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Tn7 Transposes Proximal to DNA Double-Strand Breaks and into Regions Where Chromosomal DNA Replication Terminates

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Summary

We report that the bacterial transposon Tn7 can preferentially transpose into regions where chromosomal DNA replication terminates. DNA double-strand breaks are associated with the termination of chromosomal replication; therefore, we directly tested the effect of DNA breaks on Tn7 transposition. When DNA double-strand breaks are induced at specific sites in the chromosome, Tn7 transposition is stimulated and insertions are directed proximal to the induced break. The targeting preference for the terminus of replication and DNA double-strand breaks is dependent on the Tn7-encoded protein TnsE. The results presented in this study could also explain the previous observation that Tn7 is attracted to events associated with conjugal DNA replication during plasmid DNA transfer.

Introduction

Mobile DNA often transpose with little discrimination between potential target sites. There are, however, examples of transposons that have evolved the means to actively target certain DNAs (Craig, 1997). Transposons can specifically direct their insertion into large regions of DNA, such as silenced DNA in yeast (Zou et al., 1996), or smaller regions such as upstream of tRNA genes (Boeke and Sandmeyer, 1991). The ability to identify special DNA targets could expedite transposon dispersal while minimizing the chances of the element harming its host organism. A transposon could identify target sites by recognizing a specific sequence, a sequence-independent structure, or a protein-DNA complex. The bacterial transposon Tn7 is of particular interest in the area of target site selection because it has multiple pathways of insertion (Craig, 1995). We describe here that Tn7, in addition to having a pathway that recognizes a specific sequence, also has a target recognition pathway that responds to a structure or complex found where chromosomal DNA replication terminates and around the site of DNA double-strand breaks (DSBs).

Tn7 uses distinct but overlapping sets of transposon-encoded proteins to carry out two pathways of transposition. Tn7 encodes five proteins responsible for transposition, TnsA, TnsB, TnsC, TnsD, and TnsE (TnsABCDE) (Rogers et al., 1986; Waddell and Craig, 1988; Flores et al., 1990; Kubo and Craig, 1990). The TnsA and TnsB proteins collaborate to form the transposase responsible for the DNA breakage and joining activities which underlie transposition (May and Craig, 1996; Sarnovsky et al., 1996; Biery et al., 2000a). TnsABC make up the core transposition machinery that is incapable of transposition without one of two target site selecting proteins, TnsD or TnsE (Bainton et al., 1993; Waddell and Craig, 1988). TnsC is a regulator of transposase activity, communicating between the TnsAB transposase and the target selecting proteins (Stellwagen and Craig, 1998). When the TnsABC+D proteins are functioning, insertions are directed in one orientation into a single site in the *E. coli* chromosome, called Tn7's attachment site, *attTn7* (Lichtenstein and Brenner, 1982; McKown et al., 1988). The *attTn7* site is recognized by the sequence- specific DNA binding protein TnsD, which recruits the core TnsABC transposition machinery (Bainton et al., 1993; Waddell and Craig, 1989).

Previous studies have shown that when Tn7 transposes using the TnsABC+E proteins, insertions occur preferentially into DNA during the process of conjugal DNA replication and transfer (Wolkow et al., 1996). TnsE-dependent transposition is stimulated in cells with conjugative plasmids, and >95% of the insertions occur into the conjugative plasmid. While nonconjugative plasmids are not efficiently used as transposition targets, they can become preferred targets when mobilized to conjugate by introducing an origin of transfer and providing the conjugation proteins in *trans* (Wolkow et al., 1996).

We show here that TnsABC+E insertions into the *E. coli* chromosome do not occur at randomly distributed sites. TnsABC+E insertions occur preferentially in regions where chromosomal DNA replication terminates. Because the termination of chromosomal replication is associated with DSBs (Bierne et al., 1991; Horiuchi et al., 1994; Michel et al., 1997), we directly tested the effect of inducing a DSB at specific locations in the chromosome on TnsABC+E transposition. Following induction of DSBs, Tn7 transposition is stimulated and insertions occur proximal to DNA DSBs. The ability of Tn7 to transpose into a structure or complex associated with DSBs could help Tn7 avoid essential genes when moving though cell populations.

Results

Tn7 Transposes Preferentially Where DNA Replication Terminates

The position of Tn7 insertions that occurred via the TnsABC+E pathway into the chromosome of *E. coli* was determined using the genome sequence (see Experimental Procedures). Transposition events were isolated in cells where the Tn7 transposition functions were supplied in *trans*. A miniTn7(Kan^R) was introduced on a replication- and integration-defective lambda phage vector. The analysis of 20 insertions revealed that insertion did not occur randomly into the chromosome but instead occurred with a strong regional preference. The majority of the insertions occurred in an area equidistant from the origin of DNA replication in the ~4.6 megabase circular *E. coli* chromosome in a region where chromosomal

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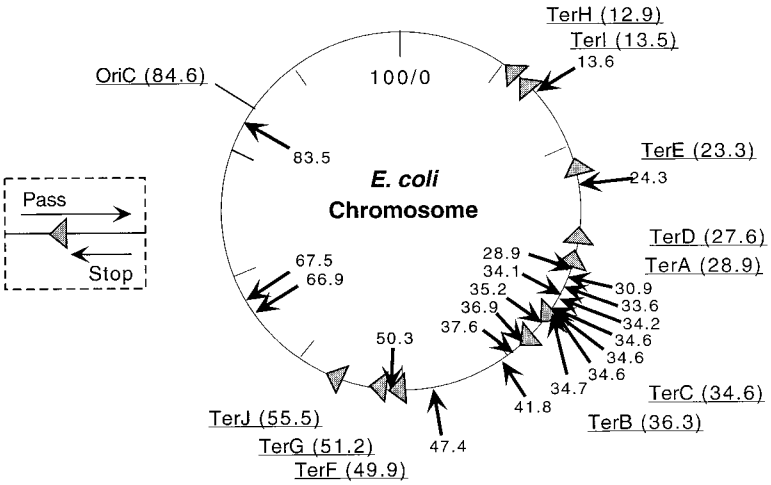

Figure 1. The Location of Tn7 Insertions in the *E. coli* Chromosome that Occurred via the TnsABC+E Pathway

