

Development of a Gene Replacement Method for the Filamentous Bacterium *Leptothrix cholodnii* SP-6

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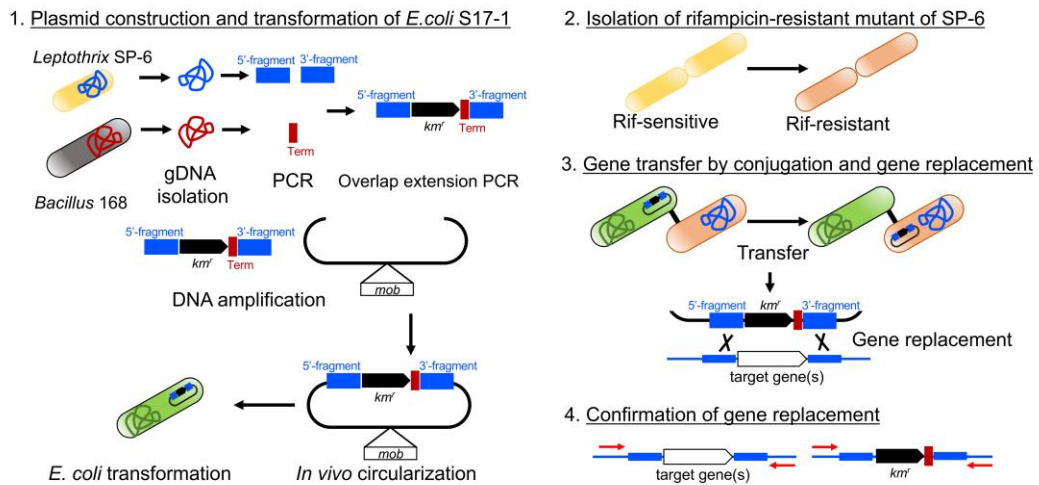
Abstract

Genetic strategies such as gene disruption and fluorescent protein tagging largely contribute to understanding the molecular mechanisms of biological functions in bacteria. However, the methods for gene replacement remain underdeveloped for the filamentous bacteria *Leptothrix cholodnii* SP-6. Their cell chains are encased in sheath composed of entangled nanofibrils, which may prevent the conjugation for gene transfer. Here, we describe a protocol optimized for gene disruption through gene transfer mediated by conjugation with *Escherichia coli* S17-1 with details on cell ratio, sheath removal, and loci validation. The obtained deletion mutants for specific genes can be used to clarify the biological functions of the proteins encoded by the target genes.

Keywords: *Leptothrix cholodnii* SP-6, Gene transfer, Conjugation, *Escherichia coli* S17-1, Calcium depletion

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Graphical overview



An overview of the entire protocol

Background

Leptothrix species are microorganisms with a long history as research targets. The type strain, *L. ochracea*, was reported in the late 18th century (Spring, 2006) and, since then, has attracted much attention from researchers in the fields of microbiology and environmental science. *Leptothrix* ubiquitously inhabits seeps, streams of iron-rich groundwater, and activated sludge. *Leptothrix* divides to produce cell chains encased with entangled nanofibrils termed *sheaths* and eventually generates mats composed of porous networks of cell chains (Spring, 2006). Over the time of mat aging, sheaths are encrusted with inorganic particles, especially iron oxide, resulting in the production of rigid microtubes (Furutani et al., 2011). Focusing on this innate characteristic, *Leptothrix* has been utilized for the removal of metal contaminations such as iron and manganese from groundwater in water purification plants (Kunoh et al., 2015a). However, overgrowth of filamentous bacteria, including *Leptothrix*, often results in clogging of water distribution pipes and bulking of activated sludge in wastewater treatment plants (van der Waarde et al., 2002). Therefore, understanding the functions of key proteins responsible for nanofibril formation and entanglement is significant for the artificial control of their filamentous growth.

The next-generation sequencing technologies that have been developed recently enable us to freely access significant data of the whole genome sequences existing in a wide range of organisms, from prokaryotes to eukaryotes. For *Leptothrix* species, the complete genome sequence of *L. cholodnii* SP-6 (hereafter SP-6) was published by the US DOE Joint Genome Institute (Copeland et al., 2008) [GenBank (accession: GCA_000019785.1)], which opened new doors for understanding the gene functions and interactions of this strain. To date, a gene replacement system for *Leptothrix* species remains unpublished, except for the sheath-less strain *L. discophora* SS1 (Bocioaga et al., 2014). Although we attempted to perform gene transfer of SP-6 mediated by conjugation with *Escherichia coli* S17-1 by following their report, the efficiency of gene replacement was quite low relative to that of SS1. We therefore considered the possibility that the sheath structure of SP-6 might interfere with the conjugation with *E. coli* cells, and eventually the incorporation of extracellular DNA into SP-6 cells.

We previously reported that sheath structure plays a critical role in holding aligned cell chains of SP-6 (Kunoh et al., 2020), and that calcium depletion frequently results in breakage of cell filaments of SP-6 due to failure in the production of sheath structures encasing the cell chains (Kunoh et al., 2021). From these findings, we hypothesized that preculturing SP-6 cells in a calcium-lacking medium might enhance the accessibility of *E. coli* cells to SP-6 cells for conjugation, which results in efficient gene transfer and subsequent gene replacement of SP-6 cells.

Here, we describe a protocol for gene replacement in SP-6 covered with sheaths (Graphic abstract), which was modified from that reported previously (Yoshihara, 2002). This protocol is expected to contribute to efficient systematic genetic analyses of SP-6, including genes responsible for sheath formation (Kunoh et al., 2022).

Materials and Reagents

Chemical compounds

1. Kanamycin sulfate (FUJIFILM Wako Pure Chemical Co., catalog number: 117-00341)
2. Ampicillin sodium salt (FUJIFILM Wako Pure Chemical Co., catalog number: 012-23303)
3. Rifampicin (Nacalai Tesque Inc., catalog number: 30259-94)
4. Ammonium sulfate [(NH₄)₂SO₄] (Nacalai Tesque Inc., catalog number: 02633-15)
5. Magnesium sulfate heptahydrate (MgSO₄·7H₂O) (FUJIFILM Wako Pure Chemical Co., catalog number: 131-00405)
6. Calcium chloride dihydrate (CaCl₂·2H₂O) (Nacalai Tesque Inc., catalog number: 031-00435)
7. Potassium dihydrogen phosphate (KH₂PO₄) (Nacalai Tesque Inc., catalog number: 28721-55)
8. Sodium phosphate dibasic heptahydrate (Na₂HPO₄·7H₂O) (Nacalai Tesque Inc., catalog number: 31725-15)
9. 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Nacalai Tesque Inc., catalog number: 17514-86)
10. Iron(II) sulfate (FeSO₄) (FUJIFILM Wako Pure Chemical Co., catalog number: 094-02942)
11. Sodium pyruvate (FUJIFILM Wako Pure Chemical Co., catalog number: 191-03061)
12. Sodium hydroxide (NaOH) (Nacalai Tesque Inc., catalog number: 31511-05)
13. Sodium chloride (NaCl) (Nacalai Tesque Inc., catalog number: 31319-45)
14. Hexadecyltrimethylammonium bromide (CTAB) (Nacalai Tesque Inc., catalog number: 07906-82)
15. Sodium dodecyl sulfate (SDS) (Nacalai Tesque Inc., catalog number: 30400-85)
16. Phenol/chloroform/isoamyl alcohol (25:24:1) (FUJIFILM Wako Pure Chemical Co., catalog number: 311-90151)
17. Chloroform (Nacalai Tesque Inc., catalog number: 08401-65)
18. Isopropanol (Nacalai Tesque Inc., catalog number: 29113-95)
19. Ethanol (Nacalai Tesque Inc., catalog number: 14713-95)
20. Sodium acetate trihydrate (CH₃COONa·3H₂O) (Nacalai Tesque Inc., catalog number: 31115-05)
21. Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (Nacalai Tesque Inc., catalog number: 35434-21)
22. Ethylenediaminetetraacetic acid tetrasodium salt (EDTA-4Na) (FUJIFILM Wako Pure Chemical Co., catalog number: 349-01885)
23. 35% hydrochloric acid (HCl) (Nacalai Tesque Inc., catalog number: 18321-05)
24. Acetic acid (Nacalai Tesque Inc., catalog number: 00212-85)
25. Polyethylene glycol 8000 (PEG8000) (MP Biomedicals LLC., catalog number: 195445)
26. Glycerol (Nacalai Tesque Inc., catalog number: 17017-35)
27. Dimethyl sulfoxide (DMSO) (Nacalai Tesque Inc., catalog number: 13407-45)
28. Potassium chloride (KCl) (Nacalai Tesque Inc., catalog number: 28514-75)
29. Magnesium chloride (MgCl₂) (Nacalai Tesque Inc., catalog number: 20935-05)
30. D-glucose (Nacalai Tesque Inc., catalog number: 16805-35)
31. Agarose S (FUJIFILM Wako Pure Chemical Co., catalog number: 312-01193)
32. 1 kb Plus DNA ladder (Thermo Fisher Scientific, catalog number: 10787018)
33. Ethidium bromide solution (10 mg/mL) (Nacalai Tesque Inc., catalog number: 14631-94)
34. Green nucleic acid stain (Midori Green Advance, Nippon Genetics Co. Ltd., catalog number: NE-MG04)
35. Liquid nitrogen (Air Water Inc., YJ code number: 799070CX1020)

