tISCpe8, an IS1595-family lincomycin resistance element located on a conjugative plasmid in Clostridium perfringens

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Short title: Analysis of tISCpe8

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**ABSTRACT**

*Clostridium perfringens* is a normal gastrointestinal organism that is a reservoir for antibiotic resistance genes and can potentially act as a source from which mobile elements and their associated resistance determinants can be transferred to other bacterial pathogens. Lincomycin resistance in *C. perfringens* is common and is usually encoded by *erm* genes that confer macrolide-lincosamide-Streptogramin B (MLS) resistance. In this study we have identified strains that are lincomycin resistant but erythromycin sensitive and have shown that the lincomycin resistance determinant was plasmid-borne and could be transferred to other *C. perfringens* isolates by conjugation. This plasmid, pJIR2774, is the first conjugative *C. perfringens* R-plasmid to be identified that does not confer tetracycline resistance. Further analysis showed that resistance was encoded by the *InuP* gene, which encoded a putative lincosamide nucleotidyltransferase, and was located on tISCpe8, a functional transposable genetic element that was a member of the IS1595 family of transposon-like insertion sequences. This element had significant similarity to the mobilizable lincomycin resistance element tISSag10 from *Streptococcus agalactiae*. Like tISSag10, tISCpe8 carries a functional origin of transfer within the resistance gene, allowing the element to be mobilized by the conjugative transposon Tn916. The similarity of these elements and the finding that they both contain an oriT-like region supports the hypothesis that conjugation may result in the movement of DNA modules that are not obviously mobile since they are not linked to conjugation or mobilization functions. This process is likely to play a significant role in bacterial adaptation and evolution.
INTRODUCTION

There has been increasing concern about the emergence of multiply antibiotic resistant strains of many common bacterial pathogens. The development of multiple resistance phenotypes has already led to compromises in the ability to successfully treat infected patients and to increased treatment costs (15). The emergence of resistant bacteria is often the result of excessive or inappropriate use of antibiotics and the ability of antibiotic resistance genes to be transferred from resistant to susceptible bacteria, either within a bacterial species, between different species within the same genus, or between different genera (14). Different types of mobile genetic elements, including conjugative plasmids, conjugative transposons, mobilizable plasmids, mobilizable transposons, non-conjugative plasmids and integrons, may encode the resistance genes (14). All of these elements have the ability to mediate the transfer of resistance genes within and between bacterial cells, either independently or cooperatively, which has significant implications for the transfer and evolution of antibiotic resistance, particularly in pathogenic bacterial species.

Clostridium perfringens is a normal gastrointestinal organism that causes food poisoning, necrotic enteritis and gas gangrene (29). It is a proven reservoir for antibiotic resistance determinants. For example, the catP chloramphenicol resistance determinant, which is located on the Tn4451/Tn4453 family of integrative mobilizable elements in C. perfringens and Clostridium difficile, has been detected in clinical isolates of Neisseria meningitidis (20, 23, 41). Similarly, genetically related variants of the macrolide-lincosamide-Streptogramin B (MLS) resistance determinant Erm(B) from C. perfringens are found in Enterococcus faecalis, Streptococcus agalactiae and C. difficile (19). It is likely that the C. perfringens determinant is the progenitor of the C. difficile determinant (18, 19, 44). Significantly, both determinants can be transferred into recipient cells by conjugation,
although the process is different (12, 19, 43). The pathogenic clostridia also carry other uncharacterized MLS resistance determinants and can potentially act as a source from which these resistance determinants may be transferred to other bacterial pathogens (10, 18).

Lincomycin belongs to the lincosamide group of antibiotics, which also includes clindamycin. The spectrum of activity of lincosamides predominantly encompasses Gram-positive bacteria and these antimicrobial agents are often used for the treatment of infections caused by anaerobic bacteria (45). These antibiotics inhibit protein synthesis by blocking the peptidyltransferase site of the 23S rRNA component of the 50S subunit of the bacterial ribosome (17). Although cross-resistance to MLS antibiotics most commonly involves N6 dimethylation of the A2058 residue of 23S rRNA, and is catalyzed by an \textit{erm}-encoded rRNA methyltransferase (24, 34, 47), specific resistance to the lincosamides is the result of modification and inactivation by a lincosamide nucleotidyltransferase encoded by the \textit{lnu} (previously \textit{lin}) gene family (5, 34, 45). This type of resistance gene is found in staphylococci and streptococci, where it is often located on plasmids or transposons (5, 45).

