Optimized protocols for ChIP-seq and deletion mutant construction in *Pseudomonas syringae*

Chromatin immunoprecipitation sequencing (ChIP-seq) is an efficient technique to identify the binding sites of transcription factors (TFs) in both eukaryotes and prokaryotes. However, its application in bacteria is very heterogeneous. In this protocol, we optimized the methods of ChIP-seq that can be widely applied to plant pathogens. We used homologous recombination to construct pK18mobsacB-Psph plasmid instead of restriction site ligation and replaced transconjugation with electroporation transformation in *Pseudomonas syringae* deletion mutant construction, which is more efficient and faster than previous methods.

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**Highlights**
This approach can be used to construct TF-overexpressed *Pseudomonas syringae* strain

Protocols for ChIP-seq library construction of *Pseudomonas syringae*

A simplified procedure to construct a deletion mutant of *Pseudomonas syringae*
Optimized protocols for ChIP-seq and deletion mutant construction in *Pseudomonas syringae*

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SUMMARY
Chromatin immunoprecipitation sequencing (ChIP-seq) is an efficient technique to identify the binding sites of transcription factors (TFs) in both eukaryotes and prokaryotes. However, its application in bacteria is very heterogeneous. In this protocol, we optimized the methods of ChIP-seq that can be widely applied to plant pathogens. We used homologous recombination to construct pK18mobsacB-*Psph* plasmid instead of restriction site ligation and replaced transconjugation with electroporation transformation in *Pseudomonas syringae* deletion mutant construction, which is more efficient and faster than previous methods. For complete details on the use and execution of this protocol, please refer to Shao et al. (2021).

BEFORE YOU BEGIN
Introduction
*Pseudomonas syringae* is a Gram-negative pathogenic bacterium, causing deadly diseases of more than 50 plants and huge economic lost in agriculture worldwide (Gonzalez et al., 2000; Hirano and Upper, 2000). *Pseudomonas syringae* relies on type III secretion system (T3SS) to invade hosts (Cunnac et al., 2009; Galan and Collmer, 1999). Transcription factors (TFs) control the rate of transcription by binding to specific downstream DNA sequences (Karin, 1990; Latchman, 1997), which are of great importance for regulation of virulence and metabolism in *Pseudomonas syringae*. We have identified the binding sites of nine T3SS TFs, RhpR, AefR, HrpS, Lon, OmpR, CbrB2, PhoP, PilR, and MgrA in *P. syringae* by ChIP-seq (Deng et al., 2010; Deng et al., 2014; Deng et al., 2009; Hua et al., 2020; Shao et al., 2021; Wang et al., 2018; Xiao et al., 2007; Xie et al., 2019; Zhou et al., 2016). However, the protocols of ChIP-seq are not unified in the study of plant pathogenic bacteria compared with eukaryotes.

In addition, phenotypes and gene expression level in deletion mutants are essential for determining the functions of TFs. In this protocol, we provided an optimized method for ChIP-seq in *P. syringae* and it can be used in other plant pathogenic bacteria. We also use an efficient homologous recombination strategy to construct the pK18mobsacB-*Psph* suicide plasmid instead of restriction site ligation, which simplifies the procedures in a previous method (McDowell, 2011).

Primer design
© Timing: 2–3 h
1. Primers design of the pHM1-TF (Labes et al., 1990) plasmids overexpressing each TF tagged by hemagglutinin (HA).

This STAR protocol uses cbrB2 (PSPPH_0857) as an example. The opening reading frame (ORF) of cbrB2 is amplified from *P. syringae* pv. *phaeseolicola* (*Psph*) chromosomal DNA using forward and reverse primers (pHM1-cbrB2-F and pHM1-cbrB2-HA-R). Primers are designed as the operation instruction of ClonExpress II One Step Cloning Kit. As shown in Figure 1A, the left homology arm of pHM1 plasmid (5′ATGACCATGATTACGCCAAGCTT3′) is added to the 5′ end of the forward primer. Then, the right homology arm of pHM1 plasmid and HA-tag sequence (5′GACCTGCAGGCA TGCAAGCTTTTAAGCGTAATCTGGAACATCGTATGGGTA3′) is added to the 3′ end of the reverse primer.

