Conjugative transposition of the vancomycin resistance carrying Tn1549: enzymatic requirements and target site preferences

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Summary

Rapid spread of resistance to vancomycin has generated difficult to treat bacterial pathogens worldwide. Though vancomycin resistance is often conferred by the conjugative transposon Tn1549, it is yet unclear whether Tn1549 moves actively between bacteria. Here we demonstrate, through development of an in vivo assay system, that a mini-Tn1549 can transpose in E. coli away from its natural Gram-positive host. We find the transposon-encoded INT enzyme and its catalytic tyrosine Y380 to be essential for transposition. A second Tn1549 protein, XIS is important for efficient and accurate transposition. We further show that DNA flanking the left transposon end is critical for excision, with changes to nucleotides 7 and 9 impairing movement. These mutations could be partially compensated for by changing the final nucleotide of the right transposon end, implying concerted excision of the two ends. With changes in these essential DNA sequences, or without XIS, a large amount of flanking DNA transposes with Tn1549. This rescues mobility and allows the transposon to capture and transfer flanking genomic DNA. We further identify the transposon integration target sites as TTTT-N6-AAAA. Overall, our results provide molecular insights into conjugative transposition and the adaptability of Tn1549 for efficient antibiotic resistance transfer.

Introduction

Pathogenic bacteria that are resistant to several antimicrobials are now common, and it is becoming challenging again to treat simple bacterial infections in humans and animals. In particular, infections caused by multidrug-resistant (MDR) bacteria, with MDR defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories (Magiorakos et al., 2012), are becoming a worldwide problem. In 2013, the Centers for Disease Control and Prevention (CDC) generated a comprehensive Antibiotic Resistance Threat Report (CDC, 2013) showing that approximately 2 million people per year become infected with antibiotic resistant bacteria in the United States and 23 000 of those die from their infection (CDC, 2013). Also, the World Health Organization (WHO) has recognised antimicrobial resistance as a global health concern and in 2015 they launched a Global Action Plan on antimicrobial resistance (WHO, 2015), which representatives of the Worlds States and Governments have agreed to follow. This shows that MDR microorganisms are a serious global issue with actions taken at many levels worldwide (UN, 2016).

Despite this attention to antibiotics, the situation is already quite concerning for some bacteria. For example, for Neisseria gonorrhoeae infections treatment failures due to resistance to the last effective antibiotic have been reported in at least 10 countries (WHO, 2015). Also, healthcare-associated Enterococcal infections are becoming a serious problem, as approximately 30% of those infections (about 20 000 per year in the U.S.) are caused by vancomycin resistant Enterococci (VRE) that are resistant to the main last resort antibiotic used against those infections (WHO, 2015). One example of an MDR-VRE strain is the clinical E. faecium C68 isolate from Ohio that is resistant to at least 11 antibiotics from 7 drug classes (Carias et al., 1998; Rice et al., 2004; Rice et al., 2010; García-Solache and Rice, 2016). Concerningly, at least two of the resistance determinants are located on mobile genetic elements, the conjugative transposons Tn916 providing TetM-based tetracycline resistance and Tn1549 providing VanB-based
vancomycin resistance. These conjugative transposons appear functional and can facilitate transfer of resistance to other bacteria (Carias et al., 1998; Rice et al., 2010). In the future, more such MDR bacteria are likely to arise through movement of mobile genetic elements with antibiotic resistance genes.

Conjugative transfer allows movement of large DNA molecules, providing an important route by which antibiotic resistance genes are spread between bacteria (Wozniak and Waldor, 2010; van Hoek et al., 2011). Two main classes of DNA elements that spread via conjugative transfer mechanisms are conjugative plasmids and conjugative transposons. In particular, one family of conjugative transposons, the Tn916 family, has successfully spread across a large number of different bacteria. Members of the family have been identified in six bacterial phyla and more than 35 genera including Gram-positive and Gram-negative bacteria as well as pathogenic and non-pathogenic bacteria (reviewed in Clewell et al., 1995; Roberts and Mullany, 2009), constituting perhaps the most abundant and wide-spread conjugative transposon family in nature. Transposons of this family have a functional modular structure with one module providing the transposition functions, a second module with the conjugative transfer functions and a third module supplying accessory functions. A commonly observed accessory function for the Tn916 family is antibiotic resistance usually against tetracycline, macrolide and aminoglycoside antibiotics (Roberts and Mullany, 2009; Roberts and Mullany, 2011; Santoro et al., 2014).

Most of our current knowledge on the mechanisms of conjugative transposons, is based on studies of Tn916 and Tn1545 from the Tn916 family. These studies have revealed two distinct but linked processes: movement from one DNA site to another by transposition (Fig. 1, steps II and IV) and transfer between bacteria via conjugative transfer (Fig. 1, step III, (Senghas et al., 1988))

The proposed transposition mechanism consists of two recombination events (Fig. 1). First, recombination of the DNA at the two transposon ends leads to excision of the element from its original location in the donor DNA. Then, recombination of the transposon into a new target DNA site results in integration. Both of these events are catalyzed by a tyrosine recombinase enzyme, named the integrase (INT) (Poyart-Salmeron et al., 1989; Poyart-Salmeron et al., 1990). For Tn916, INT has been shown to bind specific DNA sequences at the two transposon ends (also referred to as attR and attL) recombining these (Manganelli et al., 1996) to create a circular transposon junction intermediate (CI) (Scott et al., 1988; Poyart-Salmeron et al., 1990; Jaworski and Clewell, 1994). Simultaneously, the donor DNA is resealed.

By analogy to related site-specific tyrosine recombinases, INT-mediated recombination involves step-wise cleavage and exchange of four DNA strands in pairs. A conserved tyrosine residue in the INT active site cuts the DNA at each transposon end generating a covalent 3’ phosphotyrosyl protein-DNA linkage and a free 5’ hydroxyl group (Lu and Churchward, 1994; Taylor and Churchward, 1997). The 5’ hydroxyl group joins with the 3’end of the other transposon end forming a four-way Holliday junction (HJ) DNA intermediate. The HJ is then resolved by a second round of DNA cleavage and strand exchange completing one recombination event (reviewed in Rajeev et al., 2009). The two DNA strands are cleaved at staggered positions in each transposon end (Manganelli et al., 1996), generating a 6 bps hetero-duplex crossover region in the CI product [Fig 1, (Caparon and Scott, 1989)].

For integration, INT can use a highly similar pathway to catalyze recombination of the transposon CI into a new DNA site (also referred to as attB for Tn916). Here, the nucleotides from the crossover region of the CI are inserted with the transposon into the new DNA site (Trieu-Cuot et al., 1993; Scott et al., 1994). The integration sites of Tn916 have shown to be AT-rich, but they are difficult to predict as they lack clearly recognisable sequence patterns.

