Chapter 2

Detection of 2-Alkyl-4-Quinolones Using Biosensors

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Abstract

2-Alkyl-4-quinolones (AQs) such as 2-heptyl-3-hydroxy-4-quinolone (PQS) and 2-heptyl-4-hydroxyquinoline (HHQ) are quorum-sensing signal molecules. Here we describe two methods for AQ detection and quantification that employ thin-layer chromatography (TLC) and microtiter plate assays in combination with a *lux*-based *Pseudomonas aeruginosa* AQ biosensor strain. For TLC detection, organic solvent extracts of bacterial cells or spent culture supernatants are chromatographed on TLC plates, which are then dried and overlaid with the AQ biosensor. After detection by the bioreporter, AQs appear as both luminescent and green (from pyocyanin) spots. For the microtiter assay, either spent bacterial culture supernatants or extracts are added to a growth medium containing the AQ biosensor. Light output by the bioreporter correlates with the AQ content of the sample. The assays described are simple to perform, do not require sophisticated instrumentation, and are highly amenable to screening large numbers of bacterial samples.

Key words *Pseudomonas aeruginosa*, *Biosensor*, *2-Alkyl-4-quinolones*, *Pseudomonas quinolone signal (PQS)*, *2-Heptyl-4-hydroxyquinoline (HHQ)*, *pqsA*

1 Introduction

In *Pseudomonas aeruginosa*, cell-cell communication (quorum sensing, QS) controls the production of multiple virulence factors and secondary metabolites and promotes biofilm maturation. The QS system consists of two *N*-acylhomoserine lactone (AHL) regulatory circuits (*las* and *rhl*), linked to a 2-alkyl-4-quinolone (AQ) system [1]. In the *las* system, the *lasI* gene product directs the synthesis of *N*-(3-oxo-dodecanoyl)-L-homoserine lactone (3-oxo-C_{12}-HSL), which interacts with the transcriptional regulator LasR and activates target promoters. In the *rhl* system, RhlI directs the synthesis of *N*-(butanoyl)-L-homoserine lactone (C_4-HSL), which interacts with the cognate regulator RhlR and activates target gene promoters. The *las* and *rhl* systems are hierarchically connected and regulate the timing and production of multiple virulence factors including elastase, alkaline protease, exotoxin A,

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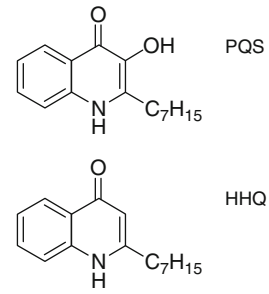

Fig. 1 Structures of the AQ signal molecules; PQS and HHQ

rhamnolipids, pyocyanin, lectins, superoxide dismutases, and biofilm development [1].

Pesci et al. [2] demonstrated that addition of a spent culture medium extract from a *P. aeruginosa* wild type (PAO1) caused induction of *lasB* (elastase) expression in a PAO1 AHL-deficient *lasR* mutant [2]. This data suggested that a non-AHL signal produced by the bacterium was capable of activating *lasB* expression which required LasR and 3-oxo-C_{12}-HSL for its biosynthesis. It was also shown that the novel signal required a functional *rhl* system for its bioactivity since *lasB* could not be activated in a *rhlI/rhlR* double mutant by PAO1 spent culture extracts. The molecule responsible for the non-AHL-mediated QS signaling pathway was purified and chemically identified as 2-heptyl-3-hydroxy-4-quinolone and termed the *Pseudomonas* quinolone signal (PQS) (Fig. 1) [2]. PQS belongs to the AQ family of compounds, which were first chemically identified in the 1940s and studied for their antibacterial properties. In addition to PQS, other AQ molecules produced by *P. aeruginosa* include 2-heptyl-4-hydroxyquinoline (HHQ) (Fig. 1), 2-nonyl-4-hydroxyquinoline (NHQ), and 2-heptyl-4-quinolone *N*-oxide (HQNO) [3]. PQS and HHQ enhance their own biosynthesis by binding to the cognate transcriptional regulator protein, PqsR, to activate the expression of the AQ biosynthetic operon, *pqsACBDE* [4, 5], that is responsible for producing over 50 AQ congeners in conjunction with the *pqsH* and *pqsL* gene products. PqsH is a mono-oxygenase that converts 2-alkyl-4-quinolones such as HHQ into 2-alkyl-3-hydroxy-4-quinolones such as PQS while PqsL is required for the biosynthesis of *N*-oxides such as HQNO [3].