2. Primers design of *Psph* deletion mutant construction (Figure 1B).

Four pairs of primers are required for each mutant construction. The sizes of upstream and downstream fragments need to differ by 100–500-bp to differentiate them in agarose gel. Generally, these two fragments are about 1,000-bp and 1,500-bp, respectively.

   a. Upstream fragment primers. A pair of Upstream fragment primers are used to amplify the upstream fragment of cbrB2. The left homology arm of pK18mobsacB suicide plasmid (5′AAACAGCTATGACATGATTACGAATTC3′) is added to the 5′ end of the forward primer (pK18-cbrB2-Upstream fragment-F). The XbaI restriction site (CCTCTAGA) is added to the 3′ end of the reverse primer (pK18-cbrB2-Upstream fragment-R).

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**Figure 1. Primers design of pHM1-cbrB2-HA plasmid and *Psph* deletion mutant construction**

(A) Primers design of pHM1-cbrB2 plasmid.

(B) Primers design of *Psph* deletion mutant construction.
b. Downstream fragment primers. A pair of downstream fragment primers is used to amplify the downstream fragment of cbrB2. The XbaI restriction site (CCTCTAGA) is added to the 5' end of the forward primer (pK18-cbrB2-Downstream fragment-F). The right homology arm of pK18mobsacB suicide plasmid (5' ACGACCGCCATGCCAAAGCCT3') was added to the 5' end of the reverse primer (pK18-cbrB2-Downstream fragment-R).

c. A pair of verification primers (cbrB2-verify-F and cbrB2-verify-R) outside the upstream fragment reverse primer and the downstream fragment forward primer are designed to amplify the connected part of the upstream and downstream fragments.

d. A pair of qRT-PCR primers (cbrB2-RT-F and cbrB2-RT-R) designed to genomic location that has been deleted are used to verify the deletion.

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| HA Tag Monoclonal Antibody (1:10000 diluted with PBST) | Invitrogen | Catalog # 26183-1MG |
| Monoclonal Anti-HA-Agarose antibody produced in mouse (1:10000 diluted with PBST) | Sigma-Aldrich | Catalog # A209S |
| Bacterial and virus strains |        |            |
| E.coli DH5α (endA hsdR17 supE44 thi-1 recA1 gyrA relA1 D(lacZYA-argF) U169 deoR (φ80dlacD (lac2) M15)) | Tiangen Biotech | Catalog # CB101 |
| Pseudomonas syringae pv. phaseolicola 1448A (Psph) | (Deng et al., 2014) | N/A |
| Chemicals, peptides, and recombinant proteins |        |            |
| XbaI               | NEB    | Catalog # R0145M |
| EcoRI-HF           | NEB    | Catalog # R3101M |
| HindIII-HF         | NEB    | Catalog # R3104M |
| T4 DNA Ligase      | Takara | Catalog # 2011A |
| Taq Master Mix for PAGE | Vazyme | Catalog # P115-01 |
| Proteinase K stock | Tiangen | Catalog # RT403 |
| dNTP Mix           | NEB    | Catalog # N0475S |
| T4 DNA Polymerase  | NEB    | Catalog # M0203L |
| Klenow DNA polymerase (1 U/μL) | NEB | Catalog # M0210L |
| T4 polynucleotide kinase | NEB | Catalog # M0201L |
| dATP               | NEB    | Catalog # N0440S |
| Klenow exo         | NEB    | Catalog # M0212L |
| Phusion High-Fidelity PCR Master Mix | NEB | Catalog # M05315 |
| NEBNext® Multiplex Oligos for Illumina | NEB | Catalog # E6612A |
| Adaptor Dilution Buffer | NEB | Catalog # B1430S |
| Critical commercial assays |        |            |
| DNA Purification Kit | Tiangen | Catalog # DP214-03 |
| Rapid Mini Plasmid Kit | Tiangen | Catalog # DP105-03 |
| ClonExpress II One Step Cloning Kit | Vazyme | Catalog # C112-01 |
| QIAquick PCR Purification Kit | QIAGEN | Catalog # 28106 |
| QIAquick Gel Extraction Kit | QIAGEN | Catalog # 28706 |
| Oligo Clean & Concentrator | Zymo | Catalog # D4061 |
| Qubit™ dsDNA HS Assay Kit | Invitrogen | Catalog # Q32851 |
| Quick Ligation Kit | NEB    | Catalog # M2200L |
| Oligonucleotides |        |            |
| pHM1-cbrB2-F: ATGACCATGATTACGGCCCAAGCT | This study | N/A |
| TACCGTCAGAGAAACTGAA | | |
Note: Dissolve in 1 L ddH₂O and adjust the pH to 7.2. Add 15 g/L agar for solidified medium and autoclave. Prepare before use.
Luria-Bertani

| Reagent        | Final concentration | Amount |
|----------------|---------------------|--------|
| Tryptone       | N/A                 | 10 g   |
| Yeast extract  | N/A                 | 5 g    |
| NaCl           | N/A                 | 10 g   |
| Total          | N/A                 | 1 L    |

Note: Dissolve in 1 L ddH₂O and adjust the pH to 7.2. Add 15 g/L agar for solidified medium and autoclave. Prepare before use.

