

Phage ϕ C2 Mediates Transduction of Tn6215, Encoding Erythromycin Resistance, between *Clostridium difficile* Strains

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ABSTRACT In this work, we show that *Clostridium difficile* phage ϕ C2 transduces *erm*(B), which confers erythromycin resistance, from a donor to a recipient strain at a frequency of 10^{-6} per PFU. The transductants were lysogenic for ϕ C2 and contained the *erm*(B) gene in a novel transposon, Tn6215. This element is 13,008 bp in length and contains 17 putative open reading frames (ORFs). It could also be transferred at a lower frequency by filter mating.

IMPORTANCE *Clostridium difficile* is a major human pathogen that causes diarrhea that can be persistent and difficult to resolve using antibiotics. *C. difficile* is potentially zoonotic and has been detected in animals, food, and environmental samples. *C. difficile* genomes contain large portions of horizontally acquired genetic elements. The conjugative elements have been reasonably well studied, but transduction has not yet been demonstrated. Here, we show for the first time transduction as a mechanism for the transfer of a novel genetic element in *C. difficile*. Transduction may also be a useful tool for the genetic manipulation of *C. difficile*.

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Clostridium difficile is an important pathogen that causes diarrhea in humans and animals and has been detected in environmental and food samples (1). *C. difficile* infection is difficult to resolve and control because antibiotic therapy is a predisposing factor, and spore formation allows the bacterium to persist in the environment. The emergence of several virulent strains in the last 10 years has prompted detailed comparative genomic analyses that have revealed considerable genome plasticity, a large percentage of which is mediated by horizontally acquired elements, such as conjugative transposons (CTn) and phages (2, 3).

Conjugative transposons are modular mobile genetic elements that contain regions required for recombination, conjugative transfer, and regulation. In addition to these core areas, they also contain accessory regions not directly required for transfer and transposition, such as antibiotic resistance genes (4). Elements initially found in *C. difficile* are able to transfer to other species; for example, Tn5397 can transfer from *C. difficile* to *Bacillus subtilis* (5) and *Enterococcus faecalis* (6).

Some *C. difficile* phages are capable of lysogeny and integration of phage genome(s) into the host chromosome. Prophage sequences are commonly found in *C. difficile* isolates, many of which form infectious particles either spontaneously or following mitomycin C induction (7, 8). The five sequenced *C. difficile* phage genomes are modular; distinct clusters of homologous genes for virion structure, cell lysis, lysogeny control, and DNA replication

and recombination were identified. Homologous genes were found in phages of other clostridia, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Lactobacillus johnsonii*, and *Bacillus cereus*. The phage genomes are also mosaic, meaning each genome is a unique composite of gene modules interspersed with nonhomologous sequences (9–13). Diversity in phage genomes can arise from recombination events involving host or other phage genomes, plasmids, or transposons. The ubiquity of prophage sequences in *C. difficile* strains suggests that phage infection is widespread, indicating the potential for transduction. The acquisition of antimicrobial resistance and virulence genes is mediated by phage transduction in several clinically important Gram-positive bacteria, such as *S. aureus* (14, 15) and *E. faecalis* (16, 17). Opportunities for phage-mediated gene transfer occur during bacterial colonization or infection, as lysogenization has been demonstrated *in vivo* for phages of *Streptococcus pyogenes* and *C. difficile* (18–20). In this work, we investigated the ability of phage to transduce antimicrobial resistance markers between *C. difficile* strains and showed that phage ϕ C2 mediates the transfer of a novel mobile element conferring erythromycin resistance.

RESULTS

ϕ C2 mediates transfer of erythromycin resistance from CD80 to CD062. Donor and recipient strains were chosen for transduction experiments based on ϕ C2 susceptibility, determined previ-

