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The Integration and Excision of CTnDOT

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Abstract

Bacteroides species are one of the most prevalent groups of bacteria present in the human colon. Many strains carry large, integrated elements including integrative and conjugative elements (ICEs). One such ICE is CTnDOT, which is 65 kb in size and encodes resistances to tetracycline and erythromycin. CTnDOT has been increasing in prevalence in *Bacteroides* spp., and is now found in greater than 80% of natural isolates. In recent years, CTnDOT has been implicated in the spread of antibiotic resistance among gut microbiota. Interestingly, the excision and transfer of CTnDOT is stimulated in the presence of tetracycline. The tyrosine recombinase IntDOT catalyzes the integration and excision reactions of CTnDOT. Unlike the well-characterized lambda Int, IntDOT tolerates heterology in the overlap region between the sites of cleavage and strand exchange. IntDOT also appears to have a different arrangement of active site catalytic residues. It is missing one of the arginine residues that is conserved in other tyrosine recombinases. The excision reaction of CTnDOT is complex, involving excision proteins Xis2c, Xis2d, and Exc, as well as IntDOT and a *Bacteroides* host factor. Xis2c and Xis2d are small, basic proteins like other recombination directionality factors (RDFs). Exc is a topoisomerase; however, the topoisomerase function is not required for the excision reaction. Exc has been shown to stimulate excision frequencies when there are mismatches in the overlap regions, suggesting that it may play a role in resolving Holliday junctions containing heterology. Work is currently under way to elucidate the complex interactions involved with the formation of the CTnDOT excisive intasomes.

Introduction

Bacteroides spp. are one of the more prevalent members of the human colonic microbiota, representing approximately 40% of the bacterial community (1). *Bacteroides* spp. are normally in symbiosis with their human hosts. Although they are usually harmless members of the gut microbiota, they can become opportunistic pathogens if released from the colon (2, 3). This most commonly occurs due to surgery, trauma or disease such as gangrenous appendicitis or malignancies (4). Among anaerobic bacteria, *Bacteroides* spp. are the pathogens most commonly isolated from clinical samples, including blood (2). The treatment of *Bacteroides* infections has become more challenging as they have acquired a variety of genes that encode resistances to antibiotics. In the 1970s, only 20–30% of

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Bacteroides spp. clinical isolates were resistant to tetracycline. By the 1990s, the prevalence of tetracycline resistance had increased to 80% (5). This increase in tetracycline resistance can be attributed to the presence of Integrative and Conjugative Elements (ICEs) that encode antibiotic resistance genes.

ICEs, formerly referred to as Conjugative Transposons, have been increasingly implicated in the dissemination of antibiotic resistance in *Bacteroides* spp. and other members of the human colonic microbiota. The best characterized ICE in *Bacteroides* spp. is the 65 kb CTnDOT. CTnDOT was discovered as a mobile element in the chromosome of a *Bacteroides* spp. strain from a local clinical isolate (N. Shoemaker and A. Salyers, personal communication).

Like other ICEs, CTnDOT encodes the genes necessary for its excision and transfer to recipient cells via conjugation, as well as integration into the recipient cell chromosome. Figure 1 shows the genes relevant to the integration, excision and transfer of CTnDOT. The *intDOT* gene encodes the integrase (IntDOT) required for both the integration and excision of CTnDOT. The excision operon contains the *xis2c*, *xis2d*, *orf3*, and *exc* genes. With the exception of *orf3*, the genes contained in this operon are (as its name implies) involved in CTnDOT excision. However, Xis2c and Xis 2d have also been identified as positive regulators of the *tra* genes that encode proteins involved in conjugation (6). In addition, Xis2d and Exc also appear to stimulate expression of the *mob* operon that encodes the relaxase and coupling proteins required for mobilization (7). CTnDOT also encodes two antibiotic resistance genes, *tetQ* and *ermF* (8, 9).

The regulation of CTnDOT excision is complex and highly coordinated. One of the novel features of the CTnDOT element is that excision and conjugative transfer are stimulated in the presence of low levels of tetracycline (10). Upon exposure to tetracycline, the *tetQ-rteA-rteB* operon (shown in orange in Figure 1) is expressed via a translational attenuation mechanism (11, 12). The *tetQ* gene encodes a ribosomal protection protein, and *rteA* and *rteB* encode a two-component regulatory system. Exactly what RteB is sensing in the environment is not yet known, although it is likely not tetracycline (13). RteB is the transcriptional activator of another regulatory protein, RteC. The function of RteC is to activate expression of the excision operon which contains the *xis2c*, *xis2d*, *orf3*, and *exc* genes (14, 15). RteC binds to two inverted repeat half-sites upstream of the excision operon promoter (16, 17).

