Role of the Origin of Transfer in Termination of Strand Transfer during Bacterial Conjugation

MRINAL BHATTACHARJEE, XIAO-MEI RAO, AND RICHARD J. MEYER*
Department of Microbiology, University of Texas, Austin, Texas 78712

Received 8 May 1992/Accepted 7 August 1992

Conjugal transfer of the broad-host-range plasmid R162 is initiated and terminated at the nic site within the 38-bp origin of transfer (oriT). Termination involves ligation of the transferred single strand by the plasmid-encoded MobA protein. Several different assays were used to identify the oriT DNA required for termination. For plasmids containing two oriTs, with transfer initiated at one and terminated at the other, the inverted repeat within oriT is important for termination. Deletion of the outer arm reduces the termination frequency; those terminations that do occur probably depend upon nicking at this oriT prior to transfer. The locations of second-site suppressor mutations indicate that base pairing between the arms of the inverted repeat is important for termination. In vitro, the inverted repeat is not required for specific cleavage of single-stranded DNA at nic, but competition experiments indicate that oriTs with the inverted repeat are preferentially cleaved. We propose that the function of the oriT inverted repeat is to trap the plasmid-encoded MobA protein at the end of a round of strand transfer, thus ensuring that the protein is available for the ligation step.

The basic steps in DNA processing during conjugal transfer are probably similar for many different plasmids in gram-negative bacteria. One of the DNA strands is first nicked within the origin of transfer (oriT), which is required in cis. The nicked strand is then linearly transferred into a recipient cell in the 5'-3' direction and subsequently recircularized. In both donor and recipient cells, the complementary strand of DNA is resynthesized to restore the duplex plasmid molecule. Many different plasmids can be isolated in the form of a relaxosome, a complex of proteins and supercoiled plasmid DNA, which can be induced to undergo site- and strand-specific nicking within oriT (for example, see references 5, 6, 12, and 16). This nicking models the initial DNA-processing step in plasmid transfer.

R162 (nearly identical to RSF1010) is a broad-host-range, IncQ plasmid that is efficiently mobilized during the conjugal transfer of IncP1 group plasmids (15, 20). The R162 oriT consists of no more than 38 bp of DNA (Fig. 1) (4). The cleavage site within oriT has been mapped previously (1, 4) and is designated nic in Fig. 1. RSF1010 DNA has been isolated as a relaxosome (16); more recently, this complex has been reconstructed from plasmid DNA and purified proteins (18). The recirculation of plasmid DNA after transfer is modeled by the recombination of directly repeated oriT copies on replicating M13 phage DNA (1, 14). In this system of recombination, it is likely that the single-stranded oriT copies are treated as intermediates in transfer and are therefore cleaved and ligated at the nic site. MobA*, encoded by a segment of the R162 mobA gene, is required for this recombination and can carry out these reactions in vitro (2).

The effects of mutations indicate that oriT consists of more than one functional domain (10). Two simultaneous C-to-T transitions at bp 30 and 32 (Fig. 1, line 2) affect the initiation of transfer but not the termination step modeled by the recombination of oriT copies in M13 (10, 14). Deletion of bp 1 to 8, which make up the outer arm of the inverted repeat in oriT (Fig. 1, line 3), has the opposite effect (1, 10). This mutation does not affect initiation but does interfere with termination and phage recombination.

We have examined in greater detail than previously the structural requirements within oriT for the termination of conjugal transfer of R162 DNA. Our results indicate that the inverted repeat within oriT is essential for efficient termination in vivo. However, an intact inverted repeat is not required for specific cleavage in vitro of single-stranded DNA within oriT by the MobA* protein. We propose that during transfer, the inverted repeat acts to initiate termination by trapping the protein required for ligation of the transferring strand.

