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Bacterial Quorum Sensing and Bioluminescence

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Abstract: The bacterium *Vibrio fischeri* produces light when it is growing symbiotically in the light organ of certain fish and squid species. The bacteria sense they are in a light organ because they are present at a high bacterial cell density, which they detect through "quorum sensing." We use several bacterial strains, each of which carry only part of the genes responsible for the quorum sensing and bioluminescence process. We use chemical and genetic complementation to determine which genes are lacking in each strain by measuring their ability to produce and/or detect a quorum-sensing signal.

Keywords: quorum sensing, bioluminescence, complementation, plasmid, transformation, competent cells

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Contents:

Introduction	202
Student Outline	203
Materials	210
Notes for the Instructor	211
Acknowledgments	212
Literature Cited	213
About the Authors	213
Appendix: Culture, Media, and Solution Preparation	214

Introduction

Quorum sensing is a mechanism of intercellular communication active in many species of bacteria. It is used by the bacteria to measure the density of their own population within their environment and to regulate their gene expression and behavior accordingly. For instance, it is used by many pathogenic species such that when they first invade the host, and are at low density, they behave in ways that allow them to evade the immune system. If they achieve high density, and are apparently overwhelming the body's defenses, then they change their behavior and progress to a full-blown disease state. In *Vibrio fischeri*, quorum sensing controls bioluminescence, the ability of the bacteria to produce light, an exciting visual phenomenon for the student lab.

The mechanism of quorum sensing involves an autoinducer synthase, LuxI in *V. fischeri*, which makes the small autoinducer molecule. The autoinducer builds up in the medium and at high concentrations will bind to a transcription regulator, LuxR in *V. fischeri*, which will then alter the gene expression. We can work with this system in *E. coli*, which is easier to handle and more predictable in the lab. We use four *E. coli* strains that carry plasmids, extrachromosomal DNA elements, that carry various parts of the *V. fischeri lux* genes. None of these strains can luminesce because they are lacking one or more parts of the *lux* regulatory system. Two of the strains have the entire system except for one gene, either *luxI* or *luxR*. The other two strains contain only *luxI* or only *luxR*.

We will examine whether any of these strains can complement each other to produce luminescence. We will test what is sometimes called "chemical complementation" in which the strains grow in an intermixed population on a plate. In this situation, the cells cannot exchange macromolecules such as DNA or protein, but they can exchange small diffusible molecules such as the autoinducer. We will also carry out genetic complementation in which plasmid DNA will be purified from two strains and transformed into the other two strains in order to try and reconstruct the entire *lux* system in one strain.

Students will learn about intercellular signaling and gene regulation. They will see the association between the gene and protein product and understand how these must be produced within the cell in order to affect the phenotype. They will understand the difference between these genetically encoded macromolecules and enzymatically produced small molecules, which can more frequently cross membranes and thus affect the phenotype of neighboring cells.

This lab is currently taught to junior/senior level students, but could potentially be adapted to a sophomore-level class. The lab is taught over two 4-hour class periods two days apart with an additional follow-up two days later for the students to observe the luminescent colonies that arise on their plates. The lab does require some extensive setup for several days in advance and some microbiology technical knowledge is required for the preparation. One possibility for greatly shortening the lab is to simply do the cross-streaking exercise in order to observe the effect of diffusion of the autoinducer.

Student Outline

Quorum sensing and bioluminescence

The phenomenon of quorum sensing is a common regulatory mechanism used by a number of bacteria. During the process of quorum sensing, a bacterial species takes a population census and thereby induces specific cellular functions only at a high cell density. An intercellular signaling molecule, commonly termed the autoinducer, is produced and subsequently sensed by the bacterial cells. Autoinducers can be thought of as pheromones: chemicals produced by an individual that can be sensed, and interpreted as a specific piece of information, by other individuals within a population.

The quorum sensing response was observed in the luminescent marine bacterium *Vibrio fischeri* in the early 1970s and now serves as a model system for understanding quorum sensing in Gram-negative proteobacteria. It has been determined that two genes are essential for this type of regulatory scheme: *luxI*, which encodes an autoinducer synthase called LuxI; and *luxR*, which encodes an autoinducer-dependent activator of the luminescence genes called LuxR. The autoinducer molecule produced by LuxI is an acylated homoserine lactone (3-oxo-hexanoyl-homoserine lactone). *V. fischeri* cells are permeable to the autoinducer, therefore the compound accumulates within the cells and in the surrounding environment at equal concentrations. When the autoinducer reaches a critical threshold concentration, LuxR-autoinducer complexes begin to form and the genes responsible for cellular luminescence (the *lux* operon) are activated. Quorum sensing thus constitutes an environmental sensing mechanism that allows the bacteria to respond to changes in their population density.

