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Requirements for Strand- and Site-Specific Cleavage within the *oriT* Region of Tn*4399*, a Mobilizing Transposon from *Bacteroides fragilis*

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Replicons that contain Tn*4399*, a conjugal mobilizing transposon isolated from *Bacteroides fragilis*, can be mobilized in the presence of broad-host-range IncP plasmids RP4 and R751 in *Escherichia coli* to *B. fragilis* or *E. coli* recipients (C. G. Murphy and M. H. Malamy, J. Bacteriol. 175:5814–5823, 1993). To identify the initial DNA processing events involved in Tn*4399*-mediated mobilization in *E. coli*, plasmid DNA from pCGM328 (a pUC7 vector that contains the mobilization region of Tn*4399*) was isolated from donor cells following the release of plasmid DNA from the relaxation complex. Site- and strand-specific cleavage within the *oriT* region of Tn*4399* was detected by denaturing gel electrophoresis and Southern hybridization analysis of this DNA in the presence or absence of IncP plasmids. Mutations in either *mocA* or *mocB*, two genes which are encoded by Tn*4399* and are required for mobilization, significantly decrease the amount of specifically nicked DNA detected. These results suggest roles for the MocA and MocB gene products in specific processing of Tn*4399*-containing plasmid DNA prior to mobilization. By isolation of the nicked strand and primer extension of this template, we mapped the precise 5′ end of the single-stranded cleavage reaction. The nucleotide position of nic_{Tn4399} is adjacent to two sets of inverted repeats, a genetic arrangement similar to those of previously characterized *oriT* regions. Two site-directed mutations which remove nic_{Tn4399} (*oriTΔ1* and *oriTΔ2*) cannot be mobilized to recipients when they are present in *trans* along with functional MocA and MocB proteins and an IncP mobilizing plasmid; they are *cis*-dominant loss-of-function mutations.

Plasmid-mediated bacterial conjugation is the process by which plasmid DNA is transferred from a bacterial donor cell to a suitable recipient by a mechanism that requires cell-to-cell contact (reviewed in reference 29). As a general rule, once effective donor-recipient interactions have been established, transfer of plasmid DNA requires two distinct events, DNA processing at the origin of transfer (*oriT*) and movement of a defined DNA strand from the donor to the recipient cell (29). For self-transmissible plasmids, such as the broad-host-range IncP plasmids RP4 and R751, plasmid-encoded functions are specifically required for donor-recipient cell interactions, DNA processing events at *oriT*, and DNA transfer (4). Another class of plasmids, which includes the IncQ plasmid RSF1010 of *Escherichia coli*, pC221 of *Staphylococcus aureus*, and pTF-1 of *Thiobacillus ferrooxidans*, contains *oriT* regions and codes for some DNA processing functions but not all of the functions required for transfer. These plasmids, however, can be mobilized in *trans* by a coresident autonomous or integrated transfer factor (2, 19, 31).

Other plasmids, such as pBFTM10 from *Bacteroides fragilis*, share some of the properties of both classes of transferable plasmids. pBFTM10 is capable of self-transfer in *B. fragilis*, but not in *E. coli*. In *E. coli*, pBFTM10 can be mobilized in *trans* by the IncPβ plasmid R751 (5, 25). Two plasmid genes, *btgA* and *btgB*, are required for self-transfer of pBFTM10 in *B. fragilis* and for mobilization in *E. coli* (5). However, the details of DNA processing and transfer of pBFTM10 have not been established. Plasmids that contain Tn*4399*, a conjugal mobilizing transposon from *B. fragilis*, resemble pBFTM10 in that they lead to self-transfer in *B. fragilis*, but not in *E. coli*. Furthermore, in *E. coli*, they can be mobilized in *trans* by a coresident IncPα plasmid (RP4) or IncPβ plasmid (R751) (6, 12). Previous studies have identified two Tn*4399* genes, *mocA* and *mocB*, and an *oriT* region contained within a sequenced fragment from the middle of the transposon which are required for mobilization by the IncP plasmids (12).

The F factor and R factors of the IncP class serve as model systems to define the mechanisms of conjugal DNA transfer in gram-negative bacteria. Several recent reviews have described a general model for processing DNA in the donor cell to initiate conjugal transfer in *E. coli* (4, 28, 30). A series of biochemical reactions at *oriT* that initiate the formation of a DNA-protein complex have been characterized. This DNA-protein complex, termed a relaxasome by Lanka and colleagues (13), is assembled by a cascade-like mechanism at *oriT*, assisted by the intrinsic bending of DNA in this region. This is followed by cleavage at a unique site within the *oriT* region (the *nic* site) by a relaxase on the strand to be transferred to the recipient (18, 30).

Tn*4399*-containing plasmids require a broad-host-range transfer factor to be mobilized in *E. coli* (12). In contrast to conjugal transposons, such as Tn*916* and Tn*1545*, in which transfer proceeds via a circularized intermediate of the excised transposon, the mobilization region of Tn*4399* promotes transfer in the absence of the ends of the transposon (12, 22, 23). Thus, unlike the situation in previously described conjugal transposons, the mechanism of transfer initiated by the isolated mobilization region of Tn*4399* is independent of the

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TABLE 1. *E. coli* strains and plasmids

Strain or plasmid	Relevant phenotype and/or genotype	Comment	Source and/or reference(s)
Strains			
BMH71-18	*mutS*		Promega (7)
DH5α	*recA*		21
DW1030	Sp^r *recA*		20
HB101	Sm^r *recA*		21
JM109	*recA1*		Promega (21)
Plasmids			
Transfer factor, pRK231	Kan^r Tet^r Tra^{+a}	IncPα plasmid	3, 10
Constructs containing derivatives of Tn*4399*			
pCGM328	Amp^r	pUC7 + 2.8-kb *Nde*I-*Hin*dIII fragment of Tn*4399*	12
pCGM349.1	Amp^r	pCGM328 + 1.0-kb insertion in *mocA*	This study
pCGM371	Amp^r	pUC7 + 199-bp *Xmn*I-*Mae*II *oriT* fragment of Tn*4399*	12
pCGM380	Amp^r	pCGM328 with site-directed mutation in *mocB*	This study
pCGM528	Tet^r	pACYC184 + 2.8-kb *Nde*I-*Hin*dIII fragment of Tn*4399*	12
pCGM600	Tet^r	pAlter vector	Promega
pCGM628	Tet^r	pAlter + 2.8-kb *Nde*I-*Hin*dIII fragment of Tn*4399*	12
pCGM610	Tet^r	*oriT* mutant derivative of pCGM628	This study
pCGM611	Tet^r	*oriT* mutant derivative of pCGM628	This study
pCGM612	Tet^r	*oriT* mutant derivative of pCGM628	This study
pDWH10	Cm^r	pACYC184 + Tn*4399*	6

[a] Tra$^+$, capable of self-transfer.

mechanism of its transposition. Tn*4555*, a mobilizable transposon isolated from *Bacteroides vulgatus*, is similar to Tn*4399* in that it can mobilize coresident plasmids in *cis* and the ends of the transposon are not required for the mobilization process (24).

