The anti-cancerous drug doxorubicin decreases the c-di-GMP content in *Pseudomonas aeruginosa* but promotes biofilm formation

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Current antibiotic treatments are insufficient in eradicating bacterial biofilms, which represent the primary cause of chronic bacterial infections. Thus, there is an urgent need for new strategies to eradicate biofilm infections. The second messenger c-di-GMP is a positive regulator of biofilm formation in many clinically relevant bacteria. It is hypothesized that drugs lowering the intracellular level of c-di-GMP will force biofilm bacteria into a more treatable planktonic lifestyle. To identify compounds capable of lowering c-di-GMP levels in *Pseudomonas aeruginosa*, we screened 5000 compounds for their potential c-di-GMP-lowering effect using a recently developed c-di-GMP biosensor strain. Our screen identified the anti-cancerous drug doxorubicin as a potent c-di-GMP inhibitor. In addition, the drug decreased the transcription of many biofilm-related genes. However, despite its effect on the c-di-GMP content in *P. aeruginosa*, doxorubicin was unable to inhibit biofilm formation or disperse established biofilms. On the contrary, the drug was found to promote *P. aeruginosa* biofilm formation, possibly through release of extracellular DNA from a subpopulation of killed bacteria. Our findings emphasize that lowering of the c-di-GMP content in bacteria might not be sufficient to mediate biofilm inhibition or dispersal.

**INTRODUCTION**

Bacterial biofilms are multicellular bacterial communities that are omnipresent in nature (Costerton *et al.*, 1995; Wahl *et al.*, 2012). The organization, composition and dynamic of the biofilm community confer to the cells protection against threats from the environment, such as natural predators or exposure to harmful substances (Chiang *et al.*, 2012; Klebensberger *et al.*, 2009; Malone *et al.*, 2010; Mishra *et al.*, 2012; Tseng *et al.*, 2013). While this mode of life appears to be beneficial for the bacterial community, it is the cause of persistent infections in medical settings and fouling problems in various industrial settings (Ciofu *et al.*, 2015; Costerton *et al.*, 1999; Dosler & Karaaslan, 2014; Fleming, 2002; Hill *et al.*, 2005). In clinical settings, bacterial biofilms are difficult if not impossible to eradicate because of their tolerance towards conventional antibiotics and the host immune system (Ciofu *et al.*, 2015; Costerton *et al.*, 1999). They are responsible for diverse chronic infections and the cause of morbidity and mortality (Flores-Mireles *et al.*, 2015; Mayer-Hamblett *et al.*, 2014; Wenzel, 2007). Consequently, there is an urgent need to find new strategies, targets and compounds that can eradicate biofilms (Abraham, 2016; Blackledge *et al.*, 2013; Rasamiravaka *et al.*, 2015; Sharma *et al.*, 2014; Tolker-Nielsen, 2014). High-throughput screening using cell-based screens is a competitive approach to identify potential drug candidates. As a proof of concept, this approach has successfully identified the benzimidazole compound ABC-1, which lowers the...
c-di-GMP content in *Vibrio cholerae* and inhibits biofilm formation of both Gram-negative and Gram-positive bacteria (Sambanthamoorthy *et al.*, 2011).

The signal molecule c-di-GMP has been shown to positively control biofilm formation in numerous Gram-negative bacteria and in some Gram-positive bacteria (Hengge *et al.*, 2016; Purcell *et al.*, 2012). c-di-GMP is synthesized by diguanylate cyclases (DGCs) containing a GGDEF domain and is degraded by phosphodiesterases (PDEs) containing either an EAL domain or an HD-GYP domain (Hengge, 2009). Those domains are conserved amongst various bacterial species, making the enzymes involved interesting new targets for biofilm prevention and dispersal (Kostakioti *et al.*, 2009). Those domains are conserved amongst various bacteria.

To identify molecules targeting the c-di-GMP metabolism in bacteria, we recently developed a fluorescent-based monitor capable of gauging the intracellular content of c-di-GMP in *Pseudomonas aeruginosa* (Rybtke *et al.*, 2012). *P. aeruginosa* is an important opportunistic pathogen capable of causing numerous severe chronic infections due to biofilm formation and is a model organism for biofilm research (Tolker-Nielsen, 2014).

In an effort to identify novel anti-biofilm compounds, we screened a chemical library containing 5000 low-molecular weight chemical compounds, using our *P. aeruginosa* monitor strain to identify compounds that are able to decrease the bacterial c-di-GMP content. One compound that was capable of lowering the output of the monitor substantially was the anthracycline doxorubicin. This compound also reduced the transcription of several genes involved in biofilm formation. However, we found that lowering of c-di-GMP levels through doxorubicin treatment did not inhibit or disperse *P. aeruginosa* biofilms. Although c-di-GMP levels were significantly reduced in the presence of doxorubicin, biofilm production was increased, most likely due to increased release of extracellular DNA (eDNA) from the bacteria.

