Chapter 22

A Coculture-Based Approach for Screening Campaigns Aimed at Identifying Novel *Pseudomonas aeruginosa* Quorum Sensing Inhibitors

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Abstract

Quorum sensing (QS) is recognized as a promising target for the identification of anti-virulence drugs hampering *Pseudomonas aeruginosa* adaptability to the host environment and pathogenicity. Consequently, a number of studies in the last decade focused on the identification of small molecules or proteins with anti-QS activity, mainly targeting the *las* QS system, which is based on *N*-3-oxododecanoyl-homoserine lactone ($3OC_{12}$-HSL) as signal molecule. Different experimental approaches have been successfully used to identify QS blockers interfering with the activity/stability of the $3OC_{12}$-HSL receptor LasR, with the functionality of the $3OC_{12}$-HSL synthase LasI, or with the stability/bioavailability of the $3OC_{12}$-HSL signal molecule itself.

Here we describe the use of a high-throughput screening system for the identification of novel *las* QS inhibitors based on the cocultivation of *P. aeruginosa* wild type and the *P. aeruginosa*-derived biosensor strain PA14-R3, in which light emission relies on the ability of the wild type strain to synthesize $3OC_{12}$-HSL and of the biosensor strain to perceive this signal molecule. With respect to other screening systems, this method has the advantage of being cost-effective and allowing the identification of compounds targeting, besides $3OC_{12}$-HSL reception, any cellular process critical for the functionality of the *las* QS system, including $3OC_{12}$-HSL synthesis and secretion.

Key words Quorum sensing inhibitors, Screening, Whole-cell biosensors, Anti-virulence drugs, Niclosamide, *Pseudomonas aeruginosa*, *lasR*, *lasI*

1 Introduction

The introduction of antibiotics into clinical practice at the middle of the twentieth century is a milestone in the history of medicine. However, the original expectation that all bacterial infections could be defeated by antibiotics has been soon disregarded by the emergence of antibiotic-resistant strains. As traditional antibiotic research appears to be helpless in coping with the emergence of

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antibiotic-resistant strains, novel approaches should be undertaken in order to identify new drugs [1, 2].

An innovative strategy to combat bacterial infections relies on specific inhibition of bacterial virulence, hence the ability to cause disease rather than bacterial growth. The use of anti-virulence drugs is expected to reduce bacterial adaptability to the host environment, facilitating the host immune system to resolve the infection, and to diminish the strong selective pressure exerted by conventional antibiotics, although this is not yet supported by direct clinical evidence [3–5].

Since in many bacteria pathogenicity is controlled and coordinated by quorum sensing (QS), this communication system is considered one of the most promising targets for anti-virulence therapies [5, 6].

The Gram-negative bacterium *Pseudomonas aeruginosa* is one of the most dreaded opportunistic pathogens, and represents a prototype of multidrug resistant bug for which effective therapeutic options are limited. The ability of *P. aeruginosa* to cause a wide range of both community- and hospital-acquired infections in humans is linked to its capacity to produce a large repertoire of virulence factors, form antibiotic-tolerant biofilms and, ultimately, respond and adapt to environmental fluctuations, including host immune responses and antibiotic treatments. For these reasons, *P. aeruginosa* infections are generally characterized by high morbidity and mortality rates [7, 8].

The pathogenic potential of *P. aeruginosa* relies on the coordinated expression of a large array of virulence factors, the majority of which are positively controlled by QS [9, 10]. The *P. aeruginosa* QS network consists of at least three different QS systems, *las*, *rhl*, and *pqs*, based on the production and perception of the signal molecules *N*-3-oxododecanoyl-homoserine lactone ($3OC_{12}$-HSL), *N*-butanoyl-homoserine lactone (C_4-HSL), and 2-heptyl-3-hydroxy-4-quinolone (PQS), respectively. *P. aeruginosa* QS is hierarchically organized, since the *las* QS system is required for optimal activation of the *rhl* and *pqs* QS systems. Overall, QS controls the expression of nearly 10% of the *P. aeruginosa* genome, including genes for secreted virulence factors, biofilm formation, and immune-modulatory and pro-inflammatory agents [10, 11].

