bio-protocol

Published: Sep 05, 2022

# A New Tool for the Flexible Genetic Manipulation of *Geobacillus kaustophilus*

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## Abstract

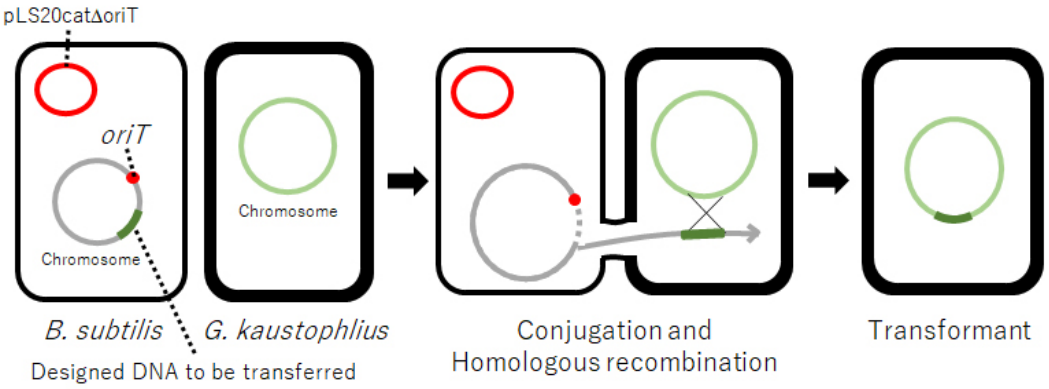
*Geobacillus kaustophilus*, a thermophilic Gram-positive bacterium, is an attractive host for the development of high-temperature bioprocesses. However, its reluctance against genetic manipulation by standard methodologies hampers its exploitation. Here, we describe a simple methodology in which an artificial DNA segment on the chromosome of *Bacillus subtilis* can be transferred via pLS20-mediated conjugation resulting in subsequent integration in the genome of *G. kaustophilus*. Therefore, we have developed a transformation strategy to design an artificial DNA segment on the chromosome of *B. subtilis* and introduce it into *G. kaustophilus*. The artificial DNA segment can be freely designed by taking advantage of the plasticity of the *B. subtilis* genome and combined with the simplicity of pLS20 conjugation transfer. This transformation strategy would adapt to various Gram-positive bacteria other than *G. kaustophilus*.

**Keywords:** *Bacillus subtilis*, Conjugation, *Geobacillus kaustophilus*, Gram-positive, Homologous recombination, Transformation

**This protocol was validated in:** Microb Cell Fact (2022), DOI: 10.1186/s12934-022-01759-8

Published: Sep 05, 2022

## Graphical abstract




## Background

*Geobacillus kaustophilus* HTA426 is a thermophilic Gram-positive bacterium isolated from sediments of the Mariana Trench (Nazina et al., 2001; Takami et al., 2004). It grows at temperatures ranging from 48 to 74 °C (optimal temperature of 60 °C) and is a promising chassis for high-temperature bioprocessing with the advantages of preventing contamination by mesophilic bacteria and lowering the cost of controlling fermentation heat. However, this bacterium is difficult to transform by natural competence or electroporation, and an approach based on a conjugation system for Gram-negative bacteria has been developed (Suzuki and Yoshida, 2012). This method has the disadvantage of not being able to select transformants that acquired mutations with adverse effects on growth. Therefore, better techniques for manipulating the genome of *G. kaustophilus* are needed.

Conjugation is one of the three main mechanisms for horizontal gene transfer in bacteria. Conjugative elements, which often are located on plasmids, encode all the proteins required for their transfer from a donor cell to a recipient cell. Conjugative elements encode adhesins or organelles facilitating interaction with recipient cells. In almost all conjugative systems only a single strand of the DNA (ssDNA) is transferred. Generation of the ssDNA is done by a relaxase, often assisted by one or two auxiliary proteins, that introduces a site- and strand-specific nick in a region of the plasmid named origin of transfer (*oriT*). After nicking, the relaxase remains covalently attached to the 5′ end of the DNA, and the generated 3′ end is used as a primer for DNA synthesis resulting in a rolling-circle mode of replication, thereby generating the ssDNA molecule that is destined for transfer into the recipient cell. Conjugative elements encode a sophisticated transferosome (type IV secretion system) connecting the donor and recipient cells. The relaxosome complex, which is composed of a number of proteins including the relaxase, is recruited to the cytosolic side of the transferosome in the donor cell, after which it guides the transfer of the ssDNA into the recipient cell (Christie et al., 2014; Christie, 2016; Waksman, 2019).

pLS20 is a 65 kb conjugative plasmid isolated from *Bacillus subtilis* var. natto (Tanaka and Koshikawa, 1977); it can transfer itself among various *Bacillus* species ( Koehler and Thorne, 1987). Contrary to most conjugative systems that require prolonged interactions between donor and recipient cells on solid medium, efficient conjugation of pLS20 is achieved by mixing donor and recipient cells in liquid medium (Tanaka and Koshikawa, 1977; Singh et al., 2013; Miyano et al., 2018). Genes involved in pLS20 conjugation (genes *28–74*) form a large operon controlled by a strong promoter named Pc. Regulation of pLS20 conjugation genes has been studied in detail (for a review, see Meijer et al., 2021). Gene *27* encodes $Rco_{pLS20}$, which is the master regulator of the conjugation operon. In its default state, conjugation is suppressed due to binding of $Rco_{pLS20}$ to its two operator sites within the Pc region, strictly repressing the Pc promoter. Gene *25* encodes an antirepressor named $Rap_{pLS20}$, which activates conjugation by relieving repression from $Rco_{pLS20}$ and forming a Rap/Rco complex. Gene *26* encodes a quorum sensing peptide, $Phr^*_{pLS20}$, that regulates the activity of $Rap_{pLS20}$. $phr_{pLS20}$ synthesizes a small pre-proprotein. After being secreted, $Phr_{pLS20}$ is proteolytically cleaved, generating the mature $Phr^*_{pLS20}$ peptide corresponding to its 5 C-terminal residues. When taken up by donor cells, $Phr^*_{pLS20}$ inactivates $Rap_{pLS20}$ by changing its conformation upon binding to it,

returning the system to its default repressed state (Singh et al., 2013). Ectopic overproduction of $Rap_{pLS20}$ increased the efficiency of conjugation by weakening the effect of $Phr^*_{pLS20}$ (Singh et al., 2013).

