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Deletion Analysis of the F Plasmid *oriT* Locus

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Functional domains of the *Escherichia coli* F plasmid *oriT* locus were identified by deletion analysis. DNA sequences required for nicking or transfer were revealed by cloning deleted segments of *oriT* into otherwise nonmobilizable pUC8 vectors and testing for their ability to promote transfer or to be nicked when *tra* operon functions were provided in *trans*. Removal of DNA sequences to the right of the central A+T-rich region (i.e., from the direction of *traM*) did not affect the susceptibility of *oriT* to nicking functions; however, transfer efficiency for *oriT* segments deleted from the right was progressively reduced over an 80- to 100-bp interval. Deletions extending toward the *oriT* nick site from the left did not affect the frequency of transfer if deletion endpoints lay at least 22 bp away from the nick site. Deletions or insertions in the central, A+T-rich region caused periodic variation in transfer efficiency, indicating that phase relationships between nicking and transfer domains of *oriT* must be preserved for full *oriT* function. These data show that the F *oriT* locus is extensive, with domains that individually contribute to transfer, nicking, and overall structure.

Conjugative plasmids can transfer chromosomal genes, insertion sequences, and transposons between bacteria, and they are responsible for the spread of antibiotic resistance in the environment (20). The classical F plasmid has been considered a paradigm for one type of conjugation system which has closely related R-plasmid counterparts. In addition to more than 20 *tra* genes required for erection of the pilus and DNA transmission, F and kindred plasmids possess a *cis*-acting locus called *oriT* (29), at which a strand-specific nick is introduced. The nicked strand is then transmitted to the recipient bacterium in the 5′-to-3′ direction, so that the *tra* genes are transferred last (12, 30).

Mechanisms for transfer origin function are being investigated for IncP (9) and IncQ plasmids (23), but the dissection of the *oriT* of F (incompatibility group FI) has not been continued beyond the seminal studies of Thompson et al. (26). Earlier deletion analyses had indicated that *oriT* is confined to a 375-bp *Bgl*II-*Hae*II fragment (7) (from bp 1 to the *Hae*II site depicted at the top of Fig. 2). The DNA sequences of F *oriT* and of *oriT* loci from related Col and R plasmids are similar in the region where nicking occurs, and the loci all contain an 80-bp region that is 80% A+T (8). The *oriT* loci of IncF plasmids contain a number of direct or inverted repetitions that may correspond to locations where *tra* proteins bind. The location of the F *oriT* nick has been remapped (27).

There are reasons to suspect that F *oriT* is a complex locus. First, *traY* and *traI* allele specificities, compared with sequences of related IncF plasmids, suggest that their gene products interact with *oriT* over an extended region (8). Second, locations of intrinsic bends and integration host factor (IHF) binding sites on *oriT* of F (28) suggest that *oriT* might form complex looped or bent structures. Finally, genetic analyses of the *oriT* from the IncQ plasmid R1162 reveal genetically distinct domains (15), which raises the possibility that functional domains might be a general characteristic of *oriT* loci.

We have generated deletions entering F *oriT* from the right, from the left, or internally. We measured the ability of these mutants to be nicked or to sponsor transfer when *tra* gene products are provided in *trans*. A domain required for nicking and regions that affect transfer efficiency to various extents were identified. Internal deletions or insertions near the right boundary of the *oriT* A+T-rich region (91 bp to the right of the nick site) indicate that relative spacing of nicking and transfer domains must be preserved for efficient transfer.

MATERIALS AND METHODS

Bacterial strains and plasmids. Donor strains were RD5042 carrying individual small plasmids having *oriT* or *oriT* deletion variants. RD5042 is RD17 [*thi-1 rel-1* Δ(*pro-lac*)$_{X111}$ λ^- *supE44 recA56* (4)] carrying F42. The F42 plasmid (classical F′ *lac* [13]) supplied the F *tra* functions. Recipient strains were either AB1157 (relevant genotype, *lacY rpsL31 recA*$^+$ [5]) or AB2463 (relevant genotype, *lacY rpsL31 recA13* [11]).

Plasmids are described in Table 1. Plasmid pXRD620 contains a 1,078-bp *Bgl*II fragment from F (includes *oriT*) cloned in the viable orientation into the *Bam*HI site of pUC8 (Fig. 1). Plasmid pXRD608 is pUC8 with the 527-bp *Bgl*II-*Sal*I fragment of *oriT* cloned into the *Bam*HI site (10a, 28). Plasmid pXRD620S was generated from pXRD620 by filling in the *Sal*I site in the polylinker with DNA polymerase I. Transformation of strains with small plasmids was conducted by the procedure of Dagert and Ehrlich (3).

Generation of deletions and insertions. Deletion was accomplished by the exonuclease III (ExoIII)-mung bean nuclease procedure of Henikoff (10) or by using nuclease BAL 31 (21). For deletion from the right, pXRD620 was digested with *Pst*I and *Sal*I (Bethesda Research Laboratories-GIBCO) to generate a linear substrate with the *Pst*I site adjoining the vector sequence at one end and the *Sal*I site (which is cleaved after *oriT* bp 527) at the other. DNA was digested with ExoIII (Bethesda Research Laboratories) for 90 s, and after quenching, a further digestion with mung bean nuclease (Bethesda Research Laboratories) was performed. DNA was then ligated for 22 h and transformed into RD17. Deletions generated by BAL 31 were constructed with pXRD620 or pXRD620S as a substrate. To generate dele-

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TABLE 1. Plasmids[a]

