Seminar Minutes of 10-21-74

I. Ron:

A. Discussion of the survival of linkage of the markers in Bacillus globigii DNA after R_I endonuclease digestion.

1. The recipient was a hybrid strain between B. globigii and B.s. The donor was B. globigii DNA.

2. Using the hybrid strain 6-2 as recipient, His_2 and Tyro cotransfer were tested after R_I digestion.

   a. It was only 19% of normal (low), if Tyr was the first selection and His_2 the second by replica plating.

   b. If His_2 was the primary selection and Tyr the second, then cotransfer was about 80%. There was thought to be a cut near His_2 somewhere.

3. A hybrid strain 38-1 that is $tryp^-$ tyr^- has been prepared, for use as recipient in transformation. The same competent cells were used for all these experiments.

   a. After transformation, selecting for Tryp first and then for cotransfer of Tryp-Tyro $\longrightarrow$ 67% cotransfer.

   b. Selecting for Tyro first and then for cotransfer of Tryp-Tyro $\longrightarrow$ 65% cotransfer. This is the same result as seen in B.s. co-transfer. Cotransfers were done by replica plating and by use of plates lacking both amino acids; results were the same.

   c. After R_I digestion of the DNA used in transformation, selection for Tryp first and then for cotransfer of Tryp-Tyro $\longrightarrow$ the cotransfer was lowered to 23%.

   d. After R_I digestion of the DNA, selection for Tyro first and then for cotransfer of Tryp-Tyro $\longrightarrow$ the cotransfer showed only 18% linkage. Linkage of Tryp and Tyro markers is preserved but is reduced by R_I digestion to about 1/3 its previous value.

   e. Using strain 6-2 as the recipient, selection for His first and then for cotransfer of His-Tyro $\longrightarrow$ a high co-transfer of 75-80% before R_I digestion, & the same value after R_I digestion of the DNA.

   f. Again using 6-2 as a recipient, selection for Tyro first and then for cotransfer of His-Tyro $\longrightarrow$ a cotransfer of 88% before R_I digestion, but a cotransfer of only 18% after R_I digestion of the DNA.

4. Implications of a.-f.:

   a. Some wierd polarity of His-Tyro (there is a difference in co-transfer depending upon which marker is first selected) effect possibly, though this should not be so, since Tryp-Tyr is symmetrical in crossing behavior (co-transfer behavior is the same regardless which marker is selected first).

   b. Do these results represent a disparity in the recombinant types or, most likely, a disparity in the way in which the selection was done?

   c. One is probably running into artifacts during the transformation and selection procedures.

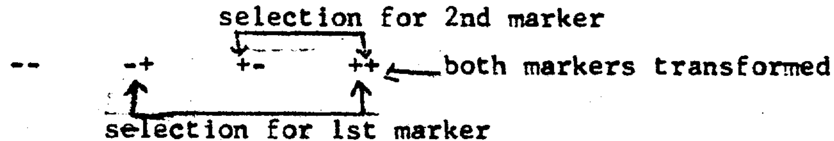
   d. Something is strange about the His marker. After R_I digestion, polarity is seen in the His co-transfer, (a difference in co-transfer depending upon which marker is first selected). The His-Tyro $\neq$ Tyro-His co-transfer frequency. His transformants are more than Tyr transformants, using R_I treated DNA not separated by electrophoresis.

5. Refer to the relative frequencies of transformation by the three modes of selection:

   a. Selection for one marker -+ (plate lacks one amino acid)

   b. Selection for other marker +- (plate lacks other amino acid)

   c. Selection for both markers ++ (plate lacks both amino acids)



$$\% \text{ of co-transfer} = \frac{++}{(+-) + (++)} = \frac{\text{incidence of co-transfer}}{\text{incid. of single transfer + of cotransf}}$$

$$\% \text{ co-transfer} = \frac{++}{(-+) + (++)} = \frac{\text{incidence of co-transfer}}{\text{incid. of single transfer + of cotransfe}}$$

Another value to measure is $\frac{++}{(+-) + (-+) + (++)}$

This has been done for Tryp-Tyr and no difference was obtained, i.e., the frequency of Tryp-Tyr double transformants was the same on +-, -+, and ++ plates. This has not yet been done for His-Tyr.

6. Polyphosphate has an augmenting effect on transformation. There is a several-fold increase in transformation, comparable to the helper effect with increase in DNA concentration (high DNA conc. → increase in co-transfer This may be a way to increase the competence of cells.

B. Continuation of the search for markers in B. subtilis DNA that survive R_I endonuclease treatment (i.e., that are resistant to R_I)

1. 5 markers have survived R_I treatment:

marker	% survival
tryp-tyro	5
his	10
uracil	7
methionine	60
adenine	8

2. lysine and glycine are completely destroyed after R_I treatment.

3. Other markers gave strange effects.

4. 5 markers are currently being tested.

5. Aro_2 cannot show its presence by transformation after R_I treatment, but can do so before such digestion; it is not destroyed but its activity is greatly decreased after R_I treatment.

