Versatile Allelic Replacement and Self-excising Integrative Vectors for Plasmid Genome Mutation and Complementation

Dereje D. Gudeta and Steven L. Foley

Division of Microbiology, National Center for Toxicological Research, Jefferson, Arkansas

National Center for Toxicological Research, Jefferson, Arkansas.
Tel:870-543-7449
E-mail: Dereje.Gudeta@fda.hhs.gov
Steven.Foley@fda.hhs.gov

Introduction

- *Salmonella enterica* is a leading cause of foodborne illness in the United States (1) and around the globe (2).
- Efficient vector tools for mutation and complementation of plasmid borne genes in *Salmonella* and other Gram-negative pathogens are lacking.
- Most of the previously described allelic replacement vectors are not efficient for plasmid genome mutation as they rely either on plasmid derived counter selection toxins, that can be neutralized by a cognate antitoxin, or they depend on other strategies such *sacB*, *tetA* and *rpsL*, which have been proven to be less efficient(3, 4).
- The lambda red recombinase system is an excellent resource for chromosomal gene mutations and in some cases for plasmids, but it has limitations related to unintended flippase (FLP) mediated genetic "scar" formation or off target mutations(4, 5).
- Only few integrative vectors are available to study *Salmonella* pathogenesis and non of them express chromophore indicators requiring laborious screening and, moreover, excision of the vector's backbone relies on introduction of FLP expressing plasmid(6).
- Thus, the aim of this study was: 1) to develop an efficient allelic replacement vector expressing chromophore indicators with an inducible potent counter selection marker, 2) to develop chromophore expressing self-excising integrative vectors that does not require FLP expressing plasmid for its excision.

Material and Methods

Construction of allelic replacement vectors

- pKOV, a previously described vector (7, Fig. 1A), was used as a backbone for construction of the allelic replacement vectors.
- pDG1 was constructed by removing *sacB* gene using inverse PCR followed by replacing the stuffer(pink, Fig. 1A) with zeocin resistance marker (*zeoR*) and thermophilic beta-galactosidase encoding gene(*bgaB*) by cloning (Fig. 1B).
- pDG2 was constructed by replacing *bgaB* in pDG1 with mCherrey (Fig. 1C).
- pDG3 was constructed by inserting *tse2*, a toxin encoding gene derived from *Pseudomonas aeruginosa's* secretory system, under *Salmonella's* rhamnose inducible promoter(salPrha, Fig. 1D).

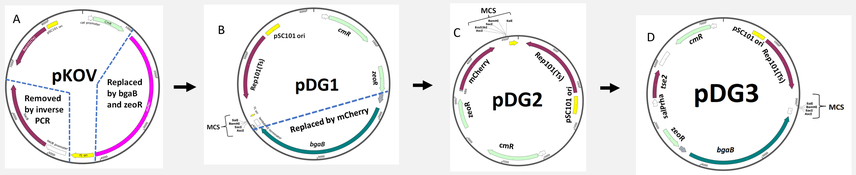


Fig. 1. Schematic representation of construction of allelic replacement vectors.

Allelic replacement procedure

- Transform cells with the recombinant plasmid and isolate colonies followed by growing a single colony at 30 °C (Fig. 2A and B).
- Dilute the 30 °C culture and grow at 42 °C (Fig. 2C) followed by plating and growth at 42 °C(Fig.2 D).
- Inoculate single colony from 42 °C plate in LB, grow at 30 °C in LB (Fig. 2E) and plate on x-gal (35 ug/mL) supplemented LB agar for pDG1 and on plain LB agar for pDG2 (Fig. 2 F).
- For pDG3; dilute the 30 °C culture in M9 salts (supplemented with 2 % rhamnose), and plate on x-gal and rhamnose (0.2%) supplemented M9 agar.
- Grow all the plates at 37 °C(no antibiotics) and screen white colonies to detect target mutants (Fig. 2G).

