Chapter 23

Methods for Detecting *N*-Acyl Homoserine Lactone Production in *Acinetobacter baumannii*

Aimee R. Paulk Tierney and Philip N. Rather

Abstract

Acinetobacter baumannii cell-cell signaling is mediated by production and release of *N*-acyl homoserine lactones (AHLs), primarily *N*-(3-hydroxydodecanoyl)-L-homoserine lactone (3-hydroxy-C_{12}-HSL). The secretion of this signal can be readily assessed via simple plate-based or thin-layer chromatography-based assays utilizing an *Agrobacterium tumefaciens traG-lacZ* containing biosensor. In this chapter, we describe methods to assay for secreted *N*-acyl homoserine lactone production in *Acinetobacter baumannii*.

Key words *Acinetobacter baumannii*, *N*-Acyl homoserine lactone, Quorum sensing, Biosensor, *Agrobacterium tumefaciens*, Thin-layer chromatography

1 Introduction

Similar to other Gram-negative bacteria, *Acinetobacter baumannii* communicates via production and secretion of *N*-acyl homoserine lactone (AHL) autoinducers utilizing a LuxI-/LuxR-type system [1]. In this system, an autoinducer synthase produces the AHL signal, which typically diffuses across the cell membrane, reenters the cell at high concentrations, and then binds and activates a transcriptional regulatory protein to mediate global changes in gene expression [2]. In *A. baumannii*, the autoinducer synthase and transcriptional regulator proteins are termed AbaI and AbaR, respectively [1].

Secretion levels of AHL signal can be qualitatively assessed by plate-based methods that utilize bacterial biosensor strains containing a reporter gene that is transcriptionally activated in the presence of AHL signals. Typical reporters include luminescence (*lux*), β-galactosidase (*lacZ*), or *Chromobacterium violaceum* purple pigment violacein [3, 4]. Here, we demonstrate detection of *A. baumannii* AHL signals using an *Agrobacterium tumefaciens* biosensor containing a plasmid-localized *traG-lacZ* fusion (pZLR4). The host biosensor strain does not make AHL signals,

Indranil Biswas and Philip N. Rather (eds.), *Acinetobacter baumannii: Methods and Protocols*, Methods in Molecular Biology, vol. 1946, https://doi.org/10.1007/978-1-4939-9118-1_23,

but exogenous AHL from other bacteria will bind to TraR, coactivating *traG* and inducing production of β-galactosidase [4, 5]. Strains of interest can be surveyed for AHL secretion by (a) cross-streaking the *A. tumefaciens* biosensor against the strain of interest on a LB plate containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal); (b) direct plating of bacterial culture, cell-free supernatants, or patched colonies onto a soft agar lawn containing the *A. tumefaciens* biosensor and X-gal; or (c) separating extracts on reversed-phase C_{18} thin-layer chromatography plates and overlaying the dried plates with a soft agar lawn containing the *A. tumefaciens* biosensor.

2 Materials

Prepare all solutions with distilled water prior to daily use. Store reagents at room temperature unless otherwise indicated.

1. Media: Luria broth (LB)—10 g tryptone, 5 g yeast extract, 5 g sodium chloride per liter.
2. Soft agar. Luria broth containing 0.7% agar.
3. Gentamicin stock solution 20 mg/mL in water.
4. Petri dishes and tubes for bacterial culture.
5. X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) solution at 20 μg/mL. Store at −20 °C.
6. Reversed-phase C-18 thin-layer chromatography (TLC) plates.
7. Glass chromatography chamber for developing TLC plates.
8. 60% methanol.

