ONLINE HOW-TO-DO IT

Use of the Phototactic Ability of a Bacterium

To Teach the Genetic Principles of Random Mutagenesis & Mutant Screening

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Research-based classroom activities have long been recognized as an effective means of teaching science to undergraduate students. Inquiry driven laboratory exercises encourage the development of critical analysis skills and provide for a better understanding of the course material while contributing to a greater overall appreciation for the processes of science. (National Science Foundation, 1996; Boyer, 1998; Rothman & Narum, 1999; Bauer & Bennett, 2003). Ideally, students not only learn the wonders of scientific discovery, but also are encouraged to think like scientists. This teaching approach may also stimulate their interest in pursuing research-oriented careers, where the skills they have acquired in the classroom setting provide a smooth transition into the laboratory environment. It is therefore desirable for instructors to utilize or design laboratory activities that not only effectively illustrate important scientific concepts, but that are also challenging and exciting for the students.

One of the most invaluable and highly utilized tools in molecular genetics is the technique of generating mutant strains of living organisms to observe the phenotypic effects of mutations. The subsequent study and analysis of these mutant strains can lead to a better understanding of how biological processes function at the molecular and genetic levels. This technique can either target specific genes for inactivation or make random changes in an organism's genome (reviewed in Hopwood, 1970). This latter technique, called random mutagenesis, involves increasing the mutation rate in organisms, using chemicals or UV irradiation (Witkin, 1976; Singer & Kusmierek, 1982; Miller, 1985). Mutagenesis is followed by procedures that allow the detection and isolation of mutants with desired phenotypes through selection and screening techniques. This approach has contributed greatly to the understanding of numerous biological processes in microbes and higher organisms. However, students have difficulty grasping the fundamental principles of mutagenesis when presented in a lecture setting.

The challenge then for the instructor is to design or employ a laboratory exercise that effectively introduces the concept of mutagenesis (generating mutant strains of organisms) while capturing the interest and imagination of students. Conducting genetic experiments with students requires that the study organism is amenable to genetic manipulation, easy to cultivate, and in the case of bacteria, is non-pathogenic. It is also desirable to use an organism that exhibits easily identifiable phenotypic traits to simplify screening strategies and that will, hopefully, captivate students. Lastly, it is obviously preferable to avoid the use of toxic chemicals or potentially harmful irradiation during the mutagenesis procedure to eliminate safety issues. We describe here an easy transposon mutagenesis experiment that addresses all of these concerns and that can be used very effectively in a classroom setting to isolate mutants of the prokaryote, *Rhodospirillum centenum*.

Transposons are genetic elements that can "jump" randomly from one site in a genome to another or from one DNA molecule to another, using a process called transposition. They range in size from several hundred base pairs up to 40 kilobase pairs, and carry genes that are required for transposition. Some also carry additional genes including resistance to antibiotics. (For reviews on transposons and their uses, see Cohen & Shapiro, 1980; Kleckner, 1981; Bennett, 2000; Hayes, 2003). Transposons are important for mutagenesis studies as they can cause genetic mutations through varied processes. These include gene disruptions, DNA rearrangements, and activation or repression of genes. Transposon mutagenesis procedures have hence proved to be powerful in the genetic and molecular analysis of a wide

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range of organisms (Kleckner et al., 1977; and for more recent examples, see: Jadoun et al., 2004; Horie et al., 2003; Zhang et al., 2003; Muller, 2004).

R. centenum is a α-purple, photosynthetic bacterium that can be found in aquatic and soil habitats. Like many purple bacteria it is metabolically versatile. In the laboratory, cultures of *R. centenum* exhibit a rich purple color as a consequence of the pigments used to capture light energy during photosynthesis. In liquid culture, individual "swimmer" cells are motile by virtue of a single flagellum located at one pole. If placed onto an agar surface, cells quickly undergo differentiation into "swarmer cells" that acquire numerous lateral flagella used to propel themselves across a solid substrate (Ragatz et al., 1994; Ragatz et al., 1995), Figure 1. The motility of swarmer cells on agar plates facilitates what may be *R. centenum's* most fascinating property: the ability to undergo phototaxis.

