Interaction of the Diguanylate Cyclase YdeH of Escherichia coli with 2',3'-Substituted Purine and Pyrimidine Nucleotides

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ABSTRACT
Diguanylate cyclases (DGCs) synthesize the bacterial second messenger cyclic 3',5'-diguanosine monophosphate (c-di-GMP), which is degraded by specific phosphodiesterases. c-di-GMP levels control the transition of bacteria from a motile to a biofilm-forming lifestyle. These bacterial communities are highly resistant to antibiotic treatment and represent the predominant lifestyle in most chronic infections. Hence, DGCs serve as starting point for the development of novel therapies interfering with the second messenger-signaling network in bacteria. In previous studies, we showed that 2'(3')-O-(N-methylanthraniloyl) (MANT)- and 2',3'-O-(2,4,6-trinitrophenyl) (TNP)-substituted nucleotides are potent adenyllyl and guanylyl cyclase inhibitors. The catalytic domain of DGCs is homologous to the mammalian adenyllyl cyclase catalytic domain. Therefore, we investigated the interaction of various MANT adenine and pyrimidine nucleotides with the model DGC YdeH from Escherichia coli. We observed strong fluorescence resonance energy transfer between tryptophan and tyrosine residues of YdeH and the MANT group of MANT-NTPs (MANT-ATP, -CTP, -GTP, -ITP, -UTP, and -XTP) and an enhanced direct MANT fluorescence upon interaction with YdeH. We assessed the affinity of MANT-NTPs to YdeH by performing competition assays with NTPs. We conducted an amino acid alignment of YdeH with the earlier crystallized Caulobacter crescentus DGC PleD and found high similarities in the nucleotide-binding site of PleD. In vitro mass-spectrometric activity assays with YdeH resulted in the identification of new MANT/TNP nucleotide-based inhibitors of DGC activity. Together, the analysis of interactions between MANT/TNP nucleotides and YdeH provides a new basis for the identification and development of DGC inhibitors and allows insights into nucleotide-protein interactions.

Introduction
Multiple species of bacteria form communities by attachment and aggregation on surfaces such as inert solid materials, living tissue, or boundary surfaces in aquatic systems (Costerton et al., 1995). These biofilms are characterized by the formation of an exopolysaccharide matrix in which the microorganisms are encased (Branda et al., 2005). One or more different bacterial species can accumulate in one single biofilm. For example, dental biofilms are estimated to contain more than 500 bacterial species (Whittaker et al., 1996). Biofilm-grown cells are highly persistent and, in contrast to planctonic cells, display increased resistance to antimicrobial agents. For example, dental biofilms are estimated to contain more than 500 bacterial species (Whittaker et al., 1996). Biofilm-grown cells are highly persistent and, in contrast to planctonic cells, display increased resistance to antimicrobial treatment and host defense. Most chronic bacterial infections result from the formation of stable biofilms. The most prominent case related to persistent biofilm formation is cystic fibrosis, where the airways of respective patients are infected by Pseudomonas aeruginosa (Whiteley et al., 2001). Biofilm formation on diagnostic or surgical medical devices also poses a serious problem for public health (Donlan, 2001). The mechanisms of antibiotic resistance are only partially understood (for review, see Mah and O'Toole, 2001; Stewart and Costerton, 2001). Hence, the development of effective antibiotics is stagnant. Few chemical compounds capable of affecting biofilm formation have been identified (Ueda et al., 2009; Antoniani et al., 2010).

The transition from a motile, planctonic lifestyle to a sessile, cooperative lifestyle is regulated by the bacterial second messenger cyclic 3',5'-diguanosine monophosphate (c-di-GMP) (for review, see Jenal and Malone, 2006). In general, elevated levels of c-di-GMP account for increased biofilm formation. c-di-GMP is synthesized by diguanylate cyclases (DGCs) via the condensation of two GTP molecules and degraded by specific phosphodiesterases to GMP via the linear intermediate pGpG. These two highly abundant protein...
families in bacteria contain the conserved GGDEF and EAL domains, respectively (Paul et al., 2004; Schmidt et al., 2005). In most cases, a single bacterial genome encodes many different members of these protein families. Hence, c-di-GMP-metabolizing enzymes, especially DGCs as key enzymes of second messenger signaling in bacteria, constitute a pharmacological target for the development of possible inhibitors capable of affecting c-di-GMP biosynthesis and biofilm formation.

