The Roles of Exc Protein and DNA Homology in the CTnDOT Excision Reaction

Running Title: CTnDOT Excision in vitro

Carolyn M. Keeton* and Jeffrey F. Gardner
Department of Microbiology, University of Illinois at Urbana-Champaign
601 S. Goodwin Avenue, Urbana, Illinois 61801, USA.
*To whom correspondence should be addressed. Email: ckeeton2@illinois.edu
Excision from the chromosome is the first step during transfer of conjugative transposons (CTn) to a recipient. We previously showed that excision of CTnDOT is more complex than excision of lambdoid phages and CTNs such as Tn916. Excision in vivo of CTnDOT utilizes four CTnDOT-encoded proteins: IntDOT, Xis2c, Xis2d, Exc and a host factor. We previously developed an in vitro excision reaction where the recombination sites, attL and attR, were located on different plasmids. The reaction was inefficient and did not require Exc suggesting that the reaction conditions did not mimic in vivo conditions. Here, we report the development of an intramolecular excision reaction where the attL and attR sites are located on the same DNA molecule. We found that Exc stimulates the reaction 3 to 5-fold. The efficiency of the excision reaction was also dependent on the distance between the attL and attR sites and on the sequences of the overlap regions between the sites of the strand exchanges. Substrates with identical overlap sequences recombined more efficiently than ones with heterologous overlap sequences. This was surprising because the integration reaction is not sensitive to heterology in the overlap regions of the attDOT and attB sites.
INTRODUCTION

*Bacteroides* spp. are Gram negative obligate anaerobes found in the human colon but can act as opportunistic pathogens if they escape from the colon during surgery or other abdominal traumas. *Bacteroides* spp. have acquired antibiotic resistance genes from transmissible elements called conjugative transposons or integrative conjugative elements (ICES) (22). One well studied example is CTnDOT, a 65kb element which carries the *tetQ* and *ermF* genes that confer tetracycline and erythromycin resistance respectively (28). The presence of tetracycline induces production of proteins that promote the excision and transfer of CTnDOT. When CTnDOT excises from the chromosome, it forms a closed circular intermediate that is nicked and transferred to the recipient cell by conjugation. Following circularization of the single stranded DNA and replication in the recipient, CTnDOT integrates into the chromosome at one of several *attB* sites (5, 21).

CTnDOT integration into the chromosome requires the CTnDOT encoded tyrosine recombinase, IntDOT, and a host factor (Ringwald and Gardner, in preparation) (5). These proteins promote recombination between two attachment sites called *attDOT* and *attB*. The *attDOT* site is 600bp and the *attB* site is 60bp (7). Presumably IntDOT and the host factor assemble a complex on the *attDOT* site called the integrative intasome which undergoes synapsis with an *attB* site. Recombination occurs by sequential strand exchanges 7bp apart in a region called the overlap sequence in each site. The first exchange requires 2 bp of DNA homology adjacent to the cleavage site in each site while the second strand exchange does not require homology (12, 16). IntDOT differs from other tyrosine recombinase in that it does not require homology in the overlap sequences for integration into the chromosome and, furthermore, there is no difference in integration efficiency if the overlap sequences are the same or different.
Presumably, the differences in the ability to resolve different overlap sequences occur during the resolution of Holliday Junctions (HJs), where IntDOT has the ability to resolve HJs with mismatches while other tyrosine recombinases lack this function.

Another difference between CTnDOT and other mobile elements is that the excision system used by CTnDOT appears to be more complex. Excision from the chromosome requires IntDOT and the host factor along with three additional CTnDOT encoded proteins Xis2c (formerly called Orf2c), Xis2d (formerly called Orf2d), and Exc which were identified by deletion analysis and complementation experiments (6, 25). Xis2c and Xis2d are small basic proteins similar to lambda Xis and other recombination directionality factors (RDFs) (14). Exc is a DNA topoisomerase III enzyme capable of relaxing DNA in vitro (26). The topoisomerase activity is mediated by the catalytic tyrosine in the active site (4). However, when the catalytic tyrosine of Exc is mutated to a phenylalanine (Y315F), there is no detectable effect on excision in vivo (26). The DNA sequences required for excision are the 420bp attL and the 220bp attR sites (9). IntDOT, the host factor, Xis2c, Xis2d, and Exc assemble excisive intasomes on the attL and attR sites that undergo synapsis which is followed by two rounds of strand exchanges to produce the attDOT and attB sites.

