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Monitoring of diguanylate cyclase activity and of cyclic-di-GMP biosynthesis by whole-cell assays suitable for high-throughput screening of biofilm inhibitors

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Abstract In Gram-negative bacteria, production of bis-(3′,5′)-cyclic diguanylic acid (c-di-GMP) by diguanylate cyclases (DGCs) is the main trigger for production of extracellular polysaccharides and for biofilm formation. Mutants affected in c-di-GMP biosynthesis are impaired in biofilm formation, thus making DGCs interesting targets for new antimicrobial agents with anti-biofilm activity. In this report, we describe a strategy for the screening for DGC inhibitors consisting of a combination of three microbiological assays. The primary assay utilizes an *Escherichia coli* strain overexpressing the *adrA* gene, encoding the DGC protein AdrA, and relies on detection of AdrA-dependent cellulose production as red colony phenotype on solid medium supplemented with the dye Congo red (CR). Presence of DGC inhibitors blocking AdrA activity would result in a white phenotype on CR medium. The CR assay can be performed in 96-well microtiter plates, making it suitable for high-throughput screenings. To confirm specific inhibition of c-di-GMP biosynthesis, chemical compounds positive in the CR assay are tested for their ability to inhibit biofilm formation and in a reporter gene assay which monitors expression of curli-encoding genes as a function of DGC activity. Screening of a chemical library using the described approach allowed us to identify sulfathiazole, an antimetabolite drug, as an inhibitor of c-di-GMP biosynthesis. Sulfathiazole probably affects c-di-GMP biosynthesis in an indirect fashion rather than by binding to DGCs; however, sulfathiazole represents the first example of drug able to affect biofilm formation by interfering with c-di-GMP metabolism.

Keywords c-di-GMP · Diguanylate cyclase · Biofilm formation · High-throughput screening · Antimicrobial drugs · Sulfathiazole

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Introduction

Most bacteria are able to switch between two different "lifestyles": single cells (planktonic mode) and biofilm, a sessile microbial community. Biofilm and planktonic cells differ significantly in their physiology, in their gene expression pattern, and even in their morphology. In particular, biofilm cells are characterized by increased production of adhesion factors and extracellular polysaccharides (EPS), lower sensitivity to antibiotics, and increased resistance to environmental stresses compared to planktonic cells (Costerton et al. 1995; Anderl et al. 2000; Harrison et al. 2007, 2009). Thus, biofilm formation is considered a virulence determinant and an important factor in antibiotic resistance and persistence in the host. Although biofilm inhibition does not usually impair bacterial growth under laboratory conditions, treatment with biofilm-inhibiting compounds can improve growth inhibition and bacterial killing by antibiotics (Jabra-Rizk et al. 2006), thus making biofilm determinants an interesting target for specific inhibitors to be used in combination with conventional antimicrobial agents.

Transition from planktonic cells to biofilm is regulated by environmental and/or physiological signals, relayed to the bacterial cell by signal molecules or "second messengers", and by intermediates or products of metabolic pathways such as indole (Di Martino et al. 2003) or uracil (Ueda et al. 2009). A second messenger, bis-(3′,5′)-cyclic diguanylic acid, better known as cyclic-di-GMP (c-di-GMP), plays a pivotal role in several processes linked to biofilm formation and maintenance, such as production of EPS and adhesion factors (Ross et al. 1991; Kader et al. 2006; Weber et al. 2006), as well as in modulation of virulence factors (Jones et al. 1999; Kulasakara et al. 2006; Hammer and Bassler 2009). Intracellular levels of c-di-GMP are determined by the opposite activities of diguanylate cyclases (DGCs), also called GGDEF proteins from the amino acid sequence found in their catalytic motif, and c-di-GMP-phosphodiesterases (reviewed in Tamayo et al. 2007). Genes encoding proteins involved in c-di-GMP biosynthesis and turnover are conserved in all Eubacteria and are present in remarkably high numbers in Gram-negative bacteria: for instance, 19 GGDEF protein-encoding genes can be found in *Escherichia coli* (Méndez-Ortiz et al. 2006). In contrast, c-di-GMP-related genes are only sporadically present in Eukarya or in Archea and totally absent in animal species (Galperin 2004), which makes enzymes involved in its biosynthesis interesting as targets for antimicrobial or anti-biofilm agents. The discovery of novel DGC inhibitors can be greatly facilitated by the development of assays suitable for high-throughput screening (HTS) of chemical compounds. HTS must be based on simple and reliable assays, suitable for automation and, whenever possible, performed in living cells, in order to select compounds able to cross the membrane barrier and to show activity in vivo. However, to our knowledge, no rapid methods for screening for DGC inhibitors based on their mode of action have yet been described in the literature. Current methods for determination of c-di-GMP levels in bacterial cells are laborious, since they require mass spectrometry or high-performance liquid chromatography (HPLC; Simm et al. 2004, 2009) and are therefore not suitable for HTS.

In this report, we describe the exploitation of a suite of well-established microbiological assays as a screening approach for inhibitors of DGC enzymes. As a primary screening method, we employed a Congo red (CR) assay, which provides a simple, qualitative, whole-cell assay to test DGC activity in living cells. Rapid secondary screening methods are provided by the crystal violet assay for semi-quantitative measurement of biofilm formation and by a quantitative reporter gene assay measuring expression of curli-encoding genes as a function of DGC activity. Screening of a chemical library using this strategy led to the identification of the inhibitory activity of c-di-GMP biosynthesis by sulfathiazole, a known antimetabolite drug.