After activation of the excision operon CTnDOT excises from the *Bacteroides* chromosome and forms a closed circular intermediate (Figure 2). The intermediate is nicked at *oriT* and a single strand is transferred to a recipient cell via conjugation. The DNA is replicated in the recipient cell to form the double stranded circular form, which then integrates into the *Bacteroides* chromosome (18, 19). CTnDOT is stably maintained even in the absence of selection, perhaps due to the complexity of its regulatory system (19). In addition, no *Bacteroides* strains have thus far been identified that lack ICEs (19). Many of the ICEs contained in *Bacteroides* are cryptic and do not carry antibiotic resistance genes. However, they can potentially acquire resistance determinants which could facilitate their transfer to other bacterial species (19). CTnDOT can also mobilize other mobile elements, including

conjugative plasmids and mobilizable transposons (20–23). In the case of mobilizable transposon NBU1, the CTnDOT-encoded RteB activates expression of *orf2x*, one of the genes required for NBU1 excision (K. Moon, unpublished results).

Originally, CTnDOT was called a conjugative transposon because it was thought that the element integrated into random target sites after transfer by conjugation. However, it was subsequently discovered that CTnDOT was site-selective because it integrates into a few target sites (24, 25). DNA sequencing of the *intDOT* gene suggested that it was a member of the tyrosine recombinase family which also includes lambda integrase (see below) (24, 26–28).

Since CTnDOT utilizes site-selective recombination during integration into and excision from the *Bacteroides* chromosome, it was initially assumed that the processes would mechanistically resemble that of bacteriophage lambda. However, recent studies have demonstrated that CTnDOT recombination differs significantly in several respects from that of the lambda system.

IntDOT

The enzyme responsible for catalyzing the integration and excision of CTnDOT is IntDOT, a tyrosine recombinase. The expression of IntDOT is constitutive, and IntDOT and a host factor are sufficient for integrative recombination (24). IntDOT was identified as a member of the tyrosine recombinase family because it contained five of six residues of a characteristic RK(H/K)R(H/W)Y motif, including a catalytic tyrosine found in tyrosine recombinases (24, 26–29). However, alignments with other tyrosine recombinases (including lambda Int) demonstrated that in place of the putative first arginine IntDOT has a serine (S259) (24, 27). Substitution of S259 to alanine or arginine had no effect on *in vitro* integration, cleavage or ligation activities (30). This amino acid substitution, along with other experimental results that will be detailed later in this chapter, were among the first indicators that IntDOT may have a unique active site structure.

The reactions catalyzed by tyrosine recombinases (including IntDOT) are described in detail (in the chapters by Makkuni Jayaram, Anca Segall, Gregory Van Duyne, and Art Landy). The reactions involve the binding of two monomers of the enzyme to each of the two DNA substrates. In the case of CTnDOT integration, the substrates are the joined ends of the excised element (*attDOT*) and the target sequence in the *Bacteroides* chromosome (*attB*) (24, 31). In CTnDOT excision, the substrates are called *attR* and *attL* (Figure 2) (24, 32, 33). Initially, two monomers on each site are active. The active monomers each cleave one strand of DNA to form a 3'-phosphotyrosyl bond with the DNA releasing free 5'-OH groups. Next, the 5'-OH groups undergo a ligation reaction by performing a nucleophilic attack on the partner phosphotyrosine linkage, forming a Holliday junction (HJ). A conformational change activates the other pair of monomers, and another round of cleavage, strand exchange and ligation occurs. The end result is a recombinant with attachment sites containing DNA from each substrate (34, 35).

Identifying residues important for IntDOT function

Like lambda Int and several other tyrosine recombinases, IntDOT is a heterobivalent DNA binding protein that contains three different domains (30, 36, 37). The core-binding (CB) and catalytic (CAT) domains bind to core-type sites (D and D' on *attDOT* and B and B' on *attB*) (Figure 2) that flank the region of cleavage and strand exchange (overlap sequence). The arm-binding (N) domain binds to distal arm-type sites (R1', R1, R2, R2', L1, L2; see below) (30, 37–39). Initially an *in vivo* screen for recombination was developed to identify randomly generated mutants deficient in integration (37, 40). Mutants were isolated with substitutions in each of the IntDOT domains and tested for deficiencies in DNA binding, cleavage, ligation, and Holliday junction resolution. The locations of the IntDOT substitutions isolated through random mutagenesis are shown in Figure 3.

Four mutants with substitutions in the N domain were isolated. Two mutants (R13C and S38N) contain substitutions located in the N domain and 2 additional substitutions (V95M and G101R) occurred in the region where the N domain meets the CB domain (Figure 3) (37). All four mutants showed reduced ability to form protein/DNA complexes in gel shift assays but displayed wild-type levels of *in vitro* cleavage and ligation (37). This result suggests that the N domain is involved in interactions with the DNA (37).

