitor screening. Additionally, we deleted the genes *pelA* and *pslBCD* in both the wild-type and *wspF* mutant, resulting in an inability to produce Pel and Psl polysaccharides. Pel and Psl are important parts of the *P. aeruginosa* extracellular matrix during biofilm growth (16), and their production is regulated positively by c-di-GMP (24, 34, 45), which results in bacterial aggregation in planktonic culture when c-di-GMP levels are elevated. Aggregation complicates the OD measurements used to monitor growth of bacteria, which is important during identification of inhibitors that are nontoxic to the cells. Therefore, we chose to work with reporters in the nonaggregating Δ*pel* Δ*psl* strains. Based on LC-MS/MS measurements, the cellular c-di-GMP content was estimated to be 10.9 (standard deviation [SD], 4.2) and 77.0 (SD, 6.5) pmol c-di-GMP/mg total protein in mid- to late-log-phase cells of strains Δ*psl*Δ*pel* and Δ*wspF*Δ*psl*Δ*pel*, respectively. The *wspF* deletion thus resulted in a 7-fold increase in the cellular level of c-di-GMP.

The Seattle series of c-di-GMP reporters (Fig. 1B) was created in a fashion similar to that for the Copenhagen reporters. The reporters were based on the plasmids pMH487 and pMH489 harboring promoterless genes encoding stable and unstable variants of *gfp*, respectively. Both plasmids harbor an RNase III splice site directly upstream of *gfp*. To regulate the expression of the *gfp* variants, the *cdrA* promoter and part of the coding sequence were inserted in front of the GFP genes, thereby creating pCdrA::*gfp*^{S} and pCdrA::*gfp*(ASV)^{S}.

The c-di-GMP reporters respond to the cellular levels of c-di-GMP. The ability of the c-di-GMP reporters to fluoresce in accordance with c-di-GMP levels was visualized by epifluorescence microscopy of 1-day-old colonies on minimal medium. Comparing the plasmid-based to the chromosome-integrated reporters, it became clear that the latter were not suited for the intended screening purposes due to a low level of fluorescence (data not shown). We decided to use plasmid-based reporters for the further development of screening assays. The *wspF* mutant harboring elevated levels of c-di-GMP displayed the brightest colonies, indicating that the fluorescence intensity of the reporters is indeed correlated with the intracellular level of c-di-GMP, as hypothesized (Fig. 2). In addition, the fluorescence intensity pictures showed that the reporters with unstable GFP fluoresced less than the reporters with stable GFP, confirming the temporal instability of GFP (ASV) (Fig. 2).

A previous study by Hickman and Harwood (23) indicated that transcription of the *cdrA* gene is repressed by the transcriptional regulator FleQ in *P. aeruginosa* and that c-di-GMP can bind to the FleQ regulator and cause derepression, releasing FleQ from the promoter. The reporters were placed in a Δ*fleQ* strain and in a Δ*wspF* strain to investigate if this regulatory pattern could be observed for our reporters as well. We found that the Δ*fleQ* strain with the reporter displayed a significantly higher level of fluores-

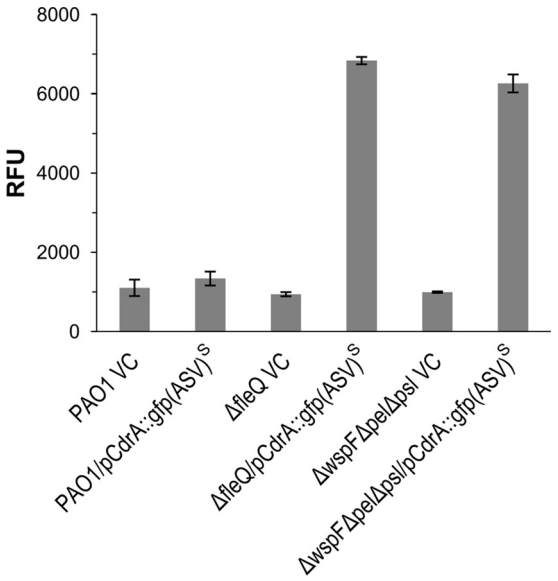

FIG 3 Fluorescence from *P. aeruginosa* PAO1/pCdrA::*gfp*(ASV)^S, Δ*fleQ*/pCdrA::*gfp*(ASV)^S, Δ*wspF*Δ*pel*Δ*psl*/pCdrA::*gfp*(ASV)^S, and vector controls (VC). RFU values are arbitrary fluorescence intensity units corrected for cell density. Results are averages of triplicate measurements on test tube cultures in mid-log growth phase.