The role of Exc in excision was opened to further question by the finding that although Exc appeared to be required for excision in vivo, it was not required in the in vitro intermolecular reaction. An intermolecular reaction was developed where the attL and attR sites were on two different plasmids (25). In this reaction, the excision frequency was low and ranged from 0.5-5%. The reaction only required IntDOT, Xis2c, Xis2d, and a Bacteroides crude extract containing the host factor (25). The lack of a role for Exc in the intermolecular reaction could be
a consequence of the DNA topology caused by the \textit{attL} and \textit{attR} sites being on two different plasmids.

An important difference between the \textit{in vitro} intermolecular reaction and the situation encountered by the excision proteins \textit{in vivo} is that the \textit{attL} and \textit{attR} sites are on different DNA molecules rather than as part of the same molecule as found in the natural form of CTnDOT \textit{in vivo}. In this paper, we developed an \textit{in vitro} intramolecular excision reaction with the \textit{attL} and \textit{attR} sites on the same plasmid. We report that when substrates containing different overlap sequences in the \textit{attL} and \textit{attR} sites are tested for excision, Exc is required for maximal recombination. Supercoiling of DNA was not required for excision to occur. A surprising finding, given that IntDOT tolerates differences in the overlap sequences during the integration reaction, was that substrates with the same overlap sequences in the \textit{attL} and \textit{attR} sites increased the efficiency of the excision reaction.
MATERIALS AND METHODS

Plasmids, Bacterial strains, Electrophoresis, and growth conditions

All plasmids and strains used in this paper are described in Table 1. The primers used in constructing the excision plasmids are listed in Table S1 in the supplemental material section. Details of purification of the excision proteins and construction of the in vitro intramolecular excision reaction substrates are described in the Supplemental Data Section (Figure S1).

Sequencing of the plasmids was done by either the UIUC Core Sequencing Facility or ACGT, Inc. Oligonucleotides were synthesized by Integrated DNA Technologies, Inc. Bacterial strains were grown in Luria–Bertani (LB) broth or on LB agar plates or MacConkey-lactose plates (23). Antibiotics were supplied by Sigma and used at the following concentrations: kanamycin (kan) 50µg/ml; chloramphenicol (cam) 20µg/ml; and ampicillin (amp) 100 µg/ml. Restriction enzymes, alkaline phosphatase, and T4 DNA ligase were supplied by New England Biolabs.

PCR reactions were either performed with KOD Hot Start DNA Polymerase from Novagen or PCR Master Mix from Fermentas. Agilent Technologies’ QuikChange XL site-directed mutagenesis kit was used for site-directed mutagenesis. The TriDye 100bp and 1kb ladder were from NEB.

Intramolecular Excision Reaction

The intramolecular excision reaction was adapted from the conditions used in the intermolecular reaction (25). The reaction mixture contained 100.0 fmol of plasmid substrate incubated in a buffer containing 20mM Tris-HCl pH 7.4, 5mM DTT, 0.05mg/ml BSA, 1% glycerol, 50mg/ml polyvinylalcohol, and 50mM KCl. Excision was not detectable without polyvinylalcohol. Each plasmid was incubated with IntDOT, the host factor, Xis2c, Xis2d or the
same mixture with the addition of Exc or F315Y Exc. All proteins were overexpressed in *E. coli* BL21(DE3) *ihfA*. Protein concentrations used were as follows; IntDOT 13.5 µM (approximately 80% pure, (8), Xis2d 200 µM (approximately 85% pure, this paper), the host factor 0.80 µM (approximately 85% pure, K. Ringwald and J. Gardner, in preparation), and Exc 0.20 µM (95% pure, (26). The concentration of Xis2c is unknown because it was in a crude extract. The concentrations of protein and DNA were optimized for optimal excision. All excision reactions were incubated overnight at 37 °C except for the time course experiments. Next, the DNA was purified from the proteins and reaction buffer using a QIAdx PCR Purification Kit. The DNA then was electroporated into DH5α::λpir+ cells which were then grown for 1 hour and plated on MacConkey-Kan plates. Red colonies contain plasmids that did not undergo excision during the *in vitro* intramolecular excision reaction (Figure 1). White colonies resulted from an excision event during the reaction. The percent excision was calculated as the number of white colonies (excised plasmids) divided by the total number of colonies on the plate (unexcised plus excised plasmids). White colonies were routinely analyzed by PCR to show that excision occurred after each reaction.
RESULTS

Characterization of an in vitro intramolecular excision reaction

Since, the excision reaction in vivo in Bacteroides utilizes direct repeat copies of the attR and attL sites that are located on the same chromosome; it was of interest to determine whether an in vitro intramolecular excision reaction is more efficient and whether Exc stimulates the reaction. Accordingly, we created an in vitro intramolecular excision reaction by constructing a series of plasmids with the lacZα gene between the attL and attR sites. If the excision reaction occurred, the attL and attR sites would undergo excision to form a plasmid containing attDOT and a closed circular product containing the attB site and the lacZα gene. After electroporation, the attB/lacZα circular fragment is lost because it lacks an origin of replication (Figure 1). Red colonies on MacConkey-lactose plates contain plasmids that did not undergo excision and still contain the attL and attR sites. White colonies contain plasmids that underwent excision and contain plasmids carrying the attDOT site.