Lincomycin resistance in \textit{C. perfringens} is relatively common, but is usually conferred as MLS resistance by \textit{erm}(B) or \textit{erm}(Q) genes (10, 11). Recent studies have shown an increase in lincomycin resistance in \textit{C. perfringens} strains isolated from chickens in Belgium (28). These researchers reported two strains that conferred resistance to lincomycin and carried the \textit{Inu}(A) or \textit{Inu}(B) genes, the first such strains reported for \textit{C. perfringens}.

In the current study we analyzed several multiply antibiotic resistant isolates of \textit{C. perfringens} and identified strains that were lincomycin resistant but were susceptible to erythromycin. We have characterized these isolates and their lincomycin resistance determinant(s) and showed that resistance could be transferred to other \textit{C. perfringens} isolates. Detailed analysis of the lincomycin resistant strain 95-949 showed that resistance
was encoded by the \textit{lnuP} gene, which was encoded by a transposable genetic element tIS\textit{Cpe8} that was located on a conjugative plasmid, pJIR2774. This plasmid is the first conjugative \textit{C. perfringens} R-plasmid to be identified that does not confer tetracycline resistance.

\textbf{MATERIALS AND METHODS}

\textbf{Bacterial strains and growth conditions.} Bacterial strains and plasmids are listed in Table 1. \textit{C. perfringens} strains were cultured at 37°C in TPG (Trypticase-peptone-glucose) broth (36), BHI (brain heart infusion) broth or agar (Oxoid), FTG (fluid thioglycolate medium, Difco), or nutrient agar (35) supplemented with the following antibiotics where appropriate: lincomycin (25 µg/ml), rifampicin (20 µg/ml), nalidixic acid (20 µg/ml) and streptomycin (100 µg/ml). Solid medium containing 1% vol/vol of potassium chlorate was made by the addition of a saturated potassium chlorate solution to the medium prior to dispensing (22). \textit{C. perfringens} cultures were grown in an atmosphere of 10% H$_2$, 10% CO$_2$ and 80% N$_2$, at 37°C in anaerobic jars (Oxoid). The \textit{Escherichia coli} strain DB10 (16) was used as the recipient for the cloning of the lincomycin resistance gene and the resultant derivative was cultured aerobically at 37°C in 2×YT media (30) in the presence of lincomycin (25 µg/ml) and ampicillin (100 µg/ml).

\textbf{Lincomycin resistance transfer experiments.} \textit{C. perfringens} to \textit{C. perfringens} conjugation experiments were performed as follows. Donor and recipient strains were incubated in FTG broth for 6 h before 100 µL aliquots of both cultures were mixed by spreading onto a thick BHI agar plate without antibiotics. These mating plates were subsequently incubated anaerobically overnight at 37°C, the bacterial growth removed with 2
to 3 ml of dilute (1 in 10) BHI broth, serially diluted, plated onto appropriate selective medium and incubated for 24 to 48 h. Donor and recipient controls were included in each conjugation experiment and donor viable counts were used to calculate the transfer frequency, which was defined as the number of transconjugants per donor cell.

DNA isolation and molecular techniques. Plasmid DNA from *E. coli* was obtained using QIAprep spin miniprep columns (QIAGEN) according to the manufacturer’s instructions. Total DNA and plasmid DNA were obtained from *C. perfringens* as described previously (25). *E. coli* (38) and *C. perfringens* (39) transformations were also performed as described previously. Standard methods for the digestion, ligation and analysis of plasmid DNA and PCR products were used (38). PCR products for nucleotide sequencing were purified from agarose gels with the QIAquick Gel Extraction Kit (QIAGEN).