In previous studies, we identified various 2′(3′)-O-(N-methylanthraniloyl) (MANT)-substituted nucleotides as potent inhibitors of the bacterial adenyl cyclase (AC) toxin edema factor and of mammalian ACs (mACs) by both enzymatic and fluorescence spectroscopy methods (Mou et al., 2006; Taha et al., 2009). Moreover, 2′,3′-O-(2,4,6-trinitrophenyl) (TNP)-substituted nucleotides are potent inhibitors of various AC isoforms and soluble guanylyl cyclase (GC) (Suryanarayana et al., 2009). Sequence similarity between the GGDEF and mAC catalytic domains has been detected (Pei and Grishin, 2001). From this homology, it was deduced that the fold of the GGDEF domain is similar to the mAC catalytic domain. This finding prompted us to investigate the interaction of different MANT-substituted nucleotides with a model DGC. For this purpose, we used the DGC YdeH from Escherichia coli. The YdeH gene has been identified as target for the carbon storage regulator CsrA, an RNA-binding protein that controls biofilm formation (Jonas et al., 2008). Later, it was shown that YdeH possesses in vitro DGC activity (Boehm et al., 2009). However, Chan et al. (2004) show that despite the similar fold of the DGC and mAC domains, the nucleotide-binding mode in the DGC PleD from Caulobacter crescentus is substantially different.

In this study, we investigate the interaction between MANT- or TNP-substituted nucleotides and the DGC YdeH by fluorimetric and mass-spectrometric means. In our first approach, we evaluated the affinity of MANT nucleotides to YdeH by performing fluorescence competition assays with nonsubstituted nucleotides and assessed structural aspects of nucleotide binding by YdeH via amino acid alignment with the DGC PleD. The DGC activity of YdeH was determined in vitro using sensitive high-performance liquid chromatography-coupled tandem mass spectrometry (HPLC-MS/MS). The in vitro analysis of a series of MANT- or TNP-substituted nucleotides for their potential inhibitory effect on DGC activity of YdeH resulted in the identification of three GTP-based YdeH inhibitors.

Materials and Methods

Chemicals. Solvents used in HPLC analysis were water, methanol, and acetonitrile (HPLC-grade gradient; Mallinckrodt Baker, Deventer, The Netherlands). Isopropl β-D-1-thiogalactopyranoside, ammonium acetate, bovine serum albumin (BSA), L-glutamic acid, L-arginine, ATP, GTP, ITP, UTP, and guanosine-13C10,15N5 were obtained from Sigma-Aldrich (Steinheim, Germany). CTP, XTP, MANT-ATP, MANT-GTP, MANT-ITP, and MANT-UTP were synthesized as described previously (Taha et al., 2009).

Expression and Purification of YdeH. C-terminally His6-tagged YdeH was expressed from a pET28 vector in the Escherichia coli Rosetta strain (provided by U. Jenal and A. Böhm, Molecular Microbiology Division, Biozentrum, University of Basel, Switzerland). Cells were grown in Luria-Bertani medium supplemented with 50 μg/ml kanamycin and 30 μg/ml chloramphenicol, and expression of YdeH was induced by the addition of 1 mM isopropyl 1-thio-β-D-galactopyranoside for 3 h at 30°C. Cells were harvested by centrifugation, resuspended in lysis buffer (50 mM NaH2PO4, pH 7.5, 200 mM NaCl, 10 mM imidazole, 50 mM L-glutamic acid, and 50 mM L-arginine) containing EDTA-free protease inhibitor cocktail (one tablet for every 50 ml of buffer), and lysed by ultrasonic treatment on ice using seven 30-s burst periods at 250 W with 30-s cooling periods in between. The lysate was cleared by centrifugation (20,000 g), and the supernatant fluid was filtered (0.22 μm). YdeH was purified by nickel-nitrioltriacetic acid-affinity chromatography using a 5-ml HisTrap FF column (GE Healthcare, Munich, Germany). After washing the column with lysis buffer (without protease inhibitors), YdeH was eluted with a linear gradient of imidazole from 10 to 500 mM. Desalting and buffer exchange of pooled fractions were performed on a 5-ml HiTrap desalting column (GE Healthcare). Final protein concentration was determined with a Bradford assay (Carl Roth).

