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Protocol for Constructing a Gene Knockout in *Pseudomonas* Using pT18mobsacB

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ABSTRACT

Gene knockout via homologous recombination is a powerful approach for investigating gene function in bacteria. Here, we present a streamlined protocol for constructing an unmarked gene knockout in *Pseudomonas* using the suicide plasmid pT18mobsacB. The method involves the insertion of upstream and downstream homologous arms into the plasmid, followed by two-step allelic exchange using tetracycline selection and sucrose counter-selection. Unlike commonly used vectors, pT18mobsacB confers tetracycline resistance, which offers broader inhibitory coverage across bacterial species and expands the host range of this system. The method enables precise marker-free knockout without leaving any antibiotic resistance cassette, allowing unrestricted downstream applications. A complete knockout can be achieved in as little as 1 week. Despite its versatility, the use of pT18mobsacB has been rarely detailed in previous literature. This protocol fills that gap by providing a fully annotated, reproducible guide that covers every experimental detail—from vector construction to mutant verification. It offers a valuable tool for functional genomics, microbial physiology, and applications ranging from food microbiology to biotechnology.

1 | Introduction

The genus *Pseudomonas* comprises versatile Gram-negative bacteria of great biological and industrial importance. *P. aeruginosa*, for example, is a prevalent opportunistic pathogen and a mainstay model organism for studying motility, biofilm formation, and virulence in Gram-negatives (Hmelo et al. 2015). On the industrial side, psychrotrophic *Pseudomonas* species such as *P. fluorescens* are notorious contaminants in the food and dairy supply chain, producing heat-resistant enzymes that spoil products and reduce shelf-life (Scales et al. 2014; Goh et al. 2023). *Pseudomonas* spp. plays beneficial roles in biotechnology (e.g., *P. putida* in bioremediation), highlighting the breadth of their impact (Weimer et al. 2020). *Pseudomonas* is also commonly found to be a biocontrol bacterium, exhibiting resistance against certain pathogenic microorganisms (Mercado-Blanco 2014). Therefore, research on the *Pseudomonas* genus holds great potential, and this genus may become an important microbial group in fields such as food safety, human health, plant life sciences, and microbial engineering (Chen et al. 2023; Yang et al. 2025). Despite extensive study, many genes in *Pseudomonas* remain uncharacterized—in *P. aeruginosa* PAO1, only 18% of ORFs have experimentally confirmed functions—underscoring the need for genetic tools to probe gene function and regulation in this genus (Hmelo et al. 2015).

A powerful strategy to determine gene function is to create a targeted gene knockout and observe the resulting phenotypic

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changes. In *Pseudomonas* and related bacteria, this is commonly achieved via a two-step allelic exchange using a non-replicating "suicide" plasmid that carries both an antibiotic resistance marker and the *Bacillus subtilis sacB* gene for counter-selection (Huang and Wilks 2017). Vectors such as pK18mobsacB and its tetracycline-resistant derivative pT18mobsacB are exemplars of this system (Hachisuka et al. 2021). In the first recombination step, the engineered plasmid—containing 500–1000 bp DNA fragments upstream and downstream of the target gene—is introduced into the *Pseudomonas* host, where it cannot replicate autonomously and thus integrates into the chromosome via a single crossover (yielding a merodiploid) (P. Wang et al. 2015). Selection on an appropriate antibiotic ensures that only integrants survive. In the second step, bacteria are grown under counter-selective conditions (typically on medium with 5%–20% sucrose and no antibiotic) to induce loss of the plasmid. Cells that undergo a second homologous recombination event will excise the plasmid sequence; depending on the crossover site, they either revert to the wild-type genotype or acquire an in-frame knockout of the target gene. The *sacB* gene product (levan sucrase) is lethal in Gram-negatives when sucrose is present, so cells retaining the plasmid (single-crossover integrants) are counter-selected and only plasmid-free segregants can form colonies (Gay et al. 1983). Screening for sucrose-resistant antibiotic-sensitive clones and confirming by PCR allows identification of successfully knocked-out mutants without any scar or resistance marker. Compared with other commonly used suicide vectors, the tetracycline-resistant derivative pT18mobsacB offers several unique advantages. First, tetracycline provides a broader inhibitory spectrum than kanamycin, thereby expanding the applicability of this system to a wider range of bacterial hosts. Second, the protocol achieves scarless gene knockout, ensuring that resulting mutants are free of antibiotic markers and can be propagated without selective pressure. Third, the procedure is relatively rapid, with complete knockout mutants obtainable in as little as 1 week. Despite these advantages, detailed applications of pT18mobsacB have rarely been reported, and current literature lacks a comprehensive step-by-step protocol.

