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Four different integrative recombination events involved in the mobilization of the gonococcal 5.2 kb beta-lactamase plasmid pSJ5.2 in *Escherichia coli*

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Abstract

We identified and characterized four different recombination mechanisms involved in the cointegrative transfer of the *Neisseria gonorrhoeae* beta-lactamase plasmid pSJ5.2 by the gonococcal 41 kb *tet(M)* and the Gram negative self-transmissible plasmids N3 and R64 *drd-33* using an *Escherichia coli recA*- background. Mobilization of pSJ5.2 by the *tet(M)* plasmid occurred by cointegration through a replicative transposition of two *IS1* elements inserted upstream from the beta-lactamase gene of pSJ5.2 and creating a *IS1::beta-lactamase* hybrid promoter. Two types of recombinational events occurred within the 1.8 kb BamH1-HindIII fragment of pSJ5.2 with the N3 and R64 plasmids. A non-homologous recombination was found at coordinates 1817 and 2849 of pSJ5.2 with sequences from R64. A non-homologous recombination combined with an *IS26*-mediated one-ended transposition was found at coordinates 1817 and 3010 of pSJ5.2 with N3. In both recombinational events, a deletion of over 1 kb of pSJ5.2 occurred. The fourth recombination event was detected in the 1.0 kb BamH1-HindIII fragment of pSJ5.2 by homologous recombination between DNA from the truncated Tn3 resolvase gene of pSJ5.2 and the resolvase sequences from R64 and N3.

Keywords

Neisseria gonorrhoeae; beta-lactamase plasmids; mobilization; *IS1*; *IS26*; cointegration; non-homologous recombination

1. Introduction

The 5.2 kb beta-lactamase plasmid from *Neisseria gonorrhoeae* (the gonococcus) is one of the three small broad-host range and mobilizable resistant (R)-plasmids associated with the worldwide spread and prevalence of Penicillinase-Producing *Neisseria gonorrhoeae* (PPNG) infections (Phillips, 1976; Ray et al., 2005; Scharbaai-Vázquez et al., 2007). The 5.2 kb R-plasmid is a *mob/oriT* deletion derivative of the parental 7.4 kb R-plasmid (Dillon et al.,

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1991; Pagotto *et al.*, 2000; Candelas, 2000). These R-plasmids in the pathogenic gonococci have the potential to be transferred by conjugation to commensal *Neisseria spp.* and other endogenous flora, creating a pool of resistance genes in healthy hosts (Kirvin and Thornsberry, 1977; Sox *et al.*, 1978; Flett *et al.*, 1981; Guinney and Ito, 1982; Mc Nichol *et al.*, 1983; Piffareti *et al.*, 1988; Roberts and Knapp, 1988; Van Passel *et al.* 2006).

The conjugal transfer of the gonococcal 7.4 kb and the 5.6 kb R-plasmids has been extensively studied (Roberts and Falkow, 1977; Sox *et al.*, 1978; Guinney and Ito, 1982; McNichol *et al.*, 1983; Tenover *et al.*, 1985; Ikeda *et al.*, 1986; Pifaretti *et al.*, 1988; Roberts and Knapp, 1988; Biswas *et al.*, 1989; Dillon and Yeung, 1989; Gauthier, 1990; Dillon *et al.*, 1991; Rodríguez-Bonano and Torres-Bauzá, 2004). These two R-plasmids carry a mobilization (*mob*) region encoding a specific endonuclease (relaxase) and an origin of transfer (*oriT*) that allow them to be efficiently mobilized with the help of either the co-resident gonococcal 38 kb or the 41 kb tetracyclineencoding *tet(M)* plasmid among gonococci and by the Incompatibility Group P (IncP-type) conjugative plasmids in *Escherichia coli* (Sox *et al.*, 1978; Roberts and Knapp, 1988; Guinney and Ito, 1982; Rodríguez-Bonano and Torres-Bauzá, 2004). In the 5.2 kb R-plasmid, which has no known *oriT*, conjugal transfer has been reported in both a *recA*⁻ *E. coli* and in the gonococcal natural host (Dillon and Yeung, 1989; Marquez *et al.*, 1999). These observations suggest that the gonococcal R-plasmids may employ alternative mechanisms for plasmid conjugal spread.

We previously demonstrated the cointegration of the 5.2 kb R-plasmid isolated in San Juan, Puerto Rico (pSJ5.2) with the genomes of the Enterobacteria-derived R64 and N3 and the gonococcal 41 kb *tet(M)* conjugative plasmids in *recA*⁻ *E. coli* (Scharbaai-Vázquez, *et al.*, 2007). Stable R64::pSJ5.2 and N3::pSJ5.2 plasmid cointegrates lost pSJ5.2-derived DNA within the 1.8 kb BamH1-HindIII fragment of pSJ5.2 indicating that cointegrates formed by recombination within that fragment (Scharbaai-Vazquez, *et al.*, 2007). *E. coli*-to-*E. coli* mobilization of pSJ5.2 using *N. gonorrhoeae* providing the *tet(M)* conjugative plasmid was observed to occur by transposomal insertion of *IS1* in the 2.4 kb BamH1-BamH1 fragment of pSJ5.2 (Scharbaai-Vázquez, *et al.*, 2007). In addition, the 1.0 kb BamH1-HindIII fragment of pSJ5.2 was capable of mobilizing a pUC19 vector (pUC1.0) at frequencies of 1.9×10^{-7} and 2.2×10^{-7} transconjugants per donor cell in the presence of R64 and N3, respectively (unpubl. obs. from our laboratory). Therefore, each of the three BamH1-HindIII restriction fragments of pSJ5.2 contained active sites for cointegrative mobilization (Scharbaai-Vazquez, *et al.* 2007). *E.coli* transconjugants containing stable R64::pSJ5.2, N3::pSJ5.2, R64::pUC1.0 and N3::pUC1.0 cointegrates were isolated for further study.

The gonococci mobilize R-plasmids DNA through cointegration with self-transmissible plasmids (Sox *et al.*, 1978; Dillon *et al.*, 1991). Moreover, conjugative transfer of gonococcal plasmid DNA by cointegration and stable integration into the gonococcal genome was reported for the cryptic 4.2 kb plasmid (Johnson *et al.*, 1983; Hagblom *et al.*, 1986). It is well known that the gonococcus take up and integrate DNA from related species by transformation and homologous recombination, respectively (Reviewed in Kline, et al., 2003; Hamilton and Dillard, 2006). However, the study of the mechanisms of recombination during conjugation has been hindered by the very low frequency of conjugal mobilization of plasmids by cointegration. In addition, plasmid cointegrates often dissociate (resolve) rapidly into their components after conjugative transfer.