Lysis buffer basic

| Reagent            | Final concentration | Amount |
|--------------------|---------------------|--------|
| NaCl (5M)          | 0.14M               | 28 mL  |
| HEPES (1M)         | 15mM                | 15 mL  |
| EDTA pH8.0 (0.5M)  | 1mM                 | 2 mL   |
| 10% sodium deoxycholate | N/A             | 10 mL  |
| ddH₂O              | N/A                 | 945 mL |
| Total              | N/A                 | 1 L    |

Note: Stock needs to be autoclaved. Store at 4°C. Stable for 1 month.

Lysis buffer complete=IP buffer

| Reagent                          | Final concentration | Amount |
|----------------------------------|---------------------|--------|
| lysis buffer basic               | N/A                 | 45 mL  |
| 10% Triton X-100                 | 1%                  | 5 mL   |
| DTT (1M)                         | 0.5mM               | 25 μL  |
| cocktail-inhibitors (Roche)      | N/A                 | 1 tablet|
| Total                            | N/A                 | 50 mL  |

Note: Store at 4°C. Stable for 1 month.

TE buffer:

| Reagent            | Final concentration | Amount |
|--------------------|---------------------|--------|
| 1M Tris-HCl pH 8.0 | 10mM                | 500 μL |
| 0.5M EDTA          | 1mM                 | 100 μL |
| ddH₂O              | N/A                 | 49.4 mL|
| Total              | N/A                 | 50 mL  |

Note: Store at 25°C. Stable for 2–3 months.

Elution buffer 1

| Reagent            | Final concentration | Amount (volume) |
|--------------------|---------------------|-----------------|
| 0.5M EDTA          | 10mM                | 1 mL            |
| 20% SDS            | 1%                  | 2.5 mL          |
| 1 M Tris-HCl       | 50mM                | 2.5 mL          |
| ddH₂O              | N/A                 | 44 mL           |
| Total              | N/A                 | 50 mL           |
Note: Store at 25°C. Stable for 2–3 months.

| Elution buffer 2 | Final concentration | Amount (volume) |
|------------------|---------------------|-----------------|
| TE               | N/A                 | 14.5 mL         |
| 20% SDS          | 0.67%               | 0.5 mL          |
| Total            | N/A                 | 15 mL           |

Note: Store at 25°C. Stable for 2–3 months.

**STEP-BY-STEP METHOD DETAILS**

This STAR protocol uses TF CbrB2 as an example. The flow diagram of overexpressed TF strain construction and ChIP are showed in Figure 2.

![Flow diagram of overexpression of cbrB2, ChIP, and library construction](image)

**Figure 2.** The flow diagram of overexpression of cbrB2, ChIP, and library construction.
Overexpressed TF strain construction

© Timing: 4 days

Construction of pHM1-cbrB2-HA plasmid

1. Amplify the ORF of Psph cbrB2 gene from Psph chromosomal DNA using 20 μL PCR reaction (18 μL Taq premix, 0.5 μL pHM1-cbrB2-F primer, 0.5 μL pHM1-cbrB2-HA-R primer and 20 ng Psph chromosomal DNA). The concentration of primers is 10 μM.
   a. The following thermocycle program is recommended for PCR amplification.

| PCR cycling conditions | Temperature | Time | Cycles |
|------------------------|-------------|------|--------|
| Initial Denaturation   | 98°C        | 5 min| 1      |
| Denaturation           | 95°C        | 30 s | 25-35 cycles |
| Annealing              | 58°C        | 30 s |        |
| Extension              | 72°C        | 1 min/Kb |        |
| Final extension        | 72°C        | 5 min| 1      |
| Hold                   | 4°C         | Forever |        |

b. The PCR product is checked by 1% agarose gel. The band is specific and at the right size (1529 bp) (Figure 3A). The PCR product was purified by DNA purification kit.

2. Digest the pHM1 plasmid by HindIII. The pHM1 is extracted from the 1 mL mid-log phase liquid culture. Incubate 2 μg pHM1 plasmid with HindIII in a 50-μL reaction at 37°C for 1 h. The digested pHM1 plasmid is separated by 0.8% agarose gel. The band of linear plasmid is above the circular plasmid (Figure 3B). Extract the linear plasmid fragment from the gel by DNA purification kit. Elute with 30 μL ddH2O.

