Applied Microbiology and Biotechnology (2024) 108:132
https://doi.org/10.1007/s00253-023-12858-w

GENOMICS, TRANSCRIPTOMICS, PROTEOMICS

IS*6* family insertion sequences promote *optrA* dissemination between plasmids varying in transfer abilities

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Received: 26 May 2023 / Revised: 25 September 2023 / Accepted: 13 November 2023

Abstract

Plasmids are the primary vectors for intercellular transfer of the oxazolidinone and phenicol cross-resistance gene *optrA*, while insertion sequences (ISs) are mobile genetic elements that can mobilize plasmid-borne *optrA* intracellularly. However, little is known about how the IS-mediated intracellular mobility facilitates the dissemination of the *optrA* gene between plasmid categories that vary in transfer abilities, including non-mobilizable, mobilizable, and conjugative plasmids. Here, we performed a holistic genomic study of 52 *optrA*-carrying plasmids obtained from searches guided by the Comprehensive Antibiotic Resistance Database. Among the 132 ISs identified within 10 kbp from the *optrA* gene in the plasmids, IS*6* family genes were the most prevalent (86/132). Homologous gene arrays containing IS*6* family genes were shared between different plasmids, especially between mobilizable and conjugative plasmids. All these indicated the central role of IS*6* family genes in disseminating plasmid-borne *optrA*. Thirty-three of the 52 plasmids were harbored by *Enterococcus faecalis* found mainly in humans and animals. By Nanopore sequencing and inverse PCR, the potential of the enterococcal *optrA* to be transmitted from a mobilizable plasmid to a conjugative plasmid mediated by IS*6* family genes was further confirmed in *Enterococcus faecalis* strains recovered from the effluents of anaerobic digestion systems for treating chicken manure. Our findings highlight the increased intercellular transfer abilities and dissemination risk of plasmid-borne *optrA* gene caused by IS-mediated intracellular mobility, and underscore the importance of routinely monitoring the dynamic genetic contexts of clinically important antibiotic resistance genes to effectively control this critical public health threat.

Key points

- *IS6 was prevalent in optrA-plasmids varying in intercellular transfer abilities.*
- *Enterococcal optrA-plasmids were widespread among human, animal, and the environment.*
- *IS6 elevated the dissemination risk of enterococcal optrA-plasmids.*

Keywords *optrA* · Insertion sequence · One Health · *Enterococcus faecalis* · Inverse PCR · Nanopore sequencing

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Introduction

Linezolid is a last-resort oxazolidinone antibiotic approved for use in humans to treat serious infections caused by Gram-positive organisms, including vancomycin-resistant enterococci (VRE) (Bozdogan and Appelbaum 2004). Florfenicol is a phenicol antibiotic used exclusively for the prevention and treatment of disease in animals (El Garch et al. 2016; Zhao et al. 2016). The *optrA* gene, which encodes the ATP-binding cassette ribosomal protection protein, confers cross-resistance to linezolid and florfenicol (Sharkey et al. 2016; Wang et al. 2015; Wang et al. 2018). While linezolid has never been approved for veterinary use worldwide, the intensive use of florfenicol in food-producing animals may also enhance linezolid resistance by selecting for the *optrA*

Published online: 15 January 2024

gene and its host bacteria (Wang et al. 2015; Wang et al. 2020; Zhao et al. 2016). From a One Health perspective, the prevalence of *optrA*-carrying bacterial pathogens in animals and the environment may ultimately threaten human health (Larsson and Flach 2022; Walsh 2018; Zhang et al. 2022).

Since the first report of the *optrA* gene in 2015 (Wang et al. 2015), this gene has been identified in various bacterial genomes. Chromosomal *optrA* gene is commonly found in transposons of both Gram-positive (Fan et al. 2016; Fan et al. 2017; Li et al. 2019) and Gram-negative bacteria (Tang et al. 2021), but its intercellular transfer was largely restricted. Integrative and conjugative elements (ICEs) and prophages have the potential for intercellular transfer once activated (Botelho and Schulenburg 2021; Johnson and Grossman 2015), but so far, *optrA*-carrying ICEs and prophages have only been identified in *Streptococcus suis* (Shang et al. 2019; Yang et al. 2022). In contrast, *optrA*-carrying plasmids have been extensively reported in various Gram-positive bacteria from diverse isolation sources (Almeida et al. 2020; Biggel et al. 2021; Li et al. 2016; Wang et al. 2015; Wu et al. 2022). Considering the vital role of plasmids in the acquisition and dissemination of antibiotic resistance genes (ARGs) among a wide spectrum of bacteria (Bennett 2008; San Millan 2018), the prevalence and dissemination of *optrA*-carrying plasmids may pose a greater risk to human health.

While the *optrA*-carrying plasmids are widely distributed among different hosts and sources, they vary in intercellular transfer abilities. Based on the completeness of the components required for plasmid conjugation (i.e., *oriT*, relaxase, T4CP, and T4SS), plasmids can be divided into three categories: non-mobilizable, mobilizable, and conjugative plasmids (Smillie et al. 2010). Non-mobilizable plasmids are unable to perform cell-to-cell conjugation, while mobilizable plasmids require the assistance of other DNA molecules within the cell for conjugative transfer (Smillie et al. 2010). In contrast, conjugative plasmids can spontaneously and independently undergo conjugative transfer and are generally considered to have a greater capacity to spread ARGs (Che et al. 2021; Smillie et al. 2010). Despite numerous studies reporting the presence of *optrA*-carrying plasmids, the classification of *optrA*-carrying plasmids has not been systematically summarized, and their distribution among various hosts and isolation sources has not been well-mapped.

In addition, long-read sequencing has provided complete plasmid sequences, enabling analysis of the overall genetic environments of the *optrA* gene. Highly similar genetic environments of *optrA* have been detected in different plasmids from different countries and sources (He et al. 2016; Schwarz et al. 2021), suggesting that the *optrA*-containing segments are exchanged between different plasmids. Once transmitted to plasmids with higher transfer abilities, the dissemination risk of the *optrA* gene may be amplified. While insertion sequences (ISs) are frequently found adjacent to the plasmid-borne *optrA* gene, only two ISs, namely, IS*1216E* from the IS*6* family and IS*Efa15* from the IS*21* family, have been reported to facilitate the intracellular mobility of the *optrA*-containing segments by forming translocatable units (TUs) (D'Andrea et al. 2019; Dai et al. 2023; Schwarz et al. 2021). However, due to the lack of a holistic view of the distribution of ISs adjacent to the *optrA* gene in different categories of plasmids, the diversity of ISs that mediate the intracellular mobility of the plasmid-borne *optrA* gene may be underestimated, and it is unclear which ISs play a central role in transmitting *optrA*-containing segments between different plasmid categories.

