Chapter 10

Quorum Sensing in *Agrobacterium tumefaciens* using N-oxo-Acyl-homoserine Lactone chemical signal

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Introduction

The phenomenon of quorum sensing was first described in the Gram-negative marine bacterium *Photobacterium (Vibrio) fisheri* in the 1990's. This process has since been recognized as the most important intracellular signaling reaction operating between bacteria. Quorum sensing uses a low molecular weight chemical signal (almost all belonging to the chemical family called N-oxo-acylhomoserine lactones (HSL) that is excreted in a very small amount per bacterial cell. When the bacterial population reaches a high cell density, especially in a confined space, the concentration of HSL produced per cell is constant. In this environment, the HSL also reaches a higher concentration inside the cells, due to its free diffusion and equilibrium across the cell membrane. At sufficient concentration, HSL inside the cell acts as an autoinducer to activate a regulatory protein, which in turn is a transcriptional activator of a series of other genes, including those responsible for HSL production. This results in even greater levels of HSL and a change in the activity of the population of organisms at high cell density.

The list of bacteria expressing quorum sensing is growing, and it includes a number of pathogens. Quorum sensing is a very important process that allows bacteria to communicate with each other, to state that they have reached a critical cell mass in a suitable (host) environment, and should modify their activities to take advantage of this environment.

Bacterial species	Activities modulated by quorum sensing
Agrobacterium tumefaciens	conjugal transfer of the Ti plasmid
Erwinia caratovora	production of protease, cellulase, pectinase, exopolysaccharide, and antibiotics
Escherichia coli	cell division, but inducer molecule is not HSL
Photobacterium fischeri	bioluminescence
Pseudomonas aeruginosa	alkaline protease, elastase, cyanide (HCN) production, hemolysin, exotoxin A, neuraminidase, pyocyanin and rhamnolipid production
Rhizobium leguminosarum	expression of rhizosphere genes
Serratia liquefaciens	swarming motility

Materials

1. PART A

Equipment for the entire class

- 50ºC Water bath (1)

Supplies require for each group

- Adjustable volume autopipettor, 1-10 L
- Micropipettor tip, 1-10 L
- tip discard
- 18x150 mm Test tube racks

Media for each group

- divided Petri plates containing 5 mL base layer of AB medium with glucose, 40mg/L X-Gal and 1.5% Agar on both side
- 5 mL AB medium with glucose, 40mg/L X-Gal and 0.7 % Agar (final concentration) for CONTROL overlay
- 2.5 mL AB medium with glucose, 40mg/L X-Gal (final concentration) and 1.5 % Agar (0.7 % Agar final concentration) for TEST overlay
- 2.5 mL overnight broth culture of *A. tumefaciens* NTL4(pZLR4): reporter for TEST overlay

Bacterial cultures for each group

- *Agrobacterium tumefaciens* NT1 negative control
- *Agrobacterium tumefaciens* NT1(pTiC58) positive control
- *Agrobacterium tumefaciens* NTL4(pZLR4) reporter
- *Erwinia caratovora, Pseudomonas aeruginosa, Pseudomonas fluorescens, Vibrio fischeri, Rhizobium meliloti, Azotobacter vinelandii, Agrobacterium tumefaciens* (wild type) test strains (1mL each)

2. PART B

Equipment for the entire class

- 3-L Bioreactor or 500-mL shake flasks for growing test culture or shaker and/or tube roller
- Eppendorf centrifuge model 5415C

Supplies for each group

- Spectrophotometer Novaspec II (1)
- Disposable cuvettes (6)
- Toluene (200 L)
- Sterile eppendorf tubes (6)
- Vortex (1)
- Micropipettor 10-100 L (1)
- Micropipettor 100-1000 L (1)
- Sterile micropipettor tips 10-100 L (1 box)
- Sterile micropipettor tips 100-1000 L (1 box)
- Tip discard (1)
- 2 mL ONPG (4mg/mL dissolved in Z buffer)
- Graph paper (1/student)

Bacterial cultures for each group

- 5 mL *A. tumefaciens* NTL4(pZLR4)
- 5 mL of 0,4,8,12 and 24 h. *A. tumefaciens* H2 culture

Notes to Instructors

The amount of HSL produced by the culture is very small and difficult to detect by physical or chemical assay. The reporter strain can be used to perform a bioassay for HSL, and relative concentration of HSL can be followed by the activity of LacZ. The presence of beta-galactosidase is monitored qualitatively by observing X-gal cleavage as indicated by the appearance of blue color in a soft agar overlay, and then quantitatively by employing the lactose analog ONPG (ortho-nitrophenyl-galactopyranoside) in a spectrophotometric assay.