**METHODS**

**Media, strains and plasmids.** Strains and plasmids used in this study are described in Table 1. Cell-based screening, c-di-GMP extractions and transcriptomic analysis were performed using *P. aeruginosa* Δ*pelA Δaps*Δ*pslBCD* transformed with the pCdrA::gfp’ plasmid (Rybtke *et al.*, 2012). Biofilm inhibition and dispersal assays were performed using either the PAO1 wild-type (Stover *et al.*, 2000) or the PAO1 Δ*pelA Δaps*Δ*pslBCD* strain (Rybtke *et al.*, 2012). Expression and purification of the WspR protein were performed using *Escherichia coli* Rosetta (GE Life Sciences). All overnight cultures were grown in lysogeny broth (LB) medium (Difco). Cell-based screening and c-di-GMP extractions were performed in ABtraceGC composed of AB medium (Clark & Maaloe, 1967) supplemented with 0.2% glucose and 0.5% casamino acids and 1:1000 (v/v) of a trace metal stock solution (Pamp & Tolker-Nielsen, 2007). Dispersal and inhibition assays were performed in ABGFe3.

**Screening process.** An overnight culture of PAO1 Δ*pelA Δaps*Δ*pslBCD* was diluted 1:100 in ABtraceCCG and 100 µM well−1 was distributed into a 96-well plate (Nunc ref. no. 265300). Compounds at a concentration of 100 µM or DMSO at a concentration of 1 % (v/v) were added to the cultures. Before starting the experiment, we measured OD590 nm and GFP fluorescence (excitation, 485 nm; emission, 535 nm) using a VICTOR plate reader (Perkin Elmer). Plates were then incubated overnight at 37 °C at 290 r.p.m. After 16 h of incubation, OD590 nm and GFP fluorescence were measured again. Activity of the compound was estimated from the relative fluorescence unit (RFU), i.e. \( \frac{(OD_{final} - OD_{0})}{(OD_{0} - OD_{b})} \), and the relative activity of the compound, i.e. \( \frac{RFU_{treated} - RFU_{untreated}}{RFU_{untreated}} \).

**Compound library.** Our library of low-molecular weight chemical compounds consists of various commercial and non-commercial sub-libraries, 5000 compounds in total. The majority of the compounds were obtained from BioFocus (Galapagos) and consist of sub-libraries targeted versus protein classes, including protein kinases, ion channels, G-protein-coupled receptors, proteases, purine-binding proteins and protein–protein interaction motifs. The library also includes the Calbiochem InhibitorSelect library of 244 pre-clinically tested protein kinase inhibitors (Merck). The molecular weight of the compounds ranges from 200 to 1000 Da, with the majority of them being between 300 and 500 Da. All compounds in the library were solubilized in DMSO at a concentration of 10 mM and stored at −20 °C.

**Compound preparation.** For screening purposes, compounds other than the screening library were dissolved in DMSO. Stock solutions were 10 mM, except for SNP (Sigma Aldrich) that was prepared to a stock solution of 5 mM. For biofilm assays, doxorubicin hydrochloride powder was dissolved in sterile milliQ water to obtain a stock solution of 80 mM. This solution was kept at 4 °C for up to a month. For MAHMA NONOate (Sigma Aldrich), a stock solution of 5 mM was freshly prepared on the day in 10 mM NaOH and kept at 4 °C.

**Biofilm inhibition and dispersal assays.** An overnight culture was diluted 200-fold in ABGFe3 and was used as inoculum to prepare successive fourfold dilution cultures, and then 200 µl well−1 was distributed into a 96-well plate (TPP no. 92096). The plate was incubated at 37 °C at 452 r.p.m. (3 mm amplitude in linear mode) into a Tecan Infinite 200Pro plate reader for 18 h. Growth was monitored over time by continuous OD590 nm measurements. In biofilm inhibition assays, compounds were added from the beginning of the experiment. In dispersal assays, compounds were added after 18 h of incubation after which incubation in the Tecan plate reader was continued.

**DNase assays.** An overnight culture of PAO1 or PAO1 Δ*pelA Δaps*Δ*pslBCD* was diluted 1:200 in ABGFe3, Briefly, 150 µl well−1 of culture was dispensed into a 96-well plate (TPP no. 92096), incubated at 37 °C with 220 r.p.m. in a shaker incubator (IKA KS 4000 ic control). After 18 h of incubation, cultures were aspirated using a multipipette, and wells were washed three times in 200 µl of 0.9% saline solution. Then 200 µl of DNase buffer without or with DNase 0.1 U µl−1 (ROMEGA) was dispensed per well. The plate was then incubated at 37 °C for 80 min at 88 r.p.m. The amount of biofilm in each well was subsequently quantified as described below.

**Biofilm quantification using crystal violet staining.** Biomass of biofilms grown in a batch system was quantified using crystal violet (CV) staining. Briefly, after incubation, cultures were aspirated using a multipipette. Biofilms were washed three times in 280 µl of milliQ water
Table 1. Bacterial strains and plasmids used in the study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant features or genotype</th>
<th>Reference or source</th>
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</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
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<tr>
<td><em>P. aeruginosa</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO1 WT</td>
<td>Sequenced PAO1</td>
<td>Stover et al. (2000)</td>
</tr>
<tr>
<td>PAO1 ΔpelAΔpslBCD</td>
<td>gfp (mut3) tagged on chromosome</td>
<td>This study</td>
</tr>
<tr>
<td>PAO1 ΔpelAΔpslBCD</td>
<td>ΔpelAΔpslBCD; impaired aggregation</td>
<td>Rybtke et al. (2012)</td>
</tr>
<tr>
<td>PAO1 Δ wspFΔpelAΔpelBCD</td>
<td>Δ wspFΔpelAΔpslBCD; high c-di-GMP; impaired aggregation</td>
<td>Rybtke et al. (2012)</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rosetta</td>
<td>DB3 optimized for protein expression</td>
<td>GE Life Sciences</td>
</tr>
<tr>
<td><strong>Plasmid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pΔΔC14::gfp (mut3)</td>
<td>pUCP22Not-PΔΔC14::rbsI1-gfp(Mut3)-T0-T1, AmpR GmR</td>
<td>Rybtke et al. (2012)</td>
</tr>
<tr>
<td>pET21::wspR-HIS</td>
<td>Expression vector for WspR WT-HIS, IPTG inducible, AmpR</td>
<td>De et al. (2008)</td>
</tr>
<tr>
<td>pGEX-6P-2::GST-wspR WT</td>
<td>Expression vector for GST-WspR WT, IPTG inducible, AmpR</td>
<td>This study</td>
</tr>
</tbody>
</table>

