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Further Evidence Concerning the Configuration of Transforming Deoxyribonucleic Acid During Entry into *Bacillus subtilis*[1]

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ABSTRACT

STRAUSS, NORMAN (State University of New York at Buffalo, Buffalo, N.Y.). Further evidence concerning the configuration of transforming deoxyribonucleic acid during entry into *Bacillus subtilis*. J. Bacteriol 91:702–708. 1966.—The appearance of linked, unselected traits with selected markers was followed as a function of time after the exposure of competent cells to transforming deoxyribonucleic acid (DNA). It was found that the per cent cotransfer of a linked, unselected trait with a single selected trait increased sharply soon after the lag period characterizing the appearance of the selected trait. Similar results were obtained when cotransfer of a linked unselected trait with a pair of selected traits was examined. The results are taken as an unequivocal demonstration that the entry of transforming DNA into competent *Bacillus subtilis* occurs in longitudinal fashion. The nature of the linkage between try_2 and his_9 was characterized. It was found that, although these two traits had been found to be unlinked on the basis of recombination tests, the saturation curves showed these two traits to be present on the same fragment of DNA.

Recent studies have provided a more detailed picture of the uptake of deoxyribonucleic acid (DNA) by competent *Bacillus subtilis*. The addition of DNA to competent cells at 28 C is followed by an unmeasurably rapid, irreversible attachment of DNA to cells (4, 6, 10). Subsequently, a period of 2.5 min ensues during which the potential transformants are sensitive to the action of deoxyribonuclease. The lag period of 2.5 min, which is independent of the single marker selected, was considered to be the amount of time necessary for the entry of a length of DNA which is long enough to participate in a recombination event. With the assumption that a cistron consists of 1,000 nucleotide pairs, this minimal length was found to be approximately five cistrons long, corresponding to a molecular weight of 3×10^6 daltons. The appearance of selected linked markers which are more than five cistrons distant was characterized by an increase in the length of the lag period. This increase appeared to be a linear function of the distance between the selected markers. These findings suggested that the DNA molecule enters the cell in lengthwise fashion (9). (For ease of discussion, it will be assumed that attainment of insensitivity to deoxyribonuclease is tantamount to entry. It must be emphasized, however, that this has not been proven.)

A more crucial test of the model would be a demonstration that the end of the lag period represents the beginning of the contribution of a different size of DNA fragment to the accumulation of the selected transformants. This can be done by an examination of transformants for the cotransfer of an unselected, linked trait. If the lag period is an artifact resulting from insensitivity of the assay, then the linkage relationship between any two markers should be constant in time. If, however, the lag represents the time for entry of a given length of DNA, the per cent cotransfer of a linked unselected trait should begin to change after the end of the lag period.

The results of such an examination are presented herein, and provide confirmation that transforming DNA enters cells in longitudinal fashion.

MATERIALS AND METHODS

Organisms. Table 1 indicates the strains of *B. subtilis* employed and the map positions of the genetic

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TABLE 1. *Strains used in the present study*

Strain	Genotype	Origin	Growth requirement
Wild-type	—	Reversion of 168 try_2^-	—
SB 222	$tyr_1^- aro_2^-$	Nester	Tyrosine, phenylalanine, tryptophan, *p*-aminobenzoic acid
SB 455	MT^R	Nester	—
Strain 30	$his_9^- try_2^- MT^S$	Zamenhof	Histidine, tryptophan
Strain 301	$his_9^- try_2^- tyr_1^-$	Transformation by DNA from aro_2^+ revertant of SB 222	Histidine, tryptophan, tyrosine

markers used. Genetic markers are indicated as follows: tryptophan, *try*; histidine, *his*; tyrosine, *tyr*; methyl tryptophan, *MT*. A subscript numeral indicates the enzymatic step affected by the genetic lesion. A genetic map of the markers used in the present study is shown in Fig. 1.

