

Chapter 3

Transposon Mutagenesis of *Rhodobacter sphaeroides*

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Introduction

The objectives of the transposon mutagenesis experiment are as follows:

1. Familiarize students with photosynthetic microorganisms, specifically the *Rhodospirillaceae*.
2. Demonstrate plasmid transfer between distantly related groups of microorganisms.
3. Introduce transposons and techniques for generating mutations with them.
4. Demonstrate mutagenesis of microorganisms.
5. Demonstrate the selection of mutants.
6. Observe different phenotypes in the mutagenized strains.
7. Expose students to specialized strains used in molecular biology.

The experiment, as presented below, is designed for advanced biology or microbiology students. The experiment is 2 weeks long and is performed over three, 2-hour lab periods with 1-week incubations in between each lab. Suggested prerequisite courses for this lab include: Introductory Genetics, Introductory Microbiology, and an understanding of aseptic technique (this can come from a previous microbiology lab or be taught to students before performing this experiment). However, we feel the experiment could be modified to be understandable to a less sophisticated group if so desired. When presenting to less advanced students, the experiment itself need not be modified, since the manipulations required are simple. It is important, however, to tailor the theoretical detail presented to suit your audience.

The microorganism used in these studies, *Rhodobacter sphaeroides*, is a relatively fast growing photosynthetic bacterium. It is easy to culture and little specialized equipment is required for the experiment. It is a facultative anaerobe, only requiring anaerobic conditions when growing under photosynthetic conditions. The pigment mutants generated from this protocol can be detected visually — a very simple screen. Other nutritional mutants can also be screened for depending on the inclination of the instructor. *Rhodobacter sphaeroides* is used instead of *E. coli* to expose students to different microbes and demonstrate the use of cutting-edge molecular biology in an organism other than *E. coli*.

Strains Used

R. sphaeroides strain 2.4.1 is the common wild-type strain used for research on this photosynthetic microorganism. S17-1A is an *Escherichia coli* strain developed by Alf Pühler's lab for the mutagenesis of various gram negative bacteria (Simon et al., 1986). It readily transfers the transposon carrying plasmid to *R. sphaeroides* with a frequency of about 1 in 10⁵. Both strains are available from the authors. Preparation for the lab takes approximately 6 hours, which includes making the media. An autoclave is required as well as equipment for mixing solutions.

Materials

Period 1

Microfuge that will hold microfuge tubes (2/class)
 Sterile microfuge tubes (4/student)
 Receptacle for supernatant of cultures, filled with disinfectant (4/class)
 Sistrom's Minimal Medium broth (5 ml) in a capped 16 × 250 mm tube (1/pair)
 Bent glass rod, L-shaped like a hockey stick (1/pair)
 Luria-Bertani Agar petri plates (4/pair)
 30°C incubator (1/class)
 Sterile 0.85% saline (50 ml) in a 200-ml bottle (1/pair)
 Sterile capped test tubes, 13 × 100 mm (8/pair)
 P1000 micropipet* (1/pair)
 P1000 micropipet tips* (1 box/pair)
 P200 micropipet* (1/pair)
 P200 micropipet tips* (1 box/pair)
 Sterile velvets in aluminum foil (4/pair)
 Replica plater (1/pair)
 Agar petri plate of Sistrom's Minimal Medium + 0.1% Yeast Extract (= SIS + YE) (4/pair)
 Agar petri plate of SIS+YE + 50 µg/ml tellurite + 30 µg/ml trimethoprim (4/pair)

* Micropipets are common now in molecular biology laboratories, however they can be expensive for an instructional lab. It is possible to perform this lab without them, substituting glass pipets instead.

Period 2

Sterile velvets in aluminum foil (4/pair)
 Replica plater (1/pair)
 Agar petri plate of SIS (4/pair)
 Agar petri plate of SIS + histidine (4/pair)
 Agar petri plate of SIS + B12 (4/pair)
 Agar petri plate of SIS + YE (8/pair)
 Agar petri plate of SIS + YE + tellurite + trimethoprim (4/pair)
 Anaerobe jar (1 for 3 students)

