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Burkholderia thailandensis: genetic manipulation

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Abstract

Burkholderia thailandensis is a Gram-negative bacterium endemic to southeast Asia and northern Australia soils. Non-pathogenic, it is commonly used as a model organism for the related human pathogens *Burkholderia mallei* and *Burkholderia pseudomallei*. *B. thailandensis* is relatively easily genetically manipulated and a variety of robust genetic tools can be used in this organism. This unit describes protocols for conjugation, natural transformation, mini-Tn7 insertion, and allelic exchange in *B. thailandensis*.

Keywords

conjugation; transformation; allelic exchange; mutation; mini-Tn7

INTRODUCTION

Burkholderia thailandensis is a Gram-negative bacterium isolated from soil and water in southeast Asia and northern Australia. It is evolutionarily related to *Burkholderia mallei* and *Burkholderia pseudomallei*, which cause the diseases glanders and melioidosis, respectively. Lacking many virulence factors found in these human pathogens but otherwise sharing significant genomic similarity (Yu et al., 2006), *B. thailandensis* is utilized for comparative analyses or as an avirulent model organism. Indeed, *B. thailandensis*, like *B. pseudomallei*, can replicate in a variety of cell types (Haraga et al., 2008), utilize actin-based motility (Stevens et al., 2005), and induce the formation of multinucleate giant cells (Kespichayawattana et al., 2000; French et al., 2011). Moreover, its ease of genetic manipulation and the wide variety of genetic tools available for *B. thailandensis* has led to increased research in recent years, particularly in the study of interbacterial interactions (Anderson et al., 2012; Schwarz et al., 2010; Chandler et al., 2009). Protocols in this unit describe the basic genetic techniques used in *B. thailandensis* research, including the introduction of plasmids by conjugation (Basic Protocol 1), site-specific chromosomal

INTERNET RESOURCES

http://www.burkholderia.com

The *Burkholderia* Genome Database contains a comprehensive collection of sequences, annotations, and comparative tools for completed and draft genomes within the *Burkholderia* genus, including *B. thailandensis*.

http://tools.nwrce.org/tn_mutants/

A useful tool for *B. thailandensis* researchers, the laboratory of Colin Manoil has generated a near-saturation transposon mutant library in *B. thailandensis* E264, consisting of 87,000 unique mutants (7.5 mutants per gene) (Gallagher et al., 2013). The Transposon Mutant Library Browser displays available mutants and provides links for information on how to request mutants.

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insertion via Tn7 transposition (Basic Protocol 2), homologous recombination by natural transformation with linear DNA (Basic Protocol 3), antibiotic marker excision (Basic Protocol 4), and allelic exchange (Basic Protocol 5).

CAUTION: Burkholderia thailandensis is a Biosafety Level 1 (BSL-1) organism. Such organisms are not known to consistently cause disease in healthy adult humans, and are of minimal potential hazard to laboratory personnel and the environment. Standard microbiological practices should be followed when working with these organisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

BASIC PROTOCOL 1 INTRODUCTION OF PLASMIDS BY CONJUGATION

Although *Burkholderia thailandensis* is naturally competent, the most effective way to introduce plasmids (particularly nonreplicative plasmids) is via conjugation. Donor *Escherichia coli* strain RHO3 (*asd* *aphA*) is a 2,6-diaminopimelic acid (DAP) auxotroph and, following conjugation, is easily selected against by the exclusion of DAP from the selection medium (López et al., 2009). However, any donor *E. coli* strain that carries conjugation machinery may be used.

Materials

B. thailandensis from a frozen stock

Donor *E. coli* RHO3 strain(s) carrying the plasmid(s) to be transferred

Sterile swabs

Low salt LB (LSLB) plates supplemented with 200 μg/ml 2,6-diaminopimelic acid (DAP) and/or appropriate antibiotics (see Reagents and Solutions; Table 1)

1 Streak *B. thailandensis* onto a LSLB plate and *E. coli* RHO3 carrying the plasmid(s) to be transferred on LSLB plate(s) supplemented with DAP and appropriate antibiotics.

2 Incubate overnight (*B. thailandensis*) or two days (*E. coli* RHO3) at 37°C.

3 Using a sterile swab, collect several large *B. thailandensis* colonies and thoroughly spread the bacteria onto approximately one half of a LSLB plate supplemented with DAP (no antibiotics).

The plate is spread evenly with a layer of bacteria, not streaked for isolated colonies.

4 Using the same technique, use a clean sterile swab to collect an equivalent amount of *E. coli* RHO3 and spread it on top of the area spread with *B. thailandensis.*

5 For triparental matings, repeat step 4 with the remaining donor *E. coli* strain.

5 To prepare control conjugations, use a sterile swab to collect fresh *B. thailandensis* and spread it alone onto a clean section (approximately one-quarter or less of the plate) of the conjugation plate.

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6. Repeat step 5 with each donor *E. coli* strain.