Currently, *optrA*-carrying plasmids have been reported in a variety of host bacteria (Biggel et al. 2021; Cai et al. 2021; Fan et al. 2017; Wang et al. 2015; Zhou et al. 2020), including *Enterococcus faecalis*, which is one of the leading causes of nosocomial infections (Arias and Murray 2012; Ch'ng et al. 2019). Animal manure is an important reservoir for *optrA*-carrying plasmids and their enterococcal hosts, and land application of animal manure has been reported to enrich the *optrA* gene in the environment (Wang et al. 2015; Wang et al. 2020; Zhao et al. 2016). Anaerobic digestion is a well-established technique for the treatment of animal manure (Khoshnevisan et al. 2021; Tian et al. 2021), while our previous study found that enterococcal *optrA* gene was persistently present in the effluents of both lab- and full-scale anaerobic digesters for swine manure (Yang et al. 2020). Persistence of ARGs in the environment has been linked to horizontal gene transfer mediated by mobile genetic elements (MGEs) (Liao et al. 2018; Lopatkin et al. 2017). For the *optrA* gene, its horizontal transfer mainly involves IS-mediated intracellular mobility and plasmid-mediated intercellular transfer (Brenciani et al. 2022; Schwarz et al. 2021). However, it remains unknown how the interactions between ISs and different plasmid categories facilitate the dissemination of the *optrA* gene during animal manure treatment.

This study aims to present holistic distribution patterns of *optrA*-carrying plasmids and to explore the key ISs involved in the dissemination of the *optrA* gene between different plasmids and the underlying mechanisms. Here, the *optrA*-carrying plasmids were collected from searches guided by the Comprehensive Antibiotic Resistance Database (CARD), and the IS-associated transfer patterns of the *optrA* gene between different plasmid categories were visualized by phylogenetic analysis and genomic comparison. Using Nanopore and Illumina whole-genome sequencing, we detected two novel *optrA*-carrying plasmids among 156 *E. faecalis* strains recovered from the effluents of anaerobic digestion systems for chicken manure treatment. By combining conjugation experiments, inverse PCR, and genomic analysis, the role of IS*6* family genes in disseminating the *optrA* gene between enterococcal plasmids with different

transfer abilities was highlighted. This study provides valuable insights into IS-mediated interactions in plasmid dissemination of the *optrA* gene, which not only informs the improvement of biological control against *optrA*-carrying bacterial pathogens, but also extends to mitigating the dissemination of clinically important ARGs in a One Health approach.

Materials and methods

Retrieval and analysis of *optrA*-carrying plasmids from CARD

Considering CARD's curated focus on ARGs, obtaining *optrA*-carrying plasmids through CARD-guided searches may be advantageous compared to direct searches in NCBI, as CARD provides a more targeted and relevant selection. A total of 52 non-redundant *optrA*-carrying plasmids were retrieved by searching CARD using the keyword "*optrA*" (as of Dec. 22, 2022), as described in Fig. 1. Duplicated sequences, i.e., plasmids with different accession numbers but identical sequence, were considered as a single plasmid sequence. Four components involved in plasmid conjugation, including *oriT*, relaxase, T4CP, and T4SS, were predicted by oriTfinder (Li et al. 2018b). Based on the integrity of these components, plasmids can be categorized into either conjugative, mobilizable, or non-mobilizable, as previously described (Che et al. 2021; Coluzzi et al. 2022; Garcillan-Barcia et al. 2009; Smillie et al. 2010). Relaxase and T4SS are the most essential components for plasmid classification (Coluzzi et al. 2022; Garcillan-Barcia et al. 2009). Conjugative plasmids contain all four components, while plasmids encoding relaxase but not T4SS are classified as mobilizable (Che et al. 2021; Coluzzi et al. 2022; Smillie et al. 2010). Chord diagrams were visualized using R packages "statnet" and "circlize". Phylogenetic analysis was conducted using the MAFFT online service (Version 7.505) (Katoh et al. 2019). Genetic contexts of sequence segments were compared using Easyfig Version 2.2.5 (Sullivan et al. 2011).

Bacterial strains isolated from anaerobic digestion systems for treating chicken manure

Lab-scale anaerobic digestion systems (continuous stirred tank reactor, CSTR) for treating chicken manure were constructed. Mesophilic (37 °C) and thermophilic (55 °C) anaerobic digestions were operated in parallel using the same feeding substrate (Fig. S1). The total solids (TS) and organic loading rate (OLR) were 100 g/L and 2.5 gTS/(L·day), respectively, with hydraulic retention time (HRT) of 20 days. A total of 75 and 81 *Enterococcus* strains were isolated from the mesophilic and thermophilic effluents of the anaerobic digestion systems, respectively.

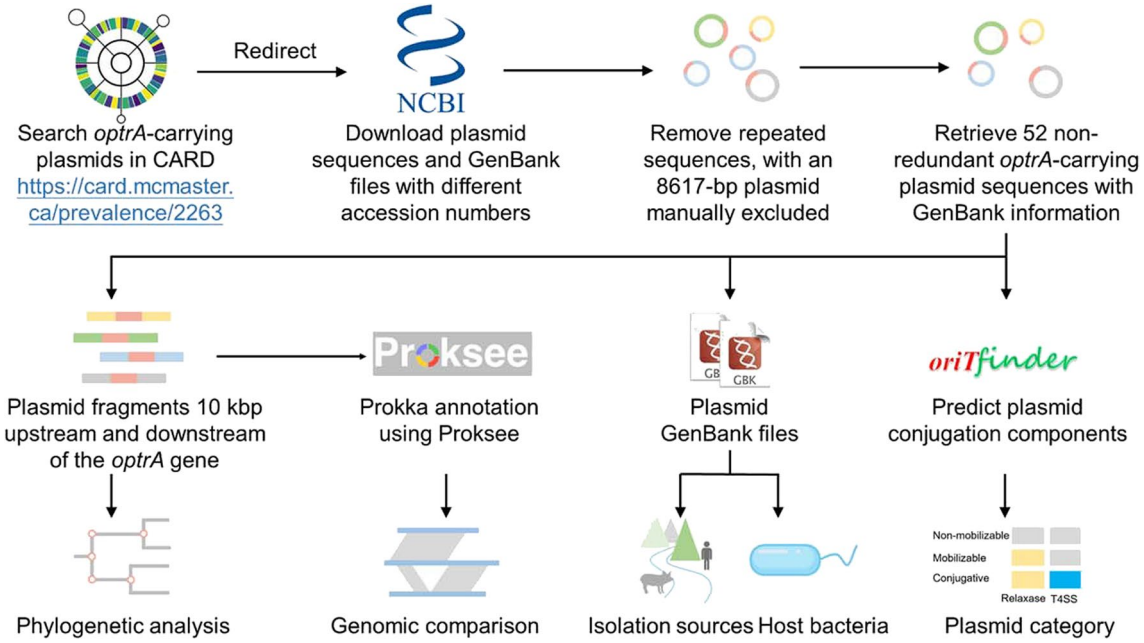


Fig. 1 Workflow for analyzing *optrA*-carrying plasmids obtained from searches guided by the Comprehensive Antibiotic Resistance Database (CARD). An 8617-bp plasmid was excluded manually for further analysis due to length limit

Illumina and Nanopore whole-genome sequencing

A total of 50 strains were randomly selected from mesophilic ($n = 25$) and thermophilic ($n = 25$) effluents for WGS using Illumina NovaSeq PE150. Oxford Nanopore MinION and Illumina NovaSeq PE150 were employed for obtaining the complete circular sequences of *optrA*-carrying *E. faecalis* of different sequence types (STs). Hybrid assembly was performed using Unicycler (Li et al. 2018a; Wick et al. 2017).

