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Copy-out-paste-in transposition of a Tn6283-like integrative element assists interspecies antimicrobial resistance gene transfer from Vibrio alfacsensis

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Copy-out-paste-in transposition of a Tn*6283*-like integrative element assists interspecies antimicrobial resistance gene transfer from *Vibrio alfacsensis*

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Short title: Copy-out integrative element carrying a beta-lactamase gene

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Abstract

The exchange of antimicrobial resistance (AMR) genes between aquaculture and terrestrial microbial populations has emerged as a serious public health concern. However, the nature of the mobile genetic elements in marine bacteria is poorly documented. To gain insight into the genetic mechanisms underlying AMR gene transfer from marine bacteria, we mated a multi-drug resistant *Vibrio alfacsensis* strain with an *Escherichia coli* strain, and then determined the complete genome sequences of the donor strain and multidrug-resistant transconjugants. Sequence analysis revealed a conjugative plasmid of the MOB_H family in the donor strain, which was integrated into the chromosome of the recipient. The plasmid backbone in the transconjugant chromosome was flanked by two copies of a 7.1 kb integrative element, designated Tn*6945*, harboring a beta-lactamase gene that conferred ampicillin resistance to the host cell. Use of a *recA* mutant *E. coli* strain as the recipient yielded a transconjugant showing ampicillin resistance but not multidrug resistance, suggesting the involvement of homologous recombination in plasmid integration into the chromosome. Polymerase chain reaction experiments revealed that Tn*6945* generates a circular copy without generating an empty donor site, suggesting that it moves via a copy-out-paste-in mode, as previously reported for Tn*6283*. Transposition of the integrative element into multiple loci in the recipient chromosome increased the resistance level of the transconjugants. Overall, these results suggest that Tn*6283*-like copy-out integrative elements and conjugative plasmids additively spread AMR genes among marine bacteria and contribute to the emergence of isolates with high-level resistance through amplification of AMR genes.

Introduction

Antimicrobials have been used globally in aquaculture to control fish diseases. Although this approach helps to maintain a stable supply of aquacultural products, misuse of antimicrobials has led to the emergence of antimicrobial-resistant microbes and the accumulation of resistance genes at aquaculture sites [1, 2]. As the spread of multidrug-resistant (MDR) bacteria and pan-drug-resistant bacteria is threating human life [3, 4], it is important to obtain clues about whether and how aquatic and terrestrial microbial populations exchange genetic materials.

Plasmids [5] and integrative conjugative elements [6] are DNA units that can move from one cell to another through conjugation machinery, including direct movement between species [7, 8] and indirectly mobilize genes on specific mobile elements [9–11]. Plasmids can be classified into families according to replicon type or mobilization machinery type for epidemiological purpose [12, 13]. Transposons are DNA units that move from one locus to another in a genome. They can move via cut-and-paste or copy-and-paste mode [14, 15] from one replicon to another replicon, such as conjugative plasmid co-occurring in the cell. Genes embedded in these mobile genetic elements are thus readily shared among bacteria and provide genetic resources for microbial adaptation in changing environments.

To increase knowledge of the genetic mechanisms underlying the spread of antibiotic resistance genes at aquaculture sites, we previously collected resistant bacteria from sediments at an aquaculture site in Kagawa, Japan [16–18]. We identified a self-transmissible MDR plasmid of the MOB_H family, named pAQU1, that can replicate in both the original host *Photobacterium damselae* subsp. *damselae* and in *Escherichia coli* [17]. Subsequently, another MOB_H-family MDR plasmid, named pSEA1, was identified in a *Vibrio*

alfacsensis isolate (previously identified as *V. ponticus*) [19]. The pSEA1 carries a 12 kb nonconjugative integrative element Tn*6283* in addition to antimicrobial resistance (AMR) genes. Although pSEA1 could not replicate in *E. coli* at 42°C, it could integrate in the *E. coli* chromosome upon conjugation via homologous recombination between two Tn*6283* copies: one on pSEA1 and another that moved from pSEA1 into the chromosome [19]. Further, Tn*6283* was identified as a new type of transposon moving via a copy-out-paste-in mode without generating an empty donor site, presumably using tyrosine site-specific recombinases. However, it is not known whether interspecies gene transfer assisted by Tn*6283*-like integrative elements is common in nature.

To obtain further insights into the mechanisms behind genetic exchange among aquaculture-associated bacteria, in this study, we mated another MDR *Vibrio* isolate, *V. alfacsensis* 04Ya249, with *E. coli* strains in the laboratory and then determined the genome sequences of both the donor and transconjugants. We identified a new Tn*6283*-like integrative element and showed that transposition of this element not only assisted the horizontal transfer of an AMR gene embedded on the plasmid but also affected the resistance levels of the recipient cell depending on the copy number integrated in the genome.

Results

Identification of a Tn*6283*-like integrative element carrying a beta-lactamase gene

V. alfacsensis strain 04Ya249 was previously isolated from sea sediment at an aquaculture site [16] and shows resistance to erythromycin, tetracycline, and ampicillin [20]. To identify active mobile elements in this strain that are relevant to its AMR, the strain was mated with

84 macrolide-sensitive *E. coli* JW0452, and the first transconjugant was selected in the
85 presence of erythromycin at 42°C (Fig 1A) in a non-quantitative manner. One transconjugant
86 was named strain TJ249. Quantitative mating assays were then performed using a
87 rifampicin-resistant recipient strain, JW0452rif, under selection with tetracycline, ampicillin,
88 and rifampicin, but not erythromycin, at 42°C. The transfer frequency of tetracycline
89 resistance from *V. alfacsensis* 04Ya249 to *E. coli* JW0452rif was determined to be
90 approximately 10^{-9} per donor, indicating a very rare event, generating 0–3 transconjugant
91 colonies per mating.
92
93 **Fig 1. Identification of the integrative element Tn*6945*.** (A) Capture of pSEA2 and Tn*6945* in
94 *E. coli* by mating assays. (B)(i) Tn*6945* insertion sites in pSEA1 and chromosome 1 of *V.*
95 *alfacsensis* 04Ya249. (ii) Schematic representation of Tn*6945* and pSEA2 insertion sites in *E.*
96 *coli* TJ249. (C) Genetic organization of Tn*6283* and Tn*6945*. The nicking sites used for
97 circularization were six bases upstream of the 5′-end of motif C and the 3′-end of motif C′
98 (black arrowheads). No strand exchange was detected on the bottom strand (white
99 arrowheads). (D) Nucleotide sequences of Tn*6945* terminal regions (*attL* and *attR*), target
100 site (*attB*), and joint region (*attTn*) on the circular form. Sequences shown in orange are
101 parts of a mobile unit. PCR products of *attTn* from strains 04Ya249 and LN95 were cloned
102 into T-vector and sequenced. The observed frequency is shown on the right side of each
103 sequence type.
104
105 The genome sequences of strains 04Ya249 and TJ249 were determined using the
106 PacBio RS II platform. The genome of strain 04Ya249 consists of four replicons: two
107 chromosomes, one putative conjugative plasmid pSEA2, and a smaller plasmid pVA249 (Fig