and the plate was air dried at 37 °C. Biofilms were stained using 250 μl of 0.1% CV well−1 for 15 min. Stained biofilms were washed three times, and the plate was air dried at 37 °C. Biofilm biomass was determined by resuspending stained biofilms in 200 μl of 30% acetic acid for 10 min, followed by OD590 nm readings using a VIKTOR plate reader (Perkin Elmer).

c-di-GMP measurements. Bacterial cultures were grown at 37 °C under continuous agitation (200 r.p.m.) in a 24-well flat, clear-bottom plate (TPP). Growth was monitored over time at OD590 nm and cells were harvested in the late exponential phase. Nucleotides were extracted using an established protocol (Spangler et al., 2010). Briefly, 1 ml of culture was spun down for 2 min at 15000 r.p.m. at 4 °C. Cells were then washed once in 1 ml of ice-cold 0.9% saline. Nucleotides were extracted using an ice-cold extraction buffer composed of acetonitrile/methanol/water (40%:40%:20%, by vol.). Resuspended cells were left on ice for 15 min followed by a heat shock (95 °C, 10 min), cooled down (ice bath, 3 min) and then spun down (15000 r.p.m., 10 min at 4 °C). Extraction steps were repeated twice with 200 μl of extraction solvent at 4 °C, omitting the heat shock step. The solvent was subsequently removed in a Speedvac at 60 °C, and quantification of c-di-GMP was performed by HPLC-coupled MS-MS as previously described (Burhenne & Kaever, 2013) with the exception that an LC-10AD VP HPLC system (Shimadzu) and an API4000 mass spectrometer (Sciex) were used. c-di-GMP concentrations were normalized to the total protein concentration. Total protein concentrations were determined using a cell pellet equivalent to the extracted cultures using the Pierce 660 nm Protein Assay (Thermo Scientific Cat. no. 22660) according to the manufacturer’s protocol.

RNA extraction. An overnight culture of PAO1 Δ wspFΔpelAΔpslBCD was diluted to an OD590 nm of 0.1 in ABtraceCG, and 100 μM doxorubicin was added for treatment. Cultures were then distributed with 2 ml per well in a 24-well plate. Cells were incubated at 37 °C at 200 r.p.m. until late exponential phase. RNA was extracted using the Qiagen miRNAeasy Mini Kit (Cat. no. 74104) and purified using the kit ‘RNA Clean and Concentrator-5’ following the manufacturer’s instructions. Approximately 10 μg of RNA per sample was treated with DNase I (Ambion) 15 min at 37 °C. This step was repeated twice to remove any DNA residues. RNA integrity was determined using the Agilent Tape Station and RNA Screen Tape. RNA extracts with an RNA integrity number above 8.0 were considered suitable for transcriptome analysis and quantified. RNA and DNA were quantified, respectively, using the Qubits kits RNA HS assays and Qubit dsDNA HS Assay Kit (Invitrogen) following the manufacturer’s protocol. Samples with an RNA:DNA ratio inferior to 10% were subjected to RNA depletion using the Ribo-Zero magnetic kit (bacteria) (Epicentre). An RNA-depleted RNA sample was used to perform transcriptome analysis.

RNA sequencing and data analysis. Gene expression analysis (three biological replicates) was conducted by RNA sequencing (RNA-Seq technology). The rRNA-depleted RNA was fragmented to 200–300 bp fragments, and then first- and second-strand cDNA was synthesized, followed by end repair and adapter ligation. After 12 cycles of PCR enrichment, the quality of the libraries was assessed using Bioanalyzer (Agilent Technologies, USA). The libraries were sequenced using the Illumina HiSeq 2500 platform with a paired-end protocol and read lengths of 100 nucleotides. The RNA-Seq data have been deposited in the NCBI Short Read Archive database with accession code SRP074218. The sequence reads were uniquely mapped to the PAO1 reference genome using CLC Genomics Workbench 8.0 (https://www.qiagenbioinformatics.com/). The following criteria were used for mapping: minimum length fraction of 0.9, minimum similarity fraction of 0.8, maximum number of two mismatches and maximum number of hits for a read being set to 1. The differentially expressed genes (fold change >2, Benjamini–Hochberg adjusted P value <0.05) between doxorubicin-treated and -untreated cells were identified by performing a negative binomial test using the R package DESeq (Anders & Huber, 2010).

