Seminar Minutes of 10-7-74

I. Dusko: Discussion of Ectopic Insertion in Transformation Experiments
The donor used was B. subtilis wild type Aro_2^+ $Tryp^+$ His^+ $Tyro^+$ and the recipient, B. subtilis Aro_2^- $Tryp^-$ His^- $Tyro^-$ made competent.

1. In experiment I the donor DNA was degraded with R_I endonuclease, and the DNA fragments were separated by a sucrose gradient. The various donor fragments were incubated with the recipient for Transformation, selection being made for the + markers of donor DNA. Three colonies out of 100 had all markers +. They differed in the following manner:
   Colony #1 showed no Tryp-Aro_2 linkage. The linkage between the markers Tryp and Aro_2 was completely broken.
   Colony #2 had the Tryp and Aro linked to about 5%, and the Tryp-His and Tryp-Tyro to about 15%. The Aro_2-His-Tyro linkage appeared normal.
   Colony #3 showed 100% linkage of Aro_2-Tryp-His-Tyro. Regardless of which one of the four markers was selected, all the others were present.
2. In experiment II the extracted donor DNA was degraded by R_I, and the DNA fragments that carry the markers were separated by gel electrophoresis (instead of sucrose gradient). In Transformation, a colony was obtained that had 100% linkage between all markers Aro_2^+--------$Tyro^+$]
3. In experiment III the extracted B.s. DNA was treated with R_I without separation of the fragments at all. From the Transformants first there was selection for $Tryp^+$ only (plating without tryp). Then replica plates were made on which selection was for Aro_2^+ and $Tryp^+$, by using plates lacking phenylalanine and tryp. Three colonies out of about 200 had Aro_2^+ $Tryp^+$ (His and Tyro may have been + or -). Two colonies showed normal linkage between Tryp and Aro_2 (~70%) one colony showed 100% linkage between Tryp & Aro_2.
4. In experiment IV the R_I treated DNA was followed by gel separation of the fragments. Transformation was checked for Tryp Tyro (using plates lacking Tryp and Tyro). Replica plates without phenylalanine were made to check for colonies that could grow without phenylalanine., i.e., selection for Aro_2. Linkage was lower than about 20%, perhaps 0% (technical problems).
5. In experiment V there was R_I treatment of DNA without separation and the transformants were selected for the entire sequence Aro_2^+ $Tryp^+$ His^+ $Tyro^+$.
   Out of 260 colonies obtained, the transformation results were:

   Aro_2 Tryp His_2 Tyro

   155 →
   25 →
   73 →

   The breakage is three times as probable between Tryp and His as between His and Tyro. In terms of distance: Tryp — His — Tyro
   3 units, 1 unit
6. There could be a manifestation of some sort of end effect. Places that are sheared closer to Tyro are almost inactive.
7. A model: Aro_2 ___ Tryp ___ His
   ↑R_{I_1} ↑R_{I_2}
   R_{I_1} is cut more often than R_{I_2}; a cut at both sites does not happen concurrently.
   With R_I Tryp is normally not linked to Aro_2 but is linked to His & Tyro. Once His is introduced there is a linkage with Tyro. Perhaps there are secondary sites for R_I digestion - sites that can be cleaved between Tryp & His and between Aro_2 & Tryp, but usually are not.

8. There are different size DNA fragments after R_I TREATMENT AND THESE HAVE different transforming efficiencies. (Longer piece Aro_2-Tryp-His-Tyro, shorter piece with Aro_2-Tryp-His, and shortest piece with Tryp).
9. The most extensively tested colony showed the normal linkage between Aro_2-His-Tyro and very low linkage with Tryp (5% of Tryp to Aro_2 and 15% of Tryp to His & Tyro). How come Tryp is now out of it? How does this DNA behave as compared to standard DNA?
10. Is the R_I treatment the crucial variable? Some new markers may have crept into the tester stocks. What is the operational variable? Is the anomalous transformation result of R_I TREATment, the result of shearing down the DNA, & would it be obtained using untreated DNA?
11. If the DNA is prepared by methods other than R_I treatment, are the same results obtained; The DNA of daughter cells must be examined; what kind of colonies are produced from daughter cells? Use the same recipient with controls of sheared DNA and check linkage.
12. Search for the site to which Tryp may be transferred; pick up a new linkage to a clear cut marker. Check linkage in some other regions loosely linked to Tryp; if rearrangement of genes occurs, a new linkage group should be established.
13. Enzymes similar to R_I might be involved in recombination. There might be certain points at which integration can occur of DNA & not at other points.
14. E. coli is modified, but try using modificationless mutants of E. coli as DNA donor with B.s. which restricts but doesn't modify DNA. Also, take DNA from 20-30 random donor bacteria and see if these will transform B.s. recipient. The DNA should be treated with R_I BUT Not separated. If a transformant is obtained, see if R_I is necessary.
15. The idea of suppressor should be considered.
    a. $Tryp^-$_____$Tryp^+$ Ectopic Insertion

    b. $Tryp^-$_____X Suppression (the suppressor establishes a $Tryp^+$ phenotype)