1A). Strain 04Ya249 has a very similar genome architecture to the database strain *V. alfacsensis* CAIM 1831 based on the average nucleotide identity and gene synteny (S1 Fig). Seven AMR genes were identified on plasmid pSEA2 (S2 Fig). One beta-lactamase gene (*CARB-19* allele in the CARD database [21]) was located within the 7.1-kb repeat region found in both chromosome 1 and plasmid pSEA2 (Fig 1B(i)), which contained four protein-coding sequences in addition to the beta-lactamase gene (Fig 1C). Each of these four coding sequences showed homology, albeit with very low gene product identity, to the four coding sequences clustered at one end of Tn*6283* from strain 04Ya108 (Fig 1C). This repeat region was confirmed to be an active integrative element and was designated Tn*6945* in the transposon registry [22].

The chromosome of *E. coli* TJ249 contained two notable insertions (Fig 1B(ii)), a smaller insertion of Tn*6945* alone and a larger insertion containing a complete copy of pSEA2 and an additional copy of Tn*6945* (Fig 1B(ii)). This indicated that Tn*6945* has at least two target sites in the *E. coli* chromosome. Sequence comparison of the Tn*6945* insertion sites in the *E. coli* genome (Fig 1D) indicated that Tn*6945* inserts its terminal inverted repeats, ending with 5'-GTA-3' (termed C and C') along with an additional 6 bp from the donor molecule into the target site (Fig 1D).

As plasmid pSEA2 was very similar to the previously reported plasmid pSEA1 (accession no. LC081338.1) from strain 04Ya108 [19], we also determined the complete genome of strain 04Ya108 for comparison with that of strain 04Ya249. As predicted from the Southern hybridization experiment in a previously study, strain 04Ya108 possessed two copies of Tn*6283*, one in chromosome 1 and the other in plasmid pSEA1. pSEA1 also carried the Tn*6945* insertion in the same locus as in pSEA2 (S2 Fig). Therefore, pSEA2 may be a precursor of pSEA1.

Transposition of Tn*6945* and plasmid integration in mating

The direct repeats of Tn*6945* in the TJ249 chromosome suggested that the plasmid was integrated into the recipient chromosome through transposition of Tn*6945*, with subsequent integration of pSEA2 into the chromosome facilitated by the homology of the Tn*6945* copy on the chromosome (referred to as a two-step gene transfer mechanism in the previous study [19]). If this scenario holds, transposition of Tn*6945* alone should occur more frequently than pSEA2 integration upon mating, and pSEA2 integration would be reduced in frequency or abolished altogether in the chromosome of a *recA*-null mutant recipient. To test these possibilities, we conducted additional mating assays.

Transposition of Tn*6945* in the recipient cell upon mating was detected based on the transfer frequency of ampicillin resistance alone, and plasmid integration was detected by the transfer of both ampicillin and tetracycline resistance (Fig 1A). Tetracycline-resistant JW0452rif transconjugants were detected only in 2 out of 4 replicated mating experiments (Fig 2). Ampicillin-resistant transconjugants were obtained at a 295-fold higher frequency than tetracycline and ampicillin-resistant transconjugants (Fig 2). Moreover, when *E. coli* LN52rif (which already carries one copy of Tn*6945*) was used as the recipient, tetracycline-resistant transconjugants were obtained at a 160-fold higher frequency than that observed when using strain JW0452rif as the recipient (Fig 2).

Fig 2. Resistance gene transfer from *V. alfacsensis*. Transfer frequency (Y axis) is the $\log_{10}$-transformed value of the transconjugant colony-forming units (CFU) divided by the donor CFU and recipient CFU. Four replicate mating experiments were performed. No

155 transconjugant was detected in any of the four replicate experiments using JW0452Δ*recA*rif
156 with tetracycline selection, or in the two replicate experiments using JW0452rif with
157 tetracycline selection. In one experiment on LN52rif, the donor CFU was not evaluated but
158 the transconjugant CFU was obtained at a comparable frequency to that observed in the
159 other three replicate experiments. The detection limit was 10^{-19}.
160
161 When the *recA*-null mutant strain JW0452Δ*recA*rif was used as the recipient, transfer of
162 tetracycline resistance was not observed in any experiment, but ampicillin-resistant
163 transconjugants were obtained (Fig 2). Collectively, these results suggested that pSEA2
164 integration, but not transposition of Tn*6945*, depends on the homologous recombination
165 system in the recipient cell. Therefore, MDR transconjugants likely emerge through two
166 distinct intracellular processes: transposition and homologous recombination.
167

168 **Copy-out of Tn*6945***

169 The previous study [19] revealed the following unique features of Tn*6283*: (i) it does not
170 generate an unoccupied donor site upon its circularization *in vivo*, and (ii) the circular form
171 of the integrative element is generated using only one strand as a template, at least in *E. coli.*
172 Although Tn*6283* encodes tyrosine recombinases but not a single transposase with a D-D-E
173 motif [23], this behavior is analogous to the copy-out-paste-in transposition mechanism of
174 insertion sequence elements [24]. To test whether Tn*6945* also moves through a copy-out-
175 paste-in mode, we created a pSEA2-free 04Ya249 derivative strain LN95 carrying only a
176 single copy of Tn*6945* in the chromosome and analyzed production of the Tn*6945* circular
177 molecule in this strain.