Plasmid	Description
Basic constructs	
pXRD620	pUC8 with 1.1-kb *Bgl*II *oriT* fragment
pXRD620S	pXRD620 with *Sal*I site in polylinker region removed
pXRD608	pUC8 with 527-bp *Bgl*II-*Sal*I *oriT* fragment
Deletions from the right[b]	
pXRD620Δ132	364/
pXRD620Δ21	325/
pXRD620Δ474	321/
pXRD620Δ93	309/
pXRD620Δ174	308/
pXRD620Δ5	287/
pXRD620Δ87	285/
pXRD620Δ112	265/
pXRD620Δ115	252/
pXRD620Δ209	249/
pXRD620Δ104	237/
pXRD620Δ79	222/
pXRD620Δ52	186/
pXRD620Δ270	167/
pXRD620Δ90	138/
Deletions in the middle[c]	
pXRD608Δ48	194/253
pXRD608Δ118	194/235
pXRD608Δ49	197/242
pXRD608Δ41	197/235
pXRD608Δ169	204/233
pXRD608Δ125	212/235
pXRD608Δ27	220/234
pXRD608Δ15	223/236
pXRD608Δ40	222/234
pXRD608Δ12	223/234
pXRD608Δ34	222/230
pXRD608Δ13	223/230
pXRD608Δ109	227/232
Insertions into the *Dra*I site[d]	
pXRD608HM	6
pXRD608H	10
pXRD608HF	14
pXRD608HMB	15
pXRD608HFE	22
pXRD608HFB	26
Deletions from the left[e]	
pXRD620Δ122	/71
pXRD620Δ101	/85
pXRD620Δ213	/99
pXRD620Δ207	/115
pXRD620Δ201	/139
pXRD620Δ103	/145

[a] All plasmids were constructed for this study except pXRD608 (10a).
[b] *oriT* DNA from bp 1 to the stated base pair is retained. /, Novel joint.
[c] *oriT* base pairs immediately adjacent to the deleted material are stated.
[d] Number of base pairs inserted is shown.
[e] *oriT* DNA from the indicated base pair rightward to bp 529 is retained. /, Novel joint.

tions from the right, pXRD620S was digested with *Sal*I to produce a linear molecule cut at *oriT* bp 527. BAL 31 digestion was conducted for 15, 20, or 25 min, and after the reaction was quenched, termini were filled in by using the Klenow fragment of DNA polymerase I. DNA was then ligated with T4 ligase and transformed into RD17 as described above. Deletions from the left were generated similarly by using pXRD620 digested with *Eco*RI. The portions of *oriT* retained in the deletants used in this study are diagrammed in Fig. 2.

Deletions extending from the *Dra*I site at bp 228 of *oriT* were constructed by the ExoIII-mung bean nuclease procedure described above. Because the vector contains three *Dra*I sites, the substrate for the reaction was purified, linear pXRD608 incompletely digested with *Dra*I. ExoIII digestion was allowed to proceed for 20, 40, or 60 s. Plasmids deleted at the desired site were identified by *Dra*I digestion and agarose gel electrophoresis.

Insertion mutations at the *oriT* bp 228 *Dra*I site in plasmid pXRD608 were generated by insertion or modification of commercial linkers (Pharmacia). Plasmid pXRD608H (10-bp insertion) was constructed by insertion of the *Hin*dIII linker 5′-CCAAGCTTGG-3′. Plasmid pXRD608HM (6-bp insertion) was constructed by mung bean nuclease digestion of purified linear pXRD608H DNA generated by incomplete *Hin*dIII digestion. Plasmid pXRD608HF (14-bp insertion) was constructed from purified, linear *Hin*dIII-digested pXRD608H. The *Hin*dIII ends were filled in with the Klenow fragment of DNA polymerase I prior to ligation. Plasmid pXRD608HMB was generated from linearized pXRD608H by digestion of the *Hin*dIII single-strand extensions with mung bean nuclease and insertion of the dodecameric *Bam*HI linker 5′-CCCGGATCCGGG-3′. An 18-bp insertion was expected, but a 15-bp insertion actually resulted. To create pXRD608HFE (contains a 22-bp insertion), pXRD608H was linearized at the *Hin*dIII site, the termini were filled in as described above, and an octameric linker (5′-GGAATTCC-3′) was inserted. Plasmid pXRD608HFB (contains a 26-bp insertion) was constructed similarly, except that the *Bam*HI linker was used instead of the *Eco*RI linker. The identities of the inserts were verified by sequencing.

Identification and characterization of mutants. Ligated deleted plasmids were transformed into RD17 or RD5042, and Amp^r transformants were selected on L agar (19) or MacConkey agar containing ampicillin (100 μg/ml). The plasmid content of purified clones was checked by using sodium dodecyl sulfate (SDS) minilysates (1), and plasmid sizes were approximately determined by comparison with supercoiled size standards (Bethesda Research Laboratories-GIBCO). Mutants were further checked by restriction enzyme digestion. For rightward deletants (generated with ExoIII), only those mutants retaining the polylinker *Hin*dIII site were used. The *oriT* portions of the mutant plasmids were sequenced by modified dideoxy methods (25) with Sequenase (United States Biochemical Corp.) and the M13 sequencing or reverse sequencing pentadecamer primers (New England BioLabs).

Nicking assays. The methods of Thompson et al. (27) were used for nicking assays. Cultures were grown with vigorous shaking to 6×10^8 cells per ml in L broth containing ampicillin (100 μg/ml), and bacteria were pelleted at 15,600 × *g* in a microfuge. Bacteria were lysed with 0.57% SDS–14% glycerol in TAE buffer (0.04 M Tris, 0.02 M sodium acetate, 0.005 M EDTA [pH 8.2]) at 70°C for 10 min. Lysates were centrifuged for 30 min at 15,600 × *g* in a microfuge to pellet most of the chromosomal DNA, and supernatants were collected. Nicked plasmid species were observed after electrophoresis in TAE buffer on 1.2 or 1.5% agarose gels at 3.5 V/cm for 5 h.

