Inhibition of Bacterial Conjugation by Ribonucleic Acid and Deoxyribonucleic Acid Male-Specific Bacteriophages

CHARLES NOVOTNY, WILLIAM S. KNIGHT, AND CHARLES C. BRINTON, JR.

Microbiology Section, Department of Biophysics and Microbiology, University of Pittsburgh, Pittsburgh, Pennsylvania 15213

Received for publication 16 October 1967

Both deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) male-specific phages, with an F-specific host range, inhibited the bacterial mating process of Escherichia coli. DNA phages prevented the formation of mating pairs but had no effect on mating pairs once they were formed. A step in RNA phage infection, prior to RNA penetration, prevented the formation of mating pairs and, in addition, prevented a fraction of existing mating pairs from completing the mating process. These findings are compatible with the hypothesis that donor cells have a single surface structure involved in both conjugation and male-phage adsorption and that this element is the F pilus.

Since the discovery of bacterial mating in Escherichia coli (23, 32), many other genera and species of gram-negative bacteria have been shown to transfer deoxyribonucleic acid (DNA) by cell-to-cell contact (17). This ability is genetically controlled by several classes of episomes or plasmids: fertility or F factors (7, 16, 22), colicinogenic factors (13), and drug resistance transfer factors (1, 34). These genetic elements also control filamentous surface structures called pili (5). Several kinds of pili have been implicated in DNA transfer by cell contact. F pili are involved in the transfer of the F factor (5) and may also be correlated with the transfer of the colicin V factor (3, 6) and with the f- species of drug resistance transfer factor (26–28). The transfer of the f+ species of drug resistance transfer factor is correlated with the presence of f- pil (29, 34), and the transfer of the colicin I, factor is correlated with the presence of I, pil (30). Thus, it is now apparent that many systems of conjugal DNA transfer involve pili.

A group of spherical ribonucleic acid (RNA) phages and a group of rod-shaped DNA phages, which infect only those bacterial strains capable of F (or related sex factor), mediated conjugation (24, 25), have been shown to adsorb to F pil (5, 6, 9). The RNA phages adsorb to the lateral surface of the F pilus, and the DNA phages, to its tip. The specificity of these phages lies in the adsorption and penetration steps of infection, since female cells can replicate them (10).

It has been proposed that F pilus function as conductors of nucleic acid into and out of male bacteria (5). Further evidence and arguments in favor of this hypothesis have been advanced for the transfer of bacterial DNA, phage DNA, and phage RNA (2–4). According to this model (the F pilus conduction model), a single structure, the F pilus, may specifically mediate three different types of nucleic acid transfer in two different directions. If this model is essentially correct, it may be reasonable to expect that the F pilus would not simultaneously conduct more than one kind of nucleic acid at any given time and that therefore interference among bacterial DNA transfer, phage RNA transfer, and phage DNA transfer would occur. Preliminary reports from other laboratories (19, 21) indicated the existence of such interference phenomena.

It is the purpose of this paper to investigate the effects of DNA and RNA male-specific phages on the F system of bacterial conjugation.

MATERIALS AND METHODS

Bacterial and viral strains. Descriptions of the strains of E. coli used are given in Table 1. We also used a spherical RNA phage (R17) and a rod-shaped DNA phage (M13) which were donated by J. Watson and P. Hofschneider, respectively.

Preparation of cells. Cells were grown aerobically at 37°C in Z medium, minimal glucose (MGlu), or MGlu supplemented with methionine (MGluMet) or threonine (MGluThr). Z medium contains,
inhibitory of bacterial conjugation

per liter: tryptone (Difco), 10 g; yeast extract (Difco), 1 g; NaCl, 8 g; glucose, 1 g; and NaOH, 0.3 g. MGlU contains per liter: MgSO₄·7H₂O, 0.025 g; (NH₄)₂SO₄, 1 g; K₂HPO₄, 7 g; KH₂PO₄, 3 g; sodium citrate·2H₂O, 0.5 g; and glucose, 2 g. MGlUMet and MGlUThr were prepared by adding 0.04 g of L-methionine or 0.08 g of L-threonine (per ml) to MGlU. Female cells were resuspended in a buffered medium, at pH 6.1 (FB), that contained, per ml: NaCl, 4 g; K₂HPO₄, 1.31 g; KH₂PO₄, 5.78 g; and MgSO₄·7H₂O, 0.2 g. The pH was adjusted to 6.1 by adding NaOH or HCl. It was shown previously that optimal pair formation occurs at pH 6.1 (2, 12). Cell concentrations were determined with a Fisher Electrophotometer that was calibrated in terms of viable cells per milliliter.

Selective media. MGlU/MStr agar and MLacThiStr agar were employed as selective media. MGlU/MStr agar is MGlU with the addition of streptomycin (Str), 2 g/liter, and Noble Agar (Difco), 30 g/liter. MLacThiStr agar is the same as MGlU except that dextrose was deleted and the following were added: lactose, 2 g/liter; thiamine, 0.01 g/liter; str, 1 g/liter; and Noble Agar (Difco), 15 g/liter (hard agar) or 7 g/liter (soft agar).

Mating systems. The two types of mating systems used were HB11 × A1 and W1895 × 200 U. Chromosomal transfer in the HB11 × A1 system was detected by selecting for thr⁺ str⁺ recombinants. Although the F factor of HB11 carries the gene(s) involved in lactose utilization, a chromosomal gene, thr⁺, was used as a selective marker. With the W1895 × 200 U system, chromosome transfer was detected by selecting for lac⁺ str⁺ recombinants.

