Experiment 10	**Bacterial genetic exchange : Conjugation of *ina (ice nucleation active)* gene from *E. coli* into luminous *V. harveyi***
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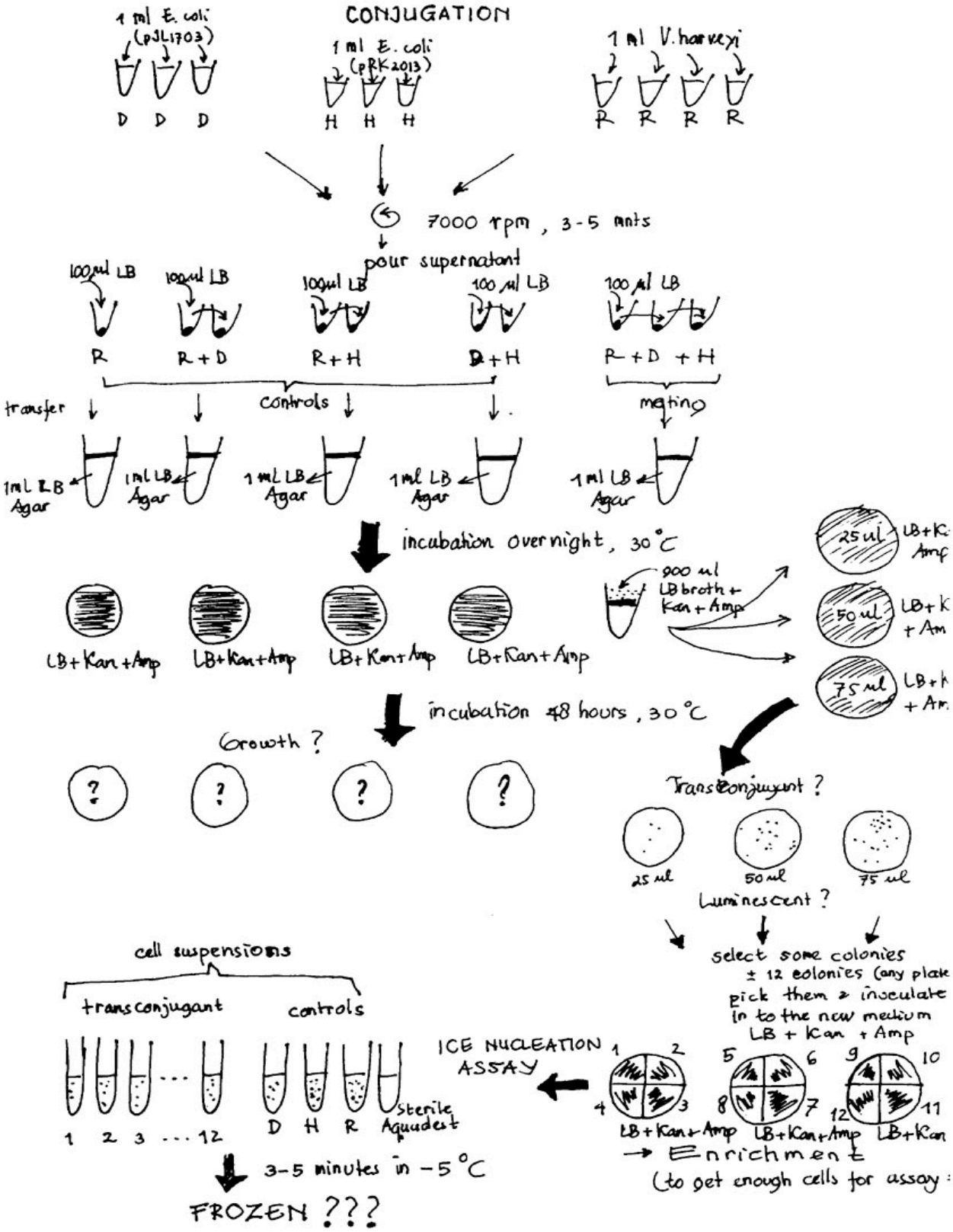
Textbook Chapters	BBOM 9th edition (1999): Chapters 9.1, 9.5, and 9.9. BBOM 10th edition (2003): Chapters 10.1, 10.5, and 10.9 BBOM: Madigan M.T., J.M. Martinko and J. Parker: **"Brock: Biology of Microorganisms"**, Prentice Hall.
Objectives	To understand a conjugation experiment (triparental mating) To learn about *ina (ice nucleation active)* gene expresssion in recipient bacteria by performing the ice nucleation assay.
Background	Several Gram-negative bacteria, including *Pseudomonas syringae, Pseudomonas fluorescens, Erwinia herbicola, Erwinia ananas,* and *Xanthomonas campestris* pv. *translucens* are able to catalyze ice formation at temperatures of -2 to -12 °C. These microorganisms efficiently catalyze ice formation at temperatures much higher than most organic and inorganic substances. They are responsible for initiating ice formation, which results in frost injury, because of their distribution on the surfaces of frost-sensitive plants. The high temperature of ice catalysis conferred by bacterial ice nuclei makes them useful in ice-nucleation-limited processes such as artificial snow production, the freezing of some food products, and possibly in future weather modification schemes (Gurian-Sherman and Lindow, 1993). The *ina* (or *ice*) gene encodes INA proteins. These proteins are arranged on the surface of the outer membrane of the bacteria and become nuclei for water crystallization. The *ina* gene can be transferred by conjugation and is expressed in other Gram-negative bacteria. The donor used in this experiment is *Escherichia coli* (pJL1703) carrying a plasmic encoding the *ice nucleation active* gene and an antibiotic (*Kanamycin*) resistance gene as molecular marker. The plasmid is mobilizable but not self-transferable because it does not contain a *tra* (*transfer*) operon. In this case, helper strain *E. coli* (pRK2013) is required, which provides the *tra* gene and the kanamicin resistance gene on its plasmid. A Gram-negative, *ina* deficient, luminous *Vibrio harveyi* strain that has been isolated from the shrimp (*Penaeus monodon*) will be used as the recipient. *Vibrio harveyi* is resistant to ampicillin, but sensitive to *Kanamycin.*
Literature	Gurian-Sherman Douglas and Lindow Steven E. Bacterial Ice Nucleation: Significance and Molecular Basis. FASEB (Federation of American Societes for Experimental Biology) Journal 7(14): 1993. 1338-1343
www. Links	http://www.hhmi.org/biointeractive/animations/conjugation/conj_frames.htm http://www.molgen.mpg.de/~ag_lanka/bacterial_conjugation.html http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/A/Avery.html

