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Mobile-CRISPRi protocol optimization for *Vibrionaceae*

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ABSTRACT Mobile clustered regularly interspaced palindromic repeats interference (Mobile-CRISPRi) is an established method for bacterial gene expression knockdown. The deactivated Cas9 protein and guide RNA are isopropyl β-D-1-thiogalactopyranoside inducible, and all components are integrated into the chromosome via Tn*7* transposition. Here, we optimized methods specific for applying Mobile-CRISPRi in multiple *Vibrio* species.

KEYWORDS CRISPR, CRISPRi, *Vibrio*, *Vibrio fischeri*, *Vibrio parahaemolyticus*, *Vibrio vulnificus*, *Vibrio campbellii*, *Vibrio cholerae*, regulation of gene expression

Vibrio bacteria are important model organisms for diverse fields of research. Their use in research has predicated the demand for increasingly creative genetic tools. While many *Vibrio* species are naturally competent (1), other models remain more genetically recalcitrant. Mobile clustered regularly interspaced palindromic repeats interference (Mobile-CRISPRi) was initially demonstrated in non-model *Vibrio caseii*, and its potential made it a target for further optimization in model Vibrio species (2, 3). Herein, we specify the optimized workflow for Mobile-CRISPRi in *Vibrio*.

Mobile-CRISPRi utilizes inducible expression of deactivated Cas9 and a single-guide RNA (sgRNA), both expressed through isopropyl β-D-1-thiogalactopyranoside-dependent induction, to facilitate conditional knockdown of target genes. This system is stably incorporated endogenously into the target organism via Tn*7* transposition (Fig. 1A). This is typically done using a tri-parental conjugation, with diaminopimelic acid (DAP) counter-selection of the plasmid donors and antibiotic selection for successful target organism integration. When optimizing this system for use in *Vibrio*, we observed a limiting deficiency in successful conjugates, typically yielding exconjugants in *Vibrio fischeri* but not the other species tested. To address this issue, we deployed an *Escherichia coli* strain containing an RP4 helper plasmid, pRK600, a pRK2013 derivative (4, 5). We found that quadraparental mating improved conjugative efficiency by ~100-fold in *Vibrio campbellii* (Fig. 1B). As a result, we have optimized this protocol for non-*fischeri Vibrio* as follows:

1. Acquire the necessary vectors from Addgene (see Fig. 1C).
2. Clone desired sgRNA into pJMP1339 following the protocol from Banta et al. (6).
    a. Note that the R6Kγ origins require the use of cloning strains that possess *pir* (e.g., λ *pir*).
3. Grow up overnight cultures of *E. coli* strains containing the following plasmids in appropriate antibiotic-containing Lysogeny Broth (LB) media:
    a. pJMP1039 (transposase).
    b. Modified pJMP1339 with the cloned sgRNA.
    c. Target bacterial strain.
    d. pEVS104 helper plasmid.

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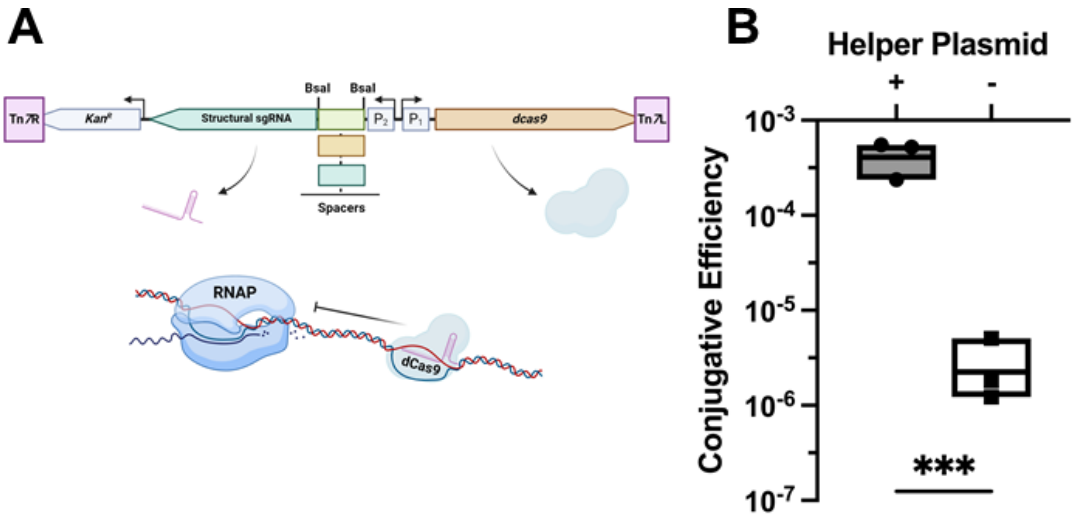