Culture media

1. BD Difco LB broth, Lennox (Thermo Fisher Scientific, catalog number: 240230)
2. BD Bacto agar (Thermo Fisher Scientific, catalog number: 214010)
3. BD Bacto yeast extract (Thermo Fisher Scientific, catalog number: 212750)
4. BD Bacto tryptone (Thermo Fisher Scientific, catalog number: 211705)
5. BD Difco nutrient broth (Thermo Fisher Scientific, catalog number: 234000)

Vitamins

1. D-biotin (Nacalai Tesque Inc., catalog number: 04822-04)
2. Folic acid (Nacalai Tesque Inc., catalog number: 16221-91)
3. Thiamine hydrochloride (HCl) (Nacalai Tesque Inc., catalog number: 36319-24)
4. D-calcium pantothenate (Nacalai Tesque Inc., catalog number: 26004-44)
5. Vitamin B12 (Nacalai Tesque Inc., catalog number: 36323-96)
6. Riboflavin (Nacalai Tesque Inc., catalog number: 36322-51)
7. Nicotinic acid (Nacalai Tesque Inc., catalog number: 24326-52)
8. Pyridoxine hydrochloride (Nacalai Tesque Inc., catalog number: 29611-81)
9. p-aminobenzoic acid (Nacalai Tesque Inc., catalog number: 01930-32)

Enzymes

1. Proteinase K (FUJIFILM Wako Pure Chemical Co., catalog number: 166-28913)
2. Ribonuclease A (FUJIFILM Wako Pure Chemical Co., catalog number: 182-01493)
3. Lysozyme from egg white (FUJIFILM Wako Pure Chemical Co., catalog number: 122-02673)
4. Tks Gflex DNA polymerase (Takara Bio Inc., catalog number: R060A)
5. PrimeStar Max DNA polymerase (Takara Bio Inc., catalog number: R045A)
6. FastDigest restriction enzymes *Bam*HI, *Hind*III, and *Sma*I (Thermo Fisher Scientific, catalog numbers: FD0054, FD0504, FD0663)

Kits

1. Gel Loading Buffer (10×) (Thermo Fisher Scientific, catalog number: 10816015)
2. Gel Extraction kit (Promega Co., Wizard® SV Gel and PCR Clean-Up System, catalog number: A9282)
3. Plasmid Extraction kit (Merck KGaA, GenElute™ Plasmid Miniprep Kit, catalog number: PLN350-1KT)
4. DNA Ligation kit <Mighty Mix> (Takara Bio Inc., catalog number: 6023)

Materials

1. 50 mL polypropylene tube (IWAKI AGC Techno Glass Co., Ltd., catalog number: 2345-050)
2. 15 mL polypropylene tube (IWAKI AGC Techno Glass Co., Ltd., catalog number: 2425-015)
3. 1.5 mL centrifuge tube (Midland Scientific, catalog number: 311-08-052)
4. PCR microtube (Bio Regenerations Co., Ltd., catalog number: BRG-DC-01)
5. φ 90 × 15 mm sterilized dish (RIKAKEN Holdings Co., Ltd, catalog number: RSU-SP915-2)
6. 10 and 30 mL syringes (TERUMO Co., Ltd., catalog numbers: SS-10ELZ, SS-30ESZ)
7. 25 G × 1 inch injection needle (TERUMO Co., Ltd., catalog number: NN-2525R)
8. Stericup quick release-HV sterile vacuum filtration system (Merck Millipore, catalog number: S2HVVU05RE)
9. MF-Millipore membrane filter, 0.22 μm pore size (Merck Millipore, catalog number: GSWP04700)
10. Toothpicks (e.g., Yanagi Products Co., Ltd., DN Tsumayouji L)
11. 0.22 μm syringe filter (Sartorius AG, catalog number: S7597-FXOSK)
12. Vacuum filtration device (Merck Millipore, catalog number: SCGPS02RE)

13. 2.0 mL stock tube (Sarstedt AG, catalog number: 72.694.107.03)
14. IWAKI 20 mL glass test tube (ϕ 16.5 × 165 mm) (AGC Techno Glass Co., Ltd., catalog number: TEST16.5NP)
15. Test-tube closures (ϕ 17–18 mm) (LÜDI SWISS AG, catalog number: 08418)
16. Erlenmeyer flask (200 mL) (AGC Techno Glass Co., Ltd., catalog number: 4980FK200)
17. Silicon stopper (Shin-Etsu Polymer Co., Ltd., catalog number: T-38)
18. 100–1,000 mL medium bottles (AGC Techno Glass Co., Ltd., catalog numbers: 1516/04D, 1516/08D, 1516/10D)
19. Cuvette for electroporation (2 mm gap) (NEPA GENE Co. Ltd., catalog number: EC-002S)

Bacterial strains

1. *Leptothrix cholodnii* SP-6 (ATCC strain 51168)
2. *Bacillus subtilis* 168 (Lab stock)
3. *Escherichia coli* S17-1 (Lab stock)
4. *Escherichia coli* DH5 α (Lab stock)
5. *Escherichia coli* SN1187 [National BioResource Project – *E. coli* Strain (National Institute of Genetics, Mishima, Japan)]

Plasmids

1. pSUP5011::Tn5-mob (Lab stock, DSMZ collection 5167)
2. pUC18 (Takara Bio Inc., catalog number: 3218)

Prepared recipes (see Recipes below)

1. MSVP (ATCC 1917) medium
2. LB medium
3. SOC medium
4. NB medium
5. 1 M MgSO₄ and 1 M MgCl₂ solutions
6. 1 M glucose solution
7. NaCl solutions (0.9%, 5 M)
8. TE buffer
9. TEN buffer
10. 10% SDS solution
11. CTAB/NaCl solution
12. 3 M sodium acetate (CH₃COONa) solution
13. 50× TAE buffer
14. 2× TSS solution
15. TSS solution
16. 50% glycerol solution
17. Rifampicin solution
18. Kanamycin solution
19. Ampicillin solution

Equipment

1. Reciprocating shakers (TAITEC Co., Ltd., models: personal-11 SM, BR-41FM)
2. Centrifuges (Eppendorf AG., models: 5430R, 5425R)
3. Thermostat (Yamato Scientific Co., Ltd., model: DG400)

4. Spectrophotometer (ERMA INC., model: MP-1200)
5. Water purification systems (ELGA LabWater, model: PURELAB Chorus 1 Complete, PURELAB Chorus 2+)
6. pH meter (HORIBA, Ltd., model: F-74)
7. Electronic scale (A&D Co., Ltd., model: HR-202i)
8. Magnetic stirrer with hot plate (Kenis, model: MI10 33220962)
9. Autoclave (TOMY SEIKO Co., Ltd., model: LBS-245)
10. Safety cabinet (DALTON Co., Ltd., model: Class 2 A1)
11. Thermal cycler (BIO-RAD, model: T-100)
12. Microwave (for example, Sharp Co., Ltd., model: RE-21G)
13. Submarine electrophoresis system (Mupid.com, model: Mupid-2plus)
14. Gel documentation system (ATTO Co., model: WSE-5400 Printgraph Classic)
15. Heat block incubator (Anatech Co., Ltd., model: 5520a)
16. NanoDrop UV-Vis spectrophotometer (Thermo Fisher Scientific, model: 2000c)
17. Mixer (Vortex Co., Ltd., model: VORTEX-GENIE 2)
18. Adjustable air displacement pipette (Gilson Inc. model: PIPETMAN P series)
19. Electroporation system (Bio-Rad Laboratories Inc., model: Genepluser Xcell)
20. -30 °C freezer (for example Nihon Freezer Co., Ltd., model: GS-5210HC)
21. -80 °C freezer (for example Thermo Fisher Scientific, model: TSX600G)
22. LED illuminator (Optocode Co., Ltd., model: LED-EXTA/GFP + RFP)
23. Ceramic tweezer (ASONE, catalog number: 7-166-03)
24. Microscope (for example Carl Zeiss, model: Axio observer Z1)

Software

1. ApE (<https://www.frontiersin.org/articles/10.3389/fbinf.2022.818619/full>) used as a DNA plasmid sequence editor
2. R software version 4.2.2 [R Core Team (2022). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. (<https://www.R-project.org/>)] used for statistics

Procedure

A. Preparation of genomic DNA from SP-6 cells

This protocol was essentially described in Kunoh et al. (2015b).

