Excision of a conjugative transposon *in vitro* by the *Int* and *Xis* proteins of Tn916

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ABSTRACT

The roles of purified *Int* and *Xis* proteins of the conjugative transposon Tn916 in excision of a deletion derivative of the closely related element Tn1545 were investigated. At a low salt concentration (37.5 mM NaCl), *Int* alone was able to promote limited excision to produce a covalently closed circular form of the transposon, showing that Tn916 *Int* can catalyze both DNA cleavage and strand exchange. This reaction was stimulated by *Xis*. At higher salt concentrations (150 mM NaCl), excision by *Int* alone was reduced to barely detectable levels and *Xis* was required for excision. The low salt, *Xis*-stimulated reaction was ~8-fold more efficient than the high salt, *Xis*-dependent reaction. These results reflect *in vivo* requirements for *Int* and *Xis* in excision.

INTRODUCTION

Tn916 (1–3), which encodes resistance to tetracycline, and its close relative, Tn1545 (4), which, in addition to tetracycline resistance, also encodes resistance to kanamycin and erythromycin, are members of a class of genetic elements called conjugative transposons (5–7). These elements are found in both Gram-positive and Gram-negative bacteria, and, as their name implies, are able to promote their own transfer from a donor bacterium to a recipient during transposition. They are important determinants of antibiotic resistance in several human pathogens, and have a very broad host range, being able to transfer between bacteria belonging to different species and genera.

During transposition, Tn916 first excises and forms a circle (8,9). This circular intermediate then transfers to a recipient bacterium using an origin of conjugal transfer that is distinct from the ends of the transposon (10). Following transfer, the circular transposon integrates into the DNA of the recipient. Unlike other bacterial transposons, there is no duplication of the target sequence upon integration. Rather, the integrated transposon is usually flanked on one side by DNA belonging to the recipient target and on the other side by a short stretch of DNA carried by

the transposon from the donor (9,11,12). These short stretches of DNA are called coupling sequences. Because the ends of Tn916 are rich in A-T base-pairs, and the transposon inserts into DNA that is rich in A-T base-pairs (12,13), it has been difficult to determine the length of the coupling sequences. In two cases where it was possible to distinguish 5 from 6 bp and 6 from 7 bp, respectively, the coupling sequences were found to be 6 bp (14).

It appears that excision is the rate limiting step in conjugative transposition, and that the coupling sequences can be major determinants of excision frequency (15,16). In a comparison of a series of donors that differed only in the coupling sequences flanking Tn916, Clewell found that the transposition frequency varied over a 1000-fold range. High frequency donors contained detectable levels of circular Tn916, while low frequency donors did not (16). Other studies have shown that in donor strains where the transposon is inserted at different sites, the amount of excised circular transposon is proportional to the frequency of conjugative transposition (15).

Two genes, close to one end of the transposon, called *int* and *xis*, play a role in conjugative transposition (17–19). The *int* gene is essential for transposition and encodes a protein that is related to the lambda integrase family of site-specific recombinases (20). The *int* genes of Tn916 and Tn1545 differ by a single base, which leads to a conservative amino-acid difference between the two proteins (3,18,21). The ends of the two transposons are identical (11,22), and their *int* genes can complement each other (23,24).

The *xis* gene encodes a small basic protein (3,18). Measurements of the loss of Tn916 from *Escherichia coli* (21) and conjugative transposition in *Enterococcus faecalis* (19) suggest that *xis* is required for excision and transposition of Tn916. However, excision of Tn1545 by both Tn1545 *Int* (18) and Tn916 *Int* (24) can occur in *E.coli* in the absence of *xis*, although *xis* stimulates excision by Tn1545 *Int* (18).

Int protein is a bivalent DNA binding protein (25). The C-terminal domain of *Int* binds to the ends of Tn916 and flanking bacterial DNA, and protects ~40 bp, centered on the coupling sequence, from nuclease cleavage (25). This domain contains Arg, His and Tyr residues that are conserved within the integrase family of recombinases (20) and that have been shown to be essential for biochemical activity in other recombinases (26–28).

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At the ends of the transposon, Int makes staggered cleavages flanking the coupling sequences, leaving a 5' hydroxyl group and forming a covalent protein–DNA complex at the 3' side of the site of cleavage similar to those formed by other integrase family recombinases (29). The N-terminal domain of Int binds to direct repeats called DR-2 close to the ends of the transposon, and protects ~40 bp of DNA from nuclease cleavage (29). These repeats are located 90 bp from one end of the transposon and 150 bp from the other (11).