TABLE 1 *C. difficile* isolates in this study

Isolate	Purpose in this study	Relevant genotype/phenotype ^a ; ribotype	Reference
CD80	Donor	$\Delta tcdA \Delta tcdB$ Erm ^r Tet ^r ; 010	This study
CD38	Recipient (transduction)	$tcdA^+ tcdB^+$	This study
CD062	Recipient (transduction)	$\Delta tcdA \Delta tcdB$; 010	(21)
CD839	Recipient (transduction)	$\Delta tcdA tcdB^+$	(21)
CD6938	Recipient (transduction)	$tcdA^+ tcdB^+$	(21)
CD062R11	Recipient (conjugation)	Rif ^r (Rif ^r mutant of CD062)	This study
CD062E1	Transductant	Erm ^r ; 010	This study
CD062R1112	Transconjugant	Erm ^r Rif ^r	This study
CD062R1146	Transconjugant	Erm ^r Rif ^r	This study
CD062R1170	Transconjugant	Erm ^r Rif ^r	This study

^a Erm^r, erythromycin resistant; Rif^r, rifampin resistant; Tet^r, tetracycline resistant.

ously (21), and on either the presence or absence of antibiotic resistance genes, determined in this study (Table 1). Phage ϕ C2 (21) was propagated in the erythromycin- and tetracycline-resistant donor strain CD80 to a concentration of 10^7 to 10^8 PFU/ml and used to infect the antibiotic-susceptible strains CD38, CD062, CD839, and CD6938 to test for transduction of resistance genes. Erythromycin-resistant CD062 cells were isolated (using either crude or purified phage) at a frequency of $1.2 \times 10^{-6} \pm 1.0 \times 10^{-6}$ (mean \pm standard deviation) per PFU. Crude and purified phage suspensions of $\leq 6 \times 10^7$ PFU/ml and a multiplicity of infection (MOI) of less than 0.02 did not result in transduction. There was no growth on control plates when no recipients were present, showing that the phage preparation did not contain any bacterial contaminants. Tetracycline resistance was not transferred to CD062, and erythromycin or tetracycline resistance transfer did not occur for the other three strains tested. The donor and all Erm^r transductants contained *erm*(B), as determined by PCR with primer pair E5/E6 (see Table S1 in the supplemental material). The amplicon sequences from the donor and

transductants were identical. A control mixture of CD80 filtrate that did not contain ϕ C2 and CD062 did not result in erythromycin-resistant derivatives of CD062, indicating that ϕ C2 is necessary for the transfer of erythromycin resistance from CD80 to CD062. Two transductants, CD062E1 and CD062E2, were randomly selected for further analysis. Both strains were lysogenized with ϕ C2, as they produced plaques after mitomycin C induction and contained ϕ C2 integrase (results not shown).

The *erm*(B) gene resides in a novel integrative mobile element, Tn6215. The DNA sequences of the transferable *erm*(B) gene and the flanking regions were obtained from the donor and transductants, and the DNA region containing the junction between the transferred element and the host genome was located by Southern hybridization (see Fig. S1 in the supplemental material) and obtained by comparing the sequence in the transductants and element-free recipients. The genetic organization of this element is shown in Fig. 1. The element had the genetic organization of a mobilizable transposon (Fig. 1) and was subsequently shown to be capable of transfer by a conjugation-like mechanism; it was as-

