Conjugating plasmids are preferred targets for Tn7

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Most transposons display target site selectivity, inserting preferentially into sites that contain particular features. The bacterial transposon Tn7 possesses the unusual ability to recognize two different classes of target sites. Tn7 inserts into these classes of target sites through two transposition pathways mediated by different combinations of the five Tn7-encoded transposition proteins. In one transposition pathway, Tn7 inserts into a unique site in the bacterial chromosome, *attTn7*, through specific recognition of sequences in *attTn7*; the other transposition pathway ignores the *attTn7* target. Here we examine targets of the non-*attTn7* pathway and find that Tn7 preferentially inserts into bacterial plasmids that can conjugate between cells. Furthermore, Tn7 appears to recognize preferred targets through the conjugation process, as we show that Tn7 inserts poorly into plasmids containing mutations that block plasmid transfer. We propose that Tn7 recognizes preferred targets through features of the conjugation process, a distinctive target specificity that offers Tn7 the ability to spread efficiently through bacterial populations.

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Transposition is a DNA rearrangement resulting in the insertion of a mobile DNA element at various positions in the genome of a host cell. Although insertions may occur into different places in the genome, many transposons insert selectively into sites containing particular features that are recognized specifically by the transposition machinery. Target-site selectivity can benefit both the host and the transposon by minimizing random insertions throughout the genome and thus reducing the possibility of deleterious insertions into essential genes.

What features can transposons use to distinguish preferred target sites in the DNA of the host? Often, sequence recognition directs transposition into preferred sites. For example, hotspots for Tn10 transposition share a consensus target sequence recognized by the Tn10 transposase (Halling and Kleckner 1982; Lee et al. 1987). Alternatively, the transposition machinery may recognize host proteins associated with particular sites in the host genome. The retrotransposon Ty3 inserts preferentially into the promoters of RNA polymerase III-transcribed genes (Chalker and Sandmeyer 1990, 1992). RNA polymerase III transcription factors positioned on the promoter DNA recruit the Ty3 integration complex to this transposition target, probably through direct protein-protein interactions (Kirchner et al. 1995). Transposons like Ty3, which collaborate with host factors to identify preferred target sites, are particularly interesting

for their ability to target transposition to the sites of biological processes such as transcription. Transposons can also insert selectively into target sites that lack obvious sequence similarity, but contain particular DNA structures. For example, some retroviruses insert preferentially into nucleosomal DNA at sites of DNA bending (Pryciak and Varmus 1992; Müller and Varmus 1994). Another theme of target selection is exhibited by transposons that avoid inserting into particular genomic regions. For instance, several bacterial transposons avoid transposition into actively transcribed DNA (Casadesu and Roth 1989; Wang and Higgins 1994). These examples show that transposons can possess many different levels of target recognition. Thus, although transposons may insert into many different regions in the host DNA, close investigation often reveals that common features attracted the transposon to each of these sites.

The bacterial transposon Tn7 displays particularly interesting targeting characteristics. Tn7 can insert into two distinct classes of target sites, and each class of Tn7 targets is selected by one of two alternative transposition pathways. These transposition pathways are mediated by different, but overlapping, combinations of the five Tn7-encoded transposition genes, *tnsABCDE* (Rogers et al. 1986; Waddell and Craig 1988; Kubo and Craig 1990; for review, see Craig 1996). One transposition pathway inserts Tn7 efficiently into *attTn7*, a unique site contained in the chromosomes of *Escherichia coli* and several other bacteria (Barth et al. 1976; Lichtenstein and Brenner 1981; Craig 1991). In contrast, the alternative

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Tn7 transposition pathway ignores *attTn7* and directs Tn7 to many different sites.

The Tn7-encoded genes required for transposition into *attTn7* are *tnsABC+D* (Rogers et al. 1986; Waddell and Craig 1988; Kubo and Craig 1990). Tn7 transposition into an *attTn7*-containing target has been reconstituted in vitro from purified proteins, enabling biochemical dissection of this reaction (Bainton et al. 1991, 1993). TnsD is a sequence-specific DNA-binding protein that recognizes DNA sequences contained in *attTn7* (Waddell and Craig 1989; Bainton et al. 1993). TnsD binds to *attTn7* and then recruits the remaining components of the Tn7 transposition machinery, TnsA, TnsB and TnsC, to this target site. A hallmark of Tn7 transposition is its remarkable dependence on the target site: The DNA breakage and joining events constituting the chemical steps of transposition do not occur until an appropriate target has been located (Bainton et al. 1991; A.E. Stellwagen and N.L. Craig, in prep.). The TnsD-*attTn7* complex can mark the target site for TnsC, which then recruits TnsA and TnsB to this target site. Together, TnsA and TnsB perform the chemical steps of the transposition reaction, removing Tn7 from the donor site and inserting Tn7 into the target site (M.C. Biery, M.A. Lopata, and N.L. Craig, in prep.; Sarnovsky et al. 1996).

The non-*attTn7* pathway for Tn7 transposition is mediated by *tnsABC+E* (Rogers et al. 1986; Waddell and Craig 1988; Kubo and Craig 1990) and displays strikingly different target site selectivity from the *tnsABC+D* transposition pathway. Although *tnsABC+D*-promoted transposition is highly selective for a particular target sequence, the *tnsABC+E* pathway inserts Tn7 into targets that share no obvious sequence similarity (Kubo and Craig 1990). However, TnsA, TnsB and TnsC are required for both the TnsD- and TnsE-mediated transposition pathways, suggesting that Tn7 insertions into each class of target sites occurs by the same overall transposition mechanism.

In this work, we show that the *tnsABC+E* transposition pathway preferentially inserts Tn7 into a distinctive class of bacterial plasmids that possess the ability to propagate through bacterial populations by conjugation. Plasmid conjugation is a special type of DNA replication during which one strand of the plasmid duplex is transferred into a recipient cell. Prior to plasmid transfer, a plasmid-encoded protein specifically nicks the origin of transfer, *oriT*, whereupon a DNA helicase separates the plasmid strands. Mating begins when a conjugal pore forms between two cells, and the nicked plasmid strand passes through this pore into the recipient cell. DNA replication, mediated by host proteins, completes the conjugation process by synthesizing the missing strands in the donor and recipient cells (for review, see Lanka and Wilkins 1995). We have found that the *cis*- and *trans*-acting factors necessary for plasmid transfer are also required for preferential Tn7 transposition into conjugable plasmids. Our results thus suggest that components of the conjugal replication process attract Tn7 to these plasmids. The ability of Tn7 to insert preferentially into conjugable plasmids provides a strategy for

rapid dissemination of Tn7-encoded genetic information through bacterial populations.

Results

The tnsABC+E pathway inserts Tn7 preferentially into conjugable plasmids

It was shown previously that the *tnsABC+E* pathway can insert Tn7 into the *E. coli* chromosome at many different sites that are apparently unrelated in structure or sequence (Kubo and Craig 1990). To further characterize the targets of the *tnsABC+E* pathway, we have examined Tn7 transposition in cells containing different bacterial replicons. Independent Tn7 insertions were selected in vivo using a λ hop assay for transposition. In the λ hop assay, a miniTn7 element, containing a gene conferring resistance to kanamycin (Km), transposes from a replication- and integration-defective λ phage into a stable replicon in cells that express Tn7 transposition proteins (McKown et al. 1988). Transposition events are selected as colonies on LB + Km plates and the frequency and distribution of Tn7 insertions is determined from independent λ infections. The transposition frequency is determined as the number of Km-resistant colonies arising per plaque forming unit (PFU) of infecting λ phage.