1. Pick SP-6 cells from a glycerol stock with a sterilized toothpick, spread on MSVP agar plate, and incubate for 3–5 days at room temperature (RT).
2. Pick several colonies of SP-6 cells on an MSVP plate with a sterilized toothpick, inoculate into 25 mL of MSVP liquid medium in a 50 mL polypropylene tube, and incubate for 2–3 days at RT by shaking at 70 rpm.
3. Collect exponentially growing SP-6 cells (total 50–100 mL) by centrifugation at $7,000 \times g$ for 5 min.
4. Wash the resultant cell pellet twice in 25 mL of TE buffer, resuspend in 5 mL of TE buffer containing 20 $\mu\text{g/mL}$ proteinase K and 0.6% SDS, and transfer to a 15 mL polypropylene tube.
5. After incubation at 65 °C for 1 h, add 1 mL of 5 M NaCl and 800 μL of CTAB/NaCl solution to the cell suspension and incubate at 37 °C for one additional hour by shaking at 190 rpm.
6. To eliminate proteins, add an equal volume (~7 mL) of phenol/chloroform/isoamyl alcohol (25:24:1) to the cell suspension, mix by inversion, and centrifuge at $7,000 \times g$ for 15 min.
7. Transfer the resultant aqueous phase to a new 15 mL polypropylene tube and perform chloroform extraction as described in step A5.

8. For precipitation of nucleic acids, transfer the resultant supernatant to a new 15 mL polypropylene tube, add an equal volume (~5 mL) of isopropanol, and incubate at -20 °C for 1 h.
9. Then, collect the pellet by centrifugation (7,000 × g, 30 min) and discard the supernatant, followed by rinsing the pellet in ~5 mL of 70% ethanol.
10. After drying up the pellet at RT for 15 min, resuspend it in 500 µL of TE buffer containing 20 µg/mL ribonuclease A by pipetting, transfer it to a 1.5 mL centrifuge tube, and incubate the suspension at 37 °C for 30 min.
11. To remove ribonuclease A from the suspension, add an equal volume (~500 µL) of phenol/chloroform/isoamyl alcohol (25:24:1), mix by inversion, and centrifuge (15,000 × g, 30 min). Then, transfer the resultant aqueous phase to a new 1.5 mL centrifuge tube. Repeat this step once more.
12. For precipitation of nucleic acids, add an equal volume (~500 µL) of ethanol and 1/10 volume (~50 µL) of 3 M CH₃COONa solution to the resultant aqueous phase.
13. After incubation at -20 °C for ~30 min, centrifuge the mixture (15,000 × g, 30 min) and remove the supernatant, followed by rinsing the pellet in 70% ethanol.
14. After drying at RT for 5 min, suspend the resultant pellet in ~100 µL of TE buffer. Then, determine DNA concentration of the suspension by using a NanoDrop 2000c.

B. Preparation of genomic DNA from *B. subtilis* 168 cells

1. Pick *B. subtilis* 168 cells from a glycerol stock with a sterilized toothpick, spread on LB agar plate, and incubate at 37 °C overnight.
2. Inoculate a single colony into 4 mL of LB medium in a test tube and incubate by shaking at 37 °C overnight.
3. Transfer cell culture to 1.5 mL centrifuge tubes and harvest the cells by centrifugation (20,000 × g, 1 min).
4. Resuspend the resultant cell pellet in 300 µL of TEN buffer containing 10 µg/mL ribonuclease A and 30 µg/mL lysozyme and incubate at RT for 20 min.
5. To lyse the cells, add 9 µL of 10% SDS solution to the cell suspension, mix gently by inversion, and incubate at 50 °C for 10 min.
6. Add 200 µL of TEN buffer and 500 µL of phenol/chloroform/isoamyl alcohol (25:24:1) to the cell lysate, mix gently by inversion, and centrifuge at 20,000 × g for 10 min.
7. Transfer the upper aqueous phase to a new centrifuge tube, add 1 mL of ethanol, and mix by inversion, followed by centrifugation at 20,000 × g for 10 min.
8. Discard the supernatant, add 1 mL of 70% ethanol for rinsing, and centrifuge at 20,000 × g for 5 min.
9. Discard the supernatant, dry the DNA pellet, and resuspend it in 200 µL of TE buffer. Dilute approximately 10-fold by TE buffer and use the resultant DNA solution as a template for PCR.

C. Construction of plasmids pUC18-*Km^r* and pUC18-*mob*

1. To construct pUC18-*Km^r*, digest 20–50 ng of pSUP5011 and pUC18 with *Sma*I and *Hind*III at 37 °C for 30 min. To construct pUC18-*mob*, digest these plasmids with *Bam*HI.
2. Purify the insert DNAs from pSUP5011 (1.3 kb *Hind*III-*Sma*I and 1.8 kb *Bam*HI fragments, respectively) and digest pUC18 by agarose gel extraction.
3. Clone the purified insert DNA into pUC18 by using DNA Ligation kit according to the manufacturer's instructions.
4. Transform *E. coli* DH5α cells with the ligation mixtures by electroporation, spread the cells on LB plates containing 50 µg/mL ampicillin sodium salt, and incubate at 37 °C overnight.
5. Pick the resultant single colonies, re-streak them on LB agar with 50 µg/mL ampicillin sodium salt, and incubate at 37 °C overnight.
6. Inoculate the resultant colonies into 4 mL of LB medium containing 50 µg/mL ampicillin sodium salt in test tubes and incubate by shaking at 37 °C overnight.
7. Harvest the cells by centrifugation (20,000 × g, 1 min) and extract the plasmid DNA (pUC18-*mob* and pUC18-*Km^r*) using the GenElute™ Plasmid Miniprep kit according to the manufacturer's instructions.

8. Confirm the plasmid DNAs by digesting the restriction enzymes.

D. Construction of the plasmid pUC18-*mob*- Δ *Lcho_0008*::*Km^r* for *Lcho_0008* gene deletion (see Figures 1 and 2)

Standard overlap extension PCR, DNA extraction from agarose gel, and iVEC3 system are described in Sections H, G, and I, respectively.

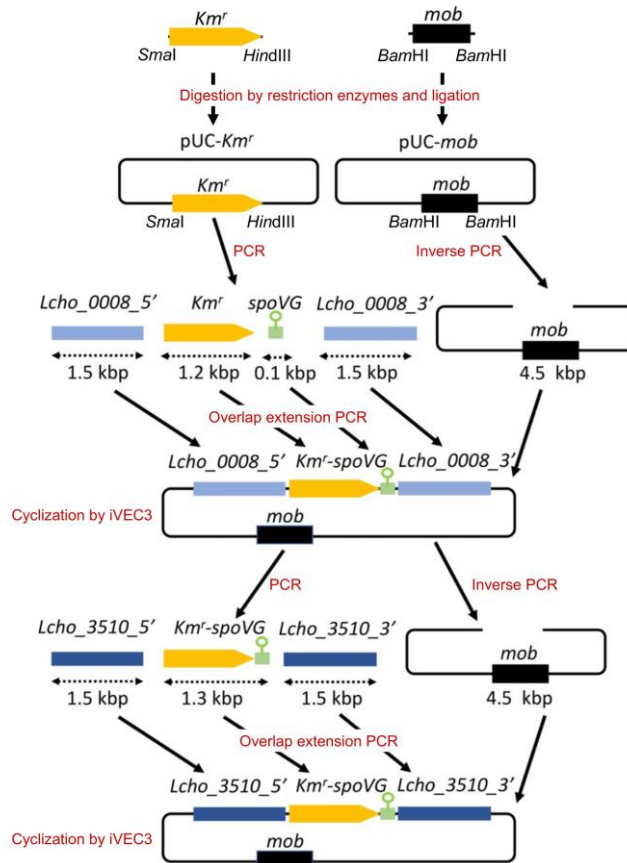


Figure 1. An overview of plasmid construction (see Kunoh et al. 2022). To construct a plasmid for the gene replacement of *Lcho_0008* with the kanamycin resistance gene (*Km^r*), we first inserted fragments containing *Km^r* and *mob* from pSUP5011 into pUC18, respectively. Then, the upstream and downstream of *Lcho_0008* (approximately 1.5 kb) and the *spoVG* terminator (approximately 0.1 kb) were amplified using genomic DNAs isolated from *L. cholodnii* SP-6 and *B. subtilis* 168, respectively. In parallel, *Km^r* was amplified using the pUC-*Km^r* plasmid as a template. These four fragments were stepwise joined by overlap extension PCRs (also see Figure 2) and cloned into the pUC-*mob* plasmid by the iVEC3 system, resulting in construction of the plasmid pUC18-*mob*- Δ *Lcho_0008*::*Km^r*. Now, the resultant plasmid can be used not only for the gene replacement of *Lcho_0008* but for the amplification of the *Km^r*-*spoVG* terminator cassette as the template to construct other plasmids, such as pUC18-*mob*- Δ *Lcho_3510*::*Km^r*. *Lcho_0008_5'* and *Lcho_0008_3'*, and *Lcho_3510_5'* and *Lcho_3510_3'* represent upstream and downstream fragments of these genes, respectively. *spoVG* represents the *spoVG* terminator.

