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Conjugal Deoxyribonucleic Acid Replication by *Escherichia coli* K-12: Stimulation in *dnaB*(ts) Donors by Minicells

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R64-11$^+$ donor cells that are thermosensitive for vegetative DNA replication will synthesize DNA at the restrictive temperature when recipient minicells are present. This is conjugal DNA replication because it is R64-11 DNA that is being synthesized. and there is no DNA synthesis if minicells that cannot be recipients of R64-11 DNA are used. The plasmid DNA present in the donor cells before mating is transferred to recipient minicells within the first 20 min of mating, but additional copies of plasmid DNA synthesized during the mating continue to be transferred for at least 90 min. However, the transfer of R64-11 DNA to minicells is not continuous because the plasmid DNA in minicells is the size of one R64-11 molecule or smaller, and there are delays between the rounds of plasmid transfer. DNA is synthesized in minicells during conjugation, but this DNA has a molecular weight much smaller than that of R64-11. Thus, recipient minicells are defective and are not able to complete the synthesis of a DNA strand complementary to the single-stranded R64-11 DNA received from the donor cell.

Genetic information can be transferred from donor to recipient cells of *Escherichia coli* K-12 by the process of conjugation, and the capacity of a cell to be a donor is determined by the presence of a conjugative plasmid such as the fertility factor (F), resistance transfer factor (R), or transferable colicin factor (Col). During conjugation, a single strand of donor cell deoxyribonucleic acid (DNA) synthesized before mating is passed to the recipient cell (see 13 for a review). It is a unique strand of the donor cell DNA that is transferred, and the 5′ end of the molecule enters the recipient first (33, 38). Furthermore, it has been demonstrated by Vapnek and Rupp (43) that the complement of the transferred strand remains in the donor cell in which the transferred strand is replaced by de novo DNA synthesis. Transfer of a unique single strand of DNA and its replacement in donor cells has also been described for both F-like and I-like R factors (42).

Bonhoeffer (3) isolated temperature-sensitive mutants that immediately stop synthesizing DNA at restrictive temperatures [*dnaB*(ts)]. Donor cells possessing these mutations, however, are still capable of transferring their DNA during conjugation (3, 4). Although it initially appeared that DNA was being transferred from such donors in the absence of DNA synthesis, Bresler et al. (5) and Marinus and Adelberg (27) demonstrated that there is a stimulation of DNA synthesis when these thermosensitive strains are mated at restrictive temperatures. Vapnek and Rupp (44) have extended these findings by showing that the DNA synthesized in F$^+$ *dnaB*(ts) cells during conjugation at 42 C is that strand of the fertility factor which is normally replicated in the donor during a mating to replace the transferred strand. Thus, vegetative replication and conjugal replication appear to be controlled separately as was predicted by the replicon model of Jacob et al. (23).

Molecular studies of the relationship between DNA transfer and replication during conjugation and the molecular mechanisms involved in these processes are complicated because the amount of conjugal DNA replication is small compared with that of vegetative replication, and two populations of cells must be studied. By using *dnaB* thermosensitive mutants, conjugal DNA replication can be studied in the

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absence of vegetative replication. However, to make meaningful comparisons of events within donor cells, including conjugal replication and the transfer of DNA of recipient cells, one must be able to separate donor and recipient cells under nonphysiological conditions. The selective lysis of one parent with phage (33, 43) not only makes it difficult to analyze both parents in a particular mating, but the time required at physiological temperatures for lysis and enzymatic degradation of the unwanted parent allows for considerable molecular changes in the other parental cell's DNA before it can be extracted and examined. For these reasons, we have explored the possibility of studying conjugal DNA replication during transfer of the resistance transfer factor, R64-11, from *dnaB*(ts) donor cells to recipient minicells. Minicells are small, anucleate cells formed by a mutant of *Escherichia coli* K-12 (2), and they can act as recipients of conjugally transferred DNA(9, 10). These minicells can be efficiently separated by sucrose gradient sedimentation at 0 to 4 C from either their parent cells or donor cells after a mating.

In this communication we report that minicells do stimulate conjugal DNA replication in *dnaB*(ts) donor cells at 42 C, and we describe the kinetics of single and multiple rounds of plasmid transfer to minicells. Subsequent papers will describe the effects of chloramphenicol, rifampin, and nalidixic acid on conjugal replication and transfer by using the mating system described in this report (17, 18).

MATERIALS AND METHODS

Bacterial strains. The genotypes and sources of the strains used in these experiments are shown in Table 1. χ1284, a *dnaB*(ts) strain carrying the derepressed R factor R64-11, which is I-like (30) and confers resistance to tetracycline and streptomycin, was prepared by adding 0.5 ml each of overnight cultures of χ1005 and χ908 to 9 ml of L-broth (25). The mixture was incubated overnight at 35 C, and dilutions were plated on minimal agar (12) containing 25 μg of oxytetracycline per ml (Chas. Pfizer and Co.). Recombinant colonies were purified and checked for inhibition of growth and DNA synthesis at 42 C and for sensitivity to the I pili-specific phage If2 (31).

Preparation of recipient minicells. All strains were grown in a mineral salts solution (12) supplemented with 0.5% Casamino Acids (Difco), 0.5% glucose, and 2 μg of thiamine hydrochloride per ml. The minicell-producing strains were diluted 1:100 into fresh medium and incubated overnight (approximately 9 h) in a rotory shaker at 35 C. The minicells were isolated from their parent cells by sucrose gradient centrifugation as described by Roozen et al. (37). The purified minicell preparation was suspended in ice-cold growth medium to an optical density of 0.4 at 620 nm in a Spectronic 20 (Bausch and Lomb) spectrophotometer and kept in an ice bath until needed for a mating. By using this procedure, 10 to 15 ml of purified minicells can be isolated from 100 ml of the original culture.

Preparation of donor cells. Cultures of χ1284 that had grown overnight at 35 C were diluted 1:20 into fresh growth medium and incubated without shaking at 35 C. If the donor cell DNA was to be labeled before mating, 100 μg of adenosine per ml and 10 μCi of [^{3}H]thymidine (20.5 Ci/mmol; Schwarz BioResearch) per ml were added to the cultures at the time of dilution. When the donor cells reached an optical density of 0.33 at 620 nm (about 3×10^8 cells/ml), the mating procedure was begun.

