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13. ABSTRACT (Maximum 200 words)

Conjugative DNA transfer is an important mechanism for the acquisition of multiple drug resistance by bacteria. The role of DNA synthesis in this process was examined under conditions where vegetative replication was strictly prevented. There was more than one round of transfer from a donor cell containing a single plasmid molecule, indicating replacement strand synthesis by a conjugation-specific mechanism. A significant time lag between rounds of transfer was detected, arguing against the spooling of DNA into recipients by a rolling-circle replication. The plasmid-encoded DNA primase, synthesized as a fusion product with one of the proteins required for conjugative mobilization, was required in the donor for transfer, but the canonical priming sites for vegetative replication could be deleted. The endogenous priming system of the host could not substitute for the plasmid-encoded system in the absence of vegetaive replication. Overall, the results suggest a plasmid-dependent, cryptic priming system active during conjugative transfer.

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**Forward:**

Bacterial conjugation involves the transfer of DNA from donor to recipient cell by a process requiring cell-to-cell contact. A pore is generated between the mating pair, and a single strand of DNA is passed from one cell to the other. Conjugation has been the subject of intense concern because it is the molecular mechanism most often responsible for the acquisition of multiple drug resistance by microorganisms. The rapid spread of these resistant bacteria dramatically decreases the useful lifetime of antibiotics and increases the mortality due to formerly treatable bacterial infections. Adding significantly to the problem, genes for resistance are often encoded by promiscuous plasmids able to cross species barriers and maintain themselves in a large number of different hosts. The intentional or inadvertent introduction of these plasmids into pathogenic organisms intended for biowarfare or terrorism can in general be easily accomplished, and their presence would obviously complicate both treatment and containment.

**Statement of Problem Studied:**

The long-term objective of this project has been to develop strategies for the containment of plasmids by interfering with the spread of these elements by conjugation. To this end, we have been investigating the mechanism of conjugation-specific DNA replication. This replication is distinct from the vegetative replication of plasmids, and understanding its molecular requirements could illuminate promising targets for new classes of antibiotics. As a model plasmid, we chose R1162 (RSF1010), because its mechanism of its vegetative replication has been intensively studied (1). In addition, this plasmid is highly promiscuous (2).

**Summary of Results:**

In order to understand the role of DNA synthesis during conjugation, it was necessary to distinguish this synthesis experimentally from normal, vegetative plasmid replication. We had devised a protocol in which R1162 plasmid DNA was electroporated into cells, and these cells then immediately used as donors in conjugative matings (3). Our results showed that the plasmid-encoded DNA primase, but not the other plasmid genes for replication, was required for transfer from the donor (3). During the period of this project, we used the electroporation and transfer assay to characterize conjugation-specific transfer, and to investigate the role of the primase in this process.

Only one strand of DNA is transferred during conjugation, and it is obvious that there must be strand replacement synthesis in the recipient cell. Less certain, for multicopy plasmids at least, is whether there is replacement of the transferred strand in the donor, since loss of the strand without replacement would have little consequence for overall plasmid stability. Thus, our initial studies were directed at determining the fate of the remaining plasmid DNA strand in the donor after the complementary strand had been transferred to a recipient cell. A “satellite plasmid” (that is, one depending on gene products provided in trans for replication and transfer) was constructed in vitro so that it contained a single base mismatch. This DNA was

electroporated into conjugation-proficient donor cells that contained either all the plasmid encoded proteins for replication, and were thus able to replicate the satellite plasmid (Rep+), or only the plasmid primase (Rep-). At the site of the mismatch, there was an NheI restriction site in the strand transferred during conjugation, and an FspI site at the same location in the other strand. Thus, the complement to the FspI strand must be synthesized before this restriction site will appear in the DNA transferred to a recipient.

When the test plasmid was electroporated into the Rep+ donor and the cells immediately mated, NheI and FspI plasmids were found with equal frequency in the pooled population of transconjugant cells. This indicates that upon entry, vegetative replication of the plasmid was rapid compared to formation of an active mating pair and strand transfer, since otherwise NheI+ plasmids would predominate. However, when individual transconjugant colonies were examined, 50% of these contained both NheI+ and FspI+ plasmid molecules, in about equal amounts. Since there were many more potential recipient cells than donor cells in the mating mixture, the possibility that one of these formed a mating pair with more than one donor cell was small. Thus, within a mating pair, there is frequently transfer of more than one plasmid molecule. Moreover, the second round of transfer must occur rapidly, since too great a lag between rounds would result in failure of the second molecule to establish, due to incompatibility with the newly-replicated molecules derived from the first round. The observation that there is often more than one round of transfer was consistent with our earlier published results (4).

Did multiple rounds of transfer from a single donor cell depend on vegetative replication, or was there a specific mechanism of strand replacement synthesis in the donor? To address this question, we next asked about the distribution of the restriction sites when the donor was Rep-. In this case, there were more NheI+ plasmids than FspI+ plasmids in the entire population of transconjugants; this was also true when the plasmid content of individual colonies was examined. Some of the FspI+ plasmids were due to mismatch repair, since the proportion of these decreased when the donor was deficient in mismatch repair (*mutL*) (5), or when the DNA was digested with deoxyadenosine methylase prior to electroporation, a treatment that inhibits this repair (6). Neither condition affected the NheI+/FspI+ ratio in the Rep+ case. Interestingly, however, a small but significant proportion of colonies contained both NheI+ and FspI+ plasmids. In contrast to the results with the Rep+ donors, the copy-number of each was not equal, but rather there were many more NheI+ plasmids in the colony. Our interpretation was that there is a lag or eclipse phase before a plasmid can participate in a second round of transfer. During this phase the transferred strand is resynthesized. Because of this lag, the first molecule transferred has time to establish in the recipient. The resident plasmid then prevented, by the normal incompatibility mechanism, the establishment of molecules entering later. Occasionally the FspI+ plasmids escape the inhibition, but the number of these in the colony is always less, due to the late start of replication This model is consistent with the observation that colonies can contain NheI+ plasmids, NheI+ and FspI+ plasmids, but not just FspI+ plasmids alone.

Our results suggested that a single plasmid molecule can recover and participate in more than one round of transfer, even in the absence of ongoing vegetative replication. To test this conclusion, we introduced (again by means of a duplex oligonucleotide with a mismatched basepair) a mutation preventing vegetative replication of the strand that must enter first during

conjugation. In this case, after synthesis of the complementary strand in the recipient, the mutation would prevent vegetative DNA synthesis and the plasmid would be lost. If incompatibility prevented subsequent rounds of transfer, then the mutation should result in a large increase in the frequency of such transfer. We found that indeed when replication of plasmids arising from first-round transfer was inhibited, the frequency of second-round transfer increased dramatically (7). Our results suggested, therefore, that there is a lag between the first and second rounds of transfer. Perhaps the strand remaining after transfer detaches from the membrane at the conjugative pore, undergoes replacement strand synthesis, and then attaches again before initiating a second round of transfer. In any case, the data were inconsistent with a widely held model that there is rapid transfer of multiple rounds due to spooling of multiple plasmid units into the recipient.

Once we showed that there was conjugation-specific replication in the donor, we turned our attention to the role of the plasmid-encoded primase. This protein is essential for vegetative replication of R1162. Synthesis is initiated from two, oppositely-oriented priming sites (*oriL* and *oriR*) at the origin of replication, and there are no other known priming sites on the plasmid (8,9). Thus, a requirement for the primase was reasonable in view of the necessity for complementary strand synthesis in the recipient. It was surprising, therefore, that the requirement for the primase could not be satisfied by providing the protein in the recipient (3).

A popular view is that replacement strand synthesis in the donor is accomplished by rolling circle replication, that is, by extension from the 3' end of the transferring strand. However, the requirement for primase in the donor suggested that instead it is this enzyme initiates such synthesis. We constructed plasmids lacking *oriL*, the site oriented for priming of donor replacement strand synthesis. Since the test plasmid no longer had a complete origin of replication, it was fused to a vector and then, prior to electroporation, cut out with restriction enzymes, and a mismatched oligonucleotide introduced as before so we could follow the fate of each strand. In addition, the plasmid contained the lambda attachment site (*att*) and the recipient cells encoded the lambda integrase protein, so that incoming molecules would be rescued by integration into the chromosome. Thus, transfer could be examined without the requirement for plasmid vegetative replication in either donor or recipient, and we could alter the vegetative priming mechanism without facing this requirement.