It appears that the reactions catalyzed by Int protein during conjugative transposition are similar to the recombination reactions catalyzed by other integrase family recombinases. The coupling sequences are analogous to the overlap regions in lambda, Flp and XerC/D recombination. One difference between Tn916 and these other integrase family members is that in normal Tn916 excision and integration, the coupling sequences of the two partners are different and the resulting recombinant products contain heteroduplex DNA (9,30). In reactions catalyzed by other integrase family members, the presence of non-complementary sequences in the overlap regions of recombining molecules inhibits recombination (31–35).

Xis protein binds to both ends of the transposon close to the DR-2 repeats and, at higher concentrations, produces a nuclease cleavage pattern that suggests that the DNA is wrapped around the protein (36). The role of Xis in conjugative transposition is currently unclear.

Having established how Int cleaves the DNA at the ends of the transposon (29), to learn more about the mechanism of Int-mediated recombination and the function of Xis we have established an *in vitro* recombination system focusing on excision because it appears to be the rate limiting step in conjugative transposition of Tn916. We report here that using a plasmid carrying a derivative of Tn1545 as substrate, Tn916 Int alone will catalyze a low level of excision under low-salt conditions. This reaction is stimulated by Xis. At higher salt concentrations, Xis is required for excision.

MATERIALS AND METHODS

Plasmids

pUC18::Tn1545del4 (18) carries a deletion derivative of Tn1545. pUCTnJ4 was constructed by amplifying the joined transposon ends of Tn916 from circular transposon DNA present in DNA extracted from the *E. faecalis* strain FB31 containing pIP501orfA::Tn916 (12). The amplification was carried out using the primers 5'-TTTGAATTCCATATTTTACTATCC-3' and 5'-GATCGGATCCGCTTGAATAAAGAGAAGC-3'. The resulting product was digested with *EcoRI* and *BamHI* and ligated to similarly digested pUC18.

Expression and purification of Xis

The oligonucleotides 5'-TCAGAATTCTAAGGAGGTAAAT-ATTATGAAGCAGACTGACATTCC-3' and 5'-TTTGAAGC-TTCTAGATTGCGTCCAATGTA-3' were used as primers in a PCR reaction to amplify the *xis* gene of Tn916. The first oligonucleotide contains a consensus ribosome binding site nine bases upstream of the initiation codon of *xis*. The resulting amplified DNA fragment was digested with *EcoRI* and *HindIII* and ligated to similarly digested pUC18 so that the cloned *xis* gene was expressed from the *lac* promoter of pUC18. The

mixture was introduced into SG22094 *lon clpP* (37) by electroporation, and resulting colonies were screened for the presence of a plasmid containing an insert of the expected size. Bacteria that contained such a plasmid were then grown overnight in LB, and screened for the production of a protein of 8 kDa, the size expected for Xis. The nucleotide sequence of the insert in several clones was determined. One clone was recovered that contained an insert with a sequence identical to Tn916 *xis*. This clone, pUC18RBSXIS was used to produce Xis.

A 1 l culture of SG22094 containing pUC18RBSXIS in LB with ampicillin (50 µg/ml) and chloramphenicol (34 µg/ml) was inoculated from an overnight culture at an OD₆₀₀ of 0.2 and allowed to grow at 37°C for 3.5 h. The cells were harvested by centrifugation and the resulting cell pellet was frozen at -80°C. The pellet was thawed on ice, and suspended in 30 ml buffer A (50 mM Tris-HCl pH 7.5, containing 0.15 M NaCl and 1 mM EDTA). PMSF was added to a final concentration of 2 mM. The cells, on ice, were then lysed by sonication using three 30 s pulses at maximum power. A 15 ml aliquot of ice cold buffer B (50 mM Tris-HCl pH 7.5, containing 3 M NaCl and 1 mM EDTA) was added, and the lysate, on ice, was stirred gently for 30 min. The lysate was then centrifuged at 20 000 r.p.m. in an SS34 rotor for 25 min. The supernatant was dialyzed against buffer A overnight at 4°C. After dialysis, the suspension was centrifuged at 10 000 r.p.m. for 10 min in an SS-34 rotor. The supernatant was loaded onto a Heparin Hitrap (Pharmacia) column equilibrated with buffer A. The column was eluted with a gradient of 0.15–1 M NaCl. Xis-containing fractions were pooled and concentrated using a Centricon 3.5 (Amicon). The concentrated Xis was then further purified by gel filtration chromatography using Superdex 75 (Pharmacia) in the presence of buffer A. The Xis protein was electroblotted onto PVDF membrane and its N-terminal sequence was determined using an Applied Biosystems pulsed-liquid sequencing system (38).

