A Type 2 A/C_2 plasmid carrying the *aacC4* apramycin resistance gene and the *erm*(42) erythromycin resistance gene recovered from two *Salmonella enterica* serovars

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Abstract

Objective: To determine the relationships between RepA/C_2 plasmids carrying several antibiotic resistance genes found in isolates of *Salmonella enterica* serovars Ohio and Senftenberg from pigs.

Methods: Illumina HiSeq was used to sequence seven *S. enterica* isolates. BLAST searches identified relevant A/C_2 plasmid contigs, and contigs were assembled using PCR.

Results: Two serovar Ohio isolates were ST329 and the five Senftenberg isolates were ST210. The A/C_2 plasmids recovered from the seven isolates belong to Type 2 and contain two resistance islands. Their backbones were closely related, differing by five or fewer single nucleotide polymorphisms. The *sul2*-containing resistance island ARI-B is 19.9 kb and also contains the kanamycin and neomycin resistance gene *aphA1*, the tetracycline resistance gene *tetA*(D), and an erythromycin resistance gene, *erm*(42), not previously seen in A/C_2 plasmids. A second 30.3 kb resistance island, RI-119, is in a unique location in the A/C_2 backbone 8.2 kb downstream of *rhs*. RI-119 contained genes conferring resistance to apramycin, netilmicin, tobramycin (*aacC4*), hygromycin (*hph*), sulphonamides (*sul1*) and spectinomycin and streptomycin (*aadA2*). In one of the seven plasmids, this resistance region contained two IS*26*-mediated deletions. A discrete 5.7 kb segment containing the *aacC4* and *hph* genes and bounded by IS*26* on one side and the IR of Tn*5393* on the other was identified.

Conclusions: The presence of almost identical A/C_2 plasmids in two serovars indicates a common origin. Type 2 A/C_2 plasmids continue to evolve via addition of new resistance regions such as RI-119 and evolution of existing ones.

Introduction

Plasmids of the incompatibility groups A and C (later combined as A/C) were among the earliest plasmids to be associated with antibiotic resistance in Gram-negative bacteria. However, the sequence of the *repA* gene in the reference plasmid RA1 differs significantly from that of most of the sequenced A/C plasmids, and they are now designated A/C_1 (RA1) or A/C_2 based on the *repA* gene sequence.[1] A/C_2 plasmids contribute to multiple antibiotic resistance in *Salmonella enterica*, a major cause of foodborne illness,[2-6] and several A/C_2 plasmids from *S. enterica* serovars Newport, Heidelberg and Typhimurium have been sequenced.[3-5, 7, 8] A/C_2 plasmids also mobilize *S. enterica* genomic island 1 (SGI1) carrying genes conferring resistance to multiple antibiotics.[9, 10]

Recently, the A/C_2 group was subdivided into two types, Type 1 and Type 2 (Figure 1a), that diverged a long time ago.[11] They have accumulated extensive SNPs and differ by two insertions or deletions (i1 and i2 in Figure 1a) within the backbone and two regions where part of a large gene has been replaced (R1 and R2 in Figure 1a).[11] The replacements give rise to two versions of the *rhs* gene (*rhs1* and *rhs2*) and open reading frames between *traA* and *traC* that predict proteins of 1832 aa (orf1832) in Type 1 and 1847 aa (orf1847) in Type 2.

We previously reported an unusual class 1 integron-associated gene cassette configuration in seven *S. enterica* isolates, two of serovar Ohio and five of serovar Senftenberg sourced in Australia from pigs. These isolates all carried an A/C_2 plasmid, and also shared resistance to apramycin, gentamicin, kanamycin, neomycin, streptomycin, spectinomycin, sulfamethoxazole and tetracycline.[6] They carry the *aacC4* gene[6], which confers resistance to apramycin, netilmicin and tobramycin.[12] However, conjugative transfer of the shared resistance genes and the A/C_2 plasmids could not be

detected.[6] Here, we have sequenced the seven isolates and completed the sequence of the A/C_2 plasmids.

