Chapter 1

Methods for Measuring the Production of Quorum Sensing Signal Molecules

Manuel Alcalde-Rico and José Luis Martínez

Abstract

One relevant aspect for understanding the bottlenecks that modulate the spread of resistance among bacterial pathogens consists in the effect that the acquisition of resistance may have on the microbial physiology. Whereas studies on the effect of acquiring resistance of bacterial growth are frequently performed, more detailed analyses aiming to understand in depth the cross talk between resistance and virulence, including bacterial communication are less frequent. The bacterial quorum sensing system, is an important intraspecific and interspecific communication system highly relevant for many physiological processes, including virulence and bacterial/host interactions. Some works have shown that the acquisition of antibiotic resistance may impair the quorum sensing response. In addition, some antibiotics as antimicrobial peptides can affect the production and accumulation of the quorum sensing signal molecules. Given the relevance that this system has in the bacterial behavior in the human host, it is important to study the effect that the acquisition of antibiotic resistance may have on the production of quorum sensing signals. In this chapter we present a set of methods for measuring quorum sensing signals based on the use of biosensor strains, either coupled to Thin Layer Chromatography or for performing automated luminometry/spectrophotometry assays. We use *Pseudomonas aeruginosa* as bacterial model because it has a complex quorum system than encloses different signals. Namely, *P. aeruginosa* quorum sensing system consists in three different interconnected regulatory networks, each one presenting a specific autoinducer molecule: the *las* system, which signal is *N*-(3-oxo-dodecanoyl)-L-homoserine lactone, the *rhl* system, which signal is *N*-butanoyl-homoserine lactone and the *pqs* system, which signals are 2-heptyl-3-hydroxy-4(1H)-quinolone together with its immediate precursor 2-heptyl-4-hydroxy-quinoline.

Key words Quorum sensing, *Pseudomonas aeruginosa*, Pseudomonas Quinolone Signal, 2-Alkyl-4(1H)-quinolones, *N*-acyl homoserine lactones, Antibiotic resistance, Thin layer chromatography, PQS, AHL, Fitness cost

1 Introduction

It is generally assumed that the acquisition of resistance is associated with fitness costs that make resistant bacteria to be less proficient for growing in different ecosystems than their wild-type counterparts. Whilst this is true in occasions [1–3], in other cases, the acquisition of resistance produces specific changes in the bacterial physiology that do not correlate with growth alter-

Stephen H. Gillespie (ed.), *Antibiotic Resistance Protocols*, Methods in Molecular Biology, vol. 1736,
https://doi.org/10.1007/978-1-4939-7638-6_1,

ations [4]. Among such changes, one of relevance for bacterial behavior consists on alterations in the quorum sensing (QS) response. Indeed, different articles have shown that some multidrug efflux pumps are able to extrude QS signal molecules (QSSMs) or their metabolic precursors. This situation makes that the acquisition of resistance due to due to the overexpression of these efflux pumps might impair the QS response, and consequently the expression of virulence factors, of resistant strains [5–10]. The QS system is dependent on cell density; serves to determine the cells concentration in a given environment. When the population density reaches a given threshold, a coordinated response is triggered, which is relevant for several bacterial processes including virulence [11, 12]. The process starts with the production of one or more low molecular weight compounds, known as "autoinducers," by the cells and their diffusion across the membrane. This diffusion results in a progressive accumulation of the signal until the signal threshold level needed to produce the signaling cascade is reached [13, 14]. This quick response is due to the efficient binding of the QSSM to its specific transcriptional regulator, which activates (or represses) the transcription of a high number QS-regulated genes [11, 12, 15, 16]. In general, among those genes positively regulated by QS are both the transcription factors that mediate the QS response and the enzymes that catalyze the synthesis of the QSSMs resulting in a positive feedback regulation [16, 17]. For this reason the QSSMs are named autoinducers too.

Since the first QS phenomenon was discovered by Nealson in 1970 [18, 19], a large number of signaling properties have been associated to several small molecules synthesized by different bacteria [17, 20–22]. The best studied signals in Gram-negative bacteria are the *N*-acyl homoserine lactones (AHLs) and the 2-alkyl-4(1H)-quinolones (AQs), whereas in Gram-positive bacteria the most important ones are the autoinducer peptides (AIPs). The different QS networks regulated by these autoinducers drive the expression of several genes involved in many biological processes as the production of antibiotics and virulence factors (elastase, proteases, siderophores, toxins, phenazines, T3SS or T6SS), biofilm maturation, bioluminescence, swarming motility, sporulation or antibiotics resistance, among others [11, 12, 14]. In addition to their role in mediating the communication among members of the same species, QS also may be involved in interspecific and even interkingdom signaling [16, 17, 23, 24].

The common structure of AHLs autoinducers is a homoserine lactone ring attached to an acyl chain through an amide bond. The number of carbon atoms of this acyl chain can vary and the third position may be modified in occasions with carbonyl or hydroxyl group [25–27]. These different structures made AHLs sufficiently different to be recognized by specific sensor proteins of the LuxR

family [27]. One of the best characterized AHLs signaling system is based on *N*-(3-oxo-dodecanoyl)-L-homoserine lactone (3-oxo-C12-HSL), which is involved, in *P. aeruginosa*, in both intraspecific signaling (i.e., elastase and exoproteases production) and interspecific signaling (i.e., with yeast or mammalian cells) [11, 23, 24]. One relevant interkingdom signaling process of this autoinducer is the interaction with human cells. Smith et al. [28] have shown that 3-oxo-C12-HSL induces the production of several pro-inflammatory chemokines, including IL-8 in human bronchiolar epithelial cells and lung fibroblast. In addition, 3-oxo-C12-HSL can act as chemoattractant for neutrophils inducing their migration to the site where the signal is released [29, 30].

