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The Conjugative Transposon Tn*5397* Has a Strong Preference for Integration into Its *Clostridium difficile* Target Site

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Tn*5397* is a conjugative transposon, originally isolated from *Clostridium difficile*. The Tn*5397* transposase TndX is related to the phage-encoded serine integrases and the *Clostridium perfringens* Tn*4451* transposase TnpX. TndX is required for the insertion and excision of the transposon. Tn*5397* inserts at one locus, $attB_{Cd}$, in *C. difficile* but at multiple sites in *Bacillus subtilis*. Apart from a conserved 5′ GA dinucleotide at the recombination site, there appears to be little sequence conservation between the known target sites. To test the target site preference of Tn*5397*, $attB_{Cd}$ was introduced into the *B. subtilis* genome. When Tn*5397* was transferred into this strain, 100% of the 50 independent transconjugants tested had Tn*5397* inserted into $attB_{Cd}$. This experiment was repeated using a 50-bp $attB_{Cd}$ with no loss of target preference. The mutation of the 5′ GA to 5′ TC in the $attB_{Cd}$ target site caused a switch in the polarity of insertion of Tn*5397*, which is consistent with this dinucleotide being at the crossover site and in keeping with the mechanism of other serine recombinases. Tn*5397* could also transpose into 50-bp sequences encoding the end joints *attL* and *attR* but, surprisingly, could not recombine into the circular joint of Tn*5397*, *attTn*. Purified TndX was shown to bind specifically to 50-bp $attB_{Cd}$, *attL*, *attR*, *attTn*, and $attB_{Bs3}$ with relative binding affinities $attTn \approx attR > attL > attB_{Cd} > attB_{Bs3}$. We conclude that TndX has a strong preference for $attB_{Cd}$ over other potential recombination sites in the *B. subtilis* genome and therefore behaves as a site-specific recombinase.

Conjugative transposons are genetic elements that can mobilize via transposition and conjugation from the genome of a donor to that of a recipient, sometimes across large phylogenetic distances. As they commonly encode antibiotic resistances, they are clinically important (7, 22, 24, 27, 29). Tn*5397* is a 21-kb tetracycline resistance-encoding conjugative transposon originally found in *Clostridium difficile* (13, 20, 21). It can transfer from *C. difficile* to *Bacillus subtilis* and back to *C. difficile* (20). It can also transfer in a model oral biofilm community, indicating that the element is likely able to transfer in natural environments (26).

Tn*5397* has been completely sequenced, revealing that it is very closely related to the extensively studied, conjugative transposon Tn*916* in the regions concerned with transfer and resistance to tetracycline (25). However, the regions required for transposition in Tn*916* and Tn*5397* are completely different. The insertion and excision of Tn*5397* are dependent on the large serine recombinase TndX, the only Tn*5397*-encoded protein required for these functions (33, 34). Tn*916*, on the other hand, requires the tyrosine recombinase (Int) for integration and Int and the accessory factor Xis for excision (15). Although Tn*916* can insert into multiple sites in most hosts, it does have preferred integration sites and, in some strains of *C. difficile*, it has one highly preferred site (33). The clostridia also contain mobilizable transposons such as Tn*4451* and its close relatives that integrate and excise via the transposase TnpX (1, 2, 9, 17–19). TnpX and TndX share about 30% amino acid identity. Because Tn*4451* transposes at a low frequency, only a limited number of insertion sites have been sequenced. However, this analysis indicates that there is a consensus target for TnpX which resembles the ends of the transposon (9).

Recombination by TndX is believed to occur using a mechanism similar to that described for the resolvase/invertases (22). In the model for excision, the ends of the transposon, *attL* and *attR*, synapse with the transposase, which is followed by concerted cleavage of all four DNA strands, forming 2-bp staggered breaks and transient covalent linkages to protein via the recessed 5′ ends. By analogy with the resolvase/invertase mechanism, a 180° rotation of one pair of half sites then occurs to configure the DNA sites into the recombinant format and the DNA backbone is relegated. Thus, the products of excision are an intact (donor) chromosome having suffered a precise deletion of the transposon and a circular form of Tn*5397* that is the transposition and conjugational intermediate (22). The recombination site in the circular form of Tn*5397*, *attTn*, is the joint where the two ends of the transposon have been ligated (Fig. 1A). When the element transfers to a new host, *attTn* recombines with the target site *attB* to establish the transposon in the genome of the recipient. Transposition of Tn*5397* has been observed in *C. difficile*, *B. subtilis*, *Enterococcus faecalis*, and *Escherichia coli* (in a genetically engineered system) (20, 35), and the sequences of the ends of the insertions suggest that the crossover has occurred between 5′ GA in the genome target and 5′ GA in *attTn*.

