Construction of *Pseudomonas syringae* pv. tomato DC3000 Mutant and Polymutant Strains

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Abstract

Redundancy between *Pseudomonas syringae* pv. tomato DC3000 virulence factors has made their characterization difficult. One method to circumvent redundancy for phenotypic characterization is to simultaneously delete all redundant factors through the generation of polymutant strains. Described here are methods by which single and polymutant strains of DC3000 can be generated through the use of the small mobilizable sucrose counter-selection vector pDONR1K18ms, FRT-flanked antibiotic marker cassettes, and Flp recombination.

Key Words: *Pseudomonas syringae*; gene deletion; polymutant; recycling antibiotic resistance; sucrose counter-selection; Flp recombinase; Conjugation.

1. Introduction

The Gram-negative plant pathogen *Pseudomonas syringae* pv. tomato DC3000 (DC3000) uses a large and well defined repertoire of type III effector proteins to cause disease in tomato, *Arabidopsis thaliana* and *Nicotiana benthamiana* [1]. These effectors are collectively essential for pathogenicity but are individually dispensable for virulence. Characterizing the functions and virulence contributions of individual type III effectors has been made extremely difficult by the apparent redundancy of function between unrelated type III effectors.

The theme of redundancy in DC3000 virulence factors extends beyond type III effector repertories. It has also been demonstrated for type III translocators and

specialized lytic transglycosylases. Genomic analysis has suggested potential interplay between several bacterial toxins in plant disease [2-4].

One strategy that has been successfully employed to dissect functional redundancy is to create polymutant strains that combine deletions of all members in a functionally redundant group. When a redundant group is deleted, a synergistic combinatorial phenotype can be revealed that greatly simplifies analyses of individual group members. Using traditional methods of marked mutation, the number of mutations that can be introduced into a single strain is limited by the number of available antibiotic markers. To move past the marker barrier in DC3000, we have adopted and adapted several genetic tools for recycling antibiotic markers and generating unmarked mutants [3, 5].

Although several tools and tool sets have been used by our group for the generation of DC3000 mutants, we have found that the most effective tool for DC3000 deletion and poly-deletion strain construction is the small, mobilizable, sucrose counter-selection vector pK18*mobsacB* [6]. To that end we have sequenced pK18*mobsacB* and deposited the full sequence in Genbank (accession FJ437239). To simplify the creation of deletion constructs we created the Gateway-compatible derivative pDONR1K18ms and a workflow based on standardized 5' primer extensions. The procedure for making a deletion of your favorite gene (*yfgX*) has two major phases. First, making the pDONR1K18ms::Δ*yfgX* deletion construct and, second, recombining that deletion into DC3000 using a selection/counter-selection strategy. There is also the optional step of marking the deletion construct with a FRT-flanked antibiotic marker cassette and removing that FRT-flanked marker after recombination into DC3000. These optional steps add robustness to the procedure by allowing selection at both the single and double cross-over steps, while still facilitating recycling of the antibiotic markers. The major steps of the protocol are diagrammed in Figure 1. These basic procedures have been used by us to delete genomic regions ranging in size

from 500-bp to 22-kb, and to combine over seven separate deletions into a single DC3000 strain.

2. Materials

2.1. Primer design

1. DC3000 genome sequence, (accessions; chromosome AE016853, pDC3000A AE016855, pDC3000B AE016854)

*2.2. Building the pDONR1K18*ms*::*∆yfgX *deletion construct*

1. NEB Phusion High-Fidelity Polymerase (New England Biolabs, Ipswich MA).
2. Phusion High Fidelity Buffer
3. 10 mM dNTPs (10mM of each dNTP)
4. DC3000 genomic DNA (~1000 ng/µl) prepared using the GENTRA Puregene Yeast/Bact. Kit (Qiagen, Valencia CA).
5. Oligonucleotides (10 pmol/µl).

   flank A forward, flank A reverse

   flank B forward, flank B reverse

   A seq forward, B seq reverse

   M13F TGTAAAACGACGGCCAGT

   PK18M13R AACAGCTATGACATGA (*see* **Note 1**)
6. Sterile PCR-grade dH_2O.
7. 0.2 mL RNase/DNase-free PCR tubes.
8. Agarose 1™ (Amresco, Solon, OH).
9. Ethidium bromide (1%).
10. 1X TBE buffer (Tris 10.8 g, boric acid 5.5 g, 0.5 M EDTA 4 ml, fill to 1L with dH_2O).

