Mobile Antibiotic Resistance Encoding Elements Promote Their Own Diversity

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Abstract

Integrating conjugative elements (ICEs) are a class of bacterial mobile genetic elements that disseminate via conjugation and then integrate into the host cell genome. The SXT/R391 family of ICEs consists of more than 30 different elements that all share the same integration site in the host chromosome but often encode distinct properties. These elements contribute to the spread of antibiotic resistance genes in several gram-negative bacteria including Vibrio cholerae, the agent of cholera. Here, using comparative analyses of the genomes of several SXT/R391 ICEs, we found evidence that the genomes of these elements have been shaped by inter–ICE recombination. We developed a high throughput semi-quantitative method to explore the genetic determinants involved in hybrid ICE formation. Recombinant ICE formation proved to be relatively frequent, and to depend on host (recA) and ICE (s065 and s066) loci, which can independently and potentially cooperatively mediate hybrid ICE formation. s065 and s066, which are found in all SXT/R391 ICEs, are orthologues of the bacteriophage λ. Red recombination genes bet and exo, and the s065/s066 recombination system is the first Red-like recombination pathway to be described in a conjugative element. Neither ICE excision nor conjugal transfer proved to be essential for generation of hybrid ICEs. Instead conjugation facilitates the segregation of hybrids and could provide a means to select for functional recombinant ICEs containing novel combinations of genes conferring resistance to antibiotics. Thus, ICEs promote their own diversity and can yield novel mobile elements capable of disseminating new combinations of antibiotic resistance genes.

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Introduction

Mobile genetic elements, including bacteriophages, conjugative plasmids and integrating conjugative elements (ICEs), are key mediators of bacterial genome evolution [1]. These elements can rapidly spread in bacterial populations and often confer to host bacteria selectable traits that are advantageous in particular environments or enable adaptation to new ecological niches. Transfer of ICEs and plasmids from donor to recipient bacteria occurs via conjugation, a process that requires direct cell-to-cell contact [2,3]. Conjugative transmission of ICEs and plasmids has limited the clinical usefulness of many antibiotics, since these mobile elements are potent vectors for dissemination of antibiotic resistance genes in bacterial populations [2,4–7].

ICEs integrate into and replicate along with the host cell chromosome, whereas plasmids exist as extra-chromosomal (usually circular) autonomously replicating DNA molecules. ICEs can excise from the donor cell chromosome and form circular molecules that are thought to be the substrates for the conjugative machinery. Similar to most conjugative plasmids [8], ICE conjugative DNA transfer is thought to be initiated at a specific cis-acting site (oriT) required for efficient translocation of the DNA to the recipient cell through the mating bridge. Within the recipient cell, host enzymes are thought to convert the translocated single-stranded DNA into double-stranded DNA that is circularized. An element-encoded recombinase (integrase) enables the integration of the ICE into the chromosome of the new host [2,9–11].

ICEs are widespread among diverse taxonomic groups of bacterial species and are able to transfer between genetically unrelated bacteria [5,10–12]. The SXT/R391 family of ICEs, which is one of the largest and most diverse set of ICEs studied, includes elements that have been detected in clinical and environmental isolates of several species of γ-proteobacteria from four continents over the past 40 years [13–20]. In Asia and Africa, this family of ICEs has played an important role in the spread of genes conferring resistance to multiple antibiotics in Vibrio cholerae, the causative agent of cholera [17,19,21–23]. Currently, nearly all isolates of V. cholerae from cholera patients from these two continents harbor SXT, a prototypical member of the SXT/ R391 family originally isolated from a 1992 Indian V. cholerae O139 isolate, or a closely related ICE [17–19,24–26].

The ICEs of the SXT/R391 family are grouped together because they all encode a highly conserved integrase (Int) that mediates the elements’ site-specific integration into the host genome in the 5′ end of pyfC, a conserved gene encoding the peptidase chain release factor RF3 [27]. Based on knowledge of the
Integrating and conjugative elements (ICEs) are a class of mobile elements found in diverse bacteria. ICEs of the SXT/R391 family have enabled the dissemination of genes conferring resistance to antibiotics among several important pathogens, including Vibrio cholerae, the agent of cholera. Here, using comparative analyses of the genomes of several SXT/R391 ICEs, we found that these elements are mosaics that have been shaped by inter-ICE recombination. We developed a plate-based method for semi-quantitative analyses of the genetic requirements for hybrid ICE formation. We discovered that hybrids form at relatively high frequencies and that both host and ICE genes can function independently and potentially cooperatively to mediate hybrid formation. The ICE-encoded recombination genes, which are found in all SXT/R391 ICEs, are related to genes that mediate recombination in bacteriophages, but have not been described previously in conjugative elements. Conjugative ICE transfer was not required for hybrid ICE formation but facilitates the segregation of hybrids. Thus, ICEs promote their own diversity and the generation of recombinant ICEs can yield novel mobile elements capable of disseminating new combinations of antibiotic resistance genes.

~100-kb genomes of several SXT/R391 ICEs [15,28–31], in addition to the conserved integrase gene (int), these elements all contain a conserved set of ~24 genes that mediate their common functions that include: excision/integration, conjugative transfer and regulation [5]. Distinct variable regions that confer element-specific phenotypes, such as synthesis of the second messenger c-di-GMP or resistance to antibiotics or heavy metals are interspersed within this conserved and syntenous SXT/R391 backbone (see Figure 1A) [5,15,22,30,32].

In some cases, SXT/R391 ICEs do not exclude one another and can be present in the same host [33–35], providing the opportunity for the generation of recombinant ICEs. For example, R391, the other prototypical member of the SXT/R391 family, which was originally derived from a 1967 South African Providencia rettgeri isolate, and SXT can reside together in the same host [33]. A cell that contains one of these two ICEs can acquire a copy of the other ICE, yielding tandem arrangements of SXT and R391 in the host chromosome [33]. Tandem repeat structures are often excellent substrates for recombination [36] and exconjugants derived from donor strains containing such tandem arrays sometimes contain hybrid ICEs with genes from both R391 and SXT [37].