Plasmid cointegrates have a profound significance in genetic diversity and evolution, as a novel combination of genes is available for transfer (Brom et al., 2004). Some R-plasmids rely on cointegration with self-transmissible plasmids to circumvent limitations of spread, such as: (1) incompatibility of the transferred plasmids with those plasmids already present in the new host cells, (2) replication deficiency of plasmids in new hosts, (3) presence of restriction-

modification systems in the new hosts, (3) functional inactivity of the resistance gene product in new hosts and (4) reduced expression of resistance genes in new hosts. The study of plasmid *oriT*-independent transfer is interesting due to the discovery of strategies to inhibit the relaxase enzyme required for *oriT*-mediated plasmid transfer (Fernández-López *et al.*, 2005; Lujón *et al.*, 2007).

We report here that pSJ5.2 utilizes four different mechanisms for *oriT*-independent cointegrative mobilization with the self-transmissible plasmids tested, and present evidence of several hot-spots for pSJ5.2 recombination with self-transmissible plasmids for its dissemination. In this paper we describe the sequence analysis of each of the recombination junctions leading to the cointegrative intermediates. Two non-homologous recombination events occurring within the 1.8 kb BamH1-HindIII fragment of pSJ5.2 with the N3 and R64 self-transmissible plasmids are described. An intermediate of a homologous recombination between the resolvase gene from the 1.0 kb BamH1-HindIII fragment of pSJ5.2 and the resolvases of N3 and R64 was identified. The insertion of approximately 800 bp comprising the *IS1* transposon observed in the pSJ5.2-insertion derivatives was cloned and used as a probe to elucidate the source of the transposon. Therefore, pSJ5.2 provides a unique model to study alternative mechanisms for gonococcal R-plasmid transfer. Given that no functional *oriT* has been identified so far in the 5.2 kb R-plasmid, it is probable that transfer mechanisms by cointegration have evolved to ensure its survival.

2. Materials and Methods

2.1 DNA isolation and cloning procedures

The plasmid constructs used in this work are listed in Table 1. Plasmid and chromosomal DNAs were purified from *E. coli* and *N. gonorrhoeae* using the Strataprep plasmid Miniprep Kit® (Stratagene, La Jolla, CA) and QIAamp DNA Mini kit® (Qiagen, Valencia, CA), respectively. Restriction digests, ligations and transformations to *E. coli* DH10B were performed as specified by the manufacturers (New England Biolabs, Beverly, MA; Epicentre, Madison, WI; and Invitrogen Corp., Carlsbad, CA). Isolation and screening of plasmids from *E. coli* transconjugant colonies was done using a chemical lysis and boiling plasmid extraction method (Colony Fast-Screen Kit ®; Epicentre) followed by agarose gel electrophoresis.

Cloning of the 2.4 kb BamH1-BamH1, 1.8 kb BamH1-HindIII and 1.0 kb BamH1-HindIII fragments of pSJ5.2 into pUC19 was done in order to analyze the minimal region required for pSJ5.2 transfer (data not shown). Among the clones generated (pUC2.4, pUC1.8 and pUC1.0, respectively), the pUC1.0 construct was selected for study of its cointegrative properties in the presence of N3 and R64 plasmids.

Plasmid pUC3.2 was constructed by cloning the 2.4 kb BamH1-BamH1 fragment of a pSJ5.2 *IS*-derivative transmitted with the help of the *tet(M)* conjugative plasmid into pUC18. This fragment contains the *IS1* insertion.

2.2 Rescue cloning of DNA surrounding the recombination junctions

Isolation of recombination junctions between pSJ5.2 and the self-transmissible plasmids was made by rescue of pSJ5.2 from the R64::pSJ5.2, N3::pSJ5.2, R64::pUC1.0 and N3::pUC1.0 cointegrates. *E. coli* transconjugants containing the cointegrates were isolated from LB plates containing both ampicillin and tetracycline and subjected to plasmid DNA extraction by alkaline lysis (Sambrook *et al.*, 1989). Residual bacterial genomic DNA and single-stranded fragments were removed from DNA lysates by incubation with an ATP-dependent DNase (Plasmid-Safe®; Epicentre). The R64::pSJ5.2 and N3::pSJ5.2 DNA lysates were digested with AlwN1 restriction enzyme, which digests the conjugative plasmid but does not cut the

integrated pSJ5.2. The BglII restriction enzyme was used for the digestion of N3::pUC1.0 DNA, since it does not cut either the 1.0 kb insert nor the pUC19 vector. Likewise, the EcoRV and BsmI restriction enzymes were used to cut the R64::pUC1.0 cointegrate. The DNA fragments were self-ligated and transformed into *E. coli* DH10B cells (Invitrogen), with subsequent selection for resistance to ampicillin. The resultant ampicillin-resistant (amp^R) colonies were then screened for plasmids larger than the size of pSJ5.2 or pUC1.0. The miniplasmids were then mapped by double digestion with BamH1-HindIII and the restriction patterns compared with those of wild type pSJ5.2 and pUC1.0. The fragments obtained upon digestion of R64::pUC1.0 and N3::pUC1.0 miniplasmids were compared with those obtained with the parental pUC1.0.

2.3 Plasmid constructs derived from the rescue of pSJ5.2 from cointegrates

Plasmid pN5.2 resulted from the rescue of pSJ5.2 from a N3::pSJ5.2 cointegrate (Table 1). The pN5.2 construct was double digested with BamH1-HindIII and the resultant restriction fragment containing a “nonconserved” 3.0 kb BamH1-HindIII fragment which replaced the wild type 1.8 kb BamH1-HindIII fragment was subcloned into pUC19, producing pUC3.0. Likewise, plasmid pR5.2 was obtained by rescue of pSJ5.2 from a R64::pSJ5.2 cointegrate. Double digestion with BamH1-HindIII produced a non-conserved 3.5 kb restriction fragment instead of the 1.8 kb fragment. Cloning of the 3.5 kb fragment into pUC19 produced pUC3.5.

Plasmids pN1.0 and pR1.0 resulted from the rescue of clone pUC1.0 from the N3::pUC1.0 and R64::pUC1.0 cointegrates; respectively.

2.4 Polymerase Chain Reaction, non-radioactive probes and Southern Blot experiments

The oligonucleotides used for the generation of non-radioactive probes, pUC-derived clones and the recombination junctions within pN5.2 and pR5.2 are listed in Table 2. Synthesis of oligonucleotides was carried out at the RCMI-Molecular Genetics Core Facility, at Medical Sciences Campus, University of Puerto Rico.

To elucidate the source of the *IS1* isolated in previous triparental crosses (Scharbaai-Vazquez, 2006), a non-radioactive probe for *IS1* was generated. Primers *IS1/F119* and *IS1/R119* were specific for the *InsB* gene of *IS1* (Table 2 and Fig. 2b). A 117 bp PCR product was generated with the primers annealed at coordinates 421-520 respective to a pSJ5.2::*IS1* derivative used as template. The probe was used in Southern Blot hybridizations involving EcoRI-digested chromosomal DNA from *N. gonorrhoeae* and *E. coli*.

Insert DNA fragments in pUC3.6, pUC3.0 and pUC3.5 were sequenced using the M13 universal primers (Invitrogen, CA). Additional walking primers were designed to assemble the sequence of *IS1* in clone pUC3.6 (Table 2).