6. Bands are 2-3 mm wide, but the gel is being cut in 1 mm slices.

   a. The Tyro marker location (determined by biological activity of transformation) is at mm 23 from the origin.

   b. His marker is at mm 39 but has transformants throughtout the spectrum = background.

   c. Aro_2 is not found; there is no transformant for phenylalanine after two days incubation of plates.

   d. Methionine is found at mm 32 → 99+ % enrichment.

7. Using whole DNA, get the enrichment ratio. After R_I and before gel separation calculate, for example, $\frac{His^+}{Meth^+}$ and $\frac{Meth^+}{His^+}$

   After gel separation, calculate the same frequencies in the respective peaks (in the His peak $\frac{His^+}{Meth^+}$ should now be much higher and in the Meth peak, $\frac{Meth^+}{His^+}$ should now be much higher).

   The ratio of the ratios after separation and before separation =

   $$\text{enrichment} = \frac{\text{the ratio after gel electrophoresis separation}}{\text{the ratio before gel electrophoresis separation}}$$

8. For Tryp. there is almost 100% separation of its biological activity. In Tryp-Tyr cotransfer there were 50 colonies/.05 ml from the peak. His_1 has a 99% separation. There were 1,200 colonies/.05 ml from the peak of the His_1 band gel slice (count of colonies = absolute # of transformants). Less than 1% His transformant colonies were elsewhere. Background of His is about 2-10/.05 ml.

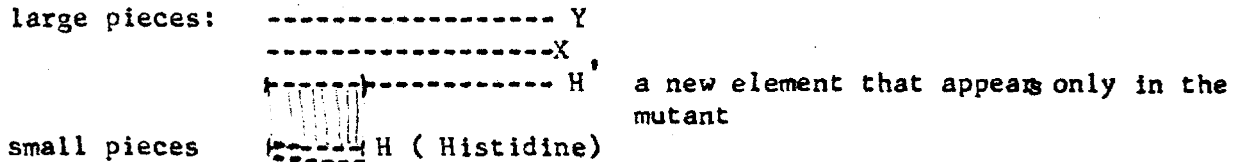
9. R_I treatment acts at specific sites. Shearing of DNA cuts randomly, Since it is the specific sites that are now being observed, the R_I may be destroying more of the activity of these specific sites than would sheering.

C. One rough test for a rough degree of purification of markers.

1. Adenine survives to a lower frequency than the other markers; it is not present in the first 2.5 cm.
2. The fractionation is good.
3. Look for size mutants - an increase in size of DNA fragment carrying a specific marker, caused by a mutation at an R_I site resulting in that cut not being cut.
4. Are there any colonies at the big end of the spectrum where not expected , or any shift; are there any colonies seen that could be the results of R_I site mutations (a change in a nucleotide so that the enzyme does not recognize that particular site).
5. Mutation may account for His_1 marker behavior; the His_1 may be in a hot spot.
6. Twice consecutively a major His peak and a minor His peak were found; the minor peak (less than 1 % of total) was found a few mm from tryp-tyr peak.
7. Extract the DNA from the expected mutants & pool the DNA. Look for the location of his_1, by locating the peak in gel electrophoresis. See if the His_1 peak can be moved over to where the minor peak is, due to a mutation in the R_1 site near His_1. Pick one colony ⟶ subculture (it may be a mutant). Pool 39 colonies ⟶ subculture (this provides a chance of finding a mutant).
8. His_1 has almost no reversion. Methionine seems very stable; it doesn't appear to have any revertants (the recipient negative marker stays negative and doesn't revert to positive).

9. Rationale for looking for mutants: It's a mapping tool; it corroborates the general picture of what is going on & in so doing, verifies the model. Any structural mutants that can be used to map the DNA have many uses.

10. Pool the R_I treated and gel-separated DNA over several intervals. For example, pool big peak His_1, then pool minor peak His_1 (which could be a major peak if a size mutant were found). What fraction of His (a short piece) will hybridize with the big band His?



A hypothetical example: A mutant is found that has histidine **activity** in a heavier band than is normally found on gels. Its DNA could then be isolated, R_I treated, & gel separated to get "pure" big mutant His molecules (H'). One could then hybridize the mutant "big His'" with the nonmutant "small His". Only a portion of the "big His'" will hybridize with the "small His" since this is a specific type of aggregation. When observed under the electron microscope the proportion of double stranded to single stranded DNA in the hybrid molecules can provide a measure of the His' length between the end of the double-stranded section & the next R_I cut - the end of the single-strand H', and also a measure of the His' length.

If a size mutant were found and its DNA isolated, R_I treated & separated on gels, there would be a change in the height of the H' band; also, the band for the small piece H would be missing or smaller. The DNA missing in the small piece should be in the big band. The absolute amount of DNA in the hybrid H' can be measured. How many fragments form one band?

11. Results may be confused by the ectopic problem.

12. Currently a way to measure the purity of the piece is being sought. Cut the gel into 1 mm pieces. Make counts of each band. How many counts per band, & how many per piece? This is a crude measure of purity.