The molecular mechanisms that enable the formation of hybrid ICEs, which may contain novel combinations of genes conferring resistance to antibiotics, have not been addressed. However, two genes, s065 and s066, which are highly conserved (≥96% identity) among all known SXT/R391 ICEs could contribute to the formation of hybrid ICEs. These genes encode proteins that are similar to the recombinase Bet (71% similarity and 55% identity) and the double-strand specific 5’ to 3’ exonuclease Exo (38% similarity and 26% identity) that are encoded by the temperate bacteriophage λ and several other phages [38] (Figure 1C). In λ, Bet and Exo, along with the Gam protein constitute an efficient recA-independent recombination system known as Red. Classic studies by Stahl and colleagues revealed many of the key features of the λ Red recombination system. They showed that efficient Red-mediated homologous recombination between λ chromosomes was almost entirely dependent on DNA replication [39], which generates a significant population of λ DNA with double-stranded breaks that serve as substrates for Red. Using replication-blocked crosses of phage λ chromosomes containing a single double-stranded cut, Stahl et al proposed that λ Red mediates recombination by a strand annealing mechanism [40]. Red Exo degrades 5’ ends of linear double-stranded DNA, creating 3’ single-stranded overhangs that can serve as templates for Red Bet to pair with complementary single-stranded DNA targets [41]. Red Gam (for which there is no SXT-encoded homologue) inactivates the E. coli exonuclease V (RecBCD), thereby protecting the ends of linear double-stranded DNA from degradation [41,42]. Besides providing significant amounts of double-stranded breaks, replication also provides a single-stranded DNA target for strand annealing on the lagging strand that is exposed by a passing replication fork [43]. Ordinarily, λ recombination is RecA-independent; however, when DNA replication is blocked, λ Red can also mediate efficient recombination via a strand invasion mechanism that is dependent upon RecA function [40,44]. Pottete et al suggested that the strand invasion pathway is a RecA-dependent salvage pathway for aborted Red-mediated recombination [45]. In recent years, the λ Red system has proven to be extremely useful for genetic engineering of Escherichia coli and closely related species [46–49]; however, investigation of the function of the Red pathway in its natural context, cells undergoing the λ lytic cycle, has several technical challenges [48]. To our knowledge, λ Red-like recombination systems have not been described previously in conjugative elements.

Here, we found that the genomes of SXT/R391 ICEs appear to be routinely shaped by inter-ICE recombination. We explored the role of the SXT and R391 bet and exo homologues (s065 and s066) and that of recA, a key host recombination gene, in the formation of hybrid ICEs. To accomplish this, we created a high throughput semi-quantitative screening assay that enabled the visual identification of exconjugant colonies containing hybrid ICEs. We found that recA mediated the formation of the majority of hybrid ICEs. Both s065 and s066 also contribute to the formation of hybrid ICEs and in the absence of recA, s065 and s066 appear to mediate the formation of nearly all hybrid ICEs. Conjugation was not essential for the formation of hybrid ICEs, suggesting that conjugative transfer acts as a means to segregate hybrid elements into new host cells. Thus, both host- and element-encoded recombination systems promote the formation of the mosaic genomes of SXT/R391 ICEs.

## Results

### Evidence for recombination between SXT/R391 ICEs

When the genomes of SXT [28] and R391 [29] were originally reported, it appeared that the variable regions in this family of ICEs (shown as colored bars underneath the set of shared genes in the gray rectangle in Figure 1A) were element-specific [50]. However, examination of the growing number of sequenced SXT/R391 ICE genomes suggests that even though some variable regions may be element-specific, others are shared by two or more ICEs (e.g. see ICE/PilSpa1 and ICE/SpuPO1 in Figure 1A), suggesting that this family of ICEs undergoes recombination. Closer analysis of conserved regions of these elements also suggested that recombination between SXT/R391 ICEs has shaped their genomes. Pairwise alignments of the genome sequence of ICE/PilSpa1, an ICE derived from the fish pathogen Photobacterium damselae subsp. piscicida, with that of SXT or R391 revealed that the majority of conserved sequences are only 95–97% identical, but that the 11.5-kb attL-tnpB and 0.6-kb s021-vmuB regions of ICE/PilSpa1 and SXT are nearly 100% identical (Figure 1B). These comparisons suggest that a relatively recent
recombination event within the 5’ end of the truncated copy of \textit{rumB} occurred between precursors of ICE\textit{PdaSpa1} and SXT, and support the idea that SXT/R391 ICE genomes are mosaics that have been sculpted by inter-ICE recombination. Exchange of DNA segments between these ICEs occurs when these elements are present in the same host cell. The tandem arrays that these ICEs can form in the host chromosome likely provide a suitable substrate for such recombination events to occur.

Detection of hybrid ICE formation

We developed a high throughput conjugation-based semi-quantitative screen to assess the genetic requirements for the formation of hybrid ICEs. The assay employs donor cells bearing tandem copies of modified SXT and R391, and was designed to distinguish between exconjugant colonies containing SXT-R391 tandem arrays, hybrid elements or single parental elements (Figure 2). The phenotypic markers \textit{lac}\zeta and \textit{gal}K were inserted between \textit{traG} and \textit{ex} in SXT and between \textit{traG} and \textit{mer}R in R391, respectively (Figure 1A and Figure 2). The position of these two loci, near the right ends of the elements, is remote from the antibiotic resistance markers that are found near the left ends of SXT (\textit{sul}II \textit{dfr}18) and R391 (\textit{aph}) (Figure 1A and Figure 2), thereby maximizing the opportunity to detect recombination events occurring within tandem arrays. Both \textit{lac}\zeta and \textit{gal}K were placed under control of the \(P_{\text{lac}}\) promoter to enable high-level \(\beta\)-galactosidase and