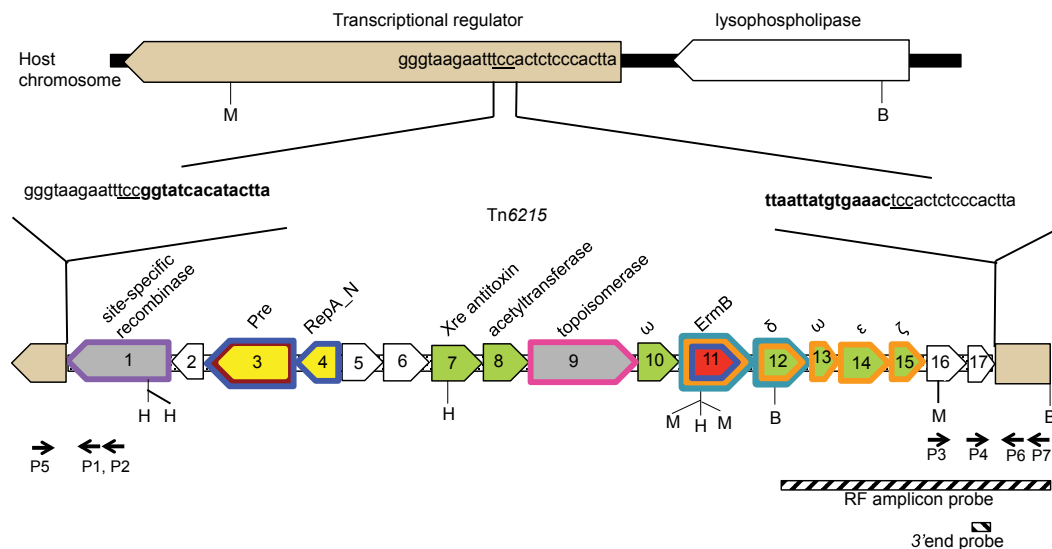


FIG 1 Genetic structure of Tn6215 and site of host chromosome integration. In recipient strain CD062, a putative transcriptional regulator gene was uninterrupted. In donor strain CD80 and transductant strain CD062E1, Tn6215 was integrated into the putative transcriptional regulator gene and the site of integration, TCC, was duplicated at either end. Predicted functional modules of the 13-kb transposon are color coded as follows: red, resistance; yellow, mobilization and replication; green, stability; gray, recombination; and white, indeterminate. Restriction sites are indicated as follows: B, BsrGI; H, Hyp99I; and M, MfeI. Probes used in this study are shown as hatched bars and are shown under their target regions. Homologues in other mobile elements and plasmids are indicated by colored outlines of putative genes as follows: purple, Tn5397; brown, pMV158; blue, CTnBST; pink, CTnDOT; orange, pSM19035; and turquoise, Tn5398. Primers P1 to P7 flanking host and transposon junctions are indicated by black arrows.

Host target site

5' TGGGTAAGAATTTCCACTCTCCCACTTAGA 3'
 3' ACCCATTTCTTAAAGGTGAGAGGGTGAATCT 5'

Host-Tn6215 junction

5' TGGGTAAGAATTTCCGGTATCACATACTTA 3'
 3' ACCCATTTCTTAAAGGCCATAGTGATGAAT 5'

Tn6215-host junction

5' ATTATGTGAAACTTCCACTCTCCCACTTAGA 3'
 3' TAATACACTTTGAGGTGAGAGGGTGAATCT 5'

Regenerated empty site

5' TGGGTAAGAATTTCCACTCTCCCACTTAGA 3'
 3' ACCCATTTCTTAAAGGTGAGAGGGTGAATCT 5'

FIG 2 Sequences of host target site and resulting host and transposon junctions in transductants. The host target site is underlined, and Tn6215 sequences are in red.

signed transposon number Tn6215 (22). It is 13,008 bp in length, with 17 open reading frames (ORFs) (Table S2). Tn6215 encodes a putative serine site-specific recombinase (SSR) (Fig. 1, ORF1) that is likely to be involved in excision and integration. However, no circular forms of the transposon in the donor or transductant were detected by PCR using outward-facing primers specific to the left and right ends of the integrated transposon. Using primers P5/P6 that are specific for the host target site, two amplicons (543 bp and 13.5 kb) were obtained from the donor and transductants,

while only the 543-bp amplicon was detected in the recipient (data not shown). Sequencing of the left and right ends of the 13.5-kb amplicons from the donor and transductants showed Tn6215 integrated within a transcriptional regulator gene (Fig. 1). The sequence of the 543-bp amplicon derived from the donor and transductants was identical to that of the target site in the recipient, indicating that either regenerated integration sites or empty sites were present along with the integrated transposon in the donor and transductants. Sequence comparisons of the transposon-host junctions, the host target site, and the regenerated integration site showed duplication of the target sequence TCC, which is present at either end of the integrated transposon (Fig. 1 and Fig. 2).

