Published in final edited form as:

Plasmid. 2013 September ; 70(2): 190–200. doi:10.1016/j.plasmid.2013.03.009.

Interactions of the Excision Proteins of CTnDOT in the *attR* Intasome

Carolyn M. Keeton, Crystal M. Hopp, Sumiko Yoneji, and Jeffrey F. Gardner

Department of Microbiology, University of Illinois at Urbana-Champaign, 601 S. Goodwin Avenue, Urbana, Illinois 61801, USA.

Abstract

Excision of the conjugative transposon CTnDOT from the chromosome of *Bacteroides spp.* involves four CTnDOT-encoded proteins: IntDOT, Xis2c, Xis2d, and Exc along with a host factor. These proteins form excisive intasomes on the *attR* and *attL* sites which undergo synapsis and recombination to form the *attDOT* and *attB* sites. We recently developed an *in vitro* intramolecular excision reaction where the *attL* and *attR* sites are on the same plasmid. This reaction requires IntDOT, Xis2c, Xis2d, and is stimulated by Exc. We used this reaction to identify the binding sites of the IntDOT, Xis2c, and Xis2d. In this paper, we show that three of the six arm-type sites are absolutely required for excision. Furthermore, we also identified two binding sites for Xis2d and two possible binding sites for Xis2c on the *attR* site. We also showed that IntDOT interacts cooperatively with the Xis2c and Xis2d proteins on the *attR* site.

INTRODUCTION

Bacteroides spp. are gram-negative obligate anaerobes found in the human colon that can act as an opportunistic pathogens outside the colon (Rasmussen et al., 1993). *Bacteroides spp.* are naturally resistant to aminoglycoside antibiotics and have acquired antibiotic resistance genes from transmissible elements called conjugative transposons or integrative conjugative elements (ICEs) over the last thirty years (Salyers et al., 1995; Shoemaker et al., 2001). One example of an ICE is CTnDOT, a 65kb element which carries the *tetQ* and *ermF* genes that confer resistances to tetracycline and erythromycin, respectively (Whittle et al., 2002).

Integration of CTnDOT into the chromosome requires the tyrosine recombinase, IntDOT, and a host factor (Cheng et al., 2000). IntDOT and the host factor bind unique sites on *attDOT* (460 bp) to assemble a complex called the integrative intasome which undergoes synapsis with an *attB* site (60bp). Recombination occurs by sequential sets of strand exchanges separated by 7 bp. The first strand exchanges require the 2bp adjacent to the cleavage sites in the overlap sequence to be the same while the second strand exchanges do not require homology. There is no difference in integration efficiencies if the overlap sequences are the same or different in the *in vitro* integration reaction (Laprise et al., 2010; Malanowska et al., 2007).

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*To whom correspondence should be addressed: ckeeton2@illinois.edu, Telephone Number: (217) 333-7289 and Fax Number: (217) 244-6697.

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The binding sites of IntDOT and the host factor have been characterized (Dichiara et al., 2007; Wood et al., 2010). Previously, the six arm-type sites bound by the N-terminal domain of IntDOT: R1, R1', R2, R2', L1, and L2 were identified by DNaseI footprinting and EMSA assays (Dichiara et al., 2007; Wood et al., 2010). These binding sites were further characterized for their role in the integration reaction by mutating a conserved 6 bp sequence found in each site on plasmids containing *attDOT* and testing these plasmids for integration efficiency in the *in vitro* integration reaction (Wood et al., 2010). Of the arm-type sites, only plasmids containing mutations in the L1 and R1' sites were unable to undergo detectable integration. However, no integration was observed in reactions containing plasmids with mutations in the R1 site and either the R2 or R2' sites, suggesting cooperative interactions between these arm-type sites during integration (Wood et al., 2010).

In the CTnDOT excision reaction, the excision proteins, IntDOT, Xis2c, Xis2d, Exc, and the host factor bind to the *attL* site (240bp) and the *attR* site (220bp) to form the excisive intasomes. The complexes then undergo synapsis and IntDOT performs the strand exchanges that form the *attDOT* and *attB* sites. Xis2c and Xis2d are small basic proteins similar to lambda Xis and other recombination directionality factors (RDFs). Exc is a DNA topoisomerase III enzyme (Keeton and Gardner, 2012; Lewis and Hatfull, 2001; Sutanto et al., 2002). Recently, an *in vitro* intramolecular excision reaction was developed where the *attL* and *attR* sites are on the same molecule. The efficiency of this reaction ranged from less than 1% to virtual completion depending on whether the substrates contained the same overlap sequences and whether Exc was present in the reaction (Keeton and Gardner, 2012). 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 where only 2 out of 7 bp were the same. When Exc was added to the reaction, excision was stimulated 3–5 fold (Keeton and Gardner, 2012).

We report here that three arm-type sites are required in the *in vitro* intramolecular excision: R1, R1', and L1. We also show that Xis2c and Xis2d bind the *attR* site and we have identified the binding sites of Xis2d and two binding sites for the Xis2c during excision. Finally, we show that the Xis2c and Xis2d proteins appear to interact cooperatively with the IntDOT on the *attR* site.