In order to understand the mechanism of transposition by Tn*5397*, we have investigated the nature of the target sites into which Tn*5397* inserts. There is a "hot spot" in *C. difficile* CD37 where Tn*5397* is always found if present, but in *B. subtilis*

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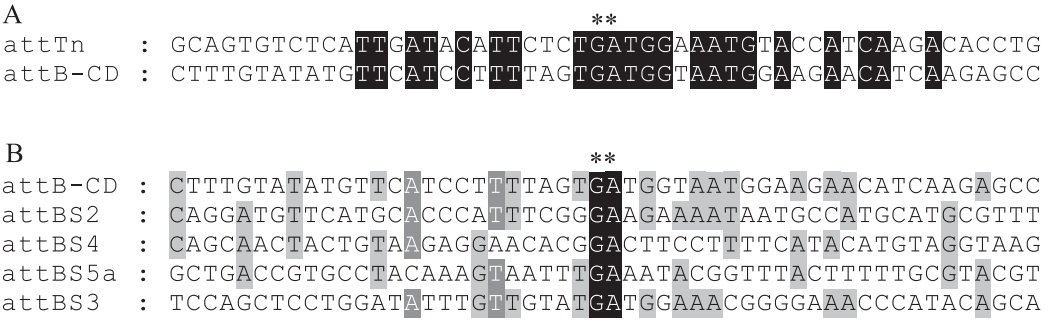

FIG. 1. Sequences of recombination substrates for TndX. (A) Comparison of the sequences of *attTn* formed within the circular joined intermediate of Tn*5397* and the preferred target site in *C. difficile*, $attB_{Cd}$. (B) Sequences of the known target sites for Tn*5397* in *C. difficile* ($attB_{Cd}$) and *B. subtilis* (att_{Bs2}, att_{Bs3}, att_{Bs4}, and att_{Bs5a}). Black shading indicates identity, dark-gray shading indicates 80% conservation, and light-gray shading indicates 60% conservation. The two asterisks above the sequences 5′ GA indicate the dinucleotides that are inferred to take part in the DNA cleavage and rejoining.

CU2189, the element enters the genome at multiple sites and there is no obvious consensus target site in this host apart from the 5′ GA dinucleotide at the center of the target (Fig. 1B) (33). In this work, we wished to resolve this apparent contradiction and further investigate the properties of TndX. We show, using an in vivo assay, that TndX does indeed have a favored target site, the original insertion site from *C. difficile* ($attB_{Cd}$), and that it binds in vitro to this target in a sequence-specific manner. This is the first demonstration of a conjugative transposon that has a preferred insertion site that it will use in two very different hosts while still being able to use other sites if the preferred site is not present.

MATERIALS AND METHODS

Bacterial strains, plasmids, and in vivo methods. The bacterial strains and plasmids used are listed in Table 1. *C. difficile* 630 was used as a donor in conjugation. *B. subtilis* CU2189 and its derivatives, containing the Tn*5397 att* sites inserted via the vector pSWEET (3), were used as recipients. Plasmids were linearized by cutting with PstI and introduced into competent *B. subtilis* (14).

TABLE 1. Plasmids and strains used in this study

Plasmid or strain	Comments[a]	Reference or source
Plasmids		
pSWEET	*B. subtilis* suicide vector for integration at *amyE*	3
pHW*attB*-CD	pSWEET containing the 436-bp *attB*-CD cloned in the BamHI site	This work
pHW*attB*-CD2	pSWEET containing two copies of *attB*-CD in direct repeat	This work
pHW*attB*-CDTC	pSWEET containing $attB_{cd}$(TC)	This work
pHW*attB*50	pSWEET containing the 50-bp *attB*-CD	This work
pHW*attL*	pSWEET containing *attL*	This work
pHW*attR*	pSWEET containing *attR*	This work
pHW*attTn*	pSWEET containing *attTn*	This work
Strains		
C. difficile CD630	Tc^r Em^r *C. difficile* strain containing Tn*5397* and Tn*5398*	12
B. subtilis CU2189	Tc^s recipient strain	6

[a] Tc, tetracycline; Em, erythromycin.

Conjugations were performed using a filter-mating procedure as described previously (33).

DNA manipulations. DNA manipulations were performed according to standard procedures (28).

