Delineation of ISEcp1 and IS26-Mediated Plasmid Fusion Processes by MinION Single-Molecule Long-Read Sequencing

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We recently reported the recovery of a novel IncI1 type conjugative helper plasmid which could target mobile genetic elements (MGE) located in non-conjugative plasmid and form a fusion conjugative plasmid to mediate the horizontal transfer of the non-conjugative plasmid. In this study, interactions between the helper plasmid pSa42-91k and two common MGEs, ISEcp1 and IS15DI, which were cloned into a pBackZero-T vector, were monitored during the conjugation process to depict the molecular mechanisms underlying the plasmid fusion process mediated by insertion sequence (IS) elements. The MinION single-molecule long-read sequencing technology can dynamically reveal the plasmid recombination events and produce valuable information on genetic polymorphism and plasmid heterogeneity in different multidrug resistance (MDR) encoding bacteria. Such data would facilitate the development of new strategies to control evolution and dissemination of MDR plasmids.

Keywords: helper plasmid, ISEcp1, IS15DI, polymorphism, plasmid fusion

INTRODUCTION

Bacterial asexual reproduction is known to involve division of its genetic traits equally into daughter cells to maintain the original phenotypic characteristics. However, bacteria may also evolve by elevating genetic plasticity via horizontal gene transfer (HGT), a process in which beneficial genetic elements are acquired from different species and passed onto the offspring of the recipient strain, whereas harmful genes are prevented from being heritable (Thomas and Nielsen, 2005; Pinilla-Redondo et al., 2018). Three processes of HGT, namely transformation, transduction, and conjugation, have been described to date (Frost et al., 2005). During the transformation process, exogenous genes are passively acquired by the recipient cells and mobile genetic elements (MGE) are not involved. Transduction is often accompanied by bacteriophage invasion, yet this infection event is usually detrimental to the recipient strain (Davison, 1999). In contrast, bacterial conjugation is a much more prevalent HGT mechanism which requires an intricate molecular structure to transfer genetic materials from a donor strain to a recipient...
Conjugation plays a significant role in dissemination of antimicrobial resistance and virulence-encoding elements in bacteria (San Millan, 2018; Xie et al., 2020). Conjugative plasmids normally harbor genes that encode genetic transfer functions and the Type IV secretion system, which are essential elements that drive the conjugation events. A number of non-conjugative plasmids also encode transfer functions via cooperation with co-resident conjugative plasmids that carry the oriT sequence, or via expression of the cognate genes to produce relaxase (Miyano et al., 2018). In previous studies, we reported the discovery of helper plasmids which mediated transmission of non-conjugative plasmids and a chromosomal DNA fragment through fusion mechanism. In these co-integration processes, the insertion sequences (ISs) IS26 and ISPa40 played a critical role in molecular interaction via replicative transposition and homologous recombination, and led to a sharp increase in the incidence of Salmonella resistance to ciprofloxacin (Chen et al., 2018, 2019a,b). In addition, these ISs, particularly IS26, exhibit a vital function in the spreading of antimicrobial resistance elements among gam-negative strains, as it is normally linked with determinants that confer resistance to various categories of antimicrobial agents (Wrighton and Strike, 1987; Kim and Aoki, 1994), or some class 1 integrons (Mirigou et al., 2005). In this work, we investigated the nature of interactions between helper plasmids and MGEs during the conjugation process of Salmonella. Plasmid pSa42-91k is a 91-kbp-conjugative plasmid originated from a strain of Salmonella Meleagridis, which exhibited high homology to pSa44-CRO (MH430883) and was considered a helper plasmid. Two prevalent mobile genetic elements, ISEcp1 and IS15DI, were cloned into the pBackZero-T vector and transferred to a strain known as Sa42, which was then used as the donor strain in conjugation experiments. Recipient strains which have acquired fusion plasmids were subjected to whole genome sequence analysis. Utilizing the long-read sequencing technology, we revealed the functional role of ISEcp1 and IS15DI in mediating recombination events that enhance the genetic plasticity of the resistance-encoding plasmids during the process of conjugative transfer, and hence the dissemination potential of the resistance genes harbored by the plasmids concerned.