Figure 1. Evidence suggesting that recombination occurs between SXT/R391 ICEs. (A) The middle gray box represents the set of genes (arrows) conserved in the 4 SXT/R391 genomes shown. Gray ORFs represent genes of unknown function, white ORFs represent genes of known function [28,52,53], and black ORFs correspond to s065 and s066. Below, variable ICE regions are shown with colors according to the elements in which they were originally described: SXT [28] (blue), R391 [29] (red), ICE\textit{PdaSpa1} [15] (green), and ICE\textit{SpuPO1} [30] (purple). (B) A close-up of the attL-s025 region of ICE\textit{PdaSpa1} (accession number AJ870986) is shown in the upper left. The variation of percentage of identity was plotted using a Multi-LAGAN pairwise comparison [63] of this ICE\textit{PdaSpa1} region with the corresponding regions of SXT (accession number AY055428) and R391 (accession number AY090559) and the mVista visualization module [64] with a sliding window of 100 bp. The minimum width and the minimum percent conservation identity that must be maintained over that width for a region to be considered conserved were set at 100 bp and 70% respectively. The dark gray area highlights the large nearly identical region conserved between SXT and ICE\textit{PdaSpa1}. (C) A comparison of s065 and s066, which are present in all SXT/R391 ICEs, to the bacteriophage \(\lambda\). Red genes (numbers represent % similarity between S065 and Bet, and S066 and Exo, respectively) is shown in the upper right, \textit{dfr}18 encodes trimethoprim resistance; \textit{flo}R encodes chloramphenicol resistance; \textit{str}AB encodes streptomycin resistance; \textit{sul}II encodes sulfamethoxazole resistance; \textit{tet}AR encodes tetracycline resistance; \textit{aph} encodes kanamycin resistance; and \textit{mer} encodes mercury resistance.

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galactokinase activities in a lacI background. Escherichia coli strains containing tandem arrays of these labeled ICEs were used as donors in mating assays using ΔgalK lacZU118 lacI42::Tn10 derivatives of E. coli MG1655 as recipient strains. Exconjugants were isolated on MacConkey indicator agar containing trimethoprim, sulfamethoxazole and tetracycline (bottom panel) reveals colonies harboring single parental ICEs (blue colonies), hybrid ICEs (red colonies), and SXT-R391 tandem arrays (purple colonies). Purple colonies may also consist of cells containing an array composed of SXT and a hybrid element on this media (e.g. exconjugant 4). Red and purple colonies are larger on this medium because they can use D-galactose as a carbon source. (A,B) SXT left and right extremities; (C,D) R391 left and right extremities, respectively amplified by primer pairs VISLF/VISLR3, VISRF/VISRR, VISLF/VISLR2, VISRF/VISRR2 [37].

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In pilot experiments, we found that the percentage of hybrid ICEs detected was influenced by which ICE’s antibiotic resistance markers were selected. A higher percentage of exconjugants harboring a hybrid ICE was isolated on Su-Tm (6.75%) than on Kn (2.70%). This is probably a consequence of the fact that the R391 transfer frequency is about 10-fold higher than that of SXT, and hence a high frequency of colonies containing hybrids are likely to contain R391 as well, and thus cannot be distinguished from strains containing tandem arrays (39.2% tandem arrays on Su-Tm vs 10.4% on Kn). Consequently, in most subsequent studies of the genetic requirements for hybrid ICE formation, we used donors harboring SXT-R391 arrays and Su Tm to select for hybrid-harboring exconjugants; however, in some experiments we were unable to obtain SXT-R391 arrays with the desired deletions and in these cases we used donors containing R391-SXT arrays.

recA enables the formation of most, but not all, hybrid ICEs

We suspected that the host recA gene might play a key role in the generation of hybrid ICEs since the SXT and R391 genomes have
more than 95% identity over nearly 64 kb of DNA distributed in 11 segments ranging from 247 bp to 12,085 bp. Hybrid ICEs could form by RecA-mediated homologous recombination either in the donor cells prior to transfer or in recipient cells after transfer of both SXT and R391 from donor cells. We carried out conjugation experiments using recA+ (GG61) or recA− (GG66) donor cells containing a tandem array of SXT and R391, and recA+ (VB30) or recA− (VB47) recipient cells (Table 1) to distinguish between these possibilities. However, since RecA is required in donor cells for SXT and R391 transfer, probably to alleviate the repression of expression of genes encoding the conjugative transfer machinery (tra genes), it was necessary to exogenously express SetC and SetD, the activators of the tra genes, in all recA donors [51]. Such exogenous activation of transfer genes generally induces a 10- to 100-fold increase in the frequency of ICE transfer ([51] and data not shown); however, since we compare the percentage of hybrids in different backgrounds, rather than the absolute frequency of hybrid formation, the increase in transfer frequency should not distort our results.

Deletion of recA from donor cells had a significant effect on the percentage of exconjugants found to contain hybrid ICEs. Conjugation assays with recA donors reduced the percentage of hybrids at least 5.6-fold relative to assays with WT donors, both when WT and recA recipients were used (p<0.001) (Figure 3A and 3B). In contrast, deletion of recA from the recipient cells did not have a significant effect on the percentage of exconjugant colonies containing a hybrid ICE when WT donor cells were used (Figure 3A). When recA donor cells were used, there was an ~2-

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Table 1. Strains of E. coli and plasmids used in this study.

| Strain or plasmid | Relevant genotype or phenotype* | Reference or source |
|-------------------|---------------------------------|---------------------|
| **Strains** |
| CAG18420 | MG1655 lacZU118 lacI42::Tn10kan (KnR) | [65] |
| CAG18439 | MG1655 lacZU118 lacI42::Tn10 (TcR) | [65] |
| VB112 | MG1655 Rf | [52] |
| VB38 | CAG18439 ΔgalK (TcR) | This study |
| VB47 | CAG18439 ΔgalK ΔrecA (TcR) | This study |
| GG55 | VB112 ΔrecA (Rf) | This study |
| GG47 | GG55 pV67 (Rf ApR) | This study |
| HW220 | CAG18439 prfC::SXT (TcR SuR TmR) | [27] |
| JO99 | CAG18439 prfC::ΔSXT (TcR KnR) | [33] |
| VB40 | CAG18439 ΔlacZ prfC::SXT::lacZ | This study |
| GG13 | CAG18439 ΔgalK prfC::ΔSXT::galK | This study |
| GG61 | VB112 prfC::R391::galK::SXT::lacZ (Rf ApR SuR TmR KnR) | This study |
| GG64 | VB112 prfC::ΔSXT::lacZ Δox65 (Rf ApR SuR TmR KnR) | This study |
| GG65 | VB112 prfC::ΔSXT::lacZ Δox66 (Rf ApR SuR TmR KnR) | This study |
| GG66 | CAG18439 ΔgalK (Δox65 Δox66) [SXT::lacZ Δox65 Δox66] | This study |
| GG69 | CAG18439 ΔgalK Δox65 [R391::galK Δox65] (Rf ApR SuR TmR KnR) | This study |
| GG70 | CAG18439 ΔgalK Δox69 [R391::galK Δox69] (Rf ApR SuR TmR KnR) | This study |
| GG102 | CAG18439 ΔgalK Δox65 Δox66 [R391::galK Δox65 Δox69] | This study |
| GG125 | VB38 prfC::R391::galK Δmobi::SXT::lacZ Δmobi (TcR SuR TmR KnR) | This study |
| GG171 | VB38 prfC::SXT::lacZ Δint::R391::galK Δint (TcR SuR TmR KnR) | This study |
| GG185 | VB38 prfC::R391::galK [SXT::lacZ] (TcR SuR TmR KnR) | This study |
| GG186 | VB38 prfC::SXT::lacZ::R391::galK (TcR SuR TmR KnR) | This study |
| **Plasmids** |
| pKD3 | CmR template for one-step chromosomal gene inactivation | [47] |
| pKD4 | KnR template for one-step chromosomal gene inactivation | [47] |
| pVI36 | SpR template for one-step chromosomal gene inactivation | [52] |
| pVI40A | pV36 BamHI::p mobI-galK | This study |
| pVI42B | pVI36 BamHI::lacZ | This study |
| pAH57 | oriR101 repA101::cI857F L.P::xylI::intII (Ts ApR) | [59] |
| pVI67 | pAH57 Δ(xylI::intII::cI857F) (Ts) | This study |
| pVI68 | pAH57 Δ(xylI::intII::cI857F) (Ts) | This study |
| pMobi-B | pBAD-TOPO mobi | [52] |

