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RECIPROCAL RECOMBINATION AND SEGREGATION IN *Escherichia Coli*

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Several problems of genetic recombination are susceptible to genetic analysis only if it is possible to ascertain the genetic constitution of more than one chromosome emerging from the group within which recombination occurs. These problems concern the reciprocality of recombination, the number of homologous chromosomes in the recombining group, the distribution of exchanges among them, and the rules governing their segregation.

In bacteriophages and bacteria, several attempts have been made to recover more than one product of recombination in order to detect reciprocality. So far, no significant indication of reciprocal recombination of vegative phages has been reported (Hershey and Rotman 1949; Jacob and Wollman 1954; Bresch, 1956). However, the occurrence of reciprocal recombination is a central assumption in Campbell's (1962) appealing and so far successful hypothesis for the integration and removal of prophages and other bacterial episomes. Indeed, Herman (1965) has obtained evidence for reciprocal recombination of the *E. coli* chromosome with an F' episome.

The present study was undertaken to examine the pattern of recombination and segregation of prophage λ markers in *E. coli* carrying λ on the chromosome and another, differently marked, λ on an F' episome. The results show conclusively that recombination of the markers is at least very often reciprocal. Also, several other features of recombination resemble those found in eukaryotes.

MATERIALS AND METHODS

Broth. Tryptone broth with 5×10^{-3} M-$MgSO_4$ was used throughout.

Plates. Tryptone agar was used for all phage platings. Eosin methylene blue agar containing galactose and streptomycin was used for all isolations of bacterial colonies.

Phage strains. The λ phages used to construct the parental diploid derive from λ "wild type" (Kaiser, 1957). The clear plaque mutation $cIII_{67}$ and the extended host range mutation h were isolated by Kaiser (1957, 1962). The minute plaque mutant mi_{20} was found in a wild type stock after nitrous acid treatment. These mutations will be referred to as c, h, and mi.

The virulent, extended host-range strain λ vir h was used to select bacteria resistant to phage λ. Phage T6 was employed to terminate conjugation in mating experiments. The RNA phage R17 was used to test bacterial strains for maleness and to select an F' λ strain with reduced ability to transfer λ.

E. coli strains. The K12 strains W3350 Str^R (Campbell 1958) and CR63 (Appleyard, MacGregor, and Baird, 1956) were used as indicators. A Str^R derivative of the $F^-gal_2^-$ Lederberg strain K12-W3102 was used to construct the parental diploid and a λ^R $T6^R$ derivative was used as recipient in mating experiments. The episome F'λ was isolated by Wollman from Hfr3.

The parental diploid constructed for the present study carries λ c mi h on the chromosome and λ + + + on the episome F'λ. The strain is unable to adsorb λ and λh and is sensitive to the male specific phage R17. It was obtained as follows: Strain W3102 Str^R was lysogenized with λ + + h and made resistant to λ by selection with λ vir h. This strain was mated to a

streptomycin sensitive donor carrying F'λ made lysogenic for λ c mi +. A Str^R gal^+/gal^- heterogenote was selected on EMB galactose streptomycin agar. On this medium, gal^+ homogenotes form black colonies, gal^- homogenotes white colonies and gal^+/gal^- heterogenotes red-brown colonies with occasional slight sectoring. The gal^+/gal^- heterogenote was found to carry both λ c mi + and λ + + h. It segregated strains carrying various prophage combinations, including strains rather stably lysogenic for three different prophage types. One of the prophages in these multiple lysogens was usually λ + + h. This suggested that the ancestral F^-(λ + + h) was at least doubly lysogenic. Therefore, a c mi h/+ + + segregant was isolated from the c mi +/+ + h heterogenote in the hope that the extra λ + + h had been eliminated. This proved to be the case. Decendents of the c m h/+ + + strain never were found to carry more than two prophage types. This strain was still heterozygous for gal. The final step in constructing the parental diploid was selection of a gal^+ homogenote still lysogenic for λ c mi h and λ + + +. Its configuration was (λ c mi h)/F'(λ + + +), as determined by zygotic induction. At 37° it transfers λ + + + to recipient cells beginning at 4.5 minutes after donor and recipient cells are mixed.