In *P. aeruginosa*, the AQ signaling pathway plays a role in the regulation of production of several virulence factors including the blue-green pigment pyocyanin, rhamnolipids, and lectin A and is involved in extracellular DNA release during biofilm development [6, 7]. AQs are also produced by other bacterial genera including *Burkholderia pseudomallei*, the causative organism of melioidosis [8], suggesting that AQ signaling may be more widespread than previously thought.

For the detection and unequivocal chemical identification of AQs, both NMR (Nuclear Magnetic Resonance) spectroscopy and LC-MS (Liquid Chromatography-Mass Spectrometry) have been used [9–11]. These methods are extremely sensitive but rely on access to expensive instrumentation and require considerable expertise. A simple alternative is to use thin-layer chromatography (TLC) to detect AQs and previously TLC assays have been used to detect PQS under UV light [6, 12, 13]. However, this method is not sufficiently discriminatory in complex bacterial supernatants that contain multiple fluorescent compounds and is not particularly suitable for 2-alkyl-4-quinolones such as HHQ, the fluorescence of which is much weaker than PQS under UV light. A third option is to use a specific AQ biosensor [14–16] as described in this chapter.

2 Materials

2.1 Bacterial Bioreporters, Growth Media, and AQ Compounds

1. The bioreporter used in this assay is PAO1Δ*pqsA* CTX-*lux::pqsA* [16]. This strain is *P. aeruginosa* PAO1 with a chromosomal deletion in the *pqsA* gene. It is AQ negative but responds via light and pyocyanin production to the presence of AQs such as PQS and HHQ. The reporter gene construct in this strain is the *pqsA* promoter (which is sensitive to both PQS and HHQ) fused to the CTX-*luxCDABE* cassette [17]. This construct was conjugated into PAO1Δ*pqsA*, resulting in a stable, chromosomal single-copy reporter. The strain can be maintained in tetracycline 125 μg/ml and stored at −80 °C in 25% (vol/vol) glycerol.
2. PQS standard, 10 mM in methanol (PQS MW 259).
3. HHQ standard, 10 mM in methanol (HHQ MW 243).
4. Lysogeny broth (LB): 1% (wt/vol) bacto-tryptone, 0.5% (wt/vol) yeast extract, 1% (wt/vol) sodium chloride in distilled water.
5. LB agar: Lysogeny broth with the addition of 2% (wt/vol) agar technical No. 3.
6. Tetracycline, 50 mg/ml in methanol.

2.2 Extraction of AQs

1. Potassium phosphate monobasic (KH_2PO_4).
2. Methanol—HPLC gradient grade.
3. Ethyl acetate—HPLC gradient grade.
4. Acetone—HPLC gradient grade.
5. Dichloromethane—HPLC gradient grade.
6. Glacial acetic acid—analytical grade.
7. Rotary evaporator or centrifugal evaporator.

2.3 Detection of AQs Using TLC

1. Normal-phase 20 × 20 cm silica 60_{F254} TLC plates.
2. TLC developing tank.
3. Soft top agar: 0.65% (wt/vol) agar technical No. 3, 1% (wt/vol) tryptone, 0.5% (wt/vol) sodium chloride.
4. UV transilluminator (312 nm).
5. Luminograph photon video camera.
6. X-ray film.