To construct pHW$attB_{Cd}$, the insertion site of Tn*5397* in *C. difficile* ($attB_{Cd}$) was amplified by PCR with primers P1 (5397RGI/Bam) (5′-GCGGGATCCGAAAACTGCTTGGATTCAGA-3′) and P2 (5397flank/Bam) (5′-GCGGGATCCGCATATTACGCATCTCATTA-3′), using CD37 genomic DNA as a template. Underlining in the sequences indicates restriction enzyme recognition sites. This fragment was digested with BamHI and cloned into the vector pSWEET (3) which was digested with the same enzyme. pHW$attB_C$d$_2$ was obtained by ligating two copies of the above-mentioned fragment in direct repeat and then ligating it to the vector. To construct pHW$attB_{Cd}$(TC) by changing the central GA to TC (top strand) and TC to GA (bottom strand), two rounds of PCRs were carried out. In the first round, primers P1 and P3 (IS/tc-ga) (5′-GTTCTTCCATTACCAGAACTAAAAGGATGAAC-3′) were used to amplify the left part of the target, while primers P4 (IS/ga-tc) (5′-GTTCATCCTTTTAGTTCTGGTAATGGAAGAAC-3′) and P2 were used to amplify the right part of the target in two independent reactions. The products were purified and used as templates in the second-round PCR with primers P1 and P4. The final product was cloned into a pSWEET vector in the same way as that for pHW$attB_{Cd}$. To generate the 50-bp *C. difficile* (*attB50*) target, two oligonucleotides, IS_{50}-top (5′-GATCCTTTGTATATGTTCATCCTTTTAGTGATGGTAATGGAAGAACATCAAGAG-3′) and IS_{50}-bottom (5′-GATCCTCTTGATGTTCTTCCATTACCATCACTAAAAGGATGAACATATACAAAG-3′), were designed. These were annealed to a double-stranded fragment with BamHI sites at both ends. This was cloned into the pSWEET vector via the BamHI site as described before to generate pHW*attB50*. To generate *attL*, two oligonucleotides, *attL*-top (5′-GATCCTTTGTATATGTTCATCCTTTTAGTGATGGAAATGTACCATCAAGACACCT-3′) and *attL*-bottom (5′-GATCCAGGTGTCTTGATGGTACATTTCCATCACTAAAAGGATGAACATATACAAA-3′), were designed. These were annealed and cloned into the pSWEET vector via the BamHI site as described before to generate pHW*attL*. To generate *attR*, two oligonucleotides, *attR*-top (5′-GATCCAGTGTCTCATTGATACATTCTCTGATGGTAATGGAAGAACATCAAGAGC-3′) and *attR*-bottom (5′-GATCCGCTCTTGATGTTCTTCCATTACCATCAGAGAATGTATCAATGAGACACTG-3′), were designed. These were annealed and cloned into the pSWEET vector via the BamHI site as described before to generate pHW*attR*. To generate *attTn*, two oligonucleotides, attTn-top (5′-GATCCAGTGTCTCATTGATACATTCTCTGA TGGAAATGTACCATCAAGACACCT-3′) and attTn-bottom (5′-GATCCAGGTGTCTTGATGGTACATTTCCATCAGAGAATGTATCAATGAGACACTG-3′), were designed. These were annealed and cloned into the pSWEET vector via the BamHI site as described before to generate pHW*attTn*. All constructs were confirmed by PCR and DNA sequencing using primers 5′Bam/SW (5′ GATGTAGCAGTGTTAAGAGAGC-3′) and 3′Bam/SW (5′CGGGCAGACATGGCCTGCCCGG-3′). To generate $attB_{Bs2}$, oligonucleotides attBBS2-top (5′-GATCAGGATGTTCATGCACCCATTTCGGGAAGAAAATAATGCCATGCATGCGTT-3′) and attBS2-bottom (5′-GATCAACGCATGCATGGCATTATTTTCTTCCCGAAATGGGTGCATGAACATCCT-3′) were annealed. To generate $attB_{Bs3}$, oligonucleotides attBS3-top (5′-GATCCCAGCTCCTGGATATTTGTTGTATGATGGAAACG

GGGAAACCCATACAGC-3′) and attBS3-bottom (5′-GATCGCTGTATG GGTTTCCCCGTTTCCATCATACAACAAATATCCAGGAGCTGG-3′) were annealed.

Genomic DNA from *C. difficile* and *B. subtilis* was prepared by using the gram-positive DNA isolation kit (Puregene). Plasmid DNA was prepared by using the QIAGEN plasmid mini-prep kit (QIAGEN). The Southern blotting experiments were performed using an ECL direct nucleic acid labeling and detection system (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

To carry out band shift assays, the instruction manual of the digoxigenin (DIG) gel shift kit (Roche) was followed with some modifications. To label the probe, 3.85 pmol of double-stranded DNA was dissolved in distilled water to make the final volume of 10 μl and then the following reagents were added: 4 μl of 5× labeling buffer (1 M potassium cacodylate, 0.125 M Tris-HCl, 1.25 mg/ml bovine serum albumin, pH 6.6 [25°C]), 4 μl of 25 mM $CoCl_2$, 1 μl of 1 mM DIG-11-ddUTP (DIG-ddUTP), and 1 μl of terminal transferase (50 units/μl). The mixture was incubated at 37°C for 15 min before being placed on ice. The reaction was stopped by adding 2 μl of 0.2 M EDTA (pH 8.0). In the band shift reaction, purified TndX was added to a mixture containing 200 mM potassium cacodylate, 25 mM Tris-HCl, 0.250 mg/ml bovine serum albumin, 1 μg of poly(dI-dC), 0.1 μg of poly L-lysine, 0.310 pmol of DIG-labeled probe and water to make up to 20 μl. Up to 100× molar ratio of specific competitor was added when required. The reaction mixture was incubated at 4°C for 20 min before being mixed with 5 μl of loading buffer (0.25× Tris-borate-EDTA buffer, 60%; glycerol, 40%; bromophenol blue, 0.2% [wt/vol]) and analyzed on a 6% acrylamide gel. After the electrophoresis, the DNA was transferred to a nylon membrane by contact blotting. The DNA was fixed to the membrane by UV cross-linking and then detected by chemiluminescent detection.

The binding affinity of TndX protein for each of the above-mentioned DNA fragments was determined quantitatively with Scion Image analyzing software (Scion Co.).

Purification of TndX. TndX was expressed in *E. coli* as a fusion to the C terminus of a 10-His tag as described before (35). The cell pellets were dissolved, and the soluble fraction was obtained by using Bug Buster master mix (Novagen). TndX was purified by using the Ni-resin (Clontech Laboratories, Inc.). The manufacturer's protocols were followed. Protease inhibitor cocktails (for use in the purification of poly-His-tagged proteins [Sigma]) were added to the supernatant. The amount used was 1 ml cocktail per 20 g of *E. coli* cell extract.

RESULTS

The target from *C. difficile* is a hot spot for Tn*5397* insertion in *B. subtilis*. Our previous work showed that Tn*5397* inserts in only a single target site in *C. difficile* but in multiple sites in *B. subtilis* (20). All of the targets analyzed have a central 5′ GA dinucleotide in common. In this work, we have employed a vector, pSWEET (3), to integrate target sites into the *amyE* locus of the *B. subtilis* genome. The *B. subtilis* strains containing the targets were then used as recipients in filter-mating experiments with *C. difficile* 630 as a Tn*5397* donor. The Tn*5397*-containing, tetracycline-resistant transconjugants were then analyzed for the presence of Tn*5397* in the target sequence. In the first experiment, a 436-bp fragment containing the *C. difficile* target $attB_{Cd}$ was amplified by PCR and inserted into pSWEET to form pHW$attB_{Cd}$, which was then introduced into *B. subtilis* to create *B. subtilis*::$attB_{Cd}$. This strain was used as a recipient in a mating experiment with *C. difficile* 630.

