Complex Multiple Antibiotic and Mercury Resistance Region Derived from the r-det of NR1 (R100)

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The sequence of the 45.2-kb multidrug and mercury resistance region of pRMH760, a large plasmid from a clinical isolate of *Klebsiella pneumoniae* collected in 1997 in Australia, was completed. Most of the modules found in the resistance determinant (r-det), or Tn*2670*, region of NR1 (also known as R100), isolated from a *Shigella flexneri* strain in Japan in the late 1950s, were present in pRMH760 but in a different configuration. The location was also different, with the Tn*2670*-derived region flanked by the transposition module of Tn*1696* and a mercury resistance module almost identical to one found in the plasmid pDU1358. This arrangement is consistent with a three-step process. First, the r-det was circularized via homologous recombination between the IS*1* elements and reincorporated at a new location, possibly in a different plasmid, via homologous recombination between the 5′-conserved (5′-CS) or 3′-CS of the In34 integron in the r-det and the same region of a second class 1 integron in a Tn*1696* relative. Subsequently, resolvase-mediated recombination between the *res* sites in the r-det and a second mercury resistance transposon removed one end of the Tn*1696*-like transposon and part of the second transposon. Other events occurring within the r-det-derived portion have also contributed to the formation of the pRMH760 resistance region. Tn*2* or a close relative that includes the *bla*$_{\text{TEM-1b}}$ gene had moved into the Tn*21* mercury resistance module with subsequent deletion of the adjacent sequence, and all four 38-bp inverted repeats corresponding to Tn*21* family transposon termini have been interrupted by an IS*4321*-like element.

One of the earliest resistance factors to be identified was NR1, subsequently also called R100, a 94.3-kb self-transmissible multiresistance plasmid isolated from a *Shigella flexneri* strain in Japan in the late 1950s (20). This IncFII plasmid confers resistance to chloramphenicol, streptomycin, spectinomycin, sulfonamides, tetracycline, and mercury, and its complete sequence is now available (GenBank accession no. AP000342). NR1 consists of two components, a resistance transfer factor (RTF) and a resistance determinant region (r-det), separated by two directly oriented copies of the insertion sequence IS*1*. The RTF contains genes for conjugative transfer (*tra*) and replication (*rep*) functions but also includes the transposon Tn*10,* which contains the *tetA*(B) tetracycline resistance determinant (30). The r-det includes the other antibiotic resistance markers of NR1 and a mercury resistance (*mer*) determinant and is essentially equivalent to the transposon Tn*2670*, which includes both copies of IS*1* (13, 16).

Tn*2670* (Fig. 1A) has a complex structure that has arisen via a series of transpositional and other events. It is composed of a transposon closely related to Tn*9* (1), with the mercury and antibiotic resistance transposon Tn*21* (19) within it. The Tn*9*-like backbone consists of the two IS*1* elements flanking a 1,547-bp central region that contains the *catA1* (formerly *cat₁*) chloramphenicol resistance gene and is identical to but 445 bp longer at the IS*1b* end than the central region of Tn*9* (GenBank accession no. V00622). Tn*21* is inserted 58 bp from the

IS*1b* end and is flanked by a 5-bp duplication of the target sequence, as is characteristic of Tn*21* transposition. However, Tn*21* is also made up of a transposon within a transposon (2, 19, 23). The class 1 integron In2, a transposon remnant, is situated 340 bp beyond the end of the Tn*21* resolution site (*res*$_{21}$) and is flanked by a 5-bp target duplication, indicating that it was also acquired by transposition. In2 contains two conserved segments (CS) flanking another mobile element, the *aadA1* gene cassette (encoding streptomycin and spectinomycin resistance), as well as a partial transposition (*tni*) module and two insertion sequences, IS*1326* and IS*1353* (2). The 5′-CS includes the *intI1* gene, the *attI1* site, and the P$_c$ promoter typical of class 1 integrons (10, 11), and the 3′-CS contains the *sul1* sulfonamide resistance gene.