Materials and methods

Bacterial strains and growth conditions Bacterial strains used in this work are listed in Table 1. When not otherwise stated, bacteria were grown at 30°C, a temperature facilitating cellulose production and biofilm formation in Enterobacteria (Zogaj et al. 2001; Robbe-Saule et al. 2006; Gualdi et al. 2008) in M9 salts supplemented with 0.5% (*w*/*v*) glucose, 0.02% peptone, and 0.01% yeast extract (M9Glu/sup medium). When needed, antibiotics were used at the following concentrations: ampicillin, 100 g/ml; chloramphenicol, 35 g/ml; and kanamycin, 50 g/ml. For growth on CR-supplemented or Calcofluor-supplemented agar media, bacteria were inoculated in M9Glu/sup medium in a microtiter plate, and the cultures were spotted, using a replicator, on CR medium (1% Casamino acids, 0.15% yeast extract, 0.005% $MgSO_4$, 0.0005% MnCl2, 2% agar) to which 0.004% CR and 0.002% Coomassie blue (for CR medium) or 0.005% Calcofluor (for Calcofluor medium) were added after autoclaving. Bacteria were grown for 18–20 h at 30°C; staining was better detected after 24–48 h of additional incubation at 4°C.

Biofilm formation assays Biofilm formation in microtiter plates was determined by the crystal violet staining assay (O'Toole and Kolter 1998; Dorel et al. 1999). Bacteria were grown overnight (ca. 18 h) in liquid M9Glu/sup medium at 30°C in polystyrene microtiter plates (0.2 ml); the liquid culture was removed, and cell density of planktonic bacteria was determined spectrophotometrically (OD_{600nm}). Cells attached to the microtiter plates were washed gently with water and stained for 20 min with 1% crystal violet, thoroughly washed with water, and dried. For semi-quantitative determination of biofilms, crystal violet-stained cells were resuspended in 0.2 ml of 95% ethanol by vigorous pipetting. The OD_{600nm} of crystal violet-stained biofilm cells was determined and normalized to the OD_{600nm} of the planktonic cells from the corresponding liquid cultures; this value is defined as "adhesion units".

Plasmid construction Plasmids and primers used in this work are listed in Table 1. For overproduction of the AdrA and YdaM proteins, the corresponding genes were amplified by polymerase chain reaction (PCR) from the *E. coli* MG1655 chromosome, and the resulting products were cloned into the pTOPO vector.

The pTOPOAdrA$_{GGAAF}$ plasmid, carrying a mutated allele of the *adrA* gene in which the DGC catalytic site is inactivated, was constructed as follows: the 5′ and the 3′ portions of the *adrA* gene were amplified by PCR using the adrA_fwr and adrA-mut_rev primers (for the 5′-portion of *adrA*) or the adrA-mut_fwr and the adrA_rev primers (for the 3′-portion of *adrA*), resulting in the following sub-

Table 1 Bacterial strains, plasmids, and primers used in this study

Escherichia coli strains		
MG1655	Standard reference strain F^-, λ^-, *rph-1*[a]	Blattner et al. (1997)[b]
PHL856	MG1655 *csgA*::*uidA-kan*[a]	Gualdi et al. (2008)[b]
AM70	MG1655 *ΔcsgA::cat*[a]	This work[b]
AM73	MG1655 *ΔcsgA::cat*, *ΔbcsABZC::kan*[a]	This work[b]
Plasmids		
pTOPO	Control vector allowing direct cloning of PCR products, ampicillin, and kanamycin resistance	Invitrogen[b]
pTOPOAdrA$_{wt}$	*adrA* gene cloned as PCR product into pTOPO vector	Gualdi et al. (2008)[b]
pTOPOAdrA$_{GGAAF}$	*adrA* allele carrying mutation resulting in GGDEF→GGAAF change in AdrA protein catalytic site	This work[b]
pTOPOYdaM	*ydaM* gene cloned as PCR product into pTOPO vector	This work[b]
Primers		
adrA_fwr	5′-GCTCCGTCTCTATAATTTGGG-3′[c]	Construction of pTOPOAdrA$_{wt}$ and *adrA* mutant[d]
adrA_rev	5′-ATCCTGATGACTTTCGCCGG-3′[c]	Construction of pTOPOAdrA$_{wt}$ and *adrA* mutant[d]
adrA-mut_fwr	5′-CTGCGCG**C**TAGCGATGTGATTGGT CGGTTT GGCGGCG**C**TG**C**GTTTG-3′[c]	Construction *adrA* mutant[e]
adrA-mut_rev	5′-CAATCACATCG**C**TAGCGCGCAG-3′[c]	Construction *adrA* mutant[d]
ydaM_fwr	5′-GCGATCGGATAGCAACAA-3′[c]	*ydaM* cloning[d]
ydaM_rev	5′-GAAGTCGTTGATCTCGAC-3′[c]	*ydaM* cloning[d]
csgA_cam_fwr	5′-TTTCCATTCGACTTTTAAATCAATC CGATGGGGGTTTTACTACCTGTGACGGAAGATCAC-3′[c]	*csgA* inactivation[d]
csgA_cam_rev	5′-AACAGGGCTTGCGCCCTGTTTCTGT AATACAAATGATGTAGGGCACCAATAACTGCCTTA-3′[c]	*csgA* inactivation[d]
cat_rev	5′-GGGCACCAATAACTGCCTTA-3′[c]	Mutant verification[d]
csgA_fwr	5′-ACAGTCGCAAATGGCTATTC-3′[c]	Mutant verification[d]

Boldface type characters indicate mutations introduced by the primer. The newly created NheI restriction site is underlined

