Biology 163 Laboratory

Prodigiosin Production in *Serratia marcescens*

(Revised Fall 2008)

Introduction

Cellular production of biochemical compounds is often accomplished through multi-step biosynthetic pathways in which intermediate compounds are produced and modified in order to create a final product. The pathway begins with a precursor molecule (or molecules) derived either from the environment or some other biosynthetic pathway. Enzymes then catalyze the conversion of the precursor molecules into intermediates, which may in turn be converted any number of times until the final product is produced (Figure 1).

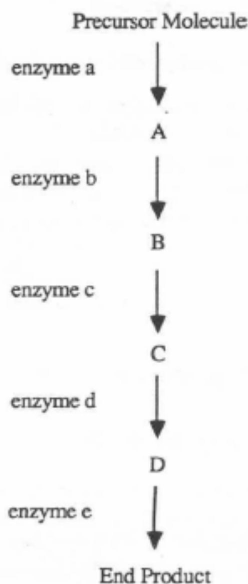

Figure 1. Hypothetical biochemical pathway showing substrates, genes, and enzymes.

The enzymes catalyzing each step of the pathways are typically specific to that step. As proteins, the "blueprint" for each enzyme is contained in the genetic code (DNA) of the cell. Many different mechanisms may be employed to control the activation of the genes responsible for enzyme production.

Defects (mutations) in the genes for a particular enzyme can result in an enzyme being non-functional. In such cases, the biosynthetic pathway is "blocked" at the point of the non-functional enzyme, and is unable to proceed to the final product. Organisms with a fully functional pathway (i.e., able to produce the final product) are referred to as the *wild-type* or *prototroph*. Those that are unable to complete the pathway as a result of mutations in one or more of the genes coding for the necessary enzymes are called *auxotrophs*. Figure 2 shows a sample four-step biosynthetic pathway for a wild-type strain as well as four auxotrophic strains, each blocked at a different point in the pathway. Since the final product is often responsible for regulating (i.e., turning off) the pathway via *feedback inhibition*, blocked pathways will often continue to produce large quantities of the intermediate molecule utilized in the blocked step.

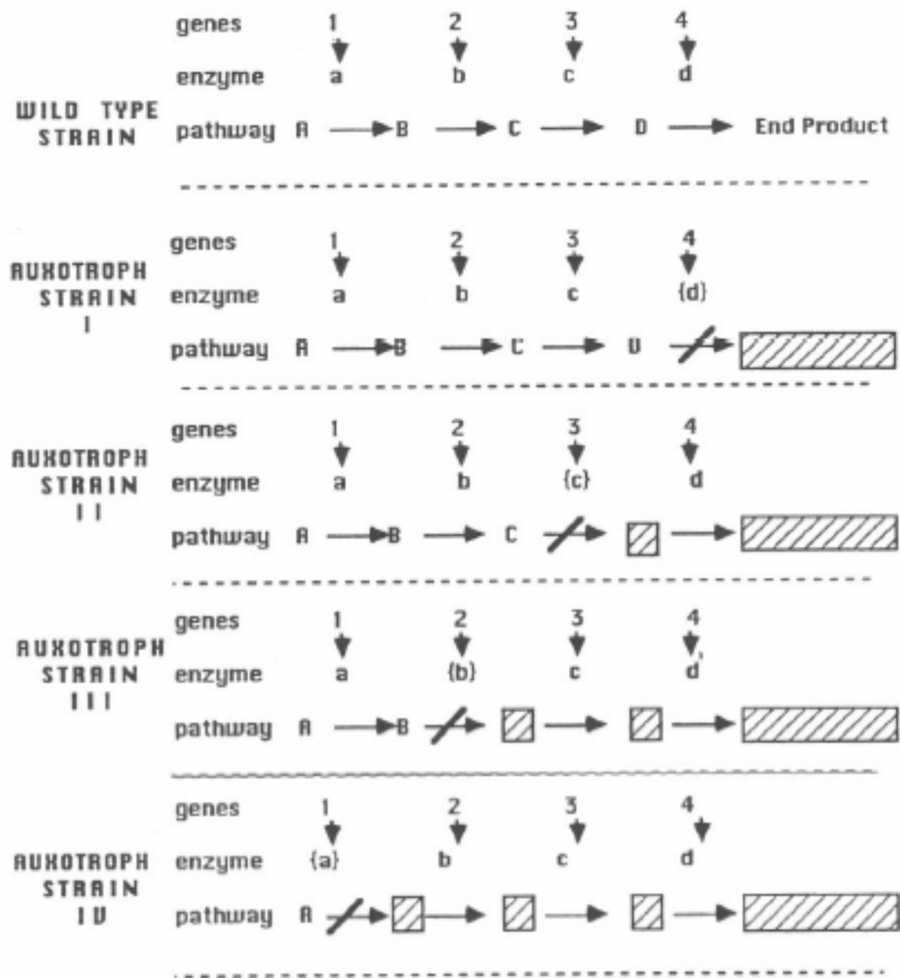

Figure 2. Four auxotrophic strains (I–IV) resulting from blocks in a simple linear biochemical pathway. Each block results from a mutation in one of the four genes controlling the pathway.

