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The N-terminus of IntDOT Forms Hydrophobic Interactions During Holliday Junction Resolution

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Abstract

DOT Integrase (IntDOT) is a member of the tyrosine recombinase family. It catalyzes the integration and excision reactions of an integrative and conjugative element (ICE) called CTnDOT. Like other tyrosine recombinases, the integration reaction proceeds by two sets of strand exchanges between the *attDOT* site on CTnDOT and an *attB* site in the host chromosome. The strand exchanges occur seven bases apart and define an overlap region. After the first strand exchanges a Holliday Junction (HJ) intermediate is formed. Previous work showed that a valine (V95) in a predicted alpha helix in the N-terminus of IntDOT is required for resolution of HJs to substrates and products. We have identified two additional hydrophobic residues in the helix (A92 and F99) that are involved in resolution of HJs. IntDOT proteins with substitutions at these residues form aberrant complexes in an electrophoretic mobility shift assay. We propose that these three residues participate in hydrophobic interactions that are involved in forming higher-order complexes and resolution of HJs.

Keywords

Conjugative transposon; Integrative and Conjugative Element; CTnDOT; Integrase

1.1 Introduction

Bacteroides are Gram-negative, obligate anaerobic bacteria that compose a major portion of the microbiome in the human gut. When trauma occurs in the colon, *Bacteroides* can escape and become opportunistic pathogens that cause abscesses in other parts of the body. These abscesses are often difficult to treat because many *Bacteroides* species carry integrative conjugative elements (ICEs) with genes that encode for resistance to antibiotics. ICEs are capable of moving from a host to a recipient by excising from the host chromosome, transferring to a recipient by conjugation, and integrating into the recipient chromosome (24, 25).

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CTnDOT is a 65kB ICE found in *Bacteroides*. IntDOT, a member of the tyrosine family of recombinases, is required for integration into and excision from the host chromosome. Like many tyrosine recombinases, IntDOT contains three domains: the catalytic domain (CAT), the core binding domain (CB), and the N-terminal arm-binding domain (N) (11, 16, 20). The CAT domain performs the site specific recombination reaction, while the other two domains bind to separate regions of the DNA. The CB domain binds to the core-type sites that appear as imperfect inverted repeats flanking the overlap region where the strand exchanges take place. The N domain binds to arm-type sites that are located adjacent to the core sites. The arm-type sites are required to coordinate and form higher order nucleoprotein complexes known as intasomes (16, 27).

During recombination, the intasome complex forms and is composed of four IntDOT monomers bound at the four core-type sites. Initially two of the IntDOT monomers are catalytically active and conduct the first strand exchanges between partner sites while the other two IntDOT monomers remain inactive. After the first strand exchanges, a Holliday Junction (HJ) intermediate is formed. After a conformational change in the complex, the other two IntDOT monomers become catalytically active and perform the second strand exchanges, and the HJ is resolved to products. Interactions between the IntDOT monomers are likely important in coordinating the strand exchanges and facilitating the resolution of the HJ intermediate (20).

Most tyrosine recombinases, like lambda Integrase, require DNA sequence identity in a seven base pair overlap sequence between the sites of the strand exchanges. A mismatch in the overlap sequences of partner sites blocks recombination (1, 2, 19). IntDOT differs from other tyrosine recombinases because the sequence identity within the overlap region is not as strict. IntDOT only requires that the first two base pairs adjacent to the sites of the first cleavages to match. Presumably, these two base pairs are required to be complementary for base pairing during the ligation reaction. However, the other five base pairs of the overlap region may contain mismatches, and recombination is still able to proceed (13, 17).

Previous studies of the lambda Integrase protein (15) indicated that two residues (R30 and D71) in the N domain were necessary for the resolution of HJs. This was surprising because the N domain is responsible for binding to arm-type sites and does not contain the catalytic site. Residue D71 is located in the linker region adjacent to a putative alpha helix and was proposed to form an ion pair with residue R30 (Figure 1) (15).