11. 10X DNA sample loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 0.1% SDS, and 30% glycerol).
12. O'GeneRuler 1-kb DNA ladder (Fermentas, Glen Burnie, MD).
13. Gel DNA Recovery Kit and Clean-up and Concentrator Kit-5 (Zymo Research, Orange, CA).
14. Sterile 1.5 mL RNase/DNase-free microcentrifuge tubes.
15. pDONR1K18ms plasmid DNA (~150 ng/µl). (*see* **Note U1**)
16. LB liquid and agar solidified media (Tryptone 10.0 g, yeast extract 5.0 g NaCl 10.0 g, 1 M NaOH 1.0 ml, fill to 1L with dH_2O add 18 g agar for solidified media, autoclave).
17. Sterile 14-ml culture tubes.
18. 1000X kanamycin (Km) 50 mg/ml filter-sterilized in dH_2O.
19. Competent *E. coli* DH5α or any standard plasmid cloning strain.
20. BsrGI restriction enzyme and 10X restriction buffer (NEB, Ipswitch, MA).
21. Qiaprep® Spin Miniprep Kit (Qiagen, Valencia, CA).

2.3. (Optional) Introduce a FRT-flanked antibiotic marker cassette between* Δ*yfgX flanks A and B.

1. Oligonucleotides (10 pmol/µl).
    i. FRT cassette forward (with XhoI, SmaI or SpeI sites)
    ii. FRT cassette reverse (with XhoI, SmaI or SpeI sites)
2. Plasmid DNA (~200 ng/µl) from either pCPP5209 (Accession EU024549) or pCPP5242 (Accession EU024551) [3].
3. XhoI, SmaI or SpeI restriction enzymes, 10X restriction buffers (NEB, Ipswitch, MA).
4. Calf Intestinal Phosphatase (NEB, Ipswitch, MA).
5. pDONR1K18ms::Δ*yfgX* plasmid DNA (~200 ng/µl).

6. 1000X Gentamicin (Gm) 5 mg/ml filter-sterilized in dH_2O.
7. 1000X Spectinomycin (Sp) 50 mg/ml filter-sterilized in dH_2O.

2.4. Conjugate the pDONR1K18ms::Δ*yfgX *deletion construct into DC3000 to get single cross-over merodiploid transconjugants.

1. Competent *E. coli* RHO5 [7](*see* **Note U2**)
2. 100 mg/mL diaminopimelic acid in 1M NaOH, filter sterilized Sigma, 2,6-diaminopimelic acid, catalog # D1377-10G (*see* **Note U3**)
3. pDONR1K18ms::Δ*yfgX* plasmid DNA (~200 ng/µl) marked or unmarked variants.
4. DC3000 strain to mutate.
5. LM liquid and solid media (Tryptone 10.0 g, yeast extract 6.0 g, NaCl 0.6 g, $MgSO_4*7H_2O$ 0.4 g, K_2HPO_4 1.5 g (*see* **Note 3**) fill to 1L with dH_2O add 18 g agar for solidified media, autoclave).
6. KB liquid and solid media (Bacto™ Proteose peptone No. 3 20.0 g, $MgSO_4*7H_2O$ 0.4 g, glycerol, K_2HPO_4 1.5 g [*see* **Note 3**] fill to 1L with dH_2O add 18 g agar for solidified media, autoclave). (*see* **Note U4**)
7. 500X Rifampicin (Rf) 20 mg/ml in Methanol.
8. Immobilon-NY+ filter cut into squares slighylt smaller than a postage stamp with the blue backing paper still attached and autoclaved (Millipore, Billerica, MA).
9. Inoculating loop (disposable or reusable)

2.5. Counter-select the integration with sucrose to recover double-crossover mutants.

1. 1M sucrose, filter-sterilized.
2. Sterile toothpicks.

2.6. Screen for the* yfgX *deletion mutant by PCR

1. GENTRA Puregene Yeast/Bact. Kit (Qiagen, Valencia CA).
2. Oligonucleotides (10 pmol/µl).

i. out A forward

ii. out B reverse

2.7.1. Removing FRT-flanked cassette using Flp recombination.

1. Marked deletion strain mutant of DC3000.

2. *E. coli* RHO5 pCPP5264 [3] (*see* **Note 4 and U2**).

4. 500X tetracycline (Tc) 5 mg/ml in 50% ethanol (*see* **Note 2**).

2.7.2. Removing a FRT-flanked marker by secondary mutagenesis.

1. Marked deletion strain mutant of DC3000.

2. *E*. coli RHO5 transformed with unmarked pDONR1K18ms::Δ*yfgX.* (*see* **Note U2**)

3. Methods

The following sections describe the protocols to make deletions in DC3000 using the Gateway-compatible small, mobilizable, sucrose counter-selection vector pDONR1K18ms (*see* **Note U1**). The final *yfgX* deletion in the DC3000 genome will look exactly like the center join of the pDONR1K18ms deletion construct. Slightly different paths should be taken with this protocol depending on the number of deletions desired in the final strain and the proximity of those genes to one another. If only a single mutation is desired, a deletion construct marked with a FRT-flanked antibiotic resistance cassette can be used and the FRT-flanked marker can be left in place. If two to five deletions in one strain are desired and the genes/regions to be deleted are not located too close to one another, the FRT-flanked cassettes can be removed by Flp-mediated recombination leaving behind FRT site scars. What defines “too close” is unfortunately hard to know universally. Any two genes without some essential gene between them may be too close together. If six or more deletions are to be combined in one strain, or if some of the genes to be deleted are located near one another, the genes can be deleted using unmarked deletion constructs or the FRT-flanked cassettes can be removed by

conducting a second mutagenesis with unmarked deletion constructs. The rationale for these recommended paths are explained in **Note 29**.