Mating-pair formation. The kinetics of mating-pair formation was measured by a dilution technique. Female cells growing exponentially in Z (200 U) or MGlUThr (A1) were centrifuged at 37 C for 10 min at 5,000 × g. Pellets were resuspended in FB to a cell concentration of 4 × 10⁸ cells/ml. Male cells were grown in Z or minimal media (as indicated in legends to figures and tables) to a cell concentration of 2 × 10⁸ cells/ml and then diluted 1:10 into homologous media at 37 C. At zero-time, 1-ml portions of the diluted male cultures were mixed with 5 ml of female cultures that had been resuspended in FB. These mating mixtures were incubated at 37 C without agitation. At various times after mixing, the mating mixtures were diluted 1:500 into the appropriate minimal medium or ZFB (1 part Z medium to 5 parts FB). Control experiments showed that no significant cell contact took place in these diluted mixtures. In the HB11 × A1 mating experiments, 1:500 dilutions were made into 50 ml of MGlU. These diluted mixtures were incubated for 60 min at 37 C to allow for the standard amount of chromosomal transfer to take place. It was necessary to plate all of the diluted mixture in order to detect a significant number of recombinants. Consequently, each diluted mixture was mixed with 50 ml of liquid MGlU agar (55 C), and the entire mixture was poured into large petri dishes (150 × 15 mm). The plates were incubated for 48 hr to allow for the growth of thr⁺ str⁺ cell colonies.

In the W1895 × 200 U mating experiments, the mating mixtures were diluted 1:500 into 10 ml of MGlU/MStrMet. These diluted mixtures were incubated for 30 min at 37 C. The diluted mixtures were then agitated for 1 min on a Vortex-Genie mixer (Scientific Industries), and portions (0.1 or 0.2 ml) were mixed with 4 ml of liquefied MLacThiStr soft agar (47 C) containing 0.04 ml of Z medium. This mixture was then poured on an MLacThiStr hard-agar plate. Plates were incubated for 48 hr to allow for the growth of lac⁺ str⁺ colonies. The number of colonies observed in both mating systems was taken to be a measure of the number of mating pairs in the mating mixture at the time of dilution.

In the experiments in which R17 and M13 bacteriophages were added, several mating mixtures were made at the same time. Phage were added (in amounts indicated in figure and table legends) to individual mixtures at different times. These mixtures, and a control mixture which did not receive phage, were assayed for recombinants as described above.

Preparation of phage stock. R17 bacteriophage was grown on E. coli Hfr. Hfr was grown in PM1 medium which contained, per liter: tryptone, 10 g; yeast extract, 1 g; NaCl, 8 g; Na₂HPO₄·7H₂O, 5.7 g; K₂HPO₄, 1.5 g; (NH₄)₂SO₄, 1.0 g; MgSO₄·7H₂O, 0.25 g; thiamine, 0.01 g; dextrose, 6 g; and gelatin (Difco), 0.01 g. A culture of Hfrg, growing exponentially in PM1 medium at 37 C and containing approximately 3 × 10⁸ cells/ml, was infected with R17 at a multiplicity of 50 phage per bacterium. At the time of R17 addition, CaCl₂ (0.3 g/liter) was added; 1 hr later, 2.85 g of Na₂HPO₄·7H₂O was added to 1 liter of culture, and the culture was incubated overnight. Chloroform was added, and cell debris was removed by centrifugation at 10,000 × g for 30 min at 4 C. The clarified lysates (phage stock) were treated with

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>HB11</td>
<td>E. coli B/r</td>
<td>F⁺ lac⁺/lac⁻ thr⁺ str⁺</td>
<td>H. Boyer</td>
</tr>
<tr>
<td>A1</td>
<td>E. coli B/r</td>
<td>F⁻ thr⁻ str⁻</td>
<td>E. Englesberg</td>
</tr>
<tr>
<td>W1895</td>
<td>E. coli K-12</td>
<td>Hfr Cavalli lac⁺ met⁺ str⁺</td>
<td>L. S. Baron</td>
</tr>
<tr>
<td>200 U</td>
<td>E. coli K-12</td>
<td>F⁺ lac⁻ str⁺ thi⁻</td>
<td>L. S. Baron</td>
</tr>
<tr>
<td>Hfr</td>
<td>E. coli K-12</td>
<td>Hfr met⁻</td>
<td>J. Watson</td>
</tr>
</tbody>
</table>

* Abbreviations: met, thr, thi designate biosynthesis of l-methionine, l-threonine and thiamine; lac, utilization of lactose; str, streptomycin.

---

TABLE 1. Strains of bacteria

Vol. 95, 1968

Downloaded from http://jb.asm.org/ on September 23, 2017 by guest
chloroform and stored at 4°C. M13 stocks were prepared in essentially the same way, except that Hfr was grown in Z medium and we did not add Na2HPO4 or 7H2O. We routinely obtained R17 titers of $2 \times 10^{12}$ plaque-forming units (PFU)/ml and M13 titers of $6 \times 10^8$ PFU/ml. R17 and M13 were assayed by the agar overlay technique. Base plates contained, per liter: tryptone, 10 g; yeast extract, 1 g; NaCl, 8 g; Ionagar (Oxoid), 8.6 g; dextrose, 5 g; and CaCl2, 0.3 g. Overlay agar contained, per liter: tryptone, 10 g; yeast extract, 1 g; and Ionagar, 3.5 g.