MATERIAL AND METHODS

Plasmids, Primers, and Reagents

All plasmids are described in Table 1. Bacterial strains were grown in Luria-Bertani (LB) broth or on LB agar plates or MacConkey-lactose plates (Silhavy et al., 1984). Antibiotics were supplied by Sigma and used at the following concentrations: kanamycin (kan) 50µg/ml, rifampicin (rif) 10µg/ml, and chloramphenicol (cam) 20µg/ml. Sequencing of the plasmids was done by the ACGT, Inc. Oligonucleotides were synthesized by Integrated DNA Technologies, Inc. Restriction enzymes, alkaline phosphatase, and T4 DNA ligase were supplied by New England Biolabs. PCR reactions were either performed using 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 Reactions and Protein Purification

The intramolecular excision reaction was performed as previously described (Keeton and Gardner, 2012). The reaction mixture contained 100 fmol of plasmid substrate. The excision protein final concentrations used and purification details were as follows; IntDOT 13.5 µM (approximately 80% pure, (Dichiara et al., 2007), Xis2d 200 µM (approximately 85% pure,

(Keeton and Gardner, 2012), the host factor 0.80 μM (approximately 85% pure), and Exc 0.20 μM (95% pure, (Sutanto et al., 2002). The concentration of Xis2c is unknown because it was supplied in a crude extract. The excision frequency was calculated by the total number of white colonies/total number of all white +red colonies on each plate.

Purification of the Xis2c Protein

The Xis2c protein was overexpressed in *E. coli* Rosetta (DE3) pLysS *ihfA*. Cells were grown to an optical density of 0.6 at A600 nm at 30°C and induced with 1 mM IPTG. After induction, cells were grown at 25°C for 20 minutes. Then, rifampicin was added to a concentration of 200 $\mu\text{g/ml}$ and the cells were shaken for 2 hours and pelleted by centrifugation. A cell pellet from a 500ml culture was resuspended in 5 ml of low salt sodium phosphate buffer [50 mM sodium phosphate, pH 7.0, 600 mM NaCl, 1 mM EDTA pH 7.0, 5% glycerol, 1 mM dithiothreitol (DTT), a Roche Complete EDTA-free protease inhibitor tablet, and lysozyme at 1mg/ml]. Cells were sonicated and the extract was centrifuged at 10000 rpm for 30 minutes. The supernatant was loaded onto a HiTrap SP HP column (GE Life Sciences) and washed with 5 column volumes of low salt sodium phosphate buffer. A stepwise salt gradient ranging from 600 mM to 2 M NaCl was used for elution and Xis2d eluted from the column at approximately 1.3 M NaCl. Active fractions were immediately dialyzed in Xis2c storage buffer (50 mM sodium phosphate, pH 7.0, 0.25M NaCl, 1 mM EDTA pH 7.0, 40% glycerol, 1 mM DTT) 2 times for 2 hours and overnight. Activity was verified by EMSA analysis. The supernatant was aliquoted, frozen in a dry ice in ethanol bath, and stored at -80°C . The identity of Xis2c was confirmed by mass spectrometry performed at the University of Illinois Mass Spectrometry lab. The protein was approximately 90% pure. The protein also lost activity with 48–72 hours of purification. The Xis2c protein preparation was frozen in 15 μl aliquots and thawed only once prior to use. Freezing the protein preparation more than once resulted in loss of activity.

Construction of the plasmids with mutations in the Arm-type sites

Plasmids containing mutations in the arm-type sites were created to examine their effect in intramolecular excision by mutagenizing a conserved 6 bp sequence identified previously (Wood et al., 2010). There are 6 arm-type sites in CTnDOT; R1', R1, R2, R2', L1, and L2. These arm-type sites were identified previously by DNaseI footprinting and were subsequently evaluated for their role in integration (Dichiara et al., 2007; Wood et al., 2010). A series of plasmids was constructed containing one of the arm-type site mutations with the same overlap sequences (pCMK972- pCMK983) as described by Wood et al. (Wood et al., 2010). The *attL* and *attR* sites are separated by 1444 bp.

Construction of the Plasmids containing mutations in the Xis2d and Xis2c sites

The Xis2d binding sites on the *attR* site were first localized to a 70 bp region on the *attR* site by DNA footprinting. To identify the bases bound by the Xis2d protein, a series of fragments were designed that contained either the wild-type *attR* sequence or systematic 8 bp mutations. These fragments were incubated with the Xis2d protein and characterized using EMSA analysis. Two Xis2d binding sites were identified which we named the D1 and D2 sites. A series of plasmids pCMK1049- pCMK1057 were created by using site-directed mutagenesis to mutate the D1 and/or D2 binding sites to its complement. These plasmids changed the identified binding sequence to its complement to generate plasmids with mutations in the D1, D2, and both the D1 and D2 sites with the same and different overlap sequences. Two tentative binding sites for the Xis2c protein was identified between the D1 and D2 sites (see text) and a series of plasmids was constructed with mutations in the C1, C2, and both the C1 and C2 sites.