3.1. Primer design

1. Eight primers will be designed for each deletion construct. Forward and reverse primers to amplify flank A upstream to *yfgX*, forward and reverse primers to amplify flank B downstream to *yfgX*. Two sequencing (seq) primers within flanks A and B, which prime internally for sequencing the deletion construct. Lastly, two outside (out) primers which are located just external to the flank primers for deletion strain confirmation. The Tm of all 8 primers should ideally be within 3°C of one another.
2. Flanks A and B may be designed to have a 100-500-bp difference in size if they will be joined by restriction digest as described in the original protocol, while this is not necessary in the current protocol it may serve as a back up strategy. (*see* **Notes 6, 7**)

The flank amplifying primers should be designed according to the following templates: (*see* **Notes U5 and U6)**

flank A forward: attB1 5'-ggggacaagtttgtacaaaaaagcaggcttc 18-25 nt identity

flank A reverse: XSS 5' **ctcgagcccgggactagt** 18-25 nt identity

flank B forward: SSX 5' **actagtcccgggctcgag** 18-25 nt identity

flank B reverse: attB2 5' ggggaccactttgtacaagaaagctgggtc 18-25 nt identity

3. Additional care should be taken in the design of flank A reverse and flank B forward if *yfgX* is in an operon. To manage the risk of introducing polar effects, the flanks should be designed so flank A includes the first 2 or 3 codons of *yfgX* followed by the joining sequence **ctcgagcccgggactagt** and flank B carries the joining sequence followed in-frame by the last two or three

codons, including the stop codon of *yfgX*. This way the deletion strain will express a small peptide in place of YfgX.

4. Seq A forward and seq B reverse primers should be designed to bind near the middles of flanks A and B and should prime towards *yfgX*. Aim for 18-25 nt each.
5. Out A forward and out B reverse primers should be located about 50-bp upstream from flank A forward and downstream of flank B reverse. Aim for 18-25 nt each.

3.2. Building the pDONR1K18ms::*∆**yfgX *deletion construct

(*see* **Note 12**)

1. Gather the Phusion polymerase HF buffer, dNTPs forward and reverse flank primers and genomic DNA template for the PCR reaction.
2. Set up a separate PCR reaction for each flank according to the following 100 µl recipe.

20 µl	5X HiFi buffer
5 µl	forward PCR primer
5 µl	reverse PCR primer
2 µl	10 mM dNTPs
1 µl	genomic DNA
1 µl	Phusion polymerase
66 µl	PCR grade dH_2O

3. Divide the PCR reaction into two 50 µl aliquots in 0.2 mL PCR tubes to run in the thermocycler. The PCR will work better with the smaller sample volume. Recombine the 50 µl aliquots after the cycling is complete. The following thermocycler program is recommended for PCR amplification.

2 m	@ 98°C
Repeat 30 cycles	
10 s	**@ 98°C**
30 s	**@ 50°C** (*see* **Note U7**)
30 s/kb	**@ 72°C**
10 m	@ 72°C
Hold	@ 4°C

4. Use 10 µl of the PCR reaction to confirm product amplification by agarose gel electrophoresis. The band should be strong and run at the correct size. There should be no obvious secondary bands in the reaction (*see* **Note 13**).
5. Use the Zymoclean Clean and Concentrate kit-5 to clean the remaining 95 µl volume. Elute with a 10 µl volume.

Join the two flanks via overlap extension PCR according to the following recipe

20 µl	5X HiFi buffer
5 µl	flank A forward primer
5 µl	flank B reverse primer
2 µl	10 mM dNTPs
5 µl	purified flank A DNA
5 µl	purified flank B DNA
1 µl	Phusion polymerase
57 µl	PCR grade dH_2O

6. Divide the PCR reaction and use the same cycle parameters as described in step 3.2.3

7. Using fresh agarose, cast a 1% agarose gel using a comb size which produces wells sufficient to fit the 50 µl PCR reaction volume.
8. Gel purify the desired A+B product from the PCR reaction by agarose gel electrophoresis of the entire volume. Use fresh TBE running buffer.
9. Cut the A+B band out of the gel with a clean razor blade.
10. Purify the DNA from the agarose block using the Zymoclean Gel Recovery kit-5. Elute with a 10 µl volume.
11. Use BP clonase II to recombine the A+B product with pDONR1K18ms

BP clonase II reaction

7 µl	purified flank A+B DNA
1 µl	pDONR1K18ms DNA (150 ng)
2 µl	BP clonase (thawed on ice, vortex briefly prior to use)

Incubate 1 h @ 25C

Add 1 µl proteinase K

Incubate 10 min @ 37C

12. Purify the DNA from the ligations with the Zymoclean Clean and Concentrate kit-5. Elute with a 6 µl volume. (*see* **Note U8**)

13. Transform the purified DNA into competent *E. coli* (DH5α, TOP10, etc) using electroporation or chemically competent heat-shock transformation by standard protocols [8].