Phototaxis is defined as movement toward or away from a light source that is independent of the intensity of irradiation. For *R. centenum*, phototaxis can be observed when colonies growing on agar plates are exposed to a light source. Remarkably, entire colonies can be induced to migrate toward infrared light (positive phototaxis) or to move away from light of the visible spectrum (negative phototaxis). In either case, colonies can move at an amazing rate of up to 75 mm/hr, so phototaxis is readily observed after a relatively short period of time (Figure 2). Jiang and coworkers (Jiang et al., 1998) demonstrated that random transposon mutagenesis of *R. centenum* generated strains that were defective for phototaxis at a high frequency. We have adapted these experiments for use in a microbiology or genetics laboratory course with the objective of introducing students to the concept of random mutagenesis (creating random mutations in the genomes of organisms) and the use of genetic screens (isolation of mutant strains with desired phenotypes), as applied in the study of a biological process. The observation that roughly 1 in 25 mutants screened was found to be defective for phototaxis (Jiang et al., 1998) simplifies the logistics of conducting this activity since relatively few strains need to be screened to isolate the desired phenotype. Moreover, the simplicity of the mutagenesis step and the genetic screen, combined with the dramatic visual observations of positive and negative phototaxis will almost assuredly appeal to inquisitive students.

This module has another advantage in that it requires relatively little specialized equipment and so could be easily adapted for use at the high school and undergraduate levels. We have adapted this activity for use in two of our upper level undergraduate biology courses at Loyola (Microbiology and Recombinant DNA Technology) as well as in Microbiology Lab and Genetics Lab courses at USD. It has also been used successfully at summer workshops at the Marine Biological Laboratory, Woods Hole in Massachusetts (Carl Bauer, personal communication).

Figure 1. The vegetative life cycle of *R. centenum* cells. In liquid culture, "swimmer" cells are short, curved and possess a polar flagellum. If cells are transferred from liquid culture to an agar plate, they become slightly elongated and acquire several lateral flagella. These "swarmer" cells are able to move across a solid surface in response to a light source (phototaxis). If used to inoculate a broth culture, swarmer cells taken from an agar plate will differentiate back into swimmer cells.

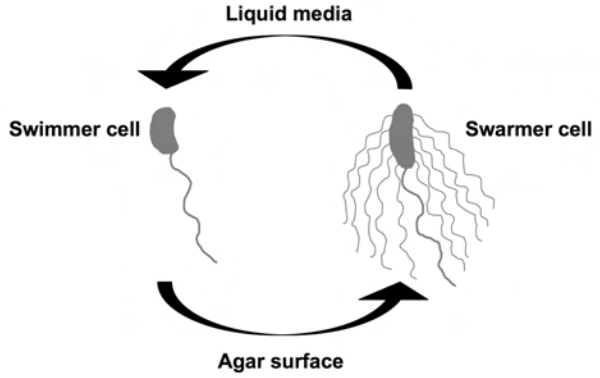
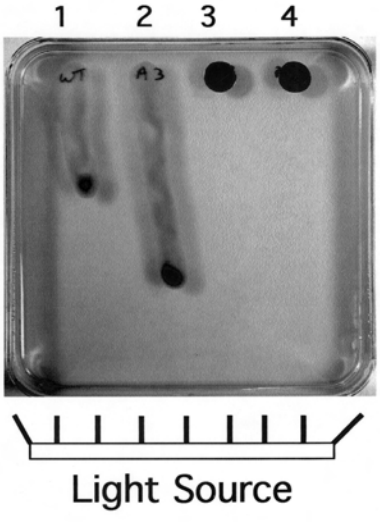


Figure 2. Phototaxis assays of wild type and mutant strains of *R. centenum*. Overnight cultures of cells were concentrated 20 fold and 10 µl was spotted onto one side of the PYVS (0.8% agar) plate. The plate was incubated in the dark at 37° C for eight hours before being moved to a 30° C incubator fitted with a 60-watt tungsten bulb. The figure shows the results of the assay after a four-hour incubation of the plate in the light. Lane 1 = Wild type cells. Lane 2 = Hyperswarmer mutant strain A3 (J. E. Berleman, unpublished). Lanes 3 and 4 = non-motile mutants B3 and RR1 respectively (J. E. Berleman, unpublished).