△ CRITICAL: The E. coli DH5α strain with pHM1 should be recovered on agar plate. The overnight (12–16) colony is cultured in 1ml LB broth to mid-log phase for plasmid extraction. Old bacteria (OD600 >1.0) are not recommended for plasmid extraction.

3. Recombine cbrB2 DNA fragment with linear pHM1 plasmid in a 10 μL reaction (50–100 ng HindIII-digested pHM1 plasmid fragment, 50 ng cbrB2 DNA fragment, 2 μL 5x CE II buffer and 0.5 μL Exnase II). Incubate the mixture at 37°C for 30 min.

4. Heat-shock transform all 10 μL recombination product into E. coli DH5α competent cell. The transformants are screened by LB agar plates with 100 μg/mL spectinomycin at 37°C for 12–16 h.

5. Choose two colonies and culture them in 2 mL LB broth with 100 μg/mL spectinomycin to mid-log phase and extract plasmids using the Rapid Mini Plasmid kit.

6. Screen the correct plasmids.
   a. The cbrB2 DNA fragment is amplified by PCR from the generated plasmids by corresponding primers (pHM1-cbrB2-F and pHM1-cbrB2-HA-R). The PCR product is checked by 1% agarose gel. The band is specific and at the right size (1529 bp) (Figure 3C).
   b. Plasmid sequencing is used to ensure the plasmid is correct.

   Note: We recommend to verify the recombinated pHM1 plasmid using PCR verification, because of poor visualization of enzyme digestion verification.

Transform pHM1-cbrB2-HA plasmid into Psph wild-type strain

8. Prepare Psph competent cell.
a. Culture Psph wild-type strain in 2 mL KB (King’B) (King et al., 1954) broth with 25 μg/mL rifampicin for 12–16 h with shaking at 220 rpm.
b. Gather bacterial cells by centrifugation at 2,400 × g for 5 min and dispose of the supernatant. Resuspend the pellets with 500 μL pre-cooled 10% aseptic glycerin. Centrifuge at 4°C (2,400 × g, 5 min) and dispose of the supernatant. Repeat at least three times.
c. Resuspend the pellets with 100 μL pre-cooled 10% aseptic glycerin and put it on ice.
9. Transform 50–100 ng pHM1-cbrB2-HA plasmid into the Psph competent cells prepared above by electroporation transformation (Electroporation Cuvettes: 0.1 cm gap, E=1.8 KV) after 20 min incubation on ice.
10. Add 1 mL KB to the reaction tube and culture it at 28°C for 2 h with shaking at 220 rpm.
11. Collect bacterial cells by centrifugation at 3,500 × g for 5 min and plate them onto KB agar plates with 25 μg/mL rifampicin and 100 μg/mL spectinomycin. Incubate the plates at 28°C for 36 h.
12. Choose single colonies and culture it in 1 mL KB with 25 μg/mL rifampicin and 100 μg/mL spectinomycin for 12–16 h with shaking at 220 rpm.

**Figure 3. The experimental results of pHM1-cbrB2-HA plasmid, ChIP, and library construction**

(A) PCR amplification of Psph cbrB2 gene. The band is specific and at the right size (1529 bp).
(B) pHM1 is digested by HindIII. The plasmid is digested to a linear fragment.
(C) PCR verification of pHM1-cbrB2-HA construction. Two pHM1-cbrB2-HA plasmids are at the right size of 1529 bp.
(D) Detection of the expression of CbrB2-HA protein in the OX-cbrB2 and Psph WT/pHM1 strain by Western blot.
(E) DNA bands after sonication.
(F) Size selection by 2% low melting point agarose gel.
(G) PCR amplification production of libraries.
Pause point: After mixing with 50% sterile glycerin by 1:1, the bacteria can be stored at −80°C.

13. Detect the expression of CbrB2-HA protein by Western blot.

Collect bacterial cells (200 μL overnight (12–16 h) KB cultures) by centrifugation at 13,800 × g for 1 min. Resuspend the pellets with 20 μL 50% SDS and incubate at 95°C for 15 min. The total bacterial lysates with same amounts of protein (50 μg) are loaded and separated by 10% SDS-PAGE to detect the expression of CbrB2-HA protein in Psph wild-type and cbrB2-overexpressing (OX-cbrB2) strain. As shown in Figure 3D, a specific band of 53.7 KDa in OX-cbrB2 was detected, while no band in Psph wild-type. Both strains are used for the subsequent ChIP-seq experiment.

