



## Analysis of the zinc finger domain of TnpA, a DNA targeting protein encoded by mobilizable transposon Tn4555

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### Abstract

The mobilizable transposon Tn4555, found in *Bacteroides* spp., is an important antibiotic resistance element encoding a broad spectrum  $\beta$ -lactamase. Tn4555 is mobilized by conjugative transposons such as CTn341 which can transfer the transposon to a wide range of bacterial species where it integrates into preferred sites on the host chromosome. Selection of the preferred target sites is mediated by a DNA-binding protein TnpA which has a prominent zinc finger motif at the N-terminus of the protein. In this report the zinc finger motif was disrupted by site directed mutagenesis in which two cysteine residues were changed to serine residues. Elemental analysis indicated that the wild-type protein but not the mutated protein was able to coordinate zinc at a molar ration of 1/1. DNA binding electrophoretic mobility shift assays showed that the ability to bind the target site DNA was not significantly affected by the mutation but there was about a 50% decrease in the ability to bind single stranded DNA. Consistent with these results, electrophoretic mobility shift assays incorporating zinc chelators did not have a significant on affect the binding of DNA target. In vivo, the zinc finger mutation completely prevented transposition/integration as measured in a conjugation assay. This was in contrast to results in which a TnpA knockout was still able to insert into host genomes but there was no preferred target site selection. The phenotype of the zinc finger mutation was not effectively rescued by providing wild-type TnpA in trans. Taken together these results indicated that the zinc finger is not required for DNA binding activity of TnpA but that it does have an important role in transposition and it may mediate protein/protein interactions with integrase or other Tn4555 proteins to facilitate insertion into the preferred sites.  
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### 1. Introduction

The *Bacteroides* comprise approximately 30% of the total bacterial population in the human large

intestine where they are essential for proper gut function; however, they also are significant opportunistic pathogens being the most frequently isolated organisms from anaerobic infections (Finegold and George, 1989; Smith et al., 2003). *Bacteroides* spp. are notoriously drug resistant due to a combination of inherent resistance and acquired resistance genes and this has serious implications in the treatment of

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anaerobic infections (Snydman et al., 1999; Falagas and Siakavellas, 2000). The emergence of drug resistance in *Bacteroides* is in part due to their armament of mobile genetic elements including conjugative transposons (CTNs), and mobilizable transposons (MTNs) which catalyze horizontal gene transfer (Whittle et al., 2002; Smith et al., 1998). The MTNs are novel genetic elements described only in *Bacteroides* and *Clostridium*. In contrast to the CTNs, these elements are not capable of self-transfer but depend on a coresident CTN to form the conjugative mating bridge. MTNs do encode mobilization proteins required for relaxosome formation and integrase proteins for transposition.

Mobilizable transposon Tn4555 from *Bacteroides vulgatus* encodes CfxA, a clinically significant broad spectrum  $\beta$ -lactamase conferring high-level resistance to cefoxitin and other  $\beta$ -lactams (Parker and Smith, 1993). The *cfxA* gene has been found in 80–100% of cefoxitin resistant, imipenem sensitive *Bacteroides* isolates (Mastrantonio et al., 1996; Parker and Smith, 1993) and more recently the gene was isolated from pathogens found in the oral cavity, *Prevotella* and *Campylobacter* (Iwahara et al., 2006; Jolivet-Gougeon et al., 2004). The *cfxA* gene in these oral isolates often is associated with the Tn4555 mobilization genes suggesting that this MTN or related elements are important in the spread of  $\beta$ -lactam resistance.

Tn4555 transposition is by a site-specific recombination mechanism mediated by Int, a lambda family tyrosine recombinase. A second protein, TnpA, was found to direct Tn4555 insertions into preferred sites on the chromosome of *Bacteroides* spp. and *Escherichia coli* (Bacic and Smith, 2005; Tribble et al., 1999). In the absence of *tnpA*, Tn4555 transposition was random and occurred at 1/40 the frequency of the wild-type transposon. TnpA is a DNA binding protein but there does not appear to be a primary DNA sequence to which the protein binds with high affinity (Bacic and Smith, 2005). Although the exact mechanism for target selection is not known TnpA also binds single stranded DNA (ssDNA) and the preferred target sites all exhibit characteristics of bent DNA (Bacic and Smith, 2005).