ORF3 is a homologue of the mobilization protein TnpZ from Tn4451 of *Clostridium perfringens* (see Table S2 in the supplemental material) (23). However, ORF3 is truncated, and the upstream DNA sequence lacks inverted repeats/palindromes for DNA binding (*oriT*/*RS_A* regions) and, therefore, probably lacks activity compared to TnpZ and the archetypal Pre (24, 25). ORF4 shares the conserved domain RepA_N with replication proteins from plasmids of low G+C Gram-positive bacteria (26). A RepA_N homologue was found in a *Bacteroides* conjugative transposon, CTnBST, which also contained *pre* and *erm*(B) (Fig. 3a) (27). ORF9 is homologous to topoisomerase I, which is required for excision in the conjugative transposons CTnDOT of *Bacteroides fragilis* and CTnPg1 of *Porphyromonas gingivalis* (28, 29). Although the amino acid sequence relatedness of the Tn6215 topoisomerase to homologues from CTnDOT and CTnPg1 (Tn6161) was only moderate (29% identity over 644 amino acids [aa] for both) they shared conserved domains for DNA and ATP binding, strand cleavage and joining sites, C4 zinc finger binding, and C-terminal repeats.

Genes associated with toxin-antitoxin systems (TAS) were identified in Tn6215. Two putative type II proteic TAS are present: a GCN5-related *N*-acetyltransferase (GNAT) family enzyme-Xre

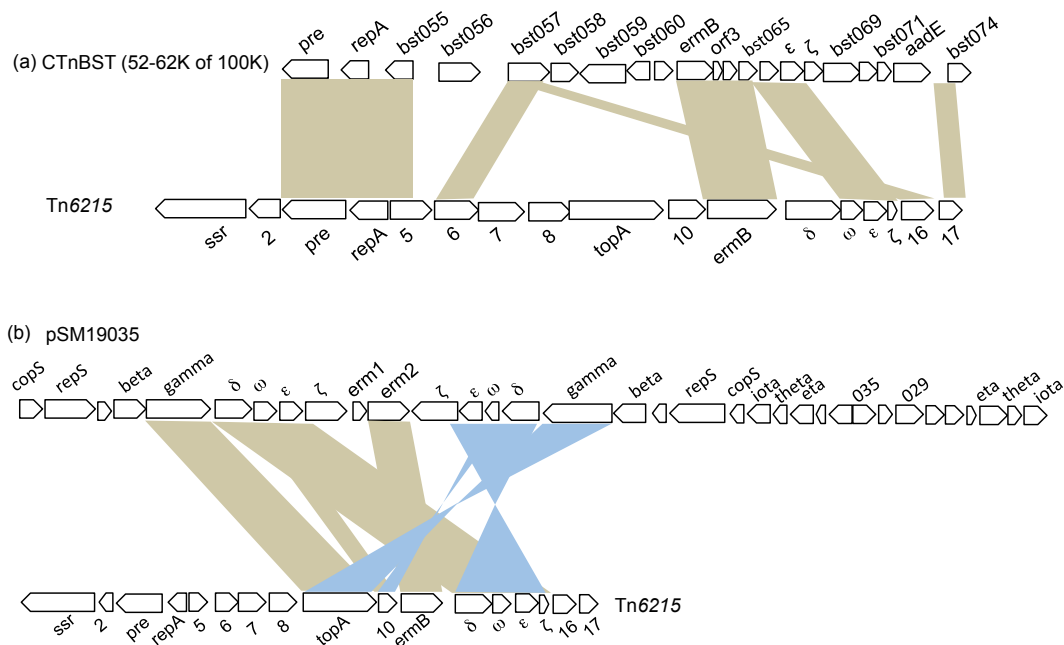


FIG 3 Nucleotide sequence comparisons of Tn6215 to the *Bacteroides* conjugative transposon CTnBST (a) and to the *S. pyogenes* plasmid pSM19035 (b). Sand-colored boxes indicate regions of similarity of >94%, and blue triangles indicate regions of inversions.