R17 antiserum. A phage stock lysate of R17 (2 $\times 10^{12}$ PFU/ml) was emulsified with an equal volume of complete Freund adjuvant (BBL), and 0.1-ml portions were injected into each toe pad of the hind feet of an albino rabbit. One month later, 1.0 ml of the emulsion was injected subcutaneously under the scapula of the rabbit. Ten days later, the serum was obtained from the marginal ear vein of the rabbit. The first-order neutralization rate constant of the serum was $10^5 \times \text{min}^{-1}$.

RESULTS

Effect of RNA male phage on the kinetics of mating pair formation. Bacterial conjugation begins with the formation of mating pairs from random collisions between donors and recipients. In this paper we define mating pairs as pairs in which DNA transfer can take place. The formation of a mating pair defined in this way requires specific collision between a fertile donor and a competent recipient and the establishment of a donor-recipient connection capable of maintaining cell contact and providing for DNA transfer.

The kinetics of mating pair formation was measured by a dilution technique. Mating mixtures were diluted at various times to prevent further cell collisions, and DNA transfer was allowed to take place in those pairs formed prior to dilution. Portions of the dilution were then plated on selective media and the number of recombinant colonies observed was taken to be a measure of the number of mating pairs in the mating mixture at the time of dilution. Formation of mating pairs as a function of time in Hfr X F- (W1895 X 200U) and F+ X F- (HB11 X A1) mating systems is shown in Fig. 1. With both the Hfr and F+ systems there was a fast initial rate of mating pair formation followed by a slower rate which lasted throughout the course of the experiments. When R17 bacteriophage was added to the undiluted mating mixtures several minutes after they were mixed, mating pair formation was inhibited. Least squares regression lines for the number of mating pairs formed, as a function of time after R17 was added, had a slight positive slope, and extrapolation to the times of R17 addition yielded recombinant levels that corresponded to approximately 75% of the mating pairs present in the mating mixtures when R17 was added. This indicates that about 25% of the existing mating pairs were lost rather quickly when R17 was added. Addition of R17 at zero-time inhibited the formation of mating pairs but the inhibition was not complete. Approximately 20% of the mating pairs which would have formed in the first 2 min in the absence of phage were able to form in the presence of phage. Again, least squares regression lines for number of mating pairs formed as a function of time had a positive slope.

In these experiments, the inhibitory effect of R17 on mating must have occurred only in the mating mixture, because dilution of a phage-free mating mixture into a diluent containing R17, at a titer of $2 \times 10^8$ PFU/ml, had no effect on recombinant formation (Fig. 2). This phage titer is 10 times the concentration carried over by dilution in a phage inhibition experiment.

![Fig. 1. Formation of mating pairs in F+ X F- (HB11 X A1) and Hfr X F- (W1895 X 200U) mating mixtures. Symbols: , control mixture, no phage; R17 added at 0 min; O, R17 added where indicated. R17 final concentration, $2 \times 10^9$ PFU/per ml of mating mixture. HB11 and W1895 were grown in Z medium.](http://jb.asm.org/
Identification of the inhibitory agent. In the preceding experiments, we inhibited mating-pair formation by adding a portion of a crude lysate of R17 to a mating mixture. Analysis of a sucrose gradient of the crude lysate shows that the inhibiting agent sedimented at the same rate as R17 PFU (Fig. 3). In addition, the supernatant fluid from a crude lysate, as well as a crude lysate heated for 10 min at 68 C and a crude lysate pretreated with R17 antiserum, did not inhibit the formation of mating pairs (Table 2). This evidence supports our supposition that R17 was the inhibitory agent.

Effect of RNA male phage on preformed mating pairs. Previous results indicated that about 25% of the mating pairs in a mating mixture were rapidly lost when R17, at a titer of 10^6 PFU/ml, was added (see Fig. 1). We observed a similar loss in mating pairs when a phage-free mating mixture was diluted, as a function of time after mixing, into either MGluThiMet or ZFB media containing R17 phage at 10^6 PFU/ml (Fig. 4). When a phage-free mating mixture was diluted into a 100-fold lower concentration of phage (2 x 10^6 PFU/ml), no loss in mating pairs was observed (see Fig. 2). In another experiment, a mating mixture was diluted, 3 min after mixing, into ZFB containing R17 at 10^6 PFU/ml, and marker (lac+) entry time was determined (Fig. 5). The diluted mixture containing R17 yielded 25% fewer recombinants than the control mixture without R17. Marker entry time, as measured by the extrapolation of the interrupted-mating curves to zero recombinants, was unaffected by the presence of phage.

Effect of pretreating males and females with RNA male phage. Pretreatment of females with R17 for 5 min prior to mating had no effect on the formation of mating pairs, but pretreatment of males for 5 min had a marked effect (Fig. 6). In both experiments, the phage were neutralized with R17 antiserum before the cells were mated. Pretreating the males did not completely inhibit the formation of mating pairs; the number of mating pairs remained constant at a low level for 2 min and then began to rise. The fact that pretreatment of male cells, but not female cells, had an effect on mating is consistent with the idea that R17 inhibits the formation of mating pairs by reacting with the male cell. The low level of mating pairs observed initially (0 to 2 min) in the male pretreatment experiment is not significantly different from that observed when R17 was added at zero time (see Fig. 1), but the increase in mating pairs observed after 2 min of contact is greater.