[a] Relevant genotype or characteristics

[b] Reference or source

[c] Sequence

[d] Utilization

stitutions: G→C at nucleotide 842 of the *adrA* gene (creation of an NheI restriction site), C→A at nucleotide 872, and C→A at nucleotide 875. The last two mutations result in the substitution of both the aspartic and the glutamic acid residues at position 291–292 of the AdrA protein to alanine residues (GGDEF→GGAAF). Both the 5′ and the 3′ portions of the mutated *adrA* gene were cloned into pTOPO, and the full-length *adrA* gene carrying the GGAAF mutation was reconstituted by subcloning the 3′ portion of *adrA* into pTOPO carrying the 5′ portion of the gene, using the newly created NheI restriction site in the mutated *adrA* gene and the XbaI site present in the pTOPO multiple cloning site. Both the wild type and mutant alleles of the *adrA* gene were verified by sequencing.

Determination of intracellular c-di-GMP concentration Overnight cultures were collected by centrifugation, and the supernatant were carefully removed. Bacterial cells were resuspended in 0.4 M $HClO_4$ at a ratio of 45 mg cells/0.35 ml and broken by sonication; cell debris was removed by centrifugation (10,000×g, 10 min, 4°C).

Supernatants were neutralized with 0.16 M K_2CO_3, kept on ice for 10 min, and centrifuged at 12,000×g for 3 min. Supernatants were filtered and injected into an HPLC system equipped with a diode-array detector. HPLC separation was essentially performed as described in Stocchi et al. (1985). A 12.5-cm Supelcosil LC-18-DB, 3 μm particle size, reversed-phase column was used, and the temperature was fixed at 18°C. Elution conditions were 9 min at 100% buffer A (100 mM potassium phosphate buffer, pH 6.0), followed by step elution to 12%, 45%, and 100% buffer B (buffer A containing 20% methanol), at a flow rate of 1.3 ml/min. Purity index of c-di-GMP peak is 0.96. Its identity as genuine c-di-GMP was determined by coelution and identical UV absorption spectra with a c-di-GMP standard (purchased from Biolog, Bremen, Germany). C-di-GMP

concentration was calculated based on an extinction coefficient (ε) of 23,700 at 254 nm (Hayakawa et al. 2003).

Other methods The *E. coli* MG1655 derivative deleted in the *csgA* gene (AM70, *ΔcsgA::cam*) was constructed using the λ Red technique (Datsenko and Wanner 2000). Target gene disruption was confirmed by PCR. P1 transduction of the *ΔbcsABZC::kan* mutation (Da Re and Ghigo 2006) was carried out as described (Miller 1972). β-Glucuronidase specific activity was measured by hydrolysis of *p*-nitrophenyl-β-D-glucuronide into *p*-nitrophenol at 405 nm (Bardonnet and Blanco 1992). Real-time reverse transcription-PCR for determination of *adrA* expression was performed as described (Gualdi et al. 2007), using 16S RNA as reference gene. Antimicrobial activity was determined as the minimal inhibitory concentration (MIC) in liquid M9Glu/sup medium, using the broth microdilution method according to the guidelines of the Clinical and Laboratory Standards Institute (2006). Inhibition of bacterial growth was determined by lack of turbidity by visual inspection. Inhibition of biofilm formation was performed on the same samples by the crystal violet assay as described above.

Results

Rational design of a Congo red-based microbiological assay for diguanylate cyclases (DGCs) inhibitors CR is a diazo-dye with strong affinity for amyloid fibers (Bennhold 1922), such as curli fibers produced by Enterobacteria (Olsén et al. 1989). In addition, CR can also bind polysaccharides (EPS) such as cellulose (Zogaj et al. 2001; Da Re and Ghigo 2006). It is well established that in Enterobacteria, production of both curli and cellulose depends on c-di-GMP biosynthesis and is mediated by specific DGCs: YdaM is required for curli production in *E. coli* (Weber et al. 2006), while AdrA is necessary for cellulose biosynthesis in *Salmonella* (Zogaj et al. 2001). In *E. coli* laboratory strains such as MG1655, red colony phenotype on CR-supplemented medium is totally dependent on curli production, due to low levels of cellulose production, and mutants in curli-encoding genes display a white phenotype on CR medium (Gualdi et al. 2008; see also Fig. 2). Thus, since in *E. coli* the red phenotype on CR medium depends on curli production, which in turn requires c-di-GMP biosynthesis by YdaM (Weber et al. 2006), exposure of *E. coli* to DGC inhibitors would result in a white phenotype on CR medium, providing an easy screening assay. However, a problem connected with using this strategy is the extremely complex regulation of curli: indeed, curli fibers production does not depend exclusively on c-di-GMP but requires a combination of environmental and physiological cues, including low salinity, slow growth, oxygen tension, etc. (Römling et al. 2000; Gerstel and Römling 2001). Exposure to chemical compounds altering general physiological conditions of the bacterial cells might lead to reduced curli production, thus generating a large number of false positives.

