Studies on the mechanism of F-promoted chromosome transfer in Escherichia coli K-12
by Louis Wallace Wendt

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Genetics
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Abstract:
The consequences of two different (but not mutually exclusive) models of chromosome transfer by Hfr donors of E. coli have been examined experimentally. The finding that normal gene transfer can be carried out by HfrtS undergoing thymineless death in the presence of submaximal concentrations of thymine does not support the proposal that transfer depends on concurrent DNA synthesis. An attempt to stimulate the fertility of Hfr cultures by bringing all the Hfr to the (hypothetical) point at which transfer may be initiated by the use of chloramphenicol was unsuccessful. However, chloramphenicol treatment was found to reduce the ability of Hfr cells to form both stable and unstable pairs. These effects, and similar effects of phenethyl alcohol, have been traced to their action on donor-specific surface structures known as F pili. The inhibition of gene transfer by the male—specific bacteriophage MS-2, which is believed to attack the donor cell via its F-pili, supports the idea that these pili are the corridors, or vehicles, of transfer. finally, an examination of the available experimental evidence suggests that transfer may, in fact, be driven by processes associated with T-pili metabolism.

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LOUIS WALLACE WENDT

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ABSTRACT

The consequences of two different (but not mutually exclusive) models of chromosome transfer by Hfr donors of E. coli have been examined experimentally. The finding that normal gene transfer can be carried out by Hfr's undergoing thymineless death in the presence of submaximal concentrations of thymine does not support the proposal that transfer depends on concurrent DNA synthesis. An attempt to stimulate the fertility of Hfr cultures by bringing all the Hfr to the (hypothetical) point at which transfer may be initiated by the use of chloramphenicol was unsuccessful. However, chloramphenicol treatment was found to reduce the ability of Hfr cells to form both stable and unstable pairs. These effects, and similar effects of phenethyl alcohol, have been traced to their action on donor-specific surface structures known as F pili. The inhibition of gene transfer by the male-specific bacteriophage MS-2, which is believed to attack the donor cell via its F-pili, supports the idea that these pili are the corridors, or vehicles, of transfer. Finally, an examination of the available experimental evidence suggests that transfer may, in fact, be driven by processes associated with F-pili metabolism.

INTRODUCTION

The genetic basis of sexual differentiation in *Escherichia coli* is understood in some detail. Genetic exchange is unidirectional, proceeding from donors to recipients during conjugal pairing of the cells. The donor property is associated with the presence in the donor cell of genetic elements called promoters (Clark and Adelberg, 1962). Among these the most thoroughly studied is the sex factor, F, which may be cytoplasmic (F^+ types), have a chromosomal location (Hfr types), or alternate between these states (F'). If F is absent the cell is a recipient only (F^-) (Jacob and Wollman, 1961).

Genetic information is also available on the nature of F promoted chromosome transfer. The chromosome in all sex types appears to be circular. During conjugation involving, for example, an Hfr and F^-, the Hfr chromosome is thought to break at a point adjacent to F. The chromosome is then passed from the Hfr to the F^- lengthwise, starting with a particular end, the origin. Genes located at different distances from the origin are thus transferred to the F^- at different times, and in a sequence depending on their order along the chromosome. F itself is transferred last; that is, it is attached to what is now the 'tail' of the chromosome (Bouck and Adelberg, 1963). A basically identical sequence of events is thought to occur during F promoted chromosmal transfer by any donor type. "Conjugation tubes" through which the chromosome may be transferred have been observed connecting cells of opposite mating type under the electron microscope.

The physiological events necessary for transfer, however, are not clear. It is known that the genetic material in _E. coli_ and the material transferred during conjugation are predominantly DNA (Jacob and Wollman, 1958; Garen and Skaar, 1958; Silver, 1963). Early work by Fisher (1957) was interpreted as showing that energy production by the donor cells only was required for transfer, and that the synthesis of neither DNA, RNA nor protein were involved. The first of Fisher's conclusions, which was based on experiments in which cells were aerated in buffer prior to mating to deplete their endogenous energy sources, has been criticized by Clark and Adelberg (1962). Since aeration in buffer may have other effects, they point out, this conclusion does not follow necessarily. Experiments by Fisher involving the use of dinitrophenol and anaerobic conditions, however, show energy production by mating _pairs_ is necessary. The second of Fisher's conclusions is, in part, the subject of the present study.