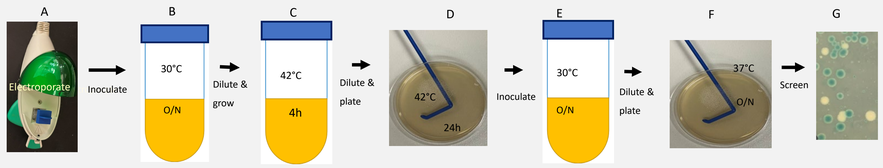
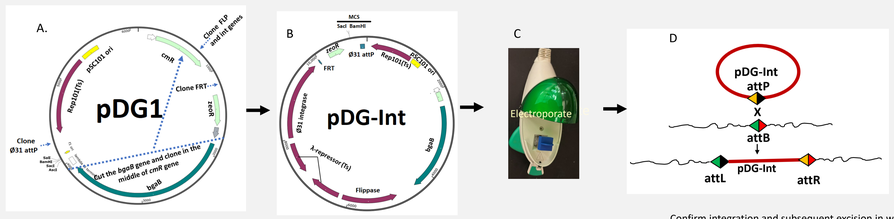


Fig. 2. Schematic representation of construction of allelic replacement procedure. Use 20 µg/mL zeocin for steps A-D and no antibiotic for steps E-G.

Construction of pDG-Int, an integrative vector, and integration to target attB site

- pDG1 was used as a backbone for construction of pDG-Int, an integrative vector(Fig. 3 A).
- *bgaB* was cut and re-inserted to the *cmR* gene on pDG1 followed by cloning of Ø31 *attB* site, FRT site, FLP and Ø31 integrase encoding genes to form pDG-Int (Fig. 3B).
- Clone target gene/operon to pDG-Int and transform to strain carrying Ø31 attB site to initiate integration and subsequent excision which, respectively, are catalyzed by the integrase and FLP encoded from the vector (Fig. 3D).



Construct pDG-Int by using cloning.

Transform pDG-Int to strain carrying target Ø31 attB site.

Confirm integration and subsequent excision in white colonies.

Fig. 3. Schematic representation of construction of pDG-Int(A-C) and its integration to target attB site(D).

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Results

Blue-white screening strategy can be used for mark-less gene deletion in *Salmonella* plasmids

- Aerobactin, a siderophore encoded by *iucABCD-iutA* operon(Fig. 4A) carried by IncFIB plasmids is thought to contributes to *Salmonella enterica* virulence.
- Here we used pDG1 to delete ~ 6 kb *iucABCD* operon (Fig. 4 A and B) and applied blue/white screening strategy to isolate colonies that lost the mutagenesis vector (Fig. 4. C).
- White colonies were screen by PCR using primers annealing ~ 0.6 kb fragment flanking the knock-out region to confirm deletion (Fig. 4 D).
- We further validated pDG1 by deleting part to of VirB4/D4 type IV secretary system, resolvase encoding genes carried by IncX4 plasmids and by creating translational/ transcriptional fusions in IncFIB plasmids.
- pDG2 was validated by replacing resolvase encoding gene in IncX4 plasmid with *bgaB* and by deleting N-terminal sequence of *lacZ* gene in *E. coli* which formed white colonies on x-gal supplemented media (Fig. 4E).

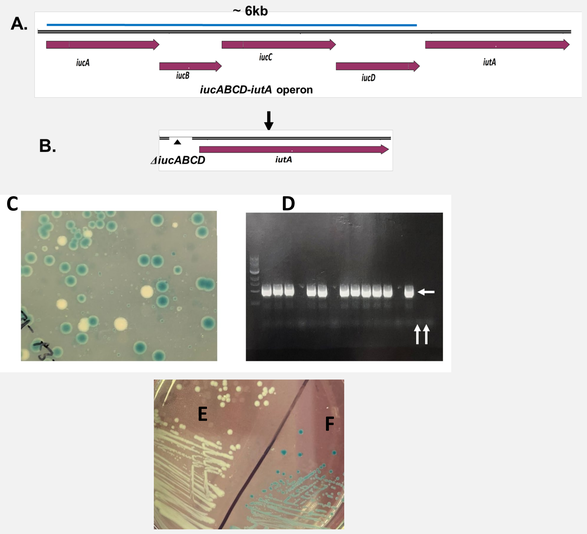

Fig. 4. Validating pDG1 and pDG2 vectors in deleting target genes. Schematic representation of *iucABCD-iutA* operon(A) and *iucABCD* operon deletion(B). Blue/white colonies following allelic replacement with pDG1(C) and verification of *iuABCD* operon deletion in the white colonies (D). The PCR bands shown by horizontal arrow are *iuABCD* deletion mutants while the upward facing arrows (left to right) indicate negative control and wild type, respectively(D). A PCR product could not be obtained for the wildtype because of its size which is ~7 kb. *E.coli lacz* (N-terminal) deletion mutant (E) and wild type *E. coli* (F) on x-gal and IPTG supplemented LB agar.