When we expanded the survey of Tn7 insertions, we were surprised to find that two plasmids stand out as highly preferred targets for the *tnsABC+E* pathway (Table 1). These plasmids, R388 and a derivative of the F plasmid called pOX-G, share the ability to conjugate between bacterial cells. In cells containing either pOX-G (60 kb in size) or R388 (33 kb), Tn7 inserts predominantly into these plasmids, virtually ignoring the *E. coli* chromosome (4700 kb). Furthermore, the presence of pOX-G stimulates the overall frequency of Tn7 transposition ~50-fold, from $[(0.34 \pm 0.16) \times 10^{-6}]$ to $[(15 \pm 6) \times 10^{-6}]$ -Km-resistant colonies/PFU. The much larger bacterial chromosome is a relatively poor target for the *tnsABC+E* pathway, as are two nonconjugating plasmids, the bacteriophage P1 lysogen, which exists as a large, single copy plasmid, and pBR322, a small, multi-copy plasmid. We have also observed that pOX-G is a preferred target for *tnsABC+E*-mediated transposition using a promoter capture assay to select transposition events (data not shown). Only the *tnsABC+E* pathway preferentially inserts Tn7 into conjugable plasmids; the frequency and distribution of *tnsABC+D*-mediated Tn7 insertions was not changed in cells containing pOX-G or R388 (data not shown).

Tn7 inserts in only one orientation into many different sites on pOX-G

We characterized the target sites of the *tnsABC+E* pathway by isolating pOX-G plasmids containing independent Tn7 insertions and determining the position and orientation of Tn7. No single position on pOX-G was an insertion hot spot; instead, Tn7 had inserted into many different sites on the plasmid (Fig. 1). Alignments of the

Table 1. The distribution of Tn7 insertions mediated by *tnsABC* + *E*

Replicon	Size ^a	Tn7 on plasmid ^b	Tn7 on chromosome ^c	Transposition frequency ^d
pOX-G	60	44/45	1/45	15 ± 6
R388	33	11/20	9/20	0.75 ± 0.37
P1	90	0/25	25/25	0.49 ± 0.30
pBR322	4	0/34	34/34	0.73 ± 0.33
Chromosome	4700	—	28/28	0.34 ± 0.16

^aReplicon size in kb.^b[Tn7 insertions into plasmid]/[total insertions examined].^c[Tn7 insertions into chromosome]/[total insertions examined].^dMean observed transposition frequency ($\times 10^6$) ± SEM; transposition frequency is defined as the number of transposition events (Km resistant colonies) per PFU; mean determined from ≥25 independent infections for each.

Tn7 target sites were performed, using 100 bp of the pOX-G sequence immediately flanking each Tn7 insertion at both ends of the Tn7 element. A consensus target sequence was not discernible at or near the point of Tn7 insertion, suggesting that Tn7 does not recognize a specific target sequence within pOX-G. However, Tn7 displays regional specificity within pOX-G, inserting preferentially into two regions of the 60-kb pOX-G plasmid. Nine Tn7 insertions (50% of those examined) were clustered within a 2-kb region near *oriT*, and five Tn7 insertions (28% of those analyzed) were located within a 3-kb region at the 3' end of the *tra* operon. For a fraction of the isolated insertions (~20%), we sequenced DNA immediately flanking both ends of the Tn7 element, and found that *tnsABC* + *E*-mediated transposition results in five base-pair target site duplications. Tn7 insertions into *attTn7*, mediated by *tnsABC* + *D*, also create five base-pair target site duplications, consistent with the suggestion that Tn7 transposition into both classes of target sites occurs by the same overall mechanism.

The most striking characteristic of Tn7 insertions into pOX-G is their extreme orientation bias. Every insertion into pOX-G was oriented such that, if conjugation were to occur, the right Tn7 end would enter the recipient cell before the left Tn7 end. Thus, although Tn7 can insert into many different sites on pOX-G, Tn7 inserts into these sites in a single orientation. Earlier reports have also described orientation-specific Tn7 insertions into other conjugable plasmids (Barth and Grinter 1977; Krishnapillai et al. 1984; Rogers et al. 1986). Tn7 also inserts into *attTn7* with a striking orientation bias (Lichtenstein and Brenner 1981). Tn7 is an asymmetric transposon, as the left and right Tn7 ends are structurally and functionally distinct (Arciszewska et al. 1989).

Two biological processes occur on pOX-G that confer polarity to the pOX-G plasmid and thus might influence the orientation of Tn7 insertions. These processes are conjugal DNA replication and transcription. The first candidate, conjugal DNA replication, is a special type of DNA replication that takes place as one strand of the plasmid duplex is transferred into a recipient cell. Tn7 could recognize proteins that are situated on pOX-G in a specific orientation as a result of the polarity of conjugal DNA replication. Alternatively, the orientation of Tn7 insertions could be influenced by the polarity of tran-

scription on pOX-G. A striking feature of pOX-G is a 33-kb transcription unit, the *tra* operon, that encodes proteins necessary for plasmid conjugation. If Tn7 interacts with components of the transcription machinery, the orientation of Tn7 insertions could be determined by the direction of *tra* transcription.

As shown in Figure 1B, many Tn7 insertions into pOX-G are found in the *tra* operon, which occupies nearly half of the pOX-G plasmid. However, Tn7 insertions also occurred outside of the *tra* operon, in sequences upstream of *oriT*. This region of pOX-G is called the leading region because, during conjugation, these sequences enter the recipient first. Three open reading frames have been mapped in this region (Loh et al. 1989). One of these open reading frames, ORF169, beginning 47 bp downstream of the *Bgl*III site near *oriT*, is oriented in the opposite direction as the *tra* operon and contains Tn7 insertions. Although little is known about the expression of this gene, the observation that Tn7 can insert into ORF169 in the same orientation as into the *tra* operon suggests that the orientation of Tn7 insertions is not determined by transcription processes.

The experiments described below establish that events of plasmid conjugation, and not transcription, attract Tn7 to pOX-G and R388. Consistent with this conclusion, we have found that, in cells containing pOX-G or R388, Tn7 transposition increases when plasmid conjugation is stimulated by the presence of recipient cells (data not shown). This observation implies that pOX-G transfer can also occur when transposition is measured in pure populations of pOX-G donor cells, as in Table 1. Most conjugable plasmids actually attempt to minimize conjugal transfer into other plasmid-containing cells by displaying plasmid-encoded proteins on the donor cell surface that block further conjugation into that cell. However, it has been shown that individual cells in the donor cell population can lose these exclusion markers and consequently, become recipient cell phenocopies able to trigger conjugation from neighboring donor cells (Peters and Benson 1995). We have measured pOX-G transfer with pure cultures of donor cells under conditions of the λ hop assay and detected conjugation between ~1% of the plasmid-containing cells (data not shown). Thus, plasmid transfer can occur during the λ infection and, possibly, during the course of selection,