Sequence analysis of *optrA*-carrying plasmids carried by *E. faecalis* survived the anaerobic digestion

Draft genome sequences generated by Illumina short-read data were mapped against the complete plasmid sequences of pEFM9-1 and pEFT30-1 using BLAST Ring Image Generator (BRIG) (Alikhan et al. 2011). Complete sequences of plasmids were annotated and visualized using Proksee (Grant et al. 2023), an online software that integrates tools such as Prokka (Seemann 2014) and CARD Resistance Gene Identifier (RGI) (Alcock et al. 2020; Alcock et al. 2023), among others. Multi-locus sequence typing (MLST) of the 50 sequenced *Enterococcus* strains was performed using an online service provided by Center for Genomic Epidemiology (https://cge.food.dtu.dk/services/MLST/).

Conjugation experiment and stability assay

To test the transferability of *optrA*-carrying plasmids, filter mating and broth mating experiments were conducted using florfenicol-resistant *E. faecalis* strains M9 and T30 as donors and rifampicin- and fusidic acid–resistant *E. faecalis* JH2-2 as the recipient. Mueller-Hinton Agar (MHA) supplemented with 50 mg/L rifampicin, 25 mg/L fusidic acid, and 16 mg/L florfenicol was used for screening enterococcal transconjugants. The presence of *optrA* in transconjugants was validated by PCR and Sanger sequencing. Conjugation frequency was determined by normalizing the colony formation units (CFUs) of transconjugants to those of recipients (Fioriti et al. 2021). In addition, *Staphylococcus aureus* RN4220 was employed as a recipient to test the transferability of *optrA*-carrying plasmids across phylogenetically distant bacteria (Text S1). Natural transformation experiment was conducted to determine the contribution of transformation to the acquisition of *optrA*-carrying plasmids during conjugation experiments (Text S2). To assess the hereditary stability of *optrA*-carrying plasmid pEFM9-1, 30-day passages of donors and transconjugants were conducted in Brain Heart Infusion (BHI) broth with or without 16 mg/L florfenicol. Stability was evaluated by comparing resistance rates between day 1 and day 30, which was calculated by normalizing the CFUs on florfenicol-supplemented plates to those on florfenicol-free plates.

Antimicrobial susceptibility testing and PCR analysis

Using *E. faecalis* ATCC29212 as a quality control strain, antimicrobial susceptibility testing (AST) was performed to test antimicrobial phenotypes of the donor, recipient, and transconjugants. Broth microdilution was conducted following the guidelines provided by the Clinical and Laboratory Standard Institute (CLSI) (CLSI 2018). Quantitative PCR was performed to determine the abundance of the *optrA* gene in anaerobic effluents. For conventional and inverse PCR, DNA was extracted from pure cultures using Magen HiPure Bacterial DNA Kit (Magen, China). Conventional PCR was performed to test the presence of the *optrA* gene in all *Enterococcus* strains. The excision of TUs from the *optrA*-carrying plasmid pEFM9-1 and pEFT30-1 was tested using inverse PCR. All the primers are given in Table S1. Gel electrophoresis and Sanger sequencing (Sangon Biotech Co., Ltd., Shanghai) of PCR products were performed to confirm the positive amplifications.

Data availability

The Illumina-generated WGS of 50 *Enterococcus* strain can be accessed by BioProject PRJNA896765 in GenBank. The complete genomes of *optrA*-carrying *E. faecalis* strains M9, M61 and T30 were available under BioProject PRJNA846573 in GenBank. The complete sequences of the plasmids pEFM9-1 and pEFT30-1 can be accessed via accession numbers CP098744.1 and CP113829.1, respectively. Raw reads and assembled sequences of the inverse PCR products are available at https://doi.org/10.6084/m9.figshare.21638606.

Results

Characterization of *optrA*-carrying plasmids obtained from CARD-guided searches

CARD is a curated database specifically for ARGs and their genomic locations and provides timely updated links to relevant genomes uploaded to the NCBI RefSeq database (Alcock et al. 2020; Alcock et al. 2023). Following the workflow described in Fig. 1, a total of 52 non-redundant *optrA*-carrying plasmids were retrieved from CARD-guided searches. These plasmids, including two novel ones identified in this study from anaerobic digestion systems, originated from nine different countries and range in size from 25 to 246 kbp (Table S2). The host bacteria of the *optrA* gene

comprised five bacterial species that belong to the phylum Firmicutes, including *Clostridium perfringens*, *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus hirae*, and *Lactococcus garvieae* (Fig. 2a, Table S2). Among these, *E. faecalis* was the most dominant host bacteria (33/52) and was found to be widely distributed in animal-, human-, and environment-related settings, as well as in pet food (Fig. 2a, Table S2). According to plasmid classification, the 52 plasmids were categorized into 24 non-mobilizable, 19 mobilizable, and 9 conjugative plasmids. *E. faecalis* and *E. faecium* were found to harbor all three categories of plasmids, with mobilizable (14/19) and conjugative (7/9) plasmids being predominantly detected in *E. faecalis* (Fig. 2b). Detailed information on the plasmids is provided in Table S2. A total of 132 IS genes, aligned to 17 specific ISs from 8 IS families, were identified within 10 kbp upstream and downstream from the *optrA* gene (Fig. 2c, Fig. S2). Among them, 86 ISs belonging to the IS*6* family, notably IS*1216E* (63/132) and IS*Enfa1* (17/132), were the most prevalent in the vicinity of the *optrA* gene in all three categories of plasmids, particularly in the mobilizable and conjugative plasmids (Fig. 2c, Fig. S2a). It was also shown that the IS*6* family was predominantly carried by *E. faecalis* (47/63) (Fig. S2b).

The *optrA*-containing segments (~ 20 kbp) from the 52 plasmids were subjected to phylogenetic analysis using the Neighbor-Joining method. For the genomic comparison, the order of *optrA*-containing segments corresponded to that of the branches in phylogenetic tree. It was found that the IS*6* family sequences were highly prevalent in three distinct clades of the phylogenetic tree (Fig. 3). In the three clades, the overlapped genomic contexts either contained or were bracketed by IS*6* family sequences (Fig. 3). This suggested that IS*6* family sequences are likely to mediate the genetic exchange of the *optrA*-containing segments among plasmids