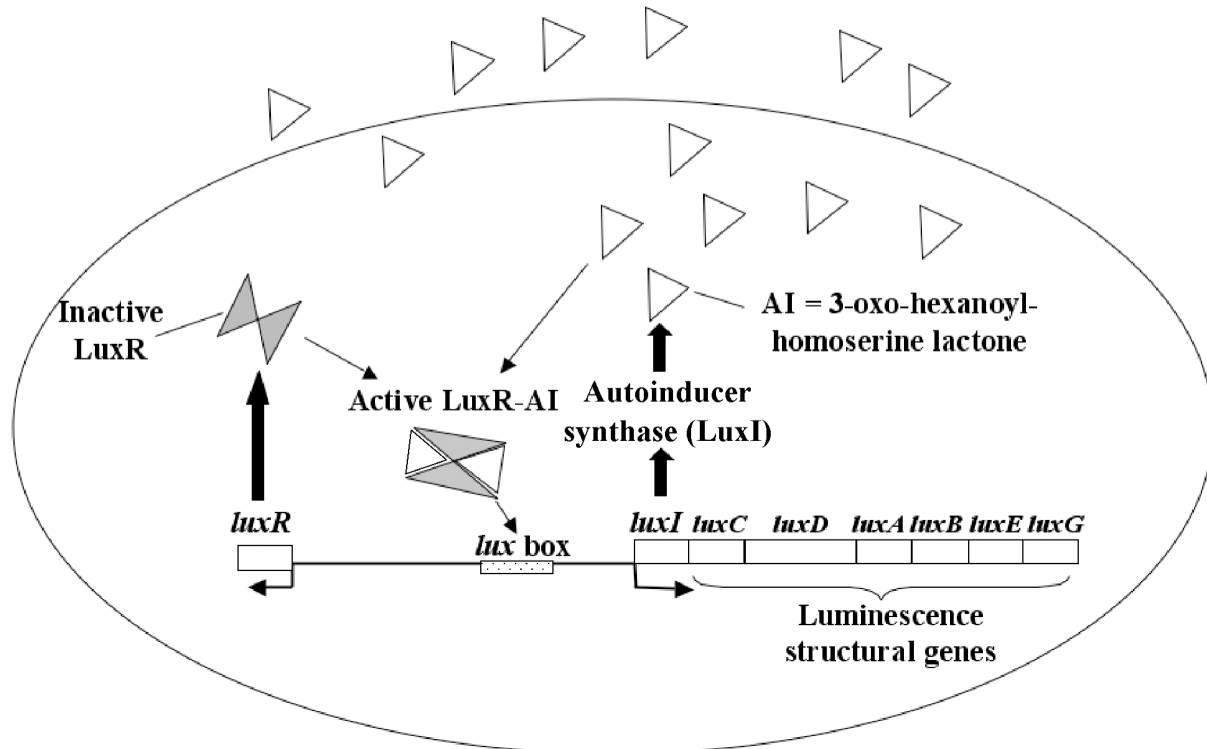


Figure 1. Model of quorum sensing in *Vibrio fischeri*. Autoinducer is produced by the product of the *luxI* gene and diffuses into and out of the cell. When the population grows to a high cell density, enough autoinducer is present to bind to and activate the product of the *luxR* gene. LuxR, in complex with autoinducer, binds to the *lux* box to activate expression of the *lux* genes, and light is produced.

In the low nutrient environment of seawater, *V. fischeri* is present at low densities; any autoinducer produced by the cells diffuses away rapidly, and cellular luminescence does not occur. However, certain species of marine animals, such as the Japanese pinecone fish (*Monocentris japonicus*) and the Hawaiian squid (*Euprymna scolopes*), have developed symbiotic relationships with *V. fischeri*. These animals contain light organs where they provide a nutrient rich environment for the bacteria to grow. When *V. fischeri* grows to a high cell density within the light organ, it induces expression of the *lux* genes and produces light. The host animal uses this light to attract prey or to avoid predation.

Molecular studies of the luminescence of *V. fischeri* has been possible by the cloning of a 9-kilobase DNA fragment that encodes all of the functions required for quorum sensing-regulated luminescence. These genes can function in the heterologous host *Escherichia coli* to produce light. The luminescence genes are arranged in two divergent transcriptional units. One unit contains *luxR*, and the other contains *luxI* and the structural genes necessary for light production (*luxCDABE*). In this study, two recombinant *E. coli* strains that contain the entire *lux* region will be provided, however, each has a null mutation in one gene that results in the loss of quorum sensing and light production. One strain carries a null mutation in *luxR* and the second strain has a null mutation in *luxI*. Plasmid DNA encoding either only *luxR* or only *luxI* will be purified from two additional strains and subsequently used to complement the genetic defect in the original mutant strains.

