Video Article

Site-specific Bacterial Chromosome Engineering: ΦC31 Integrase Mediated Cassette Exchange (IMCE)

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Abstract

The bacterial chromosome may be used to stably maintain foreign DNA in the mega-base range[1]. Integration into the chromosome circumvents issues such as plasmid replication, plasmid stability, plasmid incompatibility, and plasmid copy number variance. This method uses the site-specific integrase from the *Streptomyces* phage (Φ) C31[2,3]. The ΦC31 integrase catalyzes a direct recombination between two specific DNA sites: *attB* and *attP* (34 and 39 bp, respectively)[4]. This recombination is stable and does not revert[5]. A "landing pad" (LP) sequence consisting of a spectinomycin- resistance gene, *aadA* (*SpR*), and the *E. coli* ß-glucuronidase gene (*uidA*) flanked by *attP* sites has been integrated into the chromosomes of *Sinorhizobium meliloti, Ochrobactrum anthropi,* and *Agrobacterium tumefaciens* in an intergenic region, the *ampC* locus, and the *tetA* locus, respectively. *S. meliloti* is used in this protocol. Mobilizable donor vectors containing *attB* sites flanking a stuffer red fluorescent protein (*rfp)* gene and an antibiotic resistance gene have also been constructed. In this example the gentamicin resistant plasmid pJH110 is used. The *rfp* gene[6] may be replaced with a desired construct using *Sph*I and *Pst*I. Alternatively a synthetic construct flanked by *attB* sites may be sub-cloned into a mobilizable vector such as pK19mob[7]. The expression of the ΦC31 integrase gene (cloned from pHS62[8]) is driven by the *lac* promoter, on a mobilizable broad host range plasmid pRK7813[9].

A tetraparental mating protocol is used to transfer the donor cassette into the LP strain thereby replacing the markers in the LP sequence with the donor cassette. These cells are trans-integrants. Trans-integrants are formed with a typical efficiency of 0.5%. Trans-integrants are typically found within the first 500-1,000 colonies screened by antibiotic sensitivity or blue-white screening using 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid (X-gluc). This protocol contains the mating and selection procedures for creating and isolating trans-integrants.

Video Link

The video component of this article can be found at https://www.jove.com/video/3698/

Protocol

1. Production of Culture

1. Prepare sterile liquid media: TY[10] (5 g/l tryptone, 3 g/l yeast extract, 0.44 g/l calcium chloride dehydrate), and LB[11] (10 g/l tryptone, 5 g/l yeast extract, 5 g/ sodium chloride, pH 7).
2. Inoculate from a single colony: SmUW227 (*S. meliloti* LP-strain: construction will be described elsewhere, strain construction details available upon request) () into 5 ml of TY media with 50 µg/ml spectinomycin. Inoculate the following strains into 5 ml of LB liquid media, supplementing with the given antibiotic: *E. coli* DH5α[12] containing pJC2, the integrase expression plasmid, with 10 µg/ml tetracycline; *E. coli* MT616[13], the mobilizer, with 10 µg/ml chloramphenicol; and *E. coli* DH5α containing pJH110, the donor cassette plasmid, with 5 µg/ml gentamicin.
3. Incubate *E. coli* strains overnight at 37 °C with constant shaking. Incubate *S. meliloti* strain at 30 °C for two days with shaking. It is possible to obtain a *S. meliloti* culture overnight with a larger inoculum (1:500 subculture of a saturated culture).

2. Culture Preparation and Mixing

1. Wash 1.5 ml of culture for each strain by collecting the cell pellet by centrifugation at 17,000 x g for 30 seconds and resuspending in 1 ml sterile 0.85% NaCl; repeat once.
2. Resuspend pellet in 100 µl of sterile 0.85% NaCl.
3. Individually spot 10 µl of washed culture for each strain on a plain TY agar plate. These spots are control spots.
4. Add 40 µl of each strain excluding the *E. coli* DH5α[12] containing pJC2 in a sterile tube, mix, and spot 120 µl of this mixture on a plain TY agar plate. This spot is the no-integrase negative control.
5. Add 40 µl of each strain in a sterile tube, mix, and spot 160 µl of this mixture on a plain TY agar plate. This spot is the IMCE mating spot.
6. Allow spots to dry in a laminar flow hood.