Most attention in the past has been focused on the role of DNA synthesis (often equated with chromosome replication) in the transfer process, and two models of transfer have been proposed. Model 1, originating with A. J. Clark (Bouck and Adelberg, 1963) envisions that the break in the chromosome occurs at a particular time in its replication—at the end of what has been called the replication cycle. One of the new pair of chromosomes is then transferred to the F^- cell by an unspecified mechanism. Model 2, devised by Jacob and others (1963), proposes that transfer is driven by the replication process. The donor

chromosome (here assumed to be a DNA double-helix) is replicated from the origin by an enzymic apparatus associated with F. F and its associated "replication system" are fixed at a point inside the cell wall near the base of a conjugation tube so that the chromosome must move through this apparatus to be replicated. Early in conjugation the F system of replication is activated, and as replication begins one of the copies is diverted into the conjugation tube. Further replication pushes this copy through the tube into the recipient. The time at which the chromosome breaks is not clearly specified, but to be consistent with the model it would have to occur before, or as, the conjugal replication begins.

These models differ most clearly in the relationships they propose between DNA synthesis and the transfer process. On Model 1, DNA synthesis is required to complete chromosome replication prior to transfer, but may not be necessary during the transfer process itself. On Model 2, concurrent DNA synthesis is necessary for transfer. Model 1 also predicts a unique time in the cell division cycle during which chromosome transfer may be initiated—the period between the end of one DNA replication and the start of another. This feature may be accommodated to Model 2, but is not required by it.

The results of attempts to test these models using treatments which inhibit DNA synthesis are apparently contradictory. Bouck and Adelberg (1963) performed an experiment which is consistent with the existence of a particular time in the DNA replication cycle during

which transfer is initiated. Jacob and co-workers (1963) offer evidence that it is not. The experiments of Roeser and Konetzka (1964) were interpreted as consistent with both models, but their interpretation seems to be inconsistent with certain aspects of their own results. Reassuringly, all of these interpretations are open to objections. For example, phenethyl alcohol (PEA) has been used to inhibit DNA synthesis (Bouck and Adelberg, Jacob et al., Roeser and Konetzka), but it affects RNA and, indirectly, protein synthesis as well (Nonoyama and Ikeda, 1964; Treick and Konetzka, 1964; Rosenkranz and others, 1965). Furthermore, as already indicated, these models are not mutually exclusive. It could be that DNA synthesis is necessary for transfer, but that such synthesis must be initiated at a particular time in the cell division, or chromosome replication, cycle.

Our own experiments were aimed at testing the dependence of transfer on concurrent DNA synthesis by depriving Hfr cells of thymine during conjugation, and the existence of a unique time of initiation of transfer by the use of treatments which might synchronize cell division or DNA replication. These have led, unexpectedly, to the recognition of the importance of donor-specific surface structures known as F-pili (Brinton et al., 1964) in conjugation, and to further experiments aimed at understanding F-pili function.

MATERIALS AND METHODS

Bacterial and bacteriophage strains

The bacterial strains used were derivatives of *Escherichia coli* K-12. MSC 603 is a thymineless derivative of CS 101, which is itself a derivative of the Cavalli Hfr strain (Hfr C; Cavalli, 1950). MSC 603 thus requires thymine and methionine, ferments lactose, and is streptomycin sensitive. W1177 is a multiply-marked K12 derivative via Y53 (Lederberg, 1947). It requires threonine, leucine and thiamine, does not ferment lactose and is streptomycin resistant.

MS-2, a donor specific bacteriophage, was donated by Dr. A. J. Clark.

Media and supplements

Final concentrations are given as grams/liter of distilled water unless otherwise indicated. Media were sterilized in an autoclave at 18 pounds pressure for 20-25 minutes.