7. Incubate the conjugation plate at 37°C for 4–6 h.

8. Using a sterile swab, collect bacteria from the conjugation (section of mixed *B. thailandensis* and *E. coli*) and spread onto approximately one-half of a LBLB plate supplemented with appropriate antibiotics (selection plate).

   To select against the donor E. coli strain, the selection plate should not contain DAP.

9. Streak once for isolated colonies.

10. Using sterile swabs, collect bacteria from the control areas (sections of *B. thailandensis* and *E. coli* alone) of the conjugation plate and patch them onto clean sections of the selection plate.

11. Save the conjugation plate at room temperature overnight.

    Conjugation will continue to occur. If no colonies are observed on the selection plate, selection (steps 8–12, omitting step 11) may be repeated once from this overnight conjugation.

12. Incubate the selection plate at 37°C overnight.

    Depending on the efficiency of the mating, isolated colonies may be found in either the heavy-streaked area of the selection plate or in the area streaked for isolation (or both).

13. Observe the control conjugations on the selection plate. If growth is observed, the mating should be repeated following troubleshooting.

BASIC PROTOCOL 2 SITE-SPECIFIC CHROMOSOME INSERTION BY MINI-TN7 TRANSPOSITION

Unlike other transposons, Tn7 inserts site-specifically (and orientation-specifically) into Gram negative bacterial chromosomes at Tn7 attachment, or *att*Tn7, sites, which are located downstream of *glmS* genes (Peters, 2014). Tn7-based vectors take advantage of this biology, allowing the stable insertion of DNA at a neutral chromosomal site. A mini-Tn7-based system developed in the Schweizer laboratory works robustly in *B. thailandensis* (Choi et al., 2005). In addition, *B. thailandensis* has two *glmS* genes and two corresponding *att*Tn7 sites, each of which may be inserted with the same or different genetic material. Possible applications include mutant complementation, overexpression, introduction of gene expression reporters (*lacZ*, *gfp*), and fluorescent labeling (*gfp*, *rfp*) (Norris et al., 2010).

In this system, DNA of interest is cloned between the Tn7L and Tn7R recognition sequences on a mini-Tn7 suicide plasmid, which also contains an antibiotic resistance cassette. Together with a helper plasmid containing the required transposase, pTNS3 (Choi et al., 2008), the mini-Tn7 plasmid is introduced into *B. thailandensis* and the mini-Tn7 DNA (containing the DNA of interest and resistance cassette) is moved to an *att*Tn7 site. Once introduced, insertions at *att*Tn7 sites are stable and do not require antibiotic selection for

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maintenance (Choi et al., 2005). Moreover, many commonly-used mini-Tn7 plasmids feature Flp recombinase target (*FRT*) sequences that flank the antibiotic resistance cassette, allowing for subsequent marker excision, if desired (see Basic Protocol 4).

Materials

B. thailandensis from a frozen stock

Donor *E. coli* RHO3 carrying a mini-Tn7 plasmid (see Table 2)

Donor *E. coli* RHO3 carrying helper plasmid pTNS3 (see Table 2)

Sterile swabs

Low salt LB (LSLB) plates supplemented with DAP and/or appropriate antibiotics (see Reagents and Solutions; Table 1)

Sterile toothpicks or inoculating loops

1. Perform a triparental mating with *B. thailandensis*, *E. coli* RHO3 carrying a mini-Tn7 plasmid, and *E. coli* RHO3 carrying the helper plasmid pTNS3 (see Basic Protocol 1).

2. Using aseptic technique, streak 4–8 transconjugant colonies from the conjugation selection plate onto a fresh LSLB plate supplemented with antibiotics. Streak for isolated colonies.

   Antibiotic included in the medium should correspond to the resistance cassette carried on the mini-Tn7 plasmid that is delivered to the B. thailandensis attTn7 site (located between the plasmid's Tn7L and Tn7R sequences). Antibiotic resistance cassettes located elsewhere on the mini-Tn7 plasmid backbone will not transfer to B. thailandensis.

3. Incubate overnight at 37°C.

4. Test for *att*Tn7 site insertions in each candidate using colony PCR (see Support Protocol 1). Two colony PCR reactions should be set up for each *B. thailandensis* candidate – one testing for insertion at the *att*Tn7 site downstream of *glmS-1* and a separate reaction to test for insertion at the site downstream of *glmS-2* (Fig. 1) (Choi et al., 2005). Reaction 1 contains primers "Tn7L" (ATTAGCTTACGACGCTACACCC) and "glmS1" (GCGTTCGTCGTCCACTGGGA) and reaction 2 contains primers "Tn7L" and "glmS2" (TGTGAATGGTCAGACGCTGTTCG).

   Occasionally, a single B. thailandensis colony will contain insertions at both attTn7 sites. Depending on the downstream application, this may or may not be acceptable.

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BASIC PROTOCOL 3 HOMOLOGOUS RECOMBINATION BY NATURAL TRANSFORMATION WITH LINEAR DNA

Developed in the Manoil laboratory (Thongdee et al., 2008), this technique takes advantage of the fact that *B. thailandensis* becomes naturally competent (able to take up and incorporate exogenous DNA) when cultured in minimal glucose medium. By engineering specific DNA fragments, transformation with linear DNA is used to construct marked mutations. Using PCR and standard cloning methods, an antibiotic resistance marker is flanked by DNA homologous to the *B. thailandensis* genome so that integration of the linear DNA fragment replaces a specific chromosomal element with the resistance cassette. Alternatively, chromosomal DNA containing an antibiotic resistance marker may be used as the transformation substrate. In this way, the method can be used for both generating marked mutations (via transformation with PCR product), as well as for transferring mutations between strains (via transformation with whole chromosomal DNA).

Materials

B. thailandensis from a frozen stock

Sterile capped glass or plastic culture tubes

Low salt LB (LSLB) medium (see Reagents and Solutions)

Sterile sticks or inoculating loops

M63 minimal medium (see Reagents and Solutions)

Spectrophotometer and cuvettes

Microcentrifuge and plastic tubes

DNA containing an antibiotic resistance cassette that is flanked by >500 bp regions of homology to the *B. thailandensis* genome (may be PCR product or *B. thailandensis* chromosomal DNA). If subsequent antibiotic resistance marker removal is desired, the cassette should be flanked first by *FRT* sequences (see Basic Protocol 4).

Sterile cell spreader or glass beads

Low salt LB (LSLB) plates supplemented with appropriate antibiotics (see Reagents and Solutions; Table 1)

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1. Inoculate several *B. thailandensis* colonies into 2 ml LSLB medium.
2. Incubate overnight with aeration at 37°C.
3. Dilute the overnight culture by adding 40 µl culture to 2 ml M63 minimal medium (1:50 dilution).

   This may be scaled up depending on how many transformations are required. Adjust the resuspension volume accordingly in step 5 below.
4. Incubate with aeration at 37°C until OD_{600}=0.5 (approximately 4–5 h).

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5 Pellet culture in microcentrifuge (1 min at 15,000 × *g*), decant supernatant, and resuspend pellet in 100 μl fresh M63 (1/20th of the 2 ml culture volume).

6 Mix 50 μl cell suspension with 5 μl DNA (at least 100 ng) or water (no DNA) control.

Inclusion of a "no DNA" control with each set of transformations is important for data interpretation.

7 Incubate 30 min at room temperature with no agitation.

6 Add 2 ml fresh M63 minimal medium to each transformation and transfer each to a culture tube.

8 Incubate overnight with aeration at 37°C.

9 Pellet each culture as described in step 5, decant supernatant, and resuspend in 100 μl M63.

10 Using a sterilized cell spreader or glass beads, spread the cell suspension (100 μl) onto a LSLB plate supplemented with appropriate antibiotics (depending on the antibiotic resistance cassette included in transformed DNA).

11 Incubate plates ~20 h at 37°C.

Colonies should be visible on transformation plates, but not on "no DNA" control plates.

Check for the presence of the desired mutation in the resulting transformants by colony PCR (see Support Protocol 1).

BASIC PROTOCOL 4 FLP RECOMBINASE-MEDIATED MARKER EXCISION

Excision of antibiotic resistance markers allows markers to be recycled for subsequent genetic manipulations and provides antibiotic-sensitive bacteria for applications where this feature is desired. Flp recombinase mediates site-specific recombination between *flp* recombinase target (*FRT*) sequences and the Flp-*FRT* system has been adapted for many applications. Tools developed in the Schweizer laboratory allow an *FRT*-flanked antibiotic resistance marker to be excised from a *B. thailandensis* chromosome using a rhamnose-inducible *flp* contained on a temperature-sensitive plasmid (Choi et al., 2008). Plasmid pFlpe4 (or its Tet-resistant derivative, pFlpTet (Garcia et al., 2013)) replicates in *Burkholderia* at 30°C, but is lost at higher temperatures (42°C or 37°C).

Materials

B. thailandensis with a chromosomal *FRT*-flanked antibiotic resistance cassette

Donor *E. coli* RHO3 containing pFlpe4 or pFlpTet (see Table 2)

Sterile swabs

Low salt LB (LSLB) plates (see Reagents and Solutions)

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LSLB plates supplemented with 200 μg/ml 2,6-diaminopimelic acid (DAP), 250 μg/ml kanamycin (Kan), 50 μg/ml tetracycline (Tet), and/or 0.2% rhamnose, where appropriate (see Reagents and Solutions; Table 1)

Sterile applicator sticks or inoculating loops

1. Perform a mating with *B. thailandensis* and *E. coli* RHO3 carrying pFlpe4 or pFlpTet according to Basic Protocol 1 with the following modification: incubate the conjugation at 30°C (instead of 37°C) for 6 h.
2. Select the mating on LSLB/Kan (for pFlpe4) or LSLB/Tet (for pFlpTet) plates (see Basic Protocol 1) and incubate the selection plate 48 h at 30°C.
3. To induce *flp* expression, streak 8–16 colonies for isolation onto LSLB/Kan/rhamnose (for pFlpe4) or LSLB/Tet/rhamnose (for pFlpTet) plates.
4. Incubate the plates 48 h at 30°C.
5. Sequentially patch 3–4 colonies from each plate/sector onto LSLB containing the antibiotic to which sensitivity is desired and either LSLB/Kan (for pFlpe4) or LSLB/Tet (for pFlpTet).
6. Incubate the plates 48 h at 30°C.