2.4 Detection of AQs Using Microtiter Plates

1. 96-Well plate spectrophotometer/luminometer.
2. 96-Well, white or black, clear bottom microtiter plates.

3 Methods

3.1 Preparing Bacterial Cultures for AQ Extraction

1. Under sterile conditions, streak out a 10 μl loop of the test bacterium, *P. aeruginosa* PAO1 (AQ positive control), the AQ-negative mutant PAO1 *pqsA* (AQ-negative control) and the PQS-negative (but HHQ positive) PAO1 *pqsH* (PQS-negative control) onto fresh LB agar plates. Grow overnight at 37 °C with any appropriate antibiotics.
2. On the following day, inoculate 5 ml of LB medium containing appropriate antibiotics with single colonies of the relevant strains. Grow the cultures overnight at 37 °C with shaking at 200 rpm. It is not essential that LB medium is used for this stage; any appropriate growth medium can be used, although *P. aeruginosa* strains produce high concentrations of AQs in LB.
3. The following day, the optical densities (OD) of the cultures should be measured at 600 nm (OD_{600}). Using these readings, standardize the cultures to OD 1.0 by diluting with the growth medium used in **step 2**.
4. Transfer 0.25 ml of standardized culture into 250 ml Erlenmeyer flasks containing 25 ml LB broth (or alternative growth medium). A small volume of culture in a large flask allows good aeration of the medium. Incubate at 37 °C, with shaking at 200 rpm for 8 h (*see* **Note 1**).

3.2 AQ Extraction of Bacterial Cultures

1. Transfer a defined volume of each culture (in this example 10 ml) to a 50 ml centrifuge tube and centrifuge at 10,000 × *g* for 10 min. Extract the cells (*see* **step 2**) or the supernatant (*see* **step 3**). It is possible to carry out both procedures together.
2. For extraction of AQs from cells, centrifuge the culture at 10,000 × *g* for 10 min, remove the supernatant (save if required for supernatant extraction), and resuspend the cells

in 10 ml of fresh LB or relevant medium. Centrifuge again at 10,000 × *g* for 10 min and discard the supernatant. Repeat the media wash steps twice to remove all traces of the supernatant AQs from the cells. Add 10 ml of methanol to the cell pellet and vortex until fully resuspended. Allow to stand for 10 min to allow the cells to lyse before centrifuging again at 10,000 × *g* for 10 min. Filter the extract through sterile 0.2 μm filters into clean centrifuge tubes to remove all cell debris from the extraction mixtures. At this stage it is possible to store the cell extractions in the freezer at −20 °C for several days if required.

3. For supernatant extraction, centrifuge the culture at 10,000 × *g* for 10 min and filter the supernatants through sterile 0.2 μm filters into clean centrifuge tubes to remove any cells. Add 10 ml of acidified ethyl acetate (glacial acetic acid 0.01% (vol/vol) in ethyl acetate) to the supernatant and vortex for 30 s so the two phases are well mixed. Transfer the extraction mixtures into a separating funnel that has been previously washed with acetone and allow the extraction mixtures to settle and the two phases to separate. Transfer the top organic layer into a fresh centrifuge tube. Repeat the extraction procedure on the bottom layer twice before discarding. Pool the collected organic layers. If time is limiting, supernatant extraction mixtures can be stored in the freezer at −20 °C for several days.
4. For the cell and supernatant procedures, evaporate the mixtures to dryness, e.g., using a centrifugal evaporator or a rotary evaporator (transfer the extraction mixtures to 50 ml round-bottom flasks that have been previously washed with acetone).
5. Add 0.5 ml of methanol to the round-bottom flasks and agitate for 30 s before transferring the liquid to 2 ml glass sample vials. Repeat this step with two further additions of 0.5 ml methanol and pool each sample in each vial. If time is limiting, both cell and supernatant extraction mixtures can be stored in the freezer at −20 °C for several days.
6. Dry down the extraction mixtures in the sample vials using, e.g., a centrifugal evaporator or under a stream of nitrogen gas. Dry cell and supernatant extraction residues can be stored in the freezer at −20 °C for several months.