We will test whether these different strains, none of which can luminesce on their own, can provide each other with a component of the quorum sensing/bioluminescence system they require. If combining two strains together can result in bioluminescence, then this will indicate "complementation." We will test for chemical complementation, the ability of one strain to provide a chemical to another when they grow beside each other, and for genetic complementation, in which we will purify DNA from one strain and introduce it into the cells of another strain. We will use chemical transformation to introduce DNA into the cells.

Plasmid DNA purification

DNA is commonly purified from bacterial cells for at least three reasons. First, it is used for the transfer of genetic information from one strain to another, as we will do via chemical transformation of *E. coli* cells. Second, DNA fragments can be cloned into vectors for replication and transfer into other strains. Finally, the structure of the DNA can be analyzed by a variety of methods such as Southern blotting, polymerase chain reaction (PCR), or DNA sequencing. We will use transformation to put the DNA into a new cell background, allowing us to observe the phenotypic effects of the genetic information on the DNA.

The initial step in DNA purification must be cell breakage, releasing a mixture of DNA, RNA, proteins, membranes, and small molecules. Membranes are generally dissociated using detergents, rendering them into relatively low molecular weight components. We will first suspend our cells in a buffered solution (Solution I). We will then break the cells open and dissociate the membranes by addition of Solution II. This reagent is highly alkaline, which disrupts the bacterial cell wall, and contains a detergent (sodium dodecyl sulfate, SDS) to dissociate the membrane. Solution II also serves two other purposes. The detergent causes denaturation of much of the cell protein. When proteins are denatured their hydrophobic regions, normally folded into the internal recesses of globular proteins, are exposed and tend to aggregate. These large protein aggregates will eventually precipitate, and can be removed via centrifugation. The alkaline conditions also cause denaturation of the double-stranded DNA into single-stranded molecules. This would seem to be counter to the

goal of purifying the plasmid DNA, but it assists in the separation of the very small plasmid DNA molecules from the very large chromosomal DNA. When solution III is added, it neutralizes the pH. The large chromosomal DNA has been sheared during cell breakage and mixing, so the two complementary strands have drifted apart and cannot renature quickly upon neutralization. The small plasmid DNA, on the other hand, is still circular and wound around its complementary strand, so it immediately renatures upon neutralization. In addition, the potassium ions in solution III cause precipitation of the SDS, along with a large amount of denatured protein. The very large chromosomal DNA fragments are trapped within this precipitate, while the small plasmid DNA is not. The majority of the protein and the chromosomal DNA can then be removed by centrifugation. The small plasmid molecules remain in the supernatant. Additional protein is removed by extraction with the organic solvents phenol and chloroform. Again, these cause denaturation and precipitation of the proteins. RNA is more difficult to physically separate from DNA due to its similar structure, but it can be selectively degraded using ribonuclease (RNase) digestion. For our purposes, the RNA contamination is not important, so we ignore it to save time. A late step in nucleic acid purification is often precipitation in the presence of alcohol. A relatively high concentration of a salt (often sodium acetate) is added to neutralize the charge on the DNA and to allow it to aggregate during precipitation. An alcohol (isopropanol or ethanol) is added to cause the DNA to precipitate while most small molecules and membrane components stay in solution. Following precipitation, ethanol is removed from the DNA pellet by evaporation and the DNA is dissolved in water or a buffered solution.

Bacterial transformation

Artificial, or chemical, transformation is used to introduce DNA into *E. coli* and a few other related species. Electroporation is a related method that works with a much wider variety of species. The challenges to getting DNA into cells are neutralizing the negative charges on the DNA and the cells' membranes to allow these molecules to come into close proximity and disrupting the membrane structure in order to get the DNA across. In this exercise, we use a relatively simple procedure that is sufficient to get intact plasmid DNA into the cells. Much more elaborate procedures are generally used to obtain the more efficient transformation required for DNA that has been cut and ligated, in which only a small percentage of the resulting DNA molecules will be functional.

Treatment of the cells to render them "competent," able to take up DNA, involves removal of the growth medium by centrifugation, followed by resuspension of the cells in solutions containing the divalent cations Mg^{++} and/or Ca^{++} . The cations serve to neutralize the negative charges on the DNA and membranes, as well as disrupting the membrane structures, rendering them susceptible to transfer of DNA. The competent cells are then briefly heat shocked, which apparently disrupts the membrane further and actually allows the DNA to cross the membrane and enter the cell. These treatments render the cells fragile, so they must be treated very gently throughout the transformation procedure. Following the heat shock, the cells are incubated in rich medium in order to allow them to recover. This incubation also allows the cells to express an antibiotic resistance gene on the transforming DNA. The cells are then plated in the presence of the antibiotic in order to select for those that have successfully received and retained the transforming DNA.