*ox65 and ox66 of SXT correspond to orf68 and orf69 of R391, respectively, according to the annotation of both elements [28,29]. ApR, ampicillin resistant; CmR, chloramphenicol resistant; KnR, kanamycin resistant; RfR, rifampicin resistant; SuR, sulfamethoxazole resistant; SmR, streptomycin resistant; SpR, spectinomycin resistant; TcR, tetracycline resistant; TmR, trimethoprim resistant; Ts, thermosensitive.

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fold reduction in the percent of exconjugants with hybrid elements in recA recipients compared to WT recipients, which was not statistically significant (Figure 3B). Finally, deletion of recA from both donors and recipients reduced the percentage of hybrid ICEs detected by more than 11-fold as compared to when recA was present in both donor and recipient. Taken together, these observations suggest that recA-mediated homologous recombination generates the majority of hybrid ICEs and that these recombination events happen both in donor and recipient strains. RecA’s role is more readily discerned in donors; however, this may reflect a limitation of our assay in that conjugation facilitates detection of hybrids as discussed below. Notably, 0.60% of exconjugants contained hybrid ICEs even when both donor and recipient strains lacked recA indicating that some hybrid ICEs are generated via a recA-independent recombination pathway (Figure 3B).

s065 and s066 promote hybrid ICE formation

We explored whether s065 and s066, which encode a single-strand DNA recombinase (unpublished results and [38]) and a putative exonuclease respectively (Figure 1C), also influence the formation of hybrid ICEs, and whether they might account for recA-independent generation of these elements. Donor strains harboring tandem arrays of Δs065, Δs066, or Δ(s065-s066) SXT-R391 tandem arrays, were used as donors in these assays. The recipient strains were either E. coli VB38 (recA+) or E. coli VB47 (recA-). D/R +/− and Δindicate the recA genotype of the donor and recipient strains, respectively. SetDC was expressed from a plasmid when recA donors were used. Bars represent the percentage of exconjugants containing hybrid ICEs and were calculated by dividing the number of exconjugants containing hybrid ICEs (red TcR SuR TmR CFU) by the total number of exconjugants (TcR SuR TmR CFU). The means and standard deviations obtained from at least three independent assays are shown and the number of colonies containing a hybrid ICE counted for each assay is presented in Table S2. Note the differences in the scale of the y-axis in panels A and B. One-way ANOVA with a Tukey-Kramer post-test was used to compare the means of hybrid ICE-containing exconjugant colonies. The confidence interval for the comparisons of mutant tandem arrays relative to WT tandem arrays was P<0.001, except □ which indicates P<0.05 and ● which indicates that the difference was statistically not significant. * indicates that the percentage of exconjugants bearing a hybrid ICE was below the limit of detection (<0.01%).

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Figure 3. Involvement of recA, s065, and s066 in the formation of hybrid ICEs. recA+ (A) or recA (panel B) donor strains, which contained either wild-type (WT), Δs065, Δs066, or Δ(s065-s066) SXT-R391 tandem arrays, were used as donors in these assays. The recipient strains were either E. coli VB38 (recA+) or E. coli VB47 (recA-). D/R +/− and Δindicate the recA genotype of the donor and recipient strains, respectively. SetDC was expressed from a plasmid when recA donors were used. Bars represent the percentage of exconjugants containing hybrid ICEs and were calculated by dividing the number of exconjugants containing hybrid ICEs (red TcR SuR TmR CFU) by the total number of exconjugants (TcR SuR TmR CFU). The means and standard deviations obtained from at least three independent assays are shown and the number of colonies containing a hybrid ICE counted for each assay is presented in Table S2. Note the differences in the scale of the y-axis in panels A and B. One-way ANOVA with a Tukey-Kramer post-test was used to compare the means of hybrid ICE-containing exconjugant colonies. The confidence interval for the comparisons of mutant tandem arrays relatively to WT tandem arrays was P<0.001, except □ which indicates P<0.05 and ● which indicates that the difference was statistically not significant. * indicates that the percentage of exconjugants bearing a hybrid ICE was below the limit of detection (<0.01%).