Fluorescence Experiments for Monitoring MANT Nucleotide Binding to YdeH. Fluorescence experiments were performed using a quartz UV ultramicrocuvette (type 105.251-QS; Hellma, Mülheim, Germany) in a Cary Eclipse fluorescence spectrometer (Varian, Palo Alto, CA). Reaction mixtures contained 5 mM MnCl2 and 50 mM NaCl in 50 mM Tris-HCl, pH 8.0, followed by the sequential addition of MANT nucleotides (final concentration, 1 μM) and YdeH (final concentration, 5 μM) in a total assay volume of 100 μl. Steady-state fluorescence emission spectra of MANT nucleotides were recorded at low speed in the scan mode. In fluorescence resonance energy transfer (FRET) experiments, the excitation wavelength was 280 nm with an emission wavelength at 305 to 540 nm, whereas in direct fluorescence experiments, the MANT group was excited at 350 nm, and the emission was recorded at 380 to 540 nm. In kinetic competition experiments, MANT nucleotides were displaced from YdeH by the sequential addition of increasing concentrations of nucleotides to the assay mixture. Direct fluorescence emission of MANT nucleotides was recorded at 440 nm after excitation at 350 nm.

YdeH In Vitro Activity Assay. In DGC activity assays, 10 nM YdeH was used. MgGTP concentrations were varied between 100 nM and 1 mM. The standard reaction mixture contained 50 mM Tris-HCl, pH 8.0, 5 mM MgCl2, and 0.1% (w/v) BSA in a total assay volume of 50 μl. The temperature was set to 30°C. The reaction was initiated by the addition of YdeH and was stopped after 30 min by heat inactivation at 95°C for 5 min. According to Ross et al. (1985) and Karaolis et al. (2005), c-di-GMP is stable after 10 min of exposure to 100°C. Therefore, the activity of YdeH was not falsified during the work-up procedure of the assay. The resulting suspension was centrifuged (20,000g) to remove denatured protein. c-di-GMP concentration was determined in the supernatant by HPLC-MS/MS as described previously (Spangler et al., 2010), except for the use of the enzymatically synthesized internal standard (IS) 13C20,15N10-cyclic 3′,5′-diguanosine monophosphate (cGMP, 13C20,15N10-c-di-GMP) (see below for the synthesis protocol) instead of cyclic 3′,5′-xanthosine monophosphate (cXMP). 13C20,15N10-c-di-GMP was present in a final concentration of 200 ng/ml and was detected using selected reaction monitoring (SRM) analysis in positive ionization mode with an SRM transition of +721/162 and a collision energy of 61 eV.
Assay mixtures for the inhibition experiment of YdeH with MANT-GTP contained MANT-GTP at final concentrations of 100 and 300 nM and 1, 3, and 10 μM. Inhibition experiments with MANT-GTP and TNP-GTP also contained the inhibitors at final concentrations of 10 and 30 nM. In screening experiments for potential DGC inhibitors, MANT- or TNP-substituted nucleotides were present at a concentration of 10 μM. Inhibition experiments were performed under conditions as described above with 5 μM MgGTP as substrate and an incubation time of 15 min. All assays were performed in triplicate.