Nutrient Agar: Difco Nutrient Broth 8, NaCl 5, agar 15.

Pennassay Broth (PB): Bacto-Beef Extract 1-5, Bacto-Yeast Extract 1.5, Bacto-Peptone 5.0, Bacto-Dextrose 1.0, NaCl 3.5, K_2HPO_4 3.6, KH_2PO_4 1.3.

Eosin Methylene Blue Agar (EMB): Bacto-Casitone 8.0, Yeast Extract 1.0, NaCl 5.0, K_2HPO_4 2.0, Eosin Y 0.4, Methylene blue 0.065, sugar 10.0, agar 15.0.

Davis Minimal Agar (DMA): K_2HPO_4 7.0, KH_2PO_4 2.0, crystalline sodium citrate 0.5, crystalline $MgSO_4$ 0.1, crystalline $(NH_4)_2SO_4$ 1.0,

sugar 1.0, agar 15.0. The salts were added to 500 ml of water and sterilized. If the sugar added was glucose, it and the agar were added together to 500 ml of water and mixed with the salt solution after sterilization. If the sugar added was lactose, the agar was added to 490 ml of water alone and sterilized, while one gram of lactose was dissolved in 10 ml of sterile water and added to the agar after sterilization.

Saline: NaCl 8.6.

Streptomycin (Dihydro-streptomycin sulfate): 250 mg/ml.

Aminopterin (AM): As part of the procedure for isolating thymineless mutants AM was added to certain cultures to give a final concentration of 0.2 mg/ml.

Tris-glucose Minimal Medium (TG): NaCl 5.4, KCL 3.0, NH_4Cl 1.1, $CaCl_2$ 0.011, $MgCl_2$ 0.095, $FeCl_3$ 0.00162, KH_2PO_4 0.0872, Na_2SO_4 0.0227, Tris (hydroxymethyl) aminomethane 12.1. The pH was adjusted to 7.2 with concentrated HCl and the solution sterilized (T). Just before using, 9 parts were mixed with 1 part sterile glucose solution containing 20.0 grams/liter glucose.

TG*: TG supplemented with thymine, methionine, threonine, leucine and thiamine.

Growth factors: The following were added to DMA or TG when required: methionine 0.02, thymine or thymidine 0.02, thrionine 0.04, leucine 0.02, thiamine 0.0001.

Inhibitors: To inhibit conjugation the following were added to

liquid media to give the final concentrations indicated: chloramphenical (CAP) 0.05 mg/ml, phenethyl alcohol (PEA) 0.3%.

Storage of bacterial and bacteriophage strains and preparation of working cultures and lysates

Bacterial strains. Bacterial strains were stored on nutrient agar slants at 4° C and transferred every few months. The transfer always included single colony isolation and tests for the relevant growth requirements.

In some cases—when a particular strain was to be used daily, or to be able to observe individual colony morphology of thymineless mutants—strains were streaked from these slants onto nutrient agar plates and liquid cultures inoculated by picking patches of dense growth or many colonies. All other inoculations were made directly from the stock slants.

Unless stated otherwise cultures were incubated at 37° C. Pennassay broth cultures were grown overnight (12-16 hrs.). Log phase cultures in this medium were obtained by diluting 1/10-1/20 from an overnight culture and incubating 90-120 minutes. Overnight TC cultures were started from a heavier inoculum and incubated longer (16-20 hrs.), the exact time depending on the particular batch of medium. Log phase TG cultures were also started by 1/10-1/20 dilution from an overnight culture, but needed to be incubated 3-5 hours, depending on the particular experiment or the batch of medium. For use in conjugation experiments in TC* medium, F^- cultures were routinely grown to late log

or early stationary phase to maximize the yield of viable cells. This procedure has no apparent effect on F^- fertility.

Bacteriophage techniques. Phage lysates were obtained by the method described by Adams (1959). After collection, the phage suspensions were centrifuged to remove cells, and the supernate decanted and treated with chloroform. The supernate was again poured off and incubated in an open petri dish, or aerated for a short time, to remove the remaining chloroform. The final lysate was stored at 4° C.