When we tested for the amount of second-round transfer for plasmids lacking *oriL*. We found, surprisingly, that deletion of *oriL* reduced, but did not eliminate, second-round transfer. This suggested that *oriL* can be used as an initiation site for replacement strand synthesis, but that other mechanisms were also available to the plasmid. However, even in the absence of *oriL*, primase was required in the donor. In addition, when both *oriL* and when *oriR* were deleted, plasmid DNA was still transferred and successfully rescued in recipient cells by integrase-mediated insertion into the chromosome. Again, primase in the donor was required.

Two forms of the primase, both able to sustain vegetative replication, are encoded by R1162 (10). The larger form is a fusion product with one of the proteins for moblization, and we had shown earlier that the fusion increases the frequency of transfer under conditions where this transfer is inefficient (11). In the electroporation/transfer assay used in this project, only the larger form is active for transfer in the donor. Our current model, based upon the results with

deletions of the priming sites for vegetative replication, is that the fused primase is able to access cryptic priming sites during transfer. The DNA helix is locally melted at the origin of transfer, and this possibly allows access of the primase to a suitable template.

Despite an apparently unavoidable requirement for primase in our experiments, this protein is not in general required for transfer of the plasmid. When the R1162 genes for mobilization together with the origin of transfer, but not the primase gene, are cloned, the resulting recombinant plasmid is mobilizable by conjugation (12). In this case, we assume that replacement synthesis is primed at the initiation sites for vegetative replication of the vector, in particular the primosome assembly sites (*pas*). These sites, unlike *oriL* and *oriR*, are targets for the endogenous priming mechanisms of the cell. If a *pas* was present in our plasmid, would it eliminate the requirement for the plasmid-encoded primase? We replaced *oriL* and *oriR*, both together and individually, with primosome assembly sites. The resulting plasmid was able to replicate vegetatively in the absence of the plasmid primase, as expected (13). However, in the absence of this replication, transfer still required the plasmid-encoded primase. We suppose that the the *pas* system, unlike the plasmid-specific system, is not activated by a round of conjugative transfer. Experiments designed to activate the *pas* system, and to describe the role of the plasmid primase in transfer, are underway.

**Publications (refereed):**

Parker, C., and R. Meyer. Selection of plasmid molecules for conjugative transfer and replacement strand synthesis in the donor. Mol. Microbiol. 46 (2002) 761-768.

Parker, C., X.-L. Zhang, D. Henderson, E. Becker, and R. Meyer. Conjugative DNA synthesis: R1162 and the question of rolling-circle replication. Plasmid 48 (2002)186-192.

An additional manuscript is in preparation.

Results presented at meeting, Pittsburgh, PA 2002:

Zhang, X-L., C. Parker, E. Becker and R. Meyer (2002) Some new connections between replication and conjugal DNA transfer. Plasmid Biology 2002. Pittsburgh, PA.

<u>Reprints are included at the end of this report</u>

**Participating Scientific Personnel:**

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# Selection of plasmid molecules for conjugative transfer and replacement strand synthesis in the donor

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## Summary

**Plasmid selection and strand replacement synthesis in donor cells during conjugative transfer was examined by a procedure involving electroporation of test plasmid DNA, containing a base pair mismatch, into donor cells prior to mating. Multiple copies of the plasmid were transferred from a donor cell that allowed vegetative replication of the plasmid. Under conditions non-permissive for vegetative replication, there were further rounds of transfer after a lag period. Strand replacement in the donor did not depend solely on the initiation mechanism for vegetative replication, indicating a conjugation-specific mechanism was also available. The lag period between first and second rounds of transfer argues against the transfer of multiple copies into recipients by the spooling of copies generated on a master molecule by rolling-circle replication.**

## Introduction

Landmark early studies on conjugative transfer, conducted with a few large, self-transmissible plasmids such as F and R64, established that a single DNA strand, not duplex DNA, is passed from donor to recipient (Cohen *et al.*, 1968; Ohki and Tomizawa, 1968; Rupp and Ihler, 1968; Vapnek *et al.*, 1971). Therefore, the complement to the transferred strand must be synthesized before this DNA can be maintained as a plasmid in its new host. It was also shown that there is conjugation-specific DNA synthesis in the donor cell, to replace the strand lost during transfer (Vapnek and Rupp, 1971). If the role of strand replacement synthesis in the recipient is to restore the plasmid DNA to its normal, duplex state, what is the role of this synthesis in the donor cell? For low copy number plasmids, such as those used in the early studies of conjugation, the purpose might simply be to insure that the lost copy of the plasmid is replaced. The replacement synthesis would then be one of the many mechanisms these plasmids use to insure their stable maintenance (Nordstrom and Austin, 1989). Plasmids with higher copy numbers might then forego this synthesis, with the occasional loss of plasmid copies easily accommodated by the normal copy-control mechanisms. Synthesis might also be part of a mechanism to provide a supply of strands from a single molecule for ongoing transport out of the cell. This idea is stimulated by the similarity between the intermediates of single-stranded phage DNA replication and conjugative transfer. In particular, if new stands were being made by a rolling circle mechanism, then a single master molecule in the donor might be responsible for multiple rounds of transfer. In this paper we describe experiments to examine whether there is replacement strand synthesis in the donor during conjugative mobilization of the broad host range, multicopy plasmid R1162 and whether newly synthesized molecules are preferentially used for additional rounds of transfer.

Vegetative replication of R1162 requires three plasmid-encoded proteins (Scherzinger *et al.*, 1984). One of these, RepC, is functionally analogous to DnaA: it binds to 20 base pair iterons within *oriV* and disrupts base pairing at an adjacent, AT-rich site (Kim and Meyer, 1991) (Fig. 4). RepA, a DNA helicase (Haring and Scherzinger, 1989), presumably initiates unwinding of the DNA at this site, thus exposing two oppositely oriented, single-strand initiation sites called *oriL* and *oriR*. A third plasmid protein, RepB, is a highly specific primase active at these sites (Haring and Scherzinger, 1989). R1162 does not appear to contain additional initiation sites efficiently recognized by chromosomally encoded replication proteins (Zhou and Meyer, 1990; Becker *et al.*, 1996).

The plasmid replication proteins required for mobilization were identified by constructing a set of strains containing the self-transmissible mobilizing vector R751 (Willetts and Crowther, 1981), all the proteins required for plasmid mobilization, and different combinations of the plasmid replication proteins (Henderson and Meyer, 1999). Cells of these strains were then transformed by electroporation with a plasmid that does not encode any

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replication proteins, but does contain both the origin of replication (*oriV*) and the origin of transfer (*oriT*) of R1162. After electroporation, the cells were immediately mated, and transconjugants containing this plasmid then selected. The transferred plasmid could be maintained in the recipient, allowing the formation of colonies, because these cells encoded all the plasmid replication proteins. Alternatively, the plasmid was rescued by incorporating a $\lambda$ *att* site into the molecule, and providing the $\lambda$ integrase in the recipient.

By using the electroporation–transfer system, we found that the plasmid-encoded primase RepB, but neither the helicase RepA nor the DnaA analogue RepC, was required for transfer (Henderson and Meyer, 1999). A reasonable interpretation is that the priming mechanism for vegetative replication is used to initiate synthesis of the complement to the transferred strand. However, we could not determine whether there was complementary strand synthesis in the donor as well. We describe here experiments to detect this synthesis, and to determine whether such strands are specially designated for subsequent rounds of transfer.