In this guideline, we describe a reproducible protocol for constructing gene-knockout mutants in *Pseudomonas* using the pT18mobsacB suicide plasmid system. The procedure covers: (i) cloning of the target gene's flanking regions into pT18mobsacB to create a knockout plasmid, and (ii) introduction of this plasmid into *Pseudomonas* and execution of the two-step homologous recombination (with tetracycline selection and sucrose counter-selection) to obtain a clean knockout (Hachisuka et al. 2021; O'Malley et al. 2019). By following the steps below, researchers can efficiently generate *Pseudomonas* mutants to interrogate gene function. This protocol is robust and relatively straightforward, requiring only standard molecular biology reagents and methods (PCR, restriction cloning or seamless assembly, *E. coli* transformation, and *Pseudomonas* electroporation). The resulting mutants are marker-free, which is advantageous for downstream applications such as complementation studies or iterative genome engineering. Overall, this method provides a valuable tool for molecular food microbiology and other fields, enabling functional genomic analyses of *Pseudomonas* metabolism, virulence, and other phenotypes relevant to food safety and biotechnology.

2 | Construction of the Knockout Plasmid (pT18mobsacB + UHA + DHA)

Note: "UHA" and "DHA" refer to the 0.8–1.0 kb upstream homologous arm and 0.5–0.8 kb downstream homologous arm sequences flanking the target gene.

2.1 | pT18mobsacB Plasmid Digestion

2.1.1 | Selection of Restriction Sites

Open the pT18mobsacB plasmid map in SnapGene (or a similar DNA mapping software) and locate the multiple cloning site (MCS) (Figure 1), which contains several unique restriction sites (Boyle et al. 1985). For efficient downstream ligation or assembly, we recommend linearizing the plasmid with a double digestion (two restriction enzymes). In pT18mobsacB, it is recommended to avoid using the XbaI restriction site, if possible. Suitable sites are XmaI and PstI, as an example. These two sites are separated by 20 base pairs on the plasmid map, which helps minimize the risk of fragment self-ligation or incomplete digestion by providing distinct cohesive ends (Tolmachov 2009). Other enzyme pairs can be used if they uniquely cut the vector

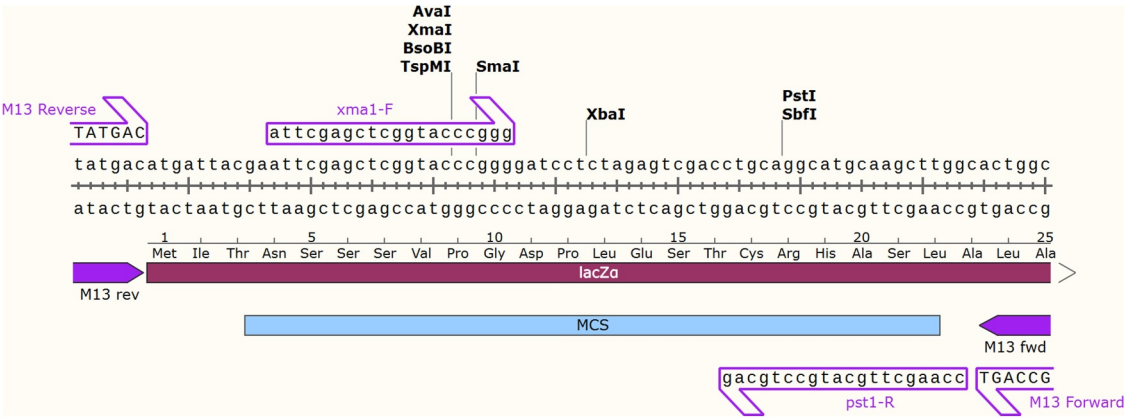

FIGURE 1 | The MCS Region of pT18mobsacB.

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outside of essential regions, but XmaI/PstI has been optimal in our experience.

2.1.2 | Plasmid Digestion

Digest the pT18mobsacB plasmid with the selected restriction enzymes to linearize it. Set up the reaction (50 μL total volume) as follows, on ice:

1. 10 × FastDigest Buffer: 5 μL
2. XmaI FastDigest enzyme: 2.5 μL
3. PstI FastDigest enzyme: 2.5 μL
4. pT18mobsacB plasmid DNA: 2.5 μg (add in an appropriate volume)
5. Nuclease-free water: add to a final volume of 50 μL

Gently mix the reaction (briefly centrifuge or flick the tube) and incubate in a thermal cycler or water bath using the following program: 37°C for 120 min (to ensure complete digestion), then 98°C for 5 min to heat-inactivate the enzymes. Finally, cool the tube to 4°C and hold (Stuchbury and Münch 2010).

2.1.3 | Recovery of Digested Plasmid

After digestion, purify the linearized plasmid DNA. If the digest was clean (only the backbone band is present), you may directly purify the product from the reaction solution using a silica column-based DNA purification kit. We use the FastPure Gel DNA Extraction Mini Kit (Vazyme, DC301) to recover the DNA from the digestion mixture. Follow the manufacturer's protocol for DNA cleanup: apply the sample to the provided spin column (the high-salt binding buffer in the kit will allow the linear DNA to bind), wash the column with the provided wash buffer to remove enzymes and small fragments, then elute the purified linear plasmid in elution buffer or water. Measure the concentration and purity of the recovered plasmid. A successful outcome is a linearized pT18mobsacB DNA with good yield and purity, ready for ligation with the homologous arms.