The efficiency of this in vitro intramolecular excision reaction ranged from less than 1% to virtual completion depending on the substrates and conditions tested (see below). IntDOT, the host factor, Xis2c, and Xis2d were able to catalyze the in vitro intramolecular excision reaction (Figure 1B). We showed previously that E. coli Integration Host Factor (IHF) can substitute for the host factor when added at high concentrations in the in vitro integration reaction (12). Presumably, IHF binds the attDOT site non-specifically and induces bends in the DNA necessary to form the intasome. However, IHF did not substitute in the intermolecular reaction and we found that IHF could not substitute for the host factor in the new in vitro intramolecular excision reaction, even at the high concentrations used in the integration reaction (25). The inability of
IHF to substitute for the host factor may be because it does not induce appropriate bend angles in the attL and attR sites that allow formation of the intasomes necessary for the excision reaction. We routinely analyzed 20 plasmids from white colonies screened after electroporation and tested for the presence of either the attDOT site or the attL and attR sites. An example of this analysis is shown in Figure 2. In this experiment, plasmids were isolated from 4 independent white colonies. They all produced a PCR band containing attDOT, which is 1200 bp as predicted (Figure 2, Lanes 1-4). A PCR product of pCMK937, the original attDOT plasmid used in creation of the excision plasmids, produced a band of 1200bp (Figure 2, Lane 5) The PCR product of pCMK1017, the plasmid substrate used in the excision reaction, is approximately 6000 bp and contains the attL and attR sites (Figure 2, Lane 8).

Following excision with the attL and attR sites with different overlap sequences, it should be possible to detect both overlap sequences in the excision products. The attL and attR sites of pCMK1017 contain overlap sequences of GCGCAAT and GCTTAGT, respectively. When excision occurs, the attDOT plasmid is predicted to contain a 5 bp mismatch that is resolved in the cell after electroporation. Therefore, both overlap sequences should be detectable in the attDOT site in the population of white colonies. Of the 15 white colonies analyzed, all contained the attDOT site. As predicted, both parental overlap sequences are found in the attDOT products. Seven contained the GCGCAAT overlap sequence and 8 contained the GCTTAGT overlap sequence (data not shown). The attDOT plasmid containing the GCGCAAT overlap could only have formed from the excision reaction because none of our laboratory strains contain this overlap sequence in attDOT. This eliminates the possibility of contamination.

Factors Affecting the Efficiency of Excision
Following the initial development of the intramolecular excision reaction, we constructed several plasmids to test three variable factors for their effects on the excision efficiency. We varied the distance between the *attL* and *attR* sites on substrates with the same or different overlap sequences in the presence or absence of Exc. We found that the distance between the *attL* and *attR* sites affects the efficiencies of excision. The bar graph on the left in Figure 3 shows the results with four excision plasmids containing 0.43kb of DNA between the *attL* and *attR* sites. The efficiency of excision ranged from 1-35% depending on whether the overlap sequences are the same or different and whether Exc is present. An intermediate distance of 1.4 kb was tested which yielded similar results to 0.43 kb of DNA (Data not shown). The least efficient recombination results from reactions containing substrates with different overlap sequences conducted without Exc while the most efficient reactions occurred when substrates contained the same overlap sequences and were incubated with Exc. When substrates containing 4.7kb of DNA between the *attL* and *attR* sites were examined the excision efficiencies ranged from 2-100% (Figure 3, right side bar graph). Thus substrates with 4.7kb of DNA between the *attL* and *attR* sites were 3 to 6 fold more efficient than the substrates with 0.43kb of DNA between the sites. It is possible that substrates with 0.43kb of DNA between the *attL* and *attR* sites are inefficient in forming the recombination complex because of the ability of the DNA to bend in the proper configuration when the *attL* and *attR* sites are in close proximity. When the distance is increased between the *attL* and *attR* sites, excision increases.