**MATERIALS AND METHODS**

**Cloning of ISEcp1 and IS15DI**

DNA segments were amplified using primer pairs targeting the ISEcp1 gene (ISEcp1-F-AGCGTTGGTAATGCTGAAACT and ISEcp1-R-TCCACAGAGCAACACTCAAT) and the IS15DI gene (IS15DI-F-TGTTGTTAATGCAAGCAGGG and IS15DI-R-AAGTCCGCCACATTCCGTCT). The PCR products were ligated to a cloning vector, pBackZero-T, respectively, yielding pBackZero-ISEcp1 and pBackZero-IS15DI, which were then transformed into E. coli DH5α by electroporation. All transformants were selected on LB plates containing 50 μg/mL kanamycin. Transconjugants named DH5α-ISEcp1 and DH5α-IS15DI were obtained, followed by confirmation of genetic identity through PCR screening with the pair of cloning primers described above.

**Conjugation Experiments**

The transmission potential of the ISEcp1, IS15DI and \( \text{bla}_{\text{CTX-M-130}} \) genes was assessed by performing a conjugation experiment using the filter mating method as previously described (Chen et al., 2018). Sa42, Sa42-TC1, and Sa42-TC2 were used as donor strains and the recipient strain was the sodium azide-resistant E. coli strain J53. The transconjugant Sa42-TC3 was selected on EM agar containing cefotaxime (2 μg/ml), and sodium azide (100 μg/ml). For donor strains Sa42-TC1 and Sa42-TC2 carrying different pBackZero-IS vectors, transconjugants Sa42-TC4 and Sa42-TC5 were selected on EM agar containing kanamycin (50 μg/ml) and sodium azide (100 μg/ml). Antimicrobial susceptibility of both parental strains and their transconjugants was determined (Table 1).

**Pulsed-Field Gel Electrophoresis**

Pulsed-field gel electrophoresis (CHEF-MAP-PER System, Bio-Rad Laboratories, Hercules, CA, United States) was performed to confirm the genetic identity of the parental strains and the corresponding transconjugants as described previously (Ribot et al., 2006). All plasmid-bearing transconjugants were analyzed by S1 nuclease PFGE.

**Analysis of Plasmidomes Based on Sequencing Data**

Total plasmid samples of strain Sa42 and the corresponding transconjugants were extracted using the Qiagen Midi Plasmid Kit and sequenced with two different platforms, namely the Illumina and Oxford Nanopore Technologies (ONT) MinION platform. Paired-end libraries were constructed and sequenced by using a 300 cycle Illumina NextSeq 500 Kit. Sequencing reads were assembled de novo with the SPAdes 3.5 tool (Bankevich et al., 2012). A Rapid Barcoding Sequencing Kit was used to construct the libraries sequenced in a MinION device as previously reported (Li et al., 2018). To determine the structure of the plasmids, Nanopore raw contigs containing pBackZero-T were selected by sequence homology searches with BLAST + makeblastdb (ver. 2.2.28) (Cock et al., 2015) and relevant sequences were extracted from the initial fasta files by faSomeRecords. Long reads assembled from Nanopore were used to align and join contigs acquired from Illumina assembly using the CLC Genomics Workbench v10 (CLC bio, Denmark) and then annotated by the Rapid Annotation using Subsystem Technology (RAST) version 2.0 (Aziz et al., 2008). The EasyFig tool was used to compare and visualize the structures of plasmids and long reads (Alikhan et al., 2011).