Tn*2670* is an active transposon (13, 16), and in NR1, it is flanked by a 9-bp target site duplication characteristic of insertion of IS*1*. However, RecA-dependent homologous recombination between the IS*1a* and IS*1b* elements of Tn*2670* can form an extrachromosomal, covalently closed circular molecule containing a single copy of IS*1* (3, 4, 29). Although the r-det circle is not a stable replicon, it could effectively act as a mobile element if it was incorporated at other locations by homologous recombination, for example, between the IS*1* in the circle and a copy of IS*1* located in a new target molecule or between the conserved regions in the integron and a second integron.

We recently reported the structure of an In2-like class 1 integron, In34 (Fig. 1B), from a clinical multidrug resistant *Klebsiella pneumoniae* strain isolated at a Sydney hospital in 1997 (25). In34 (17.53 kb) includes a backbone identical to that of In2, but the *aadB* gene cassette (encoding gentamicin, kanamycin, and tobramycin resistance) has replaced the *aadA1*

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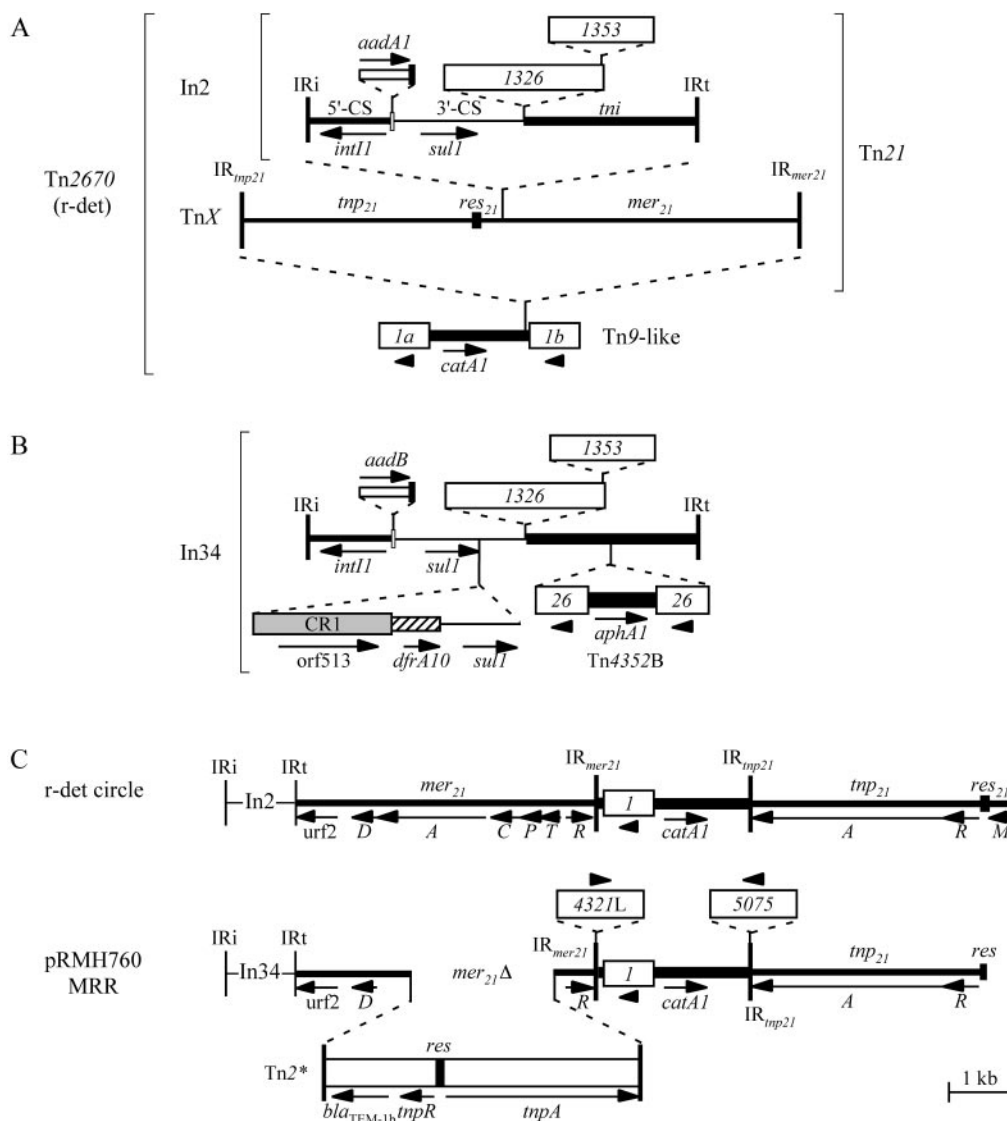
FIG. 1. Structure of (A) Tn2670 and (B) In34. IRs at transposon and class 1 integron termini are shown as solid vertical bars. The *res* sites of Tn21 (res_{21}) and Tn2* (*res*) are indicated by shorter vertical bars. The names of the various modules are indicated above the lines. Arrows below the lines indicate the positions of relevant genes. Insertion sequences are shown as open boxes containing the IS number, and, where relevant, arrowheads indicate their orientation. Gene cassettes are shown as narrow open boxes with a bar at one end to represent the 59-be, and *attI1* is shown as an open vertical bar. CR1 is a shaded box, and the *dfrA10* region is a hatched box. The Tn2670 map was constructed from the sequence in GenBank accession no. AP000342 and In34 from GenBank accession no. AY123253.1. (C) Comparison of the circular form of r-det, opened at IRi, with part of the pRMH760 MRR. The structures of the integrons In2 and In34 are as in panels A and B, respectively.