Mating. In the general conjugation procedure, donor cells and purified recipient minicells were placed into a 42 C water bath 15 min before the mating was to begin. It should be noted that minicells are equally efficient as recipients of conjugally

TABLE 1. *Bacterial strains*[a]

Strain	Plasmid genotype and phenotype	Chromosome genotype and phenotype	Derivation
χ797	F′ *lac*$^+$	Same as χ925	F′ORF-207 from χ647 into χ925
χ908	F$^-$	Prototroph T6^s λ$^-$ Str^s *dnaB*(ts)	Stallions and Curtiss (40)
χ925	F$^-$	*thr leu lacY minA* T6^s *gal* λ$^-$ *minB* Str^r *thi*	Single-colony isolate of P678-54 from H. I. Adler
χ1009	R64-11 *drd* Tc^r Sm^r	Same as χ925	R64-11 from E. Meynell into χ925
χ1041	FColVColB *trp*$^+$ [b]	Same as χ925	Fredericq factor from C. Berg into χ925
χ1100	R100-1 *drd* Tc^r Cm^r Sm^r Su^r	Same as χ925	R100-1 from Y. Nishimura into χ925
χ1284	R64-11 *drd* Tc^r Sm^r	Same as χ908	R64-11 from χ1009 into χ908

[a] The nomenclature follows the proposals of Demerec et al. (15). All mutations conferring auxotrophic requirements are listed, but some that confer inability to use carbon sources or resistance to drugs and phages are omitted for the sake of brevity. R factor drug-resistance abbreviations: Tc, tetracycline; Sm, streptomycin; Su, sulphonamid; and Cm, chloramphenicol.

[b] Referred to as Col*trp*$^+$.

transferred DNA at 35, 37, and 42 C. When DNA synthesis was to be followed during a mating, 200 μg of adenosine per ml and 20 μCi of [^{3}H]thymidine per ml were added to the donor cells 3 min before the start of the mating. To initiate the mating, equal volumes of donor cells and minicells were mixed together in a 125-ml micro-Fernbach flask. When label was added during the mating, adenosine and [^{3}H]thymidine were added at 100 μg/ml and 10 μCi/ml, respectively. Final mating volumes varied from 3 to 20 ml, depending on the needs of the experiment. Samples (50-μliters) were withdrawn immediately and at intervals throughout the mating; after absorption on Whatman 3MM filter disks for 1 min, they were precipitated with 10% trichloroacetic acid. The disks were washed twice with 5% trichloroacetic acid and twice with 100% ethanol before being air-dried, placed into 1-dram vials with about 3 ml of 5% 2,5-bis[2-(5-*tert*-butylbenzoxazolyl)]-thiophene in toluene, and counted in a Packard Tricarb scintillation spectrometer. Matings were terminated by placing all or a portion of the mating mixture into 25 ml of ice-cold buffered saline with gelatin (BSG; 12).

Separation of donors and recipients. The terminated mating mixtures were pelleted by centrifugation at 10,000 rpm in a Sorvall SS34 rotor at 4 C for 10 min. After suspension in 2 ml of BSG, the donor cells and recipient minicells were separated by sucrose gradient centrifugation (37). Both the minicells (which form a band in the upper part of the gradient) and the cells (which form a pellet at the bottom of the tube) were collected, diluted with cold BSG, sedimented in the SS34 rotor as above, suspended in 2 ml of BSG, and purified by a second sucrose gradient sedimentation. The cell and minicell fractions from the second sucrose gradient centrifugation were washed in BSG and then suspended in the same buffer. The final volumes of the suspensions equaled the original volume of mating mixture used for the purification. The optical density at 620 nm was determined for these fractions, and samples were removed to assay acid-insoluble radioactivity as described above. To correct for losses of acid-insoluble radioactivity during purification of minicells, the amount of radioactivity in purified minicells was multiplied by the optical density of the minicells in the mating mixture (=0.20) and then divided by the optical density of the purified minicells. Very few donor cells were lost during the purification, so the value for acid-insoluble radioactivity determined for the purified cells did not require correction.

Analysis of donor cell DNA. The incorporation of [^{3}H]thymidine into closed-circular plasmid DNA was assayed by a technique similar to that described by Freifelder et al. (19). Either all or a portion of the donor cell sample was put into a test tube (10 by 75 mm), and the cells were sedimented by centrifugation at 8.000 rpm in a Sorvall SS34 rotor at 4 C for 10 min. The cells were suspended in 2 ml of cold washing buffer (19) by Vortex mixing before being centrifuged again. The cell pellet was then gently suspended in 200 μliters of cold lysis buffer (19), and the cells were lysed at room temperature by the addition of 50 μliters of a solution of 1% sodium dodecyl sulfate in 0.8 M NaOH. After chromosomal DNA was sheared by 30 s of agitation on a Vortex mixer, 200 μliters of the sample was layered onto a 3.7-ml linear gradient of 5 to 20% sucrose in 0.5 M NaCl, 0.3 M NaOH, and 0.02 M ethylenediaminetetraacetic acid. Gradients were centrifuged by using an SW56 rotor in a Beckman L3-50 centrifuge for 35 min at 30,000 rpm and 20 C. Eight-drop fractions were collected from the bottom of the gradients onto Whatman 17 paper strips by the method of Carrier and Setlow (8). The strips were washed once with 5% trichloroacetic acid and twice with ethanol before being dried and counted as above.

Analysis of DNA transferred to minicells. The size of the DNA transferred to minicells was determined by sedimentation through alkaline surose gradients by a modification of the procedure of McGrath and Williams (29). All or a portion of the minicells purified from the mating mixture was washed with washing buffer and finally suspended in 200 μliters of lysis buffer by the procedure described for the donor cells. To avoid breakage of the DNA, the minicells were lysed on top of the alkaline sucrose gradients (same as described above) by first layering 50 μliters of 1% sodium dodecyl sulfate in a solution of 0.8 M NaQH over the gradient and then adding the minicell sample. After allowing 10 to 15 min for lysis, the gradients were centrifuged by using an SW56 rotor in a Beckman L3-50 ultracentrifuge for either 35 min at 30,000 rpm and 20 C to detect closed-circular plasmid DNA or 131 min at 35,000 rpm and 20 C to determine the single-strand molecular weight of the DNA. Eight-drop fractions were collected as above. The molecular weights of the DNA and the expected sedimentation position of complete single-strand molecules of R64-11 were calculated with the computer program used by Regan et al. (35).