Analysis of the genomic DNA of 28 independent, tetracycline-resistant transconjugants by Southern blotting and DNA sequencing showed that, in every case, Tn*5397* had inserted into $attB_{Cd}$ (Fig. 2A and E, lane 1). No insertions into any other *B. subtilis* targets were found. Another construct that contained two copies of the *C. difficile* target in direct repeat, *B. subtilis*::$attB_{Cd2}$ (derived by transformation of *B. subtilis* with pHW$attB_{Cd2}$), was also made. When *B. subtilis*::$attB_{Cd2}$ was used as a recipient, Tn*5397* inserted into only one of the two sites (Fig. 2B and E, lanes 2 and 3). Analysis of 22 independent transconjugants showed that the element has almost equal chances of insertion into one site or the other. In total, of the 50 independent transconjugants obtained from mating experiments with *C. difficile* and *B. subtilis* containing $attB_{Cd}$, all had Tn*5397* inserted in the *C. difficile* target. As a control, pSWEET lacking any insert was introduced into the *B. subtilis* target. When this strain was used as a recipient, we found Tn*5397* inserted in eight different sites when nine independent transconjugants were analyzed (Fig. 2F). This result is similar to those obtained when *B. subtilis* cells without the integrated vector were used as recipients (33).

The 5′ GA dinucleotide in the *C. difficile* target determines the polarity of Tn*5397* insertion. We have previously shown that Tn*5397* insertions are always flanked by 5′ GA. Moreover, *attTn* and the target sites always contain 5′ GA. It is proposed that by analogy to other serine recombinases, this dinucleotide forms the crossover site. It has recently been shown for two phage-encoded serine integrases that this dinucleotide is the sole determinant of the polarity of the recombination site (10, 11, 31). To determine whether this is also the case for TndX and as a test of the putative mechanism of this transposase, the 5′ GA in the top strand of $attB_{Cd}$ was changed to 5′ TC to form $attB_{Cd}$(TC). When *B. subtilis* cells containing $attB_{Cd}$(TC) were used as recipients, 18 of the 19 independent transconjugants analyzed had Tn*5397* inserted in this site. Further analysis by PCR and sequencing showed that in the 18 transconjugants where Tn*5397* was inserted into $attB_{Cd}$(TC), all 18 were in the opposite orientation relative to Tn*5397* inserted into $attB_{Cd}$ (Fig. 2C and E, lane 4). Only one transconjugant contained an unoccupied $attB_{Cd}$(TC), and we presume that Tn*5397* inserted elsewhere in the *B. subtilis* genome. These results indicate that the central 5′ GA dinucleotide is responsible solely for the polarity of $attB_{Cd}$ and that $attB_{Cd}$(TC) is still a preferred target.

In order to determine whether Tn*5397* was capable of excising from the att_{Cd}(TC) target, PCR for the circular form of Tn*5397* in these strains was performed (33). A PCR product of the appropriate size was produced in all of the transconjugants tested (results not shown), indicating that the element is capable of excision from this site. As excision appears to be a requirement for the transfer of Tn*5397*, (34) it is very likely that these strains will be able to act as conjugal donors of the element.

***attB*$_{Cd}$ could be reduced to 50 bp without loss of activity.** The recombination sites employed by the phage-encoded serine integrases are approximately 50 bp in length (5, 10, 23, 32). The protected sites by TnpX are approximately 68 to 93 bp (1). We decided to test whether a 50-bp $attB_{Cd}$ was still a preferred target site for Tn*5397*. Oligonucleotides encoding $attB_{Cd}$ with the crossover sequence, 5′ GA, located at the center were annealed and then ligated into pSWEET to generate pHW$attB_{Cd50}$, which was then transformed into *B. subtilis*. *B. subtilis*::$attB_{Cd50}$ transconjugants containing Tn*5397* were analyzed by Southern blotting, PCR, and DNA sequencing, which showed that all 15 transconjugants analyzed had Tn*5397* inserted into $attB_{Cd50}$ via the 5′ GA dinucleotide at the center of the 50-bp target, resulting in an element flanked by 5′ GA dinucleotides (Fig. 2D and E, lane 5). Thus, the 50-bp $attB_{Cd}$ has retained its activity as a preferred target for insertion of Tn*5397*.