Transposon insertion events were isolated from the strain NLC51 pCW15 (TnsABC) pJP104 (TnsE) where miniTn7 was introduced via a defective lambda phage (see Experimental Procedures). Three of the insertions were isolated and mapped in a previous work (Kubo and Craig, 1990). Tick marks indicate 10 min increments on the 100 min *E. coli* chromosome, the 0/100 min position is marked. One minute is approximately 45 kilobase pairs. The minute position of miniTn7 insertions (arrows) and *terA-terJ* sites (triangles) are indicated. Placement of the arrow outside the circle indicates the miniTn7 inserted in a clockwise right-to-left orientation, placement inside indicates counter-clockwise. The orientation of the triangles indicates the orientation of the *ter* site. Replisomes encountering the flat side of the triangle first are stopped, while replisomes approaching from the pointed side will pass through the terminator.

replication terminates (Figure 1). In *E. coli*, chromosomal replication initiates bidirectionally at *oriC* and terminates at a series of ten termination sites (*terA–terJ*) that halt replisomes approaching in one orientation (Hill, 1996; Coskun-Ari and Hill, 1997) (Figure 1). Over a third of the Tn7 insertions were strikingly close, within 6 kb, of *ter* sites; *terC* had four within 4 kb, *terA* had one 1 kb away, and *terI* had one 6 kb away. Transposition events around *terC* were particularly focused where insertions occurred 23, 144, 541, and 3102 bp from the *ter* consensus sequence at *terC* (Coskun-Ari and Hill, 1997). We could not identify any common DNA sequence when a 100 bp window surrounding the individual Tn7 insertion sites was examined.

To more thoroughly analyze Tn7's chromosomal insertion preference, a mapping technique was utilized where insertions were mapped to one of the 21 chromosomal NotI restriction fragments (Figure 2). The NotI restriction map can be elucidated given the sequence of the *E. coli* strain MG1655 and previous restriction analysis of the bacterial strain used in this study, MC4100 (Heath et al., 1992; Blattner et al., 1997) (see Experimental Procedures). The location of Tn7 insertions in various isolates was determined by separating NotI digested chromosomes by pulsed field agarose gel electrophoresis (PFGE) and probing with sequences specific to the transposon in Southern blots. To simplify the comparison, the fragments were grouped into 14 regions of at least ~200kb (Figure 3). To compensate for the difference in size between the regions 1–14, the insertion data is plotted as insertions per 100 kb (Figures 4A–4C). In cases where insertions occurred into NotI fragments that were too close in size to differentiate the location of the insertion, an SfiI digest was used in addition (Perkins et al., 1992).

Thirty-five new insertions that occurred via the TnsABC+E pathway were isolated and mapped to NotI fragments by Southern blotting. The fragment where *terC* and *terA* reside was the preferred transposon insertion target (region 8 in Figures 3 and 4A). Forty percent (14/35) of the insertions occurred into the region containing the *ter* sites *terC* and *terA*, a region that only encompasses 6% of the chromosome. The segments adjacent to region 8 where the *terD* and *terB* sites reside were also preferred for TnsABC+E transposition (regions 7 and 9 in Figures 3 and 4A). Insertions also occurred outside the *ter* site–containing regions. The insertion profile obtained with restriction fragment mapping was consistent with results obtained when insertions were mapped by sequence (compare Figures 1 and 4A).

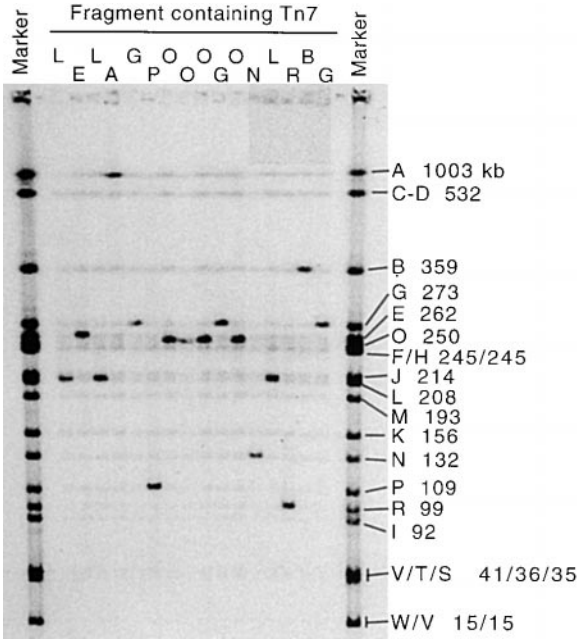

Figure 2. Southern Blot of a Pulsed Field Agarose Gel of NotI-Digested DNA Isolated from Strains with Transposon Insertions

The Southern blot was probed for the miniTn7 transposon (see Experimental Procedures). The marker lane consists of a chromosomal NotI digest labeled with ^{32}P-dCTP. Results from 16 randomly chosen miniTn7 transposition recipients are shown when a Tus$^-$ strain background was tested. The size of the various fragments is indicated in kilobases along with the letter designation given in Heath et al. (1992).

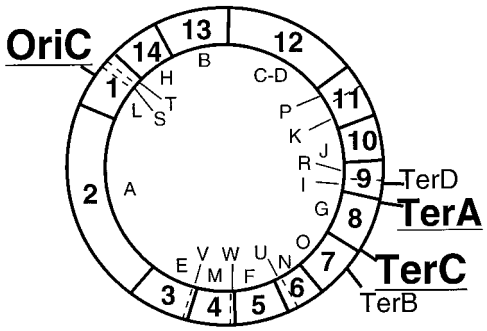

Figure 3. Schematic Breakdown of the NotI Fragments Expected from the *E. coli* K12 Strain MC4100

Solid and dashed lines indicate NotI cut sites (Heath et al., 1992; Blattner et al., 1997). Letters indicate the previous fragment designations (Heath et al., 1992); numbers 1–14 indicate the regional groupings used in Figure 4. The location of the origin of replication (*oriC*) and the four central *ter* sites (*terA–terD*) are indicated. The MC4100 strain background contains a deletion that removes one NotI site fusing fragments C and D. A NotI fragment of 4334 base pairs was identified in the DNA sequence between fragments H and B; because it is insignificant for Tn7 mapping it is not shown.

The distribution of insertions is significantly different than would be expected for a random distribution ($p < 0.01$).

TnsE Is Required to Direct Insertions into the Terminus Region

The experiment above indicated that a significant fraction of the insertions that occurred via TnsABC+ E were directed to the terminus region of the chromosome. Did insertions occur predominantly in the replication terminus region because it was the only region available to the transposition apparatus? To test this explanation, we isolated insertions generated by a core transposition machine with a gain-of-function mutant, TnsC^{A225V}, which does not require a target site selecting protein, TnsD or TnsE (Stellwagen and Craig, 1997). Previous in vivo and in vitro results with the TnsABC^{A225V} core machinery indicate that TnsABC^{A225V} catalyzes insertions with little target site selectivity (Stellwagen and Craig, 1997; Biery et al., 2000b). In vivo and in vitro results are consistent with the idea that the TnsC^{A225V} regulator protein is locked in the "on" conformation, resulting from a slowed ATPase activity as compared to the TnsC^{wt} protein (Stellwagen and Craig, 1998).