Thus, we needed a convenient "reporter" *E. coli* strain in which a red phenotype on CR medium would be a direct result of DGC activity and would be mediated by mechanisms already well characterized at the genetic and biochemical levels (Fig. 1). To this aim, we transformed AM70, a *csgA* derivative of *E. coli* MG1655 (Table 1), unable to produce curli, and thus displaying a white phenotype on solid media supplemented with CR (Fig. 2a), with a multicopy plasmid carrying the DGC-encoding *adrA* gene (pTOPOAdrA$_{wt}$). From the literature data (Zogaj et al. 2001), we expected *adrA* overexpression to activate cellulose production, thus resulting in a red phenotype on CR medium even in a *csgA* mutant of *E. coli* unable to produce curli (see also next section). An advantage of the *adrA*-overexpressing strain is that the *adrA* gene can be placed under the control of an inducible plasmid (e.g., the isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible P*lac* promoter) and expressed either in the presence or in the absence of the inducer molecule. A first round of screening can be performed on strains expressing the target protein at low concentrations (no IPTG induction), and chemical compounds showing inhibition of red coloring on CR medium can be tested on strains induced with IPTG (full AdrA overexpression). Increased amounts of the target protein should result in the need of higher inhibitor concentrations to prevent red coloring on CR plates, thus providing further limitation in selection of false positives, as represented in Fig. 1.

Validation of AM70/pTOPOAdrA$_{wt}$ as an indicator strain for screening of DGC inhibitors In order to use the AM70 (MG1655*csgA::cat*) strain transformed with the pTOPOAdrA$_{wt}$ plasmid (AM70/pTOPOAdrA$_{wt}$) in the screening for DGC inhibitors, we needed to verify whether it can display a red phenotype on CR medium and form biofilm in a manner dependent on AdrA DGC activity. Expression of the *adrA* gene from the pTOPOAdrA$_{wt}$ plasmid in the absence of IPTG induction confers a red phenotype on CR medium to AM70, while transformation of the same strain with the pTOPO control vector does not affect its white phenotype (Fig. 2a). AdrA expression stimulates cellulose production and CR binding in *Salmonella* (Zogaj et al. 2001); thus, AdrA-dependent red phenotype in a curli-deficient strain should depend on cellulose production. Indeed, pTOPOAdrA$_{wt}$ is unable to confer a red phenotype on CR medium to a *csgA*/*bcsA* double mutant strain unable

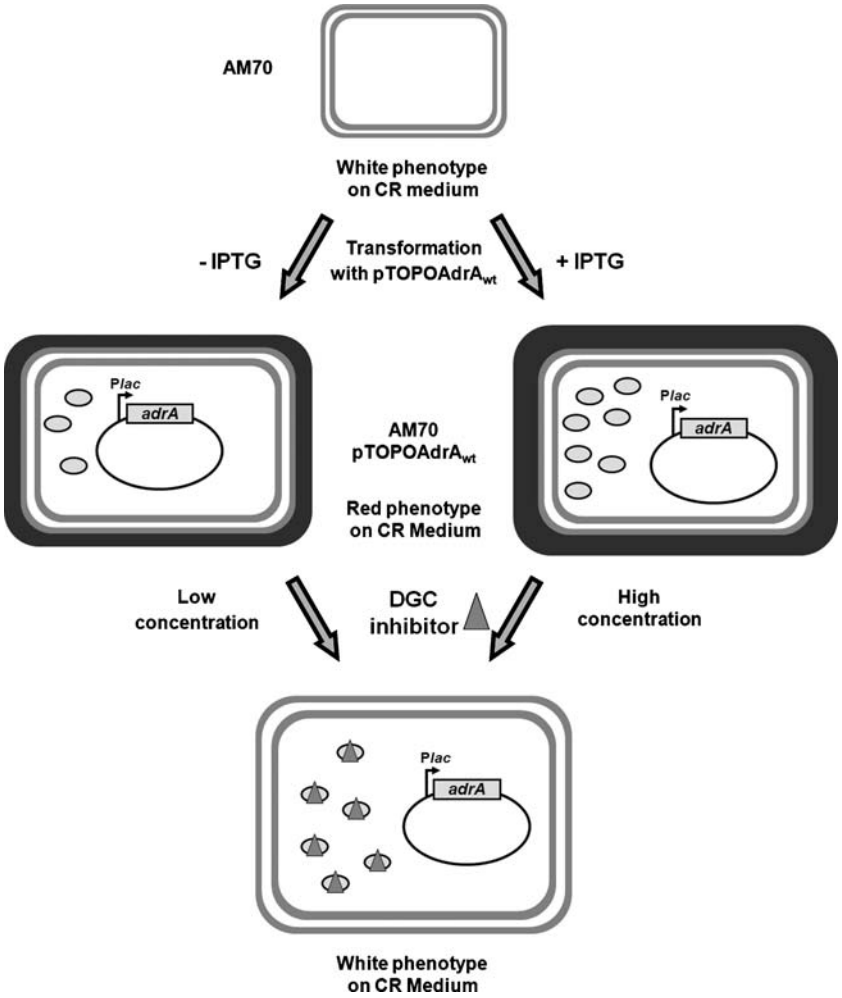

Fig. 1 Rational design of the *Escherichia coli* reporter strain used in the Congo red (CR) assays. The *ΔcsgA* AM70 strain is transformed with the pTOPOAdrA$_{wt}$ plasmid; growth either in the presence or in the absence of isopropyl β-D-1-thiogalactopyranoside can lead to different AdrA expression levels with consequent production of cellulose binding CR (indicated by the additional extracellular layer; different thickness levels only intend to indicate isopropyl β-D-1-thiogalactopyranoside-induced and uninduced cells and are not representative of actual cellulose amounts produced in either condition). Presence of a diguanylate cyclase inhibitor would block AdrA activity resulting in a white phenotype on CR medium