   Clones should be sensitive to the antibiotic corresponding to the cassette removed and resistant to either Kan or Tet.
7. Using bacteria from the LSLB/Kan (for pFlpe4) or LSLB/Tet (for pFlpTet) plates, streak 4–5 antibiotic-sensitive clones onto LSLB.
8. Incubate the plates overnight at 37°C to induce loss of pFlpe4 or pFlpTet.
9. Streak colonies from the LSLB plates onto fresh LSLB plates.
10. Incubate the plates overnight at 37°C.
11. Patch 4–5 colonies from each clone onto LSLB plates and either LSLB/Kan (for pFlpe4) or LSLB/Tet (for pFlpTet) plates.
12. Incubate the plates 48 h at 30°C.

    Growth should only be visible on LSLB plates, as colonies should be Kan- or Tet-sensitive after pFlpe4 or pFlpTet loss.
13. Confirm the loss of the antibiotic resistance marker by colony PCR (see Support Protocol 1).

BASIC PROTOCOL 5 ALLELIC EXCHANGE USING SACB COUNTERSELECTION

A common method for introducing unmarked chromosomal mutations, allelic exchange has been adapted for use in many bacterial species. This powerful method allows in-frame gene deletion, gene replacement, generation of point mutations, and introduction of sequences encoding epitope tags onto the bacterial chromosome. While several allele replacement

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systems exist for *B. thailandensis*, the pEXKm5-based system developed in the Schweizer laboratory is particularly robust, easy to use, and versatile (López et al., 2009).

In this protocol, a markerless mutation flanked by regions of homology (500 bp) is cloned into suicide vector pEXKm5. When introduced into *B. thailandensis*, the plasmid can integrate onto a chromosome via recombination at the homology regions. A kanamycin resistance cassette and *gusA* gene located on the pEXKm5 backbone allow selection and identification of colonies containing the cointegrated plasmid. Encoded by *gusA*, glucuronidase will cleave the indicator substrate X-Gluc, resulting in a blue color change and allowing for blue-white screening at several steps of the procedure. In the second stage of the protocol, subsequent loss of pEXKm5 from *B. thailandensis* by homologous recombination results in a mixture of bacteria that have either reverted to the wild-type genotype or that contain the desired mutation. Loss of pEXKm5 is achieved either through *sacB* counterselection (described here) or using I-SceI endonuclease-stimulated recombination (see Alternate Protocol 1). Located on pEXKm5, *sacB* encodes levansucrase, a *Bacillus subtilis* enzyme that is lethal for many Gram-negative bacteria when they are cultured in the presence of sucrose. Thus, *B. thailandensis* containing cointegrated pEXKm5 are sensitive to sucrose, while cells that have lost the plasmid by recombination (and which may contain the desired mutation) are rendered sucrose-resistant.

Materials

B. thailandensis from a frozen stock

Donor *E. coli* RHO3 containing pEXKm5 (see Table 2) that has been modified to contain the recombinant allele and homology regions

Sterile swabs

Low salt LB (LSLB) plates (see Reagents and Solutions)

LSLB plates supplemented with 200 μg/ml 2,6-diaminopimelic acid (DAP), 250 μg/ml kanamycin (Kan), and/or 50 μg/ml X-gluc, where appropriate (see Reagents and Solutions; Table 1)

Sterile applicator sticks or inoculating loops

YT plates supplemented with 15% sucrose and either 250 μg/ml Kan or 50 μg/ml X-gluc (see Reagents and Solutions; Table 1)

Sterile capped glass or plastic culture tubes

YT broth (see Reagents and Solutions)

Sterile cell spreader or glass beads

1. Perform a mating with *B. thailandensis* and *E. coli* RHO3 carrying the modified pEXKm5 plasmid (see Basic Protocol 1).
2. Select the mating on LSLB/Kan/X-gluc plates (see Basic Protocol 1) and incubate the selection plate overnight at 37°C.

Colonies with chromosomally-integrated pEXKm5 as the result of a single recombination event (called cointegrants or merodiploids) will be kanamycin-resistant and blue. GusA activity on X-gluc results in a subtle blue color (less pronounced than LacZ with X-Gal) that can be enhanced by incubation of the plate for several hours at 4°C.

3. Select 8–16 cointegrants and streak for isolated colonies onto LSLB/Kan/X-gluc plates.

4. Incubate the plates overnight at 37°C.

5. Check that the cointegrants contain pEXKm5 integrated at the correct chromosomal location (and determine orientation) using colony PCR and primers that anneal to the plasmid in combination with primers that anneal to the chromosome outside of the region used in homologous recombination.