Figure 1. Tn7 insertions into pOX-G. **(A)** Linear map of pOX-G showing origin of vegetative replication (*oriV*), origin of conjugal transfer (*oriT*), and *tra* operon containing conjugation factors. Positions of *traY*, *traI*, *traX*, IS3, and *gen*, conferring resistance against the antibiotic gentamicin, are noted. pOX-G conjugation begins at *oriT* and proceeds in the direction indicated by the *oriT* arrow; i.e. *oriT* is transferred first, then *oriV*, *gen*, *traX*, *traI*, etc. Nucleotide coordinates are designated with respect to published sequence of the *tra* operon (accession number U01159) which begins at the *Bgl*III site, 138 bp upstream of the *oriT* nick. *traI* is located between coordinates 26648 and 31918; *traX*, encoding a pilus subunit acetylation factor (Maneewannakul et al. 1995), maps between nucleotides 31938 and 32684. The IS3 insertion into *finO*, directly downstream of *traX*, is not included in the U01159 sequence, and is included on this map at position 33134; 33135 is position 1 of IS3. Sequences upstream of the *Bgl*III site at position (0) in the U01159 sequence are indicated as (–) numbers. Two regions of pOX-G are not represented in the available database. One region is between –13 and –4 kb on this map and the other unsequenced region is between 33.5 and 38 kb. B = *Bgl*III, E = *Eco*RI, H = *Hind*III, bp = basepairs. **(B)** The insertion sites of 18 independent *tnsABC* + *E*-mediated Tn7 insertions into pOX-G. For all Tn7 insertions, target sequences flanking the left Tn7 end were directly determined by sequencing subcloned portions of plasmids containing Tn7 insertions, using a primer complementary to the left Tn7 end. To identify the location of each insertion and the adjoining sequences flanking the right Tn7 end, the experimentally-determined sequence was used to search the non-redundant nucleotide database at NCBI using the BLAST algorithm. For a subset of these Tn7 insertions (20%), target sequences flanking the right Tn7 end were also directly determined by sequencing using a primer complementary to the right Tn7 end, to confirm the identity of the adjoining sequences and to examine the target site duplication resulting from transposition. The horizontal solid line represents the pOX-G DNA with a large arrowhead indicating *oriT*, as in upper diagram. Each short vertical arrow indicates the position of one independent Tn7 insertion; the orientation of each Tn7 insertion is indicated by the arrowhead's position above or below the horizontal line. Small numbers indicate the nucleotide position of each Tn7 insertion with respect to coordinates shown on the pOX-G map. Below is an enlarged view of the region between –2000 bp and 150 bp, including the leading region adjacent to *oriT*. Large horizontal arrows designate the positions and orientations of the three open reading frames in the leading region, ORF169 (47 bp to –462 bp), ORF273 (–1580 bp to –759 bp) and ORF95 (–1986 bp to –1699 bp), that were identified by Loh et al. (1989). Short vertical arrows represent Tn7 insertions with nucleotide positions designated as above.

producing a population of conjugating pOX-G plasmids in the cells where Tn7 transposition is evaluated.

Blocking pOX-G conjugation disrupts Tn7 transposition into pOX-G

If Tn7 inserts preferentially into plasmids that conjugate between cells, then mutant plasmids that cannot conjugate should not be preferred targets of Tn7. Specific *cis*- and *trans*-acting factors must be present in order for plasmid conjugation to occur. Plasmid transfer initiates from a site called the origin of transfer, *oriT*, where a plasmid-encoded protein specifically nicks the DNA strand to be transferred (Thompson et al. 1989). The F plasmid and pOX-G encode a protein called *TraI* that specifically nicks *oriT*; *TraI* is also a DNA helicase capable of unwinding the plasmid strands during transfer (Traxler and Minkley 1987, 1988). Without *TraI*, plasmid conjugation is blocked. Through examination of transposition in cells containing a mutant pOX-G plasmid that lacks *TraI*, we have found that Tn7 inserts less efficiently into the mutant plasmid. Furthermore, when Tn7 does insert into this conjugation-defective plasmid, the Tn7 inser-

tions lack the orientation bias of Tn7 insertions into wild-type pOX-G.

To investigate whether Tn7 is attracted to pOX-G by the conjugation process, we generated a conjugation-defective version of pOX-G, called pOXΔ*traI*, in which the gene encoding *TraI* is deleted, substituted instead with a *dhfr* gene, conferring resistance to the antibiotic trimethoprim (Devine and Boeke 1994). We examined Tn7 transposition in cells containing pOXΔ*traI*, and found that the absence of *TraI* changes Tn7 transposition in two striking ways. First, in cells containing pOXΔ*traI* and lacking a complementing source of *TraI*, pOXΔ*traI* is a relatively poor target for Tn7; the majority of Tn7 insertions are instead located in the bacterial chromosome (Table 2). In cells containing *TraI*, however, pOXΔ*traI* can conjugate, and Tn7 inserts preferentially and efficiently into pOXΔ*traI*. The second notable feature of Tn7 transposition in cells containing the nonconjugating pOXΔ*traI* plasmid is that Tn7 inserts into pOXΔ*traI* in two orientations (Fig. 2). The presence of Tn7 insertions in two orientations is in striking contrast to the orientation bias observed of Tn7 insertions into pOX-G (cf. with Fig. 1). Thus, *TraI*, a key *trans*-acting requirement

Table 2. Tn7 transposition into a non-conjugable pOX-G plasmid

Plasmids	Conjugation ^a	Transposition frequency ^b	Tn7 on pOXΔ <i>traI</i> ^c
pOXΔ <i>traI</i>	–	0.5 ± 0.3	27/130
pOXΔ <i>traI</i> + TraI ^d	+	9.6 ± 5.5	20/20

^aConjugation in the presence and absence of TraI (see Materials and Methods).

^bMean observed transposition frequency ($\times 10^6$) ± SEM; transposition frequency is defined as the number of transposition events (Km resistant colonies) per PFU; mean determined from ≥50 independent infections for each.

^c{Tn7 insertions into pOXΔ*traI*}/(total insertions examined); Tn7 insertions not located on pOXΔ*traI* are located in the chromosome.

^dTraI is provided from pSH2.

in plasmid transfer, is necessary for efficient and orientation-specific Tn7 transposition into pOX-G.

Interestingly, Tn7 insertions that do occur into non-conjugating pOXΔ*traI* plasmids display regional selectivity for target sites within pOXΔ*traI*. A large proportion of Tn7 insertions into pOXΔ*traI* are contained in a 5-kb region at the 3' end of the *tra* operon, overlapping with a cluster of Tn7 insertions into wild-type pOX-G. However, the *oriT* region on pOXΔ*traI* does not contain as high a proportion of Tn7 insertions as on pOX-G. Thus, the regional target specificity of Tn7 for the distal region of the *tra* operon appears to be separable from the global preference of Tn7 for conjugable plasmids.

Tn7 inserts into heterologous *oriT*-containing plasmids that can conjugate

The *cis*-acting requirement for conjugation of F-type plasmids is a minimal *oriT* region that includes a site specifically nicked by TraI. Conjugal DNA transfer initiates at this nick. The minimal *oriT* region of the F plasmid and pOX-G is contained in a 500-bp sequence. When inserted into a heterologous plasmid, this *oriT* region can direct conjugation of the heterologous plasmid in cells where Tra factors are provided by a coresident F

Figure 2. Tn7 insertions into nonconjugable mutant of pOX-G, called pOXΔ*traI*. The positions of 16 independent Tn7 insertions into nonconjugable pOXΔ*traI* plasmids are indicated as in Figure 1. pOXΔ*traI* contains a *dhfr* cassette substituted for *traI*; as a result of this deletion, pOXΔ*traI* is 3.9 kb smaller than pOX-G.

plasmid (Everett and Willetts 1982). We investigated whether such a heterologous, *oriT*-containing plasmid becomes a preferred target for Tn7 when Tra factors are provided. In particular, we examined Tn7 transposition into p-*oriT*⁺, a pBR322 derivative containing the 500-bp *oriT* region of pOX-G (Gao et al. 1994). For experiments examining Tn7 transposition into p-*oriT*⁺, the Tra factors needed for conjugation are supplied from a pOX-G plasmid that is unsuitable as a Tn7 target. Transposition into pOX-G was discouraged by exploiting transposition immunity, a feature of Tn7 transposition that prevents insertions into DNA molecules already containing a Tn7 element (Hauer and Shapiro 1984; Arciszewska et al. 1989). Transposition immunity acts over large distances in DNA, blocking transposition into the entire immune pOX-G plasmid. However, immune targets are only affected in *cis*; transposition into other targets is not influenced by the presence of an immune plasmid in the same cell. Therefore, a pOX-G derivative that contains mini-Tn7::Cm can provide Tra factors for conjugation but is not a transposition target for Tn7 (data not shown).