Notes for the Instructor

1. Bacterial cultures of *R. sphaeroides* and *E. coli* must be in log phase during the mating. Specific instructions on how to achieve this are given in Appendix B. If not in log phase the experiment still works, but the number of transposon mutants isolated is reduced.
2. Bring your stock cultures out of storage early. *R. sphaeroides* can take up to 4 days to form visible colonies on a plate. See Appendix B for specific instructions. *E. coli* strain S17-1A must always be grown in the presence of antibiotics. If this is not done, it will rapidly lose the plasmid carrying the transposon.
3. The students should be taught aseptic technique for handling the culture. The incubations are long (1 week) and any contaminants introduced will grow up in that time. Mold is the major problem. Try to make up the media in a dry area that is not ventilated directly by the outside environment.
4. The materials (plates, broths, sterile tubes, etc.) can be made up weeks in advance and stored. However, the Siström's Minimal Medium plates should be made no earlier than 2 weeks before the lab. *R. sphaeroides* grows poorly on dry plates.
5. Color code your plates and broths so that students have an easier time keeping track of media. Agar plates can be quickly labeled by drawing a line down a stack of them with colored magic markers.
6. Any equipment that comes in contact with bacterial culture should be sterilized before cleaning or discarding. Glassware should be autoclaved before washing and plasticware autoclaved before discarding it. Students should discard pipets or plastic tips from micropipets into disinfectant (10% Lysol or 0.5% Amphyl).
7. If desired, mutants generated by this procedure can be used in further studies. As an example, the pigment mutants could be grown under photosynthetic conditions and their absorption spectrum analyzed (from 300–900 nanometers) and compared to wild-type.

Student Outline

Background Information

The *Rhodospirillaceae*

Photosynthetic bacteria comprise a large and diverse group of microorganisms, classified taxonomically due to their ability to generate ATP from light and possession of bacterial chlorophyll. The group is divided into the purple bacteria, the green bacteria, and the Cyanobacteria. Cyanobacteria possess a “plant-like” photosynthetic apparatus, whereas the purple and green bacteria utilize a different mechanism for photosynthesis. In the purple and green bacteria photosynthesis occurs only under anaerobic conditions. Reduced sulfur compounds or in some cases organic compounds, serve as a source of electrons.

The purple and green bacteria can be further divided into four sub-groups, depending upon their tolerance for reduced sulfur compounds and the type of pigmentation they produce. Table 3.1 summarizes some important characteristics of the anoxygenic photosynthetics. The purple and green bacteria are commonly found in the anaerobic zone of aquatic habitats in a narrow band where oxygen is absent, light is present, and H₂S is available. In some lakes this band of photosynthetic

microorganisms will increase to a large enough population so that they are visible in the water column as a purple or green layer.

Table 3.1. Differentiating characteristics of the green and purple bacteria.

Common name	Green non-sulfur bacteria	Green sulfur bacteria	Purple sulfur bacteria	Purple non-sulfur bacteria
Motility	Some motile by gliding	Most non-motile	Most motile by flagella	Most motile by flagella
Modes of growth	Chemoorganotroph, photoautotroph, photoorganotroph	Strict photoautotroph	Strict photoautotroph	Chemoorganotroph, photoautotroph, photoorganotroph
Electron donors	Reduced sulfur compounds**, organics	Reduced sulfur compounds** (organics)	Reduced sulfur compounds**	Reduced sulfur compounds**, organics
Representative bacteria	<i>Chloroflexus aurantiacus</i> , <i>Chloronema gigateum</i>	<i>Chlorobium limicola</i> , <i>Pelodictyon luteolum</i>	<i>Chromatium okenii</i>	<i>Rhodospirillum rubrum</i> , <i>Rhodobacter sphaeroides</i> , <i>Rhodobacter capsulatus</i> , <i>Rhodomicrobium vanellii</i> ***
Division	Filamentous	Binary fission	Binary fission	Most by binary fission, some by budding
Oxygen relationship	Facultative anaerobe	Anaerobe	Anaerobe	Facultative anaerobe
Photosynthetic membrane organization	Vesicles attached to cytoplasmic membrane, but not contiguous		Lamellae – Photosynthetic membranes are contiguous with the cytoplasmic membrane	
Bacterial chlorophyll	a, c	c, d, e	a, (b)	a, b
CO ₂ assimilation pathway	?	Reverse TCA	Calvin Cycle	Calvin Cycle

* Often forms symbiosis with chemoorganotrophs.

** Reduced sulfur compounds include: H₂S, S, thiosulfate, and sulfite.

*** This organism has a very unusual life cycle.

The *Rhodospirillaceae*, a subset of the purple and green bacteria, are gram-negative rods, most being motile by flagella, and divide by binary fission or by budding. They are easy to isolate and have a versatile metabolism, being capable of aerobic and anaerobic respiration, fermentation, and photosynthesis (using CO₂ or organic substrates as carbon sources under photosynthetic conditions). One species, *Rhodospirillum rubrum*, is even capable of using carbon monoxide as sole carbon source! This facultative photosynthetic ability has made the *Rhodospirillaceae* indispensable tools in understanding photosynthesis, since mutations in photosynthesis can be isolated and these mutants can still be maintained using non-photosynthetic conditions. Their diverse metabolic

characteristics also make them ideal for use in the teaching laboratory. In this experiment, mutants in *Rhodobacter sphaeroides* are isolated and their characteristics studied. The next section will briefly explain mutagenesis and the protocol that we will be using.