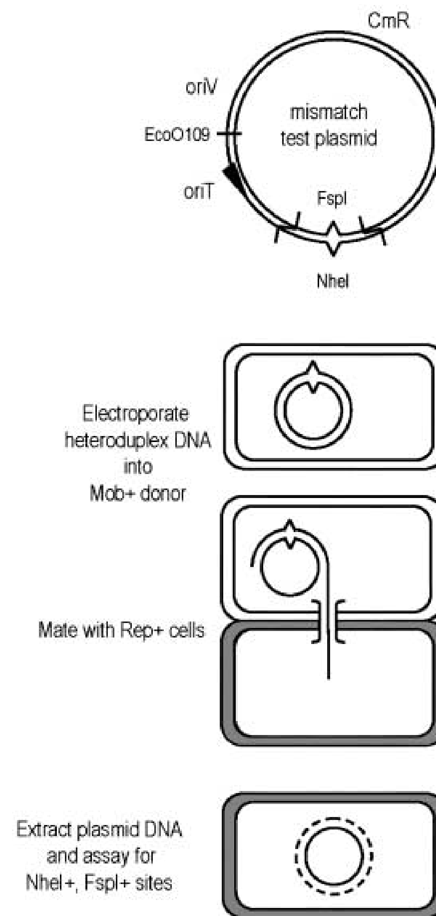



**Fig. 1.** Experimental scheme for electroporation of cells with plasmid DNA containing a base pair mismatch and for subsequent mating. The test plasmid is shown at the top of the figure. The arrow at *oriT* lies on the strand transferred and indicates the direction of transfer.

## Results and discussion

### *Multiple rounds of conjugative transfer in the absence of plasmid vegetative replication*

The outline of our experimental approach is shown in Fig. 1. Two complementary oligonucleotides having a single base pair mismatch were annealed and cloned into test plasmid DNA. The mismatch was designed so that after replication of each strand, a site for the restriction enzyme *Nhe*I would be present in one daughter molecule and an *Fsp*I site in the other. These progeny plasmids are referred to as NheI+ plasmids and FspI+ plasmids. The cloned heteroduplex oligonucleotide was oriented so that an NheI+ plasmid was formed in the recipient after synthesis of the complement to the transferred strand. Replacement strand synthesis in the donor would result in an FspI+ plasmid. If this molecule is also transferred, then FspI+ plasmids would be present in transconjugant cells.

Plasmid DNA containing the mismatch was incubated with the *dam* methylase to minimize mismatch repair (Pukkila *et al.*, 1983), then used to transform two donor strains, one containing all the plasmid proteins for vegetative replication (Rep+), and the other containing only the primase member of this group (Rep–). After electroporation, the cells were immediately mated with Rep+ cells resistant to nalidixic acid (ratio of donor to recipient, approximately 1 : 5), and then plated at one hour intervals on semi-solid medium containing nalidixic acid, and also chloramphenicol to select for the mobilized test plasmid. The transconjugant cells were allowed to grow overnight, and for each timepoint the colonies arising from the Rep+ and Rep– matings were separately pooled and the DNA extracted. To quantify the relative amounts of NheI+ and FspI+ plasmids in the populations of transconjugants, we digested equal amounts of plasmid DNA with *Eco*O109 and either *Nhe*I or *Fsp*I, which in both cases will result in a characteristic fragment of approximately 1000 base pairs, as well as a second, larger fragment (Fig. 2).

For the Rep+ donors, there were approximately equal numbers of both plasmid types for each time point (Fig. 2, top panel). We interpret this to mean that after entry of the DNA into the Rep+ donor cells, replication occurs rapidly in the presence of the pre-existing replication proteins, and predominates over transfer, so that equal numbers of NheI+ and FspI+ plasmids are produced. These molecules are then selected for mobilization. In contrast, the NheI+ plasmids were in the majority when the Rep– strain was the donor (Fig. 2, bottom panel). In addition,

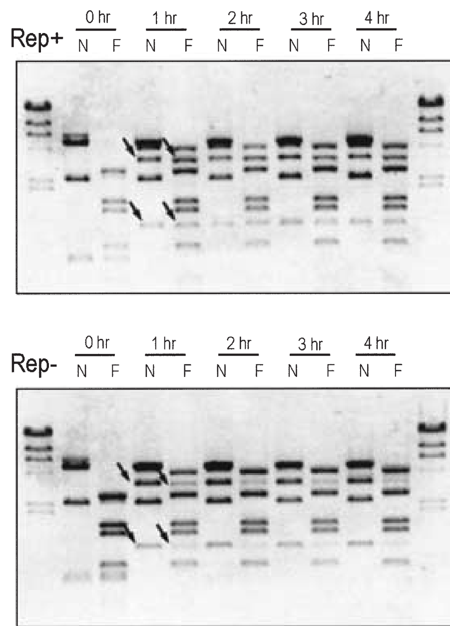
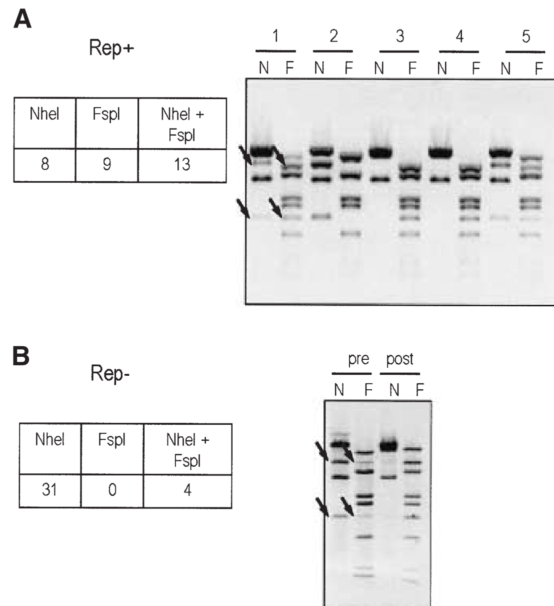



**Fig. 2.** Plasmid DNA extracted from populations of transconjugant cells after digestion with *Eco*O109 and *Nhe*I (N) or *Eco*O109 and *Fsp*I (F). Fragments were separated by electrophoresis through a 0.8% agarose gel. The bands identifying the NheI+ and FspI+ plasmids are marked by the arrows. The additional bands, those present for the 0 h samples, are due to cleavage sites in the helper plasmid DNA in the recipient. The marker in the first and last lane of each gel is *Hin*dIII-digested λ DNA.

the predominance of NheI+ plasmids indicates, as expected, that a single strand is transferred, as for the larger, self-transmissible plasmids. However, FspI+ plasmids were also detected. These could have arisen by residual mismatch repair before transfer, or by a second round of transfer after replacement strand synthesis in the donor. To distinguish between these possibilities, we examined the plasmid content of 35 transconjugant colonies derived from a Rep+ mating, and the same number from a Rep– mating (Fig. 3). The plasmid content for five, randomly selected colonies formed by transfer from Rep+ donors is also shown in Fig. 3. In the Rep+ case, colonies contained NheI+ plasmids, FspI+ plasmids, or a mixture of both, in varying proportions. Because the number of recipient cells is in excess during the mating, multiple rounds of transfer from a single donor must occur frequently.

For the transconjugants from the Rep– mating, in most cases only NheI+ plasmids were visible on the gel, although four colonies appeared to contain a small proportion of FspI+ plasmids as well. We did not find any colonies that contained only FspI+ plasmids. To confirm that FspI+ plasmids were in fact present in some colonies, we digested the pooled plasmid DNA with *Nhe*I, then dephosphorylated the ends and transformed the Rep+ recipient strain. Plasmid DNA was then digested with *Eco*O109 and *Nhe*I or *Fsp*I, and the results displayed as before. After this enrichment, FspI+ plasmids were clearly visible (Fig. 3, bottom).