The sequences of the overlap regions also affected the excision frequencies. IntDOT differs from the other tyrosine recombinases because it recombines *att* sites with different overlap sequences. Most tyrosine recombinases that have been characterized require that both *att* sites contain the same overlap sequences for recombination. In the IntDOT system, there is no
difference in efficiency of \textit{in vitro} integration between the \textit{attDOT} and \textit{attB} sites containing the same or different overlap sequences as long as 2bp of sequence identity is present at one end of the overlap region in each site (9, 12). However, in the intramolecular excision reaction this is not the case. Plasmids containing different or same overlap sequences were compared for excision frequencies (Figure 3). Excision increased 10-30 fold when the overlap sequences were the same as compared to reactions performed under the same conditions with substrates containing different overlap sequences.

We also found that Exc stimulates the \textit{in vitro} intramolecular excision reaction. Previous work using a Southern blot reaction to monitor excision \textit{in vivo} indicated that no excision was detected in the absence of Exc (6). However, Sutanto et al. found that Exc had no effect in the intermolecular reaction where the \textit{attL} and \textit{attR} sites were on different molecules (25). A comparison of excision in the absence and presence of Exc in the intramolecular excision reaction is shown in Figure 3. Excision was detectable without Exc regardless of the overlap sequences or distances between the \textit{attL} and \textit{attR} sites. When the overlap sequences were different, excision without Exc was inefficient at 1% with 0.43kb of DNA between the \textit{attL} and \textit{attR} sites and 2% when the sites were separated by 4.7kb of DNA.

However, when Exc was added to the reaction, excision increased 3-5 fold when the \textit{attL} and \textit{attR} sites were separated by 0.43 kb of DNA or when the sites contain different overlap sequences are separated by 4.7kb of DNA. When the \textit{attL} and \textit{attR} sites are separated by 4.7kb of DNA and contain identical overlap sequences, the reaction is efficient in the absence of Exc. In addition, there was no difference in excision frequencies when the mutant Y315F Exc was substituted for Exc (Figure 3). As previously seen \textit{in vivo}, the topoisomerase activity of Exc is
not required because an Exc protein containing a substitution of the catalytic tyrosine to
phenylalanine (Y315F Exc) functions as efficiently as wild type Exc (26).

We performed time course reactions using the substrates that contained 4.7kb of DNA
between the attL and attR sites with either the same or different overlap sequences. Reactions
were incubated in the absence or presence of Exc (Figure 4). There was a detectable difference in
the rate of excision when Exc was added to the excision reactions with substrates containing
different overlap sequences. The stimulatory effect of Exc could be detected after 2-4 hours. By
6 hours, the rate of excision increased when the reactions contained Exc in comparison to
reactions incubated without Exc (Figure 4A). Even when the overlap sequences were the same,
Exc slightly stimulated the reaction (Figure 4B).

In summary, the efficiency of excision ranged from 1% to completion depending on the
substrates and conditions used. The most efficient excision occurred when the plasmid contained
4.7kb of DNA between the attL and attR sites with the same overlap sequences in the presence of
Exc. The least efficient excision substrates occurred when the plasmid contained 0.47kb between
the attL and attR sites with different overlap sequences in the absence of Exc.

The Excision Proteins Inhibit in vitro Integration

Some RDFs inhibit the integration reaction of their system (14). For example, lambda Xis
inhibits integration below detectable levels when added to the in vitro integration reaction (1).
Since CTnDOT contains two RDF proteins, Xis2c and Xis2d, one or both may also inhibit
integration. We performed in vitro integration reactions in the presence of the excision proteins
to determine whether any of them inhibit integration (Figure 5). The concentrations of the
excision proteins added to the reactions were the same as used in the intramolecular excision
reaction. The addition of either Xis2c or Xis2d abolished detectable integration while addition of Exc lowered integration by 20%. Y315F Exc also inhibits the reaction by 20% (Figure 5).

**Relaxed DNA**

Some tyrosine recombinases, such as lambda Int, can use either supercoiled or relaxed DNA as substrates in excision (20). Sutanto et al. compared the efficiencies of *in vitro* intermolecular reactions using relaxed DNA substrates with both substrates supercoiled or with one substrate relaxed and the other supercoiled. They found no difference in the recombination frequencies. However, if both plasmids were relaxed, the excision frequency decreased 40 fold (25). Plasmids of pCMK1019 (same overlap sequences) and pCMK1017 (different overlap sequences), which contain 4.7kb of DNA between the *attL* and *attR* sites, were relaxed with Topoisomerase I. The relaxed plasmids were used as substrates in the reaction in the presence and absence of Exc (Figure 6). There was no difference in excision frequency between relaxed and supercoiled DNA.
DISCUSSION