Additional Reading

On bacterial luminescence and quorum sensing: Waters, C.M., and B.L. Bassler. 2005. Quorum Sensing: Cell-to-cell communication in bacteria. *Annual Review of Cell and Developmental Biology*, 21:319-346.

On DNA purification and bacterial transformation: Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (Editors). 2002. *Short Protocols in Molecular Biology*. Fifth Edition. Wiley Publishing, Hoboken, NJ, USA.

Goals

Day 1: Preparation of competent cells from two strains

Purification of plasmid DNA from two strains

Chemical complementation of mutations via cross-streaking

Day 2: Genetic complementation of mutations via transformation

Deduction of genotypes of strains and plasmids used in the study

Four recombinant *E. coli* strains will be provided to you but you will not be given their identities. From your results you will be asked to deduce which strain is which.

These strains contain the following plasmids carrying the indicated *V. fischeri lux* genes:

pHV200I *luxR* and *luxCDABE*, ampicillin resistance. This plasmid is in either strain A or B.
This plasmid is LuxI⁺ and LuxR⁺ (Pearson et al., 1994)

pAMS555 *luxICDABE*, ampicillin resistance. This plasmid is in either strain A or B.
This plasmid is LuxI⁺ and LuxR⁻

pBLH305 *luxI*, chloramphenicol resistance. This plasmid is in either strain C or D.
This plasmid is LuxI⁺ (Hanzelka et al., 1997)

pAMS121 *luxR*, chloramphenicol resistance. This plasmid is in either strain C or D.
This plasmid is LuxR⁺ (Stevens et al., 1999)

Procedure

Day 1: Preparation of competent cells for transformation:

1. Inoculate 5 ml of medium A and B with 100 μ l of overnight cultures A and B, respectively.
2. Grow the cultures with shaking or rolling at 37°C for approximately two hours. During this time, the optical density of the culture should become easily visible. While you are waiting, do the plasmid purification and cross-streaking procedures listed below.
3. After the cells have been growing for two hours, chill the cultures on ice. Once the cells are chilled make sure they remain cold through the entire protocol!
4. Harvest the cells by centrifuging at 4°C for 5 min at 2500 x g in 15 ml sterile polypropylene tubes.

5. Resuspend the cells in 1 ml cold 0.1 M MgCl₂ and transfer to a sterile microcentrifuge tube.
6. Centrifuge the cells for 5 min at 2500 x g in a refrigerated microcentrifuge.
7. Resuspend the cells in 1 ml cold CaCl₂.
8. Incubate on ice at least 20 min (longer is OK)
9. Centrifuge the cells for 5 min at 2500 x g in the refrigerated microcentrifuge.
10. Resuspend the cells in 212 µl 0.1 M CaCl₂ and 38 µl glycerol (Mix together in advance of cooling the solution and adding the cells!)
11. Freeze the cell suspension until Day 2.

Purification of plasmid DNA:

1. Harvest 1.5 ml of cultures C and D by centrifugation in a microcentrifuge tube at room temperature (30 seconds at top speed). Remove and discard the supernatants.
2. Resuspend each cell pellet in 100 µl Solution I, mix by vortexing until the entire pellet is fully resuspended.
3. Add 200 µl Solution II to each tube and mix by inversion 10 times.
4. Incubate the tubes on ice for 5 min.
5. Add 150 µl Solution III to each tube and mix by inversion 10 times.
6. Incubate the tubes on ice for 5 min.
7. Centrifuge the tubes in the microcentrifuge (5 min, 13,000 rpm).
8. Remove the clear supernatants to clean microcentrifuge tubes being careful not to transfer any white precipitate.
9. (Wearing latex gloves and working in the hood!) Add 400 µl phenol/chloroform/isoamyl alcohol solution and vortex for one full minute.
10. Centrifuge the tubes in the microcentrifuge (3 min, 13,000 rpm).
11. Remove the aqueous (top) phase to a clean microcentrifuge tube, put the phenol solution in the appropriate waste container in the hood.
12. Add 800 µl ethanol to each tube, mix by inversion 10 times, and incubate on ice for 10 minutes.
13. Centrifuge the tubes in the microcentrifuge (10 min, 13,000 rpm).
14. Pour off and discard the supernatant. Air dry the pellet until no ethanol droplets are visible.
15. Resuspend the pellets in 50 µl sterile water and store in the freezer until the next class period.

