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Miniaturized whole-cell bacterial bioreporter assay for identification of quorumsensing interfering compounds

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ABSTRACT

The continuing emergence and spread of antibiotic-resistant bacteria is worrisome and new strategies to curb bacterial infections are being sought. The interference of bacterial quorum sensing (QS) signaling has been suggested as a prospective antivirulence strategy. The AI-2 QS system is present in multiple bacterial species and has been shown to be correlated with pathogenicity. To facilitate the discovery of novel compounds interfering with AI-2 QS, we established a high-throughput setup of whole-cell bioreporter assay, which can be performed in either 96- or 384-well format. Agonistic or antagonistic activities of the test compounds against *Escherichia coli* LsrB-type AI-2 QS system are monitored by measuring the level of β-galactosidase expression. A control strain expressing β-galactosidase in quorum sensing-independent manner is included into the assay for false-positive detection.

1. Introduction

Quorum sensing (QS) is a bacterial communication system that coordinates cooperative behaviors in bacteria in a population density-dependent manner by means of small chemical signals (autoinducers) (Rutherford and Bassler, 2012). QS has been shown to affect virulence factor production and biofilm formation in several bacterial species, including clinically relevant human pathogens (Antunes et al., 2010), (Rutherford and Bassler, 2012). In contrast to conventional antibiotics, interference with QS is believed to put lower selective pressure on bacterial pathogens, reducing chances of resistance development (Rutherford and Bassler, 2012), (Rampioni et al., 2014). Bacteria utilize a diverse set of QS systems. Whereas many QS signals are specific for a certain group or even species of bacteria, autoinducer-2 (AI-2) can be produced and detected by multiple bacterial species. Therefore, AI-2-mediated QS inhibitors potentially represent broad-spectrum antivirulence agents (Guo et al., 2013).

AI-2 signal synthesis is catalyzed by the enzyme product of *luxS* gene, which is widely distributed throughout bacterial kingdom, including both Gram-positive and Gram-negative bacteria (Waters and Bassler, 2005), (Pereira et al., 2013). In this reaction AI-2-precusor, (*S*)-4,5-dihydroxy-2,3-pentanedione (DPD) is formed from S-ribosyl-l-homocysteine (SAH). Whereas *luxS* gene is highly conserved between different bacterial species, the AI-2 detection and signal transduction systems are more diverse. To date, three classes of AI-2 receptors have been described. The two best characterized are the members of LuxP family, limited to *Vibrio spp.*, and LsrB family, found in many (but not all) Gram-negative and in Gram-positive bacteria. As some bacterial species lacking a known AI-2 receptor respond to the externally added signal, additional receptors must exist (Pereira et al., 2013). The gene for LsrB receptor is a part of the AI-2-regulated *lsr* operon encoding for the proteins involved in regulation of gene expression, as well as internalization, processing and degradation of AI-2 molecules. This system is more widespread than LuxP system and is present in human pathogens such as *Shigella dysenteriae*, *Shigella flexneri, Salmonella* spp. and *Escherichia coli* (Rezzonico and Duffy, 2008). These species share common mechanism of signal detection which has been studied in detail in *E. coli*. AI-2 signal accumulation correlates with bacterial population increase, reaching the maximum level at the middle-late exponential phase (Xavier and Bassler, 2005). When threshold concentration is reached, the signal triggers expression of *lsr* operon. This results in accelerating the expression of Lsr transport system and the rapid decline of AI-2 signal in the medium (Pereira et al., 2013), (Zhao et al., 2018).