system and the ω - ϵ - ζ system. A GNAT-Xre TAS was found in plasmids by comparative genomic analyses only but has not been experimentally verified (30). The ω - ϵ - ζ system, together with δ and *erm*(B), was originally described in pSM19035 (31), a multi-drug resistance-encoding plasmid from *Streptococcus pyogenes*. This TAS is important for plasmid maintenance (31–33), has homologues in Gram-positive and Gram-negative bacteria, and is associated with antibiotic-resistant strains of *Enterococcus faecium* and methicillin-resistant *S. aureus* (MRSA) (34, 35). However, the toxin gene (ζ) in Tn6215 is truncated and is predicted to encode only 52/287 amino acids of the complete ζ in pSM19035. Nucleotide sequence comparison of pSM19035 and Tn6215 showed high sequence similarity and similar gene organization of *topA*, *erm*(B), δ , ω , ϵ , and ζ (Fig. 3b). While an *erm*(B)- δ - ω - ϵ - ζ module has not been described in transposons, an *erm*(B)- ω module was found in Tn5398 of *C. difficile* strain 630, and an *erm*(B)- ω - ϵ - ζ (truncated) module was described in a CTnI-like element in *C. difficile* strains M68 and 2007855 (36). The M68 genome has nucleotide sequence similarity to Tn6215 genes 1 to 5, 7, 10 to 12, 13 to 16, and 17 in distinct regions as determined by BLASTn (nucleotides 1760244 to 1756132, 1753494 to 1752687, 3801235 to 3803491, 3800108 to 3801236, and 1750963 to 1750400, respectively). The previously annotated *orf298* of Tn5398 (37) is predicted as δ in this study.

Tn6215 was transferred by filter mating. To determine whether Tn6215 is conjugative, we carried out filter mating experiments using the ϕ C2-free strain CD80 as the donor and the rifampin-resistant derivative of CD062, CD062R11, as the recipient. Transconjugants were selected on brain heart infusion (BHI) medium containing rifampin (25 mg/liter) and erythromycin (10 mg/liter). Erythromycin-resistant transconjugants appeared at a frequency of 1.8×10^{-9} per CFU of the donor. Southern hybridization with the RF amplicon probe (Fig. 1) containing a Tn6215-host junction confirmed the integration of Tn6215 into the transconjugants (see Fig. S1 in the supplemental material) and showed that Tn6215 entered the genome at the same site in the transconjugants as it occupied in the donor (Fig. 1; Fig. S1). Southern hybridization with a probe specific only for the right end of Tn6215 (Fig. 1) showed that there were at least two copies of the element in the donor, CD80, but only one in the CD062 transductants and transconjugants (Fig. S2). Unlike when Tn6215 is transferred by transduction, the transconjugants did not contain a copy of ϕ C2.

DISCUSSION

This is the first report of gene transfer by phage transduction between *C. difficile* strains. Erythromycin resistance contained within Tn6125 was transferred by ϕ C2 from CD80 to CD062. This element could also be transferred by a conjugation-like mechanism by filter mating, resulting in *Erm*^r *Tet*^s transconjugants but at a much lower frequency. Transductants were lysogens of ϕ C2, whereas transconjugants did not contain the phage. Once transferred, Tn6215 existed as a single copy in both transductants and transconjugants at the same integration site. Although we did not detect circularized Tn6215, empty or regenerated sites in donors and transductants were detected along with integrated Tn6215. The most likely explanation for this is that the transposon excises at a low frequency, resulting in a small number of individual cells that contain a regenerated target site. Presumably the circular form is present in too low a concentration to detect by PCR, as it

will be lost from the cell, whereas the regenerated target will remain.

There are two putative type II proteic TAS in Tn6215, a GCN5-related *N*-acetyltransferase (GNAT) family enzyme-Xre system and the ω - ϵ - ζ system. However, in the latter system, the Zeta toxin is truncated and contains only 52 amino acids of the N-terminal region, and previous work has shown that more than 79 amino acids are required for function (31). Therefore, the toxin is unlikely to be functional, but the antitoxin Epsilon is intact. A system where just the antitoxin is present has been observed in phage T4, where the Dmd protein (antitoxin) is required for successful infection of hosts containing chromosomal toxin/antitoxin systems (38). A *dmd* T4 phage mutant was unable to propagate in host cells because phage infection led to the degradation of unstable antitoxins (RlnB or LsoB) and activation of toxins (RlnA or LsoA) that resulted in inhibition of growth in host cells (38). Possibly the Tn6215 epsilon antitoxin has a similar role in stabilizing host cells under conditions that trigger toxin activity.

GNAT can have a range of enzymatic functions, including resistance to antibiotics, regulation of sporulation (39), and cell wall recovery (40), but there is no evidence in the literature of toxicity mediated by GNAT. While some transcriptional regulators and/or antitoxins have a helix-turn-helix (HTH)_XRE domain (41, 42), the putative XRE located next to GNAT suggests that it functions as a transcriptional regulator of the GNAT gene rather than as an antitoxin. Further experiments are needed to determine the functions of the genes encoding the putative TAS GNAT-Xre and Epsilon-Zeta in Tn6215.