7. Seal plates and incubate at 30 °C overnight.

3. Isolation of Trans-integrants

1. Streak control spots and IMCE spot onto TY agar plates supplemented with 200 µg/ml streptomycin (SmUW227 is based on Rm1021 which carries a mutation conferring high-level resistance to streptomycin[14]) and 30 µg/ml gentamicin.
2. For calculation of the IMCE efficiency, resuspend approximately ¼ of the IMCE mating spot in 500 µl sterile 0.85% NaCl. Make 10-fold serial dilutions to 10^{-7}. Plate dilutions 10^{-2} through 10^{-5} on TY agar plates supplemented with 200 µg/ml streptomycin and 30 µg/ml gentamicin, to select for trans-integrants. Plate dilutions 10^{-3} through 10^{-7} on TY agar plates supplemented with 200 µg/ml streptomycin, to select for both trans-integrants and potential recipients, i.e. total recipients.
3. For isolation of markerless trans-integrants resuspend approximately a quarter of the IMCE mating spot in 500 µl of sterile 0.85% NaCl. Make 10-fold serial dilutions to 10^{-6}. Plate dilutions 10^{-4} to 10^{-6} on four TY agar plates supplemented with 200 µg/ml streptomycin and 200 µg/ml X-gluc.
4. Incubate plates at 30 °C for 3 days.
5. View RFP trans-integrants under green light (525 nm) and through a red filter (>610 nm) [15].
6. Calculate the percent IMCE efficiency as CFU of trans-integrants compared to the CFU of total recipients. Approximately half of trans-integrants will have undergone true cassette exchange making them white and spectinomycin sensitive.
7. To find the markerless trans-integrants (where the donor vector does not contain Gm^r like in pJH110) screen for a white colony (an extended incubation period, 1-2 extra days at room temperature, will enhance the differentiation of colonies via X-gluc, this is necessary in *S. meliloti* SmUW227), confirm the colony's spectinomycin sensitivity by screening on an TY agar plate without antibiotics and an TY agar plate containing 100 µg/ml spectinomycin.

4. Representative Results

After three days of incubation on TY supplemented with streptomycin and gentamicin the control streaks should have no growth. The IMCE streak should have confluent growth on the head streak and many colonies on the second streak, as seen in Figure 2. The efficiency of trans-integration, expressed as the percentage of trans-integrants to total recipients, should be in the range of 0.5%. Approximately half of trans-integrants will be spectinomycin sensitive and white showing they have undergone true cassette exchange. Trans-integrants containing the tester *rfp* donor cassette from pJH110 should display discernable RFP fluorescence when viewed under green light (525 nm) and through a red filter (>610 nm) [15].

