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Inhibition of Formation of *Escherichia coli* Mating Pairs by f1 and MS2 Bacteriophages as Determined with a Coulter Counter

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The effect of male-specific filamentous deoxyribonucleic acid (f1) and isometric ribonucleic acid (MS2) bacteriophages on the formation of mating pairs in *Escherichia coli* conjugation was examined directly in the Coulter counter. When a sufficient multiplicity of infection (MOI) was used, the f1 phage immediately and completely inhibited the formation of mating pairs. On the other hand, the MS2 phage at a relatively high MOI also inhibited the formation of mating pairs significantly although not completey. The inhibitory effect of MS2 phage was dependent on the time of addition and the MOI used. At relatively low MOI (<20), the MS2 phage showed some inhibitory effect when added to a male culture prior to mixing with females, whereas no effect was observed when phages were added after mating pair formation had already commenced. At a high MOI (>400) MS2 phage disrupted the mating pairs already formed. Some preformed mating pairs were resistant to the high MOI of MS2 phages, however, and the "sensitive" (to high MOI) mating pairs seem to mature into "resistant" mating pairs as a function of time. We conclude that the tip of an F pilus is the specific attachment site for mating. The following process of mating pair formation has been formulated by deduction. (i) The sides of F pili weakly contact female cells, (ii) then the tips of F pili attach to the specific receptor sites to form initial mating pairs, and (iii) those pairs mature into mating pairs that are resistant to the high MOI of MS2 phages. The high MOI of MS2 prevents the first step, whereas f1 phages affect the second step—the binding between the tips of F pili and the receptor sites.

It has been generally accepted that the hair-like surface organelle, F pilus, is necessary for *Escherichia coli* conjugation, at least for the initial formation of pairs between male and female cells (5, 6, 11). An F pilus is a gene product of the sex factor F (6). Therefore, only those cells that harbor F factor will produce F pili on their surfaces. F pili are not only the mating arms for the formation of mating pairs but are also the specific sites for the adsorption of the male-specific phages: the isometric ribonucleic acid (RNA) phage attach to the sides, and the filamentous deoxyribonucleic acid (DNA) phage attach to the tips of F pili (6-8). Utilizing this difference in the adsorption sites of RNA and DNA phages, several investigators have attempted to determine the site of specific attachment for pair formation—the tips or the sides—by adding those phages to a mating mixture at various stages of conjugation (13, 15, 18). The results all showed that both RNA and DNA phages reduce the production of recombinants, presumably by inhibiting the formation of mating pairs (13, 15, 18). The formation of mating pairs was not measured directly, however. Other explanations are therefore possible: the infection of phages may inhibit any one stage of conjugation—such as DNA transfer or integration.

Recently, Zn^{2+} at 10^{-3} M was found to inhibit the adsorption of the filamentous DNA phage to the tips of F pili (21, 24) but not the adsorption of the isometric RNA phage (21). Furthermore, the same concentration of Zn^{2+} greatly reduced the formation of mating pairs (20). These results suggest that the tip of F pilus is the site for the attachment to form mating pairs.

In this communication we would like to report further results of experiments designed to determine the specific mating pair attachment site

on the F pilus. In our experiments we also used two different phages, the isometric RNA phage, MS2, and the filamentous DNA phage, f1, and measured directly their effect on mating pair formation in a Coulter counter. Our results strongly indicate that the tip of an F pilus is indeed the specific attachment site for the formation of mating pairs. Furthermore, there are two types of mating pairs with respect to the sensitivity to the disruption by a high multiplicity of RNA phage infection: one which is disrupted, and the other which is resistant.

MATERIALS AND METHODS

Bacterial strains and bacteriophages. The bacterial strains used are all derivatives of *Escherichia coli* K-12: strain W3011 (a Cavalli type Hfr) and F⁻W1-3, whose genetic characteristics have been described (19).

The male-specific bacteriophages used are MS2, an RNA-containing isometric phage, and f1, a DNA-containing filamentous phage. To prepare a high titer of phages, the following procedure was used. Hfr cells were grown in 10 ml of broth at 37 C to the late exponential phase ($\sim 4 \times 10^8$ cells/ml); the phages were then added to this culture with a low ($\sim 10^{-2}$) multiplicity of infection (MOI). The mixture was incubated for 20 h, and chloroform (about 2 ml) was then added to lyse the cells. The cell debris were removed by centrifugation for 10 min at $8,000 \times g$. The titer of phage suspension thus obtained was usually 5×10^{11} to 10^{12} plaque-forming units/ml.

