Simple blue-white screening strategy for *Salmonella* plasmid curing

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Introduction

- *Salmonella enterica* is a leading cause of foodborne illness in the United States (1) and around the globe (2).
- Despite the dissemination of multidrug resistance plasmids, including those carrying virulence genes in *Salmonella* spp., efficient plasmid curing tools are lacking.
- Plasmids partitioning systems such as *parA* and multimer resolution systems such resolvases are essential for even distribution of plasmids to daughter cells (3,4).
- Thus, inactivation of these systems can serve as a target for plasmid curing owing to their role in plasmid stability (3,4).
- However, plasmid curing strategies targeting these systems could be hindered by the host addiction system encoding stable toxins and relatively unstable antitoxins (5).
- As a result, in the case of plasmid loss, the relatively stable toxins would kill the bacteria through a process known as post-segregation killing (5)
- Here, we developed vector tools that can mutate the above systems while replenishing short-lived antitoxins.

Material and Methods

Approach-I: Inactivation of *parA*/resolvase encoding genes in the absence of antitoxin producing plasmid

- Construct a cocktail of antitoxin expressing temperature sensitive vector (pDG-AT) using Gipson assembly (Fig. 1A).
- Use pDG2 to knock-in a 2.3 kb *bgaB*, beta-galactosidase encoding gene, to target *parA*/resolvase encoding genes on a plasmid you wish to cure (Fig. 1B).
- Isolate blue colonies (Fig. 1C) and introduce pDG-AT to the blue colonies and screen white colonies, if any, to verify plasmid cure (Fig. 1D).

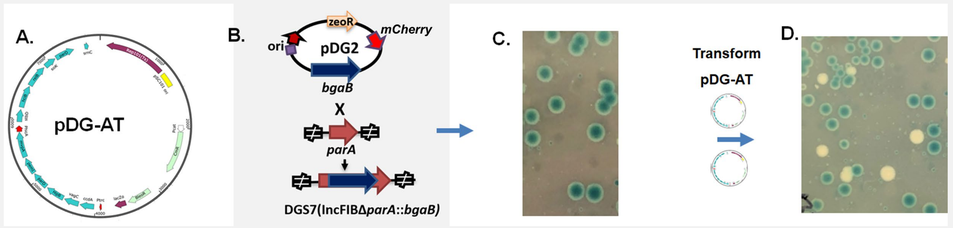

Fig. 1. Schematic representation of plasmid curing strategy-I. pDG-AT(A), generation of *parA* mutant (B), *parA* mutants expressing *bgaB* on x-gal (C) and mixture of blue and white colonies following introduction of pDG-AT to the *parA* mutants (D).

Approach-II: Inactivation of *parA*/resolvase encoding genes in the presence of antitoxin producing plasmid

- Construct a cocktail of antitoxin expressing vector with R6K$_\gamma$ origin of replication (pDG-Atπ) using Gipson assembly with pir protein under arabinose inducible promoter (ParaBAD, Fig.2 A).
- Transform pDG-Atπ to a strain carrying the plasmid you wish to cure and attempt to knock-in an N-terminal sequence of *bgaB* to target *parA* gene using pDG1 in the presence of pDG-Atπ (Fig. 2B).
- Screen white colonies for *parA* mutants (Fig. 2C).
- If there are no *parA* mutant after screening the white colonies from x-gal supplemented agar, screen the same white colonies for plasmid curing.
- If you isolated *parA* mutant in the white colonies, knock-in the C-terminal sequence of *bgaB* in-frame with the N-terminal sequence using pDG2 to generate functional *bgaB* (Fig. 2D).
- If all the white colonies are wild types, repeat the procedure.

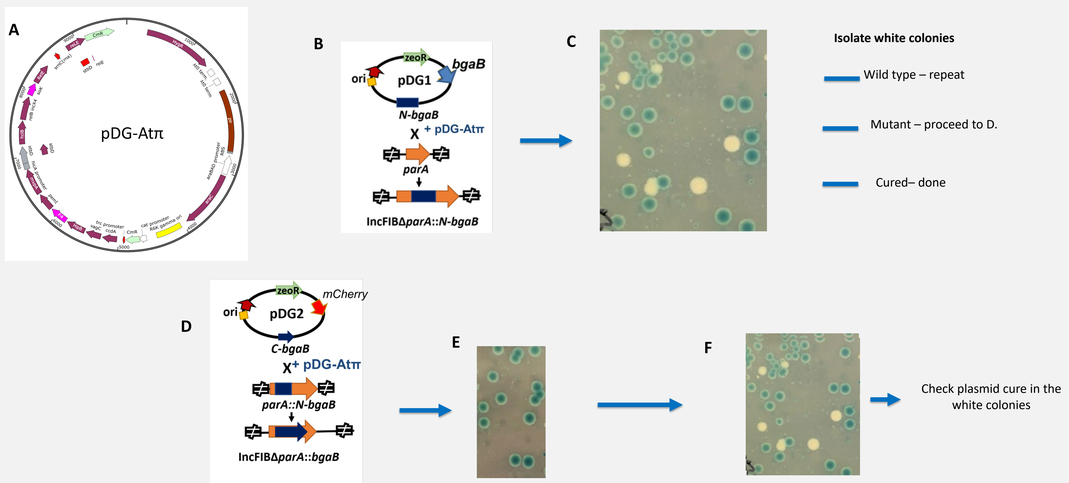

Fig. 2. Schematic representation of plasmid curing strategy-II. pDG-Atπ (A), generation of *parA* mutant in the presence of pDG-Atπ (B & C), generation of functional *bgaB* by knocking-in the C-terminal sequence of *bgaB* (D & E) and formation of mixture of blue/white colonies in the presence of pDG-Atπ (F).