ChIP

Timing: 3 days

14. Cross-linking. Culture Psph strains (OX-cbrB2 strain and WT/pHM1 strain) in 20 mL KB broth supplemented with rifampicin at 28°C for 12–16 h to OD_{600}=0.6 (Two biological replicates are required). Add 500 μL 39% formaldehyde to make the final concentration of formaldehyde 1%. Incubate at 28°C for 10 min with shaking at 220 rpm.

15. Stop crosslinking. Add 1.5 mL 2 M glycine to the Psph culture. Incubate at 28°C for 5 min with shaking at 220 rpm.

16. Collect sample. Collect bacteria by centrifugation (6,000 × g, 5 min, 25°C). Wash the pellets with 20 mL Tris buffer. Centrifuge (6,000 × g, 5 min, 25°C) to remove the supernatant. Resuspend the pellets with 1 mL Tris buffer and transfer to a new 1.5 mL EP tubes. Centrifuge (6,000 × g, 5 min, 25°C) to remove the supernatant.

Pause point: The pellets can be stored at −80°C before subsequent steps.

Fragmentation by sonication

17. Add 500 μL IP buffer to resuspend the pellets. Disrupt samples by sonication (sonicator manufacturer: SCIENTZ, model: JY96-IIN, power: 5%, mode: 5 s on, 5 s off, 3 min in total) in an ice bath.

△ CRITICAL: The sonication conditions need to be optimized according to the amount of bacteria.

18. Centrifuge the lysate solution (13,800 × g, 10 min) at 4°C. Use 1 μL supernatant to check the size of DNA fragments. The bands are between 100–300-bp by agarose gel electrophoresis (Figure 3E).

Incubate with antibody

19. Pretreat IgG1-HA-agarose-beads. Calculate the total amount of beads required (20 μL/sample). Centrifuge and remove the supernatant (13,800 × g, 1 min). Wash beads with 1 mL IP buffer at 4°C (13,800 × g, 1 min) for 5 times.

20. Add 20 μL beads to cell lysates and incubate overnight (or 6 h) with HulaMixer Sample Mixer at 4°C. Centrifuge and remove the supernatant (13,800 × g, 5 min, 4°C).

21. Wash beads.
   a. Wash beads with 1 mL lysis buffer (13,800 × g, 5 min) at 4°C for four times. Discard the supernatant.
b. Wash beads with 1 mL TE buffer (13,800 x g, 5 min) at 4°C for twice. Discard the supernatant.

22. Elution.
   a. Add 100 μL elution buffer 1 to beads. Mix well and incubate at 65°C for 10 min. Centrifuge (13,800 x g, 1 min) at 25°C and transfer the supernatant to a new 1.5 mL tube.
   b. Add 150 μL elution buffer 2 to beads and mix well. Centrifuge (13,800 x g, 1 min) at 25°C and collect supernatant to the tube in step 22a. (Total supernatant is 250 μL, named IP sample)

Cross-linking reversal

23. Incubate IP samples at 65°C for 6 h (or overnight).

Collect DNA

24. Prepare proteinase K solution (0.5 μL 20 mg/mL glycogen, 5 μL 20 mg/mL proteinase K and 244.5 μL TE).
25. Add 250 μL proteinase K solution to IP sample and incubate the mixture at 58°C for 2 h.
26. Add 55 μL 4M LiCl and mix well at 25°C.
27. Add 500 μL phenol-chloroform and mix well.
28. Centrifuge (13,800 x g, 3 min) at 25°C and collect supernatant to a new 1.5 mL tube.
29. Add 1 mL pre-cooled absolute ethanol and incubate for 30 min at −80°C. Centrifuge (13,800 x g, 30 min) at 4°C and discard the supernatant. Wash the precipitation with 1 mL 70% ethanol (13,800 x g, 5 min) at 4°C. Air-dry the precipitation with 25μL nuclease-free water.
30. Measure the DNA concentration with Qubit (model: dsDNA high sensitivity, dye: Qubit dsDNA HS Assay Kit).

Pause point: The DNA samples can be stored at −20°C.

ChIP-seq library construction

Timing: 1 days

End repair

31. Mix PCR reaction according to the following recipe. (5 μL T4 DNA ligase buffer with 10 mM ATP, 2 μL dNTP mix, 1 μL T4 DNA polymerase, 1 μL Klenow DNA polymerase (1 U/μL), 1 μL T4 polynucleotide kinase, 30 μL ChIP enriched DNA (10–30 ng, add H2O to 30 μL), 10 μL nuclease-free H2O).
32. Incubate at 20°C for 30 min.
33. Use QIAquick PCR purification kit to clean the PCR reaction. Elute with a 34 μL elution buffer.