TnpA is not related to any known family of DNA binding or other proteins in the public databases but analysis of the TnpA primary amino acid sequence revealed the presence of a putative 4-Cys zinc finger near the amino terminus (Fig. 1). Zinc fingers are common DNA binding motifs in

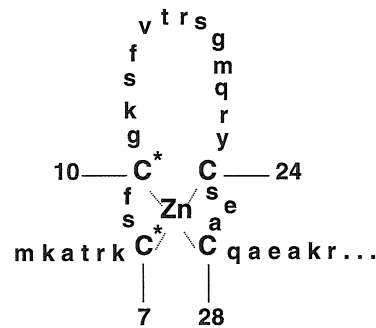


Fig. 1. Putative zinc finger motif of TnpA showing the likely Zinc-coordination site. The two cysteine residues that were changed to serines are indicated by an \*.

eukaryotes where they usually occur in multiples and have a role in genetic regulation (Rhodes and Klug, 1993). In the last few years, putative zinc finger motifs have been observed more frequently in prokaryotic sequences and some of these motifs have been shown by chemical analysis to coordinate zinc (Makarova et al., 2002; Blindauer et al., 2002; Blindauer et al., 2001; Chou et al., 1998). In order to better understand the mechanism of TnpA target site selection the role of the putative zinc finger motif was investigated.

## 2. Materials and methods

### 2.1. Mutant and plasmid construction

In order to disrupt the zinc finger motif, the TnpA/maltose binding protein fusion (M-TnpA) construct described previously (Bacic and Smith, 2005) was mutated using the QuickChange™ Site-Directed Mutagenesis kit (Stratagene, Cedar Creek, TX) per the manufacturer's instructions and primers Cys/Ser-1, 5'-ACTTGCCGGA AACTGGATTTTCTG; and Cys/Ser-2, 5'-CAGAAAA TCCAGTTTTTCCGGCAAGT. Two cysteines were changed to serines as indicated in Fig. 1. This protein, M-TnpA<sup>C-S</sup> and the wild-type M-TnpA were expressed in protease deficient *E. coli* strain ER2508 and purified using the pMAL system (New England Biolabs, Beverly, MA) as described previously (Bacic and Smith, 2005). Protein concentration was determined using the Bradford reagent with lysozyme controls (Bio-Rad, Hercules, CA).

For in vivo analysis of the TnpA zinc finger domain, the mini-Tn4555 construct, pFD660, (Tribble et al., 1999) was mutated as follows. pFD660 was digested with *NdeI* and *SstI* and the 4.8 kb fragment containing *tnpA* cloned into pUC18. The resulting construct pUC660 was used for site-directed PCR mutagenesis using primers Cys/Ser-1 and Cys/Ser-2 and the QuickChange™ Site-Directed Mutagenesis kit as described above. The mutated DNA

fragment was used to replace the wild-type fragment in the pFD660 backbone and this construct p660Z was used in mating assays. All mutations were confirmed by nucleotide sequence analysis at the University of Tennessee Knoxville DNA Sequencing Center (bioseq@utk.edu).

For complementation of the *tnpA*<sup>C-S</sup> mutated Tn4555 two plasmids containing *tnpA* were constructed. First, a 1359 bp fragment containing the entire *tnpA* gene and promoter was PCR amplified and cloned into the *EcoRI* site of the shuttle vector pFD851 (contains the cefoxitin resistance gene *cfxA*; Parker and Smith, 2004). This construct, pFD851::*tnpA*, contained from 423 bp upstream of the *tnpA* start site to the stop codon of the gene. The second construct, pFD972::*tnpA*, contained *tnpA* cloned into an expression vector pFD972 (contains tetracycline resistance gene, *tetQ*; Spence et al., 2006). The *tnpA* gene from 76 bp upstream of the start site to the stop codon of the gene (bp 283 to 1294 of Tn4555 Genbank #U75371) was cloned into the *BamHI* and *KpnI* sites just downstream of the constitutive promoter in pFD972 to allow for continuous expression of the gene.