Materials and methods

DNA sequencing and sequence analysis

The *S. enterica* isolates examined in this study are described elsewhere.[6] Genomic DNA was isolated as described previously[13] and was sequenced using an Illumina HiSeq platform at the Australian Genome Research Facility. Paired-end reads of 100 bp were assembled using Velvet,[14] yielding between 100-200 contigs with an average read depth of 47- to 60-fold. Contigs carrying parts of the A/C_2 backbone defined previously[11] were recovered using standalone BLAST (www.ncbi.nlm.nih.gov/books/NBK52640). Contigs containing resistance genes were identified using ResFinder 2.1.[15] All junctions between contigs were confirmed by PCR using 20 ng of genomic DNA. Amplicons were resolved by electrophoresis and sequenced as described previously.[16] Sequencher 5.2.3 (Gene Codes Corporation, Ann Arbor, MI, USA) was used for the final sequence assembly. Reading frames not annotated previously[11] were predicted using ORF Finder (www.ncbi.nlm.nih.gov/projects/gorf/) and annotated manually. The sequence type (ST) of each strain was determined using the Warwick MLST scheme (http://mlst.warwick.ac.uk/mlst/dbs/Senterica).

Nucleotide sequence accession number

The complete sequence of a representative plasmid, pSRC119-A/C, is deposited in GenBank under accession number KM670336.

Results

pSRC119-A/C

The sequence of pSRC119-A/C, the A/C_2 plasmid from isolate SRC119, was assembled from five contigs by using PCR to link across four copies of IS*26* (GenBank Accession Number KM670336). pSRC119-A/C is 174,068 bp long, and the backbone (Figure 1b) includes the i1 and i2 insertions together with R1-2 (orf1847) and R2-2 (*rhs2*), the Type 2 versions of R1 and R2, making it a Type 2 A/C_2 plasmid. It contains two resistance regions at the locations shown in Figure 1b. The first resistance island contains the *sul2* gene, and islands at this position were recently named ARI-B.[11] In pSRC119-A/C a deletion has removed 4477 bp from the adjacent plasmid backbone (corresponding to bases 25590 – 30066 in pRMH760, GenBank accession number KF976462). The second resistance island was named RI-119 and is 30,345 bp. We have recently shown that additional resistance islands in Type 2 A/C_2 plasmids are found in several different positions, mostly clustered within or around the *rhs* gene.[11] RI-119 is located further away at a new position, 8.2 kb downstream of the *rhs* stop codon. It replaces an 891 bp segment of the A/C_2 backbone (corresponding to bases 157537 - 158427 in pRMH760) that includes part of the *uvrD* and *kfrA* genes.

ARI-B in pSRC119-A/C

ARI-B is 19.9 kb long. The boundary at the *sul2* (RH) end, defined by comparison to pRMH760 that lacks an ARI-B island, is the same as in all other A/C_2 plasmids with ARI-B. The left hand boundary is identical to that found in the plasmids pIP1202 and pP99-018 (GenBank Accession Numbers NC_009141 and NC_008612, respectively) both of which are A/C_2 Type 2. These three plasmids have a segment from one end of GI*sul2*[17] at one end and a 4.4 kb segment sharing 98.9% nucleotide identity with the

IncN plasmid R46 (GenBank Accession Number AY046276) at the other end (Figure 1c). In addition to *sul2* (sulphonamide resistance), the GI*sul2* fragment includes a complete copy of the small mobile element CR2. In pSRC119-A/C, 4286 bp at the right end are identical to the *sul2* end of GI*sul2*[17] (bases 3902209-3906494 in GenBank Accession Number CP001918) and in pIP1202 and pP99-018 4920 bp of GI*sul2* are present (Figure 1c). Indeed, it appears that the island was originally formed via the integration of GI*sul2*. However, the internal composition of ARI-B differs. ARI-B in pSRC119-A/C also carries genes conferring resistance to kanamycin and neomycin (*aphA1b*), tetracycline (*tetA*(D)) and a novel gene, *erm*(42), that confers resistance to erythromycin, tilmicosin and clindamycin[18] and to gamithromycin and tildipirosin.[19] The *erm*(42) gene was only recently identified in *Pasteurella multocida* (GenBank Accession Numbers FR734406 and CP003022),[18] where it is part of an integrative, conjugative element (ICE) that can transfer across species and genus boundaries.[20] In GenBank there is only one other example of this resistance gene and it is found in *Photobacterium damselae* (GenBank Accession Number AB601890). Comparison of the three sequences revealed a shared boundary upstream of the gene but different divergence points downstream (Figure 1d).

