

## NOTES

# Target Immunity of the Tn3-Family Transposon Tn4430 Requires Specific Interactions between the Transposase and the Terminal Inverted Repeats of the Transposon<sup>▽†</sup>

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**Specificity of the Tn4430 target immunity signal was examined by fusing the transposase TnpA to the LacI repressor of *Escherichia coli*. The resulting chimeric proteins failed to impose immunity to DNA targets carrying copies of the *lacO* operator, though they were proficient in *lacO* binding *in vivo* and remained responsive to wild-type immunity conferred by the Tn4430 inverted repeat end. Intriguingly, the presence of *lacO* repeats within the target was found to strongly influence target site selection by Tn4430, but in a LacI-independent manner.**

Tn4430 is a transposon of the Tn3 family that was originally isolated from *Bacillus thuringiensis* (Fig. 1A) (12). Transposons of this family exhibit “target immunity,” a mechanism that prevents multiple insertion of the element into the same DNA molecule (9). Immunity has also been described for two other bacterial transposons, the bacteriophage Mu (1) and Tn7 (14). In all cases, the presence of a single copy of the transposon end is sufficient to confer immunity to the target, indicating that specific recognition of the target DNA by the transposase protein plays a central role in the process (2, 6, 8, 10). In the case of Mu and Tn7, target immunity results from the interplay between the transposase (i.e., MuA and TnsAB, respectively) and an ATP-dependent DNA binding protein involved in target capture (i.e., MuB and TnsC, respectively) (3, 4). No equivalent accessory protein is found in Tn3 family transposons, indicating that the transposase is the only transposon-encoded protein involved in immunity. The mechanism of “molecular repulsion,” underlying transposition immunity of this family of transposons, remains poorly understood.

In this study, we sought to see whether specific recognition of Tn4430 terminal inverted repeat (IR; 38 bp) by the TnpA transposase is a mandatory step in transposition immunity or whether TnpA binding to unrelated DNA sequences is sufficient to reorient target site selection. To this end, we examined whether fusion proteins between TnpA and the LacI repressor of *Escherichia coli* could confer transposition immunity to target molecules containing copies of the *lacO* operator.

**Construction and expression of the TnpA-LacI fusions.** The LacI repressor was chosen as a source for heterologous DNA

binding specificity because of its well-characterized structure and DNA binding properties, the possibility to control its activity using specific effectors such as isopropyl β-D-1-thiogalactopyranoside (IPTG), and its ability to support both N- and C-terminal fusions with other proteins (11). TnpA was fused to a truncated derivative of LacI, LacI349, lacking the C-terminal α-helix involved in tetramerization (13). This protein remains able to form dimers and bind to *lacO* in an IPTG-dependent manner. A cMyc epitope (Myc) was also added to the different chimeric proteins for immunodetection. Together, one N-terminal (LacI::TnpA::Myc) and two C-terminal (TnpA::LacI::Myc and TnpA::Myc::LacI) fusions between TnpA and LacI349 were constructed, with the two C-terminal fusions differing by the position of the cMyc epitope (Fig. 1B; also see Table S1 in the supplemental material). The constructs were placed under the control of a constitutive *plac* promoter derivative (*placO*<sup>c</sup>), in which the endogenous *lacO1* operator has been mutated to make it unresponsive to LacI (Fig. 1B) (5). Gene cassettes expressing the singly tagged TnpA (TnpA::Myc) and LacI (LacI::Myc) proteins were also constructed to be used as a control in our experiments (Fig. 1B).