Secondary structure programs predict IntDOT has an alpha helix in the N domain that contains 10 residues (Figure 2) (7). Three charged residues (E93, K94, and K96) are located in the helix. A previous study examined whether a similar charged interaction occurred in IntDOT by creating substitution mutants K94E and K96E in the alpha helix (12). The ability to resolve HJs was lost in both substitution mutants. However, proteins with substitutions in any of the negatively charged residues (D19K, D31K, D44K, and D49K) in the N-terminus region of IntDOT were unable to resolve HJs (12). A substitution mutant E93K mutant was active in the *in vivo* integration assay. Thus IntDOT does not appear to form a charged interaction like lambda integrase. However, through a random mutagenesis approach, a

substitution mutant was identified (V95M) that was also unable to resolve HJs. The substitution occurred in the putative second helix of the protein.

It appears that at least one residue in the second alpha helix of IntDOT has an effect on the ability of IntDOT to resolve HJs. This interaction appears to be different from λ integrase because the interaction may be between hydrophobic residues. In this study, we performed a mutational analysis of the helix to identify the important residues for recombination, DNA binding and the resolution of HJs.

1.2 Materials and Methods

1.2.1 Site-directed mutagenesis

The Agilent Quikchange site-directed mutagenesis kit was used to construct substitutions in pSK2. Oligonucleotides used to create mutations are listed in Table 1. The *intDOT* genes of the plasmids were sequenced to confirm correct mutations. Plasmids containing mutations were transformed into JG19002 for *in vivo* integration assay or MC1061 for expression.

1.2.2 Expression of proteins

To express wild-type and mutant IntDOT proteins, pSK2, pSK2 containing the mutated *intDOT*, or pBAD18 as an empty vector control were transformed in MC1061 cells. A 500 mL LB culture was grown until an optical density (OD₆₀₀) of 0.4 at 30° C. Arabinose was added at a concentration of 0.4% and the cells were grown at room temperature for an additional 4 hours. Cells were harvested and re-suspended in 5ml of low salt IntDOT lysis buffer [50mM NaHPO₄, pH 8.0, 1mM EDTA, 50mM NaCl, 10% glycerol, 1mM dithiothreitol (DTT)]. The extract was sonicated and spun at 4000 X *g* for 20 minutes at 4°C. The supernatants were used for the *in vitro* assays. The IntDOT proteins expressed from the wild-type or mutant genes were not detected on sodium dodecyl sulfate (SDS)-polyacrylamide gels. Quick Start Protein assays (Bio-Rad) were then used to determine the amount of protein in the crude extracts.

1.2.3 *In vivo* integration assay

Either pSK2 or pSK2 containing the mutated *intDOT* genes were transformed into *E. coli* JG19002 (11) cells. After transformation, the cells were grown for 2 hours in LB and 0.02% arabinose at 37°C before being plated on LB plates containing 0.02% arabinose, 25mg/mL Timetin, and 0.04% X-Gal and grown overnight at 37°C. Blue colonies indicated wild type activity due to the *lacI* gene being lost through the integration reaction. Colonies with IntDOT proteins with mutant phenotypes appeared white.

1.2.4 Electrophoretic mobility shift assays

A 325-bp fragment of *attDOT* was amplified from pCMK937 containing *attDOT*(9) using oligonucleotides listed in Table 1. The reaction mixture contained *attDOT* DNA (100ng/μL), IntDOT crude extracts with 7ng of protein before dilution, 7nM purified BHF_a, gel shift buffer [50mM Tris-HCl (pH 8), 1mM EDTA, 50mM NaCl, 10% glycerol, and 2μg/mL heparin sulfate] and dilutions of crude extracts of IntDOT and mutants with 7ng of protein before dilution. The samples were incubated at room temperature for 20 minutes before

being loaded on an Invitrogen 6% DNA retardation gel and run at 100V and 20mA for 1 hour. The gels were incubated with Sybr Green for 40 minutes at room temperature before being analyzed by a Fuji phosphoimager.

