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Role of simple and complex aggregates in *Escherichia coli* Hfr × F⁻ matings

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SUMMARY

Analysis of tetraparental Hfr × F⁻ matings of *Escherichia coli* strains showed that mating complexes were predominantly pairs or small aggregates of cells. Many physically associated complexes of donor and recipient cells gave rise to no recombinant cells. The observed linkage of genetic markers in recombinants is sensitive to multiple mating events, and should therefore be measured under well-defined conditions giving pairwise matings only.

1. INTRODUCTION

The mating process in *Escherichia coli* between Hfr or F′ donor cells and F⁻ recipient cells is interesting as a model of cell–cell interactions. We have shown that the efficiency with which recombinants are formed varies over a considerable range, and depends on cell concentration and on cell mobility (Collins & Broda, 1975). At high concentrations, the process is complicated by the fact that collisions of further cells with the primary mating complex may either disrupt such pairs or result in the formation of more complex mating aggregates, perhaps of the type reported by Fischer-Fantuzzi & di Girolamo (1965). Achtman (1975) has documented the existence of large aggregates of cells in mating mixes using particle size determinations with a Coulter Counter and microscopy. Since multiple aggregates might allow multiple transfer, they could be one cause of the genetic complexity observed in female exconjugants in some matings (Bresler, Lanzov & Manukian, 1967, 1973; Lotan, Yagil & Bracha, 1972). However, even single female exconjugants obtained by micromanipulation have been observed to give rise to a number of different classes of progeny (Lederberg, 1957; Anderson, 1958; Wood, 1967).

At some stage the process of aggregation must be limited by factors such as the utilization of all the potential for contact formation afforded by the pili. Certainly aggregates do not form as fast as expected on the basis of the kinetics of cell collisions (Collins & Broda, 1975); indeed, some cells appear not to participate at all in mating (Nelson, 1956; Walmsley, 1973).

We have attempted to answer the following questions about a mating between normally growing cells:

(i) what proportion of mating cells are in complex aggregates;

(ii) what proportions of the recombinant cells arise in complex and simple aggregates, respectively;

(iii) what differences can be observed between the patterns of recombinant classes arising in colonies derived from simple and complex mating aggregates, respectively?

We have used two genetically distinguishable donor strains with two genetically distinguishable recipient strains in a four-parental mating. At intervals, samples were diluted and plated on nutrient agar and agar selective for recombinant colonies. By determining the incidence of the different parental types in a number of colonies, it was possible to calculate the proportion of colonies that derive from more than one donor cell or more than one recipient cell; that is, of those formed from complex mating aggregates. We find that about 30% of donor–recipient complexes give rise to recombinants. Further, only a minority of mating complexes involve more than a single donor cell and a single recipient cell. The pattern of progeny classes in all cases could have arisen through a small number of independent recombination events.

2. MATERIALS AND METHODS

(*a*) *Strains.* The donor strains of *E. coli* used were Hfr B8, which is *metB lac*$^+$ *str*$^+$ (Broda, 1967), and a spontaneously arising Lac$^-$ derivative, ED3688. In interrupted mating experiments, the times of entry of the markers relevant here were: *proC*$^+$ 4 min; *purE*$^+$ 8 min; and *trp*$^+$ 26 min; *lac*$^+$ is transferred very late (Broda, 1974; Broda & Collins, 1974). The recipient strains were X478 which is *proC purE trp lys metE leu lac str* (Berg & Curtiss, 1947) and its Lac$^-$ derivative ED1199, made by P1 transduction.

(*b*) *Latex particles.* The calibration P.V.T. latex suspension (Coulter Electronics Ltd, Harpenden) contained particles with a diameter of 2·03 μm.

(*c*) *Matings.* The fertility of the Hfr strains was checked beforehand by testing 100 patched colonies with the recipient strains in plate matings. The proportion of fertile colonies ranged from 98 to 100%.

Cultures for mating were grown in L-broth with moderate shaking. Mating cultures were not shaken further. All dilutions were performed with L-broth using 1 ml pipettes to minimize shear. Blending and other procedures were performed as previously described (Broda, 1974).