## 2.2. Mobility shift assays

Gel shift assays were done generally as described (Bacic and Smith, 2005; Fried, 1989). Briefly, appropriate amounts of protein and [ $\alpha$ -<sup>32</sup>P]dCTP (Perkin-Elmer Life Science Inc., Boston, MA) labeled *B. fragilis* target DNA were mixed with binding buffer (BB; 25 mM Hepes pH 7.6, 100 mM KCl, 12.5 mM MgCl<sub>2</sub>, 20% glycerol, 1.0 mM DTT), mobility shift buffer (MSB; 10 mM Hepes pH 8.0, 50 mM KCl, 4 mM MgCl<sub>2</sub>, 0.1 mM EDTA pH 8.0, 2.5 mM DTT, 0.1 mg/ml BSA) and 50% glycerol at room temperature for 15 min. For the assays involving specific metals and chelators, both the BB and MSB were made without MgCl<sub>2</sub>, or KCl and then the additions shown in Fig. 2 were made. During binding, the wells of a pre-run, 5% polyacrylamide native gel (4.94% acrylamide, 0.06% bis-acrylamide, 1× Tris-borate/EDTA [TBE], 0.06% ammonium persulfate, 0.05% TEMED) were flushed with MSB and then the binding mixes quickly loaded. Gels were run in 1× TBE pH 8.4 at 4 °C for 10 h at 8 mA. After electro-

phoresis, gels were dried and exposed to X-ray film. All assays were done at least three times and representative experiments are shown in the figures. Densitometry was done on a FluoroChem™ 8800 (Alpha Innotech Corp., San Leandro, CA) imaging system and version 3.1 software.

## 2.3. Transposition assays

The model system used to study the effect of *tnpA* mutation on insertion and target site selection utilized the mini-Tn4555 vector, pFD660 (Tribble et al., 1999). This construct is a plasmid in *E. coli* but does not replicate in *Bacteroides* strains and thus transposition activity of Tn4555 can be monitored by insertion into the *Bacteroides* chromosome. The constructs, p660Z (see above) and pFD679 (Tribble et al., 1999) were transferred from *E. coli* to either *B. fragilis* 638R or *B. thetaiotaomicron* BT5482 via filter mating with helper plasmid RK231 (Bacic et al., 2005). Transconjugants were selected on media containing 10 µg/ml of erythromycin to select for the incoming vector. Transfer frequencies are the number of transconjugants per input donor cell and were determined from five independent matings.

## 2.4. Elemental analysis

The protein samples were diluted 10 times using 2% HNO<sub>3</sub> and analyzed using VG Elemental PlasmaQuad model PQII and Element 2 (Finnegan MAT, Bremen, Germany) sector field high resolution inductively coupled plasma mass spectrometers (ICP-MS). Single element standard solutions purchased from Inorganic Ventures (Lakewood, NJ) were utilized to prepare calibration and internal standard solutions. Analysis was performed using an external calibration procedure. Internal standards were included for matrix and instrumental drift corrections (Srivastava and Jain, 2002; Mirshra et al., 2003). Samples were introduced using a peristaltic pump in conjunction with an autosampler. The background counts were measured on buffer in identical conditions and using the same instrument parameters as the protein samples.

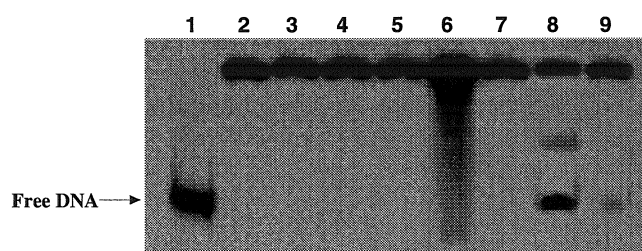


Fig. 2. Effect of various metals and EDTA on M-TnpA DNA-binding. (Lane 1), no protein; (lane 2), normal BB binding buffer containing Mg and KCl; (lane 3), no metals; (lane 4), 10 mM CaCl<sub>2</sub>; (lane 5), 10 mM CoCl<sub>2</sub>; (lane 6), 10 mM FeSO<sub>4</sub>; (lane 7), 10 mM NiCl<sub>2</sub>; (lane 8), 10 mM ZnCl<sub>2</sub>; (lane 9), 50 mM EDTA. Each lane contained 0.53 nM *B. fragilis* target DNA and (lanes 2–9) each contained 345 nM—TnpA.