Thirty-four insertions that occurred via the TnsABC^{A225V} pathway were isolated and mapped by Southern blotting. The distribution of insertions found with TnsABC^{A225V} was not significantly different than what is expected for a random distribution ($p = 0.82$) (Figure 4B). The widespread distribution found with the core machinery alone indicates that much of the chromosome is accessible to the transposase machinery. This result shows that TnsE is required to direct insertions to the *ter* region.

TnsABC+E Transposition Does Not Require the Tus Protein

Active termination of chromosomal DNA replication at *ter* sites requires the action of the Tus protein, which binds to *ter* sites and interacts with the replication helicase DnaB to halt replication forks (Hill et al., 1989; Khatri et al., 1989; Lee et al., 1989; Hill and Marians, 1990). Is active termination of chromosomal DNA replication mediated by the Tus protein required for TnsE-dependent Tn7 transposition into the terminus region? We determined the frequency of TnsABC+E transposition in a strain where the gene encoding the Tus protein had been deleted (see Experimental Procedures). The frequency of TnsABC+E transposition was modestly (30%) but reproducibly decreased when the Tus gene was deleted (data not shown). The observation of TnsE-dependent transposition in a Tus$^-$ background indicates that a direct association between Tus and TnsE is not required for targeting and that an event independent of the involvement of the Tus protein can be recognized by TnsE.

To determine any effect Tus-mediated termination had on the location of TnsE-directed insertions, we

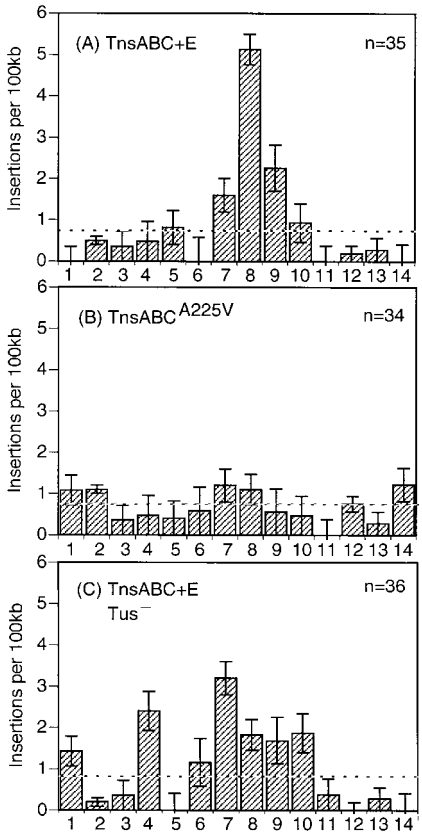

Figure 4. Distribution of Tn7 Insertions between 14 Regions of the Chromosome in Various Strain Backgrounds Graphed as Insertions per 100 kb within the Given Region

(A) Insertions were isolated using the TnsABC+E strain NLC51 pCW15 (TnsABC) pJP104 (TnsE).

(B) Insertions were isolated using the TnsABC^{A225V} strain NLC51 pCW15* (TnsABC^{A225V}) pTA106 (vector only).

(C) Insertions were isolated using the Tus$^-$ TnsABC+E strain NLC51 Δ*tus*::Amp^R pJP124 (TnsABC+E) (see Experimental Procedures). Error bars indicate the range that would result from +/− one insertion event in a given region. Dashed line indicates the average insertions per 100 kb over the entire chromosome. n = the number of isolates examined.

mapped the position of thirty-six independent insertions generated by TnsABC+E in a Tus$^-$ background using Southern blots. We found that transposition did not occur randomly; the distribution between the fragments was significantly different than would be expected for a random distribution ($p < 0.01$) (Figure 4C). Although the distribution was not random as in the case of insertions found with TnsABC^{A225V} (Figure 4B), it also did not match the distribution found in the Tus$^+$ background (Figure 4A). For example, in the Tus$^+$ background, 40% (14/35) of the insertions occurred into the fragment containing *terC* and *terA*, while in the Tus$^-$ background, only 14% (5/36) of the insertions occurred into this fragment, which encompasses 6% of the chromosome. An attractive explanation is that Tn7 continues to insert where chromosomal replication terminates in the Tus$^-$ strain background, but termination is no longer confined to a small region of the *E. coli* chromosome.

A System to Test If the TnsABC+E Transposition Pathway Recognizes DNA Double-Strand Breaks

What aspect of the termination of DNA replication is recognized by TnsE? Previous work indicates that homologous DNA recombination is highly stimulated in the *ter* region (Louarn et al., 1991). Stimulation of homologous recombination has been suggested to result from DSBs that are associated with the termination of DNA replication (Bierne et al., 1991; Horiuchi et al., 1994). Further evidence that DSBs occur in the chromosome when replication forks are paused was demonstrated by shifting a temperature-sensitive DnaB helicase mutant to the restrictive temperature (Michel et al., 1997). It is worth noting that the lambda hop transposition experiments above utilized strains deficient in the RecA protein (*recA56*) to prevent the vector backbone from integrating with endogenous lambda-like phages in the *E. coli* chromosome. While RecA is believed to be important in the repair of most DSBs, it is not unreasonable to entertain the possibility that less efficient modes of DSB repair might still be functioning in RecA$^-$ cells, or that a RecA-independent step in the DSB repair process may be recognized by TnsE (see Discussion). We were particularly interested in testing the effect of inducing DSBs at positions in the chromosome apart from the *terA-terC* region. We chose a genetic system where another bacterial transposon, Tn10, could be utilized to induce DSBs in a RecA$^+$ background.

A system for inducing DSBs was previously constructed for the study of homologous recombination (Galitski and Roth, 1997) using a class of Tn10 transposase mutants that allow the excision of the Tn10 transposon without allowing it to insert into a new target DNA (Haniford et al., 1989). A byproduct of the defective transposition event is a double-strand DNA break in the host at the position of the Tn10 (Galitski and Roth, 1997). Four different Tn10 transposase mutants were used in our study: two which allow Tn10 excision without reinsertion (G163D, P167S), a null allele (an in-frame coding sequence deletion of a 290 bp NcoI fragment, ΔNcoI), and a catalytic-defective but DNA-binding proficient Tn10 transposase (D97A/D161A) (Kleckner et al., 1996; Junop and Haniford, 1997) (see Experimental Procedures). The effect of inducing DSBs at two different positions in the chromosome, 15.1 min and 60.7 min, was examined (Nichols et al., 1998; Singer et al., 1989). The DSB sites were essentially equidistant from *oriC* and the *terA-terC* region (Figure 5A).