(*d*) *Direct observation of aggregates.* Samples of mating cultures were fixed in formaldehyde and plated on agar directly, for microscopic examination and photography. The cells were not distributed evenly in the microscopic fields but could be found in local concentrations, possibly due to channelling effects in the preparation of the slides. Additional samples prepared by the method of Achtman (1975) on gelatin slides showed the same phenomenon. It was considered important to use an internal control for these observations, which were therefore repeated with mating cultures to which a suspension of latex particles had been added. Samples were taken and plated as before, and also after a tenfold dilution in the fixative.

Many clumps of cells were noted in the undiluted samples on both agar and gelatin, but so too were clumps of latex particles, and of cells and latex particles together. In the samples diluted tenfold before plating, fewer aggregates of cells and of latex were observed. Since mating aggregates are known to be resistant to dilution, the aggregates observed in undiluted samples must have included many artifacts. Therefore microscopy can only be used to obtain qualitative information on the complexity of mating.

(*e*) *Limitations on DNA transfer after challenge with streptomycin.* A different approach involving tetraparental matings was adopted because it was free of such difficulties and also gave information on the events leading to recombinant formation. For this analysis it was necessary to establish conditions where sampling from the mating culture did not interfere with the formation of Pur$^+$ and Trp$^+$ recombinants by existing mating complexes. Otherwise the coinheritance of *trp*$^+$ among Pur$^+$ progeny would be a function of the duration of mating before plating. We therefore determined how much residual transfer from Hfr B8 to strain X478 was possible after (A) dilution into broth containing streptomycin, and holding for 30 min before plating, and (B) dilution followed by direct plating onto selective agar. The yields from samples taken at intervals and treated thus were compared to those obtained from two parallel samples (C and D) which were blended prior to dilution but otherwise treated as samples A and B.

For both Pur and Trp the data from the unblended samples (A and B) were very similar to each other. The data from the blended samples (C and D) also resembled each other but showed a characteristic displacement from the A and B values. This was more pronounced for the Trp marker, and is an index of the location on the chromosome relative to the origin of transfer. This result with C and D implies that once recipient cells have received the DNA for the nutritional marker their ability to integrate it is approximately the same in broth and on the selective agar. The similarity between the A and B results then implies that once the cells have been challenged with streptomycin, the same amount of residual DNA transfer is possible in broth or on selective agar. As the numbers of Trp$^+$ progeny rose during the course of the experiment relative to the number of Pur$^+$ progeny, we conclude that streptomycin limits Trp$^+$ DNA transfer to a greater extent than it limits Pur$^+$ DNA transfer. The introduction of streptomycin immediately after sampling does not allow us to see how the mating aggregates present at the time of sampling would have behaved if they had been left undisturbed. We have therefore chosen to study the properties of mating aggregates by diluting the samples to prevent new collisions and holding for 30 min without streptomycin to allow transfer of *trp*$^+$ to be accomplished before plating.

(*f*) *Tetraparental matings at two cell concentrations.* Exponentially growing cultures of the two donor strains were mixed to give approximately equal numbers of cells. The same was done with the two recipient strains. One generation later, 10 ml samples of the mixed donor and of the mixed recipient cultures were

combined (A) and mating proceeded without shaking. Ten min after the start of the A mating, another mating mixture (b) was established using 0·7 ml each of the mixed donor and the mixed recipient cultures, and 18 ml of prewarmed broth, giving one-tenth the cell concentration of the first mating. Other samples were diluted appropriately (at least $\times 10^4$ for cross A and $\times 10^3$ for cross B). Samples were held in broth for 30 min; portions of these samples were then plated onto selective plates for Pur$^+$ progeny without further dilution and, after dilution, on nutrient plates.