Lately, significant amounts of effort have been directed towards the discovery of compounds interfering with the AI-2 QS pathway (Galloway et al., 2011), (Guo et al., 2013). The activity of compounds can be evaluated in cell-free systems (where the interaction with the

Abbreviations: QS, quorum sensing; HTS, high throughput screening; AI-2, Autoinducer-2; DPD, (*S*)-4,5-dihydroxy-2,3-pentanedione

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target protein can be shown). In addition, the effect on the expression of virulence factors, motility or biofilm production by the pathogen of interest is typically demonstrated. However, these methods are indirect, as virulent behaviors are in many cases not solely regulated by QS. To facilitate drug discovery process, the use of reporter bacterial strains is highly beneficial, as it allows the detection of compounds showing activity through specific, QS-mediated mechanism. As bioreporters are whole-cell systems, the compounds with toxic properties or low cell permeability can be ruled out. A number of reporter strains have been established to identify novel molecules interfering with AI-2-mediated QS (Defoirdt et al., 2013). Although the majority of these strains are designed to detect QS interference with the components of LuxP system, there are a few using clinically more relevant LsrB system (Lowery et al., 2008), (Weiland-Brauer et al., 2015), (Roy et al., 2010). However, none of them has been used in high-throughput screening (HTS) format.

Here we report the optimization and validation of high-throughput whole-cell bioreporter assay for the identification of novel small molecules interfering with AI-2 quorum sensing pathway. The *E. coli* LW7 pLW11 strain has *lacZ* gene under *lsr* promoter and therefore produces β-galactosidase in response to the externally added DPD (Wang et al., 2005). The assay reveals agonistic activity (when performed in the absence of DPD) or antagonistic activity (in the presence of DPD). This strain has been previously utilized to measure the QS response of DPD analogs (Roy et al., 2010). However, the originally reported method requires high amount of test compound. Moreover, the β-galactosidase expression was measured by traditional Miller assay, which is a time-consuming multistep process. Here we adopt the simplified single-step detection procedure introduced by Schaefer et al. (Schaefer et al., 2016) and scale the assay down to 96- and 384-well plate format. As emphasized in the review by Defoirdt et al. (Defoirdt et al., 2013), one of the main limitations of using bioreporter strains as instruments to detect QS-interference is their inability to exclude compounds with unspecific (not QS-mediated) mode of action. To overcome this limitation, we incorporated into the assay a control strain where β-galactosidase is expressed in QS-independent manner.

2. Material and methods

2.1. Bacterial strains and growth conditions

E. coli LW7 pLW11 bioreporter strain was kindly provided by Prof. William E. Bentley, University of Maryland, USA. The strain does not produce either its own AI-2 or β-galactosidase. pLW11 plasmid is a pFZY1 derivative, containing *lacZ* gene under the control of quorum sensing-related *lsrACDBFG* promoter (Wang et al., 2005). The control strain *E. coli* pBAC-LacZ was a gift from Keith Joung (Addgene plasmid # 13422). It is derived from *E. coli* DH5α by introducing low copy number β-galactosidase plasmid under *lac* promoter. These strains were cultured in Lysogeny Broth (LB) medium supplemented with ampicillin 100 μg/ml and kanamycin 50 μg/ml for *E. coli* LW7 pLW11, or with chloramphenicol 12.5 μg/ml for *E. coli* pBAC-LacZ.

2.2. Chemical compounds used for assay validation

DPD analogs A1 (neopentyl-DPD), A2 (pentyl-DPD), A3 (2-methylpropyl-DPD), A4 (isopropyl-DPD), A5 (cyclopropyl-DPD), A6 (isobutyl-DPD) and A7 (cyclopentyl-DPD) were synthesized according to the procedure reported in the (Roy et al., 2010). The 4-chloro-2-phenylamino-benzoic acid (CBA) was purchased from Molport (000–162-734). The compounds were first dissolved in DMSO at 10–100 mM concentration and stored at −80 °C.

2.3. Other reagents

DPD [(*S*)-4,5-dihydroxy-2,3-pentanedione] was purchased from Carbosynth (UK), PopCulture™ reagent and rLysozyme™ from Millipore. Minimal essential medium (MEM), β-galactosidase, *o*-nitrophenyl-β-D-galactopyranoside (ONPG) and inorganic salts for buffer preparation were obtained from Sigma. The assay was performed in flat bottom clear polystyrene 96- and 384-well plates (Nunc™, Thermo Fisher Scientific). Lysogeny Broth (LB) and Tryptic Soy Broth (TSB) media were obtained from Becton Dickinson.