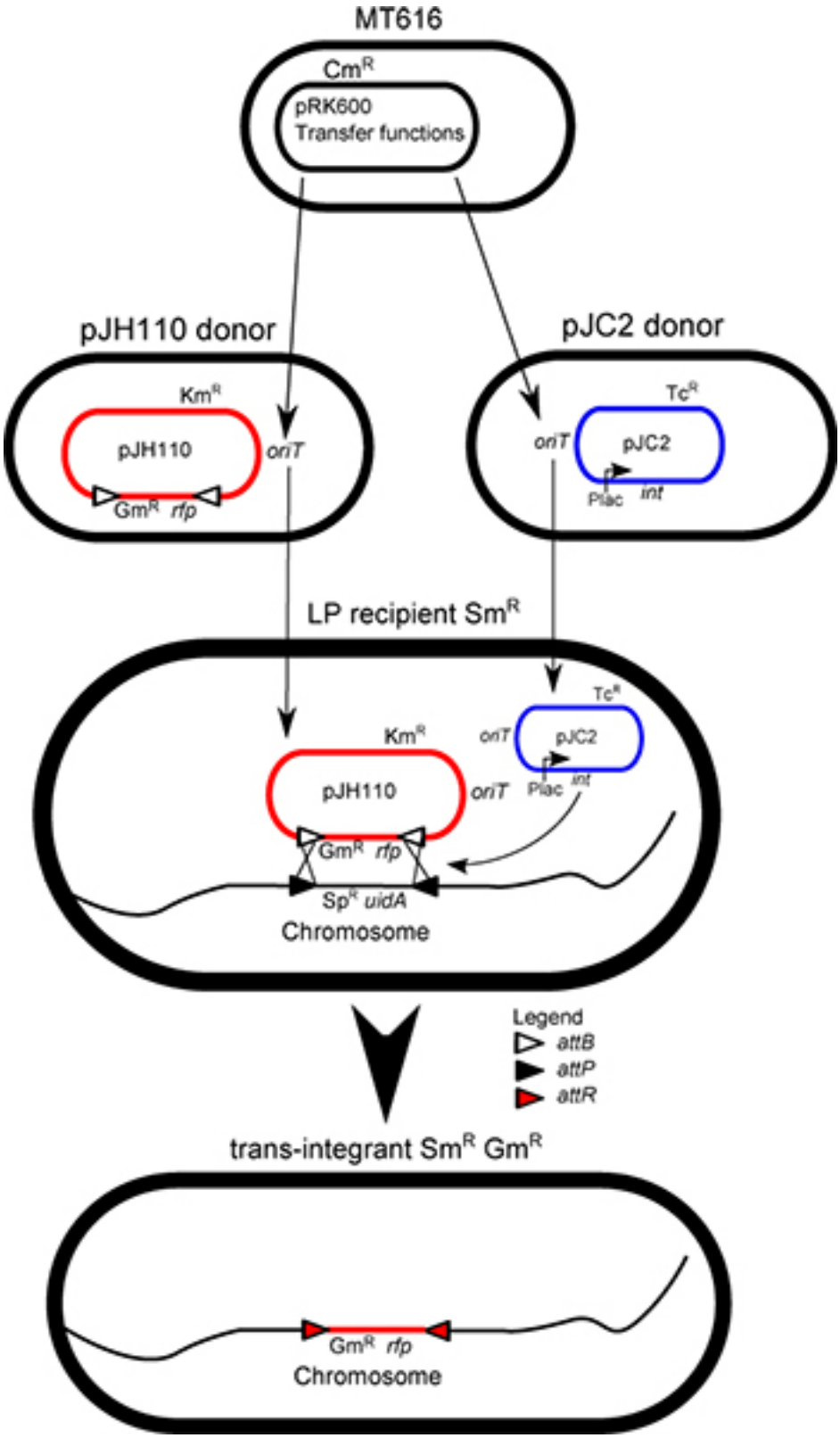

Figure 1. Conjugation mixture illustration: Aided by the expression of the transfer genes from pRK600, all of the plasmids are transferred randomly from cell to cell. This transfer results in the creation of trans-integrants in the mixture, through the LP-strain's acquisition of the two plasmids required for IMCE, the integrase (*int*) helper plasmid pJC2 and the donor plasmid pJH110. The donor cassette from the non-replicating donor plasmid (pJH110) is exchanged via ΦC31 integrase activity with the markers of the LP-cassette on the chromosome, resulting in the loss of the LP-markers (Sp^r and *uidA*) and the maintenance of the donor cassette (*rfp* and Gm^r) in the resulting trans-integrant.