Deducing Biosynthetic Pathways

The structure of a particular biosynthetic pathway can often be deduced through the use of different auxotrophs. Recall that wild-type strains regulate their pathways via feedback inhibition--the build-up of the end product eventually shuts down the pathways. The inability of auxotrophic strains to produce an end product therefore has two important consequences:

- Lack of feedback inhibition means the pathway operates continuously (but cannot be completed).
- Lack of a functional enzyme means the intermediate compound produced just prior to the blocked step will accumulate in excess and eventually be secreted by the cell.

Examine Figure 2 again and determine which substance would be secreted for each auxotrophic strain, for example, Strain I would secrete substance D. Notice that Strains II, III, and IV are normal for enzyme d and would be able to convert precursor D to the end product if it were supplied. Because of this fact, if Strain II were grown on the same medium as Strain I, it would be able to utilize secreted precursor D from Strain I to produce end product and thus grow normally (the same is true for Strains III and IV). This principle is the reason for one of the general rules for elucidation of a biochemical pathway:

Auxotrophic strains with blocks earlier in the biosynthetic pathway will be "fed" by any strains blocked later in the pathway.

and, stated another way:

Auxotrophic strains with blocks later in the biosynthetic pathway will be able to "feed" all strains with blocks earlier in the pathway.

Branched Pathways

To this point, we have only considered linear biosynthetic pathways. However, pathways may also be branched. Figure 3 shows a branched pathway in which a single precursor is split by an enzyme to produce B and C, which are then individually converted by separate enzymes to D and E. Intermediates D and E are joined by an enzyme to produce intermediate F. F is converted to G and G is changed into the end product. A branched pathway may also begin with two separate precursors.

In Figure 3 the strains are labeled I–VI and are each blocked at a single step. Strain II cannot convert compound B to D but all other enzymes in the pathway are fully functional. This means that compound B accumulates in the medium because it cannot be converted. Notice that E must be combined with D to form intermediate F, this step cannot happen because Strain II cannot produce D. Because of this, intermediate E also builds up and is secreted into the medium. Similarly, Strain III can convert B to D, but is blocked in the C to E step. Strain III therefore secretes both C and D. In this circumstance, Strain II will feed intermediate E to Strain III, and Strain III will feed intermediate D to strain II. This is the principle behind a second important rule for elucidating a biosynthetic pathway:

If a pathway is branched, “mutual feeding” will occur between all strains with blocks on opposite branches of the pathway.

Note that in the case of mutual feeding, it is not the build up of intermediate *before* the block that is important, but rather the build up of intermediate *at the end of the UNBLOCKED branch.*

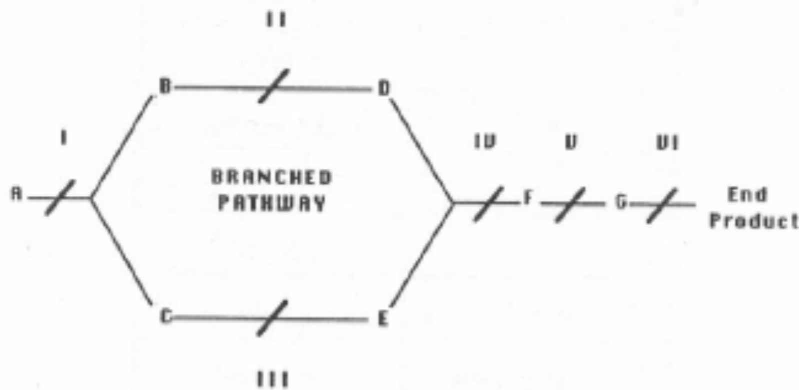

Figure 3. A representation of a simple branched biochemical pathway. Each auxotrophic strain is a result of a single mutation causing a non-functional enzyme at a single step; all other steps are functional. Pathway substances are represented by the letters A–G.

Make certain that you understand how the information in Table 1 relates to the diagram in Figure 3. This is a good time to test whether you understand the principles behind the four rules of the explanatory system.

Table 1. Important features of the six auxotrophic strains in the pathway shown in Figure 3.