Total protein extracts were prepared from *E. coli* cells grown overnight that harbored the different constructs and were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2). Coomassie blue staining of the gel revealed marked bands at the expected positions for TnpA::Myc (117 kDa) and LacI::Myc (42 kDa) (Fig. 2A). A specific band corresponding to a higher-molecular-mass protein (154 kDa) was observed for the strains harboring the vectors expressing LacI::TnpA::Myc, TnpA::LacI::Myc, and TnpA::Myc::LacI, indicating that the three chimeric proteins were efficiently expressed under the conditions used (Fig. 2A). Full-length expression of the three fusions was confirmed by Western blot analysis using a monoclonal antibody raised against the cMyc epitope (Fig. 2B). Additional bands corre-

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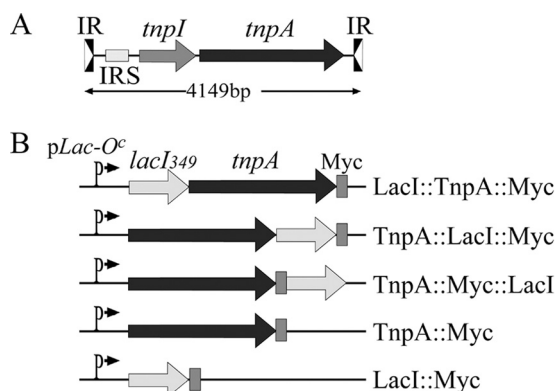


FIG. 1. (A) Genetic organization of Tn4430. The transposon (4,149 bp) is delineated by two identical inverted repeats (IR) of 38 bp that are specifically contacted by the transposase TnpA. The internal recombination site (IRS; 116 bp) is where the tyrosine recombinase TnpI acts to resolve the replicative intermediates (cointegrates) of transposition (15). (B) Schematic overview of the fusion proteins used in this study. The LacI349 and TnpA coding sequences are shown as shaded and black arrows, respectively. The position of the cMyc epitope is shown as a gray box.

sponding to N-terminally truncated forms of the proteins were also detected for the different fusions as well as for the wild-type TnpA::Myc protein (Fig. 2B).

**Transposition mediated by the TnpA-LacI chimeric proteins into *lacO*-containing targets.** A “plasmid rescue” assay was set up in *Escherichia coli* to assess the ability of the TnpA-LacI chimeric proteins to promote transposition of a Tn4430-derived mobile cassette (Mini-TnKm) into different targets *in vivo* (Fig. 3). This assay is based on the replicative transposition mechanism of Tn4430 that generates cointegrate DNA molecules in which the donor and target replicons are fused together by two copies of the transposon (12). The formed cointegrates stably accumulated within the cells as the experiments were performed in the absence of the TnpI recombinase, which normally resolves them into fully recombined transposition products (15). It relies on the presence of the following three compatible plasmids in the same strain: (i) a transposase expression vector, (ii) a donor plasmid containing the Mini-TnKm cassette, and (iii) a target plasmid with a thermosensitive replication initiation protein (Fig. 3A). Different versions of the target plasmid were used to compare transposition frequencies into a “naïve” target with those obtained for equivalent plasmids carrying a copy of the Tn4430 IR end versus 3 or 5 copies of the *lacO* operator (Fig. 3B).

*E. coli* cells of the LacI<sup>−</sup> strain HB101Δ*lacI* (see Table S1 in the supplemental material) harboring different combinations of the three different plasmids were cultured overnight and plated at both permissive (i.e., 28°C) and nonpermissive (i.e., 42°C) temperatures for target plasmid replication. Incubation of the plates at the nonpermissive temperature selected cells in which replication of the target plasmid was rescued by formation of a transposition cointegrate with the donor plasmid (Fig. 3A). The ratio of colonies growing at the nonpermissive and permissive temperatures gave an estimate of the transposition frequency in each set of experiments (Fig. 3C).

Transposition mediated by wild-type TnpA::Myc into the naïve target occurred at a relatively high frequency, generating