Band	% Counts of H^3-DNA
Tryp-Tyr	3.7
Methionine	4.2
His	4.4
Uracil	3

It is estimated that there are about 5 different fragments of DNA per band. Most of the DNA is in 3 cm; the rest is spread over 5 cm. Try to check purity using Specific Activity (biological activity/physical activity = # transformants/ml/counts/min.). Rebanding is used to obtain further purification. With rebanding, the Specific Activity of the peak goes up.

13. Tryp is still the heaviest band - the first seen with biological activity; it is probably not yet pure. What is the E.M. length?

14. Do denaturation mapping, an analytical technique, to see how multi-modal the DNA of the band is (i.e., how many different fragments are in one band). Start with the whole DNA after R_I treatment. Then use the electrophoresis fractionated DNA for comparison. Use the differential melting point (Gan's procedure). Heat, cool rapidly (that which is denatured remains so & that which is not remains so), pass through a hydroxyapatite column to separate the fragments with different degrees of denaturation (A-T rich fragments denature at a different temperature than G-C rich fragments). Clean renaturability of the material after R_I corroborates its homogeneity in a given band. A good way to get rid of nicks is to melt and renature. That DNA which comes back to the original size is the good DNA.

15. Use CsCl fractionation & calculate the enrichment ratio; a given CsCl band achieves a given enrichment.

16. Make the gel electrophoresis a preparative method for use on a large scale.

17. The methionine marker (smaller than the Tryp marker) survives well. It is in the middle and has the least edge effect (those markers at the edge are not as efficiently transformed as those more centrally situated on the chromosone).

18. Check on secondary R_I sites.

19. Size and closeness to the edge may be criteria for survivability.

20. There may be something special about the ends of R_I treated DNA so that fragments from R_I treated DNA may slide back into the chromosome differently. To destroy ends - treat with exonuclease I. Does the ectopic insertion depend on having the kind of ends obtained after R_I treatment?

21. Can the resolution be increased any further: To get still better separation consider the best conditions for doing the electrophoresis. Are these optimized?

    a. Ionic strength of buffer (0.1M Tris + borate ⟶ pH 8.7 is used; alteration of pH may cause gel shrinkage).

    b. % agarose gel (0.7 - 0.73 is being used; 1.6 - no bands, 1.3 - begin to see bands, 1 and 0.7 - good bands, below 0.7 the gels shrink)

    c. Voltage (20 volts is used. A lower voltage gives less heating effects. Slower movement may provide better separation.)

    d. Temperature (room temperature is used. Working with very thin gels, the electrophoresis can be done at 4° in the cold room. There would be a better temperature control in the cold & this might give better reproducibility.)

    e. Solutes - try adding other solutes to influence the texture of the gel and of the DNA.

22. Set up conditions for electrophoresis such that the bands stop and whatever is smaller starts traveling faster. Use step-gels; at different % agaroses different DNA fragments are sorted out, & different % agaroses can be layered one on top of the other.

D. For Publication:

1. Hone in on: level of fractionation
   rebanding
   leave the mutation work for later

2. Discuss: resolution
   measurement of resolution
   degree of fractionation
   specific activity
   number of markers

3. Include information about:

   a. sucrose gradient
      (1) to show effect of R_I treatment
      (2) to calculate mass

   b. compare bands obtained from
      (1) DNA R_I treated (bands obtained & activity increases)
      (2) sheared DNA (no bands obtained & activity decreases)

   c. computer data
      The computer simulation data agrees with the experimental data. At first it wasn't random because of the random # generator used, but now another random # generator is employed & it is more random.

   d. table of markers tested & the survival rate of the different markers

III. Hela: Crossing Experiments

1. Seven different strains were tried.
2. Six showed no growth of colonies,
3. B.s. SB 863 S^R Tyr^- Aro_2^- $Tryp^-$ Lys^+ gave no growth alone on appropriate plates, nor did SD 8 Tyr^+ Aro_2^+ $Tryp^+$ Lys^- Cys^- alone. (SD8 didn't revert at all; SD 1 reverts alot.)
4. When SB863 and SD8 were cross streaked on a plate, colonies were obtained. When colonies occur, either SB863 receives the Tyr^+ Aro_2^+ $Tryp^+$ from SD8, or SD8 receives the Lys^+ from SB863, in recombination.
5. SD8 is the result of an R_I transformation; it may have a plasmid.
6. Repeat the experiment as it was done (grow the bacteria together on the plate for 48 hours, cross-streaked). See if the result is repeatable & not a matter of contamination, but of crossing (recombination).
7. If repeatable, then mix the two kinds of bacteria together just before plating the mixture.
8. Isolate the colonies & find out their nutritional requirements.
9. Repeat with Streptomycin on the plate to find out which bac. is donating the markers.
10. Repeat with and without Cys on the plate.
11. Is DNA transfer involved? Add DNAase to the mixture.

B.C.