Electrophoretic mobility shift assays (EMSAs)

Electrophoretic mobility shift assays (EMSAs) were performed in buffer consisting of 50 mM Tris-HCl (pH 8), 1 mM EDTA, 50 mM NaCl, 10% glycerol, and 0.18 mg/ml of heparin. Substrates were incubated with various concentrations of protein for 20 minutes and then loaded onto a prerun 6% DNA retardation gel from Invitrogen. Gels were stained with Sybergreen for 40 minutes and the DNA fragments were visualized using a BioRad transilluminator.

RESULTS

Role of the Arm-Type Sites in Excision

IntDOT was previously shown by footprinting analysis to bind six arm-type sites: R1, R1', R2, R2' in the *attR* site and L1 and L2 in the *attL* site (Dichiara et al., 2007; Wood et al., 2010) (Figure 1A). We recently developed an *in vitro* excision reaction in which the *attL* and *attR* sites were arranged as direct repeats flanking the *lacZa* reporter gene. If excision occurs *in vitro*, a plasmid containing the *attDOT* site forms along with a circular intermediate containing the *attB* site and the *lacZa* reporter gene. Reactions were electroporated into DH5α pir⁺ cells and colonies were screened on MacConkey plates containing lactose. Cells containing plasmids that have undergone excision form white colonies. To test the role of the arm-type sites in the *in vitro* intramolecular excision reaction, substrates were constructed by mutating a conserved 6 bp sequence in each arm-type site. These substrates contained the *attL* and *attR* sites as direct repeats 1444 bp apart with the same overlap sequences (pCMK1007) (Table 1) (Keeton and Gardner, 2012) (Figure 1A). The excision frequency with the wild-type *attL* and *attR* sites was 33% (Figure 1B). Plasmids containing mutations in the R2, R2', and L2 sites showed no decrease in excision frequency. We conclude that these sites were not required for excision. Plasmids with mutations in the R1', R1, and L1 sites showed minimal excision with more than a 100-fold lower excision frequency than reactions containing plasmids with wild-type *attL* and *attR* sites. Thus, we conclude that the L1, R1, and R1' arm-type sites are required for intramolecular excision and are sites where IntDOT binds the DNA during excision.

EMSA analysis of the interactions of the Xis2c and Xis2d proteins with the *attL* and *attR* sites

In order to identify the possible binding sites of Xis2c and Xis2d, we performed EMSA analyses using DNA fragments containing the *attL* or *attR* sites. An EMSA analysis of the *attL* (240bp) and *attR* (221 bp) sites was conducted using the partially purified Xis2d (Figure 2). When Xis2d was incubated with DNA containing the *attR* site, multiple bands were observed at high concentrations (Figure 2A: Lanes 3 and 4). These bands formed at higher concentrations may represent both specific binding along with some possible non-specific binding observed only at high concentrations of Xis2d. As the concentration of the Xis2d was decreased, three specific bands were observed with only two of the three observed at the lowest concentration of Xis2d (Figure 2A: Lanes 5 and 6). There was no observable shift when non-specific DNA from the phage P22 was used as the template (Data not shown). When Xis2d was incubated with a DNA fragment of the *attL* site, no shift was observed (Figure 2B).

Previously, Xis2c was used as a crude extract in the *in vitro* intramolecular excision reaction (Keeton and Gardner, 2012). Recently, we developed a protocol for partially purifying Xis2c resulting in a preparation that is greater than 90% pure. The results of an EMSA analysis of Xis2c using either the *attL* or *attR* sites are shown in Figure 3. The Xis2c protein binds the *attR* site and only one band is observed at higher concentrations suggesting that there may be only one binding site or that binding of multiple Xis2c proteins to the *attR* site is highly

cooperative. The Xis2c protein does not bind the *attL* site (Figure 3B). Thus, both the Xis2c and Xis2d proteins only bind the *attR* site in our EMSA analysis and we conclude that the Xis2c and Xis2d proteins likely bind only the *attR* site during excision.

Identification of the Xis2d and Xis2c Binding Sites

To further localize the positions of the Xis2d binding sites on the *attR* site, we performed DNaseI footprinting (Zianni et al., 2006). Reactions were performed using a fragment containing the entire *attR* site incubated with different concentrations of Xis2d (Zianni et al., 2006). At higher concentrations of Xis2d, a region of protection of approximately 70 bp was identified extending from positions -145 to -75 on the *attR* site (Figure 4A). Furthermore, the entire region of Xis2d protection was lost at the same dilution during footprinting. The region of protection included the R1 and most of the R1' arm-type sites which were shown above to be bound by IntDOT during excision. Because the length of the DNA that was protected was large and overlapped the R1' and R1 arm-type sites, we were unable to identify the individual sites bound by Xis2d by footprinting analysis within this region.