The V95M protein had an additional unique phenotype. While the R13C, S38N, and G101R mutant proteins were able to resolve Holliday junctions, the V95M protein could not (40). This result was interesting, since the methionine substitution in the V95M protein is located in the N domain that binds arm-type sites. The phenotype of the V95M mutant suggested that the N domain may interact with the CB and/or CAT domains during HJ resolution. The V95M substitution is located in a putative alpha helix (H2, Figure 3) that corresponds to the coupler region of lambda Int. An alignment of the lambda Int coupler and the putative IntDOT coupler is shown in Figure 4. The lambda Int coupler is required for cooperative Int interactions necessary for arm-type site binding (41), and it is possible that the IntDOT coupler performs a similar function (37). The lambda Int coupler has also been shown to be involved in HJ resolution via a protein-protein interaction (42). Lee *et al.* (42) performed a charge switch experiment in lambda Int in which they reversed charges of two residues in the same protein. One residue was in the coupler and the other residue was in the N domain. They demonstrated that a substitution of D71 in the coupler or R30 in the N domain of lambda Int abolished HJ resolution. However, a protein containing both the D71R and R30D substitutions regained HJ resolution activity due to the restoration of an intermolecular ion bridge. They concluded that D71 and R30 make an intermolecular ion pair.

Since the V95 residue is in the alpha helix of IntDOT that is analogous to the lambda Int coupler helix, substitutions of charged residues in the coupler helix in the IntDOT helix were made. In contrast to the results found for lambda Int, it appears that an ion pair interaction is not involved in resolution of HJs by IntDOT. To ascertain whether charge interactions are similarly involved in IntDOT HJ resolution, the three charged residues (Figures 3 and 4; E93, K94, and K96) in the putative IntDOT coupler region were mutated to the reverse-charge residues and tested for DNA binding, cleavage, ligation and HJ resolution activity (40). The E93K protein showed wild-type activity in all the assays. The other two mutant

proteins (K94E and K96E) were defective for HJ resolution. However, they were also defective in DNA binding, thus making it difficult to draw a conclusion about the effect of the charge reversal on HJ resolution (40). In addition, mutant proteins that contained substitutions of each of the four negatively charged residues in the N domain (Figures 3 and 4; D19K, D30K, D44K, and D49K) were able to resolve HJs. In sum, it appears that the IntDOT coupler is involved in HJ resolution. However, unlike lambda Int, there is no evidence that IntDOT uses a charge interaction between the coupler and the N domain of a neighboring monomer.

Two mutants were isolated with substitutions in the IntDOT CB domain (T184I and P209L) (Figure 3), and both showed defects in DNA binding, cleavage, and ligation activities (37). This result demonstrates that substitutions in the CB domain can have dramatic effects on catalytic activity. Additional CB residues that are likely important for IntDOT catalytic function were identified using a predicted three-dimensional model developed through homology modeling (30). Site-directed mutagenesis was utilized to construct alanine substitutions at 15 representative residues in the CB domain. Interestingly, several of these CB domain mutants (T139A, H143A, N183A, and T194A) were defective in *in vitro* cleavage but could still perform *in vitro* ligation (30). With the exception of tyrosine recombinases with substitutions of the catalytic tyrosine, these are the only ones that are defective in cleavage but retain ligation activity. The homology modeling predicted that T139, H143, and N183 interact with the cleaved strand (Figure 5) (30). The location of N183 is striking because it is predicted to interact with DNA adjacent to the point of cleavage. T194 is predicted to interact with the uncleaved strand. Malanowska *et al.* (30) speculated that the IntDOT conformation may differ between cleavage and ligation, and that some residues, like N183, H143, and T194, are required only in the cleavage-activated conformation.

Several mutants isolated using the *in vivo* screen had substitutions located in the CAT domain. One of the interesting mutants isolated was A382V (Figure 3), which is located right next to the catalytic tyrosine (Y381). IntDOT containing the A382V substitution was defective in *in vivo* recombination but showed wild-type levels of *in vitro* DNA binding, cleavage and ligation (37). It is possible that the positioning of the catalytic tyrosine is altered in the A382V mutant while the interactions of IntDOT with DNA are unaffected (37). Another mutant, L389F (Figure 3), contains a substitution located in a predicted C-terminal tail of IntDOT. The IntDOT tail likely interacts with other IntDOT monomers during recombination (37). Like A382V, this mutant is defective in *in vivo* recombination but DNA binding, cleavage, and ligation were unaffected (37). In lambda Int, the deletion of the last eight amino acids in the C-terminal tail resulted in deficiencies in recombination but increased topoisomerase activity, suggesting that the C terminus is important for regulating catalytic activity (43). The IntDOT C-terminal tail may serve a similar function.

One of the unique features of IntDOT is the missing arginine residue in the catalytic motif. As mentioned above, IntDOT has a serine residue (S259) where an arginine is predicted to be located (27). One of the CAT domain mutants isolated in the hydroxylamine-generated mutant screen, R285H (Figure 3), was defective for *in vitro* cleavage and ligation in addition to *in vivo* recombination (37). Mutating R285 to an alanine, aspartate, or lysine residue also

resulted in severe deficiencies in recombination, cleavage, and ligation, although DNA binding was not affected (37). The IntDOT homology model suggests that R285 can enter the active site and interact with bound core-type site DNA similar to the Arg I residues of other tyrosine recombinases (Figure 6) (37). Since R285 does not align with Arg I residues, the IntDOT active site appears to be structurally different from active sites of other tyrosine recombinases.