1. PART A

Grow overnight cultures of *A. tumefaciens* NT1 (or NTL4) for negative control in AB medium containing 0.2-0.5% glucose on shaker at 200 rpm at 28ºC and *A. tumefaciens* NT1(pTiC58) for positive control in AB medium containing 0.2-0.5% glucose on shaker at 200 rpm at 28ºC.

Grow *A. tumefaciens* NTL4(pZLR4) reporter or indicator strain in AB medium containing 0.2-0.5% glucose and 30mg/L Gentamicin* on shaker at 200 rpm at 28ºC

Grow a small amount (1 mL) of cultures of each test strains selected from any of *Erwinia caratovora, Pseudomonas aeruginosa, Pseudomonas fluorescens, Vibrio fischeri, Rhizobium meliloti, Azotobacter vinelandii, Agrobacterium tumefaciens, Erwinia caratovora, Pseudomonas aeruginosa, Vibrio fischeri* cultures in suitable general growth media like Trypticase Soy broth at the required temperature on a tube roller overnight.

Cautions: If a blue spot does not appear with test strains, this could indicate that (a) HSL is not present, or (b) that a HSL is produced, but too small a quantity to be detected, or (c) that HSL is produced, but its chemical structure is not recognized by the indicator strain. HSLs with acyl-chain length of C6 to C12 are readily detected by the indicator strain. However, there are HSLs with acyl-chain lengths of C4. Other quorum sensing compounds like the cyclic peptide thiolactones formed by some Gram positive bacteria are very different chemically, but perform the same role in gene regulation at high cell density and in response to the environment.

2. PART B

Grow *A. tumefaciens* NTL4(pZLR4) reporter or indicator strain in AB medium containing 0.2-0.5% glucose, and Gentamicin* (30mg/L) on shaker at 200 rpm at 28º

Grow *A. tumefaciens* H2 TEST strain in AB medium containing 0.2-0.5% glucose on shaker at 200 rpm at 28º or in a Bioreactor. Sample at 0, 4, 8, 12, and 24 hours and record OD_{600} for each sample.

* Selection can be left out in part A and B, plasmid is sufficiently stable

Lysing cells is an important step and needs to be done thoroughly. The toluene partially disrupts the cell membrane, allowing small molecules such as ONPG, to diffuse into the cell. Yellow color development after adding ONPG to sample could be observed in 5-10 minutes. Reaction can be stopped by adding 0.5 mL of 1 M Na_2CO_3 solution.

Student Outline

Introduction

In this Exercise, we will try to observe HSL production by *Agrobacterium tumefaciens*. This is a plant pathogen that causes crown gall disease. The crown gall tissue is a tumor-like growth of undifferentiated plant tissue. This altered growth of the plant is caused by the transfer of DNA (T-DNA) from the bacterium into the plant nucleus, where it becomes integrated into a plant chromosome. The T-DNA encodes the *onc* (oncogenesis) genes, which cause tumor growth, and the *ops* genes for opine synthesis. The plant expresses the bacterial genes (is transgenic), and secretes opines into the soil, where they serve as a unique C- and N-source for use by free-living *Agrobacterium* in the rhizophere. Two of the most common opines are octopine, which is N^2- (1,3-dicarboxyethyl)-L-arginine, and nopaline, which is N^2- (1,3-dicarboxypropyl)-L-arginine. Thus, the bacterium has carried out natural genetic engineering of a plant.