Add “A” bases to the 3’ end of the DNA fragments

34. Mix the PCR reaction according to the following recipe. (5 μL Klenow buffer, 10 μL dATP, 1 μL Klenow exo (3’ to 5; exo minus), 34 μL DNA sample form Step 33).
35. Incubate at 37°C for 30 min.
36. Use Zymo Oligo Clean & Concentrator to concentrate the volume to 10 μL.

Ligate adapters to DNA fragment

37. Mix reaction according to the following recipe. (15 μL Quick ligase buffer, 1 μL Diluted oligos for Illumina (10–30 ng, dilute with adapter dilution buffer), 4 μL Quick ligase, 4 μL DNA sample)
38. Incubate at 25°C for 15 min.
39. Add 1 μL User enzyme and incubate at 37°C for 15 min.
40. Use Zymo Oligo Clean & Concentrator to concentrate the volume to 15 μL.

**Size selection**

41. Mix DNA sample with 6× loading and run a 2% low melting point agarose gel electrophoresis (90 V, 25 min) to 100–200-bp separated.
42. Cut the gel in 150–250-bp size and extract the DNA by QIAquick Gel Extraction kit. Elute with 72 μL EB. DNA bands cannot be seen at this step (Figure 3F).

**PCR amplification**

43. DNA fragments in step 42 are amplified by PCR.
   a. Mix PCR reaction according to the following recipe. (25 μL NEB Phusion high-fidelity PCR master mix, 15 μL cDNA, 3.1 μL Universal Primer (0.125 uM), 3.1 μL Index Primer (0.125 uM), 3.8 μL H2O)
   b. The following thermocycle program is recommended for PCR amplification.

| PCR cycling conditions | Temperature | Time   | Cycles |
|------------------------|-------------|--------|--------|
| Initial Denaturation   | 98°C        | 30 s   | 1      |
| Denaturation           | 95°C        | 10 s   | 17 cycles |
| Extension              | 72°C        | 30 s   |        |
| Final extension        | 72°C        | 10 min | 1      |
| Hold                   | 4°C         | forever|        |

44. Mix DNA sample with 6× loading and run a 2% low melting point agarose gel electrophoresis (90 V, 25 min). The band is between 200–300-bp (Figure 3G).
45. Cut the band between 200–300-bp from the gel and extract the DNA by QIAquick Gel Extraction kit. Elute with 30 μL EB. Measure the DNA concentration with Qubit.

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**Pause point:** The DNA samples can be stored at –20°C. The samples will be sent for sequencing by Illumina HiSeq 2000 system.

**Psph deletion mutant construction**

The flow diagram of mutant construction is showed in Figure 4.

**pK18mobsacB-TF-upstream-downstream suicide plasmid construction**

- **Timing:** 2 days

46. Amplify the cbrB2 upstream and downstream fragment form Psph chromosomal DNA by PCR.
   a. Set up a PCR reaction in a 200 μL PCR tube according to the following recipe. (18 μL Taq premix, 0.5 μL cbrB2-Upstream/Downstream fragment-F primer, 0.5 μL cbrB2-Upstream/Downstream fragment-R primer, 20 ng Psph chromosomal DNA)
   b. The following thermocycler program is recommended for PCR amplification.
c. The PCR product is checked by 1% agarose gel. The upstream and downstream fragments are specific and at the size of 1491 bp and 965 bp, respectively (Figure 5A).

d. Mix upstream and downstream fragments together. Purify upstream and downstream fragment mixture by DNA purification kit. Elute with 30 μL ddH2O.

| Steps                  | Temperature | Time     | Cycles |
|------------------------|-------------|----------|--------|
| Initial Denaturation   | 98°C        | 5 min    | 1      |
| Denaturation           | 95°C        | 30 s     | 25–35 cycles |
| Annealing              | 58°C        | 30 s     |        |
| Extension              | 72°C        | 1 min/kb |        |
| Final extension        | 72°C        | 5 min    | 1      |
| Hold                   | 4°C         | Forever  |        |
Figure A: Gel electrophoresis showing fragments of DNA.

Figure B: Gel electrophoresis of EcoRI-HindIII digested pK18mobscB.

Figure C: Gel electrophoresis of cbrB2-verify-F/R.

Figure D: Diagram indicating restriction enzyme digestion sites and distances of upstream and downstream fragments.