cassette, and additional resistance genes have been acquired (Fig. 1B). As part of the *tnp* module of Tn1696 (tnp_{1696}) (24) was found adjacent to IRi (inverted repeat at *intI1* end) and part of the mer_{21} module was found adjacent to IRt (inverted repeat at *tni* end) of In34, with precisely the same boundaries between In34 and these modules as in Tn1696 and Tn21, we proposed that this mixed transposon configuration arose by homologous recombination between the 5′-CS or 3′-CS regions of integrons in transposons related to Tn21 and Tn1696 (25). However, two additional antibiotic resistance genes, *catA1* (encoding chloramphenicol resistance) and bla_{TEM} (encoding β-lactam resistance) were identified and mapped close

to In34, indicating the presence of a larger multiresistance region (MRR) in pRMH760 (25).

As pRMH760 contains several components in common with Tn2670 from NR1 (an In2-like integron, at least part of mer_{21} and the *catA1* gene), it seemed possible that the resistance region of pRMH760 is derived from Tn2670. Also, because NR1 dates from the early days of antibiotic use and pRMH760 is a recent isolate, this derivation would indicate that large, complex MRRs persist as discrete entities but continue to evolve over time. To examine these possibilities, the remainder of the pRMH760 MRR was sequenced to determine its structure and compare it to Tn2670 and other known MRRs.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Escherichia coli* DH5α was used to propagate plasmid DNA. pRMH760 was isolated from *K. pneumoniae* strain 24120497, and subclones were constructed as described previously (25). Bacteria were routinely cultured at 37°C in Luria-Bertani medium or on Luria-Bertani agar. Appropriate antibiotics (Sigma) were added at the following concentrations: ampicillin, 100 μg ml^{-1}; chloramphenicol, 25 μg ml^{-1}; gentamicin, 8 μg ml^{-1}; kanamycin, 15 μg ml^{-1}; and tobramycin, 12.5 μg ml^{-1}.