Effect of divalent cation concentration on R17 inhibition and infection. The evidence presented indicates that R17 inhibits the formation of mating pairs and prevents about 25% of preformed mating pairs from producing recombinants. These
TABLE 2. Effect of various R17 preparations on conjugation (HB11 × A1)

<table>
<thead>
<tr>
<th>R17 preparations</th>
<th>PFU/ml of prep</th>
<th>Amt tested (ml)</th>
<th>Thr+ colonies</th>
<th>Percentage of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phage stock</td>
<td>1.5 × 10^14</td>
<td>0.06</td>
<td>27</td>
<td>29</td>
</tr>
<tr>
<td>Supernatant from phage stock</td>
<td>1.3 × 10^8</td>
<td>0.06</td>
<td>109</td>
<td>120</td>
</tr>
<tr>
<td>Resuspended phage pellet</td>
<td>1.6 × 10^14</td>
<td>0.06</td>
<td>33</td>
<td>36</td>
</tr>
<tr>
<td>Heated phage lysate</td>
<td>1.6 × 10^8</td>
<td>0.06</td>
<td>108</td>
<td>118</td>
</tr>
<tr>
<td>Phage stock diluted 1:10^6</td>
<td>1.5 × 10^11</td>
<td>0.30</td>
<td>31</td>
<td>34</td>
</tr>
<tr>
<td>Phage stock diluted 1:10 plus R17 antiserum</td>
<td>3.3 × 10^8</td>
<td>0.30</td>
<td>107</td>
<td>118</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>0.30</td>
<td>91</td>
<td>100</td>
</tr>
</tbody>
</table>

- a R17 preparations were added to mating mixtures at the time of mixing. Five minutes later, the mixtures were diluted 1:500 into MGlu and assayed for thr+ recombinants. HB11 was grown in MGlu supplemented with MgSO_4, 10^{-4} M; A1 was resuspended in FB supplemented with MgSO_4, 10^{-2} M.
- b Supernatant fluid from phage stock that was centrifuged at 4°C for 3 hr at 165,000 × g.
- c Like (b) above but pellet was resuspended in the supernatant fluid.
- d Phage stock heated in water bath at 68°C for 10 min.
- e Phage stock diluted 1:10 in Z medium.
- f Like (e) but R17 antiserum (final dilution, 1:2,500 of stock antiserum) was added.

experiments, however, do not indicate which step in R17 infection causes the inhibition. Paranchych (31) has shown that optimal R17 infection requires divalent cations greater than 7 × 10^{-4} M, and he deduced that divalent cations were required only for the intracellular penetration of R17 RNA. We investigated the ability of R17 to inhibit the formation of mating pairs in media containing 2 × 10^{-4} M Mg^{2+} and 10^{-4} M Mg^{2+} but otherwise devoid of divalent cations (Fig. 7). These results show that the Mg^{2+} ion concentration had no effect on the ability of R17 to (i) inhibit the formation of mating pairs, and (ii) inhibit a fraction of the mating pairs already present when the phage was added. Further support for the latter conclusion comes from an experiment in which a mating mixture was diluted into either ZFB (Mg^{2+}, greater than 8 × 10^{-4} M) or MGluThiMet (Mg^{2+}, 2 × 10^{-4} M) with R17 and without R17 (see Fig. 4). In both media, R17 effected about a 25% loss of mating pairs.

In contrast to the independence of mating inhibition on Mg^{2+} ion concentration, R17 infection was very dependent on the Mg^{2+} ion concentration (Fig. 8). After 10 min of incubation, in a mating mixture where R17 was added at zero-time, 50% (HB11) and 70% (W1895) of the input males in 10^{-2} M Mg^{2+} medium became infective centers, but only 9% (HB11) and 3%
mixing. At 0 dilution 1:100 containing mixtures R17, min
Five (final serum concentration, 4 × 10⁶ cells/ml) was added and mating-pair formation was measured. Female pretreatment: identical to male pretreatment except AI was treated with R17 and R17 antiserum before mixing with HB11. HB11 was grown in MGlum. Symbols: •, with R17; ○, without R17.

Fig. 6. Effect of pretreatment of male and female cells with R17 on the formation of mating pairs. Male pretreatment: R17 at a final concentration of 10⁹ PFU/ml was added to a suspension of HB11, 3 × 10⁶ cells/ml, in MGlum:FB (1:5) at -7 min. R17 antiserum was added at -2 min. At 0 min, AI (final concentration, 4 × 10⁶ cells/ml) was added and mating-pair formation was measured. Female pretreatment: identical to male pretreatment except AI was treated with R17 and R17 antiserum before mixing with HB11. HB11 was grown in MGlum. Symbols: •, with R17; ○, without R17.

Fig. 7. Effect of R17 on mating-pair formation in mating mixtures containing 2 × 10⁻⁴ M and 1 × 10⁻⁵ M Mg++. Symbols: ○, R17, 2 × 10⁹ PFU/ml added at 3 min; •, no R17. The Mg++ ion concentration of MGlum (HB11 growth medium) and FB (female resuspension medium) was obtained by varying the MgSO₄ concentration of the media.