Construction of WspR expression vector. Dr Sondermann (Cornell University) kindly provided us with the pET21::wspR-HIS plasmid. In order to optimize production and purification of the WspR protein, we changed the HIS-tag to a GST-tag. To do so, we subcloned a DNA fragment encoding the WspR protein into pGEX-6P-2. Firstly, using the primers WspR-his-fwd containing an EcoRI restriction site (italics) 5'-AAAAAATTCACAAAACCCTCATTGAGAGCAA-3' and WspR-his-rev 5'-GATCAGCAGATTCTCCTGGA-3', we generated a fragment encoding WspR. After gel purification using the kit Wizard SV Gel and PCR Clean-Up System (Cat. no. A9282) following the manufacturer’s instructions, we digested pGEX-6P-2 and the fragment with EcoRI and NotI (site lying upstream of the HIS-tag) (Sigma Aldrich). After gel purification using the kit Wizard SV Gel and PCR Clean-Up System (Promega) as previously described, we introduced the EcoRI-NotI fragment into EcoRI-NotI digested pGEX-6P-2 giving rise to pGEX-6P-2::GST-wspR. The plasmid was transformed into E. coli Rosetta, and transformants were plated on LB 1.5% agar containing 100 μg ml−1 of ampicillin. To verify the construct, we extracted the plasmid using the QiAprep Spin Miniprep Kit (Qiagen), and we sequenced it using the primer M13-fwd 5'-TAAACCCGACGCTCA-3' and WspR-seq1-rev 5'-GATCAGCAGATTCTCCTGGA-3'.
**WspR expression and purification.** The *E. coli* Rosetta strain containing the pGEX-6P-2::GST-wspR plasmid was grown at 37 °C in 11 of LB supplemented with 400 ml YTT and 50 µg ml⁻¹ ampicillin. At an OD₆₀₀ of 0.8, the growth flask was transferred to a 18 °C bath for 30 min, after which expression was induced with 0.1 mM IPTG for 16 h at 18 °C. The cell pellet was recovered by centrifugation at 3000 g for 20 min and solubilized in 40 ml lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10 % glycerol, 5 mM DTT, 0.5 % CHAPS, 2 % Triton X-100 and 1 × Complete Protease inhibitor cocktail (Roche biochemicals)] and was lysed using a French press. The soluble protein lysate was cleared by centrifugation at 20000 g for 20 min, before being passed through twice on a column with 3 ml of Glutathione-Sepharose FF (GE Healthcare). Hereafter, the resin was washed with 20 column volumes of wash buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10 % glycerol, 1 mM DTT, 0.1 % CHAPS, 0.4 % Triton X-100 and 0.1 × Complete Protease inhibitor cocktail) and 10 column volumes of Elution buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10 % glycerol and 1 mM DTT). The bound GST fusion protein was then eluted with Elution buffer supplemented with 15 mM reduced glutathione, collecting fractions of 0.5 ml. Fractions were analysed by SDS-PAGE, and positive fractions were pooled and dialysed against Elution buffer without glutathione; protein contents were measured.

**Determination of WspR DGC activity.** DGC activity of WspR enzymes was assayed by measuring the production of inorganic phosphate (Pi) using the Enzchek pyrophosphate assay kit (Invitrogen). The reaction mixtures were set up as specified by the manufacturer except that the MgCl₂ concentration was adjusted to 10 mM. Assays were performed in a 96-well optical bottom plate (Nunc, Thermo Scientific), in 100 µl of mixture per well. We incubated 1.25 µM enzyme with or without compound in the reaction mix for 40 min at room temperature before starting the reaction by the addition of 62.5 µM GTP. The formation of c-di-GMP from two GTP molecules by DGC releases two molecules of PPI, which are subsequently degraded into four inorganic phosphates (Pi) by pyrophosphatases. Pi is then used by purine nucleoside phosphorylases to degrade a substrate (2-amino-6-mercaptopurine ribonucleoside) leading to an increase in absorbance at 360 nm. The DGC activity is measured by monitoring, over time, the change in absorbance at 360 nm.

**RESULTS**

**Establishment of a screening assay for identifying compounds that lower the c-di-GMP content in *P. aeruginosa***

To identify molecules that decrease the c-di-GMP level in *P. aeruginosa*, we screened an in-house library of 5000 chemical compounds, using a *P. aeruginosa* fluorescent reporter strain capable of monitoring c-di-GMP levels (Rybtke et al., 2012). The strain contains a plasmid-based cdrA-gfp transcriptional reporter construct, which is positively regulated by c-di-GMP (Rybtke et al., 2012). For the *P. aeruginosa* wild-type, c-di-GMP levels are below the detection limit of our reporter construct during planktonic growth (Rybtke et al., 2012). However, the monitor strain is engineered with a monitor strain also harbours pelA and pslBCD mutations to avoid clumping during planktonic growth, rendering the cells unable to produce Psl and Pel exopolysaccharides. By monitoring GFP fluorescence and optical density of our reporter strain cultures at the beginning and the end of the assay, we were able to identify compounds that decrease the GFP fluorescence level without affecting growth by more than 20 %. As all of our compounds were dissolved in DMSO, a corresponding DMSO solution was used as a negative control. SNP was used as a positive control in our cell-based screening. SNP is a nitric oxide (NO) donor that has been reported to disperse the biofilm of *P. aeruginosa* by lowering the cellular c-di-GMP content (Barraud et al., 2009; Li et al., 2013), and has been shown to lower the GFP output of our c-di-GMP monitor strain (Rybtke et al., 2012). In our screening assay, SNP lowers the relative fluorescence GFP output by about 65 % (Fig. 1).