178 Two pairs of primers were designed such that one primer anneals to the inside of the
179 integrative element and the other anneals to the outside of the element, amplifying *attL*
180 (*intA* side, product 1 in Fig 3A) or *attR* (*bla* side, product 2). By changing the combination of
181 primers, the joint region (*attTn*) in the circular form of the integrative element (product 3 in
182 Fig 3A), empty donor site (*attB*), or occupied donor site (product 4) could be amplified by
183 PCR. The circular form of Tn*6945* was detected in both 04Ya249 and LN95 (Fig 3B). This
184 suggested that the chromosomal copy of Tn*6945* is functional in *V. alfacsensis*. The empty
185 donor site (*attB*) could not be detected.
186
187 **Fig 3. Detection of a copy-out event of Tn*6945*.** (A) Replicon organization of strain 04Ya249
188 and PCR assay design. Thick black lines with numbers indicate the expected PCR products.
189 The hypothetical site *attB* was not detected by PCR. The primers used are listed in Table 1.
190 (B) PCR detection of *attL*, *attR*, *attTn*, and *attB*. The PCR cycle was repeated 35 times for all
191 targets. (C) Quantitation of *attTn* in the pSEA2-free strain LN95 by quantitative PCR.
192
193 We next cloned the PCR products of *attTn*, and then sequenced 10 cloned molecules
194 to investigate the sequence variation in the spacer region between the inverted repeat
195 motifs C and C'. Only one sequence type (5'-TTTTTT-3') was detected in the pSEA2-free
196 strain LN95 (Fig 1D). Thus, the majority of the circular form is a copy of the ligated product
197 between the 5'-end of the 6 bp upstream of the motif C terminus and the 3'-end of the
198 motif C' terminus (the top strand in the gene map of Fig 1C). Two sequence types, 5'-
199 TTTTTT-3' and 5'-TTTTCT-3', were detected in strain 04Ya249 at a 1:9 ratio (examples of the
200 Sanger sequencing trace files have been posted in figshare [25]). These findings agree with
201 the top strand exchange product of chromosome 1 and pSEA2, respectively (Fig 1D).

Collectively, these observations suggest that Tn*6945* moves via copy-out-paste-in transposition without undergoing strand exchange of the bottom strand in *V. alfacsensis*.

To estimate the copy number of the circular form of Tn*6945* in the cell population, we first searched for Illumina reads of strain LN95 that spanned *attTn* or the "hypothetical" a*ttB*. Although next-generation sequencing reads were obtained at 229× chromosome coverage, no reads spanning *attTn* or *attB* were detected [25]. Quantitative PCR further revealed that the mean *attTn* to *gyrB* ratio was 0.0012 (Fig 3C). This relative copy number was consistent with the result from a previous study detecting the circular form of Tn*6283* in *E. coli*, which showed an *attTn* to chromosome ratio of 0.001 [19].

The insertion copy number of Tn*6945* affects beta-lactam resistance

The naturally occurring strains 04Ya249 and 04Ya108 carry two copies of Tn*6945* and Tn*6283* in their respective genomes. However, it remains unclear whether multiple copies of integrative elements confer an advantage to the host cell. Three copies of Tn*6945* in the transconjugant TJ249 were initially detected by genome sequencing. Southern hybridization analysis of other JW0452 transconjugants, which were maintained in the lab without tetracycline selection, revealed one to two copies of Tn*6945* in their respective genomes (S3 Fig). Therefore, we used these transconjugant strains to test whether multiple insertions of Tn*6945* can increase the resistance levels of the transconjugants.

Sixteen *E. coli* strains with or without Tn*6945* insertion were grown in Luria-Bertani broth with ampicillin, and then the minimum inhibitory concentrations (MICs) of ampicillin were determined using the broth dilution method following the Clinical Laboratory

Standards Institute (CLSI) guidelines. The MIC was the highest in the clone carrying three copies of Tn*6945*, followed by the group of transconjugants carrying two copies and the group carrying one copy, and the MIC was the lowest in JW0452 (Fig 4). Although within-group variation was also observed, these results clearly showed that the copy number of Tn*6945* in the genome positively affects the level of beta-lactam resistance of the host cell.

Fig 4. The copy number of Tn*6945* affects the resistance level. Sixteen transconjugants and a control strain (JW0452) were divided into four groups based on the Tn*6945* copy number in the genome. MIC of ampicillin is shown according to copy number.

Discussion

Vibrio is one of the major bacterial genera found in marine sediments [26], and is among the most common microbiota of wild and farmed shrimp [27] and fish [28, 29]. Several of the genus *Vibrio* are pathogens of fishes reared in aquaculture [30], while other subsets of *Vibrio* species, including *V. cholerae*, *V. parahaemolyticus,* and *V. vulnificus*, which are ubiquitous in relatively low-salinity sea water, are seafood-borne human pathogens [30, 31]. Thus, *Vibrio* can be considered as a key genus linking AMR genes between aquatic environments and human-associated environments. Indeed, the accumulation of AMR genes in this genus has attracted increased research attention, particularly in *V. cholerae* [32, 33]. However, direct experimental evidence for AMR gene transfer from *Vibrio* species to other human-relevant bacteria is limited.

Known genetic mechanisms of AMR gene transfer from *Vibrio* include integrative conjugative elements [34], A/C plasmids [35, 36], unclassified conjugative plasmids [37],

mobilizable genomic islands [38], pAQU1-type MOB_H family conjugative plasmids [39], pSEA1-type MOB_H family conjugative plasmids [19], and a combination of a chromosomal super-integron and a conjugative plasmid carrying an integron [40]. The host range of pSEA1-type MOB_H plasmids, discovered in *V. alfacsensis* (for which only one complete genome was available until the present study), is unknown. In this study, we investigated how a pSEA1-like plasmid can contribute to AMR gene transfer in a laboratory setting.

In contrast to A/C plasmids, autonomous replication of pSEA2 and pSEA1 was difficult to achieve in *E. coli,* since transconjugant selection yielded *E. coli* clones carrying plasmid DNA integrated into the chromosome. We consistently observed a two-step gene transfer mechanism that involves the transposition of a Tn*6283*-like integrative element and homologous recombination, which enabled AMR gene transfer beyond the plasmid's replication host range. This mode of horizontal gene transfer may be important among marine bacteria, since the plasmid conjugation host range is expected to be wider than the replication host range [41, 42].

Plasmid pSEA1 was found to carry both Tn*6283* and Tn*6945*. However, in the previous study, only transposition of Tn*6283* was observed, likely because we did not recognize Tn*6945* on pSEA1 and did not intend to detect its transposition [19]. The newly identified Tn*6945* is the smallest Tn*6283*-like "active" integrative element, and it is the only known element harboring an AMR gene. The beta-lactamase gene identified in this study was embedded within the mobile DNA unit without an accompanying insertion sequence element, like the case for Tn*3* [43]. This pattern is atypical for recent widespread AMR genes [44, 45], suggesting an ancient origin for the Tn*6283*-like element with the beta-lactamase gene. As Tn*6945* can have multiple target sites in a single genome, a Tn*6283*-like element may contribute to microbial adaptation to the antimicrobials used at aquaculture sites, with

diverse mechanisms beyond mediating the horizontal transmission of AMR genes, such as increasing the resistance level or gene redundancy preceding evolutionary innovation of an AMR gene [46].