In cells containing Tra factors that enable p-*oriT*⁺ to conjugate, Tn7 inserts efficiently into p-*oriT*⁺, demonstrating the selectivity of Tn7 transposition for conjugable plasmid targets (Table 3). Of 31 independent Tn7 insertions examined, 20 insertions were located in the 4.5-kb p-*oriT*⁺ plasmid. In the absence of Tra factors, when p-*oriT*⁺ cannot conjugate, Tn7 does not insert preferentially into p-*oriT*⁺, and none of 13 insertions

Table 3. Preferred Tn7 targets must contain a functional *oriT*

Plasmid	+ Tra factors ^a		– Tra factors ^b	
	Transposition frequency ^c	Tn7 on plasmid ^d	Transposition frequency	Tn7 on plasmid
p- <i>oriT</i> ⁺	1.1 ± 0.6	20/31	0.18 ± 0.12	0/13
p- <i>oriT</i> [–]	0.36 ± 0.23	0/30	0.16 ± 0.13	1/13
Vector ^e	0.31 ± 0.18	0/5	0.73 ± 0.33	0/34

^aCells contain Tra factors, from pOX-G, enabling p-*oriT*⁺ to conjugate.

^bCells lack Tra factors, thus p-*oriT*⁺ cannot conjugate.

^cMean observed transposition frequency ($\times 10^6$) ± SEM; transposition frequency is defined as the number of transposition events (Km resistant colonies) per PFU; mean determined from ≥10 independent infections for each.

^d{Tn7 insertions into plasmid}/(total insertions examined).

^epBR322.

examined was located in *p-oriT*⁺. A nearly identical plasmid, *p-oriT*⁻, that cannot conjugate because of 3-bp mutation that inhibits the *oriT* nick (Luo et al. 1995), is not a target for Tn7. We analyzed the distribution of 30 independent Tn7 insertions that occurred in cells containing both *p-oriT*⁻ and Tra factors; no insertions occurred into *p-oriT*⁻. Tn7 insertions into *p-oriT*⁺ share the same characteristics as observed in pOX-G: Tn7 inserts into the vector sequences at many different sites, and in a single orientation with respect to the direction of plasmid transfer (data not shown). Because the version of *p-oriT*⁺-containing *oriT* in the reverse orientation is unstable in cells containing Tra factors (Everett and Willetts 1982), we have been unable to examine the orientation of Tn7 insertions into plasmids conjugating in the opposite direction (data not shown). Nevertheless, the observation that the conjugable *p-oriT*⁺ plasmid is a preferred Tn7 target indicates that the conjugation process, and not the plasmid sequence, designates preferred targets for the *tnsABC* + *E* pathway of Tn7 transposition.

An oriT nick is not sufficient to attract Tn7 insertions

The previous experiments show that events requiring both *oriT* and TraI, two of the central components in conjugation, attract Tn7 insertions preferentially into conjugable plasmids. TraI acts early in conjugation, nicking *oriT* to prepare the plasmid for transfer from the donor cell (Traxler and Minkley 1988). During conjugation, the nicked strand is then transferred into the recipient cell, and host replication processes synthesize complementary strands in both cells. A plausible model is that Tn7 recognizes conjugable plasmids during an early step of conjugation, before the nicked strand enters the recipient cell. For example, Tn7 could recognize the nicked plasmid in the donor cell through interactions with TraI or host factors that process nicked DNA.

We investigated whether an *oriT* nick is sufficient to designate preferred targets for Tn7 by examining transposition into a nonconjugable plasmid that contains an *oriT* nick. This plasmid, *poriV/oriT*, is derived from pOX-G and carries *oriT* and an adjacent section of the *tra* operon that contains the gene encoding TraY. It has previously been shown that TraI and TraY are the only *tra*-encoded factors needed for *oriT* to be nicked in vivo (Everett and Willetts 1980). Thus, in cells containing TraI, *poriV/oriT* is nicked at *oriT*, but *poriV/oriT* cannot conjugate because the other Tra factors are absent (data not shown).

To determine whether an *oriT* nick is sufficient to activate targets for Tn7, we examined whether the presence of TraI could stimulate Tn7 insertions into *poriV/oriT*. As shown on Table 4, the presence of TraI does not stimulate Tn7 insertions into *poriV/oriT*, as compared with cells where TraI is absent. In cells containing TraI and *poriV/oriT*, where *oriT* can be nicked, only six of 38 independent Tn7 insertions occurred into *poriV/oriT*, and, in cells lacking TraI, four of 28 Tn7 insertions were located in *poriV/oriT*. This result suggests that the *oriT* nick is not sufficient for preferred targets for Tn7. We

Table 4. An *oriT* nick is not sufficient to attract Tn7 insertions

Plasmids	<i>oriT</i> nick ^a	Transposition frequency ^b	Tn7 on <i>poriV/oriT</i> ^c
<i>poriV/oriT</i> + TraI ^d	+	0.83 ± 0.46	6/38
<i>poriV/oriT</i> + vector ^e	–	0.64 ± 0.26	4/28

^a*oriT* nick on *poriV/oriT* assayed by Southern analysis following denaturation of plasmid DNA isolated from cells (see Materials and Methods).

^bMean observed transposition frequency ($\times 10^6$) ± SEM; transposition frequency is defined as the number of transposition events (Km resistant colonies) per PFU; mean determined from ≥30 independent infections for each.

^c[Tn7 insertions into *poriV/oriT*]/[total insertions examined].

^dTraI is provided from pSH2.

^epBR322.

have confirmed that *poriV/oriT* contains the expected *oriT* nick by directly examining the *oriT* nick site in plasmids isolated from TraI-containing cells. The *oriT* nick was detected by Southern analysis of denatured *poriV/oriT* DNA, using an *oriT*-specific probe (data not shown). We also examined the *oriT* site in pOX-G using the same technique, and found that similar levels of nicked *oriT* sites can be detected in plasmids from cells containing either pOX-G or both *poriV/oriT* and TraI (data not shown). Although caution is required in the interpretation of a negative result, the lack of preferential Tn7 insertion into *poriV/oriT*, when *oriT* nicking can occur, suggests that the *oriT* nick alone is not sufficient to activate targets for Tn7 transposition. We note that the experiments with *p-oriT*⁺ and *p-oriT*⁻ showed that an *oriT* nick is essential to activate preferred targets for Tn7. In particular, Tn7 does not insert into *p-oriT*⁻, containing a mutant *oriT* site that is not nicked (refer to Table 3). These observations indicate that Tn7 inserts into conjugable plasmids during events that occur subsequent to *oriT* nicking.