A brief description of some biological and physical characteristics of the two phages used may be useful at this point.

The DNA phage, f1, is a long filamentous phage (850 nm in length; reference 25) with a mass of 11.3×10^6 u (16) and adsorbs to the tip of an F pilus (7). Only a few, usually no more than two phages (7, 21), can attach to the tip with the adsorption rate of 3×10^{-11} ml/min (24).

The RNA phage, MS2, on the other hand, is a small spherical phage (25 nm in diameter) with a mass of 3.6×10^6 u (23) and adsorbs to the side of an F pilus (6, 8). A large number, depending on the length of the F pilus, can be attached to the adsorption site with the adsorption rate of 2.2×10^{-9} ml/min (22) which is 100-fold faster than that of f1 phage.

Media. The nutrient broth that has been described (19) was routinely used for growing and mating cells and for preparing phages. The broth was filtered twice through a membrane filter (Millipore Corp.) of 0.45-μm pore size to eliminate particles large enough to be registered by the Coulter counter.

Mating procedures. The following procedure was generally used unless otherwise mentioned. Male and female cells growing at the late exponential phase (4×10^8 to 9×10^8 cells/ml) were mixed at a ratio of 1:2, respectively, in a 25-ml Erlenmeyer flask. Mating pairs were allowed to form with gentle shaking, and samples were taken periodically. The number of pairs formed was determined by counting in the Coulter counter. The later exponential phase of growth was chosen for two reasons. (i) Late exponential-phase cells conjugate more readily (reference 5; Ou, unpublished data). (ii) Late exponential-phase cells multiply slowly (division time: 50 to 100 min) which minimizes the error in calculating the number of pairs formed. The male was usually chosen the minority parent in order to avoid the formation of homosexual pairs, i.e., male-male cell aggregation (20).

To minimize the change in the growth rate of cells due to dilution by the addition of phage, a high-titer phage suspension was used.

Coulter counter measurement. The technique of using a Coulter counter has been described in some detail (20). However, the present experiments have been performed exclusively with the Coulter counter; therefore, the technique is recounted briefly as follows.

The Coulter counter registers the impulses that are produced by the change in the electrical resistance when particles in electrolyte pass through the tiny aperture of the Coulter counter tube. The number of the monitored impulses in a unit volume of electrolyte that passes through the aperture corresponds, therefore, to the number of cells in that volume. When there are two cells close together, or clumps of cells, the counter monitors them as one. Thus, in a mating mixture of males and females, the number of impulses decreases as a function of time as more and more mating pairs are formed.

The concentration of mating pairs formed in a given time can be calculated by the following formula (20). The total number of impulses (I) in a unit volume at the time of mixing ($t = 0$) is:

$$I_0 = F_0 + M_0 \tag{1}$$

where F_0 and M_0 are the concentrations of unpaired female and male cells, respectively. At a time t, male and female cells have interacted to form pairs, triplets, quadruplets, etc. Thus, the total number of impulses in a given volume at a time, t, can be expressed as:

$$I_t = F_t + M_t + P_t \tag{2}$$

where F_t and M_t are the concentrations of unpaired female and male cells, respectively, and P_t is the concentration of female cells attached to male cells.

Thus,

$$P_t = F_0 - F_t \tag{3}$$

and

$$P_t = M_0 - M_t \tag{4}$$

Combining equations 1, 2, 3, and 4 we obtain:

$$P_t = I_0 - I_t \tag{5}$$

By measuring the total number of impulses as a function of time, as well as the individual concentrations of males and females just before mixing, one can follow the kinetics of the attachment of females to

males. Here, P_t is simply a measurement of the loss of impulses due to cell attachment and will denote the number of mating pairs. Thus, triplets will be regarded as two pairs, quadruplets as three pairs, etc.

The P_t calculated in the present report ignores the following. (i) Although cells whose division time is 50 to 100 min were used, there is an increase in cell number at the end of each experiment. In fact, we observed that the increase in cell number at the end of an experiment is usually less than 10% of the initial cell number. Since the increase in cell number increases I_t, the calculated P_t is smaller than the actual P_t. (ii) As mentioned above, minority male was used to minimize the male-male mating. However, with the ratio of 1 male to 2 females in mating mixtures, the formation of some male-male pairs cannot be avoided. Since F pili seem to be involved in the formation of these homosexual pairs (reference 20; see Fig. 3 and 4), and we are measuring the effect of f1 and MS2 bacteriophages on the F pili mediated pair formation, effort was not made to correct the number of male to male pairs from P_t.

