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METHODOLOGY ARTICLE

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An *att* site-based recombination reporter system for genome engineering and synthetic DNA assembly

Michael J. Bland^{1,2}, Magaly Ducos-Galand^{1,2}, Marie-Eve Val^{1,2} and Didier Mazel^{1,2*} 

Abstract

Background: Direct manipulation of the genome is a widespread technique for genetic studies and synthetic biology applications. The tyrosine and serine site-specific recombination systems of bacteriophages HK022 and Φ C31 are widely used for stable directional exchange and relocation of DNA sequences, making them valuable tools in these contexts. We have developed site-specific recombination tools that allow the direct selection of recombination events by embedding the *attB* site from each system within the β -lactamase resistance coding sequence (*bla*).

Results: The HK and Φ C31 tools were developed by placing the *attB* sites from each system into the signal peptide cleavage site coding sequence of *bla*. All possible open reading frames (ORFs) were inserted and tested for recombination efficiency and *bla* activity. Efficient recombination was observed for all tested ORFs (3 for HK, 6 for Φ C31) as shown through a cointegrate formation assay. The *bla* gene with the embedded *attB* site was functional for eight of the nine constructs tested.

Conclusions: The HK/ Φ C31 *att-bla* system offers a simple way to directly select recombination events, thus enhancing the use of site-specific recombination systems for carrying out precise, large-scale DNA manipulation, and adding useful tools to the genetics toolbox. We further show the power and flexibility of *bla* to be used as a reporter for recombination.

Keywords: Site-specific recombination, Tyrosine recombinase, Serine recombinase, Genetic engineering

Background

The ability to precisely and directly manipulate DNA is important for functional studies and the synthetic assembly of large genetic constructs. Site-specific recombinase (SSR) systems are widely used as tools to rearrange, insert, remove, and join DNA with virtually no upper limit in size. For biotechnology purposes, this can include the insertion of exogenous DNA into chromosomes, the fusing of DNA molecules, or the construction of synthetic gene networks [1]. The tyrosine (Y-rec) and serine (S-rec) recombination families are named for the catalytic residue of their respective integrase (Int) protein. Important members of the Y-rec family include the λ -like phage recombination systems,

which include λ and the closely related phage HK022 (hereafter referred to as HK). The Φ C31 recombinase system is an important member of the S-rec family [2]. Both HK and Φ C31 systems comprise *attB/attP* attachment sites that serve as points of recombination, and the recombinases that catalyze recombination. In each family, DNA exchange requires host-encoded proteins for recombination that differ between systems. These systems are attractive due to their directionality and stability, and both systems are functional in prokaryotic and eukaryotic organisms [3–5].

Mechanistically, *attB* and *attP* integrative recombination forms *attL* and *attR* sites. The reverse *attL* \times *attR* excisive reaction also requires Int as well as a recombination directionality factor (RDF), named Xis in the HK system and gp3 in the Φ C31 system [6], typically supplied *in trans* from a helper plasmid, a non-replicating DNA molecule, or as mRNA [7]. Structurally, HK and

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Φ C31 *att* sites differ in size, with the HK *attB* sites being generally shorter than the HK *attP* sites, 21 base pairs (bp) vs 234 bp [8, 9]; in addition, *attP* contains binding sites for Int and Xis along with host-encoded proteins Fis and IHF [8–11]. Φ C31 *attB* and *attP* sites are similar in size (~50 bp) and do not require additional proteins to carry out recombination [12].

The use of SSRs generally involves selecting the recombination event through the use of a marker gene within the inserted sequence whose presence or absence would indicate successful integration [1]. Genes can be activated following recombination through either removal of blocking DNA sequences or by bringing together physically separated congruous sequences, with the recombination site embedded within the gene or between the promoter and coding sequence. This approach has long been used with the popular *CRE/loxP* [13] and *Flp/FRT* [14] systems. The β -lactamase (*bla*) gene is an attractive marker, as it is a useful reporter gene for both pro- and eukaryotic applications [15]. Protein chimeras of β -lactamase demonstrate tolerance to exogenous peptide insertions [16], even for domains of unknown function [17]. A split gene reassembly approach using *bla* has also been developed to discover directed evolution-modified SSR enzymes capable of recombining designer sequences [18]. The *bla* signal peptide is an attractive region for peptide insertion [19], as insertions between the signal peptide sequence and the rest of the coding gene have minimal interference with protein function [20]. As we wished to expand the available molecular toolbox, we created a set of recombination reporters consisting of the *attB* of HK and Φ C31 inserted in frame with *bla*, allowing expression of the gene and enabling the direct selection of recombination events. The selective agent is not expressed when the *att* sites are in *attL* and *attR* form, as the reporter gene fragments are physically separated (Fig. 1a).

This approach has been used to explore the physical structure of the *E. coli* genome [21, 22]. Genome engineering of the two *Vibrio cholerae* chromosomes used this tool to understand the evolutionary and genetic implications of multi-chromosomal bacteria [23]. We have used HK recombination in tandem with the λ -*lacZ* system from [21] to exchange DNA between the two *V. cholerae* chromosomes in a recombination-mediated cassette exchange (RMCE), resulting in large-scale chromosomal rearrangements [23]. Because the *lacZ* reporter allows the observation of recombination events but not to select for them, we developed a reporter system for HK recombination based on antibiotic selection. We have used an HK *attB* site placed in-frame within the β -lactamase (*bla*) gene to carry out relocation of the *S10-spec- α* ribosomal locus in *V. cholerae* in order to study the consequences of essential gene positioning as it relates to

dosage [24]. We further used HK-*bla* to carry out large-scale genome inversions around the origin region (*ori*) of *V. cholerae* chromosome one (Chr1) to shift the timing of the initiation of chromosome two (Chr2) replication relative to Chr1 in order to study the mechanisms involved in bacterial chromosome replication timing [25].