3 Methods

3.1 Cross-Streak Assay

1. Inoculate 2 mL LB containing gentamicin 30 μg/mL with the *Agrobacterium tumefaciens* biosensor strain. Grow overnight at 28 °C, stationary (*see* **Note 1**).
2. Prepare a LB agar plate (15 g agar/L) with 80 μL of 20 μg/mL X-gal per 20 mL (*see* **Note 2**). Dry the plate inverted with the top off at 37 °C for at least 1 h.
3. While holding the plate at an angle, add 30 μL of the *A. tumefaciens* biosensor strain ($OD_{600} = 0.2$) and allow the culture to run down the surface of the plate to form a line of cells. Streak a loop of *A. baumannii* strain to be tested for AHL production next to the biosensor strain. Alternatively, spot 5 μL of the culture to be tested for signal production adjacent to the biosensor strain and allow the spot to soak into the agar.

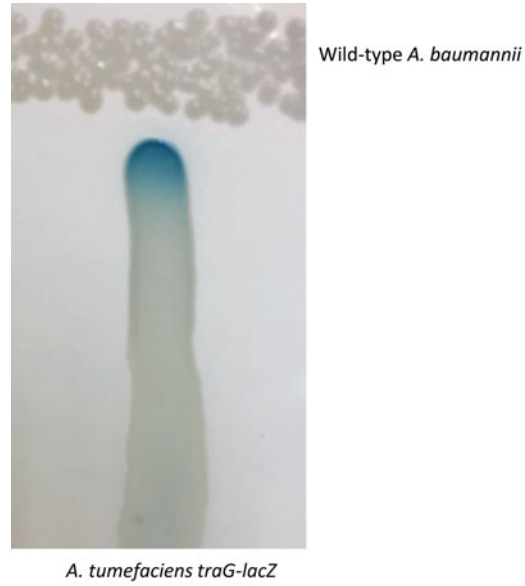

Fig. 1 Cross-streak of *Agrobacterium tumefaciens* indicator strain and *Acinetobacter baumannii*. *A. baumannii* (horizontal line, top of plate) was streaked perpendicular to the *A. tumefaciens* biosensor (vertical line). Blue pigmentation of the biosensor strain near the point of intersection to *A. baumannii* indicates production, secretion, and diffusion of AHL through the medium

4. Incubate plates at 28 °C for 12 h or until a blue color develops in the biosensor strain.

Blue pigmentation of the *A. tumefaciens* biosensor near the strain(s) of interest indicates production of the AHL signal (Fig. 1).

3.2 Soft Agar Assay

1. Inoculate 2 mL LB containing gentamicin (30 μg/mL) with the *Agrobacterium tumefaciens* biosensor strain. Grow overnight at 28 °C, stationary (*see* **Note 1**).
2. Prepare LB soft agar (0.7 g agar/L). Cool to 45 °C and add 80 μL of 20 μg/mL X-gal per 20 mL melted agar and 250 μL of the *A. tumefaciens* culture (OD A_{600} = 0.2) per 20 mL melted agar. Mix and pour plate immediately (*see* **Notes 2** and **3**).
3. Allow plate to set and then dry inverted at 37 °C for 30–45 min.
4. Plate or patch bacterial cultures, colonies, or supernatants as desired. Incubate plates at 28 °C. Strains of interest that are positive for AHL secretion will produce a blue halo (Fig. 2).

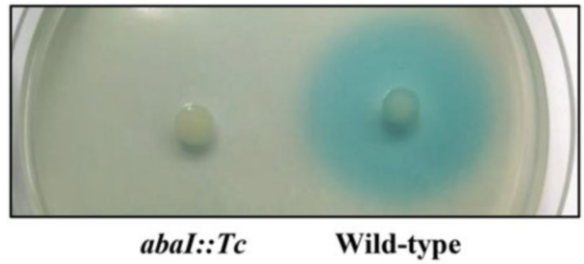

Fig. 2 Detection of AHL secretion on a soft agar lawn containing *Agrobacterium tumefaciens* biosensor strain. Colonies of an *A. baumannii* wild-type control (right) and an AHL-deficient mutant (left) were patched onto a soft agar lawn of *A. tumefaciens traG-lacZ* biosensor and X-gal. The plate was incubated for 24 h at 28 °C. The blue halo surrounding the wild-type *A. baumannii* patch indicates production, secretion, and diffusion of AHL through the medium, where it results in activation of the *traG-lacZ* fusion in the biosensor strain