PCR was performed using Taq DNA polymerase (Roche) and 0.5 µM of each primer. Denaturation (94°C for 30 s), annealing (50°C for 30 s) and extension (72°C for 3 to 5 min) steps were carried out for 30-35 cycles. DNA sequencing was carried out using a PRISM Ready Reaction DyeDeoxy Terminator cycle sequencing kit (Applied Biosystems), according to the manufacturer’s instructions. The oligonucleotide primers used for DNA amplification by PCR or for sequencing were as follows (all 5'-3'): JRP2033 (TAACATTCCGATACCTAAAGA, Forward primer, 3'-end of *lnuP*), JRP2034 (TACATTGACATTTTGGGCACTC, Reverse primer, 5'-end of *tnp*), JRP2036 (AAATAAAAAGAATGACCGAAT, Forward primer, 3'-end of *tnp*) and JRP2037 (GTGGTTGTTCATGACTTATT, Reverse primer, 3'-end of tIS*Cpe8*, between end of *lnuP* and tIS*Cpe8* right end). Sequence analysis was carried out on an Applied Biosystems 3730S Genetic Analyser and analysed using Sequencher™ 3.1 software (Genecodes Corporation). Bioinformatic analyses were performed at http://www.ncbi.nlm.nih.gov/ and http://npsa-pbil.ibcp.fr and included BLAST, Conserved
Domain Database (CDD), Clustal W and helix-turn-helix predictions. The GenBank accession number for the \textit{intP-dcm} region of pJIR2774 is DQ338473 and that of tIS\textit{Cpe8} is FJ589781.

**Cloning of the \textit{C. perfringens} lincomycin resistance gene.** Total DNA extracted from strain 95-949 was subjected to partial HindIII digestion prior to ligation to HindIII-digested pUC18. Ligated products were introduced into \textit{E. coli} strain DB10 by transformation and incubated at 37°C for 48 h in the presence of lincomycin and ampicillin. The resultant colonies were passaged once on the same selective medium to confirm the antibiotic resistance phenotype prior to plasmid DNA extraction, restriction analysis and sequencing.

**Southern hybridization.** \textit{C. perfringens} plasmid DNA was digested with EcoRI, subjected to agarose gel electrophoresis and then transferred to a Nylon H\textsuperscript{+} hybond membrane (Amersham) (38). Southern hybridization analysis was carried out using standard methods (25). DNA probes were digoxygenin (DIG)-labeled using random PCR labeling, according to the manufacturer's instructions (Roche). The blots were hybridized with a probe specific for \textit{InuP}, generated using primers JRP2036 and JRP2037.

**Transposition experiments.** The suicide plasmid pJIR1944 was introduced into \textit{C. perfringens} strain JIR325 by electroporation. Cells in which tIS\textit{Cpe8} had moved from pJIR1944 were obtained by selecting for lincomycin resistance. Total DNA was subsequently extracted and subjected to Southern hybridization analysis.

**RESULTS**

Lincomycin resistance in \textit{C. perfringens} is transferable and is plasmid-associated. Analysis of a collection of multiply resistant isolates of \textit{C. perfringens} revealed...
three strains that were lincomycin resistant but susceptible to erythromycin. Mixed plate matings showed that two of these strains had the ability to transfer their lincomycin resistance by conjugation. Specifically, when strains UAZ196 and 95-949 were used as donors in matings with the recipient strain CW504, respective transfer frequencies of $3.1 \times 10^{-5}$ and $1.6 \times 10^{-2}$ lincomycin resistant transconjugants per donor cell were obtained. By comparison, there was no detectable transfer of lincomycin resistance from strain EV3839 ($<1.0 \times 10^{-7}$ transconjugants per donor cell).

To determine if lincomycin resistance was linked to a mobile extrachromosomal element or if it was chromosomally located, total genomic DNA was isolated from strain 95-949 and used to transform *C. perfringens* strain JIR325 to lincomycin resistance. DNA preparations were also made from two CW504-derived lincomycin resistant transconjugants, JIR4344 and JIR4345, which were isolated from matings with 95-949, and used in similar transformation experiments. Transformants were consistently obtained, using independently isolated DNA preparations in separate transformation experiments. Several of the resultant independently derived transformants were then used as donors in conjugation experiments to the *C. perfringens* recipient strain JIR4394. Lincomycin resistance was transferred at frequencies similar to those observed from strain 95-949 (data not shown). Taken together, these results suggested that lincomycin resistance was encoded on a conjugative plasmid.