In this study, we have characterized the DNA processing events required to initiate mobilization of Tn*4399*-containing plasmids in *E. coli* by the basic relaxasome isolation procedure of Clewell and Helinski (1) and asked whether this transfer element from *B. fragilis* shares a transfer mechanism with the well characterized transfer elements found in *E. coli*. Since mobilization of Tn*4399* occurs at very low frequency, we have devised a new and sensitive technique to detect strand-specific cleavage within $oriT_{Tn4399}$.

MATERIALS AND METHODS

Bacterial strains and media. Relevant characteristics of the bacterial strains and plasmids used in these experiments are described in Table 1. *E. coli* strains were grown in ML broth for liquid culture (11) and selected on Luria broth (Difco Co., Detroit, Mich.) solidified with 1.5% (wt/vol) agar and supplemented with antibiotics as appropriate. The following antibiotic concentrations (in micrograms per milliliter) were used for selective media, unless otherwise specified: ampicillin, 200; chloramphenicol, 25; kanamycin, 25; spectinomycin, 50; streptomycin, 50; tetracycline, 10. Antibiotics were obtained from Sigma Chemical Co., St. Louis, Mo.

Relaxation assay. Plasmid DNA was isolated by the protocol of Clewell and Helinski (1) with modifications. Bacterial cells were harvested from 50-ml stationary-phase cultures by centrifugation at 3,000 × *g* and resuspended in 1 ml of 25% (wt/vol) sucrose–50 mM Tris-HCl (pH 8.0). Following gentle lysis of bacterial cells by the addition of lysozyme, EDTA (pH 8.0), and Triton X-100 at 4°C to final concentrations of 800 μg/ml, 63 mM, and 1% (wt/vol), respectively, with intermittent 5-min incubations on ice, chromosomal DNA and cellular membranes were pelleted by centrifugation at 88,500 × *g*. Approximately 4.5 ml of cleared lysate was collected from above the viscous pellet. This lysate, which contained plasmid DNA and bound protein complexes, was further treated to isolate specifically nicked molecules.

Release of the nicked open circular form of plasmid DNA from the DNA-protein relaxation complex was achieved following the addition of sodium dodecyl sulfate (SDS) to the cleared lysate (final concentration of 2.5% [wt/vol]). The solution was gently mixed by inversion of the tube and incubated for 5 min at 30°C. Following the addition of proteinase K to a final concentration of 100 μg/ml, gentle mixing by inversion, and incubation for 5 min at 37°C, SDS was precipitated by the addition of potassium phosphate (pH 8.8) at 4°C to a final concentration of 1 M and centrifugation at 3,000 × *g* and 4°C for 10 min. The resulting supernatant was extracted twice with an equal volume of 1:1 phenol-chloroform and once with an equal volume of chloroform to remove contaminating proteins. The aqueous phase was treated with DNase-free RNase which had been prepared as previously described (21) (final concentration of 20 μg/ml) and incubated for 60 min at 37°C. Plasmid DNA was precipitated with 2 volumes of 100% ethyl alcohol, washed with 70% (wt/vol) ethyl alcohol, and resuspended in approximately 0.4 ml of 10 mM Tris-HCl–1 mM EDTA (pH 8.0). Samples were electrophoresed on 0.7% (wt/vol) agarose gels (300 ml; 20 by 25 cm) which contained 0.1 mg of ethidium bromide (EtBr) per ml in TNE buffer (40 mM Tris-HCl, 5 mM sodium acetate, 1 mM EDTA [pH 7.9]) at 25 V for 12 h. Open circular DNA was localized by long-wavelength UV light, and the corresponding agarose region was cut from each gel, purified by using a Geneclean kit (BIO 101, La Jolla, Calif.), and treated with the appropriate restriction enzymes prior to being loaded on 0.6% (wt/vol) denaturing agarose gels.

To confirm the identifications of different forms of plasmid DNA (closed circular, open circular, and linearized), each band visualized on an EtBr-stained preparative gel was individually cut out, and the DNA was purified and analyzed for the appearance of smaller fragments under denaturing conditions (see below). Thus, on 0.7% (wt/vol) preparative agarose gels prepared with 0.1 mg of EtBr per ml, the band corresponding to the fastest-migrating plasmid DNA molecules which also was most intensely stained with EtBr was identified as the covalently closed circular form of plasmid DNA. The band corresponding to the next fastest population of migrating plasmid DNA molecules was identified as the nicked open circular form, and the slowest migrating population of DNA molecules was identified as the linear form of plasmid DNA. Additional bands which were stained less intensely than the three major forms of plasmid DNA and which migrated even more slowly than the three populations already described were probably multimeric forms of plasmid DNA (data not shown).

Denaturing 0.6% (wt/vol) alkaline agarose gels (80 ml; 9 by 11 cm) that contained 50 mM NaOH and 1 mM EDTA (pH 8.0) were prepared as previously described (21). Samples were electrophoresed onto alkaline agarose gels at 2.7 V/cm for 10 to 16 h at 4°C, with constant recycling of the buffer. Following neutralization in 1.5 M Tris-HCl (pH 7.6)–50 mM NaCl for 45 min, gels were stained in 6 μg of EtBr per ml for approximately 10 min, destained for an equal time in deionized H_2O, and photographed under long-wavelength UV light. DNA was transferred to positively charged nylon membranes (Boehringer Mannheim, Indianapolis, Ind.) for Southern hybridization experiments by alkaline transfer with a capillary blot apparatus. Nylon membranes were air dried and UV cross-linked at 120,000 μJ/cm^2.