cence than the wild-type with the reporter, indicating that the regulation of the reporter constructs works as anticipated (Fig. 3). The level of fluorescence of the Δ*wspF* strain with the reporter was comparable to that seen for the Δ*fleQ* strain with the reporter, indicating that the level of c-di-GMP present in the Δ*wspF* strain results in comparable derepression of the FleQ-regulated *cdrA* promoter activity.

Development of screening assays. To determine the kinetics of *gfp* transcription from the *cdrA* promoter, the reporters were grown in shake flasks and monitored for growth and fluorescence. Figure 4A shows that transcription from the *cdrA* promoter is related to the high levels of c-di-GMP induced during the early stationary growth phase and then kept at a constant high level over time.

To efficiently screen for compounds affecting the c-di-GMP level in *P. aeruginosa*, we developed a microtiter plate-based setup which allows screening of several compounds simultaneously. We grew dilutions of outgrown cultures in shake flasks until they reached the mid-logarithmic growth phase, after which they were transferred undiluted to a microtiter plate and incubated in a plate reader. As shown in Fig. 4B, the fluorescent kinetics resembled those seen with the shake flask setup, making the assay suitable for screening purposes.

As seen in Fig. 4, the unstable GFP (ASV) resulted in a lower ratio between strain Δ*wspF*Δ*pel*Δ*psl* and strain Δ*pel*Δ*psl* than was the case with the strains containing the stable GFP (Mut3). Consequently, to maximize the signal-to-noise level, the strains containing the stable GFP would be preferred for screening purposes. It is also evident from Fig. 4 that the high level of fluorescence obtained with the strain Δ*wspF*Δ*pel*Δ*psl* background will be better suited for screening purposes than the low level of fluorescence obtained with the strain Δ*pel*Δ*psl* background. The strain *P. aeruginosa* Δ*wspF*Δ*pel*Δ*psl*/pCdrA::*gfp* would therefore be preferred for screening in our setup.

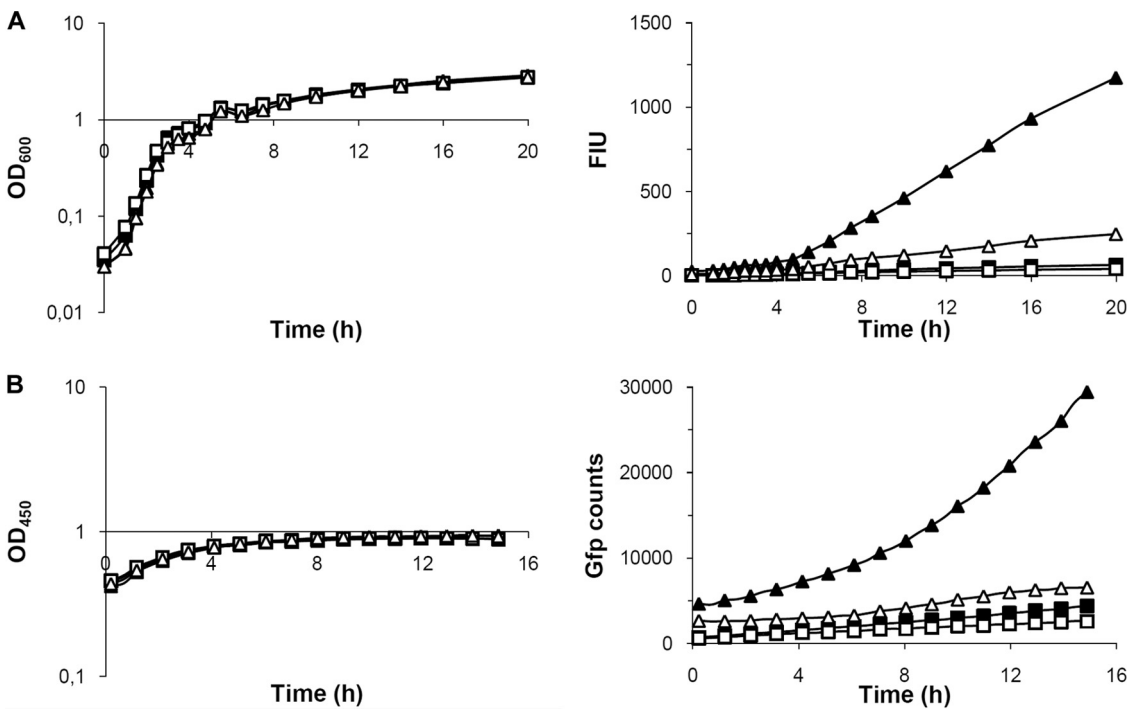

FIG 4 Test of the plasmid-based reporter strains in shake flasks (A) and microtiter plates (B). (Left) Growth measurements; (right) fluorescence measurements. Results are representative of three independent experiments. ■, *P. aeruginosa* PAO1 Δ*pel*Δ*psl*/pCdrA::*gfp*^{C} (normal c-di-GMP level, stable GFP); ▲, Δ*wspF*Δ*pel*Δ*psl*/pCdrA::*gfp*^{C} (increased c-di-GMP level, stable GFP); □, Δ*pel*Δ*psl*/pCdrA::*gfp*(ASV)^C (unstable GFP); △, Δ*wspF*Δ*pel*Δ*psl*/pCdrA::*gfp*(ASV)^C (unstable GFP).