Lysates were assayed by the agar-layer method at appropriate dilutions on nutrient agar.

Phage adsorption was measured by adding phage to an excess of cells in a growing culture and following the decrease in plaque forming units as detailed by Adams: the culture was diluted 100 fold into cold medium to stop adsorption. These dilutions were then centrifuged to sediment the cells, and then assayed by plating 0.1 ml of the supernate with 0.1 ml of an overnight culture of MSC 603 using the top layer method.

Isolation of thymineless mutants

Thymineless mutants were isolated by the general method of Okada et al. (1962). Dense cultures in TG medium and thymidine were prepared by aeration for 16 hours after inoculation from an overnight culture. These were then diluted 1/500 into the same medium lacking thymine and containing 0.2 mg/ml aminopterin and aerated for 24 hours. If, at the end of this time, the cultures were turbid, they were

discarded. If, however, there was no visible growth, 0.5 ml of thymine (2 mg/ml) was added to 5 ml of the culture and incubation continued for 48 to 72 hours more. These final cultures were streaked, or diluted and plated, on nutrient agar and incubated until colonies appeared.

Thymineless colonies of MSC 603 could be recognized immediately by a characteristic 'thin', or translucent, appearance on nutrient agar (Okada _et al._, 1962), and were tested for their thymine requirement by picking and streaking, or spotting, on minimal medium complete for this strain (i.e., lacking only thymine). The thin colony phenotype was lost in the course of repeated transfers, but no attempt to study this phenomenon has been made.

Mating procedures

Matings to determine the fertility of MSC 603 under different conditions and to follow the kinetics of transfer of certain markers were performed by the method developed by de Haan and Gross (1962). Log-phase Hfr cultures and F^- cultures were mixed to give concentrations of 1-2 x 10^7 Hfr/2-4 x 10^8 F^- per ml; when using Pennassay broth cultures the Hfr was simply diluted 1/10 into an aliquot of the F^- culture. When using TG* medium, aliquots of the F^- were centrifuged and the pellets resuspended with an appropriate volume of Hfr cells at 2 x 10^7/ml to initiate the mating. This procedure was followed to avoid using Hfr cultures which had gone out of log phase.

After mixing, the cultures were incubated five minutes to allow the cells to pair and then diluted to stop further pairing. Cultures

mated in TG* medium were diluted 100 fold into the same medium. Cultures mated in Pennassay broth were diluted into 10^{-2} PB (PB diluted 1/100 with saline). Depending on the experiment, samples were removed from the diluted cultures either at five minute intervals, or at 30 or 60 minutes to assay recombinants, and, in some cases, viable cells.

Recombinants were assayed by spreading 0.1 ml samples of the diluted culture on appropriately supplemented DMA. Where lac$^+$ transfer was measured the plates lacked glucose, and were supplemented with lactose, threonine, leucine and thiamine. Where leu$^+$ transfer was measured the plates contained glucose and were supplemented with leucine and thiamine. In all cases streptomycin was added to kill the donor cells and prevent further transfer.

Samples removed from mating cultures are usually blended in a high speed mixer before plating. This treatment is thought to be necessary to separate mating pairs and prevent transfer from being completed on the plates by those pairs which have not completed it at the time of sampling. In our own experiments blending has not been used, since it was found to be possible to obtain identical kinetics with or without it. The only difference between the data obtained in the two situations is that without blending, a few 'background' colonies may be seen on plates made at times before the marker being selected enters. It appears that the spreading of the samples and the presence of streptomycin on the plates combine to prevent transfer in greater than 90% of the pairs plated at such times.

RESULTS

The effects of thymine deprivation upon conjugation

Effects on 60 minute recombinant yield. In the absence of thymine the amount of DNA made by thymine-requiring strains of *E. coli* is small (Barner and Cohen, 1956; Korn and Weisbach, 1962; Seno and Melechen, 1964). In addition there is, typically, a 30-60 minute lag before the cells start to die, whereas DNA synthesis proceeds at its new and lower rate from time zero. If transfer depends on concurrent DNA synthesis it might then be predicted that the fertility (the number of cells able to transfer a given gene measured under some standard set of conditions) of an Hfr culture undergoing thymine deprivation would decay faster than cell viability. This assumes DNA synthesis in the donor cells is not restored in some way (e.g., by feeding) upon mixing with the F^- cells.