Practical Work	Each student group will get 3 tubes containing: 2 strains of *E. coli* and 1 strain of *V. harveyi* which have genotypes as follows: a. *E. coli* (pJL 1703) : *tra* $^{-}$, *ina* $^{+}$, Kan ^{r}, Amp ^{s}, *lux*$^{-}$ (donor) b. *E. coli* (pRK 2013) : *tra* $^{+}$, *ina*$^{-}$, Kan ^{r}, Amp ^{s}, *lux*$^{-}$ (helper) c. *V. harveyi* : *tra* $^{-}$, *ina* $^{-}$, Kan ^{s}, Amp ^{r}, *lux*$^{+}$ (recipient) The students will perform a triparental mating experiment using standard microbiological methods, e.g. preparing different combinations of mixtures of recipient, donor and helper strains. The strains will be brought together in a tube containing 1 ml LB, where the conjugation will take place. After incubation for 24-h at 30°C, plating of the mixture will be performed onto nutrient plates containing the appropriate medium (LB+Amp+Kan). Screening for the transconjugant will be done after incubating the plates during 48-h at 30°C using selective media. After observation of the luminescence in the dark, we will inoculate several luminous single colonies onto a new petri dish containing LB+Amp+Kan. This step is needed for getting enough cells for the ice nucleation assay. The plates will be incubated over night at 30°C. Ice nucleation assay (tube-assay). Before the assay, the culture should be adapted to a low temperature, by keeping it in the refrigerator for at least 1 hour. The assay will be performed by incubating the tubes containing cell suspensions in 10 ml sterilized PBS in the cooling bath (-5°C) for about 5 minutes. Details see protocols.
Materials and Experimental Protocols	Material and equipment needed: 1. Assistant will supply 24-h cultures of the following strains: *E. coli* (pJL 1703): **donor**, *E. coli* (pRK 2013): **helper**, *V. harveyi*: **recipient**. 2. Media: 5 Eppendorf tubes containing 1 ml LB agar, 2 Eppendorf tubes containing 1 ml LB broth, and 12 petri dishes containing agar solidified medium: LB+Kan+Amp(Kan: 50 ug/ml, Amp: 100 ug/ml). **LB (Luria Bertani Medium:** bacto tryptone: 10 g/l, yeast extract: 5 g/l, NaCl: 5 g/l, pH: 7.2). 2. Equipment: 1 pack sterile inoculation loops 10 ul, micro pipettes, yellow tips in tips rack (sterilized), blue tips in tips rack (sterilized), Eppendorf tubes (sterilized in jars), 3. 1 Eppendorf tube rack, 1 forcep (to handle Eppendorfs), tissue paper. 4. 16 test tubes containing sterilized Phosphate Buffered Saline (PBS): 130 mM sodium chloride, 10 mM sodium phosphate (pH 7.0). 5. Water resistant pen (permanent). 6. Waste container. **Common work station**: Centrifuge for Eppendorf tubes, vortex mixer, cooling water bath (to -10^{o} C, for ice nucleation assay), refrigerator, autoclave, 30^{o} C incubator. **Procedures: See also Appendix** **-Period 1 (mating experiment):** For some of the steps you will use cultures which were prepared by a student group the day before. Some of your cultures will be used by the group on the next day. **Please work thoughtfully!.**