In addition, to their regulatory role, QSSMs may have nonsignaling properties, including antibiotic activity or iron chelation. A good example of this antibiotic activity is shown by the lantibiotics (i.e., nisin produced by *Lactobacillus lactis* or subtilin produced by *Bacillus subtilis*), a group of antimicrobial compounds that are closely related with AIPs [16]. In the same line of reasoning, the 2-heptyl-3-hydroxy-4(1H)-quinolone (*Pseudomonas quinolone signal*, PQS) is used by *P. aeruginosa* to capture iron when growing inside an infected host, and also to steal the iron stores of other bacteria [31].

All these effects on bacterial physiology and virulence support the need of establishing clear and robust protocols to detect the QSSMs, and determine the effect of antibiotic resistance on the production of such compounds. The most useful techniques for such purpose are based on the use of biosensor strains. These biosensors do not produce any QSSMs but contain the sensor protein that recognizes the autoinducer of interest. The complex formed by QSSM and the sensor protein promotes the transcription of a reporter gene, producing a detectable signal, including bioluminescence, fluorescence, pigments production or β-galactosidase activity [32]. It is important considering the limitations of this technique, specially when working with bacterial species in which the studies on QS and on the corresponding QSSMs are scarce or even absent. In particular, it is important: (1) to know the minimal and saturating concentration of autoinducer to produce the signal of each biosensor strain, (2) to carry out positive and negative controls in the experiment to address the possibility of the presence of either quenchers or enhancers of the system under the studied conditions, (3) to establish specific conditions in which production of the QSSM to be studied is granted. In this protocol, we use as models for their detection the QSSMs produced by *P. aeruginosa* [11, 15]. The method is based on the use of Thin Layer Chromatography combined with a biosensor overlay [25, 26, 33–35]. In addition, we also describe how to quantify QSSMs with the use of an automated luminometer-spectrophotometer [26, 27, 35, 36]. More details concerning other AHLs bacterial biosensors, not described here, can be found in [32].

2 Materials

2.1 Bacterial Strains

1. *Pseudomonas aeruginosa* PAO1 wild type.
2. *P. aeruginosa ΔpqsA*.
3. *Escherichia coli* pSB1705 (LasR-based bioreporter) [27].
4. *E. coli* pSB536 (RhlR-based bioreporter) [37].
5. *P. aeruginosa* PAO1 *pqsA* CTX-*lux::pqsA* (PqsR-based bioreporter) [33].

2.2 Growth Media

1. Luria-Broth (LB).
2. LB agar plates: LB medium with bacteriological agar at 1.5% ($^{w}/_{v}$). Pour in each petri plate 20 ml of this LB agar.
3. LB agar to overlay: LB medium with bacteriological agar at 0.75% ($^{w}/_{v}$).
4. Soft top agar medium 0.65% ($^{w}/_{v}$): dissolve in H_2O milliQ agar 0.65% ($^{w}/_{v}$), tryptone 1% ($^{w}/_{v}$), and sodium chloride (NaCl) 0.5% ($^{w}/_{v}$).

2.3 Reagents

1. Acidified ethyl acetate HPLC grade 0.01% ($^{v}/_{v}$): add glacial acetic acid to ethyl acetate HPLC grade at a final concentration 0.01% ($^{v}/_{v}$).
2. Methanol HPLC grade.
3. Glacial acetic acid.
4. Dichloromethane HPLC grade.
5. Acetone for analysis.
6. Potassium dihydrogen phosphate solution 5% ($^{w}/_{v}$): KH_2PO_4 dissolved in H_2O_d.
7. Synthetic QS signal molecules: AQs suspend in methanol HPLC grade and AHLs suspend in ethyl acetate HPLC grade.
8. Ampicillin stock 100 mg/ml dissolved in H_2O milliQ.
9. Tetracycline stock 10 mg/ml dissolved in ethanol 70% for analysis.
10. H_2O milliQ (18.2 MΩ·cm at 25 °C).

2.4 Equipment

In most cases, general equipment can be purchased from different companies and a specific reference to the model used in our laboratory is not included.

1. Thin layer chromatography silica gel 60 F_{254} 20 cm × 20 cm (AQs).
2. Thin layer chromatography silica gel 60 RP-18 F_{254s} 20 cm × 20 cm (C4-HSL).
3. Thin layer chromatography silica gel 60 RP-2 F_{254s} 20 cm × 20 cm (3-oxo-C12-HSL).

4. 50 ml, 100 ml and 250 ml glass flasks.
5. HPLC glass tubes with cover.
6. Centrifuge microtubes 1.5 ml.
7. Centrifuge tubes 50 ml.
8. Spectrophotometer cuvettes.
9. 96-well white flat transparent microtiter plates.
10. Vortex.
11. Microwave oven.
12. Oven.
13. Centrifuge.
14. Shaking incubator.
15. −20 °C freezer, −80 °C freezer, and fridge.
16. Spectrophotometer.
17. Film developer machine.
18. Combined automated luminometer-spectrophotometer.
19. Bunsen burner.
20. Sterile 0.2 μm size filters.
21. 1 ml and 10 ml syringe.
22. High-sensitivity X-ray film as Curix RP2 Plus (Agfa) or BioMax (Kodak).
23. TLC developing tank.