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Conjugation is not required for the formation of hybrid ICEs

In the experiments described above, we relied on conjugal transfer to identify hybrid ICEs in exconjugant colonies. However, our observation that some hybrids appear to form in recipient cells, after elements have transferred (as indicated by differences in hybrid formation in recA and recA recipients) suggested that the conjugal process was not necessarily a component of hybrid formation. We took advantage of our previous observations that there is little, if any, conjugal transfer of SXT in broth culture [23], to begin to explore whether conjugation was required for hybrid ICE formation. We tested whether we could detect hybrid formation in a recA ΔgalK lacZ− TcR strain (GG185) bearing a wild-type R391-SXT array (the opposite array orientation as used above) in the absence of a recipient strain. GG185 was passaged with two subcultures in LB broth for 72 h (>100 generations) and then the culture was plated on MCGX indicator medium supplemented with Tc Su Tm, to identify SuR TmR hybrid ICEs (red colonies), or with Tc Kn, to identify KnR hybrid ICEs (blue colonies). KnR hybrid ICEs were detected (0.16±0.05% of colonies) at this point but SuR TmR hybrids were barely detectable (Table 2). Detection of hybrid ICE formation using this experimental system requires marker loss. We observed greater loss of SXT (20.1±4.9%) than R391 (<0.02%) in this experiment, in accord with a previous report that the ICE located at the right end of the array is more frequently lost and that in this position, R391 is more stable than SXT [33]. Thus, the few detectable SuR TmR hybrids in this experiment likely reflect the lack of loss of R391 from the tandem array in GG185.

The detection of KnR hybrids during passage of GG185 in LB broth provides support for the idea that conjugation is not essential for hybrid ICE formation. However, it is possible that there is a low frequency of conjugal ICE transfer in broth cultures. To formally exclude a role for conjugation in hybrid ICE formation, we constructed a strain harboring an R391-SXT array where the ICEs were unable to transfer due to the deletion of mobI. MobI is part of the SXT/R391 DNA processing machinery and is thought to recognize and act on oriT; deletion of mobI renders SXT and R391 non-transmissible but does not impair their excision (data not shown) or the formation of a functional conjugation apparatus [52]. We constructed a ΔmobI R391-SXT tandem array in a recA ΔgalK lacZ− TcR strain (VB38) (Table 1). The resulting strain (GG125) was cultivated for 6 days with two daily subcultures (>250 generations) in LB broth with tetracycline as the sole antibiotic. Serial passage allowed for the loss of unselected markers [37], thereby helping to reveal formation of possible hybrid ICEs.

The culture was plated at 24, 72, and 144 h post-inoculation on the indicator medium supplemented with the same antibiotics used above to identify hybrid ICEs. As noted above with GG185, loss of SXT from the R391-SXT array in GG125, yielding a single R391 (KnR) ICE, occurred much more frequently than the loss of R391 from this strain (Table 2). KnR hybrid ICEs were detectable at 24 h, when 0.11% of colonies contained a hybrid ICE, and by 144 h this percentage increased to 0.38% (Table 2). SuR TmR hybrid ICEs were only isolated after 144 h of culture and only 0.004% of colonies contained hybrids. Potential explanations for the different frequencies with which hybrids were observed are discussed below. However, the results from both selections clearly demonstrate that conjugation is not required for the formation of hybrid ICEs. Furthermore, using a variety of PCR assays [see [37]], three distinct ICE structures were identified among 19 of the KnR hybrids (data not shown). Thus, the hybrids identified in these experiments cannot be explained by clonal amplification of a single cell containing a hybrid ICE.

Excision is not required for hybrid ICE formation

Despite existing predominantly as chromosomal-encoded elements, the position of ICEs with respect to host chromosomes is highly dynamic. ICE-encoded int and xis genes allow them to excise from host chromosomes [53], and this event is thought to be an early step in conjugation. To assess whether extrachromosomal ICE DNA is a required substrate for hybrid ICE formation, we constructed a strain carrying a Δsxt SXT-R391 array (GG171) (Table 1). GG171 was used in assays similar to those described above for the ΔmobI array. After only 24 h of culture, 0.9% of colonies contained a SuR TmR glkR hybrid ICE, demonstrating that formation of recombinant ICEs does not depend on ICE excision. Thus, chromosomal tandem ICE arrays can serve as a recombination substrate.

Discussion

Comparative analyses of the genomes of several SXT/R391 ICEs revealed that these elements are mosaics that have been shaped by inter-ICE recombination (Figure 1A). The large set of core genes that are conserved among all SXT/R391 ICEs provides an ample substrate for inter-ICE recombination. Furthermore, the inherent ability of these elements to form tandem array structures [33,37] increases the opportunities for ICE recombination. Given the high degree of homology between SXT and R391, our finding that recA accounts for the generation of the majority of hybrid ICEs is understandable. However, s065 and s066, which are present in all SXT/R391 ICEs, also

Table 2. Percentage of colonies containing hybrid ICEs or single elements recovered over time from a strain initially harboring a wild-type R391-SXT, or a non-transmissible ΔmobI R391-SXT tandem array.

| Strains   | Time (h) | % colonies selected on Kn | % colonies selected on Su Tm |
|-----------|----------|---------------------------|-----------------------------|
|           |          | Hybrids*                   | Single                      |
|           |          | Hybrid*                    | Single                      |
| GG185     | 24       | 0.04±0.04                  | 11.7±3.4                    | 0.02±0.03 | 0.04±0.07 |
|           | 72       | 0.16±0.05                  | 20.1±4.9                    | 0.03±0.05 | <0.02b |
| GG125 (ΔmobI) | 24 | 0.11±0.02                  | 11.8±0.6                    | <0.01b | 0.02±0.03 |
|           | 72       | 0.21±0.03                  | 16.1±1.7                    | <0.01b | 0.02±0.03 |
|           | 144      | 0.38±0.07                  | 22.6±1.2                    | 0.004±0.007 | 0.09±0.07 |

aThe numbers of colonies containing a hybrid ICE that were counted for each assay are presented in Table S3.

bDetection limit of the assay.
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contribute to formation of recombinant ICEs. The contribution of these ICE λ, bet and exo homologues was easiest to discern in the absence of recA; in this context, s065 and s066 accounted for the formation of nearly all of the hybrids we detected. These two genes appear to function in the same recombination pathway, since deletion of s065, s066, or both genes resulted in similar reductions in hybrid formation. Neither ICE excision nor conjugative transfer proved to be essential for generation of hybrid ICEs; instead, conjugation appears to facilitate the segregation of hybrids and may provide a means to select for functional recombinant ICEs.

