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The clostridial mobilisable transposons

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Abstract. Mobilisable transposons are transposable genetic elements that also encode mobilisation functions but are not in themselves conjugative. They rely on coresident conjugative elements to facilitate their transfer to recipient cells. Clostridial mobilisable transposons include Tn*4451* and *Tn4452* from *Clostridium perfringens,* and Tn*4453a* and Tn*4453b* from *Clostridium difficile*, all of which are closely related, and Tn*5398* from *C. difficile*. The Tn*4451* group of elements encodes resistance to chloramphenicol and is unusual in that transposition is dependent upon a large resolvase protein rather than a more conventional transposase or integrase. This group of elements also encodes the mobilisation protein TnpZ that, by acting at the RS_A or *oriT* site located on the transposon, and in the presence of a coresident conjugative element, promotes the movement of the nonreplicating circular intermediate and of plasmids on which the transposon resides. The erythromycin resistance element Tn*5398* is unique in that it encodes no readily identifiable transposition or mobilisation proteins. However, the element is still capable of intraspecific transfer between *C. difficile* isolates, by an unknown mechanism. The detailed analysis of these mobilisable clostridial elements provides evidence that the evolution and dissemination of antibiotic resistance genes is a complex process that may involve the interaction of genetic elements with very different properties.

Key words. Clostridia; transposon; mobilisation; antibiotic resistance; resolvase.

The pathogenic clostridia

The clostridia are a diverse range of bacteria that are grouped together as Gram-positive anaerobic rods capable of producing heat-resistant endospores [1]. The major pathogens within the genus include the neurotoxigenic clostridia, *Clostridium botulinum* and *Clostridium tetani*, the enterotoxigenic *Clostridum difficile* and the enterotoxic and histotoxic *Clostridium perfringens* [2]. The defining feature of these species is their ability to produce potent protein toxins [1, 2]. Antibiotic resistance studies have focused on *C. perfringens* and *C. difficile* and genes encoding resistance to the tetracyclines, the macrolide-lincosamide-streptogramin B (MLS) group and chloramphenicol [3].

C. perfringens is the causative agent of clostridial myonecrosis or gas gangrene, which occurs after the infection of a traumatic or surgical wound with *C. perfringens* spores. The disease is primarily mediated by production of the extracellular α-toxin [4]. *C. perfringens* is also responsible for various gastrointestinal diseases ranging from food poisoning, which is caused by isolates that produce an enterotoxin [5], to the rare but very severe disease, enteritis necroticans [6]. *C. difficile* causes a range of gastrointestinal diseases ranging from antibiotic-associated diarrhoea (AAD) to pseudomembranous colitis (PMC), which can be lethal if untreated [5]. Both syndromes are nosocomial infections that commonly occur following antibiotic treatment. The causative *C. difficile* isolates are not necessarily resistant to the antibiotic that induces the disease but are able to sporulate and subsequently colonise the gut after treatment ceases, when the normal flora have been reduced. The organism is then able to overgrow and release large amounts of toxin A and toxin B into the lumen, causing characteristic lesions to develop [5].

There are many mobile genetic elements found in *C. perfringens* and *C. difficile*, including plasmids encoding genes for antibiotic resistance, bacteriocin production, virulence factors and some toxins [7]. *C. perfringens* is the only *Clostridium* species known to contain conjugative tetracycline resistance plasmids, the most well studied of

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which are pCW3 and pIP401 [7, 8]. Many *C. perfringens* plasmids are either identical to pCW3, or are very closely related [3, 8]. For example, pIP401 is indistinguishable from pCW3 except that it carries the chloramphenicol resistance transposon Tn*4451* [9]. A very similar transposon, Tn*4453*, is found in *C. difficile* [10] but it is located on the chromosome. *C. difficile* also carries the conjugative tetracycline resistance transposon Tn*5397*.

Antibiotic resistance in the clostridia

Resistance to tetracycline and erythromycin (a member of the MLS group of antibiotics) is the most common acquired antibiotic resistance phenotype seen in both *C. perfringens* and *C. difficile*, although resistance to chloramphenicol and lincomycin is also observed [7]. In *C. perfringens* erythromycin resistance is mediated most commonly by the *ermQ* gene [7] but may also be encoded by the *erm*(B) gene, which in one strain is located on the large, nonconjugative plasmid pIP402 [3, 7]. In *C. difficile,* erythromycin resistance is commonly mediated by an *erm*(B) gene, which can be located on the mobilisable genetic element Tn*5398* [11].

Tetracycline resistance is more widely distributed in *C. perfringens* and can be either chromosomal or located on pCW3-like plasmids [3, 7, 12]. When conferred by pCW3-like plasmids, resistance is encoded by an unusual operon that consists of two tetracycline resistance genes, the *tetA*(P) gene, which encodes an active tetracycline efflux protein, and *tetB*(P), which confers resistance by a ribosomal protection mechanism [12]. Tetracycline resistance may also be encoded by a *tet*(M) gene that is located on the chromosome within a defective Tn*916*-like element [13]. The *tetA*(P) gene is almost always found associated either with *tetB*(P), as part of an operon, or with a coresident but unlinked *tet*(M) gene. The *tetB*(P) and *tet*(M) genes have never been found in the same strain [7].

In *C. difficile*, tetracycline resistance is mediated by a *tet*(M) gene, which has been identified within the conjugative transposon Tn*5397* [14, 15]. This element shows high levels of similarity to Tn*916* and the defective CW459*tet*(M) element of *C. perfringens* [13]. Tn*5397* encodes a large resolvase, TndX, which shows significant amino acid sequence identity to TnpX from Tn*4451* and Tn*4453a* and has been shown to be the protein responsible for excision of the element from the chromosome [15].

Chloramphenicol resistance in *C. perfringens* is uncommon but is generally encoded by the *catP* gene and is located on a mobilisable transposon of the Tn*4451* family. These transposons are found on large conjugative, pCW3-like, tetracycline resistance plasmids, such as pIP401 [7, 9]. One other chloramphenicol-resistant *C. perfringens* strain has been identified; it carries a chromosomal *catQ* gene [16]. Chloramphenicol resistance in *C. difficile* is also chromosomally encoded but by a *catP* gene that is located on two mobile elements very similar to Tn*4451*, known as Tn*4453a* and Tn*4453b* [10]. These elements belong to a family of clostridial mobilisable transposons known as the Tn*4451/53* family.

Mobilisable elements from the clostridia

Conjugative transfer of plasmids and transposons occurs as a multistep process requiring specific DNA sequences and multiple gene products. These include a cis-acting origin of transfer, *oriT*, and trans-acting mobilisation or Mob proteins, which are involved in initiation of DNA transfer and replication in the recipient. In addition, other trans-acting proteins that form the conjugation pore or mating apparatus are also required. Conjugative plasmids and transposons are self-transmissible because they encode all of the proteins required for initiation, termination and for the assembly of the conjugation apparatus. Mobilisable transposons and elements have an *oriT* site but, unlike conjugative elements, do not encode all of the proteins necessary for bacterial conjugation [17, 18]. They generally carry antibiotic resistance genes and encode mobilisation proteins, which facilitate the transfer of the transposon into the recipient cell. However, transfer of a mobilisable transposon is dependent upon the conjugation machinery of a coresident conjugative plasmid or conjugative transposon [17–22].

Mobilisable elements range in size from Tn*5520* at 4.7 kb [17] to Tn*4555* at 12.1 kb [23]. Many of these elements are found within the genus *Bacteroides* [18] and include the nonreplicating *Bacteroides* units (NBUs) NBU1, NBU2 and NBU3, Tn*4399*, Tn*4555* and Tn*5520* [17, 18]. NBU1, NBU2 and Tn*4555* have been shown to form a nonreplicating circular intermediate, a single strand of which can be transferred by conjugation after it is nicked at its *oriT* site by mobilisation protein(s) encoded by the element [20–22]. Tn*4399* is capable of mobilising nonconjugative plasmids in cis and encodes the MocA and MocB proteins, which are required for recognition and nicking of the transposon at *oriT* [17]. By contrast, Tn*4555*, NBU1 and NBU2 each encode a single mobilisation protein that is responsible for both *oriT* recognition and nicking activity, unlike many other mobilisation processes which require two proteins for these tasks [24]. Tn*4555* also encodes a member of the λ integrase family of site-specific recombinases, which is responsible for excision and integration [25]. NBU1 and NBU2 have significant nucleotide sequence similarity to Tn*4555* in the region of the mobilisation gene and the *oriT* site, although the transposition mechanisms of the three elements are different [20, 22, 23, 25].

The Tn*4451*/Tn*4453* family of mobilisable transposons

The first members of the Tn*4451/53* family to be identified and characterised were Tn*4451* and Tn*4452* from *C. perfringens*. Heteroduplex analysis showed that these elements were almost identical, with one 400-bp region of nonhomology identified at the right end of both transposons [9]. Well before they were characterised as transposable genetic elements, it was observed that these DNA segments, which were normally very stable in *C. perfringens*, were lost after conjugative transfer from *C. perfringens* strains containing the conjugative tetracycline resistance plasmids pIP401 (carrying Tn*4451*) and pJIR27 (carrying Tn*4452*) [8, 9, 26]. Subsequent studies carried out in *Escherichia coli* showed that Tn*4451* excises precisely from multicopy plasmids [27]; later it was shown that Tn*4451* also excises precisely from a multicopy plasmid in *C. perfringens* [28]. Characterization of the ends of Tn*4451* showed some sequence conservation and the presence of 12-bp imperfect inverted repeats [27].

Genetic organisation of the Tn*4451/53* family

The sequence of Tn*4451* has been determined and the transposon shown to be 6338 bp in size [28]. It is bounded by directly repeated GA dinucleotides and encodes six genes (fig. 1), each of which has a consensus ribosome binding site [28]. The first gene is the site-specific recombinase *tnpX*, which is responsible for both excision and insertion. The next gene, *tnpV,* overlaps the *tnpX* gene and has limited homology to the *xis* gene from phage λ and was postulated to be involved in excision [28]. Subsequent deletion analysis showed that TnpV was not required for excision and the function of this protein remains unknown [28, 29]. The *catP* gene encodes a chloramphenicol acetyltransferase, and confers chloramphenicol resistance [28]. The next gene, *tnpY*, encodes a protein that contains a putative Walker A box, which is often found in ATP-binding proteins [28]. Consequently, TnpY may be involved in an energy-dependent function, although current studies suggest that it is not involved in either excision or insertion [D. Lyras and J. I. Rood, unpublished results]. The next gene encodes the Mob/Pre mobilisation protein, TnpZ [19]. The final gene, *tnpW*, is very small and encodes a 62-aa protein that has no significant similarity to other proteins; its role is also unknown [28]. Recent PSI-BLAST (Position Specific Iterated – Basic Alignment Research Tool) [30] analysis has revealed that TnpV has similarity to viral transcriptional regulators and that TnpY has similarity to DNA repair proteins. The significance of these findings is unknown, but the results suggest that TnpV and TnpY may have the ability to bind DNA.

Two other members of this transposon family, Tn*4453a* and Tn*4453b*, were identified from the chromosome of a single chloramphenicol-resistant *C. difficile* isolate [10]. Tn*4453a* has been completely sequenced and has 89% nucleotide sequence identity to Tn*4451* [31]. All of the Tn*4451* genes are present, with the highest degree of divergence occurring between the TnpW proteins and the TnpX proteins (table 1) [31]. Interestingly, both the TnpV and CatP proteins are completely conserved (table 1) [31]. In addition, a truncated form of these transposons has been identified in *Neisseria meningitidis*, with sequence identity extending from the very

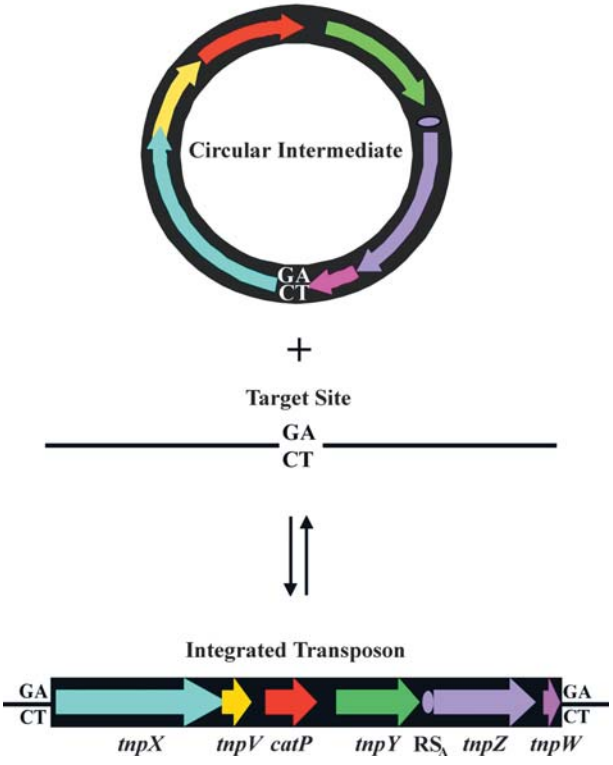


Figure 1. Mechanism of transposition of Tn*4451/53.* The Tn*4451*/Tn*4453* family of elements are excised by TnpX to form a nonreplicating circular intermediate. One copy of the duplicated GA dinucleotide is present in the circular intermediate; the other remains at the site of excision. Transposition represents the reverse of this process, with TnpX-mediated site-specific recombination leading to the integration of the element into the chromosome, with the GA residues residing at each end of the element.

Table 1. Comparison of proteins encoded by Tn*4451* and Tn*4453a*.

Protein	Size (aa)	Number of aa differences Tn*4451* vs. Tn*4453a*	Percentage aa differences Tn*4451* vs. Tn*4453a*
TnpX	707	87	12.3
TnpV	123	0	0
CatP	207	0	0
TnpY	340	29	8.5
TnpZ	421	32	7.6
TnpW	62	15	24.2

end of *tnpV* to the intergenic region between *catP* and *tnpY* [32].

The site-specific recombinase TnpX

The *tnpX* gene is 2.1 kb in length and encodes a protein of 707 aa, with a corresponding molecular size of 82 kDa. The product of the *tnpX* gene is responsible for the excision of Tn*4451* from multicopy plasmids in both *E. coli* and *C. perfringens* [28]. The sequence of the N-terminal 200 aa has similarity to members of the resolvase/invertase family of site-specific recombinases, and a region in the C-terminal domain was thought to have similarity to the λ integrase family [28]. Mutation and functional analysis of conserved residues in the later domain showed that it was not involved in transposon excision. By contrast, site-directed mutagenesis of conserved resolvase family residues resulted in a nonfunctional protein that, unlike the wild-type enzyme, was unable to excise a stable Tn*4451* derivative that carried an internal *tnpX* deletion [29]. Based on these data it was concluded that excision of Tn*4451* by TnpX involves a resolvase-mediated site-specific recombination event [29].

Resolvase/invertase proteins were until recently thought to range in size from 180 to 200 aa, with a corresponding molecular size of approximately 20 kDa. Members of the resolvase/invertase family have now been divided into five subgroups according to their domain structure. Group I and II enzymes include the small resolvases and invertases, groups III and IV include slightly larger enzymes (containing a C-terminal extension) and the newly characterised group V enzymes encompass the largest reolvase proteins (up to 772 aa). The latter group can carry out integration reactions, which were previously thought to be incapable of being catalysed by resolvase enzymes [M. Smith, personal communication]. The better-studied members of group V (table 2) include TnpX, TndX and the Int proteins from phages ϕTP901-1 and ϕC31, each of which are capable of catalysing both excision (resolution) and integration (intermolecular recombination) reactions [29, 33–35]. The catalytic mechanism for each resolvase subgroup is the same, but the formation of the synaptic complexes differs in both structure and composition, leading to alternative reaction products [36]. The small resolvases and the invertases can only catalyse cointegrate resolution and DNA inversion reactions, respectively [37–39].

The common mechanism employed by resolvase enzymes involves a DNA cleavage process that results from the formation of a covalent phosphoserine linkage between the resolvase enzyme and the 5′ end of the cleaved DNA strand [37, 39]. Simultaneous cleavage of the complementary DNA strand leads to 3′ overhangs of 2 bp, with protein-DNA links at their 5′ ends [37–40]. The two DNA strands are rotated through 180°, and the protein-DNA link is severed by nucleophilic attack via the 3′ hydroxyl from the opposite strand, rejoining the DNA backbone in the recombinant conformation [37, 38, 40].

The mechanism of transposition of Tn*4451* and Tn*4453a*

Tn*4451* and Tn*4453a* move from one site in the genome to another by a TnpX resolvase-mediated site-specific recombination mechanism that involves the formation of a circular intermediate [31]. Since movement does not involve a transposase enzyme these elements are technically not transposons, perhaps the term ‘resolvon’ would be more appropriate. The circular molecule was first observed during excision assays performed on Tn*4451* in *E. coli* [28] and more recently shown to be an essential intermediate in the transposition process [31]. This finding is consistent with a resolvase-mediated mechanism, as this reaction essentially constitutes the equivalent process to cointegrate resolution. In this case the *res* sites, the transposon ends, are located at the ends of a single element rather than in the middle of two duplicated elements. The joint of the circular intermediate resembles the ends of the element and the reconstituted target site, they all contain a GA dinucleotide [3, 29].

We have carried out extensive mutagenesis on the GA dinucleotides at the left and right ends of Tn*4451*. The results showed that when the GA residues at the left end were al-

Table 2. Group V resolvase proteins of known function.

Name	Element	Organism	Function	Size (aa)	Ref.
TnpX	Tn*4451*	*Clostridium perfringens*	excision and integration of Tn*4451*	707	29
TnpX	Tn*4453*	*Clostridium difficile*	excision and integration of Tn*4453*	707	31
TndX	Tn*5397*	*Clostridium difficile*	integration and excision of Tn*5397*	533	35
CcrA	SCC*mec*	*Staphylococcus aureus*	movement of *mec* element	448	56
CcrB	SCC*mec*	*Staphylococcus aureus*	movement of *mec* element	542	56
XisF		*Anabaena*	excision of *fdxN* element	514	57
Orf1	ϕTP901-1	*Lactococcus* sp.	integration and excision of ϕTP90-1	485	33
Int	ϕC31	*Streptomyces* sp.	integration and excision of ϕC31	613	34
SpoIVCA		*Bacillus subtilis*	excision of the skin element	500	58

tered, excision events resulted in deletent plasmids that had either a residual GA dinucleotide or the mutated dinucleotide [29]. These results are consistent with the resolvase-mediated introduction of 2-bp staggered cuts at the terminal dinucleotides of the transposon, which in the mutants results in a 2-bp heteroduplex at the deletion site and at the joint of the circular intermediate [29]. Mismatch repair or DNA replication would resolve the heteroduplex at the deletion site. These studies suggest that the GA residues derived from the right end of the transposon constitute the joint of the circular molecule. The substitution of a T for the G in the GA dinucleotide at either or both ends of the element increases the excision frequency, suggesting that the TA dinucleotide constitutes the preferred substrate for TnpX-mediated recombination [29].

The frequency of transposition was determined using *E. coli* donor strains containing chromosomal copies of Tn*4451*, Tn*4453a*, or Tn*4453b*, and pVS520, a derivative of the conjugative broad host range plasmid RP4. In this system, transposition is detected by pVS520-mediated conjugative transfer to a suitable *E. coli* recipient, selecting for chloramphenicol resistance. The transposition frequency is significantly increased by providing a wild-type *tnpX* gene on a compatible multicopy plasmid [31]. The transposition frequencies of the three elements are quite different, with Tn*4453a* showing the highest degree of transposition and transposition of Tn*4453b* being undetectable unless additional TnpX is supplied in trans [31]. The TnpX proteins from each of the elements are functionally interchangeable and result in a transposition frequency that is characteristic of the element located on the chromosome rather than the *tnpX* gene being supplied in trans [31]. When the resolvase domain mutants derived from the Tn*4451 tnpX* gene [29] were analysed, they were found to exhibit transposition frequencies below the limit of detection. This result was expected, as these proteins are unable to catalyse excision [31]. In contrast, the integrase domain mutants had no significant effect on the transposition frequency, confirming that the putative integrase domain is not involved in the transposition process [31].

Other studies have shown that the formation of the circular intermediate results in the creation of a strong promoter capable of driving expression of the *tnpX* gene [31]. This promoter is formed at the joint of the circular molecule by the fusion of the right end of the transposon, which contains the –35 box, and the left end of the element, which contains a correctly spaced –10 box and the *tnpX* gene. The circular intermediate is a nonreplicative element that would be lost from the cell if it did not reintegrate into either the chromosome or a plasmid. The formation of this strong promoter ensures that there will be enough TnpX protein available in the cell to catalyse the insertion of the circular molecule. This promoter is clearly functional since this transient molecule is capable of inserting into the chromosome of recipient cells that do not carry the transposon and which therefore contain no TnpX protein [31]. Circularisation of other elements, such as IS*911*, also leads to formation of transient promoters that result in the expression of a recombinase protein [31]. Upon insertion of the element the promoter is disrupted, leading to reduced levels of expression of the recombinase, allowing the element to remain stably integrated.

It was assumed that integration of the circular form was the reverse of the excision process because until recently integration had not been separated from the excision process experimentally. We have now developed an in vivo integration assay and shown that *tnpX* is essential for integration. Similarly, we have evidence that the other Tn*4451* proteins are not required for excision or integration [D. Lyras and J. Rood, unpublished results]. Current studies in this laboratory involve the analysis of the functional domains of the TnpX protein and the determination of the TnpX binding site.

The role of TnpZ in transposon mobilisation

Analysis of the nucleotide sequence of Tn*4451* suggested that in addition to encoding the TnpX site-specific recombinase, Tn*4451* carried a potential mobilisation gene, *tnpZ* [28]. Mobilisation proteins function by nicking the DNA at a specific site, which can then act as an origin of transfer in the presence of the appropriate conjugative element [41]. Located upstream of *tnpZ* was a site designated RS_A, which showed significant similarity to functional RS_A, or *oriT*, sites from plasmids found in other Gram-positive bacteria [28]. Subsequent studies confirmed that, in the presence of the conjugative plasmid RP4, TnpZ acts at this RS_A site to promote the in cis mobilisation of plasmids carrying either Tn*4451* or just the RS_A-*tnpZ* mobilisation cassette (fig. 2). The TnpZ protein can also function in trans to promote mobilisation of a coresident plasmid that carries an RS_A site [19]. TnpZ-mediated mobilisation occurs from an *E. coli* donor to both *E. coli* or *C. perfringens* recipients but is dependent upon the presence of an RP4-derivative in the donor [19]. The process occurs with Tn*4451*, Tn*4453a* and Tn*4453b*, although the mobilisation frequencies vary [10]. The Tn*4451* family of elements therefore are clearly defined as mobilisable transposons that in the presence of an appropriate conjugative plasmid can promote the mobilisation of nonconjugative plasmids on which they reside. This family is unique in that it represents the only group of mobilisable transposons that transpose by a resolvase-dependent mechanism.

Recent transposition studies on Tn*4453a* showed that in the presence of RP4, the nonreplicating circular intermediate can be mobilised to recipient cells, where it can subsequently insert into the chromosome (fig. 2) [31]. In this

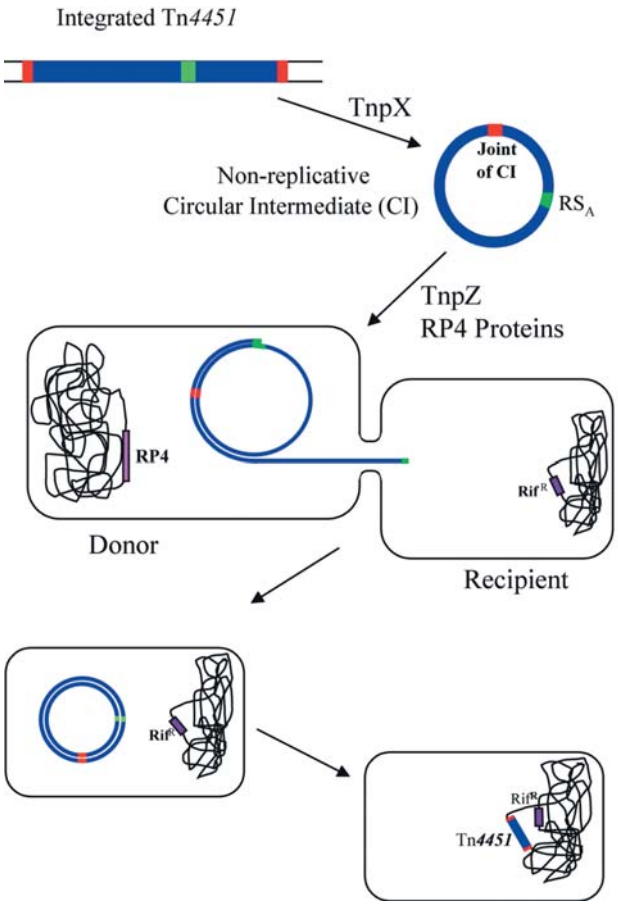


Figure 2. Mechanism of mobilisation of Tn*4451/53*. Integrated Tn*4451*/Tn*4453* elements (shown as blue rectangles with the red rectangles denoting the ends) are excised by TnpX to form the circular intermediate. This molecule is then nicked by the mobilisation protein TnpZ at the RS$_A$ site (green rectangle), and in the presence of a conjugative element such as RP4 is mobilised to a recipient cell where it must integrate into the chromosome.

respect the Tn*4451* group of elements is similar to conjugative transposons such as Tn*916*, which also produces a nonreplicating circular intermediate that is transferred to recipient cells where it then inserts into the host genome [42, 43]. The major difference is that conjugative transposons do not rely on other factors to provide the conjugation machinery. The *C. difficile* element Tn*5397* represents an intermediate between Tn*916* and Tn*4451* or Tn*4453a* since it is conjugative like Tn*916*, but its excision and insertion is mediated by a large resolvase, TndX, that is closely related to TnpX [13].

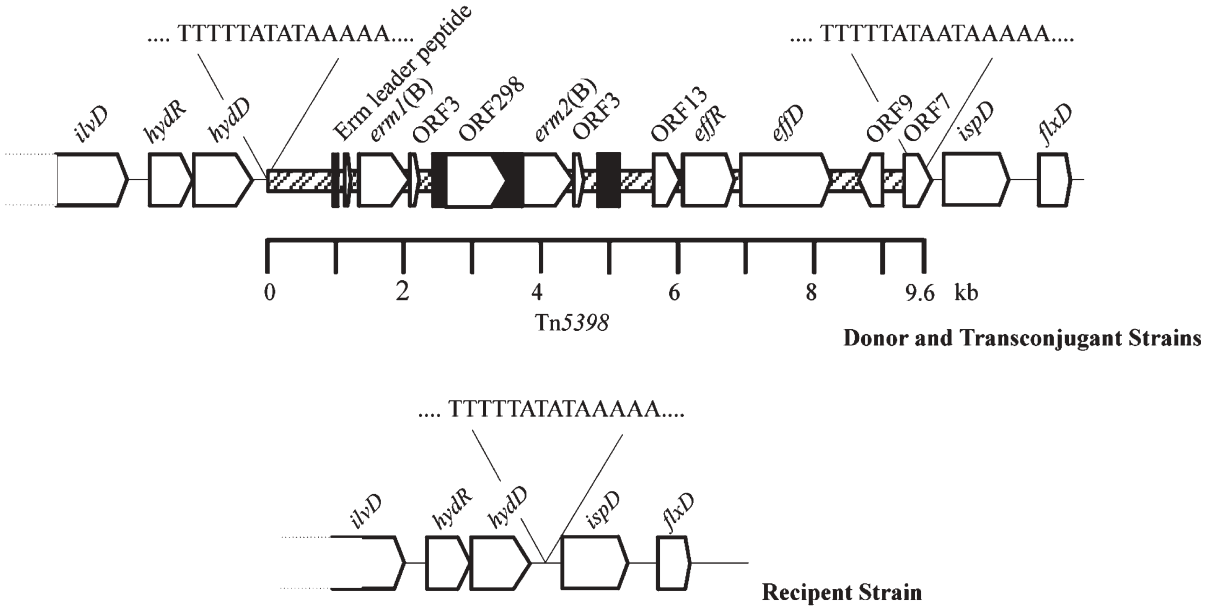
The capacity for the nonreplicating circular intermediate of the Tn*4451* family to be mobilised to recipient cells is important in an evolutionary context as it provides another means by which this family of elements can be disseminated within the bacterial population. For example, TnpZ-mediated transfer may explain why a remnant of Tn*4451* has been found in *Neisseria* [32]. Preliminary searches of the genome sequence of *C. perfringens* strain 13 and the partially completed genome sequence of *C. difficile* strain 630 suggest that RS$_A$-like sites are present within these sequences [V. Adams, D. Lyras and J. Rood, unpublished results]. Therefore, it is possible that in the presence of an appropriate conjugative element, TnpZ may act at these sites to promote the transfer of chromosomal DNA to recipient strains, in a similar manner to Hfr-mediated chromosomal transfer in *E. coli*. Further experiments are required to establish whether TnpZ can mediate chromosomal mobilisation in either *C. perfringens* or *C. difficile*.

Tn*5398*: a mobilisable erythromycin resistance element from *C. difficile*

The Erm B erythromycin resistance determinant from *C. difficile* strain 630 has been shown to be transferred by conjugation, in the absence of plasmid DNA, to *C. difficile* [44], *Staphylococcus aureus* [45] and *Bacillus subtilis* [46] recipients. Furthermore, the resistance determinant can be transferred from the *B. subtilis* transconjugants, where it integrates without site specificity, back to *C. difficile*, where it integrates into the recipient chromosome in a site-specific manner [46]. This transfer behaviour is typical of a conjugative transposon and, accordingly, the genetic element carrying the erythromycin resistance determinant was designated as Tn*5398* [46].

We have recently isolated and characterized Tn*5398* [11]. Physical and genetic analysis has revealed that Tn*5398* does not have the characteristics of a classical conjugative transposon but may represent a mobilisable genetic element [11]. Tn*5398* is approximately 9.6 kb in length and, in addition to a novel Erm B determinant that contains two directly repeated *erm*(B) genes [47], carries only four complete open reading frames (ORFs), *orf13*, *effR*, *effD* and *orf9*, and one incomplete ORF, *orf7* (fig. 3). The EffD and EffR proteins, which appear to be a potential efflux protein and its associated regulator, respectively, are unlikely to be involved in the movement of the putative transposon, as they show no homology to any proteins shown to have a role in transposition, mobilisation or conjugative transfer. The putative *orf13*, *orf9* and *orf7* products have similarity to similarly named ORFs from the conjugative transposon Tn*916*. While the Tn*916* homologues have been studied in some detail, their functions are still unknown, although ORF9 and ORF7 are postulated to have a regulatory role [48]. Therefore, if the Tn*5398* homologues have any functional role, it may be in regulation rather than mobilisation or transposition.

The only other genes that may encode proteins involved in the mobility of Tn*5398* are *orf3a*, *orf3b* and *orf298*, all of which are part of the Erm B determinant. ORF3 homologues are commonly found in association with *erm*(B) genes, but their function is unknown [49]. ORF298 has



b

Left End	AAAATTTTTATGAGC**TTTTTATATAAAAA**
Right End	**TTTTTATAATAAAAA**ATATAATGCTTGTA
Target Region	TTATGAGA***TTTTTATATAAAAA***ATATAAT

Figure 3. Genetic organisation of Tn*5398*. (*a*) Schematic diagrams of the Tn*5398* element from the *C. difficile* donor and transconjugant strains, and the target region in the *C. difficile* recipient strain CD37, are shown. The region encompassing the Tn*5398* element is backed by a cross-hatched box, and the size of the element is indicated by the scale bar below the diagram. ORFs are shown as open block arrows. The sequences of the ends of the transposon in the donor and transconjugant strains and the target region in the recipient strain are indicated. The black boxes represent regions that encompass direct repeat (DR) sequences associated with the Erm B determinant. (*b*) The nucleotide sequences of the ends of Tn*5398* and of the target region are shown. Nucleotides representing the ends of Tn*5398* are underlined and shown in bold. Nucleotides comprising the target site in the *C. difficile* recipient strain CD37 are shown in bold italics. Reproduced from [11] with the permission of the publishers.

very weak similarity to both replication proteins and proteins from the ParA and Soj families, which generally have a role in partitioning plasmid and chromosomal DNA during the replication cycle [50, 51]. It appears unlikely that either ORF3 or ORF298 have a role in the excision or integration of Tn*5398*, but this possibility cannot be completely ruled out.

The ends of Tn*5398* have been shown to consist of highly AT-rich palindromic sequences which are almost identical to the target sequence in the *C. difficile* recipient strain CD37 [11] (fig. 3). In the CD37 transconjugants the left end of Tn*5398* lies in the intergenic space downstream of the chromosomal *hydD* gene, and the right end appears to lie within the coding sequence of *orf7*.

Excision of an integrated conjugative transposon from the chromosome involves the action of site-specific recombinases of either the integrase or resolvase families. However, analysis of Tn*5398* does not reveal the presence of any genes that would encode homologues of these proteins or of transposases. The excision of Tn*5398* could be mediated in trans by other conjugative elements present in the cell, or by other proteins encoded on the genome. In addition to Tn*5398*, strain 630 contains the conjugative tetracycline resistance transposon Tn*5397*. Tn*5397* is excised from the donor chromosome through the action of the large resolvase, TndX [35]. It is possible that the TndX protein is also responsible for excision of Tn*5398* from the donor chromosome, especially since in many cases, Tn*5397* and Tn*5398* appear to be cotransferred to the recipient cell [K. Farrow, D. Lyras and J. Rood, unpublished results]. However, the sequences at the ends of these elements are different, which makes it unlikely that the TndX protein could recognize the ends of Tn*5398*, since large resolvases generally recognize specific target sequences. Note that the genome of *C. difficile* strain 630 encodes as many as five other large resolvases [M. Smith, personal

communication], one of which may potentially be responsible for the excision of Tn*5398* from the chromosome (fig. 4).

Once excised from the chromosome, most classical conjugative transposons form a nonreplicating circular intermediate, which is then nicked at an *oriT* site, and one strand subsequently transferred by conjugation from the donor to the recipient cell. In addition to catalysing its own conjugative transposition by this mechanism, Tn*916* has been shown to enhance the transfer of another homologous conjugative transposon [52] and to mobilise nonconjugative plasmids [53, 54]. The latter process does not appear to be dependent on the plasmid having a functional mobilisation gene but does require the presence of a sequence similar to the *oriT* site of Tn*916*. It is postulated that the same protein or proteins involved in nicking the Tn*916* circular intermediate nicks similar sequences present on coresident plasmids. Once nicked the plasmid assumes a relaxed form that is capable of being transferred during conjugation [54]. ORF23 of Tn*916* has similarity to the MbeA mobilisation protein of ColE1 [55] and is likely to be the Tn*916*-encoded mobilisation protein [54].

Comparative analysis has revealed that Tn*5397* from strain 630 is closely related to Tn*916*; the transposons have very similar conjugation regions (ORFs 15–23) but different insertion and excision modules, with *xis* and *int* in Tn*916* being replaced by *tndX* in Tn*5397* [13]. Furthermore, the *oriT* sites are identical, and the amino acid sequences of the ORF23 homologues have greater than 90% identity. Analysis of the Tn*5398* sequence has revealed that it has two potential *oriT* sites, each of which has similarity to the *oriT* sites on Tn*916* and Tn*5397* [11]. The first site is located within the coding sequence of *orf298*, and the second site is located in the intergenic space between *orf3b* and *orf13*. Both sites have limited similarity across the *oriT* region, while the actual *nic* site is completely conserved. If Tn*5398* was able to be excised

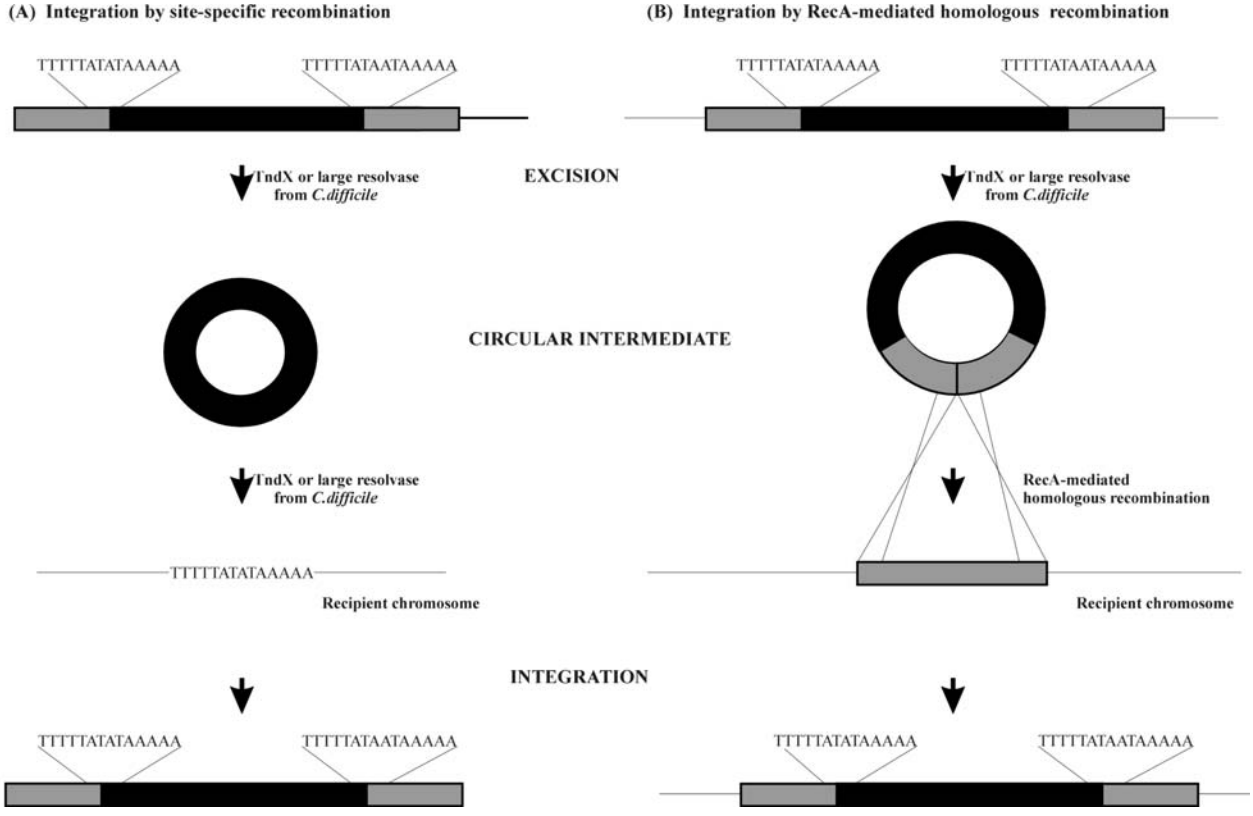


Figure 4. Models for the transfer of Tn*5398* in *C. difficile*. Schematic representations of two alternative models for the transfer of the Tn*5398* element are shown. Tn*5398* is represented by the black rectangle, and DNA flanking Tn*5398*, which is homologous to that in the recipient strain, is represented by the grey rectangles. The ends of Tn*5398* are represented by the nucleotide sequence, as is the target site in the recipient chromosome. In both models Tn*5398* is excised from the donor chromosome by a site-specific recombinase encoded on the *C. difficile* chromosome to form a circular intermediate. This molecule is then mobilised to the recipient strain by Tn*5397* or another chromosomal element. In the site-specific recombination model only Tn*5398* is excised from the donor chromosome, and it is integrated into the recipient chromosome by the same site-specific recombination mechanism. In the homologous recombination model a larger gene region, including Tn*5398* and its flanking DNA, is excised from the donor chromosome to form a circular molecule. After mobilisation this molecule is integrated into the recipient genome by RecA-mediated homologous recombination.

by a chromosomally encoded integrase or resolvase to form a circular intermediate, the resultant molecule would resemble a nonconjugative plasmid that lacks mobilisation genes. It is therefore possible that a Tn*5398* circular intermediate could be nicked and transferred to the recipient by the action of an ORF23 protein provided in trans by Tn*5397* (fig. 4).

The last step in the transfer of a conjugative transposon is integration of the element into the recipient genome. This process is generally dependent on the action of integrases or resolvases, and represents the reverse of the excision process. The mechanism by which Tn*5398* may integrate is unclear, but it is possible that as postulated for excision, the element is integrated by means of the TndX protein or another large resolvase present on the chromosome of the recipient cell (fig. 4).

Considerable sequence identity is evident between the target region of the transposon in strain CD37 and the sequence upstream of the right end of the putative Tn*5398* element [11]. It is possible that the region excised from the chromosome to form the circular intermediate is much larger than that proposed and may include genes upstream of the proposed left end (*ilvD*, *hydR* and *hydD*) and downstream of the proposed right end (*ispD*, *flxD*) of the element (fig. 4). After transfer of this region, which contains Tn*5398*, it could be integrated into the recipient chromosome by RecA-dependent homologous recombination. The end result would be the integration of Tn*5398* and some of the genes flanking the element (fig. 4). This hypothesis is supported by the position of the right end of Tn*5398*, which lies within the coding sequence of *orf7*. This position is unusual, as excision and subsequent transfer of the proposed element would interfere with the transcription and expression of this ORF. If we presume that Tn*5398* was once a fully functional conjugative transposon, which may have contained genes downstream of *orf7* that were involved in conjugative transfer of the element, a single homologous recombination event between the *orf7* gene region and the recipient genome may have resulted in a truncated form of Tn*5398*, which is no longer capable of catalysing its own transfer.

In summary, our analysis of the Tn*5398* element has raised many questions, and obviously more experimentation is required. Further studies on this interesting element await the development of efficient methods for genetic analysis in *C. difficile* so that in vivo mutagenesis of the many genes potentially involved in the intercellular movement of this interesting clostridial element can be carried out and the effects of these mutations analysed.

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