Sequencing primers REPA and REPB were designed to anneal adjacent to the BamH1 site at the *repA* gene (coordinate 1497) and to the HindIII site from the *repB* gene (coordinate 3296) of pSJ5.2 in pN5.2 and pR5.2.

The integration of pUC1.0 into the genomes of N3 and R64 plasmids during previous mating assays was confirmed with a non-radioactive probe consisting of the 1.0 kb BamH1-HindIII fragment of pSJ5.2.

All probes were randomly labeled using the Genius nonradioactive labeling and detection kit (Roche Diagnostic Corp., Indianapolis, IN). Southern analysis of both total and restricted DNA of all donor and transconjugant bacteria was done according to the manufacturer.

2.5 Determination and analysis of nucleotide sequences

DNA sequencing reactions were performed with an ABI 310 automated DNA sequencer (Applied Biosystems, Foster City, CA) at the RCMI-Molecular Genetics Core Facility, at Medical Sciences Campus, University of Puerto Rico and SeqWright, Inc. (Houston, TX).

The nucleotide and the predicted protein sequences were analyzed with Visual Cloning, version 3.0 (Redasoft Corp., Ontario, Canada) and available tools at the National Center of Biotechnology Information website (<http://www.ncbi.nlm.nih.gov>). Assembly of *IS1* DNA sequences was done with GeneStudio Professional Software (GeneStudio, Inc., Suwanee, GA).

2.6 Nucleotide sequence accession numbers

Nucleotide sequences were deposited at the NCBI GenBank database under accession nos. EF527803, EF527804, EF523805, EF523806, EF527807 and EF523808.

3. Results

3.1 The 1.8 kb BamH1-HindIII fragment of pSJ5.2 contains a site for non-homologous recombination which mediates cointegration with self-transmissible plasmids N3 and R64

Previously published physical maps of the N3::pSJ5.2 and R64::pSJ5.2 cointegrate DNA of transconjugants with BamH1 and HindIII revealed that a region within the 1.8 kb BamH1-HindIII fragment of pSJ5.2 is deleted during fusion with the R64 and N3 plasmids (Scharbaai-Vázquez *et al.*, 2007; Region B in Fig. 1). Other than this deletion, analysis showed no differences in the restriction bands, allowing us to easily delimit the recombination ends upon selection of representative transconjugants containing the N3::pSJ5.2 and R64::pSJ5.2 plasmid cointegrates. Plasmid DNA from pSJ5.2 was rescued from the stable N3::pSJ5.2 and R64::pSJ5.2 plasmid cointegrates and the resultant pSJ5.2 miniplasmids containing fragments of the conjugative plasmid (pN5.2 and pR5.2, respectively) were then mapped by double digestion with BamH1 and HindIII.

In order to test the hypothesis of a recombination occurring within the 1.8 kb BamH1-HindIII fragment of pSJ5.2, the nucleotide sequences of the 5'- and 3'- junctions for pN5.2 and pR5.2 were determined and deposited in the GenBank database under Accession Numbers EF527803, EF527804 and EF527805. Sequencing primers were designed to anneal close to the 5'-end and 3'-end recombinations, respectively. The results are summarized in Figures 1a and 1b. The analysis revealed the following: (i) the junctions at the 5'-end start at coordinate 1817 respective to pSJ5.2 and are the same for the N3::pSJ5.2 and R64::pSJ5.2 junctions tested — Accession Number EF527804, (ii) an AT-rich domain of pSJ5.2 was noted in each of the 5'-end junctions analyzed, (iii) the 5'-end junction is part of the Replicase, *repA* gene promoter and it is very close to the origin of replication 1, *oriVI* described by Pagotto and Dillon (2001) which lies at coordinate 1867 in the parental 7.4 kb “Asian” R-plasmid, (iv) the junction at the 3'-end of the R64::pSJ5.2 cointegrate - Accession Number EF527803 - was mapped at coordinate 2849 from pSJ5.2, (v) an *IS26* transposon was found at the 3'-end junction of the N3::pSJ5.2 cointegrate at coordinate 3010 — Accession Number EF527805; only the terminal inverted repeat is shown in the figure, (vi) no overlap nucleotides between pSJ5.2 and the respective conjugative plasmid sequences at the 5' and 3' — end junctions were found. These results indicate that recombination between pSJ5.2 and conjugative plasmid N3 was mediated by a non-homologous recombination at the 5'-end combined with a transposition of *IS26* at the 3'-end. Recombination between pSJ5.2 and conjugative plasmid R64 was the result of two non-homologous recombinations occurring at both 5'-end and 3'-end borders of the fusion.

The 3.0 kb and 3.5 kb fragments generated from the digestion of pN5.2 and pR5.2 with BamH1 and HindIII, respectively were analyzed by sequencing. BLAST analysis of 3,010 bp sequence

cloned into pUC19 (pUC3.0; Table 1) confirmed the presence of *IS26* at the N3 right border and at coordinates 2191 to 3010 relative to the fragment (data not shown). Sequence analysis of 3,475 bp fragment cloned into pUC19 (pUC3.5; Table 1) revealed 100% identity to R64 with no transposable element flanking either border of the junction (data not shown).

3.2 The Insertion element 1 (*IS1*) promoted cointegration of pSJ5.2 with the 41 kb *tet(M)* self-transmissible plasmid by recruiting two *IS1* elements from *E. coli*

Mobilization of pSJ5.2 by the gonococcal 41 kb *tet(M)* self-transmissible plasmid using an intergeneric triparental cross resulted in *E. coli* transconjugants containing both the self-transmissible plasmid and pSJ5.2 derivative with an insertion sequence located within the 2.4 kb BamHI-BamHI fragment (Scharbaai-Vázquez, *et. al.*, 2007; Region A in Fig. 2a). The new BamHI-BamHI fragment of about 3.6 kb was cloned in pUC19 (pUC3.6; Table 1) for sequence analysis. Sequence analysis revealed the presence of two overlapped copies of *IS1* inserted in the 2.4 kb BamHI-BamHI fragment of pSJ5.2 (Figure 2b). Moreover, the *InsA* gene from one of the *IS1* copies was found inserted at position 4565 relative to pSJ5.2 and disrupting the promoter of the beta-lactamase gene creating an *IS1*::beta-lactamase hybrid promoter (Fig. 2c; Accession Number EF527806). The presence of two overlapped copies of *IS1* inserted in the 2.4 kb BamHI-BamHI fragment of the pSJ5.2 transconjugants produced with the *tet(M)* plasmid suggests an unusual transposition event. To gain insight into the role of *IS1* in pSJ5.2 cointegrative mobilization by *tet(M)*, the genomic DNA from the bacterial strains used in the triparental cross were evaluated for the presence of *IS1* by Southern blot hybridization with an *IS1*-derived probe (Experimental Procedures). The results demonstrate that both the intermediate and recipient strains of *E. coli*, but not *N. gonorrhoeae* contained sequences that could have been the source of the two *IS1* elements (Figure 3a; Panel II).