The lincomycin resistance plasmid pJIR2774 is very similar to the prototype *C. perfringens* conjugative plasmid pCW3. Plasmid DNA was isolated from strain 95-949 and Clal digests were compared with equivalent digests of pCW3, the prototype conjugative plasmid from *C. perfringens* (1). The results indicated that a large plasmid, designated pJIR2774, was present in strain 95-949 (Fig. 1A). Southern blots confirmed that the lincomycin resistance determinant was present on the largest Clal fragment of the plasmid.
As expected, a probe specific for the lincomycin resistance gene carried on pJIR2774 did not hybridize to pCW3 (Fig. 1B). The restriction digests revealed that pJIR2774 had at least two ClaI fragments in common with pCW3, fragments that were 5,055-bp and 9,744-bp in size, as determined from the pCW3 nucleotide sequence (9). These fragments flank one another and correspond to nucleotide positions 4629 to 19428 in pCW3. This region encompasses the rep gene (located from nt 13239 to 14069), which is essential for pCW3 replication and maintenance (9), suggesting that pJIR2774 may utilize a pCW3-like replication mechanism. PCR analysis using primers specific for the pCW3 rep gene confirmed that pJIR2774 encodes a similar gene (data not shown).

Comparative PCR-walking was subsequently performed on total genomic DNA isolated from strains carrying pCW3 and pJIR2774, as previously carried out on other C. perfringens conjugative plasmids (9). These experiments utilized ten overlapping primer pairs that together encompassed the eleven transfer-related genes encoded by pCW3: intP, tcpA to tcpJ. The results showed that all of these genes were present in pJIR2774, with the exception of tcpB (Fig. 2). The differences between pCW3 and pJIR2774 were confirmed by the complete nucleotide sequencing of the intP to dcm region of pJIR2774. Two small open reading frames (ORFs), orf57 and orf55, which encoded hypothetical proteins, were identified between tcpA and tcpC, and tcpE and tcpF, respectively. The amino acid sequences of the tcp-encoded products of pJIR2774 were aligned with the corresponding pCW3-derived proteins, showing that the encoded products were very closely related (Fig. 2). Overall, when the transfer region of pJIR2774 was compared to that of pCW3, a very high degree of amino acid sequence identity was observed suggesting that the mechanism of conjugative DNA transfer is likely to be very similar in pJIR2774 and pCW3. Furthermore, when combined with the results of the comparative restriction analysis of pCW3 and pJIR2774, it appears that the
entire region from nt 4,629 to 40,030 (of a total of 47,263-bp) in pCW3 is present in pJIR2774. This analysis suggests that the similarities between the two plasmids extends well beyond the transfer-related region, although pJIR2774 is clearly larger than pCW3 and does not encode tetracycline resistance.

A lincosamide nucleotidyltransferase is responsible for lincomycin resistance.

Total genomic DNA was isolated from strain 95-949, HindIII-digested and the lincomycin resistance determinant cloned into pUC18, generating pJIR1944. Sequence analysis of the insert identified a gene whose product had 60% identity to LnuC from *S. agalactiae* (Accession number AY928180). LnuC confers lincomycin resistance by encoding a lincosamide nucleotidyltransferase that converts the antibiotic into a non-toxic form (5). The high level of sequence identity suggested that the pJIR2774-encoded lincomycin resistance gene in *C. perfringens* strain 95-948, which we designated as *lnuP*, conferred resistance by a similar mechanism. PCR analysis showed that strain UAZ196 also carried *lnuP* (data not shown), but the lincomycin resistance determinant from EV3839 could not be identified.

Lincomycin resistance is associated with an IS1595-like transposable element.