A derivative of pLS20 lacking *oriT*$_{pLS20}$, pLS20catΔoriT, is defective in conjugation (Miyano et al., 2018). However, we have recently shown that pLS20catΔoriT can mediate the transfer of large regions of the bacterial chromosome between *B. subtilis* cells when a copy of *oriT*$_{pLS20}$ is present on the chromosome in the donor cell (Miyano et al., 2018). Inspired by this, we have developed a strategy to transform *G. kaustophilus* that allows for flexible manipulation of its chromosome (Mori et al., 2022). Here, we present the protocol to transform *G. kaustophilus* by pLS20-mediated conjugative transfer of a chromosomal DNA of *B. subtilis* designed to function in *G. kaustophilus*.

## Materials and Reagents

*Note: All reagents can be stored at room temperature unless otherwise specified.*

### A. Construction of the donor strain

1. *B. subtilis* 168 (Mori et al., 2022)
2. *B. subtilis* YNB211 (Mori et al., 2022)
3. *G. kaustophilus* MK244 (Mori et al., 2022)
4. pUCG18T (Mori et al., 2022)
5. LB medium (2% agar) (BD Difco, catalog number: 240230)
6. 10% SDS (Nacalai Tesque, catalog number: 30562-04)
7. Lysozyme (Wako, catalog number: 129-06723)
8. Phenol/Chloroform/Isoamyl alcohol (25:24:1) (Wako, catalog number: 311-90151), store at 4 °C
9. 70% Ethanol
10. 99.5% Ethanol
11. KOD -Plus- Neo polymerase (Toyobo, catalog number: KOD-401), store at -20 °C
12. Agarose for electrophoresis gel (ME, Nacalai Tesque, catalog number: 01133-24)
13. *In silico* Molecular Cloning software for designing PCR primers (In Silico Biology, Inc., catalog number: IMCGF01)
14. Primer pairs used for the PCR reaction of five fragments
    a. Fragment A1:
       aprE-U-f1 ccggtacttgccaccacatcataac
       aprE-U-r cagtaacctcatcaagccaagctacctctcgctatttccgtagagactcg
    b. Fragment A2:
       aprE-D-f2 gacagaggaattagatacattcgcgttaatcaacgtacaagcagctgcac
       aprE-D-r ggccgagcagtattcgaatgtcaag
    c. Fragment B1:
       degA-U-f gtagcttggcttgatgaggttactg
       degA-U-r2 catcggtcataaaatccgtatccttggttactttcatcgctcatcattc
    d. Fragment B2:
       degA-D-f2 ctgcaaggcgattaagttgggtaacagtgaaatcgtaaggatgtgagcag
       degA-D-r cgcgaatgtatctaattcctctgtc
    e. Fragment C1:
       kan-f aaggatacggattttatgaccgatg
       kan-r2 gttacccaacttaatcgccttgcag
15. Primers used for the recombinant PCR reactions that ligate the five fragments.
    a. aprE-U-f3-nested caccgagctcatagcttgtcgcgatcacctcatcc
    b. aprE-D-r2-nested tgctttcgctgattacaacattggtgacgctgcct
16. DNA purification kit (Wizard® SV Gel and PCR Clean-Up System, Promega, catalog number: A9281)

17. 7.5 mg/mL Kanamycin sulfate stock solution in water (Wako, catalog number: 113-00343)
18. Solution A (see Recipes)
19. Solution B (see Recipes)
20. 100× Trace element (see Recipes)
21. TKE buffer (see Recipes)
22. 50× TAE buffer (see Recipes)
23. C-1 and C-2 medium for transformation (see Recipes)

### B. Conjugation and selection of transconjugant (transformant)

1. 7.5 mg/mL Kanamycin sulfate stock solution in water (Wako, catalog number: 113-00343)
2. 10 mg/mL Chloramphenicol stock solution in 70% ethanol (Wako, catalog number: 030-19452)
3. 100 mM IPTG stock solution in water (Nacalai Tesque, catalog number: 367-93-1)
4. LB medium (see Recipes)
5. LBMSM medium (see Recipes)

### C. Southern blotting analysis

1. Primers used for amplification of a template DNA for synthesizing RNA probe
   a. Km-probe-f taatacgactcactatagggtatggctctcttggtcgtc
   b. Km-probe-r tctgattccacctgagatgc
2. Agarose for electrophoresis gel (SP, Nacalai Tesque, catalog number: 01163-76)
3. DNA Molecular Weight Marker II, DIG-labeled (Roche, catalog number: 11218590910)
4. DNA purification kit (Wizard® SV Gel and PCR Clean-Up System, Promega, catalog number: A9281)
5. T7 RNA polymerase (Roche, catalog number: 10881767001), store at -20 °C
6. DIG RNA Labeling Mix, 10× conc. (Roche, catalog number: 11277073910), store at -20 °C
7. Restriction enzymes
   a. *Cla* I (Takara, catalog number: 1034A), store at -20 °C
   b. *Sac* II (Takara, catalog number: 1079A), store at -20 °C
8. DIG Easy Hyb (Roche, catalog number: 11603558001), store at 4 °C
9. Amersham Hybond-N+ (GE Healthcare, catalog number: RPN82B)
10. Hybridization bag (Hybridization Bags Hybri-Bag Hard, COSMO BIO, catalog number: S-1001)
11. Blocking Reagent (Roche, catalog number: 11096176001)
12. Anti-Digoxigenin-AP, Fab fragment (Roche, catalog number: 11093274910), store at 4 °C
13. CSPD, ready-to-use (Roche, number: 11755633001), store at 4 °C
14. 20× SSC buffer (see Recipes)
15. Maleic acid buffer (see Recipes)
16. Denaturing solution (see Recipes)
17. Neutralizing solution (see Recipes)
18. High-stringency wash solution (see Recipes)
19. Low-stringency wash solution (see Recipes)
20. Detection buffer (see Recipes)