The T-DNA is a small part of the Ti-plasmid in *Agrobacterium*, which also encodes virulence (*vir*) genes needed for T-DNA transfer into the plant, *tra* genes for transfer of the Ti plasmid between strains of *A. tumefaciens*, and genes for opine catabolism. The opine catabolism genes are matched to the type of opine produced from the T-DNA. HSL production is encoded by a gene in the *tra* gene region. When *A. tumefaciens* feeds on the kind of opine produced by a plant that has been infected by *A. tumefaciens* (for example octopine), it will grow and replicate to reach a high cell density. During growth, small amounts of *Agrobacterium* HSL (N-(3-oxohexanoyl)-L-homoserine lactone) are formed. At high cell density (indicating that *Agrobacterium* is growing in a suitable environment), HSL reaches a high intracellular concentration and binds to the TraR protein, which in turn, activates the transcription of the *tra* genes. As a result of this, *A. tumefaciens* synthesizes a conjugative pilus and other gene products required to mate with other strains of *A. tumefaciens.* In this way, the Ti-plasmid which encodes octopine catabolism can be transferred throughout the *A. tumefaciens* population (since some strains in the population may be able to use other opines, but not octopine). This is a community-oriented growth pattern which is very different from the individualistic single cell growth pattern of other bacteria, making the *A tumefaciens* population an effective competitor for rhizosphere space and nutrients.

Strains to be used:

1) *A. tumefaciens* NT1 (or NTL4): These strains lack the Ti plasmid and do not form HSL. They can be used as an HSL negative control.

2) *A. tumefaciens* NT1(pTiC58): This strain was constructed from strain NT1, by addition of a mutant Ti plasmid, which constitutively expresses the *tra* genes. This strain *(tra^c)* constituvely overproduces HSL.

3) *A. tumefaciens* NTL4(pZLR4): This strain does not have a Ti plasmid, but contains another recombinant plasmid (pZLR4). The pZLR4 vector confers resistance to gentamicin and carbenicillin, so can be maintained in strain NTL4 by antibiotic selection. Other genes on pZLR4 include a *traG::lacZ* fusion and *traR*. When HSL is added to strain NTL4(pZLR4), it will diffuse into the cell and activate the TraR protein. Transcription of the *traG* gene is then activated by TraR. However the *traG* gene has been mutated (interrupted) by the insertion of the *lacZ* gene, encoding the beta-galactosidase enzyme. Thus, LacZ (beta-galactosidase) activity can be used as a reporter of traG transcription, and hence an indicator of the presence of HSL.

Procedure

A. Plate Assay for Detection of HSL's

1. Grow the NTL4(pZLR4) reporter strain in AB medium with Gentamicin overnight at 28° C.
2. Grow strains NT1 and NT1(pTiC58) overnight in AB medium. Grow other test strains (*Agrobacterium tumefaciens*, *Synorhizobium meliloti*, *Erwinia caratovora*, *Azotobacter vinelandii*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*) in suitable medium overnight or into stationary phase
3. On the bottom of a divided Petri dish mark one side Test (T), and the other side Control (C).
4. Pour 5 mL of AB medium containing 0.2-0.5% glucose, X-gal (40 g/mL), and 1.5% agar into each side of the divided Petri dish.
5. Overlay the control side of the split plate with 5 mL of AB medium containing X-gal (40 g/mL) and 0.7% agar (final concentration). Tip the plate gently to let the soft agar overlay fully cover the Control side. *This side of the plate does not contain the reporter strain* and controls for the spontaneous or induced breakdown of X-gal.
6. Add 2.5 mL of overnight NTL4(pZLR4) reporter strain to 2.5 mL AB medium containing X-gal (40 g/mL) and 0.7% agar (final concentration). Mix, but avoid introducing bubbles.
7. Immediately overlay Test side of the plate with the reporter strain from step 6. Tip plate gently to allow the soft agar to cover the surface of the Test side.
8. Once the soft agar overlay has set, inject the test strains. Add 10 L of the test solution with micropipettes. Gently stab the soft agar overlay with the tip of the pipettor, and release the fluid while pulling the tip out of the agar. A small amount of fluid will remain on the surface. As you inject other samples, do not disturb these puddles of samples on the surface of the agar. Let the puddles absorb in the agar in discreet spots (*i.e.*, not all run together and not off to the edge of the plate!) You should be able to place one spot of strain NT1(pTiC58) and three test strains on the Test side of the Petri dish.
9. Inject the test strains (5-10 L) into the Control side of the plate. Set these up to mirror the test samples on the Test side of the plate. This will determine if the test culture or supernatant contains an activity that will cause the hydrolysis of X-gal.
10. Incubate plates (agar side down) for 24-48 h at 28°C. A blue color in the agar indicates X-gal hydrolysis. Score as + or – and indicate the strength of the reaction (*i.e.*, the intensity of blue and diameter of the zone). Refrigerate plates until next lab for sharing your results with the class.