Media. Media for the maintenance of stocks and for the transformation procedure were prepared as previously described (6). Appropriate supplements were added to permit the growth of the strains designated in Table 1.

Scoring of genotypes. The cotransfer of traits was determined by streaking colonies on the appropriate agar medium with sterile toothpicks. Whenever possible, at least 100 colonies were examined for an unselected trait. This number assures with 95% confidence that the value for per cent cotransfer so obtained will lie within 10% of the true value. Resistance to 5-methyltryptophan was assayed as previously described (9).

Transformation procedure. The transformation procedure was as previously described (6, 9). Lag periods characterizing the appearance of transformants as a function of time were determined by extrapolation of curves to infinite slope on a semilogarithm plot (9).

Preparation of DNA. Two types of preparations were used. The Marmur procedure (7) was used to obtain DNA (hereafter designated as Marmur DNA) which exhibited virtually no linkage between try_2 and his_9 as determined by recombination analysis. The method of Berns and Thomas (1) was also used to obtain DNA preparations (hereafter designated as BT DNA) which were presumed to have a higher mean molecular weight. DNA was used at the minimal saturating level in all cases unless otherwise indicated. DNA was assayed colorimetrically by the method of Burton (2).

Chemicals. Deoxyribonuclease I, one time recrystallized, was obtained from Worthington Biochemical Corp., Freehold, N.J.

RESULTS

Cotransfer of linked, unselected traits with single selected traits. When a try_2^- tyr_1^- recipient was exposed to wild-type Marmur DNA, double transformants appeared beginning at 2.6 min. Try_2^+ only appeared at about 2.2 min (Fig. 2). The occurrence of a longer lag for the simul-

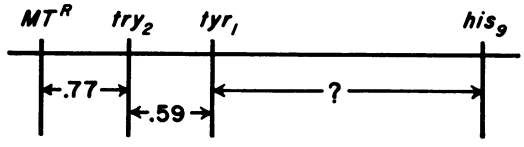

FIG. 1. *Linkage map of relevant loci. Map distances given are based on the use of the cotransfer index (r) and are given by 1 − r (8). Per cent cotransfer can be calculated from r and is given by* $\frac{2r}{1+r} \times 100$.

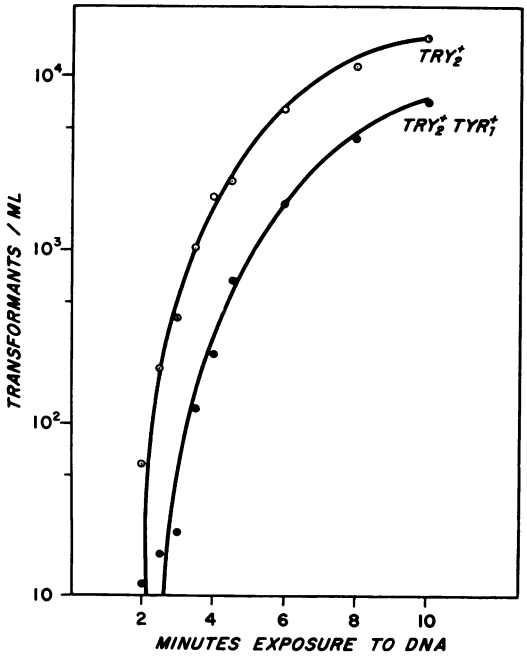

FIG. 2. *Appearance of* try_2^+ tyr_1^+ *as a function of time. Competent cells of strain 301,* $try_2^- tyr_1^- his_9^-$, *were exposed to wild-type Marmur DNA. Samples were removed at various times to tubes containing deoxyribonuclease. Portions were plated on histidine-tyrosine-agar or on histidine-agar for scoring of* try_2^+ *and* $try_2^+ tyr_1^+$ *transformants, respectively.*