The FspI+ plasmids transferred from the Rep– donors are unlikely to be the result of residual mismatch repair before transfer, as no transconjugant colonies containing only this type were found. The low proportion of these plasmids could mean that replacement strand synthesis in the donor is not robust, taking place on only a few transferring molecules. Alternatively, there might be a barrier to successful mobilization of the newly synthesized strand into the recipient. In particular, we thought that there might be a delay before this strand is available for transfer. This would allow the first strand to be established in the recipient, to reach a normal copy number, and therefore to exclude by incompatibility the establishment of late-entering molecules. In the Rep+ case, the time between successive rounds of transfer might be short, and incompatibility would not be able to prevent establishment of a second, incoming molecule. This is because the copy number of the first entering molecule would not have had a chance to increase sufficiently to exert incompatibility.




| NheI | FspI | NheI + FspI |
|---|---|---|
| 8 | 9 | 13 |

| NheI | FspI | NheI + FspI |
|---|---|---|
| 31 | 0 | 4 |

**Fig. 3.** A. Plasmid DNA content of individual transconjugant colonies after transfer of the test plasmid from Rep+ donor cells. The DNA content of five, randomly selected colonies, part of the set used to compile the table on the left, is displayed. The DNA was digested as described in Fig. 2 before gel electrophoresis.
B. DNA from the 35 colonies represented in the Rep– table were pooled, digested with *NheI* and retransformed into the recipient strain in order to show more clearly the presence of FspI+ plasmids. The plasmid content before (pre) and after (post) this enrichment step is shown as described in Fig. 2.

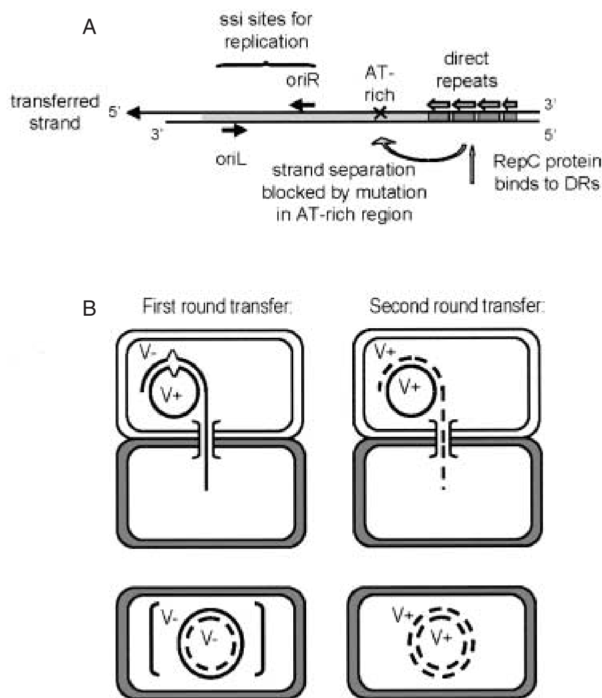



**Fig. 4.** A. Structure of R1162 *oriV*, showing location of *oriL* and *oriR*, the initiation sites for single-strand DNA synthesis in the direction indicated by the arrows, and the location of the *oriV*– mutation. The two horizontal lines represent the two DNA strands, with the arrow at the end extending from the strand transferred and indicating the direction. This strand also contains the mutation in the heteroduplex, indicated by the 'X'.
B. Results expected after first and second round transfer for a test plasmid containing the *oriV*+/oriV– heteroduplex. The non-replicating molecule is in brackets.

To distinguish between poor replication in the donor and failure to establish in the recipient, we took advantage of a point mutation in *oriV* that prevents vegetative plasmid replication (Kim and Meyer, 1991). This mutation is in the AT-rich region of the origin, and prevents localized strand separation at this site, and presumably therefore entry of the plasmid-encoded helicase (Fig. 4). We assumed that the mutation would not affect conjugative replication, where strand separation is brought about by transfer and does not require the plasmid helicase (Henderson and Meyer, 1999). A plasmid heteroduplex was constructed as before, with the mutation on the strand first transferred, and the normal base on the complementary strand (Fig. 4). As a result, the initially transferred strand is incapable of vegetative replication, and is lost from the transconjugant cell. In contrast, transfer of the replacement strand from the donor should result in a viable transconjugant.

We transformed Rep+ and Rep– donor cells with equal amounts of NheI+/FspI+ heteroduplex DNA, and separately with equal amounts of the *oriV*+/*oriV*– heteroduplex DNA. The Rep+ cells were then plated directly onto medium containing chloramphenicol to estimate the relative number of potential donors that would be created after electroporation with each of the heteroduplexes. The transformed Rep Rep– cells were mated and transconjugants selected by the usual method. We estimated the transfer frequency of the first strand as the number of transconjugants arising from transfer of the NheI+/FspI+ heteroduplex DNA, divided by the number of potential donors. The transfer frequency of the newly synthesized strand was estimated similarly, from the number of transconjugants in the *oriV*+/*oriV*– mating divided by the number of Rep+ transformants. We found that the transfer frequency for the first strand was $2.6 \times 10^{-4}$ and $1.2 \times 10^{-4}$ for the second strand. Thus, strand replacement in the donor occurs a high proportion of the time, and the resulting molecules are capable of being mobilized.

Our results led us to conclude that there is an eclipse period between successive rounds of transfer from the same molecule. During this period, other plasmid molecules in the donor can be transferred and established. This implies either that there is rapid entry of plasmid molecules at a single conjugative pore, or that there are several pores, each loaded with a plasmid molecule, and with nearly synchronous transfer of these into the recipient. In any case, our observations are not consistent with a model in which a single molecule at the pore transfers multiple rounds, with rapid regeneration of the transferred strand.

### *Donor replacement strand synthesis does not require initiation from the plasmid priming site*

Conjugative transfer in the absence of vegetative plasmid replication requires the plasmid-encoded primase, a form of which is covalently joined to MobA, the strand cleaving-rejoining protein in the relaxosome (Henderson and Meyer, 1999). The primase is highly specific, recognizing only *oriL* and *oriR* (Fig. 4; Zhou and Meyer, 1990). No efficient initiation sites for other primases have been identified on R1162 DNA. Thus, the requirement for the R1162 primase in conjugative transfer seemed reasonable, as the complement to the transferred strand must be synthesized before establishment of the plasmid DNA in the recipient cell. We asked whether the plasmid priming system was also required for the complementary strand synthesis that we had detected in donor cells.

The electroporation/transfer experiment was modified as outlined in Fig. 5. To make the plasmid independent of vegetative replication in both donor and recipient, we introduced the λ*attP* site and mated with cells that provided a supply of the λ integrase (Atlung *et al.*, 1991; Henderson and Meyer, 1999). We also used a test plasmid deleted for *oriL*, the site correctly oriented for replace-

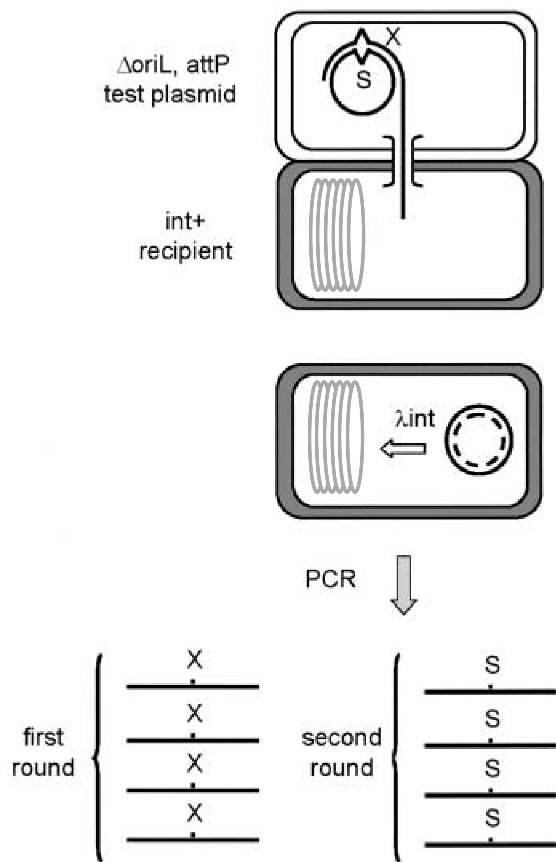



**Fig. 5.** Conjugal transfer of Δ*oriL*, *attP*+ test plasmid from Rep– donors into recipient cells containing the λ integrase. The plasmid contains an *XmnI* (X)/*SapI* (S) heteroduplex.

ment strand synthesis in the donor. This plasmid was maintained by fusing it to pBR322. Before electroporation and transfer, the pBR332 was excised by digestion with a restriction enzyme, and replaced by oligonucleotides having a mismatch designed to create either a *Xmn*I or *Sap*I site after replication. During mating the complement to the transferred strand can be synthesized because the donor cells encode the R1162 primase, and the properly oriented initiation site, *oriR*, is still present (Fig. 4). Transfer of the original strand introduced by electroporation followed by transfer will result in a plasmid integrated into the chromosome and containing an *XmnI* site. Strands replicated in the donor and then transferred will have a *Sap*I site.