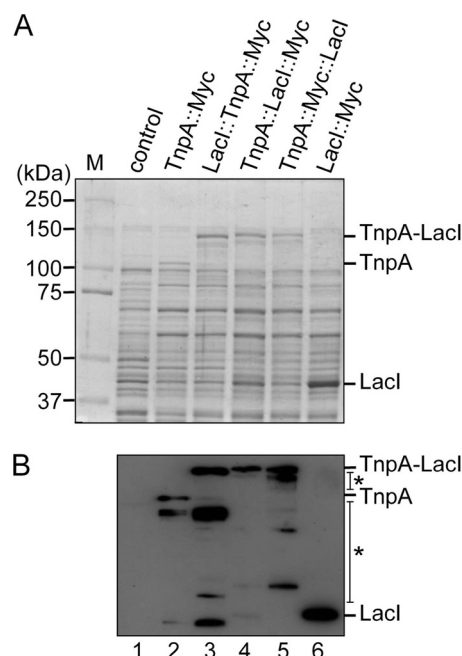


FIG. 2. Expression of the TnpA-LacI fusion proteins in *E. coli*. Crude extracts of HB101Δ*lacI* cells expressing the different fusion proteins under the constitutive *placO<sup>c</sup>* promoter were separated on a 6% SDS-PAGE gel. The gel was stained with Coomassie blue (A) and blotted with an antibody raised against the cMyc epitope (B). Positions of the LacI, TnpA, and TnpA-LacI fusion proteins are indicated to the right. Asterisks (\*) show the positions of truncated forms observed with the different proteins. “Control,” extracts of cells harboring the empty vector pGIEN001 (see Table S1 in the supplemental material); “M,” molecular mass ladder in kilodaltons.

a cointegrate product in about 5% of the cells. Similar transposition levels were measured with the *lacO*-containing target plasmids, showing that the presence of the *lacO* operator did not affect the activity of the wild-type transposase. In contrast, transposition into the plasmid containing the IR end of Tn4430 was strongly reduced, occurring at an ~50-fold lower frequency than in the naïve target (Fig. 3C). This is consistent with the fact that the 38-bp IR sequence of Tn4430 is sufficient to specify immunity to the DNA (M. Lambin et al., unpublished data).

Transposition mediated by both C-terminal fusions TnpA::LacI::Myc and TnpA::Myc::LacI into the permissive target was ~8 to 14 times less efficient than transposition mediated by TnpA::Myc, whereas the frequency measured for the N-terminal fusion LacI::TnpA::Myc was reduced by about 3 orders of magnitude (Fig. 3C). In spite of their difference in transposition activity, the three chimeras showed a further decrease in transposition into the IR-containing plasmid, displaying the same level of target immunity as wild-type TnpA::Myc (Fig. 3C). This indicates that adding LacI349 at the N or C terminus of TnpA did not affect its ability to mediate transposition immunity. However, the transposition frequency measured for the *lacO*-containing plasmids was not significantly different from that obtained with the naïve target, with any of the TnpA-LacI fusions, irrespective of whether IPTG was added to the culture or not (Fig. 3C).