Here, we describe the construction and validation of HK-*bla* and a similar tool using the serine Φ C31 *att* system (Φ C31-*bla*). We placed *attB* sites from each system immediately downstream of the *bla* signal peptide coding sequence, which directs transport of β -lactamase to the periplasm and is removed in the mature protein. β -lactamase is generally tolerant of insertions into this region. When each system is present as *attL* and *attR* sites, they are associated with fragment sequences *bla'* (the 5' region upstream of the cleavage site including the promoter and signal sequence) and *'bla* (the 3' region comprising the mature protein sequence), respectively (Fig. 1a). In addition, the cognate *att* site partners show high recombination frequencies without the presence of *bla*-resistant background from the fragmented *bla* gene. These systems are extremely useful due to their ability to directly select for recombination through resistance to β -lactam antibiotics. They also have the potential to be used within synthetic biology frameworks for constructing and precisely inserting large genetic assemblies, making them useful additions to the molecular biology toolbox for both synthetic and molecular applications.

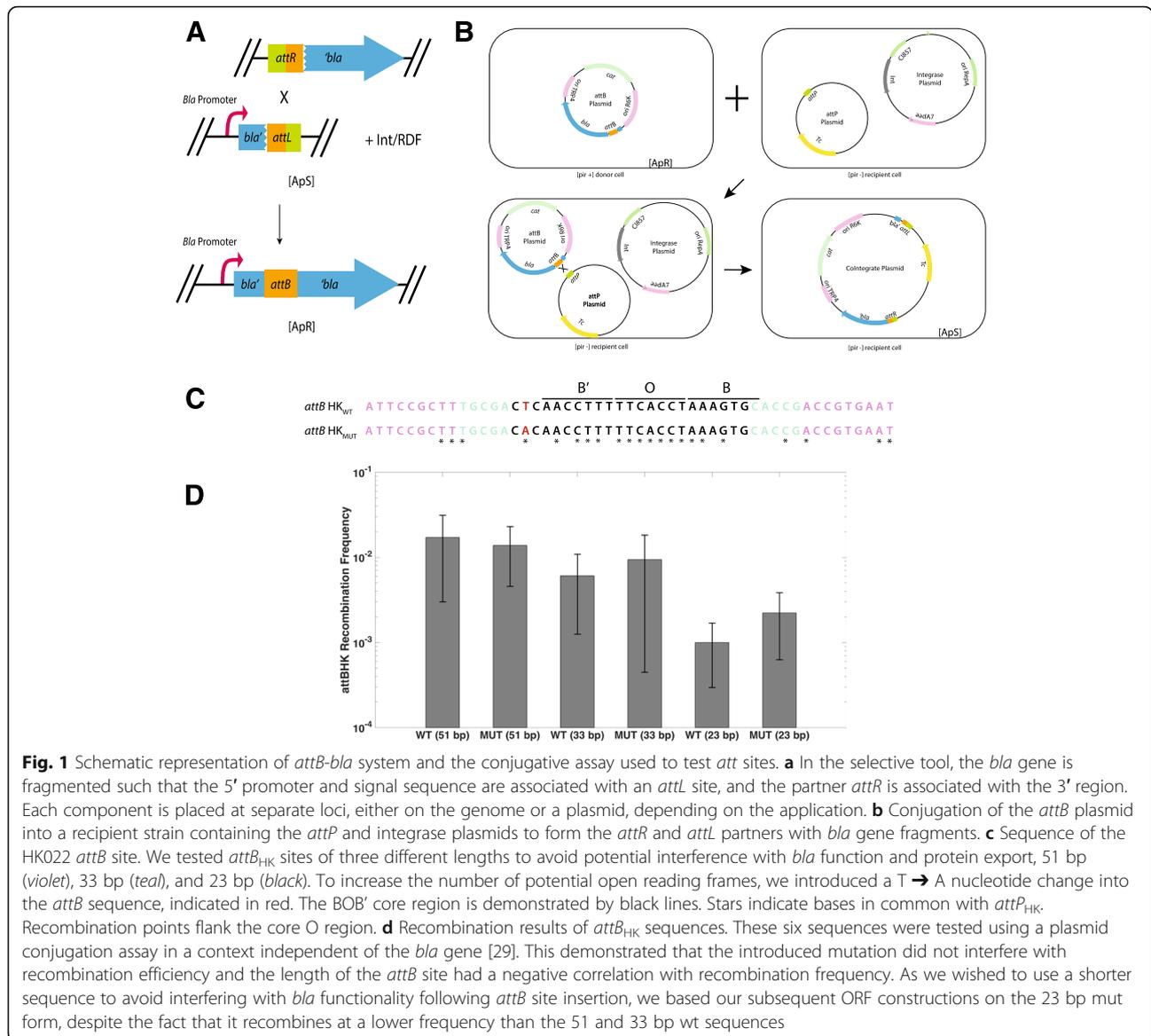
Results

In-frame insertion of *attB*_{HK} sites within the β -lactamase gene

The β -lactamase gene has a 23-amino acid (aa) signal peptide sequence for protein transmembrane transport that is cleaved during protein maturation [26]. We inserted the *attB* sequences in frame into the junction between the encoded signal sequence and the mature protein (Fig. 1a), as this region is tolerant to sequence insertions [19]. To avoid interfering with the β -lactamase coding sequence we took into account *attB* length and the amino acid sequence of the translated *att* sequence, so as to avoid frameshift or stop codon insertion.