DNA sequencing. Plasmid DNA was isolated as described previously (25). PCR was used to amplify fragments of pRMH760 to confirm boundaries between cloned fragments predicted by mapping and to obtain sequences. Amplification reactions were carried out in 50-μl volumes containing 1× PCR Master Mix (Promega), additional MgCl$_2$ to a concentration of 3 mM, 0.4 pmol of each primer, and approximately 20 ng of plasmid template. PCR conditions were generally 94°C for 3 min, followed by 40 cycles of 94°C for 1 min, 57°C for 1 min, and 72°C for 2 min, with a final incubation at 72°C for 5 min. PCR products from Tris-acetate-EDTA–agarose gels were purified for sequencing with an Ultra-Clean DNA purification kit (Mo Bio Laboratories, Inc.). Automated sequencing and DNA sequence assembly were as described previously (25). The sequence of at least one strand of inserts in subclones of pRMH760 was determined for regions for which several sequences are already available in databases (e.g., *tnp$_{21}$*, *mer$_{21}$*, *tnp$_{1696}$*, and IS*1*). Both strands were sequenced for all novel regions where ambiguities were present or if the sequence differed from published sequences.

Sequence analysis. GenBank searches were performed with the BLASTN program available through WebANGIS (Australian National Genomic Information Service). Programs in the Genetics Computer Group Wisconsin Package, version 8.1.0, were used via WAG (WebANGIS GCG) to align and analyze the level of identity of DNA sequences and to identify the boundaries of discrete regions.

Nucleotide sequence accession number. The 45.2-kb sequence of the pRMH760 MRR has been submitted to GenBank under accession no. AY123253.3, which includes 27.5 kb released previously as AY123253.1 (25), AY123253.2, and AY242531-3 (26).

RESULTS

The pRMH760 MRR contains the components of r-det/Tn*2670*. As the short (250-bp) piece of the *mer$_{21}$* sequence previously found adjacent to IRt of In34 (25) (GenBank accession no. AY123253.1) was identical to that found immediately to the right of In2 in Tn*21*, the sequence was extended on this side. A region of 15,998 bp determined here (Fig. 1C) contained the components of Tn*2670* in the order expected for the circular form of r-det reopened within the integron, but with some additions and deletions. The Tn*21* *mer* module is interrupted by a transposon, here designated Tn*2** (see below), that contains the *bla*$_{TEM-1b}$ ampicillin resistance gene. Part of *merA* together with *merC*, *merP*, and most of *merT* was missing, presumably due to Tn*2**-mediated deletion of the adjacent sequence. Both IR$_{tnp21}$ and IR$_{mer21}$ are interrupted by IS*4321*-like elements (IS*5075* and IS*4321L*) as described previously (26), and the region to the right of *res$_{21}$* that includes the gene known as *tnpM* is missing. The sequence of the Tn*2670*-derived region is identical to the corresponding parts of NR1 (AP000342), and the Tn*21*-derived region is also identical to the corrected version of the Tn*21* compilation (AF071413.3). Hence, the pRMH760 MRR backbone appears to be the result of integration of the circular form of a Tn*2670*-like transposon within a second class 1 integron.

Location of the r-det region. We had also previously found (25) that about 220 bp immediately adjacent to IRi of In34 matched the part of the *tnp$_{1696}$* module immediately adjacent to In4 (GenBank accession no. U12338) (24). This suggested that the *tnp$_{1696}$*-In34-*mer$_{21}$* configuration in pRMH760 could have arisen by homologous recombination between the 5′-CS

or the 3′-CS of two integrons, one in the same position as In4 in a Tn*1696* backbone and the other related to In2 and located in a Tn*21* backbone. If the In2-like integron, i.e., In34, was part of a circular r-det molecule, such an event would result in a structure in which two hybrid transposons containing integrons flank the *catA1* region and IS*1* (Fig. 2A). When the sequence was extended to the left of In34, a complete *tnp$_{1696}$* module was found, but an IS*4321L* element interrupted IR$_{tnp1696}$ (Fig. 2B). However, the other end was not as predicted. The segment extending from *res$_{21}$* through the r-det-derived integron to the crossover position, the remainder of the In4-like integron, and the *mer$_{1696}$* module were missing, suggesting that at least one additional step was involved in the formation of the backbone of the pRMH760 MRR. The missing region had been replaced by a *mer* module different from both *mer$_{21}$* and *mer$_{1696}$* (Fig. 2B).

Identity between the sequences of the pRMH760 MRR and Tn*21* ends within *resI*, one of the three subsites of *res$_{21}$*, and just after the AT dinucleotide (Fig. 3) at which resolvase-mediated recombinational crossover occurs (28). For 135 bp from this AT dinucleotide, the sequence is identical (Fig. 3) to the region of a partially sequenced transposon (GenBank accession no. AJ581664) immediately preceding a class 1 integron. The sequence identity may continue beyond the other end of that integron, but that sequence is not available. This indicates that a further step in the formation of the pRMH760 MRR backbone was resolvase-mediated recombination between *res$_{21}$* and the *res* site of a second transposon (Fig. 2C). Resolvase-mediated excision events occur at high frequency only when, as shown in Fig. 2C, the two *res* sites involved are in the same DNA molecule and in the same orientation (8), and this event would result in formation of the backbone structure found in the pRMH760 MRR plus an excised circular segment.

The second *mer* module. The *mer* module adjacent to the hybrid *res* site was also completely sequenced. It is 6.639 kb long and ends with a 38-bp IR identical to IR$_{tnp21}$ that is interrupted by IS*4321L* (26). It contains genes corresponding to and 79 to 85% identical to *merA*, *merD*, *merP*, *merR*, and *merT* of Tn*21*, an open reading frame that is 79% identical to *urf2*M (urf2) of Tn*X* and Tn*5060* (18, 19, 24) and a *merB* gene (Fig. 2B). The last 6.08 kb of this region is nearly identical to the available sequence of the broad-spectrum *mer* module found in the *Serratia marcesens* plasmid pDU1358 (obtained by joining sequences from GenBank accession no. M15049, M24940, and AY225348) (7, 21), which has IS*4321R* in place of IS*4321L* (26). Identities are 99.6% in the *mer* module and 97.0% in the IS.