In order to perform biochemical analyses of the CTnDOT excision system, it was important to develop an \textit{in vitro} system. We previously developed an \textit{in vitro} intermolecular reaction in which the \textit{attL} and \textit{attR} sites were on different plasmids (25). In this system, the proteins required for excision were IntDOT, Xis2c, Xis2d, and a host factor. However, Exc, which had been shown to stimulate excision \textit{in vivo}, did not stimulate excision in this version of the intermolecular reaction (25). Since CTnDOT excision \textit{in vivo} from the chromosome of \textit{Bacteroides} is an intramolecular reaction, we developed a reaction using an excision substrate containing direct repeat copies of the \textit{attL} and \textit{attR} sites on the same plasmid. IntDOT, Xis2c, Xis2d, and the host factor are required for excision while the addition of Exc to the reaction increases the excision efficiency. Thus we have successfully developed an \textit{in vitro} excision reaction that is stimulated by Exc.

Like most tyrosine recombinases, lambda Integrase and the Flp recombinase must have the same overlap sequences in both attachment sites for efficient recombination (2, 27). A strand swapping mechanism that accounted for the requirement for complementary sequences was proposed for integration and excision of lambda and a similar mechanism was proposed for Flp (13). During the first pair of strand exchanges in the recombination complex, the enzyme cleaves the top strand of each site to form a 3’ phosphotyrosine bond with the enzyme and a free 5’OH group. The homology sensitive step occurs when three bp adjacent to the sites of cleavage within the overlap sequences are paired with complementary three bp of the partner site. If the bases are complementary, the ligation reactions form a HJ intermediate (18, 21). Substrates containing
different overlap sequences cannot perform this homology dependent step so that the mismatches inhibit the integration and excision reactions. The second strand exchanges between the bottom strands also require complementary sequences at the sites of ligation.

The ability of CTnDOT to perform recombination with substrates containing different overlap sequences during the second strand exchange in the integration reaction separated it from other tyrosine recombinases systems (9, 12). Laprise et al., found that the first strand exchange in the IntDOT integration reaction requires 2bp of DNA homology adjacent to the sites of cleavage to form the HJ intermediate (12). In contrast to other tyrosine recombinases, the second strand exchange does not require homology within the other 5 bp (12). There is no effect on the integration frequency if the overlap sequences are the same (12). Similarly, in the intermolecular reaction, plasmids containing the same overlap sequences in the attL and attR sites also recombined at the same frequency as substrates containing different overlap sequences (9). Since the overlap sequences can be different, IntDOT does not use a classic mechanism of strand swapping in the integration and the intermolecular reactions.

Surprisingly, we found that the overlap sequences in the attL and attR sites affected the efficiency of the in vitro intramolecular excision reactions. In the absence of Exc the substrates with the same overlap sequences had 10 to 30 fold more recombination than substrates containing different overlap sequences (Figure 4). This finding suggests that there are differences between integration and the intermolecular reactions and the intramolecular excision reaction in the abilities of the recombination complexes to resolve HJs with mismatches in the overlap sequences to products. A model for the excision mechanism that takes our findings into account is that the first step of the intramolecular excision reaction proceeds in a manner similar to the integration reaction but there are differences in subsequent steps. After the attL and attR
intasomes are formed, a synaptic complex forms and a pair of IntDOT monomers bound to each site initiate the first set of strand exchanges. The first strand exchanges require 2 bp of homology in the partner site to proceed. Following cleavage the strands undergo homology dependent exchanges where they pair with the partner site and are ligated to form a HJ. If the excision substrates contain the same overlap sequences, the HJ intermediate is resolved efficiently to products. However, if the overlap sequences are different resolution of the HJ is less efficient. Thus, DNA homology in the overlap sequences stimulates the excision reaction (Figure 7). In contrast, heterology in the overlap region in the integration reaction does not affect the recombination efficiency as long as both sites contain 2bp of homology adjacent to the cleavage site.

We showed previously *in vitro* that there is a bias in the direction of resolution of CTnDOT HJs containing the same or different overlap sequences (10). Synthetic HJs were made that contained only the core sequences and lacked the arm-type sites. When incubated with IntDOT, HJs containing different overlap sequences were only resolved in one direction where complementary bp were exchanged to form substrates. The mismatches in the overlap sequences inhibited the strand exchanges that would result in the formation of products. However, HJs containing the same overlap sequence were resolved in both directions (10). These results indicate that IntDOT needs to interact with the arm-type sites or the accessory proteins to resolve HJs with mismatched overlap sequences to products. The differences in the roles of the arm-type sites and accessory proteins between the integration and intermolecular reaction and intramolecular excision may reflect differences in the architecture of the recombination complexes that affect their ability to process HJs to products.
We found previously that Exc did not stimulate the intermolecular reaction (25). We originally hypothesized that Exc was not required for the intermolecular reaction because it either plays a regulatory or a structural role in vivo by interacting with the attL or attR intasome. In this study we found that Exc increases the excision frequency by 5 fold in substrates that contained different overlap sequences with 4.7 kb of DNA between the attL and attR sites. Our results indicate that Exc can play a structural role in the in vitro intramolecular excision reaction. We suggest two simple possibilities for the role of Exc. It could bind DNA directly on the attL or attR sites or participate in protein-protein interactions.