Conjugating plasmids are preferred targets for Tn7 transposition that occurs in the recipient cell

After a conjugable plasmid has been nicked at *oriT*, the nicked strand is transferred into a recipient cell and the complementary, unnicked strand remains in the donor cell. Host-mediated DNA synthesis then replaces the missing plasmid strands in both cells (Vapnek and Rupp 1971; Kingsman and Willetts 1978). Because the donor and recipient cells contain template strands of opposite polarity, DNA synthesis must proceed in opposite directions with respect to the plasmid sequence in each cell. In the donor cell, the second strand can be synthesized continuously, by a leading strand mechanism, while discontinuous, lagging strand synthesis occurs in the recipient cell. The observation that Tn7 inserts into pOX-G in a specific orientation suggests an intriguing possibility: Tn7 may recognize plasmids during conjugal replication

in the donor or recipient cell. The polarity of conjugal DNA synthesis could then determine the orientation of Tn7 insertions.

To determine whether Tn7 inserts preferentially into plasmids that enter the recipient cell by conjugation, we developed a strategy for assaying Tn7 transposition exclusively in recipient cells (described in Fig. 3). In this experiment, separate cultures of donor and recipient cells were mixed together to trigger plasmid transfer from donor cells into recipient cells, and Tn7 transposition was assayed immediately after mixing the two cultures. The donor cells contain pOX Δ *traI* and a second plasmid supplying *TraI*, enabling pOX Δ *traI* to transfer into the recipient cells. However, the donor cells lack transposition proteins and, therefore, cannot promote transposition of the Tn7 element that is assayed. Tn7 transposition can occur only in the recipient cells because the recipient cells contain the Tn7 transposition proteins. With this strategy, we can determine if pOX Δ *traI* plasmids that conjugate into the recipient cells are preferred targets for Tn7. It is important to note

Figure 3. Strategy for examining targets of Tn7 when transposition occurs in recipient cells. Shaded cells represent pOX Δ *traI* donor cells, which contain a complementing source of *TraI*, but lack Tn7 transposition proteins. Unshaded cells represent recipient cells that lack a conjugable plasmid and a complementing source of *TraI*; therefore, pOX Δ *traI* plasmids transferred into the recipient cells cannot undergo subsequent conjugation out of the recipient cells. Following conjugal transfer, pOX Δ *traI* plasmids are stably maintained in the recipient cells as vegetatively-replicating plasmids. Only the recipient cells contain Tn7 transposition proteins. Tn7 transposition is assayed in the recipient cells by the λ hop transposition assay immediately after mixing separately-grown cultures of the donor and recipient cells. The distribution of Tn7 insertions is examined to determine if Tn7 inserts preferentially into transferred pOX Δ *traI* plasmids, or if the majority of Tn7 insertions occurred into the recipient cell chromosomes.

that the recipient cells lack any source of *TraI*, thus blocking pOX Δ *traI* from further transfer out of the recipient cells. After isolating cells containing transposition events, the distribution of Tn7 insertions is examined to determine if the conjugated pOX Δ *traI* plasmid is a preferred target for Tn7. If recipient conjugal replication cannot attract Tn7 to the transferred pOX Δ *traI* plasmid, then Tn7 insertions would be expected to occur primarily into the *E. coli* chromosome.

Using the strategy described above, the targets of Tn7 insertions occurring only in recipient cells were examined. We found that Tn7 inserts preferentially into the transferred pOX Δ *traI* plasmid (Table 5, line 1), indicating that conjugation of pOX Δ *traI* into the recipient cell is sufficient to make this plasmid a preferred Tn7 target. We examined the distribution of 47 Tn7 insertions from recipient cells. The majority of Tn7 insertions (37/47) had occurred into pOX Δ *traI* plasmids that had conjugated into the recipient cells. We further analyzed 27 of these insertions to determine the positions and orientations of each insertion (Fig. 4). Notably, the majority of Tn7 insertions into transferred pOX Δ *traI* plasmids occurred in one orientation. This same insertion orientation bias was observed in Tn7 insertions into pOX-G (shown in Fig. 1), suggesting strongly that Tn7 insertions into pOX-G had occurred in recipient cells. This experiment does not examine directly whether conjugable plasmids in donor cells can also be targets for Tn7 insertions; because of the possibility of plasmid conjugation between donor cells (Peters and Benson 1995), such an experiment is technically difficult. However, our results strongly suggest that transposition into conjugable plasmids occurs primarily, if not exclusively, in recipient cells.

The regional target specificity of Tn7 insertions into transferred pOX Δ *traI* plasmids is also similar to that observed for pOX-G, with insertions clustered into the distal end of the *tra* operon and the region adjacent to *oriT* (Fig. 4). We note that two sites in the distal region of the *tra* operon each contained two independent Tn7 insertions. Tn7 inserted twice into transferred pOX Δ *traI* plasmids at position 32643, once in each orientation (Fig. 4). Two insertions are also observed into position 32546, one into a nonconjugated pOX Δ *traI* plasmid (see Fig. 2) and now a second into a transferred pOX Δ *traI* plasmid (Fig. 4). Further investigation should reveal if these two sites, at positions 32643 and 32546 in the *tra* operon, are hot spots for Tn7 insertion, and provide insight into the mechanism through which Tn7 recognizes conjugable plasmids.

Discussion

Conjugable plasmids are preferred targets for Tn7 transposition

We have found that conjugating plasmids are the preferred targets for Tn7 transposition that is mediated by *tnsABC+E*. The bacterial chromosome is a much poorer target for Tn7 than conjugable plasmids, despite the

Table 5. *In recipient cells, Tn7 inserts preferentially into conjugating plasmids*

Donor cells		Recipient cells		Conjugation efficiency ^a	Transposition frequency ^b	Tn7 on pOXΔ <i>tral</i> ^c	Tn7 on chromosome ^d
pOXΔ <i>tral</i>	<i>tral</i>	<i>tnsABC</i> + <i>E</i>	<i>tral</i>				
+	+	+	–	92 (±42)%	2.4 ± 0.97	37/47	10/47
+	+	+	+	109 (±36)%	7.6 ± 3.9	22/22	0/22
+	–	+	–	0 %	0.67 ± 0.26	0/7	7/7

^aConjugation efficiency = pOXΔ*tral* plasmids transferred into recipient cells/total donor cells ± S.E.M. expressed as percent; measured during lambda infection; mean determined from ≥7 independent measurements for each.

^bMean observed transposition frequency (×10⁶) ± S.E.M.; transposition frequency is defined as the number of transposition events (Km resistant colonies) per PFU; mean determined from ≥7 independent infections for each.

^c[Tn7 insertions into pOXΔ*tral*]/[total insertions examined].

^d[Tn7 insertions into recipient cell chromosomes]/[total insertions examined].

chromosome's significantly larger size. Tn7 inserts into conjugable plasmids with a striking orientation bias that depends on plasmid conjugation; mutant plasmids that cannot conjugate contain Tn7 insertions in two orientations. The same orientation bias has been observed previously for Tn7 insertions into RP4 and R18, two closely related conjugable plasmids of the IncP family (Barth and Grinter 1977; Krishnapillai et al. 1984). In addition to a global specificity for conjugable plasmids, Tn7 displays regional target selectivity for several regions of the F plasmid derivative, pOX-G. Preferential Tn7 insertion into the distal end of the *tra* operon does not require pOX-G conjugation, as Tn7 insertions into pOXΔ*tral* are also clustered in this region, albeit in two orientations.