**Binding of the TnpA-LacI fusion proteins to the *lacO* oper-**

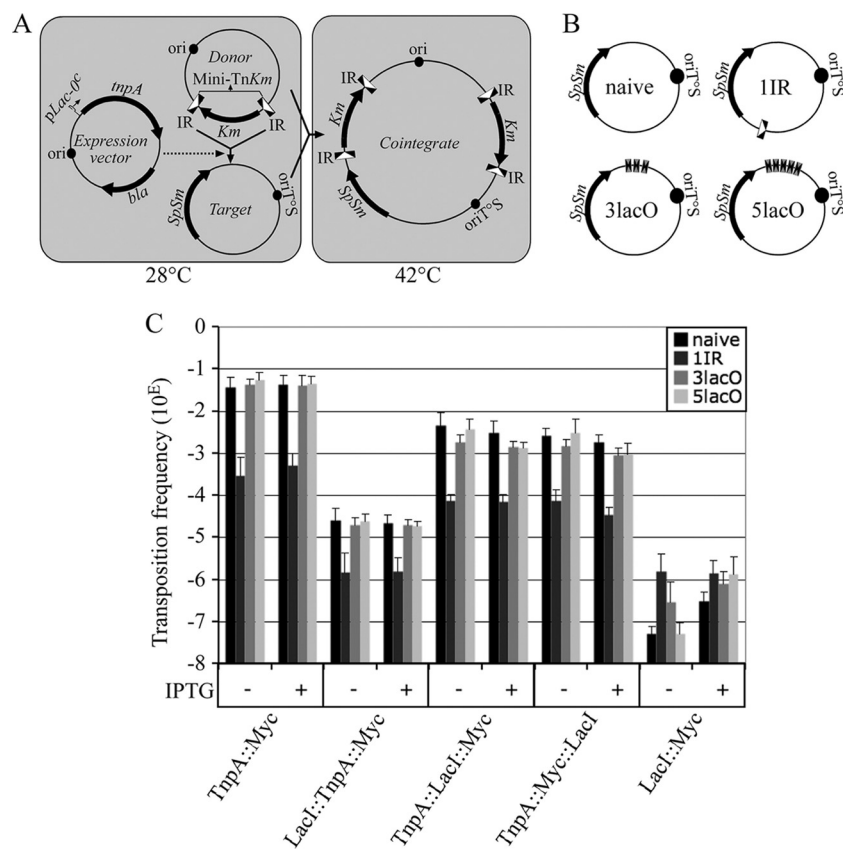


FIG. 3. Transposition and target immunity mediated by the TnpA-LacI fusion proteins. (A) The “plasmid rescue” assay measures the ability of TnpA derivatives expressed from the *placO<sup>c</sup>* promoter to promote transposition of a kanamycin (Km)-resistant Mini-Tn*Km* cassette from the donor plasmid into a spectinomycin/streptomycin (SpSm)-resistant target plasmid with a thermosensitive replication initiation protein (*ori<sup>TS</sup>*). The resulting transposition product is a cointegrate molecule in which the donor and target plasmids are fused together by directly repeated copies of Mini-Tn*Km*. The ratio of spectinomycin-resistant cells forming a colony at a nonpermissive temperature (42°C) over the total number of CFU in the culture provides an estimate of the frequency of the formation of cointegrates. (B) Transposition into a naive target (pGB2*T<sup>S</sup>*) (see Table S1 in the supplemental material) was compared to that obtained for equivalent plasmids carrying a copy of the Tn4430 IR end (1IR) versus 3 or 5 copies of the *lacO* operator (3lacO and 5lacO, respectively). (C) Transposition mediated by the different fusion proteins into the four thermosensitive targets was measured in the absence (-) or presence (+) of 1 mM IPTG. Transposition frequencies are expressed in log scale. Error bars show the standard deviations.

**ator *in vivo*.** An obvious reason for having seen no effect on transposition into the *lacO*-containing targets might be the failure of the TnpA-LacI proteins to bind to the *lacO* operator properly or with sufficient affinity. This possibility was examined using the same strains and the same conditions as those used in the transposition experiments (Table 1).

The strain HB101Δ*lacI* constitutively expresses β-galactosidase, owing to the deletion of the *lacI* gene. As expected, no

TABLE 1. Binding of the TnpA-LacI fusion proteins to the *lacO* operator *in vivo*<sup>a</sup>

| Inducer      | Target plasmid                          | Expressed protein (Miller units [SE]) |                 |                 |                 |           |
|--------------|-----------------------------------------|---------------------------------------|-----------------|-----------------|-----------------|-----------|
|              |                                         | TnpA::Myc                             | LacI::TnpA::Myc | TnpA::LacI::Myc | TnpA::Myc::LacI | LacI::Myc |
| Without IPTG | Naive (pGB2 <i>T<sup>S</sup></i> )      | 1,904 (314)                           | 17 (16)         | 241 (4)         | 281 (61)        | 5 (8)     |
|              | 1IR [pGIML01 <i>T<sup>S</sup>*</i> (T)] | 2,257 (250)                           | 14 (8)          | 220 (15)        | 254 (9)         | 3 (6)     |
|              | 3lacO (pGINC3lacO)                      | 2,327 (324)                           | 31 (20)         | 236 (102)       | 260 (21)        | 5 (5)     |
|              | 5lacO (pGINC5lacO)                      | 2,123 (867)                           | 16 (12)         | 288 (124)       | 282 (107)       | 7 (7)     |
| With IPTG    | Naive (pGB2 <i>T<sup>S</sup></i> )      | 1,728 (458)                           | 1,091 (64)      | 1,764 (727)     | 1,629 (941)     | 306 (221) |
|              | 1IR [pGIML01 <i>T<sup>S</sup>*</i> (T)] | 2,293 (37)                            | 1,077 (210)     | 1,848 (274)     | 1,460 (575)     | 345 (159) |
|              | 3lacO (pGINC3lacO)                      | 2,738 (683)                           | 1,294 (246)     | 2,071 (383)     | 1,886 (356)     | 415 (120) |
|              | 5lacO (pGINC5lacO)                      | 2,077 (181)                           | 1,086 (429)     | 1,408 (216)     | 1,439 (433)     | 365 (178) |