to produce either curli or cellulose (Fig. 2a). IPTG induction did not result in any detectable change in AM70/pTOPO or AM70/pTOPOAdrA$_{wt}$ phenotype on CR medium (data not shown). As a further verification that *adrA* overexpression results in cellulose production, we plated the AM70 strain transformed either with pTOPOAdrA$_{wt}$ or pTOPOAdrA$_{GGAAF}$ or with the pTOPO control vector on Calcofluor-supplemented plates. Calcofluor is a fluorescent whitener which binds specifically to cellulose and chitin; Calcofluor binding to cellulose can be revealed by exposure to UV lights (Perry and Miller 1989). As expected, only the AM70/pTOPOAdrA$_{wt}$ strain showed fluorescence upon UV light exposure, indicating that AdrA overexpression does indeed result in activation of cellulose production (Fig. 2b). To confirm that the *adrA*-induced phenotypic changes in CR- and Calcofluor-supplemented media were indeed due to AdrA DGC activity, we transformed AM70 with a plasmid carrying a mutated allele of the *adrA* gene, encoding an AdrA protein in which the GGDEF amino acid sequence of the DGC catalytic site was changed to GGAAF (pTOPOAdrA$_{GGAAF}$). Substitution to alanine of any residue in the GGDEF motif strongly affects DGC activity, and thus c-di-GMP biosynthesis (Simm et al. 2004; Malone et al. 2007; De et al. 2008; Jonas et al. 2008). The intracellular c-di-GMP concentration in MG1655 cells and in the cells transformed with either pTOPO or pTOPOAdrA$_{wt}$ or pTOPOAdrA$_{GGAAF}$ was determined by HPLC analysis. As shown in Fig. 2c, c-di-GMP was clearly detected in cells expressing AdrA protein at a concentration of 360 nmol/g (dry weight), in agreement with the levels measured in an AdrA-overexpressing strain of *Salmonella* (Simm et al. 2004). Intracellular c-di-GMP concentrations in MG1655 and in MG1655 carrying the control vector contained 3.5 and 2.4 nmol/g c-di-GMP, respectively.

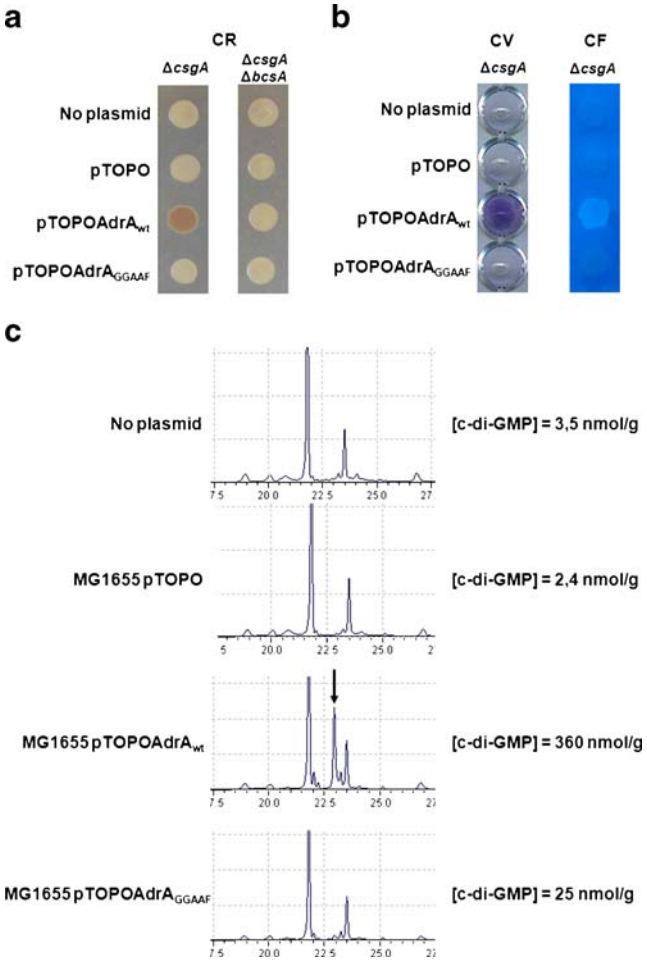

Fig. 2 **a** Effects of the expression of AdrA (either wild type or GGAAF mutant) in AM70 (*ΔcsgA*, curli-deficient) and AM73 (*ΔcsgA ΔbcsABZC*, curli- and cellulose-deficient) on Congo red binding (CR). **b** Effects of expression of AdrA$_{wt}$ and AdrA$_{GGAAF}$ proteins on biofilm formation (measured with the crystal violet assay) and on Calcofluor binding. Semi-quantitative evaluation of biofilm in crystal violet assays gave adhesion values of 19.6 for AM70/pTOPOAdrA$_{wt}$ and of 0.53 for AM70/pTOPO. **c** Determination of c-di-GMP biosynthesis in AdrA$_{wt}$ and AdrA$_{GGAAF}$ protein-expressing strains by high-performance liquid chromatography. The peak corresponding to c-di-GMP is marked by an *arrow*; the peak with a retention time of 21.8 min corresponds to NAD, while the peak at 23.5 min was not identified

Expression of the AdrA$_{GGAAF}$ protein resulted in an intracellular c-di-GMP concentration of 25 nmol/g, i.e., a >90% reduction of intracellular c-di-GMP compared to MG1655 transformed with pTOPOAdrA$_{wt}$ (Fig. 2c). Expression of the AdrA$_{GGAAF}$ protein failed to induce cellulose production when expressed in AM70 (Fig. 2a, b), as expected from its poor DGC activity. Both the AdrA$_{wt}$ and the AdrA$_{GGAAF}$ proteins were produced at similar levels, as judged by analysis of cell extracts by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown), thus indicating that lower c-di-GMP intracellular levels in MG1655 transformed with pTOPOAdrA$_{GGAAF}$ are indeed due to impaired DGC activity by the AdrA$_{GGAAF}$ protein. In order to test if AdrA-mediated cellulose production would result in biofilm formation, we performed surface adhesion experiments using the crystal violet assay (Dorel et al. 1999), which clearly showed increased adhesion to polystyrene microtiter plates by AM70 transformed with pTOPOAdrA$_{wt}$ but not with pTOPOAdrA$_{GGAAF}$ (Fig. 2b). Thus, AM70/pTOPOAdrA$_{wt}$ phenotypes on CR- and Calcofluor-supplemented plates, as well as its ability to form biofilm, totally depend on AdrA DGC activity.