RESULTS

Stimulation of conjugal DNA replication. We first determined whether recipient minicells, like recipient cells (5, 27, 44), could stimulate incorporation of [^{3}H]thymidine by *dnaB*(ts) donor cells at temperatures restrictive for vegetative DNA replication. At least 12 times more [^{3}H]thymidine was incorporated into acid-insoluble material when χ1284 [R64-11$^+$ *dnaB*(ts)] donor cells were incubated for 30 min at 42 C in the presence of minicells derived from a F$^-$ parent strain (χ925) than when they were incubated alone (Fig. 1a). When χ908 [F$^-$ *dnaB*(ts)] cells, which are not donors because they do not carry any conjugative plasmids, were incubated with or without recipient minicells at 42 C (Fig. 1b), there was only a minimal amount of precursor incorporation. This demonstrates that the incorporation observed when χ1284 cells and χ925 minicells were incubated together (Fig. 1a) was not due to incorporation by the minicells alone, and that it was dependent on the donor properties of the cells. Thus, it is likely that the stimulation of DNA synthesis observed in Fig. 1a was due to the conjugal transfer of the R factor to the minicells.

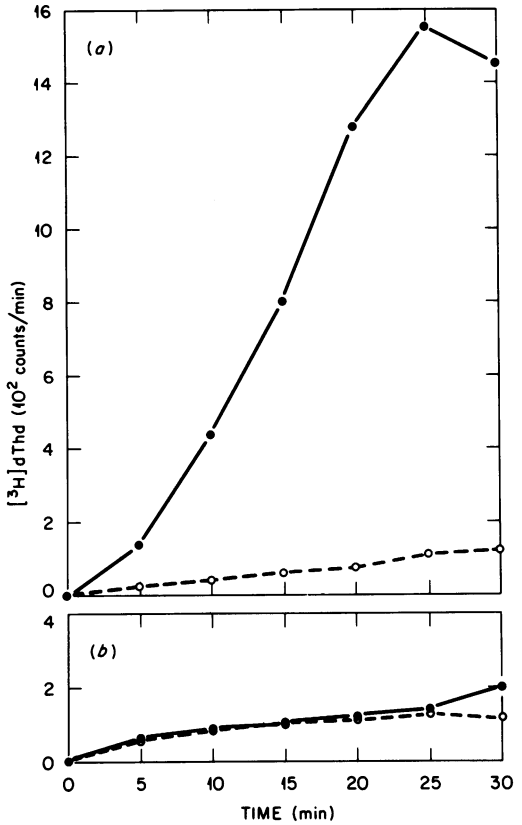

FIG. 1. *Stimulation of [³H]thymidine incorporation into trichloroacetic acid-insoluble material during conjugal transfer of R64-11 from dnaB(ts) donor cells to recipient minicells at 42 C. After 15 min of preincubation at 42 C, equal volumes of dnaB(ts) cells (about 3 × 10⁸/ml) and either recipient minicells (about 2 × 10⁹/ml) or growth media were mixed. The donor cells received 20 μCi of [³H]thymidine ([³H]dThd) per ml 3 min before the mixing. The radioactivity is plotted as counts/min in 50-μliter samples of the mating mixture. (a) Incorporation when R64-11⁺ dnaB(ts) donor cells were incubated with χ925 recipient minicells (●) or with additional growth media (○); (b) incorporation when F⁻ dnaB(ts) cells were incubated with χ925 recipient minicells (●) or with additional growth media (○).*

Specificity of the DNA replication. To confirm that the observed increase in DNA synthesis was due to conjugal transfer, we examined the newly replicated DNA from the donor cells. It has been previously demonstrated that R64-11 can be isolated as a covalently closed-circular molecule by centrifugation through alkaline sucrose gradients (34, 36, 42). If the synthesis observed (Fig. 1a) resulted from replication of the plasmid during its transfer, we should be able to demonstrate a preferential incorporation of the precursor into closed circular DNA molecules. Figure 2 shows profiles of labeled DNA extracted from the donor cells of the experiment described in the previous section. It can be seen that only the cells that were incubated with minicells incorporated a significant amount of [³H]thymidine into the rapidly sedimenting, closed-circular plasmid DNA (fractions 10 to 13). In this experiment, 32% of the label incorporated by the donor cells was in closed-circular plasmid DNA, whereas less than 1% was in that form in the unmated control cells. In a number of similar experiments, incorporation of [³H]thymidine into closed-circular plasmid DNA usually was 15 to 40% of the total for mated donor cells and 1 to 4% for the unmated controls.

Effect of entry exclusion on conjugal replication. Minicells produced from parent strains carrying certain plasmid DNA molecules contain plasmid DNA but little or no chromosomal DNA (22, 24, 26, 36). Minicells that inherit conjugative plasmids in this manner are donors of the plasmid DNA in conjugal matings with cell recipients (24, 26, 36) and, like cells carrying these plasmids, their capacity to receive a plasmid of the same mating type is greatly reduced (10, 39). The ability of these cells or minicells to prohibit the receipt of a similar plasmid has been termed exclusion (30) or entry exclusion (32). To further investigate the biological significance of the stimulation of DNA

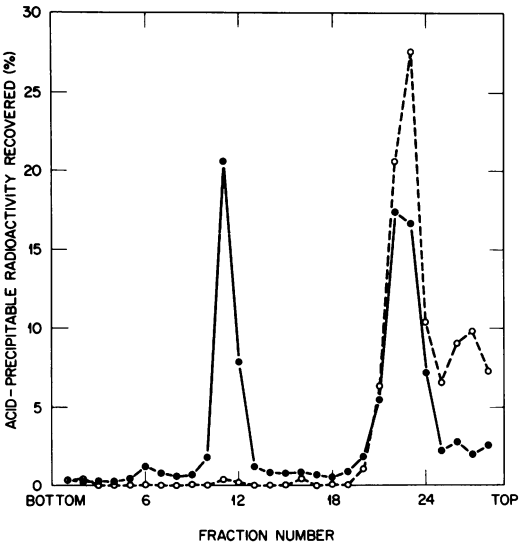

FIG. 2. *Alkaline sucrose gradient profiles of R64-11⁺ dnaB(ts) donor cell DNA. The donor cell DNA was labeled with [³H]thymidine during a 30-min incubation at 42 C with χ925 recipient minicells (●; 11,170 total counts/min) or additional growth media (○; 1,785 total counts/min).*

synthesis described above, we conducted a series of matings between χ1284 donor cells and minicells carrying a variety of conjugative plasmids (Fig. 3). When the minicells contained F'*lac*, R100-1, or Col*trp*$^+$, there was a stimulation of [^{3}H]thymidine incorporation into DNA which ranged from 30 to 60% of that achieved when the F$^-$ or plasmid-deficient minicells were used. These three plasmids are transferred by F or F-like mechanisms and thus would not be expected to exclude the R factor transferred by χ1284, which is an I-like R factor. When the minicells carried the same R64-11 factor present in the donor cells, however, there was little or no stimulation of DNA replication over the level observed when the donor cells were incubated alone. Thus, in the mating between donor cells and minicells containing isogenic plasmids, where entry exclusion should have been maximally expressed, there was no stimulation of DNA replication. This result supports the conclusion that the replication we have observed above is conjugal DNA replication.