Recombination frequency in *attB*_{HK} sites decreases with size

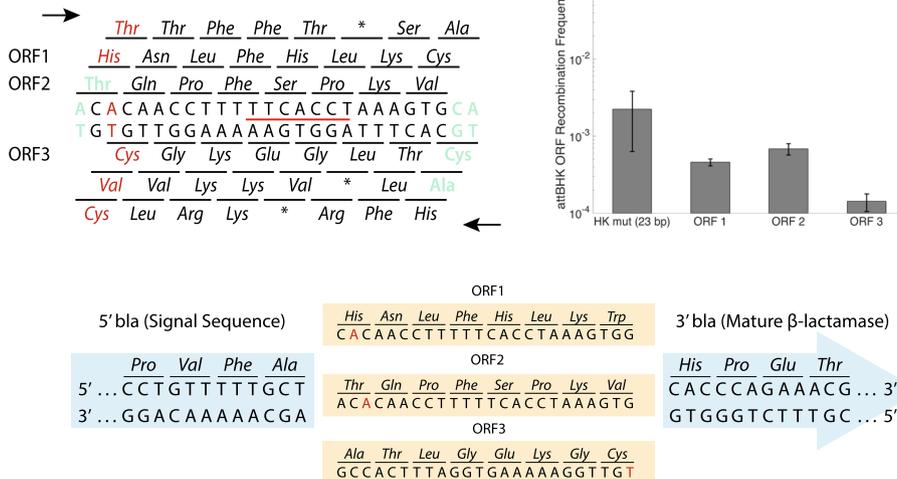
The *attB*_{HK} site comprises a 7 bp core, or overlap, (O) region where strand exchange occurs, and flanking B and B' arm regions of 7 bp each that are recognized by Int monomers to form a synaptic complex, although sites shorter than this 21 bp have been shown to be functional but with low efficiency [27]. To allow recombination, the O region between *attB* and *attP* must



perfectly overlap, and the arm regions must share similarity. Flanking the core minimal region, there are homologous nucleotides that may play an additional role in recombination efficiency [10, 28]. Insertion of *attB* into *bla* extends the gene and could affect either transport through the membrane or mature enzyme function. It is therefore necessary to test different open reading frames encoded by the *attB_{HK}* sequence to avoid unwanted interference with *bla*. The native *attB_{HK}* sequence encodes two open reading frames (ORFs) that do not have stop codons. As we wished to increase the potential sequences we could test within *bla*, we added a third potential ORF by mutating one bp just outside of the B' region (Fig. 1c; Fig. 2a) [8, 27]. We compared these “mutant” *attB* sites to the “wild-type” sites to ensure there was no loss of recombination frequency (Fig. 1d).

The 23, 33, and 51 bp “wild type” and “mutant” *attB_{HK}* sequences were tested by placing them on the conditionally replicating conjugative plasmid pSW23T containing an *oriT_{RP4}* for plasmid conjugation and *oriV_{R6KY}* for π protein replication dependence (Fig. 1b); [29]. As these plasmids do not replicate in bacterial strains not expressing the π protein, conjugation into non- π expressing DH5 α leads to plasmid loss unless *att* recombination occurs. The DH5 α recipient strain houses plasmid pHK11 Δ amp, which has the *attP_{HK}* partner site, and pHK-Int, which expresses the HK integrase under control of the temperature-dependent CI857 promoter [30]. Following conjugation, recombination frequency was calculated by measuring the ratio of recovered colonies (representing co-integrates) over the number of recipient colonies [31]. Recombination frequencies were