Although all four strains tested as recipients were susceptible to ϕ C2 infection, only one of the four acquired *erm*(B) by transduction, indicating that the transduction conditions may not be optimal. Susceptibility to infection by a transducing phage does not necessarily result in transduction, as shown in other studies (17, 43). A possible explanation for the inability to transfer Tn6215 to the other strains is that they lack a transposon integration site. However, PCR and sequencing analysis indicated that the integration site was intact. Further experiments using more donors and recipients in various growth phases and screening for the transfer of more genetic markers are needed to determine how prevalent phage transduction is as a mechanism of horizontal and antibiotic resistance gene transfer in *C. difficile*.

MATERIALS AND METHODS

Bacterial strains and phage. All *C. difficile* isolates were stored either as stocks in brain heart infusion (BHI) broth (Oxoid) with 20% (vol/vol) glycerol (Sigma) at -70°C or as working spore stocks in cooked-meat medium (Oxoid) and were grown in BHI broth or BHI agar supplemented with 5% horse blood in an anaerobic cabinet as previously described (21). The relevant properties of five clinical *C. difficile* isolates (from Sir Charles Gairdner Hospital, Perth, Western Australia) with known susceptibility to ϕ C2 infection (21) are shown in Table 1. PCR detection of *erm*(B), *tet*(M), Tn916, Tn5397, *tcdA*, and *tcdB* was carried out under the general PCR conditions described below using genomic DNA (gDNA) and the primers listed in Table S1 in the supplemental material.

The temperate phage ϕ C2 was propagated in a donor strain, CD80, using log-phase cultures and anaerobe basal agar as previously described (21). Phage suspensions were concentrated by using polyethylene glycol (PEG) and chloroform (crude phage suspension). Purified phage was prepared in a preformed CsCl density gradient (44).

Microdilution for antimicrobial MIC determination. The determination of the antimicrobial susceptibilities of *C. difficile* strains was based

on the Clinical and Laboratory Standards Institute (CLSI) guidelines (45). Twofold dilutions of antimicrobials (chloramphenicol, erythromycin, and tetracycline; Sigma) were made in BHI broth and in 100- μ l aliquots per well of a 96-well plate. The final antimicrobial concentrations ranged from 0 to 256 mg/liter. *C. difficile* cultures (16 to 18 h) were diluted to a final concentration of $\approx 10^5$ CFU/ml. The cultures were incubated anaerobically (80% N₂, 10% H₂, and 10% CO₂) at 37°C for 48 h, and the MIC of each antimicrobial for each bacterial strain was determined. The antimicrobials tested were chosen based on reports of resistance genes in *C. difficile* (5, 46, 47).

Genomic DNA preparation and PCR reactions. *C. difficile* strains were grown for 18 to 20 h in 5 ml BHI broth supplemented with appropriate antibiotics for gDNA extraction using the Genra Puregene yeast/bacteria kit (Qiagen). Three DNA polymerases, Crimson *Taq*, Phusion high-fidelity PCR master mix, and Phire II DNA polymerase (all New England Biolabs), were used for PCR reactions in this study as indicated in Table S1 in the supplemental material. The annealing temperatures and extension times of primers are shown in Table S1. The typical reaction conditions for PCRs using crimson *Taq* were 1 \times crimson *Taq* reaction buffer, 0.2 μ M deoxynucleoside triphosphates (dNTPs), 0.125 U crimson *Taq* DNA polymerase, and 20 ng DNA in a 25- μ l volume. The typical cycling conditions were 95°C for 30 s, 35 cycles of 95°C for 30 s, primer-specific temperature for 40 s, 68°C for a primer-specific number of seconds, and a final extension of 68°C for 5 min. The multiplex PCR mixture contained 0.5 μ M each primer for the gene of interest, 50 nM each primers PS13/PS14, 1 \times Crimson *Taq* reaction buffer, 0.26 μ M dNTPs, 0.625 U crimson *Taq* DNA polymerase, and 20 ng DNA in 25 μ l. The cycling conditions were 95°C for 30 s, 35 cycles of 95°C for 30 s, primer-specific temperature for 40 s, 68°C for 1 min, and a final extension of 68°C for 5 min. The typical reaction conditions for PCR using Phusion were 1 \times Phusion master mix, 0.5 μ M of each primer, and 1 to 20 ng DNA in a 20- μ l volume. The typical cycling conditions were 98°C for 30 s, 35 cycles of 98°C for 5 s, primer-specific temperature for 10 s, 72°C for a primer-specific number of seconds, and a final extension of 72°C for 5 min. The typical reaction conditions for PCR of DNA ligation reactions using Phire II DNA polymerase were 1 \times reaction buffer, 200 μ M dNTPs, 2 μ l ligation reaction buffer, 0.5 μ M each primer, and 0.4 μ l polymerase. The cycling conditions were 98°C for 30 s, 35 cycles of 98°C for 5 s, primer-specific temperature for 5 s, 72°C for a primer-specific number of seconds, and a final extension of 72°C for 1 min.