In previous work, we used multiple PCR analyses to show that exconjugants derived from conjugations with donors bearing SXT-R391 arrays occasionally contained a hybrid ICE [37]. This technique was too cumbersome to enable either quantitative or genetic analysis of hybrid ICE formation. The high-throughput semi-quantitative detection method reported here enabled more sensitive analyses of the genetic determinants involved in hybrid ICE formation. Hybrid formation was relatively frequent, as we found that almost 7% of exconjugants selected on Su and Tm contained a recombinant ICE. Since some exconjugants scored as containing a parental ICE array (purple colonies in Figure 2) actually contained a hybrid ICE and a parental ICE, 7% is an underestimation of the true frequency of hybrid formation. Thus, formation of hybrid ICEs, which may have novel combinations of genes conferring resistance to antibiotics, may be fairly common.

While hybrid ICEs were readily detectable in exconjugants using our plate-based screening method, we found that they also form in cells containing tandem arrays of non-transmissible ICEs. Detection of non-transmissible hybrid ICEs seems to depend upon the rate of post-recombinational loss of one or the other ICE, as shown by the coincident increase over time of colonies harboring hybrids (Table 2). Different frequencies of KnR vs SuR TmR hybrids formed from the non-transmissible R391-SXT array (Table 2). These differences are probably a consequence of the structure of the array used here. The relatively low frequency of hybrids in donors compared to exconjugants suggests that conjugation facilitates detection of hybrids by allowing for segregation of hybrid ICEs from parental ICEs. In nature, it is possible that conjugation serves to select for functional hybrids that are capable of transmission.

Our data indicate that both recA and s065/s066 can mediate hybrid formation independently, and potentially co-operatively as well. RecA’s role in homologous recombination has been the subject of extensive study; we assume its mechanism of action parallels that described in previous work. Our models for how s065 and s066 mediate hybrid ICE formation are largely based on prior studies of phage-borne s065 and s066 homologues. However, there is evidence that S065, like λ Bet, can mediate single-stranded DNA recombination [38] and our unpublished observations) and that S066 has double-stranded DNA exonuclease activity (Rory Watt, unpublished observations). Thus, it is reasonable to assume that S065 and S066 function in a similar fashion as RecA to repair system to promote the formation of functional ICEs capable of exiting from a damaged host and re-establishing themselves in a new host.

While numerous questions regarding the action of S065 and S066 remain to be explored, collectively our findings suggest that these genes promote the plasticity of SXT/R391 ICE genomes. Besides enhancing inter-ICE recombination, it also possible that s065 and s066 enable the incorporation of exogenous genetic material into ICE genomes, such as the DNA shown in colors in Figure 1A. Lastly, we identified orthologs of s065 and s066 in IncA/C plasmids such as pIP1202 from *T. pallidum* bv. *Orientalis*, the causative agent plague. These conjugative plasmids have recently been found to be broadly disseminated among multiply drug resistant zoonotic pathogens [6]. It will be interesting to explore whether these s065/s066 orthologs contribute to the plasticity of this family of conjugative plasmids.

### Materials and Methods

#### Bacterial strains, plasmids, and media

The bacterial strains and plasmids used in this study are described in Table 1. Bacterial strains were routinely grown in Luria-Bertani (LB) broth at 37°C in an orbital shaker and
maintained at −80°C in LB broth containing 15% (v/v) glycerol. Colonies harboring hybrid ICEs were screened by plating on MacConkey agar base (Difco) plates supplemented with 0.6% galactose, 50 mg/l X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (indicator medium MCGX) and the suitable antibiotics. Antibiotics were used at following concentrations: ampicillin (Ap), 100 mg/l; kanamycin (Km), 50 mg/l; rifampicin (Rf), 100 mg/l; spectinomycin (Sp), 50 mg/l; sulfamethoxazole (Su), 160 mg/l; trimethoprim (Tm), 32 mg/l; tetracycline (Tc), 12 mg/l.

Plasmid construction

The oligonucleotides used for construction of plasmids are described in Table S1. Plasmids pVI67 and pVI68, designed to allow conditional expression of SetDC or IntSXT, were constructed by replacing the 1,383-bp EcoRI/Ncol fragment of pVI37 [59] with either a 942-bp EcoRI/Ncol fragment containing the setDC operon of SXT or a 1,367-bp EcoRI/Vol fragment containing intSXT, respectively, and amplified by PCR using primer pairs setDF/setCR and intSF/intSR, respectively, and the DNA of E. coli HB292 as a template. Both plasmids are temperature sensitive for replication and allow the expression of the cloned genes from λPr under control of the thermosensitive repressor cI857.

Plasmids pVI40A and pVI42B were templates used in the creation of PCR products for the insertion of lacZ and galK markers into SXT and R391 with the Datsenko and Wanner protocol [47]. These templates contain galK or lacZ both under control of PgalK, introduced into the BamHI site of pVI36 [52]. The Plac-galK fragment was made by amplifying by PCR galK and the Plac-galK promoter sequence using primer pairs galK1F/galK1R and Plac3F/Plac3R, respectively, and the DNA of E. coli VB112 as a template. The resulting two fragments were fused using the Splicing by Overlap Extension protocol [60]. The Plac-lacZ of pVI42B was amplified using DNA of E. coli VB112 as a template and primer pair lacZ1R/Plac3F. The insert of all plasmids constructed for this study were sequenced by DNA LandMarks Inc (St-Jean-sur-Richelieu, QC).

Construction of chromosomal deletions and insertions

The oligonucleotides used for chromosomal deletions and insertions are described in Table S1. Deletion and insertion mutants were constructed by using the one-step chromosomal gene inactivation technique of Datsenko and Wanner [47]. All deletions were designed to be non-polar. The ΔgalK and ΔlacZ mutations were introduced in E. coli CAG18439 using primer pairs galKWF/galKWR and lacZW/B/lacZF-F, and plasmids pVI36 and pKD4 as templates. The ΔrecA mutation was introduced in E. coli VB38 and VB112 using primer pair recAWF/recAR and pVI36 as a template. The Δ065, Δ066, and Δ065-066 mutations were introduced in SXT (in strain HB292) using primer pairs 65WF/65WR, 66WF/66WR, and 65WF/66WR, respectively, and template plasmid pVI36. The corresponding mutations Δorf68, Δorf69 and Δorf68-orf69 were introduced in R391 (in strain JO99) using primer pairs betWF/betWR, exoWF/exoWR, and betWF/exoWR, respectively, and pVI36 as a template. ΔmobI and Δint mutations were created in R391 using primer pairs orfXWF/orfXR and intRF/intRW, respectively, and pKD3 as a template. SXT deletion mutants of mobI (VB119) and int (BI554) were already available [52,62].

lacZ-tagged SXT was constructed by inserting PlacZ between traG and eex using primer pair lacZWF/lacZWR and pVI42B as the template, yielding strain VB40. Similarly, galK-tagged R391 was created by inserting PlacK between traG and merR using primer pair GalWF/GalWR and pVI40A as the template, yielding strain GG13. Plac::lacZ and Plac::galK were also introduced into strains containing SXT and R391 deletion mutants, using Plac::galK as template. All deletion and insertion mutations were verified by PCR amplification using primers flanking the deletion, cloning and sequencing.