taneous appearance of try_2 and tyr_1 transformants as contrasted to try_2^+ alone does not provide, however, an unequivocal demonstration that the lag represents the entry of that length of DNA which contains both try_2 and tyr_1. The lag might be construed as being a consequence of the insensitivity of the assay or even as error. Were the linkage between these two traits to change suddenly beginning at 2.6 min, it would be clear that it took that long for sections of DNA to enter the cell that contained both the tyr_1^+ and try_2^+ traits. Any cotransfer of tyr_1^+ with try_2^+ prior to this time would be due to coincidental encounter of a single cell with two fragments of DNA, one containing the try_2^+ gene, the other the tyr_1^+ gene [the term "congression" has been coined by Nester, Schafer, and Lederberg (8) to designate this phenomenon]. Figure 3 shows the results of an experiment in which selected try_2^+ colonies were tested for the cotransfer of tyr_1^+. It is evident that the linkage relationship between these two markers changes markedly starting from 2.5 min. A similar demonstration is afforded by an examination of the cotransfer of the MT^R trait with try_2^+ (Fig. 4A). MT^R, as an unselected trait, appears about 30 sec after try_2^+.

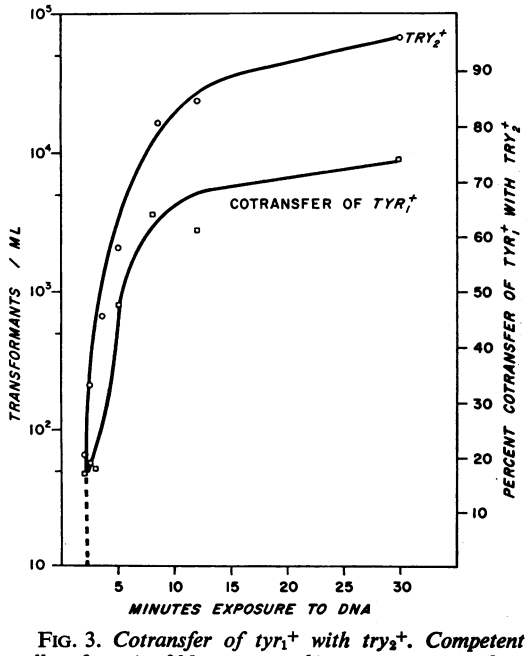

FIG. 3. *Cotransfer of tyr_1^+ with try_2^+. Competent cells of strain 301, $try_2^- tyr_1^- his_9^-$, were exposed to wild-type Marmur DNA. Samples were removed at various times to tubes containing deoxyribonuclease. Portions were plated on histidine-tyrosine-agar. The resulting try_2^+ colonies were streaked on histidine-agar to determine the proportion of try_2^+ colonies which were also tyr_1^+.*

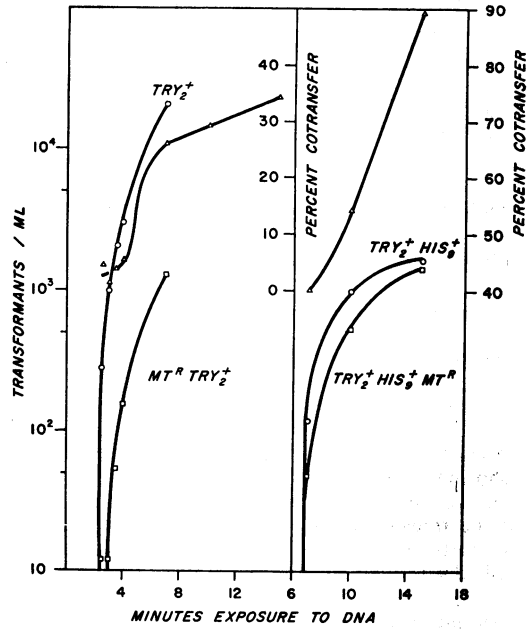

FIG. 4. *Cotransfer of MT^R with try_2^+. Strain 30 cells, MT^S try_2^- his_9^-, were exposed to SB 455 BT DNA. Samples were removed at various times to tubes containing deoxyribonuclease. Portions were plated on histidine-containing agar and on minimal agar for the enumeration of try_2^+ (A, left) and $try_2^+ his_9^+$ (B, right) transformants. These were then tested for cotransfer of MT^R by streaking colonies on an overlay containing 168 try_2^- cells.*

The incidence of cotransfer begins to change at about 3 min, rises precipitously to about 30%, and proceeds at a reduced rate.