Methods

(See Appendix for all strain and media information)

Laboratory Period 1: Mutagenesis Procedure

Objectives

The first step of the mutagenesis procedure involves mixing the *R. centenum* cells (wild-type) with *E. coli* cells (S17-1λ pir) that harbor the transposon mini-Tn5 (carrying resistance to the antibiotic, spectinomycin) on the plasmid pZJD17, which is unable to replicate in *R. centenum* (Jiang et al., 1998). The host *E. coli* and recipient *R. centenum* cells undergo a process called bacterial conjugation, or bacterial mating (Frost, 2000), whereby the plasmid pZJD17 (with the mini-Tn5 transposon), is transferred through direct cell-to-cell contact to *R. centenum*. (Figure 3). Once transfer has occurred, the transposons can jump into the chromosomes of the recipient *R. centenum*. These cells can be selected by plating the bacterial matings on media that contains spectinomycin (Figure 3). Inclusion of kanamycin in addition to spectinomycin on the selective media will eliminate the donor *E. coli* cells without affecting *R. centenum*, which is naturally resistant to this antibiotic (Figures 3 and 4).

Procedure

(See Figures 3 and 4)

Ten ml volumes of Luria broth and CENS media are inoculated with *E. coli* S17-1 λ pir /pZJD17, which carries the mini-Tn5 transposon (conferring spectinomycin resistance) and wild-type *R. centenum* respectively. The cultures are grown overnight at 37˚ C. All subsequent steps are carried out at room temperature unless otherwise stated. Both cultures are centrifuged and pelleted cells washed three times with CENS media before being resuspended in 10 ml of CENS media. One ml of *E. coli* and 5 ml of *R. centenum* cells are then mixed and filtered onto 0.2-micron membranes (Corning, 115 ml cellulose acetate filter systems, #430944). The membranes are placed onto a CENS plate that is incubated at 37˚C for four hours, or alternatively, overnight at room temperature. The membranes are then placed in a sterile test tube with 2 ml of CENS media and cells are resuspended by vortexing vigorously. One hundred µl samples of the cell suspension are plated onto 20 CENS plates, supplemented with 10 µg/ml spectinomycin and 50 µg/ml kanamycin. The spectinomycin is used to select for cells that have transposons inserted into their chromosomes and the kanamycin counter selects against the *E. coli* donor (*R. centenum* is naturally resistant to this drug). Instructors may want to do the steps leading up to the plating of the cells themselves in order to save laboratory time and then have groups of students carry out platings of the resuspended cells, with each group obtaining the samples from one mating experiment. The plates are incubated at 42˚ C and colonies usually appear after two to three days. Although *R. centenum* cannot withstand refrigeration, these plates may be stored at room temperature and colonies will remain viable for at least four weeks.

Figure 3. Steps involved in transposon mutagenesis of *R. centenum*. First, the donor *E. coli* cells harboring the mini-Tn5 transposon are mixed with the recipient wild-type *R. centenum* cells. The cells are filtered onto a cellulose-acetate membrane and placed onto an agar plate that does not contain antibiotics. The plate is incubated for a few hours (37˚ C) to overnight (room temperature) to permit conjugation (mating) and transposition. Cells are then resuspended in liquid media and plated onto agar plates supplemented with both kanamycin and spectinomycin. Because *R. centenum* is naturally resistant to kanamycin and the mini-Tn5 confers spectinomycin resistance, inclusion of these antibiotics in the growth medium selects against *E. coli* donor cells and any *R. centenum* cells that do not possess a transposon. The colonies that grow represent mutant strains that can be subjected to a screen to identify those with abnormal phototactic properties.

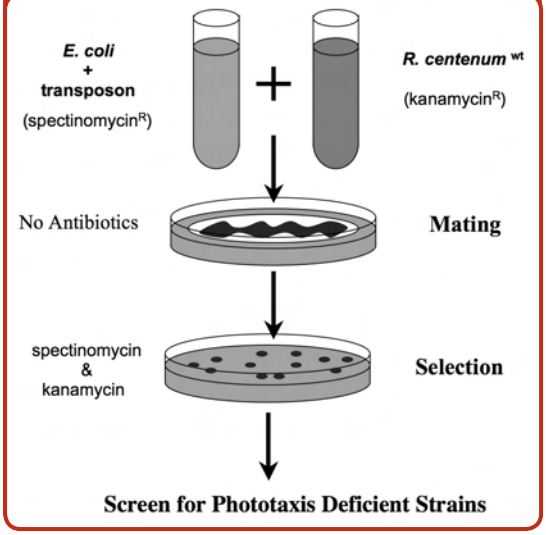
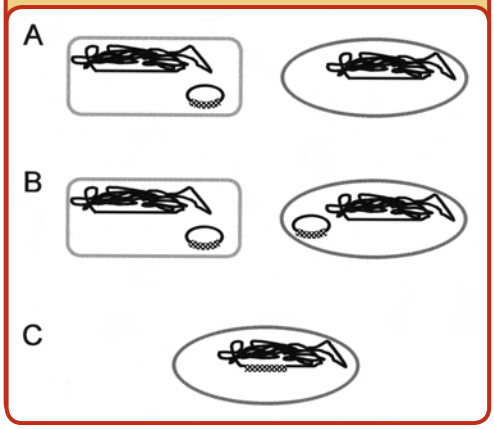