As INT catalyses both transposon excision and integration the order of the INT-based cleavage and recombination events has to be well controlled for the transposon to successfully move from one DNA site to the next. For Tn916, the reactions have been shown to be modulated by a second transposon-encoded protein, the XIS excisionase protein. XIS binds to the transposon DNA and directs the activity of INT, promoting excision (Su and Clewell, 1993; Rudy et al., 1997a, 1997b).

Given the great abundance of the Tn916 family conjugative transposons and their significant role in the development of MDR bacteria, we wanted to better understand the transposition mechanisms of these elements. Although previous studies provided important insights, the specific molecular mechanisms of the INT and XIS proteins are still incompletely understood. Also, most work has focused on Tn916 and the closely related Tn1545 elements, and it is still unclear to what extent these processes are conserved to other members of the family.

We choose to study the transposon Tn1549 (highly similar to Tn5382), in particular, because this transposon is linked to the development of MDR Enterococci in healthcare. Tn1549 provides resistance to the glycopeptide vancomycin through the vanB operon (Carias et al., 1998; Garnier et al., 2000). In addition to Enterococci, it has also been identified in Atopobium, Clostridium, Eggerthella, Ruminococcus and Streptococcus species (Ballard et al., 2005; Launay et al., 2006; Marvaud et al., 2011; Dehoux et al., 2016) and transfer of a Tn1549-like element from Clostridium symbiosum to E. faecium and E. faecalis has been demonstrated in
filter matings and the gut of gnotobiotic mice (Launay et al., 2006).

Even though Tn1549 has been shown in different hosts it is still discussed if this DNA element moves actively between bacteria via conjugative transposition or it propagates only passively, hijacking other mobile DNA elements such as plasmids (Dahl et al., 2003; Launay et al., 2006; Bender et al., 2016; García-Solache et al., 2016). Therefore, in this study we focused on reconstituting the first and the last steps of Tn1549 movement, the transposition steps. We set out to test if Tn1549 is an active transposon that can autonomously transpose, i.e., excise and integrate independently of its host. We also aimed to define the components, such as the proteins and DNA, required for its transposition. We developed in vivo assays in bacteria, and demonstrate that a suitably designed mini-Tn1549 can actively transpose in E. coli in the presence of only two transposon-encoded proteins XIS and INT. We also find that the DNA sequences flanking the transposon ends are important for efficient and correct transposition and define the nature of integration sites. In comparison with data on Tn916, our results reveal substantial similarities within this transposon family and shed new light on their mechanistic principles. The implications of these insights for antibiotic resistance transfer are also discussed.

Results

Bacterial in vivo assay for testing excision activity of the conjugative transposon Tn1549

To investigate the initial transposon excision step of Tn1549-mediated transfer of VanB resistance between

![Fig. 1. Overview of conjugative transposon movement, transferring antibiotic resistance (AR) from one bacterium to another. I. The conjugative transposon (yellow) is located on the chromosome of the donor bacterium. II. The transposon is excised from the chromosome forming a CI. Details of excision are shown in the top insert. The two transposon ends are highlighted in dark red and blue and the DNA flanking the transposon ends are shown in light red and blue. The XIS-INT proteins (shown in green) recombine the transposon ends forming a CI with a 6 bps hetero-duplex crossover region derived from the flanks. Simultaneously, the donor DNA is resealed. III. The CI is transferred as ssDNA by conjugation from the donor to a recipient bacterium in close contact. Depending on which strand is transferred, the CI contains left or right flanking DNA. IV. The CI is integrated into a new target site, here on the chromosome of the recipient bacterium. Details are shown in the bottom insert. The integration recombination event of the CI into the target DNA is performed by the INT protein. Four different integrated versions may arise upon integration into the target DNA, carrying the left or right flanking DNA on either side of the transposon.](image-url)
bacteria, we established a bacterial in vivo assay. We used a trans excision assay system in *Escherichia coli* based on two plasmids: one plasmid, the transposon donor plasmid contained a mini version of Tn1549 (Fig. 2B) and the other plasmid expressed the protein(s) needed for transposon excision (Fig. 2C).

We constructed our assay using the original conjugal transposon Tn1549 that was first described in a multi-resistant Enterococcus blood isolate, *E. faecalis* E93/268, from a hospitalised patient in the UK. This bacterial isolate carried vancomycin resistance that was transferable to the susceptible *E. faecalis* strain JH2-2 (Woodford *et al.*, 1995a, 1995b). In the resulting transconjugant, *E. faecalis* 268-10, vancomycin resistance was shown to be caused by a vanB operon (Woodford *et al.*, 1995a, 1995b) located on a conjugal transposon that was named Tn1549 [Fig. 2A, (Garnier *et al.*, 2000)].

Our mini-Tn1549, was constructed by taking DNA fragments of the Tn1549 ends from this *Enterococcus faecalis* 268-10 strain. We choose this, because Tn1549 was shown to be capable of excising and forming a circular intermediate (CI) in this strain (Garnier *et al.*, 2000), suggesting that it is active and can move from its location by transposition. In more details, the mini-Tn1549 was constructed by cloning the left transposon end (LE, 216 bps) and left genomic DNA flanking the LE (LF, 107 bps) on one side of an antibiotic resistance marker gene, and the right transposon end (RE, 224 bps).
with right flanking DNA (RF, 137 bps) placed on the other side of the resistance gene in the mini-Tn\textsubscript{1549} donor plasmid (Fig. 2B). The transposon ends LF-LE and RE-RF correspond to the \textit{attL} and \textit{attR} sites of Tn\textsubscript{916}. Although the DNA sequences flanking the transposon ends vary depending on its location, for this assay we kept the flanking DNA from the \textit{E. faecalis} 268-10 strain.

The other plasmid of the assay expressed the Tn\textsubscript{1549}-encoded proteins XIS and INT, which share sequence similarity with proteins from Tn\textsubscript{916} and Tn\textsubscript{1545} (Garnier \textit{et al.}, 2000) that were previously shown to be involved in transposon excision (Poyart-Salmeron \textit{et al.}, 1989; Su and Clewell, 1993). We cloned the genes for the two proteins from the same Tn\textsubscript{1549} element in \textit{E. faecalis} 268-10 as the ends of the transposon. To test the role of each protein, we constructed three different expression plasmids, p\textsubscript{BAD}XIS, p\textsubscript{BAD}INT and p\textsubscript{BAD}XIS-INT, by which the proteins were expressed separately or together from the arabinose inducible promoter, p\textsubscript{BAD}. On arabinose induction, the XIS and INT proteins were expressed at well detectable levels in \textit{E. coli}, both separately and together (Supporting Information Fig. S1).

In our excision assay, the mini-Tn\textsubscript{1549} donor plasmid and the protein expression plasmid were co-transformed into \textit{E. coli} (Fig. 2C) and mini-Tn\textsubscript{1549} excision from the donor plasmid was detected by PCR. We followed changes in both the mini-Tn\textsubscript{1549} and the mini-Tn\textsubscript{1549} donor plasmid using two sets of PCR primers: one set annealing to the transposon ends, and the other set annealing to the transposon donor plasmid sequences flanking the transposon (Fig. 2C). We analyzed samples taken at various time points after induction of protein expression (Fig. 3A). Before induction, PCR with the donor plasmid flank primers showed a product corresponding to a fragment of the donor plasmid containing the mini-Tn\textsubscript{1549} transposon. After induction for half an hour an additional smaller PCR product appeared corresponding to the fragment of a resealed donor plasmid lacking mini-Tn\textsubscript{1549}. PCR with the transposon end primers on the same samples showed a product that corresponds well to the expected fragment generated when the two transposon ends are directly joined in the CI. After three hours, most mini-Tn\textsubscript{1549} transposons were excised from the donor plasmids and formed CIs (Fig. 3A, lane 6, Fig. 3B).

**Fig. 3.** Effect of XIS and INT on mini-Tn\textsubscript{1549} excision.

\begin{itemize}
  \item A. Time course of Tn\textsubscript{1549} excision by XIS and INT. Samples were taken at different time points after induction of protein expression and analyzed by PCR. PCR was performed with primers detecting the mini-Tn\textsubscript{1549} CI (upper part of gel picture), or primers monitoring the donor plasmid containing the mini-Tn\textsubscript{1549} (DP) or the resealed donor plasmid without the mini-Tn\textsubscript{1549} (RDP) (lower part of gel picture). PCR fragments were run on an ethidium bromide stained agarose gel, the negatives of the images are shown. Most mini-Tn\textsubscript{1549} were excised after 3 hours. The faint PCR product above the RDP product is likely due to amplification of unrelated genomic DNA from the crude cell lysates.
  \item B. Mini-Tn\textsubscript{1549} excision analyzed with three primers sets; samples from A diluted 100 times to allow comparative analysis. The upper gel picture shows PCR with primers detecting DP and RDP. The middle and bottom gels show PCR detecting the LF-LE and the RE-RF of mini-Tn\textsubscript{1549} in the DP.
  \item C. Mini-Tn\textsubscript{1549} excision in the presence of the XIS, INT or both XIS and INT proteins showing that efficient excision requires presence of both XIS and INT. The excision assay was performed with 3 hours protein induction and the CI, DP and RDP were detected by PCR as described in A.
\end{itemize}
define the proteins and DNA components required for the first step of Tn1549 transposition.

Both XIS and INT are required for efficient transposon excision, with INT responsible for catalysis

To further explore the requirements of Tn1549 excision, we next tested the role of each of the two transposon proteins, XIS and INT, using our bacterial in vivo assay. We found that the INT protein was capable of excising the mini-Tn1549 when expressed alone (Fig. 3C, lane 9), whereas no excision was detected with expression of XIS alone (Fig. 3C, lane 8). However, excision was only efficient in the presence of both INT and XIS (Fig. 3C, lane 10).

Sequence analysis indicated that INT of Tn1549 is related to tyrosine recombinases (Pargellis et al., 1988; Esposito and Scocca, 1997; Nunes-Düby et al., 1998; Garnier et al., 2000). Since many of these proteins catalyze site-specific DNA recombination reactions to promote excision and integration of various DNA elements in bacteria (Jayaram et al., 2015), our result that INT is indispensable for Tn1549 excision suggested that INT is the enzyme catalyzing transposon excision.

To verify this, we first tested if INT’s catalytic activity is required for its function by generating an INT mutant with an inactive phenylalanine replacing the essential catalytic tyrosine. Aligning the INT protein sequence to other tyrosine recombinases (Fig. 4A and Supporting Information Fig. S2) showed that the integrases from conjugative transposons Tn916, Tn1545, Tn5386 and Tn1549 have two consecutive tyrosines (positions 379 and 380 in Tn1549) at the position of the catalytic tyrosine of canonical tyrosine recombinases. Therefore, we mutated each of the two tyrosines (INT-Y379F and INT-Y380F) and tested their effect on excision activity in our assay. This showed that INT-Y379F can excise the mini-Tn1549 transposon at levels similar to the wild type INT protein (Fig. 4B, lanes 9 and 8), whereas the INT-Y380F mutant was inactive (Fig. 4B, lane 10). This confirmed that the enzymatic activity of INT is required to promote Tn1549 excision and identified Y380 as the essential catalytic residue.

Next, we tested if INT can bind to DNA at the transposon ends. Binding studies were performed in vitro by electrophoretic mobility shift assay (EMSA) using the purified full-length protein INT and DNA oligonucleotides consisting of 25 bps of either transposon end and 25 bps of the corresponding flanking DNA (LE-LF and RE-RF). We observed protein-DNA complexes with both the LE-LF and the RE-RF sequence, confirming INT binding to both Tn1549 transposon ends (Fig. 4C).

Taken together, these results show that INT is essential for transposon excision, because it binds to the ends of the transposon DNA and catalyses excision using the conserved tyrosine nucleophile, Y380. However, transposon excision in E. coli is only efficient in the presence of both INT and XIS, showing that XIS affects INT function in vivo.

Transposon excision captures sequences of the flanking DNA

In our initial in vivo assay, we showed that the mini-Tn1549 is excited and forms a CI, consistent with previous data for the complete Tn1549 in E. faecalis (Garnier et al., 2000). For the related Tn916 transposon, it was shown that the CI contains the transposon ends connected by a junction region consisting of a 6 bases heteroduplex derived from the flanking DNA (Caparon and Scott, 1989; Rudy and Scott, 1994). To check if this was also the case for Tn1549, we sequenced the junction region of the CIs from our mini-Tn1549 excision assays and identified a 5–7 bps heterogeneous DNA sequence connecting the two transposon ends (Supporting Information Fig. S3). Sequences of 41 cloned CI junctions from separate experiments showed 5 different sequences of 5–7 nucleotides (Fig. 5A). In 46% of the cases this sequence was identical to the 6 nts of the left flanking DNA and in 49% of the cases to 6 nts (34%) or 7 nts (15%) of the right flanking DNA. The remaining two sequences (5%) contained CI junctions of 5 or 6 nts of unknown origin.

To further confirm the origin of the CI junction sequences we also cloned and sequenced the joints of the resealed transposon donor plasmid. Sequences of 42 donor plasmid joints showed 3 different sequences. In 45% of the cases the sequence revealed a loss of 6 nts from the left flanking DNA, and in 55% of the cases 6 nts (36%) or 7 nts (19%) of the right flanking DNA were lost on sealing the transposon flanks (Fig. 5B and C).

These results showed that on mini-Tn1549 excision transposon DNA is excised together with the transposon, with sequences from the left or right flank captured at similar frequencies. In all cases, exactly 6 nts from the left flank were captured, whereas the number of nucleotides captured from the right flanking DNA could vary between 6 and 7 nts.

The flanking DNA sequences on the left and the right sides of the transposon play different roles in excision

Based on our observation that flanking DNA was excised with the mini-transposon, we wanted to investigate the role of these sequences in transposon excision. First, we made several mini-Tn1549 transposon constructs, in which we removed different parts of the CIs. In 46% of the cases the sequence revealed a loss of 6 nts from the left flanking DNA, and in 49% of the cases 6 nts (34%) or 7 nts (15%) of the right flanking DNA were lost on sealing the transposon flanks (Fig. 5B and C).

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faecalis-derived flanking DNA (Fig. 6, Supporting Information Figs. S4 and S5). Remarkably, on removing the entire flanking DNA on both ends no excision was detected (Fig. 6A, construct Fmt1, Supporting Information Fig. S5A, lane 12). However, leaving just 12 nts of both flanks was enough to enable efficient and precise mini-Tn1549 excision (Fig. 6A, construct Fmt2), as we observed PCR product corresponding to the

**Fig. 4.** Identification of the tyrosine critical for INT-based excision.
A. Sequence alignment of the C-terminal part of tyrosine recombinases from different systems (see Fig. S2 for the complete alignment). Amino acid numbering for the Tn1549_INT is provided above the sequences. Tyrosine recombinases from the Tn916-like family (marked with a dot on the left) contain two conserved tyrosines (Y379 and Y380 for Tn1549_INT) marked with a box. The alignment (MUSCLE using strict CLUSTAL W 1.81) multiple sequence alignment, http://www.ebi.ac.uk/Tools/msa) includes (GenBank number is provided in brackets): Tn1549_INT (AF192329), Tn5386_INT (DQ321786.1), Tn1545_INT (X61025), Tn916_INT (U09422.1), Lambda_INT (NC_001416.1), CTnDOT_INT (CR626927.1), P1_Cre (NC_005856.1), E. coli_XerC (NC_000913.3).
B. Mini-Tn1549 excision in the presence of XIS and INT wild type (wt) or INT mutants of Y379 and/or Y380 identify Y380 as the tyrosine critical for INT based excision. The excision assay was performed with 3 hours protein induction and the CI, DP and RDP were detected by PCR and fragments were run on an agarose gel. Note the presence of faint bands at the size of the CI PCR product in some cases even at time 0. These likely result from leaky protein expression from the PBAD promoter.
C. INT binding to the Tn1549 transposon ends. Electrophoretic mobility shift assay (EMSA) was performed with different concentrations of INT on 0.07 μM DNA of the right and left ends (RE-RF and LF-LE, consisting of 25 bp of end and 25 bp flank DNA) as well as a 50 bp sequence of random DNA with a GC% similar to the end fragments.
expected CI and excision from the donor site (Supporting Information Fig. S5A, lane 11).

On removing the flanking DNA only on the right side of the transposon, excision was still detected albeit with reduced efficiency (Fig. 6A, construct RFmt1), as a considerable fraction of the donor plasmid still contained the transposon (Supporting Information Fig. S5A, lane 13). In contrast, removal of the left flanking DNA abolished proper excision (Fig. 6A, construct LFmt1) and no PCR product corresponding to the expected CI was observed. Instead, we observed a larger PCR fragment (Supporting Information Fig. S5A, lane 14), which corresponded to a CI with the transposon ends connected by approximately 650 bps instead of the usual 6 nts junction region. Sequencing of the junction regions of these large CIs (LCI) showed that they generally contained 644, 662 or 668 nts of LF DNA sequence followed by the right transposon end (Supporting Information Table S1). In addition, the 644 and 662 bps LE fragments were followed by 6 nts of the right flanking DNA before the right transposon end. These results indicated that modifications of the left flanking DNA abolished specific cleavage at the transposon LE, which in turn enabled aberrant DNA cleavage at three different positions >600 bps upstream of the left transposon end, thus joining RE to a distal position in LF and generating a CI that contains a large fragment of the donor plasmid.

Shortening the original LF DNA to just 6 nts also resulted in loss of excision (Fig. 6A, construct Fmt3, Supporting Information Fig S5A, lane 15), but leaving 12 nts of the LF DNA was sufficient to recover mini-Tn1549 excision (Fig. 6A, construct Fmt4). With this 12 nts LF segment, excision was well detectable even in the absence of any RE flank (Supporting Information Fig. S5A, lane 16), although excision was more efficient when 12 nts of the right flanking DNA were also present (Fmt2; Supporting Information Fig. S5A, lane 11).

Our observation that 12 nts of the left flanking DNA were sufficient, whereas 6 nts were insufficient for excision indicated that positions 7–12 play a critical role in transposon excision. To understand the role of these nucleotides better, we mapped the sites of DNA cleavage at both LE and RE in vitro using ‘suicide’ DNA substrates. These substrates contain a nick in the DNA backbone at various positions. When the nick is placed downstream of the cleavage site the covalent INT-DNA cleavage product is trapped and can be resolved from unmodified INT on SDS-PAGE. Nick at or upstream of the cleavage site results in no detectable product (Pargellis et al., 1988). We detected cleavage products in

Fig. 5. Flanking DNA is excised with the transposon.
A. Sequences (n = 41) of the junction region of the excised CI of mini-Tn1549. In addition to the sequences shown here two sequences of unknown origin (5%: 5'ataaat, 5'ttaaa) were found.
B. The sequence of mini-Tn1549 left and right transposon ends (LE and RE) and the left and right flanking DNA (LF and RF) in the donor plasmid (DP).
C. Sequences (n = 42) of the resealed region of the donor plasmid (RDP) after mini-Tn1549 excision. Sequences that were lost from the RDP on mini-Tn1549 excision are indicated in faint red and blue. The sequences shown in A and C were obtained by cloning PCR fragments of the CI and the RDP from four different experiments followed by sequencing of the cloned fragments from individual clones.
several conditions, further confirming the direct role of INT in catalyzing recombination. These experiments indicate that cleavage of one strand occurs near the boundary at both transposon ends, whereas cleavage of the other strand occurred 6 nts into the flanking DNA in both cases (Fig. 7). While the precise position of cleavage was unclear at the transposon end boundaries (note the presence of a small amount of cleavage product in all lanes irrespective of the positioning of the nick), cleavage in the flanking DNA mapped 5' to position 6 at both ends (note the loss of cleavage product especially on the LE top strand, Fig. 7). These cleavage positions created 6–7 nt 5'-staggered overhangs at both transposon ends. This ambiguity of cleavage site mapping is consistent with our in vivo observation that the transposon excises various lengths (6–7 nts) of flanking DNA and suggests a true ambiguity in the position of INT-mediated cleavage.

To identify which of the nucleotides position 6–12 of the LF were particularly important, we tested a number of mutations in our in vivo excision assay (Fig. 6B). We found that mutation of positions 7–9 in the LF DNA abolished excision (Fig. 6B, construct LFmt4; Supporting Information Fig. S5B, lane 16). Intriguingly, when either of these positions was mutated individually, excision was still observed with only mutation of position 7 reducing
efficiency (Fig. 6B, constructs LFm7 and 5; Supporting Information Fig. S5C, lane 13, 11). However, when both position 7 and 9 were changed accurate excision was practically abolished and only aberrant LCI excision was observed (Fig. 6B, construct LFm8; Supporting Information Fig. S5C, lane 14). Changing the corresponding nucleotides in the right flanking DNA only affected excision efficiency moderately (Fig. 6B, constructs RFm1–4; Supporting Information Fig. S5B, lane 11–13).

These data showed that specific changes to the left flanking DNA had dramatic effects on the efficiency and site of excision, whereas equivalent changes of the right flanking DNA altered excision efficiency only to a moderate extent.

**Co-operation between the left flanking DNA and the right transposon end**

On transposon excision, the transposon ends come together to form the CI. This process joins the terminal nucleotide of the right transposon end to a nucleotide 6–8 nts into the left flanking DNA and vice versa. Since we found that (i) Ts at position 7 and 9 in the LF DNA are crucial for excision in vivo and (ii) Tn\textsuperscript{1549} end cleavage occurs with a 6–7 nts stagger with one cleavage occurring near the transposon boundary and the other precisely next to position 7 in the flanking DNA, we next asked if introducing mutations at the terminal nucleotides of the right transposon end would also affect excision the same way as mutations in the LF. We tested mutations of the three terminal nts at the RE in our in vivo excision assay and found that the T at position \(-1\), the first nucleotide of the right end was essential for excision (Fig. 6B, construct REmt4 and Supporting Information Fig. S5D, lanes 10–12, 14).

As the two transposon ends need to come together in excision (Fig. 1) we also tested the effect of combining mutations of the left flank and the right transposon end (Fig. 6B, Supporting Information Fig. S5E). Intriguingly, two combinations (COmt3 and COmt4, Fig. S5E, lane 11 and 13) showed detectable levels of excision. So, those combinations of LF mutations with the RE mutant seemed to partially rescue the excision activity. The successful constructs contained compensating mutations in the left flank (position 7 or 9) and the right transposon end (position \(-1\)), indicating that the homology of the bases at these positions is important for excision.

**Bacterial in vivo assay for monitoring Tn1549 integration**

To explore integration of Tn\textsuperscript{1549} we constructed an in vivo integration assay in *E. coli* with three plasmids. Two of the plasmids, the mini-Tn\textsuperscript{1549} donor plasmid and the protein expression plasmid, were the same as in the excision assay. In addition, we introduced a third...
The conjugative plasmid pOX38-Km (an F plasmid derivative, Chandler and Galas, 1983) (Fig. 8). This conjugative plasmid was used to catch transposon integration and allow sensitive detection of integration events after conjugative transfer to a distinct recipient E. coli strain. Recipients with transferred pOX38-Km alone (transconjugants) or recipients with pOX38-Km carrying mini-Tn1549 integrations (integrants) were detected by selective plating with appropriate combinations of antibiotics (Fig. 8).

This assay also allows us to quantify integration frequency by calculating the number of integrants compared to the total number of transconjugants. We detected transconjugants in all cases where conjugations between the donor bacterium and the recipient took place. On expression of XIS and INT, integration of mini-Tn1549 into pOX38-Km occurred at well-detectable levels with an integration frequency of $1.16 \times 10^{-3} \pm 0.35 \times 10^{-3}$ integrants per transconjugants (95% confidence interval with 7 biological replicates), whereas no integration was observed when only the XIS protein or none of the proteins were expressed. Surprisingly, when only the INT protein was present, we also observed integrants with a frequency of $3.33 \times 10^{-2} \pm 2.10 \times 10^{-2}$ (95% confidence interval with 3 biological replicates), that is more than 25 times higher than the frequency obtained in the presence of both XIS and INT. This showed that mini-Tn1549 excises and integrates with only XIS and INT proteins present and provided an assay to further characterize integration.

Tn1549 integrates into various DNA sites
To determine if Tn1549 has preference for specific integration sites, we analyzed transconjugants from...
Our new heterologous in vivo integration assay described above. We identified mini-Tn1549 integration sites in the pOX38-Km plasmid by arbitrary PCR and sequencing. First, we analyzed integrants from conjugations with expression of both XIS and INT. We analyzed 51 clones from four independent experiments and identified 12 different integration sites for at least 32 independent integration events (Fig. 9A). In 7 sites, the transposon integrated with LE to RE placed in the same direction as the direction of DNA replication in pOX38-Km, in 8 sites it integrated in the reverse direction and in 3 sites integration was observed in either orientations. Together with the mini-Tn1549 transposon, additional 6 nts were integrated upstream of the left transposon end or downstream of the right transposon end. These 6 nts were identical to the DNA flanking the transposon in the original mini-Tn1549 donor plasmid. In 13 out of 17 cases the transferred donor DNA was integrated upstream of the LE and only in 4 cases we detected integration of the flanking DNA downstream of the RE. The transferred sequences originated either from RF (7 cases) or LF (10 cases) of the donor. There was no correlation between the origin of the transferred flanking DNA and its placement on either side of the transposon on integration, as we observed donor LF inserted on the right side of the transposon and vice versa. The orientation of transposon integration was also unbiased and independent of the flanking DNA sequence.

Next, we analyzed Tn1549 integration sites that resulted from conjugations with INT only. Surprisingly, sequencing from the right transposon end consistently revealed the right flanking DNA sequence as present in the mini-Tn1549 donor plasmid instead of a pOX38-Km sequence. This indicated that the mini-Tn1549 donor plasmid was co-transferred with the pOX38-Km plasmid into the recipient cells. To check this, we analyzed a number of integrants for resistance to gentamicin, a marker present on the backbone of the mini-Tn1549 donor plasmid. In assays with both XIS and INT, only 6.0% ± 2.0 (95% confidence interval, 881 colonies from seven experiments analyzed) of the integrants were resistant to gentamicin. On the contrary, with only the INT protein expressed 98% ± 1.9 (95% confidence interval, 300 colonies from 3 experiments analyzed) of the integrants were gentamicin resistant. These results confirmed co-transfer of the mini-Tn1549 plasmid, but it was unclear whether the whole mini-Tn1549 donor plasmid was integrated into the pOX38-Km or the donor plasmid simply co-conjugated to the recipient on its own. To distinguish between these co-transfer possibilities, we further analyzed 9 gentamicin resistant integrants from assays with INT only. By PCR, we first tested if the mini-Tn1549 was retained in the donor plasmid with both ends bordered by their original flanking sequences. We found that while RE remained joined to donor DNA in all 9 cases, LE did not. Subsequent sequencing of the LE showed that in all the tested clones the entire mini-Tn1549 donor plasmid had integrated into the pOX38-Km plasmid with the left end of the transposon integrating normally and the left flanking DNA serving as the right end of the enlarged ‘transposon’. Integration of the whole donor plasmid occurred at or close to the sites that we observed with normal integration of just the mini-Tn1549.

These results show that the mini-Tn1549 integrates into various sites and also inserts 6 additional nucleotides from either the LF or RF of the donor site placed mostly upstream of the LE. In the presence of both XIS and INT, the exact mini-Tn1549 is excised and integrated accurately, whereas the entire mini-Tn1549 donor plasmid is integrated when only INT is present, suggesting that XIS is important for controlling concerted excision and integration.

We also analyzed the sequences of the mini-Tn1549 integration sites. In general, the observed integration sites were AT rich (Fig. 9B). Upstream of the left transposon end, the sequence was rich in Ts, with T particularly dominating at positions 7, 9 and 10. The sequence downstream of the right transposon end was rich in As, with A dominating in position 7.

Remarkably, the consensus sequence of preferred integration sites (TTTT-N6-AAAA, with the underlined positions being most conserved, Fig. 9B) is also present at the integrated transposon termini (including transposon ends and flanking DNA, Fig. 9C), as well as in the CI junction (Fig. 9D), indicating that this sequence pattern is critical for efficient recombination during transposition. Consistently, we have shown that nucleotides at several of these positions are strictly required for transposon excision and integration.

Discussion
Our results dissect the roles of the proteins and the DNA sequences that are critical for the distinct transposition steps of Tn1549 from Enterococcus, shedding new light on its mechanism of movement and conjugative transposition in general. Our findings indicate that Tn1549 can autonomously excise and integrate in E. coli, away from its natural hosts. In the presence of just two Tn1549 encoded proteins XIS and INT we demonstrate that a mini-Tn1549 can actively transpose, and find that INT is sufficient for catalyzing transposition, whereas the excision efficiency is considerably boosted by XIS.
This critical role of INT for Tn1549 transposition is consistent with previous findings for other elements from the Tn916 conjugative transposon family, such as Tn916 and Tn1545 (reviewed in Scott and Churchward, 1995; Mullany et al., 2002; Wozniak and Waldor, 2010).

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INT proteins in Tn916-like conjugative transposons generally have two consecutive tyrosines in contrast to a single catalytic residue found in other tyrosine recombinases such as the lambda phage integrase or the Cre recombinase from the P1 phage (Pargellis et al., 1988; Gibb et al., 2010; Hickman and Dyda, 2015), but the specific functions of the two tyrosine residues were not previously known. Here, we identify the second tyrosine (Y380) as the nucleophile tyrosine, suggesting that the other one (Y379) is not essential for excision. It is possible that Y379 performs another function during transposition, which remains to be explored.

With regards to the cleavage position we found that the INT protein cleaves at ambiguous positions producing ~6 nts long 5’ overhangs at both transposon ends. Similar observations have been reported for Tn916, although different studies lead to conflicting conclusions with respect to the exact cleavage positions. Early reports predicted based on comparative analysis of various insertion sites that cleavage occurs 5 to 6 nts from the transposon end (Rudy and Scott, 1994). However, since both the transposon ends and the insertion sites are highly AT-rich, it was challenging to precisely identify the recombination sites by this approach. Later, in vitro studies (Taylor and Churchward, 1997) showed that the LE bottom strand is cleaved at the transposon boundary, whereas top strand cleavage occurs at a shifted position, 6 nts into the flanking donor DNA. Cleavage of the RE also produced 5’ overhangs, but was more ambiguous, suggesting several alternative cleavage positions (Taylor and Churchward, 1997). These results are in good agreement with our findings for Tn1549, indicating that the position of cleavage, but also its ambiguity, may be conserved in the Tn916 transposon family.

Interestingly, Tn1549 excision in vivo captures exactly 6 nts from the left flank, whereas the number of nucleotides excised from the right flanking DNA varied between 6 and 7 nts. This result is in agreement with the in vitro assays suggesting that cleavage at the two transposon ends is mechanistically similar yet distinct, which seems to be a common feature in this transposon family.

We further found that the flanking DNA at the two transposon ends have different functions: while the left flanking DNA is essential for excision, mutations in the right flank had only minor effect.

Mutating the left flanking DNA also shifted cleavage to a new position further upstream of the left transposon end, producing unusual CIs that sequester large fragments of flanking donor DNA (Fig. 10), whereas mutations in the right flanking DNA did not alter the cleavage position. These observations imply mechanistically distinct, yet coordinated and adaptable excision at the two ends.

The observed high efficiency of Tn1549 integration is similar to the ones reported for Tn1545 (Trieu-Cuot et al., 1993). We detected 12 different AT rich integration sites, with a consensus sequence of AAAA-N6-TTTT. The integration site consensus sequence pattern is highly similar to the ones reported for Tn916 and Tn1545 (Trieu-Cuot et al., 1993; Scott et al., 1994) including a recent large-scale sequencing study of Clostridium difficile (Mullany et al., 2012), attesting broad relevance of our findings.

To our surprise, we detected 25 times higher integration rates when only the INT protein was expressed, even though the absence of XIS clearly compromised excision in the excision assays. Subsequent analysis of the integration accuracy showed that XIS seems to be critical for concerted excision and integration of the two transposon ends. While LE can transpose to a new site without XIS, the correct integration of RE requires XIS (Fig. 10). The left transposon boundary region thus appears to be most critical for transposition and may in fact act as a driver of the process.