14. Plate 1/10 of the transformation and a concentrated remainder onto labeled LB Km plates. Incubate the plates overnight at 37°C. (*see* **Notes 19 and 20**)

15. Start 6 overnight LB Km cultures at 37°C. from isolated colonies (*see* **Note U9**)

16. Miniprep the plasmid DNA from the cultures using the Qiaprep Spin Miniprep kit from Qiagen.

17. Conduct a restriction digest screen on 5 µl of plasmid DNA with BsrGI in a 20 µl volume according to the following recipe. Incubate the reaction for at least 1 hour 37°C. (*see* **Note U10**)

5 µl	plasmid DNA
2 µl	10X NEB 2.1 buffer
1 µl	BsrGI
12 µl	dH_2O

18. Check by agarose gel electrophoresis. The expected bands will be 3286-bp and 2655 bp produced by the backbone and the band sizes corresponding to the insert (*see* **Note U11).** Discard any plasmids which do not have the expected band pattern.

19. Send candidate clones for sequencing with M13F, PK18M13R and the two sequencing primers designed in step 3.1.4. to check for any point mutations in the flanks and confirm that the pDONR1K18ms::Δ*yfgX* deletion construct is correct.

3.3. (Optional) Introduce a FRT-flanked antibiotic marker cassette between* ∆yfgX *flanks A and B (*see* **Note 15**).

1. Design FRT cassette primers to PCR amplify either the FRTSp^R or the FRTGm^R cassettes from pCPP5242 or pCPP5209 respectively with primers designed according to this template (*see* **Notes 8, 10, 16** and **U12**)

   FRT cassette forward primer

3-4 nt toe (restriction site XhoI, SmaI or SpeI) GTGTAGGCTGGAGCTGCTTC

   FRT cassette reverse primer

3-4 nt toe (restriction site XhoI, SmaI or SpeI) CATATGAATATCCTCCTTA

2. Conduct a PCR reaction as described in steps 3.2.1.-3.2.4. using 1 µl of pCPP5242 or pCPP5209 plasmid DNA as template.
3. Purify the PCR product with the Zymoclean Clean and Concentrate kit-5. Elute with a 6 µl volume.
4. Restriction digest the purified FRT cassette and the pDONR1K18ms::∆*yfgX* deletion construct in a total reaction volume of 50 µl with the selected restriction site enzyme. Incubate the reaction for at least 1 hour at the appropriate temperature for the restriction enzyme (most likely 37°C).

   Insert reaction
   5 µl purified FRT cassette DNA
   5 µl 10X NEB buffer
   1 µl XhoI, SmaI or SpeI
   39 µl dH_2O

   Vector reaction
   1 µl pDONR1K18ms::∆*yfgX*
   5 µl 10X NEB buffer
   5 µl 10X BSA
   1 µl XhoI, SmaI or SpeI
   1 µl NEB Calf Intestinal Phosphatase
   37 µl dH_2O

5. Purify the DNA with the Zymoclean Clean and Concentrate kit-5.
   a. Elute with a 10 µl volume.

6. 6. 15. Set up 20 µl ligation reactions and controls. Incubate the reaction overnight at 14°C.

Vector control

3 µl	purified vector DNA
4 µl	NEB 5X T4 ligase buffer
1 µl	NEB T4 ligase
12 µl	dH_2O

Ligation

3 µl	purified vector DNA
9 µl	purified insert DNA
4 µl	NEB 5X T4 ligase buffer
1 µl	NEB T4 ligase
3 µl	dH_2O

7. Purify the DNA from the ligations with the Zymoclean Clean and Concentrate kit. Elute with a 6 µl volume.
8. Prepare LB Km plates additionally augmented with either Sp or Gm.
9. Transform the purified controls and ligation into competent DH5α or any standard *E. coli* cloning strain using electroporation or chemically competent heat-shock transformation by standard protocols [8].
10. Plate 1/10 of each transformation reaction and a concentrated remainder onto labeled LB Km plates augmented with either Sp or Gm. Incubate the plates overnight at 37°C. Colonies should only grow on the ligation plates, indicating that the deletion construct now carries the FRT cassette between Flank A and B.
11. Choose three Km^R and marker antibiotic resistant colonies and start 2-ml overnight LB Km cultures at 37°C.
12. Miniprep the plasmid DNA from the cultures using the Qiaprep Spin Miniprep kit from Qiagen.
13. Conduct a restriction digest screen on 5 µl of plasmid DNA with XhoI, SmaI or SpeI in a 20 µl volume as in step 3.2.17.