MATERIALS AND METHODS

Strains and plasmids. The Escherichia coli K-12 strains used in this study are MV12 (recA56 thr-1 leuB6 lacY1 thi-1 rfd1 supE44 fhuA1 ΔargE5) (7), the nalidixic acid-resistant derivative MV12 Na+, derived by spontaneous mutation, and JM103 [Δ(lac pro) strA thi supE44 lacY1 ΔargE5 lacZAM15] (13). Bacteria were grown in 1% Bacto-Tryptone-0.5% Bacto-yeast extract-0.5% NaCl, supplemented as required with carbenicillin (200 μg/ml), trimethoprim (200 μg/ml), streptomycin (37.5 μg/ml), or nalidixic acid (25 μg/ml).

The plasmids used in the bacterial matings have the general structure shown in Fig. 2. These plasmids are derivatives of pBR322 (3) and contain two copies of oriT, one on either side of a 230-bp lacO fragment. The construction of pUT1183, which contains unmutated copies of oriT, will be described in detail elsewhere (submitted for publication); the other members of this family were derived from pUT1183 by replacement of the normal oriT in position 1 with one of the mutated oriTs shown in Fig. 1.

Mutagenesis of oriT. The isolation of mutated oriTs containing C-to-T transitions at bp 30 and 32 or deleted for bp 1 to 8 (Fig. 1, lines 2 and 3) has been described previously (4). An oriT fragment lacking bp 1 to 19 (Fig. 1, line 4) was generated by digestion of oriT DNA with AgeI. An HpaI site (GTtAAC) was introduced into oriT DNA at the location shown in Fig. 1, line 5, by oligonucleotide-directed mutagen-
Assay for relaxosome-induced plasmid nicking. Cleared lysates were prepared from 100-ml cultures grown to approximately 5 x 10^8 cells per ml by the method of Katz et al. (9). The lysates were treated first by the addition of sodium dodecyl sulfate (SDS) (0.5%) and incubated at 65°C for 10 min and then by the addition of protease K (50 μg/ml) and incubated at 37°C for 45 min. The nucleic acids were precipitated and subjected to dye-buoyant density ultracentrifugation (19). The DNA bands were removed from the centrifugation tube, extracted with CsCl-saturated isopropanol, and dialyzed against 0.05 M Tris-HCl (pH 8.0)–0.005 M Na₂-EDTA–0.05 M NaCl. The plasmids in each band and their relative amounts were estimated by digestion of the DNA with EcoRI followed by agarose gel electrophoresis.

Cleavage of single-stranded oriT DNA in vitro. The assay for cleavage of single-stranded oriT by purified MobA*-β-galactosidase hybrid protein was as previously described (2), except that reactions were carried out for 2 h at pH 8.0. Each reaction mixture contained 0.1 μg of M13 viral strand DNA, end labeled at the Bsu361 site (2) and having the cloned test oriT, and an equal amount of end-labeled viral strand DNA with the unmutated oriT as an internal control. The control DNA (drm290) contains a 42-base EcoRI fragment, derived from M13mp7 (13), inserted at the EcoRI site between oriT and the Bsu361 site, so that the cleavage product could be distinguished by its mobility during electrophoresis. After being digested with MobA*, the reaction mixture was further incubated with 1 μg of protease K at 37°C for 30 min. The DNA was then denatured, subjected to electrophoresis on an 8% polyacrylamide gel containing 8 M urea, and visualized by autoradiography.

RESULTS

Frequency of termination of conjugal transfer within oriTs containing different deletions. We constructed pBR322 derivatives containing a copy of oriT on each side of the lac operator, lacO (Fig. 2). Conjugal transfer of the intact plasmid into the lacZ⁺ recA recipient MV12 Nal' resulted in transconjugant colonies that were light blue on medium containing X-Gal, because the high lacO gene dosage titrated medium containing nalidixic acid and carbenicillin supplemented with methicillin (2 mg/ml) (21). Donor cells were enumerated by plating the mating mixture on medium containing carbenicillin.