Although we cannot rule out the binding of Exc to specific sites in the DNA, we consider this unlikely. Exc is a topoisomerase with the ability to bind and relax DNA in vitro and requires a short ssDNA template to bind DNA (3, 11, 26). However, its role in excision is independent of its topoisomerase activity because reactions containing wild type Exc and the Y315F Exc yielded the same excision frequencies. Also, Exc, by itself, does not gel shift the attL or attR sites (J. DiChiara and J. Gardner, unpublished data). Thus we propose that the direct binding of Exc to that attL or attR DNA is an unlikely mechanism.

Exc could participate in protein–protein interactions. In the first case, Exc could promote the formation of or stabilize either the attL or attR intasome. An example of this type of mechanism is the Xis and Fis proteins in the lambda excision reaction. Xis and Fis bind the attR site during excision as a nucleoprotein filament and promote the binding of Int that aids in forming the attR intasome. These proteins directly interact during this process (31,32). Exc could interact with the host factor or any of the other excision proteins in a similar manner.

Alternatively, Exc might stabilize or interact with the recombination complex and function during the first or second strand exchange of HJ resolution. This might explain why it has a
greater effect on substrates with different overlap sequences. Recombination complexes formed without Exc may be less stable but still promote excision at reduced efficiency. We are currently in the process of developing a gel based assay which could detect the HJ intermediates to determine if Exc might function during this step.

Xis2c and Xis2d also inhibit the integration reaction. This could have a biological implication *in vivo* because Xis proteins of other systems such as those of phages lambda and L5 also inhibit integration (1, 15). In *Bacteroides*, IntDOT is constitutively expressed when CTnDOT is integrated into the chromosome (5). Excision is triggered by the presence of tetracycline (5, 6, 24). After tetracycline enters the cell, it induces a regulatory cascade that stimulates the expression of the excision operon which contains the *xis2c*, *xis2d*, and *exc* genes (17, 19). CTnDOT excises and a single strand is transferred to the recipient by conjugation. Once the DNA enters the recipient cell, it circularizes and is replicated from ssDNA to dsDNA. In the presence of tetracycline, copies of CTnDOT in the donor and recipient may remain as extrachromosomal elements because the excision proteins inhibit the integration reaction. However, when the induction signal is removed, the excision proteins would ultimately be degraded and IntDOT could promote integration of CTnDOT into *attB* sites in the donor and recipient.

In conclusion, we describe here a new *in vitro* intramolecular excision reaction that is more efficient than the previously available excision reaction. The proteins required for intramolecular excision are IntDOT, the host factor, Xis2c, and Xis2d. We also found that, unlike the integration reaction, homology in the overlap sequences dramatically increases the efficiency of the intramolecular excision reaction. Exc is not required but stimulates the reaction
especially when the overlap sequences are different suggesting Exc acts after the formation of the HJ intermediate. This is the first demonstration of a role for Exc in vitro.

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REFERENCES


Figure 1. The in vitro intramolecular excision reaction. A). A plasmid, pCMK1013 or derivative, was incubated overnight with IntDOT, the host factor, and the excision proteins at 37°C. Excision results in the formation of a plasmid with attDOT and a circular fragment containing the attB site and the lacZα gene. Excision reactions were electroporated into DH5α::λpir+ cells and screened on MacConkey-lactose plates. B). In reaction 1, IntDOT and the host factor were not sufficient for excision. In reactions 2 and 3, adding Xis2d or Xis2c did not result in detectable excision by themselves. When Xis2d or Xis2c were added along with IntDOT and the host factor, Reaction 4, excision was detected. Finally in reactions 5 and 6, the addition of either WT or Y315F Exc resulted in detectable excision. The addition of the Exc protein to the reaction resulted in increased frequency of excision depending upon the overlap sequences and the distance between the attL and attR sites (See Text).