The parental diploid is rather stable when kept at 4°C on Dorset egg medium. After two years, most cells were found to have the original constitution.

Phage plating. Indicator bacteria were prepared by 3 hours aeration of a 1:9 broth dilution of an overnight culture. All eight plaque types involving c, mi, and h could usually be distinguished on strain W3350 Str^R. However, each scoring on W3350 Str^R was checked with respect to the h character by a parallel plating on a 3:1 mixture of W3350 Str^R and CR63. All h^+ type plaques are very turbid on the mixed indicator.

Recognition of the mi character is best on plates with pH in the range 6.8 - 7.0. Phages carrying mi_{20} do not make plaques above pH 8.

Episome transfer. Strains to be tested for F'λ donor ability were usually grown to 2×10^8 per ml and mixed with an equal volume of a saturated culture of W3102 Str^R λ^R $T6^R$. One ml of the mixture is kept at 37°, usually for 20 minutes. Then 10^{10} T6 phages are added and after 5 minutes the mixture is centrifuged. The sedimented bacteria are resuspended in broth at a concentration of about 2×10^7 female cells per ml. After 70 minutes of aeration at 37°, the culture is chloroformed, diluted a thousand-fold and plated with indicator bacteria.

Testing for prophage content. A fully saturated culture containing cells to be tested for prophage content is streaked out and incubated overnight at 37° C. The next day, entire colonies are picked into 1 ml of broth each and shaken at 37° for 4-5 hours to obtain full saturation. Such cultures contain approximately 2×10^9 cells and 10^7 free phages per ml, the latter produced by spontaneous induction. About 0.01 ml of each culture is transferred with a sterile glass rod to 1 ml of broth saturated with chloroform. The chloroformed dilution is kept at room temperature for at least an hour, to kill all bacteria. Then another glass rod is used to transfer 0.01 ml portions to a tube containing 0.25 ml W3350 Str^R indicator and to a tube containing 0.25 ml of mixed indicator. After five minutes at 37° C, the mixtures are plated and incubated overnight at 37° C. Plates are scored by inspection, according to the major plaque types present. The major plaque types are presumed to be the prophage types present in the streaked cell from which the plated phages ultimately decend. For example, a typical plating on W3350 Str^R showed the following distribution of plaque types.

+ + +	266	+ mi h	11	+ + h	16	+ mi +	10
c mi h	137	c + +	12	c mi +	18	c + h	8

Such a plate, and the cell from which it derives would be scored as + + +/c mi h.

PLAN OF THE EXPERIMENT

The following procedure was employed in order to determine

in the tally is an occurance of ten + + + homozygotes.

A total of 5740 cells from primary cultures were tested for prophage content. All but 20 were classified without difficulty (but not necessarily without error, see below) as containing one or, more often, two prophage types. The 20 exceptional tests showed the presence of three or four major phage types. In order to see if this resulted from cell lines stably lysogenic for more than two prophage types, the cultures grown from the unclassified cells were further investigated by testing the prophage content of five cells from each culture. None of the 110 tested cells was found to contain more than two types. The original complexity must therefore have resulted from occasional failure to isolate a single cell from a primary culture or else from the occurrence of a recombination event very early in the ancestry of the culture producing the complex phage mixture.

Fifteen of the 20 initially unclassified cells were classified on the basis of the prophage content of their five tested descendents. If at least three of the five tests revealed the same prophage content, the classification was made accordingly. Thus, all but five of the 5740 tested cells from primary cultures were classified.

In order to determine the trustworthiness of the prophage testing procedure, 216 cultures from cells classified as containing non-parental prophage combinations were further examined. Five cells from each culture were tested for prophage content. In all but seven cases, at least three of the five tests showed the same prophage content as the initial classification. The seven discrepant cases all involved errors in scoring the h character. In all seven, the five confirmatory tests agreed with one another. Apparently, h had been confused

with $\underline{h}^+$ in scoring the initial test plates of in record keeping.

RESULTS AND ANALYSIS

A total of 5735 unselected essentially independent remote descendents of the parental strain were classified according to prophage content as described above. The results are shown in Table 1.

A large number of homozygous and non-parental heterozygous cells were tested for sensitivity to phage R17. All cultures were sensitive, indicating the presence of F'. Cultures of 25 different genotypes were tested for F' mediated zygotic induction. Every heterozygous strain tested was found to transfer predominantly only one of its two phage types. This type is presumed to have been the one of the F' episome.

The parental strain is diploid for prophage λ. If the parental strain has a higher ploidy than two for prophage λ, we should expect to find among its descendents cell lines lysogenic for more than two prophage types. Although 438 non-parental type cells were found containing two prophage types, no cell lines were found with three or more. We conclude that the parental strain is truly diploid for prophage λ, in the sense that the stable unit of prophage inheritance consists of two non-sister prophages. According to the zygotic induction results, one prophage of the diploid complement is on the F' expisome, the other on the chromosome.

As for the cells found to contain only one prophage type, they are almost certainly homozygous diploids rather than

haploids. They are sensitive to phage R17 and when tested for zygotic induction they transfer λ with the same high efficiency as do heterozygous diploids.

Prophage recombination frequencies. Granting that each classified cell is indeed diploid for λ, the frequency of each prophage type may be obtained directly from the data of Table 1. The values are given in Table 2a and the corresponding recombination frequencies and map distances are shown in Table 2b. It is seen that reciprocally related recombinants occur with nearly equal frequency, although the excess of λ c + + over λ + mi h may be significant. The indicated marker order is c mi h, in accord with the established prophage map. There is a six-fold excess of double recombinants, the possible meaning of which will be considered below. The map lengths of the intervals c - mi and mi - h, corrected for an interference value of 6 are 3.0 and 3.5 centimorgans respectively. The ratio, 0.86, is not significantly different from 0.82, the ratio calculated from previously measured distances on the vegetative λ map, the order of which is sus_{11} - h - c - mi. The calculation assumes the prophage map length between sus_{11} and mi is negligable.

Symetries in the distribution of cell types. If equal numbers of episomes and chromosomes are present at the time of recombination and if all products of recombination are ultimately recovered with equal efficiency, we expect any two reciprocally related diploid types to occur with equal frequency. In Table 1, reciprocally realted pairs are indicated by short heavy lines. We see that the expected symmetries are generally found, although the excess of c + +/+ + + over + mi h/c mi h and of + + +/+ + +

over c mi h/c mi h should be noted. The possible meaning of these assymmetries will be considered below.

Recombination occurs reciprocally. The data of Table 1 show that a recombinant prophage type is often recovered with its reciprocal or with a parental type but occurs only rarely in a completely homozygous diploid or with any recombinant type other than its reciprocal.

The frequent association of recombinants with parental types is not necessarily incompatible with reciprocal recombination. Even if recombinant prophages are always produced in reciprocal pairs, they may not remain together during subsequent cell divisions. However, the rather frequent occurrence of reciprocally recombinant diploids is not, by itself, proof that recombination is significantly reciprocal. Instead, a relatively high frequency of reciprocal diploids could arise by non-reciprocal recombination if two or more different recombinant chromosomes can be formed within the same cell and if the recombination frequency in some cells is high enough often to generate a pair of reciprocally related recombinants merely by chance. The outcome could be indistinguishable from that of true reciprocity so long as only one genetic interval is under observation. However, a distinction can be mace by comparing events in the interval c - m with those in the interval m - h. We have seen that these two intervals are approximately equal. Thus, if there is no true reciprocity, we might expect the prophage c + +, for example, to be found as often with + + h as with its reciprocal + m h. More generally, the non-reciprocal diploids c + +/+ + h and + m h/c m + should occur as often as the reciprocal diploids c + +/+ mi h and c mi +/+ + h. In fact, this is not observed. There are only 16 diploids in the former class but 104 in the later. This result is conclusive evidence that recombination is at least quite often genuinely reciprocal.

Non-reciprocal diploids: recombination in a multi-strand group or episome transfer between cells? The occurrence of non-reciprocal recombinant diploids might be considered as evidence for non-reciprocal recombination. Appropriate mixtures of reciprocal and non-reciprocal recombination can be devised to explain the data of Table 1. However, we shall not follow this approach, preferring instead to analyze the data in terms of a single mode of recombination. Therefore, we suppose that recombination is always reciprocal but that the reciprocal products of exchange often separate from each other and become associated with other partners. This might be imagined to occur in two different ways.

First, if there are more than two strands present in cells at the time of exchange, a recombinant strand might often pass to a daughter cell together with a strand other than its reciprocal.

Second, reciprocal pairs might sooner or later become separated by displacement of a resident episome by a genetically different one transferred from another cell in the colony or culture.

Inspection of Table 1 shows that two conditions must be fulfilled if we are to imagine that recombinants arise only as completely heterozygous diploids and that non-reciprocal diploids are formed only secondarily, as a consequence of intercellular episome transfer. First, the probability that somewhere along its lineage a recombinant cell has undergone episome displacement with a cell of different genotype must be more than 50 per cent, for more than half of all recombinant prophages are found in non-reciprocal diploids. Second, recombinant cells must usually receive episomes from a population containing a much higher proportion of recombinant types than found in the overall distribution. For example, the reciprocal diploid c mi +/+ + h could give rise to the types c mi +/+ + + and + + h/c mi h by transfer to or from cells containing parental type prophages and could give the types c mi +/ + mi h and + + h/c + + by transfer with cells containing the recombinant

types + mi h and c + +. The observed ratio of c mi +/+ mi h and + + h/c + + diploids to c mi +/+ + + and + + h/c mi h is 0.15. However, the ratio of c + + and + mi h to parental prophages in the total population is only 0.02. Thus, to account for the observed ratio of the above diploids on the hypothesis of ipisome displacement we must suppose either that recombinant cells are more effective episome donors than non-recombinants or else that recombinants undergo episome displacement in an environment within which the proportion of recombinants is, on the average, approximately eight times greater than the overall mean. Consideration of other relevant ratios of diploids leads to the same conclusion.

Environments with an unusually high proportion of recombinants could be provided by extreme heterogeneity among primary cultures or among different regions within individual colonies. However, even if recombination intensity is very heterogeneous from colony to colony or in different regions within individual colonies, recombinant cells in different microcolonies should be partly insulated from mixing with each other both by their own sister cells and by the intervening matrix of parental bacteria in which they are presumably imbedded. But whether such shielding would be effective enough to preclude episome transfer in colonies as an explanation of homozygosis cannot be said without additional knowledge of the characteristics of colonial growth, recombination, and possible episome transfer. In the next two sections we present some experiments suggesting that episome transfer is, in fact, not very frequent.

An experiment to detect intercellular episome transfer. An experiment was carried out to measure the frequency of episome

displacement under conditions at least partly simulating those under which non-reciprocal diploids presumbably arise. Cultures of two different diploid strains derived from the parental strain were mixed together in equal amounts. One strain was c + +/ F' + + +; the other was c mi h/F' + mi h. Approximately 10^6 cells of the mixed culture were spread on a plate and incubated at 37°C. After 2, 3, and 4 hours, a few drops of broth were added, and the layer of growing cells was dispersed by rubbing with a glass rod to mix cells from different micro colonies. After the third mixing, the plate was incubated overnight. An amount of bacteria corresponding to a single overnight colony was then transferred to 1 ml of broth and aerated at 37° for approximately 5 hours. The entire process was repeated twice, using approximately 10^6 cells from the previous overnight culture to innoculate a fresh plate. Finally, 99 cells from the third overnight culture were tested for prophage content. Of these, two were found to be heterozygous at the mi and h loci; indicating that approximately two percent of the cells in the final mixed culture experienced episome transfer with a cell of the other input genotype somewhere along their lineage. Similar experiments with other mixed cultures of various genotypes gave essentially the same result. This is far too low a transfer frequency to account for homozygosis on the hypothesis of episome displacement.

Non-reciprocal diploids in a defective F' strain. As an additional means of assessing the importance of episome transfer in the formation of non-reciprocal diploids, a study was made of a nutant + + +/c mi h diploid strain with greatly reduced ability to transfer λ to sensitive female

cells. The mutant was obtained from the parental diploid by selection for resistance to the male-specific phage R17. Both broth grown cultures and broth suspensions of colonies of the mutant transfer λ with an efficiency of less than one per cent of that of the parental strain. One thousand nine hundred cells of the mutant were tested for prophage content. Only 28 recombinant type cells were found, about one seventh the proportion found with the parental strain. The cause of this reduction is unknown. However, of the 28 recombinant diploids, only 4 were reciprocal. Thus, the probability of finding a recombinant prophage associated with a prophage not its reciprocal is at least as high as seen in the parental diploid strain itself.

This result and the failure to detect episome displacement in the experiment with mixed cultures argue strongly against intercellular episome transfer as the cause of non-reciprocality among segregants from the parental diploid. Nevertheless, it must be kept in mind that neither experiment is altogether conclusive in this regard. For example, episome displacement may, for an unknown reason, occur with unusually high probability in cells which have very recently undergone recombination, even in strains otherwise defective for F' transfer. However, we will not pursue the question farther, and will assume in the ensuing discussion that non-reciprocal diploids arise by segregation from cells containing more than two prophages at the time of recombination.

Homozygosis frequencies. Since all cells possess both an episome and a chromosome, homozygosity at one or more loci can arise only if an allele originally linked to an episome becomes linked to a chromosome, or vice versa. Therefore,

if one λ locus is more tightly linked than another to whatever distinguishes chromosomes from episomes, the more tightly linked locus should become homozygous less often. The frequency of diploids homozygous for a given allele may be extracted from Table 1. Homozygosis frequencies for each allele are given in Table 3. In accordance with our earlier discussion of the nearly synetrical distirbution of complementary diploid types, the frequency of homozygosis is seen to be approximately the same for either allele at each locus. We see from the table that the total frequency of homozygosis is least at the c locus and greatest at the h locus. This gradient of homozygosis suggests that there is a site or region to the left of c which distinguishes episome from chromosome. This supposition is consistant with a property of the gal^+/gal^- diploid strain from which the parental diploid decended. Most gal homozygotes arising from this gal^+/gal^- strain are homozygous for all the λ markers. This is consistant with the established map order gal - c - mi - h if there is a region to the left of gal distinguishing episome and chromosome. Henceforth we will denote this location on the map as o giving the map order o - c - mi - h.

An estimate of the number of strands in the recombining group. Assuming that recombination occurs within a multi-strand group containing a total of n episomes and chromosomes, we shall attempt to estimate n from the data of Table 1. We assume throughout that, following recombination, the replication and segregation of all episomes and chromosomes in the group proceeds without regard to which are recombinant

and which are not, giving rise to a population of diploid cells upon which observations are made.

Consider the consequences of exchange in the interval a - b with the loci a and b in the map order o - a - b. If multiple exchange may be neglected, the proportion H_b of cells homozygous at b among all cells heterozygous at a is

$$H_b = \frac{2(n-2)}{n} R_{a-b} \qquad (1)$$

R_{a-b} is the proportion of prophages recombinant in the interval a - b in diploids heterozygous at a. The relation holds even if there are unequal numbers of episomes and chromosomes in the recombining group.

Equation (1) may be applied to the data of Table 1 for the three intervals c - mi, mi - h, and c - h. Only those diploids heterozygous at the respective proximal loci are taken into account in computing H and R. The results are given in Table 4 and plotted in Fig. 1. In the figure, lines are drawn for H/R = 4/3, 1, 2/3, and 0 - the values given by equation (1) for n = 6, 4, 3, and 2 respectively. It is seen that the observed values of H/R lie closest to the line for n = 4.

The foregoing estimate of n utilizes only part of the date of Table 1. More of the data could be taken into account if equation (1) could be applied in each of the three intervals involving o. Direct application of the equation requires that we know the three recombination frequencies R_{o-c}, R_{o-mi}, and R_{o-h}. Although these values could

have been found by testing each primary culture for F' mediated transfer of λ, this was not attempted. Nevertheless, there is another method for utilizing equation (1) to find n, which we shall now employ.

Recall that the frequency of doubly recombinant prophages is 6.8 times greater than the frequency expected if exchange in one interval were uncorrelated with exchange in the other. That is

$$\lambda_{\underline{c}\ \underline{mi}\ \underline{h}} = \frac{R_{\underline{c}-\underline{mi}} + R_{\underline{mi}-\underline{h}} - R_{\underline{c}-\underline{h}}}{2\, R_{\underline{c}-\underline{mi}}\, R_{\underline{mi}-\underline{h}}} = 6.8$$

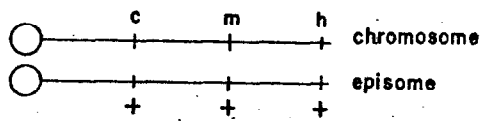
where $\underline{c}\ \underline{mi}\ \underline{h}$ is the coefficient of coincidence for the triplet of markers $\underline{c}\ \underline{mi}\ \underline{h}$. Similar relations may be written for the coincidence coefficients for each of the three triplets involving $\underline{o}$ by substituting for $R_{\underline{o}-\underline{c}}$, $R_{\underline{o}-\underline{mi}}$, and $R_{\underline{o}-\underline{h}}$ the corresponding experssions obtained from equation (1). For example, the coefficient $\underline{o}\ \underline{c}\ \underline{mi}$ may be written

$$\lambda_{\underline{o}\ \underline{c}\ \underline{mi}} = \frac{H_{\underline{c}} + \frac{2(n-2)}{n}\ R_{\underline{c}-\underline{mi}} - H_{\underline{mi}}}{2\, H_{\underline{c}}\, R_{\underline{c}-\underline{mi}}}.$$

The measured values of $R_{\underline{c}-\underline{mi}}$, $H_{\underline{c}}$, and $H_{\underline{mi}}$ are given in Tables 2b and 3. The coincidence computed from the above equation is therefore determined solely by our choice of n. Table 5 gives the value of each of the three coefficients, computed for various integral values of n. The results are plotted in Fig. 2. We now simply assume that all four coincidence coefficients under consideration are approximately

the same and we seek that value of n which brings the three indices involving o into best agreement with the observed value $\bar{\lambda}_{\underline{c}}$ $\underline{mi}$ $\underline{h}$ = 6.8. It is seen in Fig. 2 that the best choice is n = 4.

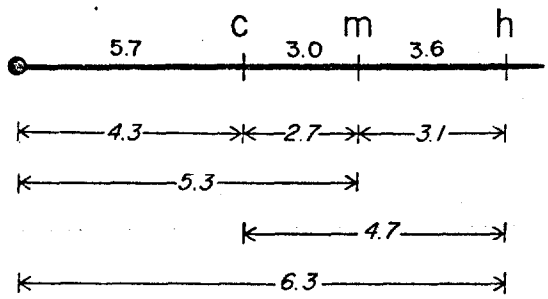
Table 1



		129	108	145	142	32	28	
+++	cmh	c++	+mh	cm+	++h	c+h	+m+	
84	4987	48	34	55	9	18	3	+++
	56	25	22	11	57	3	11	cmh
		2	39	4	7	2	0	c++
			1	8	0	2	1	+mh
				1	60	0	5	cm+
					4	1	0	++h
						0	6	c+h
							1	+m+

Table 1

not



$$I = \frac{R_1 + R_2 - R_3}{2R_1R_2}$$

fig 1