Evidence for the linkage of try_2 and his_9. If the interpretation of the experiments described above is correct, a similar result should be forthcoming when a pair of markers is selected. Selection for two widely separated markers assures a longer lag period with somewhat better resolution. Previous studies (3) have shown the try_2 and his_9 traits to be poorly linked, although in the same general region of the chromosome. A saturation curve was obtained by exposing competent $tyr_1^- try_2^- his_9^-$ cells to various concentrations of wild-type Marmur DNA and selecting for try_2^+ his_9^+ double transformants. The result of one such experiment is shown in Fig. 5. The curve obtained exhibited some quadratic properties (5), an observation consistent with a considerable amount of congressive as well as of single-fragment transformation. The saturation curve which was obtained with BT DNA (Fig. 6) revealed no quadratic character, which indicates that try_2^+ his_9^+ transformants arise predominantly,

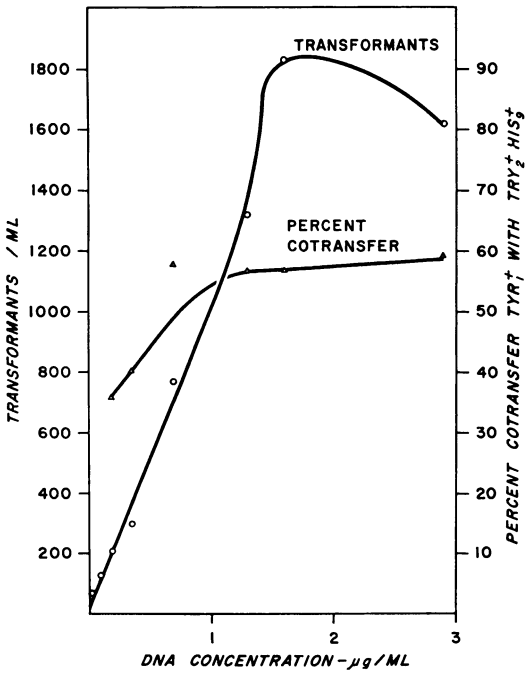

FIG. 5. *Saturation curve for Marmur DNA. Competent cells of strain 301 try$_2^-$tyr$_1^-$his$_9^-$ were exposed to various concentrations of wild-type DNA for 15 min. Deoxyribonuclease was added. Portions were plated on tyrosine-containing agar to enumerate try$_2^+$his$_9^+$ transformants. The resulting colonies were streaked on minimal medium to determine cotransfer of tyr$_1^+$ as a function of DNA concentration.*

in this case, as a result of single-fragment transformation.

The contributions of both congressive and single-fragment transformation to the total yield of try$_2^+$ his$_9^+$ transformants is indicated by an examination of the cotransfer of tyr$_1^+$ with these two traits. The per cent cotransfer of a *single* trait, C, with two selected traits, A and B, will approach a constant value as the DNA concentration approaches zero when all three traits are linked (Strauss, *unpublished data*). This technique is useful when A and B are so loosely linked that cotransfer is barely detectable. By selecting for AB double transformants and observing cotransfer of a third marker which is tightly linked to A or B as a function of DNA concentration, the relative contribution of congressive and single-fragment transformation to the total yield of double transformants can be assessed. The extent of the decrease in cotransfer between saturating and zero DNA concentrations is an indication of the contribution of congressive transformation which occurs at saturating levels of DNA to the total transformant yield. In the case of Marmur DNA (Fig. 5), congression accounts for approximately half the total try$_2^+$ his$_9^+$ transformants. With BT DNA, 15 to 20% of the double transformants arise by congression (Fig. 6).