The discovery of Tn*6945* highlights four potential core genes (*intA*, *CDS2*, *intB*, and *CDS4*) of unknown function present in Tn*6283*-like integrative elements. However, the specific roles of these gene products in copy-out-paste-in transposition remain to be determined. A notable difference between an insertion sequence and the Tn*6283*-like element is the strong strand bias unique to the latter upon strand exchange, which generates a figure-eight structure that serves as a template for the circular form [23, 24]. Unlike transposase, tyrosine recombinases usually do not generate a free 3' OH end [47]. Host factor-mediated replication on the top strand exchange product should therefore be a complex process. We propose that Tn*6283*-like elements contribute to AMR gene transmission in marine bacteria. However, we also speculate that the host range of Tn*6283* may be limited due to this unusual transpositional process. Further biochemical and bioinformatic studies on Tn*6283*-like integrative elements is needed to reveal the mechanisms of gene transfer among the genus *Vibrio* and other aquaculture-associated bacteria.

Materials and methods

Strains and culture media

We used the *Vibrio* strains 04Ya249 [18], LN95, and 04Ya108 [19] in this study. Strain 04Ya108 was previously identified as *Vibrio ponticus* based on 16S rRNA gene sequence similarity. However, determination of the complete sequence in this study revealed that

295 strain 04Ya108 shows >96% average nucleotide identity to *V. alfacsensis* strain CAIM 1831
296 (DSM 24595) (S1 Fig) [48]. Therefore, strain 04Ya108 was newly classified as *V. alfacsensis*.
297 Strain LN95 is a pSEA2-free tetracycline-susceptible derivative of strain 04Ya249. This strain
298 was generated through repeated batch culture of 04Ya249, and subsequent single-colony
299 isolation. The absence of pSEA2 in LN95 was confirmed by next-generation sequencing.
300 We used the *E. coli* strains DH5α [F^-, Φ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deoR*,
301 *recA*1, *endA*1, *hsdR*17(r_K^-, m_K^+), *phoA*, *supE*44, λ^-, *thi*-1, *gyrA*96, *relA*1], BW25113 [F^-, *Δ(araD-*
302 *araB)567, ΔlacZ4787*(::rrnB-3), *λ⁻, rph-1, Δ(rhaD-rhaB)568, hsdR514*], JW2669 [BW25113
303 *ΔrecA774::kan*], JW0452 [BW25113 *ΔacrA748::kan*] [49] and its rifampicin-resistant variant
304 JW0452rif, LN52, TJ249, and JW0452Δ*recA* and its rifampicin-resistant variant
305 JW0452Δ*recA*rif. Strain LN52, which carries a single copy of Tn*6945,* is a JW0452
306 transconjugant obtained by ampicillin selection at 42°C. TJ249 is also a JW0452
307 transconjugant obtained by erythromycin selection at 42°C. The presence of a single Tn*6945*
308 copy in the LN52 genome was confirmed by Southern hybridization (S3 Fig).
309 The *recA*-null mutant of JW0452, JW0452Δ*recA*, was constructed using the lambda-
310 Red method [50]. A DNA fragment containing the 5′ and 3′ sequences of the *recA* gene and
311 a chloramphenicol resistance gene was amplified by PCR using primers YO-175 and
312 RecA_stop_primingsite_2 and plasmid pKD3 [50] as a template (Table 1). The PCR products
313 (700 ng) were introduced into electrocompetent cells of JW0452 carrying pKD46 by
314 electroporation using a Gene Pulser Xcell™ (BioRad, Hercules, CA, USA). The occurrence of
315 recombination at the expected site was confirmed by PCR using primers CAT-584 and
316 BW25113_2815723f (Table 1). The absence of the *recA* gene was also confirmed by PCR
317 using primers LN192_recA1 and LN193_recA2.

Vibrio alfacsensis strains were cultured in BD Bacto™ brain heart infusion medium (BD237500; Becton, Dickinson, and Company, Franklin Lakes, NJ, USA) supplemented with up to 2% NaCl. *E. coli* strains were cultured in BD Difco™ LB Broth, Miller (BD244520; Becton, Dickinson, and Company). BD Difco™ Mueller Hinton Broth (BD 275730; Becton, Dickinson, and Company) was used for antibiotic susceptibility testing of *E. coli*. BD Difco™ Marine Broth 2216 (BD279110; Becton, Dickinson, and Company) was used for filter mating. Solid media were prepared by adding 1.5% agar to the broth. Antibiotics were added to the medium at the following concentrations when required: erythromycin (Nacalai Tesque, Kyoto, Japan), 100 μg/ml; tetracycline (Nacalai Tesque), 10 μg/ml; rifampicin (Sigma-Aldrich, St. Louis, MO, USA), 50 μg/ml; ampicillin (Nacalai Tesque), 100 μg/ml.

Table 1. Oligonucleotides used.

Name	Sequence (5' to 3')	Purpose
LN112	GGGTTACCTTCCCAATGCGT	Southern hybridization probe for Tn*6945 intA*
LN113	CGACTGTTGGTAGCGACTGT	Southern hybridization probe for Tn*6945 intA*
LN_142_junction2	AAGATGGTAAAAGTGTTCCA	Detection of *attTn* by qPCR
LN_143_junction2	TTTGTGTGTAGCCCTTGTG	Detection of *attTn* by qPCR
LN_150_intA2	GGTTATGTGGAGAAGTTGCC	Detection of *intA* by qPCR
LN_151_intA2	TGAGTTCGGTTTCTTGCTTC	Detection of *intA* by qPCR
LN_181_Valc_chr1_attB_L	CGAGGGTAAAGTGCCAACAT	Detection of chromosomal *attB* and *attL* by standard PCR
LN183_Valc_chr1_attB_R2	ACATCAGCAGGAGTTAGTTG	Detection of chromosomal

		attB and *attL* by standard PCR
LN184_04Ya249_gyrBf1	AACAGAATTGCACCCAGAAG	Detection of *gyrB* by qPCR
LN185_04Ya249_gyrBr1	GAAGACCGCCTGATACTTTG	Detection of *gyrB* by qPCR
LN127_cds303_r137-156	CTCTGGCTCACCGTTAGAGG	Detection of 1 kb *attTn* and *attL* detection by standard PCR
LN128_bla_r16-36	GCATTTTTGCACATGCTAATG	Detection of 1 kb *attTn* and *attR* by standard PCR
YO-175	CAGAACATATTGACTATCCGGTATT ACCCGGCATGACAGGAGTAAAAAT GTGTAGGCTGGAGCTGCTTCG	Lambda Red
RecA_stop_primingsite_2	ATGCGACCCTTGTGTATCAAACAA GACGATTAAAAATCTTCGTTAGTTT CCATATGAATATCCTCCTTA	Lambda Red
CAT-584	AAGCCATCACAAACGGCATG	Lambda Red
BW25113_2815723f	AATACGCGCAGGTCCATAAC	Lambda Red
LN192_recA1	GTTCCATGGATGTGGAAACC	Lambda Red
LN193_recA2	ATATCGACGCCCAGTTTACG	Lambda Red