Tn*5397* can integrate into *attL* and *attR* but not into *attTn*. The left (*attL*) or the right (*attR*) junction of Tn*5397* inserted

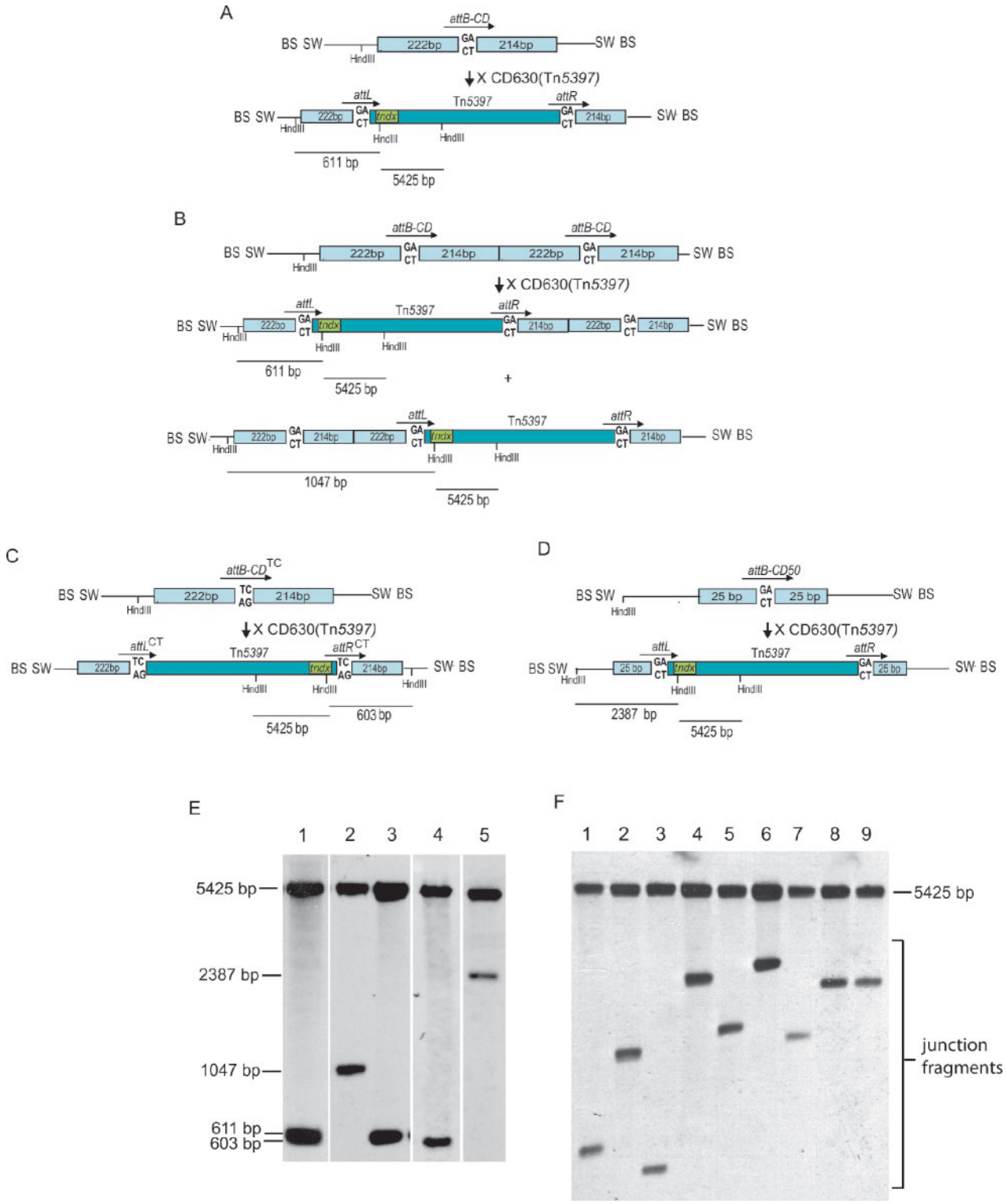

FIG. 2. Analysis of the target site choices of Tn*5397* in *B. subtilis*. (A to D) Schematic showing the insertion of Tn*5397* into different target sites used here. The annotations are as follows: the light-blue shaded boxes represent the target inserted into the *B. subtilis* chromosome and the 5′GA dinucleotide that is thought to be the crossover region. The thin bands represent the flanking vector sequence. The expected product(s) after the conjugation and insertion of Tn*5397* (dark blue), flanked by *attL* and *attR*, is shown underneath. The *tndX* gene was used as a probe in Southern blotting experiments with HindIII-digested chromosomal DNA. HindIII has one recognition site in *tndX*. If Tn*5397* had inserted into any of these targets, two bands would hybridize to the probe (as shown). The different target sites used were $attB_{Cd}$ (A), $attB_{Cd2}$ (B), $attB_{Cd}$(TC) (C), and the 50-bp *attB* target, $attB_{Cd50}$ (D). Panels E and F show results from Southern blotting of transconjugants containing various insertions of Tn*5397* at different target sites. The DNA was digested with HindIII and probed with *tndX.* For clarity, only one clone from each cross is shown here. (E) Lane 1 shows DNA from an insertion into $attB_{Cd}$ (see panel A); lanes 2 and 3 show DNA from an insertion into $attB_{Cd}$ (see panel B); lane 4 shows DNA