Kinetics of events during mating. Since we were able to isolate both the donor cells and recipient minicells from matings, we followed the incorporation of [^{3}H]thymidine into donor cells, the amount of the label in closed-circular plasmid DNA in the donor cells, and the appearance of labeled DNA in the recipient minicells during a mating. The mating was conducted at 42 C as described above, and cells and minicells were purified from 3-ml samples that were removed from the mating mixture at 10-min intervals for 1 h. After a short lag, the [^{3}H]thymidine was incorporated linearly by the mating mixture for about 40 min (Fig. 4). The

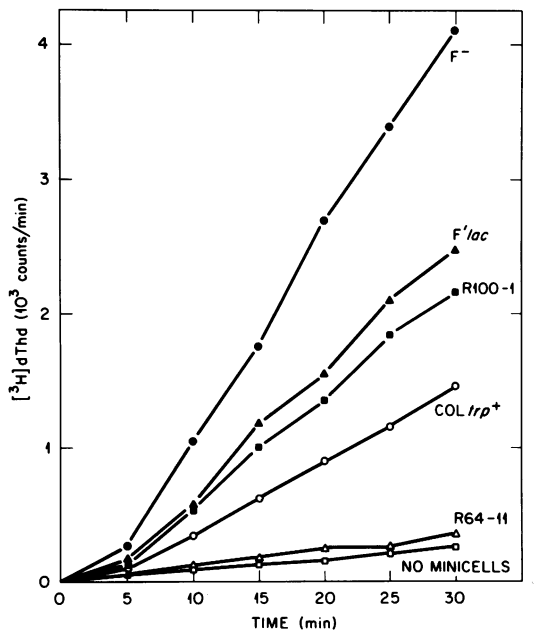

FIG. 3. *Effects of plasmid-containing minicells on the stimulation of conjugal DNA replication in R64-11$^+$ dnaB(ts) donor cells at 42 C. Minicells containing different plasmids were added as "recipients" to χ1284 donor cells in the presence of 10 μCi of [^{3}H]thymidine ([^{3}H]dThd) per ml. The minicells were from strain χ925 F$^-$ (●), χ797 F'lac$^+$ (▲), χ1100 R100-1$^+$ (■), χ1041 Coltrp$^+$ (○), and χ1009 R64-11$^+$ (△). The donor cells were also incubated without minicells (□). The radioactivity is plotted as counts/min in 50-μliter samples of the mating mixture.*

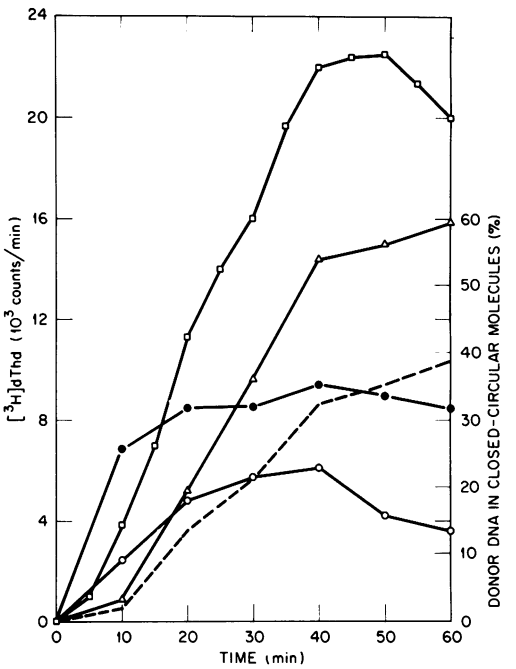

FIG. 4. *Kinetics of [^{3}H]thymidine ([^{3}H]dThd) incorporation during conjugal transfer of R64-11 from dnaB(ts) donor cells to recipient minicells at 42 C. At the indicated times, samples were withdrawn to measure the total amount of acid-insoluble radioactivity per ml of mating mixture (□). Samples (3 ml) were also taken at these times, and, after separation of the donor cells and recipient minicells, the acid-insoluble radioactivities of the donor cells (○) and the recipient minicells (△) were determined. The value for radioactivity in the minicell fraction was corrected as described in Materials and Methods to account for minicells lost during purification. The radioactivity is plotted as counts/min in 1 ml of the mating mixture. The donor cell DNA was analyzed by alkaline sucrose gradient centrifugation to determine the percentage of radioactivity in closed-circular plasmid DNA (●). The dashed line represents an estimation of the amount of the labeled DNA in the recipient minicells that was transferred from the donor cells; this correction is based on data to be described in subsequent sections of this report.*

donor cells contained more than two-thirds of the acid-insoluble radioactivity in the 10-min sample, but, by 20 min, more than half of the label was in the recipient minicells. After 20 min, the rate of incorporation by the donor cells decreased, and from 40 to 60 min the donor cells actually lost radioactivity. After a lag, the rate of appearance of isotope in the minicells was unchanged until 40 min, at which time it decreased. By 10 min of mating, about 25% of the labeled DNA in donor cells was closed-circular plasmid DNA, and from 20 to 60 min 32 to 35% was in that form. The dashed line in Fig. 4 represents an estimate of the amount of labeled DNA in the minicells that was conjugally replicated in the donor cells before being transferred. The derivation of these values is described below.