## Equipment

### A. Construction of the donor strain

1. Thermal cycler (for example, Takara Bio, Thermal Cycler Dice Touch TP350, or equivalent)
2. Centrifuge (for example, TOMY, MX-307, or equivalent)
3. Spectrophotometer (for example, GE Healthcare, NanoVue Plus, or equivalent)

4. Agarose gel electrophoresis apparatus (for example, Takara Bio, Mupid®-2plus, or equivalent)
5. Incubator (for example, PHCbi, MIR-H163, or equivalent)

### B. Conjugation and selection of transconjugant (transformant)

1. Two incubator shakers, one set at 37 °C and the other at 60 °C (TAITEC, Bioshaker BR-43FM-MR, or equivalent)
2. Centrifuge (TOMY, MX-307, or equivalent)
3. Two incubators, one is set at 37 °C and the other 60 °C (PHCbi, MIR-H163, or equivalents)
4. Spectrophotometer (SHIMADZU, UV-1800, or equivalent)
5. 300 mL baffled Erlenmeyer flask

### C. Southern blotting (or: Southern blot analysis)

1. Thermal cycler (Takara Bio, Thermal Cycler Dice Touch TP350, or equivalent)
2. Spectrophotometer (GE Healthcare, NanoVue Plus, or equivalent)
3. Agarose gel electrophoresis apparatus (Bio-Rad, Sub-Cell GT Cell, or equivalent)
4. Transfer apparatus (Bio Craft, Vacuum Transfer BS-31, or equivalent)
5. UV crosslinker (UVP, CL-1000, or equivalent)
6. Shaker (Bio Craft, Labo Shaker BC-730, or equivalent)
7. Chemiluminescence imager (Bio-Rad, ChemiDoc XRS+, or equivalent)

## Procedure

### A. Construction of the donor strain

1. Isolation of DNA from bacterial strains
   a. Grow bacterial cells in 5 mL of LB medium at 37 °C overnight with shaking.
   b. Collect bacterial cells of 1.5 mL of culture in a test tube by centrifugation at 8,152 × *g* for 3 min at room temperature.
   c. Suspend the cells in 500 µL of TKE buffer containing 1 mg of freshly added lysozyme.
   d. Incubate the tube for 30 min at 37 °C.
   e. Add 50 µL of 10% SDS and mix thoroughly.
   f. Add 500 µL of phenol/chloroform/isoamyl alcohol (25:24:1) and mix vigorously.
   g. Centrifuge the tube at 20,380 × *g* for 10 min at 4 °C, and then transfer 200 µL of supernatant to a new tube; avoid transferring the white interface.
   h. Add 500 µL of 99.5% ethanol to the tube, mix and stand on ice for 10 min, and centrifuge at 20,380 × *g* for 5 min. Discard the supernatant.
   i. Wash the DNA pellet carefully with 0.7 mL of 70% ethanol and place the sample 10 min at 37 °C to evaporate remains of ethanol.
   j. Add 100 µL of distilled water and place 10 min at 37 °C to dissolve the DNA gently.
   k. Measure the concentration of DNA using a spectrophotometer such as NanoVue Plus.
2. Setting up recombinant PCR reaction to generate the “gene cassette”
   The schematic organization of the Δ*iolQ*$_{GK}$*::kan* cassette, replacing the *iolQ*$_{GK}$ coding region of *G. kaustophilus* with a thermostable kanamycin-resistance gene, is used in this protocol as an example (Figure 1). This gene cassette was generated by recombinant PCR, ligating the five PCR fragments as follows.
   a. Amplify the five fragments individually (A1, A2, B1, B2, and C1) by PCR using KOD -Plus- neo polymerase. The specific primer pairs used to amplify each of the fragments are listed above in the Materials and Reagents section. It is strongly recommended to check the correct amplification of each PCR fragment by agarose gel electrophoresis. The PCR reactions are composed and carried out as

follows:

| PCR reaction: | | |
|---|---|---|
| Components | Volume | Final Concentration |
| 10× PCR Buffer | 5 µL | 1× |
| 2 mM dNTPs | 5 µL | 0.2 mM each |
| 25 mM $MgSO_4$ | 3 µL | 1.5 mM |
| Primer (10 µM each) | 0.75–1.5 µL | 0.15–0.3 µM each |
| Template DNA | variable | Plasmid/PCR fragment DNA ~50 ng/50 µL<br>Genomic DNA ~200 ng/50 µL |
| KOD -Plus- Neo (1 U/µL) | 1 µL | 1 U/50 µL |
| $H_2O$ | variable | |
| Total | 50 µL | |

| Thermal cycler conditions: | | |
|---|---|---|
| Initial Denaturation | 98 °C | 2 min |
| 30 cycles | 98 °C | 10 s |
| | 68 °C | 30 s/kb |
| Hold | 4 °C | |

b. Purify the PCR reactions using a DNA purification kit and determine the concentration of the purified DNA by measuring $OD_{260nm}$.
c. Mix all the amplified and purified fragments in a 1:1:1:1:1 molar ratio.
d. Run the recombinant PCR for the recombination of the five fragments using primers aprED-r2-nested and aprEU-f3-nested. The PCR reaction is the same as described above, but set the thermal cycler conditions as follows:

| Thermal cycler conditions: | | |
|---|---|---|
| Initial Denaturation | 98 °C | 2 min |
| 30 cycles | 98 °C | 10 s |
| | 68 °C | 2.5 min |
| Hold | 4 °C | |