3.3 The 1.0 kb BamHI-HindIII fragment of pSJ5.2 contains a DNA site for cointegrative transfer by homologous recombination with R64 and N3 self-transmissible plasmids

The mobilization properties of the 1.0 kb BamHI-HindIII fragment from pSJ5.2 (Region C in Fig. 1) cloned into a nonmobilizable pUC19 vector prompted us to investigate the site and mechanism responsible for this type of transfer. Fifteen pUC1.0 amp^R transconjugants produced in the presence of conjugative plasmids R64 or N3 were randomly selected for analysis. Plasmid DNA content of the representative transconjugants analyzed by agarose gel electrophoresis is shown in Fig. 4. Analysis of transconjugants did not reveal the presence of a plasmid of 3.7 kb, which is the size of pUC1.0 (Fig. 4; lanes C to E and lanes G to M in panel I). Southern Blot hybridization using a non-radioactive 1.0 kb BamHI-BamHI fragment of pSJ5.2 as probe revealed the integration of pUC1.0 into both R64 and N3 conjugative plasmids (Fig 4; Panel II; lanes c to e and g to m). Double digestion of the integrated pUC1.0 DNA with BamHI and HindIII revealed that this 1.0 kb fragment is not conserved, indicating that integration occurred within that fragment (Fig 5). A weak hybridization signal was also detected using the 1.0 kb BamHI-HindIII probe in the undigested R64 and N3 DNA from the donor *E. coli* strains (Figure 4, lanes Bb and Ff; top of gel), which may indicate some homology between DNA regions in the 1.0 kb fragment and the N3 and R64 self-transmissible plasmids. This, in turn, suggests that integration occurred between homologous sequences in the 1.0 kb fragment and the self-transmissible plasmids. To test this hypothesis, the pUC1.0-derived plasmid was rescued from the N3::pUC1.0 and R64::pUC1.0 cointegrates. The resultant miniplasmids (pN1.0 and pR1.0, respectively; Table 1) were used as DNA template for the sequencing of the N3::pUC1.0 and R64::pUC1.0 junctions using the M13 universal primers. The results are shown in Figure 6. Analysis of the R64::pUC1.0 revealed overlap of DNA sequences between the pSJ5.2 resolvase and the Tn5393 transposase from R64 (Figure 6A; diagram I; Accession Number EF527807). Likewise, the Tn3 resolvase from the pSJ5.2 plasmid was found to overlap with the Tn2 resolvase from the *IS26* in conjugative plasmid N3 (Fig. 6A; diagram II; Accession Number EF527808). Only the right arm of the inverted repeat

from *IS26* (IRR) was found, which indicates that *IS26* is partially deleted at the resolvase region. The results suggest that the N3::pUC1.0 and R64::pUC1.0 cointegrates were formed by homologous recombination between the truncated resolvases found on both plasmids.

4. Discussion

Previous reports of the gonococcal beta-lactamase plasmids mobilization have shown that different conjugative plasmids recognized different DNA sites involved in mobilization (McNichol *et al.*, 1983; Tenover *et al.*, 1985; Dillon *et al.*, 1991; Piffaretti *et al.*, 1988; Gauthier, 1990). Here we show that the mechanisms for conjugal mobilization of the 5.2 kb beta lactamase plasmid pSJ5.2 involve all three BamH1-HindIII restriction fragments and depend on cointegration with conjugative plasmids.

Cointegration can occur by: (a) recombination between homologous regions on the two plasmids; also called *rec*-dependent, and (b) non-homologous or *rec*-independent mechanisms which include: site-specific recombination mediated by integrases, resolvases, invertases; transposition of an Insertion Sequence (*IS*) element residing on either plasmid; and illegitimate recombination (Reimann and Haas, 1993). Conjugative transfer of a small R-plasmid by cointegration with a larger self-transmissible plasmid rarely involves more than one mechanism of recombination. In Gram-negative bacteria, Gram-positive bacteria and rhizobia, cointegration has been reported to occur through one type of *RecA*-dependent recombination among homologous sequences or through one specific cointegration involving transposon, insertion sequences or specific *att* sites (Merlin, *et al.* 2000; Miller and Cohen, 1980; Hille *et al.*, 1983; Leclaporn *et al.*, 1996; Vaudequin-Dransart *et al.*, 1998; Trotter *et al.*, 2004; Brom *et al.*, 2004). To our knowledge, this is the first report of a naturally-occurring small resistance plasmid that employs four different mechanisms for cointegrate formation at three different sites to ensure its spread. We had found that the type of recombination leading to the cointegration of pSJ5.2 with different conjugative plasmids occurred at different frequencies, which reflects the varied efficiencies of each event (Scharbaai-Vázquez, 2006).

In order to deduce the recombination mechanisms that occurred in the three BamH1-HindIII fragments of pSJ5.2, we analyzed representative N3::pSJ5.2, N3::pUC1.0, R64::pSJ5.2, and R64::pUC1.0 stable cointegrates and the pSJ5.2::*IS1* insertion derivatives. The use of a *recA*-deficient *E. coli* host for mating assays reduced the likelihood of mobilization via possible homologous recombination with the self-transmissible plasmids, and facilitated the trapping of stable cointegrate intermediates for the study of the plasmid-plasmid junctions. Analysis of pSJ5.2 mobilization showed that the plasmid utilizes two non-homologous recombination mechanisms in the 1.8 kb BamH1-HindIII fragment and a replicative transposition of *IS1* in the 2.4 kb BamH1-BamH1 fragment. A fourth cointegrative mechanism consisted of a resolvase-mediated homologous recombination in the 1.0 kb BamH1-HindIII fragment of pSJ5.2. This suggests that each fragment contains at least one hot-spot site for mobilization by cointegration with self-transmissible plasmids.

A hot-spot for non-homologous recombination with self-transmissible plasmids N3 and R64 was found in the 1.8 kb BamH1-HindIII fragment of pSJ5.2. Moreover, nucleotide sequence analysis of the N3::pSJ5.2 and R64::pSJ5.2 junctions (pN5.2 and pR5.2; respectively) revealed two different types of non-homologous recombination (Fig. 1b). Interestingly, nucleotide sequences at the junctions showed that both R64 and N3 recombined at the same coordinate (1817) within the 1.8 kb BamH1-HindIII fragment from pSJ5.2. We refer to coordinate 1817 as the 5'-end of the junction. The fact that two unrelated conjugative plasmids can recombine at the same spot in the pSJ5.2 lead us to search for distinctive structural characteristics in the site surrounding the 1817 coordinates. Computer analysis of the ORF flanking coordinate 1817 from pSJ5.2 (*repA*; GenBank Accession No. DQ355980) using the BLAST homology search

(Altschul *et al.*, 1997) was performed. The *repA* gene showed significant homologies with the conserved domain of the type I topoisomerases which is known to have single-strand nicking and joining activity during plasmid replication (Horowitz and Deonier, 1985; Ikeda, H., 1986; Michel and Ehrlich, 1986; Shuman, 1989; Marsin *et al.*, 2000; Zhu and Schiestl, 1996 and 2004). This activity could mistakenly join DNA strands from R64 or N3 promoting the illegitimate intermolecular recombination event with pSJ5.2. This activity is consistent with previous *in vitro* nicking assays in which a non-oriT related *nic* site was located in the 1.8 kb BamHI-HindIII fragment from the 7.4 kb gonococcal beta-lactamase “Asian” plasmid p22209 (McNichol *et al.*, 1983).