Mapping the 5′ end of the *nic* site. As previously described by Pansegrau et al. (17) for mapping the 5′ ends of IncP *nic* sites, nic_{Tn4399} was mapped by a runoff assay with minor modifications. The relaxation assay described above was scaled up 10-fold to allow the isolation of plasmid DNA from 500 ml of starting culture. After lyophilization of precipitated DNA, plasmid DNA (approximately 650 μg) was resuspended in 3 ml of 10 mM Tris-HCl–1 mM EDTA (pH 8.0) and loaded onto five agarose gels (20 by 25 cm), with each containing 300 ml of 0.7% (wt/vol) agarose–0.1 mg of EtBr per ml–TNE buffer. Following the migration of samples onto gels at 100 V, gels were covered with a glass plate and electrophoresed at 25 V for 40 to 45 h to achieve clear resolution of plasmid bands.

Bands representing the open circular form of plasmid DNA from each of these five preparative gels were cut, pooled, and purified to yield approximately 70 μg of open circular plasmid DNA. Following 24-h restriction of plasmid DNA with *Nde*I, the sample was separated on a 300-ml 0.6% (wt/vol) alkaline agarose denaturing gel (20 by 25 cm) for 36 h at 30 V and 4°C with recycling buffer. Specifically nicked single-stranded DNA was visualized and cut from the gel as described above, equilibrated in 1.5 M NaCl–1.0 M Tris-HCl (pH 7.6), and purified with a Geneclean kit according to the supplier's directions. To allow optimal binding to glass beads, the pH of the plasmid DNA solution was adjusted to 7.2 as necessary. On the basis of the results of Southern hybridization experiments with strand-specific probes (see Results), a primer complementary to the nicked strand which annealed 3′ to the *nic* region (CM16) was selected. By using the nicked strand as a template for primer extension, the DNA of the nicked strand was sequenced to the 5′ end of the *nic* site and analyzed on an 8% (wt/vol) acrylamide sequencing gel, and its sequence was compared with the wild-type sequence of the mobilization region.

The nucleotide sequence of the nick site was determined in three ways. Each method was a slight variation of the runoff assay described by Pansegrau et al. (17) and yielded the same nucleotide position for the cleavage site ± 1 bp. In the first case, the TAQuence method (version 2.0; United States Biochemical, Cleveland, Ohio) was used according to the supplier's directions. This method utilizes Δ*Taq* version 2.0 DNA polymerase to extend annealed primers at 70°C and substitutes 7-deaza-dGTP nucleotides for dGTP in deoxytriphosphate reactions. We used thermostable DNA polymerase at 70°C and the nucleotide analog for dGTP which forms a weaker secondary structure than does dGTP to reduce the secondary structure in this region and to facilitate localization of *nic*. Chain-terminating, dideoxynucleotides (G, A, T, and C) were incorporated into four respective equivalent aliquots of the primed reaction mixture to yield a sequencing ladder prior to termination of the extended primer by limiting template. In the second method, a fifth aliquot (3 μl) of primed sequencing reaction mixture from the same initial reaction was extended by using Δ*Taq* version 2.0 DNA polymerase at 70°C in the presence of 40 μM deoxynucleoside triphosphates (dNTPs) and the absence of chain-terminating dideoxynucleotides or additional 7-deaza-dGTP. This reaction was also terminated by limiting template beyond the specific cleavage site. In the third method, 6.5 μg of specifically nicked open circular template DNA was independently annealed to the same primer and extended by using Δ*Taq* version 2.0 DNA polymerase at 70°C in the presence of 40 μM dNTPs and the absence of chain-terminating dideoxynucleotides. In this reaction, 7-deaza-dGTP nucleotides were not incorporated into the initial priming or extension reaction mixtures.

Recombinant DNA techniques. Plasmid DNA isolation and restriction endonuclease analysis were performed as previously described (21). All restriction enzymes, Klenow DNA polymerase, and T4 DNA ligase were purchased from New England Biolabs (Beverly, Mass.) and used according to the supplier's directions, with the following exceptions: *Mae*II and *Esp*I were purchased from Boehringer Mannheim and United States Biochemical, respectively.

Southern hybridizations. The following oligonucleotides complementary to either strand of the mobilization region were synthesized on an Applied Biosystems DNA-RNA synthesizer (Foster City, Calif.) and purified by using Poly-Pak cartridges available from Glen Research (Sterling, Va.) according to the supplier's directions: CM10, 5′ GCCGAATCCCTTCCTCTTGG; CM11, 5′ GGATTC GGCATCTAAGCCGT; CM16, 5′ GCAGGAAAGCGGTGGAGGAT; CM23, 5′ CACATCTTTTTTAGGTTAATGTCGGCGTATTAC; CM26, 5′ CAAAAA GATGAGGGGACGATGGTCACGGACT; CM31, 5′ CACATCTTTTTTAG GATAGC; CM32, 5′ AAGTTAATGTCGGCGTATTAC; CM33, 5′ CACATC TTTTTTAGGATATCGTTAATGTCGGCGTATTACATTCC. Oligonucleotides to be used as probes in Southern hybridizations were end labeled with [γ-^{32}P]ATP (New England Nuclear, Boston, Mass.) by using T4 polynucleotide kinase (New England Biolabs). Labeled oligonucleotides were purified on Sephadex G-50 NICK columns from Pharmacia (Piscataway, N.J.) according to the supplier's directions and annealed to plasmid DNA bound to positively charged nylon membranes. The conditions for hybridizations and washes at the indicated temperatures have been described previously (21).

Radionucleotides. All radionucleotides were obtained from New England Nuclear. ^{35}S-deoxyadenosine 5′-(α-thio)-triphosphate was used for sequencing at 1,500 Ci/mmol, and γ-^{32}P-adenosine 5′-triphosphate was used for end labeling at 6,000 Ci/mmol.

Mutagenesis of *oriT*$_{Tn4399}$. Site-directed mutations within the 199-bp *oriT* region of Tn*4399* were made by using the pAlter mutagenesis kit (Promega, Madison, Wis.), as previously described (12). pCGM610, which contains the mutation *oriTΔ1*, is the result of an 8-bp deletion that spans the nick site and was introduced by oligonucleotide CM23. pCGM611, which contains the mutation *oriTΔ2*, was created by incorporation of oligonucleotide CM33. It contains a deletion of the 2 bp immediately 5′ to the nick site in addition to a C→A transversion mutation 3′ to *nic*, resulting in a new *Eco*RV restriction enzyme recognition sequence. Plasmid DNA isolated from both pCGM610 and pCGM611 was sequenced throughout the 199-bp *oriT* region to confirm the presence of these mutations and to verify that these mutations were the only ones introduced into the *oriT* region. A third mutation within the *oriT* region, introduced by oligonucleotide CM26, deletes 8 bp to the left of two sets of inverted repeats, resulting in plasmid pCGM612. This deletion removes a recognition sequence for the restriction enzyme *Nla*IV.