Sequence analysis of pJIR1944 suggested that the *lnuP* gene was located on a putative mobile element that had two potential genes, *tnp*, encoding a potential transposase, and *lnuP* (Fig. 3). Its genetic organization was almost identical to that of tISSag10 from *S. agalactiae* (4), which is a mobilizable and transposable member of the IS1595-family of transposon-like IS elements (42). Accordingly, the *C. perfringens* strain 95-949-derived element was designated as tISCpe8. PCR analysis showed that the *lnuP* gene in strain UAZ196 is also located on a tISCpe8-like element (data not shown). Comparative analysis of the putative 348 amino acid (aa) transposase from tISCpe8 showed that it had significant sequence identity to putative transposases from *Clostridium bartletti* (99% identity, tISCa1), *Clostridium*
phytofermentans (32%, tISC\textit{Clph1}), \textit{S. agalactiae} (36%, tISSag10), and \textit{Campylobacter jejuni} (36%) (Fig. 3). The other related \textit{tnp} genes were located on similar elements or remnants of such elements (Fig. 3).

To precisely delineate the ends of tISC\textit{Cpe8}, inverse PCR was used to generate PCR products that encompassed the ends of the transposon and flanking DNA from strain 95-949 and two CW504-derived lincomycin resistant transconjugants, JIR4344 and JIR4345, which were isolated from matings with 95-949. The flanking DNA sequences were then used to generate primers that were used to amplify the insertion sites from 95-459 and CW504. These PCR products were then sequenced. It was predicted that the sequence of the tISC\textit{Cpe8} insertion site in 95-949 would be the same as that from the regions flanking tISC\textit{Cpe8} in pJIR1944. Unexpectedly, the insertion site on pJIR1944 corresponded to an intergenic region located between \textit{aprX}, a putative intracellular serine proteinase, and a predicted hypothetical protein, CPE2609, located in a chromosomal region of the parent of JIR325, strain 13 (40), not to the plasmid-derived sequence. In addition, the sequences of the PCR products representing the insertion sites were very different to that of pJIR1944 (Fig. 4A), suggesting that there must more than one insertion site in 95-949. This conclusion was confirmed by Southern hybridization analysis, which showed that multiple copies of tISC\textit{Cpe8} were present in this strain (data not shown). It was also expected that the tISC\textit{Cpe8} insertion site in strains JIR4344 and JIR4345 would also match that of 95-949, since these transconjugants inherited pJIR2774 from this parent strain. However, these insertion sites were different from each other and from those of 95-949 and pJIR1944. This result suggested that tISC\textit{Cpe8} was an active transposon that moves very readily. We postulated that upon inheriting pJIR2774, tISC\textit{Cpe8} had transposed in the new host, resulting in multiple genomic copies and locations; this suggestion was confirmed by Southern hybridization analysis (data
not shown). Note that none of these insertion sites matched pCW3-derived sequences, they all were chromosomal sequences. For example, the sequence derived from strain JIR4343 was identified as another chromosomal intergenic insertion and in JIR4344 insertion occurred toward the end of the \textit{thiM} gene, located in the middle of a putative operon.

The finding that \textit{tISCpe8} had different insertion sites in the various \textit{C. perfringens} derivatives allowed the precise delineation of the element, which was found to consist of 1964-bp and to include 24-bp imperfect inverted repeats (22 of 24 bp conserved) at the termini (Fig. 4B). \textit{tISCpe8} appears to duplicate an 8-bp target sequence at the site of insertion, like other members of the \textit{ISPna2} group within the \textit{IS1595} family (42). Furthermore, insertional specificity appears to involve an AT-rich target site (Fig. 4A). The ends of the closely related \textit{tISSag10} element, and other \textit{ISPna2} elements (42), are very similar to the ends of \textit{tISCpe8} (Fig. 4B). A similar sequence was also identified immediately upstream of the related putative transposase gene from the \textit{C. jejuni} plasmid pCG8245 as well as in \textit{C. phytofermentans} and \textit{C. bartlettii}, again upstream of putative transposase genes (Fig. 4B). In \textit{C. jejuni} the right end of the putative element could not be identified.