### Enzymatic Synthesis of $^{13}$C$_{20}$,$^{15}$N$_{10}$-c-di-GMP.

For the synthesis of $^{13}$C$_{20}$,$^{15}$N$_{10}$-c-di-GMP, 2 μM YdeH was incubated with 500 μM guanosine-$^{13}$C$_{20}$,$^{15}$N$_{5}$, 5′-triphosphate in 50 mM Tris-HCl, pH 8.0, 5 mM MgCl$_2$, and 0.1 wt% BSA for 18 h at 30°C. Based on an identical experiment using unlabeled GTP substrate, we deduced that substrate turnover is complete under these conditions. The reaction was stopped by heating to 95°C for 15 min, and the suspension was clarified by centrifugation (20,000 g). The concentration of $^{13}$C$_{20}$,$^{15}$N$_{10}$-c-di-GMP in the supernatant was determined by measuring the absorption at 254 nm (ε$_{254}$ = 23,700 M$^{-1}$ cm$^{-1}$). Further purification steps are not necessary for the use of $^{13}$C$_{20}$,$^{15}$N$_{10}$-c-di-GMP as the internal standard and were therefore omitted.

### Data Analysis.

All fluorescence data shown in Figs. 1 to 3 were performed under conditions as described above with 5 μM MgGTP as substrate and an incubation time of 15 min. All assays were performed in triplicate. Error bars are standard deviations of the mean.

### Results

#### Interaction of MANT Nucleotides with YdeH in Steady-State Fluorescence Experiments.

Tryptophan and tyrosine residues represent intrinsic fluorophores in proteins and can be excited at 280 nm, resulting in endogenous fluorescence with a maximum at 350 nm, as indicated for YdeH in Fig. 1, A to F. MANT nucleotides exhibit only minimal endogenous fluorescence after excitation at 280 nm. After the addition of YdeH to all MANT nucleotides investigated, distinct new fluorescence signals appeared in the presence of Mn$^{2+}$, with a maximum ranging from 425 to 435 nm (Fig. 1, A–F). These peaks are ascribed to FRET from tryptophan and tyrosine residues to the MANT group. The extent of FRET was comparable for all examined MANT nucleotides. In the presence of Mg$^{2+}$, FRET signals were smaller or disappeared altogether (data not shown). Lower FRET intensities with MANT nucleotides using Mg$^{2+}$-containing buffers compared with those containing Mn$^{2+}$ have also been detected in earlier experiments with mAcs (Mou et al., 2005).

Although Mg$^{2+}$ is the physiologically relevant cation, all fluorescence studies were conducted with Mn$^{2+}$. The reversibility of MANT nucleotide binding to YdeH was examined by the addition of 10 μM GTP. After this addition, the FRET signals decreased considerably. In the case of MANT-GTP, only a very small effect was observed. With the applied GTP concentration, the FRET signals did not disappear completely, as indicated by the remaining shoulders between 400 and 500 nm.

We also studied the changes of direct MANT nucleotide fluorescence after interaction with YdeH at λ$_{ex}$ = 350 nm. MANT nucleotides exhibit strong endogenous fluorescence, peaking at λ$_{em}$ = 450 nm (Fig. 1, G-L). In the presence of YdeH, the fluorescence intensity was enhanced by a factor of 2 to 2.5, accompanied by a shift of the emission maximum to shorter wavelengths (blue shift) by approximately 10 to 20 nm, depending on the applied MANT nucleotide. This blue shift has been observed in binding studies of MANT nucleotides to bacterial and mammalian ACs (Mou et al., 2005; Suryanarayana et al., 2009) and is ascribed to the movement of the MANT group into a more hydrophobic environment (Hiratsuka, 1983). Apart from MANT-GTP, the addition of 10 μM GTP reduced MANT nucleotide fluorescence to almost that of the endogenous fluorescence intensities in the absence of YdeH. MANT-GTP fluorescence was only marginally decreased, which goes along with the noted small reduction of the FRET signal in the case of MANT-GTP.

### Competition Studies between MANT Nucleotides and NTPs for Binding to YdeH.

We examined the reversibility of MANT nucleotide fluorescence enhancement after interaction with YdeH in competition assays with different NTPs (ATP, CTP, GTP, ITP, and UTP) by monitoring changes in direct MANT fluorescence. Figure 2 exemplarily shows the kinetics of MANT-ATP (Fig. 2, A–C) and MANT-GTP (Fig. 2, D and E) fluorescence at λ$_{em}$ = 440 nm after excitation at λ$_{ex}$ = 350 nm. In the presence of YdeH, a pronounced fluorescence increase for both MANT nucleotides was observed. The response time of MANT nucleotide fluorescence is on a time scale of a few seconds at most. The sequential addition of increasing concentrations (0.1–50 μM) of GTP or ATP resulted in differently pronounced fluorescence decreases.