In order to localize the region of DNA bound by Xis2d in this 70 bp DNA sequence, several fragments of different lengths were constructed and tested by EMSA analysis. A 44 bp fragment, named fragment 1, was constructed containing the *attR* sequence from positions -148 to -106 (Figure 4A: Fragment 1). Fragment 1 contains the R1' and R1 arm-type sites and 24 bp of adjacent DNA towards the 3' end of the fragment as shown in Figure 4A. Although multiple bands were previously observed when Xis2d was incubated with the entire *attR* site (Figure 2A), only one band was observed when the 44 bp fragment was incubated with the Xis2d protein suggesting this fragment only contained one Xis2d binding site (Figure 4B: Lane 3). In comparison, when a non-specific fragment of the same size was incubated with Xis2d no shift was observed (Figure 4B: Lanes 8-9).

In order to identify the bases in fragment 1 bound by Xis2d, a series of fragments were constructed containing systematic blocks of 8 bp mutations starting from the 5' end of the fragment. These mutations changed the bases to their complement. Each of these fragments was tested by EMSA analysis (data not shown). Two fragments containing adjacent 8 bp mutations were only weakly shifted by Xis2d compared to the wild-type fragment shift with Xis2d. These two fragments contained 8 bp mutations with 4 bp in common. A fragment was constructed that changed all 12 bp in common to both fragments and named fragment 1-D1 (Figure 4: Lane 4). This fragment no longer shifted when incubated with Xis2d (Figure 4B: Lane 4). We named this 12bp sequence the D1 site.

In order to identify other possible Xis2d sites in the *attR* site, we constructed an entire 221bp *attR* fragment that contained mutations in all 12 bp of the D1 site. When this fragment was incubated with Xis2d, shifts were still detectable (Figure 5: Lane 4). The shifts did not contain as many bands when compared to shifts with wild-type *attR* as the substrate (Figure 5: Lane 3). However, the shift produced by an *attR* fragment containing mutations in the D1 site resembled the shift obtained using wild-type DNA with a lower concentration of Xis2d (Compare Figure 2: Lanes 5-6 to Figure 5: Lanes 4-5). Taken together, this suggests there is at least one more Xis2d binding site on the *attR* site.

Using the sequence of the D1 site as a guide, a second putative Xis2d binding site, the D2 site, was located 22 bp downstream of the D1 site. It shares 9 out of the 12 bp of identity with the D1 site (Figure 4A). In order to determine if this sequence was a Xis2d site, a fragment was constructed that contained the D2 site but not the D1 site (Figure 4A: Fragment 2). This fragment was incubated with Xis2d and a single shift was observed (Figure 4B: Lanes 5-6). However, when all 12 bp of the D2 site were changed in this fragment to their complement, no shift was observed (Figure 4B: Lane 7).

An *attR* fragment containing the mutant D2 site plus the wild-type D1 site was shifted by the Xis2d protein and produced multiple bands (Figure 5: Lane 5). When the entire *attR* site, containing mutations that changed the 12 bp sequences of identity in both the D1 and D2 sites, was incubated with the Xis2d protein, there was no detectable shift (Figure 5: Lane 6). Thus, we identified two Xis2d binding sites, the D1 and D2 sites.

Since Xis2c is unstable, we have been unable to use it for footprinting the *attR* site. EMSA analysis of the Xis2c protein produced one distinct band suggesting that the Xis2c can bind independently of the Xis2d protein (Figure 3A). After identifying the D1 and D2 sites, we examined the possible binding locations for the Xis2c that are not bound by IntDOT, Xis2d, or the host factor on the 221bp *attR* site. A likely region for the Xis2c to bind would be between the D1 and D2 sites from positions –121 to –97. To identify putative Xis2c binding sites, this 22 bp region was examined for either direct or indirect repeats. Two 10 bp conserved sequences were identified. They share 7 out of 10 bp in common and we named these sites C1 and C2 (Figure: 4A and Figure: 6A). The C1 site does not overlap with the known binding sites of IntDOT, Xis2d, or the host factor during excision. The C2 site and the D2 site overlap by 4 bp.

Three versions of *attR* were constructed that contained mutations in the C1, C2, or in both the C1 and C2 sites. These *attR* fragments were tested for their ability to shift in the presence of the Xis2c protein (Figure 6B). When an *attR* fragment containing mutations in the C1 site that changed all 10 bp to their complement was incubated with the Xis2c, a shift was still detectable. However, instead of one shift, two shifts were observed (Figure 6B: Lane 4). When an *attR* fragment containing mutations in the C2 site was incubated with Xis2c, one band was observed that migrated slightly faster than the complex formed with the wild-type *attR* fragment (Figure 6B: Lane 5). Finally, when a fragment containing mutations in both the C1 and C2 sites was incubated with Xis2c, a single shift was observed although a majority of the DNA remained unbound by Xis2c (Figure 6B: Lane 6). The change in the complexes formed between Xis2c and the different *attR* sites is consistent with the possibility that the C1 and C2 sites may be bound by the Xis2c protein. However, the shifts we observed are complicated and not easily explained by two independent binding sites. The shift observed when the C1 and C2 sites are mutated may be from interactions with a 3rd site in another part of *attR* since no non-specific binding was observed when Xis2c was incubated with the *attL* fragment (Figure 3B).