330

331

332 ## Standard molecular biology methods

333 LA Taq polymerase (TaKaRa Bio Inc., Kusatsu, Japan) was used for conventional PCR to

334 detect transposon termini and their recombination products and for TA cloning of PCR

335 products. To generate quantity standards for quantitative PCR, att_{Tn6945} and other fragments

336 (*gyrB*, *intA*) were PCR amplified from the genomic DNA of strain 04Ya249 and then cloned

337 into the pGEM-T vector using the pGEM-T easy vector system (Promega, Madison, WI, USA)
338 and transformed into DH5α competent cells. Quantitative PCR was performed using
339 THUNDERBIRD® SYBR qPCR Mix (Toyobo, Osaka, Japan) and a CFX connect Real-Time system
340 (BioRad, Hercules, CA, USA) and a two-step PCR protocol consisting of denaturation for 5 s
341 at 95°C and annealing/extension for 30 s at 60°C. Target quantity was estimated based on a
342 standard curve of the control plasmid DNA (pGEM-*gyrB*, pGEM-*intA*, pGEM-att_{Tn6945}).
343 Primers used for conventional PCR and quantitative PCR were designed based on the target;
344 the oligonucleotides used and their specific purposes are shown in Table 1.
345

346 ## Conjugation

347 The donor *Vibrio* strain and *E. coli* recipient strains (JW0452, JW0452rif, and
348 JW0452Δ*recA*rif) were grown overnight at 25°C and 37°C, respectively. A 500 µl aliquot of
349 each culture was mixed, centrifuged, and resuspended in 50 µl of Luria-Bertani broth. The
350 cell mixture was spotted on a 0.45 µm pore-size nitrocellulose filter (Merck, Millipore Ltd.,
351 Tullagreen, Ireland) placed on marine broth agar, and allowed to mate for 24 h at 25°C.
352 After incubation, the cell mixture on the filter was serially diluted in 1× phosphate-buffered
353 saline, and then 100 µl of the mixture was plated on appropriate agar medium to measure
354 the CFU. *E. coli* transconjugants were selected after 24 h of incubation at 42°C in the
355 presence of erythromycin, tetracycline, ampicillin, or both ampicillin and tetracycline,
356 depending on the purpose of the assay. The *Vibrio* donor strain was selected on brain heart
357 infusion agar with 2% NaCl supplemented with tetracycline at 25°C. The transconjugant
358 strain TJ249 was obtained by mating strain 04Ya249 with *E. coli* strain JW0452 as the donor
359 and recipient, respectively, followed by erythromycin selection at 42°C (Fig 1A).

Southern hybridization

The insertion copy number of Tn*6945* in strain LN52 and 17 ampicillin-resistant transconjugants (JW0452 derivatives) was analyzed by Southern hybridization using the 5'-end of Tn*6945 intA* as a probe. The probe was generated using the PCR DIG Synthesis Kit (Roche, Basel, Switzerland), and inserts were detected by the standard method using CDT-star® (Roche). PCR products were obtained using primers LN112 and LN113 (Table 1). Genomic DNA (2.5 µg) was double digested with either *Nde*I and *Sph*I or *Nde*I and *Hin*dIII (New England Biolabs, Ipswich, MA, USA) prior to electrophoresis.

Antimicrobial susceptibility testing

To examine the antimicrobial susceptibility of transconjugants harboring 1–3 beta-lactamase genes, the MIC of ampicillin was determined using the broth dilution method in 96 well microtiter plate format according to standard M07 of the CLSI [51]. The antibiotic concentrations tested were 1000, 500, 250, 125, 62.5, 31.25, 16, 8, 4, and 2 µg/ml. The test plates were incubated at 35°C for 24 h.

Genome sequencing

Genomic DNA was extracted from 250–500 µl of *V. alfacsensis* strains 04Ya249, 04Ya108, or *E. coli* strain TJ249 culture using the QIAGEN DNeasy blood & tissue kit (QIAGEN GmbH, Hilden, Germany). The extracted genomic DNA was sequenced on the Pacbio RS II platform at Macrogen (Tokyo, Japan). Genome assembly was conducted using HGAP v.3 [52] for *Vibrio* strains and Flye v 2.8.3-b1695 for *E. coli* TJ249 [53]. Reads were obtained at >120×

coverage for the chromosomes of each strain. Reads and the genome sequence of TJ249 has been posted to figshare [54]. Illumina reads of the pSEA2-free strain LN95 were also obtained using TruSeq PCR-free library and NovaSeq 6000 platform at NovogeneAIT Genomics Singapore Pte., Ltd. (Singapore) to confirm the loss of pSEA2 and to identify the circular form of Tn*6945*. The mapping results have been posted to figshare [25]. Genomes were compared using MUMmer3.23 [55] and GenomeMatcher [56]. AMR genes in the assembly were searched using AMRFinderPlus [57]. The average nucleotide identity was determined using fastANI [58].

Accession numbers

The complete genome sequences of strains 04Ya108 and 04Ya249 were submitted to DDBJ/NCBI/EMBL under accession numbers AP024165 (chr1 of 04Ya108), AP024166 (chr2 of 04Ya108), AP024167 (pSEA1 of 04Ya108), AP024168 (pYa108 of 04Ya108), AP019849 (chr1 of 04Ya249), AP019850 (chr2 of 04Ya249), AP019851 (pSEA2 of 04Ya249), and AP019852 (pVA249 of 04Ya249). The raw reads for strains 04Ya108, 04Ya249, and LN95 are available from Sequence Read Archive under accession numbers DRA011098, DRA008632, and DRA011762, respectively.

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569

570 Supporting information

571 **S1 Fig. Analysis of Tn*6945* insertion number by Southern hybridization.** (A) pSEA2-free
572 *Vibrio* strain LN95 and parental strain 04Ya249. (B) Nineteen *E. coli* transconjugants and
573 controls. Upper panel: digestion with *Nde*I and *Sph*I. Lower panel: digestion with *Nde*I and
574 *Hin*dIII. The color of the strain name indicates the Tn*6945* copy number: blue, three copies;
575 light blue, one copy; red, two copies.

576

577 **S2 Fig. Genetic map of pSEA2**. Locations of antimicrobial resistance (AMR) genes and
578 Tn*6945* in pSEA2. AMR genes were inferred using AMRFinderPlus [57]. Genes are visualized
579 using CLC Sequence Viewer (Qiagen, Hilden Germany). Green arch denotes the region
580 detected as a circular contig (contig1) in Pacbio RSII reads assembly of transconjugant strain
581 TJ249. (B) Location of Tn*6945* and Tn*6283* insertion positions in pSEA1.