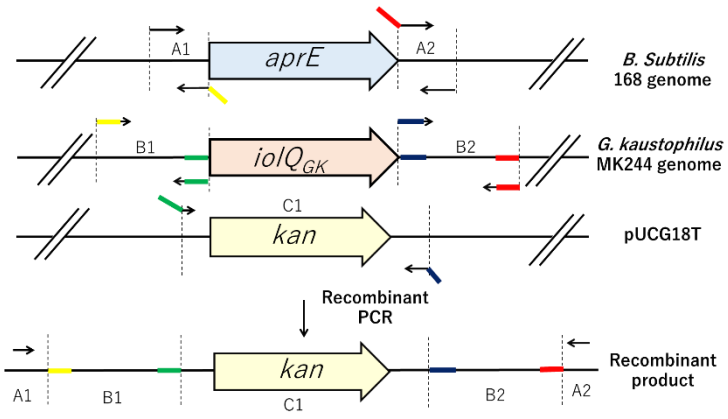



**Figure 1. Strategy to generate the Δ*iolQ*$_{GK}$::*kan* cassette (recombinant product).**
Five fragments are amplified by PCR using the primer pairs mentioned in A.14. Two fragments correspond to "upstream" and "downstream" regions of the *B. subtilis aprE* locus and are named A1 and A2,

respectively. Two other fragments corresponding to sequences located upstream and downstream of *G. kaustophilus* MK244 *iolQ*$_{GK}$ are named B1 and B2, respectively; the DNA fragment containing the kanamycin-resistance gene of pUCG18T is named C1. Fragments A1 and A2 are designed to be approximately 1 kb long, and fragments B1 and B2 are approximately 3 kb. The specific primers, indicated as arrows, are listed in the Materials and Reagents section. Colored regions of bent arrows correspond to 5′ extensions designed to create overlap with a flanking region in the final recombinant product. Overlapping sequences are indicated using different colors. The five individual PCR fragments are linked together by PCR to form one long "gene cassette" fragment.

3. Transformation

   The presence of pLS20 lowers competence levels (Singh et al., 2012). Therefore, it is recommended to generate the final *B. subtilis* donor YNB213 in two steps. First, the five-fragment recombinant product generated by PCR is used to transform competent *B. subtilis 168* cells. Second, chromosomal DNA isolated from a kanamycin-resistant transformant of this transformation is then used to transform competent *B. subtilis* YNB211 cells harboring pLS20catΔoriT and containing both the *oriT*$_{pLS20}$ and the IPTG-inducible *rap*$_{pLS20}$ present on the chromosome at the *yhfK* and *amyE* loci, respectively (Miyano et al., 2018; Mori et al., 2022). Transformation of *B. subtilis* is performed as follows:

   a. Grow a strain of *B. subtilis* to be transformed overnight (~16 h) on an LB 2% agar plate at 30 °C to form colonies.
   b. Pick up a fresh colony and use it to inoculate 5 mL of C-1 medium in a test tube to allow the cells to grow at 37 °C with vigorous shaking (at least 200 rpm) for 2.5 h.
   c. Collect the cells by centrifugation at 6,000 rpm at room temperature for 3 min.
   d. Suspend the cell pellet in 10 mL of C-2 medium in a new test tube and incubate the tube with shaking at 37 °C for 40 min.
   e. Transfer 1 mL of the C-2 medium culture into a new tube containing less than 100 µL of DNA solution (1–10 µg/mL).
   f. Incubate the tube with shaking at 37 °C for 2 h.
   g. Spread the culture onto plates containing appropriate antibiotics to select transformants.

## B. Conjugation and selection of transconjugant (Figure 2)

Based on the previous findings that the addition of sorbitol (Kananavičiūtė et al., 2015), $MgCl_2$, and maleic acid-NaOH buffer (Wyrick and Rogers, 1973) was effective in increasing the efficiency of transformation by electroporation and stabilizing L-form cells, we developed a modified medium, named LBMSM, suitable for elevating the efficiency of this transformation of *G. kaustophilus* mediated by conjugation.

1. Preparation of donor culture
   a. Grow *B. subtilis* YNB213 overnight on an LB 2% agar plate containing 10 µg/mL chloramphenicol at 37 °C to form colonies.
   b. Pick up a fresh colony and use it to inoculate 5 mL of LB liquid medium containing 10 µg/mL chloramphenicol, pre-warmed at 37 °C in a test tube.
   c. Grow overnight at 37 °C with shaking at 200 rpm.
   d. In a 300 mL baffled Erlenmeyer flask pre-warmed at 37 °C, dilute the culture in 10 mL of LBMSM medium containing 1.0 mM IPTG to reach an $OD_{600}$ of 0.05 (check using a spectrophotometer).
   e. Grow the cells at 37 °C with shaking at 200 rpm until $OD_{600}$ reaches 0.6–0.8, and use this as the donor culture.
2. Preparation of recipient culture
   a. Grow *G. kaustophilus* MK244 overnight on an LB 2% agar plate at 60 °C to form colonies.
   b. Pick up a fresh colony and inoculate it into 10 mL of LB liquid medium pre-warmed to 60 °C in a 300 mL Erlenmeyer flask.
   c. Grow the cells at 60 °C for 2 h with shaking at 200 rpm.
   d. Dilute the culture (to make $OD_{600}$ adjusted to 0.01 using a spectrophotometer) in 10 mL of LBMSM medium in a 300 mL baffled Erlenmeyer flask pre-warmed at 60 °C.

e. Allow the cells to grow at 60 °C with shaking at 200 rpm until $OD_{600}$ reaches 0.6–0.8 measured with a spectrophotometer, and use this as the recipient culture.