Cross-streaking of strains:

1. Read these instructions all the way through and carefully label the plates before you begin. Write on the part of the plate that contains the agar, and draw the lines and letters indicated in Figure 2 below. Also label with your group number and the date. *Do the streaking in exactly the order described in the next 2 steps!*
2. Using a loopful of the overnight culture of strain A, streak a single line clear across the middle of a plate of LB agar. Repeat on a second plate with strain B. Set the plate aside for at least 10 minutes until the streaks appear dry.
3. Using a loopful of the overnight culture of strain C, streak a single line clear down the left sides of the plate just inoculated with culture A being careful to cross the line previously made with strain A. STERILIZE YOUR LOOP AGAIN, then repeat using strain C to streak across the line previously made with strain B on the other plate. Repeat with strain D on the right sides of the plates.
4. Incubate the plates at 30°C (Don't grow the cultures at 37°C, the *lux* gene products won't function at this temperature!).
5. 48 hours later take your plates into a dark room and look for luminescence in different regions of each plate. You will have to wait for several minutes in the dark for your eyes to adjust before you will see the luminescence. Draw a picture showing the regions of growth and regions of luminescence in each plate.

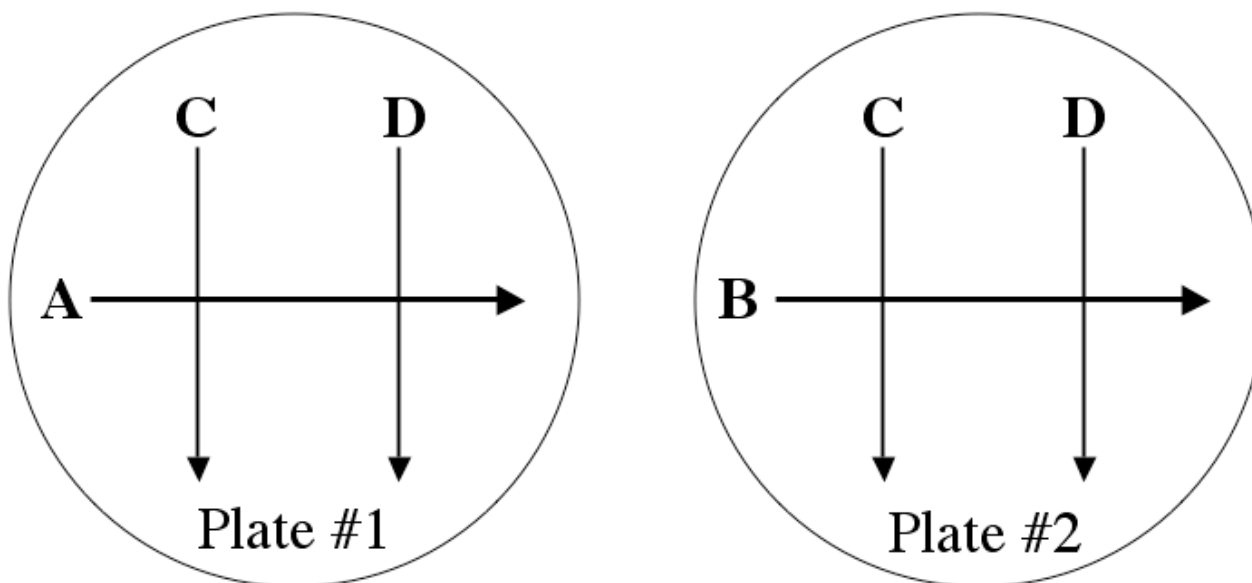


Figure 2. Streaking plates to test for chemical complementation of bioluminescence. Letters refer to the strain to be streaked as shown by the arrow. Streak strains A and B first and allow the streaks to dry. Then streak strains C and D in the direction shown.

****Dispose of all liquid cultures and plates in the provided receptacles.****

Day 2: Transformation of plasmid DNA:

1. Thaw the tubes of frozen competent cells and place on ice as soon as they are liquid.
2. Set up two sets of three sterile microcentrifuge tubes. In each set of three:
 - add 25 μ l of 0.1 M CaCl_2 and 5 μ l plasmid DNA from strain C to the first tube,
 - add 25 μ l of 0.1 M CaCl_2 and 5 μ l plasmid DNA from strain D to the second tube,
 - add just 25 μ l of 0.1 M CaCl_2 to the third tube;
 - place all the tubes on ice.
3. Add 50 μ l of the competent cell suspension from strain A to one set of three tubes, repeat with the second set using 50 μ l of the competent cell suspension from strain B
4. Keep the tubes on ice for 5 minutes.
5. Heat shock the cells by putting the six tubes in the 37°C water bath for 2 minutes.
6. Return all tubes to ice for 5 minutes.
7. Add each transformation mixture separately to a culture tube containing 1 ml LB broth.
8. Incubate the tubes with gentle shaking at 37°C for 45 minutes.
9. Spread plate 100 μ l from each transformation on LB agar containing ampicillin and chloramphenicol, incubate at 30°C.
10. 48 hours later observe the plates for colony development and bioluminescence and record your results.