RESULTS

Inhibition of the formation of mating pairs by male-specific filamentous f1 phage. The filamentous f1 phages were added to the male cell suspension immediately before the addition of the female cells. f1 phage inhibit the formation of mating pairs effectively (Fig. 1). A complete inhibition was achieved when the MOI was more than 400; 60% inhibition was observed with an MOI of 20; and even with an MOI of only 0.2, 17% inhibition was seen.

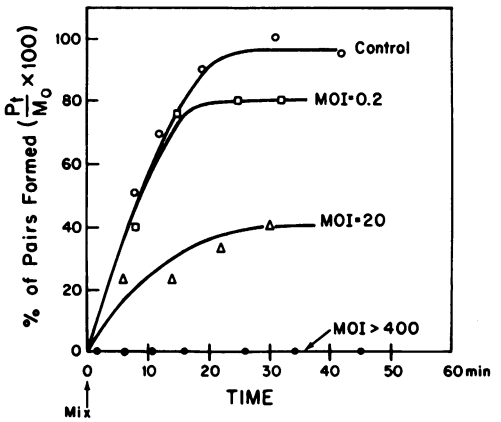

FIG. 1. *Inhibition of mating pair formation by f1 phage. At time t = −15 s, filamentous DNA phage, f1, were added to samples of HfrW3011 cultures at various multiplicities of infection (MOI). At time t = 0, the samples with and without f1 phage were mixed with F⁻W1-3 cultures at a ratio of 1:2. At various times thereafter, samples were diluted 400-fold, and impulses were monitored in the Coulter counter. The numbers of mating pairs were computed by the formula described in Materials and Methods. P_t is the concentration of mating pairs at time t and M_o is the concentration of input male cells. This figure contains three separate experiments. The concentrations of HfrW3011 were about 3 × 10⁸ cells/ml.*

Effect of male-specific isometric phage MS2 on the formation of mating pairs. Similar experiments were carried out to determine the effect of MS2 phage on the formation of mating pairs (Fig. 2). At an MOI of 20, the rate of pair formation appears to be similar to that with f1 phages at the same MOI (Fig. 1) but was never completely inhibited. When the MOI was increased to 200, the rate was reduced quickly to about one-half of the rate of the control mixture, and eventually pair formation was inhibited. At an MOI of 880, pair formation in the first 10 min was still inhibited only 50% compared to the control mixture. However, after 10 min, the number of pairs in the mixture decreased rapidly until, by 17 min, all pairs were disrupted. We shall study this phenomenon in detail in a later section.

Effect of male-specific phages f1 and MS2 on the aggregation of male cells. Male cells attach each other to form cell aggregates which are presumably formed through F pili (20). Therefore, the aggregates correspond to the mating pairs formed in the mixture of male and female cells. This aggregation is not seen in a female culture (20). The mating pairs, as well as the male-male aggregates, can be disrupted

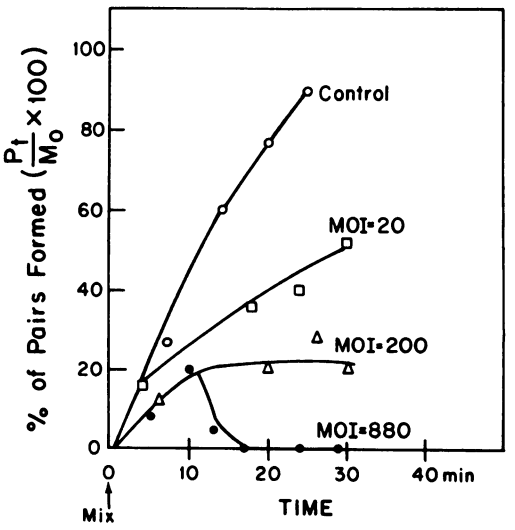

FIG. 2. *Inhibition of mating pair formation by MS2 phage. Isometric RNA phages, MS2, were used in an experiment similar to that described in Fig. 1. This figure also contains two separate experiments. The concentrations of HfrW3011 were about 2.5 × 10⁸ cells/ml.*

simply by a vigorous bubbling (19, 20; Ou, unpublished observation). The aggregation is also inhibited when an inhibitor of pair formation is present (20). The f1 phages were similarly very effective in preventing the formation of cell aggregates (Fig. 3). The decrease in the number of impulses was due to aggregation because, when a sample was bubbled, the number of impulses increased to the level of initial sample (t = 0). The increase after bubbling is shown by the arrows with dotted lines in Fig. 3. It was also observed that the higher the MOI the greater the inhibition, and when the MOI was raised to 1,000 the inhibition was complete.