Figure 2. A). Diagram of Plasmids and Primer Locations. To detect the difference in DNA before and after excision, the original substrate, plasmid pCMK1019 and the attDOT plasmid, were amplified using primers that hybridize outside the excision region. B). Plasmids obtained from white colonies isolated on MacConkey-lactose kan plates were tested for the presence of attDOT. Four plasmids were isolated from independent white colonies (Lanes 1, 2, 3, 4) and used as a template in PCR reactions (See methods). Lane 5 contains a PCR product with pCMK937, the original attDOT plasmid, as a control. Lane 7 contained a PCR reaction with no DNA template. Lane 8 contains a PCR product from the excision substrate before incubation with the excision proteins which produced a 6000bp band. Lane 6 is the TriDye 1kb DNA Ladder.

Figure 3. Characterization of the Excision Substrates. The distance between attL and attR sites is 0.43 (left graph) or 4.7 kb (right graph). The overlap sequences on attL and attR were the same (S) or different (D). Reactions were conducted in the absence of Exc (-), in the presence of wild-type Exc (WT), or in the presence of Y315F Exc (Y).

Figure 4. A Time Course of Excision Reactions. A). Time points of percent excision with 4.7 kb of DNA between the attL and attR sites containing different (D) overlap sequences conducted in the presence of Exc (Exc) or the absence of Exc (No Exc). B). Time points of percent excision with 4.7kb of DNA between the attL and attR sites conducted in the presence of Exc (Exc) or absence of Exc (no Exc) from 0 to 14 hours.

Figure 5. Effects of The Excision Proteins on the in vitro Integration Assay. Integration reactions performed with no accessory proteins are shown in (Lane 1) or with BHF (Lane 2). The addition of Xis2c, Xis2d, or Exc proteins is shown in Lanes 3-5. Combinations of Xis2c, Xis2d, and Exc are shown in Lane 6-7. The background integration obtained in Lane 1 is due to contaminating IHF protein in our IntDOT protein preparation.

Figure 6. Comparison of Relaxed and Supercoiled Plasmids. Excision reactions were performed on with either supercoiled (+) or relaxed (-) DNA with (+) or without (-) Exc using substrates with the same distance, 4.7kb between the attL and attR sites, but contain different (D) or the same (S) overlap sequences.
Figure 7. Comparison of the overlap sequences. IntDOT catalyzes strand exchange in the 7 bp overlap region. When the overlap sequences are the same, on the left, excision is much more efficient as compared to the example of different overlap sequences, on the right. When the overlap sequences are different, the recombination site contains mismatches (Right).
<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Antibiotic Resistance*</th>
<th>Description</th>
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<tbody>
<tr>
<td>pACYC184</td>
<td>Cam, Tet</td>
<td>ATCC, Inc.</td>
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<tr>
<td>pBR322</td>
<td>Amp, Tet</td>
<td>New England Biolabs</td>
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<tr>
<td>pGEMT-attDOT</td>
<td>Amin</td>
<td>Contains attDOT</td>
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<tr>
<td>pRA102</td>
<td>Kan</td>
<td>A plasmid containing the lambda attP site. (Cho et al. 1999)</td>
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<td>pCMK937</td>
<td>Kan</td>
<td>attDOT cloned into the Avall site of pRA102. The overlap sequence is GCCGAAAT. This plasmid was isolated after an excision reaction.</td>
</tr>
<tr>
<td>pCMK938</td>
<td>Kan</td>
<td>attDOT cloned into the AvaII site of pRA102. The overlap sequence is GCCGAAAT. This plasmid was isolated after an excision reaction.</td>
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<tr>
<td>pCMK1013</td>
<td>Kan</td>
<td>pCMK937 was recombined with lacZ α PCR fragment to form pCMK1013. This contains lacZα between attL and attR. attL and attR separated by 409bp. Overlap sequences are different (D). 1</td>
</tr>
<tr>
<td>pCMK1014</td>
<td>Kan, Cam</td>
<td>attL and attR separated by 1444bp. Overlap sequences are different (D). 1</td>
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<tr>
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<td>attL and attR separated by 3303bp. Overlap sequences are different (D). 1</td>
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<td>pCMK1017</td>
<td>Kan, Amp</td>
<td>attL and attR separated by 4099bp. Overlap sequences are different (D). 1</td>
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<td>Kan</td>
<td>Site directed mutagenesis of pCMK1013 to change the overlap sequences to the same (S). 1</td>
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<td>Site directed mutagenesis of pCMK1014 to change the overlap sequences to the same (S). 1</td>
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<td>pCMK1019</td>
<td>Kan, Amp</td>
<td>Site directed mutagenesis of pCMK1017 to change the overlap sequences to the same (S). 1</td>
</tr>
<tr>
<td>pCMK1042</td>
<td>Kan</td>
<td>xis2γ cloned into the NahI and XhoI sites of pET27b</td>
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<tr>
<td>pCMK1043</td>
<td>Kan</td>
<td>xis2γ cloned into the HindIII/Nhel site of pBluescriptII K II</td>
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</table>