After the addition of GTP or ATP (50 μM), the fluorescence intensity of MANT-ATP was reduced to the original level in the absence of YdeH (Fig. 2, A and B). The same result was obtained after the addition of CTP, ITP, and UTP (data not shown as a time trace). The endogenous MANT-ATP fluorescence remained constant over time in control experiments (with all NTPs examined) where buffer was added instead of YdeH at time point 1 (addition of ATP; see Fig. 2C). Figure 3A compares the potency of the examined NTPs to reduce YdeH-induced MANT-ATP fluorescence dependent on the NTP concentration present in the assay mixture. Although the increase in fluorescence intensity of MANT-ATP was almost completely abolished after the addition of NTPs at high concentrations (50 μM), slightly different potencies of the various NTPs could be deduced for concentrations lower than 50 μM: GTP > ITP > ATP > UTP > CTP.

The emission intensity of MANT-GTP was decreased to a lesser extent ranging from 70 to 40% of the initial YdeH-induced MANT-GTP fluorescence in the presence of NTPs at high concentrations (50 μM). Figure 2, D to F, exemplarily shows changes in MANT-GTP fluorescence after the addition of GTP and ATP (data for CTP, ITP, and UTP not shown as a time trace) and a representative control experiment with ATP where YdeH was replaced by buffer. Controls were performed for all NTPs tested. No changes in MANT-GTP fluorescence were detected under these conditions. The five NTPs tested showed slightly different potencies of reducing YdeH-induced MANT-GTP fluorescence: GTP > ITP > UTP > ATP > CTP, as indicated in Fig. 3B. The potency order of NTPs to reduce MANT-GTP fluorescence conforms to the reduction of MANT-ATP fluorescence except for ATP and UTP, whose order is exchanged. It is possible that MANT-ATP and MANT-GTP stabilize different conformations in the DGC, which then have different affinities for the various.
NTPs. Such concepts generally referred to as “multiple-state models” or “functional selectivity” have also been described for several G-protein-coupled receptors (Perez and Karnik, 2005; Urban et al., 2007; Seifert and Dove, 2009).

**YdeH In Vitro Activity Assay.** Assays monitoring c-di-GMP production by YdeH were performed using HPLC-MS/MS (Spangler et al., 2010). The reported method was modified in terms of using a newly synthesized IS,

Fig. 1. Analysis of MANT-NTP (NTP = ATP, CTP, GTP, ITP, UTP, and XTP) interactions with YdeH by performing both FRET and direct fluorescence studies as described under Materials and Methods. The following components were added consecutively: 1 μM MANT nucleotide (solid traces), 5 μM YdeH (dotted traces), and 10 μM GTP (dashed traces). YdeH alone (5 μM) is represented by dashed-dotted traces. Representative FRET experiments ($\lambda_{ex} = 280$ nm) and direct fluorescence experiments ($\lambda_{ex} = 350$ nm) are shown in A through F and G through L, respectively. a.u., arbitrary units.
13C20,15N10-c-di-GMP, instead of cXMP. 13C20,15N10-c-di-GMP was enzymatically produced using YdeH. Although DGCs all exhibit strong product inhibition, YdeH has a comparably high residual DGC activity (Boehm et al., 2009), which is sufficient for the production of 13C20,15N10-c-di-GMP from guanosine-13C10,15N5 5’-triphosphate. Figure 4 shows a representative chromatographic run with 13C20,15N10-c-di-GMP and unlabeled c-di-GMP. The retention time of 13C20,15N10-c-di-GMP was identical in comparison with unlabeled c-di-GMP (8.6 min). In contrast, the formerly used IS cXMP eluted earlier than unlabeled c-di-GMP at 6.1 min. Because of the structural identity and equal chromatographic behavior compared with unlabeled c-di-GMP, 13C20,15N10-c-di-GMP was used as IS for all mass spectrometric assays.