RI-119

The 30345 bp RI-119 (Figure 2a) is a mosaic that contains adjacent genes conferring resistance to apramycin, netilmicin and tobramycin (*aacC4*) and to hygromycin (*hph*), as well as the unusual class 1 integron configuration previously shown to carry an *aadA2* cassette conferring resistance to streptomycin and spectinomycin and the *sul1* gene conferring resistance to sulphonamides.[6] IRt of the 6988 bp integron is at the right-hand

boundary of the resistance island and IRi is separated from an IS*26* by 20 bp of sequence derived from Tn*21*.

The remainder of RI-119 is 23357 bp, and at the left-hand boundary of the island there are 364 bp from the *tnpA* end of Tn*1721*, with the IR adjacent to the A/C_2 backbone sequence. The *aacC4* and *hph* genes are in a 5693 bp structure (bases 138374 to 144066 in GenBank Accession Number KM670336) bounded by IS*26* at one end and IR_{tnp} of Tn*5393* at the other end (Figure 2b). This configuration is present in three other plasmids, p9134, pPWD4_103, and pK1HV (GenBank Accession Numbers KF705205, HQ114284 and HF545434, respectively) (Figure. 2c). Though the structure is within a resistance island in each of these plasmids, the surrounding sequence is different, suggesting that this may be a mobile unit that can spread between plasmids.

The IS*26*-*aacC4*-*hph*-Δ*tnpA*5393 structure in pSRC119-A/C separates two parts of a 13.5 kb segment sharing 99.8% nucleotide identity with one found in the draft genome of *Klebsiella pneumonia* strain KPNIH18 (AKA10100038). The genes in this segment encode proteins predicted to be associated with plasmid replication or conjugative transfer. In RI-119, part of this segment has been inverted, probably due to inversion of the segment between the two oppositely-oriented IS*26* (Figure 2a). An IS*26*-mediated deletion has also removed 864 bp belonging to the *trbC* and *trbD* genes found between *trbB* and *trbE* in KPNIH18.

Closely related A/C_2 plasmids in different serovars

Closure of the A/C_2 plasmids from four additional Senftenberg isolates, SRC69, SRC91, SRC102 and SRC103, and two Ohio isolates, SRC22, SRC74, revealed that the A/C_2 plasmids they carried were almost identical to pSRC119-A/C. The resistance islands

were in the same locations in all seven plasmids, and the backbones differed from pSRC119-A/C by only 2-5 SNPs. Three SNPs were shared by the two Ohio isolates, and a single SNP was shared by two of the four remaining Senftenberg isolates. However, the plasmid from SRC102 had two IS*26*-mediated deletions relative to RI-119, one of which has truncated the *hph* gene. These deletions have removed 5453 bp (bases 132921-138373 in GenBank Accession Number KM670336) and 10608 bp (bases 141081-151688 in GenBank Accession Number KM670336).

The five Senftenberg isolates were all ST210 (*aroC* 74, *dnaN* 6, *hemD* 70, *hisD* 8, *purE* 7, *sucA* 79, *thrA* 13) and the two Ohio isolates were ST329 (82-38-26-12-115-78-70). ST329 and ST210 differ at all seven alleles, and though conjugation was not detected,[6] the A/C_2 plasmid has clearly transferred between the two *S. enterica* strains.