2.4. Lysis method optimization

Overnight liquid culture of *E. coli* pBAC-LacZ was centrifuged at 3500*g* for 10 min and resuspended in PBS. Bacteria were diluted to the concentration of 5×10^8 cfu/ml and added to a 96-well plate, 100 μl/well. The plates were analyzed for the β-galactosidase expression immediately or after one freeze-thawing cycle (−20 °C), following the procedure described below (see *Measurement of β-galactosidase activity)*. Chicken egg white lysozyme (0.2, 0.5, 1 mg/ml) or rLysozyme™ (0.2–4 kU/ml) was added to the detection mix.

2.5. Evaluating QS response and unspecific β-galactosidase inhibitory activity

The assay outline is represented in Fig. 1. Overnight culture of *E. coli* LW7 pLW11 was diluted in fresh antibiotic-supplemented LB medium 1:50 and incubated at +30 °C, 200 rpm (Stuart Orbital shaker, SI500) for 4–5 h to medium/late logarithmic growth phase, as controlled by turbidity measurements using DEN-1B densitometer (Biosan). After centrifugation (3500 *g*, 10 min) bacteria were diluted in appropriate assay medium (phosphate-buffered saline, PBS, LB, TSB or MEM) to the 2× final concentration (10^9 cfu/ml in the final version of the assay). DPD (2× final concentration, 40 μM in the final version of the assay) was added to a half of the suspension. Then DPD+ and DPD- suspensions were distributed into vials (1 ml/vial), 96-well plates (50 μl/well) or 384-well plates (12 μl/well), depending on the assay format. Test compounds were prepared in appropriate assay medium (PBS, LB, TSB or MEM) to the 2× final concentration and then added to vials or well plates (1 ml/vial, 50 μl/well of the 96-well plate or 12.5 μl/well of the 384-well plate). Vials/plates were incubated for 2 h, 37 °C, with shaking at 200 rpm (for vials; Stuart Orbital shaker, SI500) or 500 rpm (for plates; Biosan PST-60HL-4 plate shaker). The samples from vials were then transferred into 96-well plates, 100 μl/well for analysis. Other samples were analyzed in the same plate which was used for the assay. Absorbance at 600 nm was measured (Multiskan GO plate reader), and the plates were frozen at −20 °C overnight before the analysis (see *Measurement of β-galactosidase activity)*.

For unspecific β-galactosidase inhibitory activity measurement the same procedure was used with the *E. coli* pBAC-LacZ strain. All the samples were tested in the absence of DPD.

2.6. Measurement of β-galactosidase activity

The β-galactosidase activity was quantified according to the procedure reported by Schaefer and coworkers (Schaefer et al., 2016), with some modifications. If the freeze/thawing step was applied, the well plates were quickly thawed in warm water and immediately proceeded to the next step. β-galactosidase detection mix (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 36 mM β-mercaptoethanol, 6.7% PopCulture™ reagent, 1.1 mg/ml ONPG) was added 150 μl/well to the 96-well plate or 38 μl/well to the 384-well plate. β-galactosidase was used as a positive control for the detection procedure. The plates were incubated for 1 h (control strain) or 2 h (bioreporter strain) at 37 °C, 500 rpm (Biosan PST-60HL-4 plate shaker), followed by absorbance measurement at 420 and 550 nm (Multiskan GO plate reader). The activity of β-galactosidase in Miller units (MU) was calculated using the following formula:

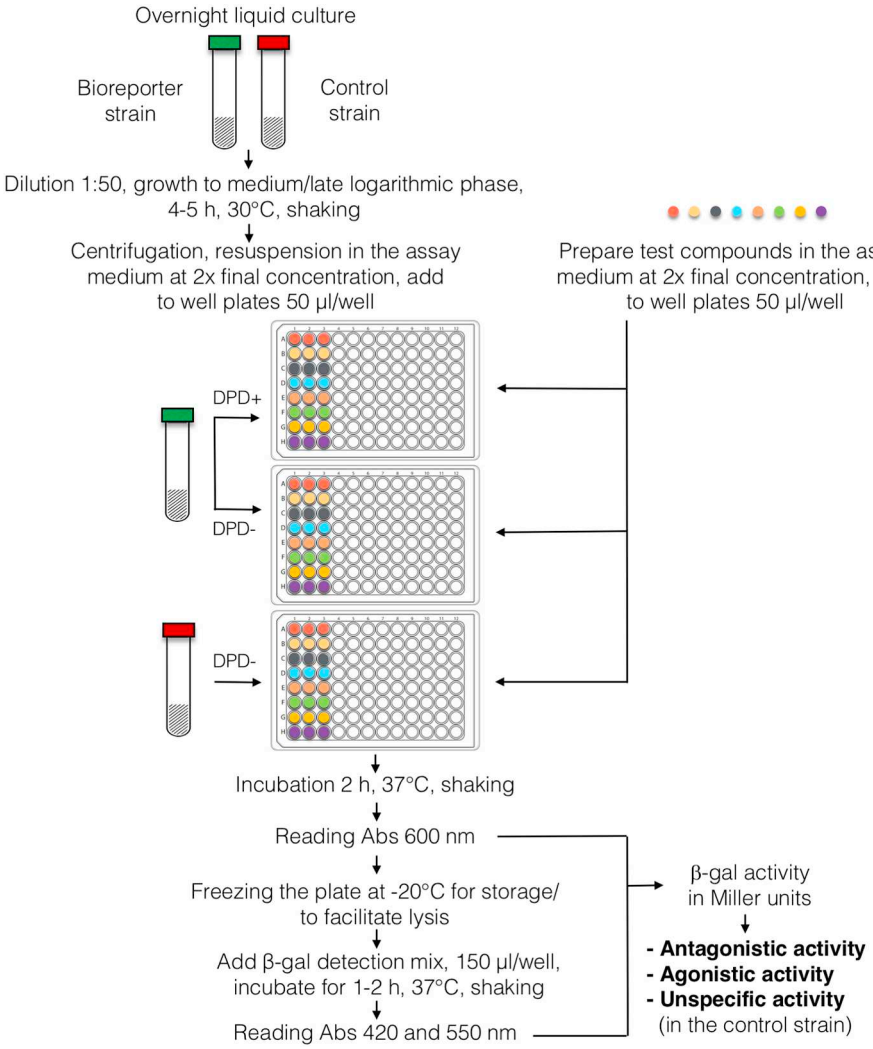

Fig. 1. Overview of 96-well format bioreporter assay.

$$\mathrm{MU} = \frac{1000*(Abs420nm - (1.75*Abs550nm)}{t*V*Abs600n\mathrm{m}}$$

wherein t = reaction time (minutes), V = volume of assayed culture (milliliters).

Antagonistic activity (AnA) was calculated using MU obtained in the bioreporter strain as % of native QS response to DPD:

$$\mathrm{AnA} = 100*\frac{\mathrm{MUs(DPD+)} - \mathrm{MUc(DPD-)}}{\mathrm{MUc\,(DPD+)} - \mathrm{MUc(DPD-)}.}$$

Agonistic activity (AgA) was determined using MU obtained in the bioreporter strain as % of activation of the native response in the absence of DPD:

$$\mathrm{AgA} = 100*\frac{\mathrm{MUs(DPD-)} - \mathrm{MUc(DPD-)}}{\mathrm{MUc\,(DPD+)} - \mathrm{MUc(DPD-)}}$$

Unspecific activity (UnA) was determined using values obtained with a control strain:

$$\mathrm{UnA} = 100*\frac{\mathrm{MUs}}{\mathrm{MUc}}$$

In the equations above s = compound-treated sample and c = DMSO control.

2.7. Assay quality assessment

The assay performance was monitored throughout experiments by calculating the screening window coefficient (Z'- factor) (Zhang et al., 1999), (Inglese et al., 2007) using following formula:

$$Z' = 1 - \frac{3*\mathrm{SDmax} + 3*\mathrm{SDmin}}{|\mathrm{MUmax} - \mathrm{MUmin}|}$$

In the equations above MU = Miller units, max = sample in the presence of DPD and min = no DPD sample.