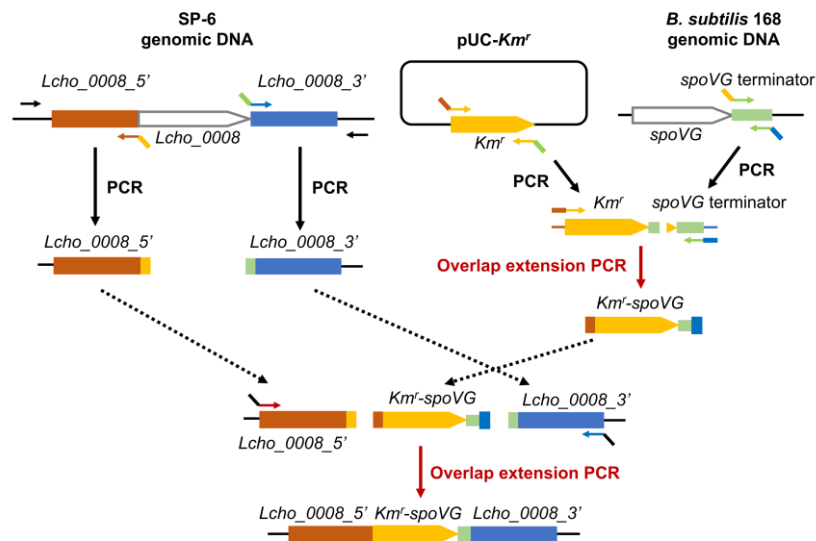


Figure 2. An overview of overlap extension PCR. The amplified PCR products contain overlapping 40 bp regions at both ends, whose T_m values were designed to be 65–76 °C (Ave. 70). These overlapping 40 bp regions are important for the overlap extension PCRs. *Lcho_0008_5'* and *Lcho_0008_3'* represent upstream and downstream fragments of *Lcho_0008*, respectively. *spoVG* represents the *spoVG* terminator.

1. Amplify the upstream (approximately 1.5 kb) and downstream (approximately 1.5 kb) regions of *Lcho_0008* gene by PCR using SP-6 genomic DNA as a template and Lc0008_F_out/Lc0008_Km_2 and term-Lc0008-1/Lc0008-R-out primer sets, respectively (see Table 1).

Table 1. Primers for the construction of pUC18-*mob*- Δ *Lcho_0008*::*Km*^r

Name	Primer sequence
Lc0008-F-out	5'-AGAAGTAGTGCAGGTGCTTGAAAATTG-3'
Lc0008-R-out	5'-TGGTCGACGAGCTGAACCGCAAATG-3'
pUC-Lc0008-1	5'-ACTCATTAGGCACCCAGGCACAGCTGCAGCCCTTCGACTG-3'
pUC-Lc0008-2	5'-AGTCGAAGGGCTGCAGCTGTGCCTGGGGTGCCTAATGAGTGAG-3'
Lc0008-Km-1	5'-GCATGAACATCCTGCCCTGAGCCGCAAGCACTCAGGGCGCAAG-3'
Lc0008-Km-2	5'-CCCTGAGTGCTTGC GGCTCAGGGCAGGATGTTTCATGCGTCGGCAG-3'
Km-term-1	5'-CGTTTTCCGGGACGCCGGCTAAAATAACCAAAAAGCAAGGAC-3'
Km-term-2	5'-CCTTGCTTTTTGGTTATTTTAGCCGGCGTCCCGGAAAACG-3'
term-Lc0008-1	5'-TTTCAAACCTAGTTGCACTCCCCGAGCGTGGCGGGCGGGTAC-3'
term-Lc0008-2	5'-ACCCGCCCGCCACGCTCGGGGAGTGCAACTAAGTTTGAAAAATCAG-3'
Lc0008-pUC-1	5'-TCGCAGCTTCAGCTCCGTGTTTTACACAGGAAACAGCTATGACCATG-3'
Lc0008-pUC-2	5'-TAGCTGTTTCCTGTGTGAAAACACGGAGCTGAAGCTGCGATGAC-3'

2. Amplify the kanamycin resistance gene (*Km^r*) (approximately 1.2 kb) and the transcriptional terminator of *B. subtilis spoVG* gene (approximately 0.1 kb) by PCR using the primers Lc0008-Km-1/Km-term-2 and Km-term-1/term-Lc0008-2 from pUC18-*Km^r* and the genomic DNA of *B. subtilis* 168, respectively.
3. Purify the four resultant PCR products (upstream and downstream regions of *Lcho_0008*, *Km^r*, and *spoVG* terminator) by agarose gel extraction.
4. Ligate these purified products of the *Km^r* and *spoVG* terminator by overlap extension PCR with Lc0008-Km-1 and term-Lc0008-2 primers, resulting in the production of *Km^r-spoVG* fragment (see Figure 2).
5. Purify the resultant PCR product by agarose gel extraction and ligate *Km^r-spoVG* fragment with upstream and downstream regions of *Lcho_0008* (steps D3, D4) by overlap extension PCR with pUC-Lc0008-1 and Lc0008-pUC-2 primers, resulting in the production of the upstream *Lcho_0008* + *Km^r-spoVG* + downstream *Lcho_0008* fragment.
6. Purify the resultant PCR product by agarose gel extraction and use as an insert DNA in the subsequent iVEC3 procedure in step D9.
7. Amplify the plasmid backbone by inverse PCR with pUC-Lc0008-2 and Lc0008-pUC-1 primers using pUC18-*mob* plasmid as a template.
8. Purify the resultant PCR product by agarose gel extraction and use it as a vector DNA for the iVEC3 method in step D9.
9. Clone the insert DNA (upstream *Lcho_0008* + *Km^r-spoVG* + downstream *Lcho_0008*) into the pUC18-*mob* vector by the iVEC3 system.
10. Pick the resultant single colonies, re-streak them on LB agar with 50 µg/mL ampicillin sodium salt, and incubate at 37 °C overnight.
11. Inoculate the resultant colonies into 4 mL of LB medium with 50 µg/mL ampicillin sodium salt in test tubes and incubate by shaking at 37 °C overnight.
12. Harvest the cells by centrifugation (20,000 × *g*, 1 min) and extract the plasmid DNA (pUC18-*mob-ΔLcho_0008::Km^r*) using the GenElute™ Plasmid Miniprep kit according to the manufacturer's instructions.
13. Confirm the construction by PCR using the extracted plasmid DNA and primer sets listed in Table 1. Sequencing especially the insert DNA of the plasmid is recommended.

Now, we are ready to use the resultant plasmid pUC18-*mob-ΔLcho_0008::Km^r* for the gene replacement of the *Lcho_0008* gene. In addition, we can employ this plasmid as a template for the construction of other plasmid(s). For example, the plasmid pUC18-*mob-ΔLcho_3510::Km^r* is constructed as follows.

E. Construction of the plasmid for *Lcho_3510* gene deletion (see Figure 1)

Detailed protocols of standard overlap extension PCR, DNA extraction from agarose gel, and iVEC3 system are described in Sections H, G, and J, respectively.

1. Amplify the upstream and downstream regions of *Lcho_3510* gene by PCR using *L. cholodnii* SP-6 genomic DNA as a template and the Lc3510-F-out/Lc3510-Km-2 and Km-Lc3510-1/Lc3510-R-out primer sets, respectively (see Table 2).
2. Amplify the *Km^r* gene and the transcriptional terminator of *B. subtilis spoVG* gene region by PCR using the pUC18-*mob-ΔLcho_0008::Km^r* as a template and Lc3510-Km-1 and Km-Lc3510-2 primers.
3. Purify the three resultant PCR products (upstream and downstream regions of *Lcho_3510* and the *Km^r*-terminator) by agarose gel extraction.
4. Ligate these purified products by overlap extension PCR with the pUC18-Lc3510-1 and Lc3510-pUC18-2 primers.
5. Purify the resultant PCR product by agarose gel extraction and use it as an insert DNA for the iVEC3 method in step E8.
6. Amplify the plasmid backbone by inverse PCR with the pUC18-Lc3510-2 and Lc3510-pUC18-1 primers using pUC18-*mob* plasmid as a template.
7. Purify the resultant PCR product by agarose gel extraction and use it as a vector DNA for the iVEC3 method in step E8.