(*g*) *Colony analysis.* Multiple platings were employed in order to produce enough colonies for further analysis. With 50 or fewer colonies per plate, the chances of coincidental platings of cells not physically associated together was calculated to be less than one per plate (assuming that cells closer than 2 mm might develop into contiguous colonies that could be mistaken for a single colony). Whole colonies from either nutrient plates or from recombinant-selective plates were emulsified in 5 ml of buffer. Samples of 0·1 ml from dilutions of 10^4-fold were spread onto the appropriate plates: minimal supplemented with methionine to allow growth of the donor strains only, minimal with appropriate supplements plus streptomycin to allow growth of the recipient strain or specific recombinant classes. A mean of 100 colonies per plate was obtained. Such plates were replicated after 2 days onto further media including lactose MacConkey plates to identify individual cell types. Since these operations took considerable time, it was necessary to show that there was no selective death within the colonies themselves; no changes in the patterns were detected from samples analysed at intervals of up to 2 weeks.

3. RESULTS

In view of the difficulties encountered with the direct method of visualization of mating aggregates (see Materials and Methods), we employed the less direct tetraparental mating method. We first showed that after dilution to prevent further aggregate formation, a holding period of 30 min sufficed to allow transfer of DNA to the last marker, *trp*, being observed in the recombinants (see Materials and Methods). We could therefore compare the behaviour of mating aggregates sampled from the mating culture at different times.

(i) *The tetraparental cross*

The yields of viable colonies on both nutrient and selective plates in the two crosses are given in Table 1. In cross *A*, the yield of Pur$^+$ recombinants rises to about 5% of the recipient colony count, and in cross *B* it reaches about 1·6%. Therefore the yields in the two crosses are not in proportion to the products of the donor and recipient cell concentrations (a ratio of 100:1 would have been expected, on the basis of Hayes (1957)). Instead the efficiency drastically declines at the higher cell concentration, as previously reported (Collins & Broda, 1975; Achtman, 1975).

(ii) *Complexity of mating aggregates*

Colonies from nutrient plates from the 50 min samples (which might be expected to contain the most complex aggregates) were emulsified and analysed for their strain composition. Only 8% and 5% of the 300 colonies examined from the A and B crosses respectively were found to contain both donor and recipient cells. Hence such mating aggregates account for only the minority of the cells

Table 1. *Pur$^+$ recombinant formation and colony-forming units on broth agar in four-parent mating (per ml of mating cultures). In cross A, the initial cell concentration was* c. *tenfold that in cross B*

Cross		Time of initial dilution (min)					
		10	15	30	35	50	55
A	Pur$^+$ recombinants ($\times 10^{-7}$)	0·41	0·77	1·4	1·7	2·2	1·8
	Donors ($\times 10^{-8}$)	1·7	—	2·3	—	2·8	—
	Recipients ($\times 10^{-8}$)	2·6	—	3·6	—	4·3	—
	Ratio: $\frac{\text{Recombinants}}{\text{Recipients}}$	0·016	—	0·039	—	0·051	—
B	Pur$^+$ recombinants ($\times 10^{-6}$)	0·1	0·2	0·6	0·5	0·8	1·5
	Donors ($\times 10^{-8}$)	1·4	—	2·5	—	3·9	—
	Recipients ($\times 10^{-8}$)	1·9	—	3·2	—	5·0	—
	Ratio: $\frac{\text{Recombinants}}{\text{Recipients}}$	0·005	—	0·019	—	0·016	—

in both crosses at this time. Mating complexes are stable during dilution, holding and plating (see below). However, during the 30 min holding period the number of recipient cells increases by a factor of 1·4 (data not shown). Therefore, the 12 and 8·5% of the recipients in the A and B crosses that were engaged in potential mating aggregates at the time of plating correspond to values of 16·9 and 12% respectively at the time of dilution.

However, it was possible that some female exconjugants were by this time (50 min) free of donor cells, so that the number of mixed colonies was an underestimate of the number of cells participating in matings. Therefore, as well as clones from all colonies that contained both donor cells and recipient cells, clones from some colonies derived from apparently uncomplexed female cells were also tested for parental types and recombinant classes present (Table 2).

Among the recipient cells apparently complexed with donors, only about 30% (in the A cross) and 20% (in the B cross) gave evidence of recombinant formation; that is, at least two-thirds of the mixed colonies gave none. Therefore many 'mating aggregates' or pairs may be non-productive. This result also means that new rounds of mating in the growing colony are rare. In contrast, only one of sixty uncomplexed recipient cells (from the A cross) and only one of fifty from the B cross contained recombinant cells. This result suggests that most recipient cells involved in successful matings remain complexed with donor cells, even

though in many such pairs DNA transfer will have stopped. Therefore our experimental procedure is an assay of past and present mating complexes.