3. Results and discussion

Bacterial QS is a potential target in antivirulence drug discovery that has been intensively investigated over the past decade (Rutherford and Bassler, 2012), (Rampioni et al., 2014). To facilitate the discovery of small molecule inhibitors of AI-2 QS system we aimed to set up an *E. coli*-based bioreporter QS interference assay in HTS format. In the course of optimization process we (1) incorporated into the assay the β-galactosidase detection procedure compatible with polystyrene plates; (2) selected optimal assay conditions (lysis method, cell number/well, DPD concentration) to be used in 96- and 384-well plates; (3) evaluated assay compatibility with different media; (4) introduced a control strain for false-positive detection; and (5) validated the assay performance using a set of known AI-2 QS inhibitors.

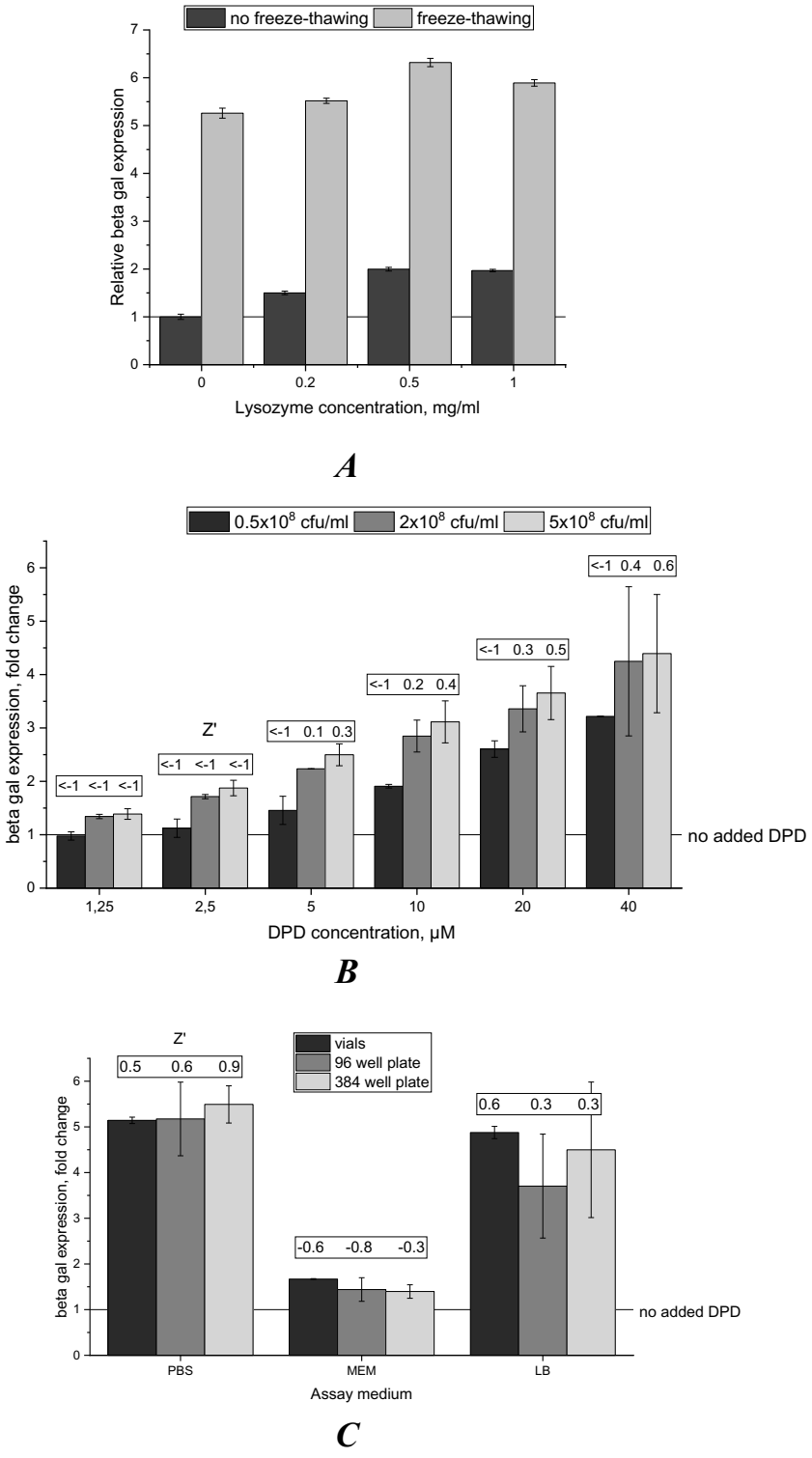

Fig. 2. (A) Bacterial lysis method optimization. The effect of lysozyme treatment and freeze-thawing on the expression of the reporter gene (β-galactosidase). The representative results from a single experiment are shown. (B) Effect of DPD concentration and bacterial number on the native response of the bioreporter strain to DPD. The data from 2 independent experiments ± SD is presented. (C) Effect of the assay medium and format on the native response of the bioreporter strain to DPD. The data from 2 independent experiments ± SD is presented. Assay quality parameter Z' represents a mean between values obtained in 2 independent experiments.

3.1. Lysis method optimization

Efficient lysis of bacterial cells is an essential prerequisite for adequate evaluation of reporter gene expression in both bioreporter and control *E. coli* strains used in this study. To adapt the assay for multiwell plate format and to reduce the number of steps, we tested the β-galactosidase detection procedure reported by Schaefer et al. (Schaefer et al., 2016). In this method all assay steps are conveniently performed in the same 96-well plate, and no sample transfer is required. The lysis step is combined with β-galactosidase detection and is achieved by a combination of PopCulture™ reagent and lysozyme added into the detection mix (Schaefer et al., 2016). However, in our hands this