(W1895) became infective centers in 2 × 10⁻⁴ M Mg++ medium.

Dependence of mating inhibition on RNA phage concentration. In the preceding experiments, we inhibited mating-pair formation by adding R17 at titers between 10¹⁰ and 5 × 10¹⁰ PFU/ml of mating mixture. Phage titers of 5 × 10⁹ PFU/ml were just as effective, but 1 × 10⁹ PFU/ml only partially inhibited mating-pair formation (Fig. 9). No inhibition was observed when 10⁸ R17 PFU/ml were added to the mating mixture.

Effect of bacteriophage M13 on conjugation. M13 phage, like R17, inhibited the formation of mating pairs when added to a mixture at a final concentration of 2 × 10⁶ PFU/ml (Fig. 10). An M13 concentration of 10⁶ PFU/ml did not inhibit the formation of mating pairs. The regression line for mating pairs, formed as a function of time after M13 (10⁶ PFU/ml) was added at 0.5 min, had a positive slope; however, unlike the results obtained with R17 (see Fig. 1), the line

Fig. 8. Formation of infectious centers in mating mixtures containing 1 × 10⁻⁴ and 2 × 10⁻⁴ M Mg++. R17, 2 × 10¹⁰ PFU/ml, was added to mating mixtures (W1896 × 200U and HB11 × AI) at the time of mixing. At indicated times mating mixtures were diluted 1:100 into MGlumThiMet containing R17 antiserum (final concentration 1:2,500 stock antiserum). Five min later, these samples were diluted 1:10 into MGlumThiMet and immediately plated on E. coli Hfr for determination of infectious centers.
extrapolated to the number of mating pairs in existence at the time of phage addition. This indicates that M13 inhibits mating-pair formation without affecting preformed mating pairs. The addition of M13 to a mating mixture at zero-time also inhibited the formation of mating pairs but the inhibition was not complete. This result is similar to that observed when R17 was added at zero-time (see Fig. 1).

In another experiment, we investigated the effect of M13 on marker (lac+) entry under conditions where (i) M13 was present during the initial contact period in a mating mixture and (ii) M13 was present after the mating mixture was diluted to prevent further mating-pair formation (Fig. 11). M13 did not affect the number of recombinants obtained when it was present after mating-pair formation had taken place. However, when the mating mixture was treated with M13 during the initial contact period, we observed a 50% reduction in the number of recombinants. Marker entry time, as measured by the extrapolation of the interrupted-mating curves to zero recombinants, was unaffected by the presence of phage.

**DISCUSSION**

In interpreting the effects of inhibitors on bacterial conjugation, it must be kept in mind that experiments designed to discriminate among the various steps in the inhibitory process, based only on a reduction in the number of recombinant colonies effected by an inhibitory treatment, are subject to several ambiguities in interpretation. An inhibitory event cannot be assigned to a particular step in the mating process unless that step can be experimentally isolated from other steps. Isolation from preceding steps can be achieved by addition of the inhibitor after preceding steps have been completed. Isolation from succeeding steps is more difficult, since the inhibitor may become immediately bound to the system but may exert its inhibitory effect at some later time.

Furthermore, a mating mixture cannot be considered to be a homogeneous population of mating pairs carrying out the mating process synchronously. Free donors, free recipients, mating pairs, and free zygotes coexist in the mixture, and each of these may or may not be inhibitor-sensitive. Mating is asynchronous in the pair population, since some pairs form before others and delays in the initiation of DNA transfer may occur (11). In addition, some donor cells may be temporarily infertile. These considerations must be taken into account if the site and mode of action of an inhibitor is to be determined.

Various steps in bacterial mating and male-phage infection have been proposed (2, 8, 15; S. Falkow, E. M. Johnson, and L. S. Baron, Ann.-Rev. Genetics, in press). In general, both...
processes involve the early steps of attachment and penetration of nucleic acid, followed by later steps leading to the formation of recombinant colonies or to phage-producing cells. We will discuss our results in these general terms first, and then relate them specifically to the F pilus conduction model of mating and male-phage infection (2).

Our first approach was to treat donor cell cultures with RNA male phage. After a 5-min treatment at high phage concentration, phage were inactivated by antiserum and female cells were added. A 75% reduction in recombinant colonies was observed. Similar treatment of recipient cells gave no reduction in recombinant colonies. Although these results indicate that phage inhibit mating by reacting with donor cells, they do not specify which step of mating is inhibited. The inability of phage pretreatment to inhibit mating completely cannot be attributed to a limited phage concentration, since we have been unable to enhance the inhibition by adding more phage. The inability of a high phage concentration to inhibit mating completely may mean that some donor cells are resistant to the phage effect.

Our second approach was to separate the effect of phage on mating-pair formation from their effect on preformed mating pairs. Phage were added at various times to a "concentrated" mixture of donor and recipient cells. A "concentrated" mixture is one in which donor-recipient collisions are frequent, and in which new mating-pair connections are continually forming. The number of recombinant colonies formed by mating pairs in existence at any particular time was determined by diluting the mixture to prevent any further collisions, incubating, and plating. Since a donor-recipient pair can separate before transfer of a specific gene occurs (20), and a gene can be transferred without becoming stably integrated into the female genome (14, 20), the number of recombinant colonies observed is always less than the number of mating pairs that have formed. Because the magnitude of these losses may be time-dependent, we kept pair separation and plating times as constant as possible. The increase of recombinant colonies with time of dilution is therefore considered to be a measure of the net rate of mating-pair formation.