Dispose of all liquid cultures and plates in the provided receptacles.

Materials

Equipment for each group

- 100-1000 μ l pipettor and one box blue tips
- 20-100 μ l pipettor and one box yellow tips
- Vortex mixer
- Bunsen burner
- Approximately 20 sterile 1.5-ml plastic microcentrifuge tubes
- Two microcentrifuge tube racks
- Ice bucket and crushed ice
- Pipeting bulb or hand pump
- Inoculating loop

Equipment for the classroom

- 37°C incubated shaker or culture tube roller
- Refrigerated microcentrifuge
- Microcentrifuge (room temperature)
- -20°C or -70°C freezer
- 30°C incubator
- 37°C water bath

Solutions, media, and cultures (numbers needed are per group unless otherwise noted)

Day 1

One 5 ml overnight culture of EACH *E. coli* strain grown in LB medium: Strains A, B, C, and D

Four culture tubes, each containing 5 ml sterile LB medium: Two labeled "A" and two labeled "B"

Two plates of LB agar

Tube containing 5 ml sterile 0.1 M $MgCl_2$

Tube containing 5 ml sterile 0.1 M $CaCl_2$

Tube containing 0.2 ml sterile 100% glycerol

7 sterile pipettes

Two 15 ml sterile polypropylene centrifuge tubes

Tube containing 1 ml Solution I (50 mM glucose, 25 mM Tris HCl pH 8.0, 10 mM EDTA)

Tube containing 1 ml Solution II (0.2N NaOH:20% SDS; 19:1 ratio)(Prepared same or previous day)

Tube containing 1 ml Solution III (60 ml 5 M potassium acetate, 11.5 ml acetic acid, 28.5 ml H_2O)

Tube containing 1 ml Phenol:Chloroform:Isoamyl alcohol (25:24:1) pH ~7.0 (This solution can be purchased from a number of vendors, including Fisher Scientific (BP1752I-100).)

Tube containing 2 ml 95% or 100% ethanol

Tube containing 1 ml sterile distilled water

Day 2

Tube containing 1 ml sterile 0.1 M $CaCl_2$

6 culture tubes of sterile LB medium, 1 ml each

6 plates of LB agar containing Ampicillin (100 μ g/ml) and Chloramphenicol (20 μ g/ml)

Notes for the Instructor

Safety

Biosafety: The *E. coli* strains used are lab strains and are entirely non-pathogenic. However, normal care should be taken to avoid ingestions (No mouth pipetting, and wash hands before leaving lab). Liquid cultures should be autoclaved or treated with bleach (dilute household bleach 100-fold into the culture) prior to disposal down the drain. Plates should be autoclaved prior to disposal in standard trash or should be placed in Biohazard waste.

Phenol:Chloroform:Isoamyl alcohol

Phenol can cause minor chemical burns, so students should wear gloves while using it.

The solution is somewhat volatile. It should be used in a fume hood if available, but not absolutely necessary. Keep exposure to a minimum.

Dispose according to your institutions chemical safety plans. This generally means incineration. Small amounts (<1 ml) in sealed plastic tubes can often be disposed in Biohazardous waste that will be incinerated.

Problem Points

This exercise generally works quite well. Pay particular attention to the following:

- 1) During the DNA preparation, the cells must be FULLY resuspended by vortexing prior to adding the Solution II.
- 2) During the streaking for chemical complementation stress that the students follow the instructions precisely. They must flame their streaking loops to sterilize them in between strains. They must let strains A and B dry on the plates before streaking strains C and D. They should not try to put too much liquid on the plates and should not move the plates until they are fully dry in order to avoid drips running across the plate.
- 3) Plates must NOT be incubated at 37°C when you want to observe luminescence. They should be incubated at 30°C. Luminescence will be maximal on these plates when the colonies are relatively large, generally after 36 to 48 hours at 30°C. After 48 hours the luminescence will fade.
- 4) In order to observe the luminescence, you need a completely dark room. You must then wait in the dark for a couple minutes for your eyes to adjust. The luminescence should appear to be light blue/green.