Twenty-five transconjugants from the mating were pooled and a part of the plasmid DNA containing the cloned oligonucleotides was amplified and tested for cleavage by *Xmn*I and *Sap*I (Fig. 6). For comparison, we carried out a similar procedure with an *oriL*+ plasmid. The results (Fig. 6) indicated that DNA containing the *Sap*I site was transferred, regardless of whether *oriL* was present. However, the proportion of product cleavable by *Sap*I was significantly greater for the *oriL*+ mating, suggesting that donor replacement strand synthesis can be initiated at *oriL*, but that other mechanisms to initiate synthesis are also available. We carried out a similar experiment in which one of the *priA* sites of pBR322 (Abarzua *et al*., 1984), oriented properly for synthesis of the donor strand, was present on the test plasmid. In this case, the DNA represented by *Sap*I cleavage predominated for both *oriL*+ and oriL– transfer (data not shown).

We performed several controls to make certain that the amplified DNA was derived from the chromosome. First, we examined the plasmid DNA content of the transconjugant cells. These contained the mobilizing vector R751 and the plasmid encoding the λ integrase, as expected, but no other plasmids were found. Moreover, mating and transformation experiments with these cells as donors indicated that the chloramphenicol resistance used for selection of transconjugants was not located on either of these plasmids. Second, transfer of the *oriL*+ and *oriL*– plasmids into cells lacking the integrase resulted in no or very few detectable transconjugants, a reduction of more than 100-fold. Lastly, PCR amplification with one primer hybridizing to the chromosome resulted in PCR product of the expected size. We conclude therefore that complementary strand synthesis in the donor can be initiated by a mechanism distinct from normal priming for vegetative replication.

How is complementary strand synthesis initiated in the

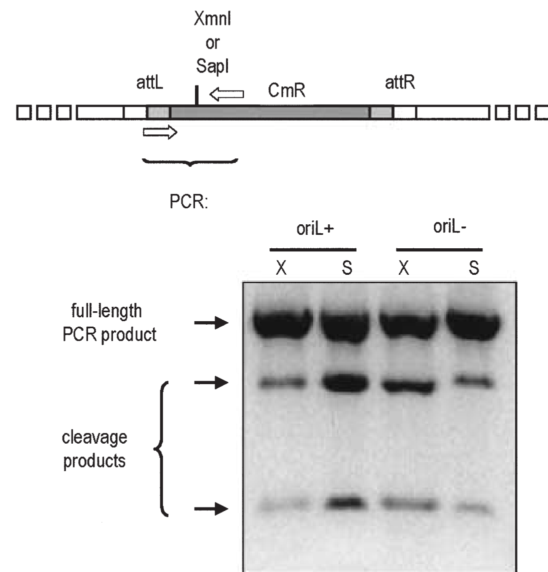



**Fig. 6.** Amplification of part of the test plasmid DNA from pooled transconjugants and digestion with *Xmn*I (X) and *Sap*I (S). The locations of the primers for the amplification are shown as open arrows at the top of the figure. DNA fragments were separated by 0.8% agarose gel electrophoresis.

donor in the absence of *oriL*? The possibilities are priming by a host-encoded protein, by the primase encoded by R751, or by extension from the 3′ end of the parental, transferred strand (i.e. rolling circle replication). Plasmids deleted for *oriL* or *oriR* are replicated poorly and are unstable in the cell but can be maintained, indicating an alternative, weak priming mechanism (Zhou and Meyer, 1990; Becker *et al*., 1996). Such a mechanism might also be operating for donor replacement synthesis, although it would have to be sufficiently active to result in the high proportion of amplified DNA containing a *Sap*I site following *oriL*– plasmid transfer.

In IncP plasmids such as RP4 and R751, a gene encoding a primase with low substrate specificity is located in one of the *tra* gene clusters (Miele *et al*., 1991). The protein is not required for replication of the plasmid, and it seems to be dispensable for transfer in *E. coli* (Lanka and Barth, 1981). Perhaps in the absence of the plasmid primase, a host-encoded mechanism for priming can substitute. The IncQ plasmid R300B, essentially identical to R1162 (Barth and Grinter, 1974), does not require the IncP primase for transfer in *E. coli* (Lanka and Barth, 1981). However, the IncP primase could substitute when the normal priming mechanism has been inactivated. This possibility is currently being tested.

Extension at the 3′ end is an attractive mechanism for strand replacement, particularly for R1162. Intrinsic to this mechanism is a requirement for cleavage of the *oriT* generated by strand extension, in order to create a unit-length molecule. Experiments with plasmids having two, directly repeated copies of *oriT* show that R1162 can do this efficiently (Bhattacharjee *et al*., 1992). However, if rolling-circle replication does occur, then termination of the first round of transfer is followed by a period during which no additional transfer is possible. This delay might reflect the sequence of events taking place at the conjugative pore. If the 5′ end of the transferred strand is piloted into the recipient by the relaxase, then the second cleavage to form a unit-length molecule would also occur in the recipient. The newly formed 5′ end of the strand might then need to be withdrawn into the donor, in order to reattach to the transport mechanism prior to the start of a new round of transfer. Rolling circle replication itself might also be self-limiting so that molecules which have undergone strand replacement are temporarily unavailable for transfer. The regulatory mechanisms might be similar to those controlling rolling-circle replication of plasmids such as pT181 or pC194. These plasmids unwind a parental strand, which becomes a new plasmid copy, and replace this strand on the parent by rolling-circle replication. However, additional rounds of replication by this mechanism are inhibited, so that the molecule is temporarily taken out of the replicating pool (Rasooly and Novick, 1993; Noirot-Gros *et al*., 1994). In a similar way, molecules of R1162 undergoing conjugative replacement synthesis in the donor might be released from association with MobA. These molecules would then have to wait for reassembly of the relaxosome before again gaining access to the conjugative pore.

## Experimental procedures

### *Strains and plasmids*

*Escherichia coli* strain MC1061 (Casadaban and Cohen, 1980), chosen because it is highly transformable by electroporation, was used to construct donors for conjugative transfer. The donors contained the IncP1β plasmid R751 (Willetts and Crowther, 1981) as the mobilizing vector and either pUT1543, which encodes all the R1162 proteins for mobilization and replication of the test plasmid, or pUT1559, which also encodes the mobilization proteins but in addition only the plasmid replicative primase. The structure of these plasmids has been described elsewhere (Henderson and Meyer, 1999). Strains containing pUT1543 and pUT1559 are referred to as Rep+ and Rep– donors respectively. The recipient in these matings was a derivative of MV12 (Hershfield *et al*., 1974) resistant to nalidixic acid. For matings involving the *NheI/FspI* and the *oriV*+ and *oriV*– mismatched oligonucleotides, recipient cells also contained a helper plasmid (pUT459, Brasch and Meyer, 1986) that encodes the R1162 replication proteins. For matings involving the *oriL*– test plasmid the recipient strain contained instead a plasmid encoding the λ integrase (Atlung *et al*., 1991).