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1https://github.com/santiagosnchez/faSomeRecords
**RESULTS AND DISCUSSION**

**Research Design**

To depict the role of ISEcp1 and IS15DI (IS26) in dissemination of antibiotic resistance genes, these IS elements were first cloned into the pBackZero-T vector to obtain pBackZero-ISEcp1 and pBackZero-IS15DI. To differentiate the original ISEcp1 from the one being cloned into pBackZero-T, only a 341 bp fragment (21–361) of ISEcp1 was cloned into pBackZero-T. These two plasmids were then transfected into Sa42, a foodborne Salmonella strain, which carried a IncI1 type plasmid, pSa42-91k, that contains the mobile element IS91k/pBackZero-IS91k to assess the conjugation potential of fusion plasmid pSa42-91k. IS elements were first cloned into the pBackZero-T vector to produce pBackZero-IS91k. To differentiate the original pSa42-91k and pBackZero-IS91k, only a 530 bp fragment (21–361) of ISEcp1 was cloned into pBackZero-T. This plasmid was used as a model plasmid to study the conjugation process mediated by IS elements. Upon transformation, two transformants were obtained, namely Sa42-TC1 and Sa42-TC2, which carried pSa42-91k/pBackZero-ISEcp1 and pSa42-91k/pBackZero-IS15DI, respectively. Strains Sa42-TC1 and Sa42-TC2 were then used as donor strains and subjected to conjugation with the recipient E. coli strain J53 to obtain transconjugants Sa42-TC4 and Sa42-TC5, which were then subjected to sequencing by both Illumina and Nanopore MinION to assess the conjugation potential of fusion plasmid pSa42-91k/pBackZero-ISEcp1 and pSa42-91k/pBackZero-IS15DI, as well as to investigate the fusion process (Figure 1).

**In vitro Plasmid Fusion and Creation of a Transmission Model**

Salmonella Meleagridis Sa42 was resistant to cefotaxime (Table 1). The cefotaxime-resistance phenotype of this strain, which was conferred by blaCTX-M-130, was found to be transferable in a conjugation experiment under the selection of cefotaxime and sodium azide. S1-PFGE revealed that it carried only one plasmid of ~90 kb, which could be conjugated to E. coli J53 under the selection of ceftazidime and sodium azide to produce transconjugant Sa42-TC3, suggesting that it was a conjugative plasmid (Figure 1E). This plasmid was subjected to whole plasmid sequencing using both the Illumina and Nanopore sequencing platform to obtain the complete plasmid map. Our data showed that this plasmid was 91,229 bp in length, exhibited a GC content of 50.9%, and comprised 98 putative open reading frames (ORFs), most of which were responsible for conjugation, replication, partition and other plasmid maintenance functions. A 530 bp β-lactamase gene blaCTX-M-130 flanked by ISEcp1 was also found in plasmid pSa42-91k at position 7434–8039 (Figure 2A).

We next cloned ISEcp1 and IS15DI into the pBackZero-T vector to produce pBackZero-ISEcp1 and pBackZero-IS15DI, respectively, followed by conjugation to the pSa42-91k-bearing strain S. Meleagridis Sa42 to produce Sa42-TC1 and Sa42-TC2. The conjugation experiment was further performed to obtain Sa42-TC4 and Sa42-TC5, which were E. coli J53 which have acquired a variety of fusion plasmids formed by interaction between pSa42-91k and pBackZero-ISEcp1 or pBackZero-IS15DI. It was expected that Sa42-TC4 and Sa42-TC5 contained the fusion plasmid of pSa42-91k and pBackZero-ISEcp1 or pBackZero-IS15DI. S1-PFGE was performed on these five transconjugants, with results showing that Sa42, Sa42-TC1, and Sa42-TC2 carried only one plasmid with a size similar to that of pSa42-91k. However, Sa42-TC4 carried only one plasmid but the size was slightly larger than that of plasmid pSa42-91k, suggesting that this plasmid should be the fusion product of pSa42-91k and pBackZero-ISEcp1. Interestingly, Sa42-TC5 carried two plasmids of different sizes and both were larger than pSa42-91k. These two plasmids should be the fusion product of pSa42-91k and pBackZero-IS15DI, but exhibited different structural arrangement (Figure 1E). Antimicrobial susceptibilities were determined for Sa42, Sa42-TC1, Sa42-TC2, Sa42-TC3, Sa42-TC4, Sa42-TC5 to confirm that these transconjugants exhibited the right antimicrobial resistance.

### TABLE 1 | Phenotypic characteristics of ISEcp1 and IS15DI-bearing strains and their corresponding transconjugants.

| Strain ID | Species | Plasmids | AMK | CIP | CRO | CTX | KAN | OLA | MRP | NAL | STR | CHL | TET | AMP | CLS |
|----------|--------|----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Sa42     | Salmonella | pSa42-91k | 4   | 0.06 | >16 | >16 | 8   | 16  | 0.06 | 8   | 8   | 2   | >64 | 2   |
| J53      | E. coli | /         | 1   | 0.015 | 0.03 | 0.03 | 1   | 8   | 0.03 | 4   | 2   | 0.5 | 0.5 | 16  | 0.5 |
| DH5a     | E. coli | /         | 8   | 0.03 | <0.03 | >0.03 | 1   | 1   | 0.06 | 32  | 1   | 1   | 0.5 | 16  | 0.5 |
| DH5a-T1  | E. coli | pBackZero-IS91k | 8   | 0.03 | <0.03 | <0.03 | >128 | 1   | 0.06 | 32  | 1   | 1   | 0.5 | 64  | 0.5 |
| DH5a-T2  | E. coli | pBackZero-IS15DI | 8   | 0.03 | <0.03 | <0.03 | >128 | 1   | 0.06 | 32  | 1   | 1   | 0.5 | 64  | 0.5 |
| Sa42-TC1 | Salmonella | pSa42-91k, pBackZero-IS91k | 8   | 0.03 | >16 | >16 | >128 | 32  | 0.12 | 4   | 8   | 1   | >64 | 2   |
| Sa42-TC2 | Salmonella | pSa42-91k, pBackZero-IS15DI | 8   | 0.03 | >16 | >16 | >128 | 32  | 0.12 | 4   | 8   | 1   | >64 | 2   |
| Sa42-TC3 | E. coli J53 | pSa42-91k | 1   | 0.015 | >16 | >16 | 1   | 16  | 0.12 | 4   | 1   | 4   | 2   | >64 | 0.5 |
| Sa42-TC4 | E. coli J53 | pSa42-TC4 | 1   | 0.015 | >16 | >16 | >128 | 32  | 0.12 | 4   | 1   | 4   | 2   | >64 | 0.5 |
| Sa42-TC5 | E. coli J53 | pSa42-TC5-92k, pSa42-TC5-96k, pSa42-TC5-117k | 1   | 0.015 | >16 | >16 | >128 | 32  | 0.12 | 4   | 1   | 4   | 2   | >64 | 0.5 |

AMK, amikacin; CIP, ciprofloxacin; KAN, kanamycin; OLA, olaquindox; STR, streptomycin; CRO, cefotaxime; TET, tetracycline; CHL, chloramphenicol; NAL, nalidixic acid; AMP, ampicillin; MRP, meropenem; CLS, colistin.
phenotypes encoded by the plasmids they harbored, namely ceftazidime resistance for pSa42-91k and kanamycin resistance for pBackZero-ISEcp1 and pBackZero-IS15DI. Antimicrobial susceptibility results showed that the resistance phenotypes of these transconjugants matched well with those encoded by the plasmids that they harbored (Table 1).

**Genetic Features of Conjugative Fusion Plasmids in Sa42-TC4 and Sa42-TC5**

The plasmids harbored by *E. coli* DH5α-T1 and DH5α-T2 were sequenced by Illumina, with results showing that pBackZero-ISEcp1 from DH5α-T1 was 3,596 bp in length and contained the pBackZero-T vector and a 341 bp DNA fragment containing ISEcp1; pBackZero-IS15DI from DH5α-T2 was 4,157 bp in length, containing the pBackZero-T vector and a 820 bp DNA fragment harboring IS15DI. Both plasmids had a GC content of 47.8%, comprised 3 coding sequences (CDSs) and harbored the kanamycin resistance gene.

In a previous study, ISEcp1 was found to mediate genetic transposition events that involve homologous recombination (Zong et al., 2010). Using the Illumina and Nanopore sequencing platforms, complete sequence of the plasmid in Sa42-TC4 was obtained. The plasmid in Sa42-TC4, designated as pSa42-TC4, was a fusion plasmid of pBackZero-ISEcp1 and pSa42-91k (Figure 2). The fusion site was found to be at ISEcp1, in which a structure of ISEcp1-pBackZero-ISEcp1 was replaced by the original ISEcp1 in plasmid pSa42-91k. To differentiate between the ISEcp1 element harbored by plasmid pSa42-91k from the one we cloned to pBackZero-T, the latter was designed to be shorter than the one in pSa42-91k, which included 21–362 bp of the full length of ISEcp1 (Figure 3A). This design will lead to production of ISEcp1 with a size of 341 bp in pBackZero-T and 530 bp in pSa42-91k, respectively (Figure 3A).
FIGURE 2 | Alignment and structure of the relevant plasmids in the donor strain and transconjugant. (A) Linear alignment of plasmids pSa42-91k, pSa42-TC4, and pBackZero-ISecp1 assembled de novo by Nanopore long reads. IS elements were highlighted in yellow and drug-resistance genes were depicted in red arrows. (B) Structure of pBackZero-ISecp1 upon being merged with the plasmid pSa42-91k. Red denoted the resistance genes and yellow denoted the ISs gene.

FIGURE 3 | Mechanism of ISecp1–mediated homologous recombination depicted by analysis of Nanopore long-reads. ISecp1 was found to exhibit a size of 530 bp in pSa42-91k. A fragment of ISecp1 was cloned into pBackZero-T vector (depicted in black). Such fragment exhibited sequence homology with ISecp1 from pSa42-91k (depicted in gray). A 21 bp and 168 bp region were located at each terminal. (A) When the homologous recombination process started from the left, various sizes of ISecp1 linked with the 21 bp sequence were observed in Nanopore long reads; oppositely oriented terminal side of a 509 bp constant size could be detected in another ISecp1. (B) Homologous recombination began in the opposite direction, Nanopore long reads were also found to contain various sizes of ISecp1 at 341 bp, which were connected to a 168 bp fragment in the original ISecp1, whereas another part of 362 bp in the original ISecp1 whose size was stable and not variable was located in the other end.

Unlike ISecp1, IS15DI was found to belong to the IS6 family and exhibited genetic characteristics similar to those of IS26, which can undergo replicative transposition during the co-integration process. In this study, two different sizes of plasmids from strain Sa42-TC5 were observed by S1-PFGE, indicating that IS15DI may target different insertion sites during conjugation. Nanopore long reads sequencing was performed to solve this problem, with sequencing reads being assembled de novo by Canu
TABLE 2 | Statistical summary of MinION nanopore long reads generated from plasmid samples of strains Sa42-TC4 and Sa42-TC5.

| Sequence statistic | pSa42-TC4 reads | pSa42-TC4 reads (>50 kb) | pSa42-TC5 reads | pSa42-TC5 reads (>50 kb) |
|--------------------|-----------------|--------------------------|-----------------|--------------------------|
| No. of sequences    | 15,483          | 376                      | 19,488          | 433                      |
| Sequence length (bp)| 112,500,802     | 29,167,802               | 132,510,505     | 33,297,717               |
| Average length (bp) | 17,487          | 77,573.94                | 6,799.59        | 76,900.04                |
| N50 length (bp)     | 12,140          | 97,133                   | 14,750          | 88,039                   |
| Maximum length (bp) | 7,985           | 80,484                   | 3,532           | 55,673                   |
| Minimum length (bp) | 500             | 50,140                   | 4,93            | 50,048                   |
| (G + C)/(G + C + A + T) (%) | 49.88 | 49.85 | 49.93 | 50.11 |

Reads (>50 kb) were extracted for further analysis of IS\(\text{Ecp1}\) activities and IS\(\text{15DI}\) target sites.

(Koren et al., 2017). Complete sequencing of the large plasmid was obtained, for which a size of 117,221 bp was consistent with the S1-PFGE result. The plasmid contained 147 CDSs with a GC content of 50.8% and was designated as plasmid pSa42-TC5-117k. Compared with the original plasmid pSa42-91k, an IS\(\text{Ecp1}\) element and an IS\(\text{15DI}\)-pBackZero-IS\(\text{15DI}\) were found to be inserted into the \(\text{tra}\) and \(\text{tra}\) genes in plasmid pSa42-TC5-96k, respectively. Another fusion plasmid in strain Sa42-TC5, designated as pSa42-TC5-96k, was formed by insertion of the IS\(\text{15DI}\)-pBackZero-IS\(\text{15DI}\) into the \(\text{tra}\) gene of pSa42-91k, which was 95,624 bp in length, contained 126 CDSs and exhibited a GC content of 50.8%. However, an extra \(\sim\)92 kb plasmid could be identified from our sequencing data and was designated pSa42-TC5-92k. This plasmid could not be observed in S1-PFGE could be identified from our sequencing data and was designated as plasmid pSa42-TC5-96k, which was 95,624 bp in length, contained 126 CDSs and exhibited a GC content of 50.8%. However, an extra \(\sim\)92 kb plasmid could be identified from our sequencing data and was designated pSa42-TC5-92k. This plasmid could not be observed in S1-PFGE as its size was similar to that of plasmid pSa42-TC5-96k. Plasmid pSa42-TC5-92k was 91,784 bp in length, contained only one IS\(\text{15DI}\) inserted into the \(\text{tra}\) gene, and exhibited a GC content of 50.9%. For the fusion plasmid pSa42-TC5-117k, in addition to the \(\text{tra}\) gene, another insertion site was also observed in \(\text{tra}\) in which a nearly 20 kb DNA sequence upstream of \(\text{tra}\) was found to be repeatedly inserted beyond the \(\text{tra}\) gene, which was responsible for causing an increase in the plasmid size to around \(\sim\)117 kb.

Mechanism That Gives Rise to IS\(\text{Ecp1}\) Polymorphisms and Enhances Dissemination Potential

To depict the detailed mechanism of interaction between the IS\(\text{Ecp1}\) elements located in two plasmids, MinION nanopore long reads were generated from Sa42-TC4 and reads baring the fusion region were extracted for further analysis (Table 2). Nanopore raw fasta reads (>50 kb) were selected and the chromosomal contamination reads were removed by matching against the pSa42-91k reference plasmid using BLASTN. To check the fusion structure in each read, two genes harbored by the plasmid pBackZero-T including \(\text{bla}_{\text{TEM}}\) and \(\text{aph}(3')-\text{III}\) were used as the marker genes to filter out reads that did not span the fusion regions of IS\(\text{Ecp1}\)-pBackZero-IS\(\text{Ecp1}\). Among the Nanopore reads from sample Sa42-TC4, 256 out of 376 reads were found to harbor complete structure of IS\(\text{Ecp1}\)-pBackZero-IS\(\text{Ecp1}\) in various sizes. Different sizes of reads were selected for BLASTN analysis against relevant sequences in donor strains (Figure 3). IS\(\text{Ecp1}\) in IS\(\text{Ecp1}\)-pBackZero-IS\(\text{Ecp1}\) structures of various sizes was observed among different reads and shown to be polymorphic by Rast tools (Overbeek et al., 2013). These reads could be grouped into two categories based on alignment to two different sizes of IS\(\text{Ecp1}\), which originated from pBackZero-T and pSa42-91k, respectively. One category of reads contained a 509 bp IS\(\text{Ecp1}\), with the first 21 bp of DNA located on the right of the reads, whereas various sizes of IS\(\text{Ecp1}\) sequences ranging from 124 to 362 bp were found located at the left side of the reads (Figure 3A). IS\(\text{Ecp1}\) elements located at the left are often truncated at different sites. These data suggested that (1) fusion of pBackZero-IS\(\text{Ecp1}\) to pSa42-91k was mediated by homologous recombination; (2) the truncated IS\(\text{Ecp1}\)-pBackZero aligned to the full length of IS\(\text{Ecp1}\) at 21 bp site, which initiated the homologous recombination; and (3) the length of the homologous region varied from 103 bp to the full length of the truncated IS\(\text{Ecp1}\) (341 bp); and upon insertion of pBackZero-IS\(\text{Ecp1}\) into the fusion plasmid, the IS\(\text{Ecp1}\) was duplicated (Figure 3B). Another category of reads had a stable 362 bp region of IS\(\text{Ecp1}\) located on the left, whereas the right side of IS\(\text{Ecp1}\) exhibited polymorphism, with sizes ranging from 320~509 bp. Sequence analysis showed that the truncated region on the left covered base 1~362 of IS\(\text{Ecp1}\), yet the terminal region of the truncated IS\(\text{Ecp1}\) was located in pBackZero-IS\(\text{Ecp1}\). The right hand side of IS\(\text{Ecp1}\) existed in various sizes and always ended at position 530, even though the start sites in IS\(\text{Ecp1}\) were variable. These findings suggest that the 3′-terminal of the truncated IS\(\text{Ecp1}\) could also align with the IS\(\text{Ecp1}\) element located in a site at 362 bp in plasmid pSa42-91k, as well as the full length of IS\(\text{Ecp1}\). Homologous recombination could occur at short sequence at any site within a 152 bp fragment (from position 210 to 362) and regions up to the start codon of IS\(\text{Ecp1}\). Upon homologous recombination, the rest of IS\(\text{Ecp1}\) (from 1 to 362) will remain at the left side of the fusion plasmid (Figure 3B). Among the reads from nanopore, the most dominant reads were 362 bp of the truncated IS\(\text{Ecp1}\) at the left side and 590 bp at the right side, which could be generated through molecular interaction by targeting either the 5′- or 3′-terminal of the truncated IS\(\text{Ecp1}\) located in pBackZero-IS\(\text{Ecp1}\) or the IS\(\text{Ecp1}\) located in pSa42-91k. Based on our understanding of the homologous recombination process, we believe that both copies of IS\(\text{Ecp1}\), located in pBackZero-IS\(\text{Ecp1}\), and pSa42-91k were identical. In this case, upon homologous recombination, the
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**FIGURE 4** | Alignment of three plasmids in transconjugant Sa42-TC5 and schematic representation of the two insertion events. (A) Alignment of various plasmids harbored by strain Sa42-TC5. Plasmids pSa42-TC5-117k, pSa42-TC5-92k, and pSa42-TC5-96k were obtained from transconjugant Sa42-TC5; pSa42-TC5-117k had two target site duplications (TSD) and was assembled de novo with Canu, the slight blue, gray, and yellow color depicted various repeated regions. Plasmids pSa42-TC5-92k and pSa42-TC5-96k with one TSD were selected directly from nanopore long reads and calibrated by matching with known plasmid sequences. (B) Genetic mechanisms of insertion of IS15DI into traJ. IS15DI led to an attack on the hot spot in the traJ gene. Resolution of traJ via transposase (Tnp) mediated cointegration, which was followed by homologous recombination (HR). (C) Proposed IS element-mediated fusion in traF only via replicative transposition event at the hot spot (CTCGTGCC). Target site duplications from different genes were underlined with bold letters. The left and right inverted repeats 14 bp (RL and IRR) of IS15DI were shown in black triangles. IS15DI was denoted in black with an arrow indicating the orientation and length of the transposase gene.
ISEcp1 located at the left could become variable in size, whereas the ISEcp1 element located at the right remains full length.