3.3 Thin-Layer Chromatography (TLC) Assay

1. Spot samples to be tested on a reversed-phase C_{18} TLC plate. Spots should be approximately 2–3 cm from the bottom of the TLC plate.
2. Allow spots to dry completely.
3. Incubate the TLC plate in a glass chamber with 50 mL of 60% methanol and allow the solvent to completely ascend to the top of the plate.
4. Allow the plate to sit at room temperature for 60 min in a fume hood to allow the methanol to evaporate.
5. Seal all four sides of the TLC plate with tape in a manner that creates a lip of at least 1/2 in. on all sides. Laboratory tape works well for this (Fig. 3A).
6. Prepare 50 mL of LB soft agar (0.7 g agar/L). Cool to 45 °C and add 160 μL of 20 μg/mL X-gal and 400 μL of the *A. tumefaciens* culture. Mix and immediately pour the agar solution over the TLC plate to completely cover the surface. Allow the agar to solidify for 30 min (*see* **Note 4).**
7. Remove the tape border and place the TLC plate on top of an 8 in. × 8 in. glass Pyrex dish that contains 100 mL of water in the bottom, Fig. 3B (*see* **Note 5**). Cover the plate with an identical 8 in. × 8 in. glass Pyrex dish (Fig. 3C). Seal the two dishes by using Saran wrap. Place the sealed Pyrex dishes in a 28 °C incubator and incubate for 24–48 h until blue spots appear indicating activation of the biosensor.
8. Air-dry the TLC plate until the soft agar lawn has become completely dehydrated. This can be done at room temperature or in a 37 °C incubator (*see* **Note 6**).