Phage transduction and analysis of transductants. Transduction was carried out anaerobically (80% N₂, 10% H₂, and 10% CO₂), except for the centrifugation steps. Log-phase cultures of the recipients were mixed with either the crude phage suspension or purified phage (see above) to achieve an MOI of 0.02 to 0.3 in a volume of 1 ml. Phage-free filtrates of the donor were used for some experiments; uninfected bacterial lawns of CD80 were resuspended in phage buffer, filtered, and processed with PEG and chloroform as for phage-infected lawns (44). Bacterial and phage concentrations were standardized to 1 $\times 10^7$ to 5 $\times 10^7$ CFU/ml and 1 $\times 10^7$ to 5 $\times 10^7$ PFU/ml, respectively. A recipient-only control containing only bacteria and phage buffer was included in each experiment. Phage mixed with the recipient was incubated for 1 h at 37°C and then centrifuged at 14,000 $\times g$ for 30 s. The supernatants were removed, and the cells were washed in 1 ml BHI broth. The washing was repeated, and the cells were resuspended in 150 μ l of BHI broth. The cells were spread plated in 50- μ l volumes onto BHI agar plates supplemented with either tetracycline (10 mg/liter) or erythromycin (50 mg/liter) and incubated for 48 to 72 h. Putative transductants were checked for the presence of *erm*(B) by using PCR primers E5/E6 (see Table S1 in the supplemental material), and two transductants were chosen for further analysis. The transduction frequency was calculated as the number of transductants per PFU. Crude phage suspensions were retrospectively inoculated onto BHIS(TA) (brain heart infusion [Oxoid] supplemented with yeast extract [5 mg/ml; Oxoid], L-cysteine [0.1% wt/vol; Sigma], and sodium taurocholate [0.1% wt/vol; Sigma]) agar and broth to check that they were free of bacterial cells/spores.

Screening for ϕ C2 in transductants by mitomycin C induction and plaque assay on strain CD062 was performed as described previously (21). In addition, PCR detection of the ϕ C2 integrase gene and integration site in transductants was carried out (see Table S1 in the supplemental material). To control for the integrity of the template, 16S rRNA primers PS13/PS14 were included in the same reaction mixture or used in separate reaction mixtures (48) as described below.

Sequencing of *erm*(B) mobile element and determination of integration site in transductants. The mobile element containing *erm*(B) was amplified from CD80 and CD062E1 in four parts and sequenced by the Sanger method (Eurofins MWG Operon, Germany). Primers SG2F/SG3R and TRR4/TPLHSout1 were used with gDNA, while primers invF2/invR2, invF2.1/invR2.1, and invF3/invR3 were used in ligation-assisted PCR (see Table S1 in the supplemental material). CD80 gDNA (0.5 to 1 μ g) was completely digested with either Hpy99I or BsrGI (New England Biolabs) and then self-ligated with T4 DNA ligase (10 U per reaction mixture volume; Fermentas) at 16°C for 18 h, and 16 to 32 ng was used in PCR. Sequences were analyzed and assembled into contigs with CLC Workbench version 6, ORFs were predicted with GeneMark, sequence alignments and homology searches were carried out using BLASTN, BLASTP, and ClustalW, and repeats/palindromes were searched for using inverted (<http://embooss.bioinformatics.nl/cgi-bin/embooss/inverted>), Inverted Repeats Finder (<http://tandem.bu.edu/cgi-bin/irdb/irdb.exe>), and REPFIND (<http://zlab.bu.edu/repfind/form.html>).