The rate of c-di-GMP production by YdeH was determined dependent on substrate concentration as indicated in Fig. 5. YdeH possesses an exceptional enzyme kinetic. DGC activity is characterized by a steep increase at low substrate concentrations, with a maximum rate constant of 2.44 min⁻¹ followed by a decreasing rate of c-di-GMP formation that then levels off and remains constant. Under the applied assay conditions, substrate turnover is higher than 10% for GTP concentrations up to 10 μM. Hence, it is likely for DGC activity to be subject to substrate depletion. Moreover, declining DGC activity determined for GTP concentrations higher than 10 μM can possibly be ascribed to product inhibition, resulting in c-di-GMP formation at a residual rate constant of approximately 0.75 min⁻¹.

The above-mentioned fluorescence assays indicate that MANT-GTP seems to have a comparably high affinity to YdeH. Therefore, we investigated the effect of MANT-GTP on the DGC activity of YdeH in detail (Fig. 6). The activity of YdeH was considerably reduced, with increasing concentrations of MANT-GTP. Under the applied assay conditions (see Materials and Methods), an IC₅₀ of 0.5 μM was determined. We also performed inhibition assays with a set of MANT- and TNP-nucleotides that were applied at a concentration of 10 μM: MANT-ATP, MANT-CTP, MANT-ITP, MANT UTP, MANT-XTP, MANT-GTP-S, and TNP-GTP. The activity of YdeH was reduced to a level near the limit of detection only in the presence of substituted GTPs (MANT-GTPS and TNP-GTP), whereas no change in DGC activity was observed.

![Fig. 2. Kinetic analysis of the competition of MANT nucleotides and NTPs for binding to YdeH in fluorescence experiments. The following components were added consecutively to cuvettes containing MANT nucleotides (A–C, 1 μM MANT-ATP; D–F, 1 μM MANT-GTP): 5 μM YdeH (1) (in C and F, buffer instead of YdeH as control) and NTPs (in A and D, GTP; in B, C, E, and F, ATP) in concentrations of 0.1 μM (2), 0.5 μM (3), 1 μM (4), 2.5 μM (5), 5 μM (6), 10 μM (7), and 50 μM (8). Excitation wavelength was set to 350 nm, and emission of MANT nucleotides was detected at 440 nm over time. a.u., arbitrary units.](https://doi.org/10.1002/jpet.238)

![Fig. 3. Potencies of NTPs to reduce YdeH-induced MANT nucleotide fluorescence. A and B show changes in fluorescence intensity (ΔF) of MANT-ATP and MANT-GTP, respectively, after the addition of ATP (●), CTP (■), GTP (stars), ITP (○), and UTP (□) compared with YdeH-induced MANT nucleotide fluorescence intensity in the absence of NTPs. Excitation wavelength was set to 350 nm, and emission of MANT nucleotides was detected at 440 nm.](https://doi.org/10.1002/jpet.238)
for all other MANT-substituted nucleotides. MANT-GTP and TNP-GTP had IC$_{50}$ values of 0.2 and 0.1 M, respectively (see Fig. 6).

Amino Acid Alignment. To evaluate the nucleotide binding mode of YdeH, we performed an amino acid alignment of PleD with YdeH (Fig. 7). The alignment was performed with ClustalX2 software (http://www.clustal.org/), and the amino acid sequences were obtained from the National Center for Biotechnology Information protein database (http://www.ncbi.nlm.nih.gov/protein/). Residues involved in GTP binding and the catalytic mechanism of c-di-GMP synthesis are conserved between the two DGCs. Hence, it is likely that the nucleotide-binding mode of YdeH is comparable with PleD. We suggest that the MANT group of MANT-NTPs is transferred into a thus far unspecified hydrophobic binding pocket as can be deduced from the fluorescence experiments. However, the exact binding mechanism of MANT nucleotides by YdeH remains to be elucidated experimentally.

Discussion

Microorganisms preponderantly exist in biofilm-forming communities, which account for the high persistence in chronic infections and may cause severe problems because of the adherence to implanted medical devices (Donlan, 2001). The development of efficient therapeutic strategies against the formation of biofilms is difficult (Jabra-Rizk et al., 2006). In this study, we assessed the inhibitory potential of MANT and TNP nucleotides on the model DGC YdeH as a basis for intervening in c-di-GMP metabolism of biofilm-forming bacteria.