582

583 **S3 Fig. Comparison of the genome structure between strain 04Ya249 with 04Ya108, CAIM**
584 **1831 or TJ249.** Structure comparison was performed using nucmer in MUMmer3 [55].
585 Purple dots indicate a match on the Watson strand, and light blue indicates a match on the
586 Crick strand. (B) Average nucleotide identity (ANI) between two strains as determined by
587 fastANI [58]. The commands used were as follows: (A) $nucmer -minmatch
588 60 ../../data/04Ya249_submission.fas ../../data/reference.fas

589 $mummerplot -x "[0,6000000]" -y "[0,22000000]" -postscript -p test out.delta; (B) $fastANI -

590 q ../../data/04Ya249_submission.fas -r ../../data/CAIM1831_Refseq.fas -o

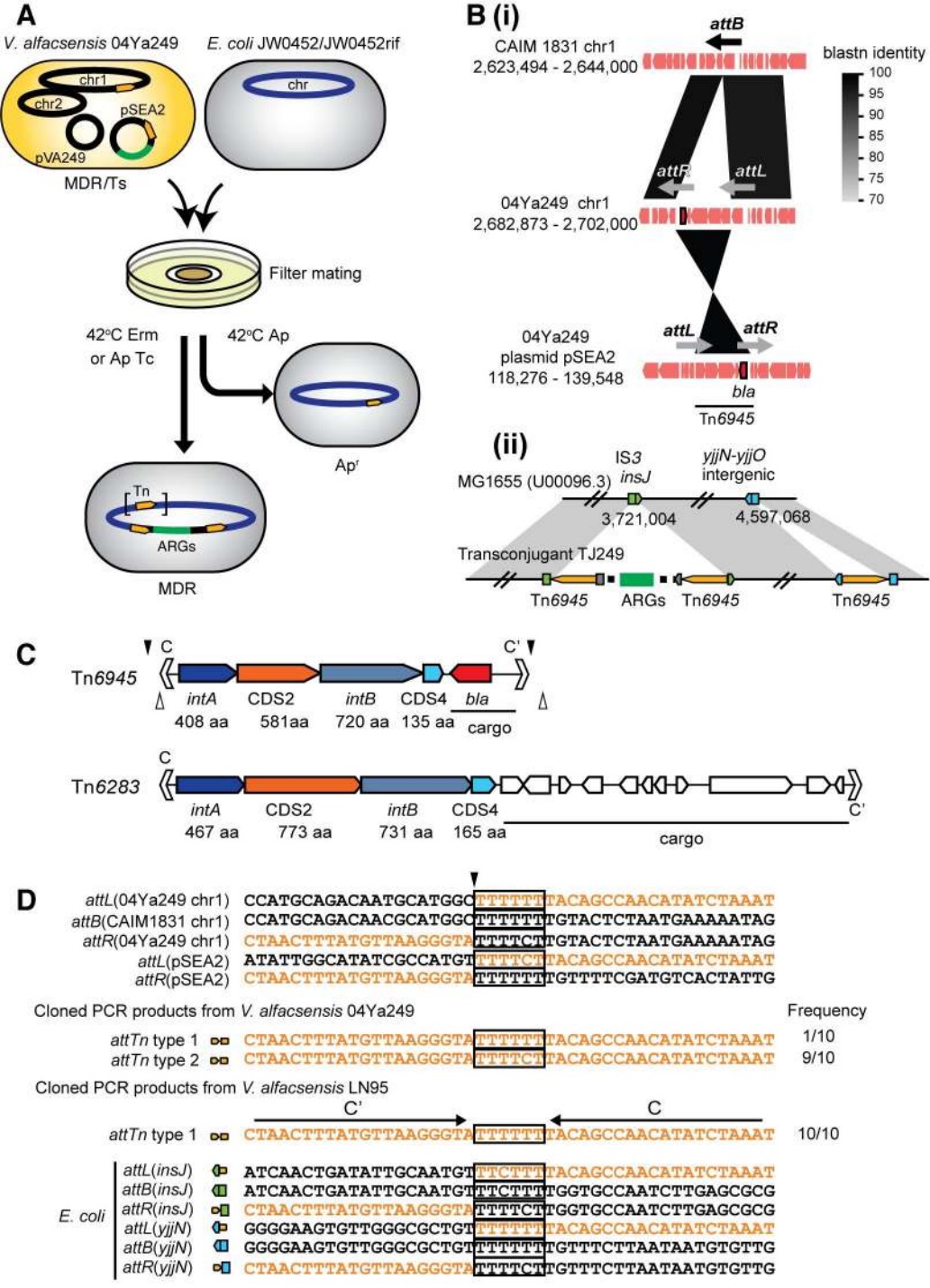
591 04Ya249vsCAIM1831.txt

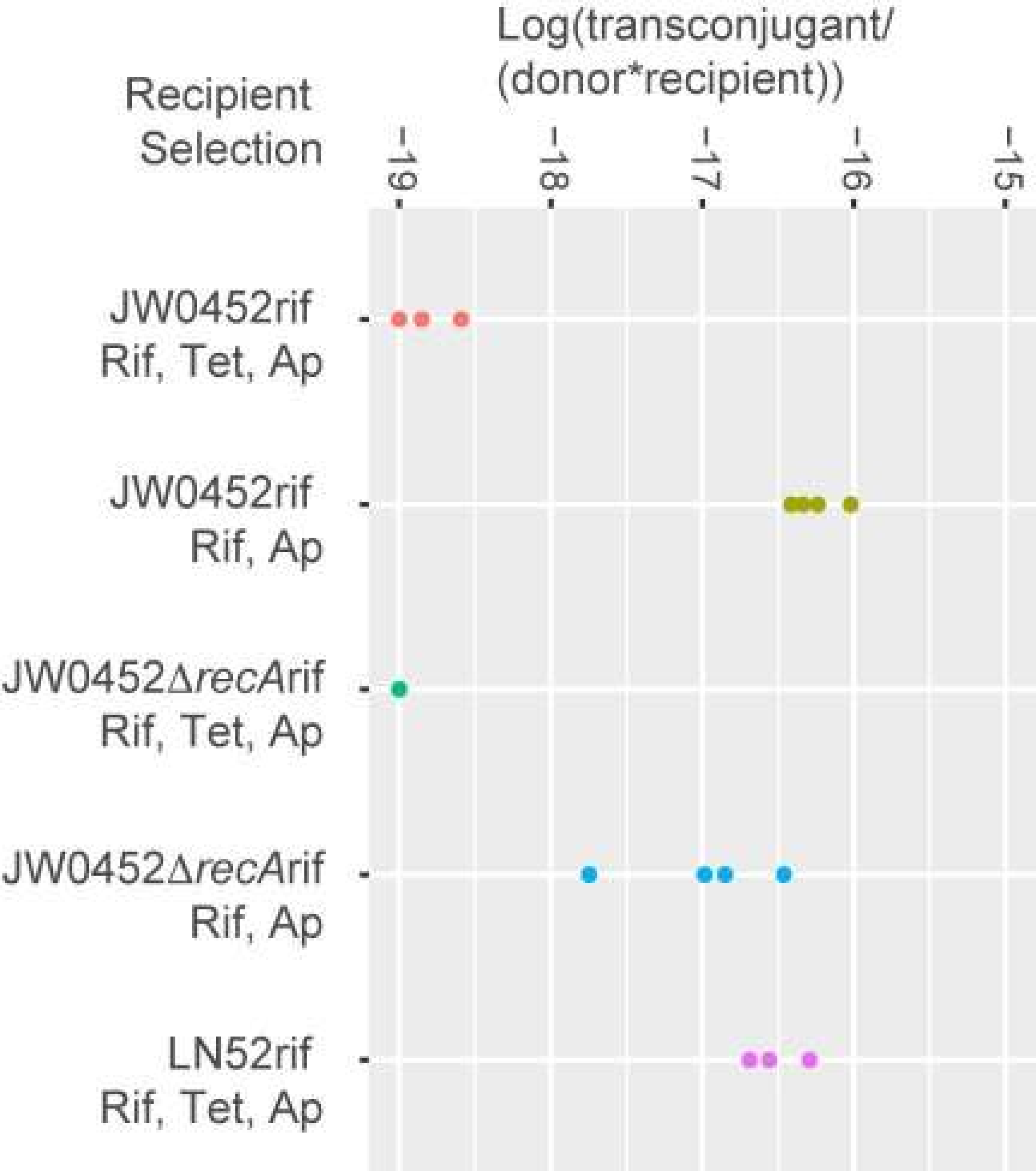
592 $fastANI -q ../../data/04Ya249_submission.fas -r ../../data/04Ya108_submission.fas -o