DNA in the minicells after conjugation. In order to obtain information about the replication of conjugally transferred R-factor DNA in minicells and its possible conversion to covalently closed-circular molecules, we examined the DNA in recipient minicells by sedimentation through alkaline sucrose gradients. Figure 5a is the profile of DNA extracted from minicells that had been mated for 1 h with χ1284 donor cells in the presence of [^{3}H]thymidine and centrifuged under the conditions used to demonstrate closed-circular plasmid DNA (see Fig. 2). There was no indication of any covalently closed plasmid molecules in the recipient minicells. If minicells do synthesize a complement to the transferred DNA molecules, they are incapable of forming a complete and covalently closed plasmid molecule. The profile of Fig. 5b was obtained by using minicells from another 60-min mating and an extended centrifugation so that the size distribution of single-stranded DNA molecules in the minicells could be examined. The arrow marked 1 in Fig. 5b marks the expected position of a DNA molecule of 38 × 10^6 daltons or one-half the double-strand molecular weight of R64-11 reported by Vapnek et al. (42). This position, calculated by reference to the sedimentation behavior of T4 bacteriophage DNA in similar gradients (35), is, within experimental error, the same as that of the zone of material that sedimented to a position just above it. Hence this material was probably intact single-strand plasmid DNA molecules. The profile also illustrates that there was little labeled DNA larger than 1 unit length of the plasmid (the arrow marked 2 shows the expected position of a single-strand molecule two times the length of the plasmid) but that there was a great deal of low-molecular-weight, labeled DNA in the minicells. All of the [^{3}H]thymidine-labeled DNA in the minicells from these experiments was synthesized during the mating. Since Cohen et al. (9, 10) found that fertility factor DNA transferred to minicells becomes partially double stranded, we thought that a portion of the labeled DNA in the recipient minicells probably was the product of the synthesis of DNA complementary to that received from the donor cells. In an attempt to distinguish the labeled DNA in the minicells that was transferred from the donor cells from any that was synthesized in the minicells, we examined the transfer to recipient minicells of DNA synthesized in the donor cells before conjugation.

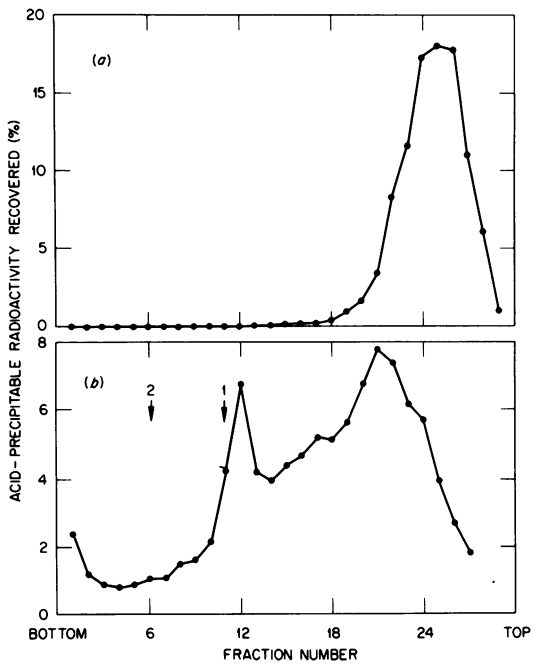

FIG. 5. *Sedimentation patterns in alkaline sucrose gradients of DNA isolated from recipient minicells after a 60-min mating with χ1284 donor cells. The DNA was labeled during the mating with 10 μCi of [^{3}H]thymidine per ml. (a) Profile after centrifugation for 35 min at 30,000 rpm to examine for the presence of closed-circular plasmid DNA; (b) profile after centrifugation for 131 min at 35,000 rpm to determine single-strand molecular weight of the DNA. The arrows mark the expected positions of monomeric and dimeric single strands of R64-11.*

Transfer of prelabeled DNA. Before mating, we labeled the donor cells with [^{3}H]thymidine and then conducted matings with minicells at 42 C. In these experiments, the donor cells had exhausted the [^{3}H]thymidine before the matings were begun. For this reason, we did not have to wash the donor cells free of isotope before starting the matings. In addition, we have found that washing the donor cells reduced

the amount of conjugal transfer (unpublished data). Data from a 30-min mating (Fig. 6a) illustrate that, after a short lag, prelabeled DNA was transferred at a constant rate for about 20 min. Extrapolation of the linear portion of the curve indicates that DNA began to reach the minicells after about 3 min of mating. In a 2-h mating (Fig. 6b), the majority of the radioactivity was also transferred in the first 20 min, and there was no indication of degradation of the transferred DNA.

Alkaline sucrose gradient profiles of the prelabeled DNA transferred to minicells after 10 and 30 min of mating are shown in Fig. 7a and b. The arrows represent the expected positions in the gradients of complete single strands of R64-11. These two profiles are essentially the same, as were those of other samples taken at 5-min intervals during the 30-min mating (data not shown). Both show a prominent band of labeled DNA that sedimented in the gradients to the expected position of complete single strands of R64-11 and labeled DNA that sedimented more slowly. The transferred DNA that was smaller than monomers of R64-11 could have been the result of either incomplete transfer of plasmid molecules due to spontaneous interruption of matings or breakage of the plasmid DNA during its isolation and sedimentation. These results indicate that complete single-strand molecules of plasmid DNA were being transferred rapidly and that the rise of radioactivity in the minicells during the first 20 min of mating was due to the asynchrony of transfer by the donor cells (14) and not to slow

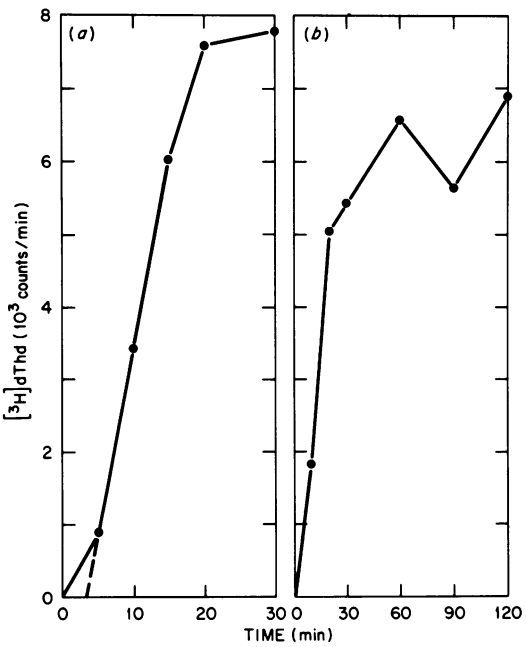

FIG. 6. *Transfer of donor cell DNA labeled before mating to recipient minicells. Overnight cultures of χ1284 donor cells were diluted 1:20 into media containing 10 μCi of [³H]thymidine ([³H]dThd) per ml and grown at 35 C for 2.5 to 3 h before being shifted to 42 C and mated with χ925 minicells. Minicells were purified from 3-ml samples withdrawn from the mating mixtures at the times indicated. The results are plotted as counts/min in the minicells of 1 ml of mating mixture. (a) 30-min mating; (b) 120-min mating.*