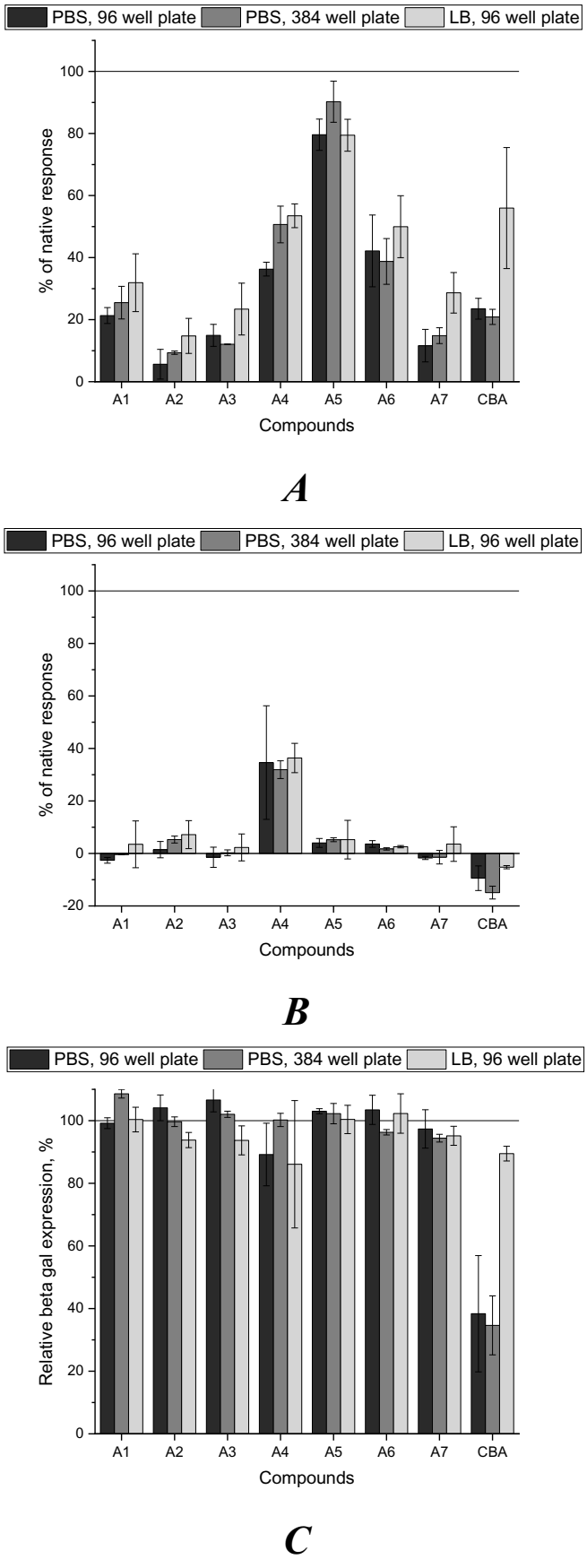

Fig. 3. Validation of AI-2 quorum sensing interference bioreporter assay using a set of DPD analogs (compounds A1-A7) and 4-chloro-2-phenylamino-benzoic acid (CBA). (A) Antagonistic activity, measured as % of native response to DPD. (B) Agonistic activity, measured as % of native response in the absence of DPD. (C) Unspecific response, measured as QS-independent inhibition of β-galactosidase expression in the control strain. Each compound was assessed in triplicate (96-well plate) or in quadruplicate (384-well plate). The data from 2 independent experiments ± SD is presented.

procedure was not sufficient for complete lysis of bacterial cells. Only about 2 times increase in β-galactosidase expression was observed with 0.5–1 μg/ml of chicken egg white lysozyme (Fig. 2A). Similarly, only minor increase in signal was observed when rLysozyme was used (data not shown). We also tested whether cell lysis is more efficient when treatment with lysozyme was performed before detection as a separate step, but no difference to the samples where lysis and detection step were combined was observed (data not shown). In contrast, performing a single freeze-thawing cycle prior to the addition of the β-galactosidase detection mix increased the signal 5–6 times (Fig. 2A). The presence of lysozyme in the detection mix did not further improve the signal of the samples subjected to freeze/thawing. Therefore, single freeze/thawing cycle in combination with the PopCulture™ reagent present in the detection mix resulted in most efficient bacterial lysis and was used in further experiments.

3.2. Optimization of cell number/well and DPD concentration