Work is currently under way to isolate crystals of IntDOT complexed with DNA, and it will be interesting to combine both genetic and structural data to generate a model for how IntDOT monomers form protein-protein interactions, bind DNA, and perform catalysis.

The role of homology in CTnDOT integrative recombination

The altered architecture of the IntDOT active site is not the only feature that distinguishes it from other tyrosine recombinases. CTnDOT integrates site-selectively into at least six *attB* sites in the *Bacteroides* chromosome (24) while most other tyrosine recombinases promote site-specific recombination reactions between two unique sites containing at least 7 bp of sequence identity. One consequence of the site-selectivity of IntDOT is that it recombines sites that contain different overlap sequences (Figure 7A). All six known *attB* sites as well as *attDOT* contain a conserved TTTGC in the top strand. The three thymines are located in the B core-type site and the G and C (known as the GC dinucleotide (31)) are located in the overlap sequence (Figure 7A) (24, 31). The strand exchanges at the top strands occur between the third T and the G in the top strands of the partner sites, and the strand exchanges at the bottom strands occur 7 bp away. Because the 5 bp between the GC dinucleotide and the sites of the second strand exchange in the overlap sequences are usually different, a heteroduplex is formed (27, 31, 44). The 5 bp of heterology that are formed is known as the coupling sequence. The exact sequence of the GC dinucleotide is not important for the integration reaction. As long as the 2 bp of identity are present between *attDOT* and *attB* at the sites of strand exchanges, wild-type levels of *in vitro* integration are observed. For example, when the GC dinucleotide was changed to AT or CG in only one of the recombination substrates (*attDOT* or *attB*), *in vitro* integration was barely detectable. However, if a complementary mutation was constructed in the partner site, such that both *attDOT* and *attB* contained identical dinucleotides, wild-type levels of *in vitro* integration were restored (44). Low levels of *in vitro* integration were also detected when only 1 bp of homology existed between *attDOT* and *attB* (31). This demonstrated that the CTnDOT integration reaction was dependent on at least 1 bp of homology between *att* sites. This is significantly different from what has been seen in other systems. Most tyrosine recombinases, including lambda Int, have strict requirements for sequence identity in the overlap sequence. For example, a mutation adjacent to one of the cleavage sites in the lambda overlap sequence significantly decreased integration frequency. However, if the same mutation was made in the overlap of the partner attachment site, recombination was restored (45).

Malanowska *et al.* (27) showed that the first strand exchanges during the integration reaction occur at the sites of identity in the top strand overlap regions. They used cleavage assays performed with nicked *attB* substrates to demonstrate that the top strands of *attDOT* and

attB adjacent to the G of the GC dinucleotide are exchanged first during recombination with the wild-type substrates (Figure 7A). To further elucidate the role of sequence identity during the IntDOT reaction, experiments were performed with *attB* substrates containing substitutions adjacent to the cleavage site in the bottom strand. In one *attB* substrate an inverted GC dinucleotide was placed next to the bottom strand cleavage site adjacent to the B' core-type site (Figure 7B, inverted overlap *attB*). This substrate recombined with wild-type *attDOT* at nearly wild-type levels, despite the absence of the GC dinucleotide at the first site of cleavage and strand exchange in the top strand of *attB* (31). Further work showed that during recombination with the wild-type *attDOT* and the inverted *attB* substrates, the first strand exchanges occur between the top strand of *attDOT* and the bottom strand of *attB*. If an *attB* substrate is constructed with the GC dinucleotides next to both sites of strand exchange in *attB* (Figure 7B, symmetric overlap *attB*), the top and bottom strands of *attB* are exchanged at approximately an equal frequency (31). Taken together, these results indicate that IntDOT first catalyzes homology-dependent exchanges at the sites of the GC dinucleotides, followed by homology-independent exchanges (31, 44). The location of the GC dinucleotide (or homology with the partner *attDOT* site) appears to dictate the order of cleavage and strand exchange in the CTnDOT system, irrespective of the location of the arm-type sites relative to the core. This is in contrast to the lambda system where locations of the arm-type sites in relation to the core dictate the order of strand exchanges (46).

The working model for CTnDOT integration is the following: First, four IntDOT monomers and the *Bacteroides* host factor assemble an intasome on *attDOT*. This is similar to the initial step proposed for lambda integration (47). Second, the intasome undergoes synapsis with a partner *attB* site. The complex is cleaved by two IntDOT protomers bound to *attDOT* and *attB*. If the partner sites are cleaved next to the GC dinucleotide in each site, the reaction proceeds and the Holliday junction intermediate is formed. The second strand exchanges occur 7 bp away to form the recombinant products. However, if synapsis occurs with the sites in the opposite orientation, the ligation reaction is aborted and the reaction is reversed to form substrate.