8. Clone the insert DNA (upstream *Lcho_3510-Km^r-spoVG* terminator-downstream *Lcho_3510*) into the pUC18-*mob* vector by the iVEC3 method.
9. Pick the resultant single colonies, re-streak them on LB agar with 50 µg/mL ampicillin sodium salt, and incubate at 37 °C overnight.
10. Inoculate the resultant colonies into 4 mL of LB medium with 50 µg/mL ampicillin sodium salt in test tubes and incubate with shaking at 37 °C overnight.
11. Harvest the cells by centrifugation (20,000 × *g*, 1 min) and extract the plasmid DNA (pUC18-*mob-ΔLcho_3510::Km^r*) using a GenElute™ Plasmid Miniprep kit according to the manufacturer's instructions.
12. Confirm the construction by PCR using the extracted plasmid DNA and primer sets listed in Table 2. Sequencing especially the insert DNA of plasmid is recommended.

Table 2. Primers for the construction of pUC18-*mob-ΔLcho_3510::Km^r*

Name	Primer sequence
pUC18-Lc3510-1	5'-ACTCATTAGGCACCCCAGGCTCGCACGCATCCGTCACCGCCTTG-3'
pUC18-Lc3510-2	5'-GGTGGGACGGATGCGTGCGAGCCTGGGGTGCTAATGAGTGAG-3'
Lc3510-Km-1	5'-CACGGATGTTTCGAGTTTTTTGAGCCGCAAGCACTCAGGGCGCAAG-3'
Lc3510-Km-2	5'-GCGCCCTGAGTGCTTGCGGCTCAAAAACTCGAAACATCCGTGATCCCCCTC-3'
Km-Lc3510-1	5'-TTTCAAACCTAGTTGCACTCCCAGCGGTTTTCCATCCTCGGTCG-3'
Km-Lc3510-2	5'-CGAGGATGGAAAACGCGGGGAGTGCAACTAAGTTTGAAAAATCAG-3'
Lc3510-pUC18-1	5'-GTTGAATCGGAGAACCGCCTTTTACACAGGAAACAGCTATGACCATG-3'
Lc3510-pUC18-2	5'-TAGCTGTTCCGTGTGAAAAGGCGGTTCTCCGATCAACCAGTTTC-3'
Lc3510-F-out	5'-TTCGATCGACCGACCCTTCAGATGAG-3'
Lc3510-R-out	5'-TCCTGATTGGAGGTTGACGCAACGGTC-3'

F. DNA amplification by PCR

1. Mix 1 µL of template DNA, 0.15 µL of 100 µM forward primer, 0.15 µL of 100 µM reverse primer, and 25 µL of PrimeStar Max Premix (x2) and fill up to 50 µL with ultrapure water.
2. Perform the PCR with the initial denaturation at 94 °C for 1 min, followed by 30 cycles of 98 °C for 10 s, 55 °C for 15 s, and 72 °C for 30 s/kb.

G. DNA extraction from agarose gel

1. Separate 10–50 µL of plasmid DNA or PCR products by agarose gel electrophoresis on 0.8%–1.5% agarose gel in 1× TAE buffer.
2. Stain the DNA in the agarose gel with Midori Green Advance for ~15 min to visualize it with a 470 nm LED illuminator.
3. Excise the appropriate DNA band and extract the DNA from the agarose gel using the Wizard® SV Gel and PCR Clean-Up System according to the manufacturer's instructions.

H. Overlap extension PCR

1. Mix 50–100 ng of gel-extracted PCR products with homologous ends and 20 μL of PrimeStar Max Premix and adjust the volume to 40 μL with ultrapure water.
2. Perform the PCR with an initial denaturation at 94 $^{\circ}\text{C}$ for 1 min, followed by 15 cycles of 98 $^{\circ}\text{C}$ for 10 s, 55 $^{\circ}\text{C}$ for 15 s, and 72 $^{\circ}\text{C}$ for 30 s/kb.
3. Mix 1 μL of the resultant PCR solution, 0.15 μL of 100 μM forward primer, 0.15 μL of 100 μM reverse primer, and 25 μL of PrimeStar Max Premix and adjust the volume to 50 μL with ultrapure water.
4. Perform the PCR with an initial denaturation at 94 $^{\circ}\text{C}$ for 1 min, followed by 30 cycles of 98 $^{\circ}\text{C}$ for 10 s, 55 $^{\circ}\text{C}$ for 15 s, and 72 $^{\circ}\text{C}$ for 30 s/kb.

I. Preparation of competent cells of *E. coli* SN1187 for the iVEC3 (in vivo *E. coli* cloning) system

1. Inoculate colonies of *E. coli* SN1187 on an LB plate into 4 mL of LB liquid medium and incubate at 37 $^{\circ}\text{C}$ by shaking at 190 rpm overnight.
2. Transfer 1 mL of the cell culture to 60 mL of LB medium in a 200 mL Erlenmeyer flask and further incubate for ~90 min.
3. Place the Erlenmeyer flask containing the cell culture on ice for ~10 min at the time of $\text{OD}_{600} = 0.4\text{--}0.5$.
4. Then, transfer the cell culture to 50 mL polypropylene tubes and centrifuge at $5,000 \times g$ for 5 min.
5. Discard the medium and suspend the resultant cell pellets in 2 mL of ice-cold LB medium.
6. Add 1.6 mL of ice-cold $2\times$ TSS solution to the cell suspension and mix gently by pipetting.
7. Add 0.4 mL of DMSO to the resultant suspension and mix gently at RT by pipetting.
8. Dispense the cell suspension (~4 mL) in 0.1 mL aliquots in centrifuge tubes.
9. Then, freeze the cell suspensions immediately in liquid nitrogen and keep at -80 $^{\circ}\text{C}$.

J. Circularization of PCR products by the iVEC3 system

The iVEC3 system was essentially reported in Nozaki and Niki (2019). Incorporated PCR products of the vector and insert are circularized in vivo using the overlapping 40 bp regions at both ends.

1. Thaw the frozen competent *E. coli* SN1187 cells in a 1.5 mL centrifuge tube on ice.
2. Add 300 fmol of the insert DNA and 60 fmol of the vector DNA to the competent cells, mix gently by pipetting, and incubate for 20 min on ice.
3. Add the suspension to 1 mL of LB medium, mix by inversion, and incubate at 37 $^{\circ}\text{C}$ for 45 min.
4. Then, centrifuge the cell suspension at $20,000 \times g$ for 1 min, discard 1 mL of the supernatant, resuspend the cell pellet with the remaining supernatant, and spread it on an LB plate containing 50 $\mu\text{g}/\text{mL}$ of ampicillin sodium salt.

K. Preparation of competent cells of *E. coli* S17-1 and DH5 α and transformation

1. Streak *E. coli* S17-1 or DH5 α cells on an LB plate from a glycerol stock using a sterilized toothpick and incubate at 37 $^{\circ}\text{C}$ for 12 h.
2. Pick a single colony, inoculate into 5 mL of LB medium, and incubate at 37 $^{\circ}\text{C}$ for 12 h by shaking at 190 rpm.
3. Transfer 200 μL of the cell culture to 80 mL of fresh LB medium in a 200 mL Erlenmeyer flask and incubate at 37 $^{\circ}\text{C}$ for an additional ~3 h by shaking at 190 rpm.
4. Place the Erlenmeyer flask containing the cell culture on ice for ~10 min at the time of $\text{OD}_{600} = 0.6\text{--}0.7$.
5. Then, transfer the cell culture to 50 mL polypropylene tubes and centrifuge at $3,000 \times g$ for 10 min.
6. For washing, suspend the resultant cell pellet in 80 mL of ice-cold sterile ultrapure water by pipetting and centrifuge at $3,000 \times g$ for 10 min.
7. Repeat step K6 once more.
8. Suspend the resultant cell pellet in 1.4 mL of ice-cold 10% glycerol solution and transfer the suspension to a 1.5 mL centrifuge.

9. Then, centrifuge the cell suspension at $7,000 \times g$ for 10 min, discard the supernatant, and suspend the resultant cell pellet in 220 μL of ice-cold 10% glycerol solution.
10. Dispense the cell suspension in aliquots of 42 μL in centrifuge tubes and keep them at -80°C until use.
11. Thaw the competent cells in a centrifuge tube on ice.
12. Add 1 μL of plasmid DNA to suspension of the competent cell and mix gently by pipetting.
13. Transfer the resultant mixture to an electroporation cuvette.
14. Perform electroporation using a GenePulser X cell at 2.5 kV.
15. Add ~ 1.0 mL of SOC medium, transfer the cell suspension to a centrifuge tube, and incubate at 37°C for 1 h.
16. Spread the resultant cell suspension on a LB plate containing 50 $\mu\text{g}/\text{mL}$ of ampicillin sodium salt (the Km^r on the constructed plasmid optionally allows us to use kanamycin for *E. coli* colony selection).