Mutational analysis of the D1, D2, C1, and C2 site

To determine if the D1, D2, C1, and C2 sites are required for excision, we constructed plasmids for the *in vitro* intramolecular excision reaction that contained the multiple mutations in each site. A diagram of the binding sites is shown in Figure 7A. Plasmids containing mutations that changed the basepairs in the D1, D2, C1, and C2 sites in *attR* to their complement were tested for excision frequencies with substrates containing the same overlap sequences and a wild-type copy of the *attL* site. Of all the sites, substrates containing mutations in the D1 site were the most defective in excision with a 5 fold decrease in detectable excision (Figure 7B). Substrates with mutations in the D2 and C1 sites showed an intermediate phenotype of a 2.5 fold decrease while plasmids containing mutations in the C2 site yielded wild-type levels of excision (Figure 7B). Plasmids were also constructed that contained either the C1 and C2 or D1 and D2 sites changed to their complement (Figure 7B). Substrates containing mutations in both the D1 and D2 sites or in both the C1 and C2 sites were severely defective in excision, showing a 30-fold decrease relative to wild-type.

Interactions of IntDOT with the Xis2c and Xis2d proteins on the attR site

The D1 site is located adjacent to the R1 arm-type site, suggesting a possible interaction between Xis2d with IntDOT (Figure 4A). To test whether Xis2d and IntDOT interact, we performed EMSAs with combinations of IntDOT and Xis2d proteins incubated with a 125bp fragment that contains the R1', R1, D1 and D2 sites but lacking the R2 and R2' arm-type sites and the core site of *attR* (Figure 8). Three different versions of this fragment were constructed that contained either the wild-type *attR* sequence or an *attR* site with mutations in all 12bp of either the D1 or D2 sites (Figure 8, A–C). The IntDOT protein, by itself, was not sufficient to shift the 125 bp fragment (Figure 8, A–C: Lane 3). When enough Xis2d was added to shift approximately half of the 125 bp fragment, two distinct complexes, which we refer to as the X1 and X2 complex, were observed (Figure 8, A–C: Lane 4). We conclude that the X1 complex represents one Xis2d bound to the *attR* fragment and the X2 complex represents two Xis2d bound. More of the X1 complex forms as compared to the X2 complex (Figure 8, A–C: Lane 4). When both the Xis2d and IntDOT proteins were incubated with the wild-type fragment, a single supershift band was observed referred to as X2I which we believe contains 2 Xis2d and 1 IntDOT (Figure 8A, Lane 5). In addition, there is a disappearance of free DNA (F) and there is a loss of the X1 complex which is correlated to an increase in what appears to be the X2 complex. However, we believe that this complex actually represents a newly formed complex of one Xis2d and one IntDOT protein (IX) that fortuitously has the same mobility as the X2 complex containing two Xis2d proteins. As the concentration of IntDOT was decreased, the X2I complex was lost (Figure 8A: Compare Lanes 5 and 6 to Lane 7).

When the same concentrations of the Xis2d and IntDOT proteins used in the EMSA shown in Figure 9A were added to the 125 bp fragment that contains mutations in the D1 site, no X2I complex was observed (Compare Figure 8B, Lane 5 with Figure 8A, Lane 5). There is a loss of some free DNA and there is an enhancement of the IX complex. However, as mentioned above, we believe this represents a newly formed complex composed of one Xis2d and one IntDOT protein.

When the same conditions were tested with a fragment containing mutations in the D2 site, the X2I complex was observed (Compare Figure 8C, Lane 5 with Figure 8A, Lane 5). However, the complex was not observed when IntDOT was diluted suggesting the interactions of the X2I complex were not as stable when compared to a complex formed on the wild-type sequence (Compare Figure 8C, Lane 6 with Figure 8A, Lane 6). There is little or no disappearance of the free DNA nor is there an accumulation of the IX complex (Figure 8C: Lanes 6 and 7).

We conclude that the IntDOT and Xis2d proteins bind cooperatively and interact with each other to form different complexes containing one Xis2d and one IntDOT (IX) or two Xis2d and one IntDOT (X2I). It appears the IntDOT and Xis2d interact at the R1 and D1 sites. However, the complex formed at the R1 and D1 sites also appears to interact with the Xis2d bound at the D2 site since complexes formed with only the R1 and D1 sites were not as stable as the wild-type complexes.