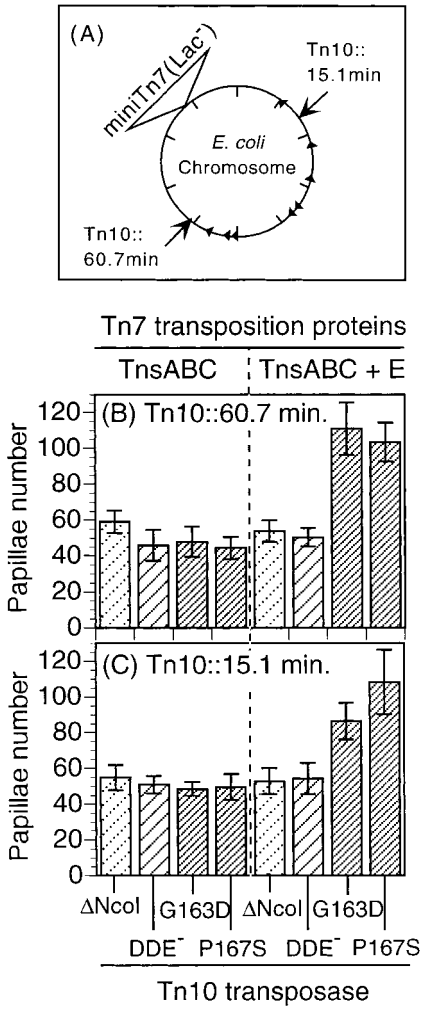

Figure 5. Inducing Double-Strand Breaks in the *E. coli* Chromosome Stimulates TnsABC+E Transposition

(A) Schematic diagram of the double-strand DNA break assay (see text for details). miniTn7(Lac$^-$) resides near *oriC* (84.2 min) in a position where the lactose utilization genes are not transcribed. Transposition proteins are provided in *trans*; a pACYC184 derivative encodes the Tn7 transposition proteins, and a pMB1 derivative encodes a mutant Tn10 transposase. Two different strains with Tn10 transposons located at chromosomal positions 15.1 and 60.7 min were tested. Triangles indicate the position and orientation of the ten *ter* sites as indicated in Figure 1.

(B and C) The number of Lac$^+$ papillae per lawn of miniTn7(Lac$^-$)-containing cells at ~70 hr post-plating at 30°C on MacConkey's indicator media (see Experimental Procedures) is shown. Strain NLC28 miniTn7(Lac$^-$)::84.2 min contained either pCW15 (TnsABC) or pJP123 (TnsABC+TnsE). Each was tested with one of four Tn10 transposase mutants: ΔNcoI, a DDE$^-$ mutant (D97A, D161A), G163D, or P167S. Strains contained a Tn10 insertion at 60.7 min (B) or 15.1 min (C). In each case 12 samples were tested; error bars show the standard deviation.

Tn7 transposition was assayed using a miniTn7 system based on promoter capture called the "papillation" assay (Stellwagen and Craig, 1997) (see Experimental

Procedures). The papillation assay provides a visual screen for transposition levels in lawns of bacteria on indicator media. The papillation assay is well suited for detecting low frequency transposition events and allows the use of RecA$^+$ strains. A miniTn7 element encoding the genes for lactose utilization but that does not have the requisite *lac* promoter, miniTn7(Lac$^-$), is located in a position and orientation in the chromosome where it is not transcribed, yielding a cell which is phenotypically Lac$^-$. If the transposition proteins are provided in *trans*, the transposon can move, introducing the miniTn7(Lac$^-$) element to new positions, some of which place the *lac* genes behind actively transcribed promoters, generating a cell that is now phenotypically Lac$^+$. On the MacConkey's lactose indicator media plates used in the experiment, red Lac$^+$ "papillae" arise from the otherwise white lawn of cells. The number of red Lac$^+$ papillae in a lawn provides a measure of transposition frequency. To insure that TnsE-dependent transposition was being monitored rather than unrelated genetic events, papillation levels in TnsABC+E strains were compared to strains with TnsABC only.

TnsE-Dependent Transposition Is Stimulated Specifically by a DNA Double-Strand Break, and Insertions Are Attracted Proximal to the Induced Break

The papillation assay was used to monitor the level of TnsABC+E transposition in cells. Inducing DSBs specifically stimulated the level of TnsE-dependent transposition; no increase was found in the absence of TnsE. The TnsE-dependent stimulation required an active Tn10 transposase (Figures 5B and 5C). A low level of Lac$^+$ papillae was found in cells without TnsE and in strains where no DSBs were induced, but these Lac$^+$ events were largely independent of Tn7 transposition events (see below). TnsABC+E transposition was stimulated when breaks were induced at either site, but the magnitude of stimulation seemed greatest when DSBs were induced at 60.7 min.

Multiple explanations existed for the DSB-induced stimulation of TnsE-dependent transposition. The stimulation of TnsABC+E transposition could have been a byproduct of the SOS induction of a protein required by TnsE for transposition. Alternatively, something associated with the DSB itself could be recognized by the TnsE protein, allowing the subsequent recruitment of the Tn7 transposition apparatus. The position of the Tn7 insertion events was examined to see if the TnsABC+E pathway directs insertions around the site of the DSB. To determine the position of the Tn7 insertion, Lac$^+$ papillae were streak purified and the Lac$^+$ allele was moved to a clean genetic background by P1 transduction (see Experimental Procedures). The position of the Lac$^+$ Tn7 insertion was determined by sequencing the DNA flanking the transposon.

A striking observation was made when transposition events stimulated by DSBs at 60.7 min were sequenced. Transposition events were directed proximal to the site of the DSB, a region not previously used for TnsE-dependent transposition (compare Figure 6A with Figure 1). In 64% (7/11) of the Lac$^+$ papillae, insertion events occurred over a region within 150 kb of the DSB. In 27% (3/11) of the papillae, transposition events occurred proximal to *ter* sites, 3 kb from *terA*, 13 kb from *terD*, and 55 kb from *terJ*. One of the eleven papillae isolates examined did not result from Tn7 transposition (see below).