When required, nicking was quantified by densitometry of photographic negatives as follows. Before staining, DNA on

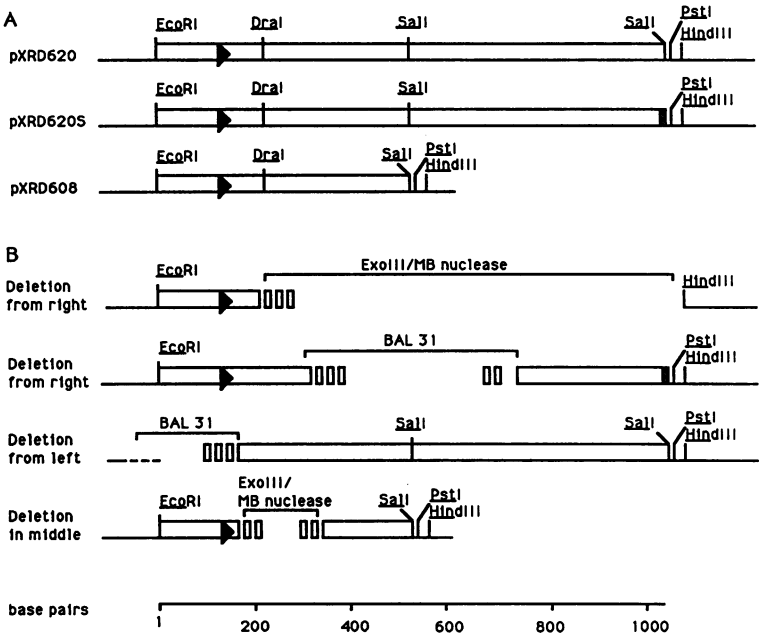


FIG. 1. (A) Plasmids used for deletion analysis and (B) locations and methods of producing deletions. Vector DNA is shown as a single line, and inserted DNA containing *oriT* is shown as an open bar. The scale line below applies to both panels. The solid triangle represents the *oriT* nick site. In panel B, portions of DNA removed are indicated by a bracket labeled with the nuclease used (MB, mung bean). Broken lines or bars indicate variable amounts of material removed. The hatched box at the right of the pXRD620S insert represents the location of the *SalI* site that was destroyed.

gels was randomly nicked with UV light at 254 nm (2 min; C61 transilluminator; UV Products, San Gabriel, Calif.) so that all species would be topologically unconstrained. After staining with ethidium bromide (0.5 μg/ml), gels were photographed (Kodak Plus-X film) beside a step density tablet (Kodak no. 2) illuminated by the fluorescence of white paper. Densitometry of the tablet and of bands on the negative allowed determination of the amount of DNA present in each band (24).

Quantitative matings. Donors were RD5042 carrying the pXRD620 mutants, and recipients were AB1157. Matings were conducted at 37°C at donor/recipient ratios of 1:10 and total cell concentrations of 1×10^8 to 2×10^8 cells per ml. Donors were not vigorously agitated prior to mating. Mat-

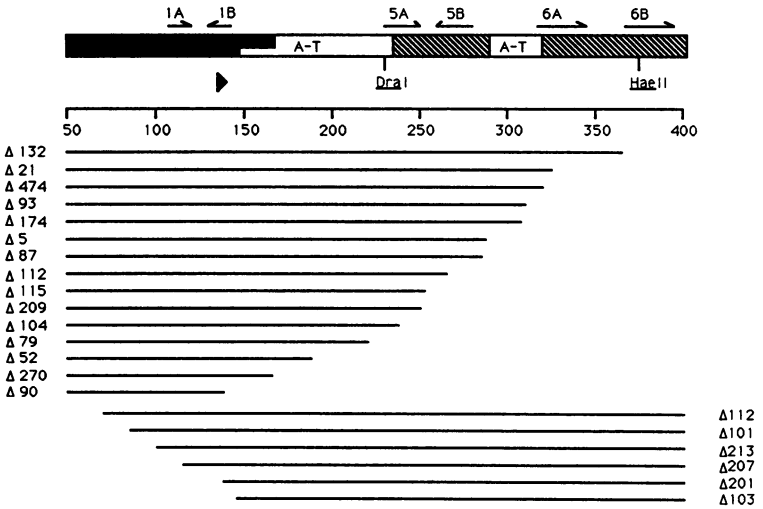


FIG. 2. *oriT* DNA retained in mutants deleted from the right (upper set of 15 mutants) or from the left (lower set of 6 mutants). Exact deletion endpoints are presented in Table 1. Structural features of *oriT* are shown in the diagram at the top. The shaded area represents DNA of F that is conserved in *oriT* loci of related IncF plasmids. Open areas represent A+T-rich sequences, and hatched areas indicate other sequence blocks unique to F. Some repeated sequences, numbered as shown in Ippen-Ihler and Minkley (12), are shown above the bar. The position of the nick is indicated by the solid triangle. The coordinate scale indicates the number of base pairs clockwise of the *BglII* site at kb 66.7 on the F map. The convention used here designates the first T in the AGATCT *BglII* recognition sequence as bp 1 (26, 30). (Other laboratories designate the first A as bp 1 [8].)

ings were conducted for 30 min or 1 h and were interrupted by vigorous agitation for 1 min with a Vortex mixer after 100-fold dilution in ice-cold minimal medium (2). Further-diluted samples were spread on MacConkey-lactose plates supplemented with streptomycin (100 μg/ml) (to contraselect donors) and ampicillin (100 μg/ml) (to select for transmission of the small plasmid).