Mutagenesis

The study of *any* microorganism's properties can be greatly enhanced by the generation of mutations in genes of interest. Creation of mutations and subsequent genetic mapping can elucidate the identity, relative size, number, and organization of genes involved in a physiological process. Also, the recognition and location of transcriptional units can be defined by mutation. In more sophisticated approaches, site-specific mutation of a gene can help to reveal the relationship of the structure of a protein to its function. Finally, mutagenesis is used to create strains with desired properties, such as the ability to overproduce a desired metabolite or enzyme. (The industrial production of penicillin and streptokinase are two examples.)

So now that you are convinced that mutagenizing microorganisms is important, interesting, and potentially profitable, how exactly is it done? In the most general terms, mutation of a gene or genes under study can be achieved by first altering the DNA of the microorganism in some fashion and then screening or selecting for the desired phenotype. For example, if you were studying the histidine biosynthetic pathway in a microorganism you might first mutagenize with a chemical mutagen and then screen for mutants that could no longer grow without histidine.

Three general treatments can be used to mutagenize microorganisms: electromagnetic radiation, chemical mutagens, and transposons. Mutation by electromagnetic radiation involves exposing the microbe to high energy electromagnetic waves (X-rays or more commonly UV light). This procedure damages the target DNA and sometimes, during repair, an improper base pair (or pairs) is incorporated in the DNA, causing a mutation.

Chemical mutagens are also employed. These compounds are added to a growing culture of an organism for a given time period and interfere with the replication of the DNA. Some mutagens achieve this by serving as base analogs, others chemically modify the DNA, and yet another class can insert or intercalate in between the base pairs of DNA causing DNA polymerase to make mistakes. In all cases, the mutagen causes incorrect copying of the DNA resulting in base substitutions (exchange of one base pair for another), insertions (addition of one or more base pairs), or deletions (removal of one or more base pairs).

Transposon Mutagenesis

The third method of mutagenesis involves the use of mobile genetic elements. The most commonly employed of these are a sub-class called transposons, which are the subject of this experiment. Transposons are relatively short pieces of DNA that replicate by inserting into other pieces of DNA (plasmids, chromosomes, viruses). They encode two sets of functions. One set is involved in regulating and performing the movement of the transposon from one piece of "host" DNA to the next (transposition functions). The other set of functions encode genes that may provide an advantage for the host of the transposon — antibiotic resistance and Hg⁺² resistance, for example.

The transposons investigated to date come in two structural flavors: compound and complex. Both compound and complex transposons contain several features in common. Each has direct or indirect DNA repeats at the end of the transposon, there are stretches of DNA that code for the transposition functions, and other areas that contain genes that usually encode the selectable function. The difference between the two depends on how the genes in the transposon are arranged,

a subject beyond the scope of this discussion. The structure of the transposon Tn5Tp, a compound transposon, is shown in Figure 3.1.

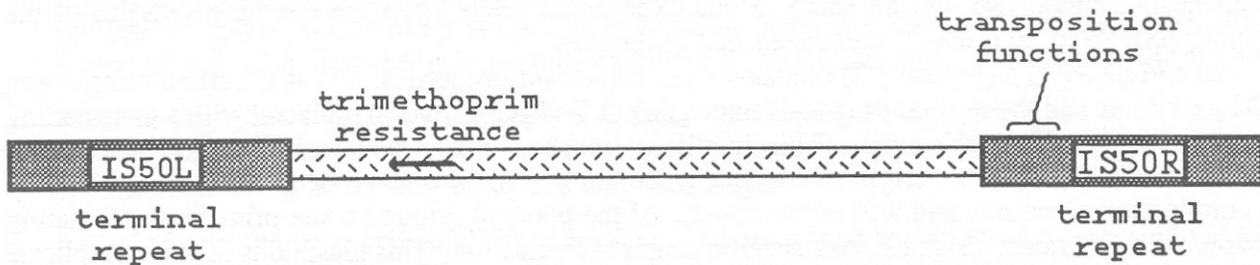


Figure 3.1. A schematic representation of Tn5Tp. The genes encoding trimethoprim resistance and the transposition functions are shown. IS50L and IS50R are insertion sequences.

When a transposon inserts in a gene, it disrupts the gene and almost always completely inactivates it. (This is referred to as a gene “knock-out”). In most cases transposon insertions cause polar mutations, meaning that genes downstream of the transposon on the same transcript are not expressed efficiently. Thus, it is possible to use transposons to infer the transcriptional organization of a set of genes.

