XII/5/74 **HELA:**

1) Continuation of quantitative Kinetics of crossing, using SB 863 and SD8 cells: Grew cells together and separately for 48 hs. in N.B., then washed cells and plated with or without N.B. added. When plated separately: no growth
" " together: no growth without N.B. after 3 days, growth after 10 days. growth after 3 days when 0.01 or 0.05 ml N.B. added.
Same results if cells were mixed after washing or if Micrococcus Nuclease was added at beginning of mixture and growth.
All growth looks like heavy confluent growth, could be cross-feeding.
The effect seems to be on plates, not on N.B.

Also diluted cells to 10^5/ml and plated with small amounts of N.B. added 1, 1λ, 3 10, 30 and 100λ, growth showed after 3 days starting at the 10λ amount.

Suggestions:

Make dilutions before plating

get rid of cross-feeding effect by using streptomycin (kill one parent) on the selective plates.

Use Sum and add 0.01 ml N.B.

Colonies that grow on selective plates, purify once on selective plate and once on nutrient agar before replica-plating to test markers.

Check nutritional requirements of SD8 everytime (it might be unstable)

2) Crossing of B.S.K auxotroph (SB1109) and Marburg auxotroph SB 863.

Under the same conditions that the above crossing, with the addition that plated not only 10^0 but also 10^{-1} and 10^{-2} dilutions.

Selective plates were -Meth, -Phe, -Tryp, -tyro and -His, -Phe, -tryp, -tyro.

When plated sepdlately, there was a very slight background. When plated together, a slightly heavier confluent growth on the 10^0 one. nothing on the 10^{-1} and 10^{-2} dilutions.

Suggestions:

Repeat crossing using same conditions as KOHIYAMA paper

3) Prepared purified DNA from B.S.K (SB1106) and K auxotroph (SB1108), and also competent cells of each.

Did Transformation experiments

a) SB1106 competent cells with SB1108 DNA
" " " " SB19 " (control)
" " " " SB1106 "

b) SB1108 " " " SB1106 "
" " " " SB19 "
" " " " SB1108 "

c) SB863 " " " SB1108 "
" " " " SB1106 "
" " " " SB19 "

Markers selected were: Sm (300γ/ml) for a) Meth and His for b) and Phe and Tyr for c)

SB863 + SB19 DNA gave Transformation:
Freq. 1.5×10^{-3} for Tyr and 1.7×10^{-4} for Phe

None of the other combinations gave Transformation

Suggestions:
Do not do any more Transformation studies with these K and K aux. strains for the moment. Concentrate on the crossing exp.

Betty:

I. Salt-Preferring Bacteria from the Baylands:
After 2 serial transfers in NB + 0.2M NaCl followed by 4 serial transfers on on N.A. + 0.2M NaCl, 18 plates were obtained with bacteria showing a definite salt preference. Of these, 15 had no growth at all on counterpart N.A. plates & 3 had far fewer & smaller colonies on such plates. Of those with no growth at all on N.A. minus salt, 10 still had no growth after a week at r.t. & 5 had growth appearing at a later stage(1 had several colonies in the heaviest streaked area after a week, 3 had colonies after half a week & 1 after a day).

A. Generation Time Studies: (of 6 plates)
1. Overnight Incubation Tubes Growth Results:
a. Spizizen + Glucose -- no growth (1 sample showed a very slight sediment & slight turbidity when shaken after 3 days)
b. N.B. -- no growth
c. Spizizen+Glucose+0.2M NaCl --
(1) 3 became turbid overnight & were used for generation curves
(2) 1 became turbid 24 hours later than is usual
(3) 2 were clear & remained so after a week
d. N.B.+ 0.2M NaCl -- all became turbid overnight & were used for generation curves.
2. Doubling Time Results:
a. In Spizizen+G+0.2M NaCl, 24' by graph, 23.52759 by computer
In N.B. + 0.2M NaCl, 22' by graph, 21.06005 by computer
b. In Spiz.+G+NaCl, no growth
In N.B. +NaCl, 30' by graph, 27.94804 by computer
c. In Spiz. + G +NaCl turbid 24 hours late & gave a very irregular step-like curve.
In N.B.+NaCl, by graph 36', 30.6413 by computer
d. The sample with very slight turbidity in Spiz+G (no salt) after 3 days
In Spiz+G+NaCl 25' by graph, 24.972 by computer
In N.B. 24' by graph, 24.47285 by computer
e. In Spiz.+G+NaCl, 24' by graph, 22.86522 by computer
In N.B+ NaCl 26' by graph, 25.29007 by computer
f. A sample that had growth on counterpart N.A. plate without salt after half a week, In Spiz+G+NaCl no growth
In N.B.+NaCl 21' by graph, 21.28596 by computer.