3.3 Preparation of TLC Plates and Running of Samples

1. Prepare normal-phase silica 20 × 20 cm 60_{F254} TLC plates (*see* **Note 2**) by soaking in a 5% (wt/vol) solution of KH_2PO_4 for 30 min before activating at approximately 100 °C for 1 h, e.g. in a hybridization oven.
2. Draw a faint line in pencil approximately 2.5 cm from the bottom of the silica TLC plate to use as a guide for spotting sample extracts.

3. Reconstitute sample extracts in 100 µl of methanol and spot 5 µl of each onto the TLC plate. The amount spotted onto the TLC depends on the concentration of AQs in the sample. *P. aeruginosa* produces high amounts of AQs and in this case 5 µl is a good starting quantity. As positive controls, 2 µl of 10 mM stock solutions of PQS and HHQ (or other AQs) in methanol can be spotted onto the TLC plate. Space each spot at 2 cm intervals along the line. A hairdryer can be used to dry samples during spotting to give a tighter spot.
4. When dry, place the TLC plate into a developing tank and run the TLC using a mixture of dichloromethane:methanol (95:5) as the mobile phase until the solvent front is 1–2 cm from the top of the plate. The TLC plate can be visualized using a UV transilluminator at 312 nm and photographed at this point (Fig. 3a).
5. Allow the TLC plate to dry and apply autoclave tape to both the underside of the TLC plate and around the edges so that the tape creates a well at least 0.5 cm deep around the TLC plate into which the nutrient agar containing the biosensor will be poured. Make sure that the autoclave tape is firmly pressed down and forms a tight seal (Fig. 2).

3.4 Overlay of TLC Plates and Detection of AQs Using a Bioreporter

1. Streak out a 10 µl loop of the AQ biosensor (PAO1Δ*pqsA* CTX-*lux::pqsA*) [16] onto fresh LB agar plates containing tetracycline 125 µg/ml and grow overnight at 37 °C.
2. The following day, inoculate a single colony into 5 ml LB medium containing tetracycline 125 µg/ml and grow overnight at 37 °C with shaking at 200 rpm.
3. The following day, gently melt 100 ml of soft top agar in a microwave and allow to cool to approximately 50 °C. Add 1 ml of the overnight culture to the soft top agar and mix gently (*see* **Note 3**).
4. Pour the agar mixture slowly into the well made around the TLC plate, being careful to minimize bubble formation in the agar and on the TLC plate (*see* **Note 4**).
5. Allow the agar to solidify around a Bunsen flame (to help keep sterile) and then incubate the plate at 37 °C for 6–8 h to view light production, or overnight to view pyocyanin production. Visualize the plates for light production using a luminograph photon video camera (or similar) (Fig. 3b) or develop using X-ray film. Alternatively simply view production of the blue/green phenazine pigment pyocyanin by eye (Fig. 3c) (*see* **Note 5**).