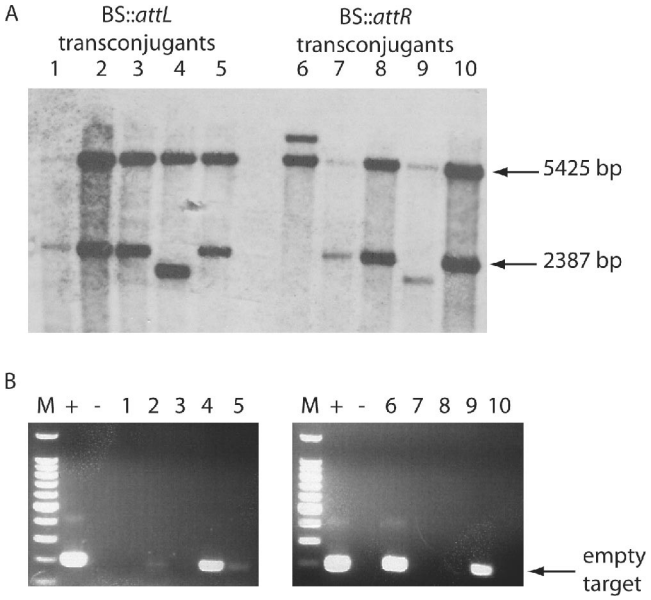

FIG. 3. Southern blot and PCR analyses of *B. subtilis* transconjugants containing either an *attL* or an *attR* site. DNA was prepared from independent transconjugants, digested with HindIII, blotted, and probed with *tndX*. If the element were integrating into the genome at random, one would expect to see two hybridizing fragments, one of 5,425 bp internal to Tn*5397* and one of variable size representing the junction region, whereas if Tn*5397* had integrated specifically into either *attL* or *attR*, bands of 5,425 bp and 2,387 bp should be observed. (A) Southern blots of five transconjugants from *B. subtilis*::*attL* (lanes 1 to 5) and *B. subtilis*::*attR* (lanes 6 to 10) (see the text for more details). If the targets are empty, they will be amplified by PCR using primers 5′Bam/SW and 3′Bam/SW, but if they are occupied by Tn*5397*, the 22-kb element will not generate a product under the conditions used. DNAs corresponding to the transconjugants used in panel A were subjected to PCR and run in agarose gels. The lane marked "+" is a positive control, where pHW*attL* or pHW*attR* was used as a template for PCR, and the lane marked "−" was a negative control with no template.

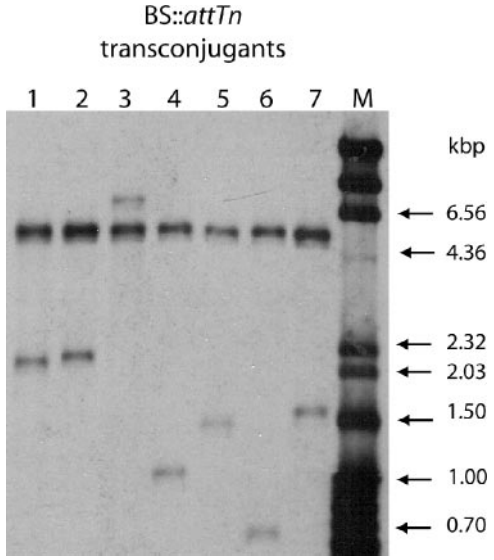

FIG. 4. Southern blot analysis of *B. subtilis* transconjugants containing *attTn*. *B. subtilis* cells containing *attTn* were used as recipients in matings with *C. difficile* containing Tn*5397*. DNA was prepared from transconjugants and digested with HindIII and probed with *tndX*. If the element were integrating into the *attTn* site, one would expect to see two hybridizing fragments, one of 5,425 bp internal to Tn*5397* (Fig. 1 and 2) and one of 2,387 bp (or 603 bp, depending the orientation of the transposon in the genome), whereas insertion elsewhere in the genome would yield the 5,425-bp band and one of variable size representing the junction region. The results of Southern blotting of eight transconjugants are shown (see the text for more details).

into $attB_{Cd}$ each contain one-half of the $attB_{Cd}$ preferred target. TndX can cause excision by recombination between *attL* and *attR* in the absence of any further Tn*5397* gene products (35). This contrasts with the phage-encoded integrases that are highly directional, being able to cause only *attP-attB* recombination in the absence of any other proteins (32). It therefore seemed likely that TndX could recombine any combination of *att* sites, including *attTn-attL* and *attTn-attR*. To test whether *attL* and *attR* could be used as targets for Tn*5397* insertion and whether they were preferred over other *B. subtilis* target sequences, pHW*attL* and pHW*attR* were constructed and the *att* sites were introduced into *B. subtilis* to form *B. subtilis*::*attL* and *B. subtilis*::*attR*, respectively. These 50-bp sites comprised, for *attL*, 25 bp of the left side of $attB_{Cd}$ and 25 bp of the right side of *attTn* and, for *attR*, 25 bp of the left side of *attTn* and 25 bp of the right side of $attB_{Cd}$. Transconjugants of *B. subtilis*::*attL* and *B. subtilis*::*attR* containing Tn*5397* were analyzed by Southern blotting and PCR and showed that Tn*5397* inserted into the *attL* in four out of five transconjugants and into *attR* in three out of five transconjugants (Fig. 3A). DNA sequencing of the PCR products generated from transconjugants containing Tn*5397* inserted at *attL* or *attR* demonstrated that insertion had occurred at the 5′ GA crossover dinucleotide. A PCR was also performed for the empty target site; a product was obtained only when the transposon had inserted into a site other than *attL* or *attR* (Fig. 3B). The insertion of Tn*5397* into *attL* or *attR* was unlikely to be due to homologous recombination, as there was only 25 bp of identity between *attL* or *attR* and Tn*5397*. Moreover, homologous recombination was not observed in experiments with two *attTn* sites (see below). Thus, *attL* and *attR* sites were recognized as preferred targets by the transposon.