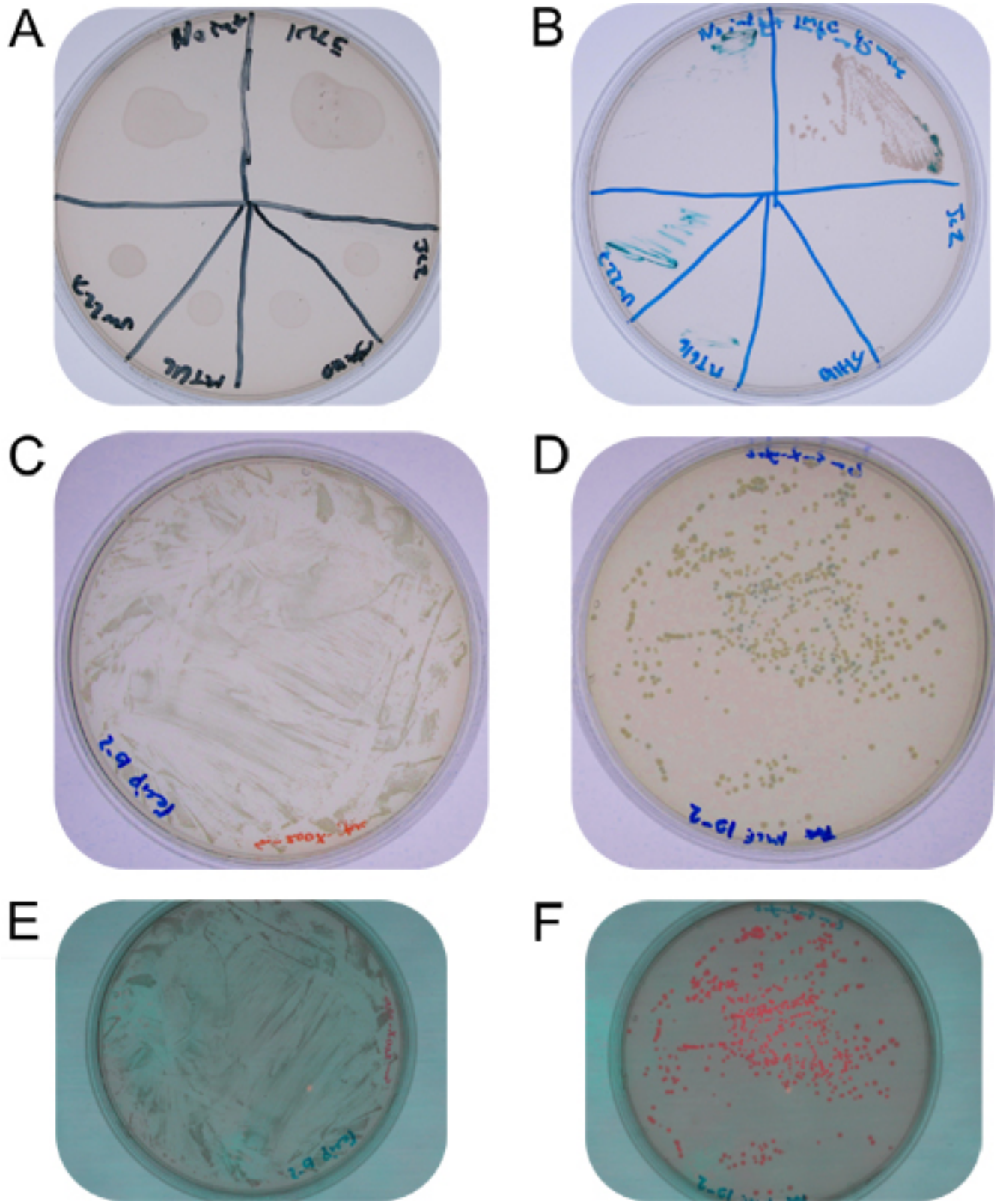

Figure 2. A: Mating spot plate on a non-selective TY plate showing dried cell mixtures on agar surface. **B**: Mating spots streaked on streptomycin-gentamicin-X-gluc plate, from top left clockwise no integrase control, IMCE into *S. meliloti*, *E. coli* DH5α containing pJC2 control, *E. coli* DH5a containing pJH110 control, *E. coli* MT616 control, and *S. meliloti* UW227 control. **C**: 10^{-2} dilution of mating spot resuspension on TY streptomycin-X-gluc agar. **D**: 10^{-2} dilution of mating spot resuspension on TY streptomycin-gentamicin-X-gluc agar (blue colonies are single recombinants, white colonies have undergone true cassette exchange). **E**: 10^{-2} dilution of mating spot resuspension on TY streptomycin-X-gluc agar showing lack of fluorescence. **F:** 10^{-2} dilution of mating spot resuspension on TY streptomycin-gentamicin-X-gluc agar showing two levels of fluorescence (brighter colonies correspond to blue colonies and have higher *rfp* expression presumably due to promoter read-though from vector

sequence, where colonies having undergone true-cassette exchange contain RFP with only its immediate promoter with no read-through from the lac promoter in the vector, which is absent.).

Discussion

The IMCE technique allows for the efficient integration of a single *attB* flanked DNA cassette into the LP-locus of a previously engineered strain. Once the desired construct is cloned in place of *rfp* to create the donor cassette, the technique does not require subsequent DNA purification and transformation, making it very robust. It is critical that appropriate growth controls are included, to be certain the antibiotic resistance is due to the creation of trans-integrants and not other factors.

IMCE produces trans-integrants at approximately 0.5% efficiency. In contrast, double crossover via homologous recombination occurs at a frequency of around 10^{-6}. Recombineering via λ-red[16], another phage based system, requires specific homology, and has varied effectiveness outside of *E. coli*[17]. Yet another option, Tn7 site specific recombination requires attTn7 sites[18], which are very rarely present outside of the γ-proteobacteria. The ΦC31 integrase has demonstrated efficient activity in disparate hosts[4,19]. Although the use of ΦC31 integrase requires prior engineering of a host, it is not limited to certain phyla. IMCE has the advantages of efficiency and modularity given that any donor cassette can potentially be integrated into any LP-strain. It is ideal for studies where a single clone library must be functionally screened in multiple hosts due to gene expression requirements or genetic complementation in single copy is desired.

The size of the construct to be integrated is limited by the size of DNA that can be successfully cloned into the donor vector via ligation. Donor vectors containing Gateway destinations[20] can be used, and are especially useful for large insert fosmid/cosmid libraries. The antibiotic resistance markers in the donor vectors may also be used to select for the assembly of overlapping DNA fragments post IMCE by crossing two different trans-integrants via transduction, or genomic electroporation[21]. This design consideration should allow for the assembly of very large constructs at the LP-locus in the LP-strain. This might be especially useful for incorporation of synthetic gene constructs for applications in metabolic engineering or synthetic biology.

Disclosures

No conflicts of interest declared.

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