L. Isolation of rifampicin-resistant mutant of SP-6 (hereafter WT*)

1. Pick several colonies of SP-6 cells from an MSVP plate with a sterilized toothpick, inoculate into 25 mL MSVP liquid medium containing 5 $\mu\text{g}/\text{mL}$ rifampicin in a 50 mL polypropylene tube, and incubate for 2–3 days at RT by shaking at 70 rpm.
2. Spread 100 μL of the cell culture on MSVP plates containing 5 $\mu\text{g}/\text{mL}$ rifampicin and incubate for 5–7 days.
3. Pick a single colony with a sterilized toothpick, inoculate into 25 mL of MSVP medium containing 10 $\mu\text{g}/\text{mL}$ rifampicin in a 50 mL polypropylene tube, and incubate for 2–3 days at RT by shaking at 70 rpm.
4. Repeat steps L2 and L3 three times using MSVP medium containing 10, 15, and 20 $\mu\text{g}/\text{mL}$ rifampicin in sequence.
5. Isolate a single colony on an MSVP plate containing 20 $\mu\text{g}/\text{mL}$ rifampicin and define it as WT*.
6. Mix a 1-day culture of WT* with an equal volume of 50% glycerol solution, dispense the resultant cell suspension to stock tubes, and store them at -80°C .

M. Gene transfer to WT* cells by conjugation with *E. coli* S17-1 (see Figure 3)

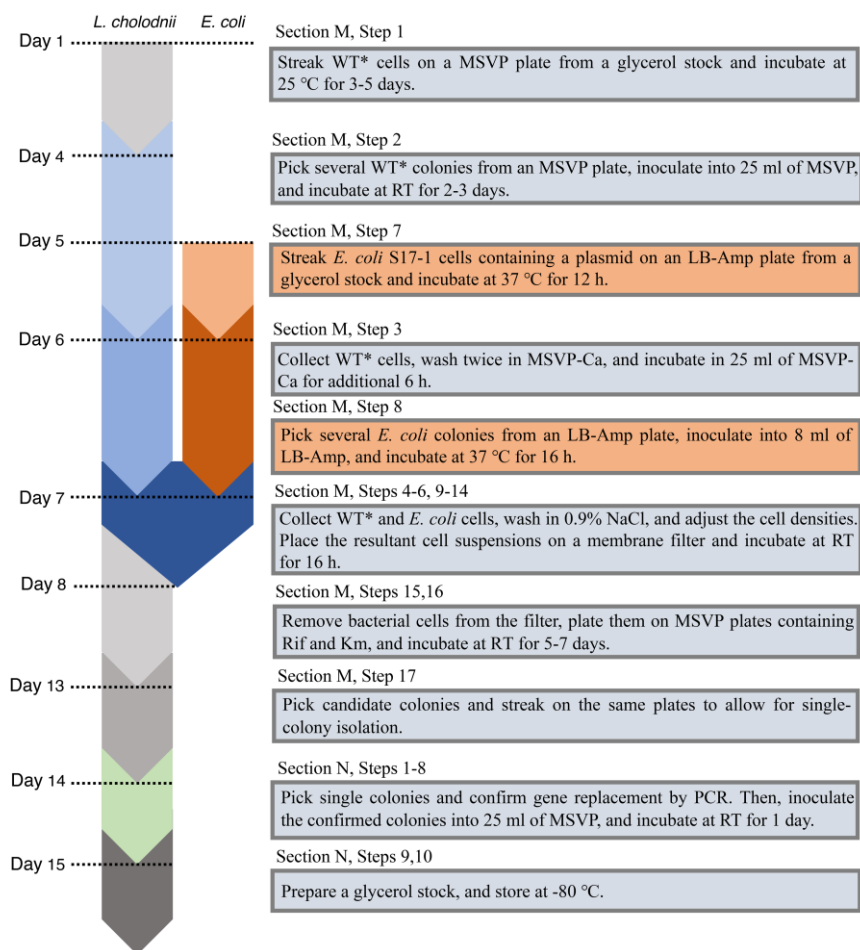


Figure 3. A detailed step-by-step chart of the culture and maintenance of *L. cholodnii* and *E. coli*

Preparation of WT* cells

1. Streak WT* cells on a MSVP plate from a glycerol stock with a sterilized toothpick and incubate at 25 °C for 3–5 days.
2. Pick several WT* colonies from an MSVP plate with a sterilized toothpick, inoculate into 25 mL of MSVP medium in 50 mL polypropylene tubes (25 mL × four tubes), and incubate for 2–3 days at RT by shaking at 70 rpm.
3. Collect exponentially growing WT* in 2-days MSVP culture by centrifugation at 7,000 × g for 5 min, wash twice in MSVP lacking Ca (MSVP-Ca), and culture in 25 mL of MSVP-Ca for additional 6 h (see Figures 7 and 8A).
4. Collect exponentially growing WT* cells by centrifugation at 7,000 × g for 5 min. Discard the medium and resuspend the cell pellet in 1 mL of 0.9% NaCl.
5. Pass the cell suspension through a needle syringe (23G × 1 inch) using a relatively high pressure to break apart cell aggregates composed of sheathed cell chains into single cells.
6. Adjust the volume of the resultant cell suspension to 2 mL with 0.9% NaCl and measure the optical density at 600 nm (OD₆₀₀) to determine the cell density.

Preparation of *E. coli* S17-1 cells containing a plasmid

7. Streak *E. coli* S17-1 cells containing a plasmid such as pUC18-*mob-ΔLcho_3510::Km^r* on an LB plate containing 50 μg/mL ampicillin sodium salt from a glycerol stock with a sterilized toothpick and incubate at 37 °C for 12 h.
8. Pick a single colony, inoculate into 4 mL of LB medium containing 50 μg/mL ampicillin sodium salt (4 mL × two tubes), and incubate at 37 °C for 16 h by shaking at 190 rpm.
9. Collect exponentially growing *E. coli* cells by centrifugation at 3,000 × *g* for 1 min.
10. Discard the medium and wash the collected cells three times with 0.9% NaCl.
11. Resuspend the washed cells in 2 mL of 0.9% NaCl and measure the OD₆₀₀ to determine cell density.

Note: To prevent damage of pilus excreted from the cell surfaces of E. coli cells, which are important for conjugation, handle the E. coli cells gently (steps M9–M11).

Conjugation and colony selection

12. Drop 500 μL of the WT* suspension (from step M6) onto a membrane filter placed on an NB plate and incubate for 1 h until there is no liquid on the membrane surface (see Figure 4).

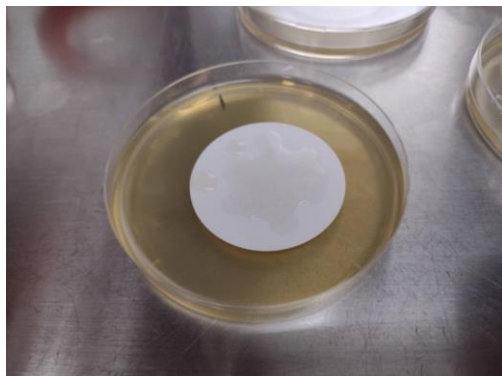


Figure 4. Image of mixing WT* and *E. coli* cells on a membrane

13. Drop 500 μL of the suspension of *E. coli* cells (from step M11) onto the same membrane filter incubated with SP-6 and incubate for 16 h.
14. Transfer the membrane filter into a 15 mL polypropylene tube with a tweezer.
15. Remove bacterial cells from the nitrocellulose membrane filter by pipetting with 10 mL of 0.9% NaCl and collect them by centrifugation at 7,000 × *g* for 5 min.
16. Resuspend the bacterial cells in 1 mL of 0.9% NaCl, plate 100 μL each onto MSVP plates containing 20 μg/mL rifampicin and 50 μg/mL kanamycin, and incubate at RT for 5–7 days.
17. Pick single colonies and streak them on the same plate to allow for single-colony isolation.

N. Confirmation of gene replacement and preparation of glycerol stock

Colony PCR (see Figure 5)

1. Mix 0.15 μL of 100 μM forward primer, 0.15 μL of 100 μM reverse primer, 10 μL of 2× Gflex PCR Buffer (Mg²⁺, dNTP plus), and 0.4 μL of Tks Gflex DNA Polymerase (1.25 units/μL) and adjust the volume to 20 μL with ultrapure water in PCR tubes.
2. Pick single colonies (~0.5 mm in diameter) from MSVP plates containing 20 μg/mL rifampicin and 50 μg/mL kanamycin (Recipes 17, 18) with sterilized toothpicks and suspend them in the colony PCR mixture.
3. Perform the PCR with the following steps: initial denaturation at 94 °C for 1 min, followed by 30 cycles of 98 °C for 10 s, 60 °C for 15 s, and 68 °C for 1 min/kb.
4. Apply and electrophorese 10 mL of PCR products into an 0.8% agarose gel.