3 Methods

3.1 Analysis of Quorum Sensing Signal Molecules by Thin Layer Chromatography and Biosensor-Based Detection

3.1.1 Culture Conditions to Extract AHLs and AQs Signal Molecules from P. aeruginosa

1. Inoculate 10 ml of LB in a 50 ml flask with a single colony of each strain to be tested from freshly grown agar plates (*see* **Notes 1** and **2**). Grow overnight at 37 °C with shaking at 250 rpm.
2. Next day, determine the optical density at 600 nm (OD_{600}) and dilute the cultures to OD_{600} = 0.01 in 100 ml flasks containing 25 ml of fresh LB medium. Incubate at 37 °C with shaking at 250 rpm.
3. To synchronize the cultures, grow to exponential phase (OD_{600} = 0.5–0.6) and dilute again in two different flasks at OD_{600} = 0.01 to have biological duplicates. Grow the cultures (*see* **Note 3**) until early stationary phase (approximately OD_{600} = 2.0) (*see* **Note 4**).
4. When two replicas of each strain are at the desired OD_{600}, mix them in a flask by gentle agitation and pass 11 ml of each culture to a centrifuge tube. Centrifuge at 6000 × *g*, 4 °C for 15 min.
5. Recover the supernatant carefully to avoid losing cellular pellet and filter it through a sterile 0.2 μm size filter. The obtained

cellular pellet will be used for the extraction of cell-associated autoinducers.

3.1.2 AHLs and AQs Extraction in Cell-Free Supernatant

1. Add 10 ml of the cell-free supernatant to a clean centrifuge tube containing the same volume of acidified ethyl acetate (10 ml) and vortex approximately 30 s until the two phases are completely mixed.
2. Centrifuge at 6000 × *g*, 4 °C for 20 min and transfer 8 ml of the upper organic layer to 250 ml flasks (*see* **Notes 5**–7).
3. Evaporate them each cell-free supernatant with a stream of nitrogen. Alternatively you can also rotary evaporate the extracts.
4. Add 4 ml of acidified ethyl acetate (AHLs extraction) or HPLC grade methanol (AQs extraction) to the flasks and agitate well for a brief time (30 s) to suspend the dry extracts (*see* **Note 7**).
5. Transfer each entire solution in aliquots of 300 μl to glass cap vials (HPLC vials for instance) (*see* **Note 7**), dry under a stream of nitrogen and store them until further use. The Subheading 3.1.2, **steps 4** and **5** can be repeated if you want to get a more exhaustive extraction (*see* **Notes 8** and **9**).

3.1.3 AQs Extraction from the Cellular Fraction (Continue from Subheading 3.1.1, Step 5)

1. Wash the pellet by adding 10 ml of LB fresh medium with a pipette and resuspend it carefully (*see* **Note 10**). Centrifuge at 6000 × *g*, 4 °C for 15 min and discard the supernatant. Repeat this step once.
2. Add 10 ml of HPLC grade methanol and resuspend the pellet by vigorous vortexing (*see* **Note 11**). Wait for 10 min until the methanol lyses the cells completely.
3. Centrifuge at 6000 × *g*, 4 °C for 20 min and transfer the supernatant (methanol containing the AQs molecules) to 250 ml flasks (*see* **Notes 6** and **7**).
4. Evaporate the extracts of each cellular pellet under a stream of nitrogen. Alternatively you can also rotary evaporate the extracts.
5. Add 4 ml of HPLC grade methanol to the flasks and agitate well (30 s) to suspend all extract (*see* **Note 7**).
6. Transfer each entire solution in aliquots of 300 μl to glass cap vials (HPLC vials for instance) (*see* **Note 7**), dry them under a stream of nitrogen and store them until further use. The Subheading 3.1.3, **steps 5** and **6** could be repeated in order to optimize the extraction (*see* **Notes 8** and **9**).

3.1.4 Preparing Reporter Strains Cultures for Overlaying TLC Plates

1. Inoculate 10 ml of LB containing either ampicillin 100 μg/ml (RhlR-based biosensor[pSB536]) [37], tetracycline 5 μg/ml (LasR-based biosensor [pSB1705]) [27] or tetracycline 125 μg/

ml (PqsR-based biosensor) [33] in 50 ml flasks with a single colony of each reporter strain from freshly grown agar plates with their respective antibiotics (*see* **Note 12**). Grow overnight at 30 °C or 37 °C under shaking at 250 rpm (*see* **Note 3**).

2. Next day, melt 100–120 ml of LB agar 0.75% (wt/vol) (AHLs reporter) or soft top agar 0.65% (wt/vol) (PqsR-based biosensor) and temperate at 50 °C.