6. Confirm the sucrose-sensitivity of cointegrants by patching each candidate sequentially onto a YT/Sucrose/Kan plate and a LSLB/Kan plate.

7. Incubate the plates 20–24 h at 30°C.

Ideal cointegrants should have heavy growth on the LSLB/Kan plate and little to no growth on the YT/Sucrose/Kan plate.

8. Select two sucrose-sensitive cointegrants for counterselection. Ideally, choose one clone that contains pEXKm5 integrated at the 5′ homology region and another clone that contains pEXKm5 integrated at the 3′ homology region (as determined by colony PCR in step 5).

9. Inoculate one colony into 1 ml YT broth.

10. Incubate with aeration for 4 h at 37°C.

11. Prepare serial dilutions from the YT culture and plate 100 μl of each dilution onto YT/Sucrose/X-gluc plates.

Which dilutions are plated depends on the incubation time of the YT culture (longer incubations will require plating more dilute cell suspension). To start, a greater number of dilutions are recommended (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) to ensure that isolated colonies are observed.

12. Incubate for 48 h at 30°C.

A mixture of blue and white colonies may be observed. Bacteria that have undergone a second recombination event will "loop out" the pEXKm5 plasmid and lose it, resulting in colonies that are sucrose-resistant, kanamycin-sensitive, and white on X-gluc.

13. Select 8–16 white colonies and streak for isolated colonies onto LSLB plates.

14. Incubate overnight at 37°C.

15. Check for sensitivity to Kan by sequentially patching each candidate onto a LSLB/Kan plate and a LSLB plate.

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16. Incubate overnight at 37°C.

    Growth should only be observed on the LSLB plate.

17. For kanamycin-sensitive clones, use colony PCR (see Support Protocol 1) to determine the genotype (mutant or wild-type) of the colony.

SUPPORT PROTOCOL 1 COLONY PCR

B. thailandensis colonies that are candidates for introduced mutations (gene deletions, insertions, or replacements) or chromosomal insertions (*ie*. *att*Tn7 site insertions) are routinely screened by PCR to determine whether or not they contain the desired genetic manipulation. PCR reactions can then be analyzed for their size or further digested with restriction enzymes, if necessary. Use of appropriate control reactions with each PCR is critical for data interpretation.

Materials

0.2 ml tubes

Sterile, deionized water

5× GoTaq® buffer (Promega), or alternative Taq buffer

10 mM dNTP mix

Primers, 10 μM each

Dimethyl sulfoxide (DMSO)

Go Taq® polymerase (Promega), or alternative Taq polymerase

B. thailandensis colonies for screening

Sterile toothpicks

Thermocycler

Restriction enzyme(s) and appropriate buffer (optional)

6× DNA gel loading dye

Agarose and 0.5× TAE buffer

Ethidium bromide or other DNA gel stain

Agarose gel electrophoresis apparatus

UV transilluminator

1. Prepare the following PCR reaction mixtures in 0.2 ml tubes:

water	14.3 μl
5× GoTaq® buffer	5.0 μl
10 mM dNTP mix	1.0 μl
Forward primer, 10 μm	1.0 μl
Reverse primer, 10 μm	1.0 μl

DMSO	2.5 μl
GoTaq® polymerase	0.2 μl

2. Using a sterile toothpick, touch a single *B. thailandensis* colony and swirl in a PCR reaction tube.

   Toothpick does not need to contain visible material from the bacterial colony.

3. Repeat step 2 for remaining reactions.

4. Place tubes in thermocycler and run the following program, where T_{anneal} = (Primer T_m) – (4–5°C):

Temperature	Time	Cycles
95°C	2 min	1
95°C	30 sec	
T_{anneal}	30 sec	30
72°C	1 min/kb expected product	
72°C	5 min	1

5. If necessary, digest the PCR product with the appropriate restriction enzyme(s).

6. Mix 2 μl DNA loading dye with 2–10 μl PCR product and run on an agarose gel.

7. Analyze the gel by UV transillumination for presence/absence of PCR product, PCR product size, or size of digested PCR products.

REAGENTS AND SOLUTIONS

Low salt LB broth (or agar)

10 g tryptone

5 g yeast extract

5 g NaCl

1 L distilled, deionized water

Autoclave

Store broth at room temperature for 3 months

For plates: add 15 g agar to broth mixture before autoclaving. Cool to 55°C and add antibiotics or other supplements, if necessary (see Table 1). Pour cooled agar into sterile plastic petri dishes. Dry plates at room temperature overnight and store at 4°C for 6 months.