**Fig. 1.** Doxorubicin decreases the output of the c-di-GMP monitor strain. The c-di-GMP monitor strain was subjected to treatment with 100 µM doxorubicin in our cell-based assay. DMSO (1 %) was used as negative control for the untreated sample, and SNP (50 µM) was added as a positive control for c-di-GMP lowering. RFU values are arbitrary fluorescence intensity units divided by the optical density of the cultures. Data are representative of three independent biological experiments. Error bar represents the variation between three technical replicates.

**Doxorubicin lowers the intracellular level of c-di-GMP in *P. aeruginosa***

By screening our compound library, we found that the anticancerous drug doxorubicin at a concentration of 100 µM decreased the GFP output of our monitor strain by 50 % (Fig. 1), without drastically affecting growth (see Fig. S1, available in the online Supplementary Material). In order to confirm that the lowering of the GFP fluorescence was due to a decrease in the cellular c-di-GMP content, we quantified the intracellular concentration of c-di-GMP using HPLCMS-MS. As shown in Fig. 2, treatment with 50–100 µM doxorubicin reduced intracellular c-di-GMP levels by up to 54 % in our *P. aeruginosa* reporter strain. However, doxorubicin
concentrations of 25 µM and below had no effect on the c-di-GMP content in *P. aeruginosa*.

**Doxorubicin does not impact DGC activity of the WspR protein**

In an attempt to gain insight into the mechanism by which doxorubicin decreases cellular c-di-GMP levels, we first hypothesized that doxorubicin might exert its effect through inhibition of c-di-GMP synthesis by preventing the enzymatic activity of DGCs. As mentioned previously, our monitor strain mainly relies on the production of c-di-GMP through the WspR. Thus, we chose to investigate whether doxorubicin was lowering c-di-GMP levels in our monitor strain by inhibiting the enzymatic activity of WspR. To produce a molecule of c-di-GMP, DGCs convert two GTP molecules into one molecule of c-di-GMP and two pyrophosphate molecules. We measured the generation of pyrophosphate released by purified WspR protein supplemented with GTP in the presence and absence of doxorubicin. We measured the generation of pyrophosphate released by purified WspR protein supplemented with GTP in the presence and absence of doxorubicin. We measured the generation of two pyrophosphate molecules.

Doxorubicin does not affect the activity of the WspR protein.

Activity of the WspR protein was assessed in the presence (square symbols) or absence (triangle symbols) of 100 µM doxorubicin until late exponential phase. As shown in Fig. 3, the cyclase activity of WspR was not noticeably impacted by doxorubicin.

**Doxorubicin promotes *P. aeruginosa* biofilm formation**

Our screening identified doxorubicin as a c-di-GMP-decreasing compound, and our transcriptomic data suggested PDE, leading to a decrease in the c-di-GMP level and to biofilm dispersal (Roy et al., 2012). As the *P. aeruginosa* genome encodes multiple genes involved in biosynthesis and degradation of c-di-GMP, we wondered whether doxorubicin was affecting the transcription of genes encoding PDEs or DGCs. Accordingly, we performed a transcriptional profiling of *P. aeruginosa* Δ wspFΔpelAΔ pslBCD cells with or without doxorubicin. The *P. aeruginosa* Δ wspFΔpelAΔ pslBCD strain was chosen for the transcriptome analysis because prior work has indicated that some biofilm dispersal pathways can only be induced in bacteria with high c-di-GMP levels (Petrova & Sauer, 2012). However, no decrease in the transcription of known DGC genes or increase in the transcription of known PDE genes was observed (Table 2). Yet, our transcriptomic data indicated that the presence of doxorubicin decreases the expression of several genes involved in biofilm formation in *P. aeruginosa* (Table 2). We observed a fivefold decrease in the transcription of the cdrAB operon (Table 2), which not only confirmed the data generated using the cdrA-gfp reporter construct but also showed that doxorubicin is able to lower the expression of an adhesin important for biofilm formation (Borlee et al., 2010). Furthermore, it was found that doxorubicin lowered the transcription of genes encoding type IV pili, Cup fimbriae and LecA lectin (Table 2), which are all shown to play a role in biofilm formation by *P. aeruginosa* (Diggel et al., 2006; Giraud et al., 2011; Klausen et al., 2003; Wei & Ma, 2013). All genes that were up- or down-regulated more than twofold in the presence of doxorubicin are listed in Tables S1 and S2.

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**Fig. 2.** Doxorubicin lowers the intracellular content of c-di-GMP in the Δ wspFΔpelAΔ pslBCD monitor strain. Cultures were grown with 0, 25, 50 or 100 µM doxorubicin until late exponential phase, and then c-di-GMP was extracted and quantified by the use of HPLC-coupled tandem mass spectrometry. Error bars represent the standard error of the mean of at least three biological duplicates. Statistical significance was calculated using a one-way ANOVA test, non-parametric data set, **P<0.01, *P<0.05.

**Fig. 3.** Doxorubicin does not affect the activity of the WspR protein. Activity of the WspR protein was assessed in the presence (square symbols) or absence (triangle symbols) of 100 µM doxorubicin, by the use of a pyrophosphatase assay. Error bars represent the variation among two technical replicates.
that it also lowers the transcription of biofilm-related genes.
To assess the potency of doxorubicin as a biofilm inhibitor
compound, we grew biofilms in the wells of microtitre trays
in the presence or absence of doxorubicin. Unexpectedly, at
50 and 100 µM, concentrations necessary to lower the c-di-
GMP content of the reporter strain, doxorubicin increased
P. aeruginosa biofilm formation markedly (Fig. 4).