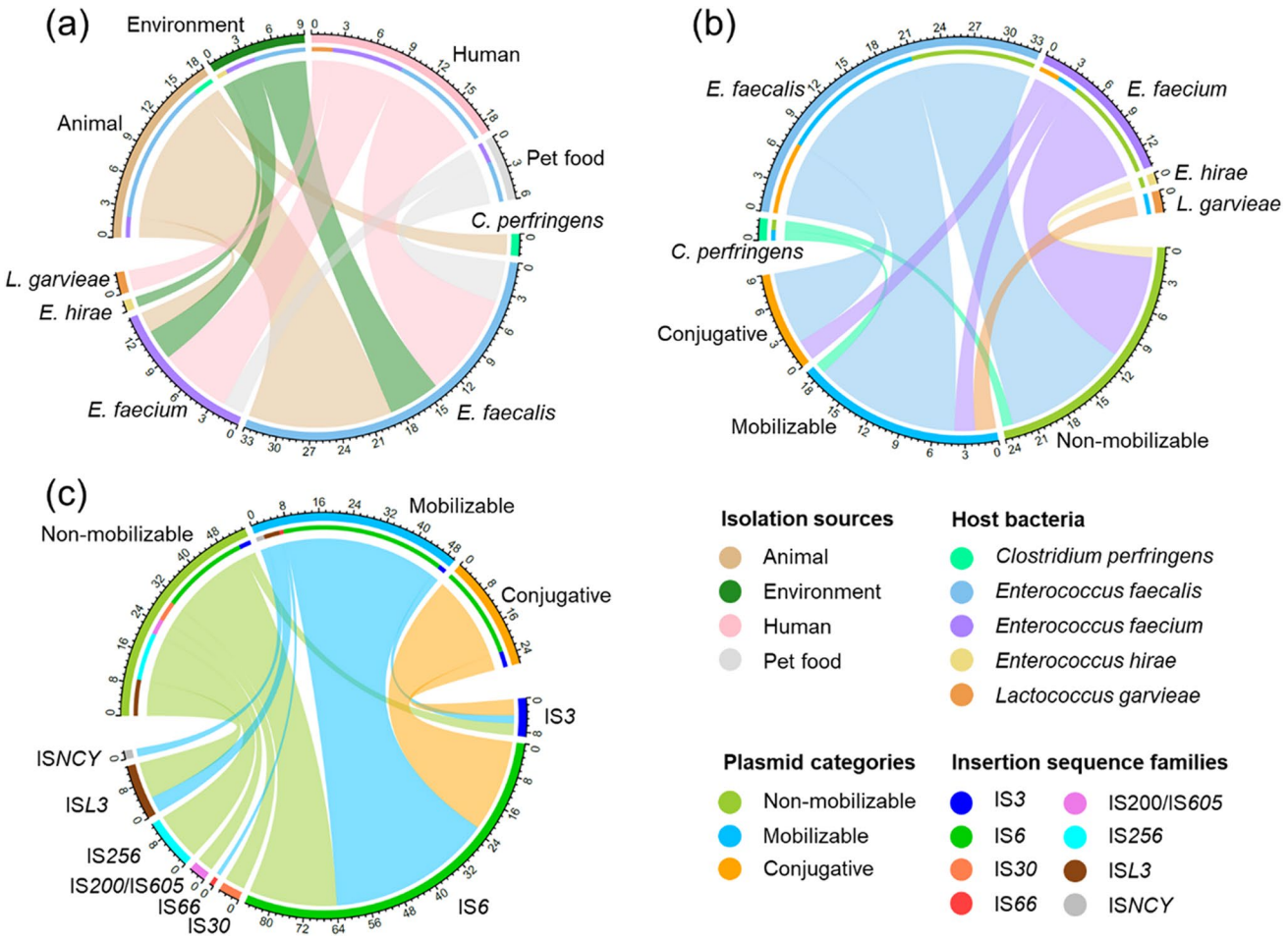


Fig. 2 Profiles of the 52 non-redundant *optrA*-carrying plasmids. **a** Distribution of host bacteria isolated from animal-, human- and environment-related settings, as well as pet food. **b** Distribution of non-mobilizable, mobilizable, and conjugative *optrA*-carrying plasmids in different host bacteria. **c** Distribution of insertion sequence (IS) families within 10 kbp from the *optrA* gene in different categories of plasmids. Distribution patterns of specific ISs are shown in Fig. S2

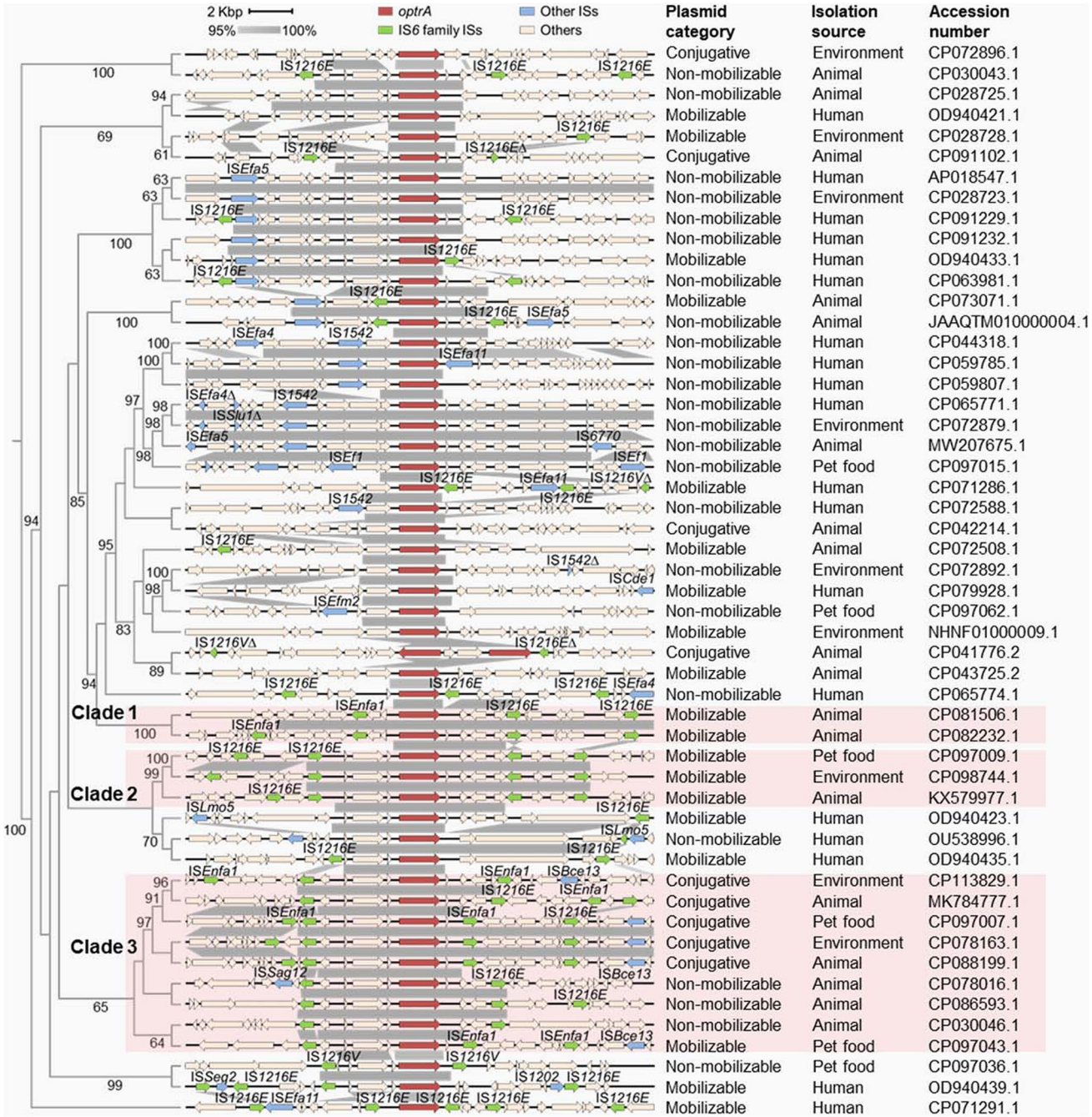


Fig. 3 Phylogenetic analysis and genomic comparison of DNA segments from 52 *optrA*-carrying plasmids. DNA segments from 10 kbp upstream to 10 kbp downstream of the *optrA* gene were selected. Segment sequences were aligned using MAFFT with FFT-NS-2 strategy. A Neighbor-Joining tree was constructed based on the 20-kbp plasmid segments, with a bootstrap value of 1000. The relative support from 1000 replications was indicated by the numbers next to the branching points, with values > 60 shown in the figure. The segments were annotated using Prokka. Genomic comparison was performed using Easyfig, with arrows indicating the orientations of genes. Details of the 52 *optrA*-carrying plasmids and selected segments are provided in Table S2