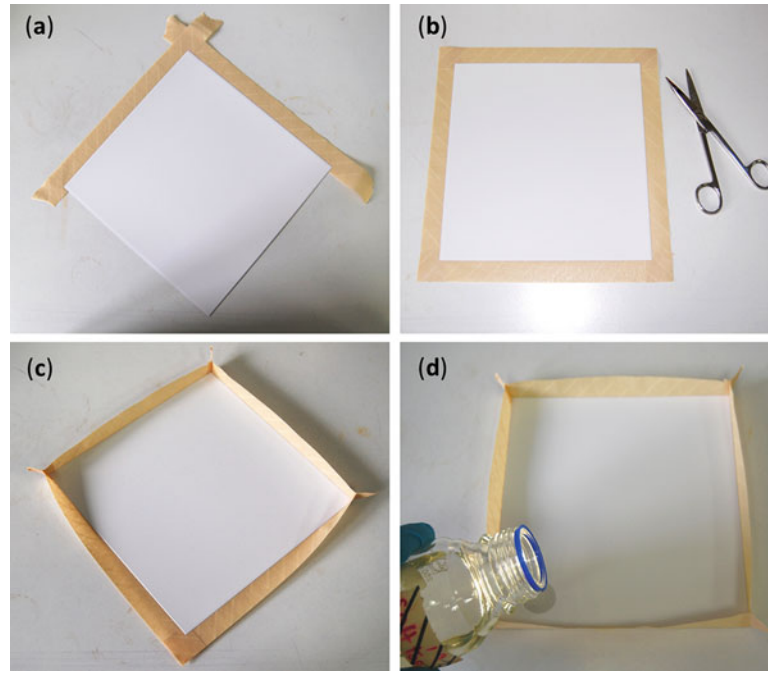

Fig. 2 Preparation of the biosensor TLC plate agar overlay. (**a**) Attach a strip of autoclave tape to the aluminum backing along each edge of the TLC plate, pressing down firmly to ensure a good seal. (**b**) Neatly trim the excess autoclave tape using scissors. (**c**) Pinch the autoclave tape at each corner of the TLC plate, creating a barrier of autoclave tape along each side of the TLC plate to create a well. (**d**) Place the TLC plate into an appropriate dish and pour into the well the molten soft top agar containing the biosensor bacteria

3.5 Testing for AQ Production Using a Microtiter Plate Assay

1. Prepare crude bacterial culture supernatants by growing the test bacterium as described in Subheading 3.1.
2. Remove 5 ml of culture and spin at 10,000 × *g* for 5 min before collecting the supernatant and passing it through a sterile 0.2 μm filter into clean tubes (*see* **Note 6**). If time is limiting, this supernatant extract can be frozen at −20 °C for a few days.
3. Grow the AQ biosensor overnight and dilute with LB medium to OD_{600} 1.0. Further dilute this standardized biosensor culture with LB medium to give (a) 1 in 50 and (b) 1 in 100 dilutions.
4. Take a sterile 96-well plate (*see* **Note 7**) and for each well mix 100 μl of the sterile test bacterial supernatant with 100 μl of the 1 in 50 dilution of the biosensor to give a final culture dilution of 1 in 100. For each negative control well, add 200 μl of the 1 in 100 dilution of the AQ biosensor. A positive control well containing a PQS or a HHQ standard at a concentration of 10 μM can be added or alternatively a *P. aeruginosa* wild-type culture supernatant (100 μl) can also be included in a well during the assay.