A HK attB Open Reading Frames



B ΦC31 attB Open Reading Frames

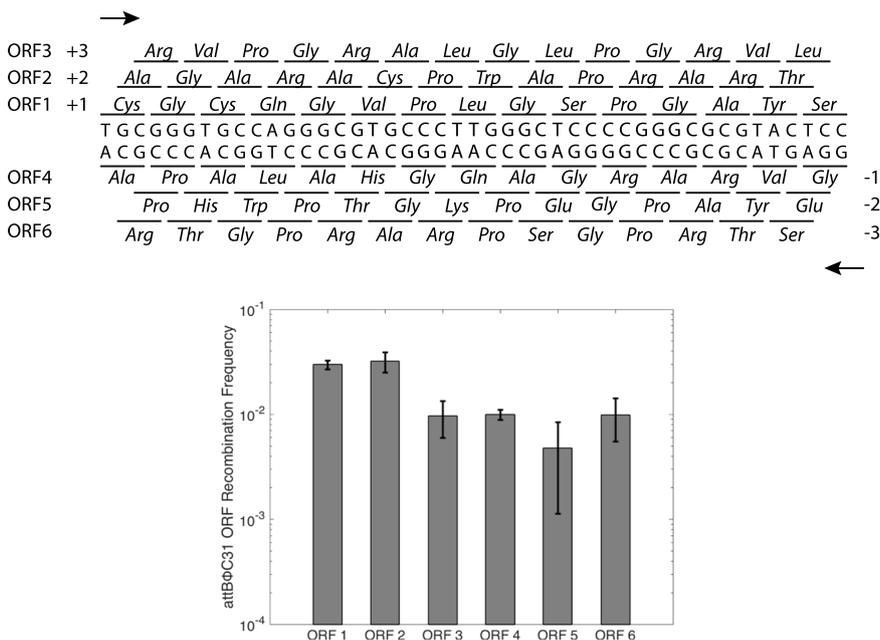


Fig. 2 Sequences and recombination frequencies of HK and ΦC31 attB sites. The three ORFs for HK and the six ORFs for ΦC31 were inserted into bla and tested using the conjugation assay as in Fig. 1a. The six open reading frames of the 23 bp attB HK site are shown. As in Fig. 1, black nucleotides represent the 23 bp HK sequence, with the corresponding amino acids also in black. The red nucleotide shows the base changed from the original attB, with the resulting amino acid changes also shown in red. Nucleotides and amino acids in teal represent sequences flanking the 23 bp site. Horizontal arrows indicate the direction of transcription, and asterisks indicate a stop codon. For both a and b, the sequence of recombination exchange is indicated by a horizontal red line. As described in the text, three open reading frames did not have a stop codon and were able to be tested for bla insertion. The recombination frequencies of these open reading frames compared to the 23 bp HK attB site are shown in the bar graph. The open reading frames are also shown in context of the bla sequence flanking the insertion site. Note that to keep the attB site in frame with bla, nucleotides were added to either the 5' or 3' end of the site, which changed the expected amino acid residue for ORFs 1 and 3 compared to the original attB. The background colors highlighting the sequence correspond to Fig. 1a. The recombination frequencies of the different ORFs were compared using 1-way ANOVA followed by a Tukey-Kramer test. Each of the HK ORF recombination frequencies are significantly different (p < 0.05). b. None of the six ΦC31 ORFs encode a stop codon. ORFs 1 and 2 recombine at a higher rate than ORFs 3–6 (p < 0.001)

similar between the different sites, with only a 10-fold reduction in recombination observed for the 23 bp sites compared to the larger attB sites (Fig. 1c). As we wished

to use a shorter sequence to avoid interfering with bla functionality following attB site insertion, we based our subsequent tests on the 23 bp attB_{HK} mutant form.

Placing a single nucleotide mutation in the 23 bp *attB*_{HK} site enables the use of three ORFs that would potentially allow *bla* function following their insertion into the gene (Fig. 2a). These ORFs were inserted separately into *bla* downstream of the signal sequence and cloned into pSW23T in a π + host. Following construction of these plasmids, we measured the ampicillin minimum inhibitory concentration (MIC) of each to test and measure *bla* function. All ORFs provided resistance to ampicillin at an MIC >256 μ g/ml (Table 1). Recombination frequencies were then tested using the conjugation assay as above. The three HK ORF constructions demonstrated a wide range of recombination efficiencies, with the ORF 2 construct recombining at the highest level, and the ORF 3 construct recombining at the lowest (Fig. 2a). Thus, we used ORF 2 for the final construction of this tool.

Φ C31 *attB* x *attP* recombination is functional in all six ORFs

We designed *attB* _{Φ C31} sites for all six possible ORFs maintaining at least the minimal sequence necessary for recombination [32] and inserted them into *bla*. Ampicillin resistance and recombination frequency were determined as with the HK system. Five of six ORFs were found to provide MICs greater than 256 μ g/ml, with the ORF 5 construction being the only sequence to interfere with β -lactamase function (MIC = 6 μ g/ml - Table 1). Φ C31 pSW23T-*bla* plasmids were conjugated into a DH5 α strain harboring plasmids p Φ C31-Int and p Φ C31-attP. All six ORFs were able to recombine successfully, with ORF constructions 1 and 2 recombining at a higher rate, on the order of 10^{-2} , than ORFs 3–6, which recombined at an average rate of 10^{-3} (Fig. 2b). We found this difference to be significant using a 1-way ANOVA ($p < 0.001$) followed by a post-hoc Tukey-Kramer test ($p < 0.001$). Additionally, all six Φ C31 ORF constructions recombined at a higher rate than HK ORFs 1–3 (Fig. 2).

Table 1 Minimum inhibitory concentration (MIC) of *attB*_{HK} and *attB* _{Φ C31} ORFs inserted into β -lactamase

| Ampicillin Resistance of <i>bla-attB</i> ORFs | MIC (μ g/ml) |
|---|-------------------|
| HK022 ORF1 | > 256 |
| HK022 ORF2 | > 256 |
| HK022 ORF3 | > 256 |
| Φ C31 ORF1 | > 256 |
| Φ C31 ORF2 | > 256 |
| Φ C31 ORF3 | > 256 |
| Φ C31 ORF4 | > 256 |
| Φ C31 ORF5 | 6 |
| Φ C31 ORF6 | > 256 |

Discussion

In this study, we describe the construction of two site-specific recombination tools useful for DNA manipulation applications. The utility of this *attB-bla* tool is based on its incorporation of the widely used HK and Φ C31 recombination systems. In the case of HK, the removal of sequences flanking the BOB' core region reduced *attB* x *attP* recombination. This reduction could be due to the removal of bases outside of the *attB* core that have homology with the *attP* sequence, which may act to stabilize the *attB/attP* complex. However, obtaining the highest possible recombination frequency was not critical for the design of this system, as our main concern was β -lactamase function following insertion of the *att* sites into the *bla* coding frame.