MS2 also inhibited male cell aggregation, although less effectively than f1 phage (Fig. 4). As with the male and female cell mixture, a high MOI (800) initially only partially inhibited aggregation, whereas after about 10 min aggregates were completely dispersed.

Effect of phages on the continuation of pair formation. Pairs were allowed to form for 10 min before phages were added to the mixture. When f1 phages (MOI of 400) were added (Fig. 5), the further formation of pairs was immediately blocked. However, when MS2 was added (MOI of 400), the formation of pairs continued at the same rate for 7 more min before the number of pairs began to decrease.

If lower MOIs were used (<19) (Fig. 6), in the case of MS2 phage there was no detectable effect at all on the formation of pairs, whereas with an MOI as high as 200 the MS2 phage inhibited the pair formation significantly (Fig. 6). There was no decrease in the number of pairs, however.

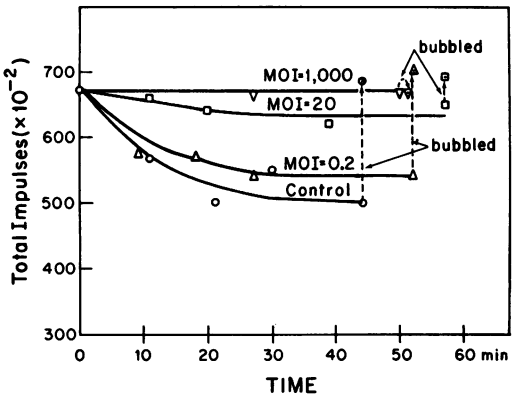

FIG. 3. *Inhibition of male cell aggregation by f1 phages. A culture of HfrW3011 was shaken to break up the aggregates of cells and was divided into four portions. At time t = 0, various numbers of f1 phages were added to three of the four samples. At various times thereafter, samples were diluted 400-fold, and impulses were counted in the counter. At the end of the experiment, each sample was vigorously bubbled to break up aggregates of cells, and the impulses in the bubbled samples were counted again (indicated by the arrows with dashed lines).*

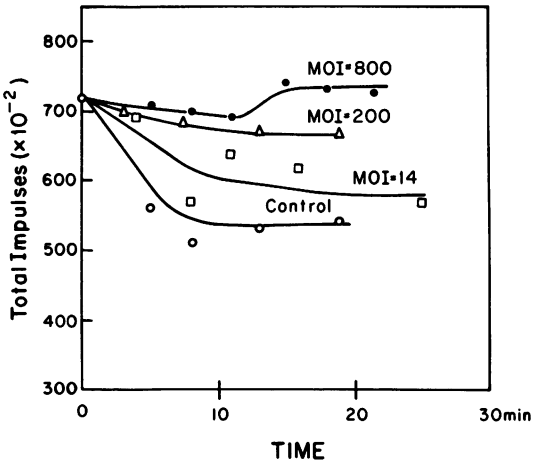

FIG. 4. *Inhibition of male cell aggregation by MS2 phages. Isometric MS2 phages were used here in an experiment similar to that described in Fig. 3.*

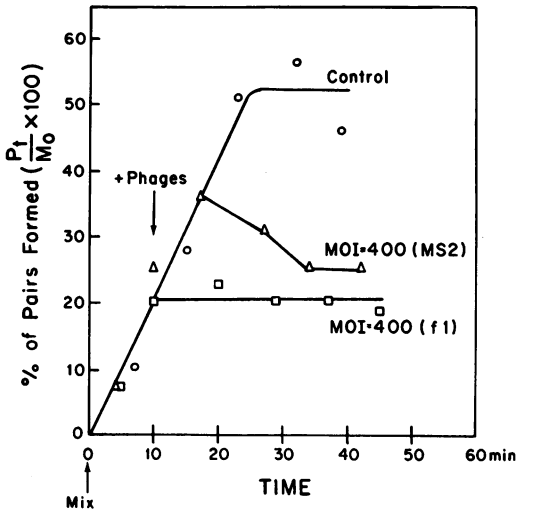

FIG. 5. *Effect of male-specific phages at high MOIs on the continued formation of mating pairs. At time t = 0, HfrW3011 and F⁻W1-3 cultures were mixed at a ratio of 2:3. The mixture was immediately divided into three fractions, and at various times samples were diluted 400-fold for measurement of impulses in the counter. At 10 min after mixing, MS2 and f1 phages at the MOI of 400 were added to two of the three fractions. Incubation and counting of impulses were continued. The percentages of mating pairs were plotted as a function of time of counting. See Materials and Methods for the computation of mating pairs.*