In analyzing the effect of phage inhibition on mating mixtures, three clear-cut possibilities can be imagined: the phage could (i) prevent mating-pair formation but have no effect on preformed mating pairs, (ii) have no effect on mating-pair formation but inhibit preformed mating pairs from forming recombinant colonies, or (iii) prevent both. In the first case, the number of recombinant colonies would remain constant at the value existing at the time of phage addition. In the second and third cases, the number of recombinant colonies would drop to zero at the time of phage addition.

The effect of DNA male phage on mating corresponded closely to the result expected if only mating-pair formation were inhibited. The effect of RNA male phage was intermediate: the least-squares regression lines fitted to the data expressing the number of recombinant colonies formed after the addition of phage showed an immediate 25% drop at the time of addition, with a subsequent increase which was slow compared to the phage-free mixture. This result indicates that 25% of existing mating pairs were inhibited by RNA phage from forming recombinant colonies, and that mating-pair formation was almost completely inhibited. The principal observation from these experiments is that the presence of either male phage greatly reduces the rate of mating-pair formation.

Our third approach was to determine the effect of male phage on mating steps subsequent to mating-pair formation. This was accomplished by adding phage to diluted mating mixtures in which cell collisions occurred at an insignificant
rate, but in which a portion of previously formed donor-recipient pairs could complete the mating process. The phage were added before the time of marker entry in order to detect possible effects on the transfer step of mating. The DNA phage had no effect on preformed mating pairs, since there was no reduction in recombinant colonies and marker entry (lac+) was normal. The RNA phage at a concentration of 10⁸ PFU/ml reduced the number of recombinants by 25% but did not affect marker entry time.

The 25% drop in the number of recombinant colonies when RNA male phage are added to mating pairs is not completely understood. This inhibition of preformed pairs may occur in several different ways. (i) A fraction of zygotcs may become phage-sensitive as a result of mating. After pair separation, these zygotcs could be infected by phage and would not produce recombinant colonies. This possibility seems unlikely, since we observed the inhibition in Mg²⁺-deficient medium where infection is negligible. Furthermore, the same degree of inhibition was observed with both Hfr and F⁺ mating systems. In the Hfr system, the recipients did not become donors, and therefore phage-sensitive, under our mating conditions. (ii) A fraction of recipient cells may become phage-infected during mating by virtue of connection to the donor cell. Again, this seems unlikely, since inhibition occurs under conditions in which infection of the donor cell does not occur. However, this possibility is not entirely eliminated, since it has not been shown that infection of the recipient cell cannot occur under these conditions. (iii) RNA phage adsorption could stop DNA transfer in 25% of the pairs, or slow DNA transfer in all pairs such that only 75% of the recipient cells would receive the marker in question. It is unlikely that the rate of transfer is retarded, since marker entry time is unaffected in those pairs which can complete transfer in the presence of phage. (iv) RNA phage adsorption could increase the probability of pair separation such as 25% of pairs would separate before marker entry.

Either an increased probability of pair separation or complete inhibition of DNA transfer in 25% of the mating pairs seems most likely at the present time.

Our fourth approach was to investigate which step of phage infection inhibited mating. The adsorption and release steps of RNA phage infection were isolated from the subsequent steps of penetration and replication by the method of Paranchych (31), who deduced that a certain class of divalent cations are absolutely required in the medium for phage RNA penetration, but not for phage adsorption, release, or replication. We found that magnesium ion concentrations which would drastically reduce the ability of phage to infect donor cells had no effect on the ability of RNA phage to inhibit mating. This finding provides support for the proposition that a step in RNA phage infection prior to RNA penetration inhibits an early step in mating. We cannot state which step in DNA male phage infection inhibits mating, since our experiments were performed under conditions in which all steps of DNA phage infection could occur.

We found that relatively high phage concentrations were necessary to inhibit the formation of mating pairs. High concentrations are needed to ensure rapid adsorption at the low donor cell concentrations (3 × 10⁶ per ml) used in our inhibition experiments. This is particularly true for the DNA phage (M13) because it has been shown that adsorption of this phage is relatively slow (33). One phage particle may be enough to inhibit but it is also possible that inhibition may require the adsorption of more than one phage particle. If this were true and if adsorption must be rapid, we could expect to inhibit mating with only high concentrations of phage.

Our results show that both DNA and RNA male phages inhibit the formation of mating pairs. However, our operational definition of a mating pair does not permit us to make precise statements as to which step in mating-pair formation is inhibited. A reasonably detailed model of mating-pair formation is required. The steps in mating-pair formation must include the expression of fertility by the donor cell, cell collision, and the establishment of a donor-recipient connection or connections which maintain cell contact and provide for DNA transfer. The F pilus conduction model of mating, proposed by Brinton et al. (5), enables precise statements to be made about each of these steps and also about the site and mode of phage interaction. This model and some alternate F pilus models will be restated here in order to compare their features with our results.