14. Check by agarose gel electrophoresis that the digest releases a fragment the same size as the FRT cassette fragment. Discard any plasmids which do not (*see* **Note 17**).

3.4. Conjugate the pDONR1K18ms::ΔyfgX deletion construct into DC3000 to get single cross-over merodiploid transconjugants.

1. Prepare an LB Km DAP 400 µg/ml plate and an LM DAP 400 µg/ml plate with no antibiotics.
2. Transform 3 µl of miniprep pDONR1K18ms ::ΔyfgX deletion construct DNA into E. coli RHO5 by electroporation or chemically competent heat-shock transformation using standard protocols. Add DAP to 200 µg/ml to the outgrowth media. The marked or unmarked pDONR1K18ms ::ΔyfgX variant can be used.
3. Plate 1/10 of the transformation onto a labeled LB Km DAP 400 µg/ml plate. Incubate O/N at 37°C
4. From fresh isolated colonies start a 5-mL LM Rf overnight culture with shaking at 30°C of the DC3000 recipient strain and a 5-mL LM Km DAP 200 µg/ml overnight culture with shaking at 37°C of the E. coli RHO5 pDONR1K18ms ::ΔyfgX donor strain. Either the marked or unmarked pDONR1K18ms ::ΔyfgX variant can be used depending on your particular strategy (see **Note 21**)
5. Transfer 1mL of overnight cultures to a 1.5 mL microfuge tube and harvest the cells by centrifugation in a microcentrifuge at 16,000 × g. for 1 min. Discard the supernatant.
6. Resuspend the cells in 1 ml of fresh LM by vortexing. Harvest the cells in a microcentrifuge 1 min at 16,000 × g. Discard the supernatant. Repeat step 3.4.6 for two total washes.

7. Resuspend the cells in a final volume of 0.2 ml LM.
8. In a third microcentrifuge tube, mix 20 µl each of the DC3000 and E. coli suspensions.
9. Using ethanol-sterilized forceps, position three sterile filter squares on a LM DAP plate, with one square each for the controls and one for the conjugation. Use the forceps to push the filter square onto the surface of the plate. Label the plate near the filter squares with the respective strains.
10. Using a micropipetor, apply 20 µl each of the DC3000 and E. coli suspensions as drops to their respective filter squares. Add all 40 µl of the conjugation mixture to its filter square (*see* **Note 22**).
11. Allow the drops of suspension to dry.
12. Invert the plates and incubate for 1 day at 30°C.
13. Prepare and label three KB Rf Km plates, one for each filter square (*see* **Note 23**).
14. Using a sterile inoculating loop, scrape the bacteria from each filter into its own to labeled 1-ml microcentrifuge tube with 1 mL of LM media.
15. Harvest the cells in a microcentrifuge 1 min at 16,000 × g. Discard the supernatant.
16. Resuspend the cells in 1 ml of fresh LM by vortexing.
17. Plate the 100 µl of resuspended cells onto the KB Rf Km plates with an ethanol-sterilized spreader.
18. Incubate at room temperature to 30°C for 3 to 6 days. RfR KmR colonies should only grow on the plate spread with the conjugation mixture.

3.5. Counter-select the integration with sucrose to recover double-crossover mutants.

1. Prepare control and counter-selection plates. The control plate is KB Rf. To make the counter-selection plate add 3 parts pre-warmed 1M sucrose to 7 parts melted KB to get approximately 10% final concentration of sucrose, add Rf as normal. If you used the marked variant of pDONR1K18ms::Δ*yfgX* in your conjugation, include the marker antibiotic in both the control and sucrose counter-selection plates.
2. Pick two Rf^R Km^R colonies from the conjugation plate with a sterile toothpick and suspend the cells in 1.9 ml KB in a 2-ml microcentrifuge tube.
3. Use an ethanol-sterilized spreader to plate 50 µl of the suspension onto the KB Rf control plate and the KB Rf 10% sucrose plate.
4. Incubate at room temperature or 30°C for two to four days. The KB Rf control plate should produce a lawn of bacteria, but the KB Rf Suc plates should produce somewhere between 50 and 500 colonies (*see* **Note 25**). In the case of the unmarked variant of pDONR1K18ms::Δ*yfgX,* these colonies will represent a mixture of deletion mutants and wild-type revertants. In the case of the marked variant of pDONR1K18ms::Δ*yfgX,* the included marker antibiotic will select for the deletion strain mutants.
5. Prepare a KB Rf and a KB Rf Km plate.
6. Using sterile toothpicks patch the Suc^R colonies onto the KB Rf and KB Rf Km plates. Patch at least 12 colonies.
7. Incubate at room temperature to 30°C overnight. The Km^S colonies have evicted the pDONR1K18ms backbone. Any colony that is Km^R is most likely a spontaneous Suc^R strain rather than a double recombinant and should not be used in any further work.

3.6. Screen for the* yfgX *deletion mutant by PCR.

(*See* **Notes 26-28**)

1. Start 2-ml KB Rf overnight cultures from at least 6 Suc^R Km^S patches.
2. Purify genomic DNA using the Gentra Puregene DNA kit for Gram-negative bacteria.
3. Gather the Phusion polymerase HiFi buffer, dNTPs, out primers and genomic DNA template for the PCR reaction
4. In PCR tubes set up a separate PCR reaction for each clone to be screened as well as a wild type control reaction using DC3000 genomic DNA according to the following recipe.

Prepare a PCR master mix by
multiplying the following recipe
1.1X the total number of PCR reactions

5 µl	5X HiFi buffer
1 µl	Out A forward primer
1 µl	Out B reverse primer
0.5 µl	10 mM dNTPs
0.25 µl	Phusion polymerase
16.75 µl	PCR grade dH_2O

Aliquot 24 µl per reaction

Add 1 µl genomic DNA

5. Run the PCR reaction in a thermocycler using the cycle parameters described in step 3.2.3.
6. Run the total volume of the PCR reaction by agarose gel electrophoresis for bands of the appropriate sizes for the wild type and the deletion strain mutant.

3.7. (Optional) Removal of the FRT-flanked marker from a marked deletion strain.

You may choose to remove the antibiotic marker from your marked deletion strain either to make that antibiotic available for other purposes or to make additional mutations in the same strain. The removal of the FRT-flanked marker can be done in two ways: Flp-mediated recombination of the FRT-flanked cassette, or a second mutagenesis with the

unmarked variant of pDONR1K18ms::Δ*yfgX*. After FRT flank removal by either method, the strain should be reconfirmed by PCR using methods similar to those described in steps 3.6.1-3.6.6 (*see* **Note 29**).

3.7.1. Removing FRT-flanked cassette using Flp recombination.

1. Similar to step 3.4.4., from isolated colonies, start a 5-ml LM Rf overnight culture with shaking at 30°C of the DC3000 marked deletion recipient strain and a 5-ml LM Tc 10 DAP 200 overnight culture with shaking at 37°C of the *E. coli* RHO5 pCPP5264 donor strain.
2. Process the overnight cultures and set up the LM DAP plate as in steps 3.4.5.-3.4.18 but plate the controls and conjugation on KB Rf Tc.
3. Patch the Tc^R pCPP5264 transformed clones on KB Rf and KB Rf with marker antibiotic looking for sensitivity to the marker antibiotic. Sensitivity indicates loss of the FRT-flanked marker and its recombination into a FRT site scar [9] (*see* **Note 30**).
4. Start 5-ml KB Rf overnight cultures with shaking at 30°C of two or three marker antibiotic sensitive colonies. Without Tc selection pCPP5264 will be rapidly lost.
5. Apply 20 µl of overnight cultures to KB Rf plates near the plate's border and spread the bacteria by three-phase streak to get isolated colonies.
6. Incubate 2-3 days at room temperature or 30°C.
7. Patch plate at least 12 isolated colonies onto KB Rf and KB Rf Tc for Tc sensitivity to confirm loss of pCPP5264.

3.7.2. Removing a FRT-flanked marker by secondary mutagenesis.

1. Repeat steps 3.4.4.-3.5.7. using your marked deletion strain as the recipient strain and *E. coli* RHO5 carrying unmarked pDONR1K18ms::Δ*yfgX* as the donor strain. When you get to step 3.5.6., patch plates for Km^S and also patch for

sensitivity to the marker antibiotic. Colonies that are sensitive to both Km and the marker antibiotic are likely to be unmarked deletion mutants.

4. Notes (Crossed out notes were relevant to the original protocol)

1. pDONR1K18ms and other pK18mobsacB derivatives have a 1-bp deletion in the M13R binding site so it requires a unique primer.
2. Tet is light sensitive. Store either in amber microcentrifuge tubes or in a light-free environment.
3. For LM and KB liquid media do not add K_2HPO_4 prior to autoclaving or it will precipitate. Mix up a 100X phosphate stock, K_2HPO_4 75 g in 500 ml dH_20 and filter sterilize. Add to the media after it cools.
4. pCPP5264 is an unstable Flp expression plasmid.
5. ~~pRK2013 is a *tra* helper plasmid.~~
6. The flanks are designed with a difference in size because they will be joined by restriction digest followed by T4 ligation of the linear products. If both flanks were the same size you could not distinguish undesired flank A+A or flank B+B products from the desired A+B product.
7. Alternative joining methods such as SOEing (splicing by overlap extension) PCR could be used instead, in which case the flanks could be designed to be identical in size [10].
8. The purpose of the 3-4 nt toe is to provide a place for the restriction enzyme to sit. Many enzymes do not cut efficiently near the end of a DNA molecule. Any base combination can be used here and it can be altered to increase or decrease the Tm of the primer, but try to avoid palindromic sequences which can lead to primer-dimers.