In directly comparing the two systems, the Φ C31 site appears to recombine at a similar frequency to the 51 bp HK sites and the 23 bp HK ORFs incorporated into *bla* have a lower recombination frequency (Fig. 2). This decrease is likely due to the reduction of size of the *attB*_{HK} site, as the recombination frequencies for the smaller HK site tested independently of *bla* insertion are not different from the frequencies obtained when they are embedded in *bla* (Fig. 1). Reported differences between recombination systems in the literature may result from differences in protocols and practices. A recent review of Φ C31 found a wide range of reported recombination frequencies for this recombinase [33]. To our knowledge, the only information comparing HK and Φ C31 recombination frequencies reports HK recombining at a higher frequency than Φ C31 [34]. However, this study used a clonetelegration technique where constructs were recombined into native *att* sites on either the *E. coli* chromosome for *attP*_{HK} or *Salmonella typhimurium* for *attP* _{Φ C31}.

While testing *bla* expression with inserted ORFs, we observed that Φ C31 ORF 5 interfered with *bla* expression, while Φ C31 recombination was not affected (Table 1, Fig. 2b). The *bla* gene used for our system originates from pBR322 and belongs to the TEM-1 class of β -lactamases. The signal sequence is recognized by the Sec export pathway that transports unfolded proteins across the cytoplasmic membrane [26, 35]. DNA secondary structures could be a source of transcription interference, as ORF 5 forms a 30 bp hairpin (ΔG at 37 °C = -9.09 kcal/mol). However, hairpins are formed in all 6 ORFs at similar ΔG , making it unlikely that this factor alone prevents *bla* expression. At the translation level, the overall charge of the first 5 amino acids following the signal sequence can influence cleavage and cross-membrane transport, as they generally have an overall negative charge [36]. For ORF 5, the overall negative charge of this region is +2. Again, however, this is unlikely to explain the loss of *bla* expression, as only ORF 1 has an overall negative charge, at -1. The

amino acids in the 1 and 2 position after the cleavage site can also influence protein function [37, 38]. For ORF 5, the first two amino acids are glycine and serine. Analysis of 307 proteins from the SPdb database [39] found that in Gram-negative bacteria, glycine occurs in the 1st position in 6.19% of proteins, and serine appears in the 2nd position in 5.54% of proteins. [40]. Additionally, two of the 307 Gram-negative proteins analyzed in this study begin with glycine-serine. Thus it is unlikely that the first two residues of the ORF 5 sequence alone interfere with protein transport. More experimental and analytical work is needed to determine the source of *bla* expression interference.

The high tolerance of *bla* to in-frame DNA sequence insertion downstream of the *bla* promoter and leader peptide sequence allows for further modifications of this system through insertion of potentially large ORFs. This approach has already been proposed as an “ORF-trap” to capture DNA encoding protein fragments [41]. Indeed, large ORFs in frame with *bla* may not greatly reduce β -lactamase function, although export to the periplasm can be inhibited [42]. Additionally, as *attB* and *attP* site reactivity can be modified through mutations to their respective core sequences, variable non-reacting “synthetic” *att* sites can be designed for sequential introduction into the bacterial chromosome [43].

Integration of exogenous DNA sequences into genomes by SSRs generally involves the recombination of an *attP* site on the inserted sequence with an endogenous chromosomal *attB* or pseudo-*attB* site [1]. The use of genome editing technologies allows the insertion of recombination sites that differ from native sites in location and sequence. Native *att* sites may be located in undesirable regions of the genome, for example, in an active gene locus, or a locus subject to silencing. Additionally, dosing effects can be observed in bacterial species dependent on a gene’s location in the chromosome [24]. Engineering *att* site recognition by Int proteins allows the creation of semi-synthetic partner sites [27, 43]. This would avoid recombination with other native *att* sites, and could allow rapid construction of synthetic gene networks. The addition of FRT sites flanking the *bla-attB* cassette would further allow for removal of the resistance selection

marker gene. Similarly, gene-editing technologies could allow the targeted insertion of *att* sites to serve as landing pads for insertion. In this way, the *bla'-attL* sequence from our system can be inserted into a genome, into which a sequence containing the partner *attR'-bla* can be inserted through *attL* x *attR* recombination. This framework has already been proposed for the construction and insertion of metabolic networks into eukaryotic cell lines [44]. Our system adds the advantage of avoiding marker expression until recombination, making it versatile for synthetic applications as well as genome-scale engineering.

Conclusions

We describe here the construction of new tools based on two different site-specific recombination systems, the tyrosine recombinase HK, and the serine recombinase Φ C31. Recombination for each system is reported based on the reconstitution of the *bla* ampicillin resistance gene, providing resistance to β -lactam antibiotics as a selective agent. Both HK-*bla* and Φ C31-*bla* are useful for selecting recombination events in a genomic context due to a high rate of recombination frequency, directionality based on the recombination proteins supplied *in trans*, and the ability to carry out *in vivo* genomic rearrangements. We have previously used this tool in our lab to carry out large-scale reorganization of the *V. cholerae* chromosomes to study the importance of chromosome size in multi-chromosomal bacteria [23], the relevance of genome position and chromosome location for gene dosage and its evolutionary importance [24], and the timing of *V. cholerae* chromosome replication [25]. The importance of these tools lie in their capacity to exist simultaneously in the cell at two separate loci without expression of the marker gene until expression of the recombination proteins is induced.