Mobilization assay. Plasmids containing the wild-type mobilization region of Tn*4399* were assayed for mobilization in *E. coli* in the presence of the IncP plasmids RP4 and R751, as previously described (12). When a mutation that might alter MocB function was introduced into the *oriT*$_{Tn4399}$ region which overlaps with the coding sequence of *mocB*, wild-type Tn*4399* was provided in *trans* on a compatible plasmid to supply MocA and MocB. Mobilization frequency is defined as the number of plasmid transconjugants/number of IncP transconjugants in the same experiment.

RESULTS

Strand-specific nicking within the mobilization region of Tn*4399*. To study early DNA processing events within the mobilization region of Tn*4399*, it was convenient to analyze this region cloned onto a high-copy-number plasmid since such a construct was found to be mobilized at high frequency in the presence of a coresident IncP plasmid in *E. coli* (12). The starting plasmid was pCGM328, a ColE1 replicon containing a 2.8-kb restriction fragment of Tn*4399*, which had previously been shown to contain functions required for mobilization. As reported, this plasmid is mobilized at a frequency of 10^{-3} in the presence of the IncPα plasmid pRK231 while control plasmids that lack Tn*4399* sequences cannot be mobilized to recipients (12).

By using a modified relaxation assay, specifically nicked DNA of plasmid pCGM328 was isolated from a *recA E. coli* mutant which also contained a coresident IncP plasmid, pRK231. This DNA was analyzed on nondenaturing and denaturing agarose gels following digestion with *Nde*I, which cleaves pCGM328 at only one site. Under nondenaturing conditions, only one band corresponding to the full-length linearized plasmid was observed (Fig. 1A, lane 2). However, on a denaturing agarose gel, the same preparation of open circular DNA gave rise to three bands. One of these bands corresponds to a full-length linearized DNA strand, 5.5 kb, while the sizes of the two smaller bands correspond to a full-length linearized DNA strand which has been cleaved into two fragments, 4.2 and 1.3 kb (Fig. 1A, lane 4). These smaller bands, particularly the fragment of 1.3 kb, appear very faint when the gel is stained with EtBr. To increase the sensitivity of this assay, DNA from this agarose gel was transferred to a nylon membrane and probed with end-labeled oligonucleotides complementary to plasmid DNA in Southern hybridization experiments. This modification of the original protocol to detect single-stranded nicking events (1) significantly increases the sensitivity of the assay, thus allowing visualization of low-frequency events. In this way, the 4.2- and 1.3-kb bands were easily detected on autoradiographs (Fig. 1B). Plasmid DNA isolated from a pUC7 vector which did not contain the Tn*4399* mobilization region showed only a single band that was equivalent to the full-length linearized vector when it was analyzed by EtBr staining and Southern hybridizations under both denaturing and nondenaturing conditions (data not shown).

To determine the strand specificity of the cleavage reaction, we performed Southern hybridization experiments in which the open circular form of pCGM328 DNA was probed with two end-labeled oligonucleotides, CM10 and CM11, each complementary to a different strand of the mobilization region. As shown in Fig. 1C, both probes hybridized to the 5.5-kb band of full-length linearized plasmid DNA, but only one probe, CM11, hybridized to the smaller 1.3-kb fragment (lane 1). Hybridization of CM11 to the 5.5-kb band is expected, since there is a large population of nonspecifically nicked plasmid DNA molecules. However, hybridization of CM11 to the 1.3-kb band represents complementarity between the sequence of CM11 and a specifically nicked population of plasmid DNA molecules on a particular strand. Thus, some of the plasmid

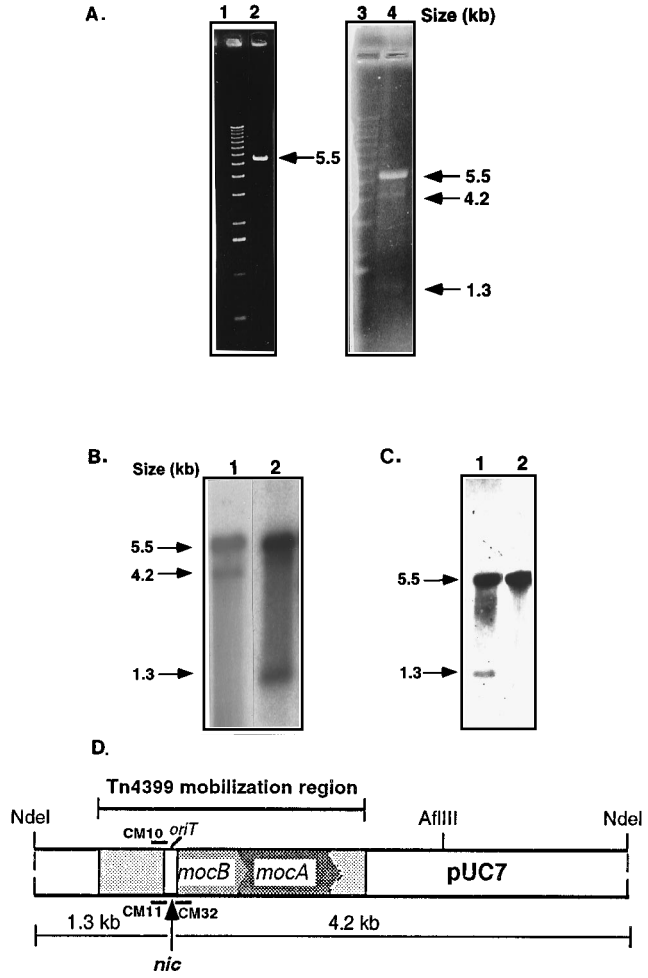

FIG. 1. Specifically nicked DNA isolated from pCGM328 (a plasmid that contains the mobilization region of Tn*4399*). (A) Nondenaturing versus denaturing agarose gel electrophoresis of the open circular form of pCGM328 DNA; DNA was isolated as described in Materials and Methods and restricted with *Nde*I. Lanes: 1 and 3, molecular size standards (1-kb ladder); 2, pCGM328, under nondenaturing conditions; 4, pCGM328, under denaturing conditions. (B and C) Southern hybridization experiments of pCGM328 DNA, as described in Materials and Methods. Probes for panel B: CM32 (lane 1) and CM11 (lane 2). Probes for panel C: CM11 (lane 1) and CM10 (lane 2). (D) Schematic representation of pCGM328 linearized with *Nde*I, including the locations of *mocA*, *mocB*, and *oriT* and of probes CM10, CM11, and CM32, as well as approximate sizes of the fragments generated by cleavage at *oriT*.

molecules that contain the mobilization region of Tn*4399* are cleaved in a site- and strand-specific manner.