Accessory factors involved in the CTnDOT excision reaction

Xis2c and Xis2d are required for excision (14, 15, 33). They are small, basic proteins that resemble other Recombination Directionality Factors (RDFs) such as Xis from the bacteriophage lambda system (33, 48, 49). The role of Orf3 in CTnDOT excision or conjugative transfer (if any) has not been elucidated, as a phenotype for an *orf3* deletion has not been detected (6, 10, 14). Exc is a type 1A topoisomerase (similar to *E. coli* DNA topoisomerase III) and utilizes an active-site tyrosine to relax negatively supercoiled DNA in the presence of Mg^{++} (50, 51). The topoisomerase function of Exc is not required for CTnDOT excision, however. If the catalytic tyrosine is mutated to a phenylalanine, excision remains unaffected (33, 51). As described below, Exc stimulates excision under certain conditions and may play a structural role in the excision reaction (15, 33). In addition to the proteins encoded by the excision operon, IntDOT and a *Bacteroides* host factor (BHFa) are also required for CTnDOT excision (14; K. Ringwald, unpublished results).

Elucidating the role of Exc

One of the major questions about CTnDOT excision is the role of Exc in the reaction. Initially, the *exc* gene was shown to be required for *in vivo* excision. When the gene was deleted, excision was not detectable *in vivo* (14, 15). However, Exc did not affect the efficiency of recombination in an *in vitro* intermolecular excision assay (15). In addition, excision frequencies with the intermolecular reaction were inefficient, usually less than 5% (15, 32, 39). The original *in vitro* excision assay involved recombination between the *attL* and *attR* sites on two different plasmids (15). Thus, this intermolecular assay utilizes the sites used in the excision reaction but the reaction does not mimic the topology of the DNA containing the sites in the *in vivo* excision reaction, where *attL* and *attR* are on the same DNA molecule. Subsequently, Keeton and Gardner created an intramolecular *in vitro* excision assay with both *attL* and *attR* sites on a single plasmid (33). Several substrates were constructed that contained varying distances between the *attL* and *attR* sites and different or identical overlap sequences. Exc was not absolutely required for the intramolecular *in vitro* excision reaction (33). Depending on the substrate used, Exc was shown to stimulate the excision frequency by up to 5-fold, although the exact extent of the stimulation was dependent on both the length of DNA between the *attL* and *attR* sites and the exact sequences of the overlap sequences. Optimal excision was obtained when Exc was present and the *attL* and *attR* sites contained identical overlap sequences. However, the magnitude of Exc stimulation of recombination between *attL* and *attR* sites was the greatest when the overlap sequences were different (33).

Although the precise role of Exc during CTnDOT excision is not known, it is unlikely that Exc interacts specifically with DNA because Exc does not appear to bind DNA in electrophoretic mobility shift assays (C. Hopp, unpublished results). Instead, Exc may participate in protein-protein interactions with IntDOT, BHF_a and/or Xis2c and Xis2d, thus facilitating excisive recombination (33). As described below, Exc may also play a role in resolving Holliday junctions during excisive recombination.

Holliday junction resolution by IntDOT

Tyrosine recombinases form Holliday junction (HJ) intermediates after the first strand exchanges. Since the overlap regions of recombination sites of most tyrosine recombinase systems are identical, it was originally thought that the first strand exchanges occur at one end of the overlap region and the HJ intermediate formed branch migrated to the other end where the second strand exchanges between the bottom strands could occur (34, 52). The branch migration would require homology or sequence identity in the overlap region. However, it was discovered that the lambda Int and Flp systems did not utilize simple branch migration to resolve synthetic HJs (53, 54). It was proposed that after the first set of strand exchanges a series of isomerization steps occur. A “strand swapping” isomerization model was proposed where two sequential swaps of three bases occur between the two partners (55). The homology-sensitive step was proposed to be at the annealing step before the ligation step could occur. Because the overlap region of a HJ formed by IntDOT contains heterology (Figure 2), the final ligation step is at sites that contain mismatches with

the partner strand (44). Thus, IntDOT differs from other tyrosine recombinases because it can perform the final ligation reactions when the sites of ligation contain mismatches.