As *E. coli* LW7 pLW11 bioreporter strain does not produce its own AI-2 signal, the expression of β-galactosidase is induced when external DPD is added. However, it must be noted that background β-galactosidase expression can be detected also in the absence of DPD. The fold increase in expression upon DPD addition is a native response to DPD. The higher native response results in the increased sensitivity of the method. We furthermore questioned how the native response to DPD is affected by the number of bacteria/well and DPD concentration. At all tested bacterial cell numbers the native response was gradually increasing with the DPD concentration (Fig. 2B). Bacterial concentrations of 2×10^8 or 5×10^8 cfu/ml demonstrated similar results, whereas at the 0.5×10^8 cfu/ml the DPD response was lower. The assay performance was monitored throughout experiments by calculating the screening window coefficient (Z'-factor) (Zhang et al., 1999) (Fig. 2B). It is suggested that the assay is suitable for HTS if the Z' is > 0.5. This criterion is met at DPD concentrations between 20 and 40 μM and bacterial numbers 5×10^8 cfu/ml. Although Z' was growing with the DPD concentration, at 40 μM increased variation was observed between biological replicates. Therefore, 20 μM DPD concentration and bacterial concentration 5×10^8 cfu/ml were selected for the final protocol.

3.3. Assay miniaturization

We furthermore compared the performance of the assay in vials, 96-well plates and 384-well plates (Fig. 2C). In PBS, similar results were obtained for all assay formats, and Z' values obtained for multiwell plates were even higher in comparison to those in vials (0.6 and 0.9 vs. 0.5). When the assay was performed in LB, larger variation between repeats was observed for both types of multiwell plates in comparison to vials. However, in all cases the assay window was high enough to enable the assay (Z' > 0.2).