B. HSL production at various times in a batch culture

1. A culture of *A. tumefaciens* H2 was grown in AB glucose medium in the fermenter for 24 hours. Samples were removed at 0 h and every 4 h thereafter. The culture turbidity was measured (OD_{600}), and samples of the culture fluid were centrifuged for 5 min in an Eppendorf centrifuge at 12,000 rpm. The supernatant of each sample time were separated from the pellet of cells and frozen.
2. A culture of *A. tumefaciens* NTL4(pZLR4) was grown in AB medium overnight.

3. A 0.5-mL sample of supernatant culture of *A. tumefaciens* H2 was added to 0.5 mL of overnight *A. tumefaciens* NTL4 (pZLR4) culture and incubated for 24 h at 28° C. If HSL was present this should induce *LacZ* production.
4. Lyse cells from mixture (in 3 above) by adding 20 L Toluene to each tube. Vortex for 3 minutes in 20-second intervals.
5. Add 0.2 mL ONPG (4 mg/mL dissolved in Z buffer) to empty Eppendorf tubes, so you have enough tubes for each test samples, plus the control.
6. Remove 0.8 mL toluenized cell suspension from each of the sample tubes by avoiding the top layer containing undissolved toluene. Add 0.8 mL of the provided AB medium to the control tube. Add sample to ONPG and immediately start incubation at 28°C up to 30 min, checking for yellow color change after every 10 minutes.
7. Measure absorbance at 420 nm in the Novaspec II. Use the control tube to blank the spectrophotometer.
8. Record OD_{600} values provided on the board from Step #1, and your values obtained in Step #7. Plot the growth (turbidity) of the culture vs. time. Plot A_{420} values per time. Does ONPG hydrolysis correlate to the onset of stationary phase (max. cell density)?

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Appendix A

Here are some helpful references:

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Boos, W. (1982). Synthesis of (2R)-Glycerol-O- -D-Galactopyranoside by -Galactosidase. Methods in Enzymology **89**:59-64

Steers, and Cuetrecases (1974). -Galactosidase. Methods in Enzymology XXXIV:350-358.

Also, check out these Web sites:
http://www.life.uiuc.edu/micro/farrand.html
www.nottingham.ac.uk/quorum
http://info.bio.cmu.edu/Courses/03441/TermPapers/99TermPapers/Quorum/intro.html

Appendix B

Bacterial AB medium:

1) add 20 mL of 20X Phosphate stock to 900 mL sterile dH_2O; for plates add 1.5% Agar for base and 0.7% agar for overlay
2) add 20 mL of 20X Nitrogen salt stock to above
3) add 1 mL of Glucose stock to 1 L media
4) add 40 g/mL X-Gal
   - 20X Phosphate stock (Autoclave and store @ room temp.)
     - K_2HPO_4 60 g/L
     - NaH_2PO_4 20 g/L
   - 20X Nitrogen-salts stock (Autoclave and store @ room temp.)
     - NH_4Cl 20 g/L
     - $MgSO_4$ $7H_2O$ 6 g/L
     - KCl 3 g/L
     - $CaCl_2$ $2H_2O$ 0.2 g/L
     - $FeSO_4$ 7H2O 0.05 g/L
   - Glucose stock:
     - 0.2 – 0.5 % glucose (Autoclave separate and store @ room temp.)
   - X-gal should be dissolved in DMFO no more than 20mg/mL concentration; no need to sterilize; store in foil covered test tube @ 4ºC

Z buffer for 1000 mL

- Na_2HPO_4 $7H_2O$ 16.1 g
- NaH_2PO_4 H_2O 5.5 g
- KCl 0.75 g
- MgSO4 7H2O 0.246 g
- ß – Mercaptoethanol 2.7 mL
- Adjust pH to 7.0. Do not autoclave, store in refrigerator