Transmission frequencies (f) for each *oriT* plasmid were calculated as number of Amp^r transconjugants divided by the number of $F42^+$ transconjugants. $F42^+$ transconjugants were identified as red colonies on MacConkey-lactose plates containing streptomycin sulfate (100 μg/ml). The transmission frequency (f_0) of pXRD608 (contains wild-type *oriT*) was determined (on the same day in most cases). The transfer efficiency for each mutant was defined as f/f_0. At least three matings were performed for each mutant, and the geometric means of the transfer efficiencies were calculated.

Transfer efficiencies for mutants carrying mutations at the *Dra*I site at bp 228 were fit to the function $\log f/f_0 = -x/A + B\{\cos[(360° \cdot x)/C] - 1\}$ by trial and error, where x is the number of base pairs inserted or deleted at the *Dra*I site, and A, B, and C are adjustable parameters. Values of C were varied until the root-mean-square deviations of the data from the values predicted by the function were minimized.

RESULTS

Boundaries of *oriT* and sequences affecting transfer. Deletions extending into *oriT* from the left (i.e., bp 1 in the *Bgl*II site at 66.7 kb on the F map) toward the nick site (after bp 137) were generated with BAL 31 (Fig. 2, lower portion). Removal of bp 1 to 84, 1 to 98, or 1 to 114 did not alter the transfer efficiency of pUC8 vectors containing these deleted *oriT* segments (data not shown), and these deletants were fully susceptible to nicking (see Fig. 6B). Removal of bp 1 to 138 (plasmid pXRD620Δ201) destroyed both nicking and transfer functions, as expected, since the site of nicking was deleted.

Deletions extending into *oriT* from the right (Fig. 2, upper portion) were generated by treatment with ExoIII and mung bean nuclease or with BAL 31. Effects on transfer are shown in Fig. 3. A deletant retaining bp 1 to 364 was transferred at wild-type levels, but transfer efficiencies for deletants whose left endpoint fell near bp 325 were reduced approximately 10-fold. Removal of an additional 16 bp to the left of bp 325 reduced transfer efficiency by an additional 10- to 20-fold. The interval from bp 285 to 309 could be deleted without further affecting transfer efficiency. Removal of DNA between bp 284 and 250 caused a further 100-fold decrease in transfer efficiency. The two regions with particularly large decreases in transfer efficiency (bp 309 to 325 and 250 to 285) may correspond to specific sequence segments needed for transfer. Plasmids pXRD620Δ104 and pXRD620Δ79 each showed low levels of residual transfer efficiency (1.35×10^{-4}), which differed significantly from the background transfer efficiency of pUC8 (1.25×10^{-5}). The transfer efficiency of plasmid pXRD620Δ52 (retains bp 1 to 186) was not significantly different from background.

Characterization of plasmids after conjugal transfer. The sizes of transferred plasmids were estimated for plasmids deleted from the right (pXRD620Δ52, pXRD620Δ79, pXRD620Δ104, and pXRD620Δ87) or from the left (pXRD620Δ101, pXRD620Δ122, pXRD620Δ207, and pXRD620Δ213) (Table 2). The plasmids deleted from the right showed 100- to 10,000-fold defects in their ability to be transferred, and we reasoned that the accuracy of circularization after transfer might have been affected, leading to aberrant transfer products. The sizes of supercoiled plasmids from donor or transconjugant colonies were determined electrophoretically. No deletants were detected for any of the mutant plasmids (resolution, ±50 bp; data not shown). Thus, at this level of resolution, the *oriT* loci deleted from the right did not generate further sequence abnormalities during or after transfer; the fidelity of nicking or circularization after transfer appeared not to be affected by these mutations. However, both donor and transconjugant clones containing

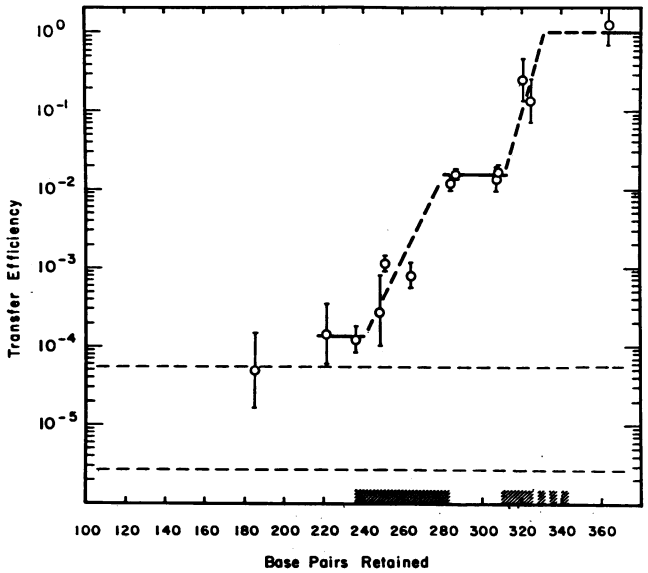
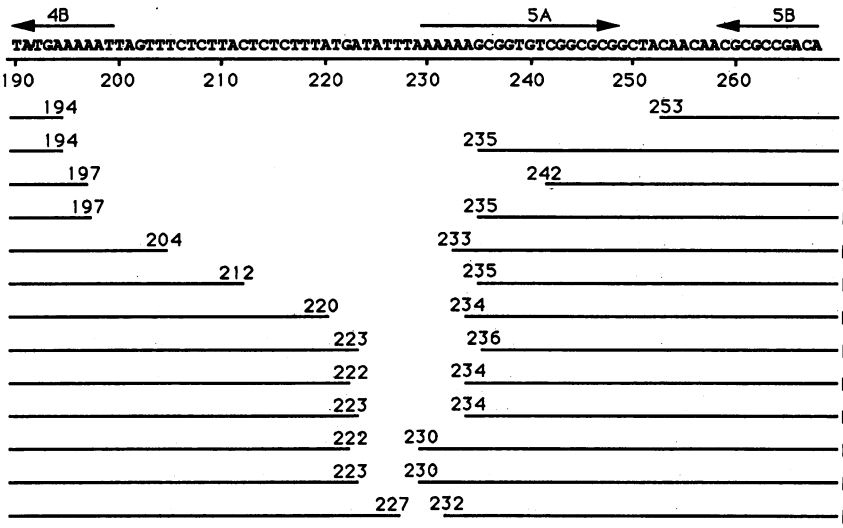