Transposon mutagenesis has several advantages over the use of other methods of mutation. First, chemicals and ionizing radiation not only can mutagenize the intended microorganism, they can also mutagenize you! Extreme caution must be exercised when employing these techniques. Transposons that mutagenize bacteria will not insert into human DNA and are much safer to work with. (Do you think there are transposons that insert into human DNA?) Second, transposons used for mutagenizing bacteria have selectable phenotypes, usually drug resistances, which can be used to isolate “transposon hops” (bacteria that have been mutagenized by the transposon hopping into the target chromosome). Once the “transposon hops” are isolated, the researcher can then screen this subset of microorganisms for the desired characteristic or deficiency. This is a very nice feature if the detection of the mutation of interest involves a difficult or expensive procedure. Also, since transposition happens at a frequency of 10^{-4} to 10^{-7} , it is important to eliminate the vast majority of organisms that do not have an insertion before proceeding. Finally, transposons cause very clean mutations. When an organism is mutagenized with chemicals or irradiated, it is possible to get more than one mutation in a bacterium (why is this an undesirable property for genetic analysis?). Transposon mutagenesis typically results in only one transposition event per individual cell.

There are, however, some disadvantages to transposon mutagenesis that must be considered when doing genetic analysis. The occupation of a transposon is to move from one site to the next and in the process increase its number. Occasionally the phenotype of a strain carrying a transposon-derived mutation may be unstable due to further hops. This problem can be minimized by preserving the transposon carrying strain in a stable manner (two good choices are freezing at -80°C in DMSO or lyophilization). Some transposons also have preferred sites for integration and their mutagenesis of a chromosome can be non-random. As an example, the transposon Tn7 has such extreme specificity that it has only one preferred site in all of the *E. coli* chromosome. This transposon is not a very good candidate to use as a mutagen. However, other transposons, such as Tn5, seem to be almost random, although detailed analysis of Tn5 insertion has shown that there can be high frequency sites for integration within each gene. Tn5 is a good mutagen and is used often in many different types of microorganisms. Finally, in most cases transposons cause gene knockouts.

If the mutation of interest involves a change in function of a protein and not its destruction, another method must be used.

In this experiment we will mutagenize *R. sphaeroides* wild-type by mating a transposon carrying plasmid from *E. coli* to *R. sphaeroides* and then selecting for the presence of the transposon. The mutagenesis protocol we will be using in this experiment relies upon the special properties of the plasmid, pSUP5Tp, and the *E. coli* strain that harbors it.

Plasmid pSUP5Tp (Figure 3.2) contains three important properties. (1) A modified transposon, Tn5Tp, that has had the normal drug resistance gene of Tn5 (kanamycin) replaced with a gene coding for trimethoprim (Tp) resistance. This modified transposon was constructed by Sasakawa and Yoshikawa (1987). (2) An origin of transfer (mob site). This sequence of DNA is recognized by the conjugation apparatus and will allow transfer of the plasmid through a sex pilus during a mating between the two strains. (3) An *E. coli*-specific origin of replication. This plasmid is unable to replicate in *R. sphaeroides* and therefore will be lost as the bacteria grows. This is termed a “suicide vector.”

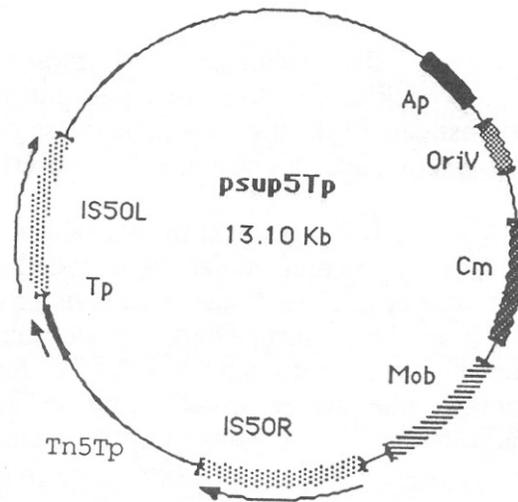


Figure 3.2. Plasmid map of pSUP5Tp. The location of genes encoding drug resistances for ampicillin (Amp), chloramphenicol (Cm), and trimethoprim (Tp) are shown. The origin of replication (OriV), the origin of transfer (mob), and the transposon Tn5Tp are also shown.