### 3. Results

#### 3.1. Effect of metals on TnpA DNA binding

TnpA is a DNA binding protein with affinity for bent DNA and a putative 4-Cys zinc finger motif at the amino terminus (Fig. 1). In a previous study TnpA and a TnpA/MBP fusion (M-TnpA) were shown to have nearly identical DNA binding properties (Bacic and Smith, 2005). Since native TnpA is unstable we used M-TnpA in mobility shift assays with various metals and chelators in order to investigate the importance of zinc in TnpA DNA binding (Fig. 2). Reactions with no metal additions showed normal binding as did mixes containing other metals except zinc. The addition of 10 mM zinc decreased the binding and densitometry showed that about 25% less DNA target was bound when compared to controls with no zinc added (Fig. 2, lanes 3 and 8). This effect seemed to be zinc dependent since there was less binding inhibition at lower concentrations of zinc down to 0.5 mM (data not shown). The addition of 50 mM EDTA had a minor affect on binding (lane 9) with about 99% DNA bound. Lower concentrations of EDTA had no effect. The effects of divalent cation chelator, *o*-phenanthroline, was also tested in binding assays because this compound has a higher affinity for zinc, however, the results were the same as with EDTA.

#### 3.2. Characterization of mutated TnpA

In order to determine if the 4-Cys motif coordinated zinc, a mutant protein was made by replacing Cys-7 and Cys-10 with serines (Fig. 1). This protein, M-TnpA<sup>C-S</sup> and the wild-type M-TnpA were purified under the exact same conditions and several samples of each were analyzed by inductively coupled plasma mass spectroscopy (ICP-MS). ICP-MS is particularly a useful technique for this study because it can determine the presence of multiple elements at very low concentrations. During ICP-MS analysis, metals are ionized by high temperature plasma and the ions are then separated on the basis of mass to charge ratio and measured. The wild-type and mutant protein samples were screened for the whole Periodic Table using two different ICP-mass spectrometers. The detected elements and their amounts in ng/ml are given in Table 1. M-TnpA samples were found to contain substantial levels of zinc while in samples of M-TnpA<sup>C-S</sup> zinc levels below the level of detection (<2 ng/g). The concen-

Table 1

Metal content of wild-type M-TnpA and the mutated M-TnpA<sup>C-Sa</sup>

Metal	M-TnpA	M-TnpA <sup>C-S</sup>
Ca	ND <sup>b</sup>	ND
Fe	6.0	6.2
Cu	1.5	13.5
Cr	ND	1.1
Zn	120.8	ND

<sup>a</sup> Metal concentrations of samples are given in ng/ml. The metal concentrations of buffer solutions were subtracted as background and the analyses were conducted on two different ICP machines. Each sample had 175 ng/ml of protein. The results are the average of two independent experiments with nearly identical results.

<sup>b</sup> ND, not detected; the concentration was below the 2 ng/g limit of detection.

tration of zinc in M-TnpA samples was about 1.85 nmol/ml (120.8 ng/ml ÷ 65.38 mw of zinc) and the concentration of M-TnpA was 2.22 nmoles/ml (175 ng/ml ÷ 78883 mw of M-TnpA). This indicates that one molecule of zinc is associated with 1 molecule of M-TnpA.