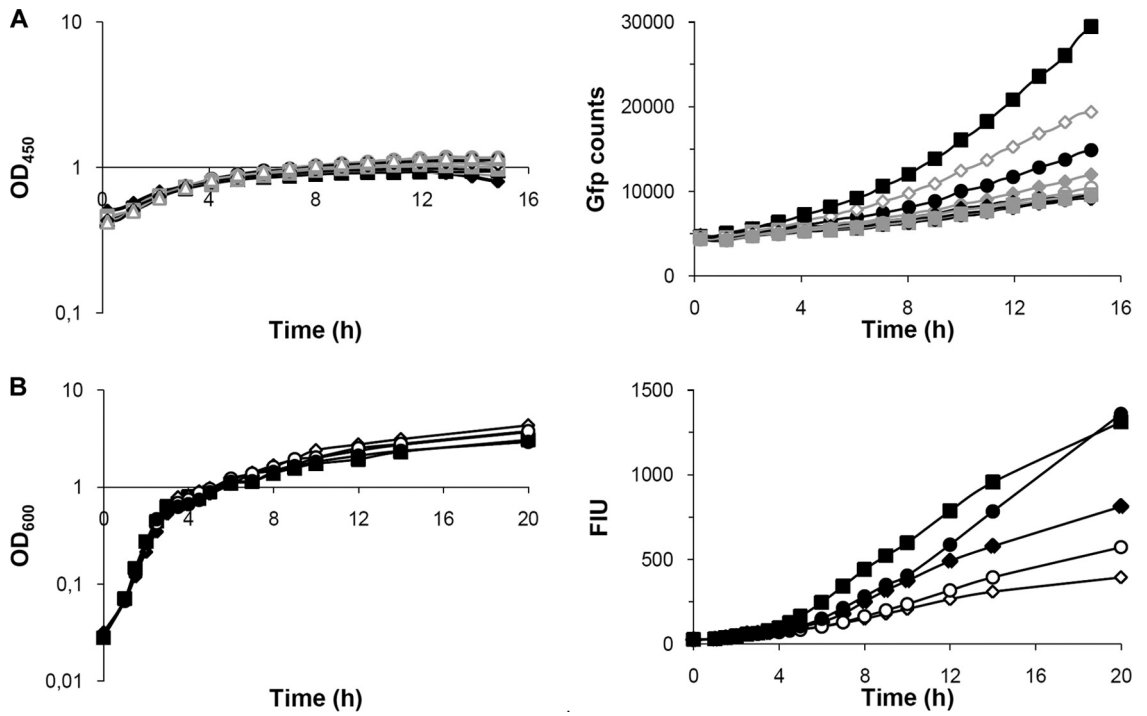

FIG 5 Treatment of *P. aeruginosa* PAO1 Δ*wspF*Δ*pel*Δ*psl*/pCdrA::*gfp*^{C} with SNP in microtiter plates (A) and shake flasks (B). (Left) Growth measurements; (right) fluorescence measurements. Results are based on the Copenhagen group of reporters and are representative of three independent experiments. ◆, 250 μM SNP; ■, 125 μM; □, 62.5 μM; ◇, 31.25 μM SNP; ▲, 15.63 μM SNP; △, 7.81 μM SNP; ●, 3.91 μM SNP; ○, 1.95 μM SNP; ◯, 0.977 μM SNP; ◆, 0.488 μM; ●, 0.244 μM; ◇, 0.122 μM SNP; ■, 0 μM SNP (untreated).

Proof of concept. We subsequently wanted to establish proof of concept that the reporter strain can be used to monitor drug-induced changes in the intracellular level of c-di-GMP. To this end, we treated the *P. aeruginosa* Δ*wspF*Δ*pel*Δ*psl*/pCdrA::*gfp*^{C} reporter with the compound sodium nitroprusside (SNP). SNP is a nitrogen monoxide (NO)-releasing compound that has been shown to induce dispersal of established *P. aeruginosa* biofilms, evidently by NO induction of a phosphodiesterase, thereby decreasing the intracellular levels of c-di-GMP (5, 6). Initially, the reporter strain was treated with 2-fold serial dilutions of SNP in the microtiter plate assay (Fig. 5A). The treatment showed that over a range of concentrations the compound decreased the fluorescent levels of the reporter without affecting growth, indicating that *cdrA*::*gfp* transcription was reduced as a result of decreased c-di-GMP levels within the bacteria. For a subset of concentrations, the results were verified with the more sensitive shake-flask setup (Fig. 5B). There was a direct correlation between the SNP concentration and decrease in fluorescence for all concentrations except the treatment with 250 μM SNP. At this high concentration, the level of NO released presumably could impose stresses on the bacteria, resulting in changes in the c-di-GMP metabolism, other than what can be attributed to PDE activation. These results indicate that the reporter can be used to monitor drug-induced changes in c-di-GMP levels.

Investigating inhibition of different DGCs. The results described above are based on a *wspF* background strain displaying a high level of fluorescence from our reporters due to the increased level of c-di-GMP stemming from WspR deregulation. However, *P. aeruginosa* contains several active DGCs that can all, presumably, affect cellular levels of c-di-GMP. It was important to determine if the reporters would respond to increased c-di-GMP levels originating from other DGCs, in addition to WspF, making it possible to test inhibitor candidates against multiple DGCs. To this end, the active DGC TpbB (33, 47) was cloned under the control of the arabinose-inducible promoter AraC-P_{BAD}, allowing conditional expression upon arabinose induction, and integrated into the chromosome for these studies. As shown in Fig. 6, overexpression of *tpbB* results in increased fluorescence from the c-di-GMP reporter at levels comparable to those for the Δ*wspF* strain. This result indicates that the reporters can be used to monitor effects on c-di-GMP levels based on the activity of additional DGCs other than WspR.

DISCUSSION

In the present paper, we have described the development of two similar series of fluorescent reporters that can form the basis for genetic and chemical biology approaches to alteration of the level of c-di-GMP in *P. aeruginosa* and potentially other bacteria as well. The reporters are based on transcriptional fusions between the c-di-GMP-responsive *cdrA* promoter and *gfp*. The *cdrA* promoter was chosen because transcriptome analyses indicate that the level of *cdrA* transcription is regulated by the level of c-di-GMP in the bacteria (9, 24). Without prior knowledge of the exact location of the regulatory elements within the *cdrA* promoter region, the Copenhagen group of reporters was constructed by amplifying 200 bp upstream of the start codon while at the same time omitting the sequence likely containing the native RBS. With a fusion of the amplified promoter region to *gfp* containing an optimized RBS, we were able to see a clear difference in the fluorescence level in the Δ*wspF* compared to the *wspF* wild-type background strain