The first 1250 bp of \textit{tISCpe8} had 98\% and 95\% identity, respectively to \textit{tISCba1} from \textit{C. bartlettii} and an isoform from \textit{Streptococcus suis} (Fig. 3) and the last 130 bp of the same elements show 90\% and 87\% identity, respectively, to \textit{tISCpe8}. The transposase gene is at the 5\’ end of all three elements. However, despite this marked similarity, the passenger gene(s) present within each element differ. \textit{tISCpe8} encodes \textit{InuP} downstream of the \textit{tnp} gene, whereas \textit{tISCba1} encodes two additional ORFs of unknown function and the putative \textit{S. suis} element encodes a chloramphenicol acetyltransferase gene.
tIS\textit{Cpe8} is capable of transposition in \textit{C. perfringens}. The detection of several different insertion sites suggested that tIS\textit{Cpe8} readily transposes in \textit{C. perfringens}. To provide direct evidence for transposition, the tIS\textit{Cpe8}-containing suicide-plasmid pJIR1944 was introduced into \textit{C. perfringens} strain JIR325 by electroporation. Several lincomycin resistant derivatives of JIR325 were isolated from independent transformation experiments. PCR analysis confirmed that these isolates carried tIS\textit{Cpe8} independently of pJIR1944, implying that the element had transposed to a different genetic location in the new host strain. Southern blotting confirmed this conclusion and also suggested that there may be a hot-spot for tIS\textit{Cpe8} insertion in JIR325. However, in other \textit{C. perfringens} hosts, such as CW504 and 95-949, multiple insertions were found (data not shown). Two copies of tIS\textit{Cpe8} were also observed in a JIR325 transconjugant carrying pJIR2774, the plasmid on which tIS\textit{Cpe8} was initially carried (data not shown). These results confirm that tIS\textit{Cpe8} is a functional transposon that is capable of transposition in \textit{C. perfringens}.

**tIS\textit{Cpe8} contains a functional ori\textit{T} site.** tIS\textit{Sag10} has a functional origin of transfer located within the 3’-end of the \textit{lnuC} gene, allowing the element to be mobilized by the conjugative transposon \textit{Tn916} (4). A similar \textit{oriT}-like region was found within the equivalent region of the \textit{lnuP} gene of tIS\textit{Cpe8} (Fig 4C). To determine if this site was functional, we introduced tIS\textit{Cpe8} into the chromosome of JIR4225 (JIR325::\textit{Tn916}) by electroporation with pJIR1944. Subsequent matings of the resultant transformants showed that \textit{Tn916} could mobilize tIS\textit{Cpe8} into the recipient strain JIR4394. All of the resultant lincomycin resistant transconjugants were susceptible to tetracycline, whereas selection for tetracycline resistance did not yield any lincomycin resistant transconjugants. These results confirmed that tIS\textit{Cpe8} was not genetically linked to \textit{Tn916} in these isolates and provided evidence that tIS\textit{Cpe8} carried a functional \textit{oriT} site.
DISCUSSION

Conjugative plasmids from *C. perfringens* have been shown to confer tetracycline resistance (2, 3, 9, 26), enterotoxin production (13, 31) and ε-toxin production (21). All of these plasmids are closely related to the prototype tetracycline resistance plasmid pCW3 and carry the same *tcp* conjugation locus. The lincomycin resistance plasmid identified in this study, pJIR2774, is also a member of this conjugative plasmid family and represents the first conjugal *C. perfringens* resistance plasmid that does not confer tetracycline resistance. Similarly, the tIS*Cpe8* lincomycin resistance element carried on pJIR2774 is the first IS-like resistance element shown to transpose in *C. perfringens*. The only other antibiotic resistance transposons detected in *C. perfringens* confer chloramphenicol resistance and are members of the Tn4451 family of integrative mobilizable elements, the excision and insertion of which is mediated by a large serine recombinase (6).

