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The excision proteins of CTnDOT positively regulate the transfer operon

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ABSTRACT

The *Bacteroides* conjugative transposon, CTnDOT, is an integrated conjugative element (ICE), found in many human colonic *Bacteroides* spp. strains. It has a complex regulatory system for both excision from the chromosome and transfer and mobilization into a new host. It was previously shown that a cloned DNA segment encoding the *xis2c*, *xis2d*, *orf3*, and *exc* genes was required for tetracycline dependent activation of the P_{tra} promoter. The Xis2c and Xis2d proteins are required for excision while the Exc protein stimulates excision. We report here that neither the Orf3 nor the Exc proteins are involved in activation of the P_{tra} promoter. Deletion analysis and electromobility shift assays showed that the Xis2c and Xis2d proteins bind to the P_{tra} promoter to activate the *tra* operon. Thus, the recombination directionality factors of CTnDOT excision also function as activator proteins of the P_{tra} promoter.

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1. Introduction

Bacteroides species compose approximately 40% of the normal microbiota of the human colon, but are capable of causing life threatening infections if they escape outside of the colon (Costello et al., 2009; Moore et al., 1978). *Bacteroides* spp. harbor many conjugative transposons (CTns), also called ICEs (integrative conjugative elements), which encode antibiotic resistance genes that can spread through horizontal gene transfer. One well-studied example of an ICE is CTnDOT, a 65-kb element containing a tetracycline resistance gene, *tetQ*, and an erythromycin resistance gene, *ermF* (2, 12, 13).

CTnDOT is stably maintained in the chromosome until the cells are exposed to tetracycline, which induces the production of proteins that promote excision and transfer. Upon exposure to tetracycline, translational attenuation increases production of proteins encoded by the three gene *tetQ* operon, *tetQ*, *rteA* and *rteB* (Wang et al., 2005, 2004). RteA and RteB proteins compose a two component regulatory system (Cheng et al., 2000, 2001; Stevens et al., 1990). The RteA protein is the sensor component and the RteB protein is the transcriptional regulator (Moon et al., 2005). The RteB protein activates expression of another regulatory gene *rteC* and RteC induces expression of the excision operon containing the *xis2c*, *xis2c*, *orf3*, and *exc* genes (Moon et al., 2005; Park and Salyers, 2011). Previous work has shown that the *orf3* gene is not needed for excision (Cheng et al., 2001). Xis2c and Xis2d are small basic proteins similar to lambda Xis and other recombination directionality factors (RDFs) (Lewis and Hatfull, 2001). Exc is a DNA topoisomerase III enzyme capable of relaxing DNA *in vitro* (26). These proteins, along with the host factor, promote excision by binding to the *attL* and *attR* sites of CTnDOT to form excisive intasomes with the tyrosine recombinase IntDOT. After the intasome forms a synaptic

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complex, IntDOT catalyzes two rounds of strand exchanges to form the *attDOT* and *attB* sites. In an *in vitro* intramolecular excision reaction, Exc is not required for excision but enhances excision 3- to 5-fold (Keeton and Gardner, 2012). The Exc protein is required *in vivo* for excision (Cheng et al., 2001).

Following excision of CTnDOT from the chromosome, a closed circular intermediate is formed. The transfer of this circular intermediate requires multiple transfer and mobilization proteins. The transfer genes are located in the *tra* operon which encodes a series of proteins TraA–TraQ. Previous work has shown that tetracycline dependent activation of this operon requires the excision operon (Jeters et al., 2009; Whittle et al., 2002). When a large portion of CTnDOT containing the mobilization region and the *tra* operon was cloned into the vector pLYL72, it was self-transmissible at a frequency of 10^{-5} to 10^{-6} transconjugants per recipient regardless of whether tetracycline was present (Li et al., 1995). However, transfer of pLYL72 was regulated by tetracycline if it was a co-resident with an intact CTn (Li et al., 1995). The genes required for this regulation were later shown to be contained within the excision operon (Whittle et al., 2002). However, it was unclear which of the excision proteins Xis2c, Xis2d, Orf3, or Exc was responsible for activation of the *tra* operon. In other excision systems, there is usually only one RDF. However, CTnDOT encodes two RDFs, Xis2c and Xis2d which are both required for excision.