- After staining the agarose gel with the ethidium bromide solution, image amplified bands using a gel documentation system.

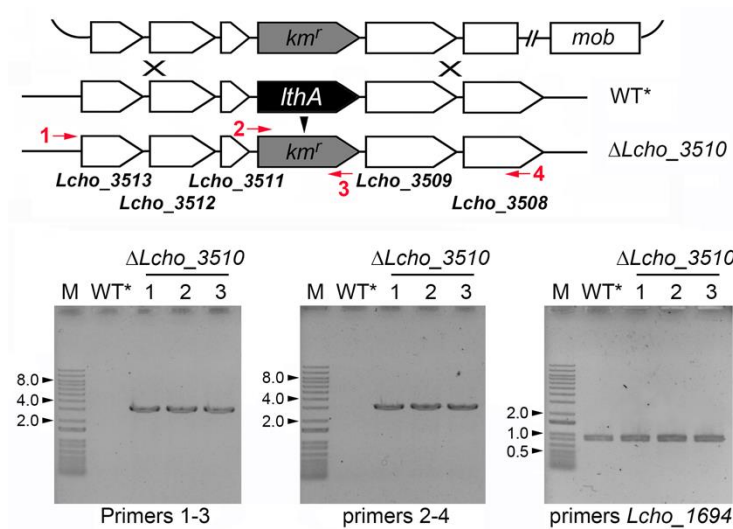


Figure 5. Confirmation of gene replacement of the *Lcho_3510* gene with *Kmr*. Lc3510-F-out/*Km*-Lc3510-2 (left), Lc3510-Km-1/Lc3510-R-out (middle), and Lcho1694-F-BamHI/Lcho1694-R-BamHI (right) primer sets were employed for colony PCR (see Tables 2, 3 and the reference Kunoh et al., 2022). Size markers (kb) determined by 1 kb Plus DNA ladder (indicated as M) are indicated by numbers and arrowheads on the left.

Table 3. Primers for colony PCR

Name	Primer sequence
Lc1694-F-BamHI	5'-CGGGATCCATGTCGAGATCGACCGCCAGCG-3'
Lc1694-R-BamHI	5'-CGGGATCCTTACTTGCGTGACGAGCGCAGC-3'

Sequencing

- Perform colony PCR using primers located outside the replaced gene as described in steps N1–N3.
- Purify the resultant PCR products by agarose gel extraction.
- Sequence the PCR products using sequencing primers listed in Table 2 (outsourced to Eurofins).

Glycerol stock

- Pick the confirmed colonies (from step M17), inoculate into 25 mL of MSVP, and incubate at RT for one day.
- Transfer 0.75 mL of the cell culture to a 1.5 mL tube, mix with an equal volume of 50% glycerol solution, and store at -80 °C.

Data analysis

Carry out statistical analyses by unpaired Welch's *t*-tests and Bonferroni-Holm method using the R software version 4.1.2 [R Core Team (2022). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. (<https://www.R-project.org/>)] (see Figures 6, 8B, and 9).

Notes

1. The genomic DNA from *B. subtilis* 168 cells can be prepared using kits such as Wizard® Genomic DNA Purification kit (Promega).
2. To fully stop the transcription of the *km^r* gene at downstream of the stop codon, we employed the *Bacillus subtilis spoVG* transcriptional terminator because it was shown to form the stable configuration (Hudspeth et al., 1992). However, other terminators can be used for this purpose.
3. For in vivo cloning (the iVEC3 system), *E. coli* strain (SN1187) is engineered to employ the exonuclease III activity of XthA for exposure of ~40 bp of the homologous region at each end that enables annealing of DNAs, while DNA polymerase I activity of PolA is used for the gap repair of annealed DNAs (Nozaki and Niki, 2019). Although we recommend this system due to its cost-effectiveness, other cloning kits such as In-Fusion® HD Cloning kit (Clontech) can be used.
4. The plasmid pUC18-mob- Δ Lcho_3510::Km^r is available from the RIKEN BRC Japan collection (RDB No.19817 pUC18_dellcho3510). Please contact us if the *L. cholodnii* strain WT* is needed.
5. As far as we examined by colony PCR and the following agarose gel electrophoresis, no long band corresponding to the size when the whole plasmid was integrated by single crossover recombination was detected (see Figure 5), indicating most of obtained colonies received double crossover recombination.
6. We employed *E. coli* S17-1 as a donor in this protocol. However, the S17-1 strain is known to transfer bacteriophage Mu silently. Therefore, we encourage using Mu-free donor strains such as MFD^{pir} (Ferrières et al., 2010).
7. We examined the conjugation efficiencies with different donor-recipient ratios. The cell suspensions of WT* (step M6) and S17-1 (step M11) were diluted prior to mixing on a membrane filter. After conjugation, the washed bacterial suspension (step M16) was serially diluted and plated on MSVP containing rifampicin and rifampicin/kanamycin to count the total WT* cells and their transconjugates, respectively. Figure 6 indicates that the efficiency tends to be highest when the WT*:S17-1 ratio is 1:1.

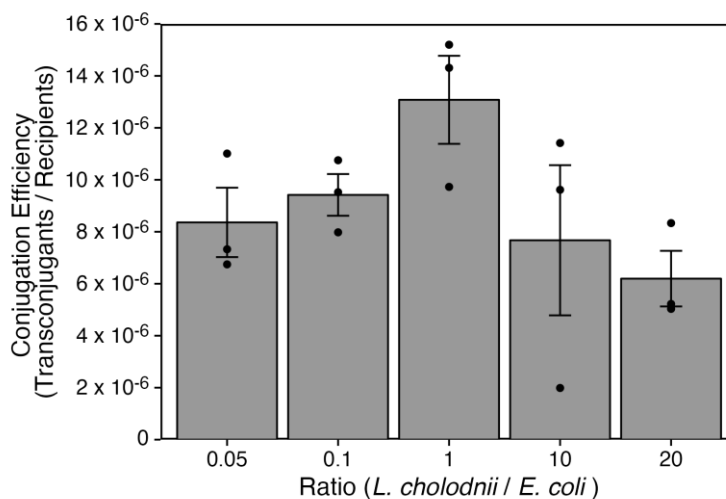


Figure 6. Conjugation efficiency with different donor-recipient ratios. Statistical analysis was performed by unpaired Welch's *t*-tests and Bonferroni-Holm method, suggesting no significant differences.

8. We considered the conjugation between WT* and S17-1 to be efficient when the cell surfaces of WT* cells were free from sheaths (Figure 7). Since we previously reported that SP-6 cells become sheath-less when cultured in calcium-free conditions (Kunoh et al., 2021), WT* cells cultured in routine MSVP were harvested and further cultured in MSVP lacking Ca (MSVP-Ca) for an additional 6 h (step M3) (Figure 8A). As expected,

Figure 8B indicates that the conjugation efficiency with additional culturing in MSVP-Ca is approximately 2-fold higher than that without additional culturing (MSVP+Ca).

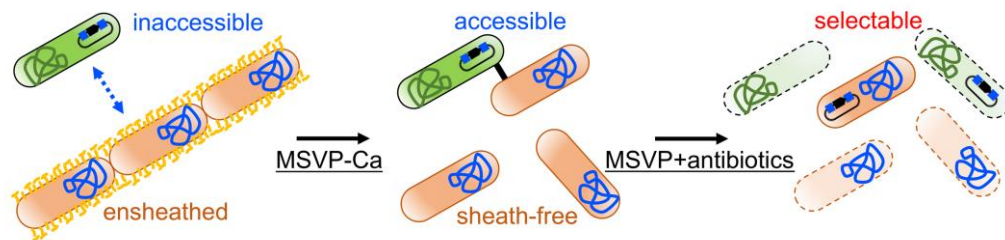


Figure 7. A strategy for enhancement of conjugation efficiency by suppressing sheath formation

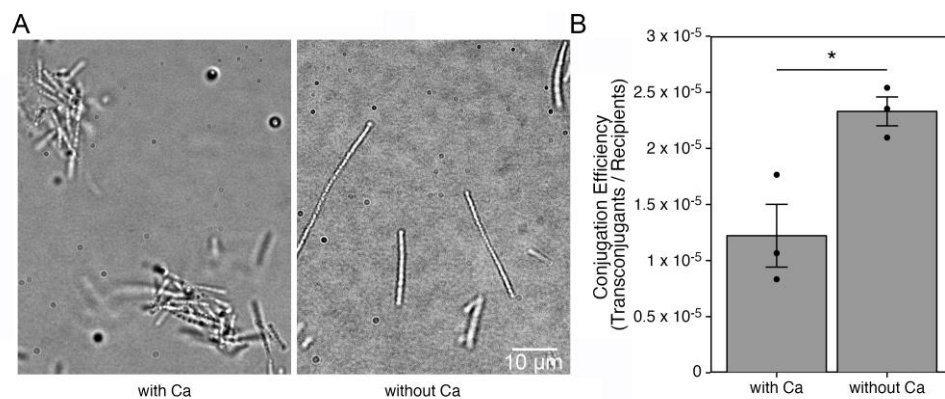


Figure 8. Conjugation efficiency with suppressing sheath formation. The brightfield images indicate that aggregate formation is reduced when cultured in the Ca-deficient condition (A). Conjugation efficiency with or without culturing cells in calcium-free medium (B). Statistical analysis was performed by unpaired Welch's *t*-tests. **p* < 0.04.