Discussion

The Type 2 A/C_2 plasmids recovered in this study have acquired three resistance genes (*erm*(42), *aacC4* and *hph*) not previously seen in A/C_2 plasmids. These genes complement genes conferring resistance to kanamycin, neomycin, tetracycline, tobramycin, sulphonamides, spectinomycin and streptomycin. The *aacC4* and *hph* genes are located in a discrete structure that appears to have moved as a unit into three different plasmids.

The ARI-B island in pSRC119-A/C belongs to a specific sub-group of Type 2 plasmids that include a fragment from the IncN plasmid R46 in ARI-B and the *erm*(42) gene has been incorporated into ARI-B. One member of this group (pP99-018) includes only an ARI-B island whereas pSRC119-A/C and pIP1202 have each acquired an additional resistance island.

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Transparency declaration

None to declare.

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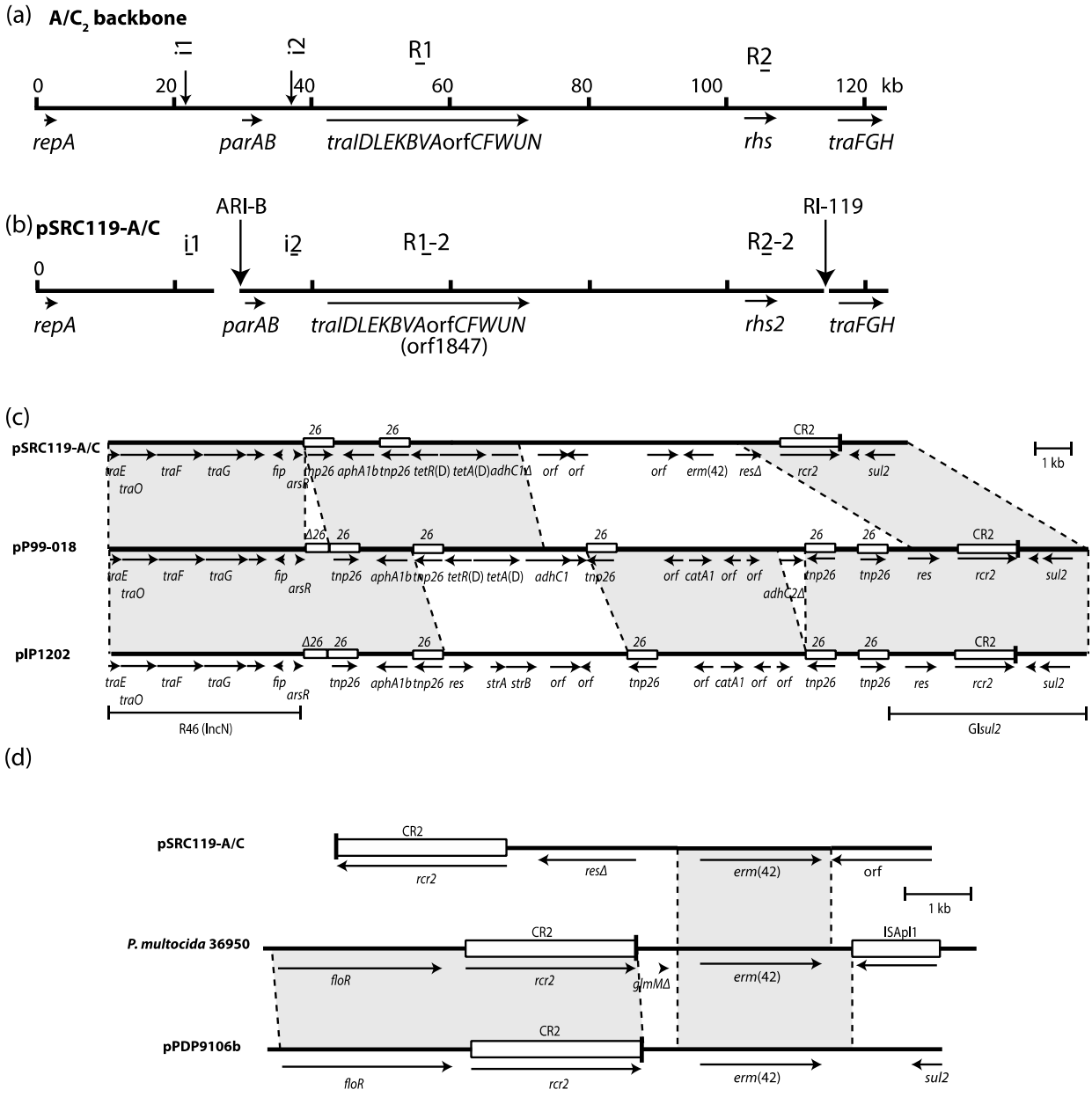
Figure Legends