The presence of IncP plasmids is not required for nicking within the mobilization region of Tn*4399*. Nicked open circular plasmid DNA from pCGM328 was isolated from cultures in the presence and absence of IncP plasmids in *trans*. Next, this DNA was restricted with either *Afl*III or *Nde*I, each of which has a single recognition site within the plasmid, and analyzed by Southern hybridizations following denaturing gel electrophoresis. As shown in Fig. 2, in both the presence and absence of pRK231, pCGM328 was specifically nicked as seen by the hybridization of probe CM16 to fragments of 1.3 and 3.3 kb, corresponding to the fragments generated by the restriction of plasmid DNA with *Nde*I (lanes 1 and 3) and *Afl*III (lanes 2 and 4), respectively. Therefore, the presence of IncP plasmids is not required for nicking within the mobilization region of Tn*4399*.

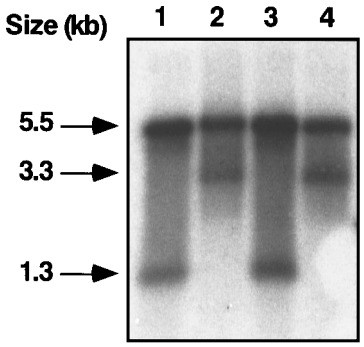

FIG. 2. Southern hybridization of pCGM328 DNA isolated from cells in the presence (lanes 1 and 2) and absence (lanes 3 and 4) of pRK231 in *trans*. Plasmid DNA, isolated as described in Materials and Methods, was probed with CM16 in Southern hybridization experiments. The position of CM16 is approximately 100 bp to the left of CM11 (Fig. 1). Lanes: 1 and 3, samples restricted with *Nde*I; 2 and 4, samples restricted with *Afl*III.

Previous experiments have established the requirement for MocA and MocB of Tn*4399* in *trans* for mobilization of nonconjugal plasmids such as pCGM371 (pUC7 + $oriT_{Tn4399}$) (12). When pCGM371 plasmid DNA was isolated by the relaxation protocol, restricted, and analyzed on a denaturing agarose gel, no bands smaller than those of the full-length linearized plasmid were detected by EtBr staining or Southern hybridization experiments (data not shown). In the presence of Tn*4399* (containing functional MocA and MocB products in *trans*), a specific band of the expected size, corresponding to the distance between the restriction site and $oriT_{Tn4399}$, was seen (data not shown). Thus, specific cleavage at $oriT_{Tn4399}$ occurs in the presence but not in the absence of *trans*-acting products of Tn*4399*. Below, we consider the individual requirements for MocA and MocB in the specific cleavage reaction at $oriT_{Tn4399}$.

MocA is required for the nicking reaction. To determine the requirement for MocA in the specific cleavage reaction within the mobilization region of Tn*4399*, a *mocA* mutant plasmid was assayed for relaxation in the presence of a wild-type *mocB* gene. Following the relaxation assay, open circular plasmid DNA was isolated and purified from pCGM349.1, a plasmid that contains a 1.0-kb insertion in *mocA*, resulting in a mobilization-deficient phenotype. This DNA was restricted with *Afl*III, for which there is a single recognition site within the plasmid. Because probe CM16 anneals to sequences between the restriction site and sequences proximal to the cleavage site and the insertion in *mocA* is distal to the cleavage site, the size of the potential specifically nicked band in pCGM349.1 should be the same as that of a wild-type plasmid (3.3 kb). However, an additional band of this size was not observed in Southern hybridizations. As shown in Fig. 3 (lane 2), a band corresponding to the full-length linearized plasmid pCGM349.1 hybridized to probe CM16, but the sample did not reveal an additional band that was smaller than the full-length restriction fragment, which would have been indicative of a specifically nicked strand. Upon prolonged exposure of autoradiographs (10 to 14 days), increased amounts of nonspecific nicking were detectable as increased smearing on the film, but distinct bands representing specific nicks were not detected. Thus, functional MocA is required for site- and strand-specific nicking of plasmid DNA that contains $oriT_{Tn4399}$.

MocB is required for the nicking reaction. We also performed the nicking assay with pCGM380, a *mocB* mutant plasmid. The *mocB* gene in pCGM380 contains a site-directed

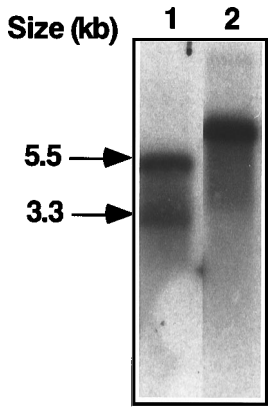

FIG. 3. Absence of nicking at $oriT_{Tn4399}$ in a *mocA* mutant plasmid. Plasmid DNA was isolated as described in Materials and Methods from pCGM328 (lane 1) or pCGM349.1 (a plasmid that contains an insertion mutation in *mocA*) (lane 2), restricted with *Afl*III, separated by denaturing agarose gel electrophoresis, and analyzed in Southern hybridization experiments with probe CM16.

point mutation outside the 199-bp region containing *oriT*, which results in the termination of *mocB* translation after approximately one-third of the coding sequence has been translated (12). This mutant shows greatly reduced mobilization efficiencies in the presence of IncP plasmids. Though this mutation lies within the $oriT_{Tn4399}$ region, pCGM380 can be complemented in *trans* by another plasmid that supplies wild-type *mocB*, indicating that the $oriT_{Tn4399}$ on this plasmid is still functional despite its mobilization-deficient phenotype. A plasmid that contained a similar mutation in *mocB* was previously shown to be defective for mobilization, but it was complemented in *trans* by a plasmid that contained only *mocB*, not *mocA*. In addition, there was no evidence of *mocB* polarity on *mocA* (12).

Aliquots of open circular plasmid DNA obtained from *E. coli* cells that contained pCGM380 were independently restricted with *Nde*I or *Afl*III. Under denaturing conditions, analysis of restricted aliquots revealed single bands corresponding to the full-length linearized plasmid. However, smaller fragments were not detected (Fig. 4, lanes 3 and 4). After prolonged exposure (14 days), these same autoradiographs revealed nonspecific smearing and a faint additional band of the same size as specifically nicked DNA in the wild-type plasmid (data not shown). Thus, a plasmid that contains a mutation in *mocB* is specifically nicked at a greatly reduced frequency, compared with that of plasmids containing wild-type *mocB*. This indicates a role for MocB in the specific nicking reaction at $oriT_{Tn4399}$.