Construction of strains containing tandem arrays of SXT and R391

Strains containing tandem arrays were constructed by successively transferring SXT::lacZ or and R391::galK (or their corresponding deletion derivatives) into VB112, yielding strains GG61 to GG65 and GG93. The recA null strains GG66 to GG70 and GG102 were created in a similar fashion except that pVI67 was introduced into GG55 prior to the transfer of the ICEs. We used the mobI expression vector pMobI-B [52] to mobilize ΔmobI ICEs in the construction of strain GG125. We verified that the deletion of mobI did not impair SXT or R391 excision using a real-time PCR quantification assay designed to determine the relative proportion of attP and attB sites per 100 chromosomes as described previously [53]. The int expression vector pVI68 was used to mobilize Δint ICEs in the construction of GG171. All strains harboring tandem arrays were tested to determine the relative positions of SXT and R391 in the tandem array by PCR amplification of the leftmost and rightmost ICE-chromosome junctions with primers pairs primer 6/prime 4 and primer 8/ primer 9 described by Hochhut et al. [33].

Conjugation assays and detection of hybrid ICEs

Conjugation assays were performed by mixing equal volumes of overnight cultures of donor and recipient strains grown overnight at 37°C. The cells were harvested by centrifugation, washed in 1 volume of LB broth and resuspended in 1/20 volume of LB broth. The mixtures were then deposited on LB agar plates and incubated at 37°C for 6 hours. The cells were recovered from the plates in 1 ml of LB broth and serial dilutions were prepared. Donors, recipients and exconjugants were selected on LB agar plates containing appropriate antibiotics.

The setDC expression vector pVI67 was used in mating assays involving recA donor strains. In these experiments, donor strains were grown overnight at 30°C and then cultures were shifted to 42°C for 15 minutes prior to contact with the recipient strain, to induce expression of SetC and SetD.

MCGX indicator agar medium plates supplemented with appropriate antibiotics were used to determine whether SXT, R391, SXT-R391 tandem arrays, or hybrid elements were present in exconjugant colonies or in donor colonies in experiments assessing the necessity of conjugative transfer or excision in hybrid ICE formation. The hybrid ICE detection technique was validated by PCR screening of exconjugant colonies using the primer pairs VISLF/VISLRR3, 10SF13/SXT1-13, YND2/ORF16, VISRF/VISRR, VISLF/VISLRR2, MER104A/MER103B and VISRF/VISRR2 as described by Burrous and Waldor [37].

Molecular biology methods

Plasmid DNA was prepared using a QIAprep Spin Miniprep kit (Qiagen) according to manufacturer’s instructions. All the enzymes used in this study were purchased from New England BioLabs. PCR assays were performed with the primers described in Table S1 in 20 μl reactions with 1 U of Taq DNA polymerase; 1 μl of a mixture of one colony resuspended in 10 μl of HyPure Molecular Biology Grade Water (HyClone) was used as a template in PCR
Hybrid ICE Formation

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Table S3 Number of colonies containing a hybrid ICE counted for each assay presented in Table 2.

Table S2 Number of colonies containing a hybrid ICE counted for each assay presented in Figure 3.

Table S1 DNA sequences of oligonucleotides used in this study.