E. coli strain S17-1A, contains the conjugal transfer genes (*tra*) on the chromosome (Simon et al., 1987). It is capable of taking any plasmid that has an origin of transfer and mobilizing it to a suitable recipient. In this experiment we will mobilize plasmid pSUP5Tp from strain S17-1A by conjugation to a *R. sphaeroides* wild-type strain (2.4.1). After the mating, the presence of the transposon in the recipient will be assayed by plating the cells on a photosynthetic medium containing Tp. Since pSUP5Tp is a suicide vector only transposons that have hopped into strain 2.4.1's chromosome will be detected. After initial isolation, we will identify three classes of mutants: mutants altered in photopigment expression, mutants unable to grow under photosynthetic conditions, and mutants that have altered nutritional requirements.

For further reading about the *Rhodospirillaceae* see Kiley and Kaplan (1988). For a background on transposons see Freifelder (1987).

Procedure

Period 1: The Mating with *E. coli*

1. You will be supplied with mid-log phase cultures of *E. coli* strain S17-1A grown in LB + Amp (50 µg/ml) and *R. sphaeroides* strain 2.4.1 grown in Sistrom's Minimal Medium (SIS). Place a total of 1.5 ml of S17-1A into each of four sterile microfuge tubes and spin for 20 seconds.
2. Remove the tubes from the centrifuge and decant the liquid, being careful not to dislodge the pellet of cells. Remove as much liquid as possible from the tube. Why? Add 1.5 ml of the 2.4.1 culture to each of the 4 tubes and centrifuge again.
3. Again, decant the tube and add 150 µl of SIS. Resuspend the co-culture by vortexing the tube until the pellet disappears. Place 120 µl of the suspension onto LB plates and spread using a completely cooled spreader (an L-shaped glass rod, sometimes referred to as a "hockey stick"). Incubate the plates, right side up (contrary to everything you have been taught previously), at 30°C for 1 hour. This is the point where the mating takes place. If all is working well pSUP5Tp is being transferred to *R. sphaeroides* strain 2.4.1.
4. Now that the bacteria have mated, it is time to select for trimethoprim-resistant *R. sphaeroides*. Replica plate each of the LB plates onto SIS + trimethoprim (30 µg/ml) + yeast extract (0.1%) + tellurite (50 µg/ml) (SIS+Tp+YE+Tel). The trimethoprim selects for microorganisms carrying the transposon. The yeast extract provides nutrients to potential auxotrophs and the tellurite, a heavy metal, selects against the donor *E. coli*. Strain 2.4.1 is naturally resistant to tellurite. These plates will allow us to identify all cells which have received the transposon.
5. Perform viable plate counts of the original 2.4.1 culture. To do this, dilute out the culture in 0.85% saline (following the directions listed below). Assume the cell density to be between 10^6 to 10^9 cells/ml. Plate onto SIS+YE.
 - (a) Label the eight 13 × 100 mm test tubes 10^{-1} to 10^{-7} .
 - (b) Using the P1000 and a blue pipet tip, pipet exactly 900 µl (0.9 ml) of sterile 0.85% saline into each of the labeled test tubes.
 - (c) Switch to the P200 and place a yellow pipet tip on its end. Remove 100 µl (0.1 ml) of the *R. sphaeroides* culture with the P200 and dispense it into the tube labeled 10^{-1} and vortex for about 5 seconds.
 - (d) Using the P200 with a fresh pipet tip, remove 100 µl from the 10^{-1} dilution and add it to the 10^{-2} dilution. Vortex the 10^{-2} dilution for about 5 seconds. Your sample is now diluted 1/100.
 - (e) Continue diluting the sample using the same procedure until the other dilution tubes are prepared. The final dilution (10^{-7}) should contain one ten-millionth as many organisms as the original sample. If you don't understand, ask your instructors.
 - (f) Label the four plates of SIS+YE 10^{-5} to 10^{-8} .
 - (g) Using the P200, pipet 100 µl of the 10^{-4} dilution onto a plate of SIS labeled 10^{-5} .
 - (h) Repeat step (g) for the 10^{-6} to 10^{-8} dilution's. Be sure to pipet each 0.1 ml onto the appropriately labeled plate.
 - (i) Take a spreader and dip the short end into 95% ethanol. Ignite the ethanol by passing the spreader through a bunsen burner flame and allow the ethanol to burn out. Do not hold the spreader in the flame!!! Allow the spreader to cool for about 20 seconds.

- (j) Now that the spreader is sterilized, use it to spread the 0.1 ml on one of the plates prepared in steps (g) and (h). Be sure to spread the liquid evenly over the whole surface of the plate. Spread the rest of the plates in a similar manner. Remember to resterilize the spreader between each plate.
 - (k) Allow the liquid to soak into the plates for about 10 minutes.
6. Incubate all plates aerobically at 30°C for 5 to 7 days.