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Resource	Purpose	Media	Marker	To Acquire
pJMP1339	dCas9 + sgRNA cloning locus under PL*lacO*1, R6Kγ origin	LB	Kan^R	Addgene: pJMP1339
pJMP1039	Transposase, R6Kγ origin	LB	Amp^R	Addgene: pJMP1039
pEVS104*	RP4 helper plasmid, a derivative of pRK2013, R6Kγ origin	LB	Kan^R	Addgene: pEVS104

FIG 1 (A) Schematic for Mobile-CRISPRi. P1 and P2 are both PL*lacO*1 (6). (B) Conjugative efficiency of plasmid pLG2 targeting *luxR* in *V. campbellii* with and without RP4 helper plasmid pRK600 (3). The line in each box represents the group mean. Each point is a biological replicate that is the average of two technical replicates. Statistical analysis performed using GraphPad Prism: unpaired *t*-test on log-transformed data. ***P = 0.0005. (C) Table outlining the conjugation resources available for this method. *pEVS104 was not the helper used in panel B.

   i. Note: While plasmid pRK600 is not available in public repositories, pEVS104 is an alternate RP4 helper plasmid modified for use in *Vibrio* that is publicly available through Addgene (Fig. 1C) (5).
4. Spin down 1 mL of each culture in a tabletop centrifuge for 5 minutes at 8,000 × *g* and resuspend in antibiotic-free LB medium.
5. Following this, co-spot 10 µL of each culture onto an LB plate. Repeat this three times to create four co-spots on the same LB plate. Incubate overnight at 30°C with the lid up.
6. The next day, resuspend all four of the co-spots in the same 1 mL of LB-Marine (LM): LB supplemented with 10 g of NaCl per liter. Streak the co-spot mix for single colonies on LM plates supplemented with polymyxin B to counter-select against *E. coli* (see note below) and 100-µg/mL kanamycin to select for successful Mobile-CRISPRi plasmid integration. Incubate plates at 30°C overnight.
   a. For *Vibrio* strains that are not naturally polymyxin B resistant, DAP auxotrophic *E. coli* should be used and the co-spots streaked on LM without DAP to counter-select against. *E. coli*.

b. We recommend plating for viability on both the plates listed above and a plate group without kanamycin to calculate conjugative efficiency out of the total viable cells. In Fig. 1B, these values were calculated by dividing the CFUs on the polymyxin B + kanamycin plates by those present on plates with polymyxin alone.

7. Upon the growth of colonies, test a small subset of exconjugants (two to three) for integration via colony PCR using a primer that sits in the *glmS* locus and one that sits within the Mobile-CRISPRi near the Tn*7*-R site.

The same concept can be applied in *V. fischeri* using conjugation and media conditions described by Stabb and Ruby (5). Use of the DAP counter-selection against the *E. coli* donor is especially useful in *V. fischeri*.

Further details of how this toolkit functions experimentally in *Vibrio* are available here (3). The use of the RP4 helper plasmid greatly expanded the efficiency of this system (Fig. 1B). This modification has made the use of Mobile-CRISPRi a reliable, robust toolkit for exploring *Vibrio* genetics (3).

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