Supporting Information

References

1. Frost LS, Leplae R, Summers AO, Toussaint A (2005) Mobile genetic elements: the agents of open source evolution. Nat Rev Microbiol 3: 722–732.
2. Burrus V, Pavlovic G, Decaris B, Guedon G (2002) Conjugative transposons: a tip of the iceberg. Mol Microbiol 46: 601–610.
3. Lawley TD, Klimke WA, Gubbins MJ, Frost LS (2003) F factor conjugation is a true IV secretion system. FEMS Microbiol Lett 224: 1–13.
4. Bennett PM (2000) Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. Br J Pharmacol 135 Suppl 1: S347–357.
5. Burrus V, Marrero J, Waldor MK (2006) The current ICE age: biology and evolution of SXT-related integrating conjugative elements. Plasmid 55: 173–183.
6. Welch TJ, Fricke WF, McDermott PF, White DG, Roso ML, et al. (2007) Multiple antimicrobial resistance in plague: an emerging public health risk. PLoS ONE 2: e209. doi:10.1371/journal.pone.0000209.
7. White G, Shoemaker NR, Salyers AA (2002) The role of Bacteroides conjugative transposons in the dissemination of antibiotic resistance genes. Cell Mol Life Sci 59: 2044–2054.
8. Gomis-Ruth FX, Coll M (2006) Cut and move: protein machinery for DNA processing in bacterial conjugation. Curr Opin Struct Biol 16: 744–752.
9. Salyers AA, Marrero J, Waldor MK (2006) The current ICE age: biology and evolution of SXT-related integrating conjugative elements. Plasmid 55: 173–183.
10. Salyers AA, Shoemaker NR, Stevens AM, Li LY (1995) In the driver’s seat: the ICE of Vibrio cholerae. Trends Genet 11: 42–49.
11. Uchida Y, Fujita A, Sato M, Higa N, et al. (2004) Environmental Vibrio spp., isolated in Mozambique, contain a polymorphic group of integrative conjugative elements and class 1 integrons. FEMS Microbiol Lett 234: 45–54.
12. Waldor MK, Tsichad H, Mekalanos JJ (1996) A new type of conjugative transposon encodes resistance to sulfamethoxazole, trimethoprim, and streptomycin in Vibrio cholerae O139. J Bacteriol 178: 4157–4163.
13. Opuntan JA, Newman MJ, Nakashodoh OA, Okeke IN (2008) Vibrio cholerae O1 from Acra, Ghana carrying a class 2 integron and the SXT element. J Antimicrob Chemother 62: 929–933.
14. Davies MA, Wilkinson A, Chung C, Lee P, Rannell SR (2000) Cloning and sequencing of the integron from a multidrug-resistant strain of Proteus vulgaris. Plasmid 43: 144–152.
15. Davies MA, Davies JR, Leader AE, Mangan HA (1995) A new type of conjugative transposon encodes resistance to sulfamethoxazole, trimethoprim, and streptomycin in Vibrio cholerae O139. J Bacteriol 178: 4157–4163.
16. Davis LM, Waldor MK (2000) CTXphi contains a hybrid genome derived from the Tn3 family of ICEs. Proc Natl Acad Sci U S A 97: 12606–12611.
17. Davis LM, Waldor MK (2001) Comparative ICE genomics: insights into the evolution of the SXT/R391 family of ICEs. Mol Cell Microbiol 66: 847–853.
18. Doherty R, Carrero A, Zhu Y, Okeke IN, Simonet F, et al. (2004) Vibrio cholerae O139 contains a novel Integrative and Conjugative Element (ICE Vch040). Plasmid 51: 1–11.
19. Doherty R, Carrero A, Zhu Y, Okeke IN, Simonet F, et al. (2004) ICE Vch040 contains a novel Integrative and Conjugative Element (ICE Vch040). Plasmid 51: 1–11.
20. Doherty R, Carrero A, Zhu Y, Okeke IN, Simonet F, et al. (2004) ICE Vch040 contains a novel Integrative and Conjugative Element (ICE Vch040). Plasmid 51: 1–11.
41. Kuzminov A (1999) Recombinational repair of DNA damage in *Escherichia coli* and bacteriophage lambda. Microbiol Mol Biol Rev 63: 751–813.
42. Unger RC, Clark AJ (1972) Interaction of the recombination pathways of bacteriophage lambda and its host *Escherichia coli* K12: effects on exonuclease V activity. J Mol Biol 70: 339–348.
43. Ellis HM, Yu D, DiTizio T, Court DL (2001) High efficiency mutagenesis, repair, and engineering of chromosomal DNA using single-stranded oligonucleotides. Proc Natl Acad Sci U S A 98: 6742–6746.
44. Poteete AR, Fenton AG (1993) Efficient double-strand break-stimulated recombination promoted by the general recombination systems of phages lambda and P22. Genetics 134: 1013–1021.
45. Poteete AR, Fenton AG, Nadkarni A (2004) Chromosomal duplications and cointegrates generated by the bacteriophage lambda Red system in *Escherichia coli* K-12. BMC Mol Biol 5: 22.
46. Yu D, Ellis HM, Lee EC, Jenkins NA, Copeland NG, et al. (2000) An efficient recombination system for chromosome engineering in *Escherichia coli*. Proc Natl Acad Sci U S A 97: 5973–5983.
47. Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci U S A 97: 6640–6645.
48. Poteete AR (2001) What makes the bacteriophage lambda Red system useful for genetic engineering: molecular mechanism and biological function. FEMS Microbiol Lett 201: 9–14.
49. Sowizralie JA, Thomason LC, Costantino N, Bubonenko M, Datta S, et al. (2007) Recombinase: in vivo genetic engineering in *E. coli*, *S. enterica*, and beyond. Methods Enzymol 421: 171–199.
50. Beaber JW, Burrus V, Hochhut B, Waldor MK (2002) Comparison of SXT and R391, two conjugative integrating elements: definition of a genetic backbone for the mobilization of resistance determinants. Cell Mol Life Sci 59: 2063–2070.
51. Beaber JW, Hochhut B, Waldor MK (2004) SOS response promotes horizontal dissemination of antibiotic resistance genes. Nature 427: 72–74.
52. Cecarelli D, Daccord A, Rene M, Burrus V (2000) Identification of the origin of transfer (oriT) and a new gene required for mobilization of the SXT/R391 family of ICEs. J Bacteriol.
53. Burrus V, Waldor MK (2003) Control of SXT integration and excision. J Bacteriol 185: 5045–5054.
54. Poteete AR (2008) Involvement of DNA replication in phage lambda Red-mediated homologous recombination. Mol Microbiol 68: 66–74.
55. Little JW (1967) An exonuclease induced by bacteriophage lambda. II. Nature of the enzymatic reaction. J Biol Chem 242: 679–686.
56. Hatfull GF (2008) Bacteriophage genomics. Curr Opin Microbiol 11: 447–453.
57. Juhala RJ, Ford ME, Duda RL, Youlton A, Hatfull GF, et al. (2000) Genomic sequences of bacteriophages HK97 and HK622: pervasive genetic mosaicism in the lambdaoid bacteriophages. J Mol Biol 299: 27–51.
58. Martinson JT, Radman M, Peit MA (2008) The lambda Red proteins promote efficient recombination between diverged sequences: implications for bacteriophage genome mosaicism. PLoS Genet 4: e1000065. doi:10.1371/journal.p-gen.1000065.
59. Haldemann A, Wanner BL (2001) Conditional-replication, integration, excision, and retrieval plasmid-host systems for gene structure-function studies of bacteria. J Bacteriol 183: 6384–6393.
60. Horton RM, Hunt HD, Ho SN, Pullen JK, Pease LR (1989) Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene 77: 61–68.
61. Hochhut B, Marrero J, Waldor MK (2000) Mobilization of plasmids and chromosomal DNA mediated by the SXT element, a consin found in *Vibrio cholerae* O139. J Bacteriol 182: 2043–2047.
62. Dower WJ, Miller JF, Ragdale CW (1988) High efficiency transformation of *E. coli* by high voltage electroporation. Nucleic Acids Res 16: 6127–6145.
63. Prudho M, Do CB, Cooper GM, Kim MF, Davydo E, et al. (2003) LAGAN and Multi-LAGAN: efficient tools for large-scale multiple alignment of genomic DNA. Genome Res 13: 721–731.
64. Frazer KA, Pachter L, Poliakov A, Rubin EM, Dubchak I (2004) VISTA: computational tools for comparative genomics. Nucleic Acids Res 32: W273–278.
65. Singer M, Baker TA, Schnitzler G, Duschsel SM, Goel M, et al. (1989) A collection of strains containing genetically linked alternating antibiotic resistance elements for genetic mapping of *Escherichia coli*. Microbiol Rev 53: 1–24.