These findings strongly suggest, therefore, that markers try$_2^+$ his$_9^+$ are to be found on the same fragment of DNA in detectable numbers in both DNA preparations.

Cotransfer of a linked, unselected trait with two selected traits. By use of BT DNA, prepared from strain SB 455 (*MT*^{R}), a time course of appearance of try$_2^+$ his$_9^+$ double transformants was made with a try$_2^-$ his$_9^-$ *MT*^{S} strain as recipient. DNA was used at slightly lower than saturating levels (0.46 μg/ml). Figure 4B depicts the appearance of the double transformant try$_2^+$ his$_9^+$. The nature of the curve obtained was consistent with linked transformation, as indicated by the results in Fig. 6. A lag period of 7 min preceded the appearance of the double transformants. An examination of these colonies for the *MT*^{R} character revealed that cotransfer increased from about 40% at 7 min to 90% at 15 min, as would be expected if it took at least 7 minutes for fragments carrying both the try$_2^+$ his$_9^+$ markers to enter the cell.

A close examination of the curves for the entry of *MT*^{R} reveals that this entry lags behind try$_2^+$ alone by some 30 sec. However, the *MT*^{R} trait appears to enter simultaneously with try$_2^+$ his$_9^+$.

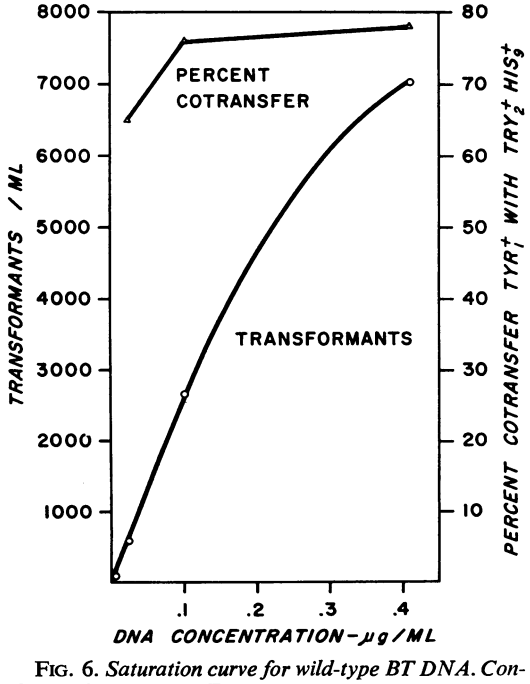

FIG. 6. *Saturation curve for wild-type BT DNA. Conditions the same as in Fig. 5.*

The explanation for this seeming contradiction lies in the frequency with which congression occurs in the two cases. Congression is insignificant in the case of the try_2^+ and MT^R traits because of the tight linkage of these two traits, but is significant in the case of try_2^+ his_9^+ MT^R. This accounts not only for simultaneous entry of MT^R with try_2^+ his_9^+ at 7 min but also for the observation that initially (i.e., at 7 min) cotransfer begins at a 40% level.

A similar experiment was carried out again with BT DNA, for an unselected linked marker, tyr_1^+, which is situated between try_2^+ and his_9^+ but is rather close to try_2^+. Once again, as shown in Fig. 7, the linkage of the unselected trait tyr_1^+ to try_2^+ his_9^+ rises from about 50% to 70% in 10 min.