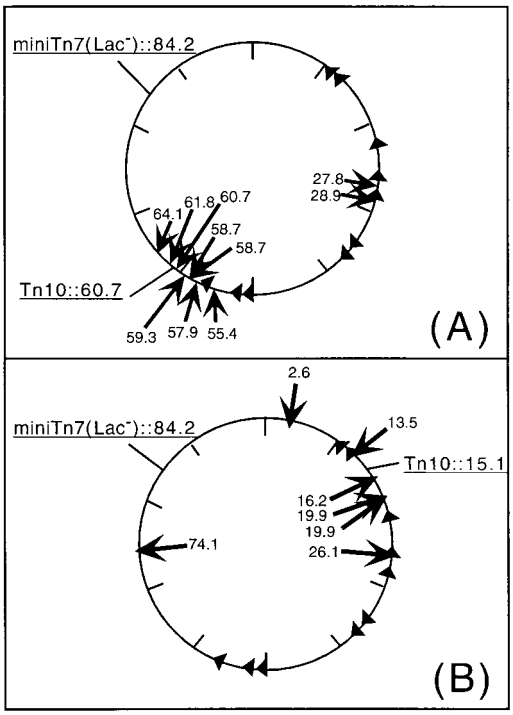

Figure 6. Position of Tn7 Insertions Following Induction of Double-Strand DNA Breaks with the Tn10 Transposase Mutant P167S.

(A) Chromosomal position of ten transposition events in a strain containing a Tn10 at 60.7 min.

(B) Chromosomal position of seven transposition events in a strain containing a Tn10 at 15.1 min. Numbers indicate the minute position of miniTn7 insertions (arrows) and *ter* sites (triangles). Placement of the arrow outside the circle indicates the miniTn7 inserted in a clockwise right-to-left orientation, placement inside indicates counter-clockwise. Triangles indicate the position and orientation of the ten *ter* sites as indicated in Figure 1.

Transposition events obtained when DSBs were induced at 15.1 min were also examined. Inducing DSBs at 15.1 min was less efficient at stimulating Tn7 transposition (Figure 5) and at attracting Tn7 transposition events proximal to the break site (Figure 6). In 88% (7/8) of the papillae, the Lac$^+$ phenotype resulted from transposition events. Insertions did not occur in the *terA-terC* region (compare Figure 6B with Figure 1) but did not show the same grouping found with excision of the Tn10 from 60.7 min (compare Figure 6B with Figure 6A). One insertion was adjacent to the nearest *ter* site, 0.6 kb from *terI*. One of the Lac$^+$ papillae was from an event independent of Tn7 transposition (see below).

In the Absence of DNA Double-Strand Breaks, Lac$^+$ Papillae Largely Result from Events Independent of Tn7 Transposition

A low level of Lac$^+$ papillae was found in all strain backgrounds (Figure 5). The background frequency of Lac$^+$ papillae was similar in cells containing TnsABC alone

and in cells containing TnsABC+E without the induction of DSBs, suggesting that the same process was involved in producing these papillae. This was not an unexpected result, given that the TnsABC+E vector used in the papillation assay produces a very low amount of TnsE, and that the frequency of TnsE-dependent transposition is greatly effected by the level of TnsE expression (data not shown).

To investigate the process that gave rise to the background level of Lac^+ papillae, we mapped insertions which occurred in cells expressing TnsABC (with and without DSBs) and cells expressing TnsABC+E without DSBs. In 83% (40/48) of Lac^+ papillae, results from restriction mapping, DNA sequencing, and P1-mediated genetic transduction indicated that the miniTn7 transposon had not moved, but an event which was genetically linked to the miniTn7 was responsible for the Lac^+ phenotype. Detailed analysis of 11 of these isolates indicated that an IS2 element endogenous to *E. coli* K12 (Blattner et al., 1997) transposed to a position where the IS2 promoter could read into the miniTn7 element to give the Lac^+ phenotype. Random IS2 insertion events adjacent to the miniTn7 element could also explain the remaining genetically linked Lac^+ events found in the absence of Tn7 movement.

In 17% (8/48) of the background Lac^+ papillae found in all strains tested, genuine Tn7 transposition occurred, and these rare insertion events were not proximal to the Tn10 (2.7, 2.9, 5.0, 84.6, 85.0, 86.2, 90.3, and 92.9 min). Two of these insertions occurred in cells where there was no TnsE, providing in vivo evidence that Tn7 can transpose at a very low frequency in the absence of TnsE or TnsD. Presumably, the low level of TnsE produced with the TnsABC+E vector used in the papillation assay was insufficient to give detectable TnsE-dependent transposition without the stimulation of DSBs. Comparison with previous results from the more quantitative but less sensitive lambda hop assay suggest that the frequency of TnsABC transposition in the absence of a target site selecting protein is down over 20-fold from TnsABC+E levels and over 10,000-fold from TnsABC+D levels (Stellwagen and Craig, 1997).

Discussion

We report that the bacterial transposon Tn7 will preferentially transpose into the region of the chromosome where DNA replication terminates. Although the insertions are distributed over a relatively large region of the chromosome, over a third of the insertion events are close to *ter* sites, strongly suggesting the involvement of a termination-related event (Figure 1). When DSBs were induced in the chromosome, TnsE-dependent transposition was stimulated and insertions occurred proximal to the DSBs (Figures 5 and 6). Given that DSBs are associated with the termination of DNA replication, a reasonable interpretation of the data is that TnsE might recognize *ter* sites by DSBs associated with termination of DNA replication.

Tn7 Inserts Proximal to DNA Double-Strand Breaks

We found that inducing DSBs stimulated Tn7 transposition, and the resulting transposition events were attracted proximal to the DSB (Figures 5 and 6). Our results indicate that either TnsE recognizes DSBs directly or some component of the DSB repair process.

It is unclear why inducing DSBs at different locations in the chromosome results in modest differences in stimulating Tn7 transposition and attracting transposition events (Figures 5 and 6). Differences between DSB sites could possibly be explained by differences in the efficiency of DSB formation or differences in the proficiency of DSB repair at different locations. One of the DSB sites was totally outside all of the *ter* sites, possibly indicating that repair of DSBs is different outside the *ter* sites.

The finding that DSBs can attract Tn7 insertion events may provide an explanation for the TnsABC+E transposition events that occurred outside the *ter* region (Figure 1). Replication fork collapse is believed to happen frequently in wild-type cells during exponential growth in rich oxygenated media, occurring in an estimated 25% of the cells (Kuzminov, 1995). Any single-strand nick will be converted into a DSB during DNA replication when half of the replication fork runs out of a contiguous template, an event called replication fork collapse. Consistent with a role for DSBs formed by replication fork collapse, it has previously been found that cells that lack DNA adenine methylase (*dam*$^-$) are specifically stimulated for Tn7 transposition in the TnsE-dependent pathway (R. DeBoy and N. L. C, unpublished observation). Cells that are *dam*$^-$ accumulate DSBs, probably as a byproduct of single-strand interruptions resulting from attempted mismatch repair being converted into DSBs by replication fork collapse (Marinus and Morris, 1975; Wang and Smith, 1986).