Methods

Bacterial strains and media

Bacterial strains used in this study are described in Table 2. All strains were grown in lysogeny broth (LB) medium at 30 °C, 37 °C, or 42 °C depending on plasmid temperature-sensitivity. Antibiotic and nutritional supplement concentrations were as follows: ampicillin (Ap):

Table 2 Bacterial strains used in this study

| <i>E. coli</i> | | |
|-----------------------------|--|-------------------------|
| Name | Genotype | Reference/Source |
| β 2163 | (F ⁻) RP4-2-Tc::Mu Δ dapA::(erm-pir) [Km ^R Em ^R] | [29] |
| π 1 | DH5a Δ thyA::(erm-pir116) [Em ^R] | [29] |
| MFDpir | MG1655 RP4-2-TC::[Mu1::aac(3)IV- Δ aphA- Δ nic35- Δ Mu2::zeo] Δ dapA::(erm-pir) Δ recA[Apra ^R Zeo ^R Erm ^R] | [47] |
| PGB-8557 | DH5a strain containing plasmids pHK Δ -Amp and pHK-Int [Tc ^R Sp ^R] | this study |
| PGB-E274 | DH5a strain containing plasmids p Φ C31-attP and p Φ C31-Int [Tc ^R Sp ^R] | this study |
| One Shot [®] Top10 | F- <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) Φ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74</i> <i>recA1</i> <i>araD139</i> Δ (<i>araleu</i>)7697 <i>galU</i> <i>galk</i> <i>rpsL</i> (StrR) <i>endA1</i> <i>nupG</i> | ThermoFisher Scientific |

100 µg/ml, carbenicillin (Carb): 100 µg/ml, kanamycin (Km): 25 µg/ml, chloramphenicol, (Cm): 25 µg/ml, tetracyclin (Tc): 15 µg/ml, spectinomycin (Sp): 100 µg/ml, erythromycin (Em): 20 µg/ml, with nutritional supplements diaminopimelic acid (DAP): 300 µM, and thymine (dT): 300 µM.

Cloning

Basic cloning steps were performed using the following tools and appropriate protocols: for DNA purification, a QIAquick PCR purification kit (QIAGEN) was used. Plasmid minipreps were performed using the GeneJET Plasmid Miniprep kit (Life Technologies). All PCR reactions for plasmid construction were performed using the Phusion High-Fidelity PCR Master Mix (Life Technologies), and all diagnostic PCR reactions were performed using DreamTaq DNA Polymerase (Life Technologies). Oligonucleotides were synthesized by Sigma-Aldrich and Eurofins Genomics. Oligonucleotides were phosphorylated by T4 polynucleotide kinase (NEB). DNA was sequenced by GATC Biotech and Eurofins Genomics.

Construction of plasmids

Insertion of *attB* sequences into pSW23T was performed by annealing phosphorylated oligos containing the respective *att* sequence with overhangs overlapping with *Bam*HI and *Eco*RI restriction sites, followed by cloning of these sequences into the pSW23T fragment. Insertion of *attB* sequences into pHK11-Amp was similarly performed. The various *attB* ORFs for both HK and ΦC31 were inserted into the β-lactamase (*bla*) by overlapping PCR, in which the 5' region of *bla* was amplified from pMP58 using oligos MV26 and the appropriate reverse *attB* oligo, and the 3' *bla* region amplified using a forward *attB* oligo and JB13. These products were gel purified and co-amplified using oligos MV26 – JB13 to form a DNA fragment containing *bla* with the inserted *attB*. This product was digested with *Eag*I and *Eco*RI and cloned into pSW23T and transformed into MFDpir. The pMP58 *bla* gene comes from pUC19.

To make plasmid pPhiC31-Int, we first deleted the *Xba*I site in pZJ7 (a kind gift of Jia Zhao and Sean Colloms) by digestion with *Spe*I-*Xba*I followed by religation to make plasmid pZJ7Δ*Xba*I. The ΦC31

Table 3 Plasmids used in this study

| Name | Description | Reference/Source |
|--------------------|--|------------------------|
| pSW23T | pSW23::oriTRP4; [Cm ^R]; oriVR6K | [29] |
| pSU38Δ | orip15A [Km ^R] | [48] |
| pHK-Int | pGB2ts::cl857-λ-P _R -HKInt, [Sp ^R] | [30] |
| pHK11-Amp | pLDR11::attP_HK, [Ap ^R ,Tc ^R] | [30] |
| pSC101 | pSC101ts, repA [Tc ^R] | [49] |
| pUC19 | oriColE1, lacZα [Ap ^R] | [50] |
| pBAD43 | oripSC101, PBAD::MCS,[Sp ^R] | [51] |
| pHK11Δamp | pHK11-Amp::attP_HK,ΔAmp, [Tc ^R] | this study |
| pMP96 | pSC101ts::cl857-λ-P _R -(HK _{Xis} -HK _{Int} λ _{Xis} -λ _{Int}), [Sp ^R] | [23] |
| pMP58 | pSC101ts::oriTRP4;repA, [Cm ^R ,Ap ^R] | this study |
| pMDG1 | pMP58;bla::attB_HK,[Ap ^R ,Cm ^R] | this study |
| pMDG2 | pSW23T::bla::attB_HK from pMDG1 | this study |
| pMDG3 | α/pSU38::attR_HK, [Ap ^R] | this study |
| pMDG4 | pSW23T::attL_HK, [Cm ^R] | this study |
| pMJM1 | pSW23T::attB_HKwt, [Cm ^R] | this study |
| pMJM2 | pSW23T::attL_HKmut, [Cm ^R] | this study |
| pMJM3 | pSW23T::attL_HK40, [Cm ^R] | this study |
| pMJM4 | pSW23T::attL_HK30, [Cm ^R] | this study |
| pJB6 | pSU38Δ::attR_HK-attL_λ, [Ap ^R] | this study |
| pJB7 | pSW23T::attR_HK-attL_λ, [Cm ^R] | this study |
| pJB8 | pBAD43::HK _{Xis} -HK _{Int} λ _{Xis} -λ _{Int} , [Sp ^R] | this study |
| pZJ7 | pBAD33::ΦC31Int, [Cm ^R] | J. Zhao and S. Colloms |
| pZJ7Δ <i>Xba</i> I | pZJ7 with <i>Spe</i> I – <i>Xba</i> I fragment deleted | this study |
| pPhiC31-Int | pGB2ts::cl857-λ-PR-ΦC31Int, [Sp ^R] | this study |
| pPhiC31-attP | pHK11Δamp::attP_ΦC31, [Tc ^R] | this study |