We report here that the deletion of either the *orf3* or *exc* genes did not reduce activation of the P_{tra} promoter. Deletion of either the *xis2c* or *xis2d* genes decreased activation of the P_{tra} promoter by over 20-fold. Using EMSA analysis we show that the RDFs of CTnDOT, Xis2c and Xis2d bind the P_{tra} promoter and activate the expression of the *tra* operon. When the Xis2c and Xis2d proteins bind the P_{tra} promoter together, the resultant complex that forms on the P_{tra} promoter does not migrate into the gel.

2. Material and methods

2.1. Plasmids, bacterial strains, and growth conditions

Plasmids and the *Bacteroides* strains used in this study are listed in Table 1 or are described in the text. All the *Escherichia coli* strains were grown in Luria Broth (LB) or on LB agar at 37 °C and the *Bacteroides* strains were grown in either Trypticase Yeast Extract Glucose (TYG) medium or Supplemented Brain Heart Infusion (BHIS) medium at 37 °C under anaerobic condition (Holdeman and Moore, 1975). Antibiotics were supplied by Sigma and used at the following concentrations: ampicillin (amp) 100 μg/ml; chloramphenicol (cam) 15 μg/ml; erythromycin (erm) 10 μg/ml; gentamicin (gen) 200 μg/ml; rifampicin (rif) 10 μg/ml; tetracycline (tet) 1 μg/ml.

2.2. Protein purification and reagents

The purification of the Xis2d proteins was described elsewhere (Keeton and Gardner, 2012). Briefly, the Xis2d protein was overexpressed in *E. coli* BL21 (DE3) *ihfA* and purified by a Heparin–Agarose Column. The Xis2d protein is approximately 80% pure. The Exc protein was prepared as previously described and is >95% pure (Sutanto et al., 2002).

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 μg/ml and the cells were shaken for 2 h and pelleted by centrifugation. A 500 ml cell pellet 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 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 salt linear 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 is approximately 90% pure. An Agilent Technologies' QuikChange XL site-directed mutagenesis kit was used for site-directed mutagenesis. PCR reactions were performed with KOD Hot Start DNA Polymerase from Novagen. The 100 bp Tri-Dye Ladder is from NEB. Sequencing of the plasmids was done by either the UIUC Core Sequencing Facility or ACGT, Inc. Oligonucleotides were synthesized by Integrated DNA Technologies, Inc.

2.3. Construction of deletions of the excision operon

The plasmid pHopp1 was constructed from the plasmid pAFD1 and encodes the entire excision operon P_E-*xis2c*-*xis2d*-*orf3*-*exc* (Smith, 1985). A series of plasmids was created containing different deletions in the excision operon to test the effect on the activation of the P_{tra}::*uidA* fusion vector, pGW40.5, which is a translational fusion of the *traA* gene and the *E. coli* β-glucuronidase (Gus) reporter gene. The P_{tra} promoter was cloned upstream of the ATG start codon of the *uidA* gene (Gardner et al., 1996; Jeters et al., 2009; Valentine et al., 1988). The plasmid pGRW53 encodes the entire excision operon P_E-*xis2c*-*xis2d*-*exc* but contains a deletion of the *orf3* gene. The plasmid pGRW53Δ2c contains an in-frame deletion of amino acids 4–91 of the *xis2c* gene. To make an in-frame deletion of the *xis2d* gene, a construct was made which deleted the *xis2d* gene from amino acid 3 to the end of the *orf3* gene to generate pGRW53Δ2d. An in-frame deletion of the *exc* gene was created leaving only 78 bp of the C-terminal amino acids to form pGRW53Δexc.