Recall that Tn7 has two pathways for transposition that insert Tn7 into two different classes of target sites. One transposition pathway inserts Tn7 efficiently into *attTn7* in the *E. coli* chromosome, and, as we have shown here, the second transposition pathway directs

Tn7 insertions preferentially into conjugating plasmids. TnsE is required to distinguish conjugable plasmids as preferred targets, while TnsD is necessary for Tn7 to insert into *attTn7*. Three additional proteins, TnsA, TnsB and TnsC, are required for transposition into both class of targets. Although the biochemical role of TnsE in transposition into conjugable plasmids remains undefined at this time, we do understand how Tn7 selects the *attTn7* target site; the mechanism of *attTn7* selection may help to illuminate possible roles for TnsE in transposition into conjugable plasmids. TnsD selects *attTn7* by binding specifically to *attTn7* sequences (Waddell and Craig 1989; Bainton et al. 1993). TnsD then recruits TnsC to *attTn7*, and TnsC, in turn, directs TnsA and TnsB to insert Tn7 into the designated target DNA (Bainton et al. 1993). Transposition into *attTn7* is dependent completely on recognition by TnsD; without TnsD, Tn7 cannot insert into *attTn7* (Rogers et al. 1986; Waddell and Craig 1988), and nucleotide changes in the *attTn7* sequence, interfering with binding by TnsD, also block transposition into *attTn7* (Gringauz et al. 1988; Waddell and Craig 1989; Bainton et al. 1993). Perhaps TnsD and TnsE function similarly, both recognizing potential targets and recruiting the transposition machinery to these sites through interactions with TnsC. A reasonable hypothesis is that TnsE may recognize host proteins that function in conjugal DNA synthesis and these proteins attract TnsE to conjugating plasmids. TnsE could then recruit TnsC to the selected target sites on conjugable plasmids. Alternatively, TnsE may modify TnsC and consequently enable TnsC to recognize target sites on conjugable plasmids. Regardless of the biochemical role of TnsE in transposition, TnsE is a critical factor for directing Tn7 transposition into conjugable plasmids.

Figure 4. Tn7 insertions into pOXΔ*tral* plasmids transferred into recipient cells where transposition occurred (as described in Fig. 3). The positions and orientations of 27 independent Tn7 insertions into transferred pOXΔ*tral* plasmids. The positions and orientations of Tn7 insertions is designated as in Figure 1. The positions of insertions into the *oriV* region were determined in the published sequence FPLMCG and this coordinate added to 13000 bp to correct for the distance between *EcoRI* site adjacent to *oriV*, where FPLMCG begins and *BglII* site in *oriT*. The boxed numbers indicate positions where two independent Tn7 insertions occurred.

Distinguishing features of conjugable plasmids

We have shown that Tn7 inserts preferentially into relatively small conjugable plasmids and virtually ignores larger DNA molecules, such as the *E. coli* chromosome, indicating that Tn7 recognizes targets through the conjugation process. Moreover, we have observed that Tn7 inserts efficiently into plasmids that have undergone conjugation into a recipient cell. An attractive hypothe-

Table 6. *Plasmids*

Plasmid	Replicon	Description	Reference
pOX-G	IncF	Identical to pOX38, a 60-kb conjugable F plasmid derivative, with a gene for gentamicin resistance inserted at HindIII	(Guyer et al. 1981; Johnson and Reznikoff 1984)
R388	IncW	33-kb trimethoprim-resistant conjugable plasmid	(Avila and De La Cruz 1988)
P1	P1	Bacteriophage P1 lysogen, which exists as a single-copy 90-kb plasmid (P1 Cm V111, $r^+ m^-$).	M. Yarmolinsky (National Cancer Institute, National Institutes of Health, Bethesda, MD)
pOX Δ tral	IncF	Nonconjugable mutant of pOX-G; <i>tral</i> substituted with <i>dhfr</i> .	This work
poriV	IncF	15-kb nonconjugable plasmid containing <i>oriV</i> and <i>gen</i> from pOX-G.	This work
poriV/ <i>oriT</i>	IncF	<i>poriV</i> with 8.6-kb <i>Eco</i> -RI fragment of pOX-G containing <i>oriT</i> , and <i>traM/JY</i> ; <i>oriT</i> is nicked when <i>Tral</i> is added	This work
pBR322	pBR322	4-kb pBR322 derivative with 346-bp deletion between <i>Hind</i> III and <i>Bam</i> HI	This work
p- <i>oriT</i> ⁺	pBR322	Contains F plasmid <i>oriT</i> (position 2-535, nicked at 141); conjugable when <i>Tra</i> factors are present; identical to pXRD647	(Gao et al. 1994)
p- <i>oriT</i> ⁻	pBR322	Nonconjugable p- <i>oriT</i> ⁺ mutant due to 3-bp substitution at nicking site; identical to pXRD647S (139-141)	(Luo et al. 1995)
pSH2	ColEI	<i>Eco</i> -RI fragment of F plasmid containing <i>traTDIX</i> and IS3.	(Achtman et al. 1978)

sis is that preferential Tn7 insertion into conjugable plasmids occurs in recipient cells, where the polarity of conjugal replication determines the orientation of Tn7 insertions. Consistent with this hypothesis, when transposition occurs exclusively in recipient cells, Tn7 inserts in predominantly one orientation into plasmids that have undergone conjugation. We observed the same orientation bias among Tn7 insertions into pOX-G and p-*oriT*⁺ in mixed populations of donor and recipient cells. Our experiments do not test directly whether Tn7 inserts into plasmids undergoing conjugation in donor cells as well. However, the possibility of plasmid transfer between donor cells (Peters and Benson 1995) complicates direct analysis of Tn7 transposition in donor cells. Nevertheless, the predominant orientation bias of Tn7 insertions into conjugable plasmids suggests strongly that transposition into conjugating plasmids occurs predominantly, if not exclusively, in recipient cells.

The observation that Tn7 inserts into conjugable plasmids in recipient cells is useful for considering features of conjugable plasmids that may constitute targets for Tn7. The *Tra* factors themselves seem unlikely candidates for attracting Tn7, as *Tra* factors from the F plasmid, and therefore pOX-G, cannot be detected in the recipient cell cytoplasm with the transferred plasmid strand (Rees and Wilkins 1990). Rather, a significant event occurring on the transferred plasmid strand in recipient cells is conjugal DNA synthesis to duplicate the parental plasmid.

Conjugal DNA synthesis occurs upon a single-stranded template and thus may differ from double-

stranded, vegetative DNA synthesis in its protein requirements or the intermediates present on the DNA. We propose that DNA synthesis upon the conjugated plasmid strand directs Tn7 insertions preferentially to conjugable plasmids. During conjugation, DNA synthesis upon the transferred plasmid strand occurs in a specific polarity, and, as a result, replication proteins and intermediates are situated asymmetrically on the plasmid. Tn7 could recognize these replication proteins, and the orientation of these proteins at the target site may dictate the orientation of the Tn7 insertion. Tn7 insertions may also be clustered where replication factors accumulate, such as regions where DNA replication initiates or stalls.

Conjugal DNA synthesis of the F plasmid is not known to require specific plasmid-encoded proteins; rather, host proteins synthesize complementary DNA during conjugation (for review, see Wilkins and Lanka 1993). Thus, Tn7 might also recognize replication processes on nonconjugating templates. Tn7 can insert into nonconjugating DNA molecules, such as pOX Δ tral and the *E. coli* chromosome, although these insertions are rare. The proteins recognized by Tn7 may be more accessible or abundant during conjugal DNA synthesis than vegetative DNA synthesis. Consequently, Tn7 could insert more frequently into conjugating plasmids. Furthermore, Tn7 insertions into non-conjugating pOX Δ tral plasmids occur in two orientations, a notable feature considering that vegetative replication of pOX Δ tral is bidirectional and occurs concurrently on both plasmid strands.