Previous experiments with Tn916 have also implicated a regulatory role for XIS in transposon excision. For example, XIS was proven to stimulate Tn916 excision (Su and Clewell, 1993) and has been shown to bind differently to the two transposon ends, enhancing excision at the left transposon end, but inhibiting excision at the right end (Hinerfeld and Churchward, 2001; Connolly et al., 2002). In Tn1549, XIS binding sites are also present near the transposon ends, but their role has yet to be unraveled. We hypothesise that XIS binds to Tn1549 DNA near the INT binding sites, helping to form a stable and active complex. Given the distinct sequences of the two transposon ends, XIS binding likely affects INT activity differently at the two ends. In the absence of XIS, RE may not be arranged properly, preventing proper excision of the transposon CI. Instead, LE acts alone directly recombining with the target DNA and integrating large parts of the flanking DNA (Fig. 10). Thus, the regulatory role of XIS is likely to be broadly conserved across Tn916-like elements, guiding both the directionality (i.e., excision or integration) and precision of INT-mediated recombination. This role of XIS is also reminiscent of propagation of the lambda phage, where accessory proteins such as XIS, IHF and FIS drive the action of the INT enzyme distinctively toward excissive or integrative recombination during the life cycle of the phage. It will be interesting to further explore the exact mechanism of XIS and its impact on Tn1549 and conjugative transposition in general.

Looking at the overall transposition process, our results show that the recombination reactions at each transposon end are unique but coupled. This feature probably ensures that the different strand exchange
reactions occur in a specific sequential order, thus driving the recombination reactions from substrates to products and enabling coordinated excision of the two transposon ends. Such control of the recombination reactions is critical to ensure efficient and accurate excision from one DNA site and successful insertion into a new DNA site, as shown for other tyrosine recombinases (Hickman and Dyda, 2015).

The mini-Tn1549 transposes with high efficiency in the Gram-negative bacterium *E. coli*, which is phylogenetically distant from its native Gram-positive hosts. This ability can probably be ascribed to its inherent simplicity requiring only two self-encoded proteins. In addition to XIS, other proteins from the host such as IHF, HU, FIS or H-NS that mainly modulate the DNA structure may also influence transposition. However, such host factor(s) should be present in both Gram-positives and *E. coli*. One example, may be an HU-like DNA binding protein present in *E. faecalis* that is similar to the HU protein in *E. coli*. The role of these host factors on transposition will also be interesting to investigate as they may play an important role in the transposition efficiency in different hosts.

Notably, several of the features discussed here for Tn1549 and the Tn916 family, such as the role of flanking DNA and specific DNA bending proteins, have also been reported in other conjugative transposon families, such as the phylogenetically distinct well-studied CTnDOT elements from Bacteroides. This indicates a broad conservation of conjugative transposition mechanisms.

Finally, we found that mini-Tn1549 can efficiently adapt to mutations in its DNA sequence. Changes to the left transposon boundary and the available proteins resulted in additional DNA being excised and transferred with the transposon (Fig. 10). This positional and sequence flexibility provides a rescue strategy for the transposon to overcome incidental mutations in its sequence. It also allows the element to capture exogenous DNA sequences from flanking genomic DNA, promoting its evolution.
Whether transposon-mediated transfer of flanking DNA is a common phenomenon in a more natural setting remains to be explored. However, our experiments show that this can happen and maybe it occurs more often than expected as this type of DNA transfer is difficult to detect and confirm through bioinformatics analysis.

The combination of the mechanistic features of Tn1549 – simplicity, host flexibility of its transposition machinery, adaptability to incidental mutations – provides a robust and adaptable system that appears to be well suited to drive gene transfer and antibiotic resistance dissemination efficiently across quite diverse bacterial populations present in nature. We envision that the mechanistic insights from our work can help tackling the emerging problem of antibiotic resistance spreading. For example, the critical roles of INT and XIS propose that inhibiting one or both of these proteins may provide a strategy to limit resistance transfer, and our results on the DNA sequence requirements of INT-mediated excision may eduate the design of specific antagonists.

**Experimental procedures**

**Strains and growth conditions**

All *Escherichia coli* strains were grown in Luria–Bertani (LB) medium with shaking at 150–170 rpm, or on LB agar plates at 37°C in ambient air. Antibiotics were provided at the following final concentrations (μg ml⁻¹): chloramphenicol (Cm) 33, gentamycin (Gm) 10, rifampicin (Rif) 50, nalidixic acid (Nal) 20 (Sigma-Aldrich), ampicillin (Ap) 100 and kanamycin (Km) 25 (Roth), when needed to select for plasmids (Supporting Information Table S3). When the strains contained a protein expression plasmid a final concentration of 0.2% glucose (D-glucose, Sigma-Aldrich) was also added to the media to repress protein expression.

Strains were grown for two hours to the mid-exponential growth phase before protein expression was induced by changing the medium to 10 ml LB with arabinose and antibiotics. Cultures were grown for two hours to the mid-exponential growth phase before protein expression was induced by changing the medium to 10 ml LB with arabinose and antibiotics and diluting the culture to OD600 of 0.1.

At specific time points, culture samples (0.5 or 1 ml) were taken for PCR or protein expression analysis. OD600 was measured and cultures were spun down at 4°C at 5000 g for 5 min. Supernatants were removed and pellets were stored at −20°C. In all experiments, except for the time course excision assay, samples were taken at time = 0 (just before shift to arabinose medium) and at 3 h after induction of protein expression.

Excision of the mini-Tn1549 transposon was detected by two PCR reactions in the following way. The excised mini-Tn1549 CI was detected by PCR with primers matching the transposon ends. In addition, the presence or absence of the mini-Tn1549 in the transposon donor plasmid was tested with PCR primers annealing to the transposon flanking DNA. (See Fig. 2C).

PCR to test for mini-Tn1549 excision: Cell pellets from −20°C were re-suspended in water (or PBS if used for testing protein expression) with correction for the OD of the sample (e.g., OD600 = 0.5, the pellet was suspended in 50 μl water). The suspensions were cooked for 10 min, cooled on ice and cell parts were pelleted by centrifugation. Finally, 0.5 μl supernatant was used as template in the PCR reaction.

PCR to detect the CI was performed using the primers P2 and P3 (see Supporting Information Table S4). PCR was performed using a reaction mix of 1 × HF-Phusion buffer, 0.4 μM dNTP, 1.5 mM MgCl₂, 0.5 mM of each primer, 0.5 μl template and 0.5 units of Phusion DNA polymerase (New England Biolabs) in a final volume of 25 μl. The reactions were run in a BioRad PCR machine at 98°C for 2 min followed by 25 cycles of 98°C at 10 s, 58°C at 10 s and 72°C at 30 s, cycles were finalized at 72°C for 2 min. Finally, the PCR products were run on a 1% agarose gel with ethidium bromide, in 1xTAE buffer (Tris-Acetate-EDTA) for 45 min to 1 h at 100 V. HyperLadder™ 1kb Plus or HyperLadder™ 1kb (Figs. 3B, SSC, E) were used as marker.

PCR to test for the presence or absence of the mini-transposon in the transposon donor plasmid was performed using the primers P1 and P4 or P46 and P47. The PCR reaction mix was the same as described above and the
reactions were run at 98°C for 2 min followed by 25 cycles of 98°C for 10 s, 55°C (with primers P1 and P4) or 60°C (with primers P46 and P47) for 10 s and 72°C for 60 s, finalized by 72°C for 2 min.