How Does TnsE Recognize DNA Double-Strand Breaks?

It is unclear which aspect of DSBs attracts TnsE-dependent transposition events. Many cell processes are required for the repair of DSBs in bacteria. The TnsE protein could specifically recognize DSBs, or TnsE could interact with DNA structures or DNA/protein complexes associated with the DSB repair process.

Any potential target for TnsABC+E transposition would have to explain why insertions are stimulated to occur over a region many kilobases from the site of the DSB. One explanation for the wide distribution of insertions around the DSB site could be that the genetic location of the DSB moves as exonucleases degrade from the initial DSB site. For example, the RecBCD helicase/exonuclease associated with the repair of DSBs is a highly processive enzyme capable of unwinding >30 kb per binding event, which would degrade DNA from the point of the DSB (Kowalczykowski et al., 1994). While the nuclease activity of the RecBCD enzyme is inactivated at chi sites found frequently in the chromosome, chi sites are not always recognized, which could allow the enzyme to travel great distances before inactivation (Kowalczykowski et al., 1994).

A second possibility holds that the wide distribution of insertions around the double-strand break could indicate that a very large structure or complex associated with the repair of DSBs is the actual target recognized by TnsE.

A third possibility that could explain the distribution of transposition events around the DSB could involve a role for long-range interactions where structures or

complexes associated with the DSB contact distal sites. It is known that other processes occurring upon DNA have been found to span regions of over 100 kb in the chromosomes of bacteria. For example, the interaction between γδ resolution sites in the chromosome can be detected over distances of >100 kb (Higgins et al., 1996). Moreover, in Tn7 transposon immunity, the immunity-conferring protein TnsC can inhibit further Tn7 insertions from occurring in a region >190 kb from the transposon in the chromosome (DeBoy and Craig, 1996).

TnsE Directs Insertions Where DNA Replication Terminates

We found that the TnsE protein is responsible for the ability of Tn7 to direct insertions to the terminus region and that this targeting is not a general propensity of the Tn7 core transposition machinery. We mapped transposition events catalyzed with a mutant transposition machine, TnsABC^{A225V}, that does not require a target site selecting protein (Stellwagen and Craig, 1997). We found that the TnsABC^{A225V} transposition events occurred throughout the chromosome, not specifically into the *ter* region (Figure 4B).

Our results indicate that Tn7 targeting does not require a direct interaction between the Tus-*ter* complex and TnsE, because Tus is not required for TnsE-directed insertion. While Tus-mediated termination is not required for TnsE-dependent transposition, the profile of Tn7 insertions does change in Tus$^-$ cells (Figures 4A and 4C). The simplest explanation for a Tus-dependent focusing of insertion into a restricted portion of the chromosome is that TnsE-directed transposition events in the Tus$^-$ background predominantly occur at the site of replication termination, but that replication termination is no longer limited to a small region of the chromosome in the Tus$^-$ background.

Tn7 May Recognize the *ter* Region by Double-Strand Breaks Associated with the Termination of DNA Replication

The intimate relationship between the termination of DNA replication and recombination has been the topic of many studies. Hyper-recombination around *ter* sites has been suggested to result from DSBs associated with the termination of DNA replication (Bierne et al., 1991; Louarn et al., 1991; Horiuchi et al., 1994). In experiments where replication forks were halted by shifting a temperature-sensitive helicase mutant to the restrictive temperature, chromosomal DSBs were found to occur (Michel et al., 1997). It therefore appears likely that DSBs occur when the DnaB helicase is halted at *ter* sites. As noted above, TnsABC+E transposition is attracted adjacent to DSBs, possibly by recognizing complexes involved in the repair of DSBs. Therefore, Tn7 may recognize the replication terminus region by DSBs associated with the termination of DNA replication. It remains formally possible that something common to both DSBs and the termination of DNA replication is the actual target for TnsABC+E transposition.

One perplexing aspect of a model where TnsE recognizes the *ter* region by DSBs associated with the termination of DNA replication is the dispensability of the RecA protein. Insertions isolated with the lambda hop assay are carried out in a RecA$^-$ (*recA56*) background, indicating that the observed targeting to the *ter* region is not RecA-dependent. The primary cellular mechanism of repairing DSBs is thought to rely heavily on the RecA protein to help establish a replication fork (Asai et al., 1994; Cox, 1998). Testing the role of RecA in Tn7 transposition is difficult because one cannot use the more quantitative lambda hop assay. Moreover, comparing RecA$^+$ and RecA$^-$ strains for transposition frequency with the papillation assay is complicated by the pleiotropic effects of RecA$^-$ mutations, e.g., cell viability. It is possible that a redundant, albeit less efficient, recombination system operating in the RecA$^-$ strains allows some cells that have experienced DSBs and a Tn7 insertion event to survive. By sequencing from the chromosome, we have found that the *recE/recT* genes do reside in our strain background (our unpublished observation), although the expression level of these proteins is unknown. The RecE/RecT proteins have been shown to allow DSB repair in RecA$^-$ strains (reviewed in Kolodner et al., 1994). Conversely, the chromosome that received the DSB and provided the signal for Tn7 insertion may be lost while the transposition is directed into a sister chromosome. For example, if a D-loop structure was recognized by TnsE, the broken chromosome could form the invading strand and be lost while insertions are directed into the sister chromosome. Future work concerning Tn7 transposition mediated by the TnsABC+E proteins could provide information concerning RecA-independent modes of DSB repair in *E. coli*.

What Does the Presented Work Say about Termination in Bacteria?

Experimental evidence suggests that the termination of chromosomal replication occurs between *trp* (28 min) and *manA* (36 min) in *E. coli* (Pelletier et al., 1988). By examining the sequence of MG1655, one might assume that replication terminates around *terC*. Polar aspects of the chromosome such as the orientation of chi sites, the orientation of DnaG priming sites used for lagging-strand DNA synthesis, and a bias in the orientation of operons all appear to converge at *terC* (Blattner et al., 1997). These polar aspects of the genome would suggest that the termination of DNA replication predominantly occurs at *terC*. Consistently we found that insertions occurred most frequently near *terC* (Figure 1). We also found insertions close to *terI* (Figures 1 and 6B), a terminator which was only identified by its DNA sequence and not by its function at its position in the chromosome (Coskun-Ari and Hill, 1997). The results found here suggest that *terI* may indeed be used to terminate DNA replication. One of the most intriguing observations concerning the termination of bacterial DNA replication is the total dispensability of the process without any apparent effect on cell fitness (Hill, 1996). It is worth noting that the effect on TnsABC+E insertion found with Tus$^-$ cells is one of the few discrete phenotypes associated with the Tus$^-$ allele.