within these respective clades. Clade 1 consisted of two segments from mobilizable plasmids originating from animals. Clade 2 contained three segments from mobilizable plasmids with origins in animals, the environment, and pet food. Finally, Clade 3 included five segments from conjugative, one segment from mobilizable, and three segments from non-mobilizable plasmids with origins in animals, the environment, and pet food. It is noteworthy that IS*6* family sequences were also found to be prevalent in several other clades. Nevertheless, the majority of these IS*6* family

sequences were situated outside of the overlapped regions. While these IS*6* family sequences might have contributed to the dissemination of *optrA*-containing segments, they might not be involved in the most recent transmission events.

The *optrA* gene and its enterococcal hosts in anaerobic digestion effluents

The database analysis revealed that IS*6* family genes were predominant in *optrA*-carrying plasmids, with *E. faecalis* being the dominant host primarily distributed in clinical and animal-related settings. However, limited knowledge exists regarding the dissemination of the *optrA* gene carried by *Enterococcus* strains in the anaerobic digestion systems for treating animal manure. In order to further understand the role of IS*6* family genes in the dissemination of the enterococcal *optrA* gene among plasmids with different transfer abilities, a total of 156 *Enterococcus* strains were isolated from the mesophilic (37 °C) and thermophilic (55 °C) effluents of anaerobic digestion systems for treating chicken manure. No significant difference in absolute and relative abundances of the *optrA* gene was observed between mesophilic and thermophilic effluents ($P > 0.05$) (Fig. S3a). PCR assays indicated that 54 out of 75 isolated *Enterococcus* strains in the mesophilic effluent, and 46 out of 81 isolated *Enterococcus* strains in the thermophilic effluent carried the *optrA* gene, respectively (Fig. S3b). Among the isolated strains, a total of 50 strains, with 25 from mesophilic effluent and 25 from thermophilic effluent, were randomly selected for whole-genome sequencing (WGS) using Illumina short-read sequencing technology. WGS-based annotations identified one *E. faecium* and 49 *Enterococcus faecalis*, assigned to nine different sequence types (STs) by multilocus sequence typing (MLST) (Table S3). All the *optrA*-carrying strains were assigned to *E. faecalis* ST368, ST631, and ST81, and it was found that strains assigned to these three STs all carried the *optrA* gene (Fig. S3c, Table S3). Three *optrA*-carrying strains, namely, *E. faecalis* M9, M61, and T30, were selected as representative strains of the three *optrA*-carrying STs, i.e., ST368, ST631, and ST81, respectively. These strains were subjected to Nanopore long-read sequencing to obtain complete genomes. Complete sequences revealed that *optrA* is located in a 66,643-bp plasmid (designated as "pEFM9-1") of *E. faecalis* ST368 strain M9, a 65374-bp plasmid (designated as "pEFT30-1") of *E. faecalis* ST81 strain T30, and the chromosome of *E. faecalis* ST631 strain M61. Illumina-generated sequences of all the 50 *E. faecalis* strains were further mapped against the two *optrA*-carrying plasmids (Fig. S4). Mapping results indicated that pEFM9-1 was carried by all the *E. faecalis* ST368 strains (Fig. S4a and b), while pEFT30-1 was carried by the *E. faecalis* ST81 strain (Fig. S4c and d).

Characterization of enterococcal *optrA*-carrying plasmids in anaerobic digestion systems treating animal manure

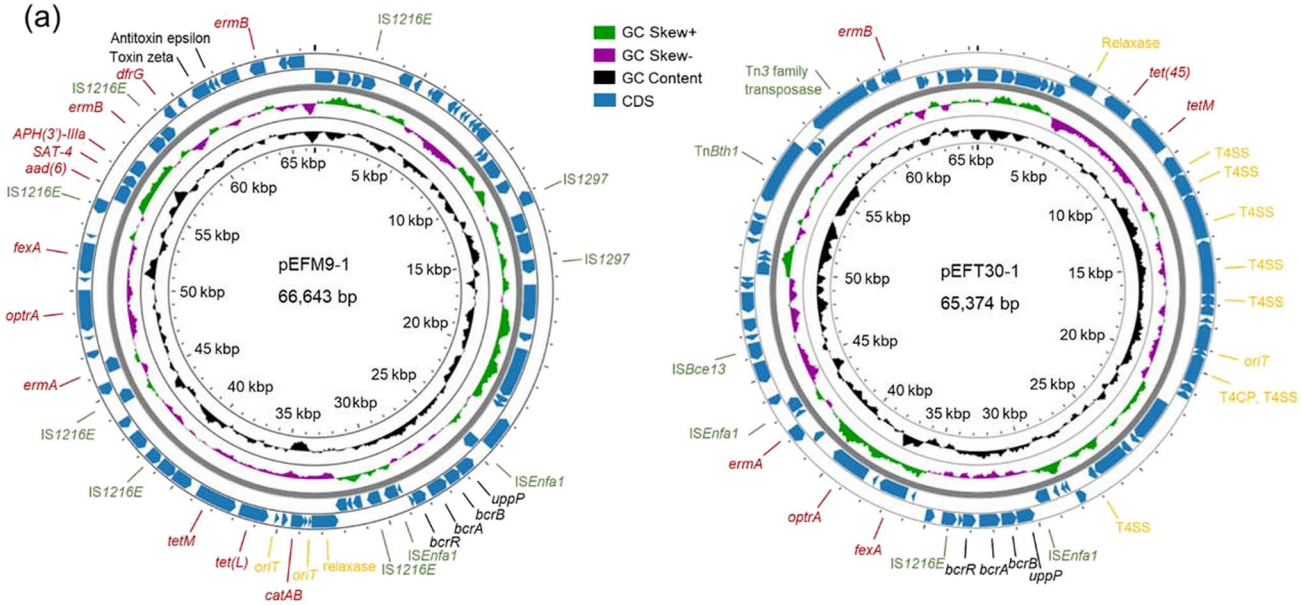
Two novel *optrA*-carrying plasmids were detected in *E. faecalis* ST368 and ST81 strains, designated as pEFM9-1 and pEFT30-1, respectively (Fig. S4). Plasmid pEFT30-1 encodes all four components, including *oriT*, relaxase, T4CP, and T4SS, and thus is categorized as a self-transmissible "conjugative plasmid" (Fig. 4a) (Che et al. 2021; Smillie et al. 2010). On the other hand, pEFM9-1 encodes only *oriT* and relaxase, and is categorized as a "mobilizable plasmid" (Fig. 4a) (Che et al. 2021; Smillie et al. 2010). We further predicted conjugation components within the genomes of *E. faecalis* M9 strain using oriTfinder. As a result, two additional plasmids were found to harbor T4CP (Table S4), which could potentially facilitate the conjugation transfer of the *optrA*-carrying plasmid pEFM9-1. It is worth noting, however, that no T4SS was found within the genomes. This absence might be attributed to the possibility that potential T4SS elements were not included in the oriTfinder database. Both filter and broth mating experiments confirmed that plasmids pEFT30-1 and pEFM9-1 can be transferred to the recipient strain *E. faecalis* JH2-2 through conjugation. The transconjugants of both plasmids showed elevated MICs of respective antibiotics compared to the recipient strain (Table S5). No transconjugants were obtained using *S. aureus* RN4220 as the recipient, indicating the two *optrA*-carrying plasmids are unable to transfer to *S. aureus* by conjugation. No transformants were obtained using *E. faecalis* JH2-2 as recipient under natural cultivation conditions, suggesting the transformation may have negligible contribution to the intraspecies conjugation frequency. The intraspecies conjugation frequency of pEFT30-1 was approximately 20 times that of pEFM9-1 on filter and 14 times that of pEFM9-1 in broth (Fig. 4b).

Although the intraspecies conjugation frequency of the mobilizable plasmid pEFM9-1 is relatively lower than that of pEFT30-1, it harbors more diverse ARGs conferring resistance to a wider spectrum of antibiotics (Fig. 4, Table S5). A 30-day stability test indicated that pEFM9-1 could be stably inherited by the offspring of the donor and transconjugant in broth culture, regardless of whether florfenicol was supplemented (Fig. S5). Additionally, pEFM9-1 is a mosaic plasmid embedded with multiple IS*6* family genes, including six copies of IS*1216E*, two copies of IS*Enfa1*, and two copies of IS*1297*.

Excision of circularizable structure flanked by IS*6* family genes in pEFM9-1 and pEFT30-1

Inverse PCR primers were designed to target three segments of pEFM9-1 and one segment in pEFT30-1 (Table S1) in

(a)



(b)

Plasmid name (Accession)	Plasmid category	Conjugation frequency (CFU/recipient cell)		Host bacteria	Bacterial sequence type	Isolation source
		Filter mating	Broth mating			
pEFM9-1 (CP098744.1)	Mobilizable	$(1.08 \pm 0.08) \times 10^{-5}$	$(1.25 \pm 0.17) \times 10^{-6}$	*E. faecalis* M9	ST368	37°C effluent
pEFT30-1 (CP113829.1)	Conjugative	$(2.20 \pm 0.30) \times 10^{-4}$	$(1.77 \pm 0.16) \times 10^{-5}$	*E. faecalis* T30	ST81	55°C effluent

Fig. 4 Enterococcal *optrA*-carrying plasmids from effluents of mesophilic (37 °C) and thermophilic (55 °C) digestion systems for treating chicken manure. **a** Circular maps of *optrA*-carrying plasmids pEFM9-1 and pEFT30-1. Antibiotic resistance genes (in red), insertion sequences (in green), and plasmid conjugation components (in yellow) were predicted by CARD RGI, Prokka, and oriT-finder, respectively. Complete sequences were obtained by performing hybrid assembly of Nanopore and Illumina sequences. **b** Details of the two *optrA*-carrying plasmids. Conjugation experiments were performed both on filter and in broth, using *E. faecalis* JH2-2 as the recipient cell

the current study. The formation of the four tested TUs was confirmed by Sanger sequencing of PCR products. As shown in the schematic diagram (Fig. S6a), "segment 1" containing *bcr* genes was circularized through recombination of two copies of IS*Enfa1*, while segments 2 and 3 containing *optrA* and multiple ARGs were circularized through recombination of two copies of IS*1216E*. The TU in pEFT30-1 containing *optrA* was formed by recombination of two copies of IS*Enfa1* (Fig. S6b). Gel images of inverse PCR products are shown in Fig. S6c.

An unnamed plasmid (accession number: LR962139.1) was retrieved by searching the sequence of pEFT30-1 against the NCBI nucleotide database. The unnamed plasmid showed perfect matching with pEFT30-1, except for a segment in the middle (Fig. 5a). This unmatched segment, which contained *optrA* and was flanked by two copies of IS*Enfa1* in pEFT30-1, was designated as the "acquired segment" (Fig. 5a). In addition, the "acquired segment" containing *bcr* genes and the *optrA* gene showed high homology with the circularizable segments 1 and 2 in pEFM9-1 (Fig. 5b), with more than 90% identity match.

Putative IS-mediated interaction between plasmids pEFM9-1 and pEFT30-1

Based on the findings, a hypothetical process of IS*6* family-mediated interactions of plasmids was proposed (Fig. 5c): An ancestral plasmid, similar to pEFT30-1 but lacking the *optrA*-containing segment, was likely capable of autonomous transfer between bacteria due to its complete conjugation components. Once this plasmid invaded an enterococcal cell that already harbored an *optrA*-carrying plasmid containing multiple IS*6* family genes, such as pEFM9-1, the *optrA*-carrying plasmid could release TUs through recombination events mediated by IS*Enfa1* or IS*1216E*. Subsequently, these TUs could integrate into the conjugative plasmid, leading to the acquisition of the *optrA* gene by the conjugative plasmid. The *optrA* gene located in the "acquired segment" of pEFT30-1 was also found to be mobilized by the two IS*Enfa1* genes in the same orientation, which facilitated further mobility of the *optrA* gene (Fig. S6b, Fig. 5c).

It should be noted that a small unmatched segment exists between the "acquired segment" and "segment 2" (Fig. 5b), suggesting that the acquisition of the *optrA* gene in pEFT30-1 may involve unknown intermediate processes. In addition, as experimental evidence supporting IS*6*-mediated integration of the *optrA*-carrying TU into the ancestral plasmid of pEFT30-1 is currently lacking, the interaction between pEFM9-1 and pEFT30-1 remains a hypothetical and potential event. However, considering that both pEFM9-1 and pEFT30-1 were detected from *E. faecalis* ST368 and ST81, respectively, in the same sample (i.e., the thermophilic effluent), and that no perfect match of the "acquired segment" of pEFT30-1 was found in the NCBI database (as of Dec. 22, 2022), the genetic exchange between pEFM9-1 and pEFT30-1 remains the most plausible hypothesis.