1.2.5 Cleavage Assay

The construction of the suicide substrate used in the cleavage assay was described previously (13). The substrate was incubated with crude extracts containing 3.5ng of protein containing wild type or mutant IntDOT in cleavage buffer [20mM Tris-HCl (pH 7.4), 0.1mM KCl, 5mM DTT, 0.05mg/ml bovine serum albumin, and 1% glycerol] for 1.5 hours at 37°C. The reaction was stopped by adding stop solution [50mM Tris HCl (pH 6.8), 2% SDS, 150mM DTT, 12.5 mM EDTA, 10% glycerol, and 0.0025% bromophenol blue] and boiling the sample for 5 minutes. The samples were electrophoresed through Novex 4-20% Tris-glycine gradient gels, exposed to phosphoimager screens and analyzed.

1.2.6 Construction of Holliday Junction intermediates

Oligonucleotides SK3, SK4, SK7, and SK8 (12) were used to make Holliday junctions with identical overlap sequences. The oligonucleotides each correspond to a SK4 was radiolabeled with ³²P. Ten µL of labeled SK4 was added with 1.2µL of unlabeled SK3, SK7, and SK8 (20µM) in annealing buffer [10mM Tris-HCl, pH 8.0, 0.1M KCl, 5mM EDTA]. The mixture was heated to 95°C then cooled to 50°C at a rate of 2°C min⁻¹ then cooled further to 22°C at a rate of 1°C min⁻¹. The sample was electrophoresed through a 5% polyacrylamide gel at 200V for 2 hours. The band containing the Holliday junction was gel purified and was suspended in low salt buffer [10mM Tris-HCl, pH8.0, 100mM NaCl, 1mM EDTA]. The solution was cleaned using DE52 column, ethanol precipitated, then re-suspended and stored in resuspension buffer [10mM Tris-HCl, pH8.0, 500mM NaCl, 1mM EDTA].

1.2.7 Holliday Junction resolution assays

The Holliday Junction resolution assay was performed as previously described (12). The reactions contained 5µL labeled Holliday Junction and 2ng IntDOT crude extract in Holliday Junction resolution buffer [50mM Tris-HCl, pH8.0, 50mM KCl, 1mM EDTA, .1mg/ml BSA, and 5mM DTT]. The reaction mixtures were incubated at 37°C for 2 hours then electrophoresed through an 8% polyacrylamide gel for 2 hours at 200V. The gel was exposed to a phosphoimager screen than analyzed using Fujifilm Image Gauge software.

1.3 Results

1.3.1 N domain of IntDOT

The secondary structure prediction of IntDOT predicts two alpha helices in the N-terminus of the protein. These helices are analogous to two alpha helices that are located on the N-terminus of several tyrosine recombinases, including lambda Integrase, and will be described here as H1 and H2 (Figure 1). Following a random mutagenesis and screen for IntDOT mutants that were unable to perform the integration reaction *in vivo*, four mutants were isolated with substitutions in the N-domain of IntDOT (12). Of these four mutants, one substitution mutant, V95M, was unable to resolve HJs. The V95M mutant was catalytically

active because it could cleave and ligate the DNA. In addition, it showed reduced ability to form protein–protein interactions on *attDOT* DNA (12). The ability to resolve HJs likely involves protein–protein interactions that coordinate the strand exchanges during resolution of the HJs. These interactions may be disrupted by the substituted residue. Since the V95 residue is located in the H2 helix of IntDOT it may function like the D71 residue in lambda Integrase although the interactions would be hydrophobic.