Figure E: Gel electrophoresis comparing ΔcbrB2 and WT chromosomal DNA.

Figure F: Bar graph showing relative mRNA levels in KB (fold change) for Psoph WT, ΔcbrB2-1, ΔcbrB2-2, and ΔcbrB2-3.
47. Digest upstream and downstream fragment mixture in step 46d with the XbaI enzyme (27 μL purified DNA, 2.6 μL cutsmart buffer and 0.4 μL XbaI enzyme). Incubate the reaction for at least 2 h at 37°C.

48. Clean the digestion reaction using DNA purification kit. Elute with 30 μL ddH2O.

49. Ligate the purified DNA fragment with T4 ligase (2.5 μL DNA, 0.5 μL T4 ligase buffer, 0.25 μL T4 ligase and 1.75 μL ddH2O). Incubate 3 h at 16°C.

50. Incubate 2 μg pK18mobsacB plasmid with EcoRI and HindIII in a 50 μL reaction at 37°C for 1 h. The digested pK18mobsacB plasmid is separated by 0.8% agarose gel. The band of linear plasmid is above the circular plasmid (Figure 5B). Extract the linear plasmid fragment from the gel by DNA purification kit. Elute with 30 μL ddH2O.

51. Recombine the ligated DNA with digested pK18mobsacB plasmid in a 10 μL reaction (100 ng lined pK18mobsacB plasmid, 3 μL ligated DNA, 2 μL 5× CE II buffer, 0.5 μL Exnase II). Incubate the reaction for 30 min at 37°C.

52. Transform the recombination products into E. coli DH5α competent cell by heat-shock transformation.

53. Transformants are screened by LB agar plates with 100 μg/mL kanamycin incubating the plates at 37°C for 12–16 h.

54. Choose two colonies and culture them in 2 mL LB broth with 100 μg/mL kanamycin culture at 37°C for 12–16 h shaking with 220 rpm.

55. Extract the plasmid DNA from the cultures by using the Rapid Mini Plasmid kit.

56. Screen the correct plasmids.
   a. PCR verification. The DNA sequence is amplified by PCR from the generated plasmids by corresponding primers (cbrB2-verify-F/R). The PCR product is checked by 1% agarose gel. The band is specific and at the correct size (411 bp) (Figure 5C).
   b. Enzyme digesting verification. Digest 2 μL of plasmid DNA by XbaI or XbaI, EcoRI, and HindIII. Incubate for at least 1 h at 37°C. The circular plasmid is digested to one linear fragment by XbaI and four linear fragments by XbaI, EcoRI and HindIII (Figure 5D).
   c. Plasmid sequencing is used to ensure the plasmid is correct.

**Psph deletion mutant construction**

© Timing: 5 days

57. Prepare Psph competent cell as above mentioned in step 8 TF-overexpressing strain construction.

58. Transform 50–100 ng pK18mobsacB-up-down plasmid into Psph-WT competent cell prepared above by electroporation transformation (Electroporation Cuvettes: 0.1 cm gap, E=1.8 KV) after 20 min incubation.

59. Add 1 mL KB with 25 μg/mL rifampicin to the reaction tube and culture it at 37°C for 2 h shaking with 220 rpm.

60. Collect the bacterial cells by centrifugation at 3,500 × g for 5 min and plate them onto KB agar plates with 25 μg/mL rifampicin and 100 μg/mL kanamycin. Incubate the plates at 28°C for 36 h.

61. Choose single colony and culture it in 1 mL KB broth with 25 μg/mL rifampicin and 100 μg/mL kanamycin for 12–16 h shaking with 220 rpm.
62. Streak plate the mutants with KB 5% sucrose agar plates with 25 μg/mL rifampicin. Incubate the plates at 28°C for 36 h.
63. Spot several colonies on KB+25 μg/mL rifampicin and KB+25 μg/mL rifampicin +100 μg/mL kanamycin agar plates. Choose colonies which grow on KB+25 μg/mL rifampicin plates but not on KB+25 μg/mL rifampicin +100 μg/mL kanamycin plates to do subsequent PCR screen.
64. Screen the correct cbrB2 mutants by PCR.
   a. The DNA fragments are amplified by PCR from the chromosomal DNA of cbrB2 mutants and WT by corresponding primers (cbrB2-verify-F/R), respectively. The PCR product is checked by 1% agarose gel. As shown in Figure 5E, the PCR product of cbrB2 mutant is 411 bp while the PCR product of WT is 1712 bp.
   b. Use qRT-PCR to check the loss of cbrB2 in the cbrB2 mutant strain by corresponding primers (cbrB2-RT-F/R). As shown in Figure 5F, the expression of cbrB2 is not detected in three deletion mutants of cbrB2.

EXPECTED OUTCOMES
We have overexpressed HA-tagged cbrB2 in P. syringae. Western blot shows CbrB2-HA expresses in the OX-cbrB2 strain (Figure 3D). Then, we have done ChIP-seq of OX-cbrB2 strain and Psph-WT/ phM1 strain. First, the DNA is between 100–300 bp size after sonication (Figure 3E). The DNA band is invisible in the DNA size selection (step 41) (Figure 3F), and DNA in 150–250 bp size is extracted. The final result indicates that ChIP-seq library DNA is between 200–300 bp for sequencing (Figure 3G). The final outcome DNA is 10–20 ng when the input DNA is 10–30 ng in step 31.

We have constructed Psph cbrB2 deletion mutant following this protocol. First, the upstream and downstream fragment of cbrB2 ORF are amplified (Figure 5B). After homologous recombination and heat-shock transformation, pK18mobsacB-cbrB2-upstream-downstream plasmid is constructed and the upstream and downstream connected part can be amplified using cbrB2 verification primers (Figure 5C). At the same time, the XbaI, EcoRI and HindIII sites on pK18mobsacB-cbrB2-upstream-downstream plasmid can be digested (Figure 5D), suggesting that the ligated upstream and downstream fragments of cbrB2 are successfully connected to the lined plasmids. In step 64, We have amplified six colonies and one Psph-WT strain using cbrB2 verification primers, and all the bands of colonies show the correct size (accuracy: 100%) (Figure 5E). Then three of cbrB2 mutants have been chosen to do qRT-PCR. As the result, the expression of cbrB2 is not detected in the correct cbrB2 mutant strain compared with the Psph-WT strain (accuracy: 100%) (Figure 5F).

In addition, compared with the previous method (McDowell, 2011), the present protocol improves the screen accuracy and simplify the experimental steps in the following ways: 1) We use the ligated DNA in step 49 directly and omit the gel purification of the ligated band (upstream-downstream fragment) in this protocol. 2) We use homologous recombination strategy to insert the ligated DNA into pK18mobsacB plasmid instead of restriction sites ligation to improve efficiency (step 51). 3) We construct clean mutants without antibiotic marker cassette. 4) Electroporation transformation is used to transform pK18mobsacB-TF-upstream-downstream plasmid to Psph instead of transconjugation. 5) Previous protocol takes 8 days to construct mutant by transconjugation, while 2 days are needed in electroporation transformation in our protocol. Besides, LM medium and E.coli S17-1 must be prepared. 6) Streak plate method should be used to isolate transconjugants instead of direct coating method.

LIMITATIONS
This protocol is mainly for ChIP-seq and mutant construction of P. syringae. The experimental conditions and materials may need to be optimized for other bacteria, including medium, plasmid, antibodies, culture conditions and sonication conditions.
TROUBLESHOOTING

Problem 1
The DNA band is not at the correct size after sonication (step 18).

Potential solution
Optimize sonication conditions (power, number of cycle and working mode) according to different bacteria and bacterial densities.

Problem 2
The weight of DNA in the IP sample (>10 ng) is not enough to do further library construction or below the detection limitation of Qubit Kit (step 30).

Potential solution
Increase the bacterial culture in step 14 or the amount of antibody in step 19. Besides, optimize sonication conditions to make the DNA band in 100–300 bp (step 17) can alleviate this problem.

Problem 3
Unexpected enrichment is observed in sequenced libraries (step 44).

Potential solution
Reduce the bacterial culture and the amount of antibody (step 14 and step 19). Reduce cross-linking time because over cross-linking can cause non-specific binding. Besides, please be care to cut the gel in 150–250-bp size (step 42).

Problem 4
The library complexity is low after sequencing.

Potential solution
Increase the amount of antibody in step 19 or use the fewest possible PCR amplification cycles during ChIP-seq library construction (step 43) to get a final DNA yield appropriate for sequencing.

Problem 5
The PCR bands are not single (step 1 and step 46).

Potential solution
Optimize PCR cycling program (increase annealing temperature) or gel purify the correct band.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Xin Deng (xindeng@cityu.edu.hk).

Materials availability
This study did not generate new unique reagents.

Data and code availability
This study did not generate new datasets or codes.

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AUTHOR CONTRIBUTIONS
X.D. and X.S. supervised the study. C.Y., X.S., and J.L. carried out the experiments. C.Y., X.S., and X.D. wrote the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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