9. ~~Using *Pvu*I or *Nhe*I for cloning will remove the M13F priming site, so a different sequencing primer will need to be used in step 3.2.24.~~

10. SmaI digests at 30°C, which can complicate double digests. XmaI is a non-blunt isoschizomer of SmaI that digests at 37°C.

11. ~~XbaI and NheI produce compatible cohesive ends.~~

12. ~~If ligating the two fragments together prior to cloning into pDONR1K18ms does not work, or if a different cloning strategy is preferred, the primer design described here can also be used to clone each fragment sequentially into pDONR1K18ms using similar restriction digest and cloning strategies to those described.~~

13. If the bands are not strong or if there are secondary bands, try to optimize your PCR or possibly redesign the flank amplification primers.

    If secondary bands are a problem, you could alternatively gel purify the correct band. To gel purify, cast a 1% agarose gel using a comb which will produce large wells to fit the 95 µl reaction volume. Use fresh agarose and running buffer. Cut out the band of the correct size with a clean razor blade. Purify the DNA from the agarose block using the Zymoclean gel recovery kit. Elute with a 6 µl volume.

14. ~~Incubation of the plates at 4°C will strengthen the blue color.~~

15. Introducing FRT-flanked cassettes into the deletion mutant strain is likely to create polar effects on downstream genes. FRT cassettes can be cloned in such a way that the FRT site scar can be read through in-frame, but to do so, the FRT cassette forward primer has to be redesigned as

    3-4 nt toe (internal restriction site) CGCTGGAGCTGCTTCGAA, and the FRT cassette will have to be cloned in the appropriate orientation.

16. These primers are based on the common primers 1 and 2 from Datsenko et al. 2000 [11].

17. The FRT cassette is cloned in a non-directional manner. You may want to determine the orientation of the FRT cassette by restriction digest or PCR screen.

18. ~~*E. coli* S17-1 is a mating strain with an integrated RP4 plasmid so it can mobilize plasmids such as pDONR1K18ms without the aid of a *tra* helper plasmid. The reason *E. coli* S17-1wasn't transformed directly is that it is not blue white compatible and is Sp^R, which would interfere with the cloning of the FRTSp^R cassette.~~

19. *E. coli* transformed with pDONR1K18ms form slightly transparent colonies which will also be sensitive to sucrose. These strains will often grow at a slower pace. and produce less turbidity in an overnight liquid culture

20. pDONR1K18ms and other sacB expressing strains transformed strains do not transition well to 4°C. It is better to keep these strains at room temperature for up to two weeks and re-streak them fresh from glycerol when needed.

21. When starting overnight cultures of DC3000 be generous with the amount of cells added to the broth.

22. To prevent cross-contamination, when applying the bacterial suspensions be very cautious not to drip onto the plate, and try to approach each filter square from its own angle of attack, not crossing over other filter squares. Hold the micropipetor tip close to, but not touching, the filter square, and depress the plunger slowly. Try to keep the entire volume of the bacterial suspension on the filter square.

23. ~~Ampicillin is included to provide additional selection against the *E. coli* donor.~~

24. ~~The best way to do this is to grab the middle of an edge of a filter square, being careful to not touch the forceps to the bacteria. Insert a corner of the filter square into the mouth of the snap cap tube and twist the filter square into the tube.~~

~~Ideally, you want the square flat against the wall of the tube with the bacteria facing inward~~.

25. DC3000 colonies grown on KB 10% sucrose plates are shiny, mucoid, semi-transparent, more strongly domed, and develop an orange color as they age.
26. If the intended deletion has a readily testable phenotype, you may want to conduct a phenotypic screen prior to the PCR screen to get some indication as to which strains are likely to be mutant or wild type. For example, a *fliC* deletion mutant does not produce a flagellum, resulting in the loss of motility on a 0.2% agar swim plate.
27. The most straightforward PCR confirmation screen is to use out A forward and out B reverse. These primers are located outside the flank amplification primers so they can screen against deletion construct integration into the incorrect genomic location. Ideally this PCR reaction will produce distinct band sizes for both wild-type and deletion mutant strains and will confirm the status of both borders simultaneously. However, there are two potential problems. If you have created a marked deletion strain, depending on the size of the gene that was deleted, the mutant and wild type may not be easily distinguishable by size. Also, if the deleted gene or genomic region was 5 kb or longer, the wild type band may be difficult or impossible to amplify. In all the alternative PCR confirmation strategies two PCR reactions are required to confirm the status of each border.

    A out forward/B seq reverse and A seq forward/B out reverse.