What Does the Current Work Tell Us about the Ability of TnsE-Dependent Transposition to Target Conjugating DNA?

Similarities exist between TnsE-dependent transposition into conjugating DNA and transposition around the

site of DSBs (Wolkow et al., 1996; this work). TnsE-dependent transposition is stimulated by both the presence of actively conjugating DNA in the cell and by DSBs (Figure 5). Transposition events are targeted into actively conjugating DNA and proximal to a DSB without sequence specificity (Figure 6). Could Tn7 recognize conjugating DNA by the presence of DSBs? DSBs may occur frequently in conjugating DNAs. Conjugating DNAs are known to be hyper-recombinogenic for homologous recombination, possibly as a byproduct of DSBs (Kuzminov, 1995). Previous work has shown that TnsABC+E transposition into conjugating DNA occurs in the recipient cell. Conjugal DNA replication in the recipient cell is carried out exclusively by lagging-strand DNA synthesis as one strand of the plasmids enters the cell in the 3′ to 5′ orientation (Wolkow et al., 1996). As DNA replication must be continuously primed in the recipient cell, frequent single strand would be expected until the RNA primers were removed and ligations complete. Lingering single-strand interruptions associated with lagging-strand DNA synthesis could be converted into DSBs by bidirectional plasmid replication in recipient cells.

What Is the Benefit of Having a Pathway of Insertion that Recognizes Places Where DNA Double-Strand Breaks Occur?

Tn7 can carry out two pathways of transposition that recognize different types of target sites. The TnsABC+D proteins allow Tn7 to transpose at a high frequency into a single site in the chromosome, *attTn7*, which is found in numerous bacterial species (Craig, 1989). The ability to transpose into a site found in many bacteria where it does not kill the host suggests that *attTn7* provides a "safe haven" for Tn7. The TnsABC+E proteins direct insertions into sites recognized by a DNA structure or a protein/DNA complex. The TnsE-dependent pathway could encourage the dispersal of Tn7. If DSBs are a general property of conjugation, directing transposition near DSBs could identify the target DNA as being capable of transporting the transposon to new hosts.

Tn7's ability to direct transposition into the terminus region could reduce the risk of inserting into essential genes in cells that lack *attTn7*. It has been shown that a large region (~340 kb) around the terminus is not essential for growth and can be deleted (Henson and Kuempel, 1985). Alternatively, the ability to target the terminus region may be an unintended by-product of being able to preferentially target conjugative plasmids.

By directing transposition adjacent to DSBs, Tn7 could "try out" target sites that may be essential for cell viability without killing the host. DSBs are likely to occur frequently when a replication fork collapses at single-strand interruptions. Transposition proximal to DSBs could therefore allow Tn7 to target collapsed replication forks. By inserting behind a replication fork, Tn7 could be insured of inserting into one of two sister chromosomes. If Tn7 inserted into an essential gene, the host would survive because another copy would reside on the sister chromosome. While Tn7 would lose the ability to colonize one of the daughter cells, Tn7 would ensure that a lethal insertion does not occur in one daughter cell.

Experimental Procedures

Bacterial Strains and Plasmids

All strains were derivatives of the NLC28, a valine-resistant derivative of MC4100 F- *araD139 Δ(argF-lac)U169 rpsL150 relA1 flb5301 deoC1 ptsF25 rbsR* (Casadaban and Cohen, 1979). Genetic markers were introduced via P1 transduction (Miller, 1992). NLC51 (NLC28 *recA56*) was constructed by first introducing a genetic marker linked to *recA* (*srlD3131*::Tn10), and then selecting for P1 transductants on minimal sorbitol plates using P1 lysates grown on a *recA56* strain and screening for the RecA-associated phenotype of UV sensitivity. The *Δtus*::AmpR allele was kindly provided by Jean-Paul Louarn (Louarn et al., 1991). The Tn10 insertion alleles (15.1 min *zbf3057*::Tn10 and 60.7 min *srlD3131*::Tn10) were from the Tn10 mapping set obtained from Nancy Trun (Nichols et al., 1998; Singer et al., 1989).

Tn7 transposition proteins were derivatives of pCW4 (Waddell and Craig, 1988). pCW15 is a Cam^R pACYC184 subclone from pCW4 of a fragment encoding TnsABC (Waddell and Craig, 1988). The pJP123 and pJP124 plasmids encode TnsABC+E and were constructed by cloning a Klenow-filled HinDIII TnsE fragment from pCW4 into the NruI site of pCW15. Plasmids pJP123 and pJP124 differ in the orientation of the TnsE-encoding HinDIII fragment. pJP123 produces very low amounts of TnsE, while pJP124 produces TnsE at higher levels (our unpublished observation). pJP104 was constructed by introducing the HinDIII fragment encoding TnsE from pCW4 into the HinDIII site pSC101 derivative, pTA106 (from Nancy Trun).

Plasmids used to induce DNA double-strand breaks were generously provided by John Roth (Galitski and Roth, 1997). The Tn10 transposase mutants are in a pZT379 backbone (Amp^R *lacIq ori*M13 *ori* pMB1) where expression is repressed in the absence of lactose. Various Tn10 transposase mutants are expressed with a Ptac promoter, pZT381 (transposase null allele, due to an in-frame NcoI deletion), pZT382 (transposase mutant G163D), and pZT383 (Transposase mutant P167S) (Haniford et al., 1989). Both the G163D and P167S mutants allow Tn10 excision without reinsertion. The Tn10 transposase mutant D97A/D161A (Kleckner et al., 1996, Junop and Haniford, 1997) was constructed into the pZT379 backbone using the pDH10 derivative pDH168 (generously provided by David Haniford) as described previously (Galitski and Roth, 1997).

Transposition was assayed with transposon derivatives, miniTn7 elements, where the *cis*-acting left and right Tn7 ends flank selectable markers. The miniTn7(Kan^R) has the Tn7 ends flanking the Kan^R cassette from pUC4K (McKown et al., 1988). The miniTn7(Lac^-) element has the Tn7 ends flanking the *lacZYA* genes without a promoter and the Kan^R cassette from pUC4K (Hughes, 1993).