M63 minimal medium

5× M63 salts solution

15 g KH_2PO_4

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35 g K_2HPO_4

10 g $(NH_4)_2SO_4$

2.5 ml 1 mg/ml $FeSO_4 \cdot 7H_2O$

pH to 7.0 with KOH

distilled, deionized water to 1 L final volume

Autoclave

Store at room temperature for 12 months

1× M63 broth

400 ml distilled, deionized water

Autoclave

Cool to 55°C and add the following:

100 ml 5× M63 salts solution

5 ml 20% (w/v) glucose (or alternate carbon source), filter sterilized

0.5 ml 1 M $MgSO_4$, sterile

Store at room temperature for 3 months

YT broth

10 g tryptone

10 g yeast extract

1 L distilled, deionized water

Autoclave

Store broth at room temperature for 3 months

YT agar with 15% sucrose

10 g tryptone

10 g yeast extract

15 g agar

700 mL distilled, deionized water

Autoclave

Cool to 55°C and add 300 ml 50% (w/v) filter sterilized sucrose. Add antibiotics or other supplements, if necessary (see Table 1). Pour cooled agar into sterile plastic petri dishes. Dry plates at room temperature overnight and store at 4°C for 2–3 weeks.

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COMMENTARY

Background Information

Occupying overlapping niches in tropical soils and water, *B. thailandensis* and the human pathogen *B. pseudomallei* are very closely related. Thus, most genetic tools developed for use in *B. pseudomallei* are also compatible with *B. thailandensis*. As a model organism, *B. thailandensis* offers greater flexibility in antibiotic marker use and ease of genetic manipulation, as, unlike *B. pseudomallei*, it is not a CDC Select Agent nor does it require BSL-3 containment.

Genome sequences of several *B. thailandensis* strains have been completed and numerous draft genome sequences are available on NCBI and other databases. Like *B. pseudomallei*, *B. thailandensis* has two circular chromosomes that, in reference strain E264, are 3.8 MB and 2.9 MB in size and are GC-rich (approximately 68% GC) (Yu et al., 2006). Phylogenetic analysis using 16S rRNA and housekeeping gene sequences suggests that *B. thailandensis* diverged from *B. pseudomallei* and *Burkholderia mallei* approximately 47 million years ago (Yu et al., 2006). Horizontal gene transfer appears to be a driver of *B. pseudomallei* and *B. thailandensis* diversity, as strains of both species contain numerous genomic islands (Yu et al., 2006; Tuanyok et al., 2008).

Robust genetic tools, including those described in this unit, have accelerated *B. thailandensis* research, particularly the ability to generate in-frame unmarked mutations using allelic exchange. Although the use of *sacB* counterselection was initially thought to be problematic in *B. pseudomallei* and related species due to the presence of endogenous *sacB* genes (Holden et al., 2004), modifications have allowed the system to function effectively. A modified *sacB* gene that includes a *Burkholderia* leader sequence (Hamad et al., 2009) and optimized culture conditions (Logue et al., 2009) enabled this method to function efficiently in *B. thailandensis* and *B. pseudomallei* (López et al., 2009). While other counterselectable markers have been used for *Burkholderia* spp., including *pheS* (Barrett et al., 2008), the pEXKm5-based *sacB* system offers several advantages (López et al., 2009). This vector enables counterselection on rich medium, supplies a useful *gusA* marker to screen cointegrants and resolved cointegrants, and provides versatility with the inclusion of an alternative I-SceI based system.

An alternative to *sacB* counterselection described in Basic Protocol 4 is the use of homing endonuclease I-SceI to stimulate recombination in pEXKm5 cointegrants. The 18 bp recognition sequence of I-SceI does not occur in any bacterial genomes sequenced to date, but is included on the pEXKm5 backbone. Generation of a double-stranded DNA break by I-SceI cleavage leads to an increased rate of intramolecular recombination and can be exploited to promote cointegrant resolution (Pósfai et al., 1999). The current tools available for I-SceI use with pEXKm5 in *B. pseudomallei* are incompatible with *B. thailandensis*, as *I-sceI* is encoded on a plasmid conferring zeocin resistance in this system (López et al., 2009). Although *B. thailandensis* is intrinsically zeocin-resistant, simple cloning to alter the resistance cassette should allow this approach to be applied to *B. thailandensis* as well, further expanding the genetic tool repertoire for this organism.

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Critical Parameters and Troubleshooting

Introduction of plasmids by conjugation—All conjugations should utilize *B. thailandensis* freshly-cultured from a frozen stock. Bacteria stored at 4°C may have decreased viability and repeated serial passages of *B. thailandensis* may allow mutation accumulation. Growth of control conjugations on the antibiotic selection plate most likely suggests that the wrong antibiotic was used for selection or that the wrong bacterial strain was used in the mating. Occasionally pinpoint colonies on the selection plate will be observed for these control conjugations (as well as for the true conjugation), especially if the plate was incubated for >24 h. These are spontaneous antibiotic-resistant mutants and are not a concern, provided that true transconjugant colonies (larger in size) are easily identified on the selection plate. Finally, frequently-used plasmids should be periodically re-transformed into fresh *E. coli* RHO3 cells because, although rare, reversion to DAP-independence has been observed for some strains.