Our results clearly suggest that the AM70/pTOPOAdrA$_{wt}$ strain can be a suitable reporter strain to measure inhibition of DGC activity via determination of its phenotype on CR medium. However, assays on solid medium are usually not amenable for HTS, mainly due to the amount of chemical compounds required for standard plates and to difficulties in automation of the assay. To overcome these limitations, we miniaturized the CR assay in 96-well microtiter plates: 200 μl of CR medium prior to solidification are distributed using a multi-channel pipette in each well; after solidification, 5 μl of an overnight culture of AM70/pTOPOAdrA$_{wt}$ are layered on top of the solidified CR medium. The 96-well microtiter plate is then incubated overnight; phenotypes are detectable immediately after overnight incubation, although they become clearer after additional 24–48 h of incubation at 4°C. The chemicals to be tested can be added in solution (5–10 μl) at various concentrations to the bottom of the microtiter plate wells prior to the addition of CR medium. This "miniaturized CR assay" was used to screen a chemical library for DGC inhibitors (see below).

DGC-dependent gene expression assays The assays described in the previous sections can be used to select for inhibitors of biofilm formation dependent on DGC activity. However, chemical compounds able to affect CR phenotype and surface adhesion in crystal violet assays might target steps in biofilm formation other than c-di-GMP biosynthesis, or they might be capable of non-specific binding to the cell surface with consequent alteration of its physico-chemical properties. Thus, a strategy for selection of DGC inhibitors should include a tertiary screening assay that can assess inhibition of DGC activity inside the bacterial cell directly.

In addition to acting as an allosteric activator of the cellulose biosynthetic proteins, the AdrA protein can activate transcription of curli-encoding *csg* genes when overexpressed in *Salmonella enterica* (Kader et al. 2006). Another DGC protein, YdaM, controls expression of curli-encoding *csg* genes through its DGC activity in *E. coli* (Weber et al. 2006; Pesavento et al. 2008). We transformed an MG1655 derivative carrying a *csgA::uidA* chromosomal fusion (PHL856, Gualdi et al. 2008), either with pTOPOA-

drA$_{wt}$ or with pTOPOYdaM. *uidA* is a reporter gene encoding β-glucuronidase, whose enzymatic activity can easily be monitored with a colorimetric assay (Bardonnet and Blanco 1992); the *csgA::uidA* fusion leads to β-glucuronidase production in response to transcription of the *csgBAC* operon, encoding curli structural subunits (Prigent-Combaret et al. 2001). β-glucuronidase experiments performed on overnight cultures grown in M9Glu/sup medium at 30°C show that *csgBAC* transcription is activated by both AdrA (ca. fourfold) and YdaM (ca. 6.5-fold; Fig. 3). Thus, measurement of DGC-dependent *csgBAC* transcription by β-glucuronidase assays provides a convenient method to test DGC inhibition by compounds showing activity in the CR and biofilm formation assays.

Identification of the antimetabolite sulfathiazole as inhibitor of c-di-GMP biosynthesis The screening strategy described in the previous sections consists of a primary qualitative assay based on a color phenotype in the miniaturized CR assay, followed by the semi-quantitative crystal violet assay to assess inhibition of biofilm formation, and by the β-glucuronidase reporter assay to verify inhibition of DGC enzymes inside the bacterial cell. Chemical compounds showing inhibitory activity in all three assays can then be tested for their ability to inhibit c-di-GMP biosynthesis in bacterial cells by HPLC. Thus, we used the miniaturized CR assay described above to screen a chemical library from Prestwick Chemicals (http://www.prestwickchemical.fr/index.php?pa=26). This library contains 1,120 chemical compounds with known biological activities, already tested for bioavailability and safety in humans, based on the Selective Optimization of Side Activities criteria for the identification of novel biological activities by known drugs (Wermuth 2006). Each chemical was tested at three concentrations (2, 10, and 50 μg/ml); the effect of 18 compounds (1.61% of total) could not be assessed, due either to CR precipitation or to medium acidification leading to loss of red color (CR is a pH indicator). One molecule, sulfathiazole, caused discoloration of AM70/pTOPOAdrA$_{wt}$ red phenotype when added to the CR medium already at the lowest concentration tested (2 μg/ml, corresponding to 7.8 μM; data not shown) and did not affect bacterial growth up to 50 μg/ml, the highest concentration tested in CR assays. Determination of sulfathiazole MIC in liquid media showed bacterial growth inhibition at ca. 70 μg/ml (275 μM, Table 2) sulfathiazole, i.e., at concentrations 35-fold higher than those inhibiting AM70/pTOPOAdrA$_{wt}$ red phenotype on CR medium. Concentrations as low as 5.8 μM inhibited biofilm formation in crystal violet assays (Table 2), thus suggesting that the sulfathiazole effect on CR phenotype correlates with its ability to prevent biofilm formation. Finally, β-glucuronidase reporter assays showed that sulfathiazole was able to inhibit both AdrA- and YdaM-dependent stimulation of *csgA* gene expression by 50% at 3.9 μM (Table 2) and by 90% at 7.8 μM (not shown), similar to the concentration needed to prevent cellulose production and biofilm formation.