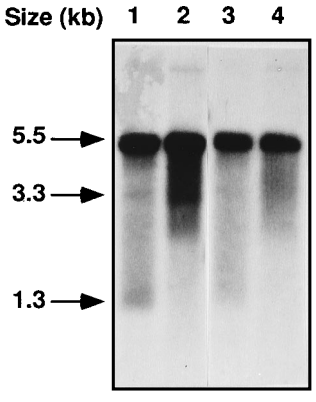

FIG. 4. Decrease in nicking at $oriT_{Tn4399}$ in a *mocB* mutant plasmid. Plasmid DNA was isolated as described in Materials and Methods from pCGM328 (lanes 1 and 2) or pCGM380 (a plasmid that contains a site-directed mutation in *mocB*) (lanes 3 and 4), restricted with *Nde*I (lanes 1 and 3) or *Afl*III (lanes 2 and 4), separated by denaturing agarose gel electrophoresis, and analyzed by Southern hybridization experiments with probe CM16.

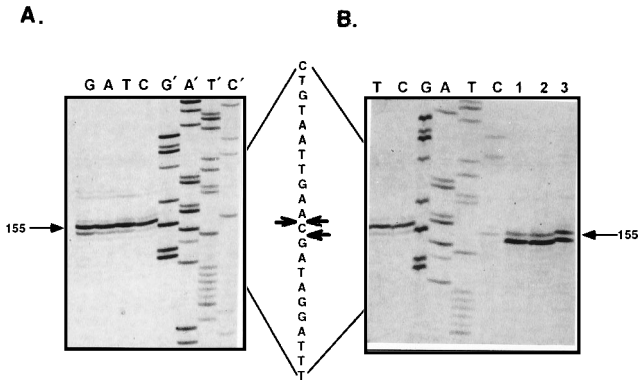

FIG. 5. Mapping the 5′ end of the cleavage site in $oriT_{Tn4399}$. Specifically nicked pCGM328 DNA was used as a template for primer extension in a sequencing reaction, and this reaction was compared with that with pCGM328 DNA which had not been specifically nicked. (A) Lanes: G, A, T, and C, nicked template, with primers extended by method 1 as described in Materials and Methods; G′, A′, T′, and C′, control unnicked template. The nucleotides in this region are shown between panels A and B, and arrows indicate the cleavage site(s). (B) Annealed primers were extended in the presence of dNTPs and the absence of chain-terminating dideoxynucleotides, as described in Materials and Methods. The first two lanes labeled T and C, respectively, are the same as the third and fourth lanes (also labeled T and C) of panel A; the next four lanes (G, A, T, and C) contained control unnicked template; lanes 1 and 2, primers extended by method 3; lane 3, primer extended by method 2.

Mapping the 5′ end of the *nic* site. To determine the precise 5′ end of the *nic* site, specifically nicked pCGM328 plasmid DNA was isolated on a denaturing agarose gel and purified as a template for primer extension. Sequencing reactions on the nicked single-stranded template (method 1 [see Materials and Methods]) were compared with the sequencing reactions of a control unnicked template of pCM328 DNA to determine the 5′ position of the cleavage site on the specifically nicked template. A sharp band appears in all four sequencing lanes at nucleotide position 155 within the $oriT_{Tn4399}$ region (Fig. 5A), indicating a limiting amount of template DNA beyond this point. This result (obtained independently in triplicate) suggests that the site at which the specific nicking reaction cleaves the template strand is between nucleotides 155 and 156 of the mobilization region sequence.

In similar experiments, the same nicked strand template was sequenced in the absence of chain-terminating dideoxynucleotides (methods 2 and 3 [see Materials and Methods]). While limiting template should theoretically lead to a single band at nucleotide position 155 (as described above), two bands of equal intensity appeared at positions 155 and 156 of the sequence when either method 2 or 3 was used (Fig. 5B). This corresponds to cleavage of the DNA strand between either nucleotides 155 and 156 or 156 and 157. To determine whether this doublet was an artifact of the reaction, a plasmid restriction fragment was sequenced to its known cleavage site by this assay. This control primer extension also resulted in a doublet in which Δ*Taq* polymerase added one base to the end of the single-stranded DNA template (data not shown).

The DNA sequence of the 199-bp *Xmn*I-*Mae*II restriction fragment of Tn*4399* that contains the *oriT* region is shown in

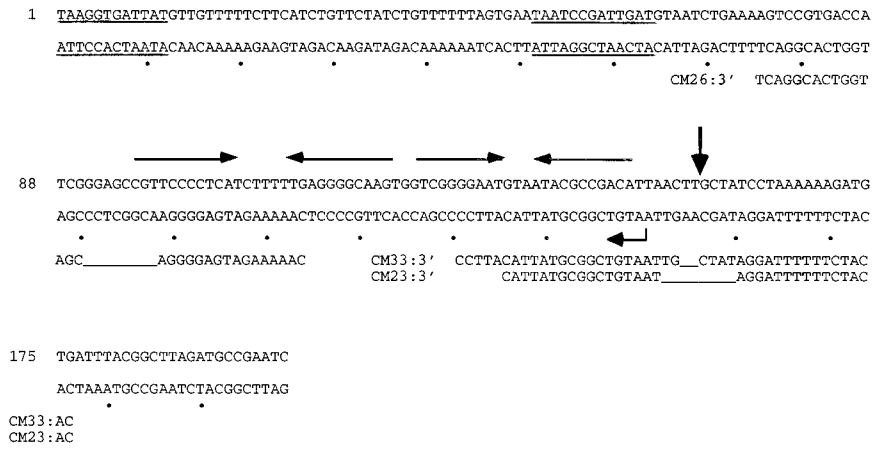

FIG. 6. Experimentally determined double-stranded sequence of $oriT_{Tn4399}$ contained within a 199-bp *Mae*II-*Xmn*I restriction fragment of Tn*4399*. The vertical arrow designates nic_{Tn4399}, the position of strand- and site-specific cleavage within Tn*4399*. Horizontal arrows above the DNA sequence indicate inverted repeat sequences. The putative translational start site of MocB is shown by a bent arrow. Potential integration host factor binding sites are underlined. The synthetic oligonucleotides CM26, CM33, and CM23 used for mapping the cleavage site and introducing mutations into the *oriT* region are designated below the corresponding sequence. __, deletion of corresponding base pairs. Cm26 gave rise to *oriT*Δ8, CM23 gave rise to *oriT*Δ1, and CM33 gave rise to *oriT*Δ2.