Genomic comparison of circularizable structure flanked by IS*6* family genes

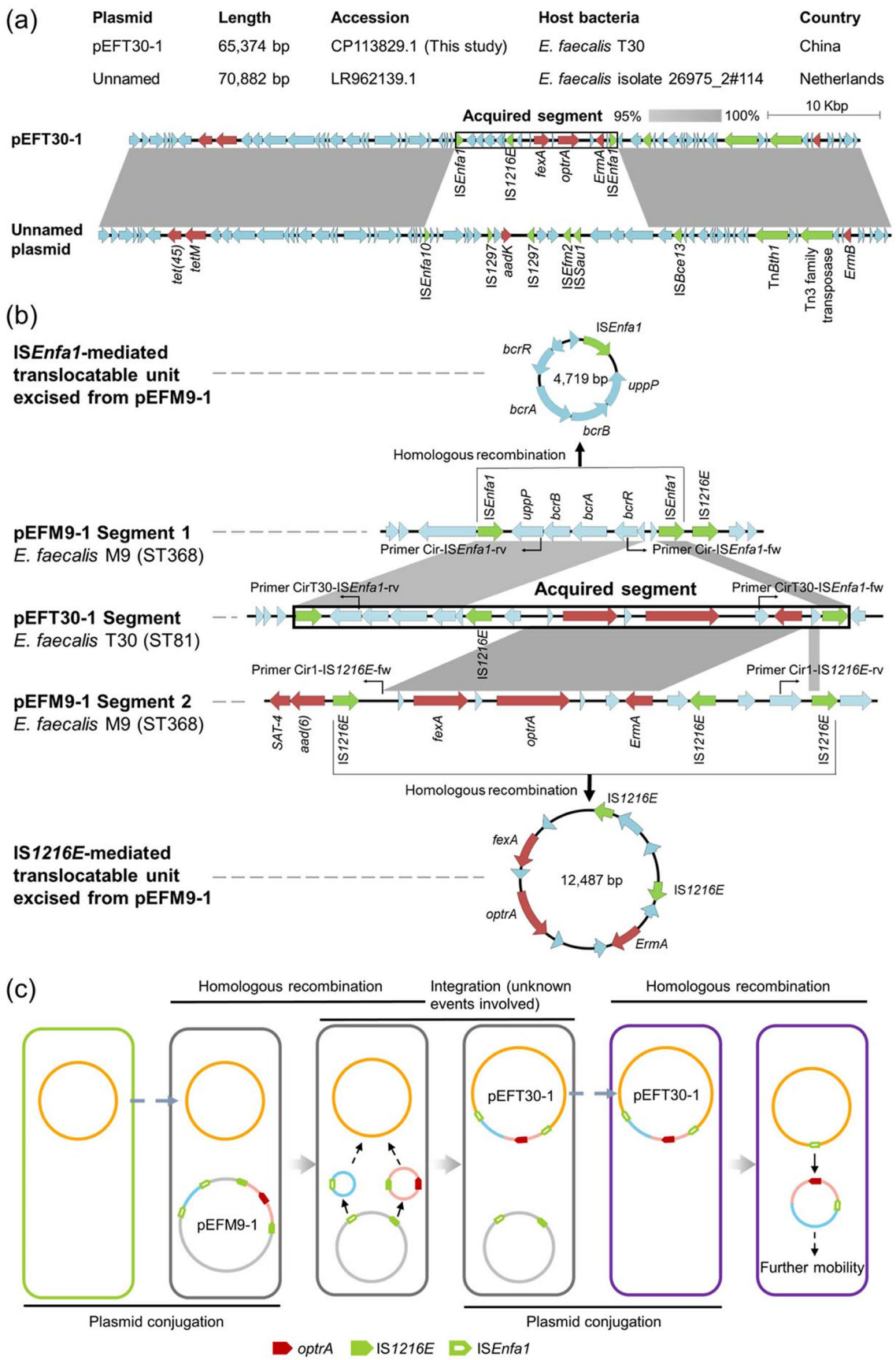
To further investigate the prevalence of IS*6* family-flanked segments, we searched the circularizable segments against the NCBI nucleotide database. To ensure representativeness, we selected only non-redundant sequences from top hits on the NCBI website as of Dec. 22, 2022. These sequences were required to share high identity with the reference sequence and are present in different host bacteria or genomic types. The results are visualized in Fig. S7. Sequences similar to "segment 1" (~ 100% coverage and identity) were identified in plasmids and chromosomes of diverse host bacteria, including *Enterococcus*, *Streptococcus*, and *Jeotgalibaca*, which were isolated from human-, animal-, and food-related samples in different countries (Fig. S7a). On the other hand, sequences perfectly matching "segment 2" were detected in plasmids of manure-borne and food-borne *E. faecalis*, with a notable finding of a "segment 2"-like sequence in the chromosome of *Fusobacterium hominis* of human origin (Fig. S7b). No highly identical matches to the "segment 3" of pEFM9-1 and the "acquired segment" of pEFT30-1 were found in the NCBI nucleotide database as of December 22, 2022.

Discussion

It has been well-established that conjugative plasmids play a key role in intercellular transfer of ARGs independently (Coluzzi et al. 2022; Smillie et al. 2010), making *optrA* gene located on conjugative plasmids of particular concern for dissemination. In contrast, non-mobilizable and mobilizable plasmids either cannot achieve cell-to-cell conjugation or require assistance from other DNA molecules, resulting in their potential to disseminate ARGs being often overlooked or underestimated. Interestingly, our study revealed that DNA segments containing the *optrA* gene with similar sequences were distributed among all three categories of plasmids, and these segments showed close phylogenetic relationships. In-depth analysis of overlapping regions of *optrA*-containing segments reveals close association between *optrA* and ISs. Key transfer units, specifically ISs-*optrA*, are conserved across various plasmid categories. Notably, this close association with ISs has significantly expanded the spread of plasmid-borne *optrA* across diverse ecological niches. For instance, IS*6*-*optrA* has been observed in isolates from animal, environmental, and human settings (Fig. 3). This implies that ISs may play a pivotal role in facilitating the genetic exchange of *optrA*-containing segments, thereby driving the dynamic evolution and dissemination of *optrA*-carrying plasmids among bacteria through a One Health approach.

Furthermore, it was found that most of the gene arrays shared by the *optrA*-containing segments, which contributed to the clustering of phylogenetic tree branches, contained or were bracketed by various ISs (Fig. 3). Among these, the IS*6* family genes were prevalent in all three categories of plasmids, and were predominant in mobilizable and conjugative plasmids (Figs. 2 and 3, and Fig. S2). This suggested that IS*6* family genes played a central role in mobilizing the *optrA* gene among plasmids. Under the mediation of ISs, the dissemination risk of the *optrA* gene may be amplified when being transmitted to plasmids with stronger transfer abilities. Once integrated into a conjugative plasmid, the *optrA* gene may spread between different strains, species, or even genera of bacteria (Schwarz et al. 2021). Therefore, although the intercellular transfer ability of non-mobilizable and mobilizable plasmids is limited, their role in disseminating *optrA* and other resistance genes cannot be ignored. It was also found that the predominant host bacteria for the *optrA*-carrying plasmids were *E. faecalis*, which were isolated not only from humans and animals but also from the environment (Fig. 2, Table S2). This suggested that *E. faecalis* could colonize diverse habitats, making it the super carrier and spreader of the *optrA*-carrying plasmids. Therefore, we hypothesize that the *optrA*-carrying plasmids harbored by *E. faecalis* can survive animal manure treatments, and IS-mediated interactions between plasmids may increase the dissemination risk of *optrA* when being discharged into the environment.