However, IntDOT cannot resolve synthetic HJ intermediates to products when the intermediate contains mismatches in the overlap region. Synthetic Holliday junctions were created *in vitro* with either homologous (identical) overlap sequences (Figure 8A) or overlaps with 5 bp of heterology in the overlap sequences (mismatched) (Figure 8B). The latter substrate is similar to the HJ that would be encountered after the first strand exchanges occur during a reaction *in vivo* (40). These substrates contained only the core-type sites and lacked the arm-type sites. Thus, interactions of IntDOT with arm-type sites cannot occur. Each synthetic HJ was incubated with IntDOT in order to determine whether it could be resolved by IntDOT and whether there would be a bias in the direction of resolution to either substrates or products. The HJ containing identical overlap sequences was resolved by IntDOT into both products (*attR* and *attL*; Figure 8A, black arrows) and substrates (*attDOT* and *attB*; Figure 8A, gray arrows) (40). Because this HJ has identical overlap sequences, a strand undergoing ligation can form a Watson-Crick basepair with the partner strand at the site of ligation at either end of the overlap sequence. Thus the HJ can be resolved to both substrates and products. Surprisingly, however, the synthetic HJ containing mismatches in the overlap sequences was resolved only back to the *attDOT* and *attB* substrates (40). In this case, the ligation reaction works only when the strand undergoing ligation contains the 2 bp GC dinucleotide and can form base pairs with the complementary GC sequence at the site of ligation to form substrates (Figure 8B, black arrows). Formation of products (*attL* and *attR*) would require ligations where the strands are mismatched. Because the HJs did not contain arm-type sites, it was proposed that IntDOT interactions with arm-type sites allow the reaction to bypass the heterology in the overlap region (40). It was also proposed that IntDOT and BHF α form an intasome on *attDOT* which undergoes synapsis with *attB* and subsequently performs the first strand exchange. This complex is able to bypass the heterology in the overlap region and perform the second set of strand exchanges to form products (40).

The effect of homology on excisive recombination

As described above, IntDOT tolerates heterology in the overlap region during the integration and excision reactions. The integration reaction works equally well whether the overlap sites are the same or different in *attDOT* and *attB* (J. Laprise, unpublished results). This observation suggests that the conversion of the HJ intermediate to a conformation that allows resolution to products is not affected by heterology in the overlap sequence.

However, an unexpected result occurred when the *in vitro* intramolecular excision reaction was performed with *attL* and *attR* substrates with identical or different overlap sequences. It was expected that the same observation would be made for the excision reaction with *attL* and *attR* sites that contain identical or different *attL* and *attR* overlap sequences. However, excision substrates containing *attL* and *attR* sites with identical overlap sequences recombined 10- to 30-fold more efficiently than substrates with mismatches in the overlap regions of the sites in the absence of Exc (33). When Exc was added to reactions when *attL* and *attR* contained identical overlap sequences, excision frequencies were nearly 100%. However, the strongest enhancement effect with Exc was seen with excision substrates

containing heterologous overlap sequences. Exc stimulated recombination between these substrates by up to five-fold (33). The mechanism behind this stimulation is unknown. One possibility is that Exc participates in a protein-protein interaction in the *attL* or *attR* intasome that somehow stimulates the ligation reaction when the sites have mismatches at the sites of ligation. The fact that sites with identical overlap sequences results in more efficient excision also highlights a major difference between the integration and excision reactions of CTnDOT.

The roles of the IntDOT arm-type sites in CTnDOT integration and excision

Several tyrosine recombinases, such as the lambda, L5, NBU1, and HP1 integrases perform directional reactions. These recombinases are typically heterobivalent DNA binding proteins that interact with two classes of binding sites. The N domain interacts with arm-type sites, which flank the sites of recombination, while the CB and CAT domains interact with the core-type sites at the sites of strand exchange (56–59). Arm-type sites have different consensus sequences that bind a monomer of the recombinase (39, 60–62). Arm-type sites play an architectural role in recombination reactions where they help form intasomes. For example, a monomer of recombinase can potentially interact simultaneously with an arm-type site and a core-type site on the same DNA molecule (intramolecular interactions) or different DNA molecules (intermolecular interactions) in an intasome (63–65). They can also interact cooperatively with each other or with accessory factors. For example, in the lambda system, 4 of the 5 arm-type sites (P1, P'1, P'2, and P'3) are required for the integration reaction, while the P2, P'1, and P'2 arm-type sites are required for the excision reaction (66–68). Xis, an accessory factor required for excision in the lambda system, bends the DNA and interacts cooperatively with an Int monomer bound to the adjacent P2 arm-type site (69–73). Xis also indirectly blocks Int from binding to the P1 arm-type site, preventing an interaction required for the integration reaction (74).

DNase I footprinting and site-directed mutagenesis identified 6 CTnDOT arm-type sites that bind IntDOT: R1', R1, R2, R2', L1, and L2 (Figure 9) (38, 39). Each arm-type site was mutated and tested in an *in vitro* integration competition assay. The results showed that mutations in either the R1' or L1 arm-type sites reduced recombination to an undetectable level, indicating that these sites are required for integrative recombination (39). Interestingly, an *attDOT* site with mutations in the R1, R2, R2', or L2 arm-type sites showed wild-type levels of integration. However, some *attDOT* sites with mutations in 2 arm-type sites were unable to undergo integrative recombination. Substrates with mutations in the R1 and R2 or R1 and R2' arm-type sites did not undergo integrative recombination at a detectable level (39). These results suggest that an IntDOT monomer binds cooperatively at the R1 arm-type site with another monomer at either the R2 or R2' arm-type sites (39). Because 5 arm-type sites appear to play a role in the integration reaction, it is possible that five IntDOT monomers may be bound to *attDOT* in the integrative intasome. Since only four monomers are likely to be necessary for catalysis, the fifth monomer could be playing an architectural role in forming the intasome (39).