3.1.5 TLC-Assay of the Extract of Supernatants for Subsequent Detection of C4-HSL or 3-Oxo-C12-HSL

1. Draw carefully a line with a pencil at 2 cm from the edge of the TLC plate (RP-18 F254s for C4-HSL and RP-2 F254s for 3-oxo-C12-HSL) and mark the points where the samples will be loaded (*see* **Note 13**).
2. Suspend the sample extracts of Subheading 3.1.2, **step 5** in 1 ml of acidified ethyl acetate and spot 5 μl of each on its place on the TLC plate (*see* **Note 14**), trying the spots to be as smallest as possible by drying samples during spotting (*see* **Note 15**).
3. Synthetic C4-HSL or 3-Oxo-C12-HSL dissolved in acidified ethyl acetate should be used as positive controls. Spot 1–2 μl of 10 μM of this solution in the TLC plate. The minimal distance recommended for optimal resolution is 2 cm between two spots (*see* **Note 14**).
4. When spots are dried, put the TLC plate into a developing chamber that contains 150 ml of the mobile phase mixture, methanol–water (60:40 [vol/vol] for C4-HSL and 45:55 [vol/vol] for 3-Oxo-C12-HSL) (*see* **Note 16**). Run the TLC by capillarity until the solvent reaches 2–4 cm from the top of the plate and then let it dry completely inside the fume hood (*see* **Note 17**).

3.1.6 TLC-Assay of the Extract of Both Cell-Fraction and Supernatants for Subsequent Detection of PQS and 2-Heptyl-4-Hydroxy-Quinolone HHQ

1. Activate the TLC sheets (silica gel 60 F_{254}): prepare 1 l of KH_2PO_4 solution 5% ($^{wt}/_{vol}$) in distilled water and transfer to a wide clean tray. Immerse the TLC sheets in KH_2PO_4 solution for 30 min at room temperature (*see* **Note 18**). Then, introduce the TLC plates into a prewarmed oven avoiding them to contact one to each other and keep them at 100 °C for 1 h (*see* **Note 19**). Once you have activated the TLC plate, you should label the position of the spots as explained above in Subheading 3.1.5, **step 1**.
2. Suspend the sample extracts of Subheading 3.1.2, **steps 5** and **6** in 100 μl of methanol HPLC grade and spot 30 μl of each on its place in the TLC sheet (*see* **Notes 14** and **15**).
3. Synthetic PQS and HHQ dissolved in methanol HPLC should be used as positive control. Spot 1–2 μl of 10 mM of each solution and a mixture of both in TLC plate (*see* **Notes 14** and **20**).
4. When spots are dried, put the TLC plate into a developing chamber that contains 100 ml of the mobile phase; dichloromethane–methanol (95:5 [vol/vol]) (*see* **Note 16**). Run the

TLC until the solvent reaches 2–4 cm from the top of the plate and let dry (*see* **Note 17**).

3.1.7 Biosensor-Based Detection of AHLs or AQs Signal Molecules in TLC-Assay

1. Once the TLC plate obtained after Subheadings 3.1.5, **step 4** or 3.1.6, **step 4** is dried, make a pool with a depth of at least 0.5 cm by rounding the TLC plate with autoclaved tape (*see* **Note 21**).
2. Inoculate the agar-containing medium from Subheading 3.1.4, **step 2** with 1.0–1.2 ml of the overnight biosensor culture obtained as described in Subheading 3.1.4, **step 1** (*see* **Note 22**) and mix carefully to avoid bubble formation (*see* **Note 23**).
3. Pour the inoculated medium over the preformed pool slowly taking care that the entire TLC plate is covered homogeneously (*see* **Note 24**). Wait until the agar is completely solidified under sterile conditions.
4. Incubate the plates 14–16 h at 30 °C for the AHLs-based bioassay or 6 h at 37 °C for the PqsR-based bioassay.
5. After incubation, you can use a luminograph photon video camera (for instance Luminograph LB 980 photon video camera from Berthold Technologies USA) to visualize the light emitted in the bioassay.
6. Alternatively, you can expose an X-ray film over the plate: put the plate in an X-ray chamber, remove with a scalpel or a razor blade the border of the tape carefully and cover the agar plate with a plastic transparent film. Place the X-ray film over the agar plate in darkness, close the X-ray chamber and expose about 2–10 min depending on the identity of the signal (*see* **Note 25**).
7. To develop the X-ray film, use a regular developer machine (Fig. 1).

3.2 Analysis of the Kinetics of QS Signal Molecules Accumulation into Supernatant Along the Cell Cycle by a Method Based in a Combined Automated Luminometer-Spectrophotometer

3.2.1 Preparing Reporter Strains Cultures for AHLs and AQs Detection by 96-Well Plate Bioassay

1. Inoculate 10 ml of LB containing either ampicillin 100 μg/ml (RhlR-based biosensor[pSB536]), tetracycline 5 μg/ml (LasR-based biosensor [pSB1705]) or tetracycline 125 μg/ml (PqsR-based biosensor) in 50 ml flasks with a single colony of each reporter strain from freshly grown agar plates with their respective antibiotics (*see* **Note 12**). Grow overnight at 30 °C or 37 °C under shaking at 250 rpm (*see* **Note 3**).
2. The next day, dilute 1:100 the overnight biosensor culture in fresh LB medium and incubate the biosensor cells with QSSMs-containing extracts in a 96-well plate as described below (Subheading 3.2.2, **step 9**).

