Conjugative Transposition of Tn916 Requires the Excisive and Integrative Activities of the Transposon-Encoded Integrase

MICHELE J. STORRS,† CLAIRE POYART-SALMERON,‡ PATRICK TRIEU-CUOT,* AND PATRICE COURVALIN

Unité des Agents Antibactériens, Institut Pasteur, 28 rue du Dr. Roux, 75724 Paris Cedex 15, France

Received 26 December 1990/Accepted 29 April 1991

Transposon Tn916 is a 16.4-kb broad-host-range conjugative transposon originally detected in the chromosome of Enterococcus faecalis DS16. Transposition of Tn916 and related transposons involves excision of a free, nonreplicative, covalently closed circular intermediate that is substrate for integration. Excisive recombination requires two transposon-encoded proteins, Xis-Tn and Int-Tn, whereas the latter protein alone is sufficient for integration. Here we report that conjugative transposition of Tn916 requires the presence of a functional integrase in both donor and recipient strains. We have constructed a mutant, designated Tn916-intl, by replacing the gene directing synthesis of Int-Tn by an allele inactivated in vitro. In mating experiments, transfer of Tn916-intl from Bacillus subtilis to E. faecalis was detected only when the transposon-encoded integrase was supplied by trans-complementation in both the donor and the recipient. These results suggest that conjugative transposition of Tn916 requires circularization of the element in the donor followed by transfer and integration of the nonreplicative intermediate in the recipient.

Antibiotic resistance involving conjugative transposons has been described in various gram-positive genera Enterococcus, Streptococcus, and Clostridium (7, 11, 15–17, 20). Conjugal transfer of these elements, referred to as conjugative transposition, consists of the movement of the transposon from a site in the genome of the donor to a new location in the genome of the recipient. Conjugative transposons have a broad host range: donor and recipient need not be of the same species or even of the same genus. These elements are large DNA segments, ranging in size from 16 to >60 kb, and all contain the tetracycline resistance determinant tetM either alone or associated with other resistance genes. They are generally found on the host chromosome, although they may transpose intracellularly to resident plasmids. The role of conjugative transposons in dissemination of drug resistance in clinically significant bacteria is thought, in some species, to be as important as that of resistance plasmids (10).

The two elements that have been best characterized are the 16.4-kb transposon Tn916, first detected in the chromosome of Enterococcus faecalis DS16 (17), and the 25.3-kb transposon Tn545, originally found in the multiply antibiotic-resistant Streptococcus pneumoniae BM4200 (11). These transposons transfer by conjugation to a large variety of gram-positive bacteria, in which they integrate at various loci in the genome of the recipient. Both elements also transpose after cloning in Escherichia coli, but conjugal transfer does not seem to occur between gram-negative bacteria (10, 12). Tn916 and Tn545 appear to be related on the basis of restriction endonuclease analysis and functional properties (4, 10, 12, 29). The nucleotide sequences at their extremities are nearly identical for at least 250 bp (5, 8). These elements are flanked by terminal imperfect (20 of 26 bp) inverted repeated sequences but, unlike most transposons, they do not generate a duplication of the target DNA upon insertion. Transposition of these elements proceeds by way of excision of a free, nonreplicative, covalently closed circular intermediate that is substrate for integration (28). The integration-excision system of these transposons is structurally and functionally related to that of lambda phages (24, 25). Excision and integration occur by reciprocal site-specific recombination between nonhomologous DNA sequences of 6 or 7 bp designated coupling or overlap sequences (6, 24, 25). Excisive recombination requires two transposon-encoded proteins, an excisionase (Xis-Tn) and an integrase (Int-Tn), whereas Int-Tn only is required for integration (25).

In this report, we demonstrate that conjugative transposition of Tn916 requires the presence of a functional integrase in both donor and recipient. We have constructed a mutant of Tn916, designated Tn916-intl, by replacing the gene directing synthesis of Int-Tn by an allele inactivated in vitro. This mutant is defective for both excision and integration. In mating experiments, transfer of Tn916-intl from Bacillus subtilis to E. faecalis was detected only when the transposon-encoded integrase was provided in trans in both donor and recipient. These results suggest that conjugative transposition of Tn916 and related elements involves circularization of the transposon in the donor, transfer, and integration of the nonreplicative intermediate in the recipient.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are listed in Table 1. E. coli strains were grown in brain heart infusion broth or on Mueller-Hinton agar. B. subtilis was grown in Luria-Bertani broth and agar, and E. faecalis was grown in brain heart infusion broth and agar. Antibiotics were used at the following concentrations (in micrograms per milliliter): for E. coli—ampicillin, 100; erythromycin (Em), 180; and kanamycin (Km), 20; for B. subtilis—chloramphenicol (Cm), 5; erythromycin, 10; kanamycin, 20; streptomycin (Sm), 1,000; and tetracycline (Tc), 10; for E. faecalis—erythromycin, 10; kanamycin, 1,000; streptomycin, 1,000; and tetracycline, 10.

Transformation and mating procedures. Recombinant plas-
were introduced by transformation into *E. coli* (27) and *B. subtilis* (13), as described previously. Filter matings between *B. subtilis* and *E. faecalis* (11) and between *E. coli* and *E. faecalis* (33) were as described before. Transfer frequency was expressed as the number of transconjugants per donor CFU after mating.

**Plasmid and DNA manipulations.** Large-scale and small-scale preparations of plasmid DNA from *E. coli* were as described previously (27). For *B. subtilis* and *E. faecalis*, lysis was carried out at 37°C for 15 min in 100 μl of TE buffer (25 mM Tris HCl, 10 mM EDTA, pH 8.0) containing sucrose (25%) and lysozyme (20 mg/ml). Cleavage of DNA with restriction endonucleases, purification of DNA fragments from agarose gels, conversion of recessed DNA ends to blunt ends, and ligation with T4 ligase were performed by standard methods (27).

**Purification of total DNA from *B. subtilis***. Cells from 5-ml overnight cultures were harvested by centrifugation and resuspended in 400 μl of TE buffer containing sucrose (25%) and lysozyme (20 mg/ml). After 15 min of incubation at 37°C, an equal volume of TE buffer containing sodium dodecyl sulfate (1%) was added and the mixture was gently mixed until clearing. Proteinase K (100 μl; 5 mg/ml) and RNase (100 μl; 5 mg/ml) were then added, and the solution was incubated at 37°C for 4 h. Total DNA was extracted twice with an equal volume of phenol-chloroform and once with chloroform. The aqueous phase was mixed with an equal volume of isopropanol and cooled to −20°C for 1 h. The DNA pellet was recovered by centrifugation and suspended in 100 μl of TE buffer.

**Southern blot analysis.** Total DNA from *B. subtilis* strains was digested with HindIII and separated by agarose gel electrophoresis. Blotting (27) and hybridization (28) were as described previously.

**DNA amplification and sequence determination.** Thirty-five cycles of polymerase chain reaction were performed as described before (26), using as primers two 21-mer oligonucleotides specific, respectively, for the left 5'-d[CGTGAAG TATCCTCTACAGT]-3' and the right 5'-d[GGATAA ATCGTCGTATC][AAG]-3' and the right 5'-d[CGTGAAG TATCCTCTACAGT]-3' extremities of Tn916 and Tn1545 (25). The resulting fragments were separated by electrophoresis through a 1% agarose gel. DNA sequence determination was performed on a purified double-stranded DNA template as described previously (22), using the two synthetic oligonucleotides.

**Construction of plasmids.** Plasmid pAT143 was constructed as follows. The 1.8-kb EcoRI-HindIII fragment containing int-Tn1545 from pAT294 was purified (24) and inserted into pBGs18+ digested with EcoRI plus HindIII, giving rise to pAT142. This plasmid has a unique *Dral* site in *int-Tn1545* that was used to inactivate this gene by insertion of the 1.2-kb BamHI-KpnI *erm* cassette purified from pAT110 (34). The resulting plasmid was designated pAT143.

Plasmid pAT144 was obtained by cloning the purified 4.0-kb *BamHI* fragment of pAT142 containing *int-Tn1545* into the *HindIII* site of pDH88 (36). The 2.5-kb PstI-EcoRI fragment of pAT144 containing *int-Tn1545* downstream from the *spac* promoter of pDH88 was cloned into the *EcoRI* site of the mobilizable shuttle vector pAT187-1 (3, 33) following addition of an *EcoRI*-PstI linker. The structure of the resulting plasmid, pAT145, is shown in Fig. 1. This hybrid shuttle replicon contains the origin of transfer of the IncP plasmid RK2 and can be transferred by conjugation to various gram-positive bacteria, provided that the *E. coli* donor contains a coresident self-transferable plasmid belonging to the IncP incompatibility group (33).

In cloning experiments, noncohesive restriction sites were made blunt ended before ligation unless linkers were used.

**Enzymes and biochemicals.** Restriction enzymes *BamHI* and *Drai* were from New England BioLabs, Inc., Beverly, Mass. Other restriction and modifying enzymes, [α-32P]dATP, and [α-32P]dCTP, and were obtained from Amersham, SA, Les Ulis, France. The Sequenase kit was purchased from US Biochemical Corp., Cleveland, Ohio. The random priming kit was from Amersham, SA. Luria-Bertani broth was from Difco Laboratories, Detroit, Mich. Other bacteriological
supplies were from Diagnostics Pasteur, Marnes-La-Coquette, France. Antibiotics were provided by the following manufacturers: ampicillin and kanamycin, Bristol, Paris, France; chloramphenicol and erythromycin, Roussel-Uclaf, Romainville, France; streptomycin, Pfizer, Paris-La Défense, France; and tetracycline, Rhône-Poulenc, Vitry, France.

RESULTS

Construction of Tn916-intl. Plasmid pAT143 (Table 1) does not replicate in gram-positive bacteria and contains int-Tn1545 insertionally inactivated by an erythromycin resistance gene known to be expressed in both gram-negative and gram-positive bacteria. This allele was designated intl-Tn. The genes coding for Xis-Tn and Int-Tn in Tn1545 and Tn916 are almost identical (9). We took advantage of this close structural relationship to replace, in Tn916, int-Tn916 by intl-Tn following homologous recombination. Competent cells of B. subtilis CKS102, which contains a single copy of Tn916 (28), were transformed with pAT143 DNA, and transformants resistant to erythromycin were selected. Total DNA from 10 randomly selected transformants was digested with HindIII and analyzed by Southern hybridization, using 32P-labeled pAM120 as a probe to detect Tn916 sequences. Tn916 contains a single HindIII site (18). In CKS102, the HindIII-generated restriction fragments exhibiting homology with Tn916 have sizes of 14 and 5 kb (Fig. 2), the smaller fragment containing int-Tn916 (28). The hybridization patterns of the 10 transformants were indistinguishable and showed a size increase of about 1.4 kb of the smaller HindIII fragment (Fig. 2 shows part of this analysis). This size increase corresponds roughly to the size (1.2 kb) of the erm cassette, which suggests that a double crossing-over event occurred leading to the replacement of int-Tn916 by intl-Tn.

One of the transformants, BM4184 (Fig. 3), was selected for further studies, and the absence of the pBGSl8+ sequence in this strain was confirmed by dot blot hybridization (data not shown). Tn916-intl is expected to be defective for both excision and integration.

Complementation of Tn916-intl for excision. It was demonstrated recently that Tn545-encoded Int-Tn and Xis-Tn are trans-acting proteins that are able to catalyze in vivo excision (Int-Tn and Xis-Tn) and integration (Int-Tn) of a deletion derivative of Tn1545 defective for both functions (24, 25). Plasmid pAT145 (Fig. 1) directs synthesis of a single transposon-encoded protein, Int-Tn, and was designed to trans-complement Tn916-intl in gram-positive bacteria. This mobilizable shuttle vector was introduced by transformation into B. subtilis CKS102 and BM4184 and by conjugation into E. faecalis BM4110, using the mobilizing E. coli strain HB101/pRK24 (31) as a donor. In each experiment, one transconjugant resistant to kanamycin and containing pAT145 was selected for further studies.

The polymerase chain reaction allows detection of circular intermediates of Tn1545 produced after excision in E. coli (25). Thus, the 21-mer oligonucleotides specific for the left and the right termini of Tn916 and Tn1545 were used to amplify the 240-bp DNA fragment spanning the joined extremities of the circular intermediates of Tn916 and of its derivative Tn916-intl. Total DNA extracted from CKS102, BM4184, and BM4184 harboring pAT145 was used as substrate for amplification, and the products of the reaction were analyzed by agarose gel electrophoresis. As expected, the 240-bp fragment corresponding to the sealed extremities of Tn916 was detected with the DNA templates of CKS102 and BM4184/pAT145 but not with that of BM4184 (Fig. 4). Specificity of the 240-bp amplification products obtained from Tn916 in CKS102 and from Tn916-intl in BM4184/pAT145 was confirmed by sequence determination (data not shown). DNAs from B. subtilis WS168 and from pAT301, a pACYC184 derivative containing the sealed extremities of Tn1545 (25), were used as negative and positive controls, respectively (Fig. 4). This analysis confirms that Tn916-intl is defective for excision and indicates that Int-Tn from pAT145 restores the capacity of Tn916-intl to excise in B. subtilis BM4184.

Conjugal transfer of Tn916-intl from B. subtilis to E. faecalis. To determine whether Int-Tn is required for conjugal transfer of Tn916, B. subtilis CKS102 and BM4184 were used as donors in mating experiments. Because natural
competence has not been demonstrated in *E. faecalis*, this species, rather than *B. subtilis*, was chosen as a recipient to avoid the possibility of transfer by transformation. In filter matings between *B. subtilis* CKS102 and *E. faecalis* BM4110, Tn916 transferred at a frequency of 10^{-5}, whereas, under similar conditions, transfer of Tn916-intl from *B. subtilis* BM4184 to *E. faecalis* BM4110 was never observed (<10^{-8}) (Table 2). Transfer of the mutant element was not detected (<10^{-8}) when int-Tn1545 was provided in trans in either the donor or the recipient. By contrast, transfer of Tn916-intl from *B. subtilis* BM4184 to *E. faecalis* BM4110 was detected at a frequency (10^{-5}) similar to that of the wild-type transposon when int-Tn1545 was provided in trans in both donor and recipient. Subsequent retransfer of Tn916-intl among *E. faecalis* strains also depended on the presence of pAT145 in both donor and recipient cells. This observation, combined with the fact that transfer of pAT145 from *B. subtilis* CKS102 to *E. faecalis* BM4110 was never detected (<10^{-8}), indicates that conjugal transposition of Tn916-intl is not due to homologous recombination between the mutant element and pAT145. The presence of pAT145 in the donor did not alter the transfer frequency of Tn916 from *B. subtilis* CKS102 to *E. faecalis* BM4110 (Table 2).

**DISCUSSION**

The current model for the nature of the movements (transposition and conjugation) of conjugative transposons such as Tn916 and Tn1545 of gram-positive cocci is that, following excision, a circular intermediate of the element can undergo intracellular transposition to a new site; can be conjugatively transferred to a new host, where it transposes; or can be lost from the progeny during cell division (18, 19). This model implies that, regardless of whether donor and recipient replicons are within the same cell, excision from the donor replicon represents the initial and rate-limiting step for transposition. In support of this model, a supercoiled intermediate of Tn916 produced in vivo in *E. coli* was identified by restriction endonuclease digestion and by Southern hybridization (28). The purified circular form of Tn916 was used to transform *B. subtilis* protoplasts and was found to retain all of its original properties: it inserted at random into *B. subtilis* chromosome and was able to promote conjugal transposition to *Streptococcus pyogenes* (28).

We demonstrated recently that excision of these elements requires two transposon-encoded proteins, Xis-Tn and Int-Tn, whereas Int-Tn is sufficient for integration (25). To test further the model described above, we have studied the conjugative ability of Tn916-intl, a Tn916 mutant with an
inactivated \textit{int-Tn} allele (Fig. 3). The mutant element was found to be defective for excision, and we have shown that the excisive activity can be restored by \textit{trans}-complementation with a plasmid encoding \textit{Int-Tn} (Fig. 1). These results demonstrate the absolute requirement of \textit{Int-Tn} for excision of this class of transposons. In mating experiments, transfer of Tn916\textit{-intl} from \textit{B. subtilis} to \textit{E. faecalis} was detected only when \textit{int}-Tn1545 was provided in \textit{trans} in both donor and recipient strains. In these conditions, Tn916\textit{-intl} transferred at a frequency similar to that of wild-type Tn916. The most likely explanation is that conjugative transposition of Tn916 and related transposons involves circularization (Xis-Tn and Int-Tn activities) of the element in the donor and then transfer, followed by integration (Int-Tn activity) of the nonreplicative intermediate in the recipient. This model implies that, as anticipated, Tn916\textit{-intl} is also defective for integration. Finally, it should be noted that our results do not exclude the possibility that Tn916 can promote conjugation similarly to \textit{F} in an Hfr donor strain (35). In this case, only the portion of Tn916 functionally homologous to the 5' leading strand is transferred into the recipient bacteria, and its rescue depends on the recombination functions of the host.

There is circumstantial evidence that conjugation of Tn925, an element closely related to Tn916, proceeds through cell fusion in \textit{B. subtilis} and in \textit{E. faecalis} (32). This process leads to the formation of a transient diploid cell in which there is no distinction between donor and recipient. However, the absolute requirement for a functional integrase in both strains of the mating pair and the fact that transfer of plasmid pAT145 was not detected when \textit{B. subtilis} CKS102 was crossed with \textit{E. faecalis} BM4110 (Table 2) rule out the possibility that transposon-mediated cell fusion occurred under our experimental conditions. It has been reported that Tn916 can mobilize certain (pC194 and pUB110), but not all (pE194), nonconjugative plasmids in matings between \textit{B. subtilis} and \textit{B. thuringiensis} (23). Mobilization of pAD2 by Tn916 was never observed in \textit{E. faecalis} (10, 17). That mobilization depends on the nature of the coresident plasmid indicates that Tn916-promoted cell fusion did not occur at a detectable frequency in these mating systems.

**ACKNOWLEDGMENTS**

We thank J. R. Scott for her participation in the beginning of this work and for helpful discussions. M. J. Storrs was a recipient of a European Commission grant under the BAP program and of a Wellcome Trust travel grant.

### TABLE 2. Transfer of Tn916 and Tn916\textit{-intl} from \textit{B. subtilis} to \textit{E. faecalis}

<table>
<thead>
<tr>
<th>Donor</th>
<th>Recipient</th>
<th>Antibiotic selection (μg/ml)*</th>
<th>Transfer frequency b</th>
</tr>
</thead>
<tbody>
<tr>
<td>CKS102/pAT145c</td>
<td>BM4110</td>
<td>Sm (1,000), Tc (8)</td>
<td>3 \times 10^{-5}</td>
</tr>
<tr>
<td>CKS102/pAT145c</td>
<td>BM4110</td>
<td>Sm (1,000), Tc (8)</td>
<td>1 \times 10^{-5}</td>
</tr>
<tr>
<td>BM4184/pAT145c</td>
<td>BM4110</td>
<td>Sm (1,000), Km (1,000)</td>
<td>2 \times 10^{-5}</td>
</tr>
<tr>
<td>BM4184/pAT145c</td>
<td>BM4110</td>
<td>Sm (1,000), Em (8)</td>
<td>&lt;10^{-8}</td>
</tr>
<tr>
<td>BM4184/pAT145c</td>
<td>BM4110/pAT145</td>
<td>Sm (1,000), Em (8)</td>
<td>&lt;10^{-8}</td>
</tr>
<tr>
<td>BM4184/pAT145c</td>
<td>BM4110/pAT187</td>
<td>Sm (1,000), Em (8)</td>
<td>2 \times 10^{-5}</td>
</tr>
</tbody>
</table>

* Sm, streptomycin; Tc, tetracycline; Km, kanamycin; Em, erythromycin.

**REFERENCES**