Only one colony (from the A cross) contained both Lac$^+$ and Lac$^-$ recipient cells. Therefore, allowing for the equal number of colonies expected to have arisen either from two Lac$^+$ or two Lac$^-$ recipient cells, we can calculate that only

Table 2. *Comparison of the genotype classes present in F$^-$ clones isolated from individual colonies on viable count plates for the 50 min samples (cf. Table 1). Colonies were tested by replicating for the Pro, Pur and Trp phenotypes*

	Cross	Recipient genotype only	Recombinant		Recipient + Recombinant		Lac$^+$	Total
			Single class	Mixed classes	Single class	Mixed classes		
(i) From mixed donor–recipient colonies	A	15	1	2	1	4	14	23
	B	12	1	0	1	1	8	15
(ii) From apparently unassociated recipients.	A	59	0	0	1	0	30	60
	B	49	0	0	1	0	26	50

about 10% of the aggregates should contain two recipient cells. Insufficient data were available to make any conclusions about the number of donor cells in a mating aggregate.

Only one among the apparently uncomplexed recipient clones tested, and none of the apparently uncomplexed donor clones tested was mixed with respect to the Lac phenotype. There must therefore be only few accidental mixed colonies (i.e. formed by coincidence on the plate).

(iii) *Complexity of recombinant colonies*

We have also assessed the complexity of Pur$^+$ recombinant colonies arising in selective medium after sampling at 10, 30 and 50 min in the same two matings. Such clones were emulsified and plated as described in Methods. In the cases where the clones were mixed with respect to recombinant classes the relative proportions of the different classes were often quite different, though no systematic bias towards particular classes was observed.

The numbers of recombinant colonies that contained Lac$^+$ and Lac$^-$ cells in the A cross were 6/94 (10 min), 9/94 (30 min) and 17/94 (50 min). The corresponding values for the B cross were 1/80, 1/93 and 2/94. Therefore with time there is a slight trend towards complexity. However, the pattern of inheritance of donor markers did not change significantly, and for this reason we present the pooled results from the A and B crosses in Table 3.

The majority of clones are pure in the sense that from each such clone only colonies with a single Pro and a single Trp phenotype were observed. This can most readily be understood if such matings are between a single donor and a single recipient and involve a single round of recombination. The genetically

mixed clones could then result from matings involving more than one recipient cell or from recipient cells (or colonies) in which more than one round of recombination had occurred. We can assess the frequency of the first type of event by analysing Pur$^+$ colonies that contain both Lac$^+$ and Lac$^-$ colonies. Of 32 such colonies in cross A, over half were mixed with respect to the donor markers. On the assumption that the most common complex aggregate has only two recipient cells, an equal number would have remained undetected because both recipient

Table 3. *The coinheritance of unselected markers among Pur$^+$ recombinants of the A and B crosses*

Low-density mating (cross B)	Colonies with single recombinant class	Recombinant classes found				Colonies with more than one recombinant class
		Pro$^+$ Trp$^+$	Pro$^+$ Trp$^-$	Pro$^-$ Trp$^+$	Pro$^-$ Trp$^-$	
(I) Colonies analysed (266)	226	4	6	21	195	40
(II) Colonies from I containing both Lac$^+$ and Lac$^-$ recipient cells (4 colonies)	1	0	0	0	1	3
(III) Corrected data from colonies believed to have arisen from simple mating pairs I$-$(2 × II) = 258 colonies	224 (86·8 %)	4 (1·5 %)	6 (2·3 %)	21 (8·1 %)	193 (74·8 %)	34 (13·2 %)
High-density mating (cross A)						
(I) Colonies analysed (282)	219	3	14	26	176	63
(II) Colonies from I containing both Lac$^+$ and Lac$^-$ recipient cells (32 colonies)	14	0	0	0	14	18
(III) Corrected data from colonies believed to have arisen from simple mating pairs I$-$(2 × II) = 218 colonies	191 (87·6 %)	3 (1·4 %)	14 (6·4 %)	26 (11·9 %)	148 (67·9 %)	27 (12·4 %)

cells were Lac$^+$ or Lac$^-$. After making allowance for such undetected complexes, we found that the remaining Pur$^+$ recombinant clones, presumably derived from simple mating pairs, were mainly genetically pure.