The test plasmid pUT1557, used for cloning the *Nhe*I/*Fsp*I mismatched oligonucleotides, has been described previously (Henderson and Meyer, 1999). It is a derivative of R1162 containing a cloned gene for chloramphenicol resistance and having a deletion that removes the genes for replication. For the *oriV*+/*oriV*– mismatch experiment, we constructed a derivative of pUT1557 with *oriV* flanked by the restriction sites *Apa*I and *Ngo*MIV. This was done by reverse PCR (Hemsley *et al*., 1989) with the primers: *Ngo*MIV, 5′-GATA ATCATGGATGGATTTTTTCAACCCCGCCGGCCCC; and *Apa*I, 5′-TAAGAATAATCCACTAGGCGCGGTTATCAGGGC CCTTGTGG. The *oriL*– test plasmid was constructed from a derivative of pUT1557 containing *attP* pUT1613 (Henderson and Meyer, 1999). The *oriV* region was modified to contain a unique *Sma*I site between *oriL* and *oriR*, by using DNA derived from pUT1101 (Becker *et al*., 1996). The *oriL* site was then excised by digestion with *Eco*O109 and *Sma*I, and the DNA resealed after blunting the ends by treatment with mung bean nuclease. The resulting plasmid was maintained by cloning at the *Bam*HI site of pBR322 (Bolivar *et al*., 1977). The *oriL*+ plasmid lacking the deletion was also maintained as a clone in pBR322.

### *Electroporation and mating*

Plasmid molecules containing the *Nhe*I/*Fsp*I mismatch were constructed by first digesting 100 ng of pUT1557 DNA with *Mfe*I and *Ban*II. The DNA was ligated to a fivefold molar excess of the annealed oligonucleotides: *Nhe*I, 5′-AGATCT GTGCGCTAGCTCGGCCGG; and *Fsp*I, 3′-TCGATCTAGA

CACGCGTTCGAGCCGGCCTTAA. Ligation was overnight at 15°C. The DNA was digested with *Eco*NI to remove the second plasmid required to maintain the test plasmid by providing the replication proteins. The DNA was then incubated with 8 U of *dam* methylase and *S*-adenosylmethionine for 1 h at 37°C. Approximately $10^9$ donor cells in 40 μl of $H_20$–10% glycerol were mixed with the DNA for electroporation at 1.8 kV, then immediately diluted in 1 ml of SOC medium (2% tryptone, 0.5% yeast extract, 20 mM glucose, 10 mM NaCl, 10 mM $MgCl_2$,10 mM $MgSO_4$, 2.5 mM KCl). Fifty microlitres of these donors cells were mixed with a fivefold excess of recipient cells, and the cells then pelleted by centrifugation, resuspended in 100 μl of SOC medium, and deposited on a broth plate. Mating was for 90 min at 37°C unless otherwise indicated. An agar plug containing the mating cells was then resuspended in 1 ml of broth. Colonies of transconjugant cells were obtained by plating on semisolid broth medium containing chloramphenicol (25 μg $ml^{-1}$) and nalidixic acid (25 μg $ml^{-1}$) and incubating overnight at 37°C. Donor cells were enumerated by plating on medium containing chloramphenicol. Electroporation and mating involving the *oriV+/oriV–* test plasmid was done in a similar way, except that the plasmid was digested with *Ngo*MIV and *Apa*I to clone the annealed oligonucleotides: 5′-TTGATAACCGCGCCTAG TGGATTATTCTTAGATAACCATGGATGGATTTTTCCAACA CCCCG and 3′-CCGGAACTATTGGCGCGGATCACCTAAT AAGAATCTATTAGTACCTACCTAAAAAGGTTGTGGGGCG GCC. Plasmid DNA of the *oriL–* and *oriL+* test plasmids was digested with *Sal*I and *Nhe*I to remove the pBR322 replicon. The remaining pBR322 DNA (422 bp) does not contain any known sites for priming of replication (Abarzua *et al*., 1984). The DNA was purified by gel electrophoresis before ligation to the annealed oligonucleotides: *Xmn*I, 5′-TCGAAGCGGAA GAACGCGTTCGCGA; and *Sap*I, 3′-TCGCCTTCTCGCGC AAGCGCTGATC.

Digestion with *dam* methylase, electroporation and mating were as described above.

## Acknowledgements

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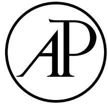
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# Conjugative DNA synthesis: R1162 and the question of rolling-circle replication

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**Abstract**

Strand-replacement synthesis during conjugative mating has been characterized by introducing into donor cells R1162 plasmid DNA containing a base-pair mismatch. Conjugative synthesis in donors occurs in the absence of vegetative plasmid replication, but with a lag between rounds of transfer, and with most strands being initiated at the normal site within the replicative origin. These characteristics argue against the idea that multiple plasmid copies are generated for successive rounds of transfer by rolling-circle replication. However, the R1162 relaxase protein can process molecules containing multiple transfer origins in the manner expected for the conversion of single-strand multimers, generated by rolling-circle replication, to unit-length molecules. This capability appears to be the result of a secondary cleavage reaction carried out by the protein. The possibility is raised that the processing of molecules with more than one origin of transfer might be a repair mechanism directed against adventitious DNA synthesis during transfer.

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It has been known for over a quarter of a century that when the F plasmid is transferred by conjugation from one cell to another, only a single DNA strand actually passes through the conjugative pore (Cohen et al., 1968; Ohki and Tomizawa, 1968; Rupp and Ihler, 1968). Therefore, the missing strand must be replaced before the incoming plasmid can be established in the recipient cell. In addition, these early studies revealed that the transferred strand is replaced by replication in the donor. However, while conjugative replication in donor and recipient for F and several other large, self-transmissible plasmids has been known for some time, the mechanisms used are not understood. Moreover, these mechanisms can be different: in the donor, a 3′ end is generated during transfer that could in principle prime replacement strand synthesis by rolling-circle replication. This idea was first suggested by Gilbert and Dressler (1968), and is so attractive that it is sometimes assumed to be true, although it has never been proven.

We have been characterizing conjugation-dependent DNA synthesis and its relationship to vegetative replication of the plasmid DNA. To do this, two conditions must be met. First, the mode of vegetative replication must be understood, with the plasmid components clearly identified. Second, there must be a way to observe conjugative synthesis when vegetative replication is strictly shut off, so that the two can be disentangled.

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These conditions are satisfied by R1162/RSF1010, two nearly indistiguishable IncQ plasmids (Frey and Bagdasarian, 1989). Work in the laboratories of Scherzinger and others, as well as in our own laboratory, has resulted in the picture of replication shown in Fig. 1 (Scherzinger et al., 1984, 1991). The origin of replication (*oriV*) is activated when the plasmid-encoded RepC protein binds to the iterons (DRs). This binding results in localized disruption of the helix within an adjacent AT-rich region (Kim and Meyer, 1991), presumably allowing entry of the plasmid-encoded helicase. DNA unwinding by the helicase makes available two single strand initiation (*ssi*) sites, *oriL* and *oriR* (Lin and Meyer, 1987), where DNA synthesis is initiated by the plasmid-encoded primase. The primase is highly specific for *oriL* and *oriR*, and there are no other known *ssi* sites efficiently utilized by the host (Honda et al., 1991; Zhou and Meyer, 1990). As a result, replication proceeds in two directions, with the intermediates containing two D-loops (Scherzinger et al., 1991).

To examine conjugative synthesis in the absence of vegetative replication, we used the protocol shown in Fig. 2. An indicator plasmid encoding chloramphenicol-resistance (CmR), and containing both the R1162 origin of conjugative transfer (*oriT*) and origin of vegetative replication (*oriV*), was introduced into donor cells by electroporation. These donor cells contain R751, a self-transmissible, IncP-1β plasmid that efficiently mobilizes R1162 (Willetts and Crowther, 1981) and a helper plasmid containing the R1162 *mob* genes. In addition, this helper plasmid contains different combinations of the plasmid *rep* genes, so that the contribution of these during conjugative transfer can be assessed. After electroporation, the cells are immediately mated with a nalidixic acid-resistant (NalR) recipient, containing a plasmid encoding the R1162 replication proteins to support replication of the reporter plasmid, and transconjugants are then selected for CmR and NalR.

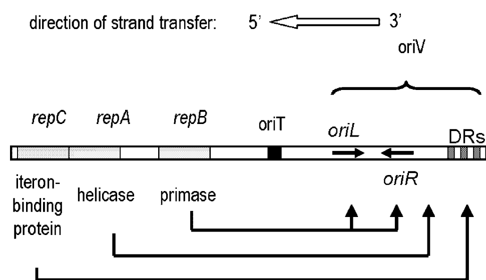


Fig. 1. Origin of replication of R1162/RSF1010 and its activation by three plasmid-encoded proteins. The direction of conjugative DNA transfer, initiated from the nearby *oriT*, is also shown.