Many zinc fingers are known to contribute to DNA binding, thus activity of the wild-type and mutant proteins were compared in mobility shift assays. During the performance of these experiments, there was no apparent difference in the stability of the wild-type and mutated purified protein samples and both maintained their characteristic binding properties through one round of freeze/thawing. The wild-type and mutant proteins both bound the double stranded target DNA (Fig. 3). M-TnpA<sup>C-S</sup> appeared to bind the target DNA somewhat more tightly than did the wild-type protein. That is, at equal molar concentrations of protein and DNA, there was more unbound DNA in assays with M-TnpA. Densitometry measurements showed that on average 10% more target DNA was bound by M-TnpA<sup>C-S</sup>.

TnpA also has been shown to bind ssDNA. A ssDNA competition assays was used to determine if there was an affect of the zinc finger mutation on binding. In this assay as the concentration of ssDNA increases there is a concomitant decrease in binding to the double stranded target DNA. The results in Fig. 4 showed that M-TnpA<sup>C-S</sup> was less efficient at binding ssDNA; 3 nM ssDNA mixed with M-TnpA<sup>C-S</sup> resulted in approximately 40% free double stranded target DNA whereas there was 75% free double stranded target DNA when wild-type M-TnpA was used in assays. These data suggest that the zinc finger mutation negatively affects TnpA's ability to bind ssDNA.

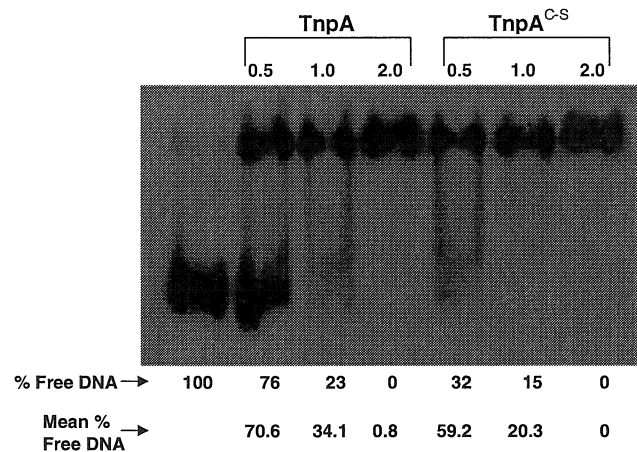


Fig. 3. DNA binding assay comparing wild-type M-TnpA and the mutated M-TnpA<sup>C-S</sup>. Each lane contained 7.0 nM of *B. fragilis* target DNA (200 bp fragment) and 0.5–2.0 μM protein as indicated. The first lane contained no protein and the remaining lanes contained either M-TnpA or M-TnpA<sup>C-S</sup> as indicated. Below the figure the % free DNA derived from densitometry of the gel is shown and the Mean % free DNA was the average obtained from at least three separate gel shift assays.

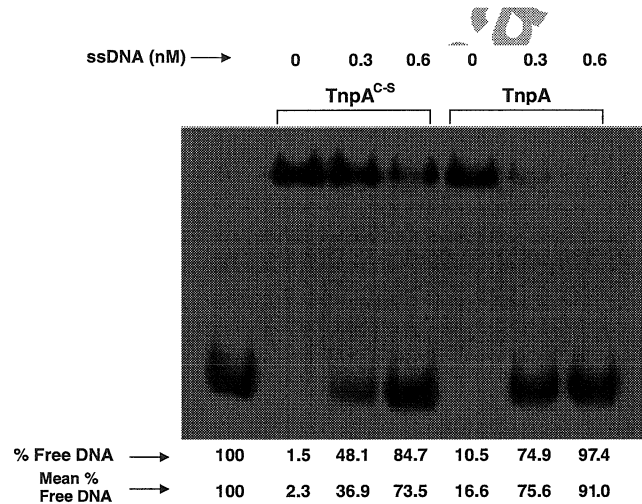


Fig. 4. Competition assay with single stranded DNA. Gel shift assays were performed as described in the text. All lanes contained 0.2 nM *B. fragilis* target DNA. The first lane had no protein or ssDNA additions. Lanes below the TnpA<sup>C-S</sup> label contained 10 nM of M-TnpA<sup>C-S</sup> while those grouped below TnpA contained 10 nM M-TnpA. ssDNA was added as indicated and all other labels are the same as in Fig. 3.