593 04Ya249vs04Ya108.txt

594 $fastANI -q ../../data/04Ya249_submission.fas -r ../../data/04Ya108_submission.fas -o

595 04Ya249vs04Ya108.txt


A
V. alfacsensis 04Ya249
E. coli JW0452/JW0452rif
chr1
chr2
pSEA2
pVA249
chr
MDR/Ts
Filter mating
42°C Erm
or Ap Tc
42°C Ap
Ap'
Tn
ARGs
MDR
B (i)
attB
CAIM 1831 chr1
2,623,494 - 2,644,000
blastn identity
100
95
90
85
80
75
70
attR
attL
04Ya249 chr1
2,682,873 - 2,702,000
attL
attR
04Ya249
plasmid pSEA2
118,276 - 139,548
bla
Tn6945
(ii)
MG1655 (U00096.3)
IS3
insJ
yjjN-yjjO
intergenic
3,721,004
4,597,068
Transconjugant TJ249
Tn6945
ARGs
Tn6945
Tn6945
C
Tn6945
C
C'
intA
CDS2
intB
CDS4
bla
408 aa
581aa
720 aa
135 aa
cargo
Tn6283
C
C'
intA
CDS2
intB
CDS4
467 aa
773 aa
731 aa
165 aa
cargo
D
attL(04Ya249 chr1) CCATGCAGACAATGCATGGCTTTTTTTACAGCCAACATATCTAAAT
attB(CAIM1831 chr1) CCATGCAGACAACGCATGGCTTTTTTTGTACTCTAATGAAAAATAG
attR(04Ya249 chr1) CTAACTTTATGTTAAGGGTATTTTTCTTGTACTCTAATGAAAAATAG
attL(pSEA2) ATATTGGCATATCGCCATGTTTTTTCTTACAGCCAACATATCTAAAT
attR(pSEA2) CTAACTTTATGTTAAGGGTATTTTTTTGTTTTCGATGTCACTATTG
Cloned PCR products from V. alfacsensis 04Ya249
Frequency
attTn type 1 CTAACTTTATGTTAAGGGTATTTTTTTACAGCCAACATATCTAAAT 1/10
attTn type 2 CTAACTTTATGTTAAGGGTATTTTCTTACAGCCAACATATCTAAAT 9/10
Cloned PCR products from V. alfacsensis LN95
C'
C
attTn type 1 CTAACTTTATGTTAAGGGTATTTTTTTACAGCCAACATATCTAAAT 10/10
E. coli
attL(insJ) ATCAACTGATATTGCAATGTTTCTTTTACAGCCAACATATCTAAAT
attB(insJ) ATCAACTGATATTGCAATGTTTCTTTTGGTGCCAATCTTGAGCGCG
attR(insJ) CTAACTTTATGTTAAGGGTATTTTCTTGGTGCCAATCTTGAGCGCG
attL(yjjN) GGGGAAGTGTTGGGCGCTGTTTTTTTTACAGCCAACATATCTAAAT
attB(yjjN) GGGGAAGTGTTGGGCGCTGTTTTTTTGTTTCTTAATAATGTGTTG
attR(yjjN) CTAACTTTATGTTAAGGGTATTTTCTTGTTTCTTAATAATGTGTTG