The data from the B cross show a simpler picture – fewer complex mating aggregates, arising slowly during the experiment, and simpler patterns of recombinant classes present in the colonies analysed. We can again deduce the behaviour of simple mating pairs (Table 3). If these deductions are valid, then the outcome of simple pairwise matings deduced from the data from each cross should be similar; the agreement is considered good.

4. DISCUSSION

We have adopted the tetraparental mating technique to assess the complexity of matings between exponentially growing cells of two well-characterized strains, Hfr B8 and X478. The mating complexes are unlikely to have been disrupted mechanically, and transfer between cells can continue first in broth and then on solid agar. Our analysis is directed at the commonest events; that is, what is the composition of the major classes of mating complex, and what is the composition of the recombinant colonies derived therefrom? We can only detect minority classes down to about 1% of the total number of recombinant cells.

For the A and B crosses, the consistent patterns of inheritance of unselected markers among the Pur^+ recombinants at the different sampling times establish that our manipulations do not interfere with zygosis. The absence of free recombinants, even after the sampling manipulations and the 30 min period of holding in broth, confirms the stability of the mating complexes even after DNA transfer may have ceased.

We find that even in the A cross, the predominant class of mating complex contains a single donor and a single recipient cell. Multiple matings can indeed the demonstrated, but probably have a significant role only late in the mating. The B cross contains even fewer multiparental matings and approximates to the ideal condition of mating, i.e. where all complexes are simple pairs. This is relevant to our consideration of progeny analyses.

Perhaps surprisingly, most presumptive mating complexes (i.e. mixed donor–recipient colonies recovered from nutrient plates) showed no evidence that genetic transfer had occurred, so that few if any rounds of mating and/or recombination take place in the colony itself within the first few generations after plating. The reason for this is unclear. However, the main implication here is that the recombinant cells found have descended from one or a few recipient cells in each colony. Post-zygotic events are therefore predominantly simple. In cross A, the presence of complex aggregates raises markedly the proportion of colonies containing mixed recombinant classes, and the proportion of colonies with a single recombinant class (particularly one of the less common ones) fall significantly.

The properties of the simple pair that does produce progeny have been deduced for crosses A and B in Table 3. Eighty-seven per cent or more show a single recombinant class observed among the *c.* 100 single cell descendants of the original zygote that could be examined by our methods. The role of multiple rounds of recombination observed when HfrH was the donor (Anderson, 1958) but much less with HfrC (Lederberg, 1957) is therefore minimal in this system. In contrast the majority of colonies in crosses A and B known to be derived by transfer into the two distinguishable recipient strains contain multiple classes of recominant (Table 3). However, the complexity of these mixed colonies was merely that expected on the assumption that in each colony two independent simple matings with the properties given in Table 3 had occurred. Complex matings raise the observed frequency of any recombinant class relative to the number

of colonies observed, but this change is much more pronounced in the case of a minor recombinant class such as *pur*$^+$ *pro*$^+$ *trp*$^+$. The data deduced for 218 simple mating pairs in cross A show that the class *pur*$^+$ *pro*$^+$ *trp*$^+$ occurred three times in 191 colonies containing a single recombinant class and three times in 27 colonies containing more than one recombinant class, i.e. six times in 218 cases (2·75%). In the 32 colonies derived from aggregates that contained zygotes from both recipient strains in cross A, the class *pur*$^+$ *pro*$^+$ *trp*$^+$ occurred four times (12·5%). The use of mating conditions in which complex aggregates are more common will therefore lead to linkage data which differ even more from those obtained under the more ideal conditions afforded by low density matings in which all recombinants are derived from simple mating pairs.

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