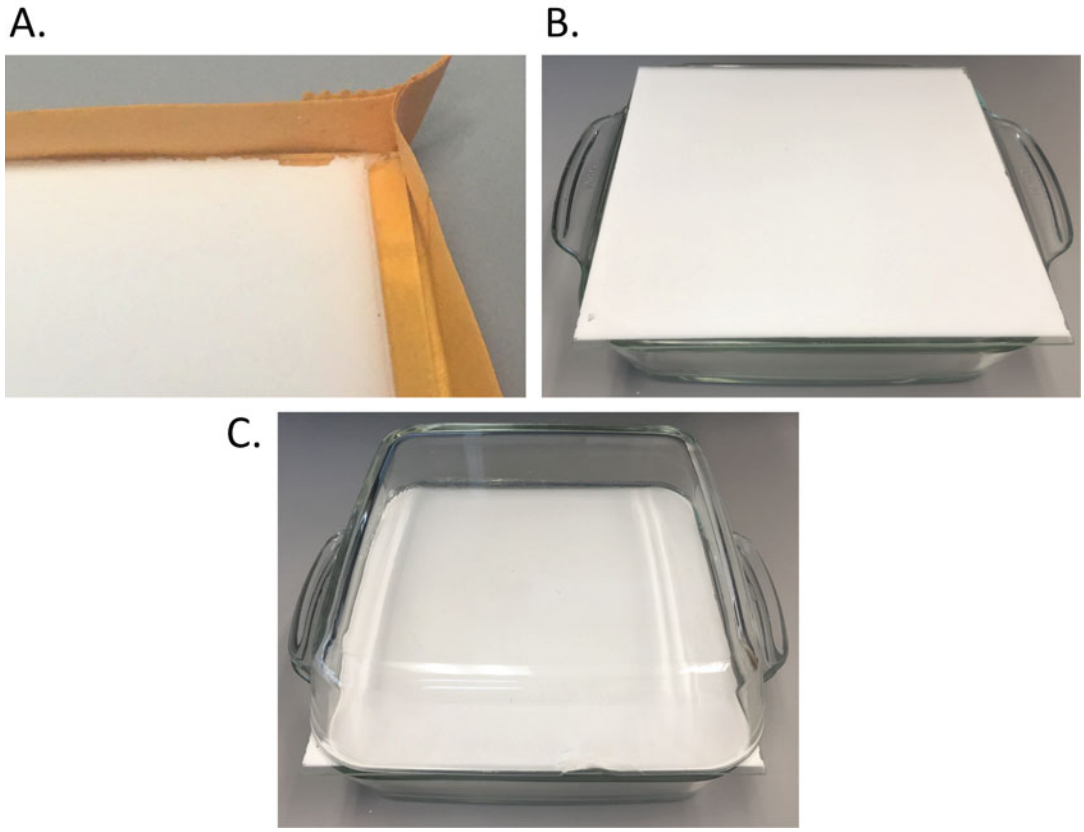

Fig. 3 Methods for incubating TLC plates with a soft agar overlay. This figure demonstrates how an enclosure can be generated with glass Pyrex dishes that can be used to incubate TLC plates in an incubator. Panel **A** demonstrates how tape can be used to create an agar enclosure for the TLC plate. Panels **B** and **C** demonstrate the use of Pyrex dishes to create a humidified enclosure

4 Notes

1. The *A. tumefaciens* with pZLR4 should be grown at 28 °C as the replication of this plasmid is temperature sensitive.
2. The concentration of X-gal can be adjusted to suit experimental needs.
3. The volume of *A. tumefaciens* culture added varies by culture density. The indicated volume of 250 μL per 20 mL agar is based on a culture at an OD_{600} of at least 0.2. It is generally better to err on the side of using a higher concentration as too low concentration will result in a sparse lawn with poor halo visualization. Troubleshooting the volume of culture needed may be required to suit experimental needs.
4. When the soft agar is added to the TLC plate, it is not necessary to have it at an even thickness in all areas of the plate. Once the

plate is done incubating and the agar overlay is dried, all changes in thickness will even out and be invisible.

5. This will allow the soft agar overlay to remain hydrated within the chamber created by the sealed Pyrex dishes.
6. When drying the soft agar lawn on the TLC plate, be aware that the dried agar/TLC matrix can sometime crack and curl up. Photograph the plates immediately after drying.

Acknowledgment

This work is supported by the following awards to P.N.R., VA Merit Award I01 BX001725 and Research Career Scientist Award IK6BX004470, both from the Department of Veterans Affairs, and R01AI072219 from the National Institutes of Health.

References

1. Niu C, Clemmer KM, Bonomo RA, Rather PN (2008) Isolation and characterization of an autoinducer synthase from Acinetobacter baumannii. J Bacteriol 190(9):3386–3392. Epub 19 Feb 2008. https://doi.org/10.1128/JB.01929-07
2. Bhargava N, Sharma P, Capalash N (2010) Quorum sensing in Acinetobacter: an emerging pathogen. Crit Rev Microbiol 36(4):349–360. Epub 18 Sept 2010. https://doi.org/10.3109/1040841X.2010.512269
3. McClean KH, Winson MK, Fish L, Taylor A, Chhabra SR, Camara M, Daykin M, Lamb JH, Swift S, Bycroft BW, Stewart GS, Williams P (1997) Quorum sensing and Chromobacterium violaceum: exploitation of violacein production and inhibition for the detection of N-acylhomoserine lactones. Microbiology (Reading, England) 143(Pt 12):3703–3711. Epub 9 Jan 1998. https://doi.org/10.1099/00221287-143-12-3703
4. Shaw PD, Ping G, Daly SL, Cha C, Cronan JE Jr, Rinehart KL, Farrand SK (1997) Detecting and characterizing N-acyl-homoserine lactone signal molecules by thin-layer chromatography. Proc Natl Acad Sci U S A 94(12):6036–6041. Epub 10 June 1997
5. Cook DM, Li PL, Ruchaud F, Padden S, Farrand SK (1997) Ti plasmid conjugation is independent of vir: reconstitution of the tra functions from pTiC58 as a binary system. J Bacteriol 179(4):1291–1297. Epub 1 Feb 1997