In vitro excision assay

Reactions were carried out in 50 mM Tris-HCl pH 7.5, containing 1 mM EDTA, 15 mM MgCl₂ and either 37.5 mM or 150 mM NaCl. Reactions contained as substrate pUC18::Tn1545del4 (50 ng/µl, 13.75 nM). Int purified from baculovirus-infected insect cells (29) was used at concentrations between 1 and 10 ng/µl (22.4–224 nM) and Xis was used at concentrations between 1 and 25 ng/µl (125 nM–3.12 µM). Samples were incubated at 37°C for different times, then SDS was added to a final concentration of 0.2%, and the samples were heated at 65°C for 20 min. Proteinase K (500 ng) was added to the samples, which were then incubated at 37°C for 2 h. The DNA in the samples was recovered by ethanol precipitation.

To detect excised circular transposon DNA, PCR reactions were carried out using as primers 5'-CTCGAAAGCACATAGA-ATAAGGC-3' and 5'-GAGTGGTTTTGACCTTGATA-3', and 5 µl of a 1:10 dilution of the DNA samples as template. PCR was carried out in the presence of [³²P]dATP for 20 cycles (94°C, 1 min; 51°C, 1.5 min; 72°C, 1 min). Each experiment also included a series of reactions containing 10, 40, 160 and 640 pg of purified pUCTnJ4 plasmid DNA as template to construct a standard curve. Following PCR, the radiolabelled product was separated from template DNA by electrophoresis on a 5% polyacrylamide gel, and quantitated using a Molecular Dynamics phosphorimager.

Figure 1. Purification of Xis. (A) Heparin chromatography of proteins extracted from SG22094 containing pUC18RBSXIS. The figure shows a Coomassie blue-stained 16.5% SDS-polyacrylamide gel. Lane A, 1 M NaCl extract; lane B, 1 M NaCl extract dialyzed against 150 mM NaCl; lane C, column flow-through; lanes 1–15, fractions eluted from the column with a 150 mM–1 M gradient of NaCl. The arrow denotes the band containing Xis which was detected by western blotting using Xis-specific antiserum. (B) Gel filtration chromatography on Superdex 75 of pooled heparin column fractions 1–3 shown in (A). The figure shows a Coomassie blue-stained SDS-polyacrylamide gel. Lane A, 1 M NaCl extract; lane B, pooled heparin column fractions applied to the Superdex 75 column; lanes 1–12, fractions eluted from the Superdex 75 column. The arrow denotes the band containing Xis which was detected by western blotting using Xis-specific antiserum.

RESULTS

Purification of Xis

Tn916 Xis was purified from *E. coli* as described in Materials and Methods by treatment of a cell lysate with 1 M NaCl, followed by heparin chromatography and gel filtration. Fractions eluted from the heparin and gel filtration columns were analyzed by SDS-polyacrylamide gel electrophoresis. The elution of Xis from a Hi-trap heparin column and a Superdex 75 column is shown in Figure 1A and B, respectively. The purified protein had a mass of 8 kDa in SDS-PAGE (39), the size expected for Xis. The N-terminal amino acid sequence of the protein was found to be Met-Lys-Gln-Thr-Asp-Ile-Pro-Ile-X-Glu-Arg-Tyr in agreement with the nucleotide sequence of the *xis* gene.

Quantitative assay for excision

We established conditions that would allow us to quantitate the amount of transposon excision using PCR. Figure 2 shows the combined results of 20 PCR reactions using as template different

Figure 2. Detection of joined transposon ends by PCR. Compilation of results from 20 different experiments using pUC18TnJ4 as template. The values for 10 pg of template DNA from each experiment were used to normalize the data. The standard deviations of the data obtained for other amounts of template DNA are shown.

amounts of pUC18TnJ4, a plasmid containing the joined ends of Tn916 cloned from the circular form of the transposon. The reactions contained [32 P]dATP, and after 20 cycles of amplification, the radiolabelled product was separated from the template on a 5% polyacrylamide gel and detected using a phosphorimager. To correct for differences in the labeling of the product and the time of exposure of the gel from experiment to experiment, for each experiment the value for the amount of product obtained with 10 pg of template was set to 1.0, and the rest of the data were normalized to this value. As shown in Figure 2, the amount of product was proportional to the amount of template containing the joined ends up to 40 pg of template per reaction.