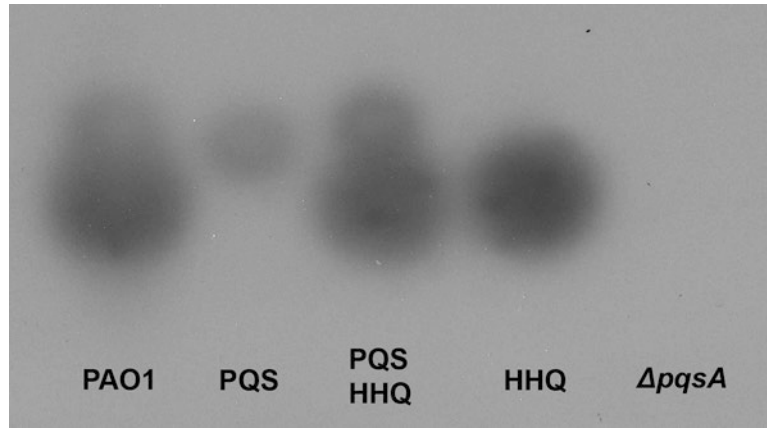

Fig. 1 TLC image of PQS and HHQ extracted from *P. aeruginosa* supernatant culture. Positive controls were included as described in methods: 2 μl from PQS-methanol 10 mM, 2 μl from HHQ-methanol 10 mM, and 2 μl from the combination of both (1 μl from each one 10 mM PQS and 10 mM HHQ). The extraction of AQs from PAO1 and *ΔpqsA* strains was performed at 8 h of incubation at 37 °C with shaking (250 rpm) and 20 μl from each extract was spotted on the TLC plate. The mobile phase used was dichloromethane–methanol (95:5). The time of incubation with de biosensor PAO1 *pqsA* CTX-*lux::pqsA* was 6 h

3.2.2 AHLs and AHQs Extraction in Cell-Free Supernatant from P. aeruginosa Culture and Detection for 96-Well Plate Bioassay

1. Inoculate 10 ml of LB in a 50 ml flask with a single colony of each strain to be tested from freshly grown agar plates (*see* **Notes 1** and **2**). Grow overnight at 37 °C with shaking at 250 rpm.
2. Next day, determine the OD_{600} and dilute the cultures to exponential phase (OD_{600} = 0.01) in 100 ml flasks containing 25 ml of fresh LB medium. Incubate at 37 °C with shaking at 250 rpm.
3. To synchronize the cultures, grow to OD_{600} = 0.5–0.6 and dilute again at OD_{600} = 0.01. Incubate at 37 °C with shaking at 250 rpm. The QS signal molecules will be extracted at different times after inoculation from the same culture. The number of extractions from each culture should be at least four for getting a robust graphic representation of results (*see* **Note 26**).
4. Transfer 1.5 ml of each culture into a microcentrifuge tube and centrifuge at 10,000 × *g*, 4 °C for 5 min. At the same time, determinate the OD_{600} of each culture. Recover the supernatants and filter them through a sterile 0.2 μm size filter.
5. To extract the AHLs signal molecules, add 900 μl of these cell-free supernatants to previously labeled microcentrifuge tubes each one containing 100 μl of HCl 1 M and mix well with a micropipette. Store the rest of the filtered supernatant (600 μl) in the freezer at −20 °C for the subsequent AQs analysis.
6. When all AHLs extractions have been performed incubate them at 20 °C for 16–18 h (overnight) with shaking at 250 rpm (*see* **Note 27**).

7. Next day, 5 μl from each sample (AHLs or AQs extract) is charged into each well of a microtiter plate. Include in the same plate at least three biological replicates of each strain and two technical replicates of each extract (*see* **Note 28**).
8. You can make a standard curve for C4-HSL and 3-oxo-C12-HSL adding 5 μl from 6 or more different concentration solutions of these autoinducers suspended with acidified ethyl acetate (*see* **Note 29**).
9. Add to each test well 195 μl of biosensor culture diluted from Subheading 3.2.1, **step 2**.
10. Include into the bioassay control wells with the bioreporter strain without quorum sensing signal molecules to measure autoluminescence and fresh LB medium without bacterium neither quorum sensing signal molecules as blank.
11. Incubate in a rocking platform at 30 °C or 37 °C with shaking at 200 rpm for 6 h (*see* **Note 3**). Every hour, the OD_{600} and bioluminescence are measured using a combined automated luminometer-spectrophotometer.

3.2.3 Analysis of the Results Obtained from Combined Automated Luminometer-Spectrophotometer 96-Well Plate Bioassay

1. Normalize the results by subtracting the background value obtained in the blank wells. Then, obtain the Relative light units (RLU) for both each well and each time by determining the bioluminescence/OD_{600} ratio.
2. Make a graphic representation of RLU variation as a function of to time and choose the optimal time at which RLUs are bigger but and technical replicas do not diverge too much.
3. The values of RLUs obtained at the optimal time point are used to make the graphic representation of RLU variation respect to OD_{600} of each tested strain in which the quorum sensing signal molecules have been extracted from cell-free supernatants (Fig. 2) (*see* **Note 30**).
4. To estimate the concentration of each QS signal molecule in every sample, make a standard curve using the RLU values obtained upon exposure of the biosensor strains to increasing concentrations of the different positive controls.

4 Notes

1. In all cases you have to work with the strains to be tested and with control strains, each one of the latter presenting a known pattern of signals production. In the case of *P. aeruginosa*, the wild type PAO1 strain and mutants in the QS-signaling pathways as *ΔlasI*, *ΔrhlI*, or *ΔpqsA* can be used.

