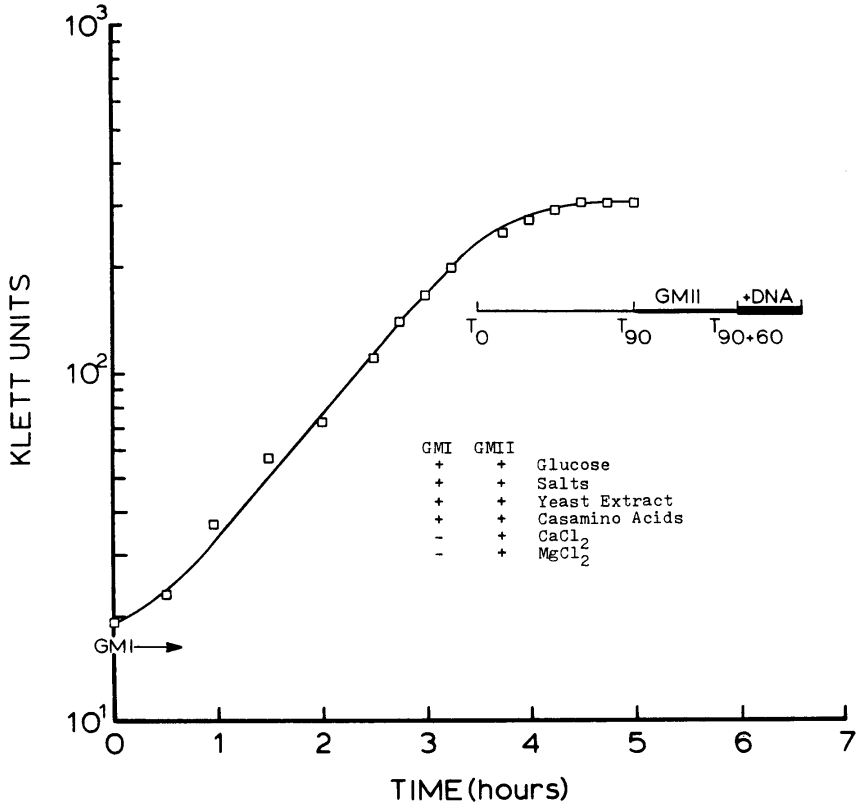
Experiment 10

Transformation of Plasmids pRIT4501 and pRIT4502 into *Bacillus subtilis*

Plasmids pRIT4501 and pRIT4502 were created by fusing the *E. coli* plasmid pUC9 with the *B. subtilis* plasmid pPL608. The recombinants therefore have origins of replication for both species and should, therefore, replicate in both hosts. pRIT4501 and pRIT4502 also contain genes for both organisms: ampicillin resistance from *E. coli* and chloramphenicol resistance and kanamycin resistance from *B. subtilis*. In this experiment, we will transform the plasmids into *B. subtilis* and in Experiment 10 we will test for heterologous gene expression in each organism.

B. subtilis, unlike *E. coli*, is naturally competent and should not have to be forced to take up exogenous DNA. However, not all of the cells in a culture are competent at any one time.. Several growth regiments have been devised for optimizing competence. In the method, which we will use, cells are grown in growth medium I (GM I) until the very end of logarithmic growth. Growth is followed by measuring optical density with a spectrophotometer (in the figure below, a Klett-Summerson spectrophotometer was used, hence the units). The cells are allowed to grown for 90 minutes past the time at which they enter stationary phase. At the end of this 90-minute period, they are diluted into GM II (GM I + Ca^{++} and Mg^{++}). Growth is continued in GM II for an additional 60 minutes. At this point, DNA is added to the cells and a 30-minute incubation is allowed for adsorption and uptake. Yeast extract is then added to shock the cells out of competence, and the cells are grown for 1 hour prior to plating on medium that will select for transformants. This final incubation is to allow time for expression of the newly acquired genes so that the cells will not die immediately when plated on selective medium.



The Lab Before

Prepare 500 ml of the following plates:

LB
LB + chloramphenicol (10 μg/m)

The Day Before

1. Inoculate cells into 10 ml of GM 1 in a side-arm flask.
2. Tilt the flask so that the cells are in the arm and incubate standing over night at 27°.

Procedure

1. Measure the optical density of the cells and record the value on a semi-log plot of "OD vs. Time." Tip the flask so that the cells run out of the arm and into the main flask. Transfer the flask to a 37º shaking incubator.
2. Early in the growth cycle, measure the OD every 30 minutes. Once the cells reach log phase, OD should be measured every 15 minutes. Keep a running graph as you follow growth. It is important to determine as precisely as possible the time when the cells leave log phase and enter stationary phase.

3. Allow the cells to incubate for 90 minutes from the time when they enter stationary phase. You won't actually know that this has occurred until about 30 minutes after the fact when you can feel certain that the graph has leveled off. You can count the time between reaching the inflection point. and the time that you realize that it is past as part of the 90 minutes.

3. At T = 90, withdraw 1 ml of cells and transfer to a second flask containing 9 ml of GM II. Incubate for a further 60 minutes.

4. Transfer 0.9 ml of cells to each of four tubes and add 100 µl of DNA as indicated. Incubate for 30 minutes.

   Tube 1 No DNA
   Tube 2 pPL608
   Tube 3 pRIT4501
   Tube 4 pRIT4502

5. Some people add 50 µl of 10% yeast extract and then incubate for an additional 60 minutes. This apparently shocks the cells out of competence and helps the cells to survive the competence regimen better. Sometimes I omit this step if I am pressed for time.

6. Serially dilute the cells using BHI broth as a diluent and plate as follows:

   LB + chloramphenicol: 10^{-1}, 10^{-2}, 10^{-3}
   LB: 10^{-6}, 10^{-7}

7. Invert the plates and incubate at 37º over night.

8. On the following day, recover your plates and count the colonies and record the numbers in your notebook. Save the colonies for use in Experiment 11.

Preparation for Experiment 10

Prepare two sets of plates as described below: You should have four plates of each. If you wish, you may collaborate with other groups to share the work.

E. coli Series	*B. subtilis* Series
+ no antibiotic	+ no antibiotic
+ ampicillin (50 μg/ml)	+ ampicillin (10 μg/ml)
+ chloramphenicol (50 μg/ml)	+ chloramphenicol (10 μg/ml)
+ kanamycin (50 μg/ml)	+ kanamycin (10 μg/ml)

10X Bacillus Salts

$(NH_4)_2SO_4$	20 g
K_2HPO_4	140 g
KH_2PO_4	60 g
trisodium citrate.$2H_2O$	10 g
$MgSO_4.7H_20$	2 g

GM I

50% glucose	1.0 ml
10X *Bacillus* salts	10.0 ml
10% yeast extract	1.0 ml
5?% casein hydrolysate	0,8 ml
1% required amino acids (50 μg/ml final conc.)…….	0.5 ml
water	90.0 ml

GM II

GM I	100.0 ml
0.1 M $MgCl_2$	2.5 ml
0.05 M $CaCl_2$	1.0 ml