Some important reminders:

- (a) Make a mark on the bottom/side of each plate in some manner so that it will be possible to compare the replica plates to each other after incubation.
- (b) Do not push down too hard on the plates when replicating. Irregular colonies will result, which can be difficult to analyze.

Period 2: Replica Plating to Selective Media

1. Observe the replica plates. Record a description of their appearance.
2. Count the total number of colonies that are transposon mutants. How? (*Hint: They will be Tp resistant.*)
3. Most of the trimethoprim resistant colonies contain the transposon. We now want to find out the phenotype of the mutants. We will screen for alteration in the pigments produced by the microorganism, loss of the ability to grow photosynthetically, loss of the ability to synthesize histidine, and loss of B12 biosynthesis.
4. Replica plate the colonies from the original selective plate (the master plate) onto the following screening medium:
 - (a) SIS + Tp,
 - (b) SIS + histidine + Tp,
 - (c) SIS + B12 + Tp,
 - (d) SIS + YE + Tp (2 plates), and
 - (e) SIS + tellurite + YE + Tp (positive control plate).Make sure the order of replica plating is the order listed above! Why?
5. Incubate one plate of SIS + YE + Tp anaerobically at 30°C under a incandescent light. Incubate all other plates aerobically in the dark at 30°C for 5 days.
6. Count the number of colony forming units (CFU) in the original 2.4.1 culture and the number of Tp-resistant CFUs to calculate the frequency of transposition. (Remember to take dilutions into account.)

Period 3: Identification and Analysis of Mutants

1. Identify the various mutants. The first step is to verify that the positive control for the experiment worked. Compare the positive control plate to the master plate. If you observe the same number of colonies in the same pattern, you may proceed. Otherwise, note the colonies that did not transfer to the positive control and do not count them in the subsequent analysis.
2. To detect pigment mutants, look for colonies on the two SIS + YE + Tp plates that are a different color than most of the colonies on the plate. If you like, wild-type cells from the viable plate count can also be used for comparison. Note the total number of pigment mutants.
3. To detect histidine auxotrophs (strains that now require histidine to grow) compare the SIS + Tp plate to the SIS + histidine + Tp plate. Any mutants that were able to form colonies on the plate containing histidine, but not on the minimal plate are histidine auxotrophs.
4. Using the same method as in step 3, determine the number of B12 auxotrophs.
5. Determine the total number of auxotrophs. (*Hint:* Compare the number of colonies on the SIS + Tp plate to the SIS + YE + Tp plate grown aerobically.)
6. Observe the number of photosynthetic-minus mutants by comparing the two SIS + YE + Tp plates. Organisms able to grow on the aerobic plate, but not on the plate incubated anaerobically in the light, are photosynthetic mutants.
7. Compile the results (a table is most convenient) and hand it in to your instructor. Be sure to calculate:
 - (a) Viable plate count for 2.4.1.
 - (b) The number of cells receiving a transposon.
 - (c) The number of the transposon containing strains that are pigment mutants.
 - (d) The number of mutants that were histidine auxotrophs.
 - (e) The number of mutants that were B12 auxotrophs.
 - (f) Total number of auxotrophs.
 - (g) The number that are unable to grow photosynthetically.
8. Class data will be annotated and handed back to you in the next lab period.

Literature Cited

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APPENDIX A
Sample Class Data and Example Calculations

Table 3.2. Class data (Spring Semester 1992).

Student group	VPC for <i>R. sphaeroides</i> (CFU/ml)	Total # transpositions	# pigment mutants	# photo minus	B12 auxotrophs	Total auxotrophs
1	2.80E+09	48.00	1.00	5.00	2.00	1.00
2	7.20E+09	66.00	1.00	6.00	2.00	6.00
3	4.30E+10	160.00	5.00	2.00	6.00	10.00
4	4.70E+09	50.00	0.00	5.00	3.00	3.00
5	7.70E+09	227.00	1.00	8.00	4.00	3.00
6	3.70E+08	107.00	1.00	19.00	4.00	5.00
7	1.00E+09	9.00	1.00	8.00	7.00	6.00
8	2.60E+07	14.00	6.00	0.00	2.00	15.00
9	1.09E+09	2.00	0.00	0.00	0.00	2.00
10	3.27E+10	10.00	3.00	0.00	3.00	2.00
11	1.80E+09	9.00	0.00	0.00	6.00	6.00
12	3.70E+09	66.00	4.00	9.00	0.00	4.00
13	2.03E+09	55.00	0.00	0.00	19.00	32.00
14	6.99E+09	11.00	0.00	11.00	0.00	7.00
15	1.73E+09	42.00	0.00	0.00	0.00	1.00
16	4.40E+08	102.00	1.00	25.00	1.00	1.00
Total or average	7.63E+09	978.00	24.00	98.00	59.00	104.00

Example Calculations

Viable Plate Count for *R. sphaeroides* 2.4.1

The concentration of bacterial cells in the original culture is calculated from the dilution plating onto non-selective medium (SIS + YE). The total dilution is calculated, its inverse is taken and then multiplied by the number of colonies on the plate:

55 colonies were observed when 0.1 ml of the 10^{-7} dilution was plated. The total dilution is: $1 \times 10^{-7} \times 0.1 \text{ ml plated} = 1 \times 10^{-8}$. The inverse of this is 1×10^8 .