The similarity between pJIR2774 and pCW3 includes the *rep* region, suggesting that the mechanism of plasmid replication is the same in these plasmids. Furthermore, ten of the eleven genes in the *tcp* conjugation region of pCW3 are also present in pJIR2774, including the *tcpA*, *tcpF* and *tcpH* genes, which are essential for the conjugal transfer of pCW3 (9, 33). The pJIR2774-encoded *tcpA* gene can complement a *tcpA* mutant of pCW3 and restore conjugal transfer (33). The *tcpB* gene is not present in pJIR2774 and also is absent in several other pCW3-like plasmids, including the conjugal tetracycline resistance plasmid pJIR26 (9) and the β-toxin plasmid pJGS1495 (9, 33), which supports the previous conclusion that *tcpB* is not required for conjugation (33). Our analysis of pJIR2774 provides further evidence that conjugal plasmids from *C. perfringens* have the same mechanism of conjugal transfer (9, 21) and are derived from a common ancestor, most likely a Tn916-like
conjugative transposon (8), but have diverged to the extent that they now carry an array of other genes, which now includes a lincomycin resistance determinant.

tIS\textit{Cpe8}, tISSag10 (4, 5) and a putative element from \textit{H. parasuis}, have a very similar genetic organization (Fig. 3), with each element carrying only two genes, encoding a putative transposase and a lincomycin resistance gene. They are all members of the IS1595 family of transposon-like IS elements and belong in the ISPna2 group (42). They appear to duplicate 8 bp sequences upon insertion and have closely related transposases. These enzymes have several common regions that are important for function, including a zinc finger motif, a putative helix-turn-helix motif, and a catalytic tetrad (42). Each element is also flanked by imperfect inverted repeats (IR) of 24 and 25 bp, respectively, with 50% identity. These regions are similar to other sequences found just upstream of similar transposase genes (Fig. 4B).

The observation that different resistance genes are located downstream of the transposase genes in these elements (Fig. 3) suggests that there may be a mechanism by which gene cassettes can be inserted or deleted from this group of elements, but there is no evidence for any integron-like structures.

tIS\textit{Cpe8} is capable of movement both within the original host strain and within recipient strains following DNA transfer by conjugation. Transposition has been demonstrated in \textit{C. perfringens}, either by the presence of multiple insertions after conjugative transfer or following electroporation-mediated introduction of the element, either in association with pJIR2774 or the clostridial suicide plasmid pJIR1944.

The genetic and functional similarity between tIS\textit{Cpe8} and tISSag10 suggests that these elements originated from a common ancestor and that DNA transfer may have occurred between \textit{C. perfringens} and \textit{S. agalactiae}, possibly via intermediate hosts. Perhaps a progenitor transposon was disseminated either by a promiscuous conjugative plasmid by
mobilization by an oriT site within the element itself. Evidence for the latter scenario is provided by studies on tISSag10, which showed that this element contains a functional oriT site and can be mobilized by Tn916 (4). We have shown that tISCpe8 has a similar oriT site and also can be mobilized by Tn916. These data reinforce the notion that conjugation may be responsible for the movement of genetic elements that are not obviously mobile since they do not encode conjugation or mobilization genes, and that this process plays a very significant role in bacterial adaptation and evolution.

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Fig. 1. Restriction analysis of pJIR2774. (A) Purified plasmid DNA was digested with ClaI and separated by agarose gel electrophoresis. The left shows λ.HindIII size standards, as indicated (kb). pCW3 was extracted from strain JIR4 and pJIR2774 from strain 95-949. (B) Southern hybridization of ClaI-digested pCW3 and pJIR2774. The blot was probed with an InuP-specific probe.

Fig. 2. Comparative analysis of the intP to dcm region of pCW3 and pJIR2774. The percentage indicates the amino acid sequence identity between the encoded orthologous proteins of pCW3 (Accession number DQ366035; (9)) and pJIR2774 (Accession number DQ338473).