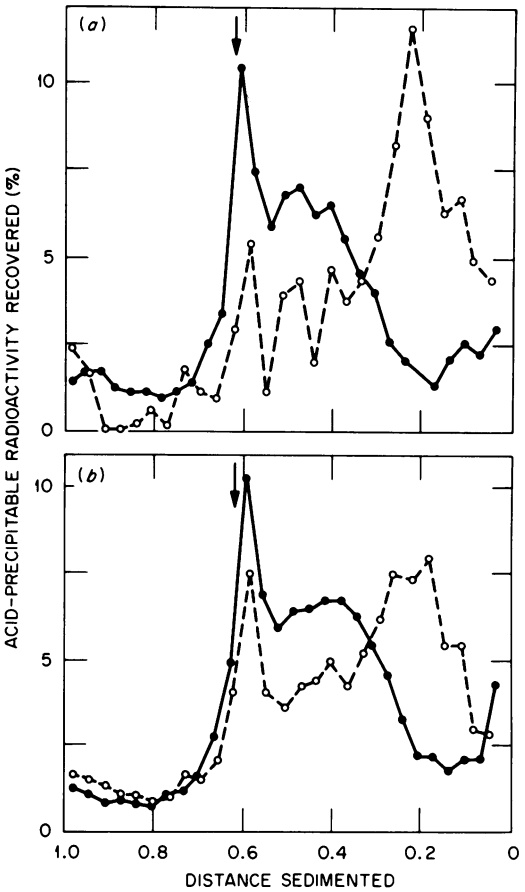

FIG. 7. *Alkaline sucrose gradient profiles of [³H]thymidine-labeled DNA extracted from recipient χ925 minicells after matings at 42 C with χ1284 donor cells. The radioactive DNA in the recipient minicells was labeled either before the mating (●) by growing the donor cells in the presence of [³H]thymidine or during the mating (○) by having [³H]thymidine in the mating mixture. (a) Minicells purified after 10-min matings (●, 6,535 total counts/min; ○, 282 total counts/min); (b) minicells purified after 30-min matings (●, 13,418 total counts/min; ○, 3,492 total counts/min). The arrows mark the expected position of monomeric single strands of R64-11.*

transfer of plasmid molecules. It is also interesting to note that the large amount of very low-molecular-weight DNA seen in Fig. 5b for an experiment in which label was present during mating is not present in minicells that have received DNA labeled before mating. For comparison, profiles of labeled DNA extracted from minicells after 10 and 30 min of mating in the presence of [^{3}H]thymidine are also shown in Fig. 7a and b. The very low-molecular-weight material is present in both of these profiles, and it is the major component of the 10-min sample. Since very low-molecular-weight DNA is only found in minicells that have received DNA while conjugating in media containing [^{3}H]thymidine, this labeled DNA is probably the result of the minicell's abortive attempt to synthesize a complete strand complementary to the incoming strand of the R factor. In this regard, both donor cells and minicells were capable of uptake of [^{3}H]thymidine, and it is known from previous work that the partially double-stranded fertility factor DNA synthesized in minicells is somewhat sensitive to degradation by the single-strand specific exonuclease I (9, 10) and contains single-stranded gaps as viewed in the electron microscope (unpublished data). In addition, we can infer that this very low-molecular-weight DNA is not synthesized in the donor and then transferred to recipient minicells, since 25% of the labeled DNA in donor cells can be isolated as covalently closed-circular molecules after 10 min of mating at a time when minicells have received little or no labeled DNA (Fig. 4), and since there is no a priori reason for suspecting that transfer of DNA labeled during mating in the donor should be subject to a higher frequency of breakdown to low-molecular-weight DNA in minicells than donor DNA labeled before mating.

For these reasons, we have made an estimate, for the data presented in Fig. 4, of the amount of labeled DNA that was conjugally transferred to recipient minicells. This was done by sedimenting the DNA from the minicell samples through alkaline sucrose gradients (see Fig. 5b and 7) to determine the fraction of the labeled DNA that had the characteristic low molecular weight of the DNA synthesized in the minicells. We elected to substract from the amount of radioactivity in the purified minicells the percentage of the DNA that sedimented in the upper 25% of the alkaline sucrose gradient. Although this calculation is somewhat arbitrary, we feel that it gives a good estimate of the amount of plasmid DNA that was synthesized in the donor cells and then transferred to the recipient minicells (dashed line in Fig. 4). From the work of Vapnek and Rupp (43, 44) we know that DNA synthesis in donor cells during conjugation replaces the strand of plasmid DNA transferred to the recipient. This newly replicated DNA should only reach the recipients when a second copy of the plasmid is transferred, and if all plasmids transfer twice, then the amounts of conjugally replicated DNA in donors and recipients should be equal. Thus, the appearance of such DNA in the minicells indicated that multiple rounds of transfer had occurred, and the corrected data in Fig. 4 show that the plasmids in the donor cells had, on the average, been transferred twice by 30 min.

Duration of conjugal replication. In Fig. 4 it was seen that incorporation of [^{3}H]thymidine by the conjugation mixture at 42 C ceased after about 40 min of mating. To determine whether that represented the end of conjugal replication, we started three identical matings between χ1284 donor cells and χ925 minicells at 42 C. The first was labeled with [^{3}H]thymidine from 0 to 30 min, the second from 30 to 60 min, and the third from 60 to 90 min of the mating. The amount of acid-insoluble radioactivity present in the donor cells at the end of each labeling period (Fig. 8) decreased almost linearly with time. By centrifugation through alkaline sucrose gradients, the donor cell DNA synthesized during the three consecutive periods was found to be 27, 25, and 17% covalently closed plasmid DNA. The decrease in both incorporation and synthesis of plasmid DNA shows that conjugal replication was declining with time. An extrapolation of the data in Fig. 8 indicates that conjugal replication ceased in donor cells between 90 and 120 min of mating. The apparent conflict between these data and those in Fig. 4 indicating cessation at 40 min was resolved by measuring the stability of the [^{3}H]thymidine in the mating mixture. We analyzed samples of the mating medium at 10-min intervals by paper chromatography (6) and found that, after 40 min of mating, all of the available [^{3}H]thymidine had been converted to [^{3}H]thymine or other unknown products (data not shown). Thus, any DNA conjugally replicated late in matings similar to the one used to obtain the data for Fig. 4 was not labeled to an appreciable extent because neither χ1284 nor χ925 can utilize exogenous thymine for DNA synthesis.