In contrast with the 5'-end junction, analysis of the 3'-end recombination junctions of N3 and R64 within the 1.8 kb BamHI-HindIII fragment of pSJ5.2 revealed structural differences which suggest differences in the mechanism of cointegrate formation. The *IS26* found at the 3'-end of the pSJ5.2-N3 junction is 820 bp in length, and is known to form cointegrates by generation of directly repeated copies of *IS26* present at each cointegrate junction by a replicative transposition mechanism (Mollet *et al.*, 1983; Brown *et al.*, 1984; Chandler and Mahillon, 2002; Kim *et al.*, 2002). It is known that *IS*-mediated cointegration can mediate the transfer of the entire DNA sequence of a nonconjugative plasmid by a conjugative one (Bennett *et al.*, 1988). The presence of one copy of *IS26* could be consistent with the formation of N3::pSJ5.2 cointegrates by one-ended transposition, which is another type of illegitimate recombination mechanism described by Reimann and Haas (1993). However, deletion of the sequence between the two recombination sites is not a consequence of such mechanism (Reimann and Haas, 1993). Moreover, no deletion of DNA associated with the transposition of *IS26* has been reported previously (Mollet *et al.*, 1983; Brown *et al.*, 1984). The *IS26*-mediated one-ended transposition occurring at the 3'-end of the fusion, combined with the non-homologous recombination event found at the 5'-end in the N3::pSJ5.2 cointegrates suggest that two recombination mechanisms occurred simultaneously at the two ends (Fig. 1b).

Although there were differences in the mechanisms for pSJ5.2 cointegration with N3 and R64 in the 1.8 kb BamHI-HindIII fragment, the net results were the same: crossover between coordinates 1817 and 2849 or 3010 led to the deletion of \approx 1,032 bp or 1193 bp from pSJ5.2 during the intermolecular R64::pSJ5.2 and N3::pSJ5.2 non-homologous recombination event, respectively (Figure 1c). Based on these observations, we conclude that the 1.8 kb BamHI-HindIII fragment of pSJ5.2 contains hot-spots for intermolecular recombination which promote the formation of pSJ5.2 fusions with conjugative plasmids N3 and R64 during conjugal transfer.

Mobilization of pSJ5.2 by the 41 kb *tet(M)* self-transmissible plasmid in *E. coli* involved *IS1*-mediated cointegration. It is known that the Insertion Sequence 1 play an active role in the formation of plasmid cointegrates and resolution by a replicative site-specific recombination mechanism (Crisona *et al.*, 1980; Masepohl and Puehler, 1983; Elhai *et al.*, 1994; Mahillon and Chandler, 1998; Martinez and De la Cruz, 1998; Shiga *et al.*, 2001; Grindley, N., 2002). The presence of two *IS1* copies within the 2.4 kb BamHI-BamHI fragment in the pSJ5.2 insertion derivatives isolated from the *E. coli* transconjugants is compatible with the presence of at least one copy of *IS1* in both the 41 kb *tet(M)* conjugative and pSJ5.2 plasmids. However, we did not find *IS1* Southern hybridization signals in the *N. gonorrhoeae* genomic and plasmid DNAs. Therefore, the genomic DNA from the *E. coli* strains used in the previous triparental matings (Scharbaai-Vázquez, *et al.* 2007) were the source of the *IS1*. To our knowledge, this type of *IS* recruitment to form plasmid cointegrates has not been reported. In addition, the results demonstrate that *IS1* is likely to play an important natural role in mobilizing plasmids across the species and generic boundaries between *Neisseria* and non-related Gram negative bacteria.

The *IS1* insertion element possesses outwardly directed -35 promoter hexamers located in the terminal inverted repeats that creates a hybrid promoter when transposing at the correct distance from a resident -10 hexamer (Iida *et al.*, 1987; Olliver *et al.*, 2005). The effect of the new hybrid promoter on the TEM-beta lactamase gene of pSJ5.2 remains to be elucidated. Other transposable elements show similar mechanisms of altering expression of adjacent genes (Barker and Matsumura, 2004; Siu *et al.*, 2003; Olliver *et al.*, 2005).

Our evaluation of recombination events occurring at the 1.0 kb BamHI-HindIII fragment of pSJ5.2 indicates that the region contains a hot spot for pSJ5.2 cointegration with self-transmissible plasmids N3 and R64. Previous BLAST computer analysis of the 1.0 kb BamHI-HindIII fragment from pSJ5.2 (GenBank Accession No. DQ355980) revealed that it contains part of the ORF which corresponds to coordinates 3908-4438 of the Tn3 resolvase, TnpR gene (Scharbaai-Vázquez, *et al.*, 2007; Fig. 6). The TnpR gene is a site-specific recombinase involved in the process of cointegrates resolution and together with the transposase, TnpA and beta-lactamase, bla genes constitute the Tn3 compound transposon. In the gonococcal R-plasmids, however, the whole TnpA and approximately 84% of the TnpR genes are truncated by a natural deletion (Candelas, 2000). Nevertheless, the remnant of the TnpR gene in pSJ5.2 can recombine with other conjugative plasmids containing Tn3 transposons such as N3 and R64, provided that their resolvase DNA regions have significant homologies with it. In the case of the R64::pUC1.0 junction, crossover between the two plasmids comprises a 155 bp homologous DNA region within the resolvase genes (Figure 6b). Similarly, the DNA homology between the resolvases from pSJ5.2 and N3 comprises a 209 bp overlapped region (Figure 6b). BLAST analysis of the pSJ5.2 Tn3 partial resolvase gene also showed between 89 to 100% similarity at the nucleotide level to several resolvases from the Tn1, and Tn2 families of resolvases (data not shown). Therefore, plasmids with resolvases that share extensive homology with the resolvase of pSJ5.2 could form cointegrates. The 1.0 kb fragment of pSJ5.2 may therefore function as a hot-spot site for homologous recombination with resolvase-encoding regions.

Analysis of the four mechanisms of recombination reported here for the conjugal transfer of pSJ5.2 by cointegration (*IS1* replicative transposition, illegitimate recombination, non-homologous combined with *IS26* transposition and homologous recombination), suggests that *N. gonorrhoeae* possesses efficient systems for both homologous and non-homologous recombination in a heterologous background. It is of particular interest of what is the role of illegitimate recombination in lateral transfer of genes in the gonococcal genome, due to the fact that *N. gonorrhoeae* can associate with non-related STD pathogens and commensal organisms from the urogenital flora (Kroll et al. 1998; Reid, 2005).