Bacterial conjugation. Donor MV12 strains contained the IncP1 plasmid R751 (8) as the mobilizing vector, R1162 (to provide the plasmid-encoded proteins necessary for mobilization), and the test plasmid containing copies of the R1162 oriT. Matings were carried out by separately growing donor and recipient cells (MV12 Nal') to mid-log phase (approximately 5 x 10^8 cells per ml). Donor and recipient cells were then mixed at a 1:10 ratio (final volume, 110 μl) and immediately pelleted in a microcentrifuge. The cells were resuspended in 40 μl of broth, placed on broth agar medium, and incubated at 37°C for 90 min. The mating mixture was then resuspended in 1 ml of broth and 100 μl of various dilutions, mixed with 50 μl of 2% X-Gal, and plated on
the chromosomally encoded lac repressor. However, the oriTs were oriented so that when transfer was initiated at oriT2 and terminated at oriT1, the lacO segment was deleted and the transconjugant colony was white. Thus, the proportion of white colonies in a mating reflects the efficiency of termination at oriT1. These plasmids are similar in design to others described earlier (10) that contain a kanamycin resistance gene instead of lacO. The advantage of the molecules used here is that colonies of cells containing the plasmid products of rare terminations can be easily identified and enumerated.

When the lacO plasmid contained two copies of unmuted oriT, the frequency of white colonies after conjugation was 56% (Table 1). We confirmed that deletion of lacO took place only during conjugation: when plasmid DNA was instead transferred from the donor strain to the recipient by transformation, only 5 of 10,456 colonies were white, a frequency of 0.05%. C-to-T transitions at bp 30 and 32 in oriT1 (the nic mutation; Fig. 1, line 2) did not significantly reduce the termination frequency (Table 1), which was consistent with the effect of this mutation on initiation only (10). The ter mutation, an 8-bp deletion of the outer arm of the inverted repeat within the R1162 oriT (Fig. 1, line 3), inhibits the termination but not the initiation of conjugal DNA transfer (10). When oriT1 contained this mutation, the frequency of termination was much lower, as expected (Table 1). However, white transconjugant colonies still occurred at a frequency of 0.12% and were readily detectable among the majority of blue colonies.

When both the inner and outer arms of the inverted repeat were deleted (Fig. 1, line 4), no termination within oriT1 was observed (Table 1). Thus, for oriTter, the inner arm of the inverted repeat is important for the remaining termination activity. The intact inverted repeat was not sufficient for termination: when oriT1 was deleted for bp 26 to 38 and contained an A-to-T mutation at bp 25 (Fig. 1, line 6), no white transconjugant colonies were formed (Table 1).

Properties of termination within oriTter. Termination within oriTter occurs at less than 1% of the normal frequency but at a rate that is still well above background (Table 1). To determine whether cleavage and ligation were nevertheless occurring at the normal position during these rare events, we constructed pUT1250, which contains both the ter and nic mutations at oriT1. This oriT contains C-to-T transitions at bp 30 and 32, which bracket the nic site. Thus, if cleavage and rejoicing of the transferred strand were occurring at the normal position, the mutation at bp 30 would be retained and the other mutation would be lost in the recombinant oriT. We extracted the plasmid DNA from three transconjugant colonies, each white on X-Gal and each derived from an independent mating, and determined the base sequences of the hybrid oriT. For each, the sequence around nic was GTGC for bp 29 to 33 (Fig. 3), indicating that recombination had taken place at the normal site.

Although termination in oriTter+nic occurs at the normal position, it does so at a frequency that is approximately 10-fold lower than that in oriTter (Table 1). In order to understand this decrease, we first examined the effect of the nic mutation on the DNA processing required for transfer. Since the nic mutation affects an early step in transfer and is located at the nic site, we thought it likely that it interferes

![FIG. 3. Base sequences around nic sites for three hybrid oriTs formed after conjugal transfer of pUT1250 (Table 1) and deletion of lacO.](http://jb.asm.org/)

![FIG. 4. Agarose gel electrophoresis of plasmid DNAs isolated from the lower (L) and upper (U) bands of a CsCl-ethidium bromide gradient and linearized by digestion with EcoRI. The molecular size marker is HindIII-digested λ DNA.](http://jb.asm.org/)
with initial nicking of the plasmid DNA. This is shown in Fig. 4.