3.4. The effect of the medium

In the original method utilized by Sintim's group, the assay was performed in phosphate buffer (Roy et al., 2010). However, in the buffer cells are under nutrient-deprived conditions. Therefore, we investigated whether the assay can be performed in other media, under

more physiological conditions. Our data demonstrate that for multiwell plates the response to DPD is smaller when the assay is performed in LB medium, when compared to PBS, and Z' values are not optimal: 0.3 in LB vs. 0.6 in PBS for 96-well plates and 0.3 vs. 0.9 for 384-well plates (Fig. 2C). However, Z' > 0 indicate that the assay can be performed in LB medium as long as adequate number of replicates (3–4) are used, which was further confirmed by our validation experiments.

It must be noted, that the assay is not compatible with glucose-containing media, as glucose is known to negatively regulate *lsr* operon (Xavier and Bassler, 2005). Indeed, no increase in the β-galactosidase expression in response to DPD was observed in MEM cell culture medium (contains 1 g/l glucose; Fig. 2C), TSB (contains 2.5 g/l glucose; Supplementary data, Fig. S1) and glucose-supplemented LB at concentrations 0.1 g/l and higher (Supplementary data, Fig. S1).

3.5. Assay validation using a panel of known AI-2 inhibitors

Assay validation was performed with a set of DPD analogs with known QS interference properties (Roy et al., 2010). To exclude false-positives, the *E. coli* pBAC-LacZ control strain was included. In this strain, the expression of β-galactosidase is under the control of *lac* promoter. The strain is used to verify that the compounds do not interfere with β-galactosidase expression in QS-independent manner. 4-chloro-2-phenylamino-benzoic acid (CBA) was demonstrated to be a false-positive compound in one of the screening campaigns performed by our group (unpublished data). Therefore, it was added to this study as a representative example.

All the analogs demonstrated AI-2 QS antagonistic activity (Fig. 3A). The lowest activity (20–30% inhibition of the native response to DPD) was demonstrated for compound A5 (cyclopropyl-DPD). These results are in line with the data of Roy et al., 2010 (Roy et al., 2010). None of the analogs inhibited β-galactosidase expression in the control strain (Fig. 3C), suggesting their specific action via QS-mediated mechanism. In the bioreporter strain, CBA showed strong antagonistic activity (Fig. 3A). However, it also strongly reduced β-galactosidase expression in the control strain (Fig. 3C), which proves the unspecific mechanism of action for this compound and shows it to be a false-positive. When added to the bioreporter strain in the absence of DPD, no increase in β-galactosidase expression was observed for any of the compounds with the exception of compound A4 (isopropyl-DPD) (Fig. 3B), demonstrating some agonistic activity (ca. 30%).

Similar results were obtained in LB and in PBS for all DPD analogs, but not for CBA. This compound inhibited β-galactosidase expression when the assay was performed in PBS, but in LB showed much lower activity in both bioreporter and control strains, demonstrating an example of the effect of the assay medium on the assay outcome.

Comparison of the results obtained in 96- and 384-well plates in PBS as an assay medium shows the same activity profile for all tested compounds (Fig. 3). The same result was obtained in LB medium (see results for 96-well plates in Fig. 3; data for 384-well plates not shown).

4. Conclusions

Here we report the optimization and validation of HTS-compatible bioreporter assay for screening of small molecule libraries for the interference with AI-2 quorum sensing pathway. The assay is based on *E. coli* strain containing a β-galactosidase reporter gene under *lsr* promoter, and therefore can be used for the detection of molecules targeting LsrB-type QS system, found in a number of clinically relevant bacterial pathogens, including *E. coli, Shigella dysenteriae*, *Shigella flexneri* and *Salmonella* spp. However, it must be noted, that the results obtained in one bacterial species cannot be always extrapolated to the other species with the QS system of the same type. For example, although LsrB receptor and AI-2 processing proteins are homologous in *E. coli* and *S. typhimurium*, these organisms respond differently to most of the DPD analogs (Roy et al., 2010). The assay can be performed in either 96- or 384-well format, therefore enhancing the discovery of new antivirulence compounds. The methodology is compatible with PBS or LB medium, but not functional in glucose-containing media, due to repression of *lsr* operon by glucose. The control *E. coli* strain is incorporated into the assay to verify that the compounds do not interfere with β-galactosidase expression in QS-independent manner.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2018.10.005.

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