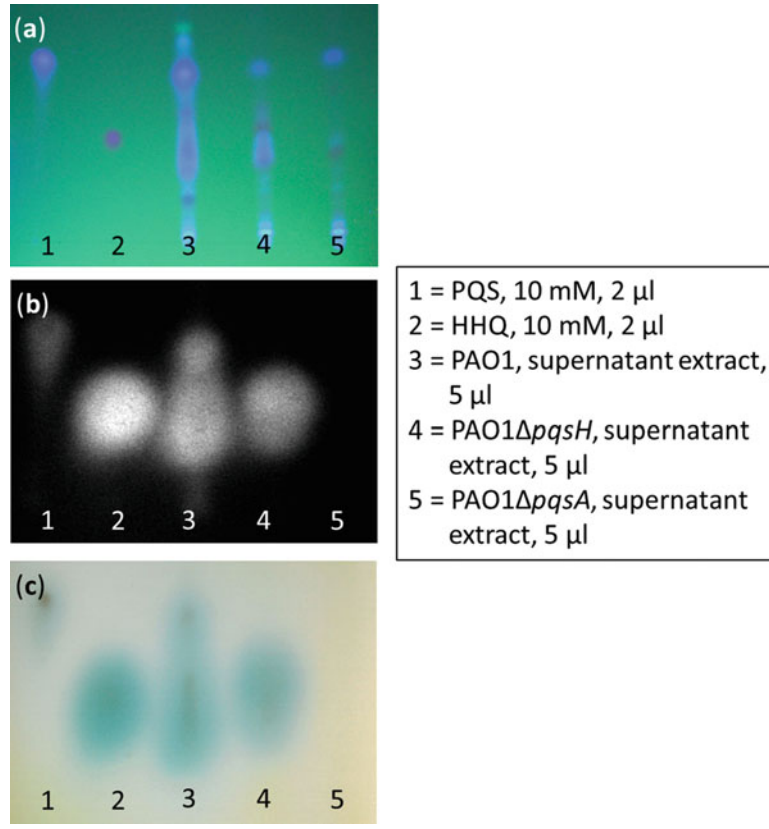

Fig. 3 TLC assay for AQs. (**a**) TLC plate run with standards of PQS and HHQ and supernatant extracts of PAO1, PAO1Δ*pqsH*, and PAO1Δ*pqsA* visualized under UV at 312 nm. (**b**) Overlay of TLC plate with soft top agar containing biosensor bacteria showing the production of light in response to AQs visualized using a luminograph photon video camera. (**c**) Overlay of TLC plate with soft top agar containing the biosensor bacteria showing production of the blue/green pigment pyocyanin, in response to AQs. TLC lanes: (1) PQS 10 mM, 2 µl; (2) HHQ 10 mM, 2 µl; (3) PAO1 supernatant extract, 5 µl; (4) PAO1Δ*pqsH* supernatant extract, 5 µl; (5) PAO1Δ*pqsA* supernatant extract, 5 µl. The AQ biosensor emits light over spots identified as PQS and HHQ in PAO1 and HHQ only in PAO1Δ*pqsH*. Light spots are absent for the AQ-negative *pqsA* mutant

5. Monitor bioluminescence and OD at 37 °C using a combined spectrophotometer/luminometer. This measures OD and bioluminescence from all wells every 30 min for 24 h. Luminescence is recorded as relative light units (RLU) per unit of OD. If an automated combined spectrophotometer/luminometer is unavailable, readings can be taken manually at defined time points by growing the bacterial cultures under specific conditions and measuring the OD and bioluminescence of culture samples using a spectrophotometer and tube luminometer, respectively.

4 Notes

1. Growth of *P. aeruginosa* cultures for 8 h in LB medium results in the bacteria reaching mid-stationary phase, which is sufficient for high quantities of AQs to be produced. If using an alternative growth medium, the time of incubation may have to be altered to account for growth rate differences. We recommend that stationary-phase cells be used when attempting to detect AQs produced by bacterial species.
2. We routinely use normal-phase 20 × 20 cm silica 60_{F254} TLC plates from Merck.
3. Make sure that the agar has cooled sufficiently before adding the bacterial reporter strain. Addition at too high a temperature will attenuate bacterial growth.
4. Pour the agar promptly or it will begin to solidify. Bubbles can be removed by gently passing a Bunsen burner flame over the surface of the agar.
5. Both PQS and HHQ will activate light production in the reporter, as both control the expression of the *pqsA* gene. In addition, both PQS and HHQ activate the production of pyocyanin.
6. In addition to cell-free culture supernatants, the solvent-extracted culture extracts described in Subheading 3.2 may also be analyzed via this method. Simply dilute 5 μl of the solvent extract in 100 μl of LB and add to 100 μl of 1 in 50 dilution of the AQ biosensor per well.
7. Specialized 96-well white or black plates need to be used when monitoring bioluminescence. The plates should not be clear-sided to reduce light scatter between wells but should have clear plastic bottoms so that an automated spectrophotometer/luminometer can detect and measure both light output and absorbance accurately.

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