- To validate whether the described method is universal for gene transfer, we employed three additional plasmids designed to target the *Lcho_1694*, *Lcho_2352*, and *Lcho_2734* loci. As Figure 9 shows that the conjugation efficiencies are comparable among the representative plasmids, we consider our method to be useful for constructions targeting different loci.

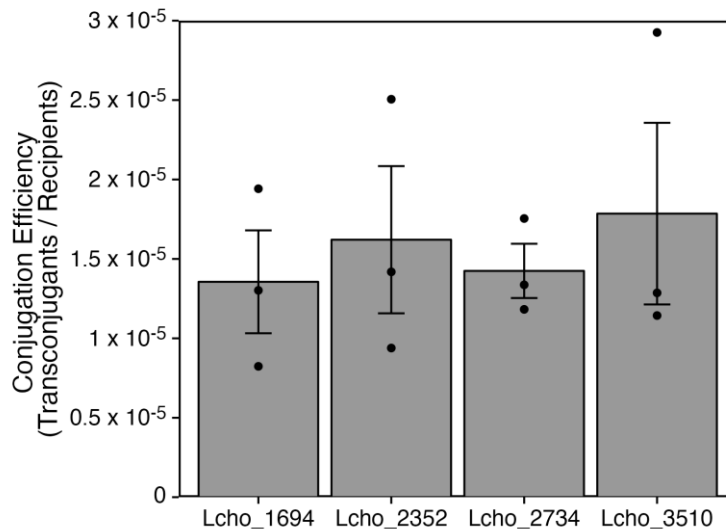


Figure 9. Conjugation efficiencies of plasmids targeting different loci. Statistical analysis was performed by Welch's *t*-tests and Bonferroni-Holm method, suggesting no significant differences.

In experiments shown in Figure 9, approximately 250 colonies on average were obtained when 25 × 4 and 8 × 2 mL cultures of *L. cholodnii* and *E. coli*, respectively, were employed for conjugation. As far as confirmed by colony PCR, the gene replacement occurred correctly in most candidate colonies. These results implicate that we can set up the mutagenesis experiments as described in this protocol.

Recipes

1. MSVP (ATCC 1917) medium

Prepare MVSP medium (Emerson and Ghiorse, 1993) for culturing SP-6 cells (see Tables 4, 5). Omit $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ from the original recipe of MSVP for a calcium-lacking medium (MSVP-Ca). To isolate spontaneous rifampicin-resistant mutant (WT*), sequentially spread the cell suspension on MSVP plates containing 5, 10, and 20 $\mu\text{g}/\text{mL}$ rifampicin. MSVP plates containing 20 $\mu\text{g}/\text{mL}$ rifampicin and 50 $\mu\text{g}/\text{mL}$ kanamycin sulfate can be used for the selection of recombinants of WT* containing the *Km^r* gene, because *E. coli* S17-1 cells are sensitive to rifampicin, while non-recombinant WT* cells are sensitive to kanamycin but not to rifampicin.

Table 4. Composition of MSVP medium

Component	Amount (g/L)	Concentration (mM)
$(\text{NH}_4)_2\text{SO}_4$	0.24	1.81
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.06	0.24
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.06	0.41
KH_2PO_4	0.02	0.15
Na_2HPO_4	0.03	0.14
HEPES	2.383	10.00

Adjust to pH 7.0 with 0.1 N NaOH, fill up to 1 L with ultrapure water, and autoclave

Component	Amount (mL/L)	Concentration (mM)
10 mM FeSO_4	1.00	0.01
20% sodium pyruvate	5.00	9.09
Vitamin stock solution (see Table 5)	1.00	-

Sterilize these solutions by filtration and add prior to use

Table 5. Composition of vitamin stock solution

Component	Amount (mg/L)	Concentration (mM)	Working concentration (nM)
Biotin	20.00	0.04	0.04
Folic acid	20.00	0.05	0.05
Thiamine·HCl	50.00	0.15	0.15
D-(+)-Calcium pantothenate	50.00	0.10	0.10
Vitamin B12	1.00	0.74 (nM)	0.74 (pM)
Riboflavin	50.00	0.13	0.13
Nicotinic acid	50.00	0.41	0.41
Pyridoxine hydrochloride	100.00	0.49	0.49
p-Aminobenzoic acid	50.00	0.36	0.36

Sterilize by filtration and add prior to use

2. LB medium

Dissolve 20 g of LB broth in 1,000 mL of distilled water and autoclave at 121 °C for 15 min. For the LB plate, add agar at 1.5%. After autoclaving, add 50 $\mu\text{g}/\text{mL}$ of kanamycin sulfate and ampicillin sodium salt as needed.

3. SOC medium

Dissolve 20 g of tryptone, 5 g of yeast extract, 0.5 g of NaCl, and 0.186 g of KCl in distilled water, adjust the pH to 7.0 by 5 N NaOH solution, and fill up to 1,000 mL with distilled water. After autoclaving at 121 °C for 15 min, add 10 mL of 1 M MgSO₄ and 1 M MgCl₂ solutions (see Recipe 5), and 20 mL of 1 M glucose solution (see Recipe 6).

4. NB medium

Dissolve 8 g of nutrient broth in 1,000 mL of distilled water and autoclave at 121 °C for 15 min. For the NB plate, add agar at 1.5%.

5. 1 M MgSO₄ and 1 M MgCl₂ solutions

Dissolve 24.65 g of MgSO₄·7H₂O and 9.52 g of MgCl₂ in 100 mL of distilled water and autoclave at 121 °C for 15 min.

6. 1 M glucose solution

Dissolve 18.0 g of D-glucose in 100 mL of distilled water and sterilize using a 0.22 μm syringe filter.

7. NaCl solutions (0.9%, 5 M)

Dissolve 0.052 or 29.22 g of NaCl in ultrapure water, adjust the volume to 100 mL, and autoclave at 121 °C for 15 min, respectively.

8. TE buffer

Add 0.61 g of Tris-HCl (10 mM) and 0.23 g EDTA-4Na (1 mM) in ultrapure water, adjust pH to 8.0 with hydrochloric acid (HCl), adjust the volume to 500 mL with ultrapure water, and autoclave at 121 °C for 15 min.

9. TEN buffer

Mix 5 mL of 1 M Tris-HCl buffer (pH 8.0), 1 mL of 0.5 M EDTA-4Na (pH 8.0), 10 mL of 5 M NaCl, and 484 mL of ultrapure water, followed by autoclaving at 121 °C for 15 min.

10. 10% SDS solution

Dissolve 10 g of SDS in ultrapure water and adjust the volume to 100 mL.

11. CTAB/NaCl solution

Dissolve 10 g of CTAB and 4.09 g of NaCl (0.7 M) in ultrapure water, adjust the volume to 100 mL, and autoclave at 121 °C for 15 min.

12. 3 M sodium acetate (CH₃COONa) solution

Dissolve 40.82 g of CH₃COONa·3H₂O in ultrapure water, adjust the volume to 100 mL, and autoclave at 121 °C for 15 min.

13. 50× TAE buffer

Dissolve 242 g of Tris and 22.6 g of EDTA-4Na in ultrapure water containing 57.1 mL of acetic acid and adjust the volume to 1,000 mL.

14. 2× TSS solution

Dissolve 4 g of PEG8000, 0.49 g of MgSO₄·7H₂O, and 4 mL of glycerol in LB medium (see Recipe 2), adjust the volume to 20 mL, and autoclave at 121 °C for 15 min.

15. TSS solution

Mix 25 mL of sterilized LB medium (see Recipe 2) and 20 mL of 2× TSS solution (see Recipe 14) with 5 mL of DMSO.

16. 50% glycerol solution

Mix 50 mL of glycerol with 50 mL ultrapure water and autoclave at 121 °C for 15 min.

17. Rifampicin solution

Mix 50 mg of rifampicin with 1 mL of DMSO and stock at 4 °C.

18. Kanamycin solution

Mix 50 mg of kanamycin sulfate with 1 mL of ultrapure water and stock at 4 °C.

19. Ampicillin solution

Mix 50 mg of ampicillin sodium salt with 1 mL of ultrapure water and stock at 4 °C.

Acknowledgments

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Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

Ethics

This study was approved by the committee for the safe handling of living modified organisms at the University of Tsukuba and was carried out according to the guidelines of the committee.

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