FIG. 3. Transfer efficiencies for plasmids containing *oriT* segments that have been progressively deleted from the right. Coordinate scale (bottom) indicates the number of base pairs retained in the mutants. Error bars correspond to standard deviations from the geometric means, which are indicated by circles. Dashed lines at the bottom are upper and lower standard deviations for the transfer efficiency of pUC8 (vector, no *oriT*). Heavy solid and broken lines represent one interpretation of the data (see text). Hatched regions beside the coordinate scale denote regions containing sequence elements that strongly affect transfer efficiencies.

TABLE 2. Fraction of donor or transconjugant clones containing predominantly monomeric *oriT* plasmids[a]

Plasmid	No. of clones with monomeric plasmids/no. tested	
	Donor	Transconjugants
pXRD620 (wild type)	12/12	24/24
Deletions from the right		
pXRD620Δ87	19/19	30/32
pXRD620Δ104	17/18	25/36
pXRD620Δ79	24/24	28/34
pXRD620Δ52	22/23	0/24
Deletions from the left		
pXRD620Δ207	22/24	5/24
pXRD620Δ213	24/24	2/24
pXRD620Δ101	12/12	3/17
pXRD620Δ122	11/12	3/24

[a] Plasmids were obtained from matings with AB2463, a *recA* strain otherwise isogenic with AB1157, the recipient used in quantitative mating experiments. Clones that did not contain predominantly monomers contained dimers, other multimers, and uncharacterized size classes.



Plasmid	Nicking Proficiency	Transfer Efficiency
pXRD608Δ48	-	0.00015
pXRD608Δ118	-	0.00014
pXRD608Δ49	-	0.00026
pXRD608Δ41	-	0.024
pXRD608Δ169	-	0.0036
pXRD608Δ125	+	0.032
pXRD608Δ27	+	0.20
pXRD608Δ15	+	0.11
pXRD608Δ40	+	0.10
pXRD608Δ12	+	0.12
pXRD608Δ34	+	0.020
pXRD608Δ13	+	0.019
pXRD608Δ109	+	0.35

FIG. 4. Deletions in the vicinity of bp 228 and their phenotypes. Span lines indicate DNA retained, with the base pairs flanking the deleted DNA indicated. Nicking proficiency and transfer efficiency for each mutant are shown at the right. The actual DNA sequence in this region (26) and repeated sequence features (labeled as in reference 12) are also shown.

pXRD620Δ104 contained somewhat higher amounts of small plasmid dimer than did clones containing pXRD620.

Transconjugants from matings with donors containing pXRD620Δ52 (this plasmid is not nicked [see below] and exhibits the same transfer efficiency as the pUC8 vector) contained no monomeric plasmids. Instead, transconjugants contained 8- to 9-kb plasmids, which have not yet been characterized. F42 contains Tn*1000*, which can transpose into pXRD620Δ52 to form cointegrates. If the cointegrates are transferred to the recipient and resolved, small plasmids having the observed, augmented size would be produced.

The plasmids deleted from the left (up to bp 114) were transferred at normal frequencies. These plasmids appeared predominantly as monomers in the donors. However, after mating of donors containing small plasmids deleted from the left, a large proportion of transconjugants contained larger multicopy plasmids. The sizes of the larger plasmids were integral multiples of the monomer sizes. Some clones contained multicopy plasmids of both monomeric and dimeric sizes in various proportions. Thus, deletions up to bp 114 did not affect transfer efficiencies, but they did alter the nature of the transferred products.

Effects of deletions and insertions near the right boundary of the major A+T-rich region. Deletants retaining only bp 1 to 222 had lost nearly all transfer proficiency. To assess the requirement for the A+T-rich region for transfer or nicking and to determine whether there were additional elements lying to the left of bp 222 that affect transfer, a set of internal deletions originating at the *Dra*I site at bp 228 were constructed. This is near the right boundary of the 80-bp A+T-rich sequence common to IncF plasmids and is adjacent to a prominent, extensive inverted repetition (feature 5 [12]) (Fig. 2). Deletions were created by using ExoIII in combination with mung bean nuclease. The rationale was that there would be sufficient asynchrony in degradation to the right and the left of the *Dra*I site to generate unidirectional deletions.