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Results

Disrupting *parA*/resolvase encoding gene with *bgaB* can impair plasmid stability resulting in plasmid cure

- Resolvase encoding gene in IncX4 and *parA* in IncFIB plasmids were disrupted by *bgaB* and the *bgaB* disrupted mutants formed blue colonies.
- Introduction of pDG-AT to the blue colonies resulted in mixture blue and white colonies with white colonies representing those who lost *bgaB* carrying plasmid and hence plasmid cure(Fig. 3A-D).
- Plasmid cure was confirmed by PCR in the white colonies (Fig. 3B & D).
- However, isolation of *parA*/resolvase mutants with this method is non-reproducible presumably due to the size of *bgaB* (2.3 kb) and/or because of post-segregation killing.

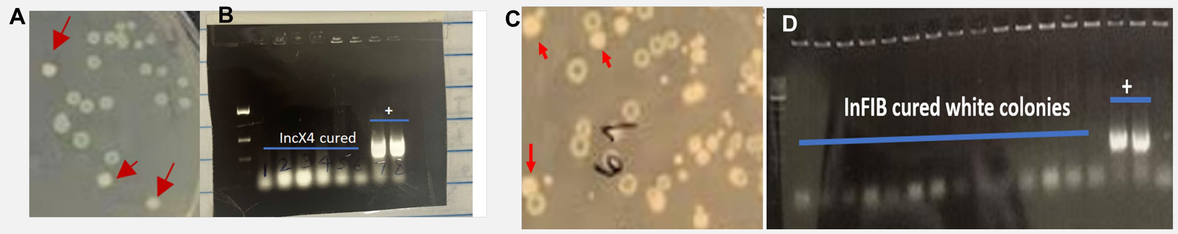

Fig. 3. IncX4 and IncFIB plasmid curing. A and C shows white colonies(arrow) obtained after introduction of pDG-AT to resolvase/*parA* mutants of IncX4 and IncFIB plasmids, respectively. B and D indicates PCR verification of IncX4 and IncFIB plasmid cure, respectively . The + sign shows positive control targeting their respective wild type plasmids.

pDG-Atπ can replicate in the absence of arabinose possibly due to pir protein "leaky" expression

- Isolation of *parA*/resolvase mutant by inserting 2.3 kb *bgaB* was extremely low and non-reproducible which leads us to knock-in the N-terminal *bgaB* (1.2 kb) to *parA* gene, in the presence of pDG-Atπ followed by an in-frame C-terminal *bgaB* knock-in to generate functional enzyme.
- Since we cannot use pDG1/pDG2 in the presence of pDG-AT, we re-constructed pDG-Atπ, antitoxin expressing plasmid, in a vector with R6K$_\gamma$ origin of replication that requires π-factor (encoded by *pir* gene) for its replication with pir gene under the control of arabinose inducible promoter (Fig. 4A), but we found that pDG-Atπ can replicate in the absence of arabinose suggesting leaky expression from the promoter (Fig. 4B).

Fig. 4. Schematic representation of araBAD promoter(A) and replication of pDG-Atπ in the absence of arabinos(B) and negative control, a vector with R6$_\gamma$ ori but without *pir* gene(C).

An attempt of *parA* mutation by inserting N-terminal *bgaB* in the presence of pDG-Atπ can be used to cure plasmid

- Here we attempted to isolate *parA* mutant IncFIB by inserting the N-terminal *bgaB* and screened 23 white colonies but none of them were *parA* mutant(mt) which should contain a band corresponding to the upper arrow in Fig. 5A, however, we could see 5 colonies retained the wild type (wt) plasmid (Fig. 5A, lower arrow).
- We then screened, PCR negative colonies using primer targeting InFIB plasmid and non of them gave positive PCR result indicating IcFIB plasmid cure(Fig. 5B).
- Similar results were obtained for IncX4 and IncA/C plasmid cure.
- We propose *parA* mutation can facilitate plasmid loss and those who lost the plasmid can be rescued by neutralizing the stable toxins using cognate antitoxin expressing pDG-Atπ.

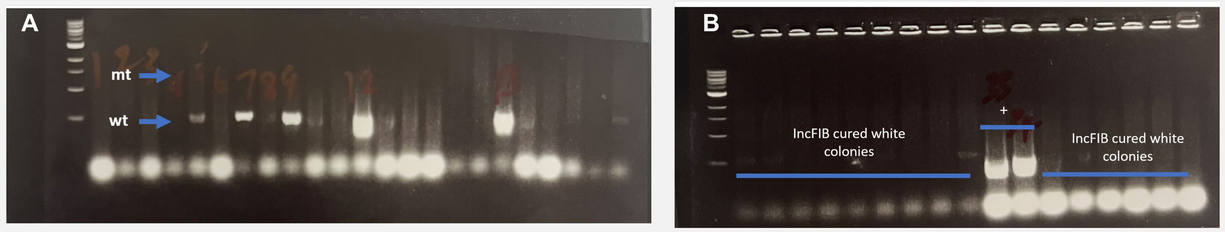

Fig. 5. IncFIB *parA* inactivation attempt and plasmid cure. A shows PCR performed to detect *parA* mutants in white colonies. The size of the PCR products for the mt and wt are shown by upper and lower arrows, respectively. B. Verification of IncFIB plasmid cure in the PCR negative white colonies checked for *parA* mutation in Fig. 5A. + sign indicates positive control targeting wt plasmid.

Conclusions

- **Isolation of viable strain carrying *parA*/resolvase mutant plasmid is extremely low.**
- **Plasmid partitioning and multimer resolution systems are an excellent target for plasmid curing strategy, but mutation of these systems should be performed in the presence of antitoxin expressing plasmid.**

References

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