An EMSA analysis of the Xis2c and IntDOT proteins was performed as described above except that the entire *attR* site was used as the substrate for the experiment (Figure 9). The amount of Xis2c protein that shifted approximately half of the *attR* fragment was used with the same IntDOT concentrations used in Figure 8A (Figure 9: Lane 3). When the Xis2c and IntDOT proteins were incubated together with the *attR* fragment, complexes were formed that do not migrate into the gel and there is a corresponding loss of free DNA (Figure 9: Lanes 4–5). This indicates that there appears to be cooperative interactions between IntDOT and Xis2c when binding the *attR* site (Figure 9: Lanes 4 and 7). As the concentration of

IntDOT is decreased, two faint bands appear above the Xis2c shifts that represent complexes of unknown stoichiometry (Figure 9: Lanes 5 and 6). We conclude that the binding of Xis2c and IntDOT to the *attR* site may be cooperative but it is not possible to determine the stoichiometry of the proteins in the complex.

DISCUSSION

Excision of CTnDOT is a complex process that requires several element-encoded proteins along with a host factor. In addition, it is the first example of a system in which two RDFs are required for excision. In order to identify the binding sites and characterize the protein-protein interactions of the excision proteins, we constructed an *in vitro* intramolecular excision reaction that we believe accurately represents the *in vivo* conditions of CTnDOT excision where the *attL* and *attR* sites are on the same DNA molecule (Keeton and Gardner, 2012). Presumably, CTnDOT excision requires four IntDOT monomers bound to the four core-type sites in a manner similar to other tyrosine recombinase systems (Chen et al., 2000; Gopaul et al., 1998; MacDonald et al., 2006; Subramanya et al., 1997). Xis2c, Xis2d, Exc, and the host factor are involved in forming intasomes on the *attL* and *attR* sites by an unknown mechanism (Keeton and Gardner, 2012; Sutanto et al., 2004). In this paper, we demonstrated the requirements for the arm-type sites and we have identified two likely binding sites for Xis2d and two binding sites for Xis2c in the *attR* site. Furthermore, we have characterized the cooperative interactions between the IntDOT and Xis2c and Xis2d proteins.

IntDOT was previously shown by footprinting analysis to bind six arm-type sites, R1, R1', R2, R2' in the *attR* site and L1, and L2 in the *attL* site (Dichiara et al., 2007). Plasmids were constructed that contained mutations in the conserved 6 bp sequence in each of the arm-type sites and the substrates were tested in the intramolecular excision reaction. Three of the six arm-type sites were required for excision, the L1, R1, and R1' armtype sites. During excision, it is likely that IntDOT binds these sites to form the excisive intasomes required for excision of CTnDOT.

We showed that the Xis2c and Xis2d proteins bind only the *attR* site during excision. EMSA analysis of the Xis2d protein with the *attR* site showed several distinct bands at high concentrations of Xis2d, and as the concentration of Xis2d was decreased, two distinct bands were observed (Figure 2). It is possible that some of these bands represent a filament forming on the *attR* site similar to the filament made by lambda Xis on the *attR* DNA (See below) (Abbani et al., 2007). Our EMSA analysis indicates that there are two sites for the Xis2d protein, the D1 and D2 sites. When both sites were mutated, only minimal excision was detected. The excision defects also appear to be correlated with the observation that Xis2d did not shift *attR* DNA containing mutations in both the D1 and D2 sites.

In the CTnDOT system, the spacing between the D1 and D2 sites is 22 bp. This region between the D1 and D2 sites was identified as a possible binding site for the Xis2c protein. Within this region, two 10 bp direct repeats were identified and named the C1 and C2 sites. The C1 site does not overlap any known binding site for the other proteins involved in excision but the C2 site overlaps the D2 site by 4 bp. However, when the Xis2c protein was incubated with *attR* DNA, only one shift was observed suggesting that there is only one binding site. Alternatively, if there is more than one binding site, multiple bands were not observed on the EMSAs because either the concentration of the Xis2c preparation was so low that only one site was occupied or because binding of the Xis2c protein to the *attR* site is highly cooperative. Substrates containing mutations in both the C1 and C2 sites showed less than 1% excision frequency. EMSA analysis of *attR* site containing mutations in either the C1 or C2 sites still produced shifts, however, the migration of these complexes was

faster than the complex with IntDOT bound to a wild-type *attR* fragment. This indicates that there is more than one site for Xis2c to bind on the *attR* site. The shift with an *attR* site containing mutations in both the C1 and C2 sites formed a complex that migrated faster than any of the complexes formed by either the wild type *attR* site or *attR* containing mutations in either the C1 or C2 sites. At the present time it appears that the Xis2c protein binds to two sites but there may be other additional binding sites.

It is likely that IntDOT and Xis2d interact at the R1 and D1 sites respectively during excision. When we performed an EMSA analysis of the binding of IntDOT and Xis2d proteins to the 125 bp *attR* DNA fragment, we observed one supershift called X2I not observed in the absence of IntDOT (Figure 8A: Lanes 5 and 6). This shift represents a complex that forms between the two Xis2d proteins and IntDOT on the 125bp *attR* site (Figure 8A: Lane 5). When this experiment was repeated with 125bp fragment that contains mutations in the 12bp sequence of either the D1 or D2 sites, the X2I supershift was absent in the fragment containing mutations in the D1 site but was only observed with the fragment containing mutations in the D2 site (Compare Lane 5, Figure 8A–C). It is likely that the X2I complex only forms when the wild-type D1 site is occupied by Xis2d. The complex formed with the wild-type D1 site and a mutated D2 site was not as stable as the wild-type complex suggesting that the D2 site contributes to the stability of the X2I complex and the X1 complex (Figure 8C: Lanes 5–7).