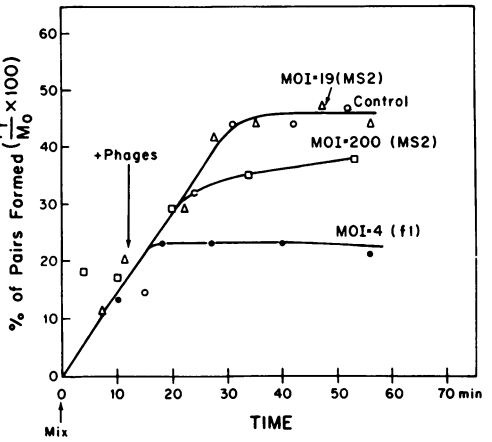

FIG. 6. *Effect of male-specific phages at relatively low MOIs on the continued formation of mating pairs. See the legend of Fig. 5 for experimental details. The ratio of male to female cells in the mixture was 1:2. The concentration of male cells in the mixture was 3 × 10⁸ cells/ml.*

In sharp contrast, when f1 phages were added with an MOI of only 4, the formation of mating pairs was stopped completely 5 min after the addition of phage. The 5 min is presumably the time required for the adsorption of phage at this low MOI.

Disruption of preformed pairs by the high MOI of MS2 phage. We have seen that MS2 phage at a high MOI (>400) disrupts some, but not all, mating pairs (Fig. 2 and 5) which suggests that there are two kinds of mating pairs: one which is sensitive to a high MOI of MS2 phage and another which is resistant. There is a possibility, therefore, that only those pairs formed in the presence of MS2 phage are unstable and can be disrupted by a high MOI. To check this possibility, the MS2 phages were added after all the pairs were formed. Pairs formed in the absence of phage were disrupted by a high MOI of MS2 (Fig. 7). There was a lag of approximately 3 min before the number of mating pairs began to decrease. (Presumably this is the time for the adsorption of enough phage to exert the disruptive effect on mating pairs.) The decrease continued for 6 min until no more were disrupted, those which remained being resistant to the action of MS2.

The kinetics of the formation of mating pairs resistant to a high MOI of MS2 are given in Fig. 8. The results indicate that sensitive mating pairs were converted to "resistant" mating pairs as a function of time. The results of a series of kinetic experiments are summarized in Fig. 9 in which the ordinate is expressed as the number of resistant mating pairs per 100 fertile males and the abscissa is the time of the addition of the MS2 phage.

There are two steps, therefore, in the formation of mating pairs. In the first step, males (M) and females (F) collide to form the initial mating pairs (P_s) that are sensitive to the disruption by a high MOI of MS2, with a rate constant k_1:

$$M + F \xrightarrow{k_1} P_s$$

The newly formed pairs (P_s) are then converted

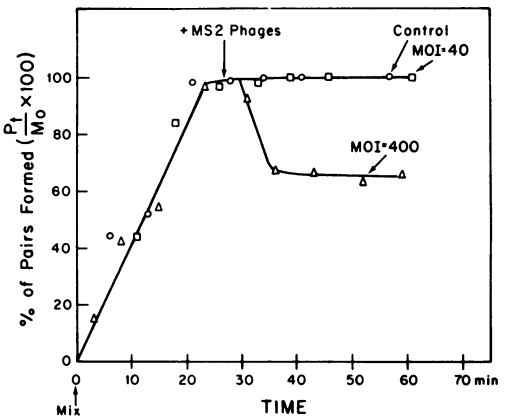

FIG. 7. *Effect of MS2 phages on preformed mating pairs. The experimental procedure was the same as in Fig. 5, except that phages were added at 27 min after mixing HfrW3011 with F⁻W1-3 cells (ratio 1:2; the concentration of HfrW3011 in the mixture was 3.8 × 10⁸ cells/ml).*