QS signal molecules can be detected in clinical samples, proving that QS is active during *P. aeruginosa* infections. Moreover, QS-defective mutants show strongly impaired virulence in several animal models of infection, corroborating the importance of QS for *P. aeruginosa* pathogenicity and its suitability as a target for the development of anti-*Pseudomonas* drugs [12, 13].

On these bases, a number of studies focused on the identification of small molecules or proteins with anti-QS activity, mainly targeting the *P. aeruginosa las* QS system. Different experimental approaches have been successfully used to identify small molecules

interfering with the activity/stability of the $3OC_{12}$-HSL receptor LasR or with the functionality of the $3OC_{12}$-HSL synthase LasI. Also the identification of enzymes that inactivate $3OC_{12}$-HSL and the development of antibodies that limit the bioavailability of this signal molecule have been reported [14, 15].

Here we describe a convenient strategy for the identification of compounds affecting the *P. aeruginosa las* QS system at multiple levels: (1) expression/activity of the signal receptor LasR; (2) activity/availability of the signal molecule $3OC_{12}$-HSL; (3) expression/activity of the signal synthase LasI [16]. This strategy is defined by a primary assay, suitable for high-throughput screening of chemical compounds, and by secondary assays, used to confirm the specific activity of the hit compounds selected in the primary assay. This approach has been successfully employed for the identification of a novel *las* QS inhibitor, the FDA-approved anthelmintic drug niclosamide [17].

As a general remark, it should be pointed out that, while we chose to illustrate a screening strategy linked to the activity of the *las* QS system in *P. aeruginosa*, some of the techniques presented here can be applied to a variety of different biological systems. For instance, reporter-based assays modelled on the one described here can be devised for the *rhl* and *pqs* QS systems of *P. aeruginosa*, as well as for different QS systems in other Gram-negatives or in Gram-positive bacteria.

2 Materials

1. Bacterial strains: *P. aeruginosa* PA14 [18] and PA14-R3 biosensor [16].
2. Growth media: Luria-Bertani broth (LB: 10 g/l NaCl, 10 g/l tryptone, 5 g/l yeast extract); Luria-Bertani agar (LA, as LB plus 15 g/l agar).
3. 1 M MOPS Buffer: 83.7 g/l 3-(*N*-morpholino) propanesulfonic acid (MOPS), 13.6 g/l sodium acetate trihydrate, 3.7 g/l ethylenediaminetetraacetic acid (EDTA) disodium salt, pH 7.0.
4. Protease Buffer: 100 mM Tris, 1 mM $CaCl_2$, pH 7.5.
5. Synthetic *N*-3-oxododecanoyl-homoserine lactone ($3OC_{12}$-HSL).
6. Elastin-Congo Red.
7. Chloroform.
8. 0.2 N HCl.
9. Black clear-bottom 96-wells microtiter plates.

10. Automated luminometer-spectrometer plate reader.
11. UV/Vis spectrophotometer.
12. Plastic microcuvettes for UV/Vis spectrophotometer.

3 Methods

3.1 Rationale of the Primary Screening

The screening system for inhibitors of *P. aeruginosa* QS is based on a biosensor strain (PA14-R3) able to detect the QS signal molecule $3OC_{12}$-HSL [16]. The PA14-R3 biosensor is available from the authors upon request.

The PA14-R3 strain carries a nonfunctional allele of the *lasI* gene, and is thus unable to synthesize $3OC_{12}$-HSL; however, it can respond to exogenous $3OC_{12}$-HSL provided either through supply of the purified molecule or by cocultivation with a wild type *P. aeruginosa* proficient in $3OC_{12}$-HSL production, such as the PA14 strain. The screening system is detailed in Fig. 1. The $3OC_{12}$-HSL signal synthesized by the wild type PA14 diffuses into the PA14-R3 biosensor and induces bioluminescence emission. The addition of a molecule with inhibitory activity towards any process related to the $3OC_{12}$-HSL-dependent QS system, namely, $3OC_{12}$-HSL synthesis, transport, and perception, will reduce light emission by the biosensor with respect to a control coculture grown in the absence of any chemical compound.