Similar experiments were done with Marmur DNA at minimal saturating levels. The results are shown in Fig. 8. It is to be noted that try_2^+ his_9^+ transformants first are evident at 5 min and that the curve does not extrapolate to infinite slope even at 4 min. These results can be attributed to the significant frequency of congressive

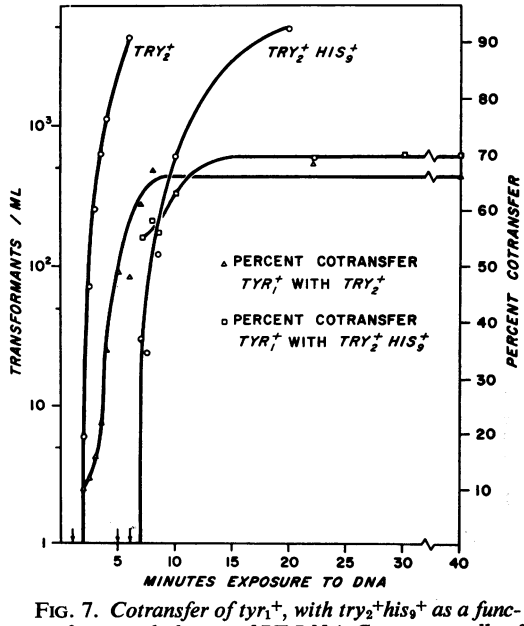

FIG. 7. *Cotransfer of tyr_1^+, with $try_2^+his_9^+$ as a function of time with the use of BT DNA. Competent cells of strain 301 $try_2^-tyr_1^-his_9^-$ were exposed to wild-type BT DNA. At various times, samples were treated with deoxyribonuclease and portions were plated on histidine-tyrosine-agar or on tyrosine-agar for the enumeration of try_2^+ and $try_2^+his_9^+$ transformants, respectively. The resulting colonies were streaked on suitable agar to determine the cotransfer of tyr_1^+ with try_2^+ or with try_2^+-his_9^+ as a function of time. Arrows indicate times of sampling for both single and double transformants.*

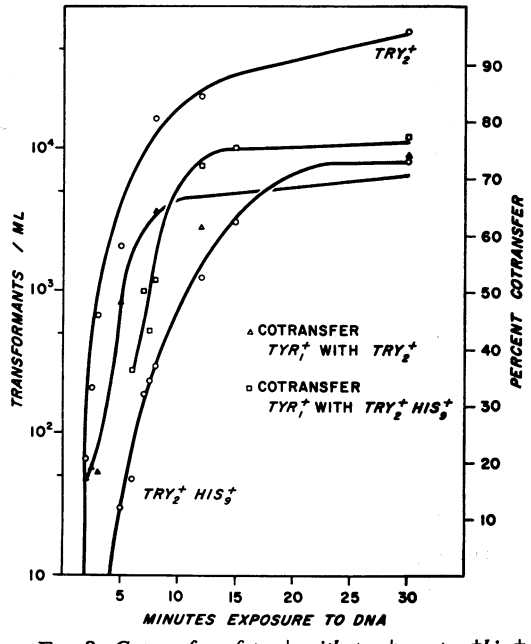

FIG. 8. *Cotransfer of tyr_1^+ with try_2^+ or $try_2^+his_9^+$ as a function of time with wild-type Marmur DNA. Procedure and conditions as for experiment in Fig. 7. Data of Fig. 3 used for comparison.*

transformation for double markers, which presumably would begin to appear about 2.5 min after the addition of DNA. Nevertheless, because of the significant contribution of single-fragment transformation at this level of DNA (Fig. 5), the cotransfer of tyr_1^+ is seen to rise from about 35% to about 75% at 15 min.

DISCUSSION

The results presented above serve to substantiate the conclusion that the lag period, as determined by extrapolation of the curve obtained by semilogarithm depiction of the appearance of transformants as a function of time to infinite slope, is an absolute lag and denotes the time necessary for the entry of a given size fragment of DNA. The rapid increase of the cotransfer frequency of an unselected linked marker beginning after the lag period for the entry of a single selected trait clearly signifies the beginning of the contribution of a different size fragment of DNA to the accumulation of transformants. Similarly, it was demonstrated that the cotransfer of an unselected trait increased rapidly after the lag period characterizing the entry of the linked selected traits try_2^+ his_9^+. The cotransfer of either of the two unselected markers, MT^R or tyr_1^+, exhibited an increase beginning after 7 min, the length of the lag period for try_2^+ his_9^+.