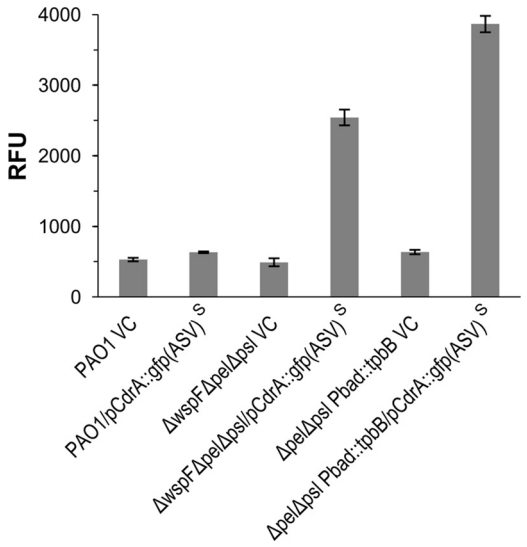

FIG 6 Fluorescence from *P. aeruginosa* PAO1/pCdrA::*gfp*(ASV)^S, Δ*wspF*Δ*pel* Δ*psl*/pCdrA::*gfp*(ASV)^S, Δ*pel*Δ*psl* P_{BAD}::*tpbB*/pCdrA::*gfp*(ASV)^S, and vector controls (VC). RFU values are arbitrary fluorescence intensity units corrected for cell density. Results are averages of triplicate measurements on test tube cultures in mid-log growth phase induced with 0.2% L-arabinose.

of *P. aeruginosa*. This difference is attributed to their known difference in internal c-di-GMP levels (24), and it was corroborated by LC-MS/MS analyses of the c-di-GMP content in the Δ*pel*Δ*psl* and Δ*wspF*Δ*pel*Δ*psl* background strains used in this study.

The Seattle group of reporters was created by amplifying a larger part of the *cdrA* promoter, including the native RBS and a small part of the coding sequence, and fusing it to promoterless GFP vectors. These reporters responded positively to an increased level of c-di-GMP in the same manner as the Copenhagen reporters, yielding a clear indication that the binding site for the c-di-GMP-responsive regulatory element had been included in both groups of constructs and that they function as expected. The regulatory element was shown to be the transcriptional regulator FleQ, as a *fleQ* mutant harboring a c-di-GMP reporter showed a high fluorescent output similar to that of a *wspF* mutant harboring the c-di-GMP reporter. This result is consistent with previous observations linking c-di-GMP to increased *cdrA* transcription via sequestration of FleQ (23). We anticipate that the c-di-GMP level reporters may work in other bacterial species that produce FleQ homologs. It is also likely that the reporters will work in other bacterial species if they are modified so that the *P. aeruginosa fleQ* gene is included in the reporter construct.

Traditionally, measurements of c-di-GMP levels have been made directly on the molecule using thin-layer chromatography or mass spectrometry on cellular extracts. These labor-intensive methods have the advantage of measuring the amount of c-di-GMP directly but are unsuitable for screening purposes. In addition, fluorescence-based measurements of c-di-GMP levels have been described recently. One study utilizes the discovery that thiazole orange fluoresces upon formation of a specific complex with c-di-GMP (38). The method requires lysis of the bacteria but is less laborious than the standard methods. However, it is sensitive to the presence of nucleic acids which bind thiazole orange and gives rise to fluorescence. Another method employs a genetically engineered c-di-GMP binding protein fused to two fluorescent proteins at the N and C termini (11). Binding of c-di-GMP induces a conformational change that decreases the amount of fluorescence observed due to fluorescence resonance energy transfer (FRET) between the fluorescent proteins. The method itself is ingenious, but the small amount of fluorescence usually obtained via FRET might be less than that required for sufficient sensitivity during screening. In contrast to these methods, we have created a cell-based c-di-GMP-sensitive reporter system similar to the ones employed in the discovery of quorum-sensing inhibitors in *P. aeruginosa*. An analogous approach has also recently been described in *E. coli* (3). The approach relies on increased binding of Congo red to cellulose produced by cells in response to elevated levels of c-di-GMP resulting from overexpression of the DGC AdrA. Compared to the present setup, the visual inspection of Congo red binding gives the assay a limited dynamic range less suitable for high-throughput screening where subtle differences in c-di-GMP levels are also of relevance.