Deletions were usually bidirectional, with endpoints at G · C base pairs (Fig. 4). The clustering of endpoints near G · C base pairs provided sets of deletions extending short distances to the right from bp 222 to 223 and to the left from bp 233 to 236. Plasmid pXRD608Δ27 (bp 221 to 233 deleted) was transferred at 0.2 times the efficiency of the parent plasmid, pXRD608. This indicates that removal of the outside 5 bp of sequence feature 5A did not drastically reduce transfer. Deletions extending from bp 233 to 235 leftward beyond bp 221 showed further loss of transfer efficiency, so that pXRD608Δ41 (deleted from bp 198 to 234) was transferred only 1/100th as well as the wild type. Deletion of only 3 bp more to the left (deletion from bp 195 to 234) caused a further 100-fold drop in transfer efficiency.

Deletions of 6 to 7 bp reduced the transfer efficiency 50- to 100-fold, but deletions of 12 or 13 bp only caused a 5-fold reduction (Fig. 4). This suggested that the transfer efficiency might vary periodically as a function of the amount of DNA deleted from the vicinity of the *Dra*I site. This type of behavior is sometimes seen for composite loci for which phase relationships between constituent parts are important. To explore this hypothesis, we introduced linker insertions at the *Dra*I site at bp 228, reasoning that these would also cause periodic changes in transfer efficiencies. The results for the deletions and linker insertions are plotted together in Fig. 5.

The transfer efficiencies for the six linker insertion mutants examined also are consistent with periodic variations. The period was estimated by fitting the pooled data for deletions and insertions to the function $\log f/f_0 = -x/A + B\{\cos[(360° \cdot x)/C] - 1\}$, where f and f_0 are the transfer frequencies for mutant and wild-type *oriT*, respectively, x is the number of base pairs deleted or inserted, and A, B, and C are adjustable parameters. f/f_0 is called the transfer efficiency. This empirical function, which was devised to allow quantitative analysis of periodicity, asserts that transfer efficiency drops exponentially with the amount of DNA deleted or inserted (first term) with a superimposed periodic variation having a period of C bp. It is seen (Fig. 5) that A = 19, B = 0.7, and C = 12.25 provide a good representation of the data for insertions as large as 26 bp and deletions as large as 28 bp, a 54-bp range. Other values for A (17 and 18 bp) give poorer fits to the data. C = 11.5, 12, or 12.5 produced poorer fits to the data. Further experiments, including a

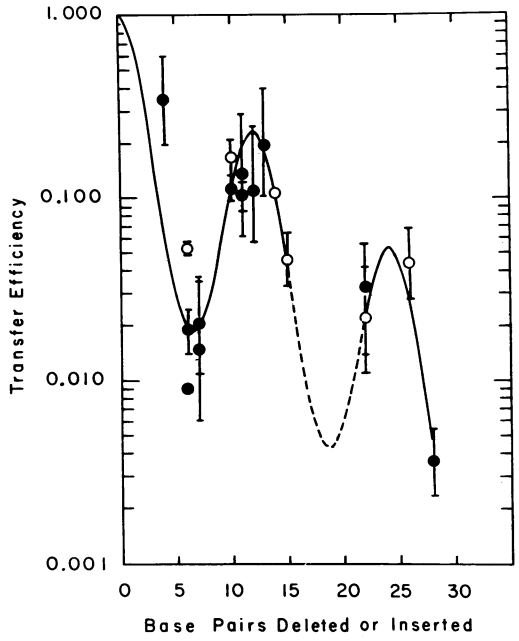


FIG. 5. Transfer efficiencies for *oriT* plasmids containing small deletions or insertions in the neighborhood of bp 228. Solid circles denote deletion mutants, and open circles denote insertion mutants. Geometric means are plotted, and error bars correspond to standard deviations from the geometric mean. Solid line corresponds to the periodic function described in the text. Broken line is the same function in a region for which no data were available.

larger collection of insertions, will be required for more accurate determination of the period.

Region of *oriT* required for nicking. Deletions extending into *oriT* from the right or from the left were tested for susceptibility to nicking by the SDS minilysate procedure (27). As shown in Fig. 6, removal of bp 1 to 114 did not affect *oriT* nicking, and removal of DNA from the right as far as bp 222 did not abolish nicking functions, even though transfer for the latter deletants was reduced by as much as 10^4-fold. Deletion of DNA down to bp 186 destroyed all susceptibility to nicking. Comparison of the nicking data with the transfer data in Fig. 3 shows that there are transfer-associated regions of *oriT* that do not participate in nicking.

Plasmids containing *oriT* deletions that originated at the *Dra*I site (bp 228) were nicked less well than the wild type. No mutants deleted beyond bp 212 displayed nicked species in the electrophoretic assay, although the transfer efficiencies of pXRD608Δ48 and pXRD608Δ118 suggest that nicking still occurs at low levels when *oriT* is deleted down to bp 194. These data show that DNA between bp 115 and 212 contains the major nicking determinants. Consistent variations in the amounts of nicked species for progressively larger internal deletions suggested periodicity, but the assay was considered insufficiently sensitive for quantitative analysis.

DISCUSSION

These results establish the presence of at least two functional domains for *oriT*: one for nicking, and one which affects transfer without affecting nicking (Fig. 7). There may be others still not identified.

The nicking domain. DNA from within feature 1A (bp 115) to bp 212 is sufficient for nicking. This region includes the nick site (after bp 137, lower strand), an IHF binding site (bp 165 to 195), and an intrinsic bend associated with two in-phase poly(A) tracts (bp 158 to 172 [28]). The abundance of nicked species is only slightly affected by removal of DNA outside this region. The region includes much of an A+T-rich region (bp 149 to 234, 80% A+T), which genetic and sequence comparisons suggest may interact with the *traY* protein (8). IHF binding site A may be needed for nicking, since an interior deletion (bp 205 to 232) approaching its right margin showed no nicked species. The position of this IHF binding site may imply protein-protein interactions between gene products bound at the nick site and to the right of IHF site A, in the A+T-rich region (28).