    Transformation with a. as donor should sometimes give normal linkage (standard base sequence should pair at standard location because of homology). Transformation with b. as donor will never give normal linkage (no homology in standard location). Super-supressor can be tested by using a number of mutants. If there is a super-suppressor, one mutation could overide a large number of other mutations. Suppressor operates by modification t-RNA which enables it to suppress gene mutations, whereas ectopic insertion has the DNA go back in the wrong position.
16. Do homology tests to see if $Tryp^+$ marker can be gotten back into its standard location. Try various treatments of DNA -very small sheared, mechanically modified by other treatments - & compare with unsheared wild type DNA - to give transformation, & try to mimic this effect.
17. Prove that these anomalies of transformation are unique to R_I TREATED DNA or are there mutations in the stocks (small colonies of B.s. mutants that grow slowly on plates lacking phenylalanine). Is R_I doing something special about integration? Use the same procedure with normal DNA or try to show that in some cases with R_I treatment standard insertions are obtained (e.g., Aro_2 Tryp together). If so, then pursue the R_I treatment results.
18. Circles may be part of the answer. R_I treated DNA circularize, whereas standard DNA is linear.
19. The extopic insertion may really be a plasmid phenomenon. Look in the supernatant for a plasmid in B.s.; Use ethidium bromide fractionation & electron microscopy. A plasmid could explain 100% linkage. If it

is a plasmid, the negative phenotype Tryo$^-$ should be obtained by use of acridine orange which eliminates the plasmid. The plasmid may be episomal with alternate states. No-one has yet picked up plasmids in B.s. or any bacillus. Plasmids may be stabilized & eventually disappear & don't remain plasmids. Plasmids may integrate into the chromosome, be at different stages of integration, may integrate at different places along the chromosome, may be in cytoplasm or on chromosome or at different chromosomal sites at different times.

20. Standardize the procedure for DNA extraction so that there are no survivors of donor B.s. cells to interfere with transformation results. Try heating for 20 minutes at 65° instead of 10 minutes. Use a high melting paraffin, warmed to 70°, but do not heat the bath while heating the material; have the paraffin bath on a cooler surface to get a temperature gradient.
21. When all amino acids are supplied except phenylalanine, the growth is different for different kinds of strains of B.s. Four strains grow big on such plates, and four strains grow small (grow slowly). The inhibition is not obtained by any of the amino acid groups. Put in all groups of amino acids in the same technical manner; there may be an inadequate concentration of the material. Make up separate plates with the amino acids.

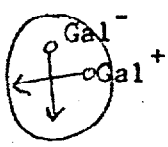
II.Hela: A. Arrival of five different bacilli from ATCC (American Type Culture Collection).

1. Assume that these are mixed cultures; do not purify this unseparated population. Give it an S.P. stock # & store in lyophile.
2. Then from each, make a single colony isolation, using nutrient agar, making sure to get a sporulating colony. When that looks pure take a single clone & give it a new S.P. # - a clonal isolate out of the S.P. stock #-.
3. Use this clone for experiments.
4. Distinguish the isolated clone from the received one.
5. Try crossing experiments. Use B.s. as recipient & grow it with these presumed prototrophs (using 0.1 ml of each parent) on minimal media with streptomycin, incubating for 48 hours. Use the highest concentration of streptomycin feasible because spontaneous mutants from the wild type at different levels of streptomycin resistance are found.
6. Check the nutrition of these bacilli, to see if they grow in Spizizen minimal within 24 hours.
7. As controls, use the two parents separately, using 0.2 ml of one parent.

B. Bacillus subtilis mutant utilizing galactose slowly (SB 19 mutant). These mutants show a very slow utilization of galactose on minimal media having galactose instead of glucose.

1. Using 10^8 cells of SB 19 mutant Gal$^-$ mixed with 10^2 SB 19 Gal$^+$ cells, no growth of either was detected. Maintaining SB 19 Gal$^+$ AT 10^2, and diluting the SB 19 Gal$^-$ to 10^7, it was still almost impossible to see Gal$^+$ CELLS. When Gal$^-$ was diluted to 10^6, both types of colonies grew and could visually be differentiated. The Gal$^+$ grow bigger and & faster & start sporulating, whereas the Gal$^-$ cells are smaller. Using 10^5 Gal$^-$ cells with the same 10^2 Gal$^+$ concentration, the difference is more apparent, & still more so when 10^3 Gal$^-$ cells are employed. The fewer Gal$^-$ cells used with the fixed amount of Gal$^+$ cells, the better the growth & the differentiation of the two types of cells.
2. Is the Gal$^-$ mutant making an inhibitor that doesn't permit the Gal$^+$ cells to grow? Transformation does not occur when a high density of Gal$^-$ cells are used. To test this, cross-streak the Gal$^-$ mutant that may be inhibiting against the Gal$^+$ on minimal media plates having galactose. If there is inhibition, depression of growth will occur.

If there is inhibition, there will be less of it the further apart the streaks.