There was also a nearly linear decrease in the radioactivity found in the minicells at the end of the three labeling periods used for the experiment presented in Fig. 8, and the size of the labeled DNA in the minicells also decreased. The profiles of the minicell DNA after sedimen-

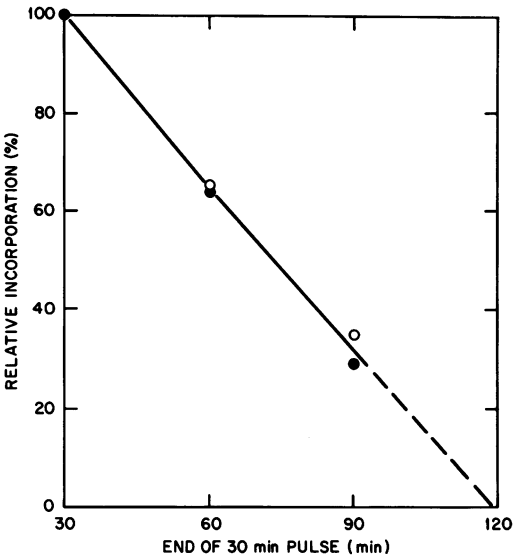

FIG. 8. *Duration of conjugal DNA replication. Three matings between χ1284 donor cells and χ925 minicells were conducted at 42 C. Tritiated thymidine (10 μCi/ml) and adenosine (100 μg/ml) were added to one mating mixture at 0 min, to another at 30 min, and to the last at 60 min. The matings were terminated 30 min after they received the [³H]thymidine, and the donor cells were purified by sucrose gradient sedimentation. The amount of acid-insoluble radioactivity in the donor cells after the 30-min incorporation periods was determined and is plotted as a percentage of the radioactivity in the donor cells labeled during the first 30 min of mating versus the times at which the matings were terminated. Data from two experiments are given.*

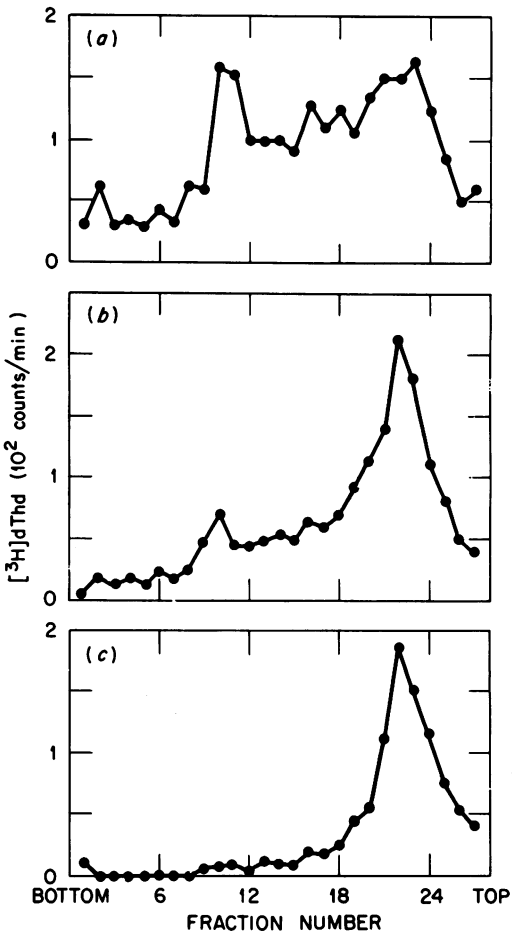

FIG. 9. *Duration of transfer of conjugally replicated R64-11 DNA to recipient minicells. Recipient minicells were purified from one of the matings described in the legend of Fig. 8, and the [³H]thymidine ([³H]dThd)-labeled DNA from these minicells was examined by sedimentation through alkaline sucrose gradients. (a) After 30 min of mating in the presence of [³H]thymidine; (b) after 60 min of mating when label was present from 30 to 60 min; (c) after 90 min of mating when label was present from 60 to 90 min.*

tation through alkaline sucrose gradients are shown in Fig. 9. The amount of labeled DNA in minicells that was the size of a full single strand of the plasmid decreased, and the low-molecular-weight material remained relatively constant. The DNA from minicells labeled during 60 to 90 min of mating (Fig. 8c) was almost totally low-molecular-weight material. This means that, after 30 min of mating, only a small portion of the DNA synthesized in the donor cells was transferred to the minicells. Thus, the ability of the plasmid molecules to be transferred more than once during successive 30-min periods decreased during the 90-min mating.

DISCUSSION

Since Cohen et al. (9, 10) demonstrated that minicells can act as recipients of conjugally transferred DNA, it was satisfying but not surprising to find that minicells, like recipient cells, are able to simulate conjugal DNA replication in *dnaB*(ts) donor cells at temperatures restrictive for vegetative DNA synthesis. This observation had permitted us to measure conjugal replication during such a mating and to correlate this synthesis with transfer of DNA to the recipient minicells. Bresler et al. (5) first noted conjugal replication when vegetative DNA synthesis was inhibited by temperature in a study using Hfr donor cells. Marinus and Adelberg (27) and Vapnek and Rupp (44) found this type of replication during the transfer of F'*lac* and F, respectively. Our study has ex-

tended these findings to the transfer of R64-11, which is an I-like R factor (30). Vapnek et al. (42) have found that the conjugal transfer of both I- and F-like R factors is similar to that of F because donor cells carrying these plasmids all transfer a unique single strand of the plasmid DNA to recipient cells. However, at least some of the gene products involved in the transfer of I-like R factors differ from those of F because Cooke et al. (11) and Willetts (45) have demonstrated the inability of I-like R factors, including R64-11, to complement transfer-deficient mutants of F and vice versa. These results differ sharply from those obtained with the F-like R factor, R100-1, which will complement mutations in 10 of the 12 cistrons of the F factor that are involved in conjugal transfer (46). We can only speculate whether all the gene products that are responsible for conjugal replication are coded for by the conjugative plasmids, but, from our data and those cited above, it is clear that conjugal DNA replication directed by both I- and F-types of conjugative plasmids is not affected by the thermosensitive *dnaB* gene product that stops vegetative plasmid and chromosome replication immediately at 42 C. Thus, as hypothesized by Jacob et al. (23), there must be a "replicator" in donor cells that is distinct from the replication system for vegetative DNA synthesis and that is activated by the presence of functional recipient cells.