We had expected that features 1A and 1B would be required for nicking, since the nick site lies in feature 1B. However, we determined that approximately half of feature 1A can be removed without affecting nicking or transfer. *oriT* point mutations at bp 141 and 146 prevent nicking and reduce transfer by 9- to 200-fold (26). These mutations lie in or immediately adjacent to feature 1B, suggesting that it constitutes at least part of the recognition site for nicking. This suggestion is supported by the present results with deletion mutations.

The transfer domain. The trends in transfer efficiency for DNA deleted from the right delineate sequences important for transfer. Extending from bp 235 to 360, this segment contains an intrinsic bend at approximately bp 265 and an IHF binding site (site B, bp 285 to 305) (28). Removal of DNA up to bp 364 caused no reduction in transfer efficiency, but further deletion to bp 325 reduced transfer efficiency by a factor of 10. The former deletion removed feature 6B, while the latter removed both 6A and 6B. One interpretation is that at least one copy of the 6A or 6B direct repetition is sufficient for full transfer activity. Deletions extending to bp 309 caused a further reduction in transfer efficiency by another factor of 10. This removes half of a sequence block that contains A or T at 29 of 30 bases and removes DNA required for binding of IHF to site B (28). It is possible that the entire effect of deletions up to bp 308 may result from damage to the IHF binding site. Deletions between bp 308 and 285 caused no further reduction in transfer.

Deletions farther than 285 (up to bp 237) caused a further drop in transfer efficiency by a factor of 10^{-2}. These deletions remove the prominent sequence features 5A and 5B. Deletions extending into feature 5A (bp 229 to 248) from the left indicate that removal of the left-most 7 bp causes a loss of 80% of the transfer efficiency. Removal of an additional 5 bp (to bp 241) more severely reduces transfer efficiency. These data strongly implicate features 5A and 5B as an important sequence element for transfer.

Possible long-range interactions. Based on genetic considerations, the central A+T-rich segment has been considered a likely location for binding of *traY* protein. In addition, our results suggest a likely structural role. Deletions emanating from the *Dra*I site at bp 228 and insertions at this site may conceivably have altered a protein-binding site. However, the partial restoration of function if deletions or insertions are enlarged from 6 to 12 bp is hard to reconcile with this simple interpretation. The transfer efficiencies for a 4-bp deletion (0.35) and the similar transfer efficiency for a 13-bp deletion (0.20) suggest that the immediate vicinity of bp 228 is not an essential, highly specific protein-binding site. Instead, deletions or insertions give a pattern of increases and decreases suggesting that (i) transfer efficiency is reduced if the size of the A+T-rich region is increased or