Complexes of supercoiled plasmid DNA and Mob nicking proteins (the relaxation complex or relaxosome) were isolated from cells. Cells contained both a pBR322 derivative with a cloned oriT having the test mutation and R1162 to provide the Mob proteins and to serve as an internal control. The DNA was then relaxed by treatment with SDS and heat, and the supercoiled ( unnicked) and open circular forms were separated by dye-buoyant density ultracentrifugation. The amount of plasmid DNA in each form was determined by gel electrophoresis after the DNA was linearized with EcoRI.

When plasmids containing either the unmutated oriT or oriTnic were tested in our assay, both supercoiled and open circular DNAs were isolated from the density gradient (Fig. 4, lanes 1 to 4). Thus, these plasmids are susceptible to relaxosome-induced nicking. Active nicking of oriTnic is in agreement with its ability to initiate transfer. In contrast, substantially less of the plasmid DNA containing oriTnic than R1162 DNA from the same preparation was converted to the nicked form (Fig. 4, lanes 5 and 6). We estimate from several experiments that the oriTnic mutation decreases relaxation complex activity by at least 90%. We conclude that the initiation defect for the nic mutation is at the nicking step of the closed circular duplex DNA.

Our results with the nic mutation suggest that it reduces termination within oriTer because it interferes with nicking of duplex oriT at this site. Alternatively, it is possible that although the nic mutation itself has no effect on termination (Table 1), it exacerbates by some mechanism the effect of the ter mutation when present in the same oriT. We used the phage recombination assay for termination (14) to distinguish between these possibilities. Two directly repeated copies of oriT, cloned into the bacteriophage vector M13mp19, recombine during propagation of the phage DNA to result in a single, hybrid oriT (Fig. 5A). This recombination models the termination step in transfer, probably because the single-stranded viral DNA resembles DNA that has been conjugally transferred (1). Relaxosome activity is not involved in the recombination, which requires only the MobA* gene product.

When oriT contained the ter mutation, the phage recombination frequency was reduced to about 10% of the normal

![Diagram A](https://example.com/diagramA.png)

**Fig. 5.** (A) Recombination assay for directly repeated copies of oriT cloned in M13mp19; (B) mutations within the oriT inverted repeat and their effects on recombination frequency.

| TABLE 2. Effect of the nic mutation on the activity of oriTter in the phage recombination assay |
|---------------------------------|---------------------------------|------------------|
| Mutation no. | Mutation in: | Blue plaques (%)b |
|----------------|----------------|------------------|
|                  | oriTter * | oriTter * |                 |
| 1                | wr        | wt              | 11               |
| 2                | ter       | wt              | 1.4              |
| 3                | nic       | wt              | 17               |
| 4                | ter + nic | wt              | 0.7              |

* See Fig. 5.

b Averages of two to four independent experiments.

c wr, wild type.
value (Table 2, mutations 1 and 2). There was only a slight decrease in the recombination frequency when oriT1 also contained the nic mutation (Table 2, mutation 4). Thus, the nic mutation, either alone (Table 2, mutation 3) or together with ter, has little effect on the cleavage of single-stranded DNA. It is likely, therefore, that the cleavage within oriTter required for the termination of conjugal transfer is generated primarily by nicking of the duplex DNA.

**Effect of base pairing between the arms of the oriT inverted repeat on termination.** We also used the phage recombination assay to examine in greater detail than previously the requirement for the inverted repeat in termination. This assay is more sensitive than transfer, because many rounds of phage propagation amplify the effects of small differences in the activities of mutated oriTs.

We showed previously that point mutations in the outer arm of the inverted repeat were similar to the ter mutation in their abilities to reduce the rate of oriT recombination from the normal frequency of 10 to 15% (1) (Fig. 5B, lines 1 to 4). All of the mutations had the greatest effects in oriT2. The results in Fig. 5B show that other point mutations, located within the inner arm of the inverted repeat, had quantitatively similar effects (Fig. 5B, lines 5 to 8). However, when the mutations in each arm were matched so that the potential for base pairing between the arms was restored, the deleterious effects of the mutations were partially suppressed (Fig. 5B, lines 9 and 10). These results suggest that base pairing per se is important for the activity of the inverted repeat in termination.