All of the recombination reactions described here use *attTn* as one of the substrates, as this is the circular joint formed after the excision of Tn*5397*. $attB_{Cd}$ and *attTn* share some sequence identity (Fig. 1A). If TndX is merely seeking out attachment sites that resemble the sequence of *attTn*, then *attTn* should be

from an insertion into $attB_{Cd}$(TC) (lane 5) DNA in which the target site had been reduced to 50 bp (see panel D). (F) Southern blot analysis of *B. subtilis* DNA (containing unmodified pSWEET integrated into the chromosome) from six transconjugants containing insertions of Tn*5397* isolated from independent mating experiments. If the element were integrating into the genome at random, one would expect to see two hybridizing fragments, one of 5,425 bp internal to Tn*5397* and one of variable size representing the junction region.

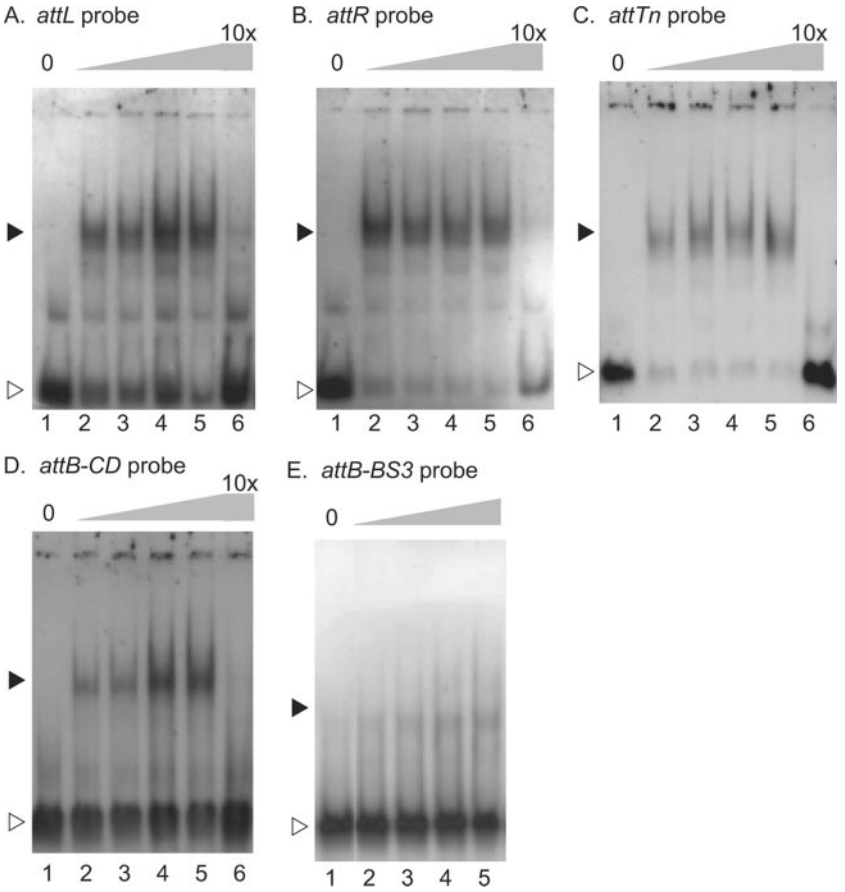

FIG. 5. TndX binding to its recombination sites. Purified TndX with a C-terminal His tag was used in gel shift assays to determine relative binding affinities to *attL* (A), *attR* (B), *attTn* (C), and $attB_{Cd}$ (D) and the pseudo-*attB* site from *B. subtilis*, $attB_{Bs3}$ (E). In all panels, the white arrowhead indicates the position of the free probe and the black arrowhead is the position of the major TndX-probe complex. (A to D) Increasing concentrations of TndX were added to the binding reactions to generate final concentrations of 0, 0.28, 0.55, 0.83, and 1.1 μM (lanes 1 to 5, respectively). Lane 6 contains the same binding reaction as does lane 5 except that 10-fold more unlabeled probe was added as a specific competitor. (E) The concentration of TndX added to each binding reaction was 0.17, 0.34, 0.68, and 1.35 μM (lanes 1 to 5, respectively).

a good target sequence for integration. Oligonucleotides encoding a 50-bp *attTn* sequence containing the joined ends of Tn*5397* as occurs in the circular form were ligated into pSWEET to form pHW*attTn*, and this was introduced into *B. subtilis*. Tn*5397* was transferred into *BS*::*attTn*, and the transconjugants were analyzed as described before. Southern blot analysis of 19 independent transconjugants showed that Tn*5397* had inserted into different sites; none of the Tn*5397* transconjugants contained a simple insertion into *attTn* (Fig. 4), as judged by PCR analysis for the empty target site (results not shown).

When a *B. subtilis* strain containing a 1.1-kb *attTn* was used as a recipient, no transconjugants were ever obtained in 30 independent filter-mating experiments. This indicates not only that this region cannot be used as a target but also that it appears to prevent other target sites in the *B. subtilis* genome from being used.