3. Mating
   a. Mix 2 mL of the donor culture and 8 mL of the recipient culture in a sterilized 300 mL Erlenmeyer flask.
   b. Stand the mixed culture at 37 °C for 90 min, and then allow the cells to grow at 60 °C for 180 min with shaking at 200 rpm.
4. Colony formation of transconjugant
   a. Transfer the mating culture into a test tube and centrifuge at 8,152 × *g* for 2 min and resuspend the pellet in 1.2 mL of LB liquid medium pre-warmed at 60 °C in a 1.5 mL tube.
   b. Spread the suspension onto LB 2% agar plates containing 7.5 µg/mL of kanamycin pre-warmed at 60 °C (200 µL on each Petri dish 9 cm in diameter).
   c. As negative control experiments, spread separately donor and recipient cells onto LB 2% agar plates containing 7.5 µg/mL of kanamycin pre-warmed at 60 °C.
   d. Incubate the plates at 60 °C overnight and score the number of colonies.
5. Colony formation of recipient
   a. Sample a small portion (~100 µL) of the mating culture, dilute it $10^7$-fold, and spread 100 µL of the dilution on LB 2% agar plates.
   b. Incubate the plates at 60 °C overnight and score the number of colonies.

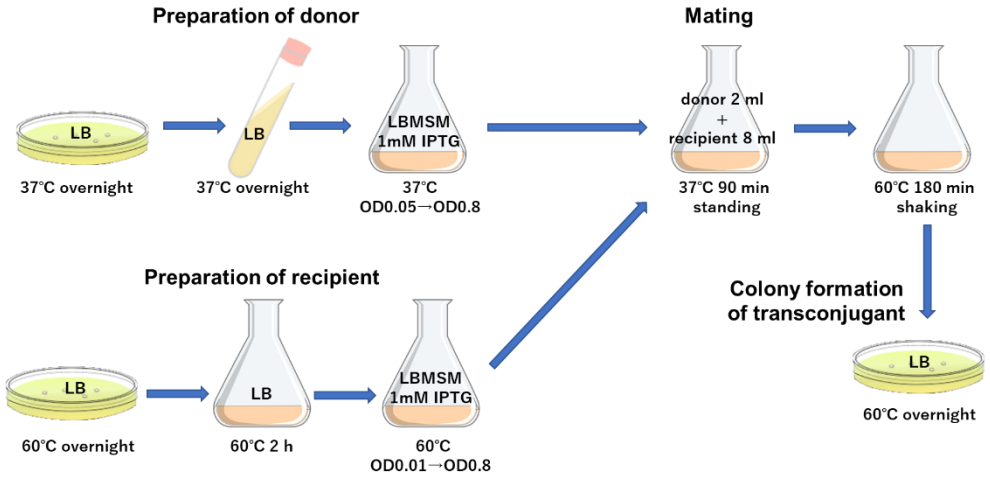



**Figure 2. A schematic presentation of the conjugation procedure.**
Donor strain *B. subtilis* YNB213 and recipient strain *G. kaustophilus* MK244 were grown independently. Mating was performed by mixing two cultures in a 1 to 4 ratio of donor and recipient cells, followed by further incubation to enhance colony formation.

## C. Southern blot analysis

Southern blot analysis allows us to detect a specific DNA sequence in chromosomal DNA samples, which usually involves four successive steps, including (i) Fragmentation of chromosomal DNA with restriction enzymes, (ii) Separation of DNA fragments by electrophoresis, (iii) Transfer of the DNA onto the membrane, and (iv) Detection of DNA on the membrane using the probe. It is important to choose appropriate restriction enzymes for the fragmentation of chromosomal DNA. In the case of this protocol, we decided on *Cla* I and *Sac* II (Figure 3). For the detection of DNA on the membrane, a fluorescence-based nonradioactive technology with a DIG-labeled RNA probe is used for the detection of target DNA.

1. Preparation of DIG-labeled RNA
   a. Run PCR to amplify the stretch corresponding to the kanamycin-resistance gene using KOD -Plus-

neo polymerase and the primer pairs Km-probe-f and Km-probe-r. The PCR reactions are composed and carried out as follows:

PCR reaction:

| Components | Volume | Final Concentration |
|---|---|---|
| 10× PCR Buffer | 5 µL | 1× |
| 2 mM dNTPs | 5 µL | 0.2 mM each |
| 25 mM $MgSO_4$ | 3 µL | 1.5 mM |
| Primer (10 µM each) | 0.75–1.5 µL | 0.15–0.3 µM each |
| pUCG18T DNA | variable | ~50 ng/50 µL |
| KOD -Plus- Neo (1 U/µL) | 1 µL | 1 U/50 µL |
| $H_2O$ | variable | |
| Total | 50 µL | |

Thermal cycler condition:

| | | |
|---|---|---|
| Initial Denaturation | 98 °C | 2 min |
| 30 cycle | 98 °C | 10 s |
| | 68 °C | 30 s/kb |
| Hold | 4 °C | |

b. Using the DNA purification kit, purify the amplified DNA as the template for *in vitro* transcription.
c. Perform the *in vitro* transcription of the template DNA using T7 RNA polymerase and DIG RNA Labeling Mix, following the protocol provided by the manufacturer. This step generates a DIG-labeled RNA probe.
d. Store the DIG-labeled RNA probe at -80 °C until being used.

2. Digestion and electrophoresis of the genomic DNA
   a. Extract genomic DNAs and measure $OD_{260}$ to determine their concentration.
   b. Digest 3 µg of genomic DNAs with appropriate restriction enzymes at 37 °C for at least 1 h to complete the digestion.
   c. Subject the digested DNAs in parallel with the size markers (DNA Molecular Weight Marker II, DIG-labeled) to 1.0% agarose gel electrophoresis.
   d. Run the electrophoresis for 1 h at 100 V.
3. Blotting, hybridization, and detection
   a. Soak the agarose gel successively in 150 mL of (i) 0.25 M HCl (30 min), (ii) denaturing solution (30 min), and (iii) neutralizing solution (30 min).
   b. Transfer DNAs in the gel onto a positively charged nylon membrane (Amersham Hybond-N+), using the transfer apparatus (Vacuum Transfer BS-31) as instructed by the supplier.
   c. Crosslink the transferred DNAs to the membrane with UV crosslinker L-1000 with accumulative radiation set to 0.07 J/$cm^2$.
   d. Transfer the membrane into a hybridization bag, and fill up the bag with 30 mL of DIG Easy Hyb at 50 °C for at least 10 min for pre-hybridization.
   e. Denature the DIG-labeled RNA probe by boiling the water solution containing the probe for 10 min and then snap-cool by placing the sample on ice.
   f. Soak the membrane in the bag filled with 30 mL of DIG Easy Hyb containing 100 ng/mL of denatured DIG-labeled RNA probe at 42 °C overnight with gentle shaking.
   g. Wash the membrane twice for 5 min with 150 mL of low-stringency wash solution at room temperature with gentle shaking.
   h. Wash the membrane twice for 15 min with 150 mL of pre-warmed high-stringency wash solution at 65 °C with gentle shaking.
   i. Wash the membrane briefly with 150 mL of maleic acid buffer with gentle shaking.
   j. Soak the membrane in blocking reagent diluted in 150 mL of maleic buffer for 30 min at room temperature to prevent non-specific binding of antibodies.