In the lambda excision system, there are 8 bp between the X1 and X2 sites in *attR*. Recently, this region has been shown to contain another binding site called the X1.5 site for lambda Xis (Abbani et al., 2007; Better et al., 1983; Mumm et al., 2006; Nash and Robertson, 1981; Numrych et al., 1991; Yin et al., 1985). Three Xis proteins have been shown to bind cooperatively to form a filament on the DNA at the X1, X1.5, and X2 sites. In the CTnDOT system, the complexes formed by both IntDOT and Xis2d or IntDOT and Xis2c are not sufficient for excision of CTnDOT. If a filament similar to the lambda system forms on the *attR* site, it is likely that it includes both Xis2c and Xis2d. Furthermore, this complex may also interact with IntDOT at the R1 arm-type site.

Clearly, IntDOT, Xis2d, and Xis2c form an intricate complex on the *attR* DNA during excision. The stoichiometry of the excision proteins in this complex is currently unknown. In the lambda system, there was difficulty in determining the stoichiometry of the complex that forms between the proteins which was only finally characterized by a crystal structure (Abbani et al., 2007; Biswas et al., 2005; Warren et al., 2003). A crystal structure of the Xis2c, Xis2d, and IntDOT proteins with *attR* DNA may be the only way to accurately determine the architecture of the *attR* intasome.

Acknowledgments

We would like to thank Abigail Salyers, Margaret Wood, Jennifer Laprise, and Ken Ringwald for suggestions and helpful comments. This work was supported by the National Institutes of Health [Grant Number GM-28717].

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Highlights

- We identified the binding sites for IntDOT, Xis2c, and Xis2d during excision
- IntDOT binds 3 of its 6 arm-type sites during excision
- 2 Xis2d and 2 Xis2c binding sites were identified on the *attR* site
- Xis2c and Xis2d interact with IntDOT cooperatively on the *attR* site.

Figure 1.

Effects of substitutions in the arm-type sites in the *in vitro* intramolecular excision reaction. A). Positions of the arm-type sites (green), on the *attL* and *attR* sites in the *in vitro* intramolecular excision reaction substrate. The overlap sequences in the *attL* and *attR* sites are the same and are designated by crimson circles, the *lacZα* gene by the blue rectangle, and the core sequences are shown by black arrows. B). Plasmids containing the wild-type *attL* and *attR* sites produced an excision frequency of 33.3%. Plasmids containing multiple mutations in the conserved 6 bp sequence in each arm-type sites were evaluated for excision frequency which ranged from not detectable (ND) to wild-type excision.

Figure 2.

EMSA analysis of the Xis2d protein binding to the *attR* and *attL* sites. A). Different concentrations of the Xis2d protein were incubated with the *attR* site (221 bp). Lane 1 contains the 100bp ladder. Lane 2 contains the *attR* site incubated without protein. Lanes 3–7 contain decreasing amounts of the Xis2d protein. The Xis2d concentrations are indicated at the top of the gels. B). The Xis2d protein was incubated with the *attL* site (240bp). Lane 2 contains DNA only while Lane 3 contains DNA incubated with the Xis2d protein.

Figure 3.

EMSA analysis of the Xis2c protein binding to the *attR* and *attL* sites. A). Different concentrations of the Xis2c protein were incubated with the *attR* site (221 bp). Lane 1 contains the 100 bp ladder. Lane 2 contains the *attR* site incubated without protein. Lanes 3–7 contains *attR* DNA incubated with decreasing amounts of the Xis2c protein. The Xis2c concentrations are indicated on the side of the gels. B). The Xis2c protein was incubated with the *attL* site (240bp). Lane 2 contains DNA only while Lane 3 contains DNA incubated with approximately 15nM of Xis2c.

Figure 4.

A). The consensus sequences of the Xis2c and Xis2d binding sites and their locations on the *attR* site. The D1 and D2 sites are located adjacent to the R1' and R1 arm-type sites. There are 22 bp between the D1 and D2 site. Two possible Xis2c binding sites were identified between the D1 and D2 site and named the C1 and C2 sites. B. Two fragments were constructed that contained either the D1 or D2 sites (shown as a blue bar in A) that either contained the wild-type sequence or all 12 bp in either the D1 or D2 site changed to the complement. Lane 1 contains the 100 bp ladder. Lane 2 contains fragment 1 without protein. Lane 3 contains the fragment 1 incubated with Xis2d. Lane 4 contains fragment 1 with all 12 bp of the D1 site mutated incubated with Xis2d. Lane 5 contains fragment 2 shown in Part A without protein. Lane 6 contains fragment 2 incubated with Xis2d. Lane 7 contains fragment 2 with all 12 bp of the D2 site changed and incubated with Xis2d. Lane 8 contains a non-specific fragment incubated without protein. Lane 9 contains the non-specific fragment with Xis2d.