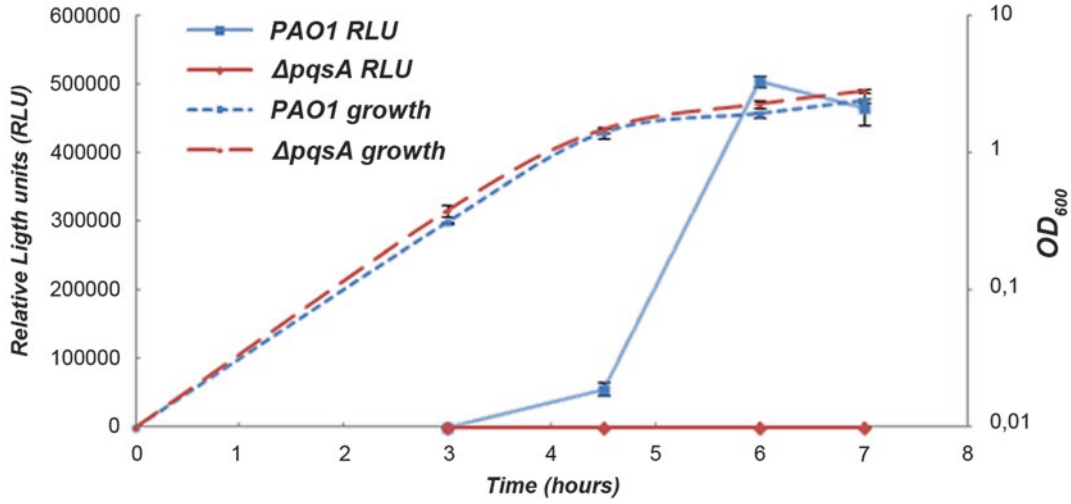

Fig. 2 Analysis of the kinetic AQs accumulation into the supernatants from *P. aeruginosa* PAO1 and *ΔpqsA* strains. The time of incubation from each extraction was 3 h, 4 h 30 min, 6 h and 7 h. The growth curve for each culture is represented in logarithmic scale together with the AHQs accumulation. The RLU values have been calculated for both each well and each time by determining the bioluminescence/OD_{600} ratio

2. To obtain a representative result, you have to repeat the extraction and TLC-bioassay at least with three different biological replicas of each strain. For 96-well plate bioassay you also need 2–3 technical replicas of each sample extraction. However, you can analyze all biological and technical replicas in the same microtiter plate.
3. The temperature used to grow AHLs reporters is 30 °C and for AQs reporter, control strain of experiment and test bacterium is 37 °C.
4. In this example, the growth media used for the extractions of QS signal is LB. However, you can use another media if needed. In the same way, the extraction process can be performed at any stage of the cell cycle.
5. For a better extraction, you can add again 8 ml of acidified ethyl acetate to the same centrifuge tube with the sample and repeat the extraction steps.
6. Extracts of supernatant and cell fraction suspended in acidified ethyl acetate and methanol respectively can be transferred to clean centrifuge tubes and frozen at −20 °C and stored for several days until the drying step.
7. All glass recipients used for the extraction have to be previously cleaned with analysis grade acetone and dried well before their use.

8. Be careful with the drying steps with N_2 to avoid splashing the walls and losing sample. If this happens, try to resuspend the extract from the walls and redry the entire sample again.
9. Dry extracts of supernatant and cell fraction can be frozen at −20 °C and stored for several weeks.
10. To resuspend the cellular pellet do not use vortex because the cells can be broken.
11. Make sure that the cellular pellet is homogenously resuspended without any remaining cell clump.
12. The concentrations of antibiotics used for growing the reporter strains are: ampicillin 100 μg/ml for the RhlR-based biosensor (*E. coli* pSB536), tetracycline 5 μg/ml for the LasR-based biosensor (*E. coli* pSB1705 or 1142) and tetracycline 125 μg/ml for the PqsR-based biosensor (*P. aeruginosa* PAO1 *pqsA* CTX-*lux::pqsA*).
13. Remember to label each loading point before spotting to avoid mistakes along the process and be careful with the pencil to avoid breaking the TLC silica plate.
14. You can modify the volume to resuspend the samples, the quantity that will be spotted and the distance recommended between each other according to its concentration.
15. For a good spotting of the extracts you have to use a tip pipette as thin as possible (i.e., you can use a sterile plastic tip pipette normally used to load thin acrylamide gels ad those used in footprinting assays) and drying the samples along the spotting.
16. The volume of the mobile phase mixture used in this example is for a 23 cm × 23 cm × 7 cm. developing chamber. The volume of mobile phase can be modified accordingly to the dimensions of the developing chamber but the proportion of the mixture cannot be changed.
17. During the running step is important that the TLC plate is equilibrated so as the front of the solvent runs as horizontal as possible.
18. You can immerse 4–5 TLC plates at the same time in KH_2PO_4 solution and heat them together in the oven but touching each other the least possible.
19. Silica gel TLC activated sheets can be stored at room temperature for several months.
20. Mix equal volumes of synthetic PQS 10 mM and HHQ 10 mM into a glass tube for obtain a final concentration of 5 mM for each other.
21. The TLC pool has to be completely sealed without any gap between the autoclave tape and the TLC sheet.