    A out forward/flank A reverse and flank B forward/B out reverse

    For marked deletion strains or deletion strains with the FRT site scar:

    A out forward/FRT check 1 and FRT check 2/B out reverse

    FRT check 1 GAAGCAGCTCCAGCCTACAC

    FRT check 2 CTTCGGAATAGGAACTAAGGAGGATATTCATATG

28. If a particular mutant has proven difficult to recover using the unmarked variant of pDONR1K18ms::Δ*yfgX* due to high recovery of wild-type revertants, it can often be forced forward by using the marked pDONR1K18ms::Δ*yfgX* variant. When the marker antibiotic is included at the sucrose counter-selection step, only the marked mutant population and spontaneous Suc^R colonies will grow. If no colonies are produced using the two colonies in 2 ml dilution strategy with the marked variant pDONR1K18ms::Δ*yfgX*, more concentrated suspensions can be spread on the sucrose counter-selective marker antibiotic selective plates. Dense suspensions of approximatly 10^8 CFU/ml have been used in this manner to isolate particularly difficult mutants.

29. The use of Flp to remove FRT-flanked cassettes is quick and straightforward, but in strains with multiple deletions there are some potential concerns. After Flp recombination of a FRT-flanked cassette, a FRT site scar is left behind in the genome. After several cycles of mutagenesis these FRT site scars will start to build up in the genome. The FRT site scars are still recombinationally active and can facilitate large scale inversions between indirect FRT sites or deletions between direct FRT sites mediated by Flp. If you intend to make more than five deletions in sequence in the same strain or if you intend to make additional deletions in genes which are located near an existing FRT site scar, you may want to consider using a second mutagenesis with unmarked pDONR1K18ms::Δ*yfgX* to remove the FRT-flanked marker rather than the use of Flp recombination. If you do make multiple deletions and use Flp to remove the FRT-flanked markers, then the status of each FRT site scar in the genome should be checked by PCR after Flp recombination to ensure that they are intact.

30. The sequence of the FRT site scar has the following structure.

GTGTAGGCTGGAGCTGCTTC**GAAGTTCCTATA**CTT*TCTAGAG***AATAGGAAC**TTC**GGAATAGGAACTAAGGAGGATATTCATATG

Bold sequences are Flp recognition sites. The sequence in italics is an *Xba*I site. Underlined sequences are primer binding sites [11].

U1. To create pDONR1K18ms the attP-GW-DONR cassette was PCR amplified from pDONR207 with Phusion polymerase and cloned into the SmaI site of pK18mosacB. As this vector caries the ccdB toxin it must be maintained in a ccdB resistant E coli strain such as DB3.1. We have also created pDONR2T18ms, a Gateway compatible derivative of the tet resistant pT18mobsacB tet resistant allelic exchange vector [12]. pDONR2T18ms can be substituted for pDONR1K18ms throughout the protocol by replacing Km selection with Tet 10 selection. We have also created pDEST1K18ms and pDEST2T18ms variants which are compatible with Gateway LR clonase.

U2. E coli RHO5 is a derivative of the conjugative delivery strain SM10 which has been engineered to have no antibiotic resistance marker genes and carry a mutation in the gene *asd* so that it requires exogenous diaminopimelic acid (DAP) to grow. DAP must be added to any media to grow RHO5 including transformation outgrowth media. RHO5 also carries the pir116 gene for high-copy number replication of R6K plasmids [7].

U3. Prepare 100mg/ml stocks in 1M NaOH and filter sterilize them. The stock will keep in the fridge at least a year. Use 200ug/ml final concentration DAP for liquid cultures and 400ug/ml for agar plates.

U4. KB media can be substituted with LM media throughout this protocol.

U5. *att*B1 and *att*B2 sites are appended with terminal GGGG sequences recommended for BP clonase recombination of PCR products.

U6. The XSS universal join region codes for the XhoI, SmaI and SpeI restriction recognition sites. These three sites are not found in pDONR1K18ms, pDEST1K18ms, pDONR2T18ms or pDEST2T18ms or the FRTGmFRT or FRTSpec.FRT marker cassettes.

U7. Typically 50°C has been effective for almost any PCR reaction I have conducted with Phusion polymerase. However, NEB suggests using higher annealing temperatures. https://www.neb.com/protocols/1/01/01/~/link.aspx?_id=CF98AC51A86D4E0BB6959DB69A93408C&_z=z

U8. I have used drop dialysis as an alternative to column cleanup using Millipore VSWP02500 filters as described here. https://www.emdmillipore.com/US/en/product/V-series-Membranes,MM_NF-C9133

U9. BP clonase recombinant clones should be seneitive to chloramphenicol 25 µg/mL.

U10. All Gateway *att* sites contain a BsrGI recognition site.

U11. Check whether you're A+B insert contains any additional BsrGI sites as this will alter the expected digest band pattern.

U12. Select from XhoI, SmaI or SpeI. Check that the chosen site is absent in your deletion flanks. If those ae not options SmaI is a blunt cuter that can be ligated to

any other blunt cutter enzyme and SpeI produces compatible ends with NheI and AvrII digests.

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