To verify that the effects of sulfathiazole on the CR phenotype, biofilm formation, and curli-encoding gene expression correlated with inhibition of c-di-GMP biosynthesis, we measured c-di-GMP intracellular concentrations both in the absence and in the presence of different sulfathiazole concentrations by HPLC. Expression of either AdrA or YdaM proteins in MG1655 strain transformed with either the pTOPOAdrA or pTOPOYdaM plasmids led to similar c-di-GMP concentrations (360 nmol/g dry weight and 341 nmol/g dry weight respectively; Fig. 2c, Table 2). However, treatment with sulfathiazole resulted in clear inhibition of c-di-GMP biosynthesis: 50% reduction in c-di-GMP intracellular levels were observed at 4.6 μM sulfathiazole, similar to the concentrations needed to inhibit biofilm formation and activation of *csgA* gene expression (Table 2). Complete inhibition of c-di-GMP production was observed at 20 μM sulfathiazole (data not shown).

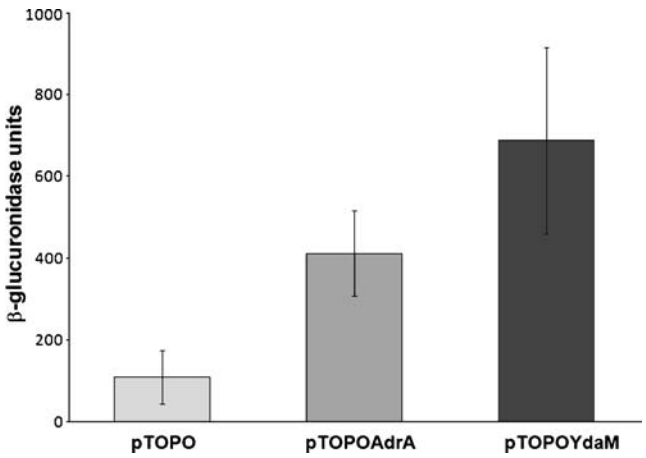

Fig. 3 β-glucuronidase assay on the PHL856 (*csgA::uidA-kan*) strain transformed with pTOPO, pTOPOAdrA$_{wt}$, or pTOPOYdaM plasmids. Average values were 109, 412, and 687 units, respectively. β-glucuronidase activity was determined on overnight cultures grown in M9Glu/sup at 30°C. Results are the average of four different experiments; error bars are shown

Discussion

In this report, we have described a novel screening strategy for inhibitors of DGCs, a class of bacterial enzymes responsible for the biosynthesis of the c-di-GMP signal molecule. C-di-GMP promotes biofilm formation through stimulation of EPS and cell surface-associated factors

Table 2 Inhibition of biofilm formation and c-di-GMP biosynthesis by sulfathiazole

Assays	Inhibition by sulfathiazole (μM)
Biofilm formation (crystal violet assay)[a]	IC_{50}^{*} = 5.8 ± 0.63
csgBAC gene expression[b] (β-glucuronidase assay)	IC_{50}^{*} = 3.9 ± 0.82
c-di-GMP biosynthesis[c] (HPLC determination)	IC_{50}^{*} = 4.6
Bacterial growth inhibition[d] (MIC determination)	MIC = 275 ± 47

IC_{50}^{*} =sulfathiazole concentration inhibiting the reaction by 50%

[a] Average of three experiments: control values for AM70/pTOPOAdrA$_{wt}$ = 19.2, for AM70/pTOPO = 0.46 adhesion units

[b] Average of three independent experiments: control values for AM70/pTOPOYdaM$_{wt}$ = 673, for AM70/pTOPO = 91 β-glucuronidase units

[c] Average of two determinations with very similar values: control values for AM70/pTOPOYdaM$_{wt}$ = 341, for AM70/pTOPO = 3.1 nmol/g dry weight

[d] Average of three independent experiments. Values determined by visual inspection

(Römling and Amikam 2006). C-di-GMP-mediated biofilm formation is an important factor in host colonization and appears to play a major role in chronic diseases such as lung infection in cystic fibrosis patients (Häussler 2004; Kulasakara et al. 2006; Cotter and Stibitz 2007; Tamayo et al. 2007). Thus, inhibition of enzymes involved in c-di-GMP biosynthesis might counteract host colonization by pathogenic bacteria and might complement antimicrobial therapies with conventional antibiotics. The screening strategy described in this report takes advantage of well defined genetic systems (Römling et al. 2000; Zogaj et al. 2001; Simm et al. 2004); and it uses simple and established assays performed on living bacteria to identify DGC inhibitors able to cross the bacterial membrane. Although the assays used in the initial steps of our screening strategy do not directly detect intracellular c-di-GMP concentrations, they can measure DGC-dependent cellulose production and biofilm formation (CR and crystal violet assays, Fig. 2) and DGC-dependent activation of gene expression (β-glucuronidase reporter gene assays, Fig. 3). The possibility of utilizing two different DGCs (AdrA and YdaM) in the β-glucuronidase assay is an additional asset, since our aim is to identify molecules active on more than one specific DGC. Unfortunately, unlike AdrA, YdaM expression does not confer a red phenotype to the AM70 *csgA* mutant strain, since it fails to activate cellulose biosynthesis (data not shown), thus making YdaM unsuitable for the CR assay in this genetic background. In addition to the AdrA- and YdaM-expressing strains described in this work, we are currently screening *E. coli* strains expressing DGCs from different pathogenic bacteria, and we have found that WspR, a DGC from *Pseudomonas aeruginosa*, can confer onto the AM70 strain a red phenotype on CR medium (data not shown), consistent with previously published results showing similar *wspR* effects in *Salmonella* (Ude et al. 2006). Thus, our screening strategy can be used for the identification of inhibitors of DGCs from pathogenic bacteria. The assays described in this report provide the first example of a screening strategy for anti-biofilm agents based on a specific mechanism of action, i.e., inhibition of DGC activity (summarized in Fig. 4). Such defined target-based screening systems are considered essential for novel drug discovery (Mills 2006). Chemicals testing positive in the target-based screening could then be tested in quantitative measurement of c-di-GMP in the bacterial cell (Table 2) or in biochemical assays using purified DGC to monitor enzymatic inhibition (Paul et al. 2004; Simm et al. 2004), as suggested in Fig. 4.