**F pilus conduction model; precontact.** The possession of at least one competent F pilus by a donor cell is necessary for fertility. Electron microscopy shows that most cells in our donor cultures, even under cultural conditions of maximal fertility, appear to have no F pilus. This is presumed to result from the small average number of F pilus per cell and their frequent loss by natural outgrowth. The loss of F pilus by natural outgrowth rather than by mechanical breakage is indicated by the presence of large numbers of detached F pilus in unagitated donor cell cultures and by the rapid appearance of new F pilus from donor cells depilated by blending (2). From this, we are led to postulate that a given donor culture...
at a given instant in time contains a large fraction of cells which phenotypically and temporarily have no F pili and are therefore phenotypically and temporarily infertile. The members of the donor cell population that constitute this fraction are constantly changing with time as new F pili appear and existing F pili detach. The absolute size and turnover rate of the F-piliated fraction at a given time depends upon the donor strain and cultural conditions.

**Collision.** The distal end of the F pilus must arrive in the vicinity of a suitable receptor site on the recipient cell surface.

**Connection.** The F pilus must react with the receptor site to form a connection which resists the forces of thermal agitation, cell motility, and gentle pipetting and dilution.

**Transfer initiation.** At the time connection is established or shortly thereafter, an event must occur which signals the donor cell to begin DNA transfer. One mechanism of initiation considers the F pilus as a rodlike virus containing F factor DNA. Contact of the F pilus tip with a recipient cell receptor site would release the enclosed DNA in a manner similar to virus infection.

**Transfer.** DNA passes from the donor to the recipient cell through the axial hole of the F pilus. The F pilus conduction model is consistent with the dimensions of the DNA molecule and with the axial hole of the F pilus. The model is also consistent with the apparent ability of F pilus to conduct viral DNA, as well as with the apparent absence of other cell surface structures correlated with fertility or male-phage sensitivity (2, 4). F pilus conduction may be analogous to the way DNA is injected through phage tails. The outgrowth of F pilus may be similar to the outgrowth of the DNA male phages (18).

**Other models involving F pili.** Other models, consistent with an F pilus requirement for mating, cannot be rejected. It is possible that the connection step in mating involves both the attachment of the F pilus to the recipient and the establishment of some other connection between the donor and recipient, DNA transfer taking place through the other connection. Accordingly, F pilus may be the stabilizing structures that hold a mating pair together, or they may be involved in the initiation of a "mating signal." A "mating signal" could be required for the establishment of a receptive state in the recipient or for the initiation of DNA transfer by the donor. Our experiments demonstrating phage inhibition of mating could not discriminate among these models and the F pilus conduction model.

An interesting feature of our experiments is the shape of the curve that describes the kinetics of pair formation (Fig. 1). This curve seemed to consist of two phases: a rapid initial rise that lasted for 1 to 3 min, followed by a slower linear rise that continued for the duration of the experiment (10 to 12 min). These kinetics are not consistent with simple collision theory, which predicts an exponential increase in number of mating pairs of the form \( (1 - e^{-kt})N \), where \( t \) = time, \( k \) = a constant, and \( N \) = the maximum number of mating pairs formed (2).

One interpretation of a biphasic curve in terms of the properties of F pilus is that the initial rapid increase in mating pairs is determined by the number of F-piliated donors in the mating mixture at zero-time. The slower secondary increase in mating pairs could reflect the rate of appearance in the donor population of new F-piliated cells which can then pair with recipients. This interpretation is consistent with our electron microscope observations which reveal that only 20 to 30\% of the donor cells have a visible F pilus at any given time and is also consistent with experiments which show that F pilus outgrowth is sufficiently rapid to account for the rate of pair formation during the second phase (2).

However, more recent results indicate that the rate-limiting factor during the secondary phase of pair formation is available oxygen and not the appearance of new F piliated cells.

Preliminary experiments indicated that mating pairs formed more rapidly in mating mixtures that were continuously stirred than in mating mixtures that were incubated without agitation. In addition, the form of the curve expressing the kinetics of pair formation was no longer biphasic and was consistent with simple collision theory. It is unlikely that the stirring enhanced F piliation and thereby increased the rate of pair formation. A more probable explanation is that stirring aerated the mating mixture.

The biphasic nature of the curve in unagitated mixtures may therefore be due to an oxygen limitation which occurs within 1 to 3 min after donors and recipients are mixed. This explanation is not unreasonable, since Fisher (12) has shown that mating pair formation does not occur under anaerobic conditions.

The fact that the rate of pair formation is partially decreased in unagitated mating mixtures should not affect any of the phage inhibition experiments reported here, since all of our experiments, including the appropriate controls, were performed under the same nonagitated conditions.

Our results regarding the inhibition of mating by male phage are consistent with the notion that a common element is involved in both conjugation and male-phage infection. This element could be the F pilus since it has been proposed that the
F pilus is the adsorption and penetration site for male phage (2). Electron micrographs show that the sides of F pilus can be completely coated with RNA phage (5) and that DNA phage attach to the distal ends of F pilus (6). The steps in male-phage infection according to the F pilus conduction model (3, 4) are outlined below.

Precontact. The F pilus is required for male-phage sensitivity. Consequently, the same considerations apply as did to the previously described precontact step in mating.

Collision and adsorption. The RNA male phage must collide with and adsorb to any of the receptor sites located along the sides of an F pilus. The DNA male phage must collide with and adsorb to sites located at the end of the F pilus. A large number of RNA phage may adsorb to the sides of a single F pilus, but only a few DNA phage adsorb to the tip.