Auxotroph strain	Substance(s) secreted due to block	Recipient strain	Donor strains
I	A	None	II, III, IV, V, VI
II	B, E	I, III	III, IV, V, VI
III	C, D	I, II	II, IV, V, VI
IV	D, E	I, II, III	V, VI
V	F	I, II,III,IV	VI
VI	G	I, II, III, IV, V	None

Prodigiosin Synthesis in *Serratia marcescens*

In this laboratory exercise you study a biochemical pathway that produces an end product called prodigiosin. This is a deep red pigment found within the bacterium *Serratia marcescens* and causes the wild-type colonies to exhibit a deep red color. You will study a number of auxotroph strains of *Serratia*, each blocked at a different step within the pathway. The auxotroph strains were produced by ultraviolet light treatment of the wild-type. Since the auxotroph strains are not able to produce prodigiosin, the color of their colonies will <u>differ from</u> the deep red color of the wild-type colonies.

You will perform pair-wise feeding trials to determine which secreted intermediates will allow completion of the prodigiosin pathway. Because of the nature of this pathway, the procedure is quite simple. Auxotrophic strains are able to grow and produce colonies that differ from wild-type only in coloration. If "feeding" occurs, the auxotrophic strain being fed will develop the deep red wild-type color. Careful examination of each pair-wise trial and comparison with coloration of the strains as they grow alone will enable you to elucidate this pathway.

Lab Research Study

You will conduct pair-wise feeding trials using three auxotrophic strains of *S. marcescens*. Auxotrophic strains are able to grow and produce colonies that differ from the wild-type only in coloration. If "feeding" occurs, the auxotrophic strain being fed will develop the deep red wild-type color. Careful examination of each pair-wise trial (and comparison with coloration of the strains as they grow alone) will enable you to deduce the structure of the prodigiosin biosynthetic pathway, as well as determine where in the pathway each mutant strain is blocked.

Procedure

This lab provides good experience in utilizing sterile procedure in order to produce plates that are not contaminated with other bacteria or fungi. <u>*Serratia* can cause skin and eye irritations so use care in handling and wash your hands when finished.</u>

Work in groups as assigned by you lab instructor. Each group will need the following materials:

--Stock plates of mutant *S. marcescens* auxotroph strains
--Stock plate of *S. marcescens* wild-type (prototroph) strain
--Sterile peptone-glycerol agar plates
--EtOH, 95% (for sterilizing work space)
--Marking pen
--Sterile, disposable inoculating loops

1) Disinfect your work area by wiping down the surface with a paper towel soaked in 95% EtOH.

2) Each of the auxotrophic strains will be plated next to each other in pair-wise combinations. Label these **feeding trial** plates on the <u>bottom</u> to indicate which strains will be plated, one on each side. Write the date in the center bottom edge of the plate and indicate your group using the initials of the group members (Figure 4).

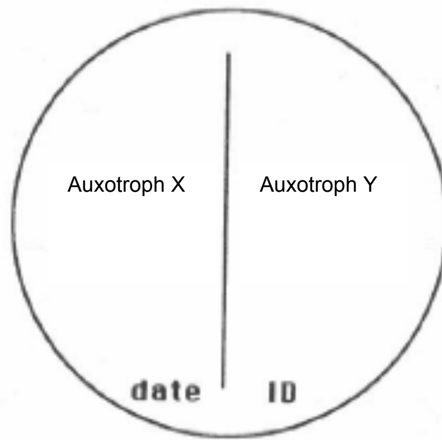

Figure 4. Before plating, make sure the bottom of your plate is carefully labelled with strains, date, and group identification.

3) In addition to the feeding trial plates, the auxotroph strains will be plated individually to serve as reference plates so that you can observe color development of these strains as they grow without being influenced by metabolites from the other strains. Because pigment color changes over time, it is important to compare your feeding trial plates with the reference plates.

4) All group members should participate in setting up feeding trial plates. To do this:
    a) Draw a line bisecting the center of each plate and perpendicular to the date at the bottom. The narrow end of the streak marks should be at the bottom above the date. See Figure 5 for an example.
    b) Have the two stock plates you will be using nearby.
    c) Procure a sterile inoculating loop from the package provided.
    d) Lift the lid of the stock plate and scrape the loop over the surface of the bacteria to gather some up (only a very small amount is needed). Try to tilt the lid up as little as possible and keep it over the plate.
    e) Using Figure 5 as a template, quickly transfer the inoculum to the feeding trial plate. Lift the lid only enough to insert the loop and carefully move the loop according to the template to form one-half of the "double L" pattern on the trial plate.
    f) Dispose of your loop and obtain a new one. Follow steps d) and e) again to transfer the second strain to your feeding trial plate. Note the two strains should be plated close, but not touching. Also note that you will not be able to see the inoculum you have plated--when plating bacteria, a little goes a long way!
    g) Continue with the procedure until all feeding trial plates have been streaked.