<sup>a</sup> Binding of the fusion proteins to the chromosomal *lacO* operator was determined by measuring the β-galactosidase activity expressed from HB101Δ*lacI* cells containing the different combinations of TnpA expression vectors and target plasmids, both in the presence and absence of 1 mM IPTG.

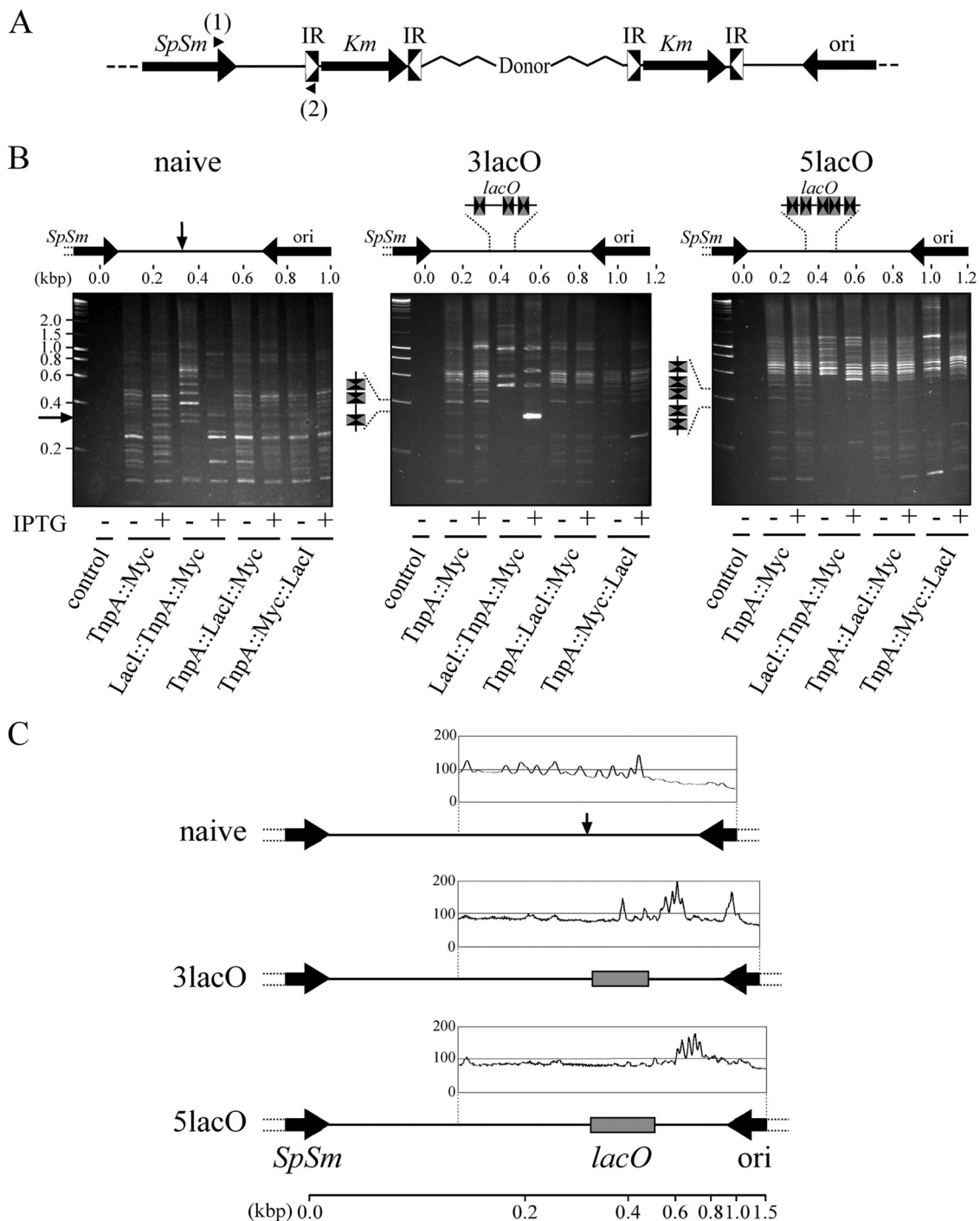


FIG. 4. Alteration of Mini-TnKm insertion pattern into *lacO*-containing targets. (A) Mini-TnKm insertions into the target plasmids were mapped by PCR using a primer corresponding to a fixed sequence within the target DNA (1) and a primer specific to the Tn4430 IR end (2). For each condition examined, PCRs were performed using a population of cointegrate DNA molecules extracted from colonies growing at 42°C in the plasmid rescue assay (see text). (B) The PCR products were separated on 6% polyacrylamide gels and stained with ethidium bromide. The gels show the insertion pattern obtained for different fusion proteins into the naive and 3 *lacO*- and 5 *lacO*-containing targets, both in the presence (+) and absence (-) of 1 mM IPTG. Control lanes correspond to PCRs performed using target DNA molecules without a Mini-TnKm insertion. A schematic map of the analyzed target region is shown at the top of each gel, showing the *lacO* repeats and the corresponding insertion site within the naive target (small arrow). The corresponding positions are reported to the left of the gel, alongside a DNA ladder of size markers (in kbp). (C) Enlarged map of the analyzed ~1-kb target region showing representative Tn4430 insertion patterns in the absence or presence of 3 or 5 copies of *lacO* (shaded boxes). The graphs show a densitometry analysis of the PCR bands pattern shown in panel B for the TnpA::Myc transposase in the presence of IPTG. PCR bands were quantified using ImageJ software (<http://rsb.info.nih.gov/ij/>).