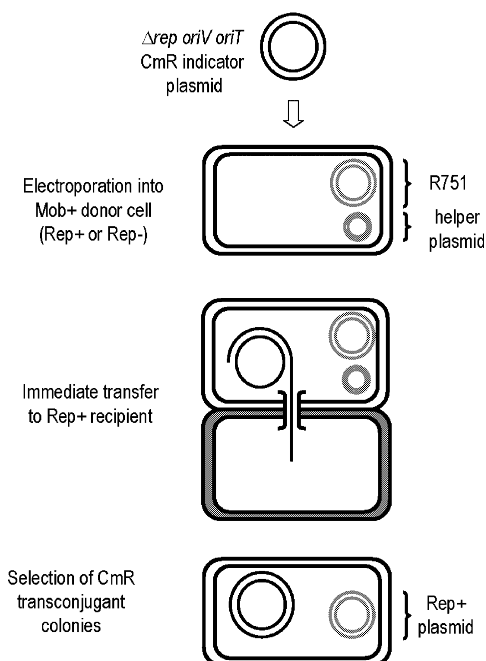


Fig. 2. Scheme for electroporation and immediate conjugative transfer of reporter plasmid DNA.

Using this electroporation and transfer system, we found that only the R1162 primase was required in the donor for transfer of the indicator plasmid (Henderson and Meyer, 1999). A requirement for the primase was not entirely surprising, since it offers the only known mechanism for initiation of replication on R1162 DNA, and complementary strand synthesis is required for plasmid establishment in the recipient. Interestingly, however, the requirement for the R1162 primase could not be met by providing this protein in recipient cells. The R1162 primase is made both as a separate protein, and also as a fusion to the R1162 relaxase, MobA (Haring and Scherzinger, 1989). The relaxase molecule that initiates transfer, and also rejoins the two ends of the transferred strand, is probably favored to prime complementary strand synthesis in the recipient (Henderson and Meyer, 1996). However, it is hard to understand why primase in the recipient cell is so ineffective in priming an incoming strand. One possibility is that primase-dependent synthesis in the donor is required for transfer. For this reason,

we decided to characterize conjugative DNA synthesis in donor cells.

For a multi-copy plasmid such as R1162, it is not obvious that there needs to be any conjugative DNA synthesis at all in the donor. The transfer of a molecule would have little impact on plasmid copy-number and could easily be accommodated by the normal mechanism for copy control. We used the electroporation and transfer system to test whether there is conjugative DNA synthesis in the donor, resulting in molecules that would then be available for a second round of transfer (Parker and Meyer, 2002). Prior to electroporation, indicator plasmid molecules were constructed that contained a base-pair mismatch, resulting in a recognition sequence for the restriction enzyme *Nhe*I in the strand initially transferred, and an *Fsp*I sequence at the same position in the complementary strand (Fig. 3). In addition, the molecules were methylated to inhibit mismatch repair in the donor cells (Pukkila et al., 1983). After mating with an excess of recipient cells, plasmid DNA was isolated from colonies of transconjugants and tested for cleavage with *Nhe*I and *Fsp*I. When plasmids were transferred from Rep+ donors (cells containing all the replication proteins of R1162), *Nhe*I and *Fsp*I plasmids were obtained with equal frequency overall, but the proportion of each varied for different colonies of transconjugants. Since about half the transconjugant colonies contained the two plasmid types, a donor usually transfers more than one molecule of plasmid DNA into a recipient cell.

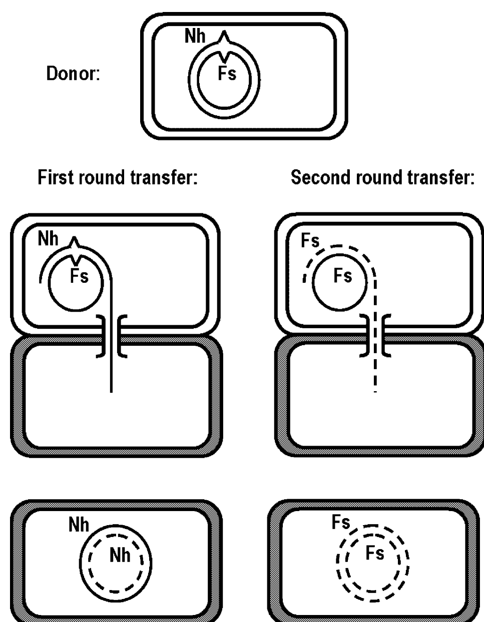


Fig. 3. Electroporation and transfer with a reporter plasmid containing a base-pair mismatch.

For transfer from a Rep+ donor, the additional molecules for transfer can be generated by vegetative replication of the indicator plasmid. In the absence of vegetative replication, would there still be multiple rounds of transfer? We repeated the experiment with a Rep− donor (containing the plasmid primase but neither of the other replication proteins). In this case, a large majority of the transferred molecules contained *Nhe*I sites, with a minority, detectable in about 10% of the transconjugant colonies, having *Fsp*I sites. This could mean that replacement strand synthesis is not routine in donor cells. The small number of *Fsp*I plasmids might then reflect residual mismatch repair or occasional, atypical replication. Alternatively, a new molecule might be synthesized, but could then be excluded from successful transfer and establishment in the recipient. This could happen if there was a time lag before the second round of transfer: the first entering molecule would have time to establish, its copy-number would increase, and subsequent entry of the second molecule would be prevented by incompatibility. We tested this possibility by repeating the experiment with an indicator plasmid having a different base-pair mismatch. This new mismatch introduced a mutation in the strand that is first transferred. The mutation prevents vegetative replication (Kim and Meyer, 1991), so that all plasmids arising after a first round of transfer are unable to establish in the recipient cell, and therefore the recipients remain empty of reporter plasmid. We then compared the frequency of second-round transfer under these conditions with the first-round transfer frequency from a Rep− donor (the frequency of transconjugant colonies containing *Nhe*I+ plasmids in the prior experiment). The frequency in each case was similar, so we concluded that there is a robust mechanism for replacement stand synthesis in donor cells, with the resulting molecules available for conjugative transfer.

What is the mechanism of replacement strand synthesis in donor cells? At present we can only provide a partial answer to this question. Since the R1162 primase is required for transfer, one possibility is that replacement synthesis is initiated from *oriL*, which is correctly oriented for this purpose (Fig. 1). To determine whether *oriL* is

involved, we made several modifications to the basic procedure shown in Fig. 2. First, the indicator plasmid was deleted for *oriL*, so that it was no longer available for priming. However, deleting *oriL* means that the plasmid will no longer replicate properly after transfer into the recipient cell. We inserted a λ *attP* site into the reporter plasmid, and replaced the Rep+ helper plasmid in the recipient with a plasmid encoding the λ integrase. As a result of these changes, after transfer many of the indicator plasmids become stably integrated into the chromosome of the recipient. Using this modified system, we carried out the mismatch experiment basically as before (Fig. 2), although with a different oligonucleotide, so that a *Xho*I restriction site indicated a first round of transfer, and a *Sap*I site subsequent rounds. The relative amounts of first- and second-round transfer were determined by amplifying by PCR integrated plasmid DNA containing the restriction sites and then separately digesting the resulting DNA with the two enzymes.

Both *oriL*+ and *oriL*− plasmids were tested for transfer from Rep− cells. In either case, however, there was no vegetative replication of the plasmid in either donor or recipient. When an *oriL*+ plasmid was transferred, the PCR-amplified DNA was cut by both enzymes, but the majority was sensitive to *Sap*I. This confirms that there are multiple (and on average probably more than two) rounds of transfer from each donor. When the *oriL*− plasmid was used, the majority of the DNA was sensitive to *Xho*I, but there was still DNA sensitive to *Sap*I. We interpret these results to mean that initiation from *oriL* contributes substantially to strand replacement synthesis in the donor, but that other mechanisms can also be used.