1.3.2 Mutational analysis of the H2 helix in the N domain of IntDOT

In order to identify more residues in the H2 helix that may affect HJ resolution, site-directed mutagenesis was employed to individually mutate all the residues in the H2 helix in the N domain of IntDOT. The plasmid pSK2, which contains *intDOT* under control of P_{BAD}, was used to create these mutants. All residues that were not alanines were mutated to alanine, while the 2 alanine residues were mutated to leucine. The first batch of mutants created was V90A, T91A, A92L, E93A, K94A, V95A, K96A, N97A, A98L, and F99A. After confirmation by DNA sequencing, these mutants were tested for integration using an *in vivo* integration assay utilizing the JG19002 indicator strain as described in the Materials and Methods. The A92L and F99A mutants were negative in the integration assay. Mutants V90A, T91A, E93A, K94A, K96A, N97A, and A98L were all able to perform the *in vivo* integration assay and were not studied further (Table 2).

Because proteins with substitutions at residues A92 and F99 had obvious phenotypes in this study and because the V95M mutant demonstrated a phenotype in an earlier study (11), more mutants were created at these positions for further analysis. Proteins with substitutions V95K and F99K were made in order to examine how a charged residue affects the protein. Proteins with substitutions at either of these charged residues were negative in the integration assay. Additionally, a substitution of T91K was also created because the residue is located next to V95 on the helical wheel prediction, but the mutant with this substitution was able to perform the integration reaction. Proteins with substitutions V95F, F99I, F99L, F99V, and F99Y were created to examine how hydrophobic residues of different sizes would affect the protein. Only the V95F mutation was unable to perform the integration reaction. The F99A protein, which lacks the large hydrophobic side chain, was also inactive in the *in vivo* integration assay. Taken together, it appears that residues at positions A92, V95, and F99 need to be hydrophobic and contain a side chain of a similar size to the original residue. All three of these residues are located on the same side of the predicted H2 helix indicating they may be providing a hydrophobic cluster necessary for the integration reaction. The other residues in the H2 helix appear to be unimportant or mutants containing substitutions at these residues are leaky enough to appear positive in the *in vivo* screen.

1.3.3 Catalytic Activity of IntDOT proteins

To examine whether the proteins could perform the first half of the strand exchange reaction, a cleavage assay was utilized as described previously (13). A suicide substrate was constructed by labeling the bottom strand of an *attB* substrate (Figure 3A). When IntDOT binds the substrate, it cleaves the DNA producing a complex containing IntDOT covalently attached to the DNA. A three base pair fragment diffuses away causing the reaction to be

irreversible. If the IntDOT protein is able to cleave the DNA it forms a product that migrates more slowly than the substrate DNA on a gel.

The mutant IntDOT proteins were tested in cleavage assay (Figure 3B). The substitution mutant A92L was able to cleave almost as efficiently as the wild type IntDOT. The V95M mutant was previously shown to cleave DNA as efficiently as wild type IntDOT as well (11). Interestingly, the V95F, V95K, F99A, and F99K proteins were all unable produce much cleavage product. Substitution mutants V95K and F99K both introduce a charge, while substitution mutants V95F and F99A have drastically different hydrophobic side chains. The large differences in the side chains may interfere with protein interactions that are required to coordinate the IntDOT protein to cleave DNA.

1.3.4 Resolution of HJs by mutant IntDOT proteins

Synthetic HJs were constructed as described previously in order to determine whether mutant proteins could resolve HJs (12) (Figure 4). The synthetic HJs contain the core-type sites but lack the arm type sites. The 5' end of the *attL* strand was radiolabeled before being annealed to the other three strands which remained unlabeled. The wild type IntDOT protein resolves the HJs to a product of 85 base pairs corresponding to *attL* or back to substrates producing a radiolabeled band of 50 base pairs corresponding to *attB* (Figure 4A).