2.2 | PCR Amplification of UHA and DHA

2.2.1 | Primer Design for Homologous Arms

Obtain the genomic DNA sequence of your *Pseudomonas* strain and identify the target gene region to be deleted. Using SnapGene or a genome browser, extract two fragments: an UHA of 800–1000 bp immediately upstream of (but not including) the target gene, and a DHA of 500–800 bp immediately downstream of the gene. It is not necessary for these arms to be directly adjacent to the gene; a small gap (up to 100 bp) between the arm and the gene is acceptable, if needed, to avoid any regulatory elements.

Design PCR primers to amplify these two fragments with the following considerations:

1. Internal primers (for genomic arms): Choose primers 18–22 bp in length that anneal at the ends of the UHA and DHA sequences. Aim for a GC content of 50%–60%, and ensure that each primer pair has a melting temperature (Tm) within 3°C of each other (Borah 2011). These primers will amplify the arms from genomic DNA.
2. Incorporate cloning sites: To facilitate insertion into pT18mobsacB, add appropriate sequences to the 5′ end of the primers. For the UHA forward primer (UHA-F), perpend the sequence containing the chosen 5′ restriction site (here, XmaI) to its 5′ end. For example, you might add 5′-*CCCGGG*-... (XmaI recognition site, *CCCGGG*, plus any necessary clamp bases) to the start of UHA-F. In our case, a 20 bp sequence *ATTCGAGCTCGGTACCCGGG* (which includes the XmaI site *CCCGGG*) was added to UHA-F as an example. Similarly, for the DHA reverse primer (DHA-R), add the sequence for the other restriction site (here, PstI) to its 5′ end (e.g., a 20 bp adapter ending in *CTGCAG* for the PstI site; an example used is *CCAAGCTTG-CATGCCTGCAG*, which contains the PstI site *CTGCAG*). After adding these adapters, UHA-F and DHA-R should each be 38–42 bp long in total.
3. Ensure overlap between UHA and DHA fragments: To allow seamless joining of UHA and DHA in the final plasmid (when using a one-step cloning kit or Gibson assembly), design the remaining two primers with overlapping sequences. Take the 18–22 bp sequence at the tail end of the UHA fragment (the region that UHA-R would bind on the genome) and append its reverse-complement sequence to the 5′ end of the DHA forward primer (DHA-F). This extended DHA-F will have a 5′ tail homologous to the end of the UHA fragment. Conversely, take the 18–220 bp sequence at the start of the DHA fragment (the region that DHA-F binds) and append its reverse-complement to the 5′ end of the UHA reverse primer (UHA-R). Now UHA-R carries a 5′ tail homologous to the beginning of the DHA fragment. By this design, the amplified UHA and DHA fragments will share an overlapping sequence, allowing them to anneal to each other. Each of these composite primers (UHA-R and DHA-F) will also be 38–42 bp long after adding the overlap sequence. (In summary, UHA-F and DHA-R include vector-binding sites, whereas UHA-R and DHA-F include an overlap between the two arms.)

Ensure that all primer sequences are unique (to avoid off-target binding) and check that adding the adapter/overlap sequences does not create secondary structures or extreme Tm differences. Synthesize the four primers (UHA-F, UHA-R, DHA-F, and DHA-R) with appropriate purification. To provide a more intuitive illustration of the homologous arm primer design method, we randomly selected a gene as a case study and presented the design approach in a Table 1.

2.2.2 | Genomic DNA Extraction

Isolate genomic DNA (gDNA) from the *Pseudomonas* strain to serve as the PCR template. Use a bacterial genomic DNA extraction kit or a standard phenol-chloroform protocol as

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TABLE 1 | A primer design example for UHA and DHA.

Sequence names	Primers
XmaI-F	*ATTCGAGCTCGGTACCCGGG*
PstI-R	*CCAAGCTTGCATGCCTGCAG*
UHA-F	*ATTCGAGCTCGGTACCCGGG*GGCAGGTAACGCGAATAGTC
UHA-R	CAGTGATAACGGTAGGCGTCCGTGTACCTCGAAAGGGGTA
DHA-F	TACCCCTTTCGAGGTACACGGACGCCTACCGTTATCACTG
DHA-R	*CCAAGCTTGCATGCCTGCAG*CCGACTTTCCAGCCTTCAGG

Note: The sequence "GGCAGGTAACGCGAATAGTC" in UHA-F is a 20 bp upstream primer derived from the 1000 bp upstream homologous arm of a specific gene in the genome. The sequence "CCGACTTTCCAGCCTTCAGG" in DHA-R is a 20 bp downstream primer derived from the 800 bp downstream homologous arm of the same gene in the genome.

preferred. For convenience, we use the FastPure Bacteria DNA Isolation Mini Kit (Vazyme, DC103) following the manufacturer's instructions. This kit includes cell lysis buffers and silica columns to purify DNA while removing RNA, proteins, lipids, and other contaminants. After extraction, verify the gDNA's quality and concentration (e.g., by Nanodrop spectrophotometry). A pure gDNA prep at a concentration of > 50 ng/μL works well for PCR amplification of the arms.