We have observed that the 3' end of the *tra* operon, on both pOX-G and pOX Δ *tral*, contains clusters of Tn7 insertions and is therefore an apparent hot spot for the *tnsABC*+*E* pathway. Although this region appears to constitute a generally preferred target for Tn7, conjugation influences both the frequency and the orientation of Tn7 insertions into this site. This region of pOX-G does not contain any sequences or structures that obviously account for preferential Tn7 insertion, with the exception of the *tra* transcription unit itself. Perhaps the transcription apparatus stalls both conjugal and vegetative replication forks in this region, creating a hotspot for Tn7 insertions. Alternatively, replication complexes may be assembled at sites contained in this distal region of the *tra* operon. A biochemical understanding the *tnsABC*+*E* pathway and its targets will enable us to identify the factors directing Tn7 to this region of pOX-G and to conjugal plasmids in general.

In addition to specific replication proteins located on plasmids during conjugation, other features of conjugating DNA may also contribute to preferential Tn7 insertion into conjugable plasmids. For example, conjugation may alter a plasmid's structure or subcellular location, making the DNA more accessible for transposition. If such accessibility differences can influence Tn7 transposition, they appear to act only through the *tnsABC*+*E* pathway; alternative Tn7 transposition pathways do not promote insertions preferentially into conjugable plasmids. For example, transposition mediated by *tnsABC*+*D*, which inserts Tn7 into *attTn7*, is not increased significantly by the presence of a pOX-G plasmid containing *attTn7*, nor are de novo Tn7 insertions redistributed dramatically into the plasmid *attTn7* site (data not shown). Furthermore, a third Tn7 transposition pathway, promoted by a gain-of-function TnsC mutant (TnsC^{A225V}) that can activate Tn7 transposition in the absence of TnsD and TnsE (A.E. Stellwagen and N.L. Craig, in prep.) does not insert Tn7 preferentially into conjugating plasmids (data not shown). Transposition mediated by TnsAB + TnsC^{A225V} directs Tn7 insertions efficiently into the bacterial chromosome, but ignores conjugable plasmids. Although structural differences could influence the accessibility of conjugating DNA to transposition proteins of the *tnsABC*+*E* pathway, such accessibility differences cannot account for the orientation specificity of Tn7 insertions into conjugating plasmids.

The biological importance of transposition into conjugable plasmids

We have found that Tn7 has the distinctive ability to insert preferentially into conjugable plasmids of bacteria, a property with clear biological consequences. Plasmid conjugation constitutes an efficient pathway for the horizontal transfer of genetic information between bacterial cells. Thus, insertion of Tn7 preferentially into conjugable plasmids provides Tn7 with access to a vehicle for disseminating its genetic information, which includes several genes for resistance to antibiotics. Several other

prokaryotic transposable elements may also exploit this strategy for dissemination. For example, Tn3 inserts more frequently into plasmids than bacterial chromosomes (Kretschmer and Cohen 1977). However, it is not known whether Tn3 responds specifically to plasmids that are conjugating, like Tn7, or whether other mechanisms, such as structural differences between plasmids and the chromosome, account for the target preference of Tn3.

Tn7 possesses the novel ability to insert efficiently into either conjugable plasmids or bacterial chromosomes, a capacity provided by the two alternative Tn7 transposition pathways. These transposition pathways complement one another to maintain Tn7 within a bacterial population. The *tnsABC*+*D* pathway efficiently inserts Tn7 into *attTn7*, a well-conserved site in the bacterial chromosome, where Tn7 can be stably maintained through future generations. The *tnsABC*+*E* pathway complements this chromosome-specific pathway by inserting Tn7 into conjugating plasmids, enabling Tn7 to disseminate through bacterial populations. The dual target specificity of Tn7 for bacterial chromosomes and conjugable plasmids means that Tn7 is well adapted to serve as a tool for the spread and maintenance of antibiotic resistance genes within bacterial populations.

Materials and methods

Media, chemicals, and enzymes

LB broth and agar were used as described by Maniatis et al. (1982); IsoSensitest Agar (Oxoid) was used with trimethoprim; MacConkey Lactose agar was from Difco; SM buffer was used as described by Miller (1972). Concentrations of antibiotics or media supplements were as follows: 100 μ g/mL carbenicillin (Cb), 30 μ g/mL chloramphenicol (Cm), 5 or 10 μ g/mL gentamicin (Gen), 50 or 100 μ g/mL kanamycin (Km), 10 or 20 μ g/mL nalidixic acid (Nal), 5 or 20 μ g/mL tetracycline (Tet), 100 μ g/mL trimethoprim (Tp), 20 mM sodium citrate, and 0.2% maltose. DNA-modifying enzymes and Proteinase K were obtained from commercial sources and used as recommended by the manufacturer. Qiaex beads (Qiagen) were used to recover DNA fragments, after agarose gel purification, for cloning.

Bacterial strains

NLC51 is *E. coli* F⁻ *araD*139 Δ (*argF-lac*) U169 *rpsL*150 *relA*1 *flbB*5301 *deoC*1 *ptsF*25 *rbsR* *val*^R *recA*56 (McKown et al. 1987); CW51 is *E. coli* F⁻ *ara*⁻ *arg*⁻ *lac* *proXIII* *recA*56 Nal^R Rif^R (Waddell and Craig 1988). Allelic replacement of *tral* in pOX-G was performed in DPB271, containing *recD*1903::minitet (Biek and Cohen 1986). CAW220 is JC3272 (F⁻ *lac* Δ X74 *gal*⁻ *his*⁻ *tyr*⁻ *lys*⁻ Str^R T6^R Su⁻) containing the plasmids pSH2 (Achtman et al. 1978) and EDFL171, a derivative of F'*lac* with a deletion of the distal region of the *tra* operon, including *tral* (McIntire and Willetts 1978).

Plasmids and Phage

λ KK1 is a derivative of λ 780 (b2::hisOGD b522 cI857 Pam80 nin5) with hisG9424::Tn10 del16 del17::attTn7(-342 to +165)::Tn7 L166 Km^r Tn7R199 (McKown et al. 1988). All plasmids used in transposition assays are described in Table 6.

Transposition proteins were supplied on derivatives of pCW4, which is pACYC184 containing a 9-kb *EcoRI* fragment from Tn7 with *tnsABCDE*. A pCW4 derivative with *tnsD* inactivated by a miniMu transposon insertion (#107) was used to study *tnsABC* + *E*-mediated transposition [Waddell and Craig 1988].

Construction of pOXΔ*traI* pSH2 is a ColEI-based plasmid containing the *EcoRI* fragment of the F plasmid that includes *traI* [Achtman et al. 1978]. The 5' half of *traI* was removed from pSH2 by digestion with *EagI*, and, after treating with Klenow, the plasmid was rejoined with a blunt-ended *dhfr* gene cassette (Devine and Boeke 1994) inserted at the blunt *EagI* sites. Trimethoprim-resistant recombinants were selected and a plasmid with *dhfr* transcription proceeding in the same direction as *tra* was identified by restriction analysis (pSH2::dhfr). The *dhfr* gene cassette adds an *EcoRI* site downstream of *dhfr*; *EcoRI* digestion of pSH2::dhfr released a 4.6-kb *EcoRI* fragment containing both *traD* (the gene directly upstream of *traI*) and *dhfr*. pCAW42 is BluescriptIIKS⁺ with an *EcoRV*/*HindIII* fragment of pOX-G fragment containing *traX* and a portion of the IS3 element; the vector's *EcoRI* site is upstream of *traX*. The *EcoRI* *traD*, *dhfr*-containing fragment of pSH2::dhfr was inserted into pCAW42 at the *EcoRI* site upstream of *traX*, reconstructing the order of the *tra* operon, with *dhfr* substituted for *traI* (pCAW43).