Figure 1. Comparison of pSRC119-A/C to other A/C_2 plasmids**.** (a) A/C_2 backbone structure showing difference between Type 1 and Type 2 groups as defined previously[11] and (b) backbone structure of Type 2 plasmid pSRC119-A/C. Regions containing genes involved in plasmid replication (*rep*), partitioning (*par*) and conjugative transfer (*tra*), and the *rhs* gene are indicated by arrows below. The remaining genes and open reading frames are annotated in GenBank Accession Number KM670336. The location of insertions i1 and i2 found in Type 2, and two regions of replacement R1 (in the orf) and R2 (in *rhs*) are shown above. In pSRC119-A/C, vertical arrows indicate the location of the ARI-B and RI-119 resistance islands. Gaps in the horizontal line indicate segments of sequence that are missing relative to the A/C_2 backbone. Figure is drawn to scale with lengths in kb shown above the line in (a). (c) pSRC119-A/C ARI-B and related ARI-B structures. Regions are drawn to scale from GenBank Accession Numbers KM670336 (pSRC119-A/C), NC_008612 (pP99-018), and NC_009141 (pIP1202). (d) Regions surrounding the *erm*(42) gene. Structures were drawn to scale from GenBank Accession Numbers CP003022 (*P. multocida*), and AB601890 (*P. damselae*). In (c) and (d), shared regions are indicated by shading. IS and the small mobile element CR2 are shown as open boxes with a vertical bar indicating the ori end of CR2. IS numbers or names are indicated above. Genes and open reading frames (orfs) are shown below the line as named arrows indicating the direction of transcription. Segments derived from the IncN plasmid R46 or GI*sul2* are indicated below.

Figure 2. RI-119. (a) Structure of RI-119. Flanking backbone is shown as a dashed line. The arrangement of genes in KPNIH18 (drawn to scale from AKA10100038) is shown below. (b) Regions surrounding the IS*26*-*aacC4*-*hph*-IR_{5393} unit. Regions are drawn to scale from GenBank Accession Numbers KM670336 (pSRC119-A/C), KF705205 (p9134), GQ114284 (pPWD4_103) and HF545434 (pK1HV). Shading indicates shared regions. Inverted repeats (IR) are shown as thick vertical lines with the origin named above, and IS are shown as open boxes with names above. Genes and open reading frames (orfs) are shown below the line as named arrows indicating the direction of transcription. The origins of individual segments are indicated above. In (b), horizontal lines with different patterns denote different genetic contexts and the direct repeats surrounding the Tn*5393*-derived portion of p9134 are shown below.