The integration site of the *erm*(B) mobile element in transductants was confirmed by Southern hybridization as described below. Primers P5/P6 (Fig. 1; see Table S1 in the supplemental material) were used to detect the integrated *erm*(B) mobile element and regenerated or empty integration sites in the donor, recipient, and transductants. Circular molecules of the *erm*(B) mobile element in transductants were searched for using four primer pairs (P2/P4, P1/P4, P2/P3, and P1/P3) reading out of the ends of the element (Fig. 1; Table S1).

Ribotyping. Ribotyping of CD80, CD062, and CD062E1 was carried out as previously described (49) except that electrophoresis was done using capillary gel electrophoresis (Qiagen) (50).

Filter mating and analysis of transconjugants compared to transductants. Filter mating assays were carried out as previously described (51) using a spontaneous rifampin-resistant mutant of CD062 as the recipient. This mutant was selected on BHI agar with 5% horse blood and 25 mg/liter rifampin to generate CD062R11 (Table 1); transconjugants were selected on rifampin (25 mg/liter) and erythromycin (10 mg/liter) after a 5-day incubation. All putative transconjugants were tested by multiplex PCR for 16S rRNA and a gene encoding an ABC-transport system (see Table S1 in the supplemental material) that is present only in the donor, as described below. The integration site of the *erm*(B) element in transconjugants was determined by PCR with primers specific for the integration site in transductants, P5/P2 for the left end and P4/P7 for the right end (Fig. 1; Table S1).

Genome sequencing to differentiate donor and recipient. As the donor and recipient strains were highly similar, we needed to find genetic markers for a PCR assay to determine real transconjugants after filter matings. Genes unique to the donor and recipients were found by whole-genome sequencing using the Illumina genome analyzer IIX at low ($\sim 100\times$) coverage. Initially, the sequence reads from the recipients were filtered by excluding those found to align to the donor. This was done using the BWA alignment package (52). The remaining reads were assembled using the A5 pipeline (53), and contigs were annotated against the genome of CD630 using the xBASE annotation pipeline (54–59). A similar process was followed to obtain donor-specific contigs. Candidate marker genes were selected to exclude hypothetical genes without a known function and genes on known transposons or phages. Finally, primers CD80C3U57F/CD80C3U57R (Table S1) were designed for a candidate marker gene with 99% (1,035/1,038) sequence similarity to a gene in CD630 encoding an ABC-type transport system (CD630_08760), present only in the donor, and experimentally verified by PCR as described

above. The PCR assay was used to distinguish transconjugants from spontaneous rifampin-resistant mutants of the donor after filter matings.

Southern hybridization. For detection of the *erm*(B) mobile element integration site in transductants and transconjugants, the RF amplicon was used as a probe (Fig. 1). The PCR conditions for obtaining the RF amplicon using invF3/invR3 are described in Table S1 in the supplemental material. For determining the copy number of the *erm*(B) mobile element, a PCR amplicon of P4/C2Tn3' end (Table S1), containing the 3' end of the mobile element, was used as a probe (Fig. 1). MfeI (Fermentas)-digested gDNA (3 µg) of CD062, CD062R11, CD80, and CD062 transductants and CD062 transconjugants served as the templates for hybridization of digoxigenin (DIG)-labeled probes, at 40°C for the RF amplicon probe and 49°C for the 3'-end probe. Detection was carried out as recommended for the DIG DNA labeling and detection kit (Roche).

GenBank accession number. The complete sequence of Tn6215 is deposited in GenBank with the accession number KC166248.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00840-13/-/DCSupplemental>.

Figure S1, PDF file, 0.3 MB.

Figure S2, PDF file, 0.5 MB.

Table S1, DOCX file, 0.1 MB.

Table S2, DOCX file, 0.1 MB.

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