Fig. 6. As depicted, there are two sets of inverted repeats within 8 and 34 bp of the biochemically determined *nic* site.

Plasmids that contain mutations in $oriT_{Tn4399}$ cannot be mobilized. By definition, an *oriT* region is the minimal *cis*-required sequence necessary for mobilization or transfer of a DNA replicon and includes essential protein binding sites and sequences required for proper assembly of relaxasome complexes. To determine the sequences that are required for cleavage of Tn*4399*-containing plasmids in addition to the cleavage site, we constructed several mutations within the *oriT* region of Tn*4399* and assayed them for mobilization in *trans* to IncP plasmids and a third plasmid with a wild-type Tn*4399* mobilization region (to provide essential MocA and MocB functions).

pCGM610, which contains an 8-bp deletion that spans the nick site (Fig. 6) (*oriTΔ1*), was not mobilized at a detectable frequency. Similarly, pCGM611, which contains a 2-bp deletion 3′ to the nick site and a C→A base pair substitution 5′ to the nick site (*oriTΔ2*), was not mobilized (Table 2). An additional mutation that deleted 8 bp 3′ to the two sets of inverted repeats, pCGM612, reduced mobilization frequency by 1 order of magnitude. This suggests that sequences to the left of the inverted repeats are also required for efficient mobilization of Tn*4399*-containing plasmids in *E. coli.*

TABLE 2. R751 mobilization of pCGM328 derivatives that contain mutations in $oriT_{Tn4399}$ in the presence of pCGM528[a]

Plasmid	Description	Mean frequency of mobilization[b]
pCGM600	Vector	$<10^{-7}$
pCGM628	Vector (pAlter_2.8)	$(1.75 \pm 0.8) \times 10^{-4}$
pCGM610	*oriTΔ1*	$<10^{-7}$
pCGM611	*oriTΔ2*	$<10^{-7}$
pCGM612	*oriT*Δ8 bp (60 bp from *nic*)	$(2.9 \pm 1.9) \times 10^{-5}$

[a] Plasmids were mobilized from DW1030(pRK231) or DW1030(R751) to HB101.

[b] Number of plasmid transconjugants/number of IncP transconjugants in the same experiment.

DISCUSSION

Transposon Tn*4399* was first shown to mobilize nonconjugal plasmids in *cis* from *B. fragilis* donor cells (6) to both *E. coli* and *B. fragilis* recipients. While Tn*4399*-containing plasmids are incapable of self-transfer in *E. coli*, these plasmids can be mobilized by broad-host-range IncPα (pRK231) and IncPβ (R751) plasmids in this background (12). Previous studies with plasmids from gram-positive and gram-negative bacteria (reviewed in references 4 and 28) have identified two classes of plasmid-encoded transfer functions which contribute to DNA processing, plasmid-specific, site-specific gene products (such as TraJ and TraK of the IncP plasmids) and generalized DNA processing functions, such as relaxase TraI, which can nick within multiple *oriT* regions, provided that the site specificity determinants which direct TraI to the cleavage site are also present.

Studies with IncP plasmids have provided biochemical details for these early DNA processing reactions. For both IncPα plasmid RP4 and IncPβ plasmid R751 (15), an *oriT*-binding protein, TraJ, binds one arm of an imperfect 19-bp inverted repeat adjacent to the specific cleavage recognition site as the first step in relaxasome formation (32). Relaxase TraI recognizes and binds 6 nucleotides 3′ to the cleavage recognition site in addition to binding TraJ (15, 27). This protein-DNA complex is stabilized by TraH via protein-protein interactions (13). In addition, several subunits of TraK bind adjacent to the cleavage recognition site. DNA binding by TraK may assist in the proper positioning of other relaxasome components or shift the thermodynamic equilibrium of the reaction toward cleavage by changing the topology of the DNA helix in this region (33). Following relaxasome assembly, TraI mediates cleavage of a single DNA strand in addition to catalyzing the cleavage-joining reaction at the termination of DNA transfer replication (15). For further details on DNA processing reactions for IncP plasmids, see references 16 and 28.

Several mobilizable plasmids, including RSF1010 and pTF-1, have been studied to determine the mechanism(s) for their mobilization by larger self-transferable plasmids and the respective contributions of each plasmid. By deletion analyses of RP4 and subsequent analyses of RSF1010 mobilization in

trans, 13 RP4 genes (the *trbB-trbL* operon, *traF*, and *traG*) were shown to be required for mobilization of RSF1010 (9). Since these genes are also required for donor-specific phage propagation, these RP4 genes were identified as genes involved in mating pair formation. Additional DNA processing functions are provided by the MobA, MobB, and MobC gene products of RSF1010 for its mobilization. In the case of pTF-1, MobL, a protein which exhibits binding activity to the 42-bp *oriT* region of pTF-1, is also required for the mobilization process (2). The model for mobilization of these plasmids includes site- and strand-specific processing of plasmid DNA by some of its own functions and utilization of the transfer apparatus contributed by the large self-transmissible plasmid.

In this study, we investigated the mechanism of initiation of DNA processing in plasmids that contain the mobilization region of Tn*4399* in *E. coli* and the potential roles of MocA and MocB products in this process. Following in vitro relaxation of pCGM328 DNA, which contains the mobilization region of Tn*4399*, three bands were visualized by EtBr staining after the separation of DNA strands under denaturing conditions. The sensitivity of this assay was greatly increased by probing the separated fragments in Southern hybridization experiments. This result is consistent with a mechanism of site- and strand-specific nicking within the mobilization region of Tn*4399* in *E. coli*. Because these three bands can be detected in open circular DNA isolated in the absence of IncP plasmids, IncP gene products are not required for the Tn*4399* initiation reaction.

By Southern hybridization analysis of plasmids that contain *mocA* or *mocB* mutations, we determined that MocA and MocB play a role in the specific cleavage reaction and can function in *trans*. This supports previous results which established that *mocA* and *mocB* are required in vivo for mobilization of nonconjugal plasmids that contain the mobilization region of Tn*4399* (12).