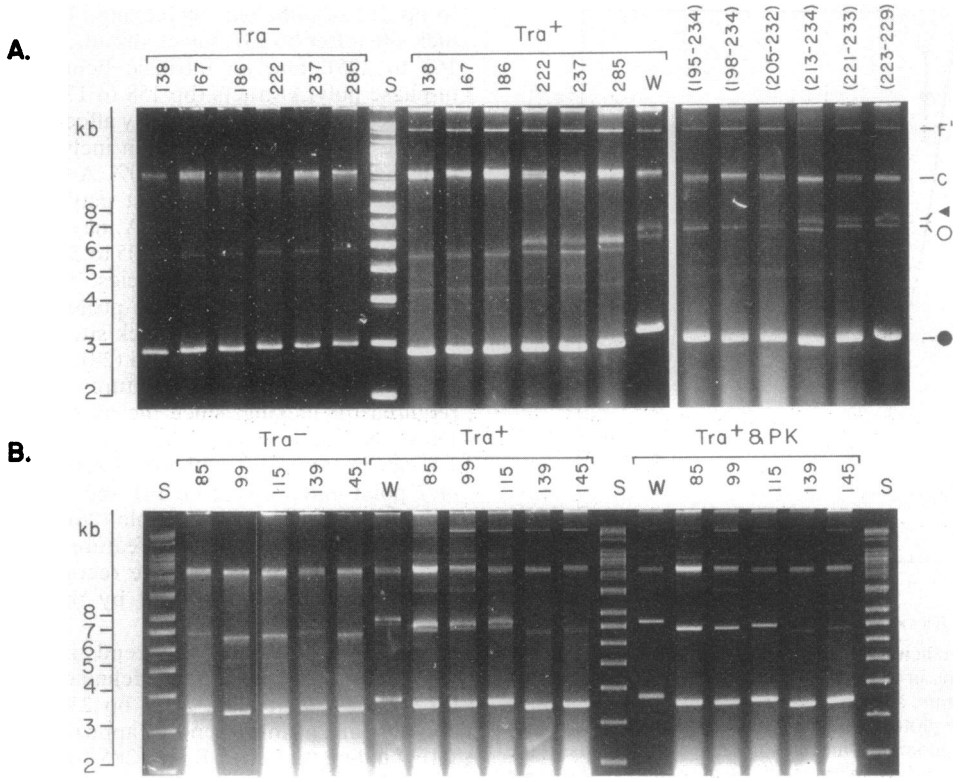


FIG. 6. Electrophoretic assay for nicking proficiency of *oriT* deletion mutants. Supercoiled (solid circle), relaxed (open circle), retarded, specifically nicked species (solid triangle), chromosomal DNA (C), and F42 supercoiled DNA (F') are identified for the samples to the right of panel A. Similar species are shown in panel B. The Tra phenotype for experiments with plasmids deleted from the left or from the right are indicated. Lanes S contained supercoiled size standards (sizes shown at left of each panel). Lanes labeled W contained pXRD608 (Fig. 1; *oriT* considered to be wild type). (A) Deletions extending into *oriT* from the right or starting at the *Dra*I site (bp 228; right part of panel). Samples for deletants at bp 228 correspond to Tra$^+$ conditions. The numbers above each lane indicate the last base pair retained for deletions from the right. The numbers in parentheses indicate the range of base pairs deleted for deletions from the *Dra*I site. (B) Deletions extending into *oriT* from the left. The numbers above each lane indicate the first *oriT* base pair appearing beyond (to the right of) the deletion endpoint. The six samples at the right of panel B were treated with proteinase K (PK) before being applied to the gel. The retarded species formed under Tra$^+$ conditions (panel B, center) are destroyed after proteinase K treatment, and more open circular DNA appears, verifying that the retarded species is a protein-DNA adduct.

decreased and (ii) phasing between sites to the left of bp 213 and to the right of bp 235 is important. Transfer is thus dependent on proper positioning of the nicking domain (leftward of bp 213) relative to a portion of the transfer domain. The period (~12 bp) is larger than the expected range for DNA (10.5 to 11 bp), which may reflect experimental error or possibly alterations in DNA pitch arising from bound proteins. We speculate that the nick site (bp 137) must be phased properly with respect to features 5A and 5B for efficient transfer. According to this speculation, the

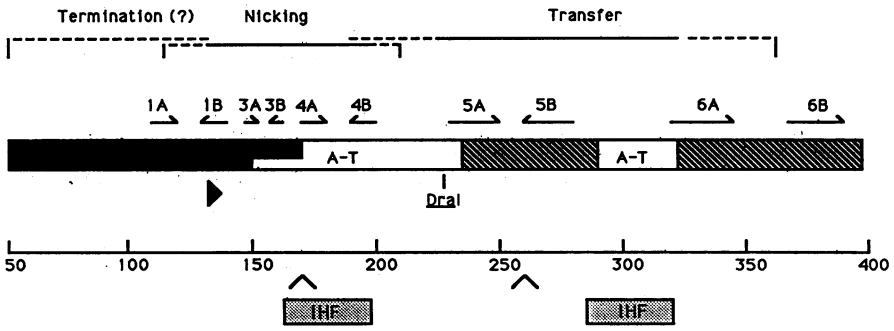


FIG. 7. *oriT* sequences required for transfer and nicking. Sequence features are designated as in Fig. 2. The locations of intrinsic bends (carets) and IHF binding sites (shaded boxes) are shown below. The extents of functional domains inferred from deletion mutants are shown at the top. Broken lines indicate regions where the extents are uncertain.

A+T-rich region is "looped" or "bent," and its role, in part, would be structural. The inclusion of IHF site A in this region is consistent with this speculation.

It is obvious that the distance between sites in these domains is also important; 20-bp insertions or deletions reduce transfer efficiency 10-fold. This large effect may arise because the spacing between the postulated sites (100 to 200 bp) is less than the Kuhn statistical segment length for DNA (300 bp [18]), hindering juxtaposition because of stiffness of the helix.

Left boundary of *oriT*. Deletions approaching the nick site from the left and up to bp 114 appear not to have an adverse effect on transfer efficiency. Deletions from the left (unlike most deletions from the right) do, however, alter the nature of the transferred products; dimers and higher-order multimers are the major species found in the recipient. Formation of multimers might reflect reduced efficiency in termination. This might not be reflected in the overall transfer efficiency, because continuation of transfer beyond the normal termination site would allow additional opportunities for termination after two, three, or more tandem copies have been introduced into the recipient.

These results may indicate that the region to the left of the nick is involved in termination (Fig. 7). However, most of the deletions from the left affect the gene X promoter, and we cannot rule out the possibility that these deletions allow enhanced transcription across *oriT* from the *lac* promoter on pUC8. Such transcription might interfere with termination.

Comparisons with other nicking loci. Other plasmid or phage loci have structures or confer phenotypes similar to those that we have observed for F *oriT*. The phage f1 origin is composed of two domains, a nicking domain and a replication enhancer domain (6, 14). The enhancer domain is approximately 70% A+T. Our data suggest that bp 213 to 234 of *oriT* (the right-hand fourth of the 84-bp central A+T-rich region in F) functions as a needed spacer. It remains to be seen whether this or the remainder of the A+T-rich block in F serves as an enhancer for nicking or transfer. The A+T-rich block shared by several IncF plasmids (8, 22) is not a general feature of *oriT* regions if other incompatibility groups are considered. For example, if the most A+T-rich regions of R64 *oriT* are deleted, 86% of the transfer efficiency is retained (16).

The *oriT* loci of plasmids from other incompatibility groups also show a modular structure. The RP4 *oriT* (IncP incompatibility group), which does not have a prominent A+T block like that of F, nevertheless has the region for nicking separated from a region responsible for efficient mobilization (9). As with F, the termination functions for *oriT* of R1162 (IncQ) are distinguishable from initiation functions (15).

Modular sequence organization may reflect the need for several biochemical steps in *oriT* function (e.g., nicking, loading of helicases, presentation of the nicked strand to a structure at the point of exit from the donor cell, termination of transfer, and religation), or it may reflect structural motifs associated with the DNA at different steps (e.g., nicking may proceed on supercoiled duplex DNA, whereas one or more termination steps may involve single-strand intermediates.) As purified proteins become available (17), further dissection of these molecular machines will be facilitated.

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