**Mechanism of Generating IS15DI Polymorphisms and Enhancing Dissemination Potential**

Similarly, nanopore long reads containing IS15DI were extracted and analyzed by the aforementioned method (Table 2). Nanopore raw fasta reads (>50 kb) were also selected and chromosomal contamination reads were screened out by mapping against the pSa42-91k reference plasmid via BLASTN. Unlike ISEcp1, IS15DI was known to undergo replicative transposition during the co-integration process like IS26, but IS15DI did not exist in the original plasmid pSa42-91k, so it can be regarded as a marker for direct selection from raw reads. Among the nanopore reads from Sa42-TC5, 222 out of 433 reads were found to harbor complete IS15DI sequences. Different forms of reads of various sizes were observed in the case of ISEcp1, among which three type of reads could be identified. The first type was a structure in which IS15DI-pBackZero-IS15DI was inserted into the traf gene by replicative transposition (Figure 4C; Arthur and Sherratt, 1979). During the process, the IS15DI element cleaved both terminal inverted repeats (TIRs), resulting in the formation of nicks in both strands of DNA and generating 3-Diols groups (3′ = -OH) that attack the hot spot of the traf gene, leading to formation of a Shapiro intermediate. The DNA complex then underwent intermolecular replicative transposition, in which the target site was duplicated. The second type involved insertion of one copy of IS15DI into the traf gene. The process probably involved the insertion of IS15DI-pBackZero-IS15DI into the traf gene and then the element was excised out, leaving a single scar copy of IS15DI in the traf gene (Figure 4B). Interestingly, a 8 bp target site duplication sequence (ATACAGAT) was found to flank the IS15DI elements, indicating that this process may occur in two steps. The first step was mediated by the attack at the hot spot of the traf gene by the insertion element IS15DI, leading to insertion of IS15DI-pBackZero-IS15DI into the traf gene, followed by the excision of pBackZero-IS15DI from the fusion plasmid through homologous recombination, leaving one single copy of the IS15DI scar in traf gene (Harmer et al., 2020). This type of plasmid contained only one copy IS15DI, which existed in a circular form observable in Nanopore long reads, but the number of such plasmid was too low to be seen in S1-PFGE. The third type is more complicated. Insertion events occurred in both the traf and traf genes and duplication of a DNA fragment from the pSa42-91k led to the formation of a plasmid of a much larger size (∼117 kb) designated as pSa42-TC5-117k (Figure 4A). The mechanism of DNA duplication in this type of plasmid is not clear and entails further investigation.

**CONCLUSION**

Insertion of MDR-encoding mobile elements into the chromosome and plasmids has been widely reported following the wide usage of the whole genome sequencing (WGS) technology. With the increasing use of MinION nanopore sequencing technology and Illumina sequencing, IS element-mediated plasmid fusion events have also been increasingly reported. However, most of these previous studies only reported the final plasmid sequences based on the assembly of the most dominant reads and contigs generated from the sequencing platforms. The underlying mechanisms of the fusion and integration processes remain unknown. In this study, we have designed a set of experiments to investigate the molecular mechanisms underlying two of the most common fusion processes mediated by ISEcp1 and IS26-like elements, which involve homologous recombination and replicative transposition, respectively. Our data showed that both the 5′- or 3′-terminal of ISEcp1 could attack another copy of ISEcp1 through interaction with homologous sequences as short as 100 bp, resulting in the formation of a truncated ISEcp1. This finding helps explain why IS elements are commonly found in MDR-encoding genetic fragments. The IS15DI element mainly attacks the hot spots located in different target genes and mediates the conjugation process of plasmids that contain the traf and traf genes. The hot spot site seems to be conservative for specific IS element, whereas the type of the hot spot site determines the mode of the integration. Findings in this study showed that detailed analysis of Nanopore reads would enable us to depict the dynamic DNA fusion processes mediated by different IS. This approach can be applied to study the mechanism of DNA fusion process mediated by other important IS elements.

**DATA AVAILABILITY STATEMENT**

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, MW567497, https://www.ncbi.nlm.nih.gov/genbank/, MW567498, https://www.ncbi.nlm.nih.gov/genbank/, MW881232, https://www.ncbi.nlm.nih.gov/genbank/, MW881230, and https://www.ncbi.nlm.nih.gov/genbank/, MW881231.

**AUTHOR CONTRIBUTIONS**

KC performed sequencing and bioinformatic analysis. MX helped with conjugation and other experiments. EC, KC, and SC participated in research design and manuscript writing. SC supervised the project. All authors contributed to the article and approved the submitted version.

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