IntDOT mutants that were deficient in integration (A92L, V95F, V95K, F99A, and F99K) were tested for their ability to resolve HJ intermediates. All mutants that could not perform integration were also unable to resolve HJ intermediates (Figure 4B). This result was similar to the previous result that showed that V95M was severely reduced in its ability to resolve HJ intermediates (11). A mixing experiment was performed where wild type IntDOT and the mutant proteins were mixed in a 1:1 ratio to examine if the mutant proteins that were unable to resolve HJs had an effect on the wild type IntDOT proteins ability to resolve HJs (Data not shown). The mutants diminished the amount of HJs that were resolved, but none of the mutants appeared to eliminate the ability of wild type IntDOT to resolve HJs altogether. This indicates interactions between WT and mutant proteins may not be functional, but interactions between WT proteins are still able to resolve HJ intermediates. Since these mutants have similar phenotypes, this provides further evidence that these residues may be providing a hydrophobic cluster that is acting similarly to the charged interaction in lambda Integrase.

1.3.5 Binding of IntDOT to DNA and formation of higher order complexes

Previous work has shown that the *Bacteroides* host factor BHFa binds and bends DNA (21). It also forms a complex with the wild type IntDOT protein. The complexes that form can be detected using an electrophoretic mobility shift assay (EMSA). BHFa forms a complex when incubated with *attDOT* DNA (Figure 5). The concentration of BHFa was added so that approximately half of the DNA was shifted. The crude extract of IntDOT by itself does not shift the *attDOT* DNA. When both BHFa and wild type IntDOT are incubated with *attDOT* DNA, they form three specific supershift complexes that correspond to multiple IntDOT monomers bound to DNA (Figure 5). These bands can correspond to IntDOT monomers forming intramolecular interactions with multiple monomers bound on the same DNA

molecule. These bands also may correspond to intermolecular interactions with multiple monomers bound on separate DNA molecules.

Mutants that were deficient in integration and HJ resolution were examined for their ability to bind *attDOT* DNA and form higher order complexes. The A92L mutant forms only two distinct complexes (Figure 5). The shift pattern of the V95M mutant demonstrated a similar pattern to the A92L mutant (Data not shown). The mutants that were defective in integration, HJ resolution, and cleavage of the DNA (V95F, V95K, F99A, F99K) all had strongly altered shift patterns with *attDOT* DNA. The shift pattern of the F99K mutant shows two distinct complexes (Figure 5), but the bands were not shifted as high as wild type or mutants A92L and V95M. It appears that these mutants are defective in the protein-protein interactions that can be formed by the wild type protein.

1.4 Discussion

During integration of CTnDOT, IntDOT undergoes a variety of protein-protein interactions that are necessary for binding to DNA, forming the intasome complex, undergoing synapses, and coordinating the strand exchanges necessary to form products. Lee et. al proposed that an interaction in the N domain of lambda integrase is required for resolution of HJs (15). The region known as the coupler contains a negatively charged residue (D71) that forms a salt bridge with a positively charged residue (R30). They found that a protein with a substitution to a residue of the opposite charge at either position could cleave DNA but was unable to resolve HJs. A mutant with substitutions R30D and D71R, which switched the charges, was able to restore the ability of the protein to resolve HJs.

A similar charged interaction does not appear to occur in IntDOT. Previous work found that mutants with substitutions of charged residues K94 and K96 in the H2 helix were unable to resolve HJs when the positive charge at these residues was changed to a negative charge (12). However, when all four of the negatively charged residues (D19K, D31K, D44K, and D49K) in the upstream N terminal region of the H2 helix were separately changed to positively charged residues, none were able to restore the protein's ability to resolve HJs.

Through a systematic mutagenesis of the H2 helix, all residues were mutated. Substitutions of all but 3 mutants were tolerated when changed to an alanine or leucine. However, mutants with substitutions at three residues, A92, V95, and F99, were found to be unable to perform the integration reaction. These residues all appear on the same face of the H2 alpha helix and may form a hydrophobic cluster.