k. Transfer the membrane into a hybridization bag and incubate for 30 min with 30 mL of blocking reagent containing 1/$10^4$ volume of Anti-Digoxigenin-AP, Fab fragment (20 mL of blocking reagent per 100 $cm^2$ of membrane should be used).
l. Wash the membrane twice with 150 mL of wash buffer for 15 min with gentle shaking.
m. Transfer the membrane into a new hybridization bag and equilibrate with 30 mL of detection buffer for 3 min.
n. Discard the detection buffer and incubate the membrane with 30 mL of detection buffer containing CSPD at 37 °C for 5–10 min (20 mL of detection buffer should be used per 100 $cm^2$ of membrane).
o. Detect the chemiluminescence signal using ChemiDoc XRS+ following the instructions of the supplier.

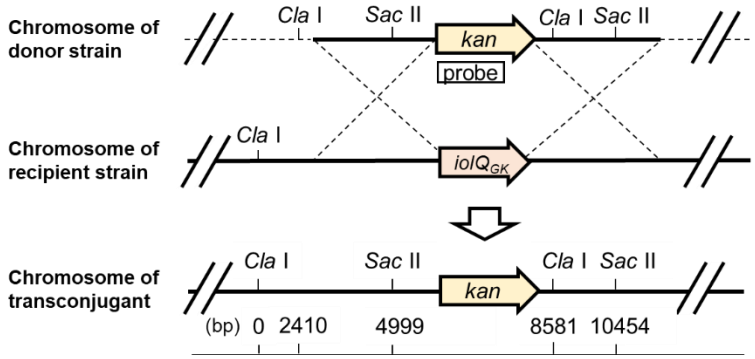



**Figure 3. Physical maps of the relevant regions of the chromosomes of donor, recipient, and transconjugant strains.**

## Data analysis

### A. Conjugation and selection of transconjugant

Evaluate the efficiency judging from the value of $CFU_{transconjugant}/CFU_{recipient}$, which usually is approximately $1.0 \times 10^{-9}$.

1. Count the number of colonies that appear on the plates containing kanamycin incubated at 60 °C overnight. Calculate $CFU_{transconjugant}$ as follows:

   $CFU_{transconjugant}$ = Mean ± SD of colony numbers per plate × 1.2 mL/200 µL/10 mL

   For example, when 3, 3, and 5 colonies were formed on three plates, respectively:
   $CFU_{transconjugant} = 3.7 \pm 1.2 \times 1.2/0.2/10 = 2.2 \pm 0.7$

2. Count the number of colonies that appear on the plates without kanamycin incubated at 60 °C overnight. Calculate $CFU_{recipient}$ as follows:

   $CFU_{recipient}$ = Mean ± SD of colony numbers per plate × $10^7$ × 1 mL/100 µL

   For example, when 18, 13, and 11 colonies were formed on three plates, respectively:
   $CFU_{recipient} = 14.0 \pm 3.6 \times 10^7 \times 1.0 /0.1 = 1.4 \pm 0.36 \times 10^9$

### B. Southern blot analysis

1. Take a picture of the chemiluminescence signal on the membrane to visualize the bands of DNA fragments containing the kanamycin-resistance gene (Figure 4).
2. Estimate the length of each band in relation to the positions of the size marker DNAs electrophoresed in parallel with the samples.
3. Check against the physical maps of the chromosome to ensure that the kanamycin-resistance gene has been inserted at the desired location. Proper insertion of the kanamycin-resistance gene, in this case, is confirmed by the appearance of a specific combination of bands on the membrane: a 5,456 bp band from both transconjugant and donor for *Sac*II digestion, an 8,581 bp band from transconjugant or a 6,172 bp band from a donor for *Cla*I digestion, and a 3,583 bp band from both transconjugant and donor for *Sac*II and *Cla*I double digestion.

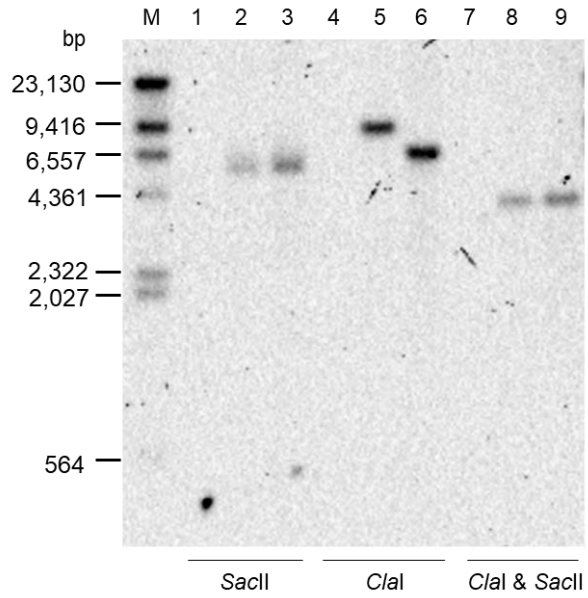



**Figure 4. Results of Southern blot analysis.**
Chromosomal DNAs of the Recipient (lanes 1, 4, and 7), Transconjugant (lanes 2, 5, and 8), and Donor (lanes 3, 6, and 9) strains digested with *Sac* II (lanes 1–3), *Cla* I (lanes 4–6), and *Cla* I & *Sac* II (lanes 7–9) were subjected to agarose gel electrophoresis in parallel with size marker DNA fragments (M) followed by Southern blot analysis probing for the kanamycin-resistance gene.