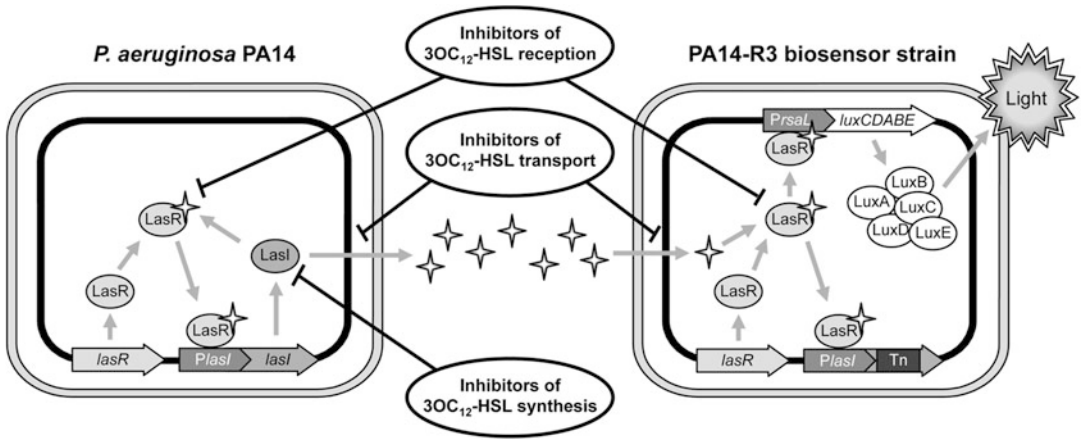

Fig. 1 Schematic representation of the PA14/PA14-R3 cocultivation screening system. The wild type PA14 produces $3OC_{12}$-HSL signal molecules that induce bioluminescence emission in the biosensor strain PA14-R3. PA14-R3 is a PA14 derivative in which a transcriptional fusion between the LasR-dependent *rsaL* promoter (P*rsaL*) and the *luxCDABE* operon was integrated at the *attB* neutral site of the chromosome. In addition, in order to avoid self-activation of the reporter strain, the *lasI* gene, encoding the $3OC_{12}$-HSL synthase LasI, was inactivated by transposon insertion (Tn). Molecules interfering with different steps of the *las* QS system are expected to cause a reduction in bioluminescence in comparison to the untreated control. Modified from [16]

3.2 Primary Screening Procedure

1. Grow *P. aeruginosa* PA14 wild type and the $3OC_{12}$-HSL reporter strain PA14-R3 (*see* **Note 1**) overnight at 37 °C on LA plates.
2. Scrape bacteria from LA plate surfaces and dilute in 1 ml of LB supplemented with 100 mM MOPS Buffer to an absorbance at 600 nm wavelength (A_{600}) of 0.09 and 0.03 for PA14-R3 and PA14, respectively (3:1 reporter:wild type ratio). Mix isovolumes of the PA14-R3 and PA14 diluted cultures, to obtain a coculture in which the A_{600} of PA14-R3 and PA14 is 0.045 and 0.015, respectively.
3. Aliquot 100 μl per well of the coculture in a 96-well microtiter black plates with clear bottom.
4. As untreated control, add 100 μl of LB in six wells containing the coculture.
5. Set up serial dilutions of the compounds to 2× the final concentrations to be tested. For example: chemical compounds to be used in the screening assays are dissolved in 10 mM DMSO and then diluted to 400 μM, 40 μM, and 4 μM in LB medium (to obtain 200 μM, 20 μM, and 2 μM final concentrations in the assay, respectively). Aliquot 100 μl of each compound in the microtiter wells containing the coculture.
6. Incubate the microtiter plate at 37 °C for 4 h with gentle shaking.
7. Measure A_{600} and light counts per second (LCPS), simultaneously with an automated luminometer-spectrometer plate reader (*see* **Note 2**).
8. Average the A_{600} and LCPS measurements of the untreated control samples. For all samples, normalize the LCPS to the A_{600} to obtain PA14-R3 reporter activity. Compare PA14-R3 reporter activity of the treated samples to one of the untreated controls (*see* **Note 3**).