The phenotype of the substitution mutant A92L is similar to the previously described V95M mutant. Adding a bulky leucine side chain abolished the ability of the protein to perform the *in vivo* integration assay and resolve HJs. However, it was active in the cleavage assay, and had a slight defect in forming higher order complexes on *attDOT*. The one major difference between the A92L mutant and the V95M mutant is that the V95M mutant demonstrates a minor ability to resolve HJs, while the A92L mutant does not appear to have that function.

Substitutions of the V95 residue demonstrate that the residue needs to be hydrophobic without a very large side chain. Substitution mutants V95K, which introduces a charge, and

V95F, which introduces a much larger hydrophobic side chain are unable to perform the *in vivo* integration assay. These substitution mutants also drastically decrease the amount of cleavage product seen in the cleavage assay and considerably alter the ability to form higher order complexes on *attDOT*.

Mutants with hydrophobic substitutions at position F99 (F99I, F99L, F99V, and F99Y) are tolerated if they have a side chain. However, substitution mutants F99K, which introduces a charge, and F99A, which removes the bulk in the hydrophobic side chain are unable to perform the *in vivo* integration reaction and resolve HJs. These substitution mutants are severely defective in the cleavage assay and drastically alter the ability to form higher order complexes on *attDOT* that the wild type IntDOT forms.

These findings are consistent with a model in which hydrophobic interactions involving residues A92, V95, and F99 interact with a hydrophobic patch on the same protein or neighboring IntDOT monomers. The mechanism behind the inability of mutants V95F, V95K, F99A, and F99K to efficiently cleave DNA is unknown. Substitution mutants A92L and V95M that slightly change the side chain are still able to cleave but are unable to resolve HJs as effectively as wild type IntDOT, so the hydrophobic region is also involved in HJ resolution. The functions altered in these mutants are possibly due to the altering of the tetrameric geometry necessary to coordinate the catalytic activity. Thus this putative hydrophobic interaction may act like the ionic interaction in lambda integrase.

The target region of IntDOT which interacts with these residues in protein-protein interactions is currently undetermined. Identification of the region may be difficult because there is no simple selection for second site revertants and multiple hydrophobic residues may be involved in the hydrophobic interaction at the target region. Hopefully, a crystal structure will become available to help identify the target region that interacts with the hydrophobic cluster identified in this work

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Highlights

- A hydrophobic pocket in H2 helix of IntDOT is identified.
- This region participates in Holliday Junction resolution.
- DNA cleavage and protein-protein interactions are also affected by this region.

Figure 1. A comparison of the N-termini of lambda integrase and IntDOT

The amino acid sequences for both proteins are shown in black. The predicted β -sheets are indicated with an E underneath, while predicted α helices are indicated with a H underneath (7). Red arrows indicate the R30 and D71 residues proposed to form a salt bridge in lambda Int (15). Black arrows indicate the residues of IntDOT proteins that are involved in HJ resolution. All three are located in the predicted second alpha helix H2.

Figure 2. Predicted helical wheel structure of H2 α helix of IntDOT

Residues in rectangles form the hydrophobic pocket. Triangles indicate mutants that have been shown to be unable perform the integration reaction if the charge was reversed in previous work (12) but no change was seen when residues were changed to alanines in this work. Residues in circles were found to have no effect. The prediction of the wheel used the Gibrat et. al method (8).

Figure 3. Cleavage Assay

(A) Three oligonucleotides are annealed to form a duplex with the D and D' core sites with a nick three nucleotides from the site of IntDOT cleavage. If IntDOT cleaves, the trinucleotide diffuses away and the protein is irreversibly bound to the substrate by a phosphoryl tyrosine bond and the resulting covalent complex migrates slower through a gel. (B) WT IntDOT cleaves the DNA product and A92L is nearly as efficient. Substitution mutants V95F, V95K, F99A, and F99K all have less cleavage product than the wild type.