### 3.3. Effect of *tnpA* mutations on integration

In order to determine if the zinc finger was important for Tn4555 insertion, a transposition assay using the mini-Tn4555 construct, pFD660 was used. The *tnpA*<sup>C-S</sup> mutation was created in pFD660 and integration into *Bacteroides* hosts was tested. The *tnpA*<sup>C-S</sup> construct, p660Z was transferred to either *B. fragilis* or *B. thetaiotaomicron* via filter matings and the results are shown in Table 2. Interestingly, p660Z never inserted into either *Bacteroides* strain suggesting that the zinc finger is critical for insertion. These results were particularly noteworthy since pFD679, a *tnpA* knockout, retained

the ability to integrate albeit at a lower frequency than pFD660 (Table 2). The *tnpA* knockout mutation resulted in the loss of target site selection and the element inserted randomly in the chromosome (Tribble et al., 1999). Thus TnpA<sup>C-S</sup> somehow interfered with insertion and prevented the integrase from catalyzing the recombination event. In order to see if integration could be rescued, plasmid constructs containing wild-type *tnpA* were introduced into the *B. thetaiotaomicron* recipient strain. The matings with p660Z were repeated and the results are shown in Table 2. When expressed from its native promoter on the pFD851::*tnpA* construct, no transconjugant colonies were obtained. It was

Table 2  
Transposition assays with TnpA mutants and complemented strains

mini-Tn4555 Construct	Relevant genotype <sup>a</sup>	Complementing plasmid	Frequency of integration <sup>b</sup>
pFD660	Wt		$2.2 \times 10^{-3}$ ( $\pm 9.4 \times 10^{-4}$ )
pFD679	<i>tnpA</i>		$1 \times 10^{-4}$ ( $\pm 6.9 \times 10^{-6}$ )
pFD660Z	<i>tnpA</i> <sup>C-S</sup>		$<10^{-8}$
pFD660Z	<i>tnpA</i> <sup>C-S</sup>	pFD851:: <i>tnpA</i>	$<10^{-8}$
pFD660Z	<i>tnpA</i> <sup>C-S</sup>	pFD972:: <i>tnpA</i>	$1.8 \times 10^{-7}$ ( $\pm 0.2 \times 10^{-7}$ )

<sup>a</sup> Relevant genotype of the mini-Tn4555 construct.

<sup>b</sup> Frequency was the average of five independent matings with *B. thetaiotaomicron* as the recipient. Similar results were seen when using *B. fragilis* as the recipient except that the frequency of integration was 1/10 that seen with *B. thetaiotaomicron*.

possible to obtain transconjugants at a low frequency when the wild-type *tnpA* was highly overexpressed in the expression vector construct (pFD972::*tnpA*).

#### 4. Discussion

Zinc fingers are heterogeneous motifs of <100 amino acids stabilized by Zn<sup>2+</sup> in a tetrahedral coordination with either cysteine, or cysteine and histidine residues. The arrangement and spacing of the cysteine and histidine residues varies between different zinc finger families but is rather constant within a family. First discovered over twenty years ago in eukaryotic transcription factors, zinc fingers have since been shown in protein-nucleic acid interactions, protein-protein interactions and protein-lipid binding (Mathews and Sunde, 2002). More recently zinc fingers have been found in prokaryotic proteins and studies have shown a wide range of functions for this versatile domain. In fact potential zinc finger motifs have been found by sequence and/or chemical analysis in many prokaryotic proteins including topoisomerases, endonucleases, DNA repair enzymes, chaperones, and protein maturation factors (Rosano et al., 2002; Makarova et al., 2002; Bouhouche et al., 2000; Hsieh et al., 2000; Moonenaar et al., 2000).