The point that recipient cells are required for the initiation of conjugal replication is supported by the fact that minicells carrying a plasmid isogenic to the one in the donor cells failed to stimulate conjugal DNA synthesis (Fig. 3). Donor cell DNA is not transferred to minicells in such matings (10, 39), so these minicells are capable of entry exclusion (30, 32) and do not act as recipients. These results mean that cells or minicells which exhibit entry exclusion either never form mating pairs with the donor cells (1) or if they do form pairs they do not generate the signal within the donor cells to begin conjugal transfer and replication.

In a study of conjugal transfer by Hfr donor cells, Fulton (20) obtained evidence of linkage between distally and proximally transferred Hfr markers. He concluded that the portion of the Hfr chromosome transferred to the recipient cell was replaced by the DNA synthesis that Jacob et al. (23) had proposed to be associated with conjugal transfer. This, he said, would give rise to a circular Hfr chromosome with no terminus that could be continuously transferred to a recipient cell and would establish linkage between donor markers that were separated by the origin of transfer. Ohki and Tomizawa (33) and Matsubara (28) examined F' and F DNA that had been transferred to recipient cells and found that linear molecules of such DNA were longer than the unit length of the episomes. Their results led Ohki and Tomizawa (33) to postulate that the transfer of episomal DNA is accompanied by a rolling-circle type of DNA replication (21) that makes possible the continuous transfer of DNA to the recipient cells. This idea was supported when Vapnek and Rupp (43) found that the strand of F not transferred during conjugation does remain in the donor cell and that its complement is asymmetrically synthesized either during or shortly after conjugal transfer. For continuous transfer of either an episome (28, 33) or an Hfr chromosome (20), this replication would have to occur concomitantly with transfer so that there would be no end to the transferred strand. From this model we can predict that during mating the first DNA to be transferred to recipient cells would be DNA that pre-existed in the donors, and that after one complete round of transfer the donor cells would then begin to transfer DNA synthesized during the first cycle. Furthermore, the amount of conjugally replicated DNA in donor cells should increase until the first round of transfer of all plasmids is complete. Continuous transfer or multiple rounds of transfer will not increase the amount of conjugally replicated DNA in donor cells because any new DNA synthesis will be offset by transfer to the recipients of DNA replicated during the previous round.

We found that the amount of conjugally replicated DNA in the donor cells did reach a maximum at about the time (20 min; Fig. 4) the first round of plasmid transfer was complete (20 min; Fig. 6) and that there was a lag of about 8 to 10 min before plasmid DNA strands synthesized in the donor cells reached the recipient minicells (Fig. 4). Our data do not, however, support the idea of continuous DNA transfer, because the great majority of DNA that reached the minicells, whether it was synthesized before or during the mating, was equal to or shorter than the unit length of R64-11. If continuous transfer did occur, we would have to postulate that the minicells were able to cut the plasmid DNA into unit lengths. This result is not limited to matings in which minicells were used as recipients, because Falkow et al. (16), in a study of conjugal transfer of an F-like R factor, found that the predominant form of plasmid DNA in the recipient cells shortly after transfer was a linear monomer of the R factor. Moreover, if we assume that R factors are transferred at about the same rate as chromosomal DNA in Hfr

matings, R64-11 which has a molecular weight equal to about 3% of the chromosome should take slightly less than 3 min to be transferred. This is calculated by using a 90-min transfer time for the entire chromosome (41) and molecular weights of 7.6×10^7 for R64-11 (42) and 2.8×10^9 for the *E. coli* chromosome (7). When donor cell DNA was labeled before mating, we found DNA the size of unit lengths of R64-11 in recipient minicells as early as 5 min after mating was initiated. Thus, the calculated transfer time of 3 min is reasonable. In addition, the first round of plasmid transfer as measured by transfer of the prelabeled donor cell DNA was completed by about 45% of the plasmids after 10 min of mating. Thus, after 10 min of mating a substantial number of the plasmids in the donor cells had transferred at least one equivalent of R-factor DNA to the minicells, and we can calculate that those plasmids that had transferred once by 5 min could have transferred almost three equivalents of plasmid DNA to the minicells by 10 min of mating time. The continuous transfer model predicts that a substantial amount of plasmid DNA synthesized in the donor cells during the first round of plasmid transfer would appear in the minicells within 10 min of mating time and that, barring its conversion to unit lengths, some of it should be two to three times the molecular weight of a single strand of R64-11. However, after 10 min of mating we found very little DNA in the minicells that had been synthesized during conjugation, and only a small portion of this seemed to be plasmid DNA that had been synthesized in the donor cells (Fig. 4 and 7a).

We propose that conjugal transfer of R-factor DNA is not a continuous process and that, after one copy of a plasmid has been transferred to a recipient, there is a lag before that plasmid enters into a second round of transfer. Such a lag would result, for example, if only one copy of a plasmid could be transferred to a minicell, because this would require the donor cell to form a mating pair with a second minicell before the plasmid could be transferred again. Alternatively, the second round of transfer to the same minicell could require the synthesis of additional structural or enzymatic components of the cellular apparatus used for the initiation or continuation of conjugal transfer and DNA replication. In another report (17) we present evidence that agents which inhibit the synthesis of RNA and protein also prevent multiple rounds of conjugal transfer. Thus, we favor the hypothesis that the donor cell must synthesize new materials before a second round of transfer can be initiated, but we cannot rule out the possibility that formation of a new mating pair may also be involved.

It is not clear whether our results are in conflict with those of Matsubara (28), Ohki and Tomizawa (33), and Fulton (20), who proposed that F-controlled conjugation was continuous. The difference may simply represent differences between conjugal transfer of F and R factors and emphasize that there is still a great deal to be learned about conjugal transfer of DNA in *E. coli*.

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