	Take 4 sterile-Eppendorf tubes, mark them with R (for recipient), 3 sterile tubes for H (helper), and 3 sterile tubes for D (donor). Fill into each of them 1.0 ml of the appropriate over night cultures. Centrifuge at 7000 rpm for 3 minutes to collect the cells, then pour off the supernatant. Take 5 Eppendorf tubes containing 1 ml LB Agar medium, mark them as: R, R+D, R+H, D+H (as controls) and R+D+H, respectively. Divide all the pellet-containing tubes into 5 groups refering to those labels. Resuspend the pelleted cells with 100 ul sterilized LB Broth (**Remember**: **fill only one tube for each group**, do not fill every tube with liquid medium), resuspend all of the pelleted cells well. Transfer suspension into tube 2, mix well, and transfer entire suspension into tube 3, mix well. Transfer entire suspension into Eppendorf tubes containing 1ml LB agar, where conjugation will now take place. Proceed accordingly with control samples. Incubate them over night at 30° C. **Period 2 (selection of the transconjugant):** Take the tubes from the incubator. Aseptically, dilute the cells of the R+D+H treatment with 0.9 ml LB containing Kan+Amp. With 100 ul micro pipette, transfer 25 ul, 50 ul, and 100 ul respectively, onto a new petri dishes containing LB+Kan+Amp. Use a new sterile loop for each plate. Spread the suspensions on the surface of the agar by skating the flat surface of the loop back and forth across the plate surface. Controls are plated undiluted onto LB +Kan+Amp plates, as well. Incubate the plates for 48 hours at 30° C. **Period 3 (enrichment of the culture).** Examine for growth of luminescent bacteria on LB+Kan+Amp and record your results. Choose 10 single colonies, aseptically pick and streak them individually onto a quadrant of a new petri dish containing LB+Kan+Amp. Incubate plates over night 30° C. **Period 4 (ice nucleation assay):** Before assaying for ice nucleation, put the incubated plates into the refrigerator (adaptation at 4° C). Scrape 2 loops full from the plate, resuspend the cells into a test tube containing 10 ml of sterile PBS. Do the same with the controls (donor *E. coli* (pJL1703) and recipient *V. harveyi* cells and **sterile PBS**). Put the test tubes into the circulating cooling bath (at -5° C) for about 3 to 5 minutes. Record your results.
Laboratory Rules & Precautions	All of the strains have antibiotic resistance genes that could be transferred unintentionally to other bacteria. Do not release your assays and cultures to the environment without sterilizing them first.
Goals & Experiences gained	Familiarity with a genetic transfer system employing cell-cell contact Understand the *ina* (or *ice*) gene expression in luminous *V. harveyi*.
Timing	90 minutes.
Reporting	keep proper notes on experimental steps and special observations in your lab book and use them for your report.

Questions to be answered	What is the antibiotic resistance of the donor strain in this practical exercise? What is the antibiotic resistance of the helper and recipient strains? What is the role of the helper strain? What are narrow-host-range plasmids and a broad-host-range plasmids? Is the donor strain still capable to nucleate ice formation after it has transferred its plasmid to the recipient? What is the antibiotic resistance of the recipient strain? If a donor bacterium successfully transfers its F plasmid containing its antibiotic resistance gene to the recipient, what should the antibiotic resistance of the recipient become? What are the phenotyphic characteristics of the transconjugant cells?

Appendix:


CONJUGATION
1 ml E. coli (pJL1703)
D D D
1 ml E. coli (pRK2013)
H H H
1 ml V. harveyi
R R R R
7000 rpm, 3-5 mnts
pour supernatant
100µl LB
R
R + D
R + H
D + H
R + D + H
transfer
controls
mating
1 ml LB Agar
incubation overnight, 30 °C
LB+Kan+Amp
900 µl LB broth + Kan + Amp
25 µl
50 µl
75 µl
incubation 48 hours, 30 °C
Growth ?
Transconjugant ?
Luminescent ?
select some colonies
± 12 colonies (any plate)
pick them & inoculate in to the new medium
LB + Kan + Amp
cell suspensions
transconjugant
controls
D H R Sterile Aquadest
1 2 3 ... 12
ICE NUCLEATION ASSAY
LB+Kan+Amp
LB+Kan
→ Enrichment
(to get enough cells for assay)
3-5 minutes in −5 °C
FROZEN ???