To test this prediction, experiments designed to measure the decay of fertility of MSC 603 in the absence of thymine were performed as follows: A log-phase culture of MSC 603 in TG* was washed twice by centrifugation, resuspended in the same medium lacking only thymine, and incubated at 37° C. Samples were removed at 30 minute intervals, diluted in saline, and spread on nutrient agar to measure viable cells. At the same times samples were removed and mated with W1177 grown in the same medium to determine relative fertility. Mating cultures in TG* lacking thymine were prepared as described in Materials and Methods. Samples (0.1 ml) were removed after one hour and plated directly on

appropriately supplemented DMA to score lac$^+$ recombinants. Thus, the criterion of fertility in these experiments is the number of lac$^+$ recombinants formed after 60 minutes of mating. By this time the number of these recombinants has reached its maximum value, as will be seen in subsequent experiments.

The results of a typical experiment are given in Figure 1. Two features of the results are immediately apparent: (1) Unexpectedly, fertility seems to be enhanced initially. (2) The rate of fertility decay appears to be less than the rate of thymineless death.

The first feature has been found to be an artifact of the washing procedure. The effect of this procedure in reducing fertility and the recovery of fertility after the final resuspension is shown in Table Ia. The experiment was performed as described above except that an additional sample was removed just before washing, and only two samples were taken after resuspension--one immediately and the other after one hour. It can be seen that the fertility was reduced to one-third of its original value by washing, but returned to this value after one hour of incubation. The results of a second experiment recorded in Table Ib show that the fall in fertility is due only to centrifugation and resuspension and not some other aspect of the washing procedure. A log-phase culture of MSC 603 in TG* was centrifuged and resuspended twice in the same medium. The centrifugation was performed in an incubator room with all materials at 36-37° C. Samples were removed to measure fertility and assay viable cells before and after each