Tn*2 and its context.** The 4.95-kb transposon containing the *bla*$_{TEM1-1b}$ gene located in the *mer$_{21}$* region of the pRMH760 MRR is 98.2% identical to Tn*1* from RP1/RP4 (L27758) (22) and 97.4% identical to Tn*3* from R1 (V00613) (14). It was designated Tn*2** because part of the 4.95-kb sequence is identical to the 1.516 kb, including the *bla*$_{TEM-1b}$ gene and part of *tnpR*, that is available for the original Tn*2* from RSF1030 (X54607) (5, 6). The remainder may also be identical. Database searches with Tn*2** identified three identical, or nearly identical, sequences. A transposon with 22 differences from Tn*2**, 8 of which would result in frameshifts, is found in the *S. flexneri* transposon TnSF1 (AF188331). High identity with the pRMH760 MRR extends on both sides of Tn*2**, from the
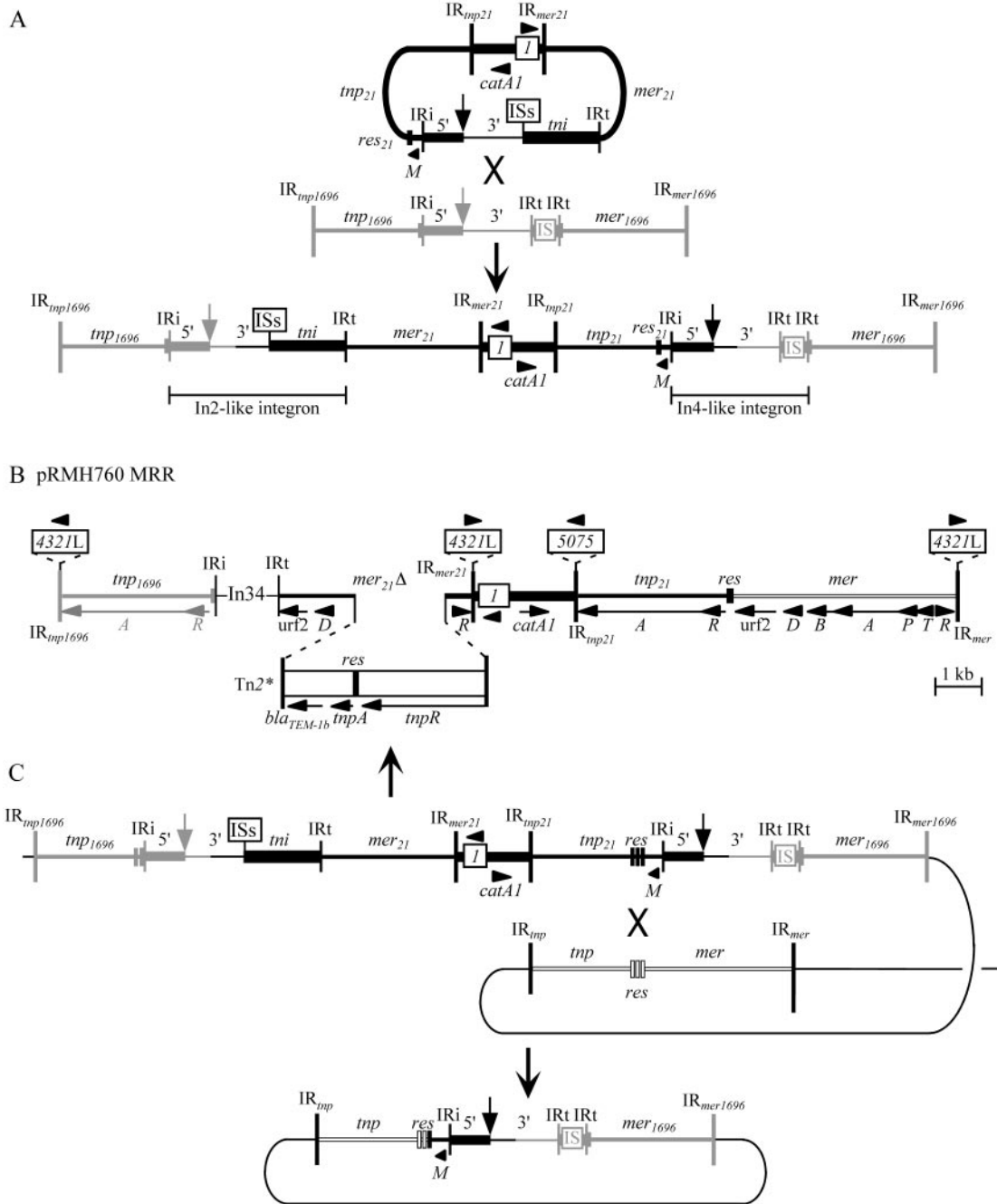
FIG. 2. Model for formation of the pRMH760 MRR. (A) Incorporation of a circular r-det by homologous recombination between two integrons, with the crossover within the 3′-CS. (B) The complete pRMH760 MRR. The full structure of In34 is given. (C) Generation of the pRMH760 MRR backbone by resolvase-mediated recombination. Resolvase-mediated excision by crossing over within *res* would result in the pRMH760 MRR structure shown in panel B plus the excised circle shown below. Features are largely as shown in Fig. 1, except that vertical arrows indicate the positions of gene cassettes, 5′ and 3′ indicate the 5′-CS and 3′-CS, and the boxes labeled ISs and IS represent IS*1326* plus IS*1353* and IS*6100*, respectively. Tn*1696*-derived regions are shown in gray, and the *mer* region related to that of pDU1358 is shown as a narrow open box. (C) Three subsites of *res* are shown (not to scale).

distal end of Tn*4352*B on the left to IR$_{mer21}$ (which is uninterrupted in TnSF1) on the right. Some of the differences in Tn*2** may be due to sequence errors in TnSF1, as three bases are also missing from the 2.39 kb of the flanking *mer* module sequence. A partial copy of Tn*2** found in the *E. coli* plasmid p1658/97 (AF550679) is truncated by IS*26* and lacks the