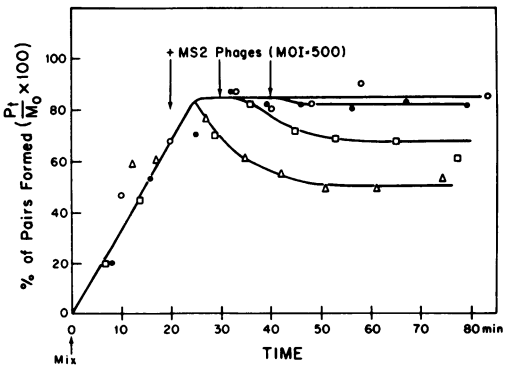

FIG. 8. *Disruption of preformed mating pairs by MS2 phages at a high MOI as a function of time of the addition of phages. See the legend of Fig. 7 for the experimental procedure. MS2 phages were added at 20 (△), 30 (□), and 40 min (●) after mixing of HfrW3011 and F⁻W1-3 cells (the ratio 1:2) at an MOI of 500. The concentration of HfrW3011 in the mixture was 3 × 10⁸ cells/ml.*

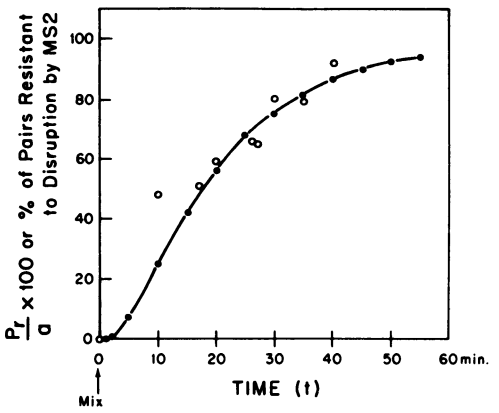

FIG. 9. *Kinetics of formation of mating pairs resistant to the disruption by a high MOI of MS2 phage. The ordinate is the number of mating pairs resistant to disruption by a high MOI of MS2 phages (P_r [measured by the asymptotic value after addition of MS2 phage, see Fig. 8]) per 100 fertile males (a [measured by the final level of mating pairs in the control mixture]), and the abscissa is the time of addition of MS2 phage. Open circles, five separate experiments; closed circles, computation with Fk_1 = 0.058/min (half-time = 12 min) and k_2 = 0.233/min (half-time = 17 min).*

into pairs (P_r) that are resistant to the disruption by a high MOI of MS2, with a rate constant k_2:

$$P_s \xrightarrow{k_2} P_r$$

In the present experiment, an excess of females (F) was always used so that the availability of female cells for conjugation (F) can be considered as constant.

Thus we obtain (1, 12):

$$P_r/a = 1 - e^{Fk_1t} - Fk_1e^{-Fk_1t}/(k_2 - Fk_1) + Fk_1e^{-k_2t}/(k_2 - Fk_1)$$

where a is the efficiency of pair formation. The factor a varies from day to day and depends on a number of variables (5, 10) such as the density of cells, the F piliation, aeration, age of bacteria, availability of receptor sites, etc. Using k_2 = 0.233/min (half-time = 12 min) and Fk_1 = 0.0578/min (half-time = 17 min) we computed the curve for P_r/a that is plotted also in Fig. 9. The points obtained from experiments fit the theoretical curve very well.

DISCUSSION

Our results indicate that the tips of F pili are the specific sites for the attachment of females. Tips of the F pili are known to be the attachment sites for the adsorption of the filamentous DNA phage, f1. Inhibition of the formation of mating pairs by f1 phage suggests that the f1 phage and females are competing for the same attachment sites, namely the tips of F pili. The phage, f1, is a long phage (850 nm) (25). Therefore, the presence of attached f1 phage around the F pili may block the accessibility of the sides of F pili for pair formation. However, the block will not be complete for two reasons. (i) f1 phages do not attach to the sides of F pili (7), and therefore the covering of the sides of F pili by the phage is not constant. (ii) The median length of F pili in a culture is 1.6 μm (14) which is twice as long as f1 phage. Thus, if the side of the F pilus could act as the mating site, one would see, in the presence of f1 phage, a continuous but decreased rate of pair formation which we consistently failed to observe (Fig. 1, 3, 5, and 6).