Site-specific chromosome insertion by mini-Tn7 transposition—Difficulty obtaining antibiotic-resistant transconjugants after conjugations with mini-Tn7 plasmids may indicate that the helper plasmid pTNS3 was omitted from the mating or the wrong antibiotic was used in the selection plate. Antibiotic-resistant colonies that lack *att*Tn7 site insertions (determined by colony PCR) can occur and likely represent spontaneous mutants or, if the mini-Tn7 plasmid contained sequences present in the *B. thailandensis* genome, bacteria that have integrated the whole plasmid into their genome. The frequency of this occurrence increases as the size (bp) of homologous *B. thailandensis* sequence present on the mini-Tn7 plasmid increases. Usually, simply screening additional colonies by PCR will identify bacteria with the desired *att*Tn7 site insertion.

Homologous recombination by transformation with linear DNA—When designing PCR products for linear DNA transformation, at least 500 bp of homologous DNA flanking the antibiotic resistance cassette must be included. For increased transformation efficiencies, 800–1000 bp may be used. For preparation of the bacteria to be transformed, it is not critical for the M63 culture to reach OD_{600}=0.5 exactly. A range of mid-late log phase culture densities (OD_{600}=0.3–0.8) can result in successful transformations, provided that sufficient numbers of cells are used. Transformant colonies may not be visible after overnight incubation, but should be visible after 20–24 h at 37°C. Spontaneous antibiotic-resistant mutants will also arise on the “no DNA” control plate, particularly after extended incubation. All plates should be checked periodically between 18–24 h to ensure that transformant colonies can be distinguished from spontaneous mutants. Incubation times >24 h are not recommended as spontaneous mutants will most likely occur and will be difficult to distinguish from transformants.

Flp recombinase-mediated marker excision—If clones are not found that are sensitive to the desired antibiotic, screen additional colonies by repeating steps 5–6 of the protocol. Alternatively (or additionally), colonies obtained in step 4 may be re-streaked onto LSLB/Kan/rhamnose or LSLB/Tet/rhamnose to re-induce Flp production and then screened for marker excision by testing antibiotic sensitivity.

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Allelic exchange using *sacB* counterselection—It is important to ensure that the cointegrants chosen for counterselection start out sucrose-sensitive, as spontaneous *sacB* mutations occur at a relatively high frequency and will negatively impact counterselection. If all tested cointegrants appear sucrose-resistant, test additional clones or simply repeat the conjugation and try again with fresh conintegrants. YT/Sucrose plates may only be stored at 4°C for 2–3 weeks. Likely due to degradation of the sucrose, prolonged storage will result in plates that are less effective in counterselection. Freshly-prepared YT/Sucrose plates are recommended for each round of allelic exchange.

In theory, homologous recombination at the 5′ and 3′ homology regions of pEXKm5 should occur with equal frequency. Thus, counterselection should yield a 50:50 ratio of clones that contain the desired mutation (having recombined at the 5′ side during cointegration and the 3′ side during counterselection, or vice versa) and those that reverted to the wild-type sequence (having recombined at the same side for both cointegration and counterselection). However, in practice this is not always the case and screening additional "loopout" candidates may be necessary to identify the desired mutant. Initiating counterselection with both cointegrants that have 5′-integrated plasmid as well as those that have 3′-integrated plasmid is often useful in overcoming this problem.

Colony PCR—If no products or nonspecific products of incorrect sizes are observed after PCR, several parameters of the reaction, including DMSO concentration and annealing temperature, may be adjusted. Use of an annealing temperature gradient may be helpful. If reaction optimization is unsuccessful, primer redesign may be necessary, paying careful attention to GC content and melting temperature.

All protocols—Particularly during *sacB*-mediated allelic exchange (but possible during any genetic manipulation), spontaneous biofilm-deficient *B. thailandensis* mutants can arise. Mutations should be complemented and marked mutations should be transferred to a fresh genetic background (see Basic Protocol 3) to ensure that phenotypes are not due to secondary mutations.

Anticipated Results

Conjugations (Basic Protocol 1) are efficient in *B. thailandensis* and should result in numerous antibiotic-resistant transconjugants. Triparental matings are generally less efficient, but still should yield at least 5–30 colonies. Basic Protocol 2 should result in *B. thailandensis* colonies having an insertion at an *att*Tn7 site. Transformation efficiency with linear DNA (Basic Protocol 3) depends on the DNA used for transformation and is expected to be approximately 2–600 transformants/ng DNA/10^9 CFU for chromosomal DNA and 0.2–1600 transformants/ng DNA/10^9 CFU for PCR fragments (Thongdee et al., 2008). Transformations usually result in 3–50 transformant colonies per plate. Marker excision frequencies (via Flp recombinase) are expected to range from 75–96% (Choi et al., 2008), but are often observed at lower rates. Allelic exchange (Basic Protocol 5) should yield *B. thailandensis* colonies that have the desired chromosomal mutation, insertion, or replacement.

