

Experiment	Bacterial genetic exchange : Conjugation of <i>ina</i> (ice nucleation active) gene from <i>E. coli</i> into luminous <i>V. harveyi</i>
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Reading	Chapters 9.1, 9.5, 9.9 in BBOM 9 th Madigan M.T., J.M. Martinko and J. Parker: "Brock - Biology of Microorganisms", 9th Edition, (BBOM, International Edition), Prentice Hall, 1999. ISBN: 0-13-085264-3.
Objectives	To understand a conjugation experiment (triparental mating) To learn about <i>ina</i> (ice nucleation active) gene expression in recipient bacteria by performing an ice nucleation assay
Background	<p>Several Gram-negative bacteria, including many pathovars of <i>Pseudomonas syringae</i>, <i>Pseudomonas fluorescens</i>, <i>Erwinia herbicola</i>, <i>Erwinia ananas</i>, and <i>Xanthomonas campestris</i> pv. <i>translucens</i>, 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. On plants, they are responsible for initiating ice formation which results in frost injury. On the other hand, the high temperature of ice catalysis conferred by bacterial ice nuclei makes them useful in ice-nucleation-limited processes such as making artificial snow, freezing certain food products, and possibly in future weather modification schemes (Gurian-Sherman and Lindow, 1993).</p> <p>The <i>ina</i> (or <i>ice</i>) gene encodes INA proteins. These proteins are arranged on the surface of the outer membrane of the bacterium and become nuclei for water crystallization. The <i>ina</i> gene can be transferred by conjugation and is expressed in other Gram-negative bacteria.</p> <p>The donor used in this experiment is <i>Escherichia coli</i> carrying a plasmid (pJL1703) encoding the ice nucleation active gene and a kanamycin resistance gene as the molecular marker. The plasmid is mobilizable. It is not self-transferable, however, because it lacks a <i>tra</i> (transfer) operon. In this case, helper strain <i>E. coli</i> (pRK2013) is required, which provides the <i>tra</i> gene and the kanamycin resistance gene on its plasmid. A Gram-negative, <i>ina</i> deficient, luminous <i>Vibrio harveyi</i> strain that has been isolated from shrimp (<i>Penaeus monodon</i>) will be used as the recipient. <i>Vibrio harveyi</i> is resistant to ampicillin, but sensitive to kanamycin.</p>
Literature	Gurian-Sherman D. and Lindow S. E. Bacterial Ice Nucleation: Significance and Molecular Basis. FASEB (Federation of American Society for Experimental Biology) Journal 7(14): 1993. 1338-1343
www. Links	http://www.molgen.mpg.de/~ag_lanka/Bacterial_Conjugation.html http://www.ultranet.com/~jkimball/BiologyPages/A/Avery.html http://www.mun.ca/biochem/courses/4103/topics/conjugation.html http://www.wsu.edu:8080/~hurlbert/pages/Chap9.html#Conjugation
Practical Work	<p>Each student group will get 3 tubes containing: 2 strains of <i>E. coli</i> and 1 strain of <i>V. harveyi</i> which have the following genotypes:</p> <ol style="list-style-type: none"> <i>E. coli</i> (pJL 1703) : <i>tra</i>⁻, <i>ina</i>⁺, Kan^r, Amp^s, <i>lux</i>⁻ (donor) <i>E. coli</i> (pRK 2013) : <i>tra</i>⁺, <i>ina</i>⁻, Kan^r, Amp^s, <i>lux</i>⁻ (helper) <i>V. harveyi</i> : <i>tra</i>⁻, <i>ina</i>⁻, Kan^s, Amp^r, <i>lux</i>⁺ (recipient) <p>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 medium, in which the conjugation will take place.</p>

	<p>After incubation for 24h at 30°C, plating of the mixture will be performed on agar plates containing the appropriate medium (LB+Amp+Kan).</p> <p>Screening for transconjugants will be done after incubating the plates for 48h at 30°C using selective media and selecting colonies that luminesce in the dark. We will inoculate several luminous single colonies onto a new petri dish containing LB+Amp+Kan in order to get enough cells for the ice nucleation assay. The plates will be incubated overnight at 30°C.</p> <p>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.</p> <p>For details see protocols.</p>
<p>Materials and Experimental Protocols</p>	<p>Material and equipment needed:</p> <ol style="list-style-type: none"> 1. Assistant will supply 24h culture of the strains as follows: <i>E. coli</i> (pJL 1703) : donor; <i>E. coli</i> (pRK 2013) : helper; <i>V. harveyi</i> : recipient. 2. Media: 5 Eppendorf tubes containing 1 ml LB agar, 2 Eppendorf tubes containing 1 ml LB broth, and 12 petri dishes containing solidified agar medium (LB+Kan+Amp: Kanamycin 50 µg/ml, Ampicillin 100 µg/ml). LB (Luria Bertani Medium): bacto tryptone 10 g/l, yeast extract 5 g/l, NaCl 5 g/l, pH 7.2, 15 g/l agar if required. 3. Material and equipment: 1 pack sterile inoculation loops 10 µl, micro pipettes, yellow tips in racks (sterile), blue tips in rack (sterile), Eppendorf tubes (sterilized in jars), Eppendorf tube rack, forceps (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. 6. Waste container. <p>Common work station: Centrifuge for Eppendorf tubes, vortex mixer, cooling water bath (to -10°C, for ice nucleation assay), refrigerator, autoclave, 30°C incubator.</p> <p>Procedures: see also Appendix</p> <p>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 following day. Please work thoughtfully!</p> <p>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 them with 1.0 ml of the appropriate overnight cultures. Centrifuge at 7000 rpm for 3 minutes to collect the cells, then pour off the supernatant.</p> <p>Take 5 Eppendorf tubes each containing 1 ml LB medium, mark them as R, R+D, R+H, D+H (as controls) and R+D+H, respectively. Divide all of the pellet-containing tubes into 5 groups referring to those labels. Resuspend the pelleted cells with 100 µl sterile LB medium (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 an Eppendorf tube containing 1 ml LB medium, where conjugation will now take place. Proceed accordingly with control samples. Incubate them overnight at 30°C.</p>

	<p>Period 2 (selection of the transconjugants): Take the tubes from the incubator. Aseptically dilute the cells of the R+D+H treatment with 0.9 ml LB medium containing Kan+Amp. With a 100µl pipette, transfer 25 µl, 50 µl, and 100 µl respectively, onto a new agar plates 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 agar plates as well. Incubate the plates for 48 hours at 30° C.</p> <p>Period 3 (enrichment of the culture). Look for growth of luminescent bacteria on LB+Kan+Amp agar plates 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 agar. Incubate plates overnight at 30° C.</p> <p>Period 4 (ice nucleation assay): Before assaying for ice nucleation, put the incubated plates into the refrigerator (adaptation to 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 <i>E. coli</i> pJL1703, recipient <i>V. harveyi</i> 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.</p>
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 <i>ina</i> (or <i>ice</i>) gene expression in luminous <i>V. harveyi</i> .
Timing	90 minutes.
Reporting	Keep good notes on experimental steps and special observations in your lab book
Questions to be answered	What are the phenotypic characteristics of the transconjugant cells? What are your own questions?

Appendix: