BACTERIAL CONJUGATION: AN ANALYSIS OF MIXED RECOMBINANT CLONES

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ABSTRACT

A fraction of recombinant colonies resulting from conjugation is heterogenetic for unselected markers. Constitutivity for alkaline phosphatase synthesis (*phoR*) is studied as the unselected marker. The frequency of *phoR* heterogeneity depends on the genetic distance between *phoR* and the selected marker. Various models are considered which explain the formation of heterogenetic colonies (mixed clones), and experiments are described which test these models. It is concluded that the Hfr fragment can replicate and participate more than once in recombination thus yielding heterogenetic colonies.

IN bacterial conjugation the Hfr parent injects a part of its chromosome into the F^- parent. The chromosome enters linearly beginning from an origin and in a direction which is specific to the Hfr parent (for Review see CURTISS 1969). The injection process occurs concomitantly with the replication of the Hfr deoxyribonucleic acid (DNA) and only one strand of the "old" DNA is injected into the F^- cell (GROSS and CARO 1966; VIELMETTER, BONHOEFFER and SCHÜTTE 1968) where it seems to acquire the complementary strand, (RUPP and IHLER 1968; OHKI and TOMIZAWA 1968; WILKINS, HOLLOM and RUPP 1971). Genetic information carried by the entering Hfr chromosomal fragment can now integrate into the F^- genome to form recombinants. Eventually this fragment is diluted out while the haploid recombinant cells continue to divide and there is evidence for the instability of this fragment (ITOH and TOMIZAWA 1971).

It has long been known that single exconjugant cells can yield more than one type of recombinant. This has been shown by single cell analyses of dividing exconjugants (LEDERBERG 1957; ANDERSON 1958) and by the analysis of colonies formed from single exconjugant cells (BRESLER, LANZOV and BLINKOWA 1967; WOOD 1967). Thus, the latter authors demonstrated that 30–50% of the recombinant colonies can be heterogenetic, i.e. are composed of more than one recombinant genotype. BRESLER *et al.* (1967) suggested that the Hfr fragment can participate more than once in recombination, yielding two or more recombinant genotypes from a single exconjugant cell. There are, however, several possibilities to explain this phenomenon. In the present paper we describe an analysis of recombinant exconjugant colonies which are heterogenetic for the mutation *phoR* (constitutive synthesis of alkaline phosphatase). Various possibilities are tested and it is concluded that, as already suggested, the Hfr fragment having

replicated, participates more than once in recombination to yield heterogenetic exconjugant clones.

MATERIALS AND METHODS

Bacterial strains: Table 1 lists the characteristics of the strains used and their origin, all being derivatives of *Escherichia coli* K12.

Media and treatment with bacteriophage T6: These were as previously described (BRACHA and YAGIL 1969; YAGIL, BRACHA and SILBERSTEIN 1970). Minimal Tris-Glucose (TG) medium (ECHOLS *et al.* 1961) contained 10^{-3} M KH_2PO_4.

Conjugation method: Both parents were grown exponentially in tryptone broth at 37°C. Only the F⁻ culture was shaken; when it reached a concentration of approximately 5×10^8 cells/ml it was mixed with the Hfr culture at a cell ratio of 5–10 F⁻ : 1 Hfr. The conjugants were kept at 37°C without shaking for 30 min (or 40 min in the cross Hfr AT2455 × F⁻ BT1), and the conjugation was interrupted by vigorous shaking for 2 min on a Vortex mixer. The exconjugants were then either plated on solid media containing streptomycin (100 μg/ml) or allowed to further segregate in broth containing streptomycin. Hfr : F⁻ input ratios were determined either by the Lac+ to Lac⁻ ratio (on MacConkey agar plates) or by the PhoR⁻ to PhoR+ ratio. Percent recombination was calculated as percent of input Hfr cells.

Determination of alkaline phosphotase activity in colonies: The plates were sprayed with a mixture of α-naphtyl phosphate (the substrate) and tetrazotized o-dianisidine (an azo dye interacting with the product) as described by BRACHA and YAGIL (1969). Colonies with a purple coloration indicated alkaline phosphotase activity; only constitutive colonies (PhoR⁻) show enzymatic activity because the high inorganic phosphate concentration in the medium represses enzyme synthesis in the wild-type (PhoR+) colonies.

TABLE 1

Characteristics of E. coli *K12 strains*

Strain	Sex	Genotype	Source or reference
AT2455	HfrH	*cysG thi* Tsx-s Str-s	A. RONEN, Hebrew University
AT2455R	HfrH	*phoR cysG thi* Tsx-s Str-s	Induction by nitrosoguanidine of a *phoR* mutation in AT2455 (TORRIANI and ROTHMAN 1961)
300P	HfrH	*thi lacY* Tsx-s Str-s	F. JACOB
300PR	HfrH	*thi lacY phoR* Tsx-s Str-s	Induction of a *phoR* mutation as with strain AT2455R
J1	HfrC	*phoR* Tsx-s Str-s	YAGIL *et al.* (1970)
JH1	HfrC	*thr phoR* Tsx-s Str-s	Induction by nitrosoguanidine of a *thr* mutation in strain J1 (ROSNER and YAGIL 1970)
C600	F⁻	*thi thr leu lacY* Tsx-r Str-r	F. JACOB
BT1	F⁻	*thr lac proC phoR trp* Tsx-r Str-r	Thi+ PhoR⁻ recombinants from the cross JH1 × RLT6 (YAGIL *et al.* 1970)
W208	F⁻	*thi thr leu lacZ* Tsx-r Str-r	A. RONEN, Hebrew University
W208R	F⁻	*thr leu lacZ phoR* Tsx-r Str-r	Thi+ PhoR⁻ recombinants from the cross J1 × W208

Abbreviations are as recommended by DEMEREC *et al.* (1966). Abbreviations used: *cys*-cysteine; *his*-histidine; *lac*-lactose; *leu*-leucine; *pho*-phosphatase; *pro*-proline; Str-s-streptomycin sensitive; *thi*-thiamine; *thr*-threonine; *trp*-tryptophan; Tsx-s-sensitivity to bacteriophage T6.

THEORETICAL CONSIDERATIONS

To outline the possibilities which may lead to a heterogenetic exconjugant cell in the simplest way let us suppose two linked genetic markers *a* and *b* and a conjugational cross in which a wild-type Hfr parent is mated with an F^- parent carrying *a* and *b* (Figure 1). Recombinant colonies are selected for the distal marker *b* and segregation for the proximal marker *a* is tested. Three types of recombinant colonies are possible: the two genetically pure ones *a*,+ and +,+ and heterogenetic clones (colonies) containing cells of *both* genotypes. In Figure 1 the possible mechanisms to obtain heterogenetic clones from an exconjugant cell are outlined; it should be pointed out that the way by which the recombination events are sketched does not imply a particular mechanism of recombination. The form we have chosen to draw crossovers is only for the sake of simplicity.

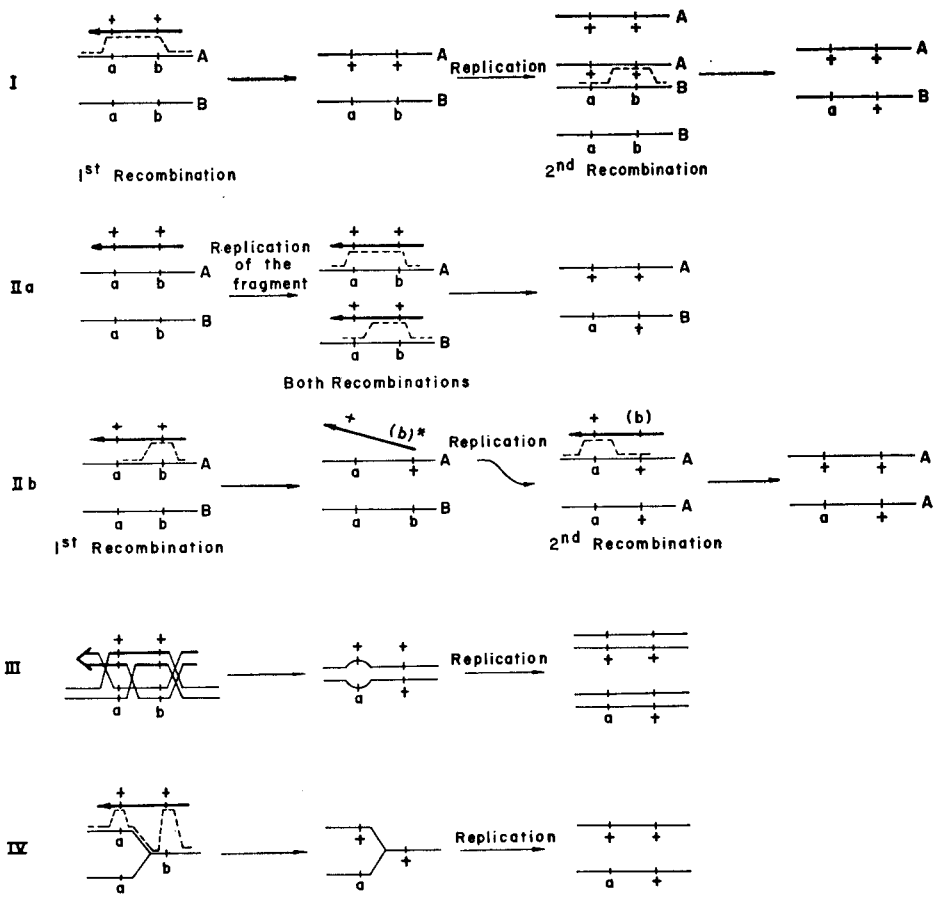

FIGURE 1.—Models describing the formation of heterogenetic clones. Thick arrows indicate Hfr fragment. See text for further details.

*Markers expected in case of a reciprocal exchange.

MODEL I

Recombination between two F⁻ nuclei: The Hfr fragment participates only once in a recombination event, the recombinant F⁻ chromosome replicates thereafter and undergoes a second recombination with a non-recombinant F⁻ chromosome in the same cell (replicating *E. coli* cells contain 2–4 chromosomes, HAYES 1968).

MODEL II

The Hfr fragment undergoes two (or more) recombinations: This is possible in two ways: either by prior replication of the fragment (Model IIa) or by replication of a recombinant F⁻ chromosome prior to the second recombination (Model IIb).

MODEL III

Unequal crossover: In the Figure both DNA strands of each parent are depicted; one of the crossovers does not occur exactly in homologous loci on each pair of strands resulting in a heteroduplex region in the recombinant molecule. (See also WHITEHOUSE 1970). A subsequent replication yields a heterogenetic exconjugant and further replication on a solid medium—a heterogenetic colony.

MODEL IV

A replication fork between the two markers: Recombination occurs while a replication fork in the F⁻ chromosome traverses between *a* and *b*. However, the two markers must integrate independently, co-integration in one piece leads to structural complications which are probably lethal. (See also CURTISS 1969).

RESULTS

The *phoR* mutation which causes constitutivity for alkaline phosphatase synthesis (ECHOLS *et al.* 1961) has been mapped in position 10.6 on the *E. coli* genetic map (Figure 2; TAYLOR 1970; ECHOLS *et al.* 1961; YAGIL *et al.* 1970). In a conjugational cross in which recombinant colonies are selected for a prototrophic marker and *phoR* is segregating, three types of colonies are obtained when sprayed for activity of alkaline phosphatase (Figure 3): stained colonies which are constitutive (PhoR⁻), unstained colonies which are wild type (PhoR⁺, enzyme formation is repressed due to the presence of excess inorganic phosphate in the medium) and heterogenetic colonies which appear sectored (arrow in Figure 3) and contain cells of both phenotypes. Figure 4 shows diagrams of two such conjugational crosses using two males, each with a different origin and with an opposite direction of chromosome transfer (HfrH, Figure 4A and HfrC, Figure 4B). In cross A the F⁻ strain is constitutive for alkaline phosphatase synthesis (*phoR*) and in cross B the Hfr strain carries this mutation. In each of these crosses recombinants were selected for various nutritional markers and were sprayed for alkaline phosphatase activity (Table 2). The relative linkage relations be-

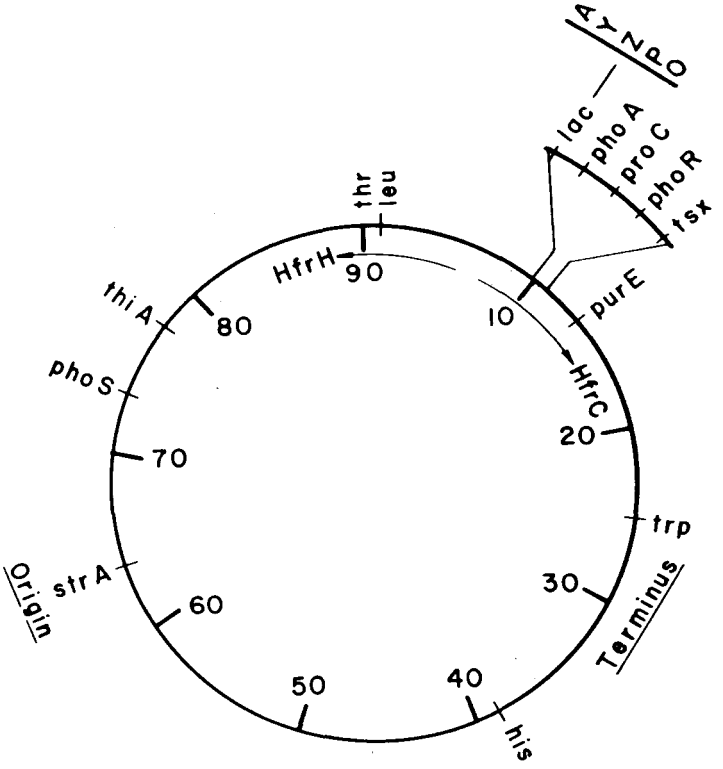

FIGURE 2.—Genetic map of *Escherichia coli*. Symbols are as recommended by TAYLOR (1970). For abbreviations see legend to Table 1. *Origin* and *Terminus* indicate approximate loci of the origin and terminus of chromosome replication (MASTERS and BRODA 1971). *phoA* indicates the locus for the structural gene and *phoR*, *phoS* indicate the regulatory genes for alkaline phosphatase synthesis.

tween *phoR* and the selected markers are confirmed and the last column of the Table shows the frequency of heterogenetic colonies. This frequency is higher in the HfrH parent than in the HfrC parent (7–20 fold for the same markers), confirming previous observations of the same kind (WOOD 1967). Furthermore,

TABLE 2

Analysis of recombinants from the crosses Hfr AT2455 × F⁻ BT1 and Hfr J1 × F⁻ C600

Cross	Selected recombinants	Number of colonies tested	Percent recombination	Percent PhoR+ colonies	Percent heterogenetic colonies
A. HfrH AT2455 × BT1	Thr+	135	6.5	34.0	6.8
	Lac+	257	2.5	85.0	3.9
	Pro+	339	2.0	86.0	2.4
	Trp+	266	0.05	52.0	10.0
B. HfrC J1 × C600	Lac+	893	2.8	14.3	0.2
	Thr+ Leu+	682	0.6	33.3	1.0
	Thi+	714	0.11	48.3	2.2

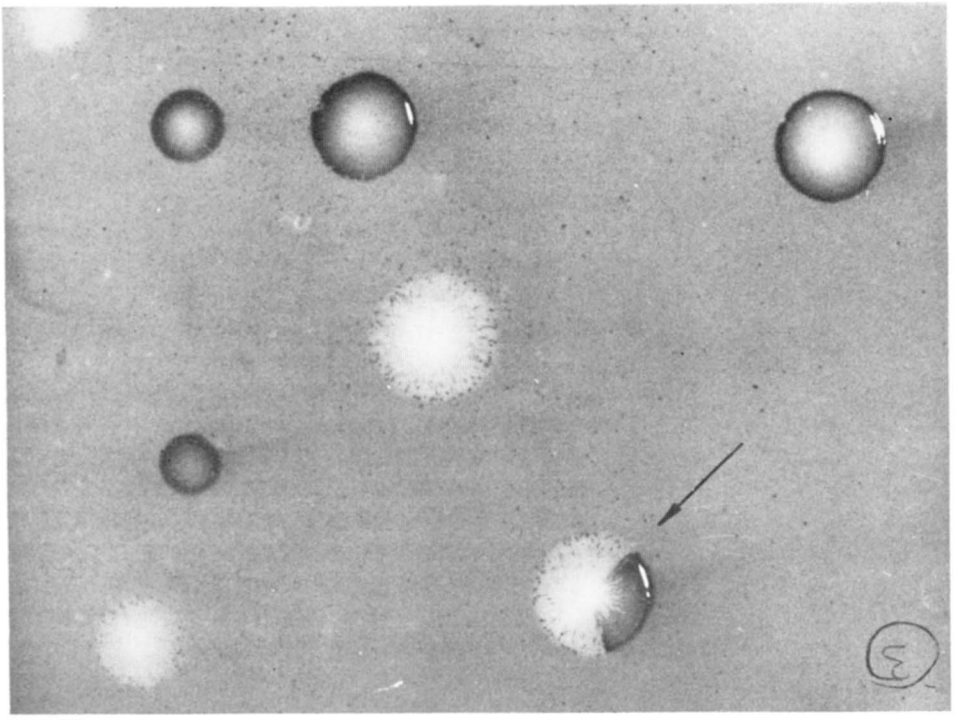

FIGURE 3.—Lac+ recombinant colonies from the cross Hfr AT2455 × F- BT1 sprayed for alkaline phosphatase activity. Dark colonies show enzyme activity. Arrow indicates a heterogenetic colony. The dark spots in the background are the F- Lac- PhoR- parental cells.

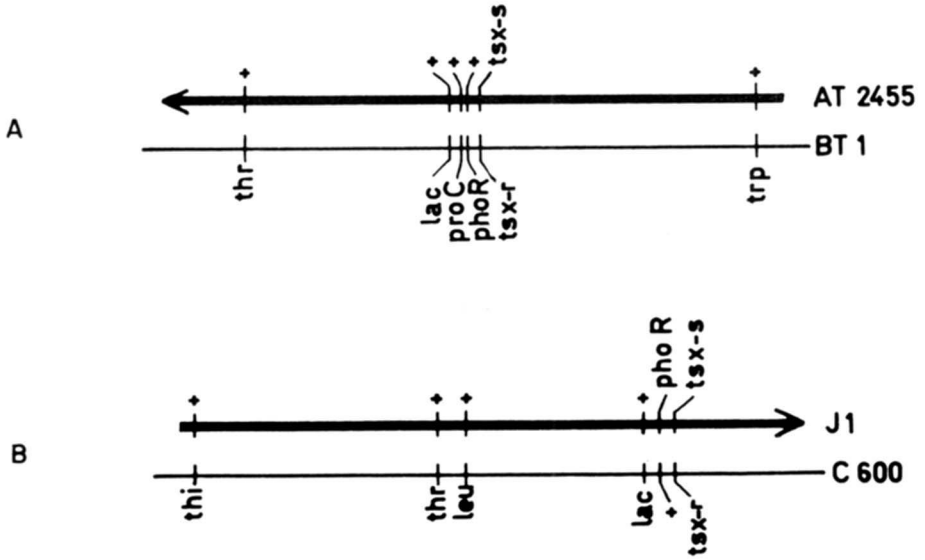

FIGURE 4.—A diagram of the cross between Hfr AT2455 × F- BT1 (A), and Hfr J1 × F- C600 (B). The arrow indicates direction of chromosomal transfer.

the closer the selected marker is to *phoR* the fewer are the heterogenetic colonies. This is expected if, for the formation of heterogenetic colonies, one of the crossovers is to occur between the selected marker and *phoR*. Because we are dealing with one segregating marker only, we observe less heterogeneity (maximum 10%) than reported by BRESLER *et al.* (1967) and by WOOD (1967), who dealt with heterogeneity for more than one unselected marker. To interpret the difference in frequency of heterogeneity between HfrH and HfrC, CURTISS (1969) proposed Model IV. It has recently been shown that chromosome replication starts at a particular initiation point on the *E. coli* chromosome (*Origin*, see Figure 2) and proceeds bidirectionally to a *Terminus* (MASTERS and BRODA 1971). However, since our genetic analysis deals only with markers on the "upper half" of the replicating chromosome we can consider this half as undergoing unidirectional replication. Model IV predicts therefore that heterogenetic colonies are expected only when the selected marker is distal to the *origin* of replication and the unselected marker is proximal. From Table 2 it is seen, however, that with both Hfr parents *phoR* heterogenetic colonies were obtained when the selected markers were proximal (Thr$^+$, Lac$^+$, Pro$^+$) to the *Origin* of replication and the segregating marker (*phoR*) was distal. These results render Model IV unlikely.

Because the ratio between the Hfr:F$^-$ parents varied between experiments we tested whether this variability has an effect on the frequency of heterogenetic colonies. Three matings with different Hfr:F$^-$ ratios were performed and the frequency of *phoR* heterogenetic colonies was scored among Lac$^+$ and Trp$^+$ recombinants (Table 3). It is seen that raising the ratio up to 2 Hfr cells per F$^-$ did not affect the frequency of heterogeneity. The possibility that heterogeneity is caused by triparental matings (2Hfr:F$^-$) is therefore rendered unlikely.

The principal difference between Models I and III *vs.* Model II (a and b) is that in the former models the Hfr fragment participates only once in a genetic recombination whereas in the latter it participates more than once in recombination. To distinguish between these possibilities we determined the frequency of *phoR* heterogeneity among Lac$^+$ recombinants in a cross between two Lac$^-$ parents (Hfr *lacY phoR* × F$^-$ *lacZ*) as compared to the control cross (Hfr *phoR* × F$^-$ *lacZ*). For the formation of Lac$^+$ recombinants in the former cross a rare crossover is required in the small region between *lacY* and *lacZ* which reduces the fre-

TABLE 3

Effect of Hfr: F$^-$ ratio on the frequency of phoR *heterogeneity*

Ratio Hfr:F$^-$	Percent phoR heterogenetic colonies among: Lac$^+$ recombinants	Trp$^+$ recombinants
1:36.6	0.93 (107)	14.3 (126)
1:2.6	3.42 (350)	9.1 (77)
1.9:1	2.32 (214)	11.6 (188)

The parental strains were Hfr AT2455 × F$^-$ BT1. The Hfr:F$^-$ ratio was changed by varying the amount of Hfr added to each of the conjugation flasks. The ratio was determined by the Lac$^+$:Lac$^-$ ratio on MacConkey agar plates. The total number of colonies tested is indicated in parentheses.

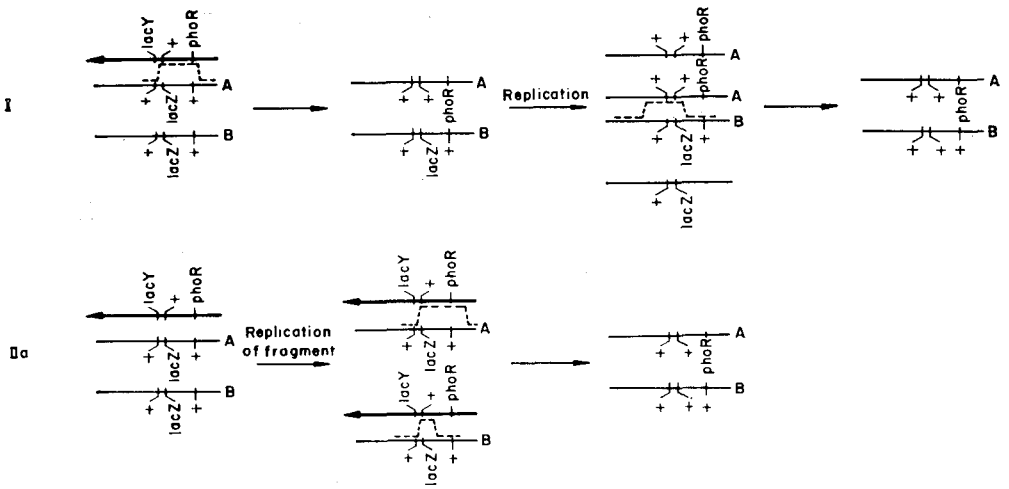

FIGURE 5.—Formation of *phoR* heterogenetic colonies in the cross Hfr 300PR (LacY⁻ PhoR⁻) × F⁻ W208 (LacZ⁻) according to Models I and IIa.

quency of Lac⁺ recombinants (Figure 5). According to Model I (Figure 1) the second recombination, which leads to heterogeneity, is no longer restricted to the region between the two closely linked *lac* loci (Figure 5) and therefore the frequency of heterogenetic colonies among the Lac⁺ recombinants should not be affected. According to Model IIa, on the other hand (Figure 5)—to obtain heterogeneity—a second recombination must occur between *LacY* and *lacZ* and therefore a reduction in the frequency of *phoR* heterogenetic colonies is expected among the selected Lac⁺ recombinants. Similarly, if one works out the expectations according to Models IIb and III (as compared to the control cross) it can be seen that no reduction in the frequency of heterogeneity is expected. Thus, only Model IIa predicts a reduction in the frequency of *phoR* heterogeneity. The results, given in Table 4A, show that of the Lac⁺ recombinants obtained (0.27% *vs.* 7.6% in the control cross) *none* showed heterogeneity for the *phoR* marker. These results favor Model IIa, i.e. the Hfr fragment replicates in the exconjugant

TABLE 4

Frequency of phoR *heterogenetic colonies among Lac⁺ recombinants*

	Cross	Genotype	Number of colonies tested	Percent recombination	Percent PhoR⁺ colonies	Percent heterogenetic colonies
A.	AT2455R × W208	Hfr *phoR* × F⁻ *lacZ* (control)	1045	7.6	33	2
	300PR × W208	Hfr *lacY phoR* × F⁻ *lacZ*	1432	0.27	25	<0.1
B.	AT2455 × W208R	Hfr wild type × F⁻ *lacZ phoR* (control)	303	10	67	4
	300P × W208R	Hfr *lacY* × F⁻ *lacZ phoR*	382	0.21	79	<0.25

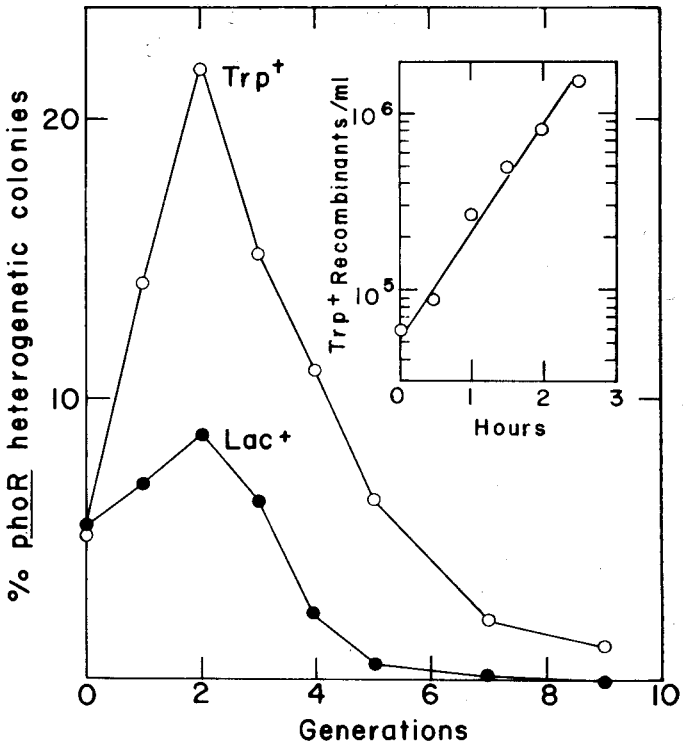

FIGURE 6.—Frequency of *phoR* heterogenetic colonies among Trp+ (○) and Lac+ (●) recombinants in a culture of segregating exconjugants. The parental strains were Hfr AT2455 × F- BT1. Following conjugation (at t = 0 min) the parents were diluted and resuspended in liquid broth containing 100 μg/ml streptomycin. At intervals samples were removed, diluted and plated for Lac+ and Trp+ recombinants. Insert shows the frequency of Trp+ recombinants in the growing culture.

cell and participates more than once in recombination. Similar results are obtained in a reciprocal cross where the *phoR* mutation is on the F- genome (Table 4B).

Finally, in order to gain more information in favor of either of the proposed Models, we determined the frequency of *phoR* heterogeneity in a segregating culture of exconjugants. Cells of the cross Hfr AT2455 × F- BT1 (Figure 4A) were mated for 40 min and were then allowed to replicate in liquid broth containing streptomycin. At intervals, samples were plated for Lac+ and Trp+ recombinants. The frequency of *phoR* heterogenetic colonies among the recombinants is shown in Figure 6. The number of Trp+ (and Lac+) recombinants increased exponentially (Figure 6–insert), as expected in a segregating culture, however, a *rise* in the frequency of heterogeneity among these recombinants occurred during the first two generations of segregation and was followed by a decline. Heterogeneity disappeared at the seventh generation in the Lac+ recombinants and even later among the Trp+ recombinants. These results, again, are best inter-

preted by Model IIa. Without replication of the fragment the rise in the frequency of heterogenetic colonies observed in Figure 6 is difficult to explain.

DISCUSSION

We have shown that the formation of heterogenetic recombinant colonies for the *phoR* marker follows Model IIa, i.e. results from replication and repeated recombination between the Hfr fragment and the genome of the F^- cell. The data of Figure 6 suggest that following mating the Hfr fragment continues to replicate for 2–3 generations but is eventually diluted out as the exconjugant cells continue to divide. The decline in the frequency of heterogeneity at the beginning of the third generation (Figure 6) is slower than exponential, indicating that more than one fragment per cell is being diluted out. ITOH and TOMIZAWA (1971) have shown that the Hfr fragment in a Rec^+ exconjugant is gradually degraded. They used a heat-inducible temperate phage as indicator for the presence of the fragment and therefore by plating for heat-inducible infective centers could not have detected a rise in the number of fragments per exconjugant cell. WLODARCZYK and KUNICKI-GOLDFINGER (1970) have shown that the capacity to form recombinants by exconjugants can develop a resistance to decay of P^{32}. BRESLER *et al.* (1967) have demonstrated the need for DNA synthesis in the exconjugants for recombinant formation. Both findings support our conclusion that the fragment not only recombines, but also replicates in the exconjugant cell.

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