By analogy to the lambda system, IntDOT and BHF α likely assemble an intasome on *attDOT* that undergoes synapsis with a naked *attB* site (47). The IntDOT proteins bound to

D and D' as well as to the R1', R1, R2 and R2' arm-type sites form the integrative intasome (39, 75). Further work will be necessary to determine the three-dimensional structure of the integrative intasome and the intramolecular and intermolecular contacts made by IntDOT.

When arm-type site mutants were tested in the *in vitro* intramolecular excision assay, the R1', R1, and L1 arm-type sites were shown to be essential for CTnDOT excision (Figure 9) (48). It is somewhat surprising that the same arm-type sites were shown to be important for both the integration and excision of CTnDOT. Again, future work will be required to determine the structures of the *attL* and *attR* intasomes as well as the synaptic complex.

Xis2c and Xis2d interactions during excision

Along with IntDOT, the other CTnDOT-encoded proteins required for excision are Xis2c and Xis2d. Both Xis2c and Xis2d are small and basic, and contain helix-turn-helix motifs. EMSA analyses demonstrated that both proteins interact specifically with *attR* (48). Footprinting analyses identified two Xis2d binding sites, denoted D1 and D2, on *attR* (Figure 9). The D1 and D2 sites are approximately 20 bp apart (48). A mutation in the D1 site had the most drastic effect on *in vitro* excision, reducing excision frequency by approximately 5-fold. Mutating both D1 and D2 reduced *in vitro* excision frequency to background levels (48), showing the importance of Xis2d binding for excisive recombination. It is possible that binding of Xis2d dimers at D1 and D2 form a DNA loop.

It is likely that a protein-protein interaction between IntDOT bound to the R1 arm-type site and Xis2d bound to the nearby D1 site is important for the excision reaction (Figure 9). EMSAs with a substrate containing the R1' and R1 arm-type sites as well as the D1 and D2 sites showed that IntDOT forms two different complexes with Xis2d. One complex was believed to contain one Xis2d dimer bound to the D1 site and one IntDOT monomer bound to the R1 arm-type site, while the other complex may contain two Xis2d dimers bound to the D1 and D2 and one IntDOT monomer bound to the R1 arm-type site (48). These results also suggested that IntDOT and Xis2d bind cooperatively to *attR*.

Xis2c shifts *attR* in an EMSA. However, interpretation of the results was not straightforward and the binding sites have not been identified conclusively by footprint analyses (48). It is possible that it interacts with the DNA between the D1 and D2 sites in the *attR* intasome.

Conclusions

The tyrosine family of recombinases contains members that are highly diverse. Experiments on the CTnDOT system have revealed some properties that highlight this diversity. For example, IntDOT catalyzes a chemical reaction characteristic of tyrosine recombinases. However, the observation that IntDOT lacks a conserved arginine residue indicates that the active site architecture likely differs from that of other family members. Presumably an arginine residue is donated to the catalytic pocket from another region of the protein.

Unlike virtually all of the other well-studied tyrosine recombinases, IntDOT does not require sequence identity in the overlap regions of partner sites. During integrative recombination the enzyme requires some sequence identity at the sites of the first strand exchanges but can

perform ligation reactions during the second exchanges where the bases undergoing ligation cannot pair with complementary bases in the partner strand. Thus IntDOT must be able to accommodate heteroduplex DNA in the HJ intermediate formed after the first strand exchanges while the other enzymes cannot form recombinants if the overlap sequences contain heterology. Interestingly, the IntN1 enzyme encoded by mobilizable transposon NBU1 can catalyze a reaction where the first strand exchanges are homology-independent (76). Thus it is possible that experiments with other members of the tyrosine recombinase family could expand further the flexibility of DNA interactions that occur during the reactions.

The excision reaction of CTnDOT is also different from those of other tyrosine recombinase systems because it is so complex. Most systems that display directionality, like the lambda system, utilize a single accessory factor encoded by the element itself. The CTnDOT excision reaction utilizes three CTnDOT-encoded proteins (Xis2c, Xis2d, and Exc) encoded in an operon that is regulated by an intricate signal cascade. In addition, the Exc protein appears to play a role in the excision reaction that is absent in other characterized systems because it may participate in the resolution of the HJ intermediate to products.

There is still much to learn about the CTnDOT system. How does the active site of IntDOT differ from the active sites of other tyrosine recombinases? Why are there differences in the role of homology in this system's integration and excision reactions, and how does Exc influence the resolution reaction? Finally, the structures of the integrative and excisive intasomes and the synaptic complexes await future investigations.

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Figure 1.