Colony forming units per ml (CFU/ml) are calculated by multiplying the dilution factor by the number of colonies: $55 \times (1 \times 10^8) = 55 \times 10^8$ or 5.5×10^9 CFU/ml.

Frequency of Transposon Mutagenesis

This is defined as the number of transposon mutants per ml divided by the total number of viable *R. sphaeroides*. The previous calculation provided the CFU/ml, here the number of transposon per ml is obtained.

1. Calculate the number of colonies per plate:

$$\frac{978 \text{ transposon mutants}}{4 \text{ plates per student} \times 16 \text{ student pairs}}$$

2. Determine the number of transposon mutants per ml of culture:

$$15.3 \times \frac{1}{0.12 \text{ ml}} \times \frac{0.15 \text{ ml}}{1.5 \text{ ml}} = 12.7 \text{ transposon mutants per ml}$$

3. Divide by the total number of viable cells:

$$\frac{12.7}{7.63 \times 10^9} = 1.67 \times 10^{-9} \text{ transposon mutants/viable plate count (VPC)}$$

Percentage of Transposon Mutant Phenotypes

This number can be calculated for any of the phenotypes that are detected. An example for the percentage of pigment mutants is given below.

$$\frac{24}{973} \times 100 = 2.46\%$$

APPENDIX B

Preparations of Media and Equipment

Replica Plating Supplies

Velveteen

Velvets can be home-made for about 75¢ each. The velveteen fabric must be 100% cotton and can be purchased at a local fabric store. Red, blue, purple, and black velveteen seem to work better than other colors. The fabric should be washed three times, once with less than 0.5 ml of mild liquid detergent (we use Ivory Snow) and the next two times with no detergent. Next, dry the fabric. (See below for specific washing instructions.) The velvet is cut into 15 × 15 cm squares and the edges sewed to prevent fraying. We paid a dressmaker in our area 50¢ a square to do this.

Washing and Sterilization

Velvets are washed in a regular washing machine. (Be careful!! The dye in the fabric runs so use an old machine if possible). A very small amount of detergent is added. This prevents accumulation of detergent which may kill cells by lysis. The velvets are re-washed to remove any traces of detergent and then sterilized.

1. Place the velvets into a washing machine and add less than 0.5 ml of Ivory Snow detergent. Run through one washing cycle on the cotton setting (this is the setting that we use, I don't know if it's critical).
2. Re-wash the velvets without adding detergent.
3. Dry the velvets in a drier. Again, I would use a dedicated machine in the department or one you don't care about. The dye runs and stains the machine.
4. Stack the velvets with the velvet side face down on a piece of aluminum foil big enough to cover them. Each aluminum wrap should contain 10–20 velvets. Wrap the aluminum foil around the velvets when you are done.
5. Autoclave the aluminum packets for 15 minutes at 17–20 pounds per square inch.
6. To dry the sterile velvets, place them in an oven overnight at 77°C (170°F).
7. Velvets prepared this way can be stored for many months before use.

Replica Platers

Many designs are possible. The only essential thing is that the diameter of the plater plus velvet should fit just inside a petri plate. We use plexiglas. Details of the construction of this apparatus are available on request from the authors.

Preservation and Growth of Bacterial Strains

***Rhodobacter sphaeroides* 2.4.1**

R. sphaeroides is preserved by mixing 0.95 ml of a turbid culture with 0.05 ml DMSO and storing at -80°C in a small screw cap vial. About 1.5 weeks before the lab is to be run, a crystal of the stock culture is streaked for isolated colonies onto SIS + YE agar and incubated for 3–5 days at 30°C. Three days before the experiment, an isolated colony from the SIS + YE plate is inoculated into 50 ml of SIS + YE broth and incubated on a shaker for 2 days at 30°C. The day before the experiment, inoculate 1 ml of this stock culture into 50 ml of SIS + YE broth in a shake flask. It is critical to get the cells in early log phase (a moderately

turbid culture) to have a successful mating. If the culture is too turbid, it can be diluted 1:1 with fresh, warm (30°C), SIS + YE broth a few hours before class starts and then incubated.