Our study demonstrates that several MANT nucleotides undergo distinct fluorescent changes in both FRET and direct fluorescence experiments after interaction with YdeH (Fig. 1). All fluorescence experiments were performed with an excess of YdeH compared with the fluorescent ligand to obtain quantitative ligand binding and sufficient fluorescence signals. It is noteworthy that an increase in fluorescence was detected for all MANT nucleotides, not only for the MANT-substituted DGC substrate GTP. Both the reduced FRET signals and decreased intrinsic MANT fluorescence after the addition of GTP to the YdeH-MANT nucleotide complexes point to the displacement of MANT nucleotides from YdeH and binding of the original substrate GTP. However, Fig. 1, C and I, indicates that displacement of MANT-GTP is clearly less pronounced compared with all other MANT nucleotides, suggesting a high affinity of the GTP group to YdeH. Competition experiments of MANT-ATP and MANT-GTP with NTPs, as shown in Fig. 2, support this finding: the direct fluorescence of MANT-ATP bound to YdeH is strongly reduced after successive additions of increasing concentrations of all NTPs tested, whereas in contrast, MANT-GTP fluorescence is decreased to a lesser extent in the same experiment. GTP is the most efficient nucleotide in the MANT-GTP competition kinetics, which can be ascribed to its function as a natural substrate of DGCs. However, given the detected interaction between YdeH and MANT-NTPs other than MANT-GTP, one can speculate that YdeH optionally accepts other NTPs as substrates. In an in vitro assay with the highly active enzyme WspR, the synthesis of c-di-dGMP and c-di-IMP from dGTP and ITP, respectively, was proposed, indicating a rather low substrate specificity of DGCs (Lory et al., 2009). These data are in accordance with our findings.

The obvious binding capability of MANT-NTPs by YdeH offers the possibility of developing DGC inhibitors. Therefore, the mechanisms of inhibitor binding have to be elucidated in detail. The inhibitory characteristics of MANT-ATP and MANT-GTP and the underlying molecular binding mechanisms have been studied intensively for mACs (Mou et al., 2005, 2006). From these studies, we know that an increase in MANT fluorescence and a blue shift of the emission maximum goes along with the interaction of the MANT fluorophore with nonpolar amino acid residues in a hydrophobic
binding pocket. The homology between mAC and DGC catalytic domains may mislead one to the assumption that the nucleotide-binding mode was the same, which, in contrast, is substantially different, as shown for the crystal structure of the DGC PleD from *Caulobacter crescentus* solved in complex with the product c-di-GMP (Chan et al., 2004). Chan et al. proposed that the positions of the guanine, ribose, and α-phosphoryl moieties in the case of GTP binding are the same as in the complex structure with c-di-GMP. It is likely that the nucleotide-binding mode of YdeH is similar, as can be deduced from the amino acid alignment presented in Fig. 7. The enzymology of DGCs representing putative inhibitor targets needs to be elucidated in detail to develop new antimicrobial therapeutics. The conversion of GTP to c-di-GMP by YdeH dependent on substrate concentration was monitored with a modified HPLC-MS/MS method. Enzymatically synthesized $^{13}$C$_2$O$_{15}$N$_{10}$ c-di-GMP serves as an ideal IS because of its identical molecular structure and retention time and further improves the described method. Hence, a reliable quantitation of c-di-GMP synthesis and DGC activity is possible. Thus far, the catalytic activity of YdeH has only been rudimentarily investigated (Boehm et al., 2009) with the objective of identifying YdeH as DGC. In the respective activity assay, YdeH was present at 2 μM, a very high concentration. The establishment of enzymatic assays is not always possible. Thus far, the catalytic activity of YdeH has only been rudimentarily investigated (Boehm et al., 2009) with the objective of identifying YdeH as DGC. In the respective activity assay, YdeH was present at 2 μM, a very high concentration. The establishment of enzymatic assays is not always possible. The active site catalytic reaction time of the assay to avoid excessive substrate conversions for low substrate concentrations. Unfortunately, incubation times of only a few seconds would have had to be chosen, which is experimentally hard to accomplish. In addition, mass-spectrometric signals would have been too small for accurate assessment. Because it is not known whether the kinetic behavior of YdeH is different in vivo, we can only speculate that the DGC works under very specific conditions where distinct substrate concentrations are possibly present. However, the intracellular GTP concentration in bacteria has not been determined so far. Moreover, it is possible that changes of free c-di-GMP concentration are related to any unknown c-di-GMP transport processes. These processes could reduce the local c-di-GMP concentration and therefore decrease product inhibition.