## Recipes

### 1. LB medium

| Reagent | Final concentration | Amount |
|---|---|---|
| LB (Difco Lennox) | 2% | 20 g |
| $H_2O$ | | |
| Total | | 1,000 mL |

Autoclave at 121 °C for 20 min.

## 2. LBMSM

| Reagent | Final concentration | Amount |
|---|---|---|
| LB (Difco Lennox) | 2% | 20 g |
| $H_2O$ | | 500 mL |
| After autoclaving the above at 121 °C for 20 min, add the following solutions sterilized separately. | | |
| 1.25 M sorbitol | 0.5 M | 400 mL |
| 0.4 M $MgCl_2$ | 0.02 M | 50 mL |
| 0.4 M maleic acid-NaOH buffer (pH 7.0) | 0.02 M | 50 mL |
| Total | | 1,000 mL |

## 3. Solution A

| Reagent | Final concentration | Amount |
|---|---|---|
| $K_2HPO_4$ | 0.16 M | 5.6 g |
| $KH_2PO_4$ | 0.088 M | 2.4 g |
| $(NH_3)_2SO_4$ | 0.03 M | 0.8 g |
| Sodium citrate·$2H_2O$ | 0.0068 M | 0.4 g |
| $H_2O$ | | |
| Total | | 200 mL |

Autoclave at 121 °C for 20 min.

## 4. Solution B

| Reagent | Final concentration | Amount |
|---|---|---|
| $MgSO_4·7H_2O$ | 0.010 M | 0.5 g |
| Glucose | 0.056 M | 2 g |
| $H_2O$ | | |
| After autoclaving the above, add the following solutions sterilized separately. | | |
| 2 mg/mL $FeCl_3·4H_2O$ | $4.02 \times 10^{-6}$ M | 80 µL |
| 0.1 mg/mL $MnSO_4·5H_2O$ | $1.66 \times 10^{-7}$ M | 80 µL |
| 100× trace element | × 0.2 | 400 µL |
| Total | | 200 mL |

## 5. 100× Trace element

| Reagent | Final concentration | Amount |
|---|---|---|
| $CaCl_2·2H_2O$ | $4.97 \times 10^{-3}$ M | 0.73 g |
| $ZnCl_2$ | $1.25 \times 10^{-3}$ M | 0.17 g |
| $CuCl_2·2H_2O$ | $2.52 \times 10^{-4}$ M | 0.043 g |
| $CoCl_2·6H_2O$ | $2.52 \times 10^{-4}$ M | 0.06 g |
| $Na_2MoO_4·2H_2O$ | $2.48 \times 10^{-3}$ M | 0.06 g |
| $H_2O$ | | |
| Total | | 1,000 mL |

Autoclave at 121 °C for 20 min.

## 6. C-1 medium

| Reagent | Final concentration | Amount |
|---|---|---|
| Solution A | | 2.5 mL |
| Solution B | | 2.5 mL |
| After autoclaving the above at 121 °C for 20 min, add the following solutions sterilized separately. | | |
| 5% yeast extract | 0.05% | 50 µL |
| 10% casamino acids | 0.10% | 10 µL |
| 5 mg/mL L-Trp | 50 µg/mL | 50 µL |
| Total | | 5 mL |

## 7. C-2 medium

| Reagent | Final concentration | Amount |
|---|---|---|
| Solution A | | 5.0 mL |
| Solution B | | 5.0 mL |
| After autoclaving the above at 121 °C for 20 min, add the following solutions sterilized separately. | | |
| 5% yeast extract | 0.05% | 10 µL |
| 10% casamino acids | 0.10% | 10 µL |
| 5 mg/mL L-Trp | 5 µg/mL | 10 µL |
| Total | | 10 mL |

## 8. 50× TAE buffer

| Reagent | Final concentration | Amount |
|---|---|---|
| Tris (hydroxymethyl) aminomethane | 2 M | 242 g |
| Acetic acid | 1 M | 57.1 mL |
| $Na_2EDTA{\cdot}2H_2O$ | 0.5 M | 18.6 g |
| $H_2O$ | | |
| Total | | 1,000 mL |

Autoclave at 121 °C for 20 min.

## 9. TKE buffer

| Reagent | Final concentration | Amount |
|---|---|---|
| 1 M Tris-HCl buffer (pH 8.0) | 0.1 M | 10 mL |
| 0.5 M $Na_2EDTA$ (pH 8.0) | 0.05 M | 10 mL |
| 1 M KCl | 0.1 M | 10 mL |
| $H_2O$ | | 70 mL |
| Total | | 100 mL |

Autoclave at 121 °C for 20 min.

### 10. 20× SSC

| Reagent | Final concentration | Amount |
|---|---|---|
| Sodium citrate·2$H_2O$ | 0.3 M | 88.2 g |
| NaCl | 3 M | 175.3 g |
| $H_2O$ | | |
| Total | | 1,000 mL |

Adjust pH to 7 with 14 N HCl.
Autoclave at 121 °C for 20 min.

### 11. Maleic acid buffer

| Reagent | Final concentration | Amount |
|---|---|---|
| Maleic acid | 0.1 M | 11.61 g |
| NaCl | 0.15 M | 8.77 g |
| NaOH | 0.18 M | 7.2 g |
| $H_2O$ | | |
| Total | | 1,000 mL |

Adjust pH to 7.5.
Autoclave at 121 °C for 20 min.