1 The overlap sequence of attL is GCCGAAAT and attR is GCTTAGT.  
2 The overlap sequence of attL is GCTTAGT and attR is GCTTAGT.  
*Cam is chloramphenicol, tet is tetracycline, amp is ampicillin, and kan is kanamycin.
Figure 1. The *in vitro* intramolecular excision reaction. A). A plasmid, pCMK1013 or derivative, was incubated overnight with IntDOT, the host factor, and the excision proteins at 37°C. Excision results in the formation of a plasmid with attDOT and a circular fragment containing the attB site and the lacZα gene. Excision reactions were electroporated into DH5α::λpir+ cells and screened on MacConkey-lactose plates. B). In reaction 1, IntDOT and the host factor were not sufficient for excision. In reactions 2 and 3, adding Xis2d or Xis2c did not result in detectable excision by themselves. When Xis2d or Xis2c were added along with IntDOT and the host factor, Reaction 4, excision was detected. Finally in reactions 5 and 6, the addition of either WT or Y315F Exc resulted in detectable excision. The addition of the Exc protein to the reaction resulted in increased frequency of excision depending upon the overlap sequences and the distance between the attL and attR sites (See Text).
Figure 2. A). Diagram of Plasmids and Primer Locations. To detect the difference in DNA before and after excision, the original substrate, plasmid pCMK1019 and the attD′OT plasmid, were amplified using primers that hybridize outside the excision region. B). Plasmids obtained from white colonies isolated on MacConkey- lactose kan plates were tested for the presence of attD′OT. Four plasmids were isolated from independent white colonies (Lanes 1, 2, 3, 4) and used as a template in PCR reactions (See methods). Lane 5 contains a PCR product with pCMK937, the original attD′OT plasmid, as a control. Lane 7 contained a PCR reaction with no DNA template. Lane 8 contains a PCR product from the excision substrate before incubation with the excision proteins which produced a 6000bp band. Lane 6 is the TriDye 1kb DNA Ladder.
Figure 3. Characterization of the Excision Substrates. The distance between attL and attR sites is 0.43 (left graph) or 4.7 kb (right graph). The overlap sequences on attL and attR were the same (S) or different (D). Reactions were conducted in the absence of Exc (-), in the presence of wild-type Exc (WT), or in the presence of Y315F Exc (Y).
Figure 4. A Time Course of Excision Reactions. A). Time points of percent excision with 4.7 kb of DNA between the attL and attR sites containing different (D) overlap sequences conducted in the presence of Exc (Exc) or absence of Exc (No Exc). B). Time points of percent excision with 4.7kb of DNA between the attL and attR sites conducted in the presences of Exc (Exc) or absence of Exc (no Exc) from 0 to 14 hours.
Figure 5. Effects of The Excision Proteins on the *in vitro* Integration Assay. Integration reactions performed with no accessory proteins are shown in (Lane 1) or with BHF (Lane 2). The addition of Xis2c, Xis2d, or Exc proteins is shown in Lanes 3-5. Combinations of Xis2c, Xis2d, and Exc are shown in Lane 6-7. The background integration obtained in Lane 1 is due to contaminating IHF protein in our IntDOT protein preparation.

<table>
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<th>Proteins</th>
<th>1</th>
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</tr>
<tr>
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<td>+</td>
<td>-</td>
<td>-</td>
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<td>+</td>
</tr>
<tr>
<td>Xis2d</td>
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<td>-</td>
<td>-</td>
<td>+</td>
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<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Percent Integration (%)</td>
<td>1.9</td>
<td>38.9</td>
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<td>Not detectable</td>
<td>10.8</td>
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<td>Not detectable</td>
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</table>
Figure 6. Comparison of Relaxed and Supercoiled Plasmids. Excision reactions were performed on with either supercoiled (+) or relaxed (-) DNA with (+) or without (-) Exc using substrates with the same distance, 4.7kb between the attL and attR sites, but contain different (D) or the same (S) overlap sequences.
Figure 7. Comparison of the overlap sequences. IntDOT catalyzes strand exchange in the 7 bp overlap region. When the overlap sequences are the same, on the left, excision is much more efficient as compared to the example of different overlap sequences, on the right. When the overlap sequences are different, the recombination site contains mismatches (Right).