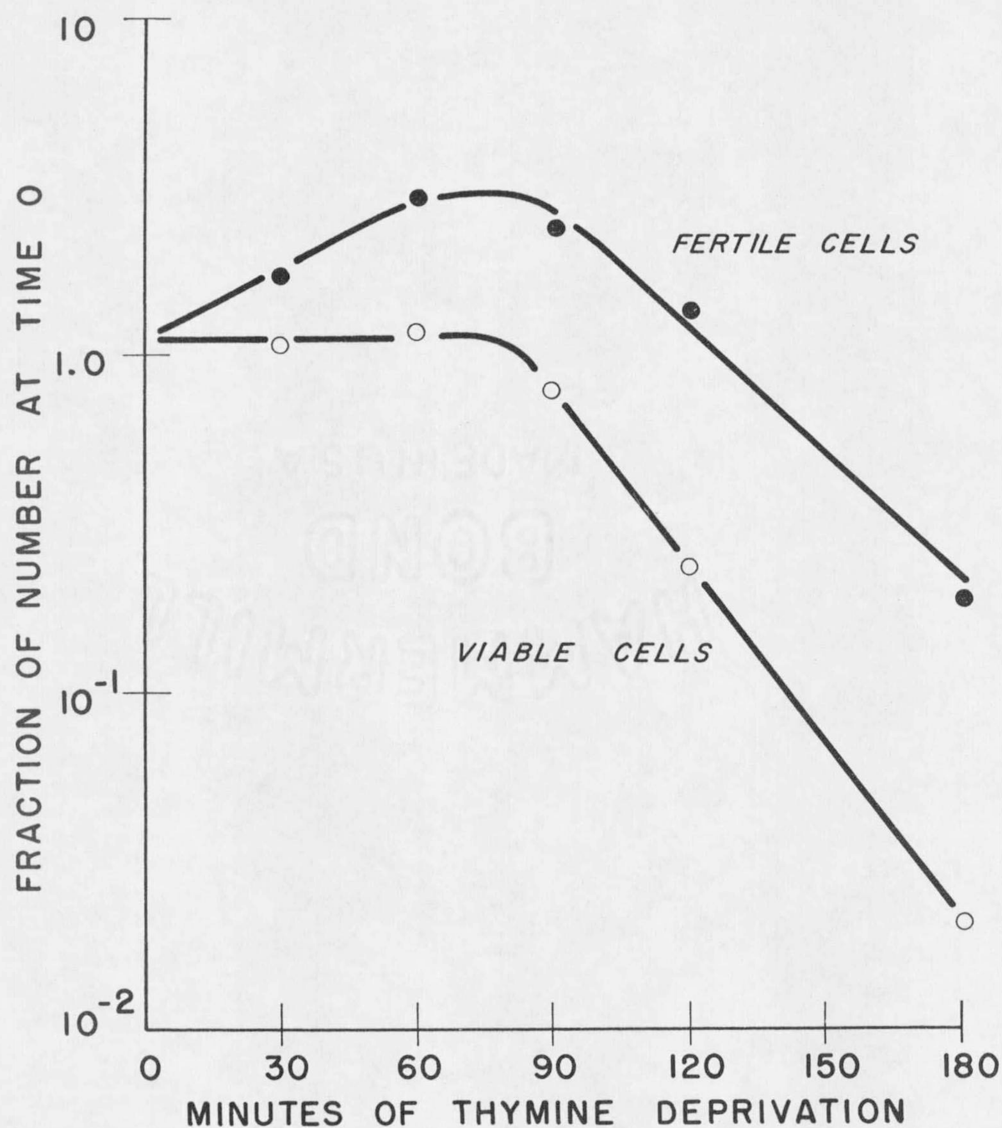

Figure 1. Decay of viability and fertility of a donor strain during thymine deprivation. Log phase MSC 603 were deprived of thymine at time 0. At intervals, samples were removed to measure viable cells and fertility. "Fertile cells" were those which yielded lac$^+$ recombinants when mated for 60 minutes with W1177. All points are averages of two independent determinations.

Table I. Effect of washing on Hfr fertility.

Time of sample	Viable cells x 10^{-5} (average of 2 independent determinations)	Recombinants x 10^{-3} (average of 3 independent determinations)	Relative Fertility (Recombinants/ Viable cells x 10^2)
a.			
Before washing	206	310	1.69
Immediately after resuspension	156	79	.51
One hour after resuspension	148	275	1.86
b.			
Before centrifugation	172	383	2.22
After first centrifugation	175	129	.73
After second centrifugation	180	107	.59

MSC 603 was grown to log phase in TG*, and a sample removed to assay viable cells, and to mate with W1177. In a. the culture was then washed by centrifugation and resuspended in medium without thymine. In b. resuspension was in medium with thymine. Samples were also removed for viable cell assay and for mating at the later times indicated.

centrifugation. After the first centrifugation the fertility dropped to about one-third of its original value. Only a slight additional fall occurred after the second centrifugation. This effect can be attributed to mechanical breakage and loss of a portion of the donor cells F-pili when the cells are packed together and subsequently resuspended. A correlation between the loss of these structures and the loss of fertility has been reported by Brinton and others (1964) and was also observed indirectly in the chloramphenicol experiments to be described later. Further information on F-pili, and experiments implicating them in gene transfer are reported in later sections of these results.

Since 60 minutes appears to be sufficient to undo the effects of centrifugation, and since thymineless death does not begin until after 60 minutes, a comparison of the kinetics of decay of fertility and viability after this time should establish their relative rates. The results of three experiments are included in Figure 2. Because of variability in fertility from experiment to experiment, the ratio between fertile and viable cells has been plotted. From this comparison it is clear that the decay in ability to form lac^{+} recombinants after 60 minutes is slower than the decay of viability, just the opposite of our prediction. This finding, in fact, suggests that cells not capable of giving rise to colonies on nutrient agar may still be competent to transfer genes.

Thymineless death in mating mixtures. The results given in