integrase gene was amplified using oligos PhiC31 IntF and PhiC31 IntR. The pHK-Int backbone was amplified using oligos JB485 and JB486. These oligos produce DNA fragments with overlapping ends, which were then joined by Gibson assembly [45]. Plasmids used in this study are listed in Table 3 and oligonucleotides in Table 4.

Recombination assay

Recombination frequencies were tested by performing a conjugation assay in which the plasmid pSW23T containing the *oriT_{RP4}* transfer region and *oriV_{R6K}* π -controlled replication origin were transferred from the $\pi+$ /DAP- donor strain MFDpir to a recipient strain

Table 4 Oligonucleotides used in this study

| Oligonucleotide | Sequence 5' – 3' |
|---------------------|--|
| PhiC31 Int F | ATGTAATACTAGAGAAGAGGATCAGAAATGGACACGTACGCGGTGC |
| PhiC31 Int R | CAAGCTGCATGCCTGCAGG |
| JB13 | AGCGGGTTCCTTCTCACTG |
| JB485 | TCTTCTAGATTAGTACATGCAACCA |
| JB486 | CGACTAGAGTCGACCTGCAGCCAAGCTTAGTAAAGCCCTC |
| MV26 | ACGGCTGACATGGGAATTGC |
| MV143 | CCTCTACGTGCCGATCAACGTCTC |
| MV145 | GCTGGTGATCCGCTTTCGCGACTCAACCTTTTTACCTAAAGTGACCCGACCGTGA |
| MV146 | ACATCAGCGATCACCTGGCAGAC |
| attBHKwtERI | AATTCGCTTTGCGACTCAACCTTTTTACCTAAAGTGACCCGACCGTGAATG |
| attBHKwtREV | GATCCATTACCGTCCGGTGCACCTTTAGGTGAAAAAGGTTGAGTCGAAAAGCGG |
| attBHKmutERI | AATTCGCTTTGCGACACAACCTTTTTACCTAAAGTGACCCGACCGTGAATG |
| attBHKmutREV | GATCCATTACCGTCCGGTGCACCTTTAGGTGAAAAAGGTTGTGTCGAAAAGCGG |
| 40wtERI | AATTCGCGACTCAACCTTTTTACCTAAAGTGACCCG |
| 40wtREV | GATCCCGGTGCACCTTTAGGTGAAAAAGGTTGAGTCGCGAG |
| 40attBHKmutERI | AATTCGCGACACAACCTTTTTACCTAAAGTGACCCG |
| 40attBHKmutREV | GATCCCGGTGCACCTTTAGGTGAAAAAGGTTGTGTCGCGAG |
| 30wtERI | AATTCACAACCTTTTTACCTAAAGTG |
| 30wtREV | GATCCACTTTAGGTGAAAAAGGTTGAG |
| 30attBHKmutERI | AATTCACAACCTTTTTACCTAAAGTG |
| 30attBHKmutREV | GATCCACTTTAGGTGAAAAAGGTTGTG |
| 30attBHKamp2ORF1min | TTTGCTCACAACTTTTTACCTAAAGTGCCACCCAGAAACGCTGGTAAAAGTAAAAGATGCTGAAGATCAGTT |
| 30attBHKamp1ORF1min | CTTTCACCAGCGTTTCTGGGTGCCACTTTAGGTGAAAAAGGTTGTGAGCAAAAACAGGAAGGCAAAATGCCGC |
| 30attBHKamp2ORF2min | TTTGCTACACAACCTTTTTACCTAAAGTGCCACCCAGAAACGCTGGTAAAAGTAAAAGATGCTGAAGATCAGTT |
| 30attBHKamp1ORF2min | CTTTCACCAGCGTTTCTGGGTGCACCTTTAGGTGAAAAAGGTTGTGTAGCAAAAACAGGAAGGCAAAATGCCGC |
| 30attBHKamp2ORF3min | TTTGCTGCCACTTTAGGTGAAAAAGGTTGTACCCAGAAACGCTGGTAAAAGTAAAAGATGCTGAAGATCAGTT |
| 30attBHKamp1ORF3min | CTTTCACCAGCGTTTCTGGGTGACAACCTTTTTACCTAAAGTGCCAGCAAAAACAGGAAGGCAAAATGCCGC |
| phiC31 ORF1 F | TTTGCTGCGGGTGCCAGGGCGTGCCCTTGGGCTCCCCGGGCGCGTACTCCACCCAGAAACGCTGGTAAAAG |
| phiC31 ORF2 F | TTTGCTGCGGGTGCCAGGGCGTGCCCTTGGGCTCCCCGGGCGCGTACTCCACCCAGAAACGCTGGTAAAAG |
| phiC31 ORF3 F | TTTGCTGCGGGTGCCAGGGCGTGCCCTTGGGCTCCCCGGGCGCGTACTCCACCCAGAAACGCTGGTAAAAG |
| phiC31 ORF4 F | TTTGCTGGAGTACGCGCCCGGGGAGCCCAAGGGCACGCCCTGGCACCCGACCCAGAAACGCTGGTAAAAG |
| phiC31 ORF5 F | TTTGCTGGGAGTACGCGCCCGGGGAGCCCAAGGGCACGCCCTGGCACCCGACCCAGAAACGCTGGTAAAAG |
| phiC31 ORF6 F | TTTGCTGGGAGTACGCGCCCGGGGAGCCCAAGGGCACGCCCTGGCACCCGACCCAGAAACGCTGGTAAAAG |
| phiC31 ORF1 R | CTTTCACCAGCGTTTCTGGGTGGGAGTACGCGCCCGGGGAGCCCAAGGGCACGCCCTGGCACCCGCAAGCAAAAACAGGAAGGCAAAATG |
| phiC31 ORF2 R | CTTTCACCAGCGTTTCTGGGTGGGAGTACGCGCCCGGGGAGCCCAAGGGCACGCCCTGGCACCCGCAAGCAAAAACAGGAAGGCAAAATG |
| phiC31 ORF3 R | CTTTCACCAGCGTTTCTGGGTGGGAGTACGCGCCCGGGGAGCCCAAGGGCACGCCCTGGCACCCGCAAGCAAAAACAGGAAGGCAAAATG |
| phiC31 ORF4 R | CTTTCACCAGCGTTTCTGGGTGGGAGTACGCGCCCGGGGAGCCCAAGGGCACGCCCTGGCACCCGCAAGCAAAAACAGGAAGGCAAAATG |
| phiC31 ORF5 R | CTTTCACCAGCGTTTCTGGGTGGGAGTACGCGCCCGGGGAGCCCAAGGGCACGCCCTGGCACCCGCAAGCAAAAACAGGAAGGCAAAATG |
| phiC31 ORF6 R | CTTTCACCAGCGTTTCTGGGTGGGAGTACGCGCCCGGGGAGCCCAAGGGCACGCCCTGGCACCCGCAAGCAAAAACAGGAAGGCAAAATG |