A schematic of CTnDOT. The genes *xis2c*, *xis2d*, *orf3*, and *exc* (shown in blue) are part of the excision operon. The genes shown in orange (*tetQ*, *rteA*, and *rteB*) are involved in regulating CTnDOT excision.

Figure 2.

A diagram showing the integration and excision reactions of CTnDOT. The D and D' core-type sites are located on CTnDOT (red), while B and B' core-type sites are located in the bacterial chromosome (purple). IntDOT makes staggered cuts 7 bp apart (vertical arrows) on *attL* and *attR* to excise CTnDOT. The element forms a closed circular intermediate containing a 5 bp heteroduplex known as the coupling sequence. The heterology is likely resolved following conjugative transfer into a recipient cell. During integration into the bacterial chromosome, IntDOT makes staggered cuts 7 bp apart on the *attDOT* site of the circular intermediate as well as the *attB* target sequence, and CTnDOT integrates into the bacterial chromosome. The 5 bp heteroduplexes that form following integration are resolved by DNA replication or repair in the recipient cell.

Figure 3.

The location of IntDOT substitution mutants isolated from hydroxylamine random mutagenesis experiments (red) or site-directed mutagenesis (black). The circled residue is the catalytic tyrosine, which was mutated to phenylalanine via site-directed mutagenesis. The N domain is shown in purple, the CB domain is shown in dark blue, and the CAT domain is shown in light blue.

Figure 4.

Alignment of the amino acid sequences and secondary structures of the N domains of *lambda Int* (top) and *IntDOT* (bottom). The helix $\alpha H2$ is the *lambda Int* coupler. The bold arrows pointing to the *lambda Int* sequence denote residues R30 and D71 that form a protein-protein interaction. The V95 residue of *IntDOT* (indicated by a bold arrow on the bottom sequence) is located in the putative *IntDOT* coupler. Thinner arrows denote aspartic acid residues that were mutated to lysines to examine possible charge interactions between *IntDOT* monomers.

Figure 5.

Locations of amino acids that, when mutated, yielded cleavage-defective phenotypes. N183 is shown in cyan, H143 is shown in blue, T183 is shown in green, and T194 is shown in purple. N183, H143, and T183 interact with the cleaved DNA strand, shown in yellow. T194 is proximal to the uncleaved strand, shown in gray. The scissile phosphate is indicated with a bold black arrow.

Figure 6.

Predicted amino acid residues and active site of IntDOT. The DNA backbone of core-type site DNA is shown in gray. The protein backbone is shown in blue and yellow. Residues 279 to 295, which contain the active site residues and interact with DNA, are represented in yellow. Alpha helices are shown as cylinders, beta sheets are shown as arrows, and regions lacking predicted secondary structure are shown as tubes. The side chains for the predicted active site residues are shown in orange except R285, which is shown in red. The “Arg I” residue from lambda Int (R212) has been superimposed on the IntDOT structure and is shown in purple. R212 aligns with S259 in IntDOT, however S259 is not involved in catalysis. The model suggests that R285 and R212 enter the active site from different positions on the peptide backbone but interact with the similar regions of DNA.

Figure 7.

(A) The core-type sites and overlap regions of wild-type *attDOT* and *attB*. The horizontal arrows denote the core-type sites D and D' on *attDOT* and B and B' on *attB*. The blue boxes designate the overlap regions, also marked with "O." The GC dinucleotides are shown in red. The vertical arrows indicate the cleavage sites. (B) Simplified schematics of the wild-type, inverted overlap and symmetric *attB* substrates utilized in homology studies. The blue

boxes indicates the heterology in the overlap regions, and the red boxes indicate positioning the GC dinucleotides in the substrates.

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Figure 8.

Synthetic Holliday junction substrates. (A) A Holliday junction containing core-type sites (not shown) and identical (homologous) overlap sequences shown with the GC dinucleotide denoted in red. The site of first cleavage and strand exchange is denoted with black arrows, while the site of second strand cleavage and strand exchange is indicated with gray arrows. The *attL* site is shown with a purple line, the *attR* is shown with a gray line, the *attB* is denoted with a blue dashed line, and *attDOT* is denoted with a red dashed line. Resolution at the sites of the black arrows form the *attDOT* and *attB* substrates while resolution at the sites of the gray arrows forms the *attL* and *attR* products. (B) A synthetic Holliday junction with two bp of homology at the GC dinucleotide and mismatches in the rest of the overlap

sequences. Resolution occurs only at the sites of the black arrows forming the *attDOT* and *attB* substrates.

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Integration

Excision

Figure 9.

The IntDOT arm-type sites and the Xis2d binding sites. Boxes denote arm-type sites and Xis2d binding sites, and ovals denote core-type sites. Binding sites shown in red are required for recombination. Blue boxes denote arm-type sites that have a stimulatory effect on the integration reaction. White boxes indicate binding sites that are not required for the given reaction. Figure not drawn to scale.