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Interactions of the excision proteins of CTnDOT in the *attR* intasome

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ABSTRACT

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

1. Introduction

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

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

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

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R2′ sites, suggesting cooperative interactions between these arm-type sites during integration (Wood et al., 2010).

In the CTnDOT excision reaction, the excision proteins, IntDOT, Xis2c, Xis2d, Exc, and the host factor bind to the *attL* site (240 bp) and the *attR* site (220 bp) to form the excisive intasomes. The complexes then undergo synapsis and IntDOT performs the strand exchanges that form the *attDOT* and *attB* sites. Xis2c and Xis2d are small basic proteins similar to lambda Xis and other recombination directionality factors (RDFs). Exc is a DNA topoisomerase III enzyme (Keeton and Gardner, 2012; Lewis and Hatfull, 2001; Sutanto et al., 2002). Recently, an *in vitro* intramolecular excision reaction was developed where the *attL* and *attR* sites are on the same molecule. The efficiency of this reaction ranged from less than 1% to virtual completion depending on whether the substrates contained the same overlap sequences and whether Exc was present in the reaction (Keeton and Gardner, 2012). Excision increased 10–30-fold when the overlap sequences were the same as compared to reactions performed under the same conditions with substrates containing different overlap sequences where only 2 out 7 bp were the same. When Exc was added to the reaction, excision was stimulated 3–5-fold (Keeton and Gardner, 2012).

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

2. Materials and methods

2.1. Plasmids, primers, and reagents

All plasmids are described in Table 1. Bacterial strains were grown in Luria–Bertani (LB) broth or on LB agar plates or MacConkey-lactose plates (Silhavy et al., 1984). Antibiotics were supplied by Sigma and used at the following concentrations: kanamycin (kan) 50 μg/ml, rifampicin (rif) 10 μg/ml, and chloramphenicol (cam) 20 μg/ml. Sequencing of the plasmids was done by the ACGT, Inc. Oligonucleotides were synthesized by Integrated DNA Technologies, Inc. Restriction enzymes, alkaline phosphatase, and T4 DNA ligase were supplied by New England Biolabs. PCR reactions were either performed using KOD Hot Start DNA Polymerase from Novagen or PCR Master Mix from Fermentas. Agilent Technologies' QuikChange XL site-directed mutagenesis kit was used for site-directed mutagenesis. The TriDye 100 bp and 1 kb ladder were from NEB.

2.2. Intramolecular excision reactions and protein purification

The intramolecular excision reaction was performed as previously described (Keeton and Gardner, 2012). The reaction mixture contained 100 fmol of plasmid substrate. The excision protein final concentrations used and purification details were as follows; IntDOT 13.5 μM (approximately 80% pure, (Dichiara et al., 2007), Xis2d 200 μM (approximately 85% pure, (Keeton and Gardner, 2012), the host factor 0.80 μM (approximately 85% pure), and Exc 0.20 μM (95% pure, (Sutanto et al., 2002). The concentration of Xis2c is unknown because it was supplied in a crude extract. The excision frequency was calculated by the total number of white colonies/total number of all white + red colonies on each plate.

2.3. Purification of the Xis2c protein

The Xis2c protein was overexpressed in *E. coli* Rosetta (DE3) pLysS *ihfA*. Cells were grown to an optical density of 0.6 at A600 nm at 30 °C and induced with 1 mM IPTG. After induction, cells were grown at 25 °C for 20 min. Then, rifampicin was added to a concentration of 200 ug/ml and the cells were shaken for 2 h and pelleted by centrifugation. A cell pellet from a 500 ml culture was resuspended in 5 ml of low salt sodium phosphate buffer [50 mM sodium phosphate, pH 7.0, 600 mM NaCl, 1 mM EDTA pH 7.0, 5% glycerol, 1 mM dithiothreitol (DTT), a Roche Complete EDTA-free protease inhibitor tablet, and lysozyme at 1 mg/ml]. Cells were sonicated and the extract was centrifuged at 10,000 rpm for 30 min. The supernatant was loaded onto a HiTrap SP HP column (GE Life Sciences) and washed with 5 column volumes of low salt sodium phosphate buffer. A stepwise salt gradient ranging from 600 mM to 2 M NaCl was used for elution and Xis2d eluted from the column at approximately 1.3 M NaCl. Active fractions were immediately dialyzed in Xis2c storage buffer (50 mM sodium phosphate, pH 7.0, 0.25 M NaCl, 1 mM EDTA pH 7.0, 40% glycerol, 1 mM DTT) 2 times for 2 h and overnight. Activity was verified by EMSA analysis. The supernatant was aliquoted, frozen in a dry ice in ethanol bath, and stored at −80 °C. The identity of Xis2c was confirmed by mass spectrometry performed at the University of Illinois Mass Spectrometry lab. The protein was approximately 90% pure. The protein also lost activity with 48–72 h of purification. The Xis2c protein preparation was frozen in 15 ul aliquots and thawed only once prior to use. Freezing the protein preparation more than once resulted in loss of activity.

2.4. Construction of the plasmids with mutations in the arm-type sites

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

2.5. Construction of the plasmids containing mutations in the Xis2d and Xis2c sites

The Xis2d binding sites on the *attR* site were first localized to a 70 bp region on the *attR* site by DNA footprinting.

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