The potential of the proposed approach was validated by a screening of a commercially available library of chemical compounds with known biological activities, a screening approach known as Selective Optimization of Side Activities (Wermuth 2006). Out of the ca. 1,120 compounds tested, we found that one molecule, the antimicrobial agent sulfathiazole, resulted in discoloration of AM70/pTOPOAdrA$_{wt}$ red phenotype on CR medium. No other molecule with antimicrobial activity present in the Prestwick chemical library (e.g., amikacin, tobramycin, dirithromycin, pipemedic acid, and ofloxacin) showed any effect on

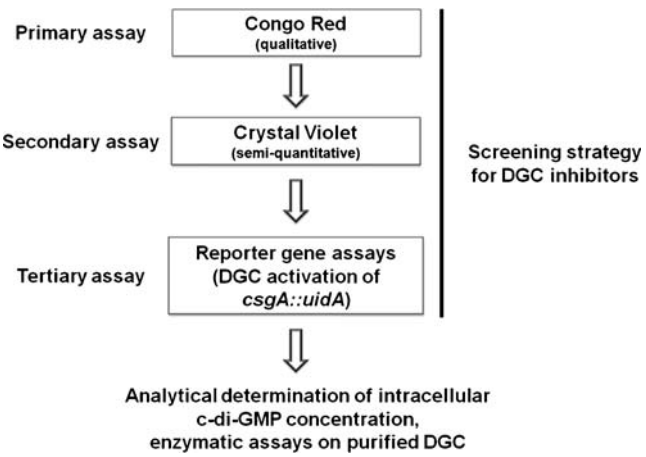

Fig. 4 Diagram summarizing the screening strategy for diguanylate cyclase inhibitors

AM70/pTOPOAdrA$_{wt}$ CR phenotype at concentrations allowing bacterial growth, suggesting that the effect of sulfathiazole in the CR assay is specific and not due to partial growth inhibition. Thus, sulfathiazole was further investigated in the secondary screening assays: sulfathiazole displayed anti-biofilm activity, inhibited DGC-mediated activation of the *csgA* gene, and was able to prevent c-di-GMP biosynthesis in bacterial cells at comparable concentrations to those used in our activity-based screening (Table 2). Sulfathiazole belongs to the sulfonamide class of antimicrobials and is an inhibitor of di- and tetrahydrofolate biosynthesis via interaction with the dihydropteroate synthase FolP (Vedantam and Nichols 1998; Haasum et al. 2001). Depletion of intracellular tetrahydrofolate in turn affects various metabolic pathways, including biosynthesis of purine nucleotides. Antimicrobial activity of sulfonamides can be overcome by growing bacteria in complex media, thus providing the end products of tetrahydrofolate metabolism. In the conditions used in our experiments (CR medium and M9Glu/sup, both supplemented with yeast extract and an amino acid source), we could only observe significant inhibition of bacterial growth by sulfathiazole at 275 μM, i.e., at concentrations 50-fold higher than needed to inhibit biofilm formation or to prevent c-di-GMP biosynthesis (Table 2). Since sulfathiazole affects tetrahydrofolate biosynthesis and nucleotide metabolism, it is likely that inhibition of c-di-GMP biosynthesis by sulfathiazole does not take place through direct inhibition of DGC activity, but through indirect effects, such as alteration of nucleotide pools, which can in turn affect DGC substrate availability. This hypothesis was confirmed by the observation that full induction of AdrA expression with IPTG in the AM70/pTOPOAdrA$_{wt}$ strain does not result in decreased sensitivity to the anti-biofilm activity of sulfathiazole in crystal violet assays (data not shown). Interestingly, another inhibitor of nucleotide biosynthesis, fluorouracil, can affect biofilm formation both in *E. coli* and *P. aeruginosa* (Attila et al. 2009), even though biofilm inhibition by fluorouracil appears to be mediated by inhibition of quorum sensing rather than c-di-GMP biosynthesis. In addition, mutations in nucleotide biosynthetic genes can impair biofilm formation and surface adhesion in *P. aeruginosa* (Ueda et al. 2009) and in *E. coli* (Landini, unpublished data), strongly suggesting that perturbation of intracellular nucleotide pools could indeed interfere with molecular signaling leading to biofilm formation. Although sulfathiazole might not inhibit c-di-GMP biosynthesis directly, it does however impair c-di-GMP intracellular levels (Table 2), indicating that the screening strategy proposed in this report is efficient for the identification of molecules affecting c-di-GMP biosynthesis. Our results, together with previous observations (Ueda et al. 2009), would suggest that development of nucleotide synthesis inhibitors could be a promising strategy for the discovery of antimicrobial agents endowed with anti-biofilm activity.

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