**Doxorubicin does not disperse P. aeruginosa biofilms**

We then queried whether the c-di-GMP decrease mediated
by doxorubicin would be sufficient to disperse established
P. aeruginosa biofilms in the same batch system used for
our biofilm inhibition assays. It has been shown that 50 µM
MAHMA NONOate, as a NO donor, could induce dispersal
of P. aeruginosa biofilms in less than 30 min (Barnes et al.,
2013). Thus, we used 50 µM MAHMA NONOate as a
positive control for our dispersal experiments. MAHMA
NONOate was found to disperse up to 50 % of 18 h-old
P. aeruginosa biofilm after 25 min of treatment (Fig. 5).
However, doxorubicin did not disperse established biofilm
after 25 min of treatment but, on the contrary, promoted
biofilm formation after 85 min of treatment (Fig. 5).

**Doxorubicin promotes eDNA-dependent P. aeruginosa biofilm formation**

In our screening set-ups, we noticed that 100 µM doxorubi-
cin, a concentration needed to lower cellular c-di-GMP con-
tents by 50 %, decreased planktonic growth yield by about
15 %. In addition to its effect on c-di-GMP, doxorubicin
seems to exert a slight cytotoxic effect on P. aeruginosa. After
the biofilm formation and biofilm dispersal assays described
above, we quantified the number of colony-forming units in

**Table 2. Effect of doxorubicin on the transcription level of selected genes involved in biofilm formation, SOS response and DNA repair**

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Locus tag</th>
<th>Fold change</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>cdrA</td>
<td>PA4625</td>
<td>−5.5</td>
<td>CdrA, secreted adhesin</td>
<td>Borlee et al. (2010)</td>
</tr>
<tr>
<td>cdrB</td>
<td>PA4624</td>
<td>−4.5</td>
<td>CdrB, putative outer membrane transporter, type 5 secretion system</td>
<td>Borlee et al. (2010)</td>
</tr>
<tr>
<td>cidA</td>
<td>PA3432</td>
<td>3.2</td>
<td>CidA, putative holin protein</td>
<td>Ma et al. (2009)</td>
</tr>
<tr>
<td>cidB</td>
<td>PA3431</td>
<td>2.5</td>
<td>CidB, putative protein</td>
<td>Ma et al. (2009)</td>
</tr>
<tr>
<td>cupE−E5</td>
<td>PA4648−</td>
<td>−2.6 to −4.9</td>
<td>CupE, pilin subunits required for fimbriae assembly</td>
<td>Giraud et al. (2011)</td>
</tr>
<tr>
<td>last</td>
<td>PA1432</td>
<td>2.4</td>
<td>Autoinducer synthesis protein</td>
<td>Passador &amp; Cook (1993)</td>
</tr>
<tr>
<td>lecA</td>
<td>PA2570</td>
<td>−2.2</td>
<td>LecA, heterophilic cell–cell adhesion</td>
<td>Diggle et al. (2006)</td>
</tr>
<tr>
<td>lexA</td>
<td>PA3007</td>
<td>15.0</td>
<td>LexA, repressor protein</td>
<td>Cirz et al. (2006)</td>
</tr>
<tr>
<td>pprA</td>
<td>PA4293</td>
<td>−2.1</td>
<td>PprA, induces cupE, involved in early step of biofilm formation</td>
<td>de Bentzmann et al. (2012)</td>
</tr>
<tr>
<td>pqsH</td>
<td>PA2587</td>
<td>2.6</td>
<td>PqsH, probable FAD-dependent monooxygenase; required for pseudomonas quinolone signal (PQS) production</td>
<td>Gallagher et al. (2002)</td>
</tr>
<tr>
<td>recA</td>
<td>PA3617</td>
<td>9.2</td>
<td>RecA, DNA repair</td>
<td>Cirz et al. (2006)</td>
</tr>
<tr>
<td>recN</td>
<td>PA4763</td>
<td>19.0</td>
<td>RecN, DNA repair</td>
<td>Rostas et al. (1987)</td>
</tr>
</tbody>
</table>

the biofilm and planktonic phase and found that 100 µM
doxorubicin reduced the final yield of the population by up
to one log under these conditions (Figs S2 and S3). This is in
agreement with the fact that doxorubicin is an antibiotic
(Arcamone et al., 2000) and has been reported to be highly
bactericidal, especially against Gram-positive bacteria such

![Fig. 4. Doxorubicin increases biofilm formation of PAO1. Biofilms were grown in microtitre plates for 18 h in the absence (untreated) or presence of 25, 50 or 100 µM doxorubicin. After 18 h of biofilm formation, the biofilm biomass was quantified by the use of a CV assay. Results are representative of two independent experiments. Error bars represent the variation among three technical replicates.](image-url)
as *Staphylococcus* (Peiris & Oppenheim, 1993). We hypothesized that the increase in biofilm formation mediated by doxorubicin could be due to release of DNA from a subpopulation of lysed bacteria, as eDNA has previously been reported to function as a biofilm matrix component (Alhede et al., 2011; Allesen-Holm et al., 2006; Barken et al., 2008; Chiang et al., 2013; Whitchurch et al., 2002; Yang et al., 2007). In that case, doxorubicin might induce biofilm formation independently of c-di-GMP-regulated biofilm matrix components such as Psl and Pel. Accordingly, we tested the ability of our PAO1 ΔpelAΔpslBCD mutant strain to form biofilm in the presence of doxorubicin. As expected, PAO1 ΔpelAΔpslBCD without doxorubicin failed to form a robust biofilm compared to the PAO1 wild-type (Fig. 6a). However, PAO1 ΔpelAΔpslBCD treated with doxorubicin formed a dense biofilm (Fig. 6a). The biofilm formed by the PAO1 ΔpelAΔpslBCD strain in the presence of doxorubicin was highly sensitive to treatment with DNase I. While the biofilm treated with DNase buffer remained, the biofilm treated with DNase I completely dispersed (Fig. 6b). These data strongly suggest that the increase in *P. aeruginosa* biofilm formation observed after treatment with 50 and 100 µM doxorubicin is due to release of eDNA from a subpopulation of dead bacteria.