2.2.3 | PCR Amplification of UHA and DHA Fragments

Perform high-fidelity PCR to amplify the UHA and DHA fragments from the genomic DNA, using the primers designed above. We recommend a high-fidelity DNA polymerase to ensure accuracy (for seamless cloning without mutations). For example, using 2 × Phanta Max Master Mix (Dye Plus) (Vazyme, P525):

Prepare two PCR reactions (one for UHA, one for DHA), each 50 μL, with the following components:

1. 2 × Phanta Max Master Mix (Dye Plus): 25 μL
2. Forward primer: 2 μL
3. Reverse primer: 2 μL
4. Template genomic DNA: 1.5 μL
5. Nuclease-free ddH_2O: add to 50 μL total volume

Mix gently and briefly spin down. Perform PCR with a program suitable for the primers and fragment lengths, for example:

1. Initial denaturation: 95°C for 3 min
2. Denaturation: 95°C for 15 s
3. Annealing: 56°C–72°C for 15 s
4. Extension: 72°C for 60–90 s (about 1 kb per minute)
5. Repeat steps 2–4 for 25–35 cycles (30 cycles is a common starting point)
6. Final extension: 72°C for 5 min
7. Hold at 4°C

After PCR, run 5–10 μL of each reaction on an agarose gel (e.g., 1% gel) to verify amplification success. You should see clear bands of the expected sizes (UHA 0.8–1 kb, DHA 0.5–0.8 kb). If non-specific bands are present, you may need to optimize the PCR conditions or perform gel extraction.

Use the FastPure Gel DNA Extraction Mini Kit (Vazyme, DC301) to purify the correct PCR product bands for UHA and DHA. Excise the DNA band from the gel (if a clean single band, you can also directly purify the PCR using a PCR cleanup kit). Follow the kit protocol to bind, wash, and elute the DNA fragments. Measure the DNA concentration of the purified UHA and DHA fragments. Typically, yields of 20–200 ng/μL can be achieved, which is sufficient for the next cloning step.

2.3 | Plasmid Assembly (Ligation)

With the linearized vector (pT18mobsacB from Section 2.1.3) and the two homologous arm fragments (UHA and DHA from Section 2.2.3) in hand, the next step is to assemble them into a single recombinant plasmid. This can be done via traditional ligation (if complementary restriction sites were used) or via a one-step seamless cloning kit. We use the ClonExpress II One Step Cloning Kit (Vazyme, C112), which employs an exonuclease-based recombination to join fragments with overlapping ends.

Set up the one-step cloning reaction (10 μL total) on ice as follows:

1. 5 × ClonExpress II Buffer: 2 μL
2. Exnase II enzyme mix: 1 μL
3. Linearized pT18mobsacB vector: 62 ng
4. Purified UHA fragment: 20 ng
5. Purified DHA fragment: 16 ng
6. DEPC-treated water: add to 10 μL total volume

Mix the reaction gently and incubate at 37°C for 30 min. Then place the reaction on ice or at 4°C. The assembled product is a plasmid (pT18-UHA-DHA) that carries the upstream and downstream arms in place of the XmaI-PstI region of pT18mobsacB. You can store the assembly mixture at 4°C temporarily if not transforming immediately (the product is relatively stable for a few hours).

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2.4 | Transformation Into *E. coli* DH5α by Heat Shock

Introduce the assembled knockout plasmid into *E. coli* DH5α to propagate it. Use chemically competent DH5α cells for a heat-shock transformation:

Take 20–50 μL of competent DH5α cells (thawed on ice) and gently mix with 5–10 μL of the assembly reaction from Section 2.3. The weight of DNA should not exceed 5% of the competent cell volume to avoid inhibiting transformation. Keep the cell/DNA mixture on ice for 30 min (Chung et al. 1989).

Heat shock the cells by placing the tube in a 42°C water bath for 45–90 s (we typically use 45 s). Immediately transfer the tube back to ice for at least 2 min to recover. Then add 200 μL of LB broth (pre-warmed to room temperature, no antibiotic) to the cells. Incubate the tube at 37°C with shaking (200–220 rpm) for 1–1.5 h to allow expression of the antibiotic resistance gene.

After outgrowth, concentrate the cells by brief centrifugation (e.g., 5000 × g for 2 min) and resuspend the pellet in 100 μL of fresh LB. Plate the entire suspension onto an LB agar plate containing tetracycline (20 μg/mL). Spread evenly and incubate the plate at 37°C overnight.

The next day, numerous tetracycline-resistant *E. coli* colonies should be visible if the assembly was successful. These colonies contain the pT18mobsacB + UHA + DHA plasmid.

2.5 | Screening and Storage of Positive Clones

Screen the *E. coli* transformants to identify those with the correct recombinant plasmid. Pick 8–10 individual colonies from the tetracycline plate and perform colony PCR. For a quick screen, colony PCR can be done using primers flanking the insert region on the plasmid (use M13 universal primers). Successful clones should yield PCR bands of the expected size (The fragment length equals the sum of UHA and DHA).