An appeal of rolling-circle replication is that it provides an easy way of visualizing multiple rounds of transfer, with successive units of plasmid DNA being generated from a single, master molecule. Our results are incompatible with this idea. First, a substantial fraction of the strands transferred from Rep− donors appear to be synthesized by a mechanism that involves initiation at *oriL*. Second, there is a lag between successive rounds of transfer from these donors. In contrast, multiple strands were transferred without a significant lag when there was vegetative replication of the plasmid in the donor cells. In our experiments, a recipient cell does not acquire several molecules because it has mated with several donor cells at once, because the ratio of recipient to donor is kept high. Either there is rapid throughput at a single pore, or donor cells contain several active pores, each injecting DNA into the same recipient at about the same time. It is unlikely that the absence of a lag between rounds in Rep+ donors is simply due to the higher copy-number of the plasmid DNA. It is hard to see how an increase in plasmid copy-number from 1 to 30, in the large, cavernous space of the cell, could have a large effect on the timing of successive rounds.

Recent studies in many labs indicate that plasmids are not uniformly distributed in the cell, but form molecular aggregates to carry out important processes such as copy control and segregation. One possibility is that plasmids are aggregated at the conjugative pore, so that after transfer of one molecule another is readily available. We asked whether plasmid molecules associate by a "handcuffing" reaction between the relaxosome proteins at *oriT*. To do this, we used a two-dimensional chloroquine gel analysis of dimer molecules, a method developed by Wu and Liu (1991) to show the tetramerization of the *lac* repressor, and subsequently by Edgar et al. (2001), to show pairing by the partitioning protein ParB, and by Park et al. (2001) to demonstrate negative regulation of P1 replication by handcuffing. This method employs a plasmid with the potential handcuffing site adjacent to a strongly transcribed gene. The transcription generates positive and negative supercoiled domains on the plasmid (Wu et al., 1988); in a dimer, handcuffing between the two sites on the plasmid prevent the topological resolution of these two domains. As a result, if the cells are treated with the gyrase inhibitor novobiocin, positive supercoils remain which can be detected by gel electrophoresis. We found that there was a large increase in the amount of positively supercoiled DNA in novobiocin-treated cells when the test plasmid contained oriT, and the Mob proteins were present in the cell (in preparation). Thus, plasmid molecules are "handcuffed" at oriT by a mechanism that involves the mobilization proteins. It is possible that this handcuffing is responsible for the delivery of more than one molecule to the conjugative pore.

Although our results indicate that rolling-circle replication is not the only mechanism for replacement strand synthesis in the donor, it has not been excluded as one of several mechanisms. In addition, characteristics of R1162 conjugative transfer seeming to support the existence of rolling-circle replication need to be taken into

account. When a plasmid is constructed to contain two, directly repeated R1162 *oriT*s, transfer can be initiated at one and terminated at the other (Bhattacharjee et al., 1992; Kim and Meyer, 1989). During transfer, such molecules are similar to intermediates of rolling-circle replication, with the trailing *oriT* representing the element that would be synthesized by extension from the 3′ end of the cleaved stand. If R1162 does not use rolling-circle replication in strand replacement, why does it have a mechanism to cleave within the trailing *oriT*, and to rejoin the resulting 3′ end to the 5′ end of the leading oriT? One simple possibility is that plasmids with two *oriT*s assemble two relaxosomes, each capable of cleaving the *oriT* DNA, but with initiation of transfer at only one of these. A recombinant *oriT* could then be formed if both *oriT*s happen to be nicked at the start of a round of transfer. In this scenario, the genetic requirements for cleavage at each *oriT* would be the same, but this is clearly not the case. An *oriT* mutation which inhibits nicking in the relaxosome has no effect on the termination of transfer at a second *oriT* (Kim and Meyer, 1989). In addition, *oriT*s cloned into single-stranded M13 phage DNA can undergo site-specific recombination at the normal cleavage site (Meyer, 1989). This reaction requires the relaxase, but not the other components of the relaxosome. Thus, R1162 encodes a mechanism to cleave single-stranded *oriT* DNA at the proper site for rejoining to another, cleaved *oriT*, the reaction needed to resolve rolling-circle intermediates.

At the beginning of transfer, the R1162 relaxase, MobA, "nicks" one of the plasmid strands by a transesterification that results in a tyrosyl phosphodiester linkage at the 5′ end of the DNA (Scherzinger et al., 1993). Presumably, after transfer the two ends of the DNA strand are rejoined by the reverse reaction. Since the primary nucleophile of the initial transesterification, the tyrosine at the 24th position from the N-terminal end of the protein (Scherzinger et al., 1993), remains joined to the DNA during transfer, a second cleavage at a trailing *oriT* must be carried out either by a second molecule of relaxase, or by a second nucleophile. Genetic evidence favors the existence of another nucleophile. A nonsense mutation in the N-terminal coding region of *mobA* decreases the frequency of transfer from a nonsuppressing donor by about 1000-fold. The residual transfer is probably due to rare suppression of the mutation by misreading (Schimmel, 1989). Interestingly, for the rarely transferred molecules the termination frequency at a second *oriT* is unaffected, remaining at 50%. Under these circumstances, it is unlikely that a second molecule is available for this reaction. The same result is found with a mutation converting the active tyrosine to a phenylalanine: again, the transfer frequency is decreased by several orders of magnitude, but the termination frequency at the second oriT is unchanged. We have purified protein with the phenylalanine substitution; although it binds *oriT* DNA normally, no additional cleavage activity has been detected. In contrast, secondary nucleophiles have been identified for the relaxases of the plasmids R388 and F (Grandoso et al., 2000; Matson et al., 2002). For the R388 protein, two tyrosines are active on a single-stranded oligonucleotide, but only one is capable of cleaving DNA in a relaxosome. One possibility is that one tyrosine is used to initiate, and the other to terminate, a round of transfer. However, when the putative terminating tyrosine was changed to a phenylalanine, there was only a 10-fold decrease in transfer frequency (Grandoso et al., 2000). Thus, despite both genetic and biochemical evidence for additional nucleophiles in relaxase proteins, their role in processing plasmid DNA for transfer remains to be elucidated.

The nature of the second cleavage reaction has implications for the model of rolling-circle replication. The nucleophiles of R388 and F are all tyrosine residues, so that cleavage of a trailing *oriT* generated by rolling-circle replication should result in protein covalently joined to the 5′ end of the DNA. This might create a difficulty in the case of R1162, if this cleavage is part of a mechanism for replacement strand synthesis in the donor. Completion of strand synthesis would result in a duplex, nicked molecule with relaxase linked at the 5′ end of the nicked strand. However, we would not expect the R1162 relaxase to seal efficiently a nick in nonsupercoiled, duplex DNA. This is because the relaxase cleaves and rejoins single-stranded DNA, and is active on double-stranded molecules only when the duplex structure has been disrupted (Zhang and Meyer, 1995).

An attractive possibility is that water, activated by an acidic amino acid in the relaxase, is the second nucleophile. In this case, the transfer intermediate would not be regenerated, and the resulting nicked, circular molecule in the donor could be sealed by the cellular ligase. Such a mechanism has been proposed for rolling-circle replication of the plasmid pC194 (Noirot-Gros et al., 1994). Cleavage by water could also account

for the lag between first and second rounds of transfer from the same molecule. After strand replacement, the molecule would be ligated and then supercoiled. The relaxosome proteins would then have to reassemble at *oriT*. The molecule would probably not remain associated with the conjugative pore during this time.

In summary, for R1162 conjugative DNA synthesis in the donor can be initiated at *oriL* and we presume at *oriR* in the recipient. Nevertheless, the transferase has properties expected of a molecule involved in rolling-circle replication, and initiation at *oriL* is not the only mechanism for strand replacement in the donor. One possible but speculative solution to this apparent paradox is that occasional extension from the 3′end is adventitious and undesirable, since it removes the correct substrate for strand-rejoining. The R1162 relaxase therefore has a mechanism for repairing such molecules, by cleaving the DNA to regenerate the correct 3′ end, thus improving the probability of a successful transfer. Whether this is correct, or whether rolling-circle replication plays a more direct role in strand replacement, remains to be discovered.

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