The reporters in this study were constructed using both stable and unstable GFP and were made plasmid based or transposon integrated. From the epifluorescence microscope observations, it became clear that the plasmid-based reporters, as expected, showed the highest level of fluorescence and were best suited for screening purposes. In addition, testing of the plasmid-based reporter strains showed that the use of stable GFP gave adequate fluorescence levels, making the plasmid-based reporter with stable GFP the reporter of choice in the screening for compounds affecting c-di-GMP metabolism. The other reporter strains could still be employed in later steps of the process. The unstable GFP could be used to monitor the change in c-di-GMP levels during longer periods of growth in flow-cell setups investigating biofilm formation over time. Experiments using these reporters should provide information regarding spatiotemporal expression of *cdrA* in *P. aeruginosa* biofilms and would be valuable in describing the antipathogenic effects of specific compounds that decrease fluorescence from the reporter in the initial screen.

The deletion of *wspF* used to increase the level of c-di-GMP causes increased polysaccharide production and therefore intense clumping of cells grown in liquid culture, making growth monitoring by means of optical density impossible. For correct measurements of growth, the background strains used here carried mutations abolishing polysaccharide production. However, the inability to produce Pel and Psl polysaccharides results in a fluorescent output from the reporters lower than what is seen with polysaccharide-producing strains harboring the reporters (data not shown). This is in accordance with previously published reverse transcription-PCR data on *cdrA* expression showing the same trend (9). It thus appears that the presence of polysaccharides somehow affects c-di-GMP metabolism in *P. aeruginosa*.

Besides being able to distinguish the two background strains with different levels of c-di-GMP on the basis of their fluorescent output, it was crucial that the reporter showed sufficient sensitivity to gauge intermediate c-di-GMP levels stemming from changes in the metabolism caused by c-di-GMP level-altering compounds. We tested this by treatment of the c-di-GMP-overproducing reporter strain with the NO-releasing compound SNP. SNP has previously been shown to reduce c-di-GMP levels 2-fold in *P. aeruginosa* at two different μM concentrations (6). In our study, we could observe different degrees of changes in the fluorescence output over a range of concentrations, indicating that the reporter was sensitive enough to distinguish small changes in c-di-GMP

levels. Compared to the untreated reporter strain, the total decrease in fluorescence was roughly 2- to 3-fold for the lower concentrations of SNP tested, which is comparable to previous observations for the cellular c-di-GMP levels (6).

P. aeruginosa contains 33 GGDEF domain proteins (including domains with degenerate motifs) (33), of which one-fourth have been found, at present, to be active DGCs (24, 33, 35, 36). This has raised the question whether the intracellular pool of c-di-GMP is compartmentalized or global and how the phenotypes related to c-di-GMP signaling are regulated. Recent research shows that the two DGCs RoeA and SadC regulate different aspects of biofilm formation (37), indicating that c-di-GMP might be sequestered as local pools that are regulated by distinct DGCs and PDEs. This suggests that potent antipathogenic drugs targeting c-di-GMP production should be able to inhibit several DGCs. This could complicate our choice of working with a reporter background where WspR is the dominating DGC, due to the *wspF* deletion, contributing to the increased level of c-di-GMP. The use of this reporter background to discover DGC inhibitors will likely result in hits that mainly affect WspR. We showed, however, that overexpression of another DGC, TpbB, caused a similar increase in the fluorescent readout from the reporters, indicating that such constructs could favorably be used to test initial drug candidates against several DGCs, determining if they display a broad activity increasing the desired antipathogenic effect.

A comparative analysis of the residues comprising the active site of the different *P. aeruginosa* DGCs shows a high degree of similarity indicating that they share the same conformation and thus should be able to bind the same inhibitor (unpublished analysis). We therefore believe that it will be possible to identify compounds that inhibit several DGCs in *P. aeruginosa* and other bacteria and thus will be suitable leads in the development of potent antipathogenic compounds. In our future work, we will use the constructed reporters in screens for antipathogenic drugs and to obtain knowledge on basic *P. aeruginosa* biofilm biology.

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