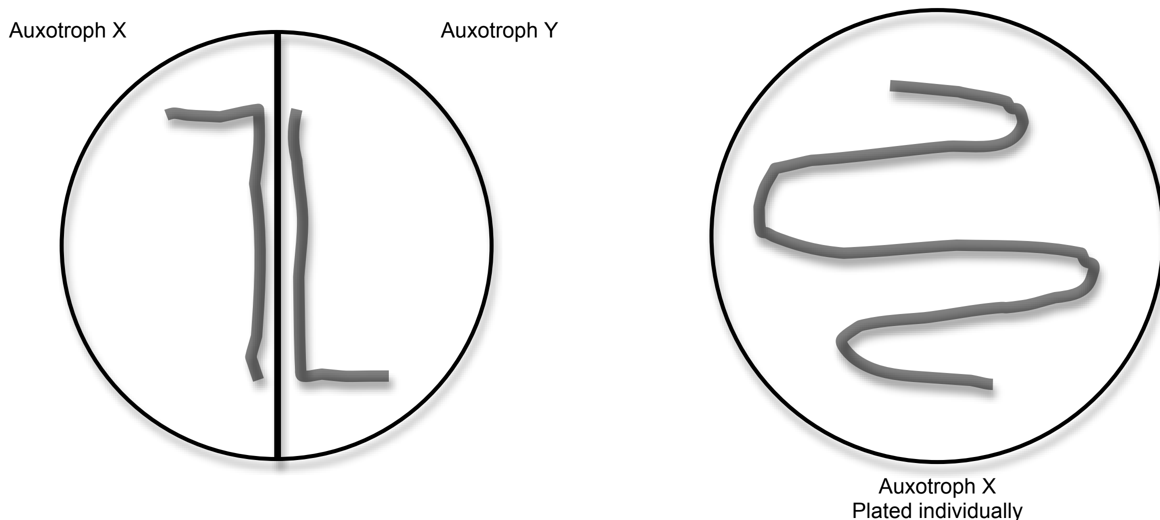

Figure 5. Examples of streak patterns for pair-wise "feeding" trials (left) and the color reference plates (right).

5) Prepare a color reference plate for each auxotrophic strain and for the wild-type strain. Use the plating pattern shown in Figure 5.

6) Store your prepared plates for incubation as directed by your lab instructor.

Analysis and Interpretation (Week 2)

In the previous laboratory session, you set up three feeding trials with auxotrophic (mutant) strains of *S. marcescens* in an effort to determine the structure and sequence of the biosynthetic pathway responsible for the production of the red pigment prodigiosin.

You also prepared four reference cultures from which you could determine the normal appearance of wild type and auxotrophic *S. marcescens* colonies not subjected to feeding from other strains.

Carefully examine the colonies from your three feeding trials to determine which auxotrophs show the formation of prodigiosin, and which do not. Record your observations in Table 1.

When making your observations, keep in mind that the distribution of prodigiosin production may vary among different colonies. For example, some cultures may produce prodigiosin uniformly, while in others it is only evident along the edge of the colony. Make a note of any such differences you observe.

Table 1. Results of feeding trials using three auxotrophic strains of *S. marcescens.* Use a plus sign (+) to indicate prodigiosin was formed, and a minus sign (-) to indicate it was not.

TRIAL 1 (I - II)	
Auxo. I	Auxo. II

TRIAL 2 (I - III)	
Auxo. I	Auxo. III

TRIAL 3 (II - III)	
Auxo. II	Auxo. III

To help guide your analysis, answer the following questions for each of your three feeding trials:

1) Is there evidence of feeding? If so, identify the donor strain(s) and the recipient strain(s). Is mutual feeding taking place?

2) What do your answers to question one tell you about the relative positions of the mutations in the prodigiosin biosynthetic pathway? (You will need to consider the "rules" presented in part one of this exercise.)

Use your answers to the above questions to construct a figure of the prodigiosin biosynthetic pathway. Be sure to clearly indicate all relevant intermediates (A, B, C, etc.) *and* indicate where in the pathway each auxotroph (I, II, III) is blocked.

Lastly, look back at the distribution of prodigiosin production noted in your feeding trials. What do these differences suggest about the chemical nature of the individual intermediates? Can you determine which intermediates are *soluble* and which are *volatile*?

References

This exercise adapted in large part from:
Schmidt, E. V. 1993. Genetic control of cell chemistry using *Serratia marcescens*. Pages 21-34, *in* Tested studies for laboratory teaching, Volume 14 (C. A. Goldman, Editor). Proceedings of the 14th Workshop/Conference of the Association for Biology Laboratory Education (ABLE), 240 pages.