E. coli S17-1A

S17-1A can be preserved in an identical manner to *R. sphaeroides* 2.4.1 or it can be kept in a screw-cap vial containing 0.3X LB agar (no ampicillin) at room temperature. However, this strain must be grown up in medium containing ampicillin at 50 µg/ml. Streak out the stock culture onto LB + 50 µg/ml ampicillin agar plates two or more days before the experiment. The day before the experiment inoculate an isolated colony from the LB-amp plate into 50 ml of LB + 50 µg/ml ampicillin in a flask and incubate at 37°C with shaking. Two hours before class, dilute the culture 1:10 into fresh, warm, (37°C) LB + 50 µg/ml ampicillin broth and keep shaking until class. This dilution before class ensures that S17-1A is in log phase. *E. coli* has the highest mating efficiency while in log phase. Dispense cultures just before class or have students dispense their own culture.

Media Recipes

The following recipe tables list the sources of the reagents used in the media formulations. The source of each component is not critical — this information is provided to help the instructor locate needed reagents. One thing that is important for the Sistrom's Minimal Medium is the use of tap distilled water. Do not use double distilled water. Something is removed by this treatment that slows the growth of *R. sphaeroides*.

Table 3.3. Sistrom's Minimal Medium, 10X.

Compound	g/liter	Source
K ₂ HPO ₄	34.8	Columbus Chem. Indust.
(NH ₄) ₂ SO ₄	5.0	Malinkroft
Succinic acid	40.0	Sigma
Glutamic acid	1.0	Sigma
Aspartic acid	0.4	Sigma
NaCl	5.0	Malinkroft
Nitriloacetic acid	2.0	Sigma
MgSO ₄ ·7H ₂ O	3.0	Malinkroft
CaCl ₂ ·2H ₂ O	0.33	Malinkroft
FeSO ₄ ·7H ₂ O	0.02	Malinkroft
Trace elements solution	1.0 ml	
Vitamin solution	1.0 ml	
Distilled H ₂ O	950 ml	

Table 3.4. Trace elements solution.

Compound	g/100 ml	Source
EDTA tetrasodium salt	1.765	Sigma
ZnSO ₄ .7H ₂ O	10.95	Merck
FeSO ₄ .7H ₂ O	5.0	Fisher
MnSO ₄ .H ₂ O	1.54	Malinkroft
CuSO ₄ .5H ₂ O	0.392	Malinkroft
Co(NO ₃) ₂ .6H ₂ O	0.248	Baker
H ₃ BO ₃	0.114	Malinkroft

Table 3.5. Vitamin solution.

Compound	g/100 ml	Source
Nicotinic acid	1.0	Sigma
Thiamin HCl	0.5	Sigma
Biotin	0.01	Sigma

Preparation of Siström's Minimal Medium

Mix all ingredients and place in two 1-liter bottles. Do not autoclave, store in a freezer. To make medium, add 1 part 10X Siström's to 9 parts distilled water. Adjust pH to 7.0 and autoclave.

Additions:

- If Yeast Extract is required add 1 g/liter and autoclave.
- If agar is required use 20 g/liter then autoclave.
- For B12 plates: Prepare a B12 stock solution by adding 2 mg of B12 to 100 ml of distilled H₂O and autoclave. After sterilizing the Siström's Medium, cool it in a 50°C water bath and add 1 ml of the B12 stock solution per liter of medium.
- For histidine plates: Prepare a histidine stock by dissolving 0.31 g histidine in 100 ml of distilled H₂O and sterilize. After sterilizing the Siström's Medium, cool it in a 50°C water bath and add 5 ml of the histidine stock solution per liter of medium.

Table 3.6. Other additions to the Siström's Minimal Medium.

Component	Stock concentration	Working concentration	Storage	Sterilization	Source
Tellurite	25 mg/ml in dH ₂ O	50 µg/ml	Room temp	None needed	
Trimethoprim	30 mg/ml in dimethyl formamide	30 µg/ml	-20°C	None needed	Sigma
Ampicillin*	50 mg/ml in dH ₂ O	50 µg/ml	-20°C	Filter sterilize	Sigma

* This is used in the LB medium for growth of *E. coli* S17-1A.

Table 3.7. Luria-Bertani Broth (for growth of *E. coli*).

Compound	g/liter	Source
Bacto-tryptone	10	Difco
Bacto-yeast extract	5	Difco
NaCl	10	
Adjust pH to 7.0		

Luria-Bertani Agar: To the broth recipe in Table 3.7 add 15 g/liter Bacto-agar and autoclave.