Protein expression test
To confirm protein expression of E. coli Top10 with protein expression plasmids were grown and induced as described for the excision assay. Samples stored at −20°C were defrosted on ice and re-suspended in PBS by wire mixing. The amount of PBS was adjusted to the OD of the sample defrosted on ice and re-suspended in PBS by wire mixing. Confrontation was initiated at 37°C by adding 1 ml donor and 0.5–1 ml recipient (the amount added was based on OD, so that the number of recipient was higher than the number of donors) in a 14 ml tube (only gentle mixing and no shaking). Confrontation was stopped after 1 h by wire-mixing and the cultures were thereafter kept on ice.

The amount of PBS was adjusted to the OD of the sample defrosted on ice and re-suspended in PBS by wire mixing.

Electrophoretic mobility shift assays
Binding of the INT protein to DNA was tested using Electrophoretic Mobility Shift Assay (EMSA) using a constant concentration of DNA with increasing protein concentrations. Double stranded DNA fragments of the left and right ends (LF-LE and RE-RF containing 25 bp of end and 25 bp of flank DNA, as well as 50 bp sequence of random DNA with a GC% similar to the end fragments) were formed by annealing the oligonucleotides LE_LfwB1 and LE_LfwB2 (top strand) (Table S4). In a 15 μl reaction volume (25 mM HEPES, pH 7.5, 100 mM NaCl, 5% glycerol, 1mM DTT, 10 mM MgCl₂, 1 mM EDTA) 40 μM INT and 20 μM DNA substrates were incubated for 2–4 h at 37°C. Samples were heated in SDS-containing sample buffer (0.002% (w/v) bromophenol-blue, 2% SDS, 2% (v/v) β-mercaptoethanol, 20% glycerol in 0.1 M Tris-HCl (pH 6.8)) and run on a 15% SDS-PAGE gel at 140V for 1 h 20 min. Finally, gels were stained with Coomassie G250 and visualized under white light.

E. coli in vivo mini-Tn1549 integration assay
The donor bacterium E. coli MC1061/pOXY38-Km was transformed with two plasmids, a protein expressing plasmid (PEP) with protein(s) expressed from an arabinose inducible promoter (PBad) and a plasmid containing the mini-Tn1549 as in the excision assay. Transformed donor bacteria were grown overnight in LB agar with 0.2% glucose and appropriate antibiotics (see Supporting Information Table S3). Also the recipient bacterium E. coli XA103 was grown overnight on LB agar plates with antibiotics. A few colonies from the plates were cultured overnight in 5 ml LB with glucose and antibiotics. With these overnight cultures, 10 ml cultures of donors were started at OD600 of 0.05 in LB with glucose and antibiotics. Also a 20 ml culture of the recipient was started at OD600 of 0.05 in LB with antibiotics. Donor cultures were grown for two hours to the mid-exponential growth phase. Medium was removed by centrifugation (3000 g, 10 min) and the cultures were re-suspended in LB with 0.2% arabinose (no antibiotics) to an OD600 of 0.2 and growth was continued. After 1 h growth with shaking, the shaking of the donor cultures was stopped, but the cultures were still kept at 37°C (to allow formation of pilii). Growth of the recipient continued with shaking. After 1 h, conjugation was initiated at 37°C by adding 1 ml donor and 0.5–1 ml recipient (the amount added was based on OD, so that the number of recipient was higher than the number of donors) in a 14 ml tube (only gentle mixing and no shaking). Conjugation was stopped after 1 h by wire-mixing and the cultures were thereafter kept on ice.

The conjugation cultures were diluted in PBS and several dilutions were plated on selective LB agar plates to count the number of donors (D), recipients (R), transconjugants
Determination of integration sites in the pOX38-Km plasmid

A two-step PCR procedure (one partly arbitrary PCR and one specific PCR) was used to identify the integration sites of mini-Tn1549 in pOX38-Km. This two-step PCR follows the random priming PCR strategy to amplify and clone trace amounts of DNA described in Zou et al., 2003. First, a colony-PCR was performed in a total volume of 50 µl using 25 µl MangoMix (BioLine), 1 µl primer P32 (anneals to RE) and 0.5 µl primer P44 (anneals to random sites and introduces a sequence to which a second primer used in the second PCR can bind) and a little amount of cells from a single colony. The reactions were run in a BioRad PCR machine at: 5 min 98°C, 10 cycles of 30 s at 98°C, 30 s at 30°C and 1.5 min at 72°C, 20 cycles of 30 s at 98°C, 30 s at 45°C and 1.5 min at 72°C and 10 cycles of 30 s at 98°C, 30 s at 48°C finalized by 10 min at 72°C.

From this PCR 2 µl was used as the template in the second PCR which was performed using a reaction mix of 1 × GC-Phusion buffer, 0.4 mM dNTP, 1.5 mM MgCl2, 0.5 mM, 2 µl of the primer P3 (anneals to RE) and P45 (anneals to the sequence introduced with the primer P44 in the first PCR), and 0.5 units of Phusion DNA polymerase (New England BioLabs) in a final volume of 50µl. The reactions were run at 98°C for 2 min and 10 cycles of 30 s at 98°C, 30 s at 50°C, 90 s at 72°C and 20 cycles of 30 s at 98°C, 30 s at 53°C, 90 s at 72°C, cycles were finalized at 72°C for 2 min.

PCR products were purified using a Sigma-Aldrich, GenElute PCR Clean-Up Kit, eluted in 30 µl water and Sanger sequenced with the primer P3 (GATC Biotech AG).

This identified the DNA flanking the right transposon end in the integrations site.

A similar PCR was performed to identify the DNA flanking the left transposon end using the primers, P31 and P44 in the first PCR and primers P2 and P45 in the second PCR.

The integration sites identified by the described PCR protocol were verified by designing primers that amplified the sequence surrounding the identified integration site in pOX38-Km. With these primers the DNA of the pOX38-Km plasmid surrounding the integrated mini-Tn1549 was amplified. These PCR fragments were purified and sequenced with the primers P2 and P3.

Logos showing the sequence of the pOX38-Km integration sites were made with the online tool on http://weblogo.berkeley.edu/logo.cgi.

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Author Contributions

L.L., O.B. designed the research and wrote the manuscript. L.L., A.R.C. and O.B. contributed to experimental design and data interpretation. L.L. designed and constructed plasmids and performed the in vivo experiments. A.R.C. designed oligonucleotides and performed the in vitro experiments. A.R.C. and K.R.P. contributed to discussion of the results and to writing the manuscript. All authors read and approved the final version of the manuscript.

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