B. Those salt-preferring bac. which did not grow in Spiz+G+NaCl overnight, incubation tubes, or grew at a delayed rate, whereas they grew to high turbidity overnight
1. in NB+NaCl were streaked on plates:
SO→no growth, NA→no growth, SO+NaCl→heavy growth, onto NA+NaCl from the SO+NaCl plates without flaming loop inbetween→heavy growth.
2. Repeat of the above in liquid media, using a heavy inoculum instead of lightly picking a single colony.
Spiz.+G→no growth; N.B.→no growth; Spiz+G+NaCl→1 no growth, 1 turbid (that had become turbid 24 hours late with a light inoculum), and 1 lightly turbid in 3 days (that had had no growth with a light inoculum); into N.B.+NaCl directly from the Spiz+G+NaCl without flaming loop inbetween→very turbid overnight.

Concl.: 1. Since the NB+salt was inoculated directly without flaming from the Spiz+salt & the NA+salt from the SO+salt, any difference in growth would not be because of an insufficient inoculum into the first tube or plate of the set. Since there was heavy & equal growth on SO+salt & NA+salt plates, but not so in comparable liquid media, it would appear that the agar provides an [illegible] ingredient for good and rapid growth of these bac. in minimal medium+salt.
2. The size of the inoculum is a factor.

Betty

B. 3. Addition of the different Amino Acid Groups to the salt-preferring bac. which didnn't grow well in Spiz+G+salt (but do well in NB+salt, SO+salt & NA+salt).

a. The sample that became turbid 24 hours late, became + without & with the various groups to the same extent and at the same time,& therefore AAs make no differen in growth enhancement.

b. The sample that showed no growth in 5 days, still exhibited no growth with each AA group (I-V & VI=Aromatic), but did with the combination of I-VI.

c. Another sample that shoed no growth in 5 days, became turbid with group I and with group IV AAs & the combined I-VI. When the twice washed cells from the group I positive tube was tested with the individual AAs of group I and IV, positives were obtained for hydroxy-proline, glutamic & aspartic acids only (of group IV). When this was repeated using cells directly from the plate, with group I, group IV & the individual AAs of these groups, no growth was obtained. Perhaps contamination was the cause of the previous +s; this required repeating.

4. Suggestions:

a. Try vitamins, nucleic acids, yeast extract.

b. Find out the optimal salt concentration . Now using 0.2M. Try other molarities within the range .05-.5M.

c. Try a variation of the ionic strength by using 10mM combined phosphate molarity instead of the current .12M

d. Vary the pH by varying the ratios of mono & dibasic phosphates. Try several different ratios maintaining the same total combined amount.

e. Make auxotrophs of the faster bac. that prefer salt.

C. Plates Exhibiting Inhibition by Salt

After 2 serial transfers in NB+salt followed by 4 on NA+salt, 5 plates had substantailly larger colonies on NA without salt than on NA+salt. These NA plates with the larger colonies were observed daily to see if smaller colonies of the type on the salt plates would appear. They didn not after several weeks & were discarded.

II. pH Changes during Generation Time Studies:

pH of Spiz+G+NaCl 7.15
pH of NB +NaCl 7.0
pH of inoculated samples 6.8
pH at end of exponentaal phase 6.8 in Spiz+G+NaCl & 7.0 in NB +NaCl
pH at end of expt., after 1320 mins. 6.6-7.0 in Spiz+G+NaCl
6.6-8.35 in NB+NaCl

III. The Problem of Soil Bacteria which grow slowly or not al all in liquid Spiz+G but grow overgint at 30C on SO plates(individual colony transfers to 3 plates beyond the initial pour plate).

A. Out of 10 different plates used for inoculating overnight Spiz+G tubes 37C, only 2 were turbid next morn., a 3rd became turbid in 2 days, a 4th slightly so in 2 days & the rest remained clear. Of the 2 that became turbid overnight doubling times were 33' and 27' . Another eight plates from similar sources resulted in 1 turbid tube overnight, which gave doubling times of 36' both at 30C & at 37C; 2 slightly turbid tubes when shaken (sediment), which gave doubling times of 38' at 30C and 30' at 37C for one and 36' at 30C and no growth at 37C (probably detergent left in flask;I now give all Klett flasks a pre-rinse with medium to be used) for the second; 1 that became turbid 8 hours later than usual, 2 in 2 days & 2 in 3 days.

Betty

B. Factors that may be involved:
   1. Temperature sensitivity: The plates had been incubated at 30C & the overnight tubes at 37C, aerated.
   2. A long lag phase - slow in getting started
   3. Growth in liquid vs agar medium.