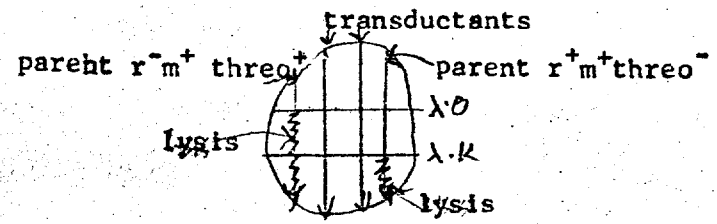
If inhibition occurs, it will be in the center where the streaks cross.

C. To get a mutant of SB 19 that will grow on lactose, lactose being the only C source.
   1. Used 0.5-5.0% lactose in Spizizen broth, the lactose being the only C source. This was followed from 24 hours - 6 days, and no change was observed except with 5% lactose. After 6 days, a little growth was seen using 5% lactose & no citrate.
   2. Spiz. broth minus both glucose & citrate resulted in no growth (no C source
   3. Spiz. broth minus citrate but with glucose resulted in growth.
   4. Spiz. broth with citrate minus glucose - no growth.
   5. In order to utilize lactose rapidly, citrate must be present in the broth.
   6. On agar plates, citrate alone without lactose provided growth in 24 hours. Citrate on agar is an adequate carbon source. Different concentrations of lactose will be tried on plates without glucose or citrate.
   7. Cells grow on agar with no carbon source other than citrate, which is a low level of C source. Citrate is also needed for chelation. Diminish the citrate to 1/10th its present level in the SO plates to see if this is a sufficient C source & sufficient for the metal need.

D. Study of Transfection Efficiency
   1. In performing transfection; the bacterial cell C600 r^+m^+ E. coli + UV inactivated T7 phage + DNA from non-inactivated T7 phage are used.
   2. T7 phage DNA of the UV inactivated phage may have a gene remaining functional (one of the first genes to enter the bacterial cell) which codes for an enzyme that inhibits the restriction system of E. coli.
   3. When cells were infected with UV inactivated phage after $CaCl_2$ treatment, there was no change of transfection efficiency when r^+m^+ competency treated cells were used. There was a decrease in transfection efficiency when r^-m^- competency treated cells were used.
   4. If cells were infected with UV inactivated phage before $CaCl_2$, there was an increase in transfection efficiency. Using a MOI (Multiplicity of Infection) of 2 and a low DNA concentration, $r^+ m^+$ cells infected before treatment showed a 13-fold increase of transfection efficiency , and r^-m^- cells a 5-fold increase.
   5. It is a possibility that whatever genetic information is destroyed by UV treatment of phage, is restored (rescued) by the DNA, also added to the cells in doing transfection = marker rescue.
   6. Try a lower MOI (less than 2) & irradiate for less time.
   7. What is needed to block restriction? Is it the expression of a gene, or the protein of phage, or something else?
   8. Try amber mutants; try using λ to knock out r^-m^-.
   9. There is a race between the restriction enzymes of the cell and the expression that blocks restriction.

III. Millie: To select for MO 671 r^-m^+ to use in future transfection & transformation experiments.
   1. MO671 (a strain of E. coli K12) originally is $r^+_k\ m^+_k$
      MO671 recB recC SbcB leu tryp$^-$ton B his$^-$ara thr$^-$thi lac mtl xyl gal pro arg str tsx sup-37 amber
      C600 r^-m^+ Ehreo$^+$ is $r^-_k\ m^+_k$ leu thi sup e44 lac tonA
   2. A transduction was done with phage P1 grown on E.coli C600 r^-m^+ threo$^+$. Plates with amino acids minus threonine, and with Vit. B_1 , maltose, and sodium citrate added which provide enhancing effects for transfection with P_1 were used.

3. The possible transductants obtainable were:
   a. threo$^+$ r$^-$ m$^+$ from the C600 parent
   b. MO threo$^+$ r$^-$ m$^+$, which is being sought
   c. revertants
4. EMB + lactose plates were streaked (2 parallel separated streaks) with loopfuls of λ vir.O (grown on parent C600 r$^-$m$^-$) and λ vir.K (grown on parent K r$^+$m$^+$). With a toothpick the transductants and the parents were streaked in the opposite direction.



Where lysis occurred (lysis occurs if host is r$^-$, having no restriction enzymes so that the phage DNA can be active; λ.O is not modified & therefore not protedted & so its DNA is destroyed by host restriction enzyme & the phage cannot replicate & cause lysis ; λ.K is modified-its bases methylated-from its former host & thereby is protected from restriction enzyme. It replicates and causes lysis.) the EMB changes color to a lighter purple. Several concentrations of the two phage were used in testing the transductants.

5. The first time the desired strain MO r$^-$m$^+$ was not obtained. Instead the first strain obtained was a threo$^+$r$^-$m$^+$ his$^+$ tryp$^+$ of C600.
6. The second time when the λ was titrated on the transductants, the desired MO 671 r$^-$m$^+$threo$^+$ was obtained as transductant.
7. Such a multi-marker strain with the r$^-$ mark is useful for transfection and transformation studies.

B.C.