Confirm the sequence of the cloned arms by Sanger sequencing. It is advisable to sequence through the junctions (at least one primer from the vector into UHA, and one from the vector into DHA, or simply UHA-F and DHA-R which will read outward into the vector) to ensure no mutations or knockouts occurred during PCR amplification or cloning.

Once a correct clone is identified (the plasmid carrying the exact UHA-DHA sequence flanking a knockout of the target gene), inoculate a culture of that *E. coli* clone and isolate the plasmid in larger quantity. This verified knockout plasmid will be used to transform *Pseudomonas*. Also, create a glycerol stock of the *E. coli* clone for long-term storage: mix 0.8 mL of overnight culture with 0.8 mL of sterile 50% glycerol in a cryovial and store at −80°C.

At this stage, we have constructed the suicide plasmid (pT18mobsacB carrying the homologous arms) for knocking out the target gene. The next steps outline how to introduce this plasmid into the *Pseudomonas* strain and isolate the gene knockout mutant.

3 | Construction of the *Pseudomonas* Knockout Mutant

3.1 | Electroporation

3.1.1 | Preparation of the Knockout Plasmid DNA

Retrieve the confirmed pT18mobsacB-UHA-DHA plasmid from the *E. coli* DH5α strain (from Section 2.5). If stored as a glycerol stock, streak it on an LB agar plate with tetracycline to obtain single colonies, then inoculate one colony into LB tetracycline broth (10 μg/mL) and grow overnight (37°C, 200 rpm). Extract the plasmid DNA using a plasmid miniprep kit (e.g., a high-purity plasmid extraction kit such as Biosharp BL1172B or any standard kit). Follow the kit's protocol to obtain a clean plasmid prep. Measure the concentration of the plasmid DNA, a yield > 300 ng/μL is desirable for efficient transformation into *Pseudomonas*. Keep the plasmid DNA on ice (or at −20°C if not using immediately).

3.1.2 | Preparation of *Pseudomonas* Electrocompetent Cells

To introduce DNA via electroporation, the *Pseudomonas* recipient must be made electrocompetent (Dennis and Sokol 1995) (capable of DNA uptake during a high-voltage pulse). The following protocol yields efficient electrocompetent cells for many *Pseudomonas* species:

1. Revive the culture: Start with a fresh culture of the *Pseudomonas* strain. If available as a glycerol stock, streak it onto an LB agar plate (no antibiotic) and incubate at an appropriate temperature (30°C for many environmental *Pseudomonas*) until single colonies form.
2. Overnight pre-culture: Inoculate a single colony into 5–10 mL of LB broth and grow overnight at 30°C with shaking (180–200 rpm).
3. Subculture into a larger volume: Next morning, dilute 1% of the overnight culture into 50–100 mL of fresh LB broth. Incubate at 30°C, shaking at 180 rpm, until the culture reaches mid-log phase (OD_{600} = 0.5–0.6). This may take a few hours (monitor the OD to avoid overgrowth).
4. Cold treatment: Immediately place the flask on ice. Keep the culture on ice for 15–30 min (All subsequent steps should be carried out with pre-chilled tubes/solutions on ice to maximize competency.)
5. Harvest cells: Transfer the culture to chilled centrifuge tubes and spin at 4°C, 5000 × g for 5 min. Carefully pour off the supernatant.
6. Wash cells: Gently resuspend the cell pellet in cold 10% glycerol (20 mL). Centrifuge again at 4°C, 5000 × g for 5 min. Remove supernatant. Repeat the wash with another

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20 mL of 10% glycerol (for a total of 3 washes). These washes remove salts that could cause arcing during electroporation and help place cells in a cryoprotective ion-free solution.

7. Final resuspension: After the final wash, resuspend the cell pellet in a small volume of cold 10% glycerol. For example, use 4 mL of 10% glycerol to concentrate the cells (this yields dense competent cells). Keep the suspension on ice.
8. Aliquot and store: Dispense 100 μL aliquots of the cell suspension into pre-chilled 1.5 mL microtubes. Work quickly to keep cells cold. Freeze the aliquots immediately in liquid nitrogen (snap-freeze) and store at −80°C. Cells prepared this way can be used immediately or stored for several months. Always thaw an aliquot on ice and use soon after thawing for best results.

3.1.3 | Electroporation of *Pseudomonas* With the Knockout Plasmid

Transform the *Pseudomonas* competent cells with the pT18mobsacB-UHA-DHA plasmid by electroporation:

1. Thaw a 100 μL aliquot of electrocompetent *Pseudomonas* cells on ice. To the chilled cells, add 5–10 μL of the purified plasmid DNA (from Section 3.1.1). Gently mix by tapping, do not pipette up and down (to avoid cell damage), and stay 10–30 min.
2. Transfer the cell/DNA mix to a pre-cooled electroporation cuvette (0.2 cm gap recommended for *Pseudomonas*). Tap the cuvette to settle the suspension evenly between the electrodes.
3. Electroporate the cells using settings optimized for your strain. A typical condition for *Pseudomonas* is 2.5 kV voltage, 25 μF capacitor, and 200 Ω resistance (which usually yields a time constant around 4–5 ms). *Pseudomonas* can tolerate 2.5 kV in a 0.2 cm cuvette.
4. Right after the pulse, immediately add 600–700 μL of ice-cold LB medium to the cuvette, and gently resuspend the cells by pipetting up and down a few times. Transfer the cell suspension to a 1.5 mL tube.
5. Incubate the cells at 30°C (or appropriate temperature for your strain) with shaking (200 rpm) for 2 h. This recovery allows the bacteria to express the plasmid's tetracycline resistance before plating.
6. After the outgrowth, concentrate the cells by centrifuging 800 μL at 6000 × g for 3–5 min. Discard 700 μL of the supernatant, leaving 100 μL. Resuspend the cells in the remaining 100 μL of medium.
7. Plate the entire 100 μL onto an LB agar plate containing tetracycline (20 μg/mL). If the medium allows, you can also spread on two plates (50 μL each) to avoid crowding. Incubate the plates at 30°C, overnight (18–24 h).

You should obtain tetracycline-resistant colonies of *Pseudomonas* on these plates. Each colony is presumed to be a single-crossover integrant where the plasmid has inserted into the chromosome (either at the UHA or DHA region). At this step, it is common to obtain only a few to a dozen single colonies on the selective plate.

3.1.4 | Verification of Single-Crossover Integrants

Confirm that the tetracycline-resistant *Pseudomonas* colonies indeed carry the integrated knockout plasmid (Huang and Wilks 2017) (and not, e.g., spontaneous Tet^R mutants, which are rare but possible). Perform colony PCR screening as follows:

1. Design a PCR strategy to distinguish single-crossover integration. One effective approach is to use a primer that binds on the plasmid outside the homologous region, paired with a primer on the chromosome within one of the homologous arms. For example, use a pT18mobsacB vector-specific forward primer (located in the plasmid backbone, just upstream of UHA insertion site in the MCS) and a reverse primer within the UHA sequence. This will amplify a junction fragment only if the plasmid has inserted via the UHA into the chromosome. Similarly, you can set up a reaction with a plasmid-specific reverse primer (downstream of DHA in the vector) paired with a forward primer within the DHA sequence, which would amplify the other junction if integration occurred via the DHA end.
2. Perform colony PCR by picking a small amount of each colony with a sterile toothpick, resuspending cells in 20 μL water, boiling (95°C for 10 min) to lyse, and using 1–2 μL of that as PCR template. Use a high-fidelity or standard Taq polymerase for a 1–2 kb amplification (adjust extension time accordingly). A correct single-crossover integrant will yield a PCR band of the expected size.

Analyze the PCR products on an agarose gel. Colonies that show the correct junction fragment are confirmed integrants. At this stage, each verified colony still contains the entire plasmid sequence integrated in its genome (the target gene is disrupted by the plasmid insertion, but not yet deleted). These integrants are tetracycline-resistant and still carry *sacB*. Choose one or two positive integrant colonies for the next step.

3.2 | Sucrose Counter-Selection

Perform counter-selection to select for clones that have excised the plasmid via a second homologous recombination (Anderson and Haj-Ahmad 2003), thereby removing the target gene:

1. Take a confirmed single-crossover *Pseudomonas* integrant colony and inoculate it into 3–5 mL of LB broth without antibiotic. Incubate at 30°C overnight with shaking. This overnight non-selective growth allows the cells to potentially undergo the second recombination and lose the plasmid (since there's no Tet pressure to keep it). The *sacB* gene is present, but in liquid LB without sucrose it is not causing lethality.

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2. The next day, dilute the culture 1:1000 in fresh LB. Spread 100 μL of the diluted culture onto an LB agar plate containing 20% (w/v) sucrose and no tetracycline. If the culture is very dense, you can also perform serial dilutions (e.g., 10^{-2}, 10^{-3}, 10^{-4}) and plate those to get well-isolated colonies. Incubate the sucrose plate at 30°C for 1–2 days.
3. After incubation, many sucrose-resistant colonies should be visible. These colonies arose from cells that lost the plasmid (since *sacB* would otherwise kill them). However, losing the plasmid can occur by two possible recombination outcomes: reversion to the wild-type gene or knockout of the target gene. We need to identify which colonies are true knockouts (target gene deleted).
4. To do this, perform a replica-plating or patching analysis for tetracycline sensitivity: pick at least 30 sucrose plate colonies and streak or patch each onto two new plates—one LB agar + tetracycline (20 μg/mL), and one LB agar + 20% sucrose (no antibiotic). Incubate both plates at 30°C overnight. Desired outcome: Clones that grew on sucrose (original plate) but do not grow on the tetracycline plate are candidates that have lost the plasmid entirely. In general, among 30 randomly selected single clones, at least one gene knockout strain can usually be obtained.

From this screening, identify colonies that are sucrose-resistant and tetracycline-sensitive (Suc^R Tet^S). These are the putative knockout mutants. Typically, if your homologous arms were designed correctly, a majority of Suc^R Tet^S clones will be the knockout of the target gene rather than the wild-type allele, but verification is needed in the next step.