Fig. 3. Comparative genetic organization of orthologous gene regions. The genetic map of the lincomycin resistance transposon tISCpe8 shows the putative transposase (tnp) and lincomycin resistance gene (InuP), their direction of transcription, and the inverted repeats located at the ends of the element (black arrowheads). The number of amino acids (aa) in the encoded proteins is indicated. The elements related to tISCpe8 include tISSag10 from S. agalactiae (5). The remaining elements were predominantly identified by searches of existing genome sequences, including those from tISCba1 (C. bartlettii NZ_ABEZ0200022), S. suis (YP_003028723.1) C. jejuni (AY701528; (32)), InsLNU from H. parasuis (NC_012661.1) and tISClph1 from C. phytofermentans (NC_010001). Related transposase genes are represented as grey arrows, conserved inverted repeats by black arrowheads and related resistance genes by black arrows. White block arrows indicate ORFs of unknown function and the chloramphenicol acetyltransferase gene (cat).
Fig. 4. Comparison of related sequences. (A) Regions flanking tISCpe8 elements. The sequenced insertion sites include those from pJIR1944, strain 95-949 and two CW504-derived transconjugants, JIR4344 and JIR4345, which were isolated after matings with 95-949. The underlined region indicates the first 8-bp of the direct repeat (DR), the bold italicized residues indicate the other 8-bp that constitute the DR, the arrows underneath the figure indicate the DR sequences and the inverted triangle indicates the position of the inserted transposon. In the absence of the transposon only one of the 8-bp sequences is present, after transposon insertion the same 8-bp is found at both ends of the transposon. (B) Alignment of inverted repeats (IR) found at the left (IRL) and right (IRR) ends of tISCpe8, tISSag10, InsLNU (H. parasuis), tISClph1 (C. phytofermentans), tISCba1 (C. bartlettii), S. suis and the left end of the C. jejuni upstream transposase sequence. (C) Alignment of putative oriT sites of tISCpe8 and tISSag10.
## Table 1: Strains and Plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Reference or source</th>
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</thead>
<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DB10</td>
<td>PR7 Ln&lt;sup&gt;+&lt;/sup&gt;</td>
<td>(16)</td>
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<tr>
<td><strong>C. perfringens</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EV3839</td>
<td>Em&lt;sup&gt;+&lt;/sup&gt;, Ln&lt;sup&gt;+&lt;/sup&gt; wild-type isolate</td>
<td>J.G. Songer</td>
</tr>
<tr>
<td>UAZ196</td>
<td>Em&lt;sup&gt;+&lt;/sup&gt;, Ln&lt;sup&gt;+&lt;/sup&gt; wild-type isolate</td>
<td>J.G. Songer</td>
</tr>
<tr>
<td>95-949</td>
<td>Em&lt;sup&gt;+&lt;/sup&gt;, Ln&lt;sup&gt;+&lt;/sup&gt; wild-type isolate, pJIR2774</td>
<td>J.G. Songer</td>
</tr>
<tr>
<td>CW504</td>
<td>Rif&lt;sup&gt;+&lt;/sup&gt;, Nal&lt;sup&gt;+&lt;/sup&gt; conjugation recipient</td>
<td>(37)</td>
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<td>JIR4</td>
<td>CW504(pCW3) Tc&lt;sup&gt;+&lt;/sup&gt;</td>
<td>(1)</td>
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<td>JIR325</td>
<td>Rif&lt;sup&gt;+&lt;/sup&gt;, Nal&lt;sup&gt;+&lt;/sup&gt; electroporation recipient</td>
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<td>JIR4225</td>
<td>JIR325::Tn916</td>
<td>(7)</td>
</tr>
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<td>Transconjugant, 95-949 X CW504</td>
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<tr>
<td>JIR4345</td>
<td>Rif&lt;sup&gt;+&lt;/sup&gt;, Nal&lt;sup&gt;+&lt;/sup&gt;, Ln&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Transconjugant, 95-949 X CW504</td>
</tr>
<tr>
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<td><strong>Plasmids</strong></td>
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<tr>
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<td>Ap&lt;sup&gt;+&lt;/sup&gt;, Cloning vector</td>
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<tr>
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<td>(37)</td>
</tr>
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<td>pJIR1944</td>
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<td>This study</td>
</tr>
<tr>
<td>pJIR2774</td>
<td>Ln&lt;sup&gt;+&lt;/sup&gt; conjugative <em>C. perfringens</em> plasmid</td>
<td>This study</td>
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pCW3

pJIR2774