5. Concluding remarks

The *Neisseria gonorrhoeae* beta-lactamase encoding (R)-plasmids have evolved into highly successful genetic elements; a characteristic that is shared by its pathogenic host. While gonococcal 7.4 kb and 5.6 kb R-plasmids depend on an *oriT* / mob system for their conjugal transfer by self-transmissible plasmids, the 5.2 kb R-plasmid has developed an impressive capability of spread and survival using multiple recombinational mechanisms. Multiple sequences that can serve as recombination targets compensate for the lack of a functional *oriT* for mobilization. This repertoire of recombination events could be a rudimentary, yet efficient mechanism for the mobilization of pSJ5.2 among gonococci. The results of this study might provide a foundation to identify strategies that would abrogate mobilization functions in the resistance plasmids (e.g., antisense RNA or agents that block *cis*-acting sites involved in recombination). On the other hand, this unique experimental system for mobilization of the naturally occurring β -lactamase plasmid pSJ5.2 can be used to provide the basis for the design of integrative plasmid recombination assays which may help to understand the role of genes

involved in DNA recombination in the gonococcus. Moreover, it can be used to design novel recombination gene delivery vectors in *Neisseria* species and other pathogenic Gram negative bacteria.

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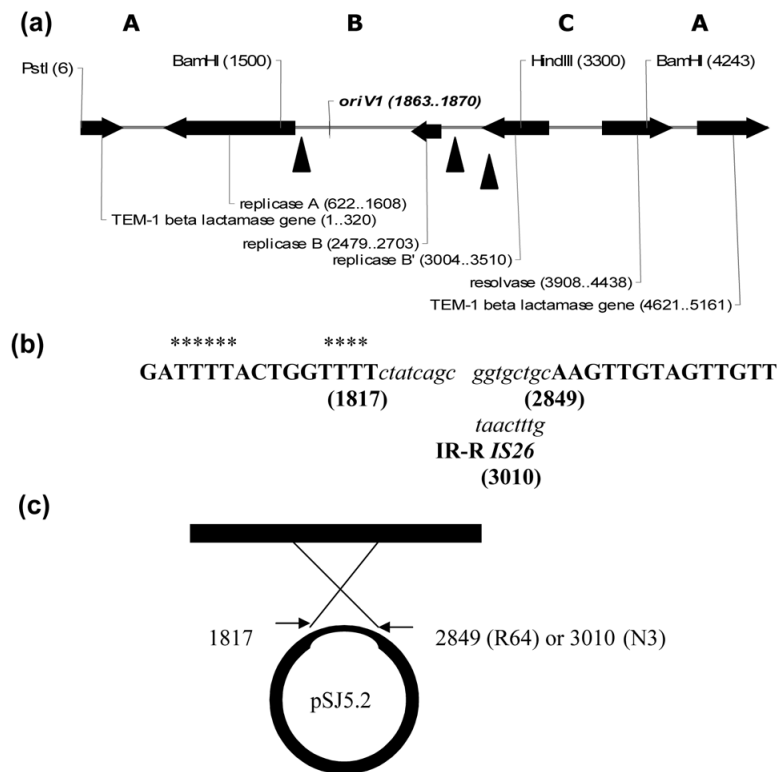
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**Figure 1.**

Hot spots for cointegration of N3 and R64 self-transmissible plasmids within the 1.8 kb BamHI-HindIII site of pSJ5.2 (B region). (a) The PstI-linearized map of pSJ5.2 showing the enzymes used in the study. Letters A, B and C refer to the 2.4 kb, 1.8 kb and 1.0 kb fragments generated by double digestion with BamHI and HindIII. Genes, origin of replication *oriV1* and restriction enzyme coordinates are in parentheses. Hot-spots for cointegration are marked by three solid black triangles. (b) DNA sequence of the three recombinational crossover points at coordinates 1817, 2849 and 3010 of pSJ5.2; they were deposited in the GenBank under Accession Numbers EF527803, EF527804 and EF527805; respectively. The partial sequence from pSJ5.2 is shown in bold capital letters; partial sequences from N3 and R64 are shown in lower case italics letters. Parentheses denote the DNA coordinate of recombination. Asterisks represents AT-rich region at the 5'-end of junction. Refer text for details. (c) Schematic representation of the structure and proposed mechanism of the N3-pSJ5.2 and R64-pSJ5.2 cointegrates at the 1.8 kb BamHI-HindIII of pSJ5.2. The solid black rectangle represent DNA insert from N3 and R64; the thick loop represents pSJ5.2 with deleted DNA indicated by the thin region of the circle at the site of recombination.

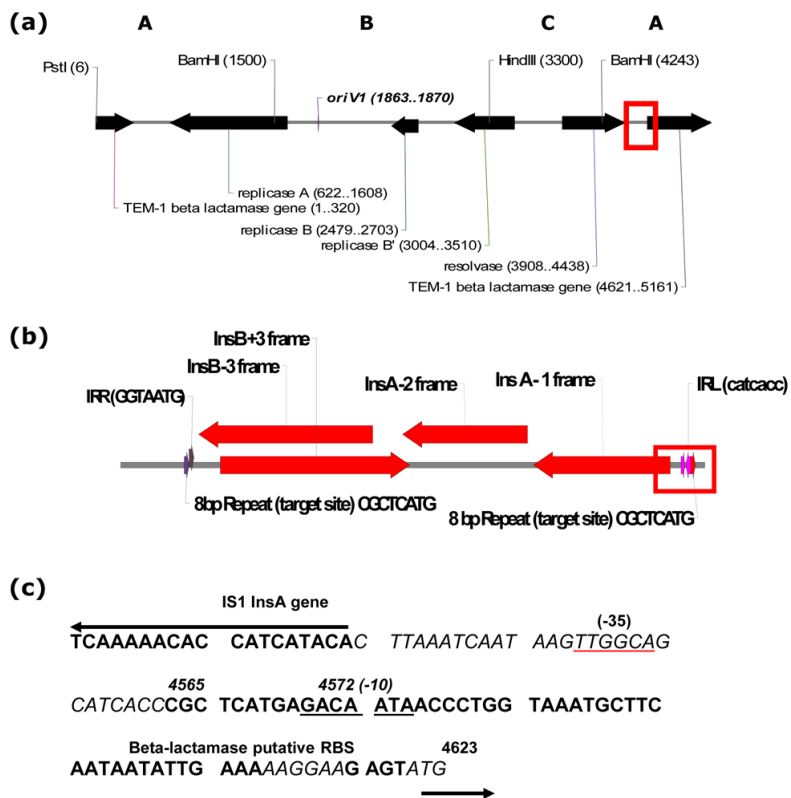


Figure 2. *IS1* integration within the 2.4 kb BamHI-BamHI site (A region) of pSJ5.2 after transfer by *tet(M)* conjugative plasmid. (a) *Pst*I-linearized map of pSJ5.2 showing the site of *IS1* insertion (open red square). Refer text for details. (b) Detail of the two overlapped *IS1* copies found inserted in the pSJ5.2 derivative. Open Reading Frames and direction of transcription of the InsB and InsA genes are shown in red solid arrows. Red open square denote the formation of an *IS1*-beta lactamase hybrid promoter. (c) The DNA sequence of the *IS1*-beta-lactamase hybrid promoter in detail (Accession Number EF527806). Only the promoter region of the InsA gene from one *IS1* and part of the pSJ5.2 beta-lactamase gene promoter are shown. Coordinates are in bp and relative to pSJ5.2. InsA gene and the 28 bp inverted repeat are shown in red and red italics, respectively. The beta-lactamase promoter region is shown in black. The 8 bp *IS1* insertion site from pSJ5.2 is shown in blue. Black arrows denote direction of both InsA and beta-lactamase gene transcription. The -35 and -10 regions of the promoter are shown underlined. RBS (Ribosome Binding Site) in italics.