Expected Results

We test what is sometimes called “chemical complementation” in which the strains grow in an intermixed population on a plate. In this situation, the cells can NOT exchange macromolecules such as DNA or protein, but they can exchange small diffusible molecules such as the autoinducer. So, the strain containing all the lux genes except *luxI*, which encodes the autoinducer synthase, can receive autoinducer from the strain that carries only *luxI*, and it can then luminesce. However, the strain lacking *luxR* cannot be complemented in this way because it needs LuxR, which can not move from one cell to another. (Figure 3)

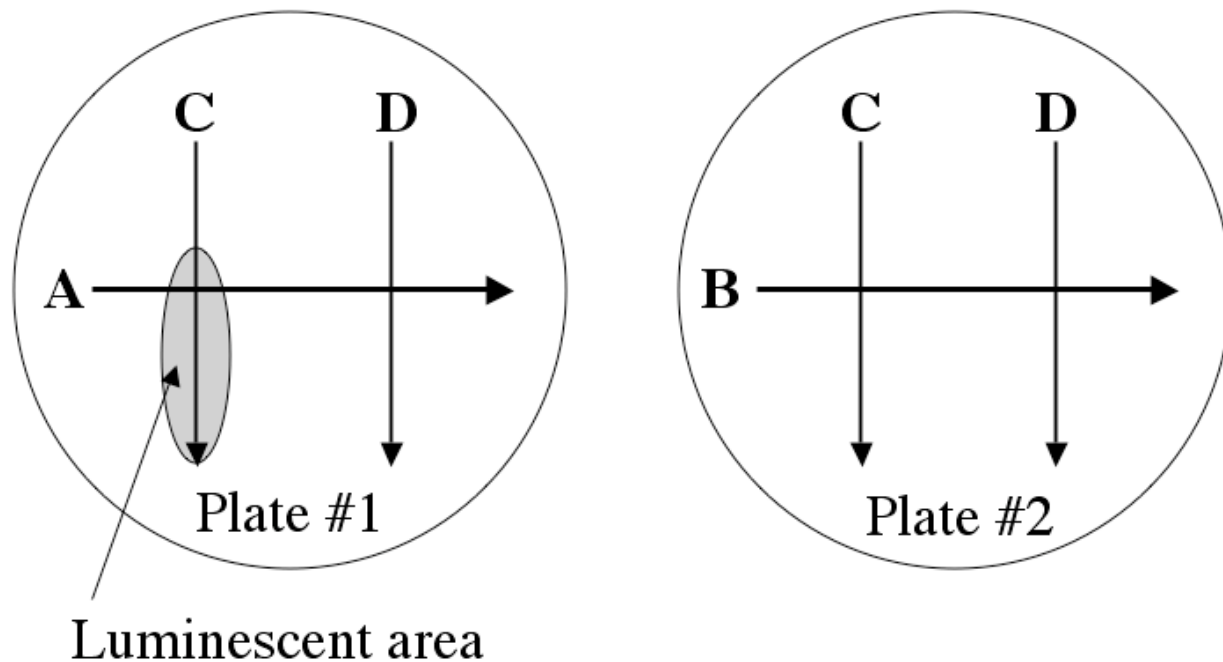


Figure 3. Streak plates to test for chemical complementation of bioluminescence. Strain C ($LuxI^+$) provides autoinducer to strain A ($LuxI^-$, $LuxR^+$) to produce bioluminescence.

We will also carry out genetic complementation in which plasmid DNA will be purified from two strains and transformed into the other two strains in order to try and reconstruct the entire *lux* system in one strain. In this case, we can get genetic complementation when the *luxR* plasmid is put into the strain lacking this gene and when the *luxI* plasmid is put into the strain lacking this gene. Colonies should arise on all four transformation plates, but only the A+C and B+D colonies should be luminescent.

Acknowledgments

We thank the members of the Virginia Tech Department of Biological Sciences microbiology prep room staff: Laura Link, Nicole Ganzala, Kathy Pennington, and Sharon Sible; for assisting in refining this exercise over the past several years. We thank Lediya Cheru, Guy E. Townsend II, and Nan Qin for constructing plasmids and strains.

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About the Authors

David Popham received a B.A. in Biology from Washington University in St. Louis and a Ph.D. in Microbiology from the University of California-Davis. He is an Associate Professor of Microbiology at Virginia Tech. David teaches Microbial Genetics in the fall and a Microbial Genetics and Physiology Lab in the spring. His research lab works on the structure and resistance properties of bacterial endospores, especially on the roles played by a specialized cell wall structure.