22. The overnight cultures of biosensor strains have always to be diluted 1:100 into agar medium overlay for TLC bioassay.
23. Before inoculating the melted agar medium to be used for the overlay, make sure that the temperature of this medium does not exceed 50 °C.
24. Be careful when pouring the overlay medium into the well to avoid bubble formation and if they form, remove them carefully with the flame of a burner. Do not expose the overlay too much time to the flame to avoid killing the cells.
25. To optimize the image, avoid leaving bubbles and creases. Additionally, depending on the signal intensity you can modify the exposure time of X-ray film to obtain the best image possible.
26. In this experiment the times of extraction are 4: T1 = 3 h [exponential phase]; T2 = 4 h [late exponential phase]; T3 = 5 h [early stationary phase]; T4 = 6 h [stationary phase]).
27. When the samples are incubated with HCl the open-ring lactones that have been formed by AHLs hydrolysis are closed. After this incubation, the AHLs samples can be stored in the fridge at 4 °C for weeks.
28. It is recommended to use a single bioreporter strain for microtiter plate assay because the emitted light is different for each of them and using two report strains in the same plate can produce wrong results because of light contamination between them.
29. For AHLs, The different concentration solutions of AHLs should be in a range of 0–20 μM.
30. Common statistic analyses (as T-test for comparing two samples or ANOVA for comparing several samples) are used to determine the statistical significance of the results.

Acknowledgments

The work in our laboratory is supported by grants BIO2011-25255 and BIO2014-54507-R from the Spanish Ministry of Economy and Competitiveness, S2010/BMD2414 (PROMPT) from CAM, Spanish Network for Research on Infectious Diseases (REIPI RD12/0015) from the Instituto de Salud Carlos III, and HEALTH-F3-2011-282004 (EVOTAR) from the European Union. MAR has been the recipient of an FPI fellowship. Special thanks are given to Miguel Cámara, Paul Williams, and Robert Hancock for providing control strains and QSSMs.

References

1. Andersson DI, Levin BR (1999) The biological cost of antibiotic resistance. Curr Opin Microbiol 2:489–493. https://doi.org/10.1016/S1369-5274(99)00005-3
2. Andersson DI, Hughes D (2010) Antibiotic resistance and its cost: is it possible to reverse resistance? Nat Rev Microbiol 8:260–271. https://doi.org/10.1038/nrmicro2319
3. Shcherbakov D, Akbergenov R, Matt T et al (2010) Directed mutagenesis of *Mycobacterium smegmatis* 16S rRNA to reconstruct the in-vivo evolution of aminoglycoside resistance in *Mycobacterium tuberculosis*. Mol Microbiol 77:830–840. https://doi.org/10.1111/j.1365-2958.2010.07218.x
4. Olivares J, Álvarez-Ortega C, Martinez JL (2014) Metabolic compensation of fitness costs associated with overexpression of the multidrug efflux pump MexEF-OprN in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 58:3904–3913. https://doi.org/10.1128/AAC.00121-14
5. Olivares J, Alvarez-Ortega C, Linares JF et al (2012) Overproduction of the multidrug efflux pump MexEF-OprN does not impair *Pseudomonas aeruginosa* fitness in competition tests, but produces specific changes in bacterial regulatory networks. Environ Microbiol 14:1968–1981. https://doi.org/10.1111/j.1462-2920.2012.02727.x
6. Köhler T, van Delden C (2001) Overexpression of the MexEF-OprN multidrug efflux system affects cell-to-cell signaling in *Pseudomonas aeruginosa*. J Bacteriol 183:5213–5222. https://doi.org/10.1128/JB.183.18.5213
7. Pearson J, Van Delden C, Iglewski B (1999) Active efflux and diffusion are involved in transport of *Pseudomonas aeruginosa* cell-to-cell signals. J Bacteriol 181:1203–1210
8. Aendekerk S, Diggle SP, Song Z et al (2005) The MexGHI-OpmD multidrug efflux pump controls growth, antibiotic susceptibility and virulence in *Pseudomonas aeruginosa* via 4-quinolone-dependent cell-to-cell communication. Microbiology 151:1113–1125. https://doi.org/10.1099/mic.0.27631-0
9. Minagawa S, Inami H, Kato T et al (2012) RND type efflux pump system MexAB-OprM of *Pseudomonas aeruginosa* selects bacterial languages, 3-oxo-acyl-homoserine lactones, for cell-to-cell communication. BMC Microbiol 12:70. https://doi.org/10.1186/1471-2180-12-70
10. Moore JD, Gerdt JP, Eibergen NR, Blackwell HE (2014) Active efflux influences the potency of quorum sensing inhibitors in *Pseudomonas aeruginosa*. Chembiochem 15:435–442. https://doi.org/10.1002/cbic.201300701
11. Williams P, Cámara M (2009) Quorum sensing and environmental adaptation in *Pseudomonas aeruginosa*: a tale of regulatory networks and multifunctional signal molecules. Curr Opin Microbiol 12:182–191. https://doi.org/10.1016/j.mib.2009.01.005
12. Withers H, Swift S, Williams P (2001) Quorum sensing as an integral component of gene regulatory networks in gram-negative bacteria. Curr Opin Microbiol 4:186–193. https://doi.org/10.1016/S1369-5274(00)00187-9
13. Swift S, Downie JA, Whitehead NA et al (2001) Quorum sensing as a population-density-dependent determinant of bacterial physiology. Adv Microb Physiol 45:199–270
14. Miller M, Bassler B (2001) Quorum sensing in bacteria. Annu Rev Microbiol 55:165–199. https://doi.org/10.1146/annurev.micro.55.1.165
15. Schuster M, Greenberg EP (2006) A network of networks: quorum-sensing gene regulation in *Pseudomonas aeruginosa*. Int J Med Microbiol 296:73–81. https://doi.org/10.1016/j.ijmm.2006.01.036
16. Mangwani N, Dash HR, Chauhan A, Das S (2012) Bacterial quorum sensing: functional features and potential applications in biotechnology. J Mol Microbiol Biotechnol 22:215–227. https://doi.org/10.1159/000341847
17. Reading NC, Sperandio V (2006) Quorum sensing: the many languages of bacteria. FEMS Microbiol Lett 254:1–11. https://doi.org/10.1111/j.1574-6968.2005.00001.x
18. Nealson K, Platt T, Hastings J (1970) Cellular control of the synthesis and activity of the bacterial luminescent system. J Bacteriol 104:313–322
19. Nealson K, Hastings J (1979) Bacterial bioluminescence: its control and ecological significance. Microbiol Rev 43:496–518
20. Keller L, Surette MG (2006) Communication in bacteria: an ecological and evolutionary perspective. Nat Rev Microbiol 4:249–258. https://doi.org/10.1038/nrmicro1383
21. Williams P, Winzer K, Chan WC, Cámara M (2007) Look who's talking: communication and quorum sensing in the bacterial world. Philos Trans R Soc Lond Ser B Biol Sci 362:1119–1134. https://doi.org/10.1098/rstb.2007.2039
22. Jayaraman A, Wood TK (2008) Bacterial quorum sensing: signals, circuits, and implications for biofilms and disease. Annu Rev Biomed