Release. The DNA or RNA of adsorbed phage is released from its protein coat.

Penetration. The DNA or RNA is conducted via the F pilus into the cell. The RNA of only one or of a few of the large number of RNA phage, which can adsorb to an attached F pilus, succeeds in penetrating the cell (4).

The difference between the manner in which the DNA and RNA male phages inhibit mating can be readily explained by the manner in which each phage reacts with F pilus. DNA phage inhibit the formation of mating pairs but do not affect preformed mating pairs. If the tip of the F pilus must attach to the recipient during mating, adsorption of a DNA phage to the tip should prevent attachment and consequently inhibit mating pair formation. Conversely, attachment of the F pilus to the recipient should prevent DNA phage adsorption and we would not expect the phage to inhibit preformed mating pairs. RNA phage inhibit the formation of mating pairs and have the additional property of being able to inhibit mating in a fraction of preformed mating pairs, as might be expected from their ability to attach to the sides of F pilus.

Since the RNA phage adsorbs only to the sides of F pilus, it is possible that the tip of the pilus could attach to the recipient despite adsorbed phage. Although connection may occur, the formation of the pilus could be altered by phage adsorption per se or by the release of phage RNA. Released RNA could block the pilus and prevent the conduction of DNA. Adsorption of RNA phage to an F pilus that is already connected to a recipient cell and carrying out its mating function could reduce the stability of the F pilus-receptor connection or interrupt the functioning of the pilus. An interaction of this sort would account for the observed inhibition of preformed mating pairs.

We also considered the possibility that male phage inhibit mating-pair formation by preventing the outgrowth of new F pilus. Electron microscope experiments have shown, however, that high concentrations of either DNA or RNA male phage have no effect on F pilus outgrowth. This observation indicates that male phage affect the functional state of F pilus rather than their synthesis or assembly.

Since, at any given time, a fraction of donor cells will not have an F pilus, the F pilus models predict that these cells will be simultaneously fertile and insensitive to male phage. The fraction of donor cells that we observed to be resistant to phage inhibition in our pretreatment experiment could represent this class. After the removal of phage, new F pilus would be produced on these donor cells, which could then form mating pairs.

However, the inability of phage to inhibit mating-pair formation completely, when added to a mating mixture at zero time (see Fig. 1), cannot be attributed to the mating of a non-F-pililated fraction of donors. (According to the F pilus models, only phage-sensitive or F-pililated donors can form mating pairs.) Since the initial rate of mating-pair formation is rapid, and since mating pairs are mainly insensitive to phage inhibition, it is more reasonable to suppose that some mating pairs form before the phage can adsorb sufficiently to inhibit. The slow rate of mating formation that occurs in the presence of phage can also be due to donor cells that produce a new F pilus and form a mating pair before the phage can inhibit.

The results of preliminary experiments by other investigators on various aspects of this problem are in general agreement with ours.

Wendt (Ph.D. Thesis, Montana State College, Bozeman, 1965) studied the effects of MS2 RNA phage on preformed mating pairs under conditions in which MS2 did not kill donor cells. He found that the yield of recombinant colonies was reduced about 30% and that "marker entry" time was unaffected. Since inhibition occurred without killing and no marked effects on host cell metabolism were produced by RNA male-phage infection until after the entry of the early marker, Wendt concluded that an event which occurs soon after RNA phage adsorption may be involved in mating inhibition.

E. Raizen (Ph.D. Thesis, University of Wisconsin, Madison, 1966) measured the attachment of DNA and RNA male phages in mating mixtures, as compared to donor cultures, and found that DNA phage attachment was significantly reduced, whereas RNA phage attachment was not.
She also found that a 60-min treatment of a donor culture with DNA phage not only reduced the ability of more DNA phage to attach but reduced donor ability to less than 0.01%. Treatment of donor cultures with an amber mutant of a DNA phage, defective in an early step in replication and unable to form infectious centers, also reduced donor ability to a low level. These results are consistent with the idea that an early step in phage infection inhibits mating and that the act of mating blocks the DNA phage adsorption site but not the RNA phage adsorption site.

Preliminary experiments by Knolle (21) indicate that ultraviolet-inactivated RNA phage can partially inhibit mating and that RNA phage adsorption is not inhibited by the mating process. In addition, he observed a partial inhibition of male-phage infection by mating. This latter finding indicates that mating inhibits an early step in phage infection subsequent to adsorption.

Ippen and Valentine (19) have published preliminary results indicating that sonic-treated fragments of a DNA phage, which can adsorb to donor cells but do not kill them, can inhibit recombiant colony formation when present in concentrated mating mixtures but have no effect on diluted mating mixtures. They also found that RNA phage, in the presence of sufficient ribonuclease to inhibit RNA penetration, could partially inhibit recombinant colony formation in concentrated mating mixtures and could inhibit about 30% of preformed mating pairs from producing recombinant colonies.

Thus, our work, and that of others, has produced a large body of evidence which indicates that an early step in male-phage infection inhibits an early step in mating. The evidence is consistent with the idea that a common element is involved in both processes and that this element is the F pilus.

ACKNOWLEDGMENTS

We thank Claude Henry, for technical assistance, and Gerald W. Stemke, for RNA phage antiserum.

This investigation was supported by Public Health Service grants AI 03242-08 and 1-S05 FR 07084-01 and by American Heart Association grant 66-778.

LITERATURE CITED