Ann Stevens received a B.S. in Microbiology from Iowa State University and M.S. and Ph.D. degrees in Microbiology from the University of Illinois. She is an Associate Professor of Microbiology at Virginia Tech. Ann teaches General Microbiology in the fall and Microbial Physiology in the spring. Her research lab works on environmental sensing and control of gene expression in bacteria. The luminescent marine bacterium *Vibrio fischeri* is used as a model for the quorum sensing mechanism of cell-density dependent gene regulation found in a variety of Gram-negative proteobacteria.

Appendix: Culture, Media, and Solution Preparation

Bacterial cultures as well as a PowerPoint presentation introducing the material can be obtained from David Popham (dpopham@vt.edu).

LB medium (Luria Bertani medium)

Tryptone	10 g
Yeast Extract	5 g
NaCl	10 g
H ₂ O	1 liter

To prepare plates, add 15 g of agar. One liter makes approximately 40 plates.

Autoclave for 30 minutes, cool to 55°C, add antibiotics if required, pour plates.

Ampicillin stock solution is 25 mg/ml in water and filter sterilized.

Store in freezer. Add 4 ml per liter of medium to obtain 100 µg/ml.

Chloramphenicol stock solution is 10 mg/ml in methanol and filter sterilized.

Store in freezer. Add 2 ml per liter of medium to obtain 20 µg/ml.

These antibiotics can be obtained from many sources.

Four days prior to day 1

Prepare the following sterile items (The amounts listed are for 6 groups):

2 bottles of 200 ml sterile LB liquid medium

16 plates of LB agar (On 2 of these, spread 100 µl of 25 mg/ml Ampicillin and on 2 of these spread 50 µl of 10 mg/ml Chloramphenicol, store all at 4°C for a day before streaking)

30 sterile 1.5 ml plastic microcentrifuge tubes

42 sterile culture tubes that can hold 5 ml of medium

36 sterile culture tubes that can hold 2 ml of medium

36 plates of LB agar containing Ampicillin (100 µg/ml) and Chloramphenicol (20 µg/ml)

Three days prior to day 1

Get out four *E. coli* strains from freezer stocks.

Strain A = pHV2000I⁻

Strain B = pAMS555

Strain C = pBLH305

Strain D = pAMS121

Streak strains A and B on LB+Ampicillin

Streak strains C and D on LB+Chloramphenicol

Incubate at 37°C overnight.

Two days prior to day 1

Verify growth of strains, store plates at 4°C.

The afternoon prior to day 1

Aliquot medium to tubes and inoculate as follows (The amounts listed are for 6 groups):

36 tubes of 1 ml LB per group (Do not inoculate, to be used on day 2) Store at room temp.

Use 18 ml out of each 200-ml bottle.

To one 200-ml bottle of LB add 725 µl of 25 mg/ml Ampicillin

Fill 30 tubes with 5 ml of this LB + Ampicillin 100 µg/ml
Inoculate 2 with strain A (pHV2000I-) = Starter cultures, shake at 37°C.
Inoculate 2 with strain B (pAMS555) = Starter cultures, shake at 37°C.
Label 12 tubes "A" and do not inoculate, store at 4°C.
Label 12 tubes "B" and do not inoculate, store at 4°C.
To one 200-ml bottle of LB add 380 µl of 10 mg/ml Chloramphenicol
Fill 12 tubes with 5 ml of this LB + Chloramphenicol 20 µg/ml
Inoculate 6 with strain C (pBLH305), shake at 37°C.
Inoculate 6 with strain D (pAMS121), shake at 37°C.

The morning of day 1

Subculture 50 µl strain A starter into 6 "A" tubes and 50 µl strain B starter into 6 "B" tubes. Shake at 37°C

Aliquot:

6 microcentrifuge tubes containing 1 ml Solution I
Prepare 1 M Tris HCl pH 8.0 and 0.5 M EDTA pH 8.0 ahead of time.
On day of use, mix together 4.5 ml H₂O, 0.25 ml 1 M Tris HCl pH 8.0, and 300 µl of 20% SDS
50 mM glucose, 25 mM Tris HCl pH 8.0, 10 mM EDTA
6 microcentrifuge tubes containing 1 ml Solution II (0.2 N NaOH, 1% SDS)
Prepare 1 N NaOH and 20% SDS (sodium dodecyl sulfate) ahead of time.
On day of use, mix together 4.5 ml H₂O, 1.2 ml 1 N NaOH, and 300 µl of 20% SDS
6 microcentrifuge tubes containing 1 ml Solution III (5 M potassium acetate solution)
Mix together 6 ml 5 M potassium acetate, 1.15 ml acetic acid, and 2.9 ml H₂O
6 microcentrifuge tubes containing 1 ml sterile high purity water.
Store at room temperature until use.