Transposition Assays

In vivo transposition was carried out using either the "lambda hop" assay or a "papillation" assay. In the lambda hop assay, a defective lambda phage containing miniTn7(Kan^R) is used that cannot replicate or integrate in NLC51 (McKown et al., 1988). The phage is introduced into a strain where the Tns proteins are provided in *trans*. Transposition frequency was determined by scoring the number of Kanamycin resistant colonies per infectious phage particle as measured by plaque forming units.

The papillation assay provides a visual screen for transposition levels in lawns of bacteria on indicator media (Huisman and Kleckner, 1987; Stellwagen and Craig, 1997). A miniTn7 element encoding the genes for lactose utilization but that does not have the requisite *lac* promoter, mTn7(Lac^-), is located in a position and orientation in the chromosome where it is not transcribed, yielding a cell which is phenotypically Lac^-. If the transposition proteins are provided in *trans*, the transposon can move, introducing the miniTn7(Lac^-) element to positions behind actively transcribed promoters, allowing a cell which is now phenotypically Lac^+. On the MacConkey's lactose indicators plates used in the experiment, red Lac^+ "papillae" arise from the otherwise white lawn of cells. The number of red Lac^+ papillae in a lawn provides a measure of transposition frequency.

Mapping Tn7 Insertion by Pulsed Field Gel Electrophoresis

Pulsed field gel electrophoresis was carried out with a Bio-Rad CHEF-DRIII apparatus as described previously (Heath et al., 1992;

Perkins et al., 1992). DNA was prepared and digested with restriction enzymes in InCert (FMC) agarose as described previously (Heath et al., 1992). Following digestion with NotI (15 units, NEB) or SfiI (40 units, NEB), samples were incubated in TE (pH 8.0) to remove the restriction buffer, melted at 75°C, and loaded into wells of a 1% pulsed field grade agarose (Bio-Rad) gel in 0.5× TBE. To separate the entire NotI-digested *E. coli* chromosome on a 21 cm (long) × 14 cm (wide) gel, samples were run for 40 hr at 14°C with a ramping swichtime from 5–40 s at 6 volts/cm and a fixed angle of 120°. For the SfiI digest, samples were run for 24 hr under the same conditions as the NotI digest.

Southern blots were used to determine which fragment contained the miniTn7 insertion. The probe was specific to the Kan^R determinant in miniTn7, a HinDIII-XhoI fragment from pUC4K, prepared as described previously (Sarnovsky et al., 1996) The NotI chromosomal digest marker lane was produced by filling the recessed ends using the Klenow fragment of DNA Pol I from Boehringer Mannheim Biochemicals (BMB) and [^{32}P]CTP; unincorporated nucleotides and buffer were removed from the DNA-containing gel slice by incubating in TE (pH 8.0).

The genome sequence of the *E. coli* strain MG1655 indicates that NotI cuts the chromosome of *E. coli* 23 times (Blattner et al., 1997). The strain currently under study, NLC28 (MC4100 background), differs slightly from MG1655 and its NotI restriction pattern was clarified by Heath et al. (1992). The C and D restriction fragments are fused in MC4100 as a result of a large chromosomal deletion (Δ(*argF-lac*)*U169*). The fragments F and I both contain uncharacterized deletions altering their size. Fragment I is smaller in MC4100, reduced from 90 to 78 kb in size (Heath et al., 1992). In the work of Heath et al., they noted that either the F or O fragment received a deletion. Using insertions that were mapped by sequencing and by Southern blotting, we determined that fragment F was around 245 kb, not the MG1655 size of 251 kb (data not shown) (Heath et al., 1992). To statistically test if the insertions occurred randomly, paired chi-square tests were used to compare "expected" versus "found" frequencies of insertions per 100 kb in each of the 14 segments of the chromosome (Figures 4A–4C).

Determining Tn7 Insertion Sites by DNA Sequencing

In many cases, the position of Tn7 insertions was determined by sequencing on an ABI automatic sequencer. Primers specific to the transposon ends were used to determine the DNA sequence flanking the transposon, NLC95-ATA ATC CTT AAA AAC TCC ATT TCC ACC CCT (left end) or NLC646-ACT TTA TTG TCA TAG TTT AGA TCT ATT TTG (right end). The sequencing template was either the insertion cloned from the chromosome or a template produced by PCR (see below). In either case, insertions were localized to the *E. coli* MG1655 chromosome sequence version M52 (http://www.genetics.wisc.edu).

Tn7 insertions were obtained by first isolating chromosomal DNA (Ausubel et al., 1988) and cloning the insertions into pACYC184 using enzymes which do not cut in the transposon, EcoRI for miniTn7(Kan^R) and/or NcoI for miniTn7(Lac^-).

PCR templates were produced using primers specific to the transposon in conjunction with primers encoding random sequences to anneal to the region flanking the transposon. The procedure was essentially as described previously, where two PCR reactions are utilized (Chun et al., 1997). In the first PCR reaction, the transposon-specific primer NLC272-ATT TTC GTA TTA GCT TAC GAC GCT ACA CCC (left end) or NLC236-ACG AGC GTA ATG GCT GGC CTG TTG AAC AA (right end) was used with the primer NLC771-GGC CAC GCG TCG ACT AGT ACN NNN NNN NNN GCT GG in a 20 μl reaction (1× BMB PCR buffer + 10 mM Mg^{2+}, 2.5 mM each NTP, 1.25 units Taq DNA polymerase [BMB]). The template for the first reaction was 1 μl of a single colony suspended in 50 μl ddH2O and PCR carried out in multiple steps (step 1, [1×], 95°C, 5 min; step 2, [6×], 95°C, 30 s, 42°C, 30 s, 72°C, 1 min; step 3, [25×], 95°C, 30 s, 65°C, 30 s, 72°C, 1 min; step 4, 4°C park). A second PCR reaction was used to amplify PCR products containing the transposon end and its flanking DNA sequence. In the second PCR reaction, NLC95 (left end) or NLC99- TCG GTG AGT TTT CTC CTT CAT TAC AGA AA (right end) was used with a primer that was identical to the 5′ region of the "random" primer. The same PCR reaction components were used except the template was provided from a 1 μl sample of a 1:5 dilution of the first PCR reaction (step 1, [1×], 95°C, 1 min; step 2 [30×], 95°C, 30 s, 65°C, 30 s, 72°C, 1 min; step 3, [1×], 72°C, 5 min; step 4, 4°C park). PCR samples were cleaned with the Qiagen PCR cleanup minicolumns.

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