In all subsequent experiments, the substrate for transposon excision was a plasmid carrying Tn1545del4, a deletion derivative of Tn1545 whose ends are identical to those of Tn916. Excised circular transposon was detected by PCR using the same primers as in the experiment shown in Figure 2. In addition, in each experiment, a series of PCR reactions was carried out using serial dilutions of pUC18TnJ4 to construct a standard curve which was used to calculate the amount of product in each experimental sample. The normalization procedure described above allowed us to compare directly the amount of excision from experiment to experiment.

Optimization of salt conditions for excision

To determine the optimal concentration of NaCl required for excision of Tn1545del4, a series of reactions was carried out using 10 ng/ μ l Int and 10 mM MgCl₂ (Fig. 3A). We found that above 37.5 mM NaCl, the amount of excision decreased as the salt concentration increased so that at salt concentrations of 150 mM and higher, excision was barely detectable. We then performed two series of reactions with 10 ng/ μ l Int, and different concentrations of MgCl₂, in 37.5 and 150 mM NaCl, respectively. As shown in Figure 3B, at both NaCl concentrations, the maximum amount of excision was found at 15 mM MgCl₂, which was the concentration used in all subsequent experiments.

Transposon excision by Int alone

Excision reactions with different concentrations of Int were performed in both 37.5 and 150 mM NaCl. As shown in Figure

Figure 3. Effect of salt concentration on excision of Tn1545del4. (A) Excision of Tn1545del4 in different concentrations of NaCl. Reactions were carried out for 16 h at 37°C with 10 ng/μl Int. (B) Excision of Tn1545del4 in different concentrations of MgCl₂. Reactions were carried out for 16 h at 37°C with 10 ng/μl Int.

4, at 37.5 mM NaCl, the amount of excision of Tn1545del4 was approximately linear with time up to 4 h but there was little increase in product after this time. Comparison of the amount of excision after 4 h with different amounts of Int showed that excision of Tn1545del4 was proportional to the amount of Int added to the reaction over the range of Int concentrations used, which varied from 0.5 to 5 ng/μl. At 150 mM NaCl, the amount of excision was reduced to barely detectable levels, which were not increased using 10-fold higher concentrations of Int (data not shown).

Stimulation of excision by Xis

To determine if Xis stimulated excision of Tn1545del4 at low salt concentrations, reactions were carried out in 37.5 mM NaCl using different concentrations of Xis and either 1 or 2 ng/μl of Int. As shown in Figure 5A and B, a low concentration of Xis (1 ng/μl) had no effect on the amount of excision, but higher concentrations of Xis (5 and 25 ng/μl) stimulated excision at both concentrations of Int. The results in Figure 5B show that higher concentrations of Xis stimulated the reaction up to ~8-fold. In addition, the

Figure 4. Excision of Tn1545del4 by different amounts of Int in low salt buffer. Reactions were carried out for different times at 37°C in 37.5 mM NaCl.

higher concentrations of Xis accelerated the reaction, so that with 2 ng/μl of Int (Fig. 5B) the majority of the product was produced within 1 h. In the presence of 2 ng/μl of Int and 25 ng/μl of Xis, ~9% of the substrate DNA underwent recombination. The molar ratio of Xis to Int under these conditions was 70:1.

We found that Xis was required for excision of Tn1545del4 in the presence of 150 mM NaCl. As shown in Figure 6, in the presence of 20 ng/μl Int, excision was only observed at the highest amount of Xis (25 ng/μl). Comparing the results shown in Figures 5B and 6, the amount of excision observed in the presence of Xis in 150 mM NaCl was 8–10-fold less than the level observed in low salt conditions. As was the case in low salt conditions in the presence of Xis, the majority of the product was produced within 1 h.

DISCUSSION

We have found that in low salt conditions, Tn916 Int protein can catalyze a low level of excision of Tn1545del4 from a plasmid substrate. The assay we used detects only circular transposon DNA with one or both strands covalently closed. Therefore Int alone is capable of performing strand exchange in addition to DNA cleavage (29).