change in  $\beta$ -galactosidase activity was observed in strains containing the wild-type TnpA::Myc expression vector (Table 1). In contrast, expression of the TnpA-LacI fusions was found to affect *lacZ* expression to different levels and in an IPTG-dependent manner (Table 1). Strains expressing the C-terminal fusions TnpA::LacI::Myc and TnpA::Myc::LacI showed an 8-fold decrease in  $\beta$ -galactosidase activity when IPTG was omitted in the cultures, while expression of the N-terminal fusion LacI::TnpA::Myc was found to repress *lacZ* to the same extent as the LacI::Myc protein, reducing  $\beta$ -galactosidase activity by about 2 orders of magnitude (Table 1). This shows that the different fusions bind to the chromosomal copies of *lacO*, thereby repressing the endogenous *lac* operon.

No change in  $\beta$ -galactosidase activity was observed in strains expressing the same protein in the presence of different targets. In particular, strains harboring the *lacO*-containing plasmids showed the same levels of *lacZ* repression as the strains harboring the naive and IR-containing targets (Table 1). This indicates that the level of proteins was not limiting in the transposition experiment and was not titrated out by additional copies of *lacO* on the target plasmids and that variations in  $\beta$ -galactosidase activity measured for the different constructs rather reflect their specific affinity for *lacO*. The weaker DNA binding activity measured for the TnpA::LacI::Myc and TnpA::Myc::LacI fusions compared to LacI::TnpA::Myc and LacI::Myc may come from the fact that attaching LacI349 at the C terminus of TnpA interfered with the structure of the protein and/or its access to DNA.

**Alteration of the Mini-Tn*Km* insertion pattern into *lacO*-containing targets.** In a number of recent studies, specific DNA binding domains of various origins were fused to the transposase/integrase of different elements in order to direct their integration into predetermined DNA sequences (7). This represents a promising approach for the development of new tools in biotechnology or gene therapy.