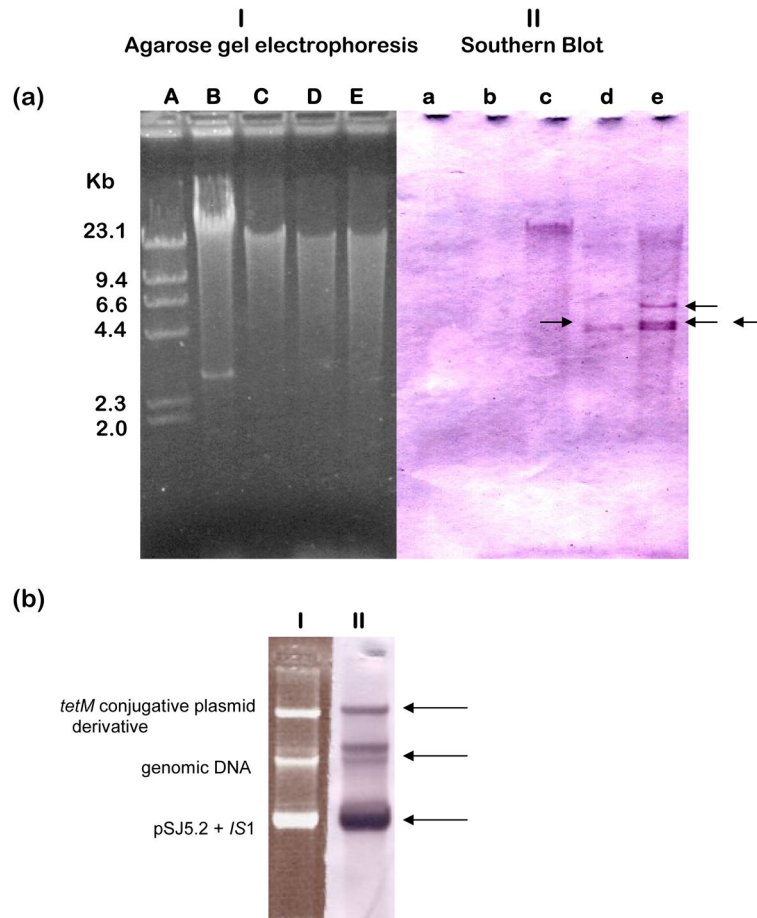


Figure 3. Copy number and distribution of the Insertion Sequence *IS1* in the *Neisseria gonorrhoeae* and *Escherichia coli* strains used in the study. (a) Agarose gel electrophoresis of *EcoRI*-digested genomic DNA's (Panel I) and Southern Blot hybridization with an *IS1*-derived non-radioactive probe (Roche, IN; Panel II). Lane Aa, Lambda + Hind III control (NEB Labs); Lane Bb, *N. gonorrhoeae* 15315 (showing the undigested 4.2 kb cryptic plasmid); Lane Cc, *E. coli* DH5 α ; Lane Dd, *E. coli* DH10B; Lane Ee, *E. coli* HMS174. Black arrows indicate the *IS1* hybridization signals. (b) Undigested DNA from a representative *E. coli* HMS174 transconjugant showing the conjugative *teM* and pSJ5.2 both resolved (Panel I) and with at least one *IS1* hybridization signal each (black arrows in Panel II).

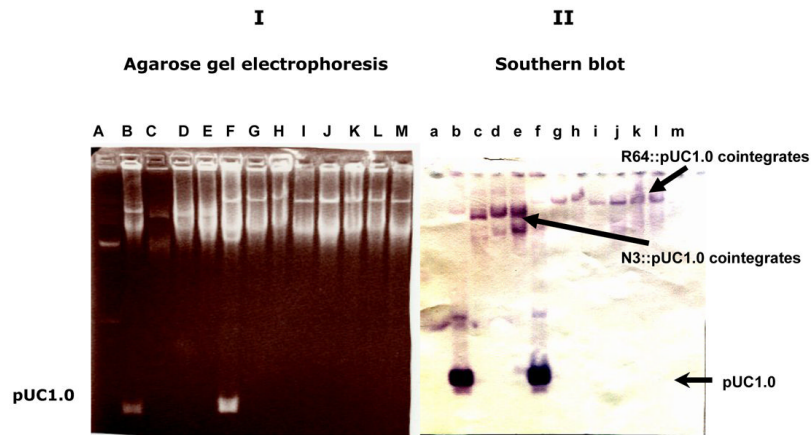


Figure 4.

Formation of N3::pUC1.0 and R64::pUC1.0 cointegrates during mobilization of pUC1.0 with self-transmissible plasmids N3 and R64. (I) Agarose gel electrophoresis of undigested DNA. (II) Southern Blot hybridization with a 1.0 kb BamH1-HindIII probe from pSJ5.2. Lanes Aa - BacTracker supercoiled DNA ladder (Epicentre, Madison WI); Lanes Bb - Donor cell with pUC1.0 and N3 plasmids located at the bottom at top of gel, respectively; Lanes Cc to Ee — representative N3/pUC1.0 transconjugants; Lanes Ff — Donor cell with pUC1.0 and R64 plasmids located at bottom and top of gel, respectively; Lanes Gg to Mm — Representative R64/pUC1.0 transconjugants.