containing an *attP* plasmid and a helper plasmid expressing the appropriate integrase gene under control of the temperature-sensitive CI857 promoter. Prior to conjugation, strains were diluted 1/100 from an overnight starter culture and grown to $OD_{600} = 0.3$. Conjugations were performed by two techniques: for the *attB* HK_{WT/MUT} strains, 0.5 ml of donor was mixed with 4.5 ml of recipient and applied to a 0.45 μ m filter (Millipore) by vacuum-filtration through a glass column. The *attB* ORF insertions into *bla* were performed by mixing 0.2 ml of donor with 1.8 ml of recipient, and following centrifugation at 6000 RPM for 5 min, ~1.8 ml of supernatant was removed, the pellet resuspended in the remaining liquid media, and similarly placed onto a 0.45 μ m filter. For both techniques, the filters were then incubated on an LB-DAP plate for approx. 16 h prior to resuspension and plating. Recombinants were recovered by selecting for Cm resistance in DAP-free media, and recombination frequencies were measured as the ratio of recovered recombinants over donor CFUs. Each *att* site was tested three times.

Minimum inhibitory concentration (MIC)

The MICs of *E. coli* strains containing plasmids with either *attB* inserted into *bla*, or *bla* fragments associated with *attL* and *attR* were performed by plating and aspirating 2 ml of a 1/100 dilution of an overnight culture onto an LB/DAP agar petri dish. An Etest (bioMérieux) ampicillin antibiotic strip was placed onto the plate and incubated overnight at 37 °C, and the level of antibiotic resistance was scored the following day.

Data analysis

Recombination frequencies were analyzed for statistical significance using MATLAB software (The MathWorks, Inc., Natick, MA). 1 and 2-way analysis of variance (ANOVA) tests were performed using the *anova1* and *anova2* functions. Tukey-Kramer post-hoc tests were performed using the *multcompare* function.

DNA folding and protein structure analysis

Secondary DNA structures were analyzed using the *mfold* software [46]. Protein residue charges were calculated by counting negatively charged residues Asp and Glu as -1 , and positively charged His, Lys, and Arg as $+1$.

Abbreviations

Bla: β -lactamase; HK022: HK; Int: Integrase; MIC: Minimum inhibitory concentration; O region: Overlap region; ORF: Open reading frame; RDF: Recombination directionality factor; S-rec: Serine recombinase; SSR: Site-specific recombinase; Y-rec: Tyrosine recombinase

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Availability of data and materials

Raw data are available from the corresponding author on request.

Authors' contributions

MJB, MEV, and DM conceived and designed the experiments; MJB and MDG carried out the experiments; MJB, MDG, MEV, and DM analyzed the data; MJB and DM wrote the paper. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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