To determine whether fusing TnpA to LacI349 affected transposition targeting, the pattern of Mini-Tn*Km* insertions into the DNA region enclosing the *lacO* repeats was compared to that obtained for the corresponding region of the naive target (Fig. 4). For the different targets, representative populations of transposition products were extracted from a pool of colonies selected in the plasmid rescue assay and analyzed by PCR using a primer corresponding to a fixed sequence within the target and a second primer complementary to the Tn4430 IR end (Fig. 4A). Amplification products were then separated on a 6% polyacrylamide gel and revealed by ethidium bromide staining (Fig. 4B).

Transposition mediated by the different TnpA derivatives into the naive target produced a ladder of regularly spaced bands, indicating that Mini-Tn*Km* insertion was more or less random, occurring at multiple positions of the analyzed region (Fig. 4B and C). However, some target sites were more frequently selected than others, as judged from the relative amounts of the amplified products. A similar insertion pattern was found for the immune target, suggesting that natural target immunity conferred by the IR end of Tn4430 does not qualitatively alter the intrinsic insertion site specificity of the transposon (data not shown). In contrast, the presence of *lacO* within the target region was found to dramatically affect target site selection by the different proteins (Fig. 4B and C). Com-

pared to the naive target, most of the insertions were clustered in a distal region with respect to the fixed primer locus, generating an array of hotspots after the position where the *lacO* repeats were inserted (Fig. 4B and C). The same clustering of Mini-Tn*Km* insertions was found for the TnpA-LacI fusions and for the wild-type TnpA::Myc protein, and it was independent of the presence or absence of IPTG in the culture (Fig. 4B and C). Thus, alteration of the insertion pattern occurred irrespective of whether TnpA was able to bind to *lacO* or not. The more variable patterns obtained for the LacI::TnpA::Myc protein likely reflects the fact that the product sample collected for this protein was not totally representative due to its low transposition activity (Fig. 3C and 4B).

**Conclusion.** Specific interaction between the TnpA protein and the transposon IR end is thought to provide the signal for triggering target immunity in Tn3 family transposition mechanism. Using chimeric proteins between the TnpA protein of Tn4430 and the LacI repressor of *E. coli*, we provide evidence that simply “attracting” TnpA to a target DNA molecule is not sufficient to make it refractory to transposition. The constructed TnpA-LacI fusions were found to bind to the “surrogate” *lacO* binding sites with different efficiencies *in vivo*. However, none of them conferred transposition immunity to target molecules carrying copies of *lacO*, though they were as proficient as wild-type TnpA at conferring immunity to DNA targets containing the IR end of Tn4430. As the different TnpA-LacI fusions were prone to some levels of proteolysis *in vivo*, we cannot totally rule out the possibility that this phenotype resulted from the production of truncated forms of the proteins that would have masked the activity of the full-length fusions. However, we do not believe that this possibility could satisfy all the observed phenotypes since there was no direct correlation between the activities measured for the wild-type and fused proteins and their pattern of degradation as determined by Western blot analysis. In particular, both C-terminal fusions showed very similar transposition and immunity activities, though their proteolytic patterns were totally different (Fig. 2B).

Thus, the simplest interpretation of our data is that establishment of immunity requires the formation of a specific complex between TnpA and its natural cognate site. Formation of this complex may be necessary to bring TnpA in a competent conformation for immunity. Consistent with this idea, we recently identified mutations affecting transposition immunity in different domains of the TnpA protein (M. Lambin et al., unpublished data). Developing sensitive *in vitro* assays to characterize these mutants will most certainly help to understand the mechanism of target immunity.

Another intriguing finding of this study is the observation that the presence of *lacO* arrays in the target DNA dramatically altered Tn4430 insertion in the flanking sequences. This effect was independent from LacI DNA binding activity, suggesting that intrinsic features of the *lacO* sequence locally affected the structure of adjacent DNA. Further experiments will be performed to investigate how these structural changes arise and how they influence transposition.

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