Runoff experiments with specifically nicked single-stranded DNA as the sequencing template indicated a strand-specific cleavage site within the mobilization region at nucleotide position 155 or 156 (Fig. 5). In a primer extension assay performed with a known restriction fragment, the observation of a doublet with one extra base supports the likelihood that the doublet seen in Fig. 5B is an artifact of the reaction and that the specific nick site in $oriT_{\mathrm{Tn}4399}$ is between nucleotides 155 and 156. However, it remains formally possible that cleavage can occur at either position because of relaxed specificity for the *nic* site or slippage of a DNA-binding protein in this region. Further analysis of site-directed mutants in this region may determine which of these possibilities is correct.

Adjacent to $nic_{\mathrm{Tn}4399}$ are two sets of inverted repeats, a common feature of *oriT* regions. As described for the *oriT* regions of IncP plasmids and several other mobilizable plasmids, inverted repeats contain potential DNA binding sites for proteins directly involved in the nicking reaction. Additionally, inverted repeats promote secondary structure within the *oriT* region which may be essential to the initiation reaction. The inverted repeats within the *oriT* region of Tn*4399* lie approximately one helical turn from the proposed nick site. This arrangement is similar to the positioning of inverted repeats within the RP4 nick region according to Ziegelin and colleagues (33). Two potential binding sites for host-encoded integration host factor protein were also detected within 90 and 145 bp of the nick site, though the requirement for integration host factor and other host proteins has yet to be determined. Another characteristic feature of *oriT* regions which is present in the $oriT_{\mathrm{Tn}4399}$ region is a high percentage of A+T nucleotides, which allows melting of the DNA double helix prior to transfer of a single strand to the recipient cell. The A+T content within the 199-bp restriction fragment (Fig. 6) is 60%.

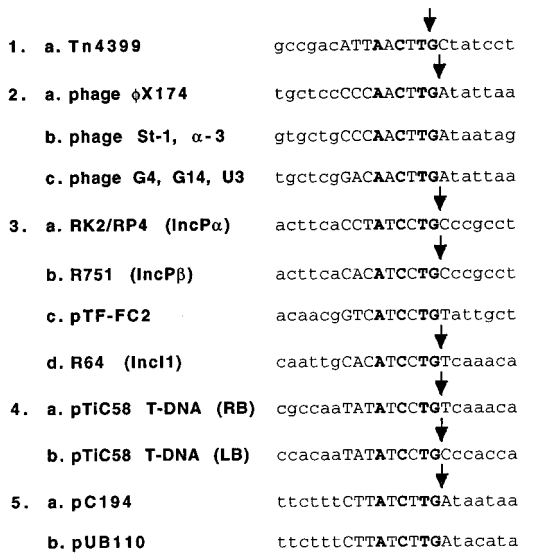

FIG. 7. Comparison of DNA sequences in the nick regions of Tn*4399* (1), φX174-like bacteriophages (2), gram-negative plasmids (3), Ti plasmids (4), and gram-positive plasmids (5) according to the scheme of Waters and Guiney (26). Nick sites, when known, are designated by arrows. Conserved nucleotides are in boldface, and consensus nucleotides for each group of related plasmids or bacteriophages are given in capital letters.

While no significant homology was detected at the nucleotide level between the *nic* region of $oriT_{\mathrm{Tn}4399}$ and other *oriT* *nic* sites, it is of interest that nucleotides at four positions proximal to the nick site (A-C-TG) are absolutely conserved in the *nic* sites of Tn*4399* and representative plasmids of gram-negative and gram-positive bacteria, Ti plasmids, and φX174-like bacteriophages (Fig. 7). The reason for the conservation of these 4 nucleotides is unknown. These nucleotides may indicate a cleavage site motif, though additional DNA binding sites and secondary structure are also presumably required. The absence of significant homology between $nic_{\mathrm{Tn}4399}$ and other nick regions can be explained if Tn*4399* contains its own specific DNA-binding protein to direct the relaxase to the *nic* site. MocB, a Tn*4399*-encoded protein that is required for mobilization, is a candidate for this type of specificity determinant. One possibility is that MocA and MocB form a heterodimer which acts as a nicking complex. Alternatively, MocA may bind DNA within the *oriT* region and cleave at $nic_{\mathrm{Tn}4399}$ while MocB functions in another role to augment the site- and strand-specific cleavage reaction.

In experiments of DNA processing in IncP and IncI1 plasmids, the relaxase remains covalently attached to the 5′ phosphate of the nick site. In this position, it can catalyze the cleavage-joining reaction at the termination of DNA transfer upon recognition of a reconstituted nick site (18). Preliminary experiments indicate that the 5′ end of the specific cleavage site in pCGM328 plasmid DNA is blocked from phosphorylation by [γ-^{32}P]ATP in a phosphate exchange reaction catalyzed by T4 polynucleotide kinase. This result suggests that the relaxase remains covalently attached to the 5′ phosphate of the nick site within the mobilization region of Tn*4399*.

Because of the absence of nicking within a plasmid that contains a *mocA* mutation and the striking homology between MocA and other DNA relaxases within a 14-amino-acid domain previously shown to be involved in the enzyme-active site

(12), we propose that *mocA* of Tn*4399* encodes a relaxase that nicks in a site- and strand-specific manner to initiate DNA processing for mobilization. In addition, MocA may remain covalently attached to the 5′ end of the nick site, as has previously been described for the TraI of RP4 (18).

On the basis of our results and comparisons with the mechanisms of initiation of DNA processing in other plasmid-mediated conjugal systems, we propose a model for DNA processing and initiation within the mobilization region of Tn*4399* in *E. coli*. This model includes expression of *mocA* and *mocB* in this organism and subsequent formation of a DNA-protein complex at sequences surrounding the *nic* site. The binding of host factors may assist in the formation of this DNA-protein complex or the bending of DNA in this region. MocA may then recognize the specific strand and site at which to nick (nucleotide 155 or 156 [Fig. 5 and 6]) and cleave at this site, independent of a coresident transfer plasmid. Additional transfer functions contributed in *trans* by a coresident IncP plasmid would establish effective mating contacts with recipient cells and provide a generalized transfer apparatus to support transfer of the nicked strand to the recipient cell in a 5′-to-3′ direction. Replacement strand synthesis would occur in the donor cell, and the transferred strand would serve as a template for DNA synthesis in the recipient cell by utilizing additional host-encoded functions. Reconstitution of the nicking reaction at $oriT_{\mathrm{Tn}4399}$ with purified MocA and MocB proteins in vitro will help to confirm this model. Thus, we propose that the transfer mechanism mediated by Tn*4399* is similar to other plasmid-mediated transfer mechanisms in *E. coli* and that the transfer properties contained within Tn*4399* share an evolutionary relationship with the transfer apparatuses of a wide variety of bacterial systems (2, 8, 14).

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