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Time Considerations

B. thailandensis colonies should be visible on plates after overnight growth at 37°C, although slightly longer incubation (20–24 h) may be required for plates containing antibiotics. Two days of growth are required for *B. thailandensis* plates incubated at 30°C or for *E. coli* RHO3 strains incubated at 37°C. With the exception of colony PCR, which will take several hours, all of the protocols described in this unit are completed over multiple days. Conjugation and mini-Tn7 insertion can be completed in 4 days (including streaking the strains from frozen stocks) and transformation with linear DNA will take 4 days to complete (5 days including streaking *B. thailandensis* from a frozen stock). Removal of an antibiotic resistance cassette by *flp*-recombination can be completed in 11 days. Allelic exchange using *sacB* counterselection can be completed 11 days and, although more time may be required if additional screening steps are required (for example, screening for sucrose-sensitive colonies).

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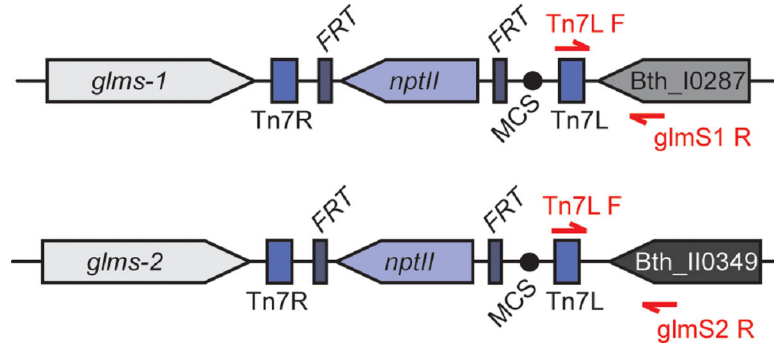

Figure 1. Mini-Tn7 insertions at *B. thailandensis* E264 *att*Tn7 sites

The two *att*Tn7 sites are located 3′ to *glmS-1* (Bth_I0288/Bth_RS13655) on chromosome I and 3′ to *glmS-2* (Bth_II0348/BTH_RS01805) on chromosome II. Primers (red arrows) that anneal to the Tn7L region of mini-Tn7 and either Bth_I0286 (Bth_RS13660) or Bth_II0349 (Bth_RS01810) are used to confirm a*tt*Tn7 insertion (for primer sequences, see Basic Protocol 2). In this example based on insertion from pUC18T-miniTn7T-Km (Choi et al., 2005), *nptII* (conferring kanamycin-resistance) carried on mini-Tn7 is flanked by *flp* recombinase target (*FRT*) sequences for marker excision, leaving the multiple cloning site (MCS) intact.

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Table 1

Antibiotic and media supplement concentrations for use with *B. thailandensis* genetic tools.

Supplement	Solvent	Stock conc.[a]	Final conc.[b]
2,6-diaminopimelic acid (DAP)	1 M NaOH	100 mg/ml	200 μg/ml
Chloramphenicol	Ethanol	20 mg/ml	20 μg/ml
Kanamycin	Water	125 mg/ml	250 μg/ml
Tetracycline[c]	Ethanol	10 mg/ml	50 μg/ml
Ampicillin[d]	Water	100 mg/ml	100 μg/ml
X-gluc[c,e]	DMF or DMSO	50 mg/ml	50 μg/ml
Sucrose	Water	50% (w/v)	15% (w/v)
Rhamnose[c]	Water	20% (w/v)	0.2% (w/v)

[a]Most stock solutions should be filter-sterilized (0.22 μm filter) and stored at −20°C. Sucrose and rhamnose solutions may be stored at room temperature.

[b]Final working concentration in liquid medium or agar plates. Media should be cooled to 55°C before the addition of antibiotics or supplements.

[c]Light-sensitive. Stock solution and prepared media should be protected from light.

[d]For use with *E. coli* only. *B. thailandensis* is resistant to ampicillin.

[e]X-gluc, 5-bromo-4-chloro-3-indoxyl-beta-D-glucuronide

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Table 2

Plasmids used routinely for genetic manipulation of *B. thailandensis*

Plasmid	Description/features	Antibiotic resistance	Reference
pTNS3	Helper plasmid for mini-Tn7, *oriT*, R6K *ori*	Amp	(Choi et al., 2008)
pUC18T-miniTn7T-Km	*att*Tn7 site delivery plasmid with MCS	Kan (mini-Tn7), Amp (backbone)	(Choi et al., 2005)
miniTn7-kan-*gfp*	Delivers *gfp* to *att*Tn7 site	Kan	(Norris et al., 2010)
mini-Tn7-kan-*rfp*	Delivers *rfp* to *att*Tn7 site	Kan	(Norris et al., 2010)
pFlpe4	Rham-inducible *flp*, TS *ori*	Kan	(Choi et al., 2008)
pFlpTet	Rham-inducible *flp*, TS *ori*	Tet	(Garcia et al., 2013)
pEXKm5	Allelic exchange vector	Kan	(López et al., 2009)

MCS, multiple cloning site; TS, temperature-sensitive; Rham, rhamnose; Amp, ampicillin; Kan, kanamycin; Tet, tetracycline