Depending upon the salt conditions, Xis either stimulates excision of Tn1545del4 (at 37.5 mM NaCl) or is required for the reaction (at 150 mM NaCl). Similar observations have been made for phage lambda (40). Excision of phage lambda can occur in the absence of Xis at low salt conditions (40 mM NaCl), and this reaction is stimulated by Xis. At higher salt concentrations (140 mM NaCl), Xis is required for lambda Int-mediated excision. Our results for Tn916 reflect observations made *in vivo* in different hosts, where *int* alone can promote excision, and *xis* either stimulates or is required for the reaction (18,19,21,24). The concentration of Xis necessary for stimulation of excision under low salt conditions is lower than the concentration required for excision to occur at higher salt concentrations. In both low and high salt conditions, stimulation of excision by Xis occurs at protein concentrations that are higher than those required for specific binding at each end of Tn916 (36). The high molar ratios of Xis to Int that were required for stimulation of recombination may mean that a significant fraction of the Xis preparation was

Figure 5. Stimulation of Tn1545del4 excision in low salt conditions by Xis. (A) Reactions were carried out for different times at 37°C in 37.5 mM NaCl with 1 ng/μl of Int. (B) Reactions were carried out at 37°C for different times in 37.5 mM NaCl with 2 ng/μl Int.

inactive. However, the stimulatory concentrations of Xis are comparable to those previously observed to produce an extended pattern of regularly spaced, enhanced nuclease cleavages around the specific binding sites (36). Unless the stimulatory activity of Xis can be destroyed without affecting its DNA binding properties, this suggests that wrapping of the DNA around Xis may be important for its activity.

One of the most unusual features of conjugative transposons like Tn916 is their very broad host range. Tn916 transfers between hosts belonging to different species and genera with the same frequency that it transfers between different strains of the same species (1,23). This implies that excision and integration can occur with similar efficiencies in widely differing host bacteria. The ability of Int alone to promote excision under low salt conditions, while Xis is required at higher salt concentrations, could reflect the use of different reaction pathways for excision. Because conjugative transposons operate in many intracellular environments, they may use different factors in different hosts.

During excision in the related phage lambda system, the ends of lambda apparently come together in different ways in the presence and absence of Xis (41), and the same may be true for Tn916. In the presence of lambda Xis, intramolecular recombination occurs between the prophage ends, *attL* and *attR*, when they are present on the same plasmid. Intermolecular recombination

Figure 6. Requirement of Xis for excision of Tn1545del4 in high salt conditions. Reactions were carried out for different times at 37°C in 150 mM NaCl with 20 ng/μl Int.

can also occur when the prophage ends are present on different plasmids. In the absence of Xis, only intramolecular recombination can occur. The products of intramolecular recombination in the presence of Xis are two catenated circles whereas in the absence of Xis the products are free circles. These differences suggest that the mechanism by which the *att* sites come together is determined by the structure of complexes formed between the sites and recombination proteins, which in turn depends upon the nature of the recombination proteins.

For Tn916, both the low salt, Xis-stimulated and high salt, Xis-dependent reactions are inefficient, and excision occurs from only a small fraction of the substrate molecules. This observation may mean that another accessory protein apart from Xis is required for excision, especially in high salt conditions, where significantly lower levels of excision occur. Since Int and Xis are the only transposon-encoded proteins that have been shown to play a role in excision, this implies that a host factor is involved in conjugative transposition. Because of the wide host range of Tn916 a single factor would have to be highly conserved in a large number of hosts or conjugative transposition would have to occur in the presence of different host factors. If excision of Tn916 does require a host factor, then the factor available in a particular host may determine which reaction pathway is used.

In summary, the development of a quantitative assay for excision *in vitro* of Tn916 is an important step in understanding the mechanism of conjugative transposition. We have shown that Tn916 Int is capable of catalyzing DNA strand exchange in addition to DNA cleavage. The Tn916-encoded Xis protein stimulates excision in low salt conditions and is required for excision in high salt conditions. These results are similar to observations made *in vivo*, and may reflect the ability of Tn916 to operate in a wide variety of intracellular environments. The assay for excision described here should allow us to understand the factors that permit conjugative transposition to occur in such a broad range of bacterial hosts.

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