Expression of an inducible tse2 toxin from pDG3 vectors can serve as a potent counter selection marker

- Her we used pDG3 to delete *iucABCD-iutA* operon using tse2, *Pseudomonas aeruginosa*-derived type VII secretion system toxin, as a counter selection.
- No blue colony was detected on x-gal and rhamnose supplemented M9 agar (Fig. 5A) indicating that tes2 can serve as a potent counter selection indicating that mutagenesis by pDG3 can be done in absence of x-gal.
- 14 randomly picked colonies from M9 agar containing 0.2 % rhamnose(no x-gal) was checked for deletion of *iucABCD* as above (Fig. 5B).

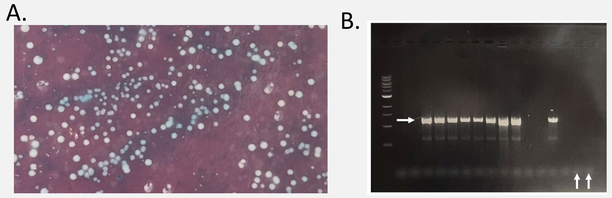

Fig. 5. Allelic replacement with pDG3 using tse2 as a counter selection. White colonies obtained on M9 media supplemented with 0.2 % rhamnoses plus x-gal (A) and verification of *iucABCD* deletion from non-x-gal supplemented M9 agar(B).

pDG-Int can integrate into the *Salmonella* chromosome and self-excise its backbone without the need of flippase expressing vector.

- Integration of pDG-Int to target attB site, which often forms light blues colonies, generates attL and attR recombination sites(Fig. 6A–F).
- Re-streaking of those blue colonies form mixture of blue and white colonies (Fig. 6F).
- Excision of the vector backbone can be confirmed in the white colonies using PCR (Fig. 6C).

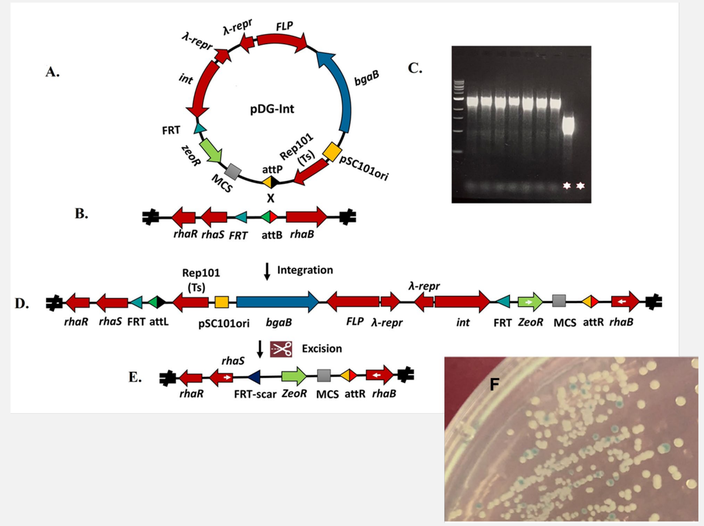

Fig. 6. Integration and excision of pDG-Int vector. pDG-Int vector map (A) and DGS49 with knocked-in chromosomal FRT and attB site(B), PCR confirming integration of pDG2-Int vector to DGS49 chromosome and subsequent excision(upper band in C). Positive and negative controls (left to right, respectively) are indicated by star. Schematic representation of chromosomal integration of pDG-Int2(D) and subsequent excision (E). Primer target site for integration/excision verification are depicted by white arrow. Blue/white colony formation following re-streaking of light blue colonies (F).

Conclusions

- **The allelic replacement vectors can be used for genetic deletions, point mutation and protein tagging.**
- **The integrative vector can be used for inserting relatively large fragments more efficiently than convectional knock-in vectors.**
- **The vectors express chromophores for detection of target gene modification or colony isolation, avoiding time consuming screening procedures.**
- **These vectors can serve as an invaluable tool for assessment of the role of plasmid/chromosomally encoded virulence factors for *Salmonella* pathogenesis.**

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Disclaimer

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