3.3 | Screening and Sequencing of Knockout Mutants

Finally, confirm the gene knockout by PCR and sequencing. For each Suc^R Tet^S candidate from Section 3.2, perform diagnostic PCR with two sets of primers:

1. Knockout check primers: Use the outer homologous arm primers (the same UHA-F and DHA-R designed in Section 2.2.1, which anneal outside the target gene region) to amplify across the deleted region. In a true knockout mutant, these primers will amplify a shorter fragment (approximately the combined length of UHA + DHA, because the internal gene is deleted). In the wild-type genome, the same primers would amplify a larger fragment (UHA + target gene + DHA). For example, if the target gene was 1.2 kb, a knockout mutant might give a PCR band of 1.3 kb (just UHA + DHA plus any small scar if present), whereas wild-type would give 2.5 kb. On an agarose gel, compare the size to wild-type DNA PCR (run as a control if available) to see if the gene is gone.
2. Gene-specific primers: Use a primer pair that binds within the target gene's coding sequence (or one in the gene and one flanking, etc.) to check for the presence of the gene. In a knockout where the gene is deleted, this PCR should yield no product (or a very small fragment if only a portion remains). In the wild-type, it would yield the gene band (for instance, 1.2 kb). For example, you could use the original primers that were used to clone the arms (UHA-R and DHA-F which originally annealed just inside the gene boundaries)—in a successful knockout, those two would no longer find their binding sites correctly and would not amplify the full gene.

Perform these PCRs for each candidate clone. Analyze by electrophoresis: *Pseudomonas* knockout candidates should show the shorter UHA-DHA band and absence of the gene band, indicating the gene has been removed. For additional confidence, include a positive control (wild-type genomic DNA) in the PCR to ensure the gene-specific primers work (they should amplify the gene from wild-type but not from the mutant) (Figure 2).

Confirm one or two of the PCR-positive knockout strains by Sanger sequencing (Yao et al. 2017). Extract genomic DNA or PCR product from the candidate and sequence using, for example, the UHA-F and DHA-R primers (which will read

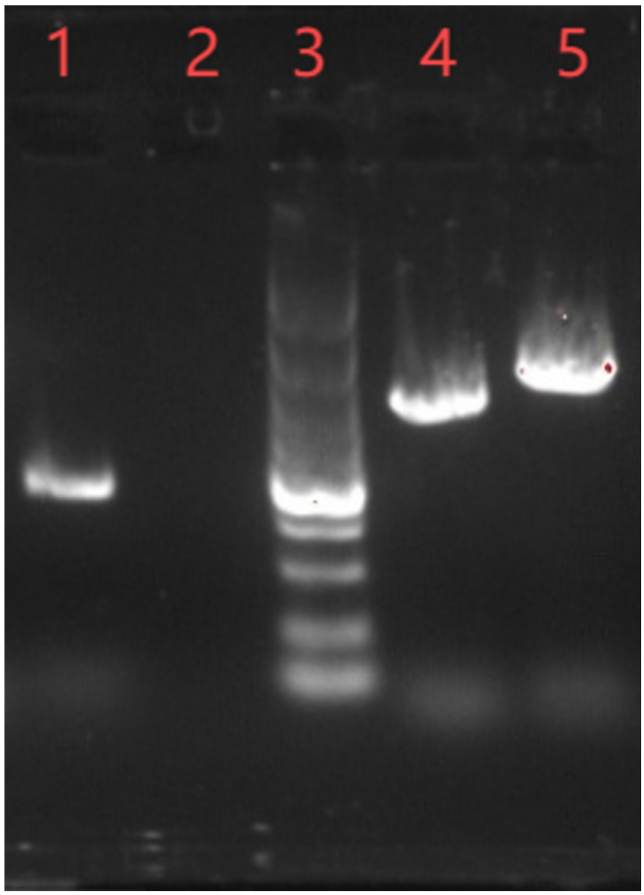

FIGURE 2 | Gel electrophoresis of gene knockout strains verified using two primer pairs (target gene ~1000 bp). Lane 1, PCR product (~1000 bp) amplified from the wild-type strain using gene-specific primers. Lane 2, No visible band from the knockout strain using the same gene-specific primers, indicating successful deletion. Lane 3, 5000 bp DNA marker. Lane 4, PCR product (~1800 bp) amplified from the knockout strain using UHA-F and DHA-R primers. Lane 5, PCR product (~2800 bp) amplified from the wild-type strain using UHA-F and DHA-R primers.