bla$_{TEM}$ end. The remaining 2.956 kb is identical to Tn*2** and is followed by a sequence identical to that of the pRMH760 MRR up to and including IS*1*, except that IR$_{mer21}$ is not uninterrupted by an IS*4321* or IS*5075* element. The third copy, found in a *Salmonella enterica* serovar Enteritidis strain (AB103092) (17), is identical to Tn*2**, but the sequence is
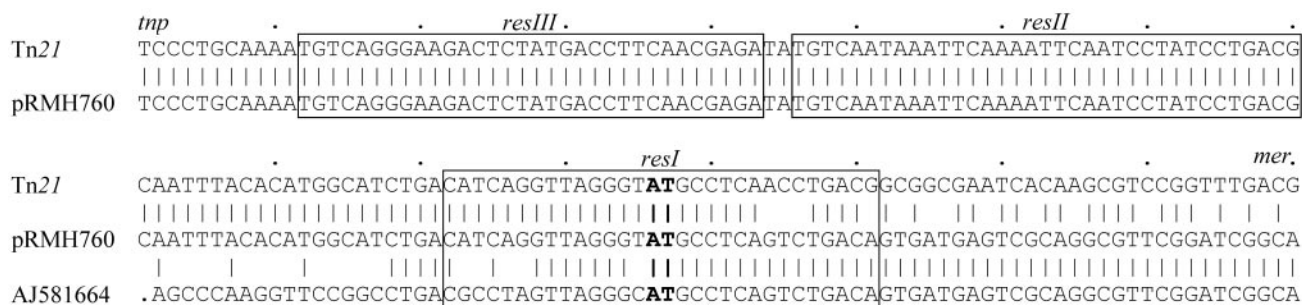
FIG. 3. Comparison of *res* sites. The sequences of the complete *res* sites of Tn*21* (GenBank accession no. AF071413), the pRMH760 MRR, and the partial *res* site in GenBank accession no. AJ581664 are aligned, with vertical lines indicating identical bases. The first nucleotide shown corresponds to position 3,579 from the start of IR$_{tnp21}$. The *res* subsites (12) are boxed, and the AT dinucleotide at which resolvase-mediated recombination takes place is shown in boldface. *tnp* and *mer*, regions adjacent to each end of the *res* site.

incomplete, with 59 bp missing from the *bla*$_{TEM}$ end. It is located in a context different from that of the other three. The fact that the context of Tn*2** is the same in pRMH760, TnSF1, and on one side in p1658/97 suggests that Tn*2** may have moved into Tn*2670* or a relative prior to its circularization and reincorporation to yield the configuration in the pRMH760 MRR

DISCUSSION

The 45.2-kb MRR of pRMH760 has a complex structure made up of a number of types of mobile elements. However, it appears to have evolved in relatively few steps from other known regions. A 33.5-kb segment that includes In34 (17.53 kb) and all of the antibiotic resistance genes is clearly derived from a transposon related to Tn*2670*, but a new order of components has been created by circularization of this transposon, followed by incorporation of the circle at a new location via homologous recombination occurring within a region different from that used to form the circle. This part of the MRR has also diverged from Tn*2670* by the acquisition of a number of DNA segments containing additional antibiotic resistance genes and of insertion sequences. Two of the additional resistance genes are found within In34 (Fig. 1B), as reported previously (25), and one, the *bla*$_{TEM-1b}$ gene in Tn*2**, is within the region examined here. Two short segments of the Tn*2670*-derived region have been lost, one that appears to be due to a deletion in the *mer*$_{21}$ module adjacent to Tn*2** and one that consists of the region of Tn*21* found between *res*$_{21}$ and the IRi end of In2. While most of these events could have occurred either before or after the r-det excision and integration events, loss of the *tnpM* segment must have occurred subsequently and is clearly the result of a resolvase-mediated excision event (Fig. 2).

The role of homologous recombination in the dissemination of antibiotic resistance genes is frequently overlooked. However, many small circles can be formed by homologous recombination between pairs of identical sequences in direct orientation, with the circular r-det molecule and the circular form of the common region 1 (CR1)-*dfrA10-sul1* region of In34 described previously (25) being just two examples. Integration of these circles either by opening them at a different position, as occurred in the formation of the pRM760 MRR backbone, or at an equivalent position as demonstrated previously in the

case of the CR1-containing circle (25), can also involve homologous recombination. Replacement of the *aadA1* cassette found in In2 by the *aadB* cassette in In34 is also likely to have occurred via homologous recombination between the flanking 5′-CS and 3′-CS, and similar exchanges have been demonstrated experimentally (23). These examples clearly illustrate the importance of homologous recombination in evolution of the pRMH760 MRR and other MRRs.