The increase in cotransfer of MT^R was particularly striking, and strongly implicated single fragments of DNA containing all three traits as the predominant source of triple transformants.

The unequivocal demonstration that the lag period is a measure of the time necessary for the entry of a given length of DNA, and that a linear relation exists between the lag period and the distance between marker loci (9), clearly indicates a longitudinal attainment of deoxyribonuclease insensitivity by the transforming DNA fragment. It may be claimed *sensu strictu* that this linear relation between map distance and lag period means simply that a large piece of DNA takes longer to become deoxyribonuclease-insensitive than does a small piece, and does not necessarily imply longitudinal entry. It is difficult to conceive, however, how this linear relation might be obtained if the rate of attainment of deoxyribonuclease insensitivity were based on any measure of size other than length.

A number of interesting questions have been posed by the findings regarding the linkage of the try_2 and his_9 loci and the frequency of cotransfer of markers located either between or outside these two loci. The linkage of try_2 and his_9 was found by Ephrati-Elizur et al. (3) to be extremely loose. Selection for these two traits as a function of DNA concentration, however, results in kinetics which indicate that most of the double transformants arise not from congression but from encounter of a cell with a single fragment containing both markers (Fig. 5 and 6). How, on the basis of recombination data, can these markers be classed as unlinked yet appear to be linked on the basis of transformant frequency as a function of DNA concentration? Although extensive shearing accompanies the preparation of DNA, the frequency of cotransfer for tightly linked traits will be a function primarily of those intracellular events which are concerned with the insertion of donor DNA into the host chromosome. For the case of loosely linked markers, however, the cotransfer of two traits will be affected to a greater extent by the disruption of the DNA incurred during preparation of the latter (5). In the case of BT DNA, the try_2 his_9 loci have been disjoined to the extent that the ratio of double to single transformants is less than 0.10. However, the number of fragments containing both markers may be sufficiently high that the probability of transformation by such fragments is greater than the probability of transformation by congression (Fig. 6). With Marmur DNA, shearing is probably even more extensive during preparation, as indicated by the slight quadratic characteristic of the saturation curve shown in Fig. 5. The contribution of single-fragment transformation is about the same as the contribution of congressive transformation. One can envisage, for example, that 99% of all try_2^+ his_9^+ pairs have been disrupted during the preparation of the DNA. The probability of transformation by the remaining 1% of intact pairs may nevertheless be as high as the probability of congressive transformation by the 99% of disrupted pairs.

The results shown in Fig. 4B were unexpected. The location of the MT^R marker, it must be remembered, is external to the traits try_2 his_9 (Fig. 1). Why the cotransfer of MT^R with try_2 alone should have been 50% lower than the cotransfer of MT^R with try_2 his_9 is not clear. Certainly, a difference such as this indicates that the linkage relation between MT^R and try_2 depends on the kind of try_2 fragment one examines. This finding may be explained either by the assumption that the presence in a recipient of longer lengths of DNA, as would be the case if both try_2 and his_9 were selected, might decrease the probability of a crossover event between the try_2 and MT^R loci, or that the fragments in the DNA preparation have a bimodal size distribution.

In the case of tyr_1, the unselected linked trait that lies between the try_2 and his_9 loci, the per cent cotransfer of tyr_1^+ with try_2^+ and his_9^+ was not much greater than the per cent cotransfer of tyr_1^+ with try_2^+ as the selected trait. This finding again was surprising, it being expected that selection for two loci would increase the probability of occurrence of transformants for loci lying between the two selected ones. These observations are now under investigation.

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