3.3 Rational of the Secondary Assays: $3OC_{12}$-HSL, Elastase, and Pyocyanin Production

As previously described, compounds reducing light emission in the primary screening could hamper the *las* QS system at different levels, including $3OC_{12}$-HSL synthesis, transport, and perception. Inhibition of any of these steps would limit $3OC_{12}$-HSL synthesis in PA14 wild type, as well as the production of $3OC_{12}$-HSL-dependent virulence factors such as elastase and pyocyanin.

Elastase is a *P. aeruginosa* secreted protease that mainly targets mammalian elastin and plays a key role in virulence [19]. Transcription of the elastase gene, *lasB*, is strongly activated by the *las* QS system [20].

Pyocyanin is a redox-active phenazine responsible for the blue-green color characteristic of *P. aeruginosa* cultures. Besides its role in virulence, pyocyanin has been also recognized as a signalling

molecule, as an electron shuttle for bacterial respiration, and as an antibacterial and antifungal agent [21, 22].

Therefore, a convenient method to validate the *las* inhibitory activity of a hit compound is to measure its effect on $3OC_{12}$-HSL, elastase, and pyocyanin production, in comparison with an untreated control.

The assay for $3OC_{12}$-HSL production by PA14 wild type is based on the use of the same PA14-R3 biosensor strain used in the primary screening [16].

The elastase assay is based on the Elastin-Congo Red reagent, a water-insoluble powder in which elastin is bound to the Congo Red dye. Hydrolysis of elastin causes the release of the dye in the aqueous phase. The amount of released (soluble) dye is proportional to the level of hydrolyzed elastin and, ultimately, to the level of elastase present in a *P. aeruginosa* culture supernatant. The method here described is modified from ref. 23.

Pyocyanin can be easily quantified in chloroform extracts of *P. aeruginosa* culture supernatants by spectrophotometric analysis. The method here described is modified from ref. 24.

3.4 Quantification of $3OC_{12}$-HSL

1. Inoculate *P. aeruginosa* PA14 in 5 ml of LB and grow the culture overnight at 37 °C with 200 rpm shaking.
2. Refresh the overnight culture to an A_{600} of 0.015 in 30 ml of LB supplemented with 50 mM MOPS Buffer, in the presence of increasing concentrations of the test compound. Incubate at 37 °C with 200 rpm shaking.
3. Withdraw 7 ml of bacterial cultures every 3 h, up to 9 h. Measure A_{600} of the samples, harvest the cells by centrifugation, and recover the culture supernatants (*see* **Notes 4** and **5**).
4. Scrape bacteria from the surfaces of an LA plate of the biosensor strain PA14-R3 and dilute in 2 ml of LB. Measure the A_{600} of this bacterial suspension and use it to prepare an inoculum of the biosensor strain in LB supplemented with 50 mM MOPS Buffer to an A_{600} of 0.045.
5. Aliquot 195 μl per well of the biosensor culture in a 96-well microtiter black plates with clear bottom.
6. Add 5 μl of each culture supernatant from **step 3** in three wells containing the reporter culture. As untreated control, add 5 μl of LB in six wells containing the reporter culture.
7. For the calibration curve, set up 1:3 serial dilutions of synthetic $3OC_{12}$-HSL in LB, from a maximal concentration of 120 μM to a minimum concentration of ~18 nM. Add 5 μl of each diluted $3OC_{12}$-HSL sample in three wells containing the reporter culture.
8. Incubate the microtiter plate at 37 °C for 4 h with gentle shaking.

9. Measure A_{600} and LCPS, simultaneously (*see* **Note 2**). Average the A_{600} and LCPS measurements of the replicates. Normalize the averaged LCPS to the averaged A_{600} to obtain PA14-R3 reporter activity. Extrapolate $3OC_{12}$-HSL concentration in the treated and untreated supernatants based on the values obtained for the calibration curve (*see* **Note 6**).