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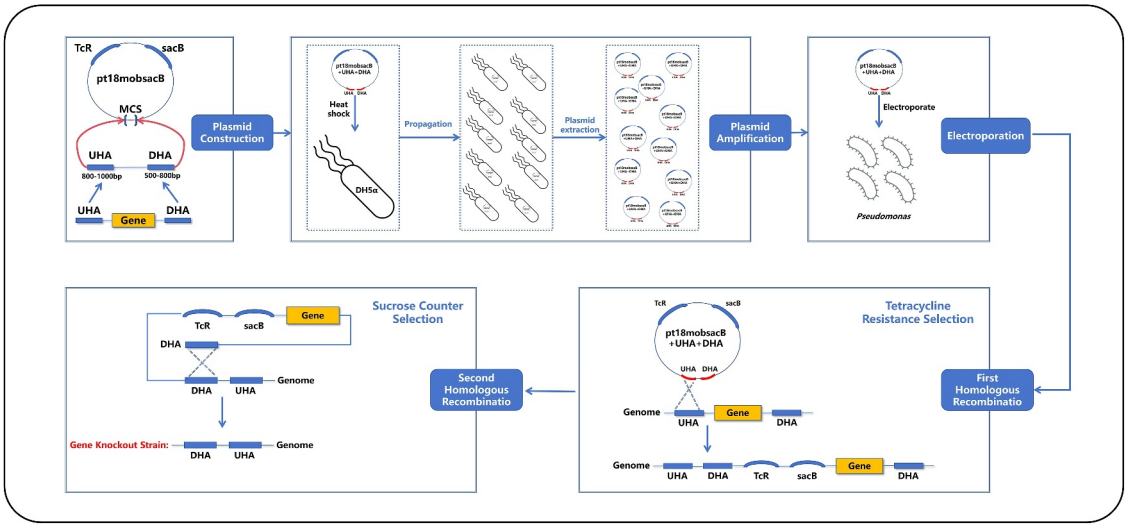

FIGURE 3 | Flowchart of gene knockout in *Pseudomonas* using the pT18mobsacB plasmid.

through the junction of the knockout). Align the sequence to the reference genome to verify that the target gene's coding region is absent and that the upstream and downstream arms are joined correctly with no unintended mutations.

Once confirmed, the gene knockout mutant is complete. It is prudent to cure the mutant of any plasmid remnants (in our case, the plasmid is already lost; the strain is tetracycline-sensitive). Finally, preserve the verified knockout strain by mixing a fresh culture with sterile glycerol (final 25%–50% glycerol v/v) and storing at −80°C.

Thus, we have completed the gene knockout, which involves five key components and principles (Figure 3). We hope that the flowchart will facilitate other researchers' understanding of the entire experimental procedure.

4 | Conclusion

In this guideline, we have presented a step-by-step method for creating an unmarked gene knockout in *Pseudomonas* using the pT18mobsacB suicide plasmid system. The protocol covers the construction of a recombinant plasmid carrying the flanking homologous arms of the target gene, as well as the procedures for transforming *Pseudomonas* and isolating knockout mutants via double-crossover homologous recombination. By following this method, researchers can reliably obtain *Pseudomonas* mutants with precise seamless gene knockouts. We demonstrated how to use positive selection (tetracycline resistance) to integrate the plasmid and negative selection (sucrose/*sacB*) to subsequently excise it, yielding mutants that lack the gene of interest while carrying no foreign resistance genes. This approach is highly reproducible and has broad applicability to various *Pseudomonas* species and other Gram-negative bacteria that accept the *mob-sacB* plasmids.

The ability to generate clean knockouts enables functional genomics investigations in *Pseudomonas*. Researchers can dissect metabolic pathways or regulatory networks by deleting specific genes and observing the resulting phenotypic changes. For instance, gene knockouts have been used to identify virulence factors in *P. aeruginosa* (by showing loss of toxin production and pathogenicity) (Shen et al. 2021) or to confirm the role of enzymes in biodegradation pathways (e.g., eliminating a key enzyme and abolishing the breakdown of a substrate) (Hachisuka et al. 2021). In the context of food microbiology, this protocol is valuable for exploring how certain microbial genes affect food spoilage, safety, or processing. One could knock out genes encoding heat-stable proteases in a spoilage *Pseudomonas* and assess the impact on dairy product shelf-life, or delete quorum-sensing regulators to study their role in biofilm formation on food equipment (Z. Wang et al. 2023). The insights gained can inform strategies to mitigate contamination and improve food preservation.

Overall, the gene knockout protocol described here provides a foundational tool for molecular microbiologists. Its utility and effectiveness have been demonstrated by the successful construction of *Pseudomonas* knockout strains in a relatively short time frame (often 1–2 weeks from plasmid construction to confirmed mutant). By enabling precise genetic knockouts, this method helps link genotype to phenotype, thereby advancing our understanding of *Pseudomonas* biology. We anticipate that the availability of such robust genetic techniques will continue to propel research in microbial physiology, pathogenesis, and biotechnological applications, ultimately contributing to innovations in food safety and biotechnology.

Author Contributions

Han Yang: writing – original draft, writing – review and editing, data curation, conceptualization, methodology, software. **Manxi Wu:** conceptualization, methodology, writing – review and editing, writing – original draft, data curation, software. **Jinping Cao:** resources, project administration, funding acquisition, supervision. **Chongde Sun:** supervision, resources, project administration, funding acquisition. **Yue Wang:** supervision, resources, visualization, funding acquisition, formal analysis, investigation, validation.

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Ethics Statement

The authors have nothing to report.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

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