When the isometric RNA phages, MS2, were added to the males just before mixing with females, they too inhibited the formation of mating pairs (Fig. 2). However, the inhibition was incomplete and different from that when f1 phages were used. Thus the isometric phages do not seem to block the specific mating site, but they do reduce the chance to form mating pairs.

MS2 phages presumably interfere with the formation of mating pairs by adsorbing to the sides of the F pilus. There may be some step in mating pair formation, therefore, which involves the sides of the F pilus which facilitates the formation of mating pairs, but is not obligatory. For instance, the sides of an F pilus may contact weakly to the surface of a female and bring the tip of the F pilus close to the specific receptor site. Two cells bound by such nonspecific weak contact, however, would not be stable enough to register as a mating pair in the Coulter counter.

This mechanism would account for the observation that, when MS2 phages were added at an MOI of <19 after the formation of mating pairs had begun, there was no effect on the subsequent formation of mating pairs. It may be that at the time of the addition of MS2 phage, most male cells had already bound to females near the receptor sites through a weak contact between the sides of the F pilus and the surface of the female cell. However, at a high MOI, MS2 phages could cover the immediate vicinity of the tips of the F pilus and block the attachment to the specific receptor sites for the formation of the mating pairs. This explains the observation that a reduced rate of pair formation was obtained when MS2 phages were added at a MOI of 200 after the mixing of male and female cells. At this MOI, 95% of total MS2

phage or approximately 190 MS2 phages per bacterium were adsorbed in 8 min (Fig. 6) (computed with the rate of adsorption, $k = 2.2 \times 10^{-9}$ ml/min and the male cell concentration of 3×10^8 cells/ml). Assuming that the average number of F pili per cell is 2 (14), there are 95 MS2 phages adsorbed to each F pilus in 8 min.

The mechanism by which high multiplicities of MS2 phages disrupt some of the preformed mating pairs is unknown. The disruption is presumably caused by the attachment of a large number of MS2 phages to an F pilus which is participating in pair formation. The adsorption of MS2 phages throughout the entire length of F pili may (i) cause a conformational change in the F pilus that results in detachment from the female, (ii) weaken the resistance of the F pilus to shearing forces, or (iii) stop the retraction of F pilus for close wall to wall contact (2, 4, 9, 14, 17); and the force of retraction may cause the detachment of the F pilus from mating cells.

Thus there are probably two types of mating pairs. (i) When a mating pair is initially formed, it is susceptible to the disruptive force of MS2 phages at high MOI; (ii) the pair then becomes resistant to the high MOI of MS2. Mating pairs become resistant to MS2 with a half-time of 17 min (Fig. 9).

Ou and Anderson (20) also reported that there were Zn^{2+}-sensitive and Zn^{2+}-resistant mating pairs and that, as a function of time, the Zn^{2+}-sensitive converted into Zn^{2+}-resistant pairs. Those mating pairs may correspond to the two types of mating pairs reported here. The two distinct states of mating pairs have been previously observed (2, 19, and 20). The initial mating pairs are presumably those that are connected by the F pilus (5, 19). The resistant mating pairs may correspond to those pairs that are connected by the cellular bridges observed by Anderson et al. (3).

The process of mating pair formation may have as many as three steps: (i) a nonspecific contact of the sides of the F pilus to a female; (ii) the F pilus slides along to the specific attachment site to form a "separate" (19) mating pair connected by an F pilus; and (iii) the "separate" mating pair is then gradually pulled together to form a "close" (19) wall-to-wall mating pair that is connected by the cellular bridge (3). The mechanism of pulling two cells together has been proposed to be achieved by the retraction of the F pilus (9, 17). Earlier observations and recent studies seem to support this retraction model (2, 4, 14, 17).

We have reported that 10^{-3} M Zn^{2+} affects the formation of mating pairs (20). It was also reported that 10^{-3} M Zn^{2+} inhibits the adsorption of DNA phage (21, 24) but not the adsorption of RNA phage (21). Taking these observations together with those presented here we conclude: (i) that the tip of an F pilus is most likely the specific and necessary site for the formation of mating pairs; (ii) that the side of an F pilus also plays a non-obligatory role in the formation of mating pairs, probably by bringing the F pilus closer to the specific site of attachment; (iii) that there are two types of mating pairs, i.e., the pairs that are sensitive and the pairs that are resistant to the high MOI of RNA phages; and (iv) that all pairs will eventually mature into resistant pairs.

ACKNOWLEDGMENTS

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