### 12. Denaturing solution

| Reagent | Final concentration | Amount |
|---|---|---|
| NaCl | 1.5 M | 87.6 g |
| NaOH | 0.5 M | 20 g |
| $H_2O$ | | |
| Total | | 1,000 mL |

### 13. Neutralizing solution

| Reagent | Final concentration | Amount |
|---|---|---|
| 1 M Tris-HCl buffer (pH 7.5) | 0.5 M | 500 mL |
| NaCl | 1.5 M | 87.6 g |
| $H_2O$ | | |
| Total | | 1,000 mL |

### 14. Low-stringency wash solution

| Reagent | Final concentration | Amount |
|---|---|---|
| 20× SSC | 0.1× SSC | 30 mL |
| 10% SDS | 0.2% | 12 mL |
| $H_2O$ | | |
| Total | | 600 mL |

### 15. High-stringency wash solution

| Reagent | Final concentration | Amount |
|---|---|---|
| 20× SSC | 2× SSC | 180 mL |
| 10% SDS | 0.2% | 12 mL |
| $H_2O$ | | |
| Total | | 600 mL |

### 16. Detection buffer

| Reagent | Final concentration | Amount |
|---|---|---|
| Tris | 0.1 M | 12.1 g |
| NaCl | 0.1 M | 5.84 g |
| $MgCl_2$ | 0.05 M | 4.76 g |
| $H_2O$ | | |
| Total | | 1,000 mL |

Adjust pH to 9.5.

## Acknowledgments

The authors thank Valeria Verrone and Anil Wipat for their indispensable contribution to the original research paper where this protocol was derived from Mori et al. (2022). This study was supported by KAKENHI 18H02128.

## Competing interests

This protocol is related to a patent and is only permitted for use in academic research. In particular, the distribution of the strains YNB211 and YNB213 requires the conclusion of an appropriate material transfer agreement.

## References

Christie, P. J., Whitaker, N. and Gonzalez-Rivera, C. (2014). Mechanism and structure of the bacterial type IV secretion systems. *Biochim Biophys Acta* 1843(8): 1578-1591.

Christie, P. J. (2016). The Mosaic Type IV Secretion Systems. *EcoSal Plus* 7(1).

Kananavičiūtė, R., Kanišauskaitė, I., Novickij, V. and Čitavičius, D. J. (2015). Geobacillus stearothermophilus NUB3621R genetic transformation by electroporation. *Biologija* 61(3-4): 101-108.

Koehler, T. M. and Thorne, C. B. (1987). *Bacillus subtilis* (natto) plasmid pLS20 mediates interspecies plasmid transfer. *J Bacteriol* 169(11): 5271-5278.

Meijer, W. J. J., Boer, D. R., Ares, S., Alfonso, C., Rojo, F., Luque-Ortega, J. R. and Wu, L. J. (2021). Multiple Layered Control of the Conjugation Process of the *Bacillus subtilis* Plasmid pLS20. *Front Mol Biosci* 8: 648468.

Miyano, M., Tanaka, K., Ishikawa, S., Takenaka, S., Miguel-Arribas, A., Meijer, W. J. J. and Yoshida, K. I. (2018). Rapid conjugative mobilization of a 100 kb segment of *Bacillus subtilis* chromosomal DNA is mediated by a helper plasmid with no ability for self-transfer. *Microb Cell Fact* 17(1): 13.

Mori, K., Fukui, K., Amatsu, R., Ishikawa, S., Verrone, V., Wipat, A., Meijer, W. J. J. and Yoshida, K. I. (2022). A novel method for transforming *Geobacillus kaustophilus* with a chromosomal segment of *Bacillus subtilis* transferred via pLS20-dependent conjugation. *Microb Cell Fact* 21(1): 34.

Nazina, T. N., Tourova, T. P., Poltaraus, A. B., Novikova, E. V., Grigoryan, A. A., Ivanova, A. E., Lysenko, A. M., Petrunyaka, V. V., Osipov, G. A., Belyaev, S. S., et al. (2001). Taxonomic study of aerobic thermophilic bacilli: descriptions of *Geobacillus subterraneus* gen. nov., sp. nov. and *Geobacillus uzenensis* sp. nov. from petroleum reservoirs and transfer of *Bacillus stearothermophilus*, *Bacillus thermocatenulatus*, *Bacillus thermoleovorans*, *Bacillus kaustophilus*, *Bacillus thermodenitrificans* to Geobacillus as the new combinations *G. stearothermophilus*, G. th. *Int J Syst Evol Microbiol* 51(Pt 2): 433-446.

Singh, P. K., Ramachandran, G., Duran-Alcalde, L., Alonso, C., Wu, L. J. and Meijer, W. J. (2012). Inhibition of *Bacillus subtilis* natural competence by a native, conjugative plasmid-encoded comK repressor protein. *Environ Microbiol* 14(10): 2812-2825.

Singh, P. K., Ramachandran, G., Ramos-Ruiz, R., Peiró-Pastor, R., Abia, D., Wu, L. J. and Meijer, W. J. (2013). Mobility of the native *Bacillus subtilis* conjugative plasmid pLS20 is regulated by intercellular signaling. *PLoS Genet* 9(10): e1003892.

Suzuki, H. and Yoshida, K. (2012). Genetic transformation of *Geobacillus kaustophilus* HTA426 by conjugative transfer of host-mimicking plasmids. *J Microbiol Biotechnol* 22(9): 1279-1287.

Takami, H., Takaki, Y., Chee, G. J., Nishi, S., Shimamura, S., Suzuki, H., Matsui, S. and Uchiyama, I. (2004). Thermoadaptation trait revealed by the genome sequence of thermophilic *Geobacillus kaustophilus*. *Nucleic Acids Res* 32(21): 6292-6303.

Tanaka, T. and Koshikawa, T. (1977). Isolation and characterization of four types of plasmids from *Bacillus subtilis* (natto). *J Bacteriol* 131(2): 699-701.

Waksman, G. (2019). From conjugation to T4S systems in Gram-negative bacteria: a mechanistic biology perspective. *EMBO Rep* 20(2).

Wyrick, P. B. and Rogers, H. J. (1973). Isolation and characterization of cell wall-defective variants of *Bacillus subtilis* and *Bacillus licheniformis*. *J Bacteriol* 116(1): 456-465.