Log(transconjugant/
(donor*recipient))
Recipient
Selection
-6
-8
-7
-9
-5
JW0452rif
Rif, Tet, Ap
JW0452rif
Rif, Ap
JW0452ΔrecArif
Rif, Tet, Ap
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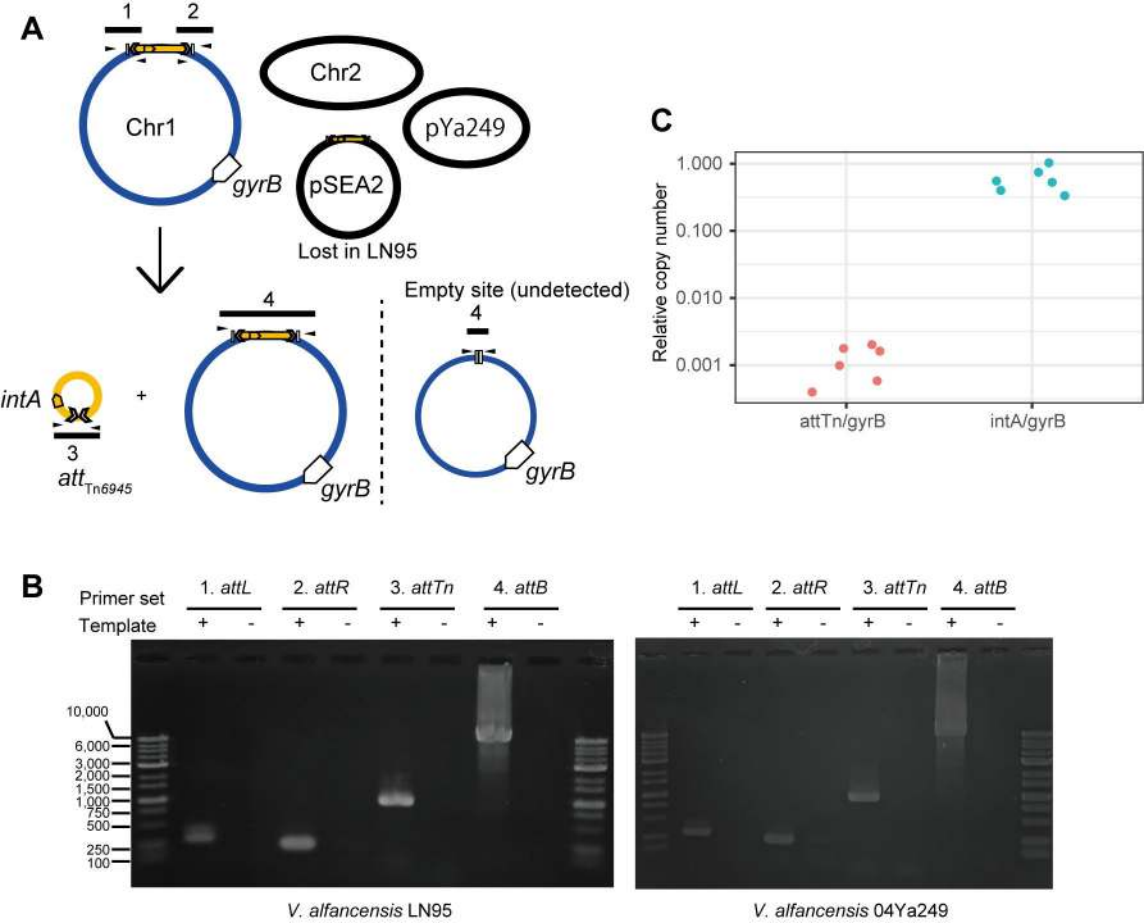
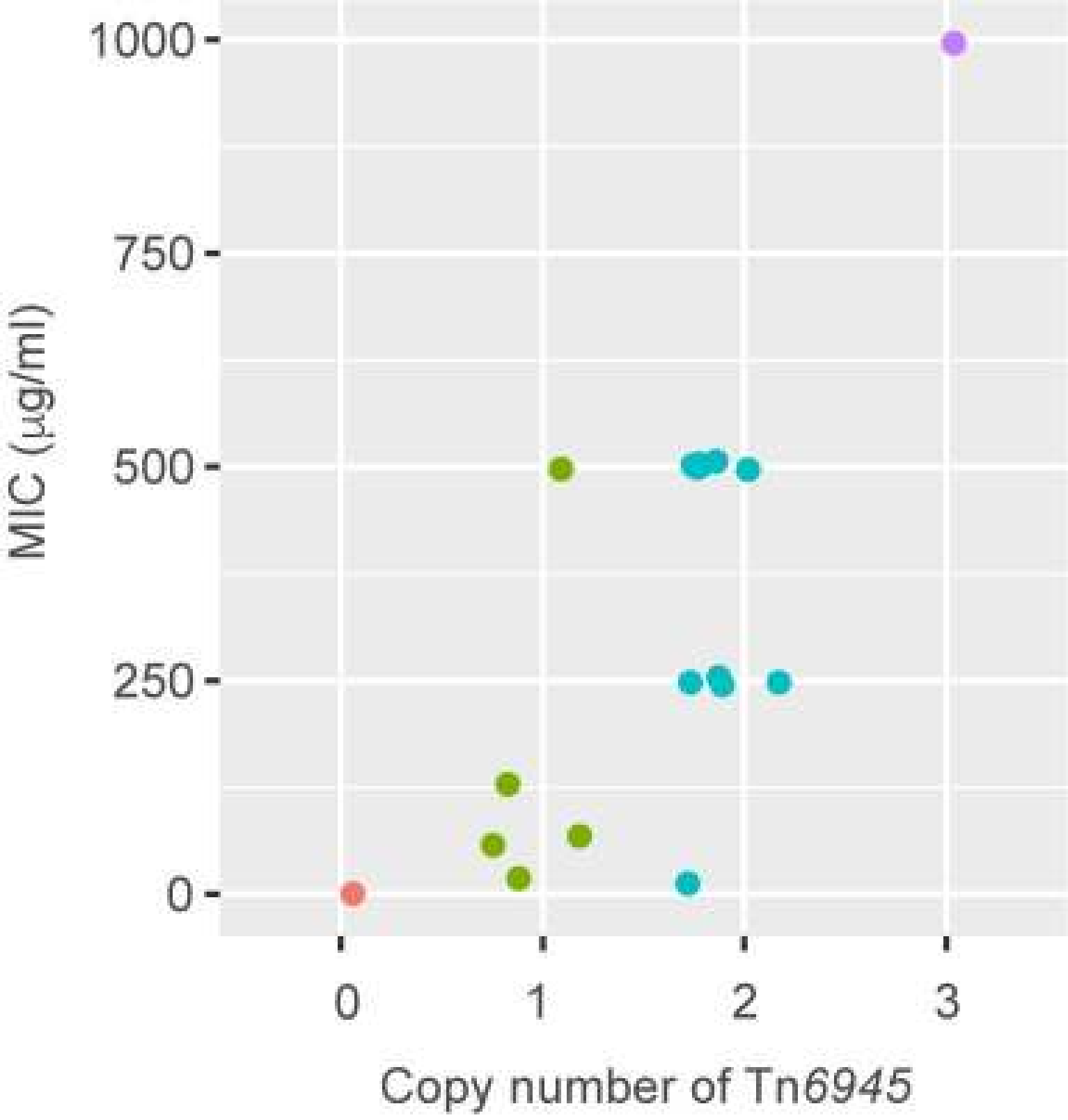
Rif, Ap
LN52rif
Rif, Tet, Ap


A
1
2
Chr1
gyrB
Chr2
pYa249
pSEA2
Lost in LN95
4
intA
+
3
att Tn6945
gyrB
Empty site (undetected)
4
gyrB
C
Relative copy number
1.000
0.100
0.010
0.001
attTn/gyrB
intA/gyrB
B
Primer set
Template
1. attL
2. attR
3. attTn
4. attB
+ - + - + - + -
10,000
6,000
3,000
2,000
1,500
1,000
750
500
250
100
V. alfancensis LN95
V. alfancensis 04Ya249


1000
750
500
250
0
MIC (μg/ml)
0
1
2
3
Copy number of Tn6945