Both the requirement for the two *res* sites to be on the same molecule and in the same orientation and the phenomenon of transposon exclusion constrain the ways in which resolvase-mediated events can contribute to plasmid evolution. However, the resolution systems that normally complete the transposition process of transposons that form cointegrate intermediates are very efficient. Hence, when two directly oriented *res* sites are introduced into the same DNA molecule, resolution is likely to occur rapidly. Compatible but nonidentical *res* sites are also known to recombine efficiently (9). Thus, homologous recombination followed by resolution, as seen here in the formation of the pRMH760 MRR, may be a common theme in the evolution of MRRs, when intact *res* sites are found in both of the molecules brought together by homologous recombination.

The wide distribution of class 1 integrons provides regions of homology between many different plasmids and has provided the incorporation site for the circular r-det described here. However, the targeting of class 1 integrons to *res* sites means that they often inactivate the *res* sites of transposons such as Tn*1696* and Tn*1403* that contain them (27). Hence, the Tn*1696* relative that appears to have been the target of the r-det integration event would not be able to participate in a resolvase-mediated event to remove the region between *res*$_{1696}$ and *res*$_{21}$ in the initial integration product (Fig. 2A). However, because In2 is a short distance from the *res* site in Tn*21*, its resolution system remains active (24) and has interacted with a third transposon only part of which remains in the final MRR. Other combinations of homologous and resolvase-mediated recombination processes can also occur; for example, the circular molecule created by the resolvase-mediated excision step of pRMH760 MRR evolution (Fig. 2C) could be incorporated at new locations by homologous recombination.

Two of the transposons in the pRMH760 MRR, Tn*2** and Tn*4352B*, are found with exactly the same flanking sequences

in the Tn*21*-derived transposon Tn*SF1* from *S. flexneri* (Gen-Bank accession no. AF188331). As transposition to precisely the same location is unlikely to occur twice and the changes to the structure surrounding the transposon, such as the deletion adjacent to Tn*2**, are also most likely to have arisen only once, this may indicate that Tn*SF1* and the pRMH760 MRR have a common ancestor. However, other explanations are also possible. For example, these transposons can also potentially be spread by homologous recombination into any region containing the sequence that flanks them. Hence, the two multidrug resistance regions cannot necessarily be assumed to be related to one another by direct lineal decent.

The reason why antibiotic resistance genes are often found clustered within a single large region made up of transposons within other transposons, as seen in Tn*2760*, the pRMH760 MMR, Tn*SF1*, and other unrelated regions has been the subject of some speculation. However, it may simply reflect the fact that in the compactly organized genomes of plasmids there are few places to go that are without deleterious consequences. Insertion of DNA into the *rep* region of a plasmid would prevent replication leading to loss of the plasmid, and insertion into a *tra* region would destroy the ability to transfer into new hosts, restricting its spread by horizontal gene transfer. In contrast, insertion of additional resistance genes into a transposon already in the plasmid would not affect vital plasmid functions. Under the selective pressure of antibiotic use, the additional resistance genes will confer a selective advantage on cells containing the MRR, favoring its retention. The genesis of other clusters of antibiotic resistance genes that have been identified, such as those in the SXT elements of *Vibrio cholerae* (15), may reflect the same constraints in operation. However, once formed, these complex structures can, as found here, move in a block to a new location and continue to exchange genes with and acquire genes from related regions via homologous recombination. Further examination of the broader context of genes originally found in Tn*2670* and its known relatives in both historic and modern-day bacterial isolates should shed further light on the evolutionary pathways involved in creating and spreading derivatives of Tn*2670* and how prevalent they are in modern day bacteria.

ACKNOWLEDGMENTS

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