The arms of the oriT inverted repeat naturally contain a
FIG. 7. Competition for cleavage by MobA* protein between φrm290 (unmutated oriT) and φrm147 (oriT deleted for both arms of inverted repeat) (A) or φrm13 (oriT* deleted for both arms of inverted repeat) (B). Each reaction mixture contained 0.1 μg of each labeled DNA and 0 (lanes a), 0.1 (lanes b), 0.2 (lanes c), 0.4 (lanes d), 0.8 (lanes e), 1.6 (lanes f), 3.2 (lanes g), and 6.4 (lanes h) μg of unlabeled φrm20 DNA (unmutated oriT). The molecular size marker was HindIII-digested, denatured pBR322 DNA.

single mismatch, which is at bp 3 and 21 (Fig. 5B). We asked whether repairing this mismatch by introducing an A-to-G transition at bp 3 would affect the frequency of recombination of oriT. The results (Fig. 5B, lane 11) show that there is a significant increase in the recombination frequency when the mutation is in the oriT* position.

Cleavage of mutated oriTs in vitro by MobA* fusion protein. MobA* is the amino-terminal segment of the large protein encoded by the R1162 mobA gene (2) and is the only R1162-encoded protein required for termination (14). In vitro, MobA*, as a β-galactosidase fusion protein, cleaves one of the two oriT single strands at nic and also slowly ligates this DNA (2). We tested different mutated oriTs for cleavage by this protein (Fig. 6). Linear M13mp19 viral strand DNA containing a mutated oriT was incubated with MobA* fusion protein. The DNA was end labeled so that cleavage at the nic site would generate a 245-base labeled fragment. As an internal control, each reaction mixture also contained M13 viral DNA with an unmutated oriT (φrm290). This DNA has a 42-base insertion between oriT and the labeled end, so that the labeled cleavage product, which comigrates with the 298-base marker fragment, is separated from that of the test DNA after electrophoresis.

The results of incubating different mutated oriT DNAs with fusion protein are shown in Fig. 6. We found that MobA* cleaved oriT deleted for either the outer arm of the inverted repeated, bases 1 to 8, or the entire inverted repeat, bases 1 to 19 (φrm147 and φrm13) (Fig. 6B, lanes b and c). Cleavage was specific and occurred at the site cleaved in the unmutated oriT in φrm20 (Fig. 6B, lane a). MobA* was also active on oriT nic in φrm74 (Fig. 6B, lane f), which was expected, since the mutations in this oriT do not affect termination.

A mutated oriT with two base changes outside the inverted repeat, at positions 25 and 28, was constructed (Fig. 1, line S). This oriT was cleaved by MobA* (φrm136) (Fig. 6B, lane d). The mutations create an HpaI site, and a 6-base insertion at this position to generate φrm293 (Fig. 1, line 7) prevents cleavage (Fig. 6B, lane e). We conclude that the inverted repeat is not required for specific cleavage of oriT single-stranded DNA at nic. However, disruption of oriT DNA adjacent between the inverted repeat and the nic site did affect cleavage.

Different results were obtained when the cleavage reactions were repeated with fusion protein that had first been heated at 70°C for 10 min. The heated protein still cleaved oriT in φrm20 and φrm290 (Fig. 6C, lane a'), but cleavage of oriTs lacking part or all of the inverted repeat was strongly reduced (Fig. 6C, lanes b' and c'). Cleavage of either oriT nic or oriT with the HpaI site was unaffected by the heat treatment (Fig. 6C, lanes d' and f'), and the oriT with the 6-base insertion remained inactive (Fig. 6C, lane e').