This *dhfr*-substituted segment in pCAW43 was recombined into pOX-G by homologous recombination in a *recD* host [Bick and Cohen 1986; Hagemann and Craig 1993]. A *Bam*HI–*Hind*III fragment of pCAW43 containing *traD*, *dhfr*, *traX*, and IS3 was electroporated into competent DPB271 cells containing pOX-G (18 kV/cm, 25 μF, 200ω). Recombinant pOX-G plasmids were selected in the presence of Tp and confirmed by restriction analysis. These plasmids were also tested for their ability to conjugate; the conjugation frequency of the mutant plasmid (pOXΔ*traI*) alone was <0.00012%. When a complementing source of *TraI* was supplied in trans, pOXΔ*traI* conjugated efficiently (>100% of plasmids were transferred into recipient cells). All conjugation experiments were carried out according to standard protocols [Miller 1972].

Construction and analysis of *poriV/oriT* The *oriT*-containing substrate, *poriV/oriT*, was constructed in two steps. First, pOXΔ*traI* was digested with *EcoRI* and the 11-kb fragment containing *oriV* and *gen* was gel purified and recircularized (*poriV*). *poriV* was linearized with *EcoRI*, and joined to the 8.6-kb *oriT*-containing *EcoRI* fragment isolated from pOX-G and recombinant plasmids selected on Gen. Restriction analysis confirmed that *oriT* is situated in the same relative orientation to *oriV* as in pOX-G (*poriV/oriT*).

We confirmed that a DNA nick actually occurred at *oriT* in cells containing both *poriV/oriT* and *TraI* (provided from pSH2) by *SalI*-digesting plasmid DNA isolated from cells as described for p-*oriT*⁺ (see below), releasing a 2.5-kb fragment containing *oriT*. The DNA strands were separated by denaturing acrylamide gel electrophoresis, and the *oriT* nick was detected by Southern analysis using as a probe an *EcoRI*–*SalI* fragment from p-*oriT*⁺ containing *oriT* [Gao et al. 1994]. The nicked strand of *poriV/oriT* is 396 nucleotides in length following *SalI* digestion and denaturation from the unnicked complementary strand, and hybridizes with the *oriT* probe.

λ hop assay for Tn7 transposition

Transposition was evaluated with a λ hop assay in NLC51 cells [McKown et al. 1988]. Cultures of cells were grown in LB broth with antibiotics and 0.2% maltose, collected when OD₅₉₅ was between 0.3 and 0.9, concentrated to 1.6×10⁹ cells/mL in 10

mM MgSO₄, and kept on ice. Aliquots of cells were combined with λKK1 (MOI=0.1), and incubated for 15 min at 37°C. Infections were terminated with the addition of 10 mM citrate in LB and the cells were allowed to recover at 37°C for 1 hr on a roller drum spinning at ~70 rpm. Cells with transposition events were selected on LB plates containing Km and citrate. After growth overnight at 37°C, the transposition frequency was determined as the number of Km-resistant colonies arising per PFU of λKK1.

When transposition was examined in mixtures of plasmid donor and recipient cells, each strain was grown separately, concentrated to 1.6×10⁹ cells/mL and mixed gently in appropriate ratios at room temperature. Aliquots of the cell mixtures were combined with λKK1 at MOI=0.1 with respect to the cells containing transposition proteins, and incubated as described above. During the one-hour growout, agitation was reduced to 35 rpm, and the conjugation frequency was measured by standard procedures [Miller 1972].

Analysis of Tn7 insertions

Cells with independent transposition events were purified twice and the distribution of Tn7 insertions determined in the following ways:

pOX-G, R388 Tn7 insertion was scored by conjugating pOX-G or R388 from the transposition strain into CW51 recipient cells and examining transconjugants for comobilization of the Tn7 element.

P1 Tn7 insertion was scored by transforming cells containing transposition events with pALA18, containing *incA* of P1 [Abel et al. 1984] and selecting cells in the presence of Cb to select for pALA18-containing cells. pALA18 destabilizes P1, which confers resistance against Cm. Therefore, cells with Tn7 insertions on P1 are both Km^s and Cm^s after loss of P1.

pOXΔ*traI*, *poriV/oriT* Tn7 insertion was scored after exclusion of the pOX-G-based plasmid by EDFL171, a *tra*⁺ derivative of F'*lac* [McIntire and Willetts 1978]. EDFL171 was introduced into cells containing transposition events from the donor strain CAW220, which contains pSH2 [Achtman et al. 1972] to complement the *tra* defect in EDFL171. EDFL171-containing transconjugants were selected on MacConkey lactose plates containing Tct and assayed for the loss of pOXΔ*traI* or *poriV/oriT*, as applicable, on Gen. All cells containing Tn7 on pOX-G-based plasmids were both Gen^s and Km^s after loss of pOXΔ*traI* or *poriV/oriT* in this fashion.

p-*oriT*⁺ and other pBR322-based plasmids Tn7 insertion was scored by physical analysis on agarose gels of plasmid minipreps and also by transformation of plasmids into competent DH5 cells. Plasmid-containing cell lysates were made from cells containing transposition events according to Thompson et al. [1989]. The lysates were digested with Proteinase K for 1 hr at 37°C, followed by extraction with phenol/chloroform/isoamyl alcohol (25:24:1) and ethanol precipitation prior to analysis.

Sequence analysis of Tn7 insertions into pOX-G and pOXΔ*traI*

Plasmids containing Tn7 were purified from cells by genomic minipreps with CTAB [Maniatis et al. 1982], and digested with *EcoRV*, which cuts pOX-G in many places but does not cut Tn7. The *EcoRV*-digested minipreps were cloned into BluescriptIIKS⁺ and recombinant plasmids containing the Tn7 insertion

and adjacent target sequences were selected in the presence of Km. Plasmids were minipreped and sequenced on an ABI automatic sequencer, using primer NLC95 which is complementary to the left Tn7 end and is extended into the adjacent target sequence [NLC95 = 5'-ATAATCCTTAAAACTCCATTTC-CACCCCT-3']. To confirm the presence of 5-bp target site duplications characteristic of Tn7 transposition, the target sequences adjacent to the right Tn7 end were also determined for a subset (20%) of the insertions using primer NLC94 (5'-AAAGTCCAGTATGCTTTTTCACAGCATAAC-3'). To determine the nucleotide position of each Tn7 insertion in the pOX-G plasmid, the sequences of the Tn7 insertion sites were used to search the nonredundant nucleotide database at NCBI using the BLAST algorithm. Two regions of pOX-G lack complete sequence information. With respect to the map shown on Figure 1, the unsequenced regions are located between -13 to -4 kb upstream of *oriT* and 33.5 to 38 kb at the end of the *tra* operon. Because of the absence of these sequences in the available database, Tn7 insertions into these regions would have recovered no matches in BLAST searches. ~20% of the Tn7 insertions into pOX-G were located in target sites that are not represented in the Genbank database.

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Conjugating plasmids are preferred targets for Tn7.

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