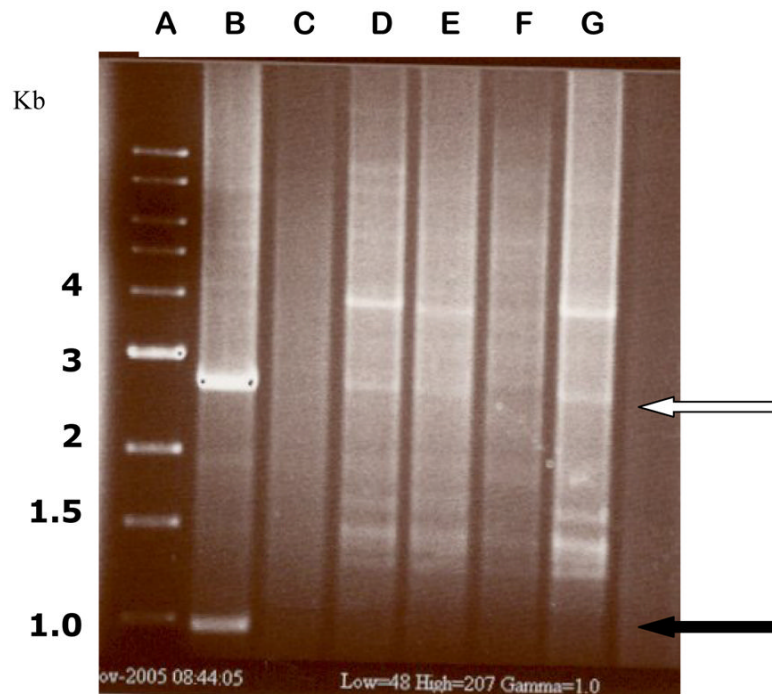


Figure 5. BamHI-HindIII restriction analysis of representative ampicillin-resistant *E. coli* transconjugant colonies containing N3::pUC1.0 and R64::pUC1.0 plasmid cointegrates. The DNA's were analyzed with a rapid boiling DNA extraction and restriction screening method (Epicentre; Madison, WI). Lane A - 1 kb control ladder; Lane B - pUC1.0 control with the 2.9 kb band from pUC19 (white arrow) and the 1.0 kb fragment derived from pSJ5.2 (solid black arrow); Lane C - digested R64::pSJ5.2 DNA; D and E - R64::pUC1.0 transconjugants; Lanes F and G - N3::pUC1.0 transconjugants (the 2.9 kb band from pUC19 is barely visible in lane F). The 1.0 kb BamHI-HindIII fragment is not visible in the cointegrates analyzed from lanes D to G, confirming that integration occurred within that fragment.

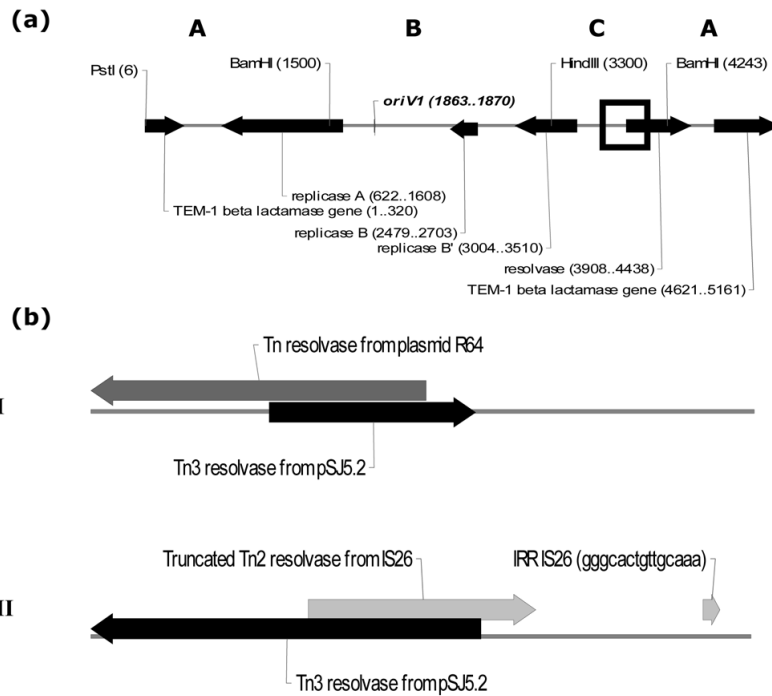


Figure 6. Resolvase-mediated homologous recombination between the conjugative plasmids N3 and R64 and the 1.0 kb BamHI-HindIII fragment of pSJ5.2 (C region). (a) PstI- linear map of pSJ5.2 showing the site of recombination (open black square). (b) Description of the recombination intermediates recovered in the R64::pUC1.0 (I) and N3::pUC1.0 (II) plasmid cointegrates. The sequences were deposited in the GenBank database under Accession Numbers EF527807 and EF527808, respectively. The Tn3 resolvase gene of pSJ5.2 is shown in solid black arrow. The Tn5393 resolvase from R64 and the IS26-derived resolvase from N3 are shown in dark and light gray arrows, respectively. (IRR) = Right Inverted Repeat from IS26. Note that the scale is different in the two panels.

Table 1

Plasmids used in the study

Plasmids	Relevant characteristics	Reference or source
	Plasmid vectors	
pUC19	<i>E. coli</i> cloning vector; amp ^R , lacZ α	Invitrogen, CA
	Recombinant plasmids	
pUC3.6	2.4 kb BamH1-BamH1 fragment plus an insert isolated from a pSJ5.2 insertion derivative and cloned into pUC19	This study
pUC1.0	1.0 kb BamH1-HindIII fragment of pSJ5.2 cloned into pUC19	Our laboratory
	Clones derived from plasmid rescue experiments and subclones	
pN5.2	Miniplasmid obtained by rescue of pSJ5.2 from a N3::pSJ5.2 cointegrate	This study
pUC3.0	3,010 bp BamH1-HindIII fragment from pN5.2 cloned into pUC19	This study
pR5.2	Miniplasmid obtained from rescue of pSJ5.2 from a R64::pSJ5.2 cointegrate	This study
pUC3.5	3,475 bp BamH1-HindIII fragment from pR5.2 cloned into pUC19	This study
pN1.0	Miniplasmid obtained from the rescue of pUC1.0 from a N3::pUC1.0 cointegrate	This study
pR1.0	Miniplasmid obtained from the rescue of pUC1.0 from a R64::pUC1.0 cointegrate	This study

Table 2

Oligonucleotides used in the study.

Oligonucleotides	Description / (Coordinates, bp)	Nucleotide sequence (5' – 3')	Source
Primers for PCR amplification of IS1			
IS1F119	<i>InsB</i> gene from <i>IS1</i> (421) *	AGCTCCACCGATTTTGAGAA	This study
IS1R119	<i>InsB</i> gene from <i>IS1</i> , reverse (520) *	AAGCTGCACGTAATCAGCAA	
Sequencing Primers for pUC19-derived clones			
M13F	Universal Primers	GTTTCCAGTCACGAC	Invitrogen
M13R		CAGGAAACAGCTATGAC	
Walking Primers for Sequencing <i>IS1</i> in <i>pUC3.6</i>			
M13FI	Primer based on M13 right border sequence	AGCTCCACCGATTTTGAGAA	This study
M13FII	Primer based on M13 FI right border sequence	TCTCTGCTCTCACTGCCGTA	This study
TEM-P 1R	Primer based on TEM-1 <i>beta</i> <i>lactamase</i> flanking sequence and used to close the gap between <i>beta-lactamase</i> and <i>IS1</i>	CGCCGCATACACTATTCTCA	This study
Primers for sequencing junctions within pN5.2 and pR5.2			
REPA	Anneal adjacent to BamH1 site from pSJ5.2 <i>repA</i> gene (1497)	TATTGCGTGAATTGCCCTTT	This study
REPB	Anneal adjacent to HindIII site from pSJ5.2 <i>repB</i> gene (3296, reverse)	TGCGGATTCTGGCTTAACT	This study

* Coordinates 421 to 520 are relative to the *IS1* mapped within the pSJ5.2 insertion derivatives