TndX binds sequence specifically to 50-bp $attB_{Cd}$, *attL*, *attR*, *attTn*, and $attB_{Bs}$, but with different affinities. The data presented above strongly suggest that TndX is acting as a site-specific recombinase, targeting Tn*5397* to a specific site in the *C. difficile* genome. If this is the case, then TndX will bind to $attB_{Cd}$ in a sequence-specific manner and with an affinity not too dissimilar to those for its other substrates (*attTn*, *attL*, and *attRs*). A C-terminal His-tagged derivative of TndX was purified from *E. coli* and used in gel shift assays (Fig. 5) (35). The DNA probes were DIG labeled. Under the conditions used, TndX bound with the highest affinities to *attTn* and *attR*. In each case, even with the lowest concentration of protein used (0.275 μM), nearly the entire probe was shifted to form a complex with slower mobility (Fig. 5). With the *attL* probe, approximately half of the probe was bound at 0.275 μM TndX, suggesting a slightly lower relative affinity, and with the $attB_{Cd}$ probe, about 50% of the probe was bound in the presence of 1.1 μM TndX. TndX bound to all of these probes in the presence of competitor DNA, and binding was greatly diminished if an excess of unlabeled probe was added. In contrast, TndX bound very poorly to $attB_{Bs3}$, with only a very small amount of bound probe observed in the presence of 1.35 μM TndX. These data confirm that TndX binds to all its substrate recombination sites, *attTn*, *attL*, *attR*, and $attB_{Cd}$, in a sequence-specific manner and that there is a binding preference, $attTn \approx attR > attL > attB_{Cd} > attB_{Bs3}$.

DISCUSSION

In this paper, we set out to determine whether Tn*5397* has a specific target or whether it integrates into random targets when it transfers from host to host. The element does indeed have a preferred target site, $attB_{Cd}$, a sequence originating from *C. difficile.* In the absence of this site, however, Tn*5397* can insert into alternative target sites, all of which have the crossover dinucleotide sequence, 5′ GA, and some of which have limited sequence similarity with $attB_{Cd}$ (Fig. 1). The transfer of Tn*5397* from *C. difficile* to *B. subtilis* is a rare event, and in each filter-mating experiment, only a few transconjugants were obtained. Thus, the relative frequency of use of these targets could not be determined. However, we observed that $attB_{Cd}$ is the preferred insertion site for at least 50 independent mating experiments, which is indicative of site-specific insertion. Although we have not demonstrated the minimum sequence required for the use of $attB_{Cd}$ by TndX, 50 bp was sufficient to maintain site-specific insertion by Tn*5397* in *B. subtilis*. This is comparable to the attachment sites used by the phage-encoded serine integrases, such as ϕC31, Bxb1, and ϕRV1 (4, 11, 16, 30, 31, 32). The ability to switch the polarity of insertion of Tn*5397* by changing the crossover sequence in $attB_{Cd}$ to 5′ TC is also strongly reminiscent of the phage integrases and implies that the same mechanism of recombination is employed by TndX. These data support the idea that during integration, the 5′ GA dinucleotide in the $attB_{Cd}$ is cleaved at the 3′ end and exchanged with the similarly cleaved *attTn* site to generate the *attL* and *attR* sequences. The reverse occurs for excision. For the preferred site $attB_{Cd}$, the sequences that flank the 5′ GA dinucleotide are probably recognized in a sequence-specific manner by TndX.

The experiments described here indicate similarities between the properties of the phage-encoded serine integrases and the transposase TndX. These similarities extend to the use of a preferred target site for integration. In vitro binding experiments with the TndX recombination sites indicated an order of preference, $attTn \approx attR > attL > attB_{Cd}$, and that binding was sequence specific. Although the apparent affinity for $attB_{Cd}$ was the lowest out of these four substrates, the affinity was only about fourfold less than that for *attL*. When this specific target site is not present in the genome, however, there is sufficient flexibility in target site recognition such that other, pseudo-*attB* sites can be used, as is the case for *B. subtilis*, e.g., $attB_{Bs3}$. The use of pseudo-*attB* sites by the phage-encoded serine integrases has also been shown to occur (8). Binding assays with TndX indicated very low affinity for one of its pseudo-*attB* sites, $attB_{Bs3}$ (50% binding requires greater than 1.35 μM TndX), and this is consistent with it being occupied only in the absence of $attB_{Cd}$. These data are in contrast to the results obtained with the related enzyme TnpX, responsible for the integration and excision of the mobilizable clostridial transposon Tn*4451* that had at least a 40-fold-higher affinity for the ends of the element than it did for its targets (1). It is possible that Tn*4451* also has a preferred target that has not yet been identified.

Despite the similarities between the properties of TndX with the phage integrases, TndX is fundamentally different from the phage integrases as it alone can catalyze both excision and integration. We therefore expected TndX to be less selective in its use of different combinations of *att* sites for recombination. In fact, TndX could utilize *attL* and *attR* as targets for Tn*5397* insertion. In these assays, insertions into other *B. subtilis* target sites were observed (one out of five for *attL* and two out of five for *attR*), suggesting that *attL* and *attR* are not as highly preferred as $attB_{Cd}$ but are still preferred. The use of *attL* and *attR* by Tn*5397* would imply that tandem insertions of Tn*5397* may be obtained occasionally. In support of this idea, we observed that when Tn*5397* is transferred to *C. difficile* CD37, two copies of the element are indeed found at specific sites (20). To our surprise, we observed that *attTn* was not used as a target for Tn*5397* integration and appeared to be avoided. TndX bound to *attTn* and *attR* with the highest affinities, yet no recombination was observed between *attTn* and *attTn* and recombination between *attTn* and *attR* was arguably less preferred than that between *attTn* and $attB_{Cd}$. Therefore, it is not the strength of binding that determines the frequency of recombination. Instead, we propose that it is the conformation adopted by TndX when bound to its recombination sites that determines whether recombination occurs. This inability to recombine *attTn-attTn* is reminiscent of the phage integrases that also do not recombine *attP-attP* (or *attB-attB*, etc.) and this property may reflect a fundamental feature of the mechanism of recombination by the large serine recombinases.

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