C. Comparison of Growth in Spiz+G, Spiz+G over agar slant, Spiz+G+Vitamin Mix and NB.
   A heavy inoculum per tube was used & tubes were incubated at 30C in water bath with shaking.
   1. For seven of the samples set up simultaneously, addition of vitamin mix made no difference. Inoculation into Spiz+G over a thin agar slant provided heavy turbidity overnight, more so event than occured in NB. The control of agar slant +Spiz+G remained clear. Concl.:The agar provided a growth substance.
   2. Another 10 samples also showed that vitamins were non-enhancing. With this batch though with or without agar were about the same. Better turbidity was obtained earlier with the overnight incubation at 30C than at 37C. Use of a large inoculum provides turbidity earlier.
   3. Suggestions:
      a. Use agar slants in the liquid medium for pre-enrichment in the isolation of rapid growing bac. The agar may act as a chelator & mineral source.
      b. Obtain the doubling times of these soil bac. in complete media.
      c. Test a few of the slow growing soil bac. & the previously isolated 24-27' CGI bac. in various ionic strenghts by employing combined mono-& dibasic phosphate molarities in the Spiz. of 5,10,25,and 50mM

Dec. 5, 1974

Grace:

(1) History of colonies #1 - #70.

1070 $\xrightarrow{RI}$ X1023 $\longrightarrow$ 260 colonies obtained from primary selection for Aro_2 and Tryp.

By replicate plating**, the 'phenotypes' of the 260 colonies were:

Aro_2 $tryp_2$ His_2 $Tyro_1$ Lys

155

25

70

3

Colonies with 'phenotype' Aro^+-$Tryp^+$-His^+-$Tyro^+$-Lys^- are numbered #1 through #70, and the linkage of each colony will be tested by using the same recipient strain 1023.

(2) Strange linkage relation obtained with colony #52.

#52 (mixed*) ——— X 1023 $\longrightarrow$ > % (1101) 'phenotype'

#52 (mixed*) $\xrightarrow{\text{sheared}}$ X 1023 $\longrightarrow$ > % (1101) 'phenotype'

#52 (mixed*) ——— X 419 $\longrightarrow$ 14% His^- 'phenotype'

(His^+, inh^R) (His^+, inh^S)

*, ** : #52 DNA used in these experiments were from unpurified patch on plate obtained directly from the replicate plate** (AA-tyro+shk) in (1).

(3) An attempt to isolate a single clone from #52 population was made, in order to distinguish whether a pure clone is unstable or the population mixed.

(i) By strea\king #52 directly from the replicate plate** on min+lys+cys. Big and small colonies were obtained and there was a clear region around the big colonies (perhaps some sort of killing effect). The big and small colonies (used single colonies) were restreaked on min+lys+cys, the same phenomena was obtained for both big and small colonies.

(ii) By resuspending a loopful of #52 obtained directly from the replicate plate**, in Spiz +1% glucose (50%), and plating 10^0, 10^{-1} on N.A. and on min+lys+cys. Only a few cells (less than 1%) grew on min+lys+cys compared to growth on N.A.

(4) Used one big colony from (3) (i), to check the linkage.
#52(Big, single?) ——— X 1023 ———> 1% (1101)

(5) (1101) ———X 1023 ———> no His^+ transformants, even at 10X the DNA concentration ordinarily used (5 λ).

Suggestions:

(1) In the #52 population, look for a clone that gives > % (1101) transformants. Check the linkage of both big and small colonies, how many genotypes are there ?

(2) Which supplement can be added to the min. medium to no longer see two different sizes of colonies.

(3) Purify the colony on complete medium first so that every cell has a chance to grow. Then use replicate plating to distinguish and select different phenotypes. In this way it is more possible to isolate clones that arise from single cells. If this method does not work, the final recourse to prove that segragation from a single cell gives a mixed clone, is by use of the microscope.

(4) See if the (1101) transformants are real His^-, the appearance of being His^- may be due to a deletion. Test this possibility by making (1101) competent, comparing the transformation efficiency with 1023. (This has now been tested and there is no difference in the transformation efficiency of the two recipients.

However, (1101) showed a high frequency of reversion to His^{+} ($\sim 10^{-5}$), which is an argument against deletion as a possibility.

(5) Distinguish between:

(i) DNA molecules which preserve a linkage to an exceptional extent after RI (i.e. reassociation occurs after the RI cut; exo-exo:the individual pieces recombine in the tube and then enter the cell, endo-endo: the individual pieces enter the same cell and then recombine).

(ii) the failure of RI to cut at particular locations (i.e., sites not cut, so that the DNA enters the cell in one piece).

To distinguish between these two possibilities, look specifically for the establishment of an Aro_2- Meth linkage.

(A) Select for Aro_2^{+}-me^{+}cotransfer; then check if this new linkage has been established.

(B) Use

$$\begin{matrix} Aro_2^{+}\ Tryp^{-} \\ Aro_2^{-}\ Tryp^{+} \end{matrix} \xrightarrow{RI} \times\ Aro_2^{-}\ Tryp^{-} \xrightarrow[Tryp^{+}]{Aro^{+}} F_1$$

If the DNA segments are cut by RI, the frequency of Aro_2^{+}-$Tryp^{+}$ cotransfer should be high because of recombination. This experiment should be done at limiting DNA concentration to avoid possibility of several DNA peices entering the same cell.

(iii) An alternative hypothesis is to question our model, the RI sites may not be fixed, they may instead be migrating.