Figure 4. Holliday Junction substrates and resolution

(A) The HJ substrate contains the core type sites and an identical overlap region. The *attL* strand was labeled with ^{32}P . The site of the first strand exchange is indicated with black arrows. The site of the second strand exchange is indicated by a dashed black areas. Resolution forward to products results in a 85 base pair labelled band (*attL*). Resolution back to substrates results in a 50 base pair labelled band (*attB*). (B) resolution of *attL* labelled HJ by wild type IntDOT and mutant proteins (A92L, V95F, V95K, F99A, and F99K). The wild type was able to resolve HJ to either *attL* or *attB*, while none of the mutants were able to resolve HJs. EV corresponds to the empty vector control.

Figure 5. *attDOT* binding by wild type IntDOT, A92L mutant IntDOT protein, and F99K mutant IntDOT protein
BHFa alone shifts the DNA, while IntDOT alone does not. When BHFa and wild-type IntDOT are incubated with DNA together, a supershift is seen with multiple bands being observed. The A92L and F99K IntDOT mutants do not form the same super shift pattern as wild type IntDOT.

Table 1

A summary of strains and oligonucleotides used.

Strains and plasmids	Phenotype	Source or Reference
JG19002	W3110(<i>lacI</i>)Kan::pSK1	10
MC1061	<i>hsdR2 hsdM+ hsdS+ araD139 (ara-leu)7697 (lac)X74 galE15 galK16 rpsL (StrR) mcrA mcrB1</i>	Lab Strain
DH5α	<i>F- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80dlacZ' M15 (lacZYA-argF)U169, hsdR17(rK- mK+), γ-</i>	Lab Strain
pSK2	pBAD18 with <i>intDOT</i> cloned between XbaI and HindIII sites	10
pSK2 derivatives	pSK2 plasmid with mutated <i>intDOT</i> genes	This work
pBAD18	Empty vector control for pSK2	Lab plasmid
Oligonucleotides for mutations	Sequence	
V90A F	GAG CGT GAC AAC TTC GCC ACG GCG GAG A	This work
V90A R	CAC CTT CTC CGC CGT GGC GAA GTT GTC A	This work
T91A F	CGT GAC AAC TTC GTC GCG GCG GAG AAG G	This work
T91A R	CTT CAC CTT CTC CGC CGC GAC GAA GTT GT	This work
T91K F	CGT GAC AAC TTC GTC AAG GCG GAG AAG G	This work
T91K R	CTT CAC CTT CTC CGC CTT GAC GAA GTT GT	This work
A92L F	GAC AAC TTC GTC ACG TTG GAG AAG GTG A	This work
A92L R	GTT CTT CAC CTT CTC CAA CGT GAC GAA GT	This work
E93A F	AAC TTC GTC ACG GCG GCG AAG GTG AAG A	This work
E93A R	GGC GTT CTT CAC CTT CGC CGC CGT GAC G	This work
K94A F	TTC GTC ACG GCG GAGGCG GTG AAG AAC G	This work
K94A R	AAA GGC GTT CTT CAC CGC CTC CGC CGT G	This work
V95A F	GTC ACG GCG GAG AAG GCG AAG AAC GCC	This work
V95A R	GAG AAA GGC GTT CTT CGC CTT CTC CGC C	This work
V95F F	GTC ACG GCG GAG AAG TTC AAG AAC GCC T	This work
V95F R	GAG AAA GGC GTT CTT GAA CTT CTC CGC C	This work
V95K F	GTC ACG GCG GAG AAG AAA AAG AAC GCC	This work
V95K R	GAG AAA GGC GTT CTT TTT CTT CTC CGC CG	This work
K96A F	AAA GGC GTT CTT CAC CGC CTC CGC CGT GAC GAA G	This work
K96A R	CTT CGT CAC GGC GGA GGC GGT GAA GAA CGC CTT C	This work
N97A F	GCG GAG AAG GTG AAG GCT GCC TTT CTC G	This work
N97A R	CAA TCC GAG AAA GGC AGC CTT CAC CTT C	This work
A98L F	GAG AAG GTG AAG AAC CTC TTT CTC GGA TTG GAA	This work
A98L R	TTC CAA TCC GAG AAA GAG GTT CTT CAC CTT CTC	This work
F99A F	AAG GTG AAG AAC GCC GCT CTC GGA TTG G	This work
F99A R	GTG TTC CAA TCC GAG AGC GGC GTT CTT C	This work
F99I F	AAG GTG AAG AAC GCC ATC CTC GGA TTG G	This work