Eng 10:145–167. https://doi.org/10.1146/annurev.bioeng.10.061807.160536

23. Martínez JL (2014) Interkingdom signaling and its consequences for human health. Virulence 5:243–244. https://doi.org/10.4161/viru.28073
24. Williams P (2007) Quorum sensing, communication and cross-kingdom signalling in the bacterial world. Microbiology 153:3923–3938. https://doi.org/10.1099/mic.0.2007/012856-0
25. Shaw P, Ping G, Daly S (1997) Detecting and characterizing N-acyl-homoserine lactone signal molecules by thin-layer chromatography. Proc Natl Acad Sci U S A 94:6036–6041
26. Yates EA, Philipp B, Buckley C et al (2002) N-acylhomoserine lactones undergo lactonolysis in a pH-, temperature-, and acyl chain length-dependent manner during growth of *Yersinia pseudotuberculosis* and *Pseudomonas aeruginosa*. Infect Immun 70:5635–5646. https://doi.org/10.1128/IAI.70.10.5635
27. Winson M, Swift S, Fish L (1998) Construction and analysis of luxCDABE-based plasmid sensors for investigating N-acyl homoserine lactone-mediated quorum sensing. FEMS Microbiol Lett 163:185–192
28. Smith RS, Fedyk ER, Springer TA et al (2001) IL-8 production in human lung fibroblasts and epithelial cells activated by the *Pseudomonas* autoinducer N-3-oxododecanoyl homoserine lactone is transcriptionally regulated by NF-kappa B and activator protein-2. J Immunol 167:366–374. https://doi.org/10.4049/jimmunol.167.1.366
29. Zimmermann S, Wagner C, Müller W et al (2006) Induction of neutrophil chemotaxis by the quorum-sensing molecule N-(3-oxododecanoyl)-L-homoserine lactone. Infect Immun 74:5687–5692. https://doi.org/10.1128/IAI.01940-05
30. Wagner C, Zimmermann S, Brenner-Weiss G et al (2007) The quorum-sensing molecule N-3-oxododecanoyl homoserine lactone (3OC12-HSL) enhances the host defence by activating human polymorphonuclear neutrophils (PMN). Anal Bioanal Chem 387:481–487. https://doi.org/10.1007/s00216-006-0698-5
31. Diggle SP, Matthijs S, Wright VJ et al (2007) The *Pseudomonas aeruginosa* 4-quinolone signal molecules HHQ and PQS play multifunctional roles in quorum sensing and iron entrapment. Chem Biol 14:87–96. https://doi.org/10.1016/j.chembiol.2006.11.014
32. Steindler L, Venturi V (2007) Detection of quorum-sensing N-acyl homoserine lactone signal molecules by bacterial biosensors. FEMS Microbiol Lett 266:1–9. https://doi.org/10.1111/j.1574-6968.2006.00501.x
33. Fletcher MP, Diggle SP, S a C et al (2007) A dual biosensor for 2-alkyl-4-quinolone quorum-sensing signal molecules. Environ Microbiol 9:2683–2693. https://doi.org/10.1111/j.1462-2920.2007.01380.x
34. McClean K, Winson M, Fish L (1997) Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of N-acylhomoserine lactones. Microbiology 143(Pt 12):3703–3711
35. Diggle SP, Winzer K, Lazdunski A et al (2002) Advancing the quorum in *Pseudomonas aeruginosa*: MvaT and the regulation of N-acylhomoserine lactone production and virulence gene expression. J Bacteriol 184:2576–2586. https://doi.org/10.1128/JB.184.10.2576
36. Winzer K, Falconer C, Garber NC et al (2000) The *Pseudomonas aeruginosa* lectins PA-IL and PA-IIL are controlled by quorum sensing and by RpoS. J Bacteriol 182:6401–6411. https://doi.org/10.1128/JB.182.22.6401-6411.2000
37. Swift S, Karlyshev A, Fish L (1997) Quorum sensing in *Aeromonas hydrophila* and *Aeromonas salmonicida*: identification of the LuxRI homologs AhyRI and AsaRI and their cognate N-acylhomoserine. J Bacteriol 179:5271–5281