Figure 4. Conjugation and transposition events of the *R. centenum* mutagenesis procedure at the cellular level. (A) The process begins with a mixture of *E. coli* donor cells (rod-shaped) that contain a plasmid (ring structure) bearing the mini-Tn5 transposon (hatched box) with the recipient wild-type *R. centenum* cells (oval-shaped). (B) Conjugation on the cellulose-acetate membrane results in transfer of the plasmid to *R. centenum* cells. (C) Because the plasmid is unable to replicate in *R. centenum,* it will soon be lost from recipient cells. However, it will be present long enough for transposition to occur in some cells, resulting in the random insertion of the transposon into the *R. centenum* chromosome. The mini-Tn5 transposon carries a gene that confers resistance to spectinomycin. Consequently, growth on agar plates containing kanamycin and spectinomycin will select for *R. centenum* cells with transposons inserted in their chromosomes.



Data Collection & Discussion

The student groups should count the number of kanamycin/spectinomycin resistant *R. centenum* colonies that grow on their selection plates. Typically this procedure can yield a total of 10^2 to 10^4 colonies, depending on the efficiency of the mating. One other interesting observation that can be made by students is that a few of the mutant colonies will exhibit colors that differ from the wild-type strain. Pink, green, and yellow-orange colonies will result from mutations in enzymes that are responsible for the various pigments found in *R. centenum*. (Deisenhofer et al., 1985; Youvan & Marrs, 1987; Schlegel & Bowien, 1989). The instructor can lead discussions on the random nature of transposon mutagenesis (Cohen & Shapiro, 1980; Kleckner, 1981; Bennett, 2000), the procedures used to select for *R. centenum* cells that now carry a transposon (i.e. plating the products of the bacterial matings on media that only allows recipient cells that carry the transposon to survive), and the importance of counter-selection against the *E. coli* strain (by incorporating the antibiotic kanamycin in the selective media plates). The discussions could then transition into the next part of the experiment, which is the screening procedure, used to detect and isolate mutant strains with the desired phenotypes. This requires that a suitable detection system or "screen" be set up so that mutant strains can be distinguished. Students should be made aware that at this point a number of different screens could be used, depending on what biological questions are being asked, and hence which genetic mutants are to be collected. A discussion of how these screens might be designed can be included at this point.

Laboratory Period 2: Positive Phototaxis Assays

Objective

An assay system or screen must be employed to find mutants of *R. centenum* that are affected in their response to light. This is easily achieved by exposing colonies of mutagenized *R. centenum* cells to an infrared light source and then identifying colonies that show an altered phototactic response as compared to the wild-type strain.

Procedure

Colonies of *R. centenum* should be chosen at random from the mutagenesis plates and used to inoculate 3 ml of CENS media. These cultures are then grown at 37˚ C overnight. One ml of each overnight culture is transferred to a sterile microfuge tube and concentrated by centrifugation (five minutes, 14K). The pelleted cells are resuspended in 50 µl of spent CENS media and 10 µl is spotted approximately one inch from one side of square culture plates (100 mm x 100 mm) containing PYVS medium solidified with 0.8% agar (Figure 2). The spots are allowed to dry by leaving the plates undisturbed at room temperature for 15 to 30 minutes. The plates are then incubated in the dark at 37˚ C until large colonies are observed (at least eight hours or overnight). This incubation is crucial to allow time for swimmer cells to differentiate into swarmer cells (Figure 1). Again, these steps can be carried out by the instructor, the night and morning before the class meets, or can just as easily be carried out by the students themselves outside of class time. During the regular class meeting time the plates can then be shifted to an incubator set at 30˚ C fitted with a 60-watt tungsten bulb. The plate is oriented so that colonies are on the side furthest from the light source. Individual colonies can then be scored for their ability to undergo positive phototaxis after incubating approximately four hours. To make this a fun activity, the students could set up colony races to see which are able to swarm to a particular position on the plate first. Note that the mutagenesis procedure could produce strains that are "hyperswarmers" (Jiang et al., 1998), as illustrated in Figure 2, and this would make the races more exciting!