**DISCUSSION**

Several studies have shown that it is possible to modulate c-di-GMP metabolism in bacteria to impact on biofilm formation. In *P. aeruginosa*, previous studies have demonstrated that overexpression of the PDE YhjH lowers c-di-GMP levels and leads to biofilm inhibition and dispersal (Christensen et al., 2012). NO has been shown to activate the PDE NbdA (Li et al., 2013), while an upshift in glutamate availability has been shown to activate the DGC NicD, leading to activation of the PDE DipA (Basu Roy & Sauer, 2014). In both cases, the activation of endogenous PDEs causes a decrease in c-di-GMP content leading to biofilm dispersal. In *P. aeruginosa*, the biofilm dispersal effect mediated by low c-di-GMP levels has been shown to be multifactorial. Low c-di-GMP levels have been shown to reduce the production of biofilm matrix components such as Psl, Pel, CdrA and Cup fimbria, and activate bacterial motility (Chua et al., 2014; Hickman et al., 2005). Evidence has also been provided that low c-di-GMP levels lead to up-regulation of the activity of the periplasmic LapG protease that cleaves off the surface-associated protein CdrA, which tethers the cells to the Psl matrix during biofilm formation (Cooley et al., 2015; Rybtke et al., 2015).

In this study we developed a successful high-throughput strategy to identify compounds that lower the c-di-GMP level in *P. aeruginosa*. Our screening showed that doxorubicin, a Food and Drug Administration-approved anticancerous drug, lowered the output of our c-di-GMP monitor by 50 % at a concentration of 100 µM. This result was confirmed by LC-MS-MS measurements of the c-di-GMP content in doxorubicin-treated and -untreated *P. aeruginosa ΔwspFApelAΔpslBCD* cells. Because our c-di-GMP monitor strain has elevated c-di-GMP levels due to reduced c-di-GMP content in doxorubicin-treated cells compared to untreated cells, we conclude that the observed decrease in c-di-GMP levels is due to treatment with doxorubicin. This finding is consistent with previous studies showing that doxorubicin decreases c-di-GMP levels in *P. aeruginosa* (Christensen et al., 2012). Our results suggest that doxorubicin may be a promising candidate for the development of new strategies to prevent biofilm formation in *P. aeruginosa*. Further studies are needed to investigate the mechanism by which doxorubicin decreases c-di-GMP levels and its potential as a therapeutic agent for biofilm-related infections.
constitutive activation of the DGC WspR, we first queried whether c-di-GMP lowering caused by doxorubicin was due to direct inhibition of WspR DGC activity. However, no significant doxorubicin-mediated inhibition of WspR activity was observed using an in vitro enzymatic assay. We then queried whether doxorubicin could exert its effect on c-di-GMP content by modulating at a transcriptional level the expression of enzymes implicated in c-di-GMP metabolism. No effect at the transcriptional level on any known DGC or PDE gene was revealed by our transcriptomic analysis. However, the drug decreases the transcription of multiple genes involved in biofilm formation and maintenance, notably those encoding the CdrA adhesin, Cup fimbria, LecA lectin and type IV pili (Barken et al., 2008; Borlee et al., 2010; Diggle et al., 2006; Giraud et al., 2011; Klausen et al., 2003). In addition to corroborating the decrease in cellular c-di-GMP content observed under doxorubicin treatment, this result was promising regarding the effect of the drug on biofilms. The cdrAB and pel genes are co-regulated (Borlee et al., 2010); however, we performed our transcriptome analysis on our c-di-GMP monitor strain, P. aeruginosa ΔwspF-ΔpelAΔpslBCD, and consequently could not monitor pel and psl transcripts.

Despite its effect on the c-di-GMP level and genes known to be involved in biofilm formation, doxorubicin promoted biofilm formation of P. aeruginosa. Doxorubicin is known to cause death of eukaryotic cells by preventing DNA transcription through intercalating into the DNA (Tacar et al., 2013), and also is known to act as an antibiotic targeting especially Gram-positive bacteria. We wondered whether the drug could have a cytotoxic effect on P. aeruginosa cells resulting in release of DNA. Accordingly, we found that doxorubicin at a concentration of 100 µM decreased the yield of P. aeruginosa cultures by about 15 %. For many bacteria, eDNA is a major biofilm matrix component (Allesten-Holm et al., 2006; Ma et al., 2009; Qin et al., 2007; Whitchurch et al., 2002). In the case of P. aeruginosa, eDNA originating from temporal and controlled cell lysis of a small subpopulation of the bacteria through activation of the PQS system has been reported to contribute to biofilm maturation (Alhede et al., 2011; Allesten-Holm et al., 2006; Das & Manefield, 2012; Ma et al., 2009). Doxorubicin treatment of P. aeruginosa cultures might result in lysis of a small subpopulation of the bacteria due to antibiotic properties, or through activation of the PQS system, as our transcriptome analysis indicated that pqs and las genes were up-regulated in the presence of doxorubicin (Table 2). Our transcriptome

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**Fig. 6.** (a) Doxorubicin increases PAO1 biofilm formation independently of Pel and Psl. Biofilms of the PAO1 wild-type and ΔpelAΔpslBCD mutant were grown in microtitre plates in the absence (dark grey bars) or presence of 100 µM doxorubicin (light grey bars). Data are representative of two independent experiments. Error bars represent the standard deviation among six technical replicates. (b) Doxorubicin-promoted biofilm formation of PAO1 ΔpelAΔpslBCD is dependent on eDNA. Biofilms of PAO1 ΔpelAΔpslBCD were grown in microtitre plates for 18 h in the absence (Untreated) or presence of 100 µM doxorubicin. The biofilms grown with doxorubicin were subsequently treated with DNase I (Doxorubicin+DNase) or with DNase I buffer (Doxorubicin) for 80 min. The remaining biofilm biomass was quantified using CV. Error bars represent the variation between two biologically independent experiments.
analysis also indicated the up-regulation of the genes PA3431–3432 encoding a holin-like protein involved in cell autolysis (Ma et al., 2009) (Table 2). Doxorubicin-mediated release of DNA might promote biofilm formation of P. aeruginosa independently of c-di-GMP-regulated biofilm factors. Accordingly, while a P. aeruginosa ΔpelAΔpslBCD mutant was unable to form biofilm, it did form biofilm in the presence of doxorubicin. In accordance with a role of eDNA in doxorubicin-promoted biofilm formation by the ΔpelAΔpslBCD mutant, this biofilm could be entirely dissolved by DNase treatment. We conclude that sub-inhibitory concentrations of doxorubicin trigger biofilm formation independent of the two major exopolysaccharides Pel and Psl, and most likely are caused by an increase in eDNA release. It is likely that the eDNA released under doxorubicin treatment overrides the effect of low c-di-GMP levels on biofilm formation and dispersal.

Our experimental data did not allow us to conclude about the mechanism underlying doxorubicin-mediated reduction of the c-di-GMP level in P. aeruginosa. Our transcriptomic data did not reveal an effect of doxorubicin on the transcription of any of the DGC and PDE encoding genes in P. aeruginosa. In addition, doxorubicin did not affect the activity of the WspR DGC. It has been shown that the expression of some DGCs is controlled at the post-transcriptional level (Moscoso et al., 2014) and that the expression of some PDEs is activated at the post-translational level in P. aeruginosa (Li et al., 2013; Phippen et al., 2014). Based on our data, we cannot exclude that doxorubicin exerts its effect on the c-di-GMP content by affecting enzymes involved in c-di-GMP metabolism at either the post-transcriptional or post-translational level.

Doxorubicin has been shown to act as a DNA intercalating agent (Tacar et al., 2013). As c-di-GMP is a purine compound with structural similarities to DNA, it is possible that the observed lowering of c-di-GMP levels could have been caused by binding of doxorubicin to c-di-GMP, which would block both its bioavailability to FleQ (the repressor of cdrA-gfp transcription) and its retention time in the HPLC-MS-MS-based c-di-GMP quantification. To test this hypothesis, we cultivated our c-di-GMP monitor strain in the presence of 100 μM doxorubicin and 100 μM extracellular c-di-GMP. We hypothesized that if doxorubicin binds c-di-GMP, then it should not be able to lower the fluorescent output of our c-di-GMP monitor strain in the presence of a high concentration of extracellular c-di-GMP. However, doxorubicin exerted the same effect on our c-di-GMP monitor strain in the absence or presence of extracellular c-di-GMP (Fig. S4), indicating that the effect of doxorubicin on the c-di-GMP level in P. aeruginosa is not caused by binding of c-di-GMP.

It is possible that doxorubicin might lower the c-di-GMP content in P. aeruginosa through an indirect mechanism. A recent study showed that, to survive sub-lethal concentrations of antibiotics, E. coli cells up-regulate their DNA/RNA repair (Belenky et al., 2015). This is thought to drain the nucleotide pools as they are immediately used for DNA/RNA repair. This leads us to suggest that induction of DNA/RNA repair could result in decreased c-di-GMP production due to a lack of GTP substrate. Our transcriptomic data revealed that recA and lexA, encoding enzymes involved in nucleotide repair and SOS response (Curz et al., 2006), are up-regulated 9.2- and 15-fold, respectively, in response to doxorubicin treatment (Table 2). These data suggest that doxorubicin may lead to an increase in DNA/RNA repair, resulting in decreased c-di-GMP levels as a side effect of stress caused by the drug.

In conclusion, we provide evidence that doxorubicin lowers the c-di-GMP content and transcription of several genes encoding biofilm matrix components in P. aeruginosa, but stimulates biofilm formation. Doxorubicin only decreases the c-di-GMP content in P. aeruginosa at concentrations where it reduces the culture yield and likely causes killing of a subpopulation of the bacteria. The stimulation of biofilm formation mediated by doxorubicin is most likely due to release of eDNA from a subpopulation of bacteria that are killed by the compound. Thus, our study shows that compounds lowering the c-di-GMP content in bacteria will not necessarily mediate biofilm inhibition or dispersal. These findings should be of interest to researchers engaged in developing anti-biofilm drugs.

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