Figure 1


(a) A/C2 backbone
i1
i2
R1
R2
0
20
40
60
80
100
120 kb
repA
parAB
traIDLEKBVAorfCFWUN
rhs
traFGH
(b) pSRC119-A/C
ARI-B
RI-119
i1
i2
R1-2
R2-2
0
repA
parAB
traIDLEKBVAorfCFWUN
(orf1847)
rhs2
traFGH
(c)
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pP99-018
pIP1202
CR2
1 kb
traE
traF
traG
traO
fip
arsR
tnp26
aphA1b
tetR(D)
tetA(D)
adhC1
orf
erm(42)
resΔ
rcr2
sul2
res
catA1
strA
strB
R46 (IncN)
GIsul2
(d)
pSRC119-A/C
P. multocida 36950
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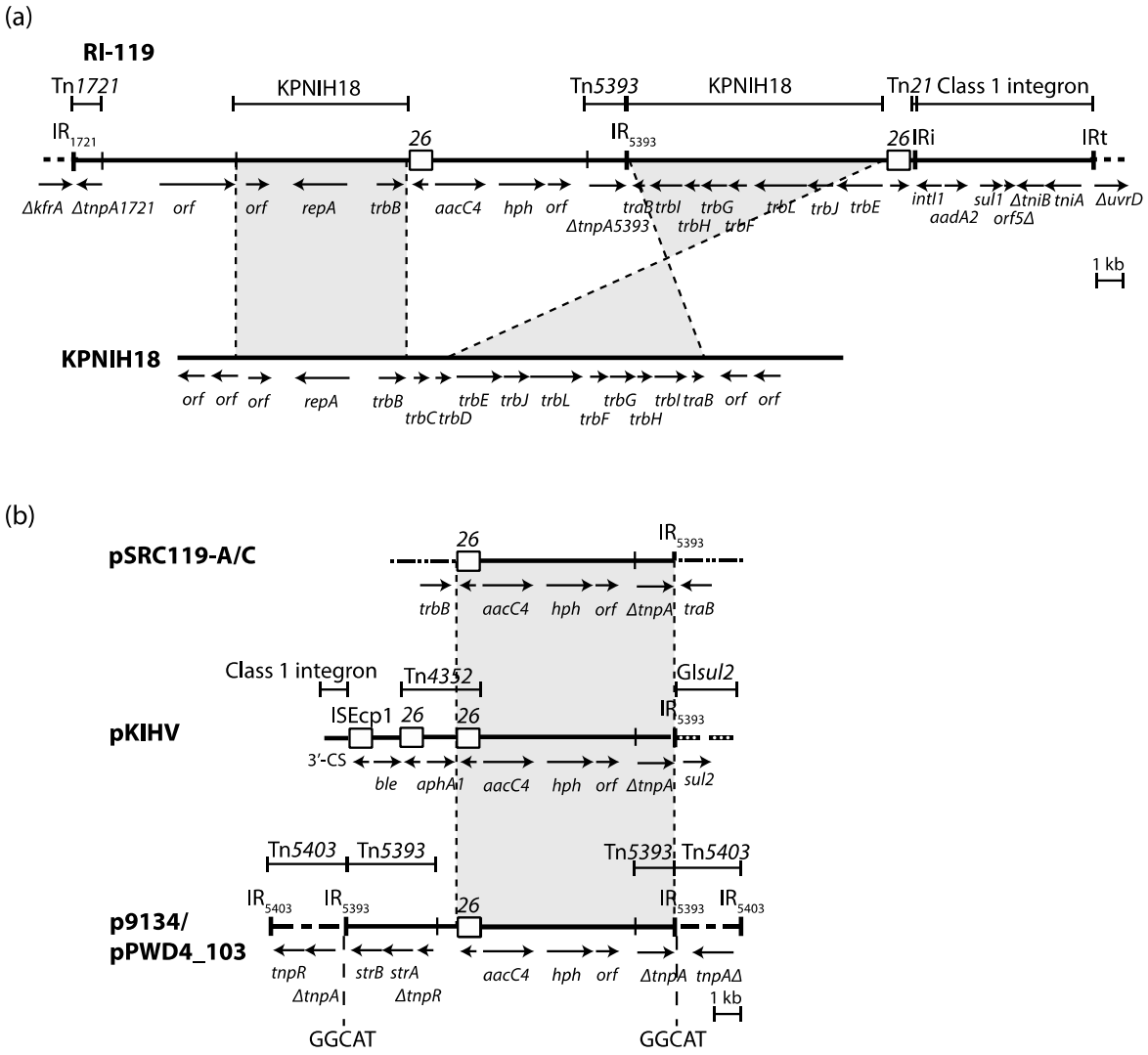
floR
glmMΔ
sul2

327 Figure 2



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