In this study the primary amino acid sequence of TnpA together with the ICP-MS analysis established that the putative zinc finger coordinates zinc and is probably a 4-Cys zinc finger with a spacing pattern CX<sub>2</sub>CX<sub>13</sub>CX<sub>3</sub>C. Typically zinc fingers are involved in DNA binding yet DNA binding assays using chelators and various metals suggested that metal coordination is not a crucial part of TnpA DNA binding. These data were confirmed by the binding assays with the M-TnpA<sup>C-S</sup> mutated protein. Although the mutated protein no longer coordinated zinc, it still bound DNA as well or slightly

better than the wild-type. The addition of zinc in the binding assays caused some free DNA to appear and these data are puzzling. There are two other cysteine residues and several histidine residues closer to the C-terminus of the TnpA sequence, and it is possible that with excess zinc improper folding occurs involving these other amino acids and resulting in an inactive protein.

Although not required for DNA binding *in vitro*, the zinc finger was required for transposition *in vivo*. As shown in Table 2, disruption of the zinc finger prevented detectable chromosomal insertion of the mini-Tn4555. This was a dominant affect since it required over-expression of the wild-type TnpA in order to observe even low frequency transposition of the *tnpA*<sup>C-S</sup> mutated transposon. In fact merely having a trans copy of *tnpA* under the control of its native promoter was not sufficient and the use of an expression vector was required to produce enough TnpA to allow for low frequency insertion.

The question remains then as to the function of the TnpA zinc finger. The zinc fingers of some proteins are involved in protein-protein interactions. For example, SecA is a part of a secretory system that transports peptides across the cytoplasmic membrane. The C-terminus of SecA has a zinc finger that aids in recognition and binding of SecB, the chaperone that delivers peptides to SecA for translocation (Matousek and Alexandrescu, 2004). Since TnpA selects the site of insertion and Int catalyzes the integration, it is conceivable that the two proteins function together and the zinc finger mediates this interaction.

The mechanism by which TnpA directs Tn4555 to preferred targets in the chromosome is still unclear but results obtained from this study of the zinc finger motif provide some additional clues to its function. TnpA is a multi domain protein with two significant motifs in the N terminus; there is the zinc

finger domain located at amino acid residues 7–28 and then a putative helix-turn-helix (HTH) motif located about 30 amino acids downstream at residues 59–80. The HTH motif was found with 100% probability using the modified algorithm of Dodd and Egan, 1990 ([http://npsa-pbil.ibcp.fr/cgi-bin/npsa\\_automat.pl?page=/NPSA/npsa\\_server.html](http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_server.html)). This zinc finger / HTH architecture is found in a number of enzymes involved in DNA metabolism including the IS1 transposase (Ohta et al., 2004) and the mobile intron endonuclease *I-TevI* (Dean et al., 2002) both of which have spacing between the zinc finger and HTH similar to TnpA. Previously we had shown that TnpA is a DNA binding protein with specificity for DNA targets which appear to share a common feature in that they are bent DNA (Bacic and Smith, 2005). It was also shown that TnpA can bind ssDNA but there does not appear to be a specific base recognition sequence required for binding.

Taking from the “clamp” and “organizer” models of *I-TevI* (Dean et al., 2002) we propose the following model. Since the zinc finger does not appear to add significantly to the binding energy (as also found for *I-TevI*) it seems likely that the HTH motif provides the initial recognition and binding to the target sites. The primary role of the zinc finger may be to mediate intramolecular protein-protein interaction and as such it may tether the C-terminal domain of TnpA on the DNA so that it can recruit Integrase in the correct orientation for DNA cleavage. This is not to say that the zinc finger does not contact the DNA and it may mediate some binding. This binding could be enhanced by the HTH motif which would further bend the DNA when it binds to the target site which in turn will result in some local unwinding of the DNA allowing TnpA to bind ssDNA. The dominant negative phenotype of *tnpA<sup>C-S</sup>* in vivo is consistent with this model since one would predict that in the absence of the zinc finger, TnpA<sup>C-S</sup> would bind to DNA targets and recruit Integrase but that the integrase would not be in an orientation appropriate for cleavage and integration at the site. Hence all Integrase would be tied up in unproductive interactions.

Future studies will examine the role of the HTH motif in DNA binding. In addition, we will examine the ability of TnpA to interact with Integrase.

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