Figure 5. EMSA analysis of the Xis2d protein binding to either the full length wild-type *attR* site or to *attR* sites containing mutations in the D1, D2, or in both the D1 and D2 sites. Lane 1: the 100 bp ladder. Lane 2: the wild-type *attR* site without protein. Lane 3: the wild-type *attR* site incubated the Xis2d protein. Lanes 4–6 : Xis2d incubated with the *attR* site containing multiple mutations in either the D1, D2, or in both the D1 and D2 sites. The Xis2d concentration is approximately 10nM.

A).

C1	GCTTTTGCTG
C2	GATTGTACTG
Consensus	GM T TK R CTG * * * * *

B).

Figure 6.

Identification of putative Xis2c sites. A). Two possible Xis2c binding sites were identified between the D1 and D2 site and called the C1 and C2 sites. The consensus sequence for Xis2c is 5' GNTTNTNCTG 3' B). EMSA analysis of the Xis2c protein binding to either the wild-type *attR* site or to *attR* sites containing mutations in the C1, C2, or in both the C1 and C2 sites. Lane 1: the 100 bp ladder. Lane 2: the wild-type *attR* site without protein. Lane 3: the wild-type *attR* site incubated the Xis2c protein. Lane 4–6: Xis2c incubated with the *attR* site containing mutations in either the C1, C2, or in both the C1 and C2 sites. The Xis2c concentration is approximately 8 nM.

Figure 7.

A). A diagram of the binding sites of the IntDOT (green) and the Xis2c (orange) and Xis2d proteins (black). The core type sites are shown as arrows, the overlap sequences are shown as circles (crimson), and the arm-type sites are shown as rectangles. B). Excision frequencies with substrates containing mutations in the D1, D2, C1, C2, or multiple sites. In this reaction, the excision of plasmids containing a wild-type *attR* site was 33.3%. Plasmids containing mutations in the Xis2d or Xis2c binding sites were evaluated for excision frequency which ranged from not 0.9% to wild-type excision.

Figure 8.

Interactions between the Xis2d and IntDOT proteins with the *attR* site. A 125 bp DNA fragment was constructed that contained the R1', R1, D1 and D2 sites but lacked the R2, R2', and the core site. Each gel above represents either the wild-type *attR* (A) sequence or a fragment that contains 12 bp mutations in either the D1 (B) or D2 sites (C). Lane 1 contains the 100 bp ladder. Lane 2 contains the 125 bp *attR* fragment with either the wild-type (A), D1 (B) or D2 fragment without protein (F). Lane 3 contains the *attR* fragment with the IntDOT protein. Lane 4 contains the *attR* fragment and the Xis2d protein. Two shifts are observed (X1 and X2). Lanes 5–7 contain the IntDOT and Xis2d proteins. The concentration of the Xis2d protein is constant while the concentration of IntDOT is varied.

Figure 9.

EMSA analysis of binding of IntDOT and the Xis2c proteins on the *attR* site. Lane 1 contains the 100bp ladder. Lane 2 contains the *attR* site without any additional proteins. Lanes 3–7 contain enough Xis2c protein to shift half of the *attR* fragment. In Lanes 4–7, IntDOT was added in decreasing concentrations.

Table 1

Plasmids used in this paper.

Plasmids	Antibiotic Resistance	Description
pRA102	Kan	A <i>pir+</i> dependent plasmid containing the lambda <i>attP</i> site. (Cho et al. 1999)
pCMK1018	Kan, Cam	<i>attL</i> and <i>attR</i> separated by 1444bp. Overlap sequences are different. ¹
pCMK1007	Kan, Cam	Site directed mutagenesis of pCMK1018 to change the overlap sequences to the same. ²
pCMK978	Kan, Cam	pCMK1007 with mutated R1' arm-type site.
pCMK979	Kan, Cam	pCMK1007 with mutated R1 arm-type site.
pCMK980	Kan, Cam	pCMK1007 with mutated R2 arm-type site.
pCMK981	Kan, Cam	pCMK1007 with mutated R2' arm-type site.
pCMK982	Kan, Cam	pCMK1007 with mutated L1 arm-type site.
pCMK983	Kan, Cam	pCMK1007 with mutated L2' arm-type site.
pCMK1049	Kan, Cam	pCMK1007 with mutated D1 site.
pCMK1050	Kan, Cam	pCMK1007 with mutated D2 site.
pCMK1051	Kan, Cam	pCMK1007 with mutated D1D2 site.
pCMK1061	Kan, Cam	pCMK1007 with mutated C1 site.
pCMK1062	Kan, Cam	pCMK1007 with mutated C2 site.
pCMK1063	Kan, Cam	pCMK1007 with mutated C1C2 site.

¹The overlap sequence of *attL* is GCGCAAT and *attR* is GCTTAGT.

²The overlap sequence of *attL* is GCTTAGT and *attR* is GCTTAGT.