3.5 Elastase Assay Procedure

1. Set up 1.5 ml tubes each one containing 20 mg of Elastin-Congo Red and 1 ml of Protease Buffer (*see* **Note 7**).
2. Add 100 μl of culture supernatant collected in Subheading 3.4, **step 3** (*see* **Notes 5** and **8**) to the tube containing the Elastin-Congo Red suspension. Prepare a control sample (blank) by adding 100 μl of sterile LB instead of the culture supernatant.
3. Incubate 2 h with gentle shacking at 37 °C.
4. Centrifuge for 5 min at 11,000 × *g* at room temperature.
5. Measure absorbance at 495 nm wavelength (A_{495}) of the clear supernatants in plastic microcuvettes, using as blank the control sample (see above). Normalize with respect to the A_{600} of the corresponding culture measured in Subheading 3.4, **step 3**.

3.6 Pyocyanin Assay Procedure

1. Add 3 ml of chloroform to 15 ml conical tubes containing 5 ml of the supernatants collected in Subheading 3.4, **step 3** (*see* **Notes 5** and **8**). Mix vigorously by vortexing for 10 s. As control sample (blank), use 5 ml of sterile LB in place of the bacterial supernatant.
2. Centrifuge the tubes at 3,000 × *g* for 5 min.
3. Transfer 2 ml of the lower organic phase in clean 15 ml conical tubes (the lower organic phase is blue if pyocyanin is present) and add 1 ml of 0.2 N HCl. Mix vigorously by vortexing for 10 s.
4. Centrifuge the tubes at 3,000 × *g* for 5 min.
5. Transfer 800 μl of the upper aqueous phase in plastic microcuvettes (the upper aqueous phase is pink if pyocyanin is present).
6. Measure the absorbance at 520 nm wavelength (A_{520}), using as blank the control sample (see above). Normalize with respect to the A_{600} of the corresponding culture measured in Subheading 3.4, **step 3**.

4 Notes

1. By using bacterial biosensors in which light emission is induced by exogenous C_4-HSL, PQS, or other QS molecules different from $3OC_{12}$-HSL, similar coculture-based approaches can be designed to identify inhibitors of other QS systems. Please

consider that growth of biosensor strains different from PA14-R3 may require LB supplementation with antibiotics for plasmid selection. Moreover, the use of other biosensor strains may require preliminary optimization of experimental parameters, including wild type/biosensor ratio, A_{600} of the coculture at t_0, and incubation time of the coculture at 37 °C.

2. We routinely use a Wallac 1420 Victor^{3V} multiplate reader (Perkin-Elmer) as automated luminometer-spectrometer plate reader. For the Wallac 1420 Victor^{3V} multiplate reader relevant parameters for bioluminescence measurement are: emission aperture, large; counting time, 1 s. Relevant parameters for absorbance measurement are: filter 595/60; excitation aperture, normal; reading time, 0.1 s.
3. The criteria used for the selection of hit compounds in [17] were: (a) ≥50% inhibition of PA14-R3 reporter activity; (b) ≤10% reduction of growth with respect to the untreated controls. The latter criterion was aimed at avoiding any unspecific effect of impaired growth on the QS response.
4. This step is just for harvesting cells, so speed and times for centrifugation can vary. Our standard conditions are 6,300 × *g* for 5 min at room temperature.
5. For the $3OC_{12}$-HSL quantification assay, the supernatants can be stored at −20 °C. Conversely, for elastase and pyocyanin assays it is recommended to process supernatants as soon as possible.
6. The same method can be used to quantify the amount of $3OC_{12}$-HSL produced by different strains of *P. aeruginosa* (*e.g.*, to compare a wild type and a mutant) or of *P. aeruginosa* grown in different media. Also quantification of $3OC_{12}$-HSL in *P. aeruginosa* clinical isolates has been described [16].
7. Avoid the preparation of a stock suspension of Elastin-Congo Red. This powder is highly insoluble and aliquots of a suspension could contain different amounts of the reagent. We have observed that aliquoting the powder in each sample tube enhances the assay reliability.
8. Under these conditions, *P. aeruginosa* usually starts to produce detectable levels of elastase and pyocyanin at around $A_{600} \approx 3.0$, therefore consider using only supernatants collected after 6 and 9 h incubation in Subheading 3.4, **step 3**.

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