Strains and plasmids	Phenotype	Source or Reference
F99I R	GTG TTC CAA TCC GAG GAT GGC GTT CTT CA	This work
F99K F	AAG GTG AAG AAC GCC AAG CTC GGA TTG G	This work
F99K R	GTG TTC CAA TCC GAG CTT GGC GTT CTT CA	This work
F99L F	AAG GTG AAG AAC GCC TTG CTC GGA TTG G	This work
F99L R	GTG TTC CAA TCC GAG CAA GGC GTT CTT CA	This work
F99V F	AAG GTG AAG AAC GCC GTT CTC GGA TTG G	This work
F99V R	GTG TTC CAA TCC GAG AAC GGC GTT CTT CA	This work
F99Y F	AAG GTG AAG AAC GCC TAC CTC GGA TTG G	This work
F99Y R	GTG TTC CAA TCC GAG GTA GGC GTT CTT CA	This work
Oligonucleotide Cleavage Assay	Sequence	
T4	CAC TTT TTT GTA GTA ACT TTG C	12
JG01B	ACT CTT TTC GTA ACT TCA CTA AGC AAA GTT ACT ACA AAA AAG TG	12
JG01T	TTA GTG AAG TTA CGA AAA GAG T	12
Oligonucleotide HJ Resolution	Sequence	
SK3 <i>attDOT</i> (94bases)	CAA GAG CGA TTA ACC TAC GCT CAT TTC CAA TAA ATT ACA CTC TTT TCG TAA CTT CAC TAA GCA AAG TTA CTA CAA AAA AGT GAA ATG CGG AAA G	11
SK4 <i>attL</i> (85 bases)	GCT CGG AAA TTT GCA GTA AAT TTG CTT AGT GAA GTT ACG AAA AGA GTG TAA TTT ATT GGA AAT GAG CGT AGG TTA ATC GCT CTT G	11
SK7 <i>attB</i> (50 bases)	AAT TAC TGT TTA GTA TTT TAA CTA AGC AAA TTT ACT GCA AAT TTC CGA GC	11
SK8 <i>attR</i> (59 bases)	CTT TCC GCA TTT CAC TTT TTT GTA GTA ACT TTG CTT AGT TAA AAT ACT AAA CAG TAA TT	11

All primers listed 5'→3'.

Table 2

A summary of substitutions that had no detectable effect on the integration reaction.

Mutant	Base Change
V90A	GTC→GCC
T91A	ACG→GCG
T91K	ACG→AAG
E93A	GAG→GCG
K94A	AAG→GCG
V95A	GTG→GCG
K96A	AAG→GCG
N97A	AAC→GCT
A98L	GCC→CTC
F99I	TTT→ATC
F99L	TTT→TTG
F99V	TTT→GTT
F99Y	TTT→TAC

Table 3

A summary of mutants tested for integration, DNA binding, HJ resolution, and cleavage.

Mutant	Base Change	Integration	Binding	HJ Resolution	Cleavage
WT	–	+	+	+	+
A92L	GCG→TTG	–	Altered	–	+
V95F	GTG→TTC	–	Altered	–	–
V95K	GTG→AAA	–	Altered	–	–
V95M	GTG→ATG	–	Altered	+/-	+
F99A	TTT→GCT	–	Altered	–	–
F99K	TTT→AAG	–	Altered	–	–

+ means the activity is at or near WT. – means the activity is not seen in the mutant. +/- means the activity is severely decreased in the mutant protein. Altered refers to the pattern of binding being different from WT while still being present.