Anaerobic digestion is one of the well-established techniques for treating animal manures (Khoshnevisan et al. 2021). However, in the current study, even after anaerobic digestion with HRT of 20 days, an *optrA*-carrying plasmid, designated as "pEFM9-1" and categorized as a mobilizable plasmid, was detected in all the *E. faecalis* ST368 strains that survived in the mesophilic and thermophilic effluents (Fig. S4a and b), indicating that pEFM9-1 could be stably inherited by the offspring. The heredity stability of the plasmid pEFM9-1 was further confirmed by the 30-day passage experiment (Fig. S5) and could be genetically explained by the presence of a toxin-antitoxin


(a)
Plasmid
Length
Accession
Host bacteria
Country
pEFT30-1
65,374 bp
CP113829.1 (This study)
E. faecalis T30
China
Unnamed
70,882 bp
LR962139.1
E. faecalis isolate 26975_2#114
Netherlands
Acquired segment
95%
100%
10 Kbp
pEFT30-1
Unnamed plasmid
(b)
ISEnfa1-mediated translocatable unit excised from pEFM9-1
4,719 bp
Homologous recombination
pEFM9-1 Segment 1
E. faecalis M9 (ST368)
Primer Cir-ISEnfa1-rv
Primer Cir-ISEnfa1-fw
Primer CirT30-ISEnfa1-rv
Acquired segment
Primer CirT30-ISEnfa1-fw
pEFT30-1 Segment
E. faecalis T30 (ST81)
Primer Cir1-IS1216E-fw
Primer Cir1-IS1216E-rv
pEFM9-1 Segment 2
E. faecalis M9 (ST368)
IS1216E-mediated translocatable unit excised from pEFM9-1
12,487 bp
(c)
Homologous recombination
Integration (unknown events involved)
Homologous recombination
pEFM9-1
pEFT30-1
pEFT30-1
Further mobility
Plasmid conjugation
Plasmid conjugation
optrA
IS1216E
ISEnfa1

◂**Fig. 5** Genetic contexts of *optrA*-carrying plasmids pEFM9-1 (categorized as "mobilizable plasmid") and pEFT30-1 (categorized as "conjugative plasmid"). **a** Comparison between the complete sequences of pEFT30-1 and a plasmid deposited in the NCBI nucleotide database (accession number: LR962139.1). Plasmid pEFT30-1 shows high identity to the other plasmid, except for a unmatched segment (in the open rectangle, designated as "acquired segment") flanked by two IS*Enfa1*. **b** Comparison of the sequence segments of pEFM9-1 and pEFT30-1. Arrows in red, green, and blue indicate the position and orientation of antibiotic resistance genes, mobile elements, and other genes, respectively. Folded arrows indicate the starting position and orientation of primers for inverse PCR. Note that the sizes of translocatable units were calculated assuming a common case: Only homologous recombination of paired IS*6* family genes occurred. **c** A putative illustration for the IS*6* family-mediated intracellular mobility and plasmid-mediated intercellular transfer of the *optrA* gene. Same-colored rounded rectangles represent the same cell. Processes validated by inverse PCR are indicated by solid black arrows, while putative processes are indicated by dotted arrows. Circles in orange represent the "conjugative plasmid," while those in gray represent the "mobilizable plasmid"

(TA) system in pEFM9-1 (Fig. 4a), which is a DNA module responsible for plasmid maintenance (Partridge et al. 2018; Van Melderen and Saavedra De Bast 2009; Wein and Dagan 2020). The TA system sets a stable stage for the *optrA*-carrying plasmid to interact with other DNA molecules in the host cell, favoring the dissemination of *optrA* even under harsh anaerobic digestion conditions. At the same time, another *optrA*-carrying plasmid, designated as "pEFT30-1," was detected in *E. faecalis* ST81 from the thermophilic effluent. The pEFT30-1 was categorized as a self-transmissible conjugative plasmid, with conjugation frequency approximately 14–20 times that of the mobilizable plasmid pEFM9-1 (Fig. 4b). This indicated that pEFT30-1 was more efficient in horizontally transferring the *optrA* gene compared to pEFM9-1.

Interestingly, interactions between pEFM9-1 and pEFT30-1 mediated by IS*6* family genes might have occurred, which was inferred based on at least three hints. Firstly, an unnamed plasmid deposited in NCBI showed a perfect match with pEFT30-1, except for an *optrA*-containing segment flanked by IS*Enfa1* (arranged as IS*Enfa1-uppP-bcrBAR*-IS*1216E-fexA-optrA-ermA*-IS*Enfa1*) (Fig. 5a). This suggested that pEFT30-1 and the unnamed plasmid shared a common ancestral plasmid, and the IS*Enfa1*-flanked *optrA*-containing segment was likely acquired, hence referred to as the "acquired segment." Secondly, the IS*Enfa1-uppP-bcrBAR*-IS*Enfa1* segment was found to be present in various host bacteria from different origins (Fig. S7a). This suggested that the segment was relatively conserved, and thus, the arrangement of the "acquired segment" in pEFT30-1 was likely formed by inserting an IS*1216E-fexA-optrA-ermA* segment into the IS*Enfa1-uppP-bcrBAR*-IS*Enfa1* segment (Fig. 5b). Thirdly, the IS*Enfa1-uppP-bcrBAR*-IS*Enfa1* segment (segments 1) and the IS*1216E-fexA-optrA-ermA*-IS*1216E*-IS*1216E* segment (segment 2) in pEFM9-1 were confirmed to form TUs by inverse PCR (Fig. S6a and c), and these circularizable segments showed high homology to the "acquired segment" in pEFT30-1 (Fig. 5b). As there were no paired IS*1216E* genes in the "acquired segment," the IS*1216E-fexA-optrA-ermA* segment was unable to excise from pEFT30-1 in principle. Hence, if it occurred, the *optrA* gene would be transmitted unidirectionally from the mobilizable plasmid pEFM9-1 to the conjugative plasmid pEFT30-1, amplifying the risk of *optrA* dissemination. Additionally, the *optrA* gene in the conjugative plasmid pEFM9-1 was also confirmed to be mobilized by recombination of two copies of IS*Enfa1* (Fig. S6b and c), which was first reported, enabling further mobility of the *optrA* gene between genomes. It is worth noting that there is a lack of evidence regarding the direct interaction between *optrA*-carrying plasmids and the IS*6*-mediated integration of *optrA*-carrying TUs. In order to capture the dynamic interactions mediated by ISs in the *optrA* transfer process, it is imperative to conduct more comprehensive whole-genome screenings across a broader spectrum of isolates in the future study.

In our study, the role of IS*6* family genes in amplifying the risk of horizontal transfer of the plasmid-borne *optrA* gene was highlighted. However, it is noteworthy that the IS*6* family genes may also facilitate the genetic exchange of various clinically important ARGs, including but not limited to the *optrA* gene (Che et al. 2021; Dai et al. 2023; Partridge et al. 2018; Schwarz et al. 2021; Shan et al. 2020). This exchange can occur not only between plasmids but also between other genomic elements such as chromosomes (Fig. S7b) and ICEs (Shang et al. 2019; Yang et al. 2022). This suggests that the IS*6* family genes play a significant role in the dissemination of antibiotic resistance genes. Therefore, routine surveillance to capture the dynamic IS-mediated mobility of ARGs is essential for understanding and controlling the dissemination of antibiotic resistance from a One Health perspective.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00253-023-12858-w.

Author contribution SL and YZ conceived and designed research. SL and XY conducted experiments. RL and SW contributed new reagents or analytical tools. SL analyzed data. SL wrote the manuscript. YZ, RL, ZH, and MY modified the manuscript. All authors read and approved the manuscript.

Funding This study was supported by National Natural Science Foundation of China (No. 32141002).

Data availability All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval This work does not involve any human participants nor live animals performed by any of the listed authors.

Conflict of interest The authors declare no competing interests.

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