Although oriT DNAs lacking part or all of the inverted repeat are cleaved by unheated MobA* fusion protein, they compete poorly with unmutated oriT DNA for this protein. End-labeled φrm290 DNA, with an unmutated oriT, was mixed with an equimolar amount of similarly labeled φrm147 or φrm13 DNA (Fig. 7). The mixtures were then digested with MobA* protein in the presence of increasing amounts of unlabelled φrm20 phage DNA, which also contains the unmutated oriT. As the amount of unlabelled DNA was increased, φrm147 and φrm13 became progressively less able to compete with φrm290 as a substrate in the cleavage reaction (Fig. 7).

DISCUSSION

Our observations suggest the following model for the termination of conjugal R1162 DNA transfer. During mobilization, the MobA protein is covalently bound to the 5' end of the transferring strand (18). We propose that the protein is also positioned (either the donor or the recipient cell) so that the DNA strand passes by MobA as it is being transferred. When the oriT inverted repeat moves near MobA, it traps the protein and termination is initiated. Because a mutation in the inverted repeat is suppressed by a second mutation restoring base complementarity between the arms, we believe that base pairing is important for trapping by the inverted repeat. Indeed, repair of the naturally occurring mismatch in the oriT arms results in a greater rate of termination, as measured by the phage recombination assay.

Possibly, the oriT sequence has evolved so that in conjuga-
tion there is a balance between binding of the protein and its subsequent release after ligation of the transferred strand.

Although there is no termination in oriT deleted for the entire inverted repeat, there is a low rate of termination in oriT ter, which lacks only the outer arm of this repeat. The termination frequency in oriT ter is greatly affected by the nic mutation, and therefore it probably requires prior nicking of the duplex oriT ter DNA. Since transfer can be initiated...
within \textit{oriT}_{\text{ter}} and since \textit{oriT}_{\text{ter}} allows an active relaxation complex (Fig. 4), duplex \textit{oriT}_{\text{ter}} DNA can be nicked in vivo. Termination at \textit{oriT}_{\text{ter}} would then depend on molecules that had been nicked at both \textit{oriT}s prior to transfer. The possibility of doubly nicked molecules is surprising, since one might expect that after being nicked at one site, the open circular DNA would not longer be susceptible to relaxosome activity. In this connection, it is interesting that Scherzinger et al. have shown that linear DNA is a substrate for relaxosome-dependent nicking in vitro (18).

In vitro, trapping of MobA protein by the inverted repeat is reflected in competition experiments by the intact \textit{oriT} having a greater rate of cleavage than the \textit{oriT} deletion derivatives lacking part or all of the inverted repeat. However, the inverted repeat is not itself required for specific cleavage of single-stranded \textit{oriT} DNA at the \textit{nic} site. Protein that has been heated to 70°C shows greater specificity, preferentially digesting those \textit{oriT} molecules having the inverted repeat. One way of rationalizing these observations is by noting that under our reaction conditions, active protein is in excess. When the protein is either heated or titrated with excess unlabeled DNA, it becomes limiting in the reaction. Under these conditions, molecules with the inverted repeat are better able to trap the protein and are more likely to be cleaved. We cannot rule out other explanations for our observations, however. For example, it is possible that the protein molecules in our preparations are present in two forms, only one of which requires the inverted repeat for cleavage at \textit{oriT}. In this case, titration with excess \textit{oriT} DNA would have a greater effect on cleavage of molecules without the inverted repeat. Heating might promote refolding of the protein into the form requiring the inverted repeat, so that the overall specificity of the preparation would be increased.

**ACKNOWLEDGMENT**

This work was supported by a grant from the National Institutes of Health (GM37462).

**REFERENCES**

1. Barlett, M. M., M. J. Erickson, and R. J. Meyer. 1990. Recombination between directly repeated origins of conjugative transfer cloned in M13 bacteriophage DNA models ligation of the transferred plasmid strand. Nucleic Acids Res. 18:3579–3586.

2. Bhattacharjee, M. K., and R. J. Meyer. 1991. A segment of a plasmid gene required for conjugal transfer encodes a site-specific, single-strand DNA endonuclease and ligase. Nucleic Acids Res. 19:1129–1137.

3. Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heyneker, H. W. Boyer, J. H. Cross, and S. Falkow. 1977. Construction and characterization of amplifiable multicopy DNA cloning vehicles. II. A multiple cloning system. Gene 2:95–113.

4. Brasch, M. A., and R. J. Meyer. 1987. A 38 base-pair segment of DNA is required in cis for conjugative mobilization of broad host-range plasmid R1162. J. Mol. Biol. 198:361–369.

5. Clewell, D. B., and D. R. Helinski. 1969. Supercoiled circular DNA-protein complex in Escherichia coli: purification and induced conversion to an open circular DNA form. Proc. Natl. Acad. Sci. USA 62:1159–1166.

6. Guiney, D., and D. Helinski. 1979. DNA-protein relaxation complex of the plasmid RK2—location of the site-specific nick in the region of the proposed origin of transfer. Mol. Gen. Genet. 176:183–189.

7. Hershel, Y., H. W. Boyer, C. Yanofsky, M. A. Lovett, and D. R. Helinski. 1974. Plasmid ColE1 as a molecular vehicle for cloning and amplification of DNA. Proc. Natl. Acad. Sci. USA 71:3455–3459.

8. Johanputra, R. S., and N. Datta. 1974. Trimethoprim R factors in enterobacteria from clinical specimens. J. Med. Microbiol. 7:169–177.

9. Katz, L., D. T. Kingsbury, and D. R. Helinski. 1973. Stimulation by cyclic adenosine monophosphate of plasmid deoxyribonucleic acid replication and catabolite repression of the plasmid deoxyribonucleic acid-protein relaxation complex. J. Bacteriol. 114:577–591.

10. Kim, K., and R. J. Meyer. 1989. Unidirectional transfer of broad host-range plasmid R1162 during conjugative mobilization. Evidence for genetically distinct events at \textit{oriT}. J. Mol. Biol. 208:501–505.

11. Kunkel, T. A. 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA 82:488–492.

12. Kupersztoch-Portnoy, Y. M., M. A. Lovett, and D. R. Helinski. 1974. Strand and site specificity of the relaxation event for the relaxation complex of the antibiotic resistance plasmid R6K. Biochemistry 13:5484–5490.

13. Messing, J., R. Crea, and P. H. Seeberg. 1981. A system for shotgun DNA sequencing. Nucleic Acids Res. 9:309–321.

14. Meyer, R. 1989. Site-specific recombination at \textit{oriT} of plasmid R1162 in the absence of conjugal transfer. J. Bacteriol. 171:799–806.

15. Meyer, R., M. Hinds, and M. Brasch. 1982. Properties of R1162, a broad-host-range, high-copy-number plasmid. J. Bacteriol. 150:552–562.

16. Nordheim, A., T. Hashimoto-Gotoh, and K. N. Timmis. 1980. Location of two relaxation nick sites in R6K and single sites in pSC101 and RSF1010 close to origins of vegetative replication: implication for conjugal transfer of plasmid deoxyribonucleic acid. J. Bacteriol. 144:923–932.

17. Norrander, J., T. Kempe, and J. Messing. 1983. Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. Gene 26:101–106.

18. Scherzinger, E., R. Lurz, S. Otto, and B. Dobrinski. 1992. In vitro cleavage of double- and single-stranded DNA by plasmid RSF1010-encoded mobilization proteins. Nucleic Acids Res. 20:41–48.

19. Stougaard, P., and S. Molin. 1981. Vertical dye-buoyant density gradients for rapid analysis and preparation of plasmid DNA. Anal. Biochem. 118:191–193.

20. Willetts, N., and C. Crowther. 1981. Mobilization of the non-conjugative IncQ plasmid RSF1010. Genet. Res. 37:311–316.

21. Wong, E. M., M. A. Muesing, and B. Polsky. 1982. Temperature-sensitive copy number mutants of ColE1 are located in an untranslated region of the plasmid genome. Proc. Natl. Acad. Sci. USA 79:3570–3574.