Data Collection & Discussion

Students can measure the rate at which selected mutant strains move, if at all. They can also make observations about any strains exhibiting enhanced phototaxis (i.e., "hyperswarmers"). The class discussions can then focus on topics such as the success of the screening process as well as the frequency at which mutations have occurred in genes that involve phototaxis, and possibly how the functions of these genes can be determined. Depending on the level at which the class is being taught, the instructor could also discuss how DNA primers that hybridize to flanking transposon sequences can be used to identify mutated genes through Southern hybridization or inverse PCR (Watson et al., 1992; Old & Primrose, 1994; Palmer & Paszko-Kolva, 2000; Zyskind, 2000), both commonly used techniques in molecular genetics.

Conclusions

We have presented a laboratory activity that relies on the use of a very versatile bacterial system to introduce the concept of how mutagenesis can be used for molecular and genetic analysis of living organisms. We have used the techniques of random mutagenesis and selection/screening to obtain strains of the organism *R. centenum* that are defective in phototaxis. The activity we have described takes advantage of just one phenotype of *R. centenum*. However, it is possible for the instructor to easily adapt these experiments to hunt for mutants of *R. centenum* that exhibit altered metabolic activity, or for mutants that are unable to carry out photosynthesis, for example. The use of transposons for the mutagenesis procedure is an added advantage if the instructor wishes to make this a more extensive laboratory module where students might conduct independent or group experiments that identify and characterize the genes that have been disrupted. Hence this simple bacterial system may be used to illustrate the power of a number of genetic and molecular techniques, while impressing upon students how truly remarkable bacteria can be.

Acknowledgments

We would like to thank Carl Bauer (Indiana University) for his constant support and invaluable advice on developing this laboratory activity, and David Rivers (Loyola College) for critical reading of this manuscript.

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Appendix.

Strain & Media

The *R. centenum* strain used in this study is wild-type swarmer (obtained through the American Type Culture Collection, ATCC #51521) and the *E. coli* strain used for the mutagenesis is S17-1 λ pir /pZJD17 (Jiang et al., 1998). Both strains are available from the authors upon request.

Growth Media

Unless otherwise stated, all chemicals can be obtained through the Sigma Chemical Company. The growth media used for *R. centenum* has been designated CENS and relies on pyruvate as a carbon source. This media contains:

- 4 g bacto-soytone (Difco, 243620)
- 2.2 g sodium pyruvate
- 0.9 g K_2HPO_4
- 0.6 g KH_2PO_4
- g NH_4Cl
- 5 mg disodium EDTA
- 200 mg $MgSO_4.7H_2O$
- ml trace element solution (see next column)
- 75 mg $CaCl_2.2H_2O$
- 2 ml chelated iron solution (see next column)
- 20 µg vitamin B_{12}
- 15 µg biotin
- 0.5 g $Na_2S_2O_3.5H_2O$

in a liter of deionized water.

The pH should be adjusted to 6.8 with NaOH prior to autoclaving (using standard sterilizing techniques of 121˚ C, 30 minutes). Chelated iron solution may be made by combining 1 g $FeCl_2.4H_2O$ and 2 g disodium EDTA and 3 ml of concentrated HCL, in a liter of deionized water. Trace elements solution contains:

- 2.5 g disodium EDTA
- 0.2 g $MnCl_2.4H_2O$
- g H_3BO_3
- g $Na_2MoO_4.2H_2O$
- 50 mg $ZnCl_2$
- 50 mg $NiCl_2.6H_2O$
- 20 mg $CoCl_2.H_2O$
- 10 mg $CuCl_2.2H_2O$
- 5 mg $NaSeO_4$
- 5 mg $NaVO_3.nH_2O$ (meta)

in 250 ml of deionized water.

PYVS swarm plates, used in the phototaxis assays, are made as follows: To 500 ml of distilled water, add:

- 3 g bacto-peptone (Difco, 0118-17-0)
- 3 g yeast extract (Difco, 5-212750)
- 4 g bacto-soytone (Difco)
- 1 ml vitamin B12 (20 µg/ml stock solution)
- ml d-Biotin (150 µg/ml stock solution).

The volume is brought up to 1000 ml with distilled water, and 8 g of agar are added (Difco, 5-214050) to the media before autoclaving (using standard sterilizing techniques of 121˚ C, 30 minutes). The media is poured into square plates (100 mm x 100 mm, Fischer Scientific ICNLX4021X) and allowed to dry at room temperature for 24 hours before use.