Facing the unusual enzyme kinetic of YdeH, we performed in vitro inhibition assays under fixed conditions. To our knowledge, effective inhibitors of DGC activity have not been identified so far. Some recently described compounds are supposed to repress biofilm formation in an indirect fashion rather than binding directly to DGCs (Antoniani et al., 2010). We clearly identified direct inhibitors of YdeH activity in mass-spectrometric inhibition experiments. The higher affinity of MANT-GTP compared with MANT-GTP compared may be due to stronger interactions of the bulky sulfur with surrounding amino acid residues in the binding pocket of YdeH. The TNP group is quite rigid and relatively polar (Hiratsuka, 2003). It can be speculated that all large and polar compounds in the binding pocket of YdeH and, thus, is responsible for the higher affinity of TNP-GTP compared with MANT-GTP compared may be due to stronger interactions of the bulky sulfur with surrounding amino acid residues in the binding pocket of YdeH. The mass-spectrometric signals would have been too small for accurate assessment. Because it is not known whether the kinetic behavior of YdeH is different in vivo, we can only speculate that the DGC works under very specific conditions where distinct substrate concentrations are possibly present. However, the intracellular GTP concentration in bacteria has not been determined so far. Moreover, it is possible that changes of free c-di-GMP concentration are related to any unknown c-di-GMP transport processes. These processes could reduce the local c-di-GMP concentration and therefore decrease product inhibition.

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tested purine and pyrimidine nucleotide derivatives. In contrast to DGCs, mACs exhibit a broad base specificity along with high conformational flexibility (Mou et al., 2006; Suryanarayana et al., 2009). Hence, we suggest that GTP serves as an auspicious core structure for the development of potent DGC inhibitors, which can act specifically on DGCs rather than other cyclase families. However, sufficient cell membrane permeability often represents a great challenge with regard to the establishment of efficient therapeutic strategies. Hence, the development of lipophilic pronucleotide inhibitors can offer new perspectives in the treatment of persistent biofilm-related infections.

Most of the examined nucleotide derivatives (except for GTP derivatives) have a very low affinity to YdeH, and an inhibitory effect could not be shown in mass-spectrometric activity assays. In contrast, the described fluorescent experiments are very suitable for detecting binding events even in case of low-affinity inhibitors. In addition, competition assays with NTPs can provide a fast estimation of affinities. To our knowledge, this is the first fluorescence-based analysis of a DGC, and it offers an elegant possibility to circumvent the problems arising from the unusual enzyme kinetics. As a first approximation, the identification of newly developed inhibitors via binding to DGCs can be accomplished straightforwardly by fluorescence spectroscopy in a high-throughput manner given that YdeH can be purified in large quantities.

In conclusion, our present study gives insights into the interaction of MANT nucleotides with the DGC YdeH. Binding events were monitored via FRET-based and direct fluorescence experiments, and the influence of potential DGC inhibitors was analyzed by sensitive HPLC-MS/MS. We identified direct DGC inhibitors based on GTP derivatives. Hence, our results provide a promising starting point for the development of effective DGC inhibitors with the objective of inhibiting the formation of highly persistent biofilms via the influence of intracellular c-di-GMP metabolism.

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Authorship Contributions
Participated in research design: Spangler, Kaeve, and Seifert.
Conducted experiments